Tissue specific expression of ABA and GA metabolic genes during grain development and with respect to dormancy and germination in barley

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ABSTRACT

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Seed development, germination and dormancy, considered as the most important phenomena in seeds, are regulated by several plant hormones; gibberellin (GA) and abscisic acid (ABA) being the major players acting antagonistically. The regulation of these seed related processes by GA and ABA is dependent partly on the endogenous levels of the two hormones, which in turn are determined by the balance between their biosynthesis and catabolism. This thesis investigated the spatial and temporal expression patterns of several members of the GA and ABA biosynthetic and catabolic gene families during grain development using a non-dormant cultivar and during imbibition using grains collected from dormant and non-dormant cultivars of barley. In addition, the thesis examined the effect of exogenous ABA treatment, and after-ripening of seeds collected from dormant cultivars on the expression patterns GA and ABA metabolism genes during grain development and imbibition, respectively. The results suggest that specific members of the gene families related to the metabolic pathways of the two hormones exhibit distinct spatial and temporal roles in the regulation of barley grain development, dormancy and germination.

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ABBREVIATIONS

AAO	ABA aldehyde oxidase
ABA	abscisic acid
ABA-GE	ABA glucosyl ester
BZ-AR	after-ripened barley cv. Betzes
BZ-D	dormant barley cv. Betzes
CPS	*ent*-copalyl diphosphate synthase
DAA	days after anthesis
DAP	days after pollination
DPA	dihydrophaseic acid
FP	forward primer
GA	gibberellin
GA20ox	GA 20-oxidase
GA2ox	GA 2-oxidase
GA3ox	GA 3-oxidase
GGDP	geranyl geranyl diphosphate
GGPP	geranylgeranyl pyrophosphate
IPP	isopentenyl diphosphate
KAO	*ent*-kaurenoic acid oxidase
KO	*ent*-kaurene oxidase
KS	*ent*-kaurene synthase
LDL	low density lipoprotein
LEA	late embryogenesis abundant
MEP	methylerythritol phosphate
MVA	mevalonic acid
MX	barley cv. Morex
NCED	nine-*cis* epoxycarotenoid dioxygenase
PA	phaseic acid
PIMT	Protein L-isoaspartyl methyltransferase
PSY	phytoene synthase
RP	reverse primer
ZEP	zeaxanthin epoxidase

FORWARD

This thesis is written in manuscript style. A general introduction about research project and literature review precedes manuscripts. An abstract, introduction, material and methods, results and discussion form each manuscript. The manuscripts are followed by a general discussion and conclusions, a list of reference and appendices.

1.0 GENERAL INTRODUCTION

Seed development, germination and dormancy are the most important phenomena in seed biology. Seed development is usually divided into three phases: embryo morphogenesis, cell enlargement and reserve accumulation, and desiccation and developmental arrest. Following maturation non-dormant seeds are able to germinate as long as the environmental requirements are met. Seed germination is defined as the initial emergence of the radicle through the seed coat (Bewley, 1997; Finkelstein *et al*., 2008). Seed embryo is surrounded by two covering layers – the endosperm and testa. For the radicle to completely protrude during germination, it is necessary for cell elongation to occur, and this requires fundamental endogenous factors such as plant hormones and exogenous factors such as water, oxygen and a suitable temperature. Seeds are said to be dormant if their germination is interrupted by intrinsic and/or extrinsic factors (Gubler *et al*., 2008). Certain level of dormancy is necessary for seeds of cereals including barley to minimize the problem of precocious germination. Barley (*Hordeum vulgare* L.) is one of the major cereal crops cultivated in a wide range of environmental conditions for various purposes such as food for human, livestock feed and malting (Shen *et al*., 2010). However its production can be affected by many stress factors including field sprouting.

Of the many different hormones, gibberellin (GA) and abscisic acid (ABA) are key players in regulating plant development processes including seed development, seed germination and dormancy. Gibberellin is a diterpenoid compound well known in controlling seed development and promoting germination. It plays a role in the regulation of fertilization and embryo growth during seed development (Kucera *et al*., 2005) as well as inducing the activity of enzymes such as those involved in the hydrolysis of storage starch during seed germination (Olszewski *et al*., 2002). The production of GA is catalyzed by a number of

enzymes, most of the enzymes involved in the late stage of GA biosynthesis, GA 20 oxidase and GA 3 oxidase, are encoded by multigene family (Sun, 2008). The GA level in seeds is regulated not only by its biosynthesis but also by its catabolism, which is mainly catalyzed by GA 2-oxidases, encoded by multigene family (Thomas *et al*., 1999). The significant role of these genes in seed development, dormancy and germination has been studied extensively in the model plant Arabidopsis and other dicot species (Graeber *et al*., 2012).

Abscisic acid is the other classical hormone that plays important role in regulating seed development as well as the induction and maintenance of seed dormancy in a wide range of plant species (Grappin *et al*., 2000). The requirement of ABA for the accumulation of seed storage and late embryogenesis abundant proteins and for the induction of seed desiccation during seed maturation has been demonstrated (Finkelstein *et al*., 2008). In addition, the degree of dormancy is well related to the amount of ABA in seeds, and blocking ABA formation or increasing ABA inactivation leads to the repression of dormancy (Nambara and Marion-Poll, 2005). The level of ABA in seeds is influenced mainly by two regulatory enzymes, the biosynthetic enzyme, 9-*cis*-epoxycarotenoid dioxygenase, and the catabolic enzyme ABA 8'-hydroxylase (Finkelstein *et al*., 2008), and these two enzymes are also reported to be encoded by multigene family members. Mutational analysis has shown that genes encoding these enzymes as the major players in determining ABA level in seeds (Nambara *et al*., 2010). Phytohormones do not work alone in plants rather function through interaction. For example, seed germination and dormancy is largely affected by the balance between two phytohormones, the germination promoter GA and the germination inhibitor ABA.

In addition to the intrinsic factors, several extrinsic or environmental factors are involved in regulating seed germination and dormancy. It is a well-known that dormancy can

be broken through dry storage of seeds, which is referred to as after-ripening. The process of after-ripening is accompanied by a number of changes; range of temperature required for germination, seed phytohormone levels, and seed sensitivity to the phytohormones (Kucera *et al*., 2005). Previous studies have shown that the expression of GA biosynthesis genes and seed sensitivity to GA increase during imbibition of after-ripened seeds while the ABA level and seed ABA sensitivity show decreases (Finkelstein *et al*., 2008).

2.0 LITERATURE REVIEW

2.1. Barley: as a major cereal crop and a good source of starch

Barley (*Hordeum vulgare* L.) is one of the most important crops in the world. It has generally been considered as the fourth important cereal crop next to maize, rice, and wheat (Zohary and Hopf, 1993). It belongs to the genus *Hordeum* in the tribe Triticeae of Poaceae, which is the largest family in monocotyledonous plants. The family Poaceae consists of other temperate crops including wheat, rye and triticale as economically important food crops, and forage plants such as ryegrass, fescue, and Kentucky bluegrass (Varshney *et al*., 2007). The main purpose of barley cultivation was for the supply of human food in the past but the usage of barley has been extended as livestock feed, malt products and as a cover crop in crop rotation practices mainly to suppress weeds (Kremer and Ben-Hammouda, 2009; Marwat *et al*., 2012). Furthermore, barley is accepted as a model crop in genetic studies due to its relatedness to crops in the Triticeae tribe such as bread wheat and rye (*Secale cereale*) as well as its relatively simple diploid genome (Schulte *et al*., 2009; Saisho and Takega, 2011). Although the best condition for barley production is temperate climate with reasonably cool and dry condition, it is widely cultivated in various environmental conditions where other cereal crops cannot grow properly due to the harsh environmental conditions such as in Himalayas, Alaska, Ethiopia, Tibet and Morocco (OECD, 2004; Baik and Ullrich, 2008; Kremer and Ben-Hammouda, 2009).

2.2. Origin of barley and history of its domestication

Domestication of barley for food resource occurred 10,000 years ago in the Fertile Crescent, a region that includes the present day Israel, Jordan, Syria, Turkey, Iran and Lebanon (Badr *et*

al., 2000). It belongs to the species *Hordeum vulgare* L. which can be categorized into two subspecies: *Hordeum vulgare* L. ssp. *Spontaneum* (C. Koch) and *Hordeum vulgare* L. ssp. *Vulgare*. The sub-species *H. Spontaneum* is best known as the wild ancestor of cultivated barley and is frequently referred to as wild barley. Although both species mentioned above have similar morphological characteristics, *H. Spontaneum* is characterized by two-rowed with triple spikelets as a result of disarticulation of a mature inflorescence. Even though the emergence of six-rowed barley (*Hordeum vulgare L. ssp. Vulgare*) occurred about 8,000 years ago as a mutant, this species lacks the ability to adapt to harsher environmental conditions, such as rough soil conditions, displayed by the wild barley (Zohary, 1963; von Bothmer *et al*., 1995). This is the reason why six-rowed barley was introduced mainly as a cultivated crop through cereal crop domestication. The main differences between wild and domesticated barley are seed size and yield. Despite the same number of spikelets in both species, only the central spikelet of the two-rowed barley is fertile while the two lateral spikelets are sterile. In the case of cultivated barley, however, three spikelets consisting of a central and two lateral spikelets are fertile and develop into grains (Komatsuda *et al*., 2007).

2.3. Scale of barley production and its utilization

Barley as one of the ancient crops is an important cereal crop world-wide. It is characterized as a short-life cycle plant. Although barley is a cool season crop, it can be grown in high temperature and low humid conditions. These are the reasons why barley becomes an important crop in different parts of the world and used for various purposes in the form of animal feed, malting and food (Shen *et al*., 2010). Approximately 144 million tons of barley was produced on about 49 million hectares around the globe in the year 2013. Russian federation ranked first (with 15 million tons) in barley production followed by Germany (10

million tons) and France (10 million tons). In Canada, approximately 10 million tons of barley was harvested on about 2.6 million hectares (FAOSTAT, 2013). The amount of barley production varies from province to province; approximately 4.8 million tons of barley was produced in Alberta on about 1.4 million hectares in 2013, followed by Saskatchewan (3.3 million tons on about 1 million hectares), and Manitoba (0.6 million tons on about 0.1 million hectares; Statistics Canada, 2013).

Barley produced around the world is classified into a wide range of classes based on different criteria. Based on grain composition, barley is further classified by the type of starch, the level of lysine and β-glucan from normal to high (Baik and Ullrich, 2008). Furthermore, other factors such as the purpose (feed or malting), growth habitat (winter or spring), and row-type (6- or 4- or 2-row) are used to classify barley varieties. Barley grains are not only fed to animals but also used as a source of food for humans in various ways (Kremer and Ben-Hammouda, 2009). Barley malt is mainly used in the brewing industry, and also in bakery products as a good source of amylases relative to wheat flour. Malt extracts and malt syrups are added in small amounts to improve the flavor and color of food products such as cereals and bakery products. Malted barley is also used for barley infused goods such as malted coffee and barley tea. Moreover, barley starch has various industrial applications such as its use in paper production, as sweetener in the food industry, and also as an additional ingredient, with malted barley, for beer production (OECD, 2004).

A barley grain is mainly comprised of starch (about 65- 68%). It also consists of protein (10-17%), β-glucan (4-9%), free lipids (2-3%) and minerals (1.5-2.5%) (Baik and Ullrich, 2008; Fox *et al*., 2003). The β-glucan in barley grains is a low-digestible carbohydrate that is effective in improving immune system through increasing macrophages activity (Rondanelli *et al*., 2009). β-glucan has also a role to lower blood glucose level and

blood cholesterol. Another constituent of a barley grain is tocols; containing all eight chemically distinct isoforms (four tocopherols and four tocotrients) belonging to vitamin E, which is a fat-soluble vitamins group having antioxidant properties. Both tocopherols and tocotrients have positive effect in reducing serum low density lipoprotein (LDL) cholesterol level. For these reasons, barley products are believed to be beneficial for those who suffer from high level of blood cholesterol and blood glucose.

2.4. Factors posing limitation to barley production

Although some food manufacturers have a favorable impression on the use of barley as a healthy food source due to its higher content of soluble dietary fiber and lower level of LDL than the other cereal crops such as wheat; the unique flavor, texture and mouth-feel of barley grain are considered as limitations to the further use of barley grains (OECD, 2004). In addition, competition from food products prepared from other cereal crops such as wheat and rice negatively influences the use and ultimately the production of barley (Baik and Ullrich, 2008).

Biotic and abiotic stresses are other factors that negatively affect the grain yield and quality of barley. Among the common abiotic factors that lowers barley crop yield are water deficiency and undesirable grain germination on the mother plant, which is referred to as preharvest sprouting (Tester and Bacic, 2005; Ceccarelli *et al*., 2010). Preharvest sprouting is caused by occurrence of cool and wet weather conditions before harvest, and it causes starch degradation and grain quality loss (Black *et al*., 1987). Furthermore, it is difficult for barley to grow properly in acidic soil condition due to the fact that it is more sensitive to acidity than other cereal crops. In addition to the several abiotic factors, diseases caused by pathogens including fungi and viruses can also cause yield and quality loss, in turn reduced income to

barley producers. In western Canada, scald (*Rhynchosporium secalis*), netted (caused by *Drechslera teres* (Sacc.) Shoemaker) and spotted (caused *Drechslera teres* f. maculata Smedeg) forms of net blotch, and spot blotch (caused by Cochliobolus sativus (Ito & Kuribayashi) Drechs. ex Dastur) are common foliar diseases, which negatively affect barley yield and quality. No specific strategy is able to mitigate the negative effects of these diseases on barley production effectively as its pathogens have life cycles that are different from one another. Therefore, optimizing and combining several strategies are required in order to achieve sustainable barley production with enhanced yield and quality (Turkington *et al*., 2011).

2.5. Seed development

Seed development is initiated by double fertilization which leads to the formation of triploid endosperm resulting from the fertilization event of two polar nuclei in the embryo sac by a sperm cell nucleus and a diploid embryo as a result of the fertilization of egg cell by the other sperm cell nucleus (Chaudhury *et al*., 1998; Berger, 1999). The triploid endosperm and the diploid embryo eventually form a mature seed, a sophisticated structure that includes the nucellus, which is maternal tissues surrounding the embryo sac, and are protected by two layers of integuments. The endosperm comprises a large proportion of the mature seed, especially in cereals (Opanowicz *et al*., 2011).

Seed development is usually divided into three phases: embryo morphogenesis, cell enlargement and reserve accumulation, and desiccation and developmental arrest (Finkelstein, 2010). In the case of barley, grain development is divided into three phases: prestorage (morphogenesis), storage (maturation) and desiccation (late maturation) (Sreenivasulu *et al*., 2010). During morphogenesis, as the first phase of the embryo development, the endosperm

and zygote are formed by fertilization and the zygote undergoes an asymmetric cell division and differentiation for apical and basal cells formation. In Arabidopsis, the model plant for molecular genetics studies, the cell division and differentiation are almost completed by 7 days after fertilization, and the endosperm development coincides with the extensive cell division of the zygote (Dubreucq *et al*., 2009). The final seed size may be influenced by this initial endosperm development and enlargement (Brink and Cooper, 1947). The second phase of seed development is represented by cell enlargement and a massive storage compound accumulation. The transition from cell division to cell enlargement for storage compound accumulation has been considered to be the turning point to seed maturation. In the grains of barley, completion of endosperm cellularization indicates the transition from prestorage phase to storage phase (around 7 days after fertilization) and starch accumulation starts within the starchy endosperm (Sreenivasulu *et al*., 2006). In Arabidopsis, triacylglycerol and storage proteins are the major components of the storage compound. Finally, seeds undergo water loss to obtain desiccation tolerance in order to survive until they are exposed to suitable environmental conditions for germination (Dubreucq, 2009).

2.5.1. *Regulation of seed development by plant hormones*

Gibberellin plays a significant role during seed development including fertilization, embryo growth and the prevention of seed abortion in tomato, pea and *Brassicaceae* (Kucera *et al*., 2005). According to previous studies (White *et al*., 2000; Holdsworth *et al*., 2008), biologically active GAs are accumulated during embryogenesis until before dormancy is initiated. For example, in developing maize kernels, GA_1 and GA_3, which are bioactive GAs, are found to be abundant between 15 and 17 days after pollination (DAP) prior to the ABA peak, which occurs at 21 DAP. Besides, the GA-deficient *ls-1* and *lh-2* mutants of pea exhibit

decreased seed size, and increased rate of seed abortion (Swain *et al*., 1997). Consistent with this finding, the expression of *GA3ox1* which is responsible to produce bioactive GAs was found to be high during early pea seed development, 0 to 7 days after anthesis (DAA) (Nadeau *et al*., 2011). In addition to that, GAs are present during early embryo development in wheat and barley.

Abscisic acid is known to regulate seed formation, for example high ABA level was detected at fertilization in barley (Frey *et al*., 2004). The level of ABA in developing seeds shows fluctuation (Karssen *et al*., 1983). The first and main peak of ABA accumulation is found during the middle phase, cell enlargement and reserve accumulation/storage phase, in barley of seed development (Sreenivasulu *et al*., 2010) and is mainly derived from mother plant, indicating its role in regulating the rate of seed filling (Yang *et al*., 2006). Consistently, a reduction of endosperm and retarded seed development is found in ABA deficient *Nicotiana plumbaginifolia* (Frey *et al*., 2004). The other peak of ABA is observed during the last phase, desiccation phase, and is required for acquisition of dormancy. The ABA observed during the desiccation phase of seed development is synthesized in both embryo and endosperm tissues (Karssen *et al*., 1983). ABA is required for accumulation of seed storage and late embryogenesis abundant (LEA) proteins and induction of seed desiccation tolerance during the seed maturation phase (Ooms *et al*., 1993).

In Arabidopsis, two genes involving in ABA biosynthesis, *NCED6* and *NECE9* are expressed in developing seeds. *NCED6* is expressed only in the endosperm while *NCED9* in the endosperm as well as in the embryo tissues (Lefebvre *et al*., 2006). In barley, the expressions of ABA biosynthetic genes such as *NCED2* and ABA aldehyde oxidase (*AAO*) in developing embryo and endosperm, respectively, suggest the synthesis of ABA during early to middle phase of seed development/maturation. However, it is the ABA:GA ratio that is

considered as a critical factor controlling normal seed maturation (Chono *et al*., 2006; Sreenivasulu *et al*., 2006). Therefore, in addition to high amount of ABA during development, maintaining low level of GA is very important so as to induce dormancy and prevent premature germination. The ABA:GA ratio during seed development is regulated by transcriptional factors such as FUS3 (Gazzarrini *et al*., 2004; Finkelstein, 2010).

2.6. Seed germination and dormancy

Seed germination and dormancy are considered as the two critical events in seeds to be an independent organism and serve as a source of new plant. Seed germination can be defined as a process comprising of all events from the absorption of water by quiescent dry seed to the penetration of the tissues around the embryo by the radicle (Rajjou *et al*., 2012). It is accompanied by several sub-events such as operation of necessary cellular metabolic activities, loosening of the surrounding tissues such as testa, and initiation of mobilization of stored reserves.

Water together with other factors such as oxygen, light and temperature critically affect the process of germination (Bewley and Black, 1994). Water uptake by quiescent dry seeds is characterized by three phases; the first swift movement of water into seeds as phase I, and the subsequent relatively slower uptake of water as phase II, also known as lag phase followed by increased absorption of water, which occurs after radicle protrusion, as phase III. The rapid water uptake by the dry seeds during phase I reinitializes several metabolic processes including respiratory activity, and repair of mitochondria and DNA damaged during seed desiccation/maturation. Seed maturation induced cellular damages may also include proteins and membranes, which needs to be repaired to initiate successful germination (Nonogaki *et al*., 2010). During the lag phase, phase II, when rate of water uptake becomes

slow and constant, the re-initiated metabolic activities continue to operate (Bewley, 1997; Bradford and Nonogaki, 2007). Repair of damaged DNA is accompanied by expression of DNA ligase, which is induced upon imbibition of seeds. Damaged proteins could be produced due to conversion from normal aspartyl amino acid to abnormal isoaspartyl amino acid, which could cause protein misfolding during maturation and rehydration phases. Protein L-isoaspartyl methyltransferase (PIMT) is a key enzyme that is able to repair damaged proteins in seeds. The PIMT catalyzes a reaction to form normal aspartyl form from abnormal isoaspartyl amino acid residues (Ogé *et al*., 2008). In addition, activities related to repairing damaged DNA and protein, respiration and energy production are also too important to be neglected for seed germination. Although extant mitochondria are present in mature dry seeds, the energy is produced through anaerobic respiration due to their poorly differentiated membranes (Bewley, 1997). Aerobic respiratory activity increases after phase I of seed imbibition because of the repair and replication of mitochondria mainly occur during phase II of water uptake. The emergence of radicle through a seed coat takes place at the end of phase II and this marks the starting point of phase III, which is a post-germination phase. The growth of the radicle is driven by the division and expansion of cells. Phase III is characterized by the mobilization of reserves from storage tissue to support the establishment of a seedling.

In contrast to seed germination, seed dormancy refers to the failure of a viable seed to germinate under suitable environmental conditions. It is, however, a developmental state acquired during seed development to prevent germination until the seed completes its maturation and gets exposure to desirable condition to germinate (Penfield and King, 2009; Rajjou *et al*., 2012). Therefore, seed dormancy is also considered as an adaptive characteristic that increases the survival of seedlings by avoiding the occurrence of germination under

unfavorable environmental conditions. Seed dormancy can be classified into different types for example, based on its onset and its causes (see the discussion below).

2.7. Types of seed dormancy

Seed dormancy is generally categorized into two types based on the onset of dormancy, primary and secondary. Primary dormancy can be induced during seed development mainly by ABA (Bewley and Black, 1994). Seeds in primary dormancy can be germinated when seeds are exposed to appropriate treatments/conditions such as specific light and temperature that break dormancy. For example, dark and low temperature treatments promote the germination of primary dormant cereal grains including barley, wheat and oat, and germination is inhibited under high temperature condition (Corbineau and Côme, 1996). Moreover, several treatment conditions including after-ripening, stratification and scarification, can reduce or break primary dormancy (Bewley, 1997). On the contrary, secondary dormancy is induced in non-dormant seeds when they are exposed to conditions undesirable for germination. Secondary dormancy, however, can be released when the optimum environmental conditions are met (Finch-Savage and Leubner-Metzger, 2006).

In addition to this general category of seed dormancy based on the onset of dormancy, a comprehensive classification system has been suggested by Baskin & Baskin (1998, 2004). According to this classification, seed dormancy is grouped into five types, including physiological, morphological, morphophysiological, physical and combinational. Among the five types of dormancy, the most common one is physiological dormancy, and it is the most abundant in seeds of temperate crops as well as several model species including Arabidopsis, sunflower, lettuce, tomato, oat and tobacco (Baskin and Baskin, 2004). This type of dormancy is further classified into deep, intermediate and non-deep, based on the depth of

dormancy. Deep primary dormancy is characterized by excised dormant embryo producing abnormal seedling and the requirement of a relatively longer period of stratification (7 to 14 weeks) and pre-treatment with after-ripening (about 3-4 months) to break dormancy (Nikolaeva, 1969). Excised embryo producing normal seedling and requiring a shorter period of stratification for promoting germination is a feature prominent for intermediate level of primary dormancy. The non-deep level of primary dormancy consists of two components based on the cause of dormancy, as embryo induced dormancy and seed coat induced dormancy. In regards to embryo dormancy, the success of germination is interrupted by the embryos which are dormant. In other cases, the seeds exhibit dormancy due to the tissues surrounding the embryos in the form of testa and endosperm that prevent the completion of germination, although the embryos are not dormant (Bewley, 1997; Baskin and Baskin, 2004). In the case of seed coat imposed dormancy, water and oxygen permeability of the seed coat to the embryo are important factors as these processes are regulated by seed coat components (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). Consistently, the seed coat including glumellae and pericarp have been believed as a factor forcing oxygen availability down through oxidation of phenolic compounds in the seed coat of barley and oat (Corbineau *et al*., 1986; Lenoir *et al*., 1986).

In the case of morphological dormancy, the embryo is not fully developed and thus it requires either some period of time or treatments to break such type of dormancy, such as after-ripening and/or stratification, and treatment with GA. Morphophysiological dormancy is characterized by under-developed embryo that also exhibits physiological dormancy. The other two types of dormancy are physical dormancy and combinational dormancy (combination of physical and physiological dormancy), and are caused by the outer layer of seeds that lacks water permeability (Finch-Savage and Leubner-Metzger, 2006). The

combinational dormancy is also characterized by physiological dormancy in addition to the water impermeable nature of the seed coat.

2.8. Regulation of seed dormancy and germination

2.8.1. *Environmental factors*

Seed germination and dormancy can be regulated by several environmental factors including oxygen, moisture, light, temperature and nitrogen (Finch-Savage and Leubner-Metzger, 2006). Oxygen is an essential external factor affecting dormancy and germination. Reduced oxygen supply to the embryo due to the seed-covering layers has been shown to increase the sensitivity of barley seeds to high temperature and ABA, germination inhibiting factors (Bradford *et al*., 2008). Beside, two-fold increase in ABA level is reported. Temperature is the other important exogenous factor affecting dormancy and germination. Seeds of cereals crops including barley, wheat and oat germinate in the dark at low temperature, but germination is inhibited by a relatively higher temperature (Lenoir *et al*., 1986; Corbineau and Côme, 1996). Similarly, seeds of barley grown under high temperature condition exhibit earlier ABA peak during their development compared to seeds derived from those grown under low temperature (Goldbach and Michael, 1976), implying the effect of growing temperature on dormancy induction. Previous studies have also shown that light regulates seed dormancy. For example, the germination of barley seeds is inhibited by blue light exposure while no effect on germination is observed under red and far-red lights (Gubler *et al*., 2008). In *Brachypodium*, seed germination is inhibited by blue and far-red light, while red light promotes germination (Barrero *et al*., 2012). The effect of light in regulating dormancy and germination is associated with its effect on ABA and GA metabolism. Light

stimulates not only GA synthesis but also the degradation of ABA in imbibing lettuce seeds (Toyomasu *et al*., 1993; Sawada *et al*., 2008). Stratification, which refers to seed imbibition under low temperature condition, and after-ripening, which refers seed storage under dry conditions, also regulates seed dormancy and germination (Finkelstein *et al*., 2008).

2.8.2. *After-ripening*

Seed after-ripening is another factor that also induces seed dormancy release and germination. It is defined as dry storage of seeds at room temperature for several months. Temperature, seed moisture content and layers surrounding seeds are considered as factors affecting after-ripening (Debeaujon *et al*., 2000; Probert, 2000; Finch-Savage and Leubner-Metzger, 2006). Although the optimal moisture content for after-ripening is not the same in all species, in general, 8 to 15% of seed moisture content is ideal for after-ripening, and the best moisture content of oil seeds tend to be lower than starch seeds (Graeber *et al*., 2012; Hoang *et al*., 2014). This process triggers a number of changes including the range of temperature for germination, and seed hormone content and sensitivity (Kucera *et al*., 2005). In A*rabidopsis* seeds, for instance, the expression of GA biosynthesis genes and the sensitivity of seeds to GA increase by after-ripening (Finkelstein *et al*., 2008). Furthermore, such a treatment decreases ABA level and sensitivity during imbibition (Gubler *et al*., 2008). A sharp decline in embryo ABA level is detected during imbibition of after-ripened barley seeds as compared to the dormant barley seeds. A significant increase in GA_1 and low ABA level is also found after 18 hours of imbibition in after-ripened barley seeds (Jacobsen *et al*., 2002). In addition, after-ripening promoted ABA catabolism and reduced ABA sensitivity in the coleorhiza tissue of barley seed (Gubler *et al*., 2008). The coleorhiza is a covering layer of seminal roots in grass seeds, and has been considered as a barrier for elongation roots (Barrero *et al*., 2009).

It has been shown that after-ripening promotes testa rupture in imbibed tobacco seeds since it causes increased transient expression of β-1,3-glucanase gene in the testa (Finch-Savage and Leubner-Metzger, 2006). Although gene transcription has been believed to occur in seeds during after-ripening (Bove *et al*., 2005; Leymarie *et al*., 2007), a recent study indicated that this process does not occur in dry state (Meimoun *et al*., 2014).

2.8.3. *Plant hormones*

In addition to the external factors, germination and dormancy are regulated by intrinsic factors including plant hormones (Koornneef *et al*., 2002). Seed germination and dormancy are regulated mainly by the antagonistic interaction of GA and ABA (Koornneef *et al*., 1982). GA enhances germination and seedling growth, whereas ABA represses these processes. In cereal seeds, GA promotes germination by inducing α-amylase gene expression and starch degradation, however, ABA inhibits the expression of α-amylase genes and also germination (Fincher, 1989; Lanahan *et al*., 1992). GA is mainly synthesized in the embryo of germinating cereal seeds. In addition to embryo as a site of GA release, scutellum cooperates with aleurone for secretion of hydrolytic enzymes for efficient starch breakdown in the endosperm (Olszewski *et al*., 2002). The level of ABA in embryos and seeds increases during development of cereal grains until the seeds enter a maturation phase. The increase in the amount of ABA in embryos and seeds appears to prevent the precocious germination until embryos are ready to grow. Developing embryos isolated from barley and wheat grains in culture media containing ABA remained in a developmental state (Morris *et al*., 1985; Walker-Simmons, 1987). In contrast, embryos cultured in a medium that contains no ABA exhibited precocious germination (Robertson *et al*., 1989). Therefore, hormonal balance between GA and ABA is an important factor to regulate dormancy and germination, and this

balance depends on the rate of biosynthesis and catabolism of each of these hormones.

In addition, other plant hormones such as auxin, cytokinin and ethylene are involved in controlling dormancy and germination (Kucera *et al*., 2005). Although auxin has been known as a key player in several growth and developmental processes, it has not been thought to involve in the control of seed germination. However, auxin has been found in the radicle tip during and after germination as well as a change in auxin level is detected in sorghum grains during imbibition (Dewar *et al*., 1998). Furthermore, recent study revealed that inhibition of AUXIN RESPONSE FACTOR10 by microRNA160 is important for seed germination (Liu *et al*., 2007). Another role of auxin is the regulation of GA biosynthesis reported by the fact that an application of an auxin transport inhibitor causes the reduction of GA_1 in stems of pea and tobacco (Olszewski *et al*., 2002; Mano and Nemato, 2012). Ethylene also plays a role in regulating dormancy release and seed germination, for example, by interfering with ABA signaling (Kucera *et al*., 2005). Cytokinin is able to promote seed germination by interacting with other plant hormones such as ABA. Cytokinin promotes germination of embryo from lettuce seeds, which is inhibited by ABA treatment (Bewley and Fountain, 1972). Cytokinin is also considered as an important plant hormone regulating seed germination by reducing several abiotic stresses (Peleg and Blumwald, 2011).

2.9. Gibberellin

Gibberellin a is very well-known plant hormones which influence various developmental processes such as seed germination, stem elongation, leaf expansion, flower induction, and seed and pericarp growth (Hedden, 1997; Olszewski *et al*., 2002).

The discovery of GAs had begun because of a fungal disease termed as *Bakanae* or foolish seedling disease, a well-known disease of rice, especially in Japan. This disease is

caused by a pathogenic fungus called *Fusarium fugikuroi*. This fungus was known to produce a chemical compound that caused the rice plants to grow too tall, and this makes the plant be vulnerable to lodging, which severely decreases grain yields (Sun, 2008). Japanese researchers later found the chemical produced by the fungus, and named it as *gibberellin A*. This *gibberellin A*, which is capable of stimulating plant growth, consisted of three components, GA_1, GA_2 and GA_3, and the structure of these compounds was determined by researchers in the UK, US and Japan. GA_3 was the predominant component and is called gibberellic acid. The application of GA_3 to dwarf mutant pea and maize plants results in the restoration of normal phenotype (Phinney, 1956; Ingram *et al*., 1984). Although 136 GAs have been discovered from higher plants and fungi to date, only a few, GA_1, GA_3, GA_4 and GA_7 are biological active GAs (Hedden and Phillips, 2000; Sun, 2008; Davies, 2010). The metabolic pathway of GA has been unveiled by the use of various approaches including GA content analysis using gas chromatography-mass spectrometry, purification of enzyme involved in GA metabolism, isolation and characterization of genes and mutants related to GA metabolism (Olszewski *et al*., 2002; Sun, 2008).

2.10. Gibberellin metabolism pathway

Gibberellins are tetracyclic diterpenoid compounds whose biosynthesis in plants is composed of three stages. The synthesis of *ent*-kaurene from geranyl geranyl diphosphate (GGDP) through a two-step cyclization reaction in proplastids is the first stage. This stage is catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) via *ent*-copalyl diphosphate as the intermediate, each of which are encoded by single gene (Olszewski *et al*., 2002). GGDP is known as a common precursor for diterpenes and carotenoids. High amount of *CPS* mRNA is found in rapidly growing tissues of Arabidopsis including shoot apices, root

tips, developing anthers, and seed. Although the expression patterns of the two genes appear to be similar, the total amount of *CPS* mRNA is much lower than that of *KS* (Olszewski *et al*., 2002).

The second stage of GA biosynthesis involves oxidation of *ent*-kaurene, which is catalyzed by *ent*-kaurene oxidase (KO) and subsequently by *ent*-kaurenoic acid oxidase (KAO) to produce GA_{12} in endoplasmic reticulum. Previous study suggested that KO and KAO are located in the outer membrane of plastid and in the endoplasmic reticulum, respectively (Helliwell *et al*., 2001). In Arabidopsis, KO is encoded by a single gene, *CYP701A1*, while KAO by two genes, *CYP88A3* and *CYP88A4*. The conversion of GA_{12} to bioactive GAs, mainly GA_1 and GA_4 as the final step of GA biosynthesis takes place via 13-hydroxylation or non-13 hydroxylation, respectively, and several subsequent oxidation steps in the cytoplasm. The oxidation steps in the final step of GA synthesis are catalyzed by 2-oxoglutarate-dependent dioxygenases, GA 20-oxidases (GA20ox) and 3-oxidases (GA3ox) (Sun, 2008). In contrast to CPS, KS and KO, the late GA biosynthetic enzymes, GA20ox and GA3ox, appear to be encoded by multigene family members (Sun, 2008).

In addition to its biosynthesis, the level of GA in plant tissues is regulated by its catabolism in which the bioactive GAs are converted to inactive forms. GA 2-oxidation is the major form of GA catabolism, and this reaction is catalyzed by GA 2-oxidase (GA2ox) (Thomas *et al*., 1999). Like the other dioxygenases, GA2ox is encoded by multigene family members. Recent studies have shown the presence of two more GA deactivation mechanisms identified: GA 16, 17-epoxidation and methylation (Yamaguchi, 2008). A study on a recessive rice mutant, *elongated uppermost internode* (*eui*), indicated the presence of high levels of bioactive GAs in the mutants. *EUI* encodes a P450 enzyme, CYP714D1, which catalyzes 16α, 17-epoxidation of non-13-hydroxylated GAs including GA_4, GA_9 and GA_{12}

(Itoh *et al*., 2004; Zhu *et al*., 2006). Another study in Arabidopsis has shown *GAMT1* and *GAMT2* as the two genes coding for GA methyltransferases. These genes are mainly expressed in developing and germinating seeds. In the double mutant of these two genes, the levels of bioactive GAs in seeds during development are highly increased. In addition, the seeds appear to contain more amount of bioactive GAs, as a result the double mutant seeds are resistant to GA biosynthesis inhibitor (Varbanova *et al*., 2007).

2.11. Gibberellin enhances seed germination

Gibberellin is a very important in regulating the seed germination in many plant species. The physiological function of GA in activating germination is attributed to its effect in enhancing embryo growth potential and overcoming tissues surrounding the radicle and act as barriers to restrict germination (Kucera *et al*., 2005). Several studies that involved GA deficient mutants have clearly demonstrated that GA is required by seed for completion of their germination process. For example, the GA deficient *ga1-1* and *ga1-3* mutants of tomato and Arabidopsis, respectively, cannot germinate without application of exogenous GA (Groot and Karssen, 1 987; Karssen *et al*., 1989). Furthermore, seed treatment with chemical inhibitors of GA biosynthesis enzymes represses germination (White *et al*., 2000). GA 3-oxdiase is responsible for conversion of inactive GA intermediates to bioactive forms of GAs (Davies, 2010). This GA3ox is encoded by four genes (*GA3ox1–4*) in Arabidopsis. *AtGA3ox1* and *AtGA3ox2* are expressed in the embryo during seed imbibition, and thus are believed to be important genes for regulating germination. Consistently, severe reduction in germination potential has been reported by the loss of function of these two genes (Holdsworth *et al*., 2008).

In the model plant Arabidopsis, a study by Ogawa *et al*. (2003) has shown increased expression of GA biosynthesis genes both in the earlier and later parts, including genes

encoding KO, GA20ox and GA3ox. In line with their function, the expression of the *GA3ox* genes which showed increased expression during seed imbibition, has been shown to be localized in the embryo axis (Yamaguchi *et al*., 2001), implying that active synthesis of GA takes place in the embryos of germinating seeds.

Dry storage of seeds plays a role in altering the metabolic aspects of different plant hormones including GA at both the biochemical and molecular levels (Iglesias-Fernández *et al*., 2011). Comparison of the expression of GA metabolic genes in imbibing barley seeds before and after dry storage revealed that dry storage triggers increased expression of GA biosynthetic genes such as *GA3ox2* (Gubler *et al*., 2008), and this is suggestive of increased GA level to activate germination in after-ripened seeds.

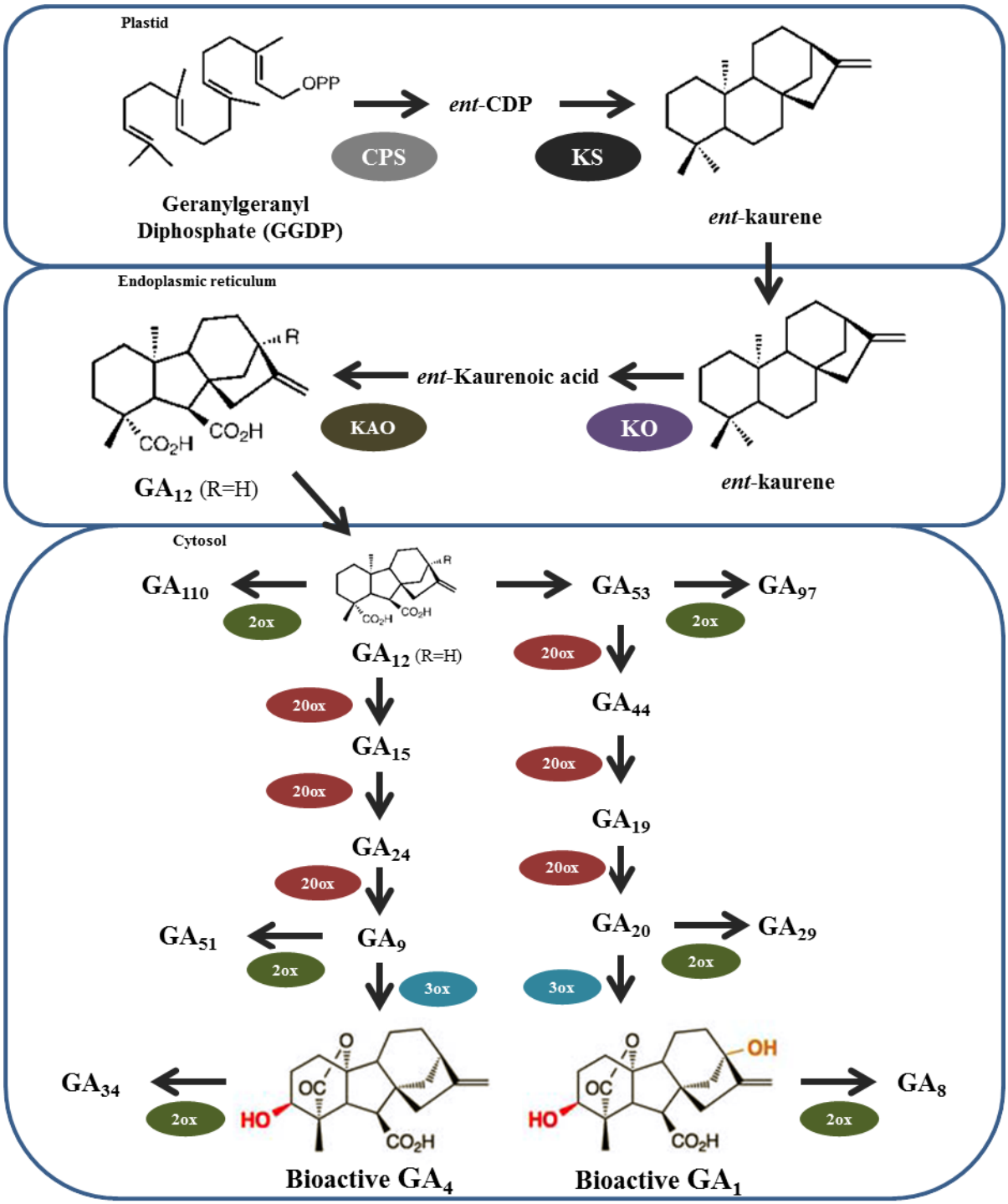

Figure 2.1 Gibberellin biosynthetic and catabolic pathway in plants.

2.12. Abscisic acid

Abscisic acid is one of the classical plant hormones belonging to one group of plant secondary metabolites referred to as isoprenoids. It is involved in various plant growth and developmental processes including storage reserve depositions during seed formation, preventing precocious germination, inducing desiccation tolerance and primary dormancy during seed maturation (Kermode, 2005). Since ABA biosynthesis begins with isopentenyl diphosphate (IPP) in chloroplasts and amyloplasts, naturally occurring ABA in plants is detected in almost all cells containing chloroplasts and amyloplasts. The regulation of ABA level in plant by its biosynthesis and degradation is very important process (Nambara and Marion-Poll, 2005). Both ABA biosynthesis and catabolism are affected by various factors such as developmental stages, environmental conditions including light and water as well as temperature (Davies, 2010).

2.13. Abscisic acid metabolism

Abscisic acid is synthesized from IPP, which contains 5 carbon atoms, and this IPP can be synthesized through two pathways, the mevalonic acid pathway and the methylerythritol phosphate (MEP) pathway. However, ABA is synthesized from carotenoids that derive from IPP produced by the MEP pathway (Eisenreich *et al*., 2004). IPP is converted to GGPP, a C20 compound, by two enzymes, IPP isomerase and GGPP synthase. Two molecules of GGPP are condensed to produce C_{40} carotenoid, phytoene, by phytoene synthase. This step has been believed to be the first committed step in carotenoid synthesis, before the ABA-specific biosynthesis pathway. Subsequently, linear molecule of lycopene, which is formed from phytoene by the action of phytoene desaturase and zeta-carotene desaturase, is converted into β-carotene through cyclization reaction. This cyclization reaction is catalyzed by lycopene β-

cyclase. Hydroxylation of β-carotene that is catalyzed by β-carotene hydroxylase, produces zeaxanthin, and this marks the beginning of ABA specific biosynthesis pathway (Cunningham and Gantt, 1998; Hirschberg, 2001).

The biosynthesis of ABA can be classified into three major regulatory steps. Zeaxanthin is converted to violaxanthin as the first regulatory step of ABA biosynthesis, and this reaction is catalyzed by zeaxanthin epoxidase (ZEP). This enzyme catalyzes the epoxidation reaction, and is the first plant ABA biosynthetic enzyme isolated and characterized (Seo and Koshiba, 2002; Xiong and Zhu, 2003). The reaction catalyzed by ZEP is reversed by violaxanthin de-epoxidase (VDE) to produce zeaxanthin whenever there is a need for this metabolite. Presumably there are enzymes such as a neoxanthin synthase (NSY) and an isomerase involved in the production of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, although they have not been isolated yet (Nambara and Marion-Poll, 2005). Xanthoxin, which is C15 intermediate, is produced by cleavage of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, and this reaction is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes as the second regulatory step of ABA biosynthesis. During this step, C_{25} metabolite is produced as a byproduct of oxidation. The gene encoding NCED, which is involved in the oxidative cleavage of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin, was first identified in the maize *viviparous 14* mutant (*vp14*). In addition to *vp14* mutant, a tomato ABA deficient mutant *notabilis* was reported to be impaired in a gene related to *Vp14* (*LeNCED1*) (Burbidge *et al*., 1999). Among the nine *NCED* genes identified in Arabidopsis, five (*AtNCED2*, *3*, *5*, *6*, *9*) are predominantly involved in ABA biosynthesis (Seo and Koshiba, 2002; Nambara and Marion-Poll, 2005). To date, *NCED*s have been isolated and characterized from several plant species, and they form a multigene family. Xanthoxin produced from the cleavage of 9-*cis*-epoxycarotenoid is subsequently converted to ABA by enzymatic reactions. Abscisic

aldehyde is produced from xanthoxin by a short chain alcohol dehydrogenase/ABA deficient 2 (ABA2) enzyme encoded in Arabidopsis by *ABA2* (González-Guzmán *et al*., 2002). The oxidative reaction of abscisic aldehyde to produce ABA is the last regulatory step of ABA biosynthesis in the cytosol. This reaction is catalyzed by abscisic aldehyde oxidases (AAO). Among the four aldehyde AAOs of Arabidopsis, AAO3 is considered as the most active enzyme to produce ABA from abscisic aldehyde (Seo *et al*., 2000).

The amount of ABA is also controlled by the rate of its catabolism, which involves both hydroxylation and conjugation (Davies, 2010). Carbon atoms at 7′, 8′ and 9′ position of ABA are the potential targets for catabolic reaction to produce the oxidation products, 7′-hydroxy ABA, 8′-hydroxy ABA, and 9′-hydroxy ABA, which are relatively unstable compared to ABA (Cutler and Krochko, 1999). The production of 8′-hydroxy ABA is catalyzed by ABA 8′ hydroxylase, a cytochrome P450 monooxygenase (P450). The 8′-hydroxy ABA is subsequently converted to phaseic acid (PA), which is considered not to have biological activity. The PA is occasionally further metabolized to dihydrophaseic acid (DPA) known as hormonally inactive ABA catabolite (Zhou *et al*, 2004). Unlikely PA and DPA, which are the most widespread and biologically inactive ABA catabolites, the intermediates 7′-OH ABA and 8′-OH ABA are reported to have hormonal activity similar to ABA. In *Brassica napus* embryo, 8′-OH ABA has been shown to positively affect the expressions of genes related to lipid and storage protein such as those involved in the biosynthesis of long chain fatty acid (Saito *et al*, 2004; Zhou *et al*, 2004). Whereas the 7′-OH ABA negatively affects germination through inhibiting α-amylase activity in barley seeds (Hill *et al*., 1995).

In addition to the hydroxylation reaction, conjugation of ABA inactivates ABA. Possible targets for conjugation with glucose are the carboxyl group at the carbon atom 1 position and the hydroxyl groups of ABA. Although several conjugation products have been

found, ABA glucosyl ester (ABA-GE) is considered as the predominant product (Seiler *et al*., 2011). Of the ABA metabolic steps, the ones catalyzed by NCED and ABA8'OH are considered in general as regulatory.

2.14. Abscisic acid: dormancy inducing hormone

It has been established by several studies that ABA plays important role in inducing and maintenance of seed dormancy in a wide range of plant species (Holdsworth *et al*., 2008; Okamoto *et al*., 2010; Seiler *et al*., 2011). The degree of dormancy is well related to the amount of ABA in seeds, and blocking ABA formation by using mutations or chemical inhibitors such as fluridone represses dormancy. Although the ABA biosynthetic step catalyzed by NCED is reported to be regulatory, previous studies have indicated that the expression of this gene is not consistent with the level of ABA in seeds (Millar *et al*., 2006). In contrast, a clear relationship between the expression of ABA catabolic genes (*CYP707A* genes) and ABA level was observed in imbibing seeds in both the dicot species such as Arabidopsis and the monocot species such as barley. However, the members of the *CYP707A* gene family that appeared to be important in regulating ABA level and thereby dormancy and germination appeared to be different between the monocot and dicot species. In Arabidopsis, *CYP707A2* gene is upregulated following imbibition, whereas in barley *CYP707A1* showed significant induction in its expression following imbibition (Rodríguez-Gacio *et al*., 2009). These results indicate the activity of ABA8'OH in controlling ABA level in seeds of different species is controlled by different members of the *CYP707A1* genes.

Similar to that observed for GA, dry storage of seeds (after-ripening) also leads to change in ABA metabolism in terms of gene expression, enzyme activity and ABA level. Comparative analysis of after-ripened and non-after-ripened seeds of barley and Arabidopsis

showed that dry storage upregulated the expression of ABA catabolic genes during imbibition and in turn led to decreased accumulation of ABA (Kushiro *et al*., 2004; Millar *et al*., 2006). Such a difference in the expression of *CYP707A1* genes was not apparent in dry seeds, suggesting that the gene expression is activated by imbibition.

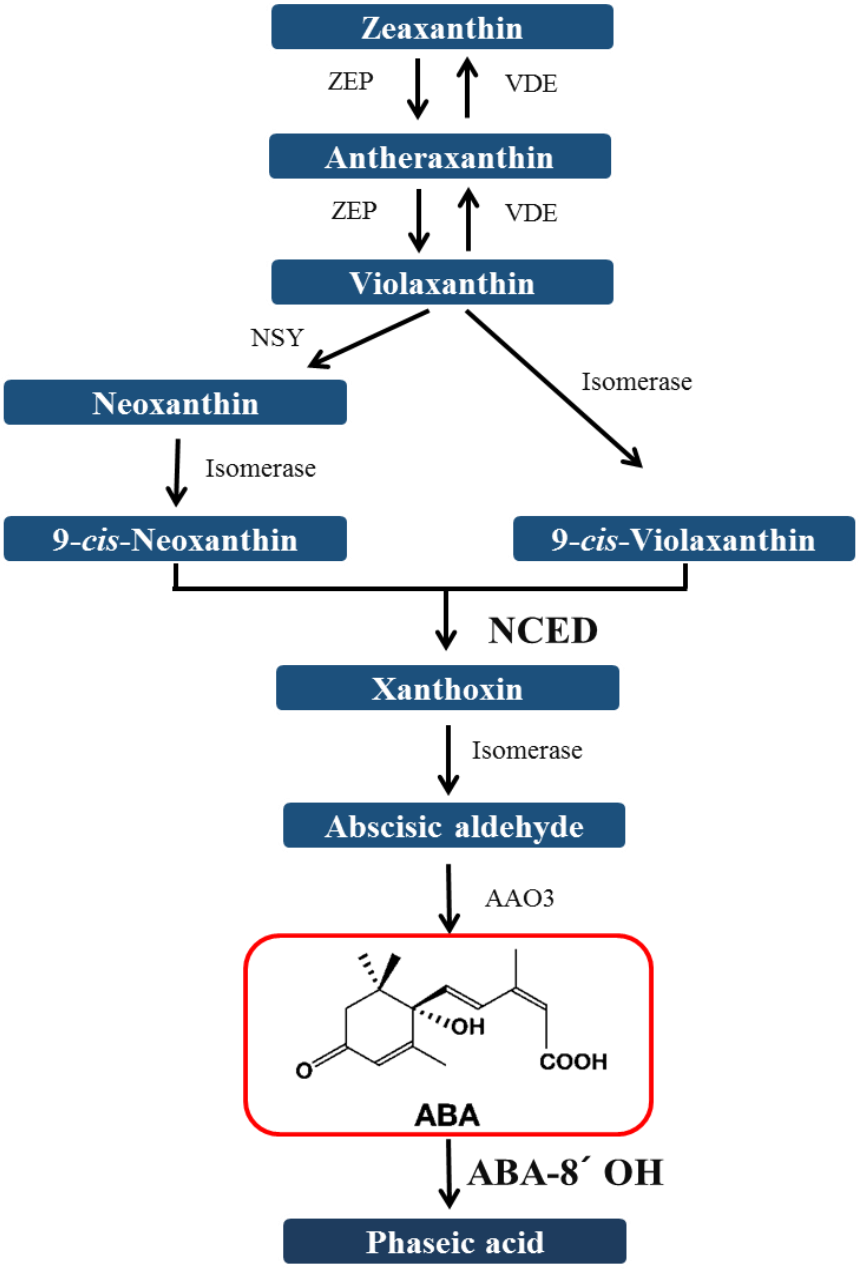

Figure 2.2 Abscisic acid biosynthetic and catabolic pathway in plants.

2.15. Role of abscisic acid and gibberellin interaction in regulating seed development and germination

Gibberellin and ABA are the two well-known plant hormones that interact antagonistically to regulate plant developmental processes including seed development and germination (Finch-Savage and Leubner-Metzger, 2006). Endogenous ABA levels are strongly influenced by GA. There are increases and decreases of ABA biosynthetic and catabolic gene expression, respectively, in *ga1* mutant of Arabidopsis, which is GA deficient (Oh *et al*., 2007). In addition, exogenous GA application to lettuce seeds induces lower ABA level and downregulated *NCED4* gene expression (Toyomasu *et al*., 1994; Sawada *et al*., 2008). Conversely, GA levels are negatively affected by ABA in Arabidopsis thus germination is inhibited (Seo *et al*., 2006). For example, increased gene expression of *GA3ox1* and *GA3ox2* are found in Arabidopsis *aba2-2* mutant, which is ABA deficient. Additional evidences are also revealed by studies using fluridone and paclobutrazol, which are ABA and GA biosynthesis inhibitors, respectively (Koornneef *et al*., 1982). Previous study that involved the use of paclobutrazol revealed that *nced6* and *nced9* seeds of Arabidopsis requires lower amount of bioactive GAs for germination compared to wild-type (Leon-Kloosterziel *et al*., 1996). In addition, GA and ABA metabolisms can be affected by light. White and blue lights induce activation of *NCED1* expression, thus these lights inhibit barley seed germination (Gubler *et al*., 2008). Red light which promotes germination induces *GA3ox1* and *ABA8ox4* in lettuce, and *GA3ox1*, *GA3ox2* and *CYP707A2* in Arabidopsis, while downregulating *GA2ox2*, *NCED2* and *NCED4* in lettuce and *GA2ox2*, *NCED6* and *NCED9* in Arabidopsis. However, all these effect of red light are inhibited by far-red light which inhibits germination (Nakaminami *et al*., 2003; Oh *et al*., 2006; Seo *et al*., 2006; Sawada *et al*., 2008).

To gain a better understanding of the role of ABA and GA on seed development,

germination and dormancy in barley grains, this study investigated the spatial and temporal expression patterns of GA and ABA metabolism genes in developing grains and during grain imbibition. Furthermore, responsiveness of the GA and ABA metabolism genes to ABA and the effect of after-ripening of dormant grains on the GA and ABA metabolism were also characterized.

3.0 Tissue specific expression patterns of gibberellin and abscisic acid metabolism genes during grain development in barley

Abstract

Gibberellin and abscisic acid regulate seed development but are known to act antagonistically. This chapter of the thesis examined the spatio-temporal expression patterns of GA and ABA metabolism genes during grain development in barley cv. Morex, and also the responsiveness of the genes under consideration to ABA to gain better understanding of the effects of ABA on developing grains. The data revealed *GA20ox3*, *GA3ox1* and *GA2ox5* as the major GA metabolic genes expressed at whole grain level of developing grains, suggesting that these genes are the major modulators of GA level in maturing barley grains. Tissue specific analysis showed that all the above genes are mainly expressed in the endosperm tissues relative to the embryo. However, *GA3ox2* was found to exhibit higher expression in the embryo than in the endosperm, reflecting the tissue specific roles of these genes. With respect to ABA, the biosynthetic gene, *NCED1*, and the catabolic gene, *CYP707A1*, appeared to show high expression at whole grain level, implying their role in regulating ABA level in maturing seeds. Tissue wise, the two *NCED* genes, *NCED1* and *NCED2*, are expressed at higher level in the endosperm than in the embryo while the two *CYP707A* genes, *CYP707A1* and *CYP707A2* showed similar expression in both tissues. Exogenous ABA treatment study indicated that the level of grain ABA might have influence in modulating the dynamics of both GA and ABA metabolic genes during grain maturation.

3.1. Introduction

Cereal grains (caryopsis), distinct feature of the Poaceae, have been considered as the major source of carbohydrates for the human consumption as well as livestock feed. Furthermore, it is attracting attention due to its importance as the source of biofuels (Varshney *et al*., 2007). The physiological and developmental characteristics of cereal grains have been extensively modified through domestication processes to improve yield and quality (Shewry, 2009).

The grain resulting from the double fertilization event is composed of the diploid embryo, triploid endosperm and the surrounding maternal tissues including the nucellus protected by two layers such as the inner and outer integuments (Opanowicz *et al*., 2011). The diploid embryo is derived from the fertilization of egg cell with a pollen sperm and the triploid endosperm is from the fertilization of a sperm nucleus with two polar nuclei (McClintock, 1978). Seed development is initiated from the moment of the double fertilization followed by embryo morphogenesis as the first phase of developmental process. A single zygote cell is formed and the formation of embryo structure is completed at the end of the heart stage during the embryo morphogenesis. The embryo grows and fills the embryo sac during the growth phase as the next phase of seed developmental processes and eventually cell division is ceased in the embryo at the end of the phase, thus the embryo growth is arrested (Raz *et al*., 2001). This is followed by the seed maturation phase. During maturation phase, the accumulation of storage reserves is initiated and seed acquires desiccation tolerance and dormancy is induced (Goldberg *et al*., 1994). As compared to dicot species such as Arabidopsis, endosperm of cereals is persistent and is composed of three major tissues: the starch endosperm, the aleurone layer and the transfer layer. The starchy endosperm tissue is storage space to accumulate starch and protein bodies. The transfer layer is specialized to transport nutrient derived from maternal tissues to the developing endosperm,

and the aleurone, which is the endosperm covering layer, has a role for starch degradation during seed germination through producing hydrolytic enzymes such as α- and β-amylases (Becraft, 2001).

Gibberellin and ABA are well-known phytohormones that play significant roles during seed development (Kucera *et al*., 2005). GAs is present during cereal seed development (Jacobsen and Chandler, 1987), and has been shown to interact antagonistically with ABA to regulate dormancy level in several species such as maize (McCarty *et al*., 1991). Bioactive GAs such as GA_1 and GA_3 are found in developing maize kernels (White *et al*., 2000) and high abundance of GA biosynthetic genes such as *GA20ox2*, *GA20ox3*, *GA3ox1* and *GA3ox4* are detected in developing Arabidopsis siliques and *GA20ox2*, *GA20ox3* and *GA3ox4* in developing Arabidopsis seeds (Kim *et al*., 2005). Analysis of mutants defective in GA metabolism or signaling have been used as tools for understating the decisive roles of GA in plant development processes including seed development (Ross *et al*., 1997). GA deficiency in Arabidopsis and tomato results in abnormal development of anther, consequently male sterility (Nester and Zeevaart, 1988; Goto and Pharis, 1999). GA deficient *gib1* mutant of tomato and *ls-1* and *lh-2* mutants of pea are used to reveal the role of GAs on normal seed growth and survival (Groot and Karssen, 1987; Swain *et al*., 1997). Besides, impaired pollen tube elongation and seed abortion are found in Arabidopsis due to overexpression of the GA inactivating gene of pea, *GA2ox2* (Singh *et al*., 2002).

Abscisic acid, the other well-known phytohormone, is known to involve in a wide range of seed developmental processes such as the synthesis of storage products, preventing precocious germination, inducing desiccation tolerance and dormancy (Kermode, 2005) as well as preventing the programmed cell death of aleurone cells in barley (Bethke *et al*., 1999). ABA deficiency and ABA accumulation during seed development lead to the absence of

primary dormancy and enhancing seed dormancy, respectively (Nambara and Marion-Poll, 2003; Kushiro *et al*., 2004). Two peaks of ABA accumulations are reported during the middle and desiccation phases of seed development in Arabidopsis, respectively (Frey *et al*., 2004; Karssen *et al*., 1983).

The first ABA peak that occurs during the middle phase of seed development is mainly derived from maternal tissues and is translocated to the embryos via phloem (Ober and Setter, 1990). In developing wheat and barley grains, high amount of ABA content is found and this level significantly declines as maturation and desiccation phases are initiated (Benech-Arnold *et al*., 1999; Jacobsen *et al*., 2002). Consistently, the gene expression and quantification analysis revealed that the changes in amount of ABA during seed development is affected by the expression of ABA biosynthetic gene *NCED2* during the early to middle phases and the expression of ABA catabolic gene *CYP707A1* in the late phase of grain development in barley (Chono *et al*., 2006). During seed development, cell division is inhibited and maturation phase is induced by the maternal ABA (Ober and Setter, 1990). A retarded growth of endosperm and delay of seed development in *Nicotiana plumbaginifolia* have been shown to be resulted from maternal ABA deficiency early in seed development (Frey *et al*., 2004).

The second ABA peak results from the ABA *de novo* synthesized in the embryo (Kanno *et al*., 2010), and only such embryonic ABA synthesized during seed development is able to induce seed dormancy. This has been demonstrated by reciprocal crosses between wild-type and ABA deficient mutants in several species such as Arabidopsis, tomato and *Nicotiana plumbaginifolia* (Kucera *et al*., 2005). Consistent with two ABA content fluctuations, expression of several ABA biosynthesis genes is found in young embryos of Arabidopsis such as *ZEP*, *NCED5*, and *NCED6* (Audran *et al*., 2001). In addition, the highest

expression levels of *NCED5* and *NCED6* are detected during the mid to late developmental phases in Arabidopsis embryos (Tan *et al*., 2003). Besides, *NCED9* is expressed in the embryo and endosperm tissues in developing Arabidopsis seeds (Lefebvre *et al*., 2006). During the desiccation phase, decrease in the ABA level is associated with low *ZEP* expression (Audran *et al*., 1998). In developing barley grains, *AO1* and *AO2*, which encode aldehyde oxidase, are expressed in the endosperm and embryo tissues, respectively, indicating *de novo* ABA synthesis in developing barley grains (Sreenivasulu *et al*., 2006).

This study characterized the spatial and temporal expression patterns of GA and ABA metabolism genes during barley grain development in order to gain a better understanding of the dynamics of GA and ABA metabolism during seed development. In addition, the responsiveness of the GA and ABA metabolism genes to exogenous ABA was analyzed to gain further understanding of the effects of ABA on developing grains.

3.2. Materials and Methods

3.2.1. Plant materials and growth conditions

3.2.1.1. Plant material

Barley (*Hordeum vulgare* L.) cv. Morex was used in this study. Seeds were germinated in Petri dishes with two layers of Whatman # 1 filter paper and 7 mL sterile deionized water. Successfully germinated seeds were transplanted into pots (1 seed/pot) containing LA4 sunshine mix (Sungro Horticulture, Bellevue, WA, USA) and fertilizer (Cornell mixture: 100 g calcium carbonate, 150 g osmocote, 120 g superphosphate, 2 g fritted trace elements, 15 g chelated iron [13.2%], 0.7 g chelated zinc [14%]). Plants were grown in a growth chamber

(day and night; 16/8 hours photoperiod; 22°C/18°C). Plants were watered every day until maturity and supplied with N-P-K (20:20:20) mix of fertilizer once a week.

3.2.2. Tissue harvesting and preparation

3.2.2.1. Seed development

Developing grains of barley cv. Morex were harvested from the primary and secondary spikes at 20, 30, 40 and 50 days after anthesis (DAA). For experiment involving ABA treatment, spikes were cut from the mother plant and dipped in water immediately. After a section of the peduncle below the flag leaf was cut off, the remaining section of the peduncle and the spike was kept sterile by treatment with 70% ethanol and 1.1% sodium hypochlorite with Tween-20 (0.05%, v/v) sequentially and finally washed with sterile water three times. Then the sterile spike was dipped in a falcon tube containing liquid MS media and ABA (25 µM) and incubated for 24 h in a growth cabinet (day and night; 16/8 hours photoperiod; 22°C/18°C). Three independent biological replicates of embryo and endosperm tissues were collected in liquid nitrogen from grains harvested from the middle regions of control or ABA treated spikes, and then stored at –80℃ until further use.

3.2.3. RNA extraction

Total RNA from embryo tissues (~100 mg) was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. For whole seed and endosperm tissues (~200 mg), RNA was extracted using a method described in Li and Trick (2005) with minor modifications. Tissues were ground into fine powder using Mortar and Pestle frozen with liquid nitrogen, and the ground tissue was mixed with 800 µL extraction

buffer I (100mM Tris, 150mM LiCl, 50mM EDTA, 1.5% SDS, 1.5% β-mercaptoethanol). After thorough mixing, 500 µL phenol: chloroform mixture (1:1) was added and mixed by inversion for 5 min. Supernatant of the mixture centrifuged at 13,000x g for 15 min at 4℃ was transferred to a new 2 mL tube containing same volume of extraction buffer II (2.1M guanidinium sulphate, 25mM sodium citrate, 0.5% lauryl sarcosine, 1M sodium acetate) and mixed by gentle inversion. After 10 min incubation at room temperature, 400 µL chloroform: isoamyl alcohols (24:1) were added and the mixture was incubated for 2 min. Following centrifugation at 13,000x g for 15 min at 4℃, the supernatant was separated and mixed with isopropanol and 1.2 M NaCl. The mixture was then incubated on ice for at least 30 min. Following centrifugation at 13,000x g for 15 min at 4℃, the supernatant was discarded by pipetting and the remaining RNA pellet was washed with 70% ethanol twice, air dried and then re-suspended with 35 µL of RNase free water. Total RNA was purified by using DNA-*free* Kit (Ambion, Austin, TX, USA) for real-time qPCR. Briefly, 1 µL DNase I and 5 µL 10X DNase I buffer were added to 10 µg of total RNA to a total reaction volume of 50 µL. The mixture was incubated for 30 min at 37℃. DNase inactivation reagent was subsequently added to the mixture and incubated for 2 min at room temperature to cease the mixture reaction. Following centrifugation at 10,000x g for 1.5 min the supernatant was transferred to a tube for further use. The integrity and purity of the RNA was determined by analysis with RNA gel and spectrophotometer, respectively.

3.2.4. cDNA preparation

Total RNA (1 µg) was used for complementary DNA (cDNA) synthesis using iScript Reverse Transcription Supermix (Bio-Rad, Hecules, CA, USA) according to the manufacturer's protocol. Briefly, 4 µL of 5X iScript reverse transcription supermix was mixed with 1 µg of

total RNA and nuclease-free water to a total reaction volume of 20 µL. The reverse transcription condition was 5 min at 25℃ for priming, followed by 30 min at 42℃ for reverse transcription, and 5 min at 85℃ for inactivation of the reaction. The cDNA products were diluted 8X and stored at -80℃ until further use.

3.2.5. Primers

All primers used in this experiment were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). Primer specificity was verified by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RT-PCR.

3.2.6. Real-time qPCR

The qPCR assay was carried out (in duplicate for each biological replicate) with a total reaction volume of 20 µL containing 5 µL of the diluted cDNA, 0.4 µL of forward primer (5 µM; final concentration 100 nM), 0.4 µL of reverse primer (5 µM; final concentration 100 nM), 10 µL of SsoFast Eva Green Supermix (Bio-Rad) and 4.2 µL of water. The following PCR conditions were applied: initial denaturation and DNA polymerase activation at 95℃ for 5 min, and then 40 cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 30 s and extension at 72℃ for 30 s. The expression of the target genes was normalized with *β-Actin*, and the relative transcript level determined by using the method described in Livak and Schmittgen (2001).

Table 3.1. Primer sequences used for analysis of the target genes by qRT-PCR

Gene	Type	Quantifying (5´ to 3´)
GA20ox1	FP[z]	CCCTGGAAGGAGACCCTCT
	RP[y]	GGCTCATCTCCGAGCAGTAG
GA20ox2	FP	AAGCTTCCCTGGAAGGAGAC
	RP	TCCCCTAGGTGCATGAAGTC
GA20ox3	FP	CGCTCACCTTCTTCCTCAAC
	RP	GAACTCACGCCAAGTGAAGTC
GA3ox1	FP	AGCACTACCGCCACTTCTCT
	RP	TACGAGGAACAGCTCCATCA
GA3ox2	FP	GCCCATCTCCTCCTTCTTCT
	RP	CCCTGTGGAACTCCTCCAT
GA2ox1	FP	AAAGCTAGCCAGGTCATGG
	RP	TTGAGTTGCAGGCAATCTGT
GA2ox3	FP	GTGGCCAACAGCCTAAAGTC
	RP	GTACTCGCCCCATGTGAAGT
GA2ox4	FP	CGCGTGTCGATGATCTACTT
	RP	TAGGCCGCCTTCTTGTATTC
GA2ox5	FP	GGTGTCCATGATCTTCTTCG
	RP	TGGGTGCTGCTCTTGTACTC
GA2ox6	FP	AGTGGTGGCCAACTACAAGG
	RP	CTACGGTACTCCCCGAAGGT
NCED1	FP	ACCTCTGGAACTCGTGGGA
	RP	CGTCCGTGTCGTTGAAGAT
NCED2	FP	GCTTCTGCTTCCACCTCTG
	RP	GCACTCGTCCGACTCGTT
CYP707A1	FP	CCATGACCTTCACCCGCAAG
	RP	GGACACTGACGGATGGAGAAC
CYP707A2	FP	TGACGCACAGGGTGATTT
	RP	CCCTTGGGGATAAGAAACC
β-Actin	FP	CCAGGTATCGCTGACCGTAT
	RP	GCTGAGTGAGGCTAGGATGG

[z]*FP: Forward primer,* [y]*RP: Reverse primer*

3.3. Results

3.3.1. Expression analysis of gibberellin metabolism genes at whole grain level and in response to abscisic acid during grain development

3.3.1.1. Expression of gibberellin biosynthesis genes

Of the three *GA20ox* genes of barley, the transcripts of *GA20ox2* and *GA20ox3* were expressed during grain development except that the transcripts of *GA20ox2* was not detected at 20 and 50 DAA, while that of *GA20ox3* at 50 DAA (Figure 3.1, Appendix 1-1). No transcript of *GA20ox1* was observed at any of the developmental stages covered in this study. Comparison of the expression levels between *GA20ox2* and *GA20ox3* revealed that *GA20ox3* is expressed at higher level than *GA20ox2* (Appendix 1-1).

The expression of *GA20ox2* in the developing barley grains remained at very low and constant level, whereas *GA20ox3* showed relatively higher expression at earlier stages (20 DAA) with a gradual decrease to undetectable level by maturity (50 DAA). Treatment of developing grains with ABA led to a drastic increase in the expression of *GA20ox2* at the later stages of development, 40 and 50 DAA (Figure 3.1A). No effect of ABA was observed in the expression of *GA20ox3* (Figure 3.1B).

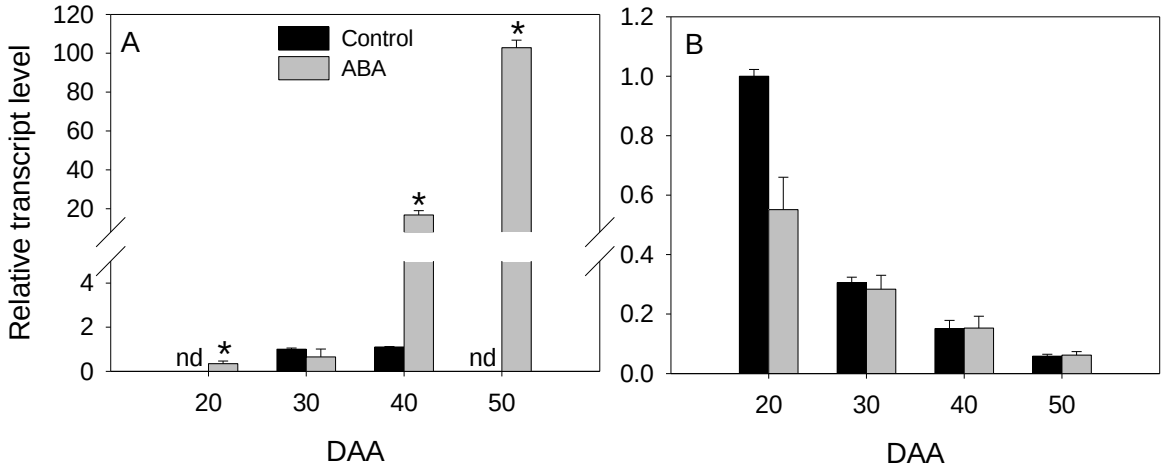

Figure 3.1 Relative abundance of *GA20ox2* (A) and *GA20ox3* (B) transcripts during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundances for *GA20ox2* and *GA20ox3* were expressed relative to transcripts detected in the 30 DAA control seed sample and 20 DAA control seed sample, respectively, which were set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

The transcripts of both *GA3ox1* and *GA3ox2* were detected during grain development (Figure 3.2; Appendix 1-2); however, the expression of *GA3ox1* at any stage of grain development was greater than that of gene *GA3ox2* (Appendix 1-2). The expression of *GA3ox1* was maintained at a relatively higher level during the earlier periods of grain development (20 and 30 DAA) but showed a decline during the later stages, 40 and 50 DAA. Furthermore, ABA treatment did not affect the expression of this gene in most stages of grain development considered in this study (Figure 3.2A).

Although no transcript of *GA3ox2* was detected at 20 and 50 DAA, developing grains at 30 and 40 DAA exhibit detectable levels of transcripts for this gene. ABA treatment led to a substantial increase in the abundance of *GA3ox2* transcripts at 50 DAA; and moderate effects of ABA on the expression of this gene were apparent at the other stages (Figure 3.2B).

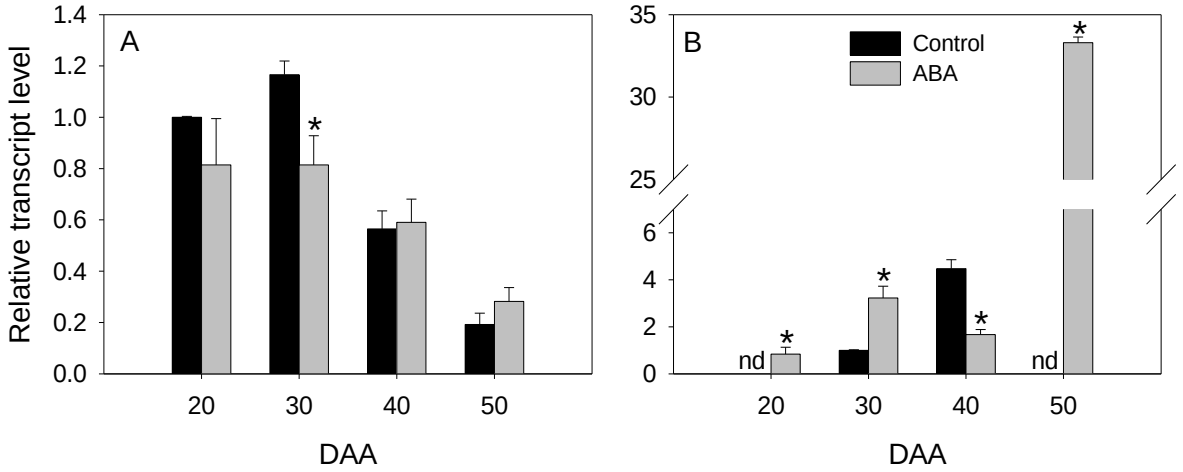

Figure 3.2 Relative abundance of *GA3ox1* (A) and *GA3ox2* (B) transcripts during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundances for *GA3ox1* and *GA3ox2* were expressed relative to transcripts detected in 20 DAA control seed sample and 30 DAA control seed sample, respectively, which were set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.1.2. Expression of gibberellin inactivating genes

Our qPCR analysis detected the transcripts of all the five GA inactivating genes studied including *GA2ox1*, *GA2ox3*, *GA2ox4*, *GA2ox5* and *GA2ox6* during barley grain development except that no transcript of *GA2ox4* was found at 20 DAA (Figure 3.3, Appendix 1-3). Of the five genes, *GA2ox5* was expressed at a higher level as compared to the other family members followed by *GA2ox3*; the expression level of these two genes showed a decrease as the grains develop (Appendix 1-3). The transcript abundances of the other three GA inactivating genes remained at low and almost constant level throughout grain development except that *GA2ox4* showed enhanced expression from 40 to 50 DAA.

ABA treatment resulted in substantial increase in the expression of *GA2ox1* (68-fold) and *GA2ox3* (44-fold) at 50 DAA (Figure 3.3). In the case of *GA2ox4*, ABA caused increased

expression at 40 DAA (24-fold) and 50 DAA (4-fold). It also induced enhanced expression of *GA2ox6* at all stages of grain development except at 40 DAA, when a similar expression level was present between the control and ABA treated samples. No effect of ABA was observed on the expression of *GA2ox5*.

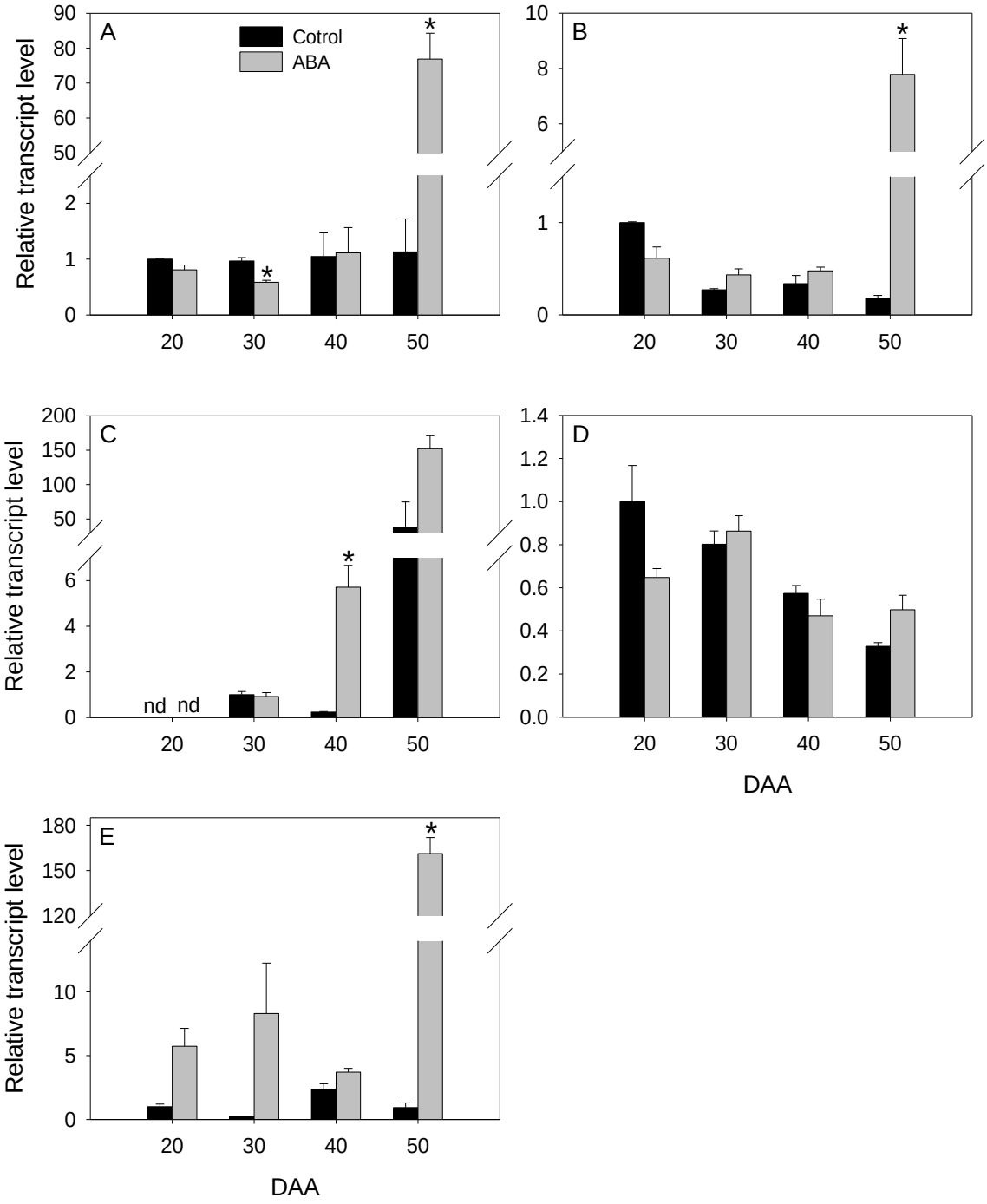

Figure 3.3 Relative abundance of *GA2ox1* (A), *GA2ox3* (B), *GA2ox4* (C), *GA2ox5* (D) and *GA2ox6* (E) transcripts during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of three biological replicates and the error bars represent standard errors. Transcript abundances for *GA2ox1*, *GA2ox3*, *GA2ox5*, and *GA2ox6* were expressed relative to transcripts detected in 20 DAA control seed sample, and the transcript abundance for *GA2ox4* was expressed relative to transcripts detected in 30 DAA control seed sample, which were set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.2. Expression analysis of abscisic acid metabolism genes at whole grain level and in response to abscisic acid during grain development

This study also examined the expression patterns of ABA metabolic genes, *NCED* and *CYP707A* and their respective family members in the developing grains of barley cv. Morex in order to assess the dynamics of ABA metabolism. In addition, the regulation of the expression of these genes by ABA during grain development was examined using exogenous ABA treatment.

3.3.2.1. Expression of the abscisic acid biosynthetic genes

The transcripts of *NCED1* but not that of *NCED2* was detected at all developmental stages (Figure 3.4; Appendix 1-4). The level of *NCED1* expression exhibited a gradual increase from 20 to 40 DAA (2.7-fold) but showed a decrease from 40 to 50 DAA (5-fold). Treatment of the developing grains with exogenous ABA affected the expression of *NCED1* only at 50 DAA, leading to a 19-fold increase (Figure 3.4). ABA induced the expression of *NCED2* only at 20 and 50 DAA.

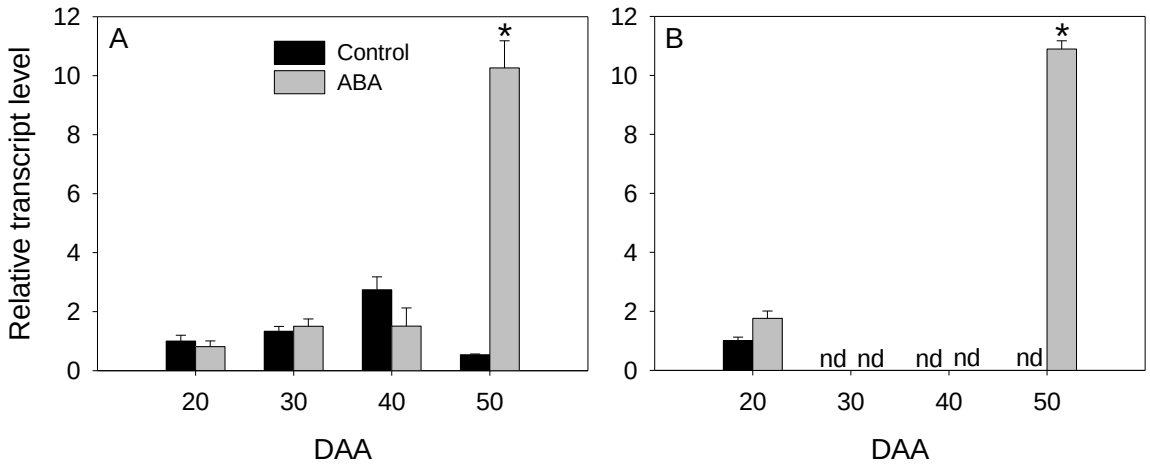

Figure 3.4 Relative abundance of *NCED1* (A) and *NCED2* (B) transcripts during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance for each gene was expressed relative to transcripts detected in 20 DAA control seed sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.2.2. Expression of the abscisic acid catabolic genes

The expression patterns of two *CYP707A* genes, *CYP707A1* and *CYP707A2* were also characterized; and our analysis revealed that their respective transcripts were detected at any stage of grain development (Figure 3.5, Appendix 1-5). The two genes were expressed at similar levels at the earlier stages of grain development. While a substantial increase in the abundance of *CYP707A1* transcript was observed as the grain developed from 30 to 50 DAA, the transcript level of *CYP707A2* decreased to undetectable level (Figure 3.5). ABA treatment induced increases in the expressions of both genes at 50 DAA (Figure 3.5).

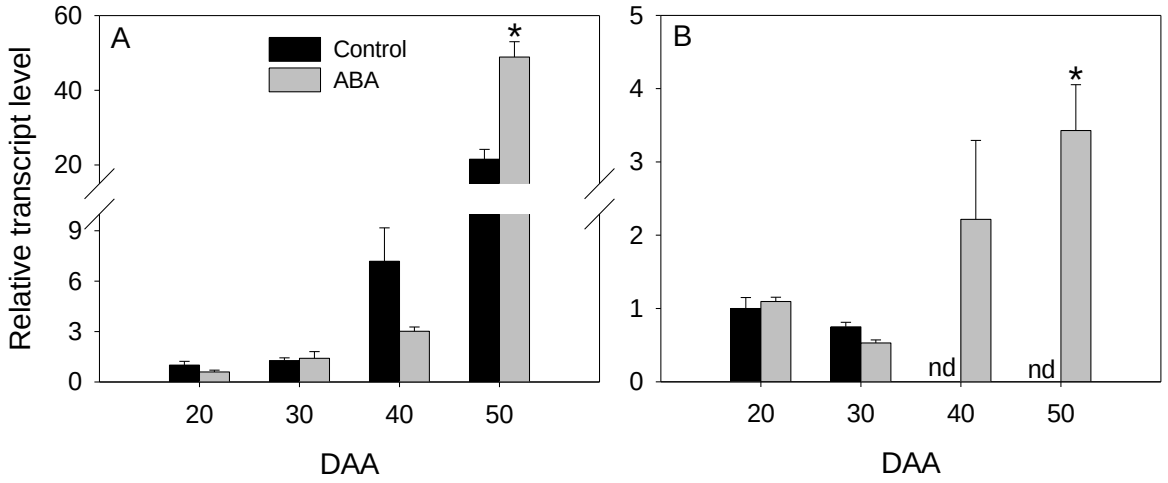

Figure 3.5 Relative abundance of *CYP707A1* (A) and *CYP707A2* (B) transcripts during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance for each gene was expressed relative to transcripts detected in 20 DAA control seed sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.3. Spatiotemporal expression analysis of gibberellin and abscisic acid metabolism genes during grain development and their regulation by abscisic acid

The expression patterns of GA (members of the *GA20ox*, *GA3ox* and *GA2ox* families) and ABA (members of the *NCED* and *CYP707A* families) metabolic genes were investigated in different tissues, endosperm and embryo, during grain development of barley cv. Morex. This study was also accompanied by ABA treatment to examine the tissues specificity of ABA effect on the expression of the targeted genes.

3.3.3.1. Expression analysis of gibberellin biosynthetic genes

Of the three *GA20oxs* (*GA20ox1*, *GA20ox2* and *GA20ox3*), only the two genes, *GA20ox2* and *GA20ox3*, were found to be expressed in both embryo and endosperm tissues except that

GA20ox2 was not detected at 20 DAA in both tissues (Figure 3.6). Comparison between the two genes showed that the expression of *GA20ox3* is substantially higher in both embryo (at 20 and 30 DAA) and endosperm tissues (at all stages) than that of *GA20ox2*. The transcript abundance of *GA20ox2* in the endosperm was higher than that detected in the embryonic tissue. The expression of this gene in the embryonic tissue showed a gradual increase with development while in the endosperm a relatively higher expression was detected at 40 DAA as compared to the other stages of development. Treatment with ABA induced upregulation of *GA20ox2* in the embryonic and endospermic tissues at 20 and 40 DAA, respectively. However, ABA was shown to repress the expression of this gene to non-detectable level in the endosperm at 50 DAA.

Similarly, much higher abundance of *GA20ox3* transcripts was detected in the endosperm than that found in the embryo. In contrast to that observed for *GA20ox2*, the expression of *GA20ox3* in both tissues showed a relatively higher expression during the earlier stages (at 20 DAA), which decreased gradually to a low level with maturation. ABA did not appear to affect the expression of this gene in both tissues, except it causes repression in the embryo at 40 DAA but upregulation in the endosperm at 40 DAA.

The expression of *GA3ox1* was detected throughout grain development in both embryo and endosperm tissues of barley grains; *GA3ox2* was found to be expressed only in the embryo tissue but only after 30 DAA (Figure 3.7). The expression of *GA3ox2* in the embryo at 30 and 40 DAA was found to be higher than that of *GA3ox1*.

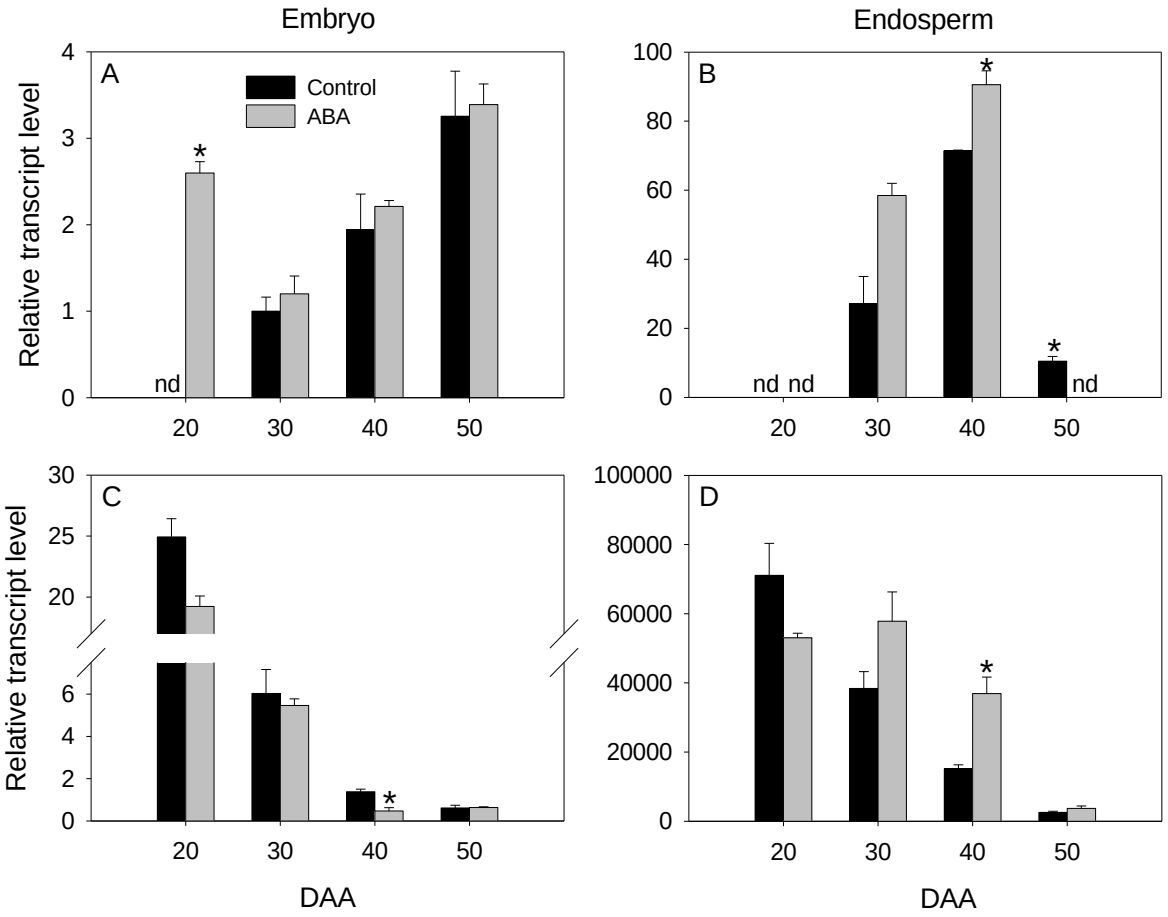

Figure 3.6 Relative abundance of *GA20ox2* (A, B) and *GA20ox3* (C, D) transcripts in the embryo (A, C) and endosperm (B, D) tissues during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *GA20ox2* transcripts detected in 30 DAA control embryo sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

Much higher level of *GA3ox1* expression was observed in the endosperm relative to that found in the embryo (Figure 3.7A, B). *GA3ox1* exhibited higher abundance of transcripts at 20 DAA in the embryo tissues, but showed a decline to a low level as maturation progresses. The high transcript level of *GA3ox1* detected in the endosperm at 20 DAA was maintained through 30 DAA, and showed a gradual decline afterwards. The temporal

expression pattern of *GA3ox2* in the embryo is different from that of *GA3ox1*, as its expression showed slight increase from 20 to 40 DAA, after which it showed a slight decline. Treatment with ABA caused reduction (2-fold) in the expression of *GA3ox1* in the embryo at 20 DAA. In the endosperm, the ABA treatment led to slight decreases in the expression of *GA3ox1* at both 20 and 30 DAA but an increase towards maturation, at 40 DAA and 50 DAA. With respect to the *GA3ox2* gene, ABA treatment induced upregulation at 20 DAA and 30 DAA in the embryo.

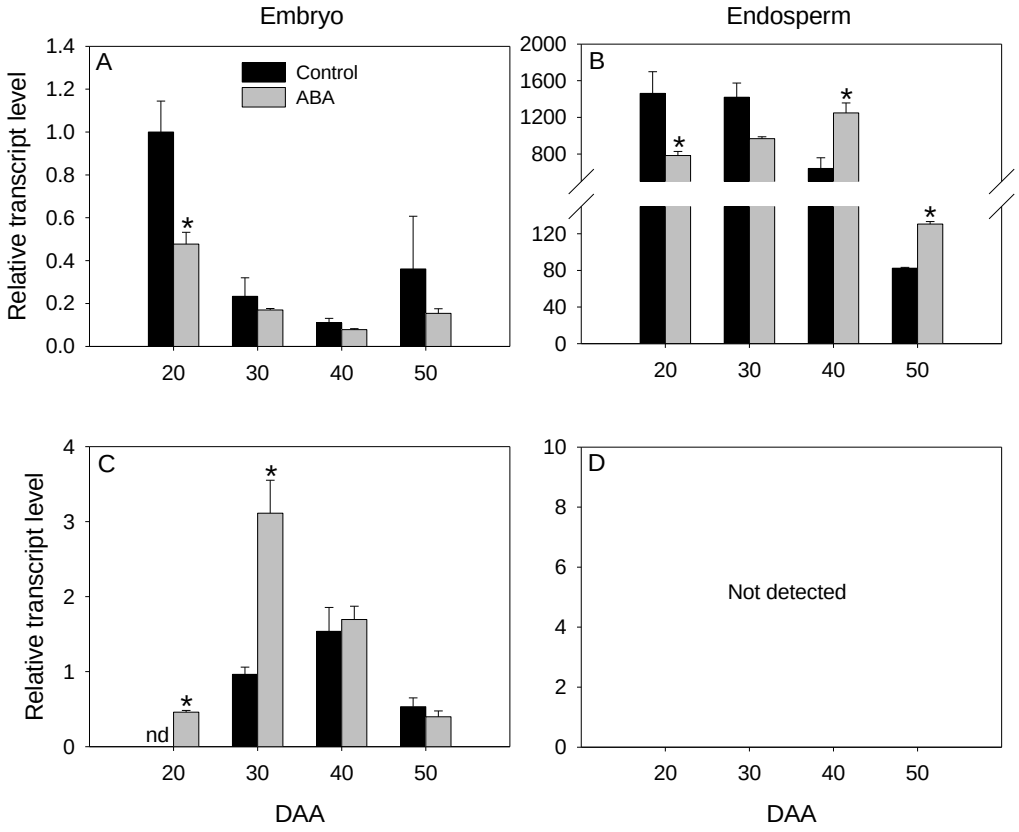

Figure 3.7 Relative abundance of *GA3ox1* (A, B) and *GA3ox2* (C, D) transcripts in the embryo (A, C) and endosperm (B, D) tissues during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *GA3ox1* transcripts detected in 20 DAA control embryo sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.3.2. Expression analysis of gibberellin inactivating genes

The transcripts of all the five GA inactivating genes were detected in both tissues of barley grain during development except that no detectable transcript of *GA2ox1*, *GA2ox4* and *GA2ox6* (Figure 3.8). All genes except *GA2ox1* showed higher expression in the endosperm as compared to the embryo; *GA2ox1* showed comparable expression level in both embryo and endosperm tissues. *GA2ox1*, *GA2ox3* and *GA2ox5* genes had similar expression patterns in the embryo, showing a relatively higher expression at 20 DAA followed by a continuous decline as the grains mature. The expressions of *GA2ox4* and *GA2ox6* genes, on the other hand, were maintained at similar level in the embryo during grain development. Unlike that observed in the embryo, the expressions of *GA2ox1*, *GA2ox4*, *GA2ox5* and *GA2ox6* in the endosperm showed increases from 20 to 40 DAA, after which a substantial decrease was apparent. The endosperm derived *GA2ox3*, however, showed an expression pattern similar to that observed in the embryo, a decrease from 20 to 50 DAA. ABA treatment caused a decline in the transcript abundances of embryonic *GA2ox1* (3.4-fold), *GA2ox3* (2.2-fold), *GA2ox5* (3.6-fold) only at 20 DAA with no effect at other stages. ABA did not affect the transcript levels of these genes in the endospermic tissue, except that it slightly downregulated the transcript level of *GA2ox1* at 50 DAA and *GA2ox3* at 30 DAA, and upregulated the transcript level of *GA2ox5* at 40 DAA. ABA led to increased transcript level of *GA2ox4* in the embryo at all stages (but not at 40 DAA), however, the effect was evident only at 30 DAA in the endosperm. In the case of *GA2ox6*, upregulation and downregulation were detected only in the endosperm at 30 and 40 DAA, respectively.

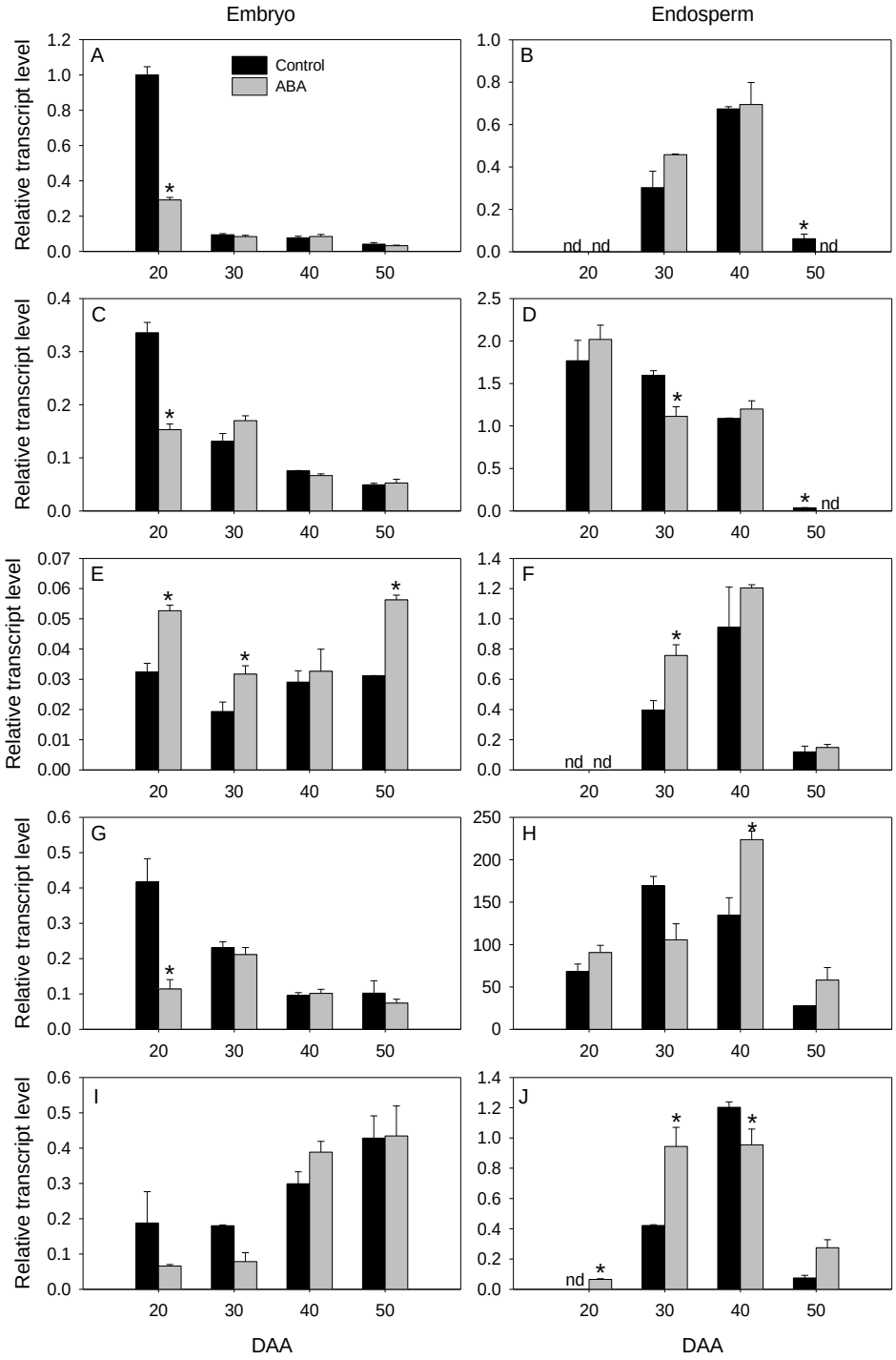

Figure 3.8 Relative abundance of *GA2ox1* (A, B), *GA2ox3* (C, D), *GA2ox4* (E, F), *GA2ox5* (G, H) and *GA2ox6* (I, J) transcripts in the embryo (A, C, E, G, I) and endosperm (B, D, F, H, J) tissues during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *GA2ox1* transcripts detected in 20 DAA control embryo sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.3.3. Expression analysis of the abscisic acid biosynthetic genes

The transcripts of two known *NCED* genes of barley (*NCED1* and *NCED2*) were detected in the developing grains; relatively higher amounts of transcript for both genes were found in the endosperm as compared to in the embryos (Figure 3.9). The expressions of both genes in the embryo appeared to be constant except that the transcripts of *NCED2* were not detected at 20 DAA. In the endosperm, the expression of *NCED1* increased from 20 to 30 DAA (6-fold) and remained at a similar level through 40 DAA before showing a decline (2.6-fold) as grain maturation progresses from 40 to 50 DAA. The other *NCED* gene, *NCED2* showed a relatively higher level of expression towards maturity, at 40 and 50 DAA. Treatment of ABA did not affect the expression of *NCED1* in both tissues except repression in the embryo at 40 DAA and upregulation in endosperm at 20 DAA. On the other hand, ABA caused substantial increase in the expression of *NCED2* in the embryo at 20 DAA and in the endosperm at 50 DAA. However, the endospermic *NCED2* was downregulated by ABA at 40 DAA.

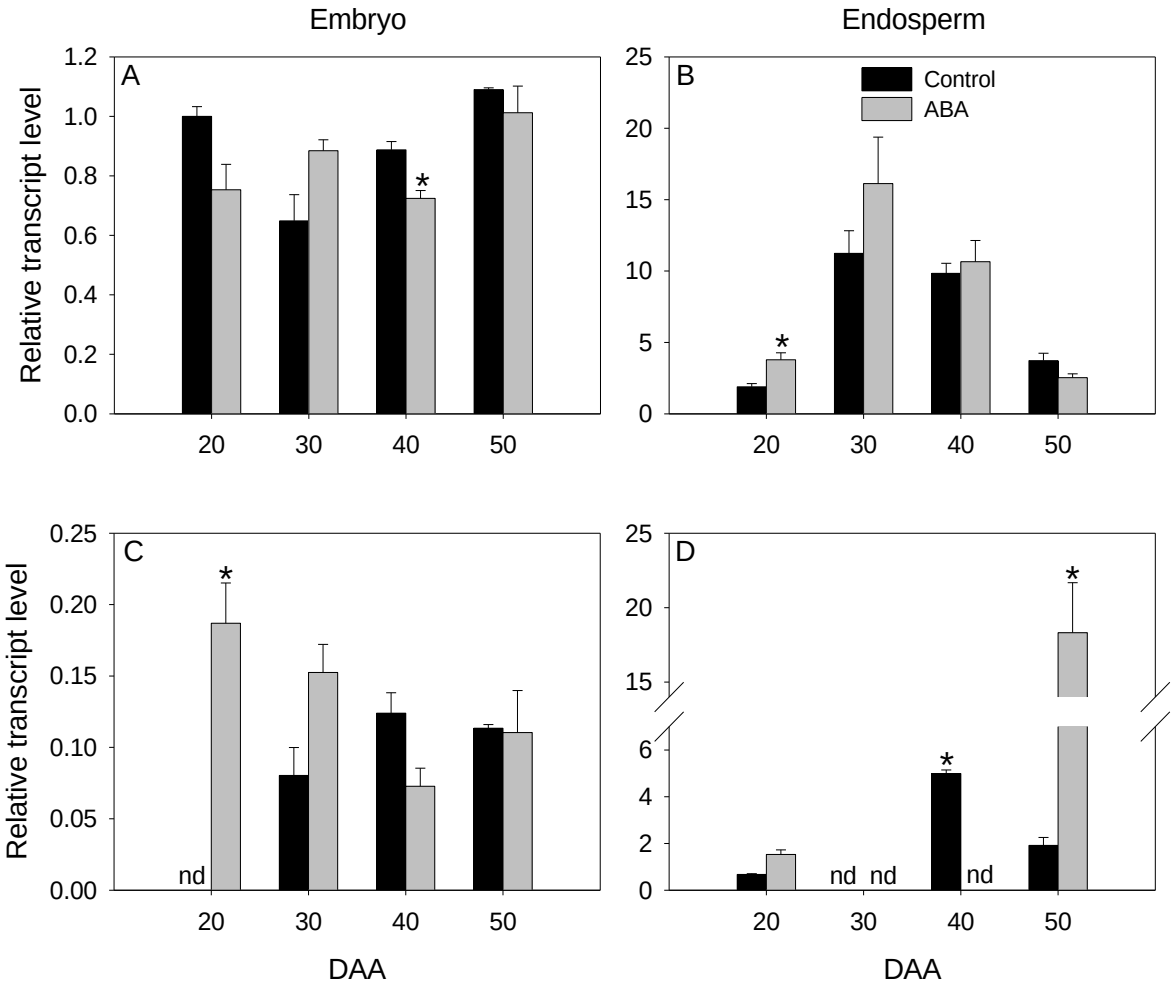

Figure 3.9 Relative abundance of *NCED1* (A, B) and *NCED2* (C, D) transcripts in the embryo (A, C) and endosperm (B, D) tissues during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *NCED1* transcripts detected in 20 DAA control embryo sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.3.3.4. Expression of the abscisic acid catabolic genes

The transcript level of *CYP707A1* in the embryo showed a continuous increase as the grains develop from 20 to 50 DAA. In the endosperm, this gene was expressed relatively at low level in 20 and 30 DAA but at 40 and 50 DAA a substantial abundance of its transcript was evident (Figure 3.10). With respect to *CYP707A2*, higher expression was observed at 20 DAA

in the embryo, which declined to a very low level from 20 to 30 DAA, after which its transcript level remained at a similar level. Likewise, higher expression level of endospermic *CYP707A2* was evident during the earlier relative to the later stages of grain maturation. ABA treatment appeared to decrease the expression of *CYP707A1* in both the embryo (~2-fold) and endosperm (14-fold) at 40 DAA; however, it caused a slight increase in both embryo at 20 and 30 DAA, and in the endosperm at 50 DAA. ABA also affected the expression of *CYP707A2* in the endosperm at 30 and 40 DAA.

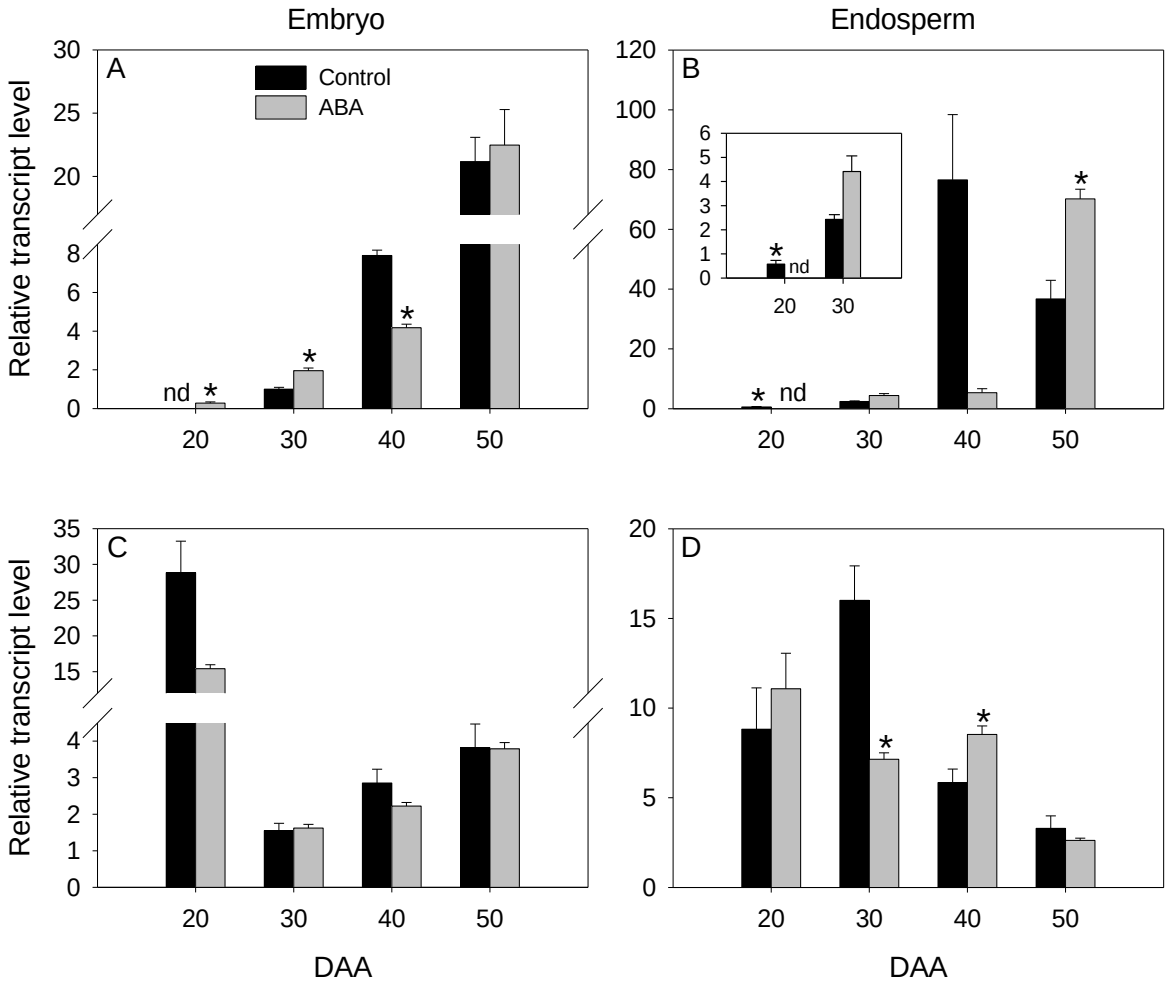

Figure 3.10 Relative abundance of *CYP707A1* (A, B) and *CYP707A2* (C, D) transcripts in the embryo (A, C) and endosperm (B, D) tissues during grain development in barley cv. Morex with and without ABA treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *CYP707A1* transcripts detected in 30 DAA control embryo sample, which was set a value of 1. Asterisks indicate statistically significant difference in a Student's t-test ($P < 0.05$); nd = not detected.

3.4. Discussion

To gain a better understanding on the role of GA and ABA in regulating grain maturation in barley, this study investigated changes in the expression of GA and ABA metabolism genes at both whole grain and tissue specific level. Furthermore, the ABA responsiveness of all the genes considered in this study was investigated.

Gibberellins are known to be involved in the control of seed developmental processes such as fertilization, embryo growth and the prevention of seed abortion in a number of species including tomato, pea and *Brassicaceae* (Kucera *et al*., 2005). The amount of endogenous GAs in plant tissues is regulated by the balance between its biosynthesis and catabolism, and these processes are regulated mainly by GA20ox, GA3ox and GA2ox enzymes, respectively, and genes encoding these enzymes have been identified from different species including barley (Yamaguchi, 2008). With respect to GA biosynthesis genes, *GA20ox3* showed not only a relatively higher expression than the other *GA20ox* genes during grain development at whole grain level but also exhibited highest expression at the earlier phases of grain maturation, implicating its significance in the synthesis of bioactive GA required for the completion of the grain development. Whereas, the transcripts of *GA20ox1* and *GA20ox2* were not either detected or remained at very low level during the grain maturation period. Of the two *GA3ox* genes, *GA3ox1* was highly expressed especially during the earlier stages of seed maturation, implying that *GA3ox1* plays important role in maintaining sufficient bioactive GA level required for proper grain development. Consistently, the GA-deficient *ls-1* and *lh-2* mutants of pea failed to exhibit proper seed development (Swain *et al*., 1997). Accumulation of bioactive GAs such as GA_1 and GA_3 has also been observed during maize seed development until dormancy is induced (White *et al*., 2000; Holdsworth *et al*., 2008). In rice, relatively high bioactive GA_3 has been observed during the early stage of grain development (Liu *et al*., 2014). Furthermore, the expression of *GA3ox1* which is a key gene for GA biosynthesis is reported to occur in developing pea seeds (Nadeau *et al*., 2011). Among the GA inactivating genes studied here, *GA2ox5* appeared to be the predominant gene whose transcripts of are more abundant at earlier than later phases of grain maturation, reflecting the role of this gene in regulating the pool of bioactive GA, and thereby

facilitating the grain desiccation process.

Abscisic acid regulates seed formation, the rate of seed filling and is required for acquisition of dormancy during seed development (Frey *et al*., 2004; Yang *et al*., 2006). At whole grain level only *NCED1* showed expression during grain maturation. This result might imply that *NCED1* is responsible for the production of ABA to induce seed desiccation tolerance. Consistently, it has been established that ABA is required for accumulation of seed storage and late embryogenesis abundant (LEA) proteins, and for induction of seed desiccation tolerance during seed maturation phase as well as prevention of programmed cell death of barley aleurone cells (Ooms *et al*., 1993; Bethke *et al*., 1999). The gradual increase in the level of *NCED1* from early to middle phases of grain maturation (20 to 40 DAA) found in this study suggests the occurrence of increased ABA synthesis during grain maturation. *NCED1* is suggested to play a role in the induction of seed dormancy during barley grain maturation (Leymarie *et al*., 2008), however the work reported by Chono *et al*. (2006) indicated the importance of *NCED2* for ABA synthesis in developing barley grains, and the expression of *NCED1* to be dependent on surrounding conditions during barley grains development. Although similar level of expression was detected between the two *CYP707A* genes at the earlier stages of seed maturation (20 to 30 DAA), higher level of *CYP707A1* expression was observed in the later phase (40 to 50 DAA), suggesting that the *CYP707A1* gene is responsible for inactivation of excess ABA during the final phases of grain maturation. Consistently, the expression of *CYP707A1* has been shown to be positively correlated with ABA catabolism (Chono *et al*., 2006).

The increase in the expressions of GA biosynthetic genes (*GA20ox2* and *GA3ox2*) in response to ABA towards the later stages of grain development may imply enhanced capacity for GA synthesis and accumulation. However, ABA also led to the induction of most of the

GA inactivating genes, and this overlaps temporally with that observed for *GA20ox2* and *GA3ox2* genes. With respect to the ABA biosynthetic genes, ABA led to enhanced expression of both *NCED1* and *NCED2* genes by the time of desiccation (at 50 DAA), and this was accompanied by increased expression of the ABA inactivating genes, *CYP707A1* and *CYP707A2*. These results suggest the overall maintenance of GA and ABA homeostasis. Previous reports have also shown that exogenous ABA induces upregulation of ABA metabolic genes in Arabidopsis (Kushiro *et al*., 2004; Saito *et al*., 2004). Given that the ABA: GA ratio rather than their respective absolute concentration is considered as a more decisive factor during seed development (Chono *et al*., 2006; Sreenivasulu *et al*., 2006), maintenance of the ABA homeostasis is required to balance the ABA: GA ratio in spite of the ABA exogenously applied to the grains. Consistently, low and high ABA: GA ratios have been found during grain development in non-dormant and deep-dormant rice cultivars, respectively (Liu *et al*., 2014).

Comparative analysis of the expression of GA metabolic genes between the embryo and the endosperm tissues revealed that all the genes whose transcripts are highly abundant during grain development (*GA20ox3*, *GA3ox1* and *GA2ox5*) are mainly expressed in the endosperm tissues relative to the embryo; *GA3ox2* exhibits higher expression in the embryo than in the endosperm reflecting their tissue specific roles. Previous study has also reported the presence of higher transcript abundance of GA metabolic genes in the endosperm of developing barley grains as compared to that found in the embryo (Sreenivasulu *et al*., 2008). ABA treatment led to over 3-fold increase in the expression of *GA3ox2* in the embryo (at 30 DAA), and this was not accompanied by induction of GA inactivating genes, suggesting the role of exogenous ABA in inducing the activation of GA biosynthesis most likely to maintain the balance between the two hormones with antagonistic functions (Debeaujon and

Koornneef, 2000).

With respect to their tissue specificity, both the *NCED1* and *NCED2* genes are highly expressed in the endosperm than in the embryonic tissues of maturing grains. Irrespective of the differential expression of these genes between the tissues, our result is in agreement with the synthesis of ABA in both embryo and endosperm tissues during the desiccation phase of seed development (Karssen *et al*., 1983). Interestingly, no significant changes in the transcript abundance of *NCED*s were detected in embryo tissues throughout grain development while in the endosperm the expression of the two genes was relatively elevated during the mid-phase of seed maturation as compared to the earlier and later phases, suggesting the temporal difference between the two tissues in terms of their requirement of ABA to induce processes related to desiccation tolerance and dormancy induction (Lefebvre *et al*., 2006). Relatively high level of *CYP707A2* expression was evident in both the embryo and endosperm tissues during the early phase of grain maturation process. Strong expression of *CYP707A1* in both endosperm and embryo tissues is rather detected during the late phase of grain development (peak at 40 DAA and 50 DAA in endosperm and embryo, respectively). Our gene expression result implies temporal specific role of the two genes, and thereby the operation of ABA catabolism during the entire phase of grain maturation for inactivation of any excess ABA. Similarly, the transcripts of *CYP707A1* of Arabidopsis are highly abundant in the embryo during the mid-maturation phase and are also detected in the endosperm tissues (Okamoto *et al*., 2006).

In summary, the high expression of GA metabolic genes including *GA20ox3*, *GA3ox1* and *GA2ox5* in endosperm, and *GA3ox2* in embryo of maturing seeds (Figure 3.11) suggest the regulatory roles of these genes in maintaining endogenous bioactive GAs for proper seed developmental events. In addition, the expressions of ABA metabolism genes

including *NCED1* and *NCED2* in endosperm during the middle phase of seed development, and *CYP707A1* in both endosperm and embryo during the late phase of seed development (Figure 3.11) indicate the important roles of these genes on the regulation of ABA level for proper barley grain maturation. Furthermore, the result from exogenous ABA treatment study suggests that ABA affects the metabolism of both GA and ABA during seed development.

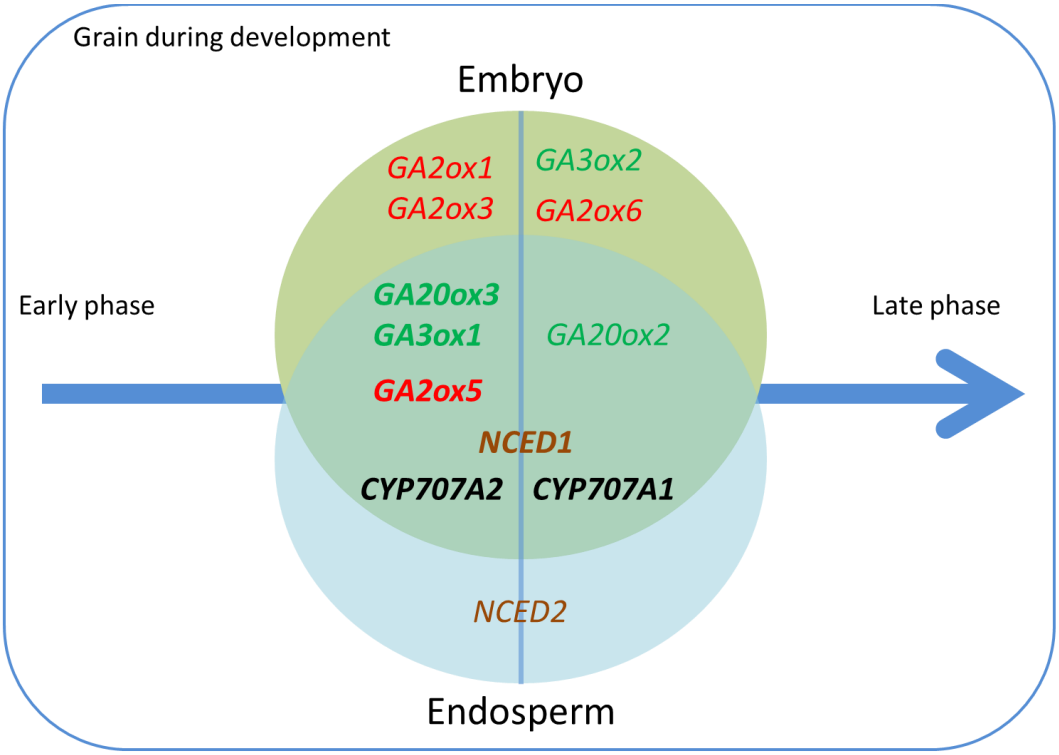

Figure 3.11 Schematic summary of tissue-specific expression patterns of GA and ABA metabolism genes during grain development. The vertical line divides the grain developmental phase, which is shown by the blue arrow, into early and late phases. Genes in bold are highly expressed in both tissues at specific stage or in both stages while genes not in bold showed a relatively higher expression in the indicated tissue(s) at specific stage or in both stages. GA biosynthesis genes: *GA20ox2*, *GA20ox3*, *GA3ox1*, and *GA3ox2*; GA inactivation genes: *GA2ox1*, *GA2ox3*, *GA2ox5*, and *GA2ox6*; ABA biosynthesis genes: *NCED1* and *NCED2*; ABA catabolic genes: *CYP707A1* and *CYP707A2*.

4.0 Analysis of gibberellin and abscisic acid metabolism gene transcriptional patterns with respect to dormancy and germination in barley

Abstract

Seed germination and dormancy are the most critical developmental events for seeds to be established as a new plant and are regulated by the plant hormones, gibberellins and abscisic acid. This chapter of the thesis analyzed the spatiotemporal expression patterns of GA and ABA metabolic genes with respect to dormancy and germination by employing barley cultivars exhibiting different depth of dormancy, and a dormancy breaking treatment, after-ripening. The results showed that *GA20ox3* and *GA3ox1* appeared to be expressed in endosperm tissues of imbibing dormant and non-dormant barley grains, suggesting the role of these genes in regulating GA biosynthesis in endosperm irrespective of dormancy status. *GA20ox2* and *GA3ox2*, however, showed relatively higher expression in the embryo of imbibing grains derived from non-dormant barley cultivar and after-ripened seeds of the dormant cultivar, implying that these genes regulate bioactive GA synthesis, and in turn dormancy in barley grains. The presence of elevated expression of *GA2ox3* and *GA2ox6* in the embryo of non-dormant grains might reflect their roles in regulating bioactive GA level and thereby germination. Irrespective of its regulatory role in ABA biosynthesis, the expression of *NCED*s was found not to be associated with dormancy or germination phenotype. Relatively higher expression of *CYP707A1* was detected during imbibition in both endosperm and embryos of grains from non-dormant cultivars and after-ripened grains of the dormant cultivar, indicating the role of this gene in regulating endogenous ABA during seed imbibition, and in turn seed dormancy and germination.

4.1. Introduction

Seed germination and dormancy are the most important phenomena in terms of establishing a new plant. Water absorption by a quiescent mature seed can be marked as an initial point of seed germination and protrusion of radicle through the embryo surrounding tissues as a germination terminal point (Rajjou *et al*., 2012). Water is one of the important factors for germination process and water uptake by seeds during germination is composed of three phases such as water uptake phase I, II and III. A rapid water uptake by seeds is the characteristic of the phase I followed by the phase II which is a plateau phase in terms of water movement into seeds (Bewley, 1997). The degree of water absorption into seeds increases during phase III, which is considered as post-germination event. Several metabolic processes such as respiratory activity and repair of cellular damages induced during seed desiccation/maturation are initiated and operated by the germinating seeds during water uptake to successfully complete the germination processes (Bradford and Nonogaki, 2007; Nonogaki *et al*., 2010). The penetration of embryo surrounding tissues by the radicle marks the initial point of phase III during which majority of storage reserve mobilization takes place to support seedling growth.

Seed dormancy refers to germination failure of a viable seed under favorable conditions (Bewley, 1997). Primary seed dormancy is acquired mainly during the desiccation/maturation phase of seed development (Bewley and Black, 1994; Finch-Savage and Leubner-Metzger, 2006). Seed dormancy can be regulated by extrinsic factors such as oxygen, moisture, light, temperature and nitrogen, and intrinsic factors including plant hormones such as GA and ABA (Koornneef *et al*., 2002; Finch-Savage and Leubner-Metzger, 2006). The amount of ABA in seeds is a well-known factor related to the depth of dormancy. Close association between the expression of ABA catabolic genes and the level of ABA in

imbibing seeds of several species has been demonstrated by previous studies, even though the ABA specific biosynthesis step catalyzed by NCED is considered as regulatory step (Kushiro *et al*., 2004; Lefebvre *et al*., 2006; Okamoto *et al*., 2006). In Arabidopsis, increased expression of *CYP707A2* is found to be well correlated with dramatic decrease in ABA level during germination, and relative to the wild-type seeds over 6-fold more ABA was found in the seeds of *cyp707a2* mutants (Kushiro *et al*., 2004). Furthermore, enhanced expressions of *CYP707A1* have been reported in germinating barley grains (Millar *et al*., 2006).

After-ripening, which refers storage of seeds under dry condition, is one of the factors regulating seed dormancy, and it has been demonstrated that after-ripening extends the range of temperature suitable for germination and also alters endogenous hormone levels and tissue sensitivity to phytohormones (Kucera *et al*., 2005). In after-ripened barley grains, a significant decrease in the amount of embryonic ABA is found during germination as compared to the dormant grains (Jacobsen *et al*., 2002). Upregulation of *CYP707A1* and decrease in the amount of ABA has been shown during germination of after-ripened barley grains (Chono *et al*., 2006). Furthermore, increased expression of the ABA catabolic gene *CYP707A1* and stimulation of ABA catabolism, and decrease in ABA sensitivity has been reported in the coleorhiza of after-ripened barley seeds during imbibition (Barrero *et al*., 2009). In *Brachypodium*, a newly emerged diploid model for temperate cereals, significant reduction in *NCED1* expression has been reported in after-ripened grain after imbibition even though there was similar level of its expression in dormant and after-ripened grain before imbibition. In after-ripened *Brachypodium* grain, increased expression of *CYP707A1* has also been found during imbibition (Barrero *et al*., 2012).

According to previous study by Ogawa *et al*. (2003), expression of Arabidopsis genes involved in GA biosynthesis including *KO1*, *GA20ox3* and *GA3ox1* are upregulated upon

seed imbibition. The expression of *GA3ox1* and *GA3ox2* are found in the embryo of Arabidopsis seeds during imbibition, and significantly reduced germination potential has been reported due to the loss of function of the two genes (Holdsworth *et al*., 2008), implying the role of these genes in regulating germination. Furthermore, correlation between the expression pattern of *GA3ox2* and the amount of bioactive GA_4 was found at later imbibition time, suggesting the specific role of this gene for changes in endogenous GA_4 (Ogawa *et al*., 2003). Furthermore, another study has shown increased seed sensitivity to GA in germinating Arabidopsis seeds (Derkx and Karssen, 1993). In barley, upregulation of *GA3ox2* has been shown during imbibition of after-ripened as compared to the dormant grains (Gubler *et al*., 2008). In addition, *GA20ox2* is expressed in the embryo tissues of barley grains during early imbibition (Sreenivasulu *et al*., 2008). In rice, the expression of *GA20ox1* is detected in non-dormant rice cultivar as compared to the dormant ones, indicating the occurrence of de novo GA synthesis during the rice grain germination (Liu *et al*., 2014).

Antagonistic interaction between GA and ABA in regulating seed dormancy and germination has been reported by several studies. For example, in the GA deficient *ga1* mutant of Arabidopsis, increase in the expression of ABA biosynthesis genes (*ABA1*, *NCED6* and *NCED9*) and decrease in the expression of ABA catabolism gene (*CYP707A2*) have been observed, indicating that GA negatively affects the level of ABA (Oh *et al*., 2007). In addition to this, Seo *et al*. (2006) showed increased expression of GA biosynthetic genes including *GA3ox1* and *GA3ox2* in the ABA deficient *aba2-2* mutant, and this suggests that germination of Arabidopsis seeds is inhibited due to the antagonistic effect of ABA on the level of GA (Seo *et al*., 2006).

In this chapter, the spatial and temporal expression patterns of GA and ABA metabolism genes were investigated during imbibition of barley grains from varieties with

varying degree of dormancy in order to gain more insights into the role of GA and ABA. The effect of after-ripening of dormant grains on the expression of GA and ABA metabolism genes during germination was also analyzed.

4.2. Materials and Methods

4.2.1. Plant materials and seed germination

Seeds were harvested from two cultivars with contrasting dormancy behavior; Morex (non-dormant) and Betzes (dormant), grown in a greenhouse conditions (day and night; 16/8 hours photoperiod; 22^{o}C/18^{o}C). Mature seeds were collected from the middle regions of primary and secondary spikes of each cultivar. To break dormancy in the seeds of cv. Betzes, half of the seed lot collected was stored at room temperature (after-ripened) for 11 months. Mature dry seeds of cv. Morex, and dormant and after-ripened seeds of cv. Betzes were imbibed for 0, 6, 12 and 24 h at room temperature under darkness using a Petri-plate with two layers of Whatman # 1 filter paper and 7 mL deionized water. After imbibition, the seeds were dissected into embryonic and endospermic tissues, immediately frozen in liquid nitrogen and then stored at -80℃ until further use. Germination of the seeds was also monitored daily for 5 days, and emergence of the coleorhizae was used as indicator for the completion of germination.

4.2.2. RNA isolation

Total RNA was isolated from tissue samples as described in chapter 3. After RNA extraction step, DNA-*free* Kit (Ambion, Austin, TX, USA) was applied to eliminate genomic DNA from the extracted total RNA. Up to 10 μg of total RNA was prepared to digest genomic DNA with

1 µL DNase I, 5 µL 10X DNase I buffer for total 50 µL volume of reaction. The mixture was incubated for 30 min at 37°C and then 5 µL inactivation buffer was added to terminate the mixture reaction.

4.2.3. cDNA synthesis

The iScript Reverse Transcription Supermix (Bio-Rad, Hecules, CA, USA) was used to synthesize complementary DNAs from 1 µg of total RNA following the manufacturer's instruction. Briefly 1 µg of total RNA was mixed with 4 µL of 5X iScript reverse transcription supermix in a total of 20 µL reaction volume. Reaction protocol was same as decribed in chapter 3 (5 min, 25℃ for priming; 30 min, 42℃ for reverse transcription; 5 min, 85℃ for inactivation of the reaction). The synthesized cDNAs were diluted 8X and then stored at -80℃ until the RT-qPCR analysis.

4.2.4. Gene expression analysis

Real-time qPCR analysis was performed with 5 µL of the diluted cDNA, 1.2 µL of forward and reverse primers (5 µM; final concentration 300 nM), 2.6 µL of water and 10 µL of SsoFast Eva Green Supermix (Bio-Rad), a total reaction volume of 20 µL. The reactions were run following the protocol described in chapter 3. Specific primers used for gene expression analysis are described in Table 3.1.

4.3. Results

4.3.1. Seed germination

Monitoring germination for 5 days indicated that the mature seeds collected from cv. Morex and the after-ripened mature seeds of cv. Betzes exhibited 72% and 89% of germination within 2 days of seed imbibition, respectively (Figure 4.1). By 5 days after imbibition, over 90% of seeds from the two samples were germinated. In contrast, the dormant or non after-ripened seeds of cv. Betzes showed almost no sign of germination within 2 days of imbibition. Only 8% of the seeds displayed coleorhiza emergence following 5 days of imbibition.

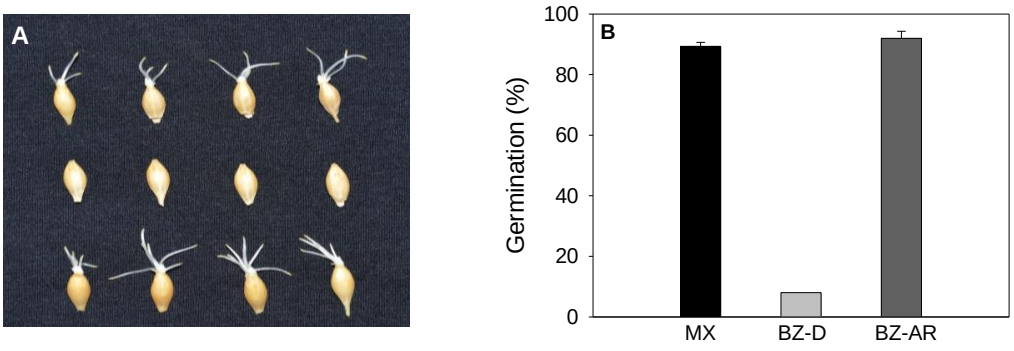

Figure 4.1 Barley grains of cv. Morex (top) and non after-ripened (middle) and after-ripened (bottom) grains of cv. Betzes imbibed for 2 days (A), and comparison of their germination percentage after 5 days of imbibition (B). MX=Morex; BZ-D=Betzes dormant; BZ-AR=Betzes after-ripened.

4.3.2. Spatiotemporal expression analysis of gibberellin and abscisic acid metabolism genes during grain imbibition and their regulation by after-ripening

The spatiotemporal expressions of GA and ABA metabolic genes were examined in endosperm and embryo tissues at 0, 6, 12 and 24 h after imbibition (HAI) in two cultivars of barley that exhibit different degree of dormancy, the non-dormant cv. Morex (MX) and the

dormant cv. Betzes (BZ-D). Furthermore, the expression patterns of the GA and ABA metabolic genes were examined during imbibition of after-ripened grains cv. Betzes (BZ-AR).

4.3.2.1. Expression analysis of gibberellin biosynthetic genes

Of the three *GA20ox* genes, the transcripts of two genes, *GA20ox2* and *GA20ox3*, were detected in both embryo and endosperm tissues of all imbibing grain samples except that of *GA20ox3* were not detected in MX embryo at any stage of imbibition (Figure 4.2). In addition, the transcripts of *GA20ox3* were detected only at 6 and 24 HAI in BZ-AR and BZ-D embryos, respectively. Comparison between the two genes revealed that the expression of *GA20ox3* is substantially higher (over 2.5-fold) in the endosperm tissues of BZ-D, BZ-AR and MX grain samples than that of *GA20ox2* at all stage of imbibition (Figure 4.2B, D). *GA20ox2* and *GA20ox3* exhibited higher transcript abundance in the endosperm (over 3.6 fold) than the corresponding embryo tissue of dry grains (Figure 4.2). Both genes showed similar expression pattern in the endosperm across all grain samples; expressed at high level in dry grains but declined to a low level as imbibition continued, except that *GA20ox2* expression in the endosperm of BZ-D increased with imbibition from 0 to 12 HAI, but declined to a very low level afterwards. Although imbibition induced a decline in their expression, the transcript abundances of *GA20ox2* and *GA20ox3* in the endosperm were still higher than that detected in the embryo; except for MX samples from 6 to 24 HAI, BZ-D samples at 24 HAI and BZ-AR samples at 12 HAI where there was comparable or higher expression of *GA20ox2* in the embryo.

Between the two *GA20ox* genes, *GA20ox2* appeared to be predominant in the embryonic tissue of all grain samples (Figure 4.2A, C). The expression of *GA20ox2* in the embryos of imbibing non-dormant grain samples (MX and BZ-AR grains) was higher than

that found in the dormant grain samples (BZ-D) at 6 and 12 HAI, while in the BZ-D grains, a higher transcript abundance of this gene (than that of MX and BZ-AR) was observed only after 24 h imbibition. Although not detected in MX embryos, upregulation of *GA20ox3* was also observed in BZ-AR grains at 6 h after imbibition, but this occurred only after 24 h after imbibition in BZ-D grains.

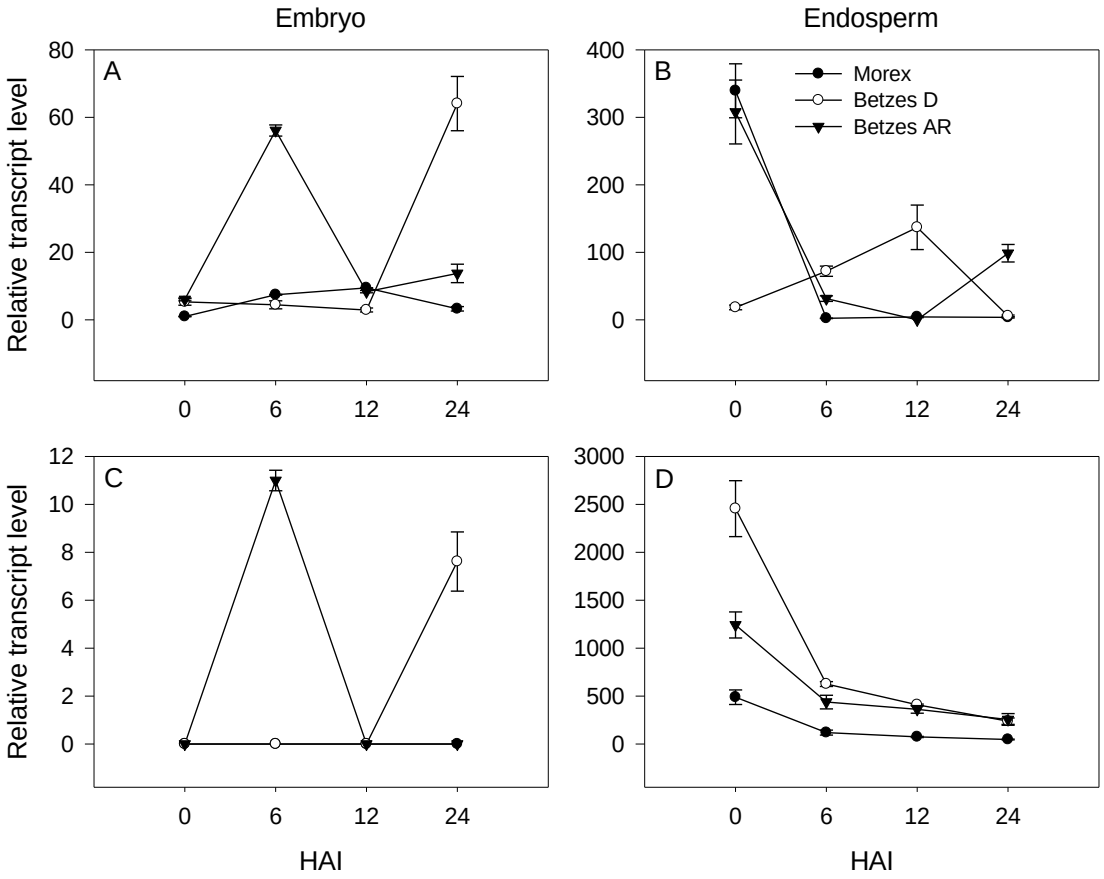

Figure 4.2 Relative abundance of *GA20ox2* (A, B) and *GA20ox3* (C, D) in the embryo (A, C) and endosperm (B, D) tissues during grain imbibition in barley cv. Morex and Betzes with and without after-ripening treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *GA20ox2* transcripts detected in the 0 HAI embryo sample of cv. Morex, which was set a value of 1.

The transcripts of two known *GA3ox* (*GA3ox1* and *GA3ox2*) genes of barley were detected throughout grain imbibition except that transcript of *GA3ox1* were not detected in MX embryos. In addition, the transcripts of *GA3ox1* were detected only at 6 and 24 HAI in BZ-AR and BZ-D embryos, respectively. *GA3ox1* exhibited much higher expression (over 9-fold) in the endosperm of all grain samples than that of *GA3ox2* at every stage of imbibition (Figure 4.3A, C). Endospermic *GA3ox1* showed higher transcript abundance during imbibition than that found in the embryo of both cultivars, irrespective of dormancy status (Figure 4.3A, B). When compared across samples, the expression of *GA3ox1* in the endosperm was in general in the following order; BZ-AR > BZ-D > MX. In contrast, *GA3ox2* appeared to be expressed at comparable level in both embryo and endosperm tissues, although transcripts were not detected in the embryo of BZ-D samples from 0 to 12 h imbibition, and in the endosperms at some points of imbibition. Overall, the expression of *GA3ox1* in the endosperm of the three samples appeared to be constant during imbibition, while the expression pattern of *GA3ox2* varied with samples. In non-dormant grains (BZ-AR and MX) the transcript abundance of *GA3ox2* in embryo tissues increased with imbibition. In contrast, no transcript of this gene was detected during imbibition of dormant sample (BZ-D) except that some transcripts were evident at 24 HAI. Expression of *GA3ox1* in embryo tissues was detected only at 6 HAI in BZ-AR and after 24 h imbibition in BZ-D.

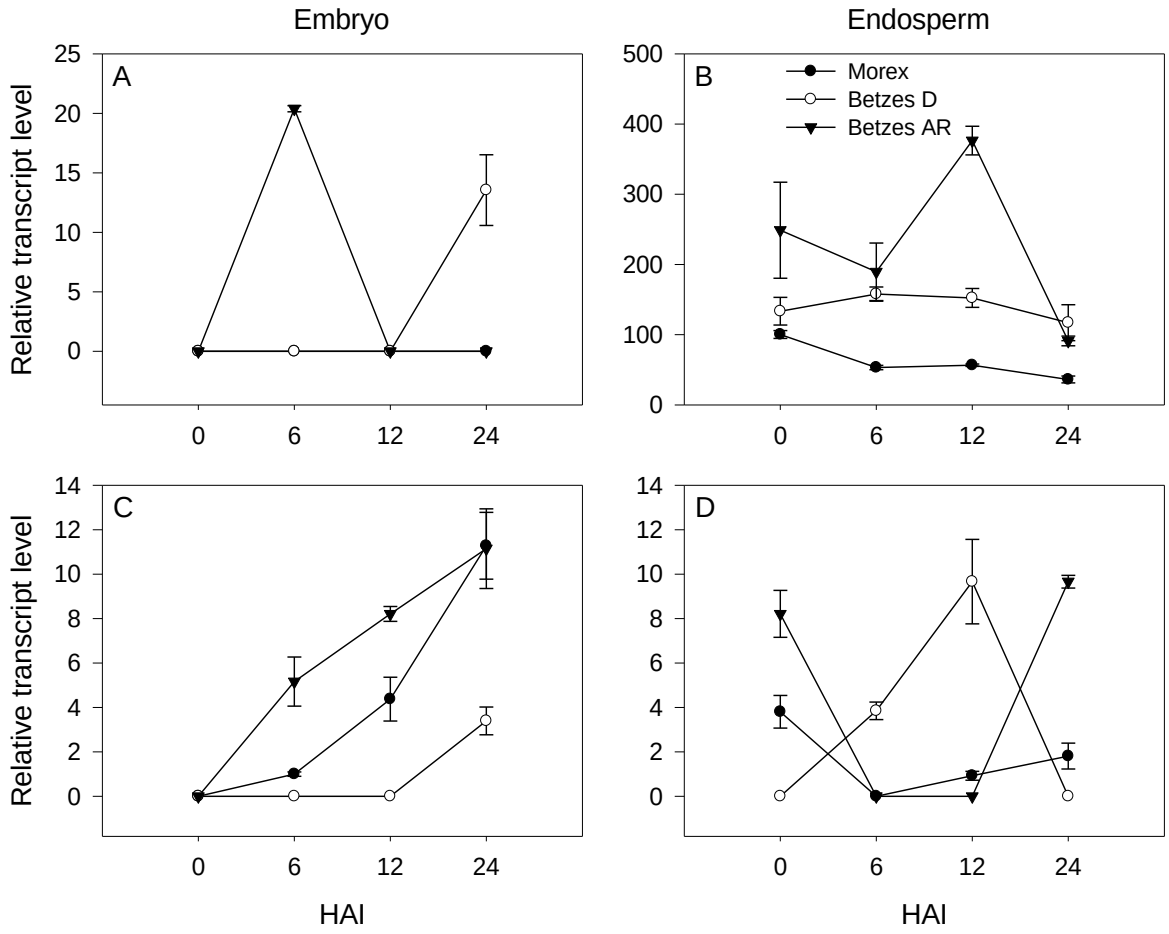

Figure 4.3 Relative abundance of *GA3ox1* (A, B) and *GA3ox2* (C, D) in the embryo (A, C) and endosperm (B, D) tissues during grain imbibition in barley cv. Morex and Betzes with and without after-ripening treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *GA3ox2* transcripts detected in the 6 HAI embryo sample of cv. Morex, which was set a value of 1.

4.3.2.2. Expression analysis of gibberellin inactivating genes

All the five *GA2oxs* (*GA2ox1*, *GA2ox3*, *GA2ox4*, *GA2ox5* and *GA2ox6*) were found to be expressed in both tissues (embryo and endosperm) of imbibing grain samples derived from both cultivars (Figure 4.4), except that the transcripts of *GA2ox1*, *4* and *5* were not detected in the embryo at some imbibition time points. In the dry grains of all samples, all of the

GA2ox genes appeared to have substantially higher level of transcripts (over 3-fold) in the endosperm than in the embryo; and after-ripening of BZ-D grains led to enhanced expression of endospermic *GA2ox1* (31-fold), *GA2ox3* (23-fold), *GA2ox4* (30-fold), *GA2ox5* (3-fold) and *GA2ox6* (19-fold) genes in the dry (0 HAI) grains. As the grain imbibition progressed, the expression of all endospermic *GA2ox* genes declined except that in BZ-D grains these genes exhibited a different expression pattern; exhibiting an increase as imbibition progressed from 0 to 12 HAI but a decline afterwards (Figure 4.4B, D, F, H, J). Despite the decline with imbibition, endospermic *GA2ox5* showed relatively higher transcript abundance throughout the grain imbibition period relative to the other *GA2oxs*.

The transcript abundance of *GA2ox3* and *GA2ox6* in the embryo of MX grains increased from 0 to 12 HAI followed by drastic decline to low level by 24 HAI; the other *GA2ox* genes exhibit only minimal expression. Elevated expression of *GA2ox3* and *GA2ox6* was also detected in BZ-D grains at 24 HAI (Figure 4.4 C, I) While the *GA2ox1*, *GA2ox4* and *GA2ox5* genes exhibited substantial increase in their transcript abundance in the embryo of BZ-AR grains at 6 HAI, and induction of the expression of these genes was evident only after 24 h imbibition in BZ-D (Figure 4.4 A, E, G).

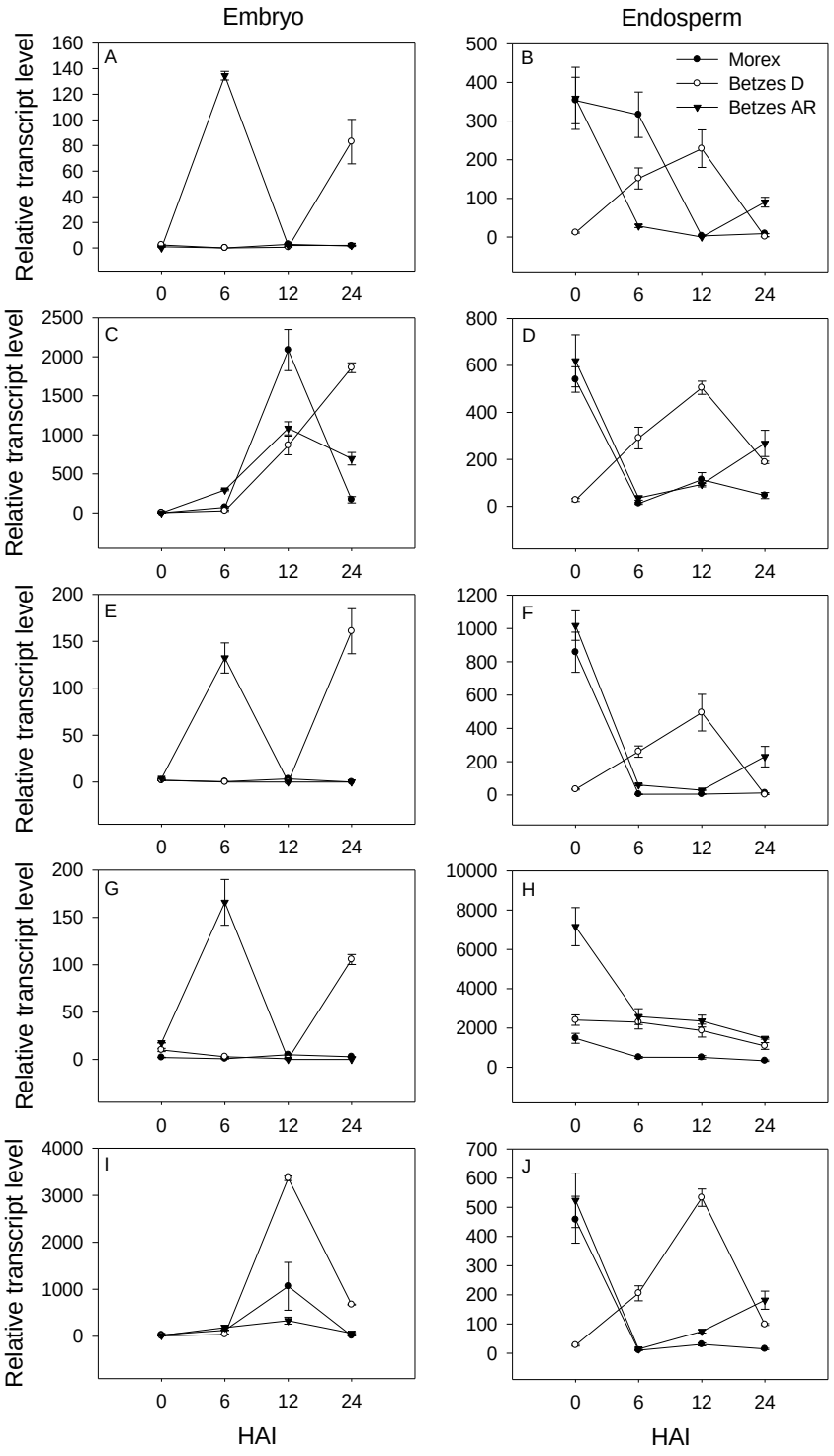

Figure 4.4 Relative abundance of *GA2ox1* (A, B), *GA2ox3* (C, D), *GA2ox4* (E, F), *GA2ox5* (G, H) and *GA2ox6* (I, J) in the embryo (A, C, E, G, I) and endosperm (B, D, F, H, J) tissues during grain imbibition in barley cv. Morex and Betzes with and without after-ripening treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *GA2ox1* transcripts detected in the 0 HAI embryo sample of cv. Morex, which was set a value of 1.

4.3.2.3. Expression analysis of the abscisic acid biosynthetic genes

Of the two known *NCED* genes of barley (*NCED1* and *NCED2*), only *NCED1* was found to be expressed in the embryonic and endospermic tissues of all grain samples except that no transcript was detected in the endosperm of BZ-AR and BZ-D at 12 and 24 HAI, respectively. In addition, the transcripts of *NCED1* were not detected in the embryo tissues of BZ-D and BZ-AR at 6 HAI and following 12 HAI, respectively. In the dry grain of all samples, *NCED* was found to be having greater transcript abundance in the endosperm (~3-fold) relative to the embryo. However, the expression level decreased markedly with imbibition. On the contrary, *NCED1* expression in the endosperm of BZ-D exhibited over 2-fold increase as imbibition progressed from 0 to 12 h, although no transcript was detected at 24 HAI. The transcription of embryonic *NCED1* was maintained at low level in the embryos of all grain samples except that an increase was detected in BZ-AR and BZ-D at 6 and 24 HAI (Figure 4.5).

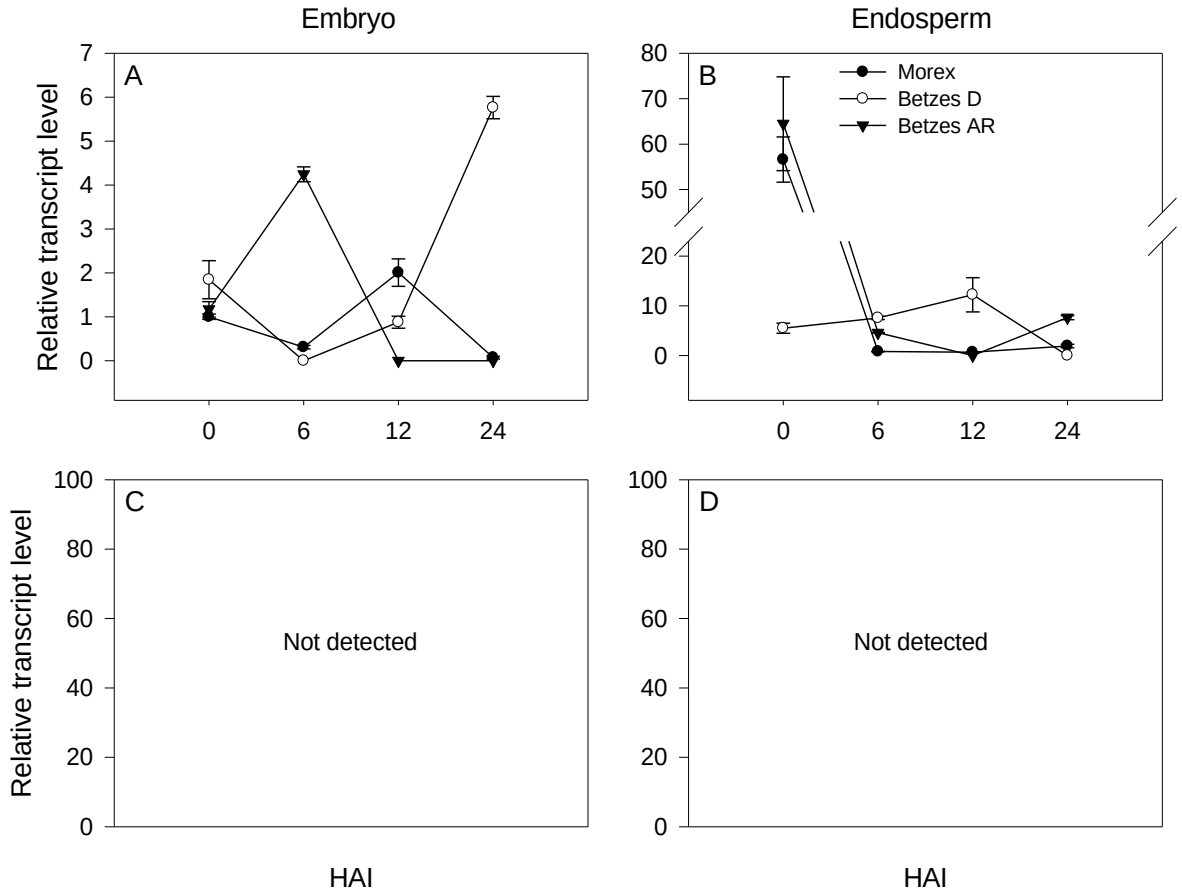

Figure 4.5 Relative abundance of *NCED1* (A, B) and *NCED2* (C, D) transcripts in the embryo (A, C) and endosperm (B, D) tissues during grain imbibition in barley cv. Morex and Betzes with and without after-ripening treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *NCED1* transcripts detected in the 0 HAI embryo sample of cv. Morex, which was set a value of 1.

4.3.2.4. Expression analysis of the abscisic acid catabolic genes

Comparison between the two ABA catabolic genes (*CYP707A1* and *CYP707A2*) across the three grain samples revealed that *CYP707A1* was expressed at a relatively higher level during imbibition in both tissues; no or very low level of *CYP707A2* transcripts were detected in both embryo and endosperm tissues of imbibing grains (Figure 4.6). The endosperm and embryo tissues of dry grains of MX and BZ-AR contain slightly more transcripts of

CYP707A1 than that of BZ-D. As imbibition continued from 0 to 12 HAI, upregulation of both embryonic and endospermic *CYP707A1* was evident in all grain samples, however, the degree of upregulation of this gene in BZ-D was lower than that observed for MX and BZ-AR samples (Figure 4.6A, B). Following 24 HAI, the transcript abundance of *CYP707A1* decreased except that it was maintained at a similar level observed at 12 HAI in the embryo of BZ-D and endosperm of BZ-AR.

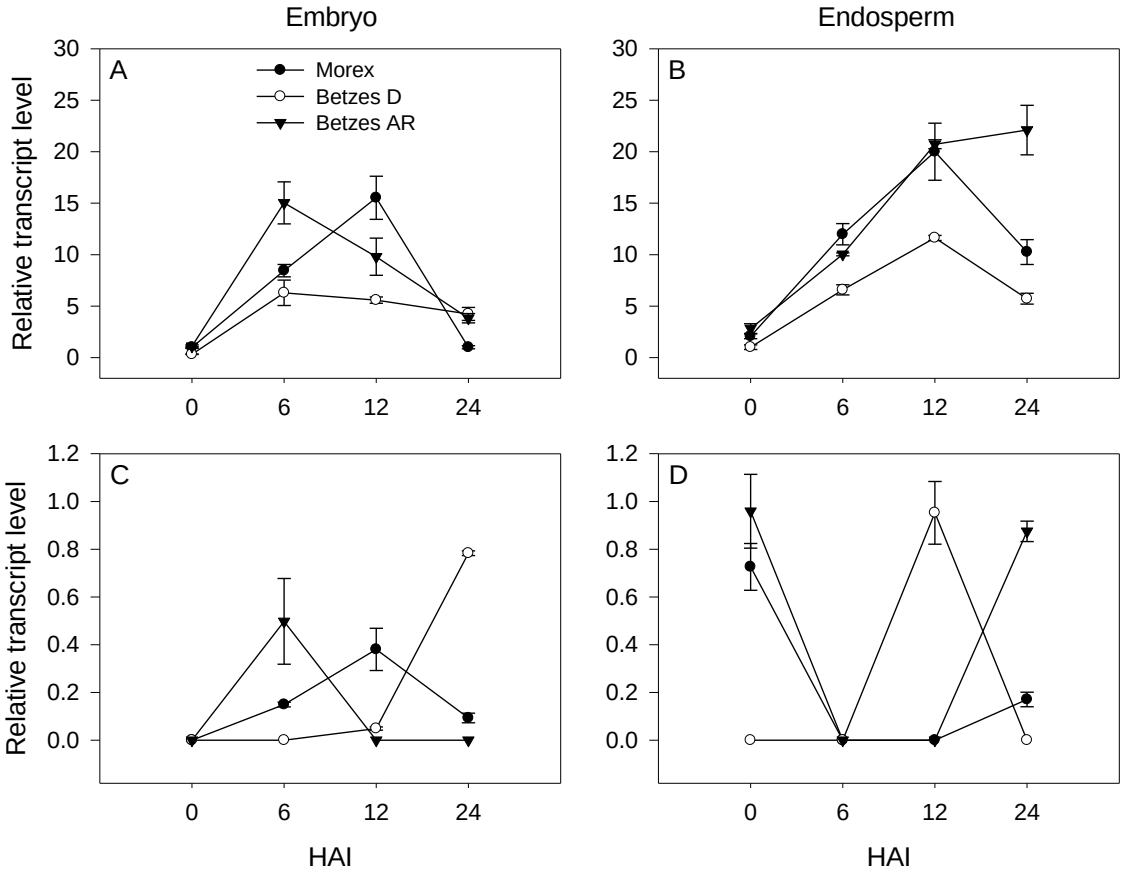

Figure 4.6 Relative abundance of *CYP707A1* (A, B) and *CYP707A2* (C, D) transcripts in the embryo (A, C) and endosperm (B, D) tissues during grain imbibition in barley cv. Morex and Betzes with and without after-ripening treatment. The data presented are means of two to three biological replicates and the error bars represent standard errors. Transcript abundance was expressed relative to *CYP707A1* transcripts detected in the 0 HAI embryo sample of cv. Morex, which was set a value of 1.

4.4. Discussion

Seed germination and dormancy, which are the critical events in seeds, are mainly regulated by the interaction of GA and ABA in antagonistic manner (Koornneef *et al*., 1982). In order to gain insights into the role of GA and ABA in regulating dormancy and germination in barley grains, this study investigated the spatiotemporal expression of GA and ABA metabolic genes using barley cultivars showing different degree of dormancy and a dormancy releasing treatment called after-ripening. Seed after-ripening triggers a number of changes including gene expression, and seed hormone content and sensitivity (Kucera *et al*., 2005).

The relatively high expression of *GA20ox3* and *GA3ox1* detected in the endosperm of imbibing grains derived from all the samples; grain of cv. Morex, after-ripened seeds of cv. Betzes and dormant grains of cv. Betzes (Figure 4.2D; Figure 4.3B) might suggest the occurrence of GA synthesis in this tissue regardless of the state of dormancy. The *GA20ox2* and *GA3ox2* genes appeared to show prominent expression in the embryo tissues of non-dormant grains of cv. Morex and after-ripened grains of cv. Betzes (Figure 4.2A; Figure 4.3 C). Previous study that involved after-ripening has also revealed the induction of *GA3ox2* expression in after-ripened grains of barley as compared to the corresponding dormant grains (Gubler *et al*., 2008). Likewise, upregulation of GA biosynthetic genes (*GA20ox1* and *GA3ox2*) by after-ripening has been reported in imbibing wheat grains (Liu *et al*., 2013). The expression of GA biosynthesis genes and the sensitivity of seeds to GA have also been shown to increase by after-ripening of Arabidopsis seeds (Finkelstein *et al*., 2008). Given that GA is mainly synthesized in the embryo of germinating cereal grains (Bewley, 2001), our results suggest that the synthesis of bioactive GA in the embryos and in turn germination of non-dormant grains is subjected to transcriptional regulation of *GA20ox2* and *GA3ox2*. Consistently, the expression of *GA20ox2* has been reported in the embryo of barley grains at

early stage of grain imbibition (Sreenivasulu *et al*., 2008) and *GA20ox1* was found to be expressed during germination of non-dormant grains of rice (Liu *et al*., 2014). Relatively higher transcript abundance of a gene encoding GA 20-oxidase as well as an accumulation of bioactive GA_4 was reported in embryo tissue of imbibing grains derived from non-dormant sorghum cultivar as compared to those from dormant cultivar (Pérez-Flores *et al*., 2003). The transcript abundance of *GA3ox2* in the embryo tissues of non-dormant grains of cv. Morex and after-ripened grains of cv. Betzes showed continuous increase with imbibition and peaked at 24 HAI of grain imbibition (Figure 4.3C), suggesting the specific role of *GA3ox2* in regulating the completion of germination. Similarly, the expression of *GA3ox2* and the level of endogenous bioactive GA_4 have been found to be high at later stages of seed imbibition in Arabidopsis (Ogawa *et al*., 2003).

The high expression of embryonic *GA2ox3* and *GA2ox6* genes during imbibition of non-dormant grains of cv. Morex (Figure 4.4C, I), might reflect the role of these genes in controlling the pool of bioactive GA in the embryo tissue. Imbibition mediated increased expression of *GA2ox3* gene was also found in the embryo tissue derived from non-dormant barley grains (Gubler *et al*., 2008). The presence elevated transcript abundance of endospermic *GA2ox5* in imbibing grains may suggest the importance of this gene in catabolizing active GAs, which may be essential to control endogenous level of GA, specifically in the endosperm of imbibing grains. The role of *GA2ox* in regulating the level of bioactive GAs during germination has been also been reported in other species such as Arabidopsis (Yamauchi *et al*., 2007; Rieu *et al*., 2008). After-ripening appeared to induce increased expression of all *GA2ox* genes in the endosperm of dry grain, however, the expression of these genes declined as seed imbibition continued (Figure 4.4). This result may suggest the role of after-ripening in the regulation of GA biosynthesis (Gubler *et al*., 2008)

but also its catabolism to modulate grain GA level.

The involvement of ABA in regulating seed dormancy and germination is well established (Nambara *et al*., 2010) in which ABA induces/maintains dormancy or inhibits germination. However, no significant changes in the expression of *NCED*s were detected in both endosperm and embryo tissues of all grain samples except the presence of a relatively high abundance of *NCED1* transcript in the endosperm of dry grains derived from cv. Morex and after-ripened grains of cv. Betzes, which subsequently showed a rapid decline with imbibition (Figure 4.5B). This result suggests the absence of correlation between ABA biosynthesis and the level of ABA during barley grain imbibition, irrespective of the regulatory role of NCED in catalyzing ABA biosynthesis (Taylor *et al*., 2000). In contrast, substantial decline in the transcript abundance of *NCED1* in after-ripened *Brachypodium* grains has been reported during grain imbibition (Barrero *et al*., 2012), implicating that *NCED1* regulates endogenous ABA level and thereby dormancy. It is likely that the role of NCED in regulating dormancy is species specific.

The elevated expression of *CYP707A1* in both endosperm and embryo tissues derived from non-dormant grains of cv. Morex during imbibition (Figure 4.6) implies enhanced catabolism of bioactive ABA, thereby a decrease in ABA level. After-ripening also increased the expression of endospermic and embryonic *CYP707A1*; especially induction of *CYP707A1* in the embryo occurred earlier during imbibition (Figure 4.6A). Consistently, a sharp decline in embryo ABA level has been detected during imbibition of after-ripened barley grains as compared to the dormant counterparts (Jacobsen *et al*., 2002). In barley, the initial ABA catabolic activity is suggested to be localized in the coleorhiza, which is non-vascularized multicellular embryonic tissue (Millar *et al*., 2006). Consistently, upregulation of *CYP707A1* and enhanced ABA catabolism, and reduced seed ABA sensitivity has been

reported in the coleorhiza of imbibing after-ripened barley grains (Barrero *et al*., 2009). Furthermore, increase in the transcript abundance of *CYP707A1* appeared to occur in imbibing after-ripened grains of *Brachypodium* (Barrero *et al*., 2012). Overall, a clear relationship has been demonstrated between the expression of ABA catabolic genes and ABA content of imbibing seeds in both dicot such as Arabidopsis and the monocot species such as barley (Millar *et al*., 2006; Okamoto *et al*., 2006), indicating the significance of ABA catabolism in modulating ABA level in imbibing seeds for completing germination.

In summary, by comparing the spatio-temporal expression patterns of GA and ABA metabolic genes between grains derived from dormant and non-dormant cultivars of barley, and between after-ripened and the respective dormant grains, the finding of this thesis provides further insights into the roles of GA and ABA biosynthesis and catabolism in regulating dormancy and germination. This study identified that *GA20ox2*, *GA3ox2*, *GA2ox3* and *GA2ox6* genes as regulators of endogenous GA level in the embryo while *GA20ox3*, *GA3ox1* and *GA2ox5* in the endosperm of imbibing barley grains (Figure 4.7). Of the ABA metabolism genes, the *CYP707A1* appears to play a significant role for regulating ABA level in imbibing barley grains, thereby dormancy and germination.

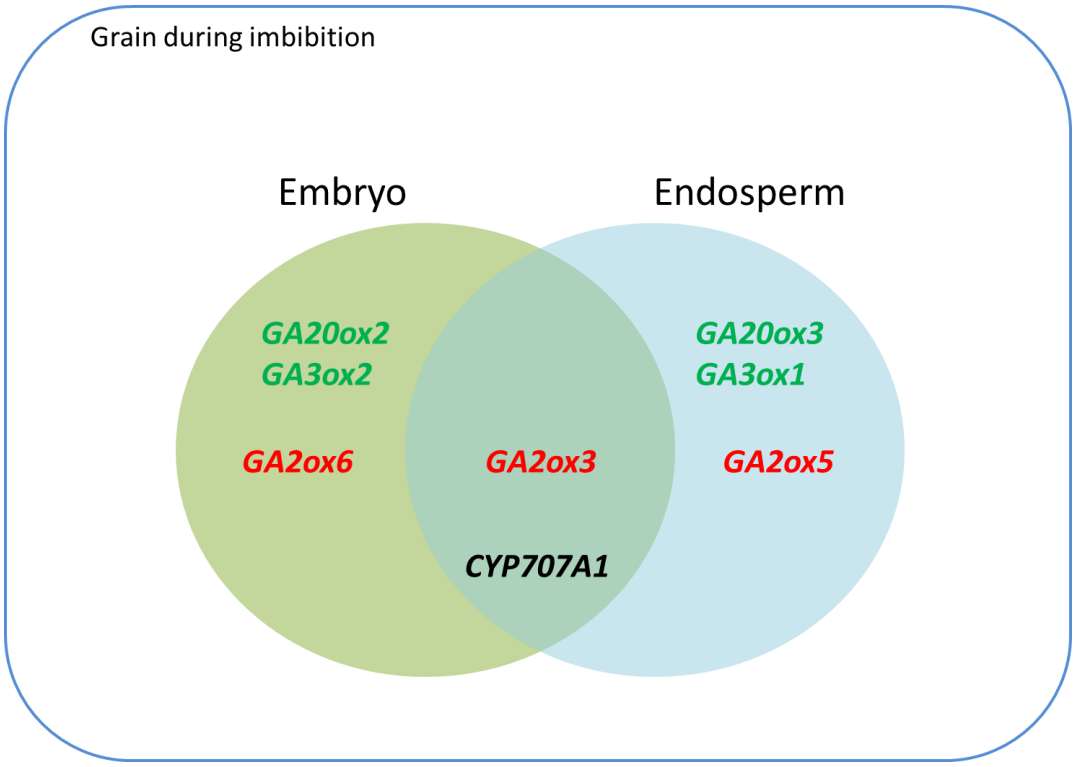

Figure 4.7 Schematic summary of the tissue-specificity of GA and ABA metabolism genes differentially expressed between dormant and non-dormant grains during imbibition. GA biosynthesis genes: *GA20ox2*, *GA20ox3*, *GA3ox1*, and *GA3ox2*; GA inactivation genes: *GA2ox3*, *GA2ox5*, and *GA2ox6*; ABA catabolic genes: *CYP707A1*.

5.0 General discussion and conclusions

Seed development/maturation, germination and dormancy are the most critical processes in seeds because of their major effect on the establishment of a new plant from mature seeds. Seed development comprises three different phases encompassing embryo morphogenesis, cell enlargement and reserve accumulation, and desiccation and developmental arrest. Germination of a quiescent mature seed is initiated by water uptake and terminated by radical emergence. On the other hand, seed dormancy refers to the failure of seed germination even under suitable environmental condition. Seed development, germination and dormancy are regulated by various environmental factors such as oxygen, moisture, light, temperature and nitrogen. Furthermore, these seed related processes are influenced by endogenous signaling factors including plant hormones. Among the several plant hormones known to date, GA and ABA are well known in regulating seed development, dormancy and germination of seeds thorough antagonistic interaction. In this study, the spatial and temporal expression patterns of GA and ABA metabolic genes were investigated during grain development, and imbibition with respect to dormancy and germination. Furthermore, the effect of exogenous ABA on the expression of GA and ABA metabolic genes during maturation of barley grains was examined.

The transcripts of nine out of the 10 GA metabolic genes and three out of the four ABA metabolic genes were detected during grain maturation, indicating the roles of these genes during this process. Relatively higher expression of *GA20ox3*, *GA3ox1* and *GA2ox5* in endosperm, and *GA3ox2* in embryo of developing barley grains suggest the tissue specific importance of these genes in regulating the level of bioactive GA required for appropriate grain development. The transcription of the ABA biosynthetic genes, *NCED1* and *NCED2*, in the endospermic tissue during the middle phase of grain maturation, and that of the ABA catabolic gene *CYP707A1* in both endospermic and embryonic tissues during the late stages

of grain maturation implies the spatial and temporal roles of these genes on the modulation of ABA level in maturing seeds. This study also indicated that exogenous ABA treatment alters the expression patterns of GA and ABA metabolic genes during grain maturation implicating that the metabolisms of GA and ABA phytohormones in maturing barley grains are subjected to regulation by seed ABA level.

This study furthermore characterized the spatiotemporal expression patterns of GA and ABA metabolism genes in imbibing barley grains with respect to dormancy and germination. Of the GA biosynthesis genes examined, two genes namely *GA20ox3* and *GA3ox1* were found to show higher level of expression in endosperm tissues of imbibing barley grains irrespective of dormancy status, indicating their role for occurrence of GA biosynthesis in this tissue of both dormant and non-dormant grains of barley during germination. On the other hand, the embryonic tissue of non-dormant grains showed high expression of other family members of *GA20ox* and *GA3ox* genes, namely *GA20ox2* and *GA3ox2*, implying that these genes play important role in controlling the synthesis of GA in the embryo. Tissue specific expression of selected members of the GA inactivating gene *GA2ox*, namely *GA2ox3* and *GA2ox6* was observed in the embryo of non-dormant grains, reflecting their participation in mediating GA catabolism as an additional process in the regulation of embryonic GA level. Our data also showed that such a role is played by another family member, namely *GA2ox5* in the endosperm of imbibing grains. With respect to the expression patterns of ABA metabolic genes, only a member of the ABA catabolic gene, *CYP707A* (*CYP707A1*) is found to exhibit association with seed dormancy and germination. It showed higher expression of in the embryo tissues from non-dormant barley grains (including those generated by after-ripening) during grain imbibition, implying a decline in seed ABA level due to increased ABA catabolic activity and in turn enhanced seed

germination. Similar expression pattern of this gene was observed in the endospermic tissue, reflecting the occurrence of ABA catabolism in this tissue and this may have importance in the regulation of germination in non-dormant grains.

In conclusion, this study provides further insights into the tissue specific role of different members of the GA and ABA metabolic gene families in regulating the three developmental events in seeds, development/maturation, dormancy and germination, and also enhances our understanding of the effect of ABA on the metabolic pathways of ABA and GA, and thereby seed maturation. The findings of this thesis overall suggest the potential for the GA and ABA metabolic genes differentially expressed between dormant and non-dormant seeds to be used as tools for molecular breeding to deal with preharvest sprouting in barley.

LITERATURE CITED

Audran C, Borel C, Frey A, Sotta B, Meyer C, Simonneau T, Marion-Poll A (1998) Expression studies of the zeaxanthin epoxidase gene in *Nicotiana plumbaginifolia*. Plant Physiology **118**: 1021-1028.

Audran C, Liotenberg S, Gonneau M, North H, Frey A, Tap-Waksman K, Vartanian N, Marion-Poll A (2001) Localisation and expression of zeaxanthin epoxidase mRNA in *Arabidopsis* in response to drought stress and during seed development. Australian Journal of Plant Physiology **28**(12) 1161-1173.

Badr A, Miller K, Schafer-Pregl R, Rabey HE, Effgen H, Ibrahim HH, Pozzi C, Rohde W, Salamini F (2000) On the origin and domestication history of barley (*Hordeum vulgare*). Molecular Biology and Evolution **17**(4): 499-510.

Baik BK, Ullrich SE (2008) Barley for food: characteristics, improvement, and renewed interest. Journal of Cereal Science **48**: 233-242.

Barrero JM, Jacobsen JV,Talbot MJ,White RG, Swain SM, Garvin DF, Gubler F (2012) Grain dormancy and light quality effects on germination in the model grass *Brachypodium distachyon*. New Phytologist **193**: 376-386.

Barrero JM, Talbot MJ, White RG, Jacobsen JV, Gubler F (2009) Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. Plant Physiology **150**: 1006-1021.

Baskin CC, Baskin JM (1998) Seeds – ecology, biogeography, and evolution of dormancy and germination. San Diego, CA, USA: Academic Press.

Baskin JM, Baskin CC (2004) A classification system for seed dormancy. Seed Science Research **14**: 1-16.

Becraft PW (2001) Cell fate specification in the cereal endosperm. Seminars in Cell and Developmental Biology. **12**(5): 387-394.

Benech-Arnold RL, Giallorenzi MC, Frank J, Rodriguez V (1999) Termination of hull-imposed dormancy in developing barley grains is correlated with changes in

embryonic ABA levels and sensitivity. Seed Science Research **9**: 39-47.

Berger F (1999) Endosperm development. Current Opinion in Plant Biology 2**:** 28-32.

Bethke PC, Lonsdale JE, Fath A, Jones RL (1999) Hormonally regulated programmed cell death in barley aleurone cells. Plant Cell **11**: 1033-1046.

Bewley JD (1997) Seed germination and dormancy. Plant Cell **9**: 1055-1 066.

Bewley JD, Black M (1994) Seeds: physiology of development and germination. 2nd edn. New York: Plenum Press.

Bewley JD, Fountain DW (1972) A distinction between the actions of abscisic acid, gibberellic acid and cytokinins in light-sensitive lettuce seed. Planta **102**: 368-371.

Black M, Butler J, Hughes M (1987) Control and development of dormancy in cereals. In: Mares DJ, ed. Proceedings of the Fourth International Symposium on Preharvest Sprouting in Cereals. Colorado: Westview Press, 379-392.

Bove J, Lucas P, Godin B, Ogé L, Jullien M, Grappin P (2005) Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. Plant Molecular Biology **57**: 593-612.

Bradford KJ, Benech-Arnold RL, Côme D, Corbineau F (2008) Quantifying the sensitivity of barley seed germination to oxygen, abscisic acid, and gibberellin using a population-based threshold model. Journal of Experimental Botany **59**(2): 335-347.

Bradford KJ, Nonogaki H (2007) Seed development, dormancy and germination. Oxford; Ames, Iowa : Blackwell Publishing.

Brink RA, Cooper DC (1947) The endosperm in seed development. Botanical Review **13**: 423-541.

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB (1999) Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*. The Plant Journal **17**: 427-431.

Ceccarelli S, Grando S, Maatougui M, Michael M, Slash M, Haghparast R, Rahmanian M, Taheri A, Al-Yassin A, Benbelkacem A, Labdi M, Mimoun H, Nachit M (2010) Plant breeding and climate change. Journal of Agricultural Science **148**: 627-637.

Chaudhury AM, Craig S, Dennis E, Peacock W (1998) Ovule and embryo development, apomixis and fertilization. Current Opinion in Plant Biology **1**: 26-31.

Chono M, Honda I, Shinoda S, Kushiro T, Kamiya Y, Nambara E, Kawakami N, Kaneko S, Watanabe Y (2006) Field studies on the regulation of abscisic acid content and germinability during grain development of barley: molecular and chemical analysis of pre-harvest sprouting. Journal of Experimental Botany **57**(10): 2421-2434.

Corbineau F, Côme D (1996) Barley seed dormancy. Boissons Bios Conditionnement **261**: 113–119.

Corbineau F, Lecat S, Côme D (1986) Dormancy of three cultivars of oat seeds (*Avena sativa L.*). Seed Science and Technology **14**: 725-735.

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annual Review of Plant Physiology and Plant Molecular Biology **49**: 557-583.

Cutler AJ, Krochko JE (1999) Formation and breakdown of ABA. Trends in Plant Science **4**: 472-478.

Debeaujon I, Koornneef M (2000) Gibberellin requirement for Arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. Plant Physiology **122**: 415-424.

Debeaujon I, Léon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. Plant Physiology **122**: 403-413.

Davies, Peter J (Ed.) (2010) Plant hormones. Kluwer Academic Publishers 3rd edn.

Derkx MPM, Karssen CM (1993) Effects of light and temperature on seed dormancy and gibberellin-stimulated germination in *Arabidopsis thaliana*: studies with gibberellin-deficient and –insensitive mutants. Physiologia plantarum **89**: 360-368.

Dewar J, Taylor JRN, Berjak P (1998) Changes in selected plant growth regulators during germination in sorghum. Seed Science Research **8**: 1-8.

Dubreucq B, Baud S, Debeaujon I, Dubos C, Marion-Poll A, Miquel M, North H, Rochat C, Routaboul JM, Lepiniec L (2010) Seed Development. Plant Developmental Biology - Biotechnological Perspectives **1**: 341-359.

Eisenreich W, Bacher A, Arigoni D, Rohdich F (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway. Cellular and Molecular Life Sciences **61**: 1401-1426.

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. New Phytologist **171**: 501-523.

Finkelstein R (2010) The Role of Hormones during Seed Development and Germination. Plant Hormones, pp. 549-573.

Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. Annual Review of Plant Biology **59**: 387-415.

Fox GP, Panozzo JF, Li CD, Lance RCM, Inkerman PA, Henry RJ (2003) Molecular basis of barley quality. Australian Journal of Agricultural Research **54**: 1081-1101.

Frey A, Godin B, Bonnet M, Sotta B, Marion-Poll A (2004) Maternal synthesis of abscisic acid controls seed development and yield in *Nicotiana plumbaginifolia*. Planta **218**: 958-964.

Fincher GB (1989) Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Annual Review of Plant Physiology and Plant Molecular Biology **40**: 305-346.

Gazzarrini S, Tsuchiya1 Y, Lumba S, Okamoto M, McCourt P (2004) The transcription factor *FUSCA3* controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. Developmental Cell **7**: 373-385.

Goldbach H, Michael G (1976) Abscisic acid content of barley grains during ripening as affected by temperature and variety. Crop Science **16**: 797-799.

Goldberg RB, de Paiva G, Yadegari R (1994) Plant embryogenesis: zygote to seed. Science **266**: 605-614.

González-Guzmán M, Apostolova N, Bellés JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodnguez PL (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. Plant Cell **14**: 1833-1846.

Goto N, Pharis RP (1999). Role of gibberellins in the development of floral organs of gibberellin-deficient mutant, *ga1-1*, of *Arabidopsis thaliana*. Canadian Journal of Botany **77**: 944-954.

Graeber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJJ (2012) Molecular mechanisms of seed dormancy. Plant, Cell and Environment **35:** 1769-1786.

Grappin P, Bouinot D, Sotta B, Miginiac E, Jullien M (2000) Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. Planta **210**: 279-285.

Groot SPC, Karssen CM (1987) Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. Planta **171**: 525-531.

Gubler F, Hughes T, Waterhouse P, Jacobsen J (2008) Regulation of dormancy in Barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. Plant Physiology **147**: 886-896.

Hedden P (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annual Review of Plant Physiology and Plant Molecular Biology **48**:431-60.

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends in Plant Science **5**: 523-530.

Helliwell CA, Sullivan JA, Mould RM, Gray JC, Peacock WJ, Dennis ES (2001) A plastid envelope location of *Arabidopsis ent*-kaurene oxidase links the plastid and

endoplasmic reticulum steps of the gibberellin biosynthesis pathway. The Plant Journal **28**: 201-208.

Hill RD, Liu JH, Durnin D, Lamb N, Shaw A, Abrams SR (1995) Abscisic acid structure-activity relationships in barley aleurone layers and protoplasts. Plant Physiology **108**: 573-579.

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Current Opinion in Plant Biology **4**: 210-218.

Hoang HH, Sechet J, Bailly C, Leymarie J, Corbineau F (2014) Inhibition of germination of dormant barley (*Hordeum vulgare* L.) grains by blue light as related to oxygen and hormonal regulation. Plant, Cell and Environment **37:** 1393–1403.

Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytologist **179**: 33-54.

Iglesias-Fernández R, Rodríguez-Gacio MC, Matilla AJ (2011) Progress in research on dry afterripening. Seed Science Research **21**(2): 69-80.

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, Macmillan J (1984) Internode length in *Pisum*. The *Le* gene controls the 3β-hydroxylation of gibberellin A_{20} to gibberellin A_1. Planta **160**: 455-463.

Itoh H, Tatsumi T, Sakamoto T, Otomo K, Toyomasu T, Kitano H, Ashikari M, Ichihara S Matsuoka M (2004) A rice semi-dwarf gene, *Tan-Ginbozu (D35)*, encodes the gibberellin biosynthesis enzyme, *ent*-kaurene oxidase. Plant Molecular Biology **54**: 533–547.

Jacobsen JV, Chandler PM (1987) Gibberellin and abscisic acid in germinating cereals. In PJ Davies, ed, Plant Hormones and their role in plant growth and development. Martinus Nijhoff, Boston, pp 164-193.

Jacobsen JV, Pearce DW, Poole AT, Pharis RP, Mander LN (2002) Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley.

Physiologia Plantarum **115**: 428-441.

Kanno Y, Jikumaru Y, Hanada A, Nambara E, Abrams SR, Kamiya Y, Seo M (2010) Comprehensive hormone profiling in developing Arabidopsis seeds: examination of the site of ABA biosynthesis, ABA transport and hormone interactions. Plant and Cell Physiology **51**: 1988-2001.

Karssen CM, Brinkhorstvanderswan DLC, Breekland AE, Koornneef M (1983) Induction of dormancy during seed development by endogenous abscisic-acid - studies on abscisic-acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. Planta **157**: 158-165.

Karssen CM, Zagorski S, Kepczynski J, Groot SPC (1989) Key role for endogenous gibberellins in the control of seed germination. Annals of Botany **63**: 71-80.

Kermode AR (2005) Role of abscisic acid in seed dormancy. Journal of Plant Growth Regulation **24**: 319-344.

Kim YC, Nakajima M, Nakayama A, Yamaguchi I (2005) Contribution of gibberellins to the formation of *Arabidopsis* seed coat through starch degradation. Plant Cell Physiology **46**: 1317-1325.

Komatsuda T, Pourkheirandish M, He C, Azhaguvel P, Kanamori H, Perovic D, Stein N, Graner A, Wicker T, Tagiri A, Lundqvist U, Fujimura T, Matsuoka M, Takashi M, Yano M (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proceedings of the National Academy of Sciences **104**: 1424-1429.

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. Current Opinion in Plant Biology **5**: 33-36.

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficent mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidoposis thaliana* (L.) Heynh. Theoretical and Applied Genetics **61**: 385-393.

Kremer RJ. Ben-Hammouda M (2009) Allelopathic plants. 19. barley (Hordeum vulgare L). Allelopathy Journal **24**(2): 225-242.

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. Seed Science Research **15**: 281-307.

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The Arabidopsis cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. The EMBO Journal **23**: 1647-1656.

Lanahan MB, Ho TH, Rogers SW, Rogers JC (1992) A gibberellin response complex in cereal *α*-amylase gene promoters. The Plant Cell **4**: 203-211.

Lefebvre V, North H, Frey A, Sotta B, Seo M, Okamoto M, Nambara E, Marion-Poll A (2006) Functional analysis of Arabidopsis *NCED6* and *NCED9* genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. The Plant Journal **45**: 309-319.

Lenoir C, Corbineau F, Côme D. (1986) Barley (*Hordeum vulgare*) seed dormancy as related to glumella characteristics. Physiologia Plantarum **68**: 301-307.

Leon-Kloosterziel KM, Gil MA, Ruijs GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JA, Koornneef M (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. The Plant Journal **10**: 655-661.

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F (2007) Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. Journal of Experimental Botany, **58** (3): 425-437.

Leymarie J, Robayo-Romero ME, Gendreau E, Benech-Arnold RL, Corbineau F (2008) Involvement of ABA in induction of secondary dormancy in barley (*Hordeum vulgare* L.) seeds. Plant and Cell Physiology **49**(12): 1830-1838.

Liu PP, Montgomery TA, Fahlgren N, Kasschau KD, Nonogaki H, Carrington JC (2007) Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed

germination and post-germination stages. The Plant Journal **52**: 133-146.

Liu A, Gao F, Kanno Y, Jordan MC, Kamiya Y, Seo M, Ayele BT (2013) Regulation of wheat seed dormancy by after-ripening is mediated by specific transcriptional switches that induce changes in seed hormone metabolism and signaling. PLoS ONE **8**(2): e56570.

Liu Y, Fang J, Xu F, Chu J, Yan C, Schläppi M, Wang Y, Chu C (2014) Expression patterns of ABA and GA metabolism genes and hormone levels during rice seed development and imbibition: a comparison of dormant and non-dormant rice cultivars. Journal of Genetics and Genomics **41**: 327-338.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25**:402-408.

Mano Y, Nemoto K (2012) The pathway of auxin biosynthesis in plants. Journal of Experimental Botany 1-20.

Marwat SK, Hashimi M, Khan KU, Khan MA, Shoaib M, Fazal-ur-Rehman (2012) Barley (*Hordeum vulgare* L.) a prophetic food mentioned in ahadith and its ethnobotanical importance. American-Eurasian journal of agricultural & environmental sciences **12** (7): 835-841.

McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK (1991) The *Viviparous-I* developmental gene of maize encodes a novel transcriptional activator. Cell **66**: 895-905.

McClintock B (1978) Development of the maize endosperm as revealed by clones. In: Subtelny S, Sussex IM, eds. The clonal basis of development. New York: Academic Press, Inc., 217–237.

Meimoun P, Mordret E, Langlade NB, Balzergue S, Arribat S, Bailly C, El-Maarouf-Bouteau H (2014) Is gene transcription involved in seed dry after-ripening? PLoS ONE **9**(1): e86442.

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F

(2006) Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8´-hydroxylase. The Plant Journal **45**: 942-954.

Morris PC, Maddock SE, Jones MGK, Bowles DJ (1985) Changes in the levels of wheat- and barley-germ agglutinin during embryogenesis *in vivo*, *in vitro* and during germination. Planta **166**: 407-413.

Nadeau CD, Ozga JA, Kurepin LV, Jin A, Pharis RP, Reinecke DM (2011) Tissue-specific regulation of gibberellin biosynthesis in developing pea seeds. Plant Physiology **156**: 897-912.

Nakaminami K, Sawada Y, Suzuki, M, Kenmoku H, Kawaide H, Mitsuhashi W, Sassa, T, Inoue, Y Kamiya Y, Toyomasu T (2003). Deactivation of gibberellin by 2-oxidation during germination of photoblastic lettuce seeds. Bioscience Biotechnology Biochemistry **67**: 1551-1558.

Nambara E, Marion-Poll A (2003) ABA action and interactions in seeds. Trends in Plant Science **8**: 213-217.

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annual Review of Plant Biology **56**: 165-85.

Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y (2010) Abscisic acid and the control of seed dormancy and germination. Seed Science Research **20**: 55-67.

Nester JE, Zeevaart JAD (1988). Flower development in normal tomato and a gibberellin-deficient (*ga-2*) mutant. American Journal of Botany **75**: 45-55.

Nikolaeva MG (1969) Physiology of deep dormancy in seeds. Izdatel'stvo "Nauka," Leningrad [Translated from Russian by Z. Shapiro, National Science Foundation, Washington, DC].

Nonogaki H, Bassel GW, Bewley JD (2010) Germination-still a mystery. Plant Science **179:** 574-581.

Ober ES, Setter TL (1990) Timing of kernel development in water stressed maize: water potentials and ABA concentrations. Annals of Botany **66**: 665-672.

Organization for Economic Co-operation and Development (OECD) (2004). Consensus document on compositional considerations for new varieties of barley (*Hordeum vulgare* L.): Key food and feed nutrients and anti-nutrients. Report No. 12, Environment Directorate, OECD, Paris.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during Arabidopsis seed germination. Plant Cell **15**: 1591-1604.

Ogé L, Bourdais G, Bove J, Collet B, Godin B, Granier F, Boutin JP, Job D, Jullien M, Grappin P (2008) Protein Repair L-Isoaspartyl Methyltransferase1 Is Involved in Both Seed Longevity and Germination Vigor in *Arabidopsis*. Plant Cell **20**: 3022-3037.

Oh E, Yamaguchi S, Hu JH, Yusuke J, Jung B, Paik I, Lee HS, Sun TP, Kamiya Y, Choi G (2007) PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the *GAI* and *RGA* promoters in Arabidopsis seeds. Plant Cell **19**: 1192-1208.

Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung W I, Choi G (2006) Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in Arabidopsis. The Plant Journal **47**: 124-139.

Okamoto M, Kuwahara A, Seo M, Kushiro T, Asami T, Hirai N, Kamiya Y, Koshiba T, Nambara E (2006) CYP707A1 and CYP707A2, which encode abscisic acid 8'-hydroxylases, are indispensable for proper control of seed dormancy and germination in Arabidopsis. Plant Physiology **141**: 97-107.

Okamoto M, Tatematsu K, Matsui A, Morosawa T, Ishida J, Tanaka M, Endo TA, Mochizuki Y, Toyoda T, Kamiya Y, Shinozaki K, Nambara E, Sek M (2010) Genome-wide analysis of endogenous abscisic acid-mediated transcription in dry and imbibed seeds of Arabidopsis using tiling arrays. The Plant Journal **62:** 39-51.

Olszewski N, Sun T-p, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell **14** (Suppl.): S61-S80.

Ooms JJJ, Leon-Kloosterziel KM, Bartels D, Koornneef M, Karssen CM (1993) Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana* (a comparative study using abscisic acid-insensitive abi3 mutants). Plant Physiology **102**: 1185-1191.

Opanowicz M, Hands P, Betts D, Parker ML, Toole GA, Mills EN, Doonan JH, Drea S (2011) Endosperm development in *Brachypodium distachyon*. Journal of Experimental Botany **62**(2): 735-748.

Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Current Opinion in Plant Biology **14**: 290-295.

Penfield S, King J (2009) Towards a systems biology approach to understanding seed dormancy and germination. Proceedings of the Royal Society B **276**: 3561-3569.

Phinney BO (1956) Growth response of single-gene dwarf mutants in maize to gibberellic acid. Proceedings of the National Academy of Sciences of the United States of America **42**: 185-189.

Probert RJ (2000) The role of temperature in the regulation of seed dormancy and germination. In Seeds: the ecology of regeneration in plant communities (ed. M. Fenner), pp. 261–292. CAB International, Wallingford.

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D (2012) Seed germination and vigor. Annual Review of Plant Biology **63**: 507-33.

Raz V, Bergervoet JHW, Koornneef M (2001) Sequential steps for developmental arrest in Arabidopsis seeds. Development **128**: 243-252.

Rieu I, Eriksson S, Powers SJ, Gong F, Griffiths J, Woolley L, Benlloch R, Nilsson O, Thomas SG, Hedden P, Phillips AL (2008) Genetic analysis reveals that C_{19}-GA 2-oxidation is a major gibberellin inactivation pathway in *Arabidopsis*. Plant Cell **20**: 2420-2436.

Robertson M, Walker-Simmons M, Munro D, Hill RD (1989) Induction of α-amylase inhibitor synthesis in barley embryos and young seedlings by abscisic acid and

dehydration stress. Plant Physiology **91**: 415-420.

Rodríguez-Gacio MC, Matilla-Vázquez MA, Matilla AJ (2009) Seed dormancy and ABA signaling: the breakthrough goes on. Plant Signaling and Behavior **4**(11): 1035-1048.

Rondanelli M, Opizzi A, Monteferrario F (2009) The biological activity of beta-glucans. Minerva Medica. **100**(3): 237-45.

Ross JJ, Murfet IC, Reid JB (1997) Gibberellin mutants. Physiologia Plantarum **100**: 550-560.

Saisho D, Takeda K (2011) Barley: emergence as a new research material of crop science. Plant and Cell Physiology **52**(5): 724-727.

Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata K, Mizutani M (2004) Arabidopsis *CYP707As* encode (1)-abscisic acid 89-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiology **134**: 1439-1449.

Sawada Y, Aoki M, Nakaminami K, Mitsuhashi W, Tatematsu K, Kushiro T, Koshiba T, Kamiya Y, Inoue Y, Nambara E, Toyomasu T (2008) Phytochrome- and gibberellin-mediated regulation of abscisic acid metabolismduring ermination of photoblastic lettuce seeds. Plant Physiology **146**: 1386-1396.

Schulte D, Close TJ, Graner A, Langridge P, Matsumoto T, Muehlbauer G, Sato K, Schulman AH, Waugh R, Wise RP, Stein N (2009) The international barley sequencing consortium - at the threshold of efficient access to the barley genome. Plant Physiology **149**: 142-147.

Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N (2011) ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. Journal of Experimental Botany **62**(8): 2615-32.

Seo, M, Hanada, A, Kuwahara, A, Endo, A, Okamoto, M, Yamauchi, Y, North, H, Marion-poll, A, Sun, T. P, Koshiba, T, Kamiya, Y, Yamaguchi, S, & Nambara, E. (2006). Regulation of hormone metabolism in Arabidopsis seeds: hytochrome

regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. The Plant Journal **48**: 354-366.

Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends in Plant Science **7**(1): 41-48.

Seo M, Peeters AJM, Koiwai H, Oritani T, Marion-Poll A, Zeevaart JAD, Koornneef M, Kamiya Y, Koshiba T (2000) The Arabidopsis aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proceedings of the National Academy of Sciences of the United States of America **97**: 12908-12913.

Shen Y, Philip Lansky E, Nevo E (2010) Wild barley—harbinger of biodiversity, biodiversity **11**: 3-4, 19-25.

Shewry PR (2009) Wheat. Journal of Experimental Botany **60**: 1537-1553.

Singh DP, Jermakow AM, Swain SM (2002) Gibberellins are required for seed development and pollen tube growth in Arabidopsis. The Plant Cell **14**: 3133-3147.

Sreenivasulu N, Borisjuk L, Junker BH, Mock HP, Rolletschek H, Seiffert U, Weschke W, Wobus U (2010) Barley grain development toward an integrative view. International Review of Cell and Molecular Biology **281**: 49-89.

Sreenivasulu N, Usadel B, Winter A, Radchuk V, Scholz U, Stein N, Weschke W, Strickert M, Close TJ, Stitt M, Graner A, Wobus U (2008) Barley grain maturation and germination: metabolic pathway and regulatory network commonalities and differences highlighted by new MapMan/PageMan profiling tools. Plant Physiology **146**: 1738-1758.

Sreenivasulu N, Radchuk V, Strickert M, Miersch O, Weschke W, Wobus U (2006) Gene expression patterns reveal tissue-specific signaling networks controlling programmed cell death and ABA-regulated maturation in developing barley seeds. The Plant Journal **47**: 310-327.

Sun T-p (2008) Gibberellin metabolism, perception and signaling pathways in Arabidopsis. The Arabidopsis Book 6:e0103.

Swain SM, Reid JB, Kamiya Y (1997) Gibberellins are required for embryo growth and seed development in pea. The Plant Journal **12**(6): 1329-1338.

Tan BC, Joseph LM, Deng WT, Liu LJ, Li QB, Cline K, McCarty DR (2003) Molecular characterization of the *Arabidopsis* 9-cis epoxycarotenoid dioxygenase gene family. The Plant Journal **35**: 44-56.

Taylor IB, Burbidge A, Thompson AJ (2000) Control of abscisic acid synthesis. Journal of Experimental Botany **51**: 1536-1574.

Tester M, Bacic A (2005) Abiotic stress tolerance in grasses. From model plants to crop plants. Plant Physiology **137**: 791-793.

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proceedings of the National Academy of Sciences of the United States of America **96**: 4698-4703.

Toyomasu T, Tsuji H, Yamane H, Nakayama M, Yamaguchi I, Murofushi N, Takahashi N, Inoue Y (1993) Light effects on endogenous levels of gibberellins in photoblastic lettuce seeds. Journal of Plant Growth Regulation **12**: 85-90.

Toyomasu T, Yamane H, Murofushi N, Inoue Y (1994) Effects of exogenously applied gibberellin and red light on the endogenous levels of abscisic acid in photoblastic lettuce seeds. Plant Cell Physiology **35**: 127-129.

Turkington TK, Tekauz A, Xi K, and Kutcher HR (2011) Foliar diseases of barley: don't rely on a single strategy from the disease management toolbox. Prairie Soils and Crops **4**:142-150.

Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochov R, Yu F, Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Rodermel S, Weiss D, Pichersky E (2007) Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. Plant Cell **19**: 32-45.

Varshney RK, Langridge P, Graner A (2007) Application of genomics to molecular

breeding of wheat and barley. Advances in Genetics **58**: 121-155.

Von Bothmer, Salomon B, Linde-Laursen I (1995) Variation for crossability in a reciprocal, interspecific cross involving *Hordeum vulgare* and *H. lechleri*. Euphytica **84**:183-187.

Walker-Simmons M (1987) ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiology **84**: 61-66.

White CN, Proebsting WM, Hedden P, Rivin CJ (2000) Gibberellins and seed development in Maize. I. evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. Plant Physiology **122**: 1081-1088.

Xiong L, Zhu JK (2003) Regulation of Abscisic Acid Biosynthesis. Plant Physiology **133**: 29-36

Yamaguchi S (2008) Gibberellin metabolism and its regulation. The Annual Review of Plant Biology **59**:225-51.

Yamaguchi S, Kamiya Y, Sun T-p (2001) Distinct cell-specific expression patterns of early and late gibberellin biosynthetic genes during *Arabidopsis* seed germination. The Plant Journal **28**, 443-453.

Yamauchi Y, Takeda-Kamiya N, Hanada A, Ogawa M, Kuwahara A, Seo M, Kamiya Y, Yamaguchi S (2007) Contribution of gibberellin deactivation by *AtGA2ox2* to the suppression of germination of dark-imbibed *Arabidopsis* thaliana seeds. Plant Cell Physiology **48**: 555-561.

Yang J, Zhang J, Liu K, Wang Z, Liu L (2006) Abscisic acid and ethylene interact in wheat grains in response to soil drying during grain filling. New Phytologist **171**: 293-303.

Zhou R, Cutler AJ, Ambrose SJ, Galka MM, Nelson KM, Squires TM, Loewen MK, Jadhav AS, Ross ARS, Taylor DC, Abrams SR (2004) A new abscisic acid catabolic pathway. Plant Physiology **134**: 361-369.

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z (2006) *ELONGATED*

UPPERMOST INTERNODE encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. Plant Cell **18**: 442-456.

Zohary D (1963) Spontaneous brittle six-row barleys, their nature and origin. Barley Genetics I; Proceedings of the first International Barley Genetics Symposium, Wageningen 27-30. Pudoc, Wageningen.

Zohary D; Hopf M (2001) Domestication of Plants in the Old World: The Origin and Spread of Cultivated Plants in West Asia, Europe, and the Nile Valley. Oxford University Press.

APPENDIX

Appendix 1-1 Relative abundance of *GA20ox*s transcripts during grain development in barley cv. Morex.

Gene	DAA[z]	Relative transcript level	SE[y]
GA20ox1	20	n.d.[x]	
	30	n.d.	
	40	n.d.	
	50	n.d.	
GA20ox2	20	n.d.	
	30	1.000000	0.0555
	40	1.102803	0.0174
	50	n.d.	
GA20ox3	20	2470.605764	55.7322
	30	755.551242	45.2774
	40	373.408757	67.8266
	50	n.d.	15.6775

[z]DAA: Days after anthesis, [y]SE: Standard error, [x]n.d.: not detected

Appendix 1-2 Relative abundance of *GA3ox*s transcripts during grain development in barley cv. Morex.

Gene	DAA[z]	Relative transcript level	SE[y]
GA3ox1	20	1.000000	0.0035
	30	1.165856	0.0533
	40	0.564740	0.0701
	50	0.192068	0.0446
GA3ox2	20	n.d.[x]	
	30	0.001509	0.0000
	40	0.006743	0.0006
	50	n.d.	

[z]DAA: Days after anthesis, [y]SE: Standard error, [x]n.d.: not detected

Appendix 1-3 Relative abundance of *GA2ox*s transcripts during grain development in barley cv. Morex.

Gene	DAA[z]	Relative transcript level	SE[y]
GA2ox1	20	1.000000	0.0086
	30	0.967677	0.0585
	40	1.047361	0.4236
	50	1.129352	0.5897
GA2ox3	20	16.506931	0.1428
	30	4.496834	0.2021
	40	5.591434	1.4552
	50	2.890722	0.5964
GA2ox4	20	n.d.[x]	
	30	1.597997	0.2197
	40	0.378976	0.0363
	50	60.417598	58.4979
GA2ox5	20	824.466513	135.6728
	30	661.296990	49.7690
	40	473.210583	29.5702
	50	270.928201	13.5805
GA2ox6	20	0.778867	0.1620
	30	0.154871	0.0075
	40	1.854554	0.3145
	50	0.732086	0.2692

[z]DAA: Days after anthesis, [y]SE: Standard error, [x]n.d.: not detected

Appendix 1-4 Relative abundance of *NCED*s transcripts during grain development in barley cv. Morex.

Gene	DAA[z]	Relative transcript level	SE[y]
NCED1	20	1.000000	0.1988
	30	1.336872	0.1625
	40	2.740364	0.4418
	50	0.537958	0.0270
NCED2	20	n.d.[x]	
	30	n.d.	
	40	n.d.	
	50	n.d.	

[z]*DAA: Days after anthesis,* [y]*SE: Standard error,* [x]*n.d.: not detected*

Appendix 1-5 Relative abundance of *CYP707A*s transcripts during grain development in barley cv. Morex.

Gene	DAA[z]	Relative transcript level	SE[y]
CYP707A1	20	1.000000	0.2262
	30	1.273968	0.1595
	40	7.187388	1.9839
	50	21.536965	2.6193
CYP707A2	20	1.701645	0.2605
	30	1.273519	0.1089
	40	n.d.[x]	
	50	n.d.	

[z]DAA: Days after anthesis, [y]SE: Standard error, [x]n.d.: not detected