Investigating the role of jasmonates and their interaction with gibberellins and abscisic acid in regulating wheat seed dormancy and germination

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Thesis submitted to the Faculty of Graduate Studies of

the University of Manitoba

in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

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Winnipeg

ACKNOWLEDGEMENT

I would like to express my sincerest thanks to my PhD. advisor, Dr. Belay T. Ayele for his great support and continued guidance throughout the course of my program. I would also like to thank Drs. Claudio Stasolla, Mark Jordan and Michele Piercey-Normore for serving on my advisory committee and providing me with valuable suggestions and comments.

I would like to extend my gratitude to all members of Dr. Ayele's group for their support and valuable discussions. It is also a pleasure to thank technical and administration staff of the Department of Plant Science.

I extend my appreciation to NSERC and Sir Gordon Wu Scholarships for financial support.

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ABSTRACT

Nguyen, Tran-Nguyen. PhD. The University of Manitoba, January, 2021. Investigating the role of jasmonates and their interaction with gibberellins and abscisic acid in regulating wheat seed dormancy and germination. Ph.D. Supervisor: Dr. Belay T Ayele.

Induction, maintenance and release of seed dormancy are crucial in cereal crops such as wheat (*Triticum aestivum* L.) to avoid precocious germination and at the same time make them quickly ready for industrial processing or planting. Seed dormancy is regulated mainly by two phytohormones; abscisic acid (ABA) induces and maintains seed dormancy whereas gibberellin (GA) releases it and, as a result, ABA–GA balance decides the dormancy status of seeds. Other hormones are also known to regulate seed dormancy mainly via their influences on ABA–GA balance. Jasmonates (JAs) have been reported to have apparent effects on seed dormancy in several plant species; however, only little is known if the effect of JAs on dormancy regulation involves ABA and GA. To this effect, this thesis investigated changes in the expression patterns of ABA, GA and jasmonate metabolism and signaling genes and the levels of the three hormones during seed maturation by using dormant and non-dormant genotypes, and in response to hormone, after-ripening or cold treatments. The findings of this thesis provide important insights into the roles of JAs and their interaction with ABA and GA in the regulation of dormancy in wheat.

FOREWARD

This thesis is written in manuscript style. A general introduction about the research project and a literature review precedes three manuscripts. An abstract, introduction, materials and methods, results and discussion form a complete manuscript. The manuscripts are followed by a general discussion and conclusions, a list of references and appendices. The three manuscripts that form the thesis are being finalized for submission to journals.

1 GENERAL INTRODUCTION

Seed dormancy is an important adaptive trait for the survival of plants in nature as well as for production of high-quality crop. Seeds of cultivated crops need to have sufficient level of dormancy at maturity to avoid precocious germination and also the ability to release their dormancy after harvest to allow their use for planting and industrial processing (Rodríguez et al., 2015; Tuan et al., 2018). Dormancy in cereal seeds can be caused by seed coat and/or embryo, although it is a complex trait controlled by several genetic and environmental factors. The coat of cereal seeds, which includes testa, pericarp and hull, can cause seed dormancy by interfering with the supply of substances required for germination or limiting the release of compounds that inhibit germination. For example, the hull of barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) seeds can limit oxygen supply to the embryos and prevent the seeds from releasing inhibitory substances such as ABA (Jacobsen et al., 2002; Bradford et al., 2008; Mendiondo et al., 2010; Hoang et al., 2013). Moreover, many phenolic compounds, which accumulate in the testa, pericarp and hull, have been found to be able to inhibit germination of barley, sorghum (*Sorghum bicolor* [L.] Moench) and wheat seeds (Krogmeier and Bremner, 1989). Another crucial factor that causes seed dormancy in cereals is the embryo, mainly due to hormone-induced suppression of the cellular activities required for germination (Bewley et al., 2013). In addition, the covering of the embryonic root, which is referred to as the coleorhiza or root sheath, can prevent the emergence of embryonic root, leading to dormancy of the embryos (Barrero et al., 2009; Jacobsen et al., 2013).

Seed dormancy and germination are regulated by several plant hormones, in which ABA, GA and their antagonistic interaction play key roles (Linkies and Leubner-Metzger, 2012; Shu et al., 2016). The roles of these two phytohormones in germination and dormancy are well characterized; ABA induces and maintains seed dormancy while GA releases dormancy and

promotes germination (Kucera et al., 2005). Studies mainly in the model plant Arabidopsis also revealed the roles of other phytohormones such as ethylene and JAs in the regulation of germination and dormancy (Linkies and Leubner-Metzger, 2012). For example, ethylene is considered as a germination-promoting hormone (Linkies and Leubner-Metzger, 2012) and its role in this regard has been shown to be mediated by its synergistic and antagonistic interactions with GA and ABA, respectively (Kucera et al., 2005; Holdsworth et al., 2008; Linkies et al., 2009). The molecular mechanisms responsible for the roles of ABA and GA in regulating seed dormancy in wheat and other cereal crop species have been examined by several studies (Rodríguez et al., 2015). However, the roles of other phytohormones hormones such as JAs in regulating seed dormancy in wheat and their interactions with ABA and GA has been scarcely studied.

Jasmonates, which include jasmonic acid (JA) and its derivatives such as methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile), and the sensitivity of seed tissue to JAs have been shown to have effects on seed dormancy and germination in Arabidopsis and other species, however their effects appear to be different depending on plant species/genotypes as well as the dormancy status of seeds (Linkies and Leubner-Metzger, 2012). Exogenous JA or MeJA could break seed dormancy in several plant species including tartaric maple (*Acer tataricum* L.) (Berestetzky et al., 1991), apple (*Malus domestica* Borkh.) (Ranjan and Lewak, 1992; Yildiz et al., 2007), brown mustard (*Brassica juncea* L.) (Sharma et al., 2018), pear (*Pyrus communis* L.) (Yildiz et al., 2007), Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) (Jarvis et al., 1997) and wheat (Jacobsen et al., 2013). However, other studies suggested that endogenous jasmonate content and sensitivity do not play important roles in the regulation of dormancy and germination. For example, seeds of Arabidopsis jasmonate biosynthesis and signaling mutants that are characterized by reduced jasmonate level and sensitivity are found to exhibit a germination

phenotype similar to that of wild-type (Staswick et al., 1992; Berger et al., 1996; Ellis and Turner, 2002; Footitt et al., 2002; Abe et al., 2003; Oh et al., 2009; Gangappa et al., 2010; Dave et al., 2011; Chen et al., 2012). Moreover, there is evidence that endogenous jasmonate levels and sensitivity might function as negative regulators of gemination. For example, jasmonate content was found to decrease during germination of Arabidopsis (Preston et al., 2009; Dave et al., 2011) and wheat (Liu et al., 2013; Kashiwakura et al., 2016; Martinez et al., 2016; Xu et al., 2016) seeds and germination is inhibited in seeds of Arabidopsis mutants that are characterized by increased jasmonate levels or sensitivity (Abe et al., 2003; Dave et al., 2011; Goossens et al., 2015). In agreement with these results, germination of tomato (*Solanum lycopersicum* L.) (Miersch et al., 2008), rapeseed (*Brassica napus* L.), flax (*Linum usitatissimum* L.) (Wilen et al., 1991), and yellow lupin (*Lupinus luteus* L.) (Zalewski et al., 2010) seeds can be inhibited by exogenous JAs.

Previous reports have shown the occurrence of both synergistic and antagonistic interactions between JAs and ABA in the regulation of seed dormancy and germination (Wilen et al., 1991; Staswick et al., 1992; Ellis and Turner, 2002; Nambara et al., 2010). Synergistic interaction between JAs and ABA in the regulation of dormancy and germination has been observed in Arabidopsis seeds overexpressing *MYC2* which encodes a positive regulator of jasmonate signaling; the seeds exhibited increased sensitivity to ABA and thereby inhibition of germination (Abe et al., 2003; Goossens et al., 2015). Whereas JAs appear to have antagonistic interaction with ABA in wheat seeds. For example, exogenous MeJA released wheat seed from dormancy and this was found to be associated with a decrease in ABA level which resulted from the changes in expression patterns of the ABA biosynthetic *9-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED*) and ABA catabolic *ABA 8′-HYDROXYLASE* (*CYP707A*) genes (Jacobsen et al., 2013). Despite this, the interaction between JAs with GA in the regulation of

wheat seed dormancy is less studied and the molecular mechanisms underlying the interaction of JAs with ABA and GA in wheat seeds are scarcely understood. Therefore, the underlying hypothesis of this thesis is that JAs have synergistic interaction with GA in breaking dormancy in wheat seeds. To test this hypothesis, this thesis investigated JAs, ABA and GA metabolism and signaling in wheat seeds during maturation in dormant and non-dormant genotypes and imbibition of seeds subjected to different dormancy releasing treatments. The specific objectives of the study are to 1) examine the metabolism and signaling of JAs, GA and ABA in maturing seeds in dormant and non-dormant genotypes; 2) analyze the changes in expression level of metabolic and signaling genes of JAs, GA and ABA in response to treatments with exogenous JAs and GA; and 3) investigate interaction between JAs, and GA and ABA during dormancy release by after-ripening and cold treatment.

2 LITERATURE REVIEW

2.1 Wheat is an important crop

Wheat is one of the most important cereal crops in the world since it is used as a major staple food by 2.5 billion people globally (Wheat, 2020). The Food and Agriculture Organization (FAO) of the United Nations (FAO, 2020) report indicated that, in 2018, wheat was the third most produced cereal crop globally with the total production of 734 million tons next to the 1148 million tons total production of maize (*Zea mays* L.) and 782 million tons total production of rice (*Oryza sativa* L.). However, the food supply quantity of wheat has been always higher than that of rice and maize since 1961 when FAO started recording the data. For example, in 2013 alone, the food supply quantity of wheat was 458 million tons while that of rice and maize were 377 and 125 million tons, respectively. Wheat is grown on approximately 97 million ha in Asia followed by 60.6, 35.5 and 10.2 million ha in Europe, Americas and Africa, respectively (FAO, 2020). In Canada, wheat has been the most produced crop since the 1920s, and its annual production is over 30 million tons in the last 5 years as compared to 20 and 14 million tons total production of rapeseed and maize, which are the second and third most important crops of Canada, respectively (Statistics Canada, 2020).

Like other crop species, wheat production has been faced with a variety of biotic and abiotic stresses such as diseases (Figueroa et al., 2018), insects (Dedryver et al., 2010), drought, and temperature stress (Abhinandan et al., 2018). Another major problem with production of quality wheat grain in both temperate and tropical areas has been pre-harvest sprouting, which causes an annual worldwide loss of US$ 1 billion (Gubler et al., 2005; Bewley et al., 2006). There has been a strong evidence that pre-harvest sprouting is associated with low level of seed dormancy at harvest in cereals (Mares and Mrva, 2001; Ogbonnaya et al., 2008; Mares et al., 2009; Gao and Ayele, 2014), highlighting the importance of understanding of wheat seed dormancy in order to

limit damages caused by pre-harvest sprouting. However, studying the molecular basis of the regulation of such physiological events in common wheat has been a challenge mainly due to the availability of very limited genome sequence information since it has a very large genome that is made up of highly repetitive sequences, and each gene has three homoeologs. This is because common wheat is a hexaploid species consisting of three subgenomes A, B and D in which the A and D subgenomes are derived from *Triticum urartu* (Thumanjan ex Gandilyan) and *Aegilops tauschiii* (Coss.), respectively; while the B subgenome is known to be related to *Aegilops speltoides* (Tausch).

2.2 Seed development, dormancy and germination

2.2.1 Seed development

Seed development is an important process in the life cycle of flowering plants. It can be divided into three main stages on the basis of dry matter accumulation including pre-filling, filling and post-filling (Fig. 2.1) (Leprince et al., 2016). The major process during the first stage is embryogenesis, the second stage is characterized mainly by organ expansion and maturation, and accomplishment of maturation is the key process during the last/third stage of seed development. The beginning and completion of seed filling stage is marked by the start and end of an increase in seed dry weight, respectively, and the seeds become physiologically mature at the end of this stage. The timing and duration for the start and end of each seed developmental stage, which are calculated as percentage of total seed development time, are different among species (Leprince et al., 2016). For example, the seed filling stage starts before 15% of the total seed development time in rice (Ellis et al., 1993), wild carrot (*Daucus carota* L.) (Gray et al., 1988), and camelina (*Camelina sativa* [L.] Crantz) (Rodríguez-Rodríguez et al., 2013), but it starts after 40% of the seed development time in cotton (*Gossypium hirsutum* L.) (Galau et al., 1987). The process of seed

maturation includes accumulation of storage reserves and acquisition of desiccation tolerance, longevity, dormancy and germination capacity. Accumulation of the raffinose family oligosaccharides and degradation of chlorophyll can start during filling stage, for example, in Brassicaceae species including Arabidopsis (Baud et al., 2002; Jolivet et al., 2011), or during post-filling stage, for example, in barrel clover (*Medicago truncatula* Gaertn.) (Righetti et al., 2015). Desiccation begins during the seed filling stage, and the developmental processes are supposed to be ceased when the seed moisture content is less than 20%. The process of desiccation continues until moisture equilibrium is attained between the seed and ambient air, and at this point the seed attains ripened stage in which its moisture content is about 10% (Leprince et al., 2016).

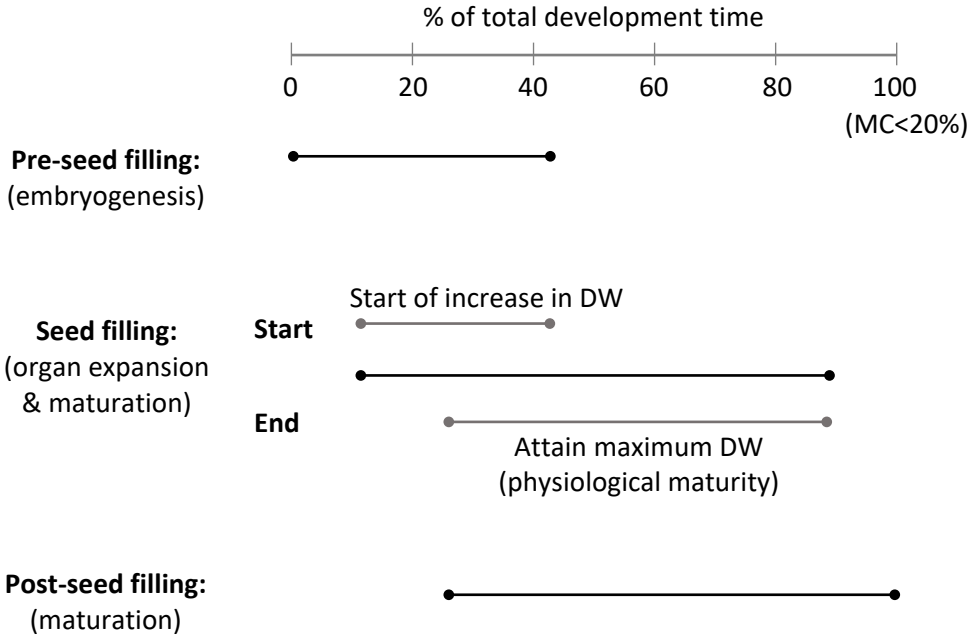

Figure 2.1. Schematic diagram of the three seed developmental stages and their relative starting and completion times. The relative time of a given stage, which is calculated as % of total development time, is different among species. The lines indicate relative time periods during which a developmental stage occurs [based on studies from 20 plant species; Leprince et al. 2016]. Major events during each stage are indicated in brackets. DW, dry weight; MC, moisture content.

Wheat seeds physiologically mature and attain maximum dry weight at 40 days after anthesis (DAA) or later depending on growth conditions and cultivars. Rogers and Quatrano (1983) indicated the presence of five stages of seed development in cv. Chinese Spring grown at 19ºC/12ºC (day/night) and 18/6-h photoperiod. The first 0 to 7 DAA is the stage that involves no embryo differentiation. Embryo differentiation occurs in the second stage (7-14 DAA), and embryonic tissues including primary root primordium, coleorhiza, leaf primordia, coleoptile, ventral scale and scutellum are observed by the end of this stage. Stage three (14-21 DAA) is characterized mainly by the presence of lateral root primordia and primary root cap. In stage four (21-31 DAA), primary leaves and lateral root caps appear, embryo almost accomplishes its growth, and scutellum starts to adhere to the endosperm. Finally, the adhesion of embryo to the surrounding tissue occurs in stage five (31-50 DAA). The seed fresh weight in this study reached a maximum value at 24 DAA and then noticeably reduced through 35 DAA; the seed dry weight, i.e. mainly for endosperm, started to increase at 10 DAA and continued to increase for many days after the fresh weight attained the maximum value. In another study performed by Wan et al. (2008), wheat cv. Hereward grown at 18ºC/15ºC (day/night) and 18/6-h photoperiod, the seeds attained a maximum fresh weight and dry weight at 35 and 42 DAA, respectively. Comparative analysis of the fresh weight of developing seeds of two different wheat cultivars, namely Jimai 20 and Zhoumai 16, grown under field conditions indicated the difference in duration of seed development depends on cultivars (Guo et al., 2012). In this study, Zhoumai 16 seeds were shown to reach their maximum fresh weight at 20 DAA while those of cv. Jamai 20 exhibited their maximum fresh weight at 31 DAA or later. Generally, a period of 3-5 weeks is required for wheat seeds to attain their maximum fresh weight, and then an additional period of 1-2 weeks is required for the seed to reach physiological maturation. Two important physiological processes are reported to occur during wheat seed maturation, which include quick desiccation of embryo and aleurone layer, and programmed cell death of starchy endosperm cells that is regulated mainly by ethylene (Young

and Gallie, 1999). The desiccation process continues after physiological maturity of the seed until moisture equilibrium with the ambient air is established (Fincher, 1989; Rodríguez et al., 2015).

2.2.2 Seed dormancy and its release

Seed dormancy is a characteristic feature by which viable intact seeds do not geminate or delay their germination under favorable conditions (Hilhorst et al., 2010). Dormancy can be classified as primary dormancy and secondary dormancy. Primary dormancy is set during development and maturation of the seeds (Fig. 2.2), and released by after-ripening or cold-stratification (Hilhorst, 2007; Hilhorst et al., 2010; Shu et al., 2016). This is a common phenomenon found in many plant species (Bewley et al., 2013). In wheat, the seeds obtain the highest degree of primary dormancy at their physiological maturity when they have maximum dry weight and the spikes and flag leaf turn yellow (Hanft and Wych, 1982; Gerjets et al., 2009). This kind of dormancy can be further classified based on the mechanisms underlying the induction of dormancy as reported by Baskin and Baskin (2004), and this classification includes physiological dormancy, morphological dormancy, morphophysiological dormancy, physical dormancy, and combinational dormancy. Secondary dormancy can develop in seeds either with or without primary dormancy in response to environmental conditions that are unfavorable for germination (Chen et al., 2014; Rodríguez et al., 2015).

In response to environmental signals, seeds can set several cycles of secondary dormancy, inducing and relieving dormancy or tuning dormancy level repeatedly, until the environment is optimum for seedling establishment (Fig. 2.2) (Footitt et al., 2011; Née et al., 2017). Soil temperature and moisture, light and nitrate are among the main environmental factors affecting secondary dormancy. Soil temperature and moisture influence seed dormancy level and seed sensitivity to other environmental signals such as light and nitrate. The induction of secondary dormancy has been reported for cereal seeds imbibed at high (30ºC) or low temperature (10-15ºC)

(Rodríguez et al., 2015). In addition, environmental factors such as blue light are other factors that are able to induce secondary dormancy in cereals (Hoang et al., 2014).

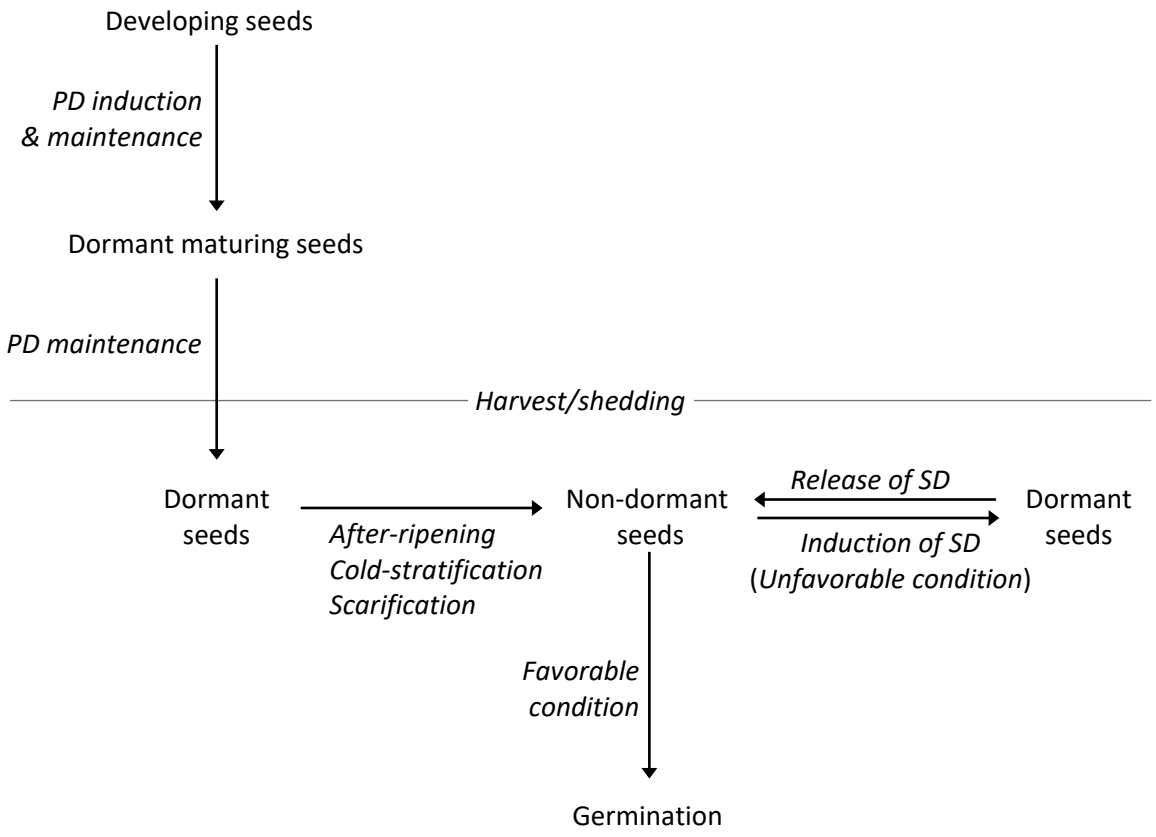

Figure 2.2. Schematic diagram of induction, maintenance and release of seed dormancy. Primary dormancy (PD) is set during seed development. Secondary dormancy (SD) can be induced and released repeatedly in response to changes in environmental factors. Seeds can be released from PD and/or SD by different treatments including after-ripening, cold-stratification or scarification.

Dormancy attains its highest level at the physiological maturity of the seeds. Seeds often maintain this elevated level of dormancy until their harvest maturity, and then release of dormancy occurs during dry storage, which is known as dry after-ripening (Rodríguez et al., 2015). Seed moisture content plays an important role in dormancy release through after-ripening. In general, dormancy is effectively released at seed moisture content ranging from 5 to 18% and lower

moisture level can prevent dormancy release (Bazin et al., 2011). Other methods to rapidly release dormancy include cold-stratification (seeds are imbibed under cold condition), and seed scarification (Finkelstein et al., 2008). In contrast, dormancy loss may start right after physiological maturity, or even before seeds obtain this status, which is often found in varieties that are susceptible to pre-harvest sprouting (Rodríguez et al., 2015).

2.2.3 The need of appropriate level of dormancy

Dormancy is a trait that is important for the survival of plants in nature. It blocks the germination of seeds due to the occurrence of a short period of favorable environmental condition during a season that is not conducive for seeding establishment and growth. This ensures that the seeds germinate when the condition is favorable not only for germination but also for the later stages of growth (Bewley, 1997; Ishibashi et al., 2017). A variation in dormancy level has been reported among individual seeds in a population, even when they are from a single plant (Simons and Johnston, 2006; Burghardt et al., 2016). This variation could protect the siblings of a germination season from an overall destruction due to a short period of extreme condition. It is also a mechanism to alleviate competition among the siblings (Nonogaki, 2014; Née et al., 2017). In contrast to the strong and various levels of dormancy as survival advantages in wild plants, weak dormancy at grain maturity is required to induce uniform germination, a phenotype that facilitates the use of mechanization in the cultivation and management of field crops. Industrial processing, which involves germination, such as malting, requires grains that have low level of dormancy (Rodríguez et al., 2015). As a result, weak dormancy has been a preferred crop breeding trait and the requirement of such weak dormancy level in seeds led to selection against dormancy (Harlan, 1992; Ishibashi et al., 2017). For example, it is understood that there is a link between pericarp color and dormancy level in rice, and selection against red pericarp color during rice domestication,

therefore, involved selection against seed dormancy (Sweeney et al., 2006; Gu et al., 2011).

Weak dormancy is often accompanied by the risk of pre-harvest sprouting which is a serious agronomic problem damaging the yield, grain quality, grain viability, and grain longevity (Rodríguez et al., 2015; Shu et al., 2015). Mature seeds with low level of dormancy can geminate on the mother plant before harvesting when they are exposed to wet conditions, particularly when it is associated with cooler temperatures (Biddulph et al., 2005; Bewley et al., 2013). Genetic studies have provided strong evidence that weak dormancy at harvest is the predominant cause of pre-harvest sprouting in cereals (Mares and Mrva, 2001; Ogbonnaya et al., 2008; Mares et al., 2009). Seeds of cereal crops damaged by pre-harvest sprouting have high activity of α-amylases and proteases; this is because the germination of seeds due to wet conditions triggers the expression of these enzymes, which initiates the degradation of starch and protein reserves in the endosperm for use by the growing embryo (Gualano et al., 2007; Rodríguez et al., 2015). Seeds with severely degraded endosperm often are downgraded to animal feed because of their poor quality for high value products such as bread and beer (Clarke et al., 1984). Damages caused by pre-harvest sprouting have been recorded for most of the world's major cereal crops such as rice (Juliano et al., 1987), wheat (King, 1983), maize (Neill et al., 1987), and sorghum (Maiti et al., 1985). It is estimated that the annual worldwide loss caused by pre-harvest sprouting for winter wheat is estimated to be up to US$ 1 billion (Bewley et al., 2006)

Given all these factors, seed of cultivated crops, especially grains, need to have a sufficient level of dormancy at maturity to avoid pre-harvest sprouting and an ability to release their dormancy quickly enough to allow their use for industrial processing or planting to obtain next season's crop.

2.2.4 Seed germination

The process of germination is completed with radicle protrusion, which marks the initiation of seedling establishment. It starts at the beginning of imbibition, however, it is the endogenous and environmental conditions during the early phase of imbibition which decide that a seed would germinate or not (Née et al., 2017). In cereals, GA produced in the embryo or scutellum diffuses into the aleurone layer to enhance the synthesis and release of α-amylase that degrades endospermic starch granules (Martinez et al., 2016). The buildup of adenosine triphosphate (ATP) in germinating seeds might reflect their high respiratory activity (Møller, 2001), and ATP serves as source of energy to fuel the germination process. Bewley and Black (1994) described germination as a two-stage process, of which the first stage is characterized by the rapid depletion of oxygen inside the seeds, and in the second stage, the seed internal conditions are almost anaerobic. It has been suggested that, for example in barley seeds, the second stage starts within few hours (3-5 h) after imbibition and lasts until radicle protrusion which occurs from 12-24 hours after the start of imbibition (HAI). Radicle protrusion gradually brings the oxygen conditions inside the seed to aerobic, which facilitates the active mobilization of storage reserves necessary for seedling development (Ma et al., 2016). Higher respiration rate in germinating seeds could also be accompanied by a higher production of reactive of oxygen species such as superoxide from the side reactions of mitochondrial metabolism (Møller, 2001).

2.3 Factors regulating seed dormancy and germination

Seed dormancy and germination are regulated by universal factors as well as species-specific factors. These regulatory factors could be hormonal or non-hormonal, and endogenous or exogenous factors (Fig. 2.3) and they often interact with one-another when performing their regulatory roles (Nonogaki, 2014). Several previous studies have provided strong evidence that

ABA and GA are the core and highly conserved hormonal factors regulating seed dormancy and germination among plant species (Nonogaki and Nonogaki, 2017).

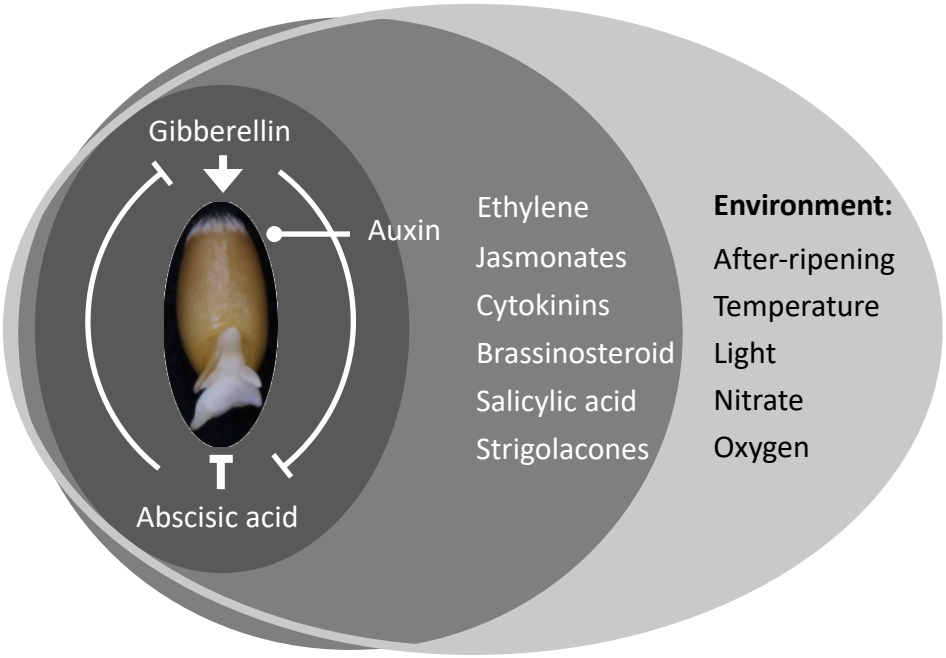

Figure 2.3. Endogenous and exogenous factors regulating seed dormancy and germination. Gibberellins (GA) and abscisic acid (ABA) exert major influences in which GA promotes germination while ABA inhibits it, and they antagonize each other's biosynthesis and signaling. Auxin is also considered as a major regulator of dormancy and germination; although its influences still need to be characterized. Other hormones, and environmental factors regulate germination mainly through GA and ABA.

2.3.1 Embryo and seed coat

2.3.1.1 Embryo dormancy

Seed dormancy can result from embryo-based dormancy and/or coat-imposed dormancy (Bewley et al., 2013). In some plant species, such as coffee (*Coffea arabica* L.) and celery (*Apium graveolens* L.), embryos are not mature when they are shed from mother plants. Germination, therefore, cannot occur until the embryo enters the maturation phase and finishes its growth in the

dispersed seed. In other species, mature embryos can be dormant. This type of dormancy is termed as “physiological dormancy”, and it is maintained by suppression of several embryonic cellular activities that are needed for germination (Bewley, 1997). It appears that ABA–GA balance is the key regulator of this suppression, making physiological dormancy reversible, i.e. primary dormancy can be lost and several cycles of secondary dormancy can be induced (Bewley et al., 2013). Coleorhiza, the embryonic tissue that covers the seminal roots of monocot seeds, is the main cause of dormancy in embryos of cereal seeds such as barley as it inhibits root emergence. Dormancy release by after-ripening in these seeds is associated with the change in ABA metabolism and sensitivity in the coleorhiza (Barrero et al., 2009).

2.3.1.2 Mechanical and physical restraints by the seed coat

The seed coat or layers covering the embryo includes dead or alive tissues of seed and fruit such as endosperm, perisperm, testa, pericarp and hulls. The coat can regulate seed dormancy through a mechanical, physical and/or chemical mechanisms, and if the embryos are not dormant, they can grow when the coat is removed (Rodríguez et al., 2015). The coat can simply place mechanical restraint that is strong enough to suppress normal embryo growth, or it can interfere with water uptake, gas exchange and substance diffusion required for germination. In Arabidopsis and tomato, the embryo is covered by testa and endosperm of which the latter plays an important role in germination. Endosperm weakening, particularly at the region surrounding the radicle tip, can have significant effects on radical emergence (Nonogaki, 2014).

In cereals such as barley and oat, the hull-imposed dormancy results from the limited diffusion of oxygen into the embryo during imbibition, which is mainly due to oxidative reaction in the hulls and this can increase the sensitivity of the embryo to ABA and alter ABA metabolism in the embryo. The same condition, in contrast, reduces embryo sensitivity to GA (Bradford et al.,

2008; Hoang et al., 2013; Rodríguez et al., 2015). Moreover, the hull can act as a barrier preventing the release of inhibitory substances, such as ABA, accumulated in the seed of these species during imbibition (Jacobsen et al., 2002; Mendiondo et al., 2010).

2.3.1.3 Chemical effects of the coat

The coat can release chemical substances that have inhibitory effects on germination. In cereal seeds, the testa, pericarp and hull accumulate various phenolic compounds, of which many have been found to be able to inhibit germination of barley, sorghum and wheat seeds (Krogmeier and Bremner, 1989). The red, brown, purple or black color of maize, rice or wheat seed coat is conferred by phenolic compounds (Weidner et al., 2000; Tian et al., 2004), and the dormancy level in wheat tend to be higher in cultivars with red pericarp than those with white pericarp (Paterson and Sorrells, 1990). It is, therefore, possible that some of the coat phenolic compounds contribute to dormancy regulation by diffusing into embryo during imbibition (Rathjen et al., 2009).

2.3.1.4 Genetic effects of the coat

Maternal expression of seed dormancy can be caused by the coat (Nonogaki and Nonogaki, 2017). Maternal effects on seed dormancy through properties of testa which is derived from a maternal tissue have been reported (Debeaujon et al., 2000). Alternatively, genomic imprinting in the endosperm, which occurs during seed development, is possibly involved in seed dormancy and germination. Piskurewicz et al. (2016) identified a group of imprinted genes which are expressed in Arabidopsis endosperm during imbibition. Ninety-three out of these imprinted genes were maternally expressed genes, of which nine genes including *SULFOTRANSFERASE 1* (*SOT1*), *PRX16, BYPASS3* (*BPS3*), *ATP-BINDING CASSETTE G30* (*ABCG30*), *CYSTEINE PROTEINASE1* (*CP1*), *HISTIDINE-CONTAINING PHOSPHOTRANSMITTER 3* (*AHP3*), *CELL*

WALL/VACUOLAR INHIBITOR OF FRUCTOSIDASE 2 (*C/VIF2*), *KINESIN-LIKE PROTEIN 1AT3G44730* (*KP1*) and *JUMONJI DOMAIN-CONTAINING PROTEIN 20* (*JMJ20*), were shown to regulate dormancy or germination. Importantly, seven of these genes were identified in dormant seed coats while only two genes were identified in non-dormant seed coats. The regulation of dormancy or germination by *SOT1, PRX16, ABCG30* and *AHP3* were related to ABA. These suggest that the implementation of maternal inheritance of dormancy can happen through preferentially expressing maternal alleles.

2.3.2 Abscisic acid and gibberellins

2.3.2.1 Abscisic acid and gibberellins metabolism and signaling components

The level of ABA in plant tissues is regulated by its biosynthesis and catabolism. NCED and CYP707A are important enzymes in ABA synthesis and catabolism, respectively (Fig. 2.4a) (Xiong and Zhu, 2003). NCEDs catalyze a committed step which convert 9'-cis-neoxanthin to xanthoxin in the ABA biosynthesis pathway. By contrast, CYP707As catalyze the first step of a major pathway which convert ABA to phaseic acid, a biologically inactive compound (Schwartz et al., 2003). Components of ABA signaling pathway (Fig. 2.4b) include PYRABACTIN RESISTANCE-LIKE (PYL) that acts as ABA receptor, PROTEIN PHOSPHATASE 2CS (PP2C), SNF1-RELATED PROTEIN KINASE2 (SnRK2) and ABSCISIC ACID INSENSITIVE (ABI)3, 4 and 5, among which only PP2Cs negatively regulate the response of plant tissue to ABA (Hubbard et al., 2010).

The enzymes, which function in GA metabolic pathway (Fig. 2.4c), are *ENT*-COPALYL DIPHOSPHATE SYNTHASE, *ENT*-KAURENE SYNTHASE, *ENT*-KAURENE OXIDASE, *ENT*-KAURENOIC ACID OXIDASE, GA 2-OXIDASE (GA2ox), GA 13-OXIDASE, GA 20-OXIDASE (GA20ox) and GA 3-OXIDASE (GA3ox). The final steps in the synthesis of biologically active GA_1 and GA_4, are catalyzed by GA20ox and GA3ox enzymes; and these active

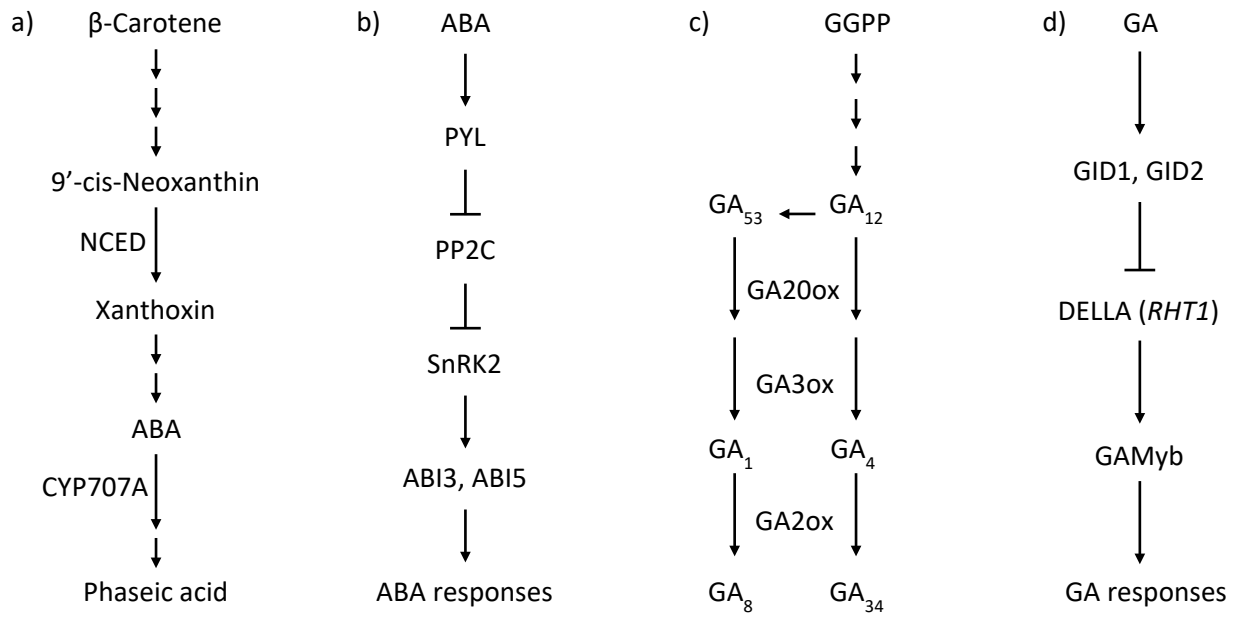

Figure 2.4. Simplified pathways of abscisic acid (ABA) and gibberellin (GA) metabolism and signaling. The pathways of ABA metabolism (a), ABA signaling (b), GA metabolism (c), and GA signaling (d). GA_1 and GA_4 are known bioactive GA in plants. ABI, ABSCISIC ACID INSENSITIVE; GA2ox, GA 2-OXIDASE; GA3ox, GA 3-OXIDASE; GA20ox, GA 20-OXIDASE; GGPP, geranylgeranyl diphosphate; GID, GIBBERELLIN INSENSITIVE DWARF; NCED, 9-CIS-EPOXYCAROTENOID DIOXYGENASE; PYL, PYRABACTIN RESISTANCE-LIKE; RHT, REDUCED HIEGHT; SnRK, SNF1-RELATED PROTEIN KINASE.

GA are inactivated mainly by GA2ox (Yamaguchi, 2008; Salazar-Cerezo et al., 2018). In Arabidopsis seeds, *GA3ox1* has been reported to be a rate-limiting gene of GA biosynthesis (Nonogaki, 2014). However, in rice seeds, *GA3ox1* has been found to be insufficient and *GA3ox2* is essential for synthesis of biologically active GA (Kaneko et al., 2002). An important element of GA signaling (Fig. 2.4d) is DELLA which functions as a negative regulator in GA signaling pathway and is subjected to degradation in response to elevated level of bioactive GA. Other components of GA signaling include GIBBERELLIN INSENSITIVE DWARF1 (GID1), GID2, and the transcription factor GAMyb, which positively regulate GA signaling. GID1 is a GA receptor, and its binding to GA makes GID1 able to interact with DELLA. GID2 is a F-box protein

of S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1) - CULLIN1 (CUL1) - F-BOX PROTEIN (SCF) E3 ubiquitin ligase complex which can bind to phosphorylated DELLA. The interactions of DELLA with GID1 and GID2 lead to the degradation of DELLA (Woodger et al., 2003; Gomi et al., 2004; Davière and Achard, 2013).

2.3.2.2 The balance between abscisic acid and gibberellins

Abscisic acid and GA play key antagonistic roles in seed dormancy and germination (Rodríguez et al., 2015; Shu et al., 2016). Therefore, it is the balance between ABA and GA which regulates the germination capacity and the induction/re-induction, expression and release of dormancy. This balance works in embryos and endosperm during seed development and imbibition. It involves the ABA and GA biosynthesis and catabolism, the sensitivity of seed tissues to ABA and GA, and the antagonistic relationship between the two hormones in their metabolism as well as signaling. An imbalance in favor of ABA is required for inducing and maintaining dormancy while that in favor of GA is required for dormancy release and germination (Finkelstein et al., 2008; Graeber et al., 2012; Gazzarrini and Tsai, 2015).

Abscisic acid is synthesized and accumulated in embryos to impose and maintain seed dormancy during seed development or imbibition (Kucera et al., 2005; Nambara and Marion-Poll, 2005). In seeds of cereal crops such as barley, sorghum and wheat, the embryonic ABA content peaks at around physiological maturity and sharply decreases by harvest maturity (Walker-Simmons, 1987; Steinbach et al., 1997; Benech-Arnold et al., 1999). Gibberellins play a vital role in the regulation of development and growth in plants (Yamaguchi, 2008). In developing seeds of Arabidopsis and maize, GA level peaks at two different stages, the embryo differentiation and the end of the maturation, reflecting its important role in promoting cell growth and expansion and

seed germination, respectively (Locascio et al., 2014). However, there is ample evidence for the role of ABA–GA balance, not ABA or GA alone, in establishing dormancy plan for the seed since the early stage of its development (Finch-Savage and Leubner-Metzger, 2006; Tuan et al., 2018). In maize, the seeds of mutants that are defective in ABA synthesis germinate on the ear when the kernel just reaches halfway in its development. However, when the appropriate ABA–GA balance is re-established via GA deficiency early in seed development, the seeds mature normally and acquire desiccation tolerance and storage longevity (White et al., 2000). Nevertheless, dormancy in developing sorghum grains has been reported not to show any correlation with either endogenous ABA or GA content (Benech-Arnold et al., 2000). Figure 2.5 shows the schematic diagram of ABA and GA accumulation during seed development and desiccation (Locascio et al., 2014). The antagonism in their accumulation could results in the peak of ABA/GA ratio occurring around physiological maturity of the seeds.

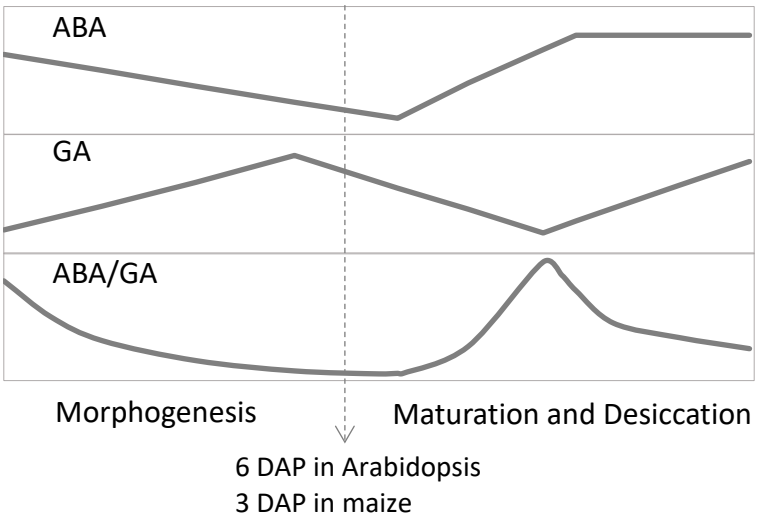

Figure 2.5. Schematic diagram of abscisic acid (ABA) and gibberellins (GA) accumulation during seed development. Created using data reported in Locascio et al. (2014). ABA/GA, ratio of ABA to GA; DAP, day after pollination.

2.3.2.3 Abscisic acid in the regulation of dormancy

Changes in dormancy level have been reported to be linked with both ABA content and ABA sensitivity; however, dormancy level in some species has been shown to depend only on ABA sensitivity (Kawakami et al., 1997; Rikiishi and Maekawa, 2010; Schramm et al., 2012). ABA catabolism, which is mainly catalyzed by CYP707As (Fig. 2.4a), plays an important role in regulating ABA level (Kushiro et al., 2004). Arabidopsis plants expressing the *TaCYP707A1-B* of wheat was shown to exhibit reduced ABA level and enhanced germination phenotypes (Son et al., 2016). Wheat plants with deletion mutation in *TaCYP707A1-A* and an insertional mutation in *TaCYP707A1-D* show a higher ABA content in developing embryos and an increase in dormancy (Chono et al., 2013). ABA synthesis is subjected to positive feedback regulation, i.e. ABA induced expression of the rate-limiting ABA synthesis gene *NCED* (Fig. 2.4a) in Arabidopsis. Nonogaki et al. (2014) have demonstrated that a chimeric *NCED* gene can be transformed to Arabidopsis seeds to enhance ABA synthesis and expression of native *NCED* genes as well leading to amplification of ABA synthesis and consequent hyperdormancy in the seeds. In many cases, however, without the involvement of ABA sensitivity, the difference in endogenous ABA content can hardly explain the differences in dormancy dynamics. For example, both ABA content and ABA sensitivity at late maturation stages of embryos have been reported to be correlated with differences in timing of dormancy release between two barley cultivars (Benech-Arnold et al., 1999). Similarly, the expression of primary and secondary dormancy during imbibition of mature seeds of different plant species such as Arabidopsis, oat and barley is associated with the maintenance of both higher ABA content and ABA sensitivity (Corbineau et al., 1993; Millar et al., 2006; Leymarie et al., 2008).

Although dormancy is regulated by both ABA content and seed sensitivity to ABA, several

reports indicate the role of ABA sensitivity alone in the regulation of dormancy. For example, differences in dormancy level between short-lived and long-lived dormancy genotypes of sorghum and wheat are poorly linked to ABA content. Rather, seeds from short-lived dormancy genotypes show precocious germination and reduced embryo sensitivity to ABA during late stages of maturation as compared to seeds from long-lived dormancy genotypes (Walker-Simmons, 1987; Steinbach et al., 1997). The importance of ABA sensitivity in regulating dormancy has been shown in three ABA hypersensitive mutants of white spring wheat cv. Zak that display strong dormancy and slower dormancy release by after-ripening as compared to the wild-type Zak (Schramm et al. (2013). Furthermore, Martinez et al. (2016) found no significant change in embryo ABA content in wheat *ENHANCED RESPONSE TO ABA8* (*ERA8*) mutant, which shows elevated level of dormancy, as compared to the corresponding wild-type Zak, and this suggests that the increased dormancy in the mutant is due to increased ABA signaling. Other studies have also shown that dormancy of intact grains in other species such as barley, oat, rice, sorghum and wheat exhibit close correlation with the sensitivity of their isolated embryos to ABA (Corbineau et al., 1991; Steinbach et al., 1995; Benech-Arnold et al., 2006; Gianinetti and Vernieri, 2007; Gerjets et al., 2009)

Embryo sensitivity to ABA in seeds depends on several factors related to ABA signaling pathway (Fig. 2.4b) including the activity and metabolism of ABA signaling components (Benech-Arnold et al., 2006). The transcription factor VIVIPAROUS1 (VP1), which is also referred to as ABI3 (Fig. 2.4b), positively regulates ABA signaling in developing seeds of maize (McCarty et al., 1989). It has been reported that mis-splicing of the genes encoding ABI3 in wheat (*TaABI3*) and rice (*OsABI3*) genotypes causes earlier dormancy loss (Fan et al., 2007), and it appears that *TaABI3* contributes to pre-harvest sprouting resistance by enhancing dormancy in wheat

(McKibbin et al., 2002; Wilkinson et al., 2005; Huang et al., 2012). However, its expression level in embryos of developing seed of sorghum has been shown not to be related to dormancy levels (Carrari et al., 2001). In developing embryo of rice, VP1 up-regulates the expression of *SEED DORMANCY 4* (*Sdr4*) encoding a protein (Os07g0585700) which is necessary for germination inhibition by ABA. Furthermore, seeds expressing *Sdr4-k*, a strong dormancy allele, have been reported to be more sensitive to exogenous ABA than those expressing the weak dormancy allele, *Sdr4-n* (Sugimoto et al., 2010). Reduction in ABA sensitivity along with decrease in ABA content through catabolic activity of CYP707A1 has also been observed in the coleorhiza of barley during imbibition of after-ripened seeds, and this reduction in ABA sensitivity is mediated through the regulation of LIPID PHOSPHATE PHOSPHATASE (LPP) and ABA-INTERACTING PROTEIN2 (AIP2), the two negative regulators of this hormone signaling pathway (Katagiri et al., 2005; Zhang et al., 2005).

2.3.2.4 Gibberellin in the regulation of dormancy and its interaction with ABA

Dormancy dynamics can also be linked to GA content and sensitivity. However, GA is known to have a negative relationship with dormancy level, and this relationship is less tight when compared to the relationship of ABA with dormancy (Miransari and Smith, 2014). The main functions of GA in releasing dormancy in the seeds of dicot species such as Arabidopsis are related to the inductions of endosperm weakening, mobilization of endospermic stored reserves to embryos, and expansion of embryonic cells via antagonizing ABA effects (Miransari and Smith, 2014). Seeds form GA-deficient *ga1-3* mutants of Arabidopsis exhibit enhanced dormancy level and require GA applications to complete their germination (Ariizumi and Steber, 2007). Changes in the transcript abundance of GA biosynthesis and catabolism genes in barley seeds expressing high temperature-induced secondary dormancy suggest a decrease in GA content in these seeds (Hoang

et al., 2013). However, Benech-Arnold et al. (2000) could not correlate GA content to the varietal difference in dormancy of developing sorghum seeds. It has been shown by Martinez et al. (2016) that the higher level of primary dormancy in wheat mutant *ERA8* as compared to the corresponding wild-type is related simultaneously to higher ABA sensitivity and lower GA sensitivity. Furthermore, the slower dormancy loss by after-ripening or cold-stratification in the seeds of this mutant is associated with a slower increase in GA sensitivity as well as a slower decrease in ABA sensitivity. Nevertheless, *ERA8* mutant does not exhibit dwarf phenotype suggesting that its enhanced dormancy mainly results from increased ABA signaling rather than a GA signaling defect (Schramm et al., 2013; Martinez et al., 2014). In general, a significant change in dormancy status was found only in a few GA-response mutants of cereals (Rodríguez et al., 2015).

The antagonistic ABA–GA interaction is established by genes and proteins involved in ABA and GA biosynthesis and signaling pathways. Previous studies in Arabidopsis have shown that the DELLA protein REPRESSOR OF GIBBERELLIC ACID1 LIKE (RGL2), which serves as a negative regulator of GA signaling pathway (Fig. 2.4d), and ABA synthesis participate in a feedback regulatory loop in which RGL2 stimulates ABA synthesis and ABA induces RGL2 expression. Furthermore, RGL2 promotes activity of ABI5, a positive regulator in the ABA signaling pathway (Fig. 2.4b) (Piskurewicz et al., 2008). On the other hand, expression of *GA2ox3*, a GA catabolism gene (Fig. 2.4c), is regulated by ABI4 and ABI5 in the monocots (Crocco et al., 2013) while expression of ABI3 and ABI5 in Arabidopsis *ga1-3* mutant are found to be repressed by GA treatment (Ogawa et al., 2003).

2.4 Jasmonates

2.4.1 Active jasmonates and their functions

Jasmonates are among the plant hormones that have diverse functions in plant immunity system,

development and growth, and secondary metabolism (Davies, 2010; Zhai et al., 2017). The term jasmonates include JA and its derivatives. Jasmonoyl-isoleucine, a conjugate of JA and amino acid isoleucine, have been widely known as the most biologically active jasmonate in plants. Others, such as JA (Fonseca et al., 2009), jasmonoyl-amino acids (other than JA-Ile), lactones of 12-hydroxy-JA-Ile, JA-glucosyl ester, 12-*O*-glucosyl-JA and 12-*O*-glucosyl-JA-Ile have been considered to be active or partially active. The 12-oxophytodienoic acid (OPDA), a precursor of JA, and its conjugate OPDA-Ile are also known to be biologically active and induce the expression of OPDA-specific responsive genes (Wasternack and Song, 2017; Zhai et al., 2017).

Jasmonates are known to regulate plant defense against attackers such as chewing insect, herbivores, necrotrophic pathogens, and mechanical wounding (Campos et al., 2014; Song et al., 2017; Chini et al., 2018). In contrast, JAs can weaken plant defense in response to colonizers, which facilitate the symbiotic association between plant and fungi, for example mycorrhiza, but it makes the plant less resistant to biotrophic pathogens. There is ample evidence that JAs are involved in plant tolerance to abiotic stresses such as salt (Toda et al., 2013; Wu et al., 2015) and cold (Hu et al., 2013; Zhao et al., 2013).

Jasmonates are also important for plant growth and development including flower and seed development, tuber formation and induction of root hair development (Zhu et al., 2006; Cai et al., 2014; Domínguez and Cejudo, 2014; Zhai et al., 2015). They also promote fruit ripening, senescence of green tissues (Ueda and Kato, 1980; Jiang et al., 2014; Zhu et al., 2015; Song et al., 2017) and flower abscission (Oh et al., 2013). In contrast, JAs inhibit seedling (Dathe et al., 1981; Song et al., 2017; Sharma et al., 2018) and root growth (Norastehnia et al., 2007; Miersch et al., 2008; Chen et al., 2011; Zhu et al., 2011; Sharma et al., 2018). Jasmonates appear to have effects on seed dormancy and germination in Arabidopsis and other species as their contents show changes during germination; however, contrary effects are observed in different plant species with

respect to seed dormancy status (Linkies and Leubner-Metzger, 2012). Many transcription factors, which regulate the production of secondary metabolites in Arabidopsis and medicinal plant species, have been reported to be activated or modulated by JAs (De Geyter et al., 2012; Goossens et al., 2016). Given these functions, high levels of JAs were found in developing reproductive organs such as flowers and fruit; and their levels in leaves are shown to increase in response to biotic and abiotic stimuli (Creelman and Mullet, 1997; Wasternack and Hause, 2002).

2.4.2 Jasmonate metabolism

Jasmonic acid is synthesized from linolenic acid or hexadecatrienoic acid, through a pathway that involves nine enzymes (Fig. 2.6) (Schaller and Stintzi, 2009). The first three enzymes including 13-LIPOXYGENASE (LOX), ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE (AOC) act in the plastid and convert linolenic acid and hexadecatrienoic acid to OPDA and dinor-OPDA (dnOPDA), respectively. OPDA and dnOPDA are transferred to the peroxisome where they are converted to JA by the subsequent steps in the pathway starting with OPDA REDUCTASE3 (OPR3) which converts OPDA and dnOPDA to 3-oxo-2-(20(Z)-pentenyl)-cyclopentane-1-octanoic (OPC8) and 3-oxo-2-(20(Z)-pentenyl)-cyclopentane-1-hexanoic (OPC6), respectively. OPC8 and OPC6 are then converted to OPC8-CoA and OPC6-CoA, respectively, by the action of OPC8: CoA LIGASE1 (OPCL1). The reactions which convert OPC8-CoA and OPC6-CoA to JA-CoA are catalyzed by ACYL CoA-OXIDASE (ACX), MULTIFUNCTIONAL PROTEIN (MFP) and L-3-KETOACYL-COA-THIOLASE (KAT). Finally, JA-CoA is converted to JA by ACYL-THIOESTERASES (ACH). Jasmonic acid is then exported to the cytosol where it is converted into JA-Ile by JASMONOYL-ISOLEUCINE SYNTHETASE, also known as JASMONATE RESISTANT1 (JAR1). The nuclear entry of JA-Ile has been shown to be mediated by JASMONATE TRANSPORTER1 (JAT1), suggesting the

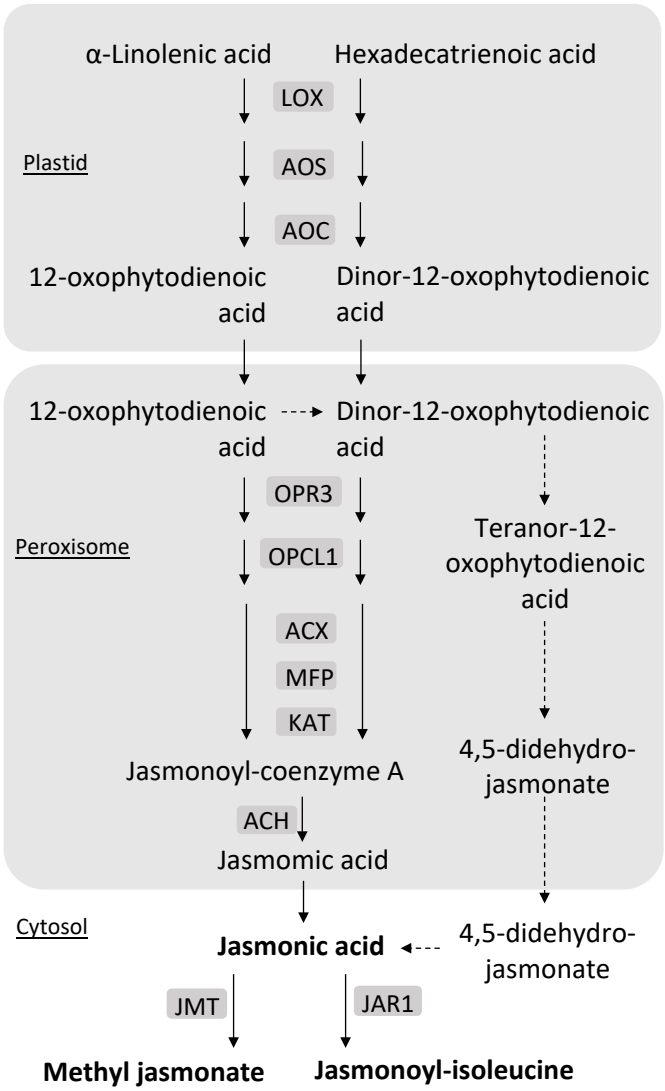

Figure 2.6. A simplified pathway of jasmonate metabolism.ACH, ACYL-THIOESTERASES; ACX, ACYL COA-OXIDASE; AOC, ALLENE OXIDE CYCLASE; AOS, ALLENE OXIDE SYNTHASE; JAR, JASMONATE RESISTANT; JMT, JASMONIC ACID CARBOXYL METHYL TRANSFERASE; KAT, L-3-KETOACYL-COA-THIOLASE; LOX, 13-LIPOXYGENASE; MFP, MULTIFUNCTIONAL PROTEIN; OPCL, 3-OXO-2-(20(Z)-PENTENYL)-CYCLOPENTANE-1-OCTANOIC COA LIGASE; OPR, 12-OXOPHYTODIENOIC ACID REDUCTASE; dashed arrows denote OPR3-independent pathway.

existence of another mechanism to regulate jasmonate activity (Li et al., 2017). Jasmonate biosynthesis mutants which include mutants of genes encoding JAR1 and five other JA biosynthetic enzymes have been identified in Arabidopsis, rice and tomato (Wasternack and Song, 2017; Xiong et al., 2017; Zhai et al., 2017). Although the AOS, AOC and OPR3 enzymes have been shown to be committed to the pathway (Schaller and Stintzi, 2009), a recent report indicated the presence of OPR3-independent JA biosynthesis pathway in Arabidopsis (Fig. 2.6) (Chini et al., 2016).

The expression of jasmonate biosynthesis genes encoding LOX, AOS, AOC, OPR3 and ACX is up-regulated in response to jasmonate signal creating a positive feedback loop, one of the major regulatory factors of jasmonate biosynthesis (Wasternack and Hause, 2013). A transcription factor designated as MYC2 has been reported to regulate jasmonate-responsive genes by binding to the jasmonate-responsive G-box (CACGTG) in the promoter region (Fig. 2.7), and therefore induces the expression of jasmonate biosynthetic genes that are responsive to JA-Ile (Dombrecht et al., 2007; Kazan and Manners, 2013), such as *LOX* in Arabidopsis (Hou et al., 2010) and tomato (Yan et al., 2013).

The activity of OPR3 and AOCs on the other hand can be subjected to post-translation regulation. For example, homodimer formation in tomato OPR3 leads to loss of its activity through a self-inhibition mechanism (Breithaupt et al., 2006). In addition, Stenzel et al. (2012) found that all the four Arabidopsis AOCs can interact among each other and form homomers and heteromers, suggesting that this heteromerization can serve as an additional layer of controlling AOC activity.

2.4.3 JA-Ile signaling

In plants, the JA-Ile signaling pathway has been described well (Browse, 2009; Fonseca et al., 2009; Koo and Howe, 2009; Pauwels and Goossens, 2011). The major components of JA-Ile signaling pathway include JA-Ile receptor complex SCF^{COI1} with CORONATINE

INSENSITIVE1 (COI1) as F-box protein, repressor JASMONATE ZIM DOMAIN (JAZ), co-repressors NOVEL INTERACTOR OF JAZ (NINJA) and TOPLESS (TPL), activator (transcription factor) MYC2 and subunit MEDIATOR25 (MED25) of MEDIATOR complex (Fig. 2.7). Under low level of JA-Ile, the complex consisting of JAZ, NINJA and TPL binds transcription factor MYC2 and prevents MYC2 form activating the expression of jasmonate-responsive genes (Fig. 2.7b). When JA-Ile is elevated, receptor complex SCF^{COI1} receives JA-Ile, making COI1 able to interact with JAZ leading to the degradation of JAZ via proteolytic destruction by 26S proteasome (Fig. 2.7c). The JA-Ile-induced degradation of JAZ allows MED25 to bind to the transcription factor MYC2 and de-repress MYC2, activating expression of jasmonate-responsive genes (Goossens et al., 2015; Chiangga et al., 2016; Mao et al., 2017; Song et al., 2017; Howe et al., 2018; Wasternack and Strnad, 2018).

2.4.3.1 JA-Ile level-dependent regulation of JAZs

The JAZs negatively regulate jasmonate-responsive genes under cellular JA-Ile levels that are lower than a threshold (Howe et al., 2018). JAZ-mediated repression of jasmonate-responsive genes includes two mechanisms, forming a repressor complex with a MYC2 transcription factor and recruitment of co-repressors (Fig. 2.7b). To activate the expression of jasmonate-responsive genes, the transcription factor MYC2 needs to bind to the G-box motif of target genes as well as the activator interacting domain of MED25 (Zhang et al., 2015; An et al., 2017) which is also a positive regulator of jasmonate-response (Fig. 2.7c) (Kidd et al., 2009; Çevik et al., 2012; Chen et al., 2012). Under low JA-Ile level, JAZs accumulate and competitively inhibit the MYC2-MED25 coupling. In addition to dismissing MED25, JAZs recruit co-repressor TPL through NINJA (Fig. 2.7b) (Pauwels et al., 2010). TPL has been shown to silence gene expression through different mechanisms (Pauwels et al., 2010; Ke et al., 2015).

Elevated JA-Ile levels, in response to development, environment or secondary metabolite

cues (Wasternack and Strnad, 2018), de-repress the transcriptional activation function of MYC2 by removing the binding of JAZs to it. Jasmonoyl-isoleucine promotes the recruitment of JAZs to SCF^{COI1} (SCF E3 ubiquitin ligase complex with COI1 as F-box protein) by facilitating the specific interaction between COI1 and JAZ and thereby forming a stable COI1–JA-Ile–JAZ complex, in which COI1–JAZ interaction acts as a JA-Ile coreceptor (Fig. 2.7c). The $SCF^{COI1–JA-Ile}$-bound JAZs are subjected to polyubiquitylation, and subsequent proteolytic destruction by 26S proteasome (Thines et al., 2007; Katsir et al., 2008; Sheard et al., 2010). The JA-Ile-dependent degradation of JAZs can be affected by multiple levels of regulation of COI1 including proteolysis (Sheard et al., 2010; Yan et al., 2013).

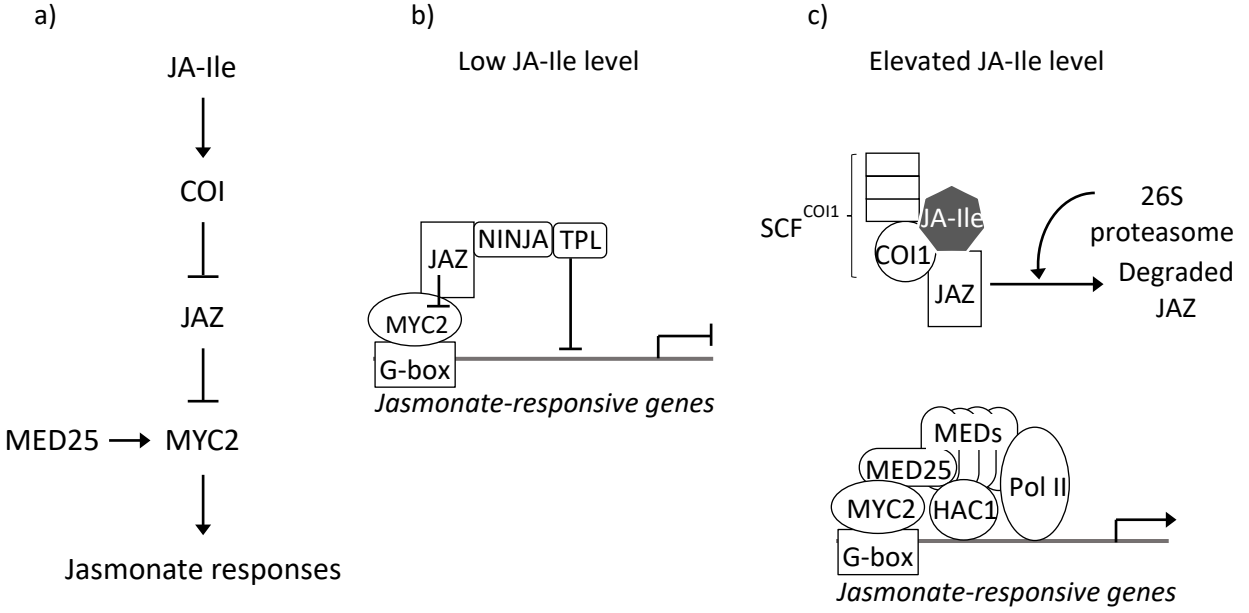

Figure 2.7. A simplified jasmonoyl isoleucine (JA-Ile) signaling. The overall JA-Ile signaling pathway (a). Under low JA-Ile level, JAZ–NINJA–TPL complex binds to MYC2 and repress the expression of jasmonate-responsive genes (b) while under elevated JA-Ile levels, SCF^{COI1} mediates the proteolytic degradation of JAZ allowing MYC2 to activate the expression of jasmonate-responsive genes (c). COI, CORONATINE INSENSITIVE; JAZ, JASMONATE ZIM DOMAIN; HAC1, transcription factor HAC1; MED25, MEDIATOR25; MEDs, MIDIATOR subunits; NINJAZ, NOVEL INTERACTOR OF JAZ; Pol II, RNA POLYMERASE II; SCF, S-PHASE KINASE-ASSOCIATED PROTEIN1- CULLIN1–F-BOX PROTEIN; TPL, TOPLESS.

JA-Ile-induced degradation of JAZ leads to dissociation of NINJA–TPL from the promoters of jasmonate-responsive genes. On the other hand, it releases the MED25 binding site on MYC2 which allows MED25 to bind MYC2 (Fig. 2.7c). This MED25–MYC2 interaction, in turn, recruits additional coactivators, such as bZIP transcription factor HAC1, and engages RNA POLYMERASE II to form the transcription pre-initiation complex, which completes the switch from repressive to activated transcriptional state of jasmonate-induced MYC2 target genes (Howe et al., 2018). Accordingly, the expression of primary jasmonate-responsive genes has been shown to be induced rapidly, in less than 15 minutes, in response to elevated JA-Ile level (Chung et al., 2008; Glauser et al., 2008; Koo et al., 2009).

2.4.3.2 Redundancy and specificity in JAZ-mediated jasmonate regulation

A large number of JAZ family members results in redundancy and specificity in jasmonate regulation. Among the components of JA-Ile signaling pathway, JAZ appears to have largest number of family members. For example, Arabidopsis has thirteen JAZs, and all of them except JAZ7 can directly interact with MYC2 (Fig. 2.7) (Howe et al., 2018), suggesting the redundancy in function of JAZs. In fact, many single *jaz* loss-of-function mutants, such as that of *JAZ1, JAZ2, JAZ7, JAZ9*, and *JAZ12* do not display expected phenotypes related to jasmonate responses (Thines et al., 2007; Demianski et al., 2012; Pauwels et al., 2015; Chini et al., 2016). However, recent studies highlight that the specificity in JAZ-mediated jasmonate response can be achieved by various mechanisms. For example, the ability to interact with COI1 (Fig. 2.7) varies among JAZs. Some JAZ proteins, which are called stable JAZs, may not interact with COI1 strongly enough for its effective JA-Ile-dependent degradation even under high cellular JA-Ile level (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Shyu et al., 2012; Thireault et al., 2015). These finding led to

the suggestion that regulation of the level of stable JAZs is among the mechanisms used by plants to regulate jasmonate-responsive genes (Howe et al., 2018). The higher JA-Ile level would be needed to combine a more stable JAZ into COI1–JA-Ile–JAZ complex, by which plants may be able to sense a dynamic range of cellular JA-Ile level and, consequently, produce appropriate responses.

2.4.3.3 Positive and negative feedback regulations, and transient burst of JA-Ile level

The MYC2 transcription factor up-regulates its own gene and most of JA biosynthesis genes including *LOX*, *AOS*, *AOC*, *OPR3* and *ACX* (Fig 2.6), in response to elevated JA-Ile level (Fig. 2.7), which can result in a positive feedback to jasmonate biosynthesis as well as jasmonate signaling (Wasternack and Hause, 2013; Wasternack and Song, 2017). However, there is also evidence supporting the occurrence of negative feedback regulation in the JA-Ile signaling pathway. Genes encoding several negative regulators of this signaling pathway including JA-Ile catabolic enzymes, JAZ and JASMONATE-ASSOCIATED MYC2-LIKE (JAM) transcription factors, are jasmonate-responsive genes (Howe et al., 2018).

JA-Ile can be hydrolyzed to JA by IAA-ALA RESISTANT3 (IAR3) and IAA-LEU RESISTANCE1-LIKE6 (ILL6) (Woldemariam et al., 2012; Bhosale et al., 2013; Widemann et al., 2013; Zhang et al., 2016). On the other hand, JA-Ile can be oxidized to 12-hydoxy-JA-Ile and 12-dicarboxy-JA-Ile by the members of CYP94 subfamily (Koo et al., 2011; Heitz et al., 2012; Koo et al., 2014). Both the hydrolysis and oxidation of JA-Ile increased in response to JA-Ile signaling via a COI1- and JAR1-dependent manner (Heitz et al., 2012; Widemann et al., 2013; Zhang et al., 2016). Other suggested feedback mechanisms that can reduce JA-Ile level are the diversion of JA flux from JAR1-catalyzed reaction (Miersch et al., 2008; Stitz et al., 2011; Caarls et al., 2017; Smirnova et al., 2017) and the transport of jasmonate within and between cells by jasmonate

transporters (Li et al., 2017; Nguyen et al., 2017).

Elevated JA-Ile also induces expression of genes encoding JAM. Like MYC2 (Fig. 2.7), JAMs can bind to the G-box in the promoters of jasmonate-responsive genes, but unlike MYC2, JAMs cannot activate transcription even when they are not bound by JAZs (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013; Fonseca et al., 2014). Therefore, increased expression of *JAMs* appears to promote JAM to competitively antagonize MYC2, and consequently weaken JA-Ile sensitivity.

Although elevated JA-Ile leads to both positive and negative feedback regulations (Howe et al., 2018; Wasternack and Strnad, 2018), the JA-Ile burst occurs transiently in many cases. For example, a rapid reduction in JA-Ile levels following wound-triggered JA-Ile burst has been well reported (Miersch et al., 2008; Heitz et al., 2012; Widemann et al., 2013). This is consistent with the observations that plant immunity triggered by JAs can physiologically antagonize growth and reproduction (Havko et al., 2016; Züst and Agrawal, 2017). The involvement of both positive and negative feedback regulatory mechanisms in the JA-Ile signaling pathway enable plant to, on one hand, boost up jasmonate responses, and on the other hand, appropriately terminate these responses.

2.4.4 Cross-talk of JAs with GA and ABA

Plant developmental processes and their interaction with the environment are regulated by several hormones working together in which they can synergize and/or antagonize each other's effects through complex cross-talk mechanisms. Plant development is regulated mainly by interaction among auxin, brassinosteroid and GA (Depuydt and Hardtke, 2011) while cross-talk among salicylic acid, JAs and ethylene play crucial role in plant defense against environmental stress (Robert-Seilaniantz et al., 2011). With respect to the seed dormancy and germination, it is well

known that ABA and GA are the major regulators, however, regulation of these developmental events involve interaction of ABA and GA with other hormones as described above (Fig. 2.3) (Shu et al., 2016).

2.4.4.1 Cross-talk between JAs and GA in plant development events

The major factors involved in the cross-talk between JAs and GA are the JAZs and DELLA proteins. Similar to the functions of JAZs in jasmonate signaling (Fig 2.7), DELLAs are negative regulators of GA signaling (Fig. 2.4d). Since they have mutual target transcription factors, JAZs and DELLAs can mediate the synergistic cross-talk between JAs and GA (Fig. 2.8a) (Song et al., 2014). Previous studies have indicated that trichome initiation in leaves (Qi et al., 2014), synthesis and emission of sesquiterpene in flowers (Hong et al., 2012), and stamen development (Mandaokar et al., 2006; Cheng et al., 2009) in Arabidopsis are controlled by JAs–GA synergistic interaction.

In contrast to the synergism, JAZs and DELLAs are also responsible for signaling antagonism between JAs and GA. The bindings of JAZs and DELLAs to each other can prevent both of them from regulating their downstream transcription factors (Fig. 2.8b). Therefore, jasmonate-induced JAZ depletion can promote DELLA activities, which represses the expression of GA-responsive genes; and GA-induced degradation of DELLAs can promotes JAZ to negatively regulate jasmonate-responsive genes (Song et al., 2014). It has been shown that the PHYTOCHROME-INTERACTING FACTOR3 (PIF3) transcription factor promotes elongation of Arabidopsis hypocotyl, and the JAZ–DELLA interaction or GA-induced degradation of DELLA allows the PIF3 to be free to function. By contrast, jasmonate-induced degradation of JAZs release DELLAs which then bind and repress PIF3 activity (Yang et al., 2012).

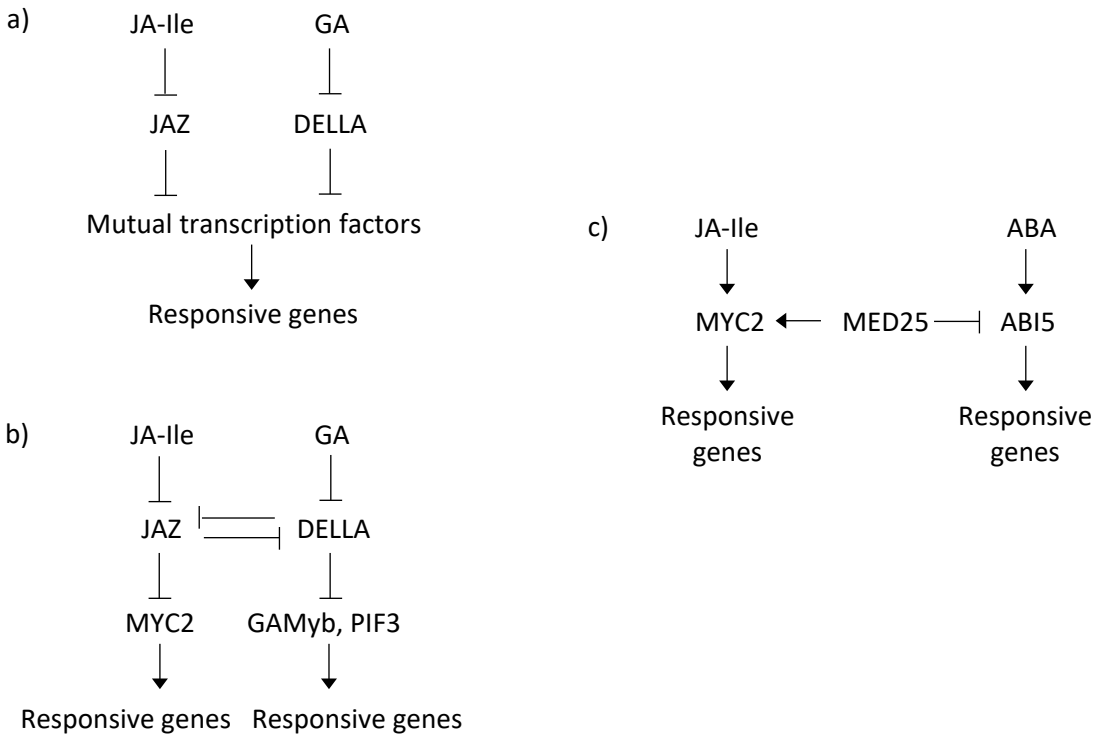

Figure 2.8. A simplified diagram of cross-talks of jasmonates (JAs) with abscisic acid (ABA) and gibberellins (GA). JAs could have synergistic (a) and antagonistic (b, c) cross-talks with GA (a, b) and ABA (c). ABI, ABSCISIC ACID INSENSITIVE; JAZ, JASMONATE ZIM DOMAIN; MED25, MEDIATOR25; PYL, PYRABACTIN RESISTANCE-LIKE

2.4.4.2 Cross-talk between JAs and ABA in plant developmental events

Previous reports have also shown the occurrence of both synergistic and antagonistic interactions between JAs and ABA. For example, Arabidopsis transcription factor MYC2 is a component of jasmonate signaling and *MYC2* gene is a jasmonate-responsive gene (Fig. 2.7). The expression of *MYC2* is also induced by ABA (Lorenzo et al., 2004). Moreover, MYC2 appears to have positive effects on ABA signaling since the Arabidopsis *myc2* knockout plants have been shown to display a decrease in ABA sensitivity (Abe et al., 2003; Yadav et al., 2005; Gangappa et al., 2010). In contrast, plants overexpressing *MYC2* are ABA hypersensitive (Abe et al., 2003; Lorenzo et al., 2004; Goossens et al., 2015). As the result, increased MYC2 activity was associated with

decreased germination efficiency and reduced growth of geminated seedlings (Goossens et al., 2015). The induction of *MYC2* expression by both JAs and ABA (Lorenzo et al., 2004) is consistent with the reports that JAs exhibits synergistic effect with ABA to promote the inhibition of germination in different species including Arabidopsis (Wilen et al., 1991; Staswick et al., 1992; Ellis and Turner, 2002).

The involvement of MED25-mediated JAs–ABA antagonism in the regulation of seed germination and seedling growth has been reported previously (Chen et al., 2012). MED25 binds to MYC2 and positively regulates the transcription of jasmonate-responsive genes but when binding to ABI5, MED25 has a negative effect on the transcription of ABI5-regulated genes (Fig. 2.8c) (Chen et al., 2012). It has been reported that exogenous MeJA-induced dormancy loss in wheat seeds was accompanied by decreases in embryo ABA content, which is mediated by a decrease in expression of *TaNCED1*, an important ABA synthesis gene (Fig. 2.4a), and an increase in the expression level of ABA catabolic gene *TaCYP707A1* (Jacobsen et al., 2013).

2.5 Roles of JAs in seed maturation, dormancy and germination

2.5.1 JAs and seed maturation

Previous studies implicated the role of JAs in seed maturation. A study in Arabidopsis that involved the use of *pxa1-1* mutant and wild-type plants has shown the occurrence of changes in the levels of different forms of JAs during seed maturation (Dave et al., 2011); PEROXISOMAL ADENOSINE TRIPHOSPHATE-BINDING CASSETTE-TRANSPORTER1 (PXA1), also known COMATOSE (CTS), functions in transporting substrates for β-oxidation into peroxisome (Zolman et al., 2001; Footitt et al., 2002). The results of this study showed a slight increase in OPDA content by the end of maturation in wild-type seeds while the contents of JA and JA-Ile decrease during late phases of seed maturation. The contents of OPDA, JA and JA-Ile in the

corresponding *pxa1-1* seeds were slightly to greatly higher at later stages of maturation as compared to the wild-type seeds. In addition, mature dry seeds of *pxa1-1* exhibit much higher levels of OPDA, JA and JA-Ile than that observed in the corresponding wild-type seeds. These results might reflect changes in the metabolism of JAs have been initiated before the onset or during seed maturation, forming a mechanism underlying differences in the contents of JAs and most likely their roles during seed maturation.

A study by Kanno et al. (2010) also examined changes in the contents of JA and JA-Ile during seed maturation in Arabidopsis using *aba2-2* mutant and wild-type plants; ABA DEFICIENT (ABA2) encodes a short-chain dehydrogenase/reductase that is involved in ABA synthesis, and the *aba2-2* mutant exhibits much lower ABA level in maturing seeds as compared to the wild-type. Results of this study showed a decrease in JA content during the late phases of seed maturation in wild-type seeds, which is in agreement with that reported by Dave et al. (2011). However, the temporal pattern of change in JA-Ile content in the wild-type seeds, which exhibited an increase during late phases of seed maturation, was contrary to that reported by Dave et al. (2011), and no significant difference in contents of JAs was observed between the *aba2-2* mutant and wild-type seeds during seed maturation.

Results from these studies in general suggest that seed maturation is associated with minimal reduction in the level of bioactive JAs; the content of JA is reduced but the seeds appear to maintain or exhibit increases in the levels of more bioactive forms of JAs, i.e. OPDA and JA-Ile in mature seeds as compared to immature seeds (Wasternack and Song, 2017; Zhai et al., 2017). Although ABA plays an important role in seed maturation (Locascio et al., 2014), there was no difference in contents of seed JAs between the ABA deficient mutant *aba2-2* and wild-type seeds during their maturation, suggesting that the function of JA in seed maturation might be ABA-

independent.

2.5.2 The role of JAs in germination and dormancy

Jasmonate are implicated to have roles in the regulation of dormancy and germination (Linkies and Leubner-Metzger, 2012). Previous studies have shown that the levels of JA and JA-Ile decrease during imbibition in non-dormant seeds, in which the level of JA shows a decrease in the first few hours of imbibition while the JA-Ile level declines thereafter (Preston et al., 2009; Dave et al., 2011). Seeds of many Arabidopsis mutants that are deficient in JA or exhibit reduced sensitivity are reported to display a germination behavior that is similar to the wild-type seeds. For example, the levels of OPDA, JA and JA-Ile are undetectable in freshly harvested seed of the jasmonate biosynthesis mutants of Arabidopsis, *aos* and *opr3-1*, while the wild-type seeds exhibit relatively higher amounts of these compounds. Despite these differences in OPDA, JA and JA-Ile levels between the mutants and the wild-type seeds, no difference in germination phenotype was observed between seeds freshly harvested from both the mutants and wild-type (Dave et al., 2011). Furthermore, seed of the jasmonate biosynthesis mutant *jar1* (Fig. 2.6) and the jasmonate signaling mutant *coi1-16* (Fig. 2.7) exhibit a germination phenotype similar to the wild-type seeds (Ellis and Turner, 2002; Dave et al., 2011). It has been shown that PHYTOCHROME INTERACTING FACTOR 3-LIKE5 (PIL5), a phytochrome-interacting bHLH transcription factor, inhibits germination of Arabidopsis seed by regulating ABA- and GA-related genes (Oh et al., 2004; Oh et al., 2009). PIL5 directly binds the promoter of *JAZ1* (Fig. 2.7) and represses its expression. Therefore, Arabidopsis *pil5* mutant is suggested to be less sensitive to JAs, however, seeds of *pil5* exhibit germination behavior that is similar to wild-type seeds (Oh et al., 2009). In addition, no significant difference in germination was observed between mutants of genes involved in jasmonate signaling (Fig. 2.7) including *myc2*, *pft1-1*, *pft1-2* and *med25-4* mutants and the wild-

type seeds; *pft1-1*, *pft1-2* and *med25-4* are mutant alleles of Arabidopsis *MED25* (Berger et al., 1996; Abe et al., 2003; Gangappa et al., 2010).

On the other hand, germination has been shown to be reduced in seeds of mutants exhibiting increased jasmonate level and jasmonate signaling. Seeds of Arabidopsis mutants that contain significantly higher levels of OPDA, JA and JA-Ile such as *kat2* and *acx1-2 acx2-1* (double mutant) as compared to the wild-type seeds show reduced germination phenotype (Dave et al., 2011). This is well supported by the results reported for seeds of other mutants such as *cts* and *lacs* that exhibit elevated jasmonate levels but reduced germination (Dave et al., 2011). These results suggest a negative relationship between germination and seed contents of JAs. Germination has also been shown to be reduced in mutants exhibiting increased jasmonate signaling. For example, although seeds from *MYC2* overexpressing Arabidopsis plants exhibit a similar germination phenotype with wild-type seeds (Abe et al., 2003), overexpressing a *MYC2* homolog with a point mutation that leads to increased MYC activity has been shown to result in reduction in germination as compared to the wild-type (Goossens et al., 2015). Similar to that observed in mutants with increased jasmonate signaling, exogenous application of JAs such as MeJA to wild-type seeds was shown to inhibit germination (Oh et al., 2009).

2.5.2.1 Changes in jasmonate activity is accompanied by changes in ABA sensitivity

Previous studies have shown that changes in activity of JAs in seeds triggers changes in seed ABA sensitivity (Dave et al., 2011; Dave et al., 2016). It has been shown previously that increased jasmonate signaling leads to increased sensitivity to ABA. For instance, seeds overexpressing the jasmonate signaling gene *MYC2* (Fig. 2.7) are shown to be more sensitive to ABA and this leads to inhibition of germination (Abe et al., 2003). In addition, overexpression of *MYC2* in Arabidopsis was found to lead to decreased germination in response to exogenous ABA (Goossens et al., 2015).

In agreement with this, application of exogenous MeJA can make seeds more sensitive to germination inhibition by ABA (Staswick et al., 1992). Other studies have also shown that a decrease in jasmonate activity leads to decreased sensitivity to ABA. For example, seeds of *aos* mutant exhibit lower level of JAs and lower sensitivity to exogenous ABA in inhibition of germination (Dave et al., 2011; Dave et al., 2016). Likewise, seeds of *myc2* mutant, which are less sensitive to JAs, exhibit lower sensitivity to exogenous ABA in inhibition of germination (Abe et al., 2003; Gangappa et al., 2010). Consistent with this result, ABA-induced reduction in germination was shown to be more significant in wild-type than *myc2-3* seeds (Yadav et al., 2005).

Several reports propose that MYC2 (Fig. 2.7) can have an important role in JAs–ABA synergism for inhibiting seed germination. In this connection, seeds of *MYC2* knockout mutants display a decrease in ABA sensitivity (Abe et al., 2003; Yadav et al., 2005; Gangappa et al., 2010) while those overexpressing *MYC2* showed increased ABA sensitivity (Abe et al., 2003; Goossens et al., 2015). Although the expression of *MYC2* could be induced by both JAs and ABA, the ABA-induced expression of *MYC2* appears to be mediated by jasmonate signaling pathway (Lorenzo et al., 2004). Given that promoting the expression of *MYC2* is among early jasmonate-responsive events (Wasternack and Hause, 2013), it is likely that high jasmonate activity during imbibition, which results from increased jasmonate level or jasmonate signaling or due to treatment with exogenous JAs, stimulate the expression of *MYC2* and consequently increases sensitivity to ABA. Moreover, JAs can promote the expression level of the gene encoding ABA receptor *PYL4* (Fig. 2.4b) (Lackman et al., 2011).

Despite all these reports, synergism between jasmonate activity and ABA sensitivity are not always obvious. For example, application of ABA, which affects germination in wild-type seeds, has been shown not to affect germination of *AOS* over-expressing seeds which exhibit

increases in jasmonate levels (Singh et al., 2017).

2.5.2.2 Jasmonates and seed dormancy and germination in wheat

A number of studies examined changes in JA and JA-Ile level during seed germination in wheat. Using a whole seed system, Liu et al. (2013) showed that the JA and JA-Ile levels decrease with imbibition of after-ripened seeds of cv. AC Domain. A study by Xu et al. (2016) also examined changes in JA and JA-Ile contents in the embryo during imbibition of after-ripened seeds of wheat cv. Sunstate; imbibition caused reduction in levels of JA and JA-Ile within 6 h of imbibition. Similarly, embryo contents of JA and JA-Ile were much lower in imbibed seeds than that observed in dry mature seeds of two Japanese wheat genotypes Gifu-komugi (Gifu) and OS38 (Kashiwakura et al., 2016). Reduction in the levels of JA and JA-Ile in the embryos and aleurone was also observed during seed imbibition in other wheat cultivars, cvs. Zak and Brevor (Martinez et al., 2016). All these studies imply that level of JAs decrease during imbibition.

Exogenous MeJA has been shown to break dormancy in wheat, for example, dormancy induced by blue light (Jacobsen et al., 2013), and this effect of MeJA is mediated by down-regulation of ABA biosynthetic gene *TaNCED1* and up-regulation of the ABA catabolic gene *TaCYP707A1* (Fig. 2.4a) during imbibition, which led to a decrease in ABA level in embryos (Jacobsen et al., 2013). Further analysis revealed that exogenous MeJA during seed imbibition leads to delay in root emergence (Jacobsen et al., 2013). Given that exogenous JAs can release the dormancy in wheat seeds, it is possible that less dormant seeds have higher jasmonate content and/or jasmonate sensitivity – which is supposed to speed up the process of dormancy release. In agreement with this, the less dormant cv. Gifu was shown to exhibit higher level of embryonic JAs and JA-Ile in dry and imbibed seeds as compared to that observed in the dormant genotype OS38 (Kashiwakura et al., 2016).

Cold-stratification is one of the dormancy breaking treatments. Jasmonate content in the embryo during cold treatment was found to be lower in less dormant wheat cv. Sunstate than in the more dormant cv. AC Barrie (Xu et al., 2016). These results suggest that less dormant wheat seeds have lower jasmonate content or jasmonate content is elevated or maintained at higher level during cold-stratification in the more dormant wheat seeds as a mechanism of dormancy release. A previous study has found higher embryo JA-Ile content during imbibition in the ABA hypersensitive mutant *ERA8* as compared to cv. Zak from which the mutant is derived. It has been proposed that the presence of increased JA-Ile content in the *ERA8* mutant is required to counteracts the effects of its ABA hypersensitivity phenotype on dormancy (Martinez et al., 2016). However, the increase in JA-Ile content was unable to overcome the enhanced dormancy induced by its ABA hypersensitivity, suggesting the absence of strong relation between seed dormancy and jasmonate level in seed tissues.

2.5.2.3 Jasmonates and dormancy release by after-ripening in wheat seeds

After-ripening and cold-stratification treatments, which releases seeds from dormancy, affect seed jasmonate levels (Kashiwakura et al., 2016; Xu et al., 2016). A previous study has shown that dry seeds of the less dormant wheat cv. Gifu which contain higher amounts of JA and JA-Ile, need shorter duration of after-ripening for dormancy release as compared to the seeds of dormant genotype OS38. In addition, higher levels of JAs were found during imbibition of seeds of cv. Gifu than that observed in genotype OS38. However, the effects of after-ripening on embryonic/aleurone jasmonate levels are found to be variable. Reports available so far indicate that the levels of JA and JA-Ile in embryo of wheat seeds can exhibit changes or remain unaffected in response to after-ripening. For example, 20 and 40 days of after-ripening release dormancy in Gifu and OS38 wheat genotypes, respectively, and the after-ripening treatment reduced embryo

contents of JA and JA-Ile in the dry seeds of the less dormant wheat cv. Gifu but appears to have different effects in JA and JA-Ile contents of the same cultivar during imbibition; an increase in JA-Ile content but a decrease in JA content. In contrast, after-ripening didn't cause any marked change in embryo contents of JA and JA-Ile in dry and imbibed seeds of the dormant wheat genotype OS38 (Kashiwakura et al., 2016).

After-ripening was not able to cause any change in embryo JA-Ile content in dry seeds of cv. Sunstate in which dormancy was induced by treatment with blue light, although it caused a change in the level of JA. However, the change in JA level was not associated with the increase in germination triggered by after-ripening (Xu et al., 2016). These results suggest that the embryo JA and JA-Ile levels in dry seeds of wheat cv. Sunstate might not play any important roles in releasing blue light-induced dormancy by after-ripening. In addition, after-ripened seeds of cv. Sunstate showed increases in embryo JA and JA-Ile contents during imbibition (Xu et al., 2016), and these pattern of changes in jasmonate content appeared to be different from after-ripening-induced changes in the content of JA and JA-Ile in imbibed seeds of other wheat genotypes, namely Gifu and OS38 (Kashiwakura et al., 2016).

A previous study with other wheat cultivars such as Zak show that after-ripening increases the contents of JA in the embryo and aleurone tissues during imbibition with no remarkable effect on the level of JA-Ile (Martinez et al., 2016). After-ripening for a shorter (4 week) and longer (10 week) periods led to similar effects on JA and JA-Ile contents while exhibiting different effects on germination rate (Martinez et al., 2016). This result might suggest that the increase in JA content due to after-ripening doesn't have significant effect on the regulation of dormancy release possibly due to its weak biological activity (Wasternack and Song, 2017). Contrary to these results, after-ripening of the seeds of *ERA8* mutant, a ABA hypersensitive mutant derived from cv. Zak,

increased JA contents in embryo and aleurone during earlier stages of imbibition (8 HAI) but either did not affect the JA content of the embryo or caused its reduction in the aleurone during the later stages of imbibition (18 HAI) (Martinez et al., 2016). After-ripening treatment was also found not to affect embryo JA-Ile content during the earlier stages of imbibition, which showed a slight increase in aleurone. However, reduction of JA-Ile content was observed in both embryo and aleurone tissues of after-ripened seeds during the later stages of imbibition. A study on a different cultivar, cv. Brevor, showed that after-ripening reduces JA and JA-Ile in embryos during imbibition, and this reduction was shown to be greater in seeds after-ripened for longer than shorter durations (Martinez et al., 2016). The same study showed that after-ripening treatment does not have effect on aleurone JA-Ile content during imbibition, and its effects on aleurone JA content was also not consistent. Together the results from these studies suggest that content JAs in seed tissues might not be significantly involved in dormancy release by after-ripening in wheat seeds, and the analysis of jasmonate signaling is needed to understand the role of JAs in this process.

2.5.2.4 Jasmonates and dormancy release by cold-stratification in wheat seeds

In addition to after-ripening, cold-stratification breaks dormancy in seeds. Cold-stratification of wheat seeds was shown to induce similar peaks of embryo jasmonate level in non-dormant and dormant wheat cultivars (Tuttle et al., 2015; Xu et al., 2016). As compared to cold-untreated seeds, cold-stratification was shown to increase embryo JA-Ile content and decrease aleurone JA-Ile content during imbibition of dormant seeds of wheat cv. Brevor (Tuttle et al., 2015). However, JA level in the embryo of blue light-induced dormant seeds of cv. Sunstate was reduced during cold-stratification, that is due to imbibition of seeds at 4^{o}C as compared to the level observed in the dry seeds. This reduction in JA level was similar to that occurred during imbibition of seeds at optimal temperature (20^{o}C) with no prior stratification. In a study that involved seed imbibition at optimal

temperature following cold-stratification, peaks of jasmonic and JA-Ile levels in the embryos were observed within 8-12 h after the end of cold-stratification (Xu et al., 2016). This cold-stratification-induced pattern of change in JA-Ile embryo contents was found in seeds with different levels of dormancy including in non-dormant Sunstate seeds (imbibed in dark), dormant Sunstate seeds (imbibed under blue light), and dormant wheat cv AC Barrie (Xu et al., 2016). Therefore, it appears that similar patterns of cold-stratification-induced changes occur during imbibition irrespective of the dormancy level present in the seeds, suggesting that induction of jasmonate level due to cold-stratification is independent of seed dormancy status. Moreover, the observation of responses to cold-stratification of seeds from dormant cv. AC Barrie and non-dormant cv. Sunstate showed that while cold-stratification effectively breaks dormancy in the seeds of cv. AC Barrie, less noticeable cold-stratification-induced embryo JAs peak was evident in this cultivar as compared to the non-dormant cv. Sunstate (Xu et al., 2016). These results suggest that cold-stratification-induced embryo JAs peaks, which occur within 8-12 h of imbibition at optimal temperature of the cold treated seeds might not be a key factor in dormancy loss by cold-stratification.

2.5.2.5 Relationship between changes in JAs, ABA and GA levels in response to release of dormancy in wheat seeds

Dormancy release by after-ripening or cold-stratification is accompanied by a decrease in ABA/GA content ratio in imbibed seeds whereas the relationship between dormancy release and jasmonate levels has been reported to be inconsistent. As a result, changes in jasmonate levels appeared to have positive or negative relationship with changes in ABA and GA levels. In some instances, changes in jasmonate levels occur independent of changes in the levels of the two hormones.

Analysis of after-ripened seeds from different cultivars including cv. Zak, which is a pre-

harvest sprouting susceptible soft white spring wheat cultivar, *ERA8*, which is an ABA hypersensitive mutant derived from cv. Zak, and cv. Brevor, which is a dormant soft white winter wheat cultivar (Tuttle et al., 2015), revealed the existence of a negative relationship between embryo ABA content during imbibition and seed germination. However, no close relationship was found between embryo ABA content with that of JA and JA-Ile contents. When the data from each genotype are analyzed separately, embryo ABA content during imbibition still exhibits a strong negative relationship with germination in all genotypes whereas ABA content appeared to have negative relationships with that of JA and JA-Ile contents in cv. in Zak and *ERA8* mutant but positive relationships in cv. Brevor. These results suggest that the embryo JA and/or JA-Ile contents of imbibed seeds of wheat might not be a universal parameter to elucidate the role of JAs–ABA interaction in regulation of seed dormancy release by after-ripening.

After-ripening of seeds of cv. Gifu, which is a standard sprouting-tolerant wheat landrace, and OS38, which is a highly sprouting-tolerant wheat line, for 20 and 40 days released dormancy in Gifu and OS38, respectively (Kashiwakura et al., 2016). The ratio of ABA to GA_1 contents in the embryos of imbibed seeds in both genotypes was found to be much lower in after-ripened seeds than in the control seeds that are not subjected to after-ripening. However, the decrease in ratio of ABA/GA_1 content in cv. Gifu was accompanied by a decrease in JA content with no change in JA-Ile content while the decrease in the ratio of ABA/GA_1 content in OS38 line was accompanied by the absence of any change in both JA and JA-Ile levels (Kashiwakura et al., 2016).

Seed imbibition at lower temperature (15ºC) was found to release dormancy in seeds of cv. Gifu but not in the seeds of OS38, and it caused reduction in the ratio of ABA/GA_1 content in Gifu but an increase in OS38 (Kashiwakura et al., 2016). However, imbibition at 15ºC led to an increase in embryo JA and JA-Ile contents during imbibition of seeds in both genotypes. Imbibition at 15ºC

also partly releases dormancy in OS38 seeds which had been after-ripened for 24 days, and this release of dormancy by a combination of after-ripening and low temperature imbibition was accompanied by a marked reduction in the ABA/GA_1 content ratio of the embryos in imbibed seeds with no change in JA and JA-Ile contents (Kashiwakura et al., 2016). Furthermore, dormancy release in seeds of cv. Gifu by imbibition at 15ºC exhibited a relationship between changes in the levels of JAs, ABA and GA in the embryo, which was different from that observed in dormancy release by after-ripening; the after-ripening-induced decrease in ABA/GA_1 content ratio was accompanied by a decrease in JA content while the decrease in ABA/GA_1 content ratio by imbibition at 15ºC was accompanied by an increase in JA content (Kashiwakura et al., 2016).

2.5.2.6 After-ripening and cold stratification induce different relationships between JAs and ABA levels in wheat seeds

Previous studies have shown that after-ripening of dormant wheat seeds does not induce consistent changes in embryo JA/ABA and JA-Ile/ABA content ratios in dry seeds of different wheat cultivars (Kashiwakura et al., 2016; Xu et al., 2016). After-ripening has been shown to reduce JA/ABA content ratios in dry seeds of Gifu and OS38 genotypes while it almost did not cause any change of JA/ABA content ratio in seeds of cv. Sunstate. In contrast, after-ripening increases JA-Ile/ABA content ratio in dry seeds of cv. Sunstate, but decrease this ratio in the corresponding seeds of Gifu and OS38 genotypes. Although after-ripening of cv. Sunstate seeds for different duration – 14 and 60 days – leads to the observation of a similar germination index (GI) of 0.82 and 0.85, respectively, the JA/ABA ratio in dry seeds was found to be higher in 14 days after-ripening than in 60 days after-ripening seeds, indicating that dormancy release by after-ripening in wheat seeds doesn't exhibit a close association with changes in JAs/ABA ratio.

Seeds of cv. Brevor are dormant at maturity, and cold stratification at 4ºC for 72 h was

required to release dormancy in seeds of this cultivar that are after-ripened for 1 week (Tuttle et al., 2015). Alternatively, dormancy release in seeds of cv. Brevor by after-ripening alone required a longer duration (over 2 months) of after-ripening (Martinez et al., 2016). As discussed previously, reduction in embryo ABA content during imbibition of after-ripened seeds of cv. Brevor was accompanied by reduction in embryo contents of JA and JA-Ile. In contrast, reduction in embryo ABA content during imbibition of cold-stratified seeds of Brevor was accompanied by an increase in embryo JA-Ile content with no apparent change in JA content (Tuttle et al., 2015). Interestingly, the JAs and ABA interaction in the aleurone was found to be opposite of that observed in the embryos; reduction in ABA content of the aleurone during imbibition of cold stratified seeds of cv. Brevor was accompanied by a decrease in JA-Ile content (Tuttle et al., 2015).

Based on the central hypothesis that seed dormancy release involves synergistic interaction of JAs with GA, the specific objectives of this thesis are 1) examine the metabolism and signaling of JAs, GA and ABA in maturing seeds of dormant and non-dormant genotypes; 2) analyze changes in expression level of metabolic and signaling genes of JAs, GA and ABA in response to treatments with exogenous JAs and GA; and 3) investigate interaction between JAs, and GA and ABA during dormancy release by after-ripening and cold treatment.

3 JASMONATES AND THEIR INTERACTION WITH GIBBERELLINS AND ABSCISIC ACID IN REGULATING WHEAT SEED DORMANCY DURING SEED MATURATION

3.1 Abstract

Regulation of seed dormancy induction during seed maturation plays an important role in conferring tolerance against precocious germination in wheat. By measuring gene expression and hormone content in the seeds of dormant (cv. AC Domain) and non-dormant (RL4452) genotypes, we showed that developmental transitions from immature (20 and 30 DAA) to mature phase (40 and 50 DAA) are associated with decreases in contents of JA-Ile and ABA and increases in expression levels of jasmonate metabolic and signaling genes including *TaLOX6*, *TaAOS2*, *TaAOC1*, *TaJARs*, *TaCOIs*, *TaJAZ*s, *TaMYC*s and *TaMED25*. The two genotypes exhibited similar dormancy levels in 40 DAA seeds, however, transition from 40 to 50 DAA reduced the dormancy level only in the non-dormant genotype, which is associated with decreases in levels JA-Ile and ABA; this transition was also associated with increased expression levels of the GA signaling genes *TaGID2* and *TaGAMyb*, and the jasmonate signaling genes *TaJAZ*s and *TaMED25*, which might enhance GA sensitivity and reduce ABA sensitivity of the embryos. The reduction of ABA content in the non-dormant seeds during transition from 40 to 50 DAA was accompanied by down-regulation of *TaNCED2* expression and up-regulation of *TaCYP707As* expression. The transition did not affect the expression of ABA signaling genes in the dormant genotype while it increased that of *TaPYL5*, *TaSnRK2* and *TaABI5* in the non-dormant genotype. The results highlight that maturation of wheat seeds can be characterized by the changes in embryo jasmonate content and signaling; particularly by changes in expression of signaling regulators JAZs and MED25 during the last phase of maturation, suggesting the involvement of synergistic and antagonistic interactions of JAs with GA and ABA, respectively, in regulation of dormancy during seed maturation.

3.2 Introduction

Maintenance of adequate level of seed dormancy, which is defined as the inability of seeds to complete germination under optimal conditions, is critical in the production of cereal crops (Rodríguez et al., 2015; Tuan et al., 2018). However, the prevalence of low level of dormancy at seed maturity to attain uniform germination and seedling establishment, which facilitate cultivation and industrial production, have been the dominant breeding traits during domestication of cereal crops (Harlan, 1992; Ishibashi et al., 2017). Because of such domestication practice, most of the modern cultivars of cereal crops exhibit a low level of dormancy at maturity. Therefore, under wet summer condition, particularly when it is associated with cooler temperatures, maturing seeds can germinate on the mother plant before harvesting (Biddulph et al., 2005; Bewley et al., 2013). This phenomenon, which is called pre-harvest sprouting, can lead to the loss of seed quality since germination triggers the accumulation of α-amylase that initiates the degradation of storage starch in the endosperm for use by growing embryos (Gualano et al., 2007; Rodríguez et al., 2015). Consequently, the grains cannot be used for making high value end products such as bread, pasta and beer (Clarke et al., 1984). On the other hand, establishment and maintenance of strong dormancy during and after maturation can be a disadvantage since it increases production cost due to the need of extra storage time (Carter et al., 2015) and negatively affects the uniformity of seed germination and subsequent seedling establishment (Harlan, 1992; Ishibashi et al., 2017).

Developing wheat seeds reach physiological maturity when they attain a maximum dry weight, which is usually marked by the turning of spikes and the flag leaf to yellow (Hanft and Wych, 1982; Gerjets et al., 2009). The time period between anthesis and seed maturation varies with genotypes and growth conditions (Rogers and Quatrano, 1983; Wan et al., 2008; Guo et al., 2012). In general, wheat seeds attain physiological maturity approximately 40 DAA, and wheat

seeds are suggested to obtain the highest degree of primary dormancy during their physiological maturity (Hanft and Wych, 1982; Gerjets et al., 2009). The three major events that occur during the maturation phase of wheat seeds include induction of dormancy, quick dehydration of embryo and aleurone layer, and programmed cell death of starchy endosperm cells. The dehydration process continues through after physiological maturity of the seed, that is during ripening, until a moisture equilibrium with ambience is established (Fincher, 1989; Rodríguez et al., 2015). Physiologically mature seeds of wheat genotypes exhibit different levels of dormancy, which can be determined by different dormancy breaking treatments such as cold-stratification, which refers to seed imbibition under cold temperature, and after-ripening, which refers to seed dry storage at room temperature (Finkelstein et al., 2008; Rodríguez et al., 2015). In general, highly dormant seeds require longer durations of stratification and after-ripening than seeds that display weak dormancy (Tuttle et al., 2015; Martinez et al., 2016).

It is well established that the balance between ABA and GA, including their levels as well as signaling, regulate seed germination, and induction and maintenance of dormancy during seed development. Dormancy release and germination are induced by an imbalance in favor of GA while dormancy induction and maintenance are promoted by an imbalance in favor of ABA (Finkelstein et al., 2008; Graeber et al., 2012; Gazzarrini and Tsai, 2015). The embryonic levels of ABA in the seeds of cereal crops, such as wheat, barley and sorghum are reported to peak around physiological maturity of the seed then decline sharply through harvest maturity (Walker-Simmons, 1987; Steinbach et al., 1997; Benech-Arnold et al., 1999). However, the onset of dormancy in seeds appeared to occur during the earlier phases of seed maturation, prior to attaining physiological maturity (Finch-Savage and Leubner-Metzger, 2006). For example, seeds of an ABA deficient maize mutant exhibit germination when they are halfway during their development;

and this was inhibited by repressing GA synthesis in developing seeds of the mutant via treatment with GA synthesis inhibitors to re-establish optimal ABA–GA balance (White et al., 2000).

Jasmonates include JA and its derivatives, of which JA-Ile, a conjugate of JA and amino acid isoleucine, have been widely known as a major biologically active jasmonate in plants (Kazan and Manners, 2008; Wasternack and Song, 2017; Zhai et al., 2017). Previous studies provided evidence for the involvement of JAs, including JA, JA-Ile and MeJA, in the regulation of seed maturation. For example, immature Arabidopsis seeds (15-19 DAP) exhibited higher content of JA and JA-Ile compared to mature seeds (21 DAP) (Dave et al., 2011); in contrast, a much lower JA level was observed in immature soybean seeds as compared to that found in ripened seeds (Creelman and Mullet, 1997).

Other studies highlighted the role of JAs in seed dormancy. For instance, imbibing seeds with exogenous JAs, even for a brief period, can release seeds of many plant species including wheat from the state of dormancy (Berestetzky et al., 1991; Ranjan and Lewak, 1992; Jarvis et al., 1997; Yildiz et al., 2007; Yildiz et al., 2008; Jacobsen et al., 2013); however, previous studies also reported JAs as inhibitors of germination of non-dormant seeds in some species (Wilen et al., 1991; Krock et al., 2002; Norastehnia et al., 2007; Yildiz et al., 2007; Miersch et al., 2008; Oh et al., 2009; Zalewski et al., 2010; Dave et al., 2011; Jacobsen et al., 2013; Sharma et al., 2018). Dave et al. (2011) reported that exogenous JA and OPDA, a precursor of JA, both inhibited gemination of non-dormant Arabidopsis seeds, but JA was much less effective than OPDA. Moreover, seeds with different levels of dormancy exhibit different levels of endogenous JAs and their sensitivity to the JAs, although their relationship could be either positive (Ellis and Turner, 2002; Preston et al., 2009; Dave et al., 2011; Liu et al., 2013; Kashiwakura et al., 2016; Singh et al., 2017) or negative (Dave et al., 2016; Martinez et al., 2016; Xu et al., 2016). The higher levels of JAs and/or

hypersensitivity to JAs observed in seeds exhibiting higher level of dormancy have been hypothesized not to function as the underlying causes for the level dormancy displayed rather they might act as a mechanism to antagonize dormancy (Martinez et al., 2016). Evidence for interaction between JAs and ABA in regulation of dormancy and germination has been reported. For examples, exogenous JAs reduces embryo ABA level and alleviate the germination inhibitory effect of exogenous ABA (Staswick et al., 1992; Ellis and Turner, 2002; Jacobsen et al., 2013). In addition, alterations in seed jasmonate content or sensitivity in many mutants have been shown to be associated with changes in seed sensitivity to ABA during germination (Staswick et al., 1992; Berger et al., 1996; Ellis and Turner, 2002; Abe et al., 2003; Cerdán and Chory, 2003; Lorenzo et al., 2004; Kidd et al., 2009; Dave et al., 2011; Chen et al., 2012; Goossens et al., 2015).

Components of the jasmonate signaling pathway in plants, including COI, JAZ, MYC and MED25, have been shown to participate in the cross-talk of JAs with ABA and GA. The Arabidopsis COI1 might mediate the JAs–ABA synergism, which upregulates the expression of *MYC2*, a jasmonate-responsive gene (Lorenzo et al., 2004). In Arabidopsis, loss of function mutation in *MYC2* causes a decrease in ABA sensitivity (Abe et al., 2003; Yadav et al., 2005; Gangappa et al., 2010) while overexpression of *MYC2* leads to ABA hypersensitivity (Abe et al., 2003; Lorenzo et al., 2004; Goossens et al., 2015). As a result, increased MYC2 activity is associated with inhibition of germination and reduced growth of Arabidopsis seedlings (Goossens et al., 2015). Gibberellins is also implicated to regulate MYC2 positively in the synthesis of sesquiterpenes (Hong et al., 2012) and negatively in the inhibition of root growth (Hou et al., 2010). On the other hand, MED25 has been reported to mediate JAs–ABA antagonism in the regulation of seed germination and seedling growth. It has been shown that MED25 binds to MYC2 and positively regulates transcription of jasmonate-responsive genes but when binding to

ABI5, a transcription factor in ABA signaling pathway, MED25 has a negative effect on transcription of ABI5-regulated genes (Chen et al., 2012). The JAZs appear to have complex roles in the interaction of JAs with GA and ABA. Both JAZ and DELLA negatively regulate MYC2 and WD-repeat/bHLH/MYB complex in the regulation of sesquiterpene synthesis (Hong et al., 2012) and trichome initiation (Qi et al., 2014), respectively. However, DELLA suppresses JAZ in inhibiting root growth (Hou et al., 2010) while JAZ suppresses DELLA in the regulation of hypocotyl elongation (Yang et al., 2012); and it is likely that they suppress each other to create an appropriate balance between plant growth and defense under stress conditions (Huot et al., 2014; Song et al., 2014; Howe et al., 2018). Arabidopsis has 13 JAZs (Howe et al., 2018), and JAZ1, JAZ9 and JAZ12 are among the JAZ proteins that function redundantly in jasmonate signaling (Thines et al., 2007; Demianski et al., 2012; Pauwels et al., 2015; Chini et al., 2016). ABA promotes degradation of JAZ12 at a greater level as compared with JAZ9, and it has no any apparent effect on JAZ1 and JA10 (Larrieu et al., 2015; Pauwels et al., 2015), indicating that the JAs–ABA cross-talk is mediated by specific JAZs.

Induction and maintenance of seed dormancy during seed maturation plays an important role in conferring tolerance of seeds against precocious germination and thereby produce high quality seeds. In addition to the balance between GA and ABA, the well-known regulators of seed dormancy and germination, other plant hormones such as JAs have a considerable role in the regulation these developmental events through their interaction with ABA and GA. However, the molecular mechanisms underlying the role of jasmonate interaction with ABA and GA in the regulation of dormancy during maturation of wheat seeds are scarcely understood. With a specific hypothesis that JAs participate in the regulation of dormancy during seed maturation through synergistic interaction with GA, this chapter of the thesis examined JAs, ABA and GA metabolism

and signaling in maturing seeds of two wheat cultivars that exhibit contrasting dormancy phenotype.

3.3 Materials and methods

3.3.1 Plant material

This study used two genotypes of wheat, AC Domain and RL4452. AC Domain is a Canada Western Red Spring wheat cultivar exhibiting high level of pre-harvest sprouting tolerance (Townley-Smith and Czarnecki, 2008). This cultivar is widely grown and used as parent for wheat breeding in western Canada (McCartney et al., 2005). RL4452 is an unregistered backcross derivative of the pedigree 'Glenlea' *6/'Kitt' and exhibits a low to moderate level of pre-harvest sprouting tolerance (Rasul et al., 2009). Seeds of the two genotypes were imbibed for three days, then germinated seeds were transferred to 3.8 L pot placed in a growth chamber. The pots contained Super Mix, and 18 g of fertilizers (ACER®nt 13-12-12) were applied to each pot prior the transplanting. The chamber was set at 22/18ºC (day/night) and 16/8 h photoperiod. The spikes were individually tagged at anthesis and harvested at different time points, including 20, 30, 40 and 50 DAA, and stored at -80ºC until tissue dissection. For analysis of gene expression and hormone levels, embryos were dissected from developing seeds, frozen immediately in liquid nitrogen and stored at -80ºC until further use. Three to four independent biological replicates of developing seeds (~100 seeds per 2 to 3 plants per replicate) were harvested from each genotype at each stage.

3.3.2 Seed treatment and germination

Seeds freshly harvested at 40 and 50 DAA were tested for germination (25 seeds per Petri-dish as a replicate with a total of three biological replicates). Seeds harvested at 50 DAA were also

subjected to cold-stratification or after-ripening prior to the germination test. The after-ripening treatment was applied by storing the seeds at room temperature (RT) for 4 weeks whereas the cold-stratification treatment was conducted by incubating hydrated seeds at 4ºC for 48 h under darkness. Seeds were surface sterilized either before cold-stratification (cold-stratified seeds) or before imbibition (after-ripened seeds), and both after-ripened and cold-stratified seeds were imbibed at 22ºC under darkness as described in Gao et al. (2012). Germinated seeds, which were identified by the emergence of coleorhiza through the coat, were counted daily for 7 days. Germination index (GI) was calculated as described in Tuttle et al. (2015) using following formula:

$$\mathrm{GI} = \frac{7}{\mathrm{N}} \times \sum_{n=1}^{7} (7 + 1 - n) \times \mathrm{S}_n$$

where N denotes the total number of seeds planted, and S_n refers to the number of newly germinated seeds in the n^{th} day of imbibition.

3.3.3 Gene expression analysis

Isolation of total RNA from tissues and its digestion with DNAse (DNA-free Kit; Ambion, Austin, TX, USA) to remove genomic DNA was performed as described previously (Mukherjee et al., 2015). After digestion with DNAse, total RNA was used for a cDNA synthesis using iScript cDNA synthesis kit (iScript Reverse Transcription Supermix for PT-qPCR; Bio-Rad, CA, USA) in a total reaction volume of 20 µL containing 4 µL iScript RT Supermix, 5 µL RNA solution and 11 µL water. The cDNA samples, after 20x dilution, were used for real-time qPCR assay which consists of 1 µl forward primer (300 nM final concentration), 1 µl reverse primer (300 nM final concentration), 5 µl of the diluted cDNA, 10 µL SsoFast EvaGreen Supermix (Bio-Rad, CA, USA) and 3 µl sterile water in a total reaction volume of 20 µL. Two replicates of each sample were

loaded to CFX97 Real-Time PCR system using 97-well optical reaction plates (Bio-Rad). The thermocycling conditions, which were described previously (Izydorczyk et al., 2017), consist of an initial denaturation at 95ºC for 3 min and 40 cycles each with denaturation at 95ºC for 30 s, primer annealing at 60 or 65ºC for 10 s, and primer extension at 72ºC for 30 s. The annealing temperature was set at 60 or 65ºC dependent on the melting temperature of the primers. *β-actin* was used as the reference gene for normalization, and the relative transcript levels were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

To identify the sequences of the target genes, the respective mRNA or protein sequences in wheat or other species were used as query sequences to BLAST search against International Wheat Genome Sequencing Consortium (IWGSC) RefSeq V1.1 database (Johnson et al., 2008; Mayer et al., 2014). All qPCR primers for interested transcripts were newly designed using Primer3 (Untergasser et al., 2012) except for *TaGA20ox2* and *TaGID1*; the primers reported by Pearce et al. (2015) were used for these two genes (Table 1S-4S). The specificity of qPCR primers was tested by blasting against IWGSC transcript database and verified by melting curve analysis and gel electrophoresis analysis. Primers for all target genes, except for *TaAOS2* and *TaJAR2*, were designed from regions that are conserved across the three homoelogous genes from all three genomes. The IWGSC accession number and the corresponding primers used for qPCR analysis are shown in Tables 1S-4S.

3.3.4 Hormone level analysis

Hormone levels were measured following the method described in Izydorczyk et al. (2017). In brief, freeze dried embryos were ground and homogenized with extraction buffer containing 80% (v/v) acetonitrile, 1% (v/v) acetic acid and internal standards. The ABA, GA and JA-Ile and their

respective internal stands were separated from extraction buffer using three columns as described previously (Izydorczyk et al., 2017). The level of each hormone was analyzed by LC-ESI-MS/MS system (Agilent 1260–6430; Agilent, Santa Clara, CA, USA) using the protocol described in Yoshimoto et al. (2009).

3.3.5 Statistical analysis

Significant differences among samples were tested by two-way ANOVA analysis at a probability of $P<0.05$ while difference between two samples was examined using LSD test at the same probability. Logarithm- or square root-transformation was used when needed to satisfy the statistical assumptions of normality and homogeneity of variances.

3.4 Results

3.4.1 Dormancy phenotype

The seed developmental stages considered in this study include stages from late part of storage reserve accumulation to seed desiccation, and were divided into three phases as early (20 DAA), mid (30–40 DAA) and late (50 DAA) phases of seed maturation as described previously (Yamasaki et al., 2017). Most of the spikes, peduncles and flag leaves of both genotypes started turning yellow at about 40 DAA, indicating the physiological maturity of the seeds. Developing seeds harvested at 40 DAA from both genotypes exhibited similar GI values of less than 0.05, indicating that seeds of both genotypes exhibited a similar level of dormancy at their physiological maturity (Fig. 3.1). While the RL4452 seeds harvested at 50 DAA showed a 3-fold increase in their GI value as compared to that observed for seeds harvested at 40 DAA, no change in GI value was observed between AC Domain seeds harvested at 40 and 50 DAA. By 50 DAA, the seeds of

RL4452 exhibited 3-fold higher GI value than the corresponding AC Domain seeds, although the GI value of the RL4452 seeds harvested at 50 DAA was still less than 0.2. Consistently, differences in GI values were observed between the 50 DAA seeds of the two genotypes in response to cold-stratification for 2 days or after-ripening for 4 weeks. While both the cold and after-ripening treatments increased the GI values of the RL4452 seeds to over 0.90, the two treatments were found to increase the GI values of the AC Domain seeds to only 0.71 and 0.44, respectively.

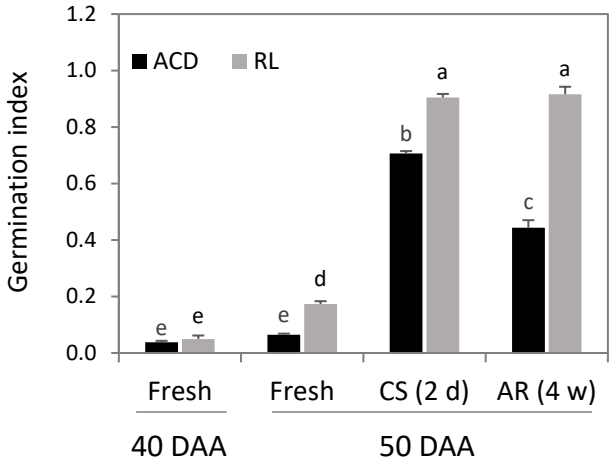

Figure 3.1. Germination index of AC Domain (ACD) and RL4452 (RL) seeds harvested at 40 and 50 days after anthesis (DAA). Freshly harvested seeds (Fresh) were cold-stratified (CS) at 4ºC for 2 days or after-ripened (AR) at room temperature for 4 weeks. Germination index was calculated over 7 days of imbibition. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test)

3.4.2 Expression patterns of jasmonate metabolic genes

The present study analyzed the expression patterns of jasmonate biosynthetic genes including *LOX*, *AOS*, *AOC* and *JAR*. The expression levels of all the JA (*TaLOX, TaAOS,* and *TaAOC*) and JA-Ile biosynthetic genes (*TaJAR*), in general, exhibited increases with seed maturation in both genotypes (Fig. 3.2). Comparative analysis of their expression patterns between the two genotypes

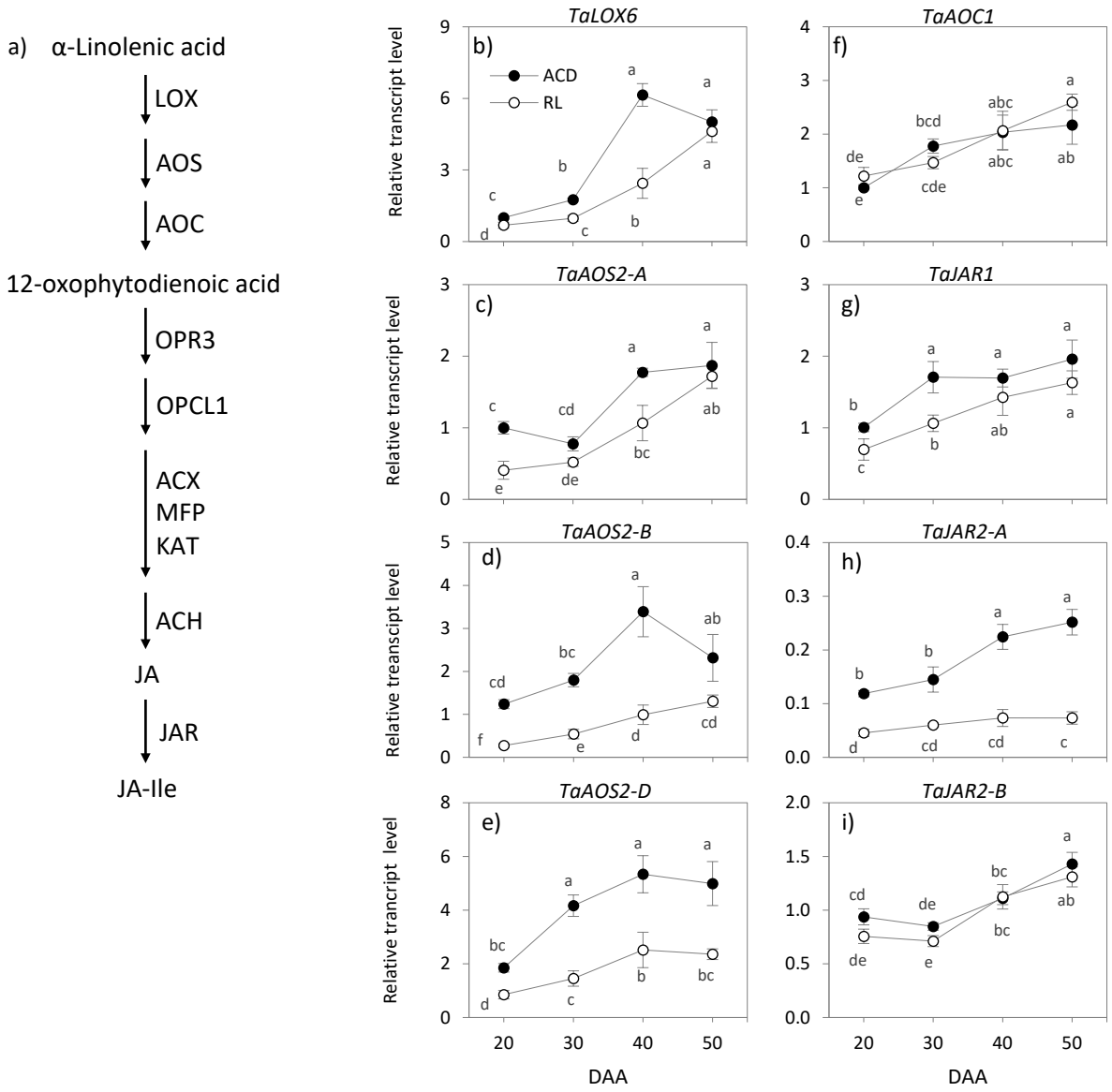

Figure 3.2. Expression of jasmonate biosynthetic genes during seed maturation. A simplified pathway of jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) biosynthesis (a); and relative transcript levels of *TaLOX6* (b), *TaAOS2-A* (c), *TaAOS2-B* (d), *TaAOS2-D* (e), *TaAOC1* (f), *TaJAR1* (g), *TaJAR2-A* (h), *TaJAR2-B* (i) in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaLOX6*, *TaAOS2*s, *TaAOC1* and *TaJARs* were expressed relative to the transcript level of *TaLOX6*, *TaAOS2-A*, *TaAOC1* and *TaJAR1* in 20 DAA samples of ACD, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

revealed that all the genes except *TaAOC1* and *TaJAR2-B* showed higher levels of expression in AC Domain embryos at one or more time points than that observed in RL4452. Of all the genes studied, *TaLOX6*, *TaAOS2-B*, *TaAOS2-D* and *TaJAR2-A* consistently exhibited over 2-fold higher expression levels in AC Domain than RL4452 in most or throughout all the stages studied.

3.4.3 Embryonic JA-Ile level

In general, the JA-Ile levels in both genotypes exhibited decreases with seed maturation (Fig. 3.3). The major reduction in the JA-Ile levels was evident during the mid-phase of seed maturation in both genotypes except that the reduction in RL4452 was prevalent at both early- and mid-phases of maturation. The two genotypes showed similar levels of JA-Ile at 30 and 40 DAA. However, RL4452 embryos contained higher JA-Ile (2.1-fold) levels at 20 DAA while the 50 DAA embryos of AC Domain contained 3-fold higher level than that observed in RL4452 embryos.

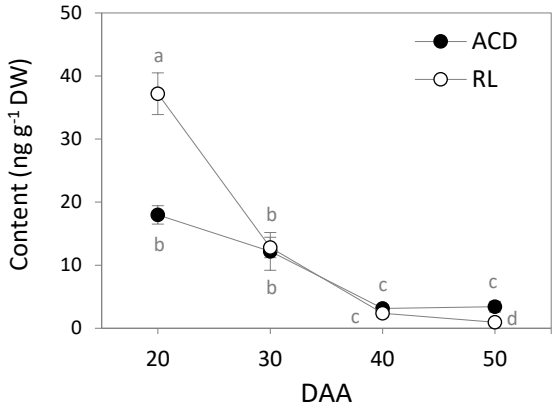

Figure 3.3. Content of jasmonoyl-isoleucine (JA-Ile) during seed maturation. Content of JA-Ile in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

3.4.4 Expression patterns of jasmonate signaling genes

Analysis of the expression patterns of jasmonate signaling genes including *COI, JAZ*, *MYC* and *MED25* was also conducted. Similar to that observed for jasmonate metabolic genes, the jasmonate signaling genes exhibited increases in their expression level as seed maturation progressed from 20 DAA to 50 DAA (Fig. 3.4). However, the major increases in the expression levels of the jasmonate signaling genes occurred during transition from the early to mid phases and/or during the mid phase of seed maturation. Generally, the jasmonate signalling genes showed similar expression levels between maturing seeds of the two genotypes. However, differential expression levels of specific jasmonate signaling genes were evident between the two genotypes at specific seed maturation stages. Higher expression levels of *TaCOI2* (over 1.6-fold) and *TaCOI3* (over 1.3-fold) were apparent in AC Domain than RL4452 at 20 and 30 DAA, and 30 and 40 DAA, respectively. Furthermore, the *TaJAZ4*, *TaJAZ7* and *TaJAZ10* genes showed higher level of expression (~1.7-fold) in AC Domain than RL4452 at 30 and/or 40 DAA while *TaMYC2*, *TabHLH13* and *TabHLH13*-like genes showed higher expression levels (over 1.3-fold) at 20 and/or 30 DAA. Higher expression level of *TaMED25* (over 1.4-fold) was also evident in AC Domain than in RL4452 throughout the seed maturation stages studied except at 50 DAA.

3.4.5 Expression patterns of ABA metabolic genes and content of ABA in embryos

The expression patterns of genes encoding the ABA biosynthetic enzyme NCED and those encoding the ABA catabolic enzyme CYP707A were analyzed during seed maturation in both genotypes (Fig. 3.5). The expression level of *TaNCED1* exhibited an increase or was maintained at almost similar level during seed maturation in AC Domain and RL4452, respectively; however, its level was consistently over 2-fold higher in AC Domain than in RL4452. The expression level

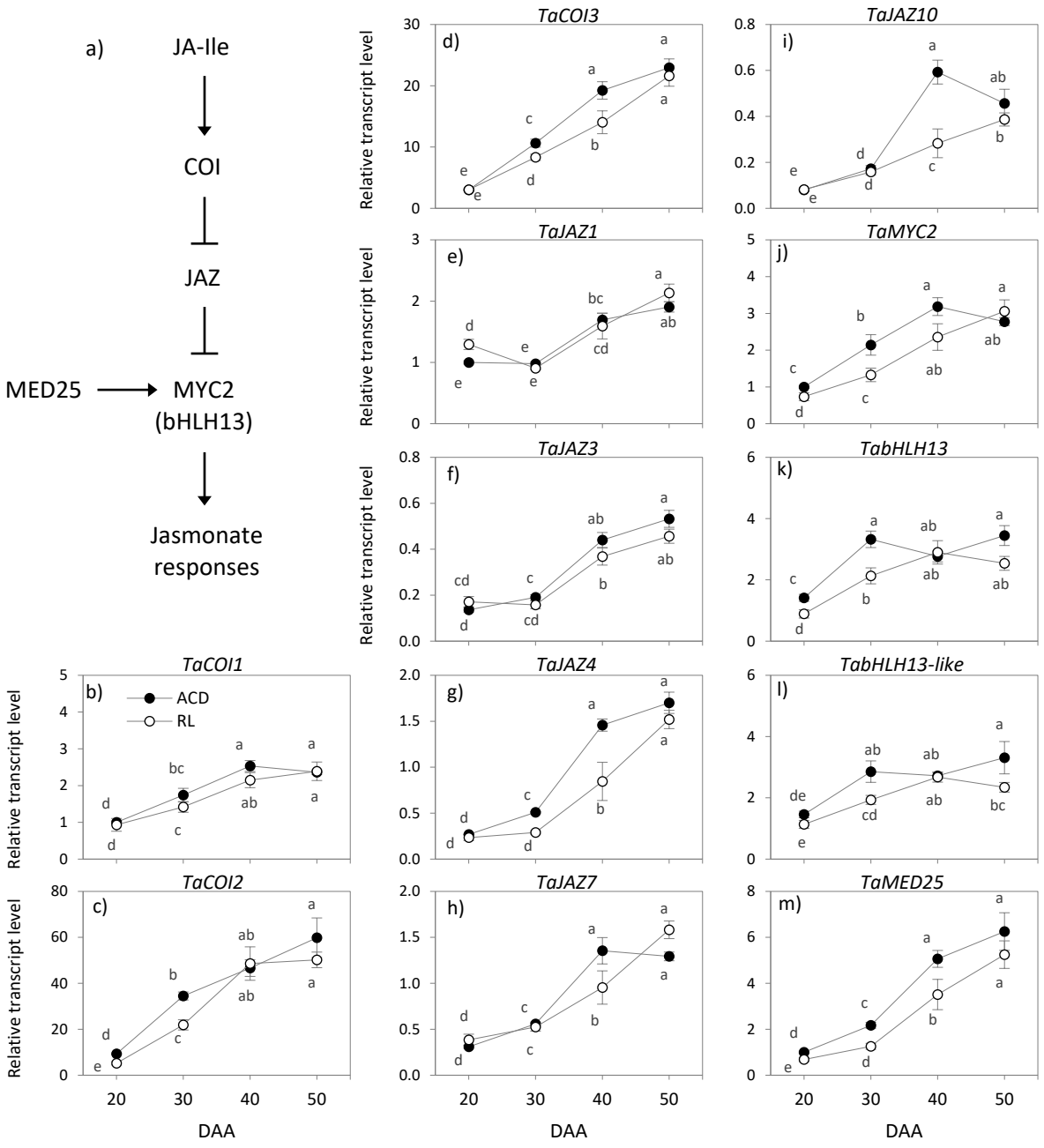

Figure 3.4. Expression of jasmonate signaling genes during seed maturation. A simplified pathway of jasmonoyl-isoleucine (JA-Ile) signaling (a); and relative transcript levels of *TaCOI1* (b), *TaCOI2* (c), *TaCOI3* (d), *TaJAZ1* (e); *TaJAZ3* (f), *TaJAZ4* (g), *TaJAZ7* (h), *TaJAZ10* (i), *TaMYC2* (j), *TabHLH13* (k), *TabHLH13-like* (l) and *TaMED25* (m) in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOIs*, *TaJAZ*s, *TaMYCs* (*TaMYC2, TabHLH13* and *TabHLH13-like*) and *TaMED25* were expressed relative to the transcript levels of *TaCOI1*, *TaJAZ1*, *TaMYC2* and *TaMED25* in 20 DAA samples of ACD, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

of *TaNCED2* decreased during seed maturation with no difference between the two genotypes except that it showed 1.7-fold higher level of expression in AC Domain than in RL4452 at 20 DAA.

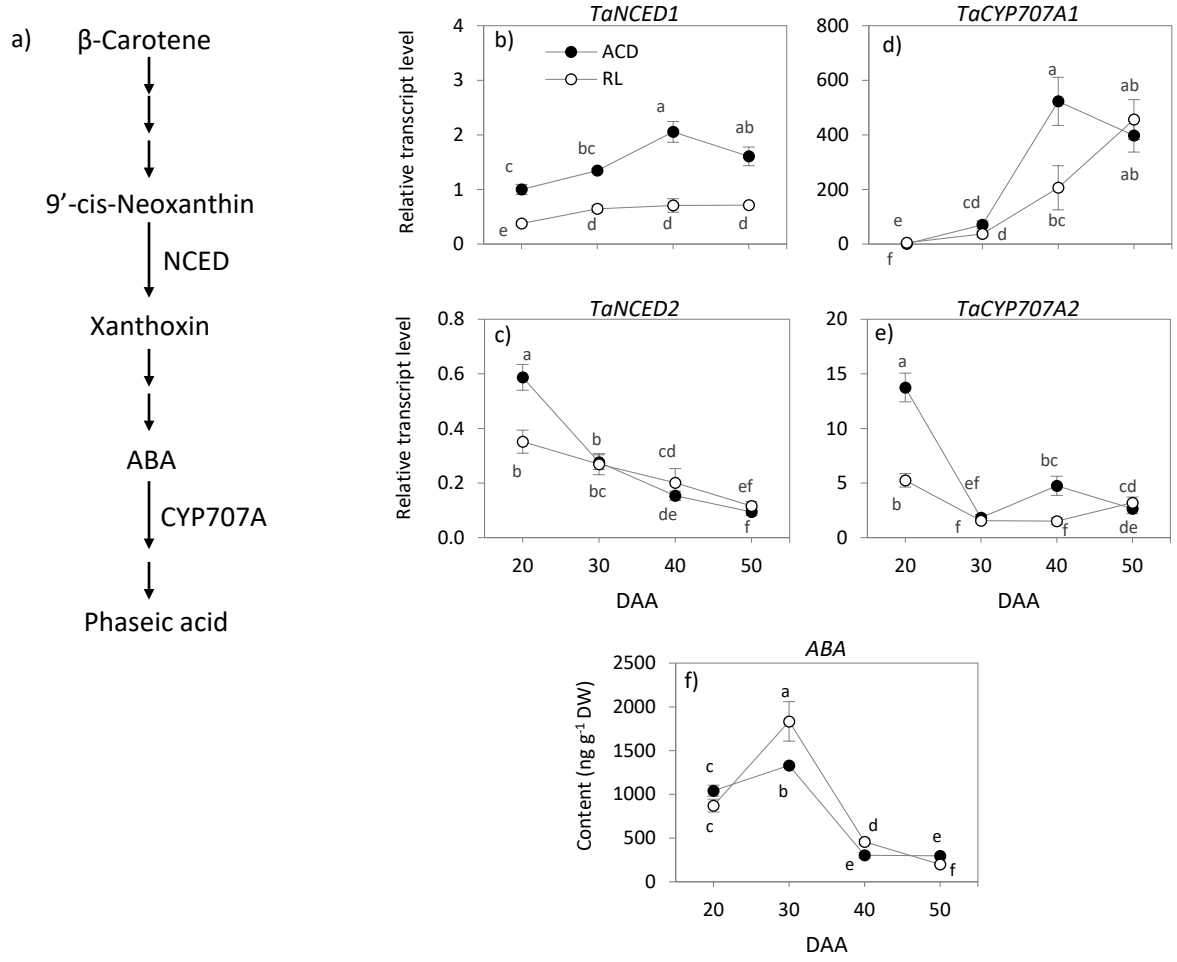

Figure 3.5. Expression of abscisic acid (ABA) metabolic genes and content of ABA during seed maturation. A simplified pathway of ABA metabolism (a); and relative transcript levels of *TaNCED1* (b), *TaNCED2* (c), *TaCYP707A1* (d) and *TaCYP707A2* (e) and ABA content (f) in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaNCEDs* and *TaCYP707As* were expressed relative to the transcript level of *TaNCED1* and *TaCYP707A1* in 20 DAA samples of ACD, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three to four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

Low level of *TaCYP707A1* expression was detected at the early stage of seed maturation (20 DAA) but its expression level showed substantial increase with seed maturation in both genotypes (Fig. 3.5). Whereas, high expression level of *TaCYP707A2* was prevalent at early stage of seed maturation and the level decreased with seed maturation (Fig. 3.5). Comparison between the two genes revealed that the expression level of *TaCYP707A1* is much higher than that of *TaCYP707A2*. AC Domain exhibited higher expression levels (over 2.5-fold) of *TaCYP707A1* and *TaCYP707A2* at 20 and/or 40 DAA compared with RL4452.

Higher ABA level was observed at 20 and 30 DAA in both genotypes, after which the ABA level decreased substantially (over 4-fold) (Fig. 3.5). Comparative analysis of the ABA level between the two genotypes revealed that RL4452 had slightly higher ABA level during the mid phase of seed maturation; however, significantly higher ABA level was prevalent in AC Domain than in RL4452 at the late phase of seed maturation (50 DAA).

3.4.6 Expression patterns of GA metabolic genes and content of GA in embryos

The expression patterns of GA metabolic genes including *GA20ox*, *GA3ox* and *GA2ox*, and content of gibberellins A1 (GA_1) and A_4 (GA_4) were analyzed during seed maturation in both genotypes. GA_1 and GA_4 are known bioactive GA in plants.

The expression of all the GA metabolic genes studied exhibited increases with seed maturation in both genotypes except that expression level of *TaGA3ox2* showed a decrease (over 3-fold) from the mid to late phase of seed maturation (40 to 50 DAA) in AC Domain (Fig. 3.6). The major increases in the expression levels of all of the genes in AC Domain were observed as seed maturation transitioned from early to mid-phase (*TaGA3ox2* and *TaGA2ox6*) and during the mid phase of seed maturation (*TaGA3ox2*, *TaGA2ox3* and *TaGA2ox6*). Whereas, the expression levels of *TaGA20ox1* and *TaGA20ox2* genes showed gradual increases with seed maturation.

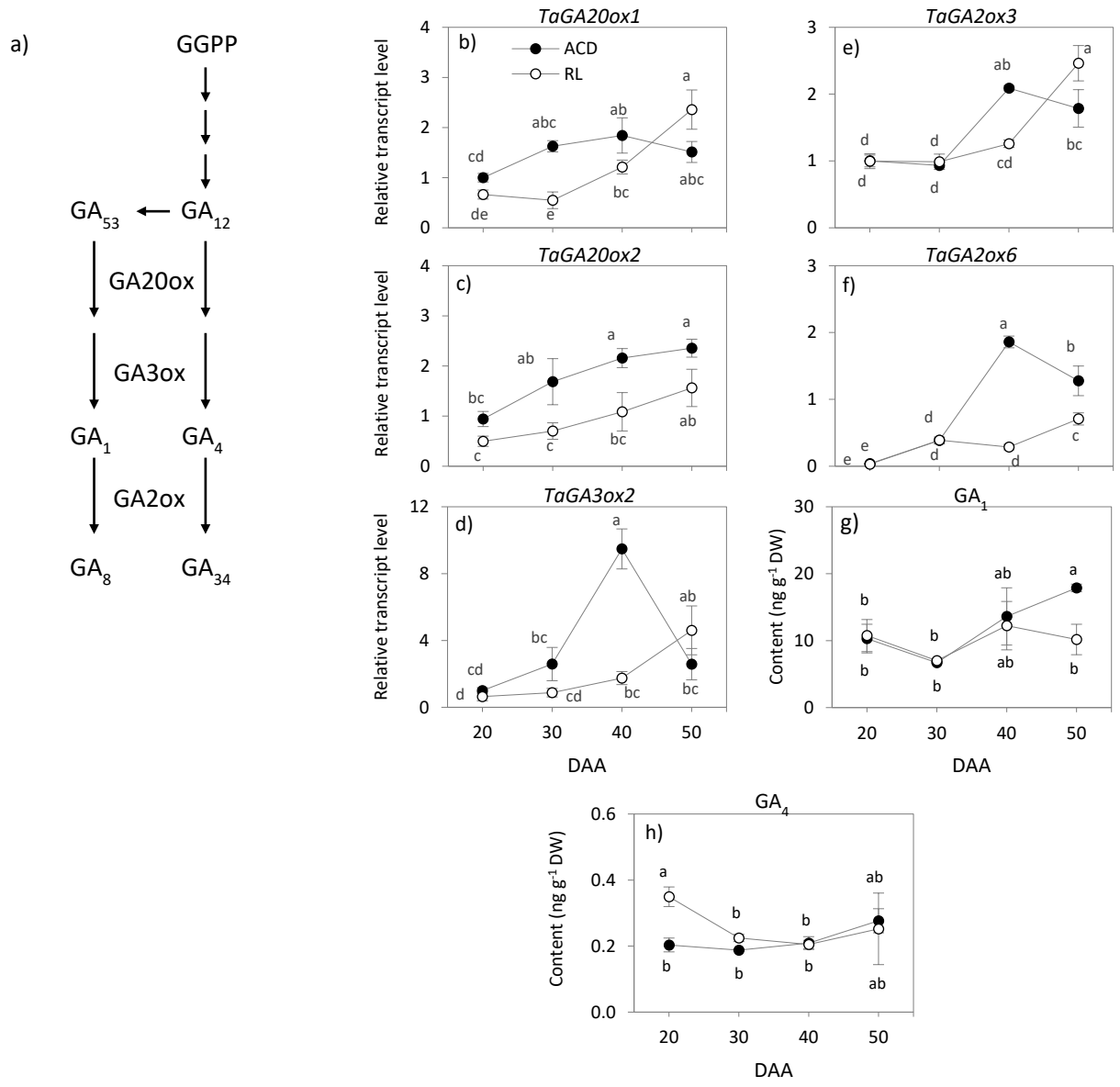

Figure 3.6. Expression of gibberellins (GA) metabolic genes and content of GA during seed maturation. A simplified pathway of GA metabolism (a); and relative transcript levels of *TaGA20ox1* (b), *TaGA20ox2* (c), *TaGA3ox2* (d) *TaGA2ox3* (e) and *TaGA2ox6* (f) and contents of GA_1 (g) and GA_4 (h) in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaGA20ox*s, *TaGA3ox2* and *TaGA2ox*s were expressed relative to the transcript level of *TaGA20ox1, TaGA3ox2* and *TaGA2ox3* in 20 DAA samples of ACD, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three to four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test). GGPP, geranylgeranyl diphosphate.

Increases in the expression levels of the GA metabolic genes in the RL4452 genotype were prevalent mainly during the mid phase (*TaGA20ox1*) and as the seed transitioned from early to mid phase (*TaGA2ox6*) and mid to late phase (*TaGA2ox3* and *TaGA2ox6*) of seed maturation (Fig. 3.6). The expression levels of *TaGA20ox2* and *TaGA3ox2* showed gradual increases throughout seed maturation in RL4452. Comparison between the two genotypes revealed that all the GA metabolic *TaGA2ox6* was also higher in AC Domain than in RL4452 at late phase of seed maturation (50 DAA). Only the *TaGA2ox3* gene showed higher level of expression in RL4452 at late phase of seed maturation (50 DAA).

The level of gibberellin A1 (GA_1) remains almost unchanged during the entire phases of seed maturation in both genotypes, and no difference in GA_1 level was evident between the two genotypes except at the late stage of seed maturation (50 DAA), when GA_1 level in AC Domain was 1.8-fold higher than that observed in RL4452 (Fig. 3.6). Similar level of gibberellin A4 (GA_4) was also maintained during seed maturation and the two genotypes exhibited no difference in their GA_4 level except at early stage of seed maturation (20 DAA) when RL4452 had 1.8-fold more GA_4 than AC Domain (Fig. 3.6).

3.4.7 Expression pattern of ABA signaling in embryos

To examine changes in ABA sensitivity during seed maturation, we examined the expression levels of ABA signaling genes including *PYL5*, *PP2C*, *SnRK2*, *ABI3* and *ABI5*.

The expression level of all the ABA signaling genes overall increased with seed maturation in both genotypes (Fig. 3.7). Increases in the expression level of ABA signaling genes in AC Domain occurred during transition from early to mid phase (*TaPP2C*, *TaSnRK2*, *TaABI3* and *TaABI5*) and during the mid phase of seed maturation (*TaPYL5*, *TaSnRK2*, *TaABI3* and *TaABI5*). In the RL4452 genotype, increases in the expression level of ABA signaling genes occurred during

transition from early to mid phase (*TaSnRK2*, *TaABI3* and *TaABI5*), during the mid phase (*TaPYL5*, *TaSnRK2*, *TaABI3* and *TaABI5*) and during transition from mid to late phase (*TaPYL5*, *TaSnRK2* and *TaABI5*) of seed maturation. The expression level of *TaPP2C* in RL4452 showed a gradual increase throughout the entire phases of seed maturation.

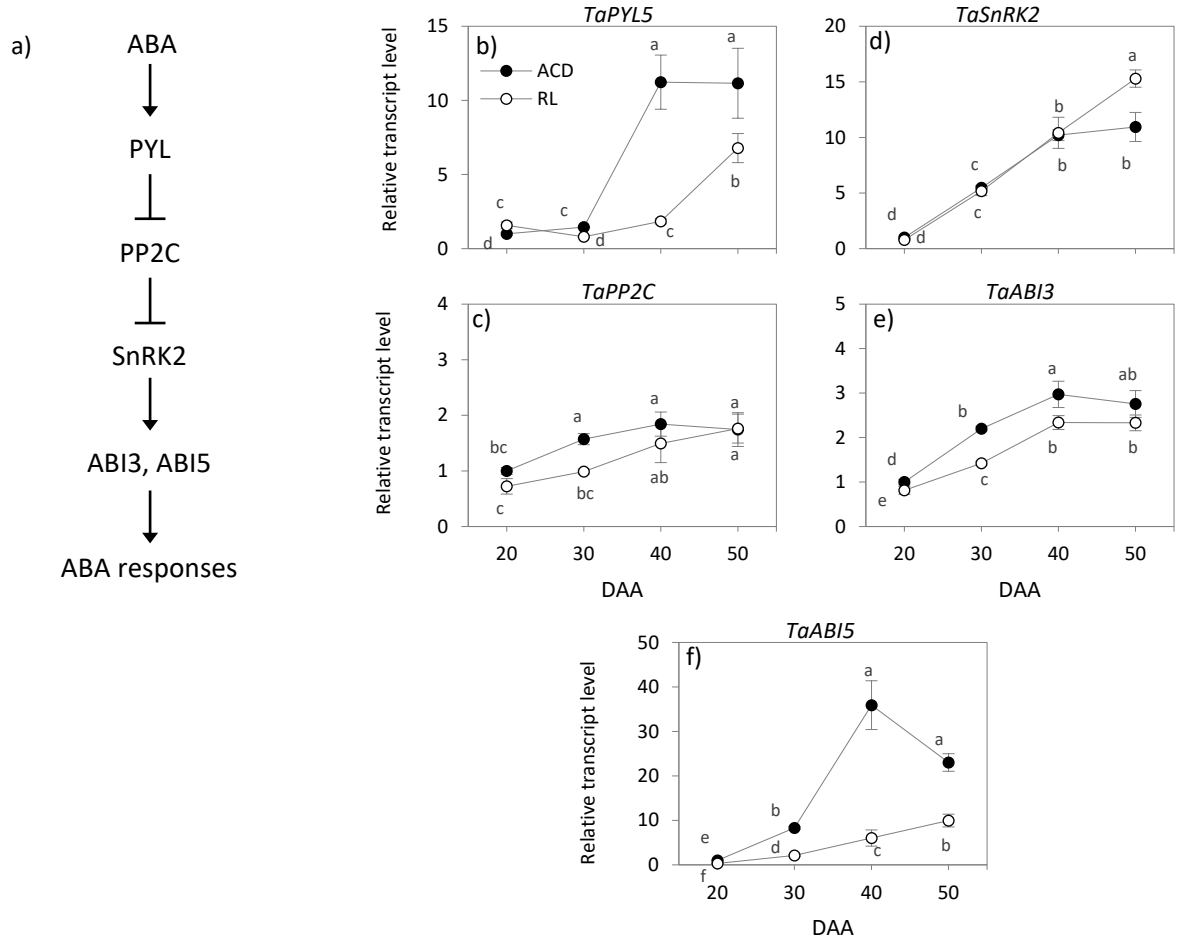

Figure 3.7. Expression of abscisic acid (ABA) signaling genes during seed maturation. A simplified pathway of ABA signaling (a); and relative transcript levels of *TaPYL5* (a), *TaPP2C* (b), *TaSnRK2* (c), *TaBAI3* (d) and *TaBAI5* (e) in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript level of each gene was expressed relative to their transcript level in 20 DAA samples of ACD, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

Comparison between the two genotypes showed the presence of higher expression levels of *TaPYL5* and *TaABI5* at all stages of seed maturation in AC Domain than RL4452 except for *TaPYL5* which showed slightly higher expression at early phase (20 DAA) in RL4452 than AC Domain (Fig. 3.7). The highest differential expression of the two genes between the two genotypes was evident at the later stages of seed maturation (at 40 DAA followed by 50 DAA). The expression level of *TaABI3* was also found to be higher in AC Domain during seed maturation except at the late stage (50 DAA) when its expression level between the two genotypes was similar.

3.4.8 Expression pattern of GA signaling in embryos

The expression patterns of GA signaling genes including *GID*, *RHT* and *GAMyb* in embryos were analyzed during seed maturation in both genotypes. The expression levels of GA signaling genes showed increases with seed maturation in both genotypes except for *TaRHT1* in AC Domain (Fig. 3.8). Major increases in the expression levels of *TaGID1*, *TaGID2*, *TaRHT1* and *TaGAMyb* genes in both genotypes occurred during transition from early (20 DAA) to mid (30 DAA) phase and during mid phase (30 to 40 DAA). Increases in the expression levels of *TaGID2 and TaGAMyb* also occurred during transition from mid (40 DAA) to late (50 DAA) phase of seed maturation in genotype RL4452.

At the early phase (20 DAA), expression levels of GA signaling genes were not different between AC Domain and RL4452 except for *TaGAMyb*; expression level of *TaGAMyb* was higher (3-fold) in AC Domain than in RL4452 (Fig. 3.8). During mid phase (30 to 40 DAA), the expression level of *TaGID1, TaGID2* and *TaGAMyb* in AC Domain were similar to or higher (over 1.3-fold) than that in RL4452 while the expression level of *TaRHT1* in AC Domain was similar to or lower (2.6-fold) than that in RL4452. No difference in expression level of GA signaling between the two genotypes was found at later phase (50 DAA) except that the expression of *TaGAMyb* was higher (3.7-fold) in RL4452 as compared to AC Domain.

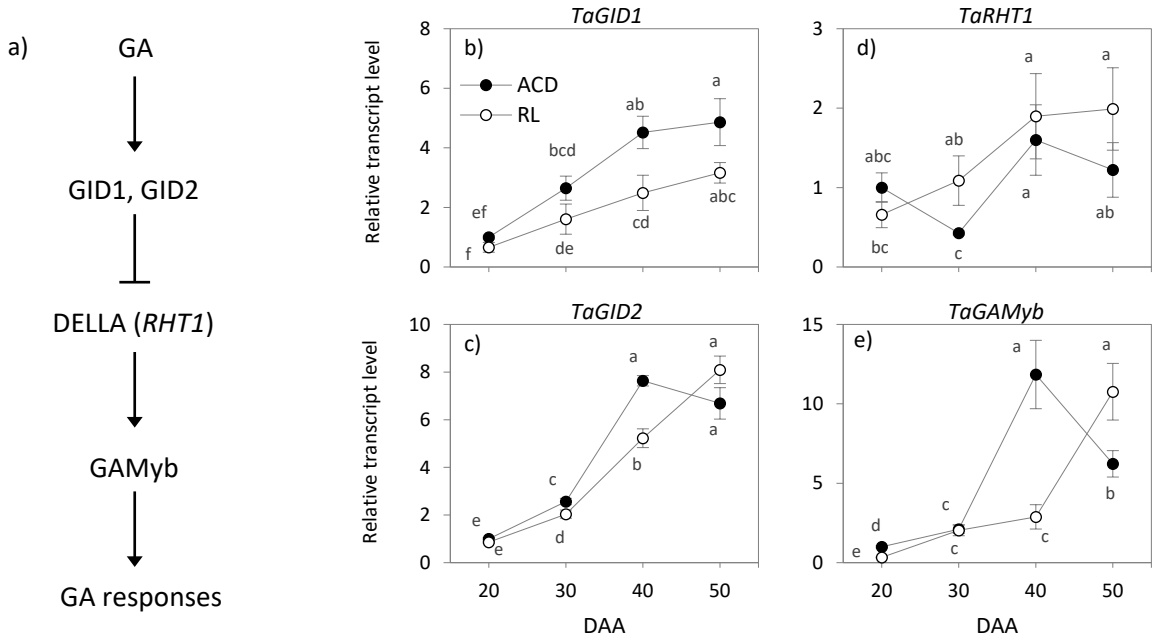

Figure 3.8. Expression of gibberellin (GA) signaling genes during seed maturation. A simplified pathway of GA signaling (a); and relative transcript levels of *TaGID1* (b), *TaGID2* (c), *TaRHT1* (d) and *TaGAMyb* (e) in embryos of AC Domain (ACD) and RL4452 (RL) seeds collected at 20 to 50 days after anthesis (DAA). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript level of each gene was expressed relative to their transcript levels in 20 DAA samples of ACD, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

3.5 Discussion

Physiological maturity of a wheat grain in general occurs 30-50 DAA depending on variety and growth condition (Rogers and Quatrano, 1983; Wan et al., 2008; Guo et al., 2012). By 40 DAA, panicles, peduncles and flag leaves of both genotypes turned yellow indicating physiological maturity of the grains and onset of their post-maturity/ripening phase. The JA-Ile contents of the embryo after physiological maturity (40 and 50 DAA) were much lower than that observed in the immature seeds (at 20 and 30 DAA) in both genotypes (Fig. 3.3), suggesting that the

developmental transition from immature to mature phases is characterized by changes in the levels of JAs in the embryo. It has been shown previously that Arabidopsis seeds attain physiological maturity by 20 DAP (Goldberg et al., 1994; Le et al., 2010). Similar to our result, the contents of JA and JA-Ile in the whole seed of Arabidopsis were found to be lower in mature (21 DAP) and ripened seeds than in immature seeds (15-19 DAP) (Dave et al., 2011). In contrast, a much higher JA level was found in ripened than immature soybean seeds (Creelman and Mullet, 1997). When the seeds transitioned from immature to mature phases, the rate of decrease in JA-Ile level was more pronounced in the non-dormant than the dormant genotype, leading to the presence of relatively higher level of JA-Ile in mature seeds of the dormant genotype AC Domain (Fig. 3.3). This result implies that JAs would play a role in the regulation of dormancy during seed maturation in wheat.

The difference in temporal pattern of embryo JA-Ile level between the two genotypes during maturation, particularly the difference in mature seeds, were associated with the expression pattern of *TaJAR2-A* (Fig. 3.2), suggesting the role of *TaJAR2* in the regulation of embryo JA-Ile level and thereby dormancy during seed maturation in wheat. Such a regulation of JA-Ile level by a specific *JAR* gene family member has been reported previously (Wakuta et al., 2011). For example, the accumulation of JA-Ile in rice in response to wounding was shown to be accompanied by the increased expression of both *OsJAR1* and *OsJAR2* genes while the response to blast fungus infection was accompanied by increased expression of *OsJAR1* only. Consistent with our observation, a higher level of embryonic JA was evident in the more dormant than less dormant wheat cultivar during cold-stratification and imbibition (Xu et al., 2016). However, Liu et al. (2013) reported that dormant seeds and non-dormant (after-ripened) seeds from AC Domain exhibited similar level of whole seed JA in dry seeds, and when the seeds were imbibed for 24 h,

dormant seeds possessed a lower JA level than non-dormant seeds. Furthermore, seeds of the Arabidopsis *aos* mutant have been reported to exhibit lower levels of JA and reduced dormancy (Dave et al., 2011; Dave et al., 2016) while dry seeds of Arabidopsis plants overexpressing the jasmonate biosynthesis gene *AOS* exhibit higher level of JA-Ile and lower level of dormancy as compared to their wild-type counterparts (Singh et al., 2017). In contrast, embryos of freshly harvested dry or imbibed seeds of a less dormant Japanese wheat cv. Gifu have been shown to exhibit higher levels of JA and JA-Ile as compared to that observed in the dormant genotype OS38 (Kashiwakura et al., 2016). Our germination phenotype data (Fig. 3.1) indicated that initiation of the decay of dormancy in the seeds of non-dormant phenotype was initiated prior to the 50 DAA stage, leading to the prevalence of different levels of dormancy in the seeds of the two genotypes at maturity and in response to cold-stratification and after-ripening treatments. Consistent with these results, comparative analysis of pre-harvest sprouting tolerance in the two genotypes identified AC Domain as more tolerant to pre-harvest sprouting than RL4452 (Townley-Smith and Czarnecki, 2008; Rasul et al., 2009). Our results overall indicate the contribution of jasmonate level in the differential expression of dormancy level in wheat seeds.

No significant difference in dormancy level (Fig. 3.1) as well as in embryonic ABA (Fig. 3.5) and GA levels (Fig. 3.6) was observed during transition of seeds from 40 to 50 DAA in the dormant genotype AC Domain, whereas reduction in dormancy and ABA level was apparent as the seeds of the non-dormant genotype transitioned from 40 to 50 DAA. Although no apparent change was evident in the level of GA during this transition, the change in ABA level leads to a change in ABA–GA balance and thereby seed dormancy. These results are consistent with the fact that an imbalance between ABA and GA contents in favor of GA is needed for reduction in the level of dormancy (Finkelstein et al., 2008; Graeber et al., 2012; Gazzarrini and Tsai, 2015). The

reduction in ABA level in RL4452 from 40 to 50 DAA was accompanied by the decrease in expression level of ABA biosynthesis gene *TaNCED2* and the increase in expression level of ABA catabolic genes *TaCYP707A1* and *TaCYP707A2* (Fig. 3.5). This implies a role of transcriptional regulation of ABA biosynthesis and catabolism in controlling level of ABA in later phase of RL4452 seed development. Transcriptional regulation of the *TaNCED1* and *TaCYP707A1* genes was previously demonstrated to be involved in the reduction of embryo ABA level caused by exogenous JAs during imbibition of wheat seeds (Jacobsen et al., 2013).

As observed for ABA level (Fig. 3.5), the maintenance of a similar level of dormancy during seed transition from 40 to 50 DAA in the dormant genotype AC Domain (Fig. 3.1) was accompanied by the maintenance of similar levels of JA-Ile (Fig. 3.3). Moreover, reduction in dormancy level during the same period in RL4452 was closely associated with decreases in the levels of JA-Ile. These results suggest synergistic interaction between JAs and ABA in regulating dormancy during seed maturation in wheat and the role of JAs as additional factors that participate in the ABA–GA mediated control of dormancy. Although exogenous JAs have been shown to break seed dormancy in wheat (Jacobsen et al., 2013) and other species (Berestetzky et al., 1991; Ranjan and Lewak, 1992; Jarvis et al., 1997; Yildiz et al., 2007; Yildiz et al., 2008), several studies with seeds of different species have also demonstrated the role of JAs in inhibiting the germination of non-dormant seeds of Arabidopsis (Oh et al., 2009; Dave et al., 2011), wheat (Jacobsen et al., 2013), maize (Norastehnia et al., 2007), tomato (Miersch et al., 2008), rapeseed (Wilen et al., 1991) and some other species (Krock et al., 2002; Yildiz et al., 2007; Zalewski et al., 2010; Sharma et al., 2018).

The maintenance of a similar level of dormancy after physiological maturity in the seeds of the dormant genotype AC Domain (Fig. 3.1) was closely associated with the maintenance of

similar expression levels all the *JAZ* genes, which encode a negative regulator of jasmonate signaling, analyzed in this study (Fig. 3.4). Likewise, the loss of dormancy during transition from 40 to 50 DAA in the non-dormant genotype RL4452 (Fig. 3.1) was associated with the prevalence of enhanced expression level of the *JAZ* genes (Fig. 3.4). These results suggest the role of transcriptional regulation of jasmonate signaling in the control of seed dormancy during seed maturation, and thereby provide additional support for the involvement of JAs in the control of dormancy in wheat seeds.

The expression patterns of GA and ABA signaling genes in RL4452 embryos during the transition from 40 to 50 DAA (Figs. 3.7, 3.8) suggest that the sensitivity of embryos to GA, whose increase could weaken dormancy level, and the sensitivity of embryos to ABA, whose increase could promote dormancy level, both might be increased by the transition. The expression level of gene encoding DELLA (*TaRHT1*; Fig. 3.8), a negative regulator in GA signaling pathway, was maintained unchanged from 40 to 50 DAA in RL4452 embryos while the expression of genes encoding positive regulators GID2 and GAMyb of the signaling pathway were remarkably increased (Fig. 3.8). Similarly, the transition from 40 to 50 DAA in RL4452 embryos did not change the expression level of gene encoding PP2C (Fig. 3.7), a negative regulator in ABA signaling pathway, but it increased expression level of genes encoding PYL5, SnRK2 and ABI5, all of which are positive regulators of ABA signaling pathway (Fig. 3.7). However, the increase in sensitivity to ABA is not in agreement with the difference in dormancy phenotype observed between 40 and 50 DAA seeds.

It has been shown previously that JAZ proteins interact with different regulatory proteins including DELLAs (Chini et al., 2016), and JAZ–DELLA antagonistic interaction has been suggested to play a key role in the JAs–GA cross-talk (Song et al., 2014). For example, it has been

shown that JAZs, by suppressing DELLA, could enhance the role of GA in regulating hypocotyl elongation positively (Yang et al., 2012). In the present study, the expression of the gene encoding DELLA, *TaRHT1*, were maintained unchanged during the transition from 40 to 50 DAA in both AC Domain and RL4452 embryos (Fig. 3.8). The expression level of all of *TaJAZs* measured were also maintained unchanged from 40 to 50 DAA in AC Domain, except for that of *TaJAZ1* which was lightly higher in 50 DAA embryos than in 40 DAA embryos (Fig. 3.4). By contrast, less-dormant 50 DAA embryos of RL4452 exhibited higher expression levels of all of *TaJAZs* as compared to dormant 40 DAA embryos, except for *TaJAZ3* as its increase was not significant (Fig. 3.4). These suggest that dormancy release in wheat seeds might be associated with up-regulation of *TaJAZs* which could enhance the activity of JAZ proteins, acting as a mechanism to antagonize DELLA action to improve GA sensitivity (Yang et al., 2012; Song et al., 2014; Chini et al., 2016). Moreover, previous reports have shown that MED25 can bind to ABI5, the downstream transcription factor in ABA signaling, and repress ABA signaling, leading to MED25 mediated JAs–ABA antagonism in the regulation of germination and seedling growth in Arabidopsis (Chen et al., 2012). Thus, the increased expression level of *TaMED25* in RL4452 embryos during transition from 40 to 50 DAA (Fig. 3.4) might suggest that dormancy release is associated with reduction in ABA sensitivity.

In addition to the substantial decrease in the levels of JA-Ile, the transition of wheat seed from immature to mature stages in both dormant and non-dormant genotypes of wheat was characterized by substantial decreases in embryonic ABA. The temporal changes in ABA level (Fig. 3.5) and the expression patterns of the ABA signaling genes (Fig. 3.7) suggest the role of embryo sensitivity to ABA in the regulation of seed maturation associated physiological processes such as acquisition of desiccation tolerance, and maintenance and release of dormancy. On the

other hand, the temporal expression pattern of the ABA metabolic genes (Fig. 3.5) suggests the role of transcriptional regulation of ABA biosynthesis and catabolism in controlling the level of ABA in mature seeds of wheat. Application of exogenous JAs during imbibition of wheat seeds has been shown to cause reduction in embryo ABA level through transcriptional regulation of the *TaNCED1* and *TaCYP707A1* genes (Jacobsen et al., 2013). In the present study, the levels of both ABA (Fig. 3.5) and JA-Ile (Fig. 3.3) were low in physiologically mature seeds of both genotypes. It is therefore likely the prevalence of up-regulation of the *TaCYP707A1* gene in maturing wheat seeds is not governed by the level of JAs.

In contrast to the reduced level of JA-Ile (Fig. 3.3) and ABA (Fig. 3.5), the transition of AC Domain seeds from immature to mature stage was accompanied by the increased level of GA_1 (Fig. 3.6). Although only 50 DAA embryos exhibited higher level of GA_1 compared to those in 20 and 30 DAA embryos, the expression pattern of GA metabolic genes (Fig. 3.6), particularly *TaGA3ox2*, suggest that the onset of the increase in level of GA might occur prior to 40 DAA. In Arabidopsis seeds, *GA3ox1* are rate-limiting gene in GA biosynthesis (Nonogaki, 2014). In wheat the suppression of GA synthesis in seeds exposed to suboptimal temperature during imbibition was accompanied by the repression of *GA3ox2* (Izydorczyk et al., 2017).

In conclusion, our results suggested that the maturation process in wheat seeds could be characterized not only by the changes in ABA and GA level and signaling in embryos which is the main factors regulating its dormancy, but also by the changes in jasmonate levels and signaling which could significantly interact with both ABA and GA (Fig. 3.9). Especially, the increases in expression of jasmonate signaling regulators JAZs and MED25 during the last phase of maturation suggest the involvement of synergism and antagonism of JAs toward GA and ABA, respectively, via JAZ–DELLA and MED25–BAI5 interactions, respectively, in the onset of dormancy loss after

physiological maturity in RL4452 seeds. However, changes in the levels of seed dormancy status can occur after harvest by using different types of treatments including after-ripening, the dry storage of seeds, and treatments with exogenous hormones. To better understand the role of JAs in such treatments, the second chapter of the thesis investigated the changes in expression level of metabolic and signaling genes of JAs, GA and ABA during dormancy release by after-ripening and treatments with exogenous JAs and GA.

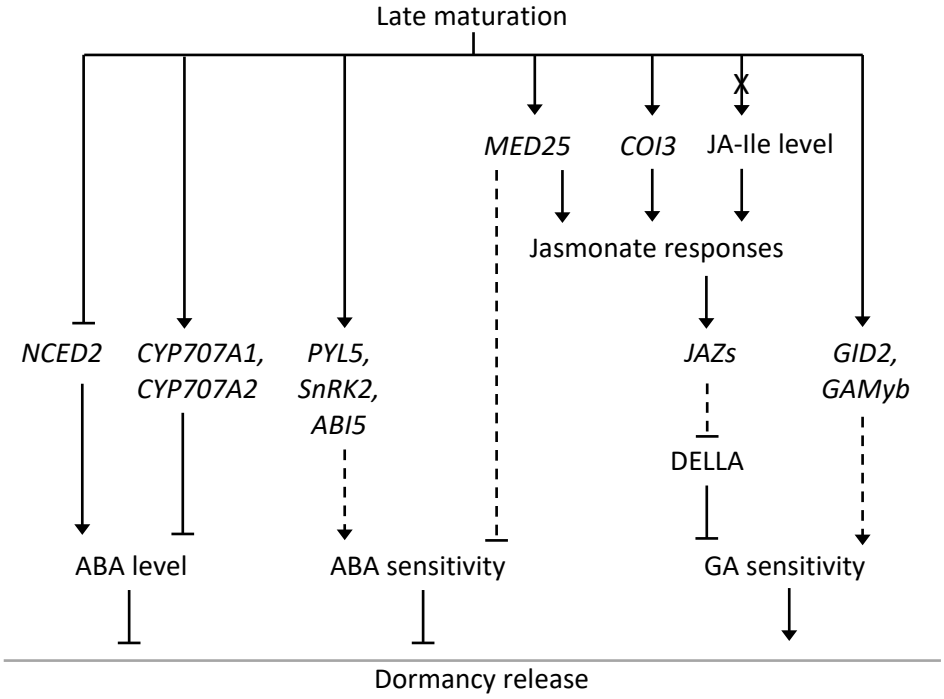

Figure 3.9. A schematic diagram of regulation of wheat seed dormancy release during the late phase of seed maturation based on wheat genotype RL4452. Up-regulation of the jasmonate signaling genes *JAZs* can lead to an increase in GA sensitivity; and the up-regulation of *JAZs* is not associated with increase in JA-Ile level rather it is associated with up-regulation of jasmonate signaling genes *COI3* and *MED25*. Dashed lines indicate expected effects; an arrow with "X" indicates absence of positive effect.

4 RELATIONSHIP BETWEEN JASMONATE RESPONSE AND GIBBERELLIN SENSITIVITY DURING SEED DORMANCY RELEASE IN WHEAT

4.1 Abstract

Interaction of JAs with ABA and GA are crucial in the regulation of plant developmental processes. The present study examined changes in the contents of the three hormones and expression of their metabolic and signaling genes during imbibition of dormant seeds exposed to hormone and after-ripening treatments. Results of this study showed that exogenous MeJA broke dormancy as effectively as exogenous gibberellin (GA_3) and after-ripening. Dormancy release by MeJA was associated with increased expression of jasmonate signaling and/or responsive genes *TaAOS2, TaAOC*, *TaCOI2*, *TaJAZs* and *TaMYC2*. The MeJA-induced enhanced expression of *TaJAZs* suggest an increase in GA sensitivity owing to the repression of DELLA by JAZs. Moreover, MeJA induced increases in the expressions of GA synthesis and signaling genes *TaGA3ox2*, *TaGID1* and *TaGAMyb*, and ABA catabolic genes *TaCYP707A*s. Treatment with MeJA plus GA_3 caused a higher level of dormancy decay as compared to GA_3 alone, adding evidence for the synergistic actions of JAs and GA. Whereas, treatment with MeJA plus paclobutrazol, a GA synthesis inhibitor, was less effective in breaking dormancy as compared to MeJA alone, implying that MeJA-induced dormancy release is partly dependent on GA. Exogenous GA and after-ripening decreased ABA level and sensitivity by reducing expression levels of *TaNCED1* and *TaABI5* and increasing those of *TaPP2C*. However, they did not affect the expression levels of most of the jasmonate biosynthesis and signaling genes. These results highlight that increases in jasmonate level and sensitivity could break dormancy in wheat seeds mainly by increasing GA level and sensitivity and decreasing ABA level. However, JAs might function only as an additional factor in the regulation of seed dormancy.

4.2 Introduction

Jasmonates are involved in regulating several plant growth and developmental processes including flower and seed development, tuber formation and induction of root hair development (Zhu et al., 2006; Cai et al., 2014; Domínguez and Cejudo, 2014; Zhai et al., 2015), and these effects of JAs are regulated by both its level and signaling. The level of JAs in plants is controlled by its biosynthesis, in which bioactive JAs are produced from linolenic acid and hexadecatrienoic acid, and this process involves several enzymes operating in different cell organelles including plastids and peroxisomes. In the plastid, the linolenic acid and hexadecatrienoic acid are converted to OPDA and dnOPDA, respectively, by the actions of three enzymes which include LOX, AOS and AOC. In the peroxisome, OPDA and dnOPDA are converted to JA by the actions of OPR3, OPCL1, ACX, MFP, KAT, and ACH. Jasmonic acid can be converted to JA-Ile and MeJA by the actions of JAR and JASMONIC ACID CARBOXYL METHYLTRANSFERASE (JMT), respectively. While JA-Ile has been well recognized as a biologically active form of JAs in plants (Wasternack and Song, 2017; Zhai et al., 2017), MeJA is often considered as biologically inactive (Stitz et al., 2011).

The signaling pathway in which JA-Ile triggers jasmonate response has been studied using several biochemical, structural, and genetic approaches (Browse, 2009; Fonseca et al., 2009; Koo and Howe, 2009; Pauwels and Goossens, 2011). The major elements of JA-Ile signaling include co-receptor F-box protein COI1, repressors JAZ, co-repressors NINJA and TPL, activators MYC2 (bHLH-Zip transcription factor), and co-activator MED25 (a subunit of MEDIATOR complex). When JA-Ile level is low, JAZ, NINJA and TPL form a complex with transcription factor MYC2 and repress MYC2 from activating the transcription of jasmonate-responsive genes. In contrast, under alleviated JA-Ile levels, JA-Ile mediates the formation of a stable COI1–JA-Ile–JAZ

complex leading to the degradation of JAZ and consequent de-repression of the transcription of MYC2-regulated genes (Howe et al., 2018; Wasternack and Strnad, 2018). The transcription factor MYC2 is known to regulate the expressions of its own gene (*MYC2*) and a number of jasmonate biosynthesis and signaling genes including *LOX, AOS, AOC, OPR3*, *ACX* and *JAZ* (Wasternack and Hause, 2013; Wasternack and Song, 2017; Howe et al., 2018). Therefore, increased transcript levels of these genes, particularly that of *JAZ* and *MYC2*, are proposed to indicate an alleviated JA-Ile response. This, on one hand, equips the plant tissues with a mechanism to quickly acquire an appropriate level of JAs, and on the other hand, it offers a way to examine JA-Ile response.

The role of JAs in regulating seed dormancy has been demonstrated in several plant species. For example, exogenous JA induces germination of dormant embryos/seeds of tartaric maple (Berestetzky et al., 1991), apple (Ranjan and Lewak, 1992; Yildiz et al., 2007) and pear (Yildiz et al., 2007). Similarly, MeJA exhibited the ability to release seed dormancy in Douglas fir (Jarvis et al., 1997) and wheat (Jacobsen et al., 2013). Jasmonates have also been implicated in reducing seed sensitivity to factors that induce secondary dormancy in seeds of different plant species. Exogenous JA or MeJA reduces imidacloprid-induced secondary dormancy in brown mustard (*Brassica juncea* L.) seeds (Sharma et al., 2018), alleviates dormancy caused by blue light in wheat (Jacobsen et al., 2013), and antagonizes the action of ABA in non-dormant seeds of Arabidopsis (Ellis and Turner, 2002). In addition, acetylsalicylic acid (ASA), which inhibits JA biosynthesis in plants (Pena-Cortés et al., 1993), has been reported to inhibit not only the release of dormancy in response to cold treatment of dormant wheat seeds (Xu et al., 2016) but also the germination of non-dormant wheat seeds (Jacobsen et al., 2013). However, JAs have also been reported to inhibit germination of non-dormant seeds in several plant species. Treatment with JA or MeJA has been found to inhibit germination of non-dormant seeds in Arabidopsis (Oh et al.,

2009; Dave et al., 2011), wheat (Jacobsen et al., 2013), rapeseed, flax (Wilen et al., 1991) and maize (Norastehnia et al., 2007). The germination inhibitory effect of exogenous JAs appeared to be in agreement with previous observations that implicate endogenous JAs as negative regulators of germination. For example, jasmonate content has been shown to decrease during germination in Arabidopsis (Preston et al., 2009; Dave et al., 2011) and wheat (Liu et al., 2013; Kashiwakura et al., 2016; Martinez et al., 2016; Xu et al., 2016) seeds. Furthermore, seeds of Arabidopsis mutants such as *aos*, *opr3-1*, *cts-1*, *jar1*, *coi1-16* and *myc2*, that are characterized by reduced jasmonate level and sensitivity, are found to exhibit a germination phenotype similar to that of wild-type (Staswick et al., 1992; Berger et al., 1996; Ellis and Turner, 2002; Footitt et al., 2002; Abe et al., 2003; Oh et al., 2009; Gangappa et al., 2010; Dave et al., 2011; Chen et al., 2012) while inhibition of germination is evident in mutants such as *kat2*, *acx1-2 acx2-1* (double mutant), OE-MYC2D^{1052}A and OE-MYC2D^{1052}B, that exhibit increased jasmonate level or sensitivity (Abe et al., 2003; Dave et al., 2011; Goossens et al., 2015).

A previous study has shown that exogenous MeJA antagonizes ABA biosynthesis in embryos by regulating the expression levels of the ABA biosynthesis (*NCEDs*) and catabolism (*CYP707As*) genes during imbibition of wheat seeds, contributing to jasmonate-mediated alleviation of dormancy induced by blue light (Jacobsen et al., 2013). It is well established that the balance between ABA and GA play a key role in controlling seed dormancy and germination through regulating the induction/re-induction, expression and release of dormancy as well as the germination capacity (Rodríguez et al., 2015; Shu et al., 2016). An imbalance in favor of GA is required for dormancy release and germination whereas that in favor of ABA is needed for the induction and maintenance of dormancy. Importantly, not only the contents of ABA and GA but also the sensitivity of seed tissues to these hormones is involved in the regulation of ABA–GA balance (Finkelstein et al., 2008; Graeber et al., 2012; Gazzarrini and Tsai, 2015). Therefore,

investigating the effects of JAs on the ABA and GA metabolism and signaling in wheat seeds is critical to understand the roles of JAs in the regulation of seed dormancy and germination. Based on a specific hypothesis that JAs enhance seed sensitivity to GA during the breaking of dormancy, this part of the thesis studied transcriptional regulation of JAs, ABA and GA metabolism and signaling genes in response to dormancy release by after-ripening and treatment with exogenous JAs and/or GA.

4.3 Materials and methods

4.3.1 Plant material and germination assay

Wheat cultivar AC Domain, a Western Red Spring wheat cultivar that exhibits high level of pre-harvest sprouting tolerance (Townley-Smith and Czarnecki, 2008), was grown in a growth chamber as described in chapter 3. The seeds were harvested and immediately stored at -80ºC. A portion of the seeds were allowed to maintain dormancy by maintaining storage at -80ºC while the remaining seed samples were subjected to after-ripening via dry storage at RT for different durations – 0, 4, 8 and 12 weeks – in which the 0 week after-ripening refers to seeds not subjected to the after-ripening treatment. Germination experiments of the different seed samples (25 seeds per Petri-dish as a replicate with a total of three to four biological replicates) were performed as described in chapter 3. Imbibed seeds were collected at 6, 12, and 24 HAI. Embryos were dissected, frozen immediately in liquid nitrogen and stored at -80ºC for analysis of gene expression and hormone level.

4.3.2 Hormone treatments

The stock solutions of individual hormones (MeJA, ABA and GA_3) or inhibitors (acetylsalicylic acid [ASA], inhibitor of jasmonate biosynthesis; fluridone [FLU], inhibitor of ABA biosynthesis; PAC, inhibitor of GA biosynthesis) were prepared using dimethylsunfoxide (DMSO) and stored

at -20ºC. The treatment solutions were prepared fresh immediately before use. The hormone/inhibitor treatments used in this experiment include control (water), MeJA (200 µM), GA_3 (10 µM), ABA, (5 µM), ASA (10 mM), PAC (300 µM), FLU (10 µM), MeJA + GA_3 (200 µM MeJA + 10 µM GA_3), and MeJA + PAC (200 µM MeJA + 300 µM PAC). Every treatment solution, including the control, contained 0.1% DMSO (v/v). Seeds were surface sterilized and imbibed with treatment solution as described above.

4.3.3 Gene Expression and hormone level analyses

Gene expression and hormone level analysis were performed as described in chapter 3. The accession number of the genes studied in this chapter and their respective qPCR primers are described in Tables 1S-4S.

4.3.4 Statistical analysis

Significant differences among samples were tested by two-way ANOVA analysis at a probability of $P<0.05$ while difference between two samples was examined using LSD test at the same probability. Logarithm- or square root-transformation was used when needed to satisfy the statistical assumptions of normality and homogeneity of variances. There were 3 and 4 biological replicates in the hormone experiment and after-ripening experiment, respectively.

4.4 Results

4.4.1 Germination of dormant seeds in response to hormone and after-ripening treatments

4.4.1.1 Hormone treatment

Germination index of the dormant seeds was only 0.1 (Fig. 4.1a); however, treatment with MeJA or GA_3 effectively released the seeds from dormancy, resulting in GI values of 0.8 and 0.9, respectively; and the GI value increased to the maximum value of 1 by treatment with MeJA plus

GA_3. Treatment of seeds with PAC along with MeJA reduced the effect of MeJA by 2-fold. Although the GIs values were calculated based on imbibition for 7 days, the differences in GI values were mainly attributed to the difference in germination percentage within the first 2 days of imbibition (Fig. 4.1b). None of the control seed and seeds treated with MeJA plus PAC germinated at 1 day after the start of imbibition (DAI) while the seeds treated with MeJA, GA_3 and MeJA plus GA_3 showed approximately 24%, 52% and 79% germination, respectively. All of the seeds treated with MeJA plus GA_3 germinated within 2 DAI when only 8% of the control seeds germinated. The germination percentages for seeds treated with GA_3, MeJA, and MeJA plus PAC at 2 DAI were approximately 37%, 77% and 87%, respectively.

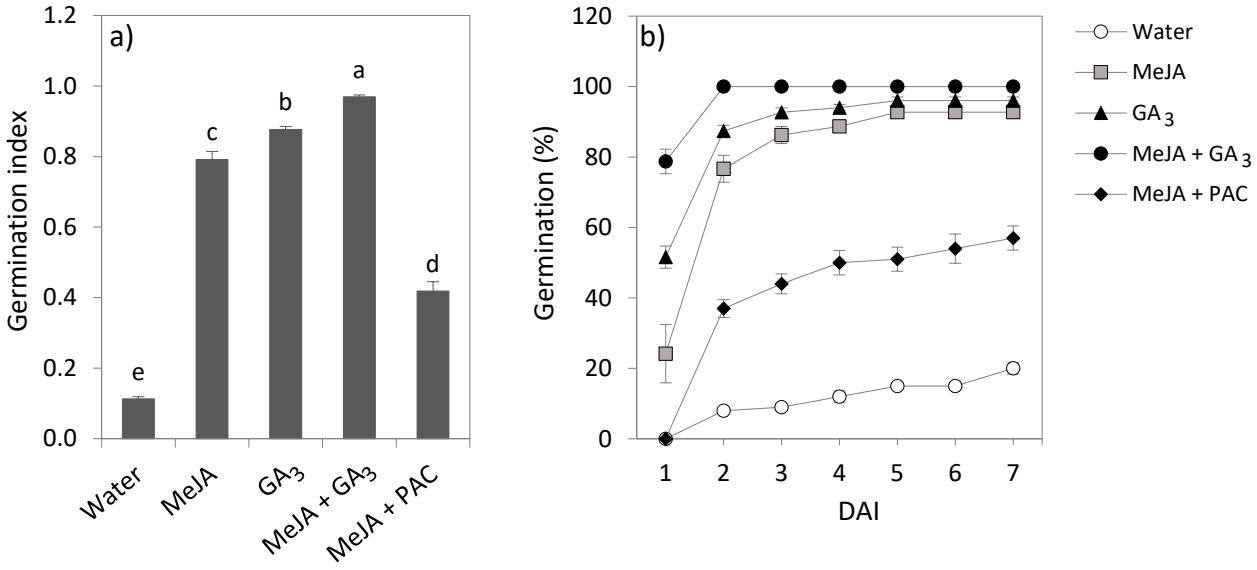

Figure 4.1. Germination of dormant seeds in response to hormone treatment. Gemination index (a) and percentage (b) of seeds imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Germination index was calculated over 7 days of imbibition. Data are mean ± SE of three biological replicates. Different letters show significant difference at $P<0.05$ (LSD test); DAI, day(s) after the start of imbibition.

4.4.1.2 After-ripening treatment

Germination percentage and GI of seeds increased with increasing duration of after-ripening from 0 to 12 weeks (Figs. 4.2, 1S). Although seeds after-ripened for 8 or more weeks attained ~100% germination at 2 DAI, only 50% to 70% germination was observed at 1 DAI. The increases in GI values were greater in the control after-ripened seeds (hormone/inhibitor untreated seeds) and those treated with PAC and ABA (over 5.6-fold) while the increases in GI values in seeds treated

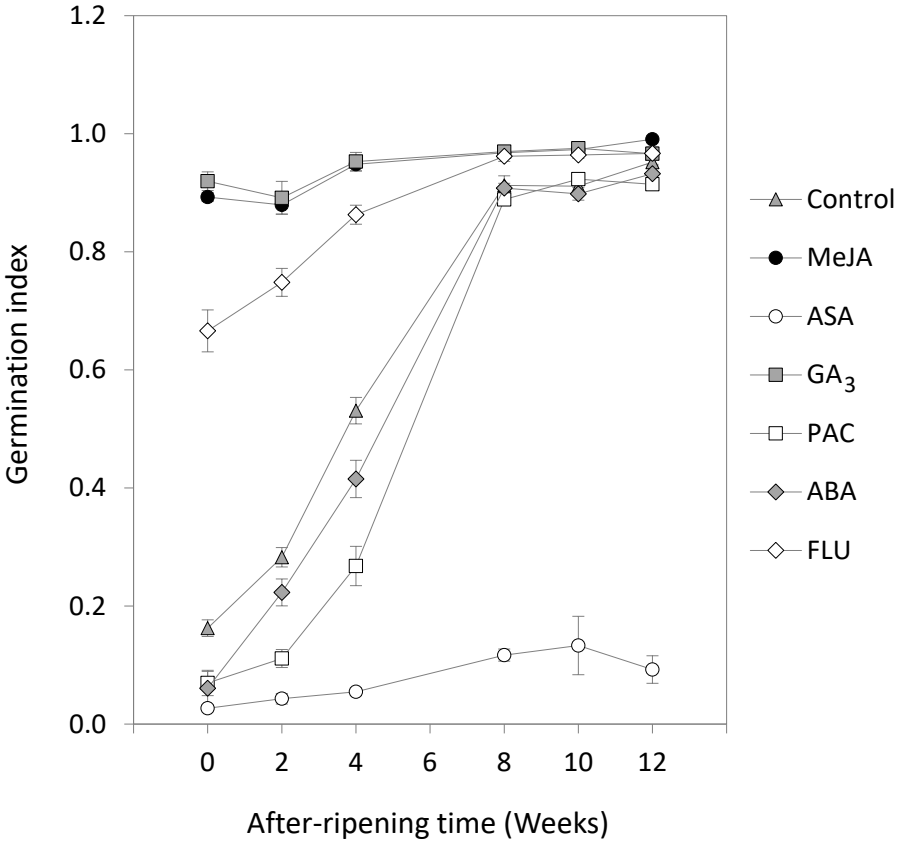

Figure 4.2. Germination of dormant seeds in response to after-ripening and hormone treatment. Germination index of seeds after-ripened for different periods of time and then imbibed without or with abscisic acid (ABA), acetylsalicylic acid (ASA), fluridone (FLU), gibberellic acid (GA_3), methyl jasmonate (MeJA) or paclobutrazol (PAC). Gemination index was calculated over 7 days of imbibition. Data were means and ±SE of four biological replicates.

with GA_3, MeJA and ASA were small. In comparison to the control, treatments with GA_3, MeJA and FLU significantly increased the GI of seeds after-ripened for 0 to 10 weeks; the effects of MeJA and GA_3 on GI were quite similar and greater than that of FLU. The effects of treatments with GA_3 and MeJA on GI appeared to be independent of after-ripening as the GIs were over 0.89 in 0-week after-ripened seeds. Whereas treatments with ASA and PAC significantly decreased the GI of seeds after-ripened for 0 to 12 weeks, and ASA exhibited greater negative effect on GI than PAC. Treatment with ABA also reduced GI but only for seeds after-ripened for 0 to 4 weeks, and its effect was lower than that of ASA and PAC except for seeds after-ripened for 0 weeks in which ABA and PAC treatments have similar effects.

4.4.2 Expression patterns of jasmonate metabolic genes and content of JA-Ile

The present study analyzed the expression patterns of jasmonate metabolic genes including *AOS*, *AOC*, *OPR*, *ACX*, *KAT* and *JAR* and also measured changes in JA-Ile levels (Fig. 4.3a).

4.4.2.1 Hormone treatment

Relative to that observed for the control samples, treatment with MeJA alone increased the expression levels of *TaAOS2-A*, *TaAOC1*, *TaOPR2* and *TaACX1* (over 1.6-fold) in imbibing embryos (Fig. 4.3). Whereas, treatment with GA_3 alone did not affect the expression patterns of these genes except that it caused changes in the expression level of *TaAOC1* – an increase at 6 HAI (1.5-fold) and a decrease at 12 and 24 HAI (over 1.3-fold). Relative to that observed in response to treatment with MeJA alone, treatment with MeJA plus GA_3 decreased the expression levels of the four jasmonate metabolic genes at 12 and/or 24 HAI (over 1.4-fold) while treatment with MeJA plus PAC either increased (*TaOPR2*; over 1.5-fold) or reversed the effect of combining MeJA with GA_3 on the expression level of the jasmonate metabolic genes particularly at 12 and/or

24 HAI. The hormone treatment also caused significant changes in the expression level of other jasmonate metabolic genes including *TaAOS2-B, TaAOS2-D*, *TaKAT2* and *TaJARs* (Fig. 2S).

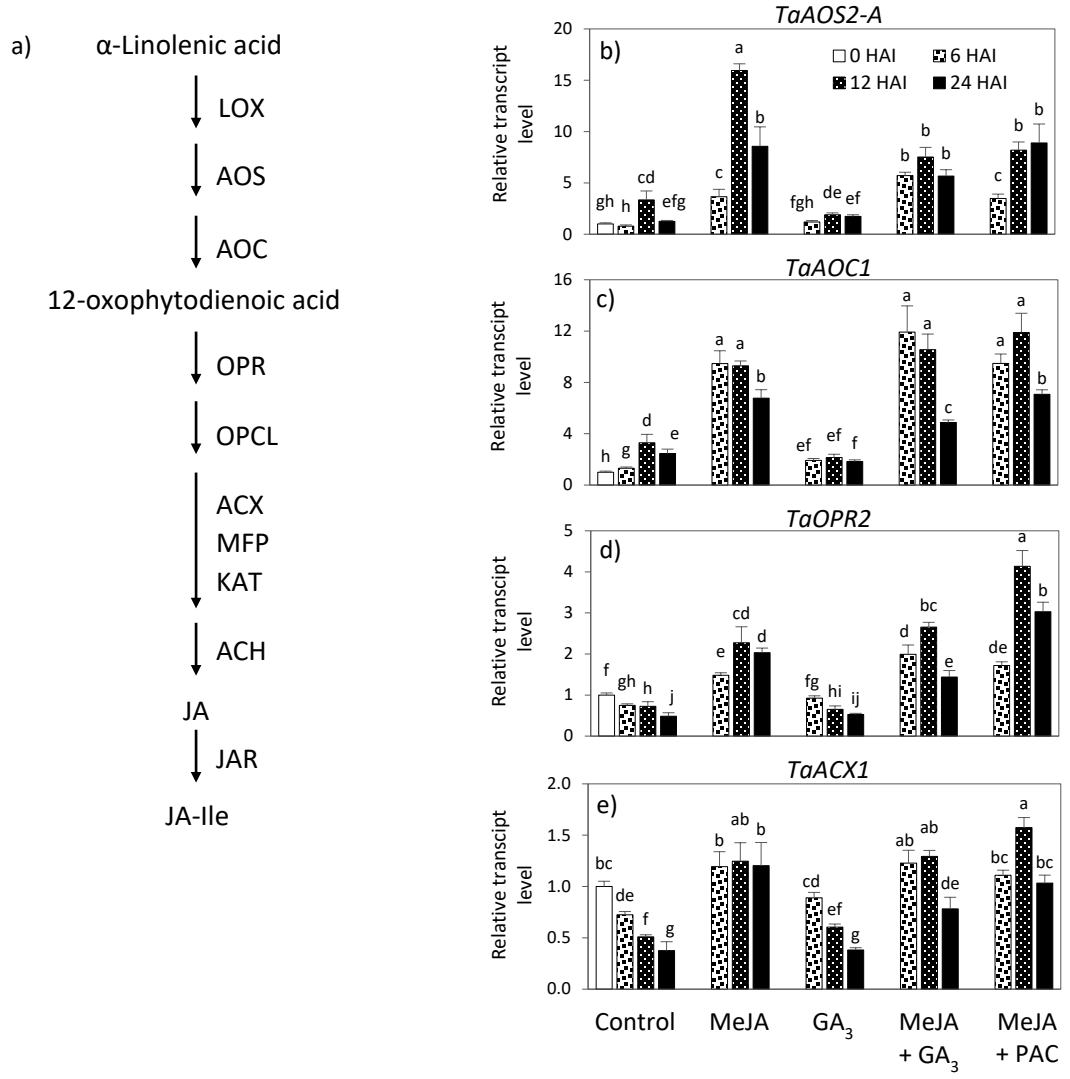

Figure 4.3. Expression of jasmonate biosynthetic genes in response to hormone treatment. A simplified pathway of jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) biosynthesis (a); and relative transcript levels of *TaAOS2-A* (b), *TaAOC1* (c)*, TaOPR2* (d) and *TaACX1* (e) in embryos of dormant seeds after different hours of imbibition (HAI). Seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

4.4.2.2 After-ripening treatment

Overall, the expression levels of *TaAOS2-A*, *TaAOC1* and *TaJAR2-B* showed increases with imbibition irrespective of the duration of after-ripening except for *TaJAR2-B* in 8 weeks samples whose expression decreased at 24 HAI after the increase at 12 HAI (Fig. 4.4). After-ripening for 4 and 8 weeks did not have any effect on the expression levels of jasmonate biosynthetic genes in the embryos of dry seeds and seeds imbibed for 12 h except the slight increases observed for *TaAOS2-A* in dry seeds (1.6-fold) and for *TaJAR2-B* in 12 h imbibed seeds as after-ripening

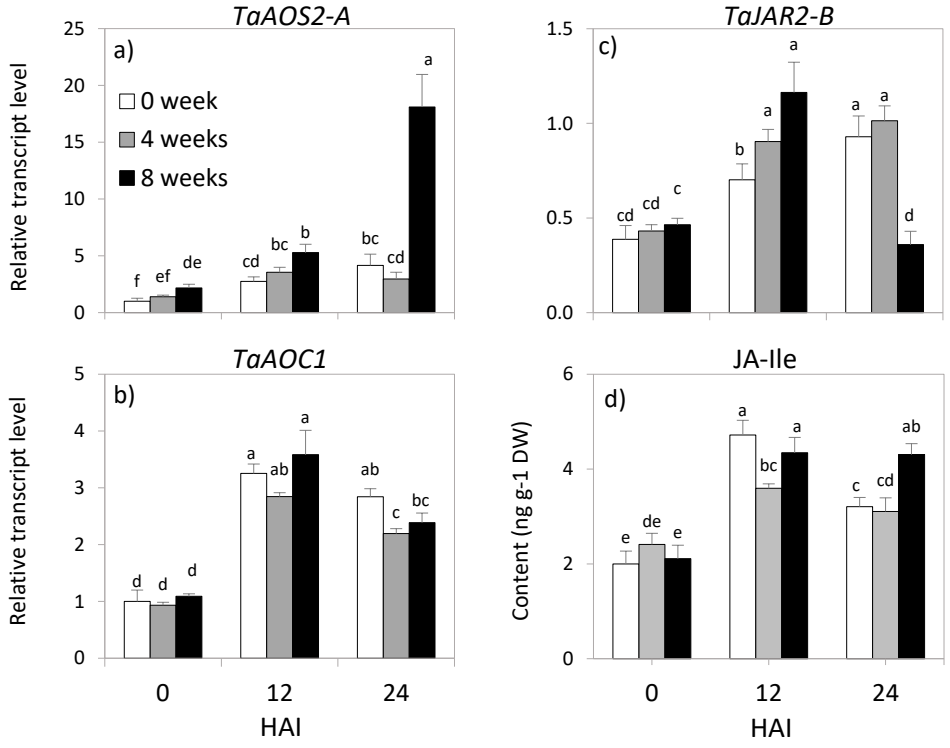

Figure 4.4. Expression of jasmonate biosynthetic genes and content of jasmonoyl isoleucine (JA-Ile) in response to after-ripening. Relative transcript levels of *TaAOS2-A* (a), *TaAOC1* (b) and *TaJAR2-B* (c) and content of JA-Ile (d) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior to imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaAOS2-A*, *TaAOC1,* and *TaJAR2-B* were expressed relative to the transcript levels of *TaAOS2-A*, *TaAOC1,* and *TaJAR1* (Fig. 3Sc) in 0 HAI samples of seeds after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

progressed from 0 to 8 weeks (1.7-fold). Imbibition for 24 h led to a substantial increase in the expression levels of *TaAOS2-A* (4.4-fold) in the embryos of seeds after-ripened for 8 weeks as compared to that observed in the seed samples after-ripened for 0 and 4 weeks. After-ripening had no significant effect on the expression level of *TaAOS2-B*, *TaAOS2D*, *TaJAR1* and *TaJAR2-A* in most of samples measured (Fig. 3S).

After-ripening did not change embryonic JA-Ile in dry seeds. However, the level of JA-Ile exhibited increases with imbibition in all after-ripened seed samples (Fig. 4.4d). Imbibition for 12 h did not have effect on the embryonic JA-Ile levels irrespective of the duration of after-ripening except the slight decrease of JA-Ile observed in seeds after-ripened for 4 weeks. Imbibition for 24 h, on the other hand, led to an increase in level of embryonic JA-Ile (1.3-fold) in seeds after-ripened for 8 weeks as compared to that detected in seeds after-ripened for 0 and 4 weeks.

4.4.3 Expression patterns of jasmonate signaling genes

The expression patterns of jasmonate signaling genes including *COI*, *JAZ*, *MYC2* and *MED25* were analyzed for embryos sample from seeds treated with hormones and/or after-ripening (Fig. 4.5a).

4.4.3.1 Hormone treatment

Treatment with MeJA or GA_3 did not have any effect on the expression levels of *TaCOI2* at 6 and 12 HAI except that MeJA increased (1.4-fold) the expression level at 6 HAI while both treatments reduced (over 1.4-fold) the expression level of *TaCOI2* at 24 HAI (Fig. 4.5). In comparison to that observed for MeJA treatment, the expression level of *TaCOI2* was decreased by treatment with MeJA plus GA_3 at 24 HAI (2.3-fold); however, this effect was reversed by treatment with MeJA plus PAC. Our data showed a negative correlation between the expression level of *TaCOI2* and germination induced by treatments studied at 24 HAI (Fig. 4.6). MeJA treatment led to increases

(over 2.2-fold) in the expression levels of *TaJAZ1*, *TaJAZ7* and *TaMYC2* during imbibition (Fig. 4.5). Treatment with GA_3 alone, on the other hand, did not affect the expression levels of *TaJAZ*s

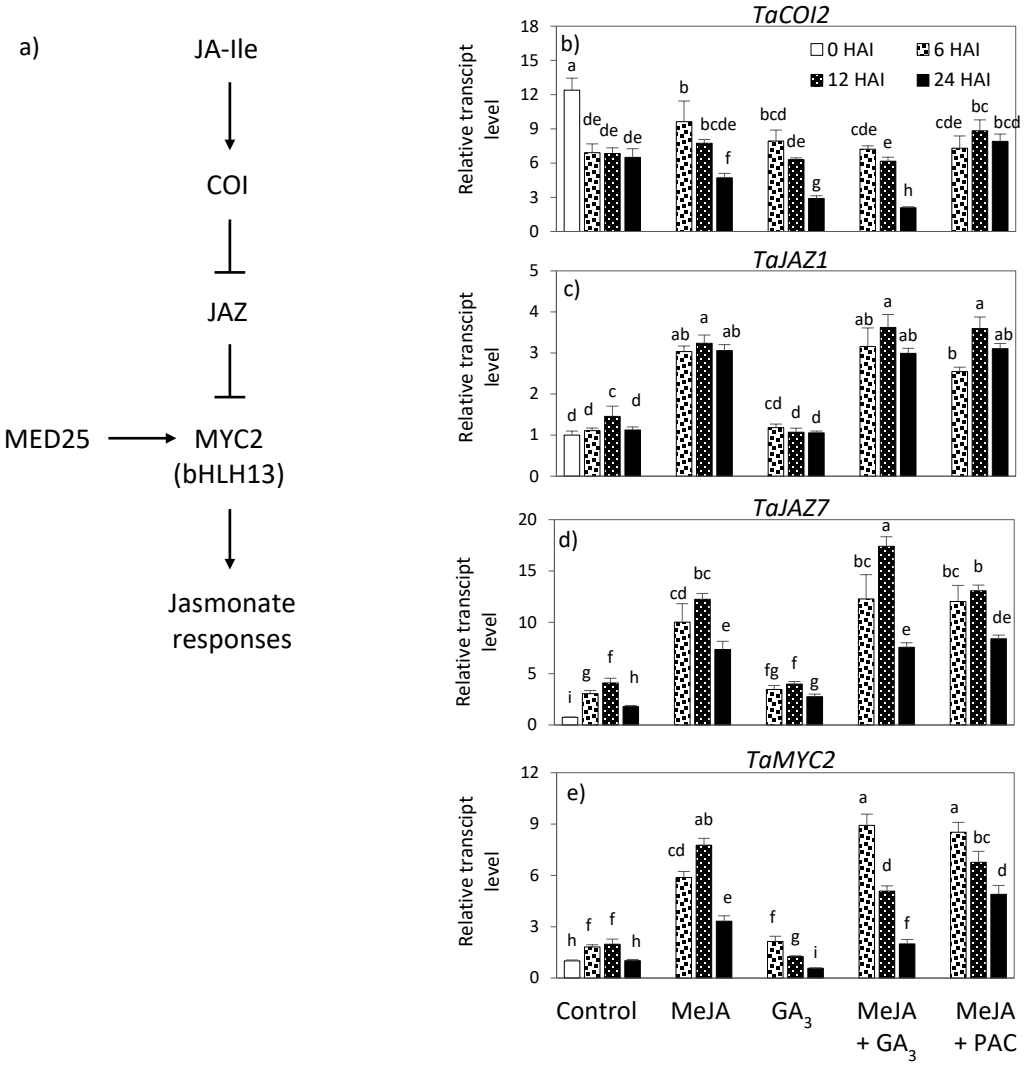

Figure 4.5. Expression of jasmonate signaling genes in response to hormone treatment. A simplified pathway of jasmonoyl-isoleucine (JA-Ile) signaling (a); and relative transcript levels of *TaCOI2* (b), *TaJAZ1* (c), *TaJAZ7* (d), and *TaMYC2* (e) in embryos of dormant seeds at different hours after imbibition (HAI). Seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOI2*, *TaJAZ*s and *TaMYC2* were expressed relative to the transcript levels of *TaCOI1* (Fig. 4S), *TaJAZ1* and *TaMYC2* in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

and *TaMYC2* except that the treatment increased (1.5-fold) the expression level of *TaJAZ7* at 24 HAI and reduced (over 1.4-fold) the expression levels of *TaJAZ1* and *TaMYC2* at 12 and/or 24 HAI. As compared to that observed in response to MeJA treatment, combining MeJA with GA_3 decreased (over 1.5-fold) the expression levels of *TaMYC2* at 12 and 24 HAI. Combining MeJA with PAC, on the other hand, increased (over 1.5-fold) the expression levels of *TaMYC2* relative to treatment with MeJA alone at 6 and 24 HAI. The hormone treatments also caused significant changes in expression levels of *TaJAZ4* and *TaJAZ10*; however, they had only minor effects on expression levels of *TaCOI1*, *TaCOI3*, *TabHLH13* and *TaMED25* (Fig. 4S).

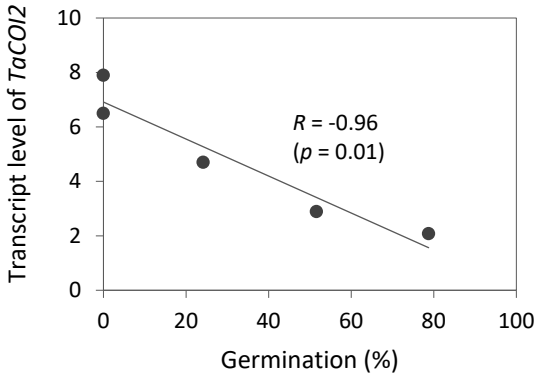

Figure 4.6. Relationship between the expression level of *TaCOI2* and germination in response to hormone treatment. Correlation between relative transcript level of *TaCOI2* in 24 HAI samples (Fig 4.5b) and germination percentage at 1 day after imbibition (Fig. 4.1b).

4.4.3.2 After-ripening treatment

Relatively high transcript levels of *TaCOI2* were detected in the dry seeds and this level was maintained through 12 HAI (Fig. 4.7); however, seed imbibition for 24 h led to decreases in the expression levels of the gene irrespective of the duration of after-ripening. The *TaJAZ7*, *TaJAZ10*, and *TaMYC2* genes showed similar expression patterns during imbibition in all after-ripened seed

samples; their expression levels increased by imbibition for the first 12 h but exhibited decreases as seed imbibition continued for 24 h (Fig. 4.7).

Almost no difference in the expression levels of *TaCOI2*, *TaJAZ7*, *TaJAZ10* and *TaMYC2* genes was observed in the embryos of dry seeds amongst the different durations of after-ripening (Fig. 4.7). Irrespective of the duration of after-ripening, the expression levels of *TaCOI2*, *TaJAZ10* genes in the embryos of seeds imbibed for 12 h remained unaffected while the expression levels of *TaMYC2* exhibited slight increases as after-ripening progressed from 4 to 8 weeks, and the

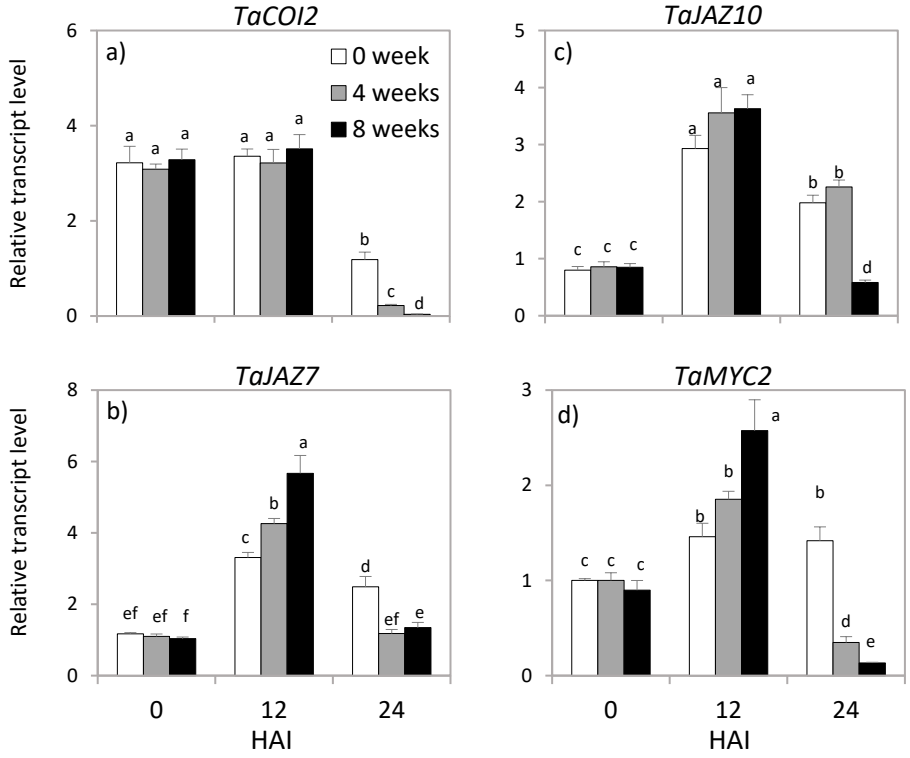

Figure 4.7. Expression of jasmonate signaling genes in response to after-ripening. Relative transcript levels of *TaCOI2* (a), *TaJAZ7* (b)*, TaJAZ10* (c) and *TaMYC2* (d) in embryos of dormant seeds at different hours after imbibition (HAI). The seeds were after-ripened for 0, 4 or 8 weeks prior to imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOI2*, *TaJAZ*s and *TaMYC2* were expressed relative to the transcript levels of *TaCOI1* (Fig. 5Sa), *TaJAZ1* (Fig. 5Sc) and *TaMYC2* in 0 HAI samples of seed after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

expression levels of *TaJAZ7*, on the other hand, showed constant increases as duration of after-ripening increased from 0 to 4 and from 4 to 8 weeks. The expression levels of embryonic *TaCOI2* and *TaMYC2* in seeds imbibed for 24 h exhibited continuous decreases as the duration of after-ripening increased from 0 to 4 and 4 to 8 weeks. The expression levels of *TaJAZ7* in the embryos of 24 h imbibed seeds decreased by the first 4 weeks of after-ripening and remained unaffected by extension of the duration of after-ripening to 8 weeks while the expression level of *TaJAZ10* showed a decrease only after 8 weeks of after-ripening. The expression level of other jasmonate signaling genes including *TaCOI1*, *TaCOI2*, *TaJAZ1*, *TaJAZ3*, *TaJAZ4*, *TabHLH13* and *TabHLH13-like* were also affected by after-ripening (Fig. 5S).

4.4.4 Expression patterns of ABA metabolic and signaling genes and ABA content

This study analyzed the expression patterns of genes encoding the ABA biosynthetic enzyme NCED and ABA catabolic enzyme CYP707A (Fig. 4.8a), and those encoding major components of ABA signaling components – PYL5, PP2C, SnRK2, ABI3 and ABI5 (Fig 4.8b). We also analyzed the changes in ABA contents in embryos.

4.4.4.1 Hormone treatment

Relative to the control, treatment with MeJA increased the expression levels of *TaNCED1* only at 6 HAI (2.4-fold) while treatment with GA_3 alone reduced their expression levels (over 2.7-fold; Fig. 4.8). As compared to that observed in response to MeJA alone, treatment with MeJA plus GA_3 reduced the expression levels of *TaNCED1* at 12 and 24 HAI (over 2.2-fold) while treatment with MeJA plus PAC increased the expression levels of *TaNCED1* at 12 and 24 HAI (over 1.5-fold).

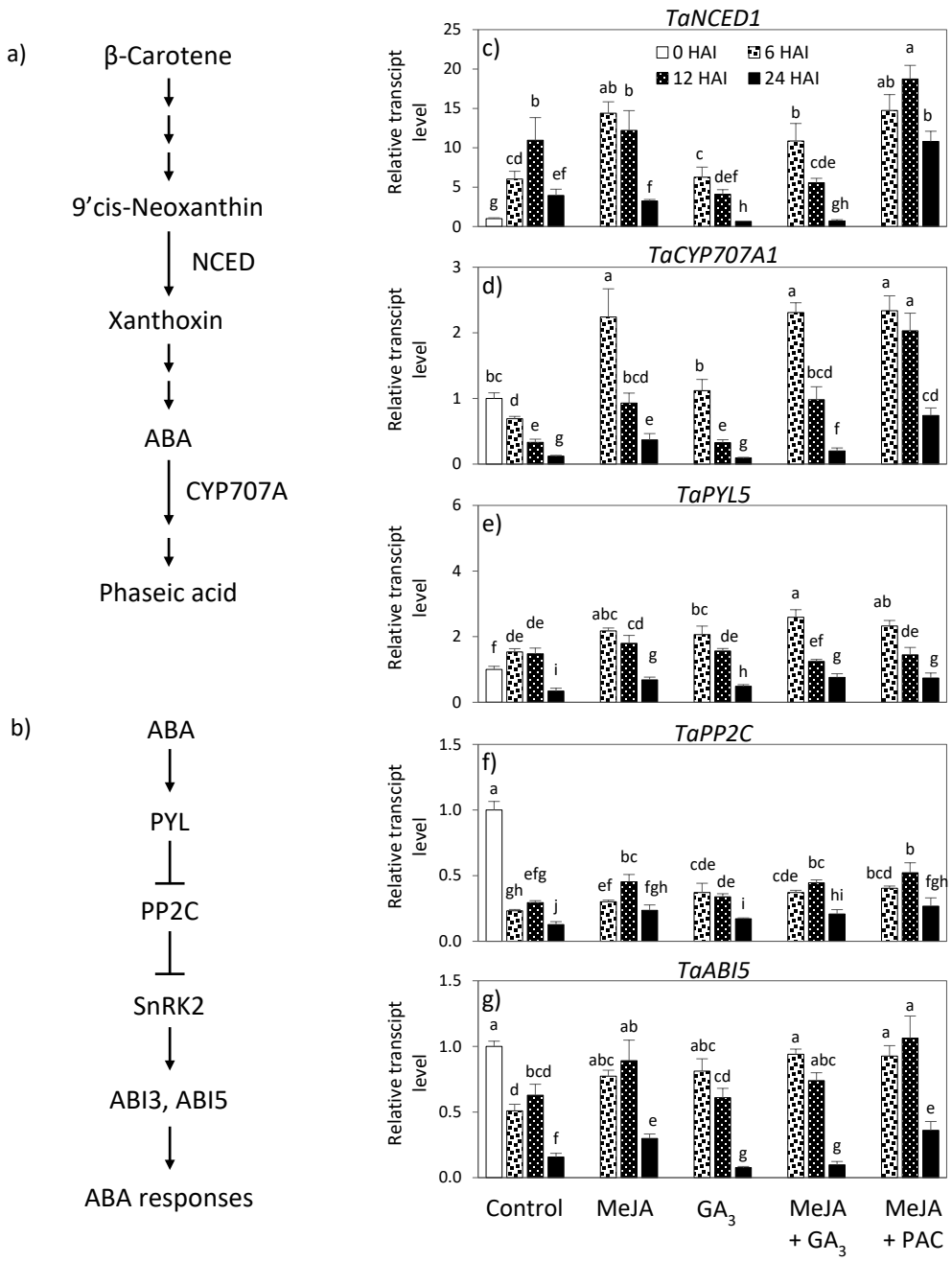

Figure 4.8. Expression of abscisic acid (ABA) metabolic and signaling genes in response to hormone treatment. Simplified pathways of ABA metabolism (a) and signaling (b); and relative transcript levels of *TaNCED1* (c), *TaCYP707A1* (d), *TaPYL5* (e), *TaPP2C* (f) and *TaABI5* (g), in embryos of dormant seeds collected at different hours after imbibition (HAI). The seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

Treatment with MeJA increased the expression level of *TaCYP707A1* during imbibition (over 1.7-fold; Fig. 4.8) while treatment with GA_3 alone increased the expression level of *TaCYP707A1* at 6 HAI (1.6-fold). As compared to treatment with MeJA alone, treatment with MeJA plus GA_3 reduced the expression levels of the *TaCYP707A1* gene at 24 HAI (1.9-fold) while treatment with MeJA plus PAC led to an increase in the expression level of *TaCYP707A1* at both 12 and 24 HAI (over 2.0-fold).

When compared to the control, treatment with MeJA increased the expression levels of *TaPYL5*, *TaPP2C* and *TaBAI5* at 6 and/or 12 and 24 HAI (over 1.3-fold; Fig. 4.8). Treatment with GA_3 alone also increased the expression levels of these genes at 6 and 24 HAI (over 1.3-fold) except for *TaBAI5* in which its expression level at 24 HAI was decreased (2.1-fold). As compared to that of MeJA alone, treatment with a combination of MeJA and GA_3 reduced the expression level of *TaPYL5* at 12 HAI (1.4-fold) and *TaABI5* at 24 HAI (3.0-fold) while treatment with MeJA plus PAC reversed these effects of MeJA plus GA_3. Either MeJA plus GA_3 or MeJA plus PAC did not have effect on the MeJA induced expression of *TaPP2C* except that MeJA plus PAC increased it at 6 HAI (1.3-fold).

The hormone treatment also caused changes in expression level ABA metabolic genes *TaNCED2* and *TaCYP707A2*, and ABA signaling genes *TaSnRK2* and *TaABAI3* (Fig. 6S).

4.4.4.2 After-ripening treatment

Transcript levels of *TaNCED1* and *TaCYP707A1* were detected in the embryos of dry seeds after-ripened for different durations (Fig. 4.9). Seed imbibition for 12 h increased the expression level of *TaNCED1* irrespective of the duration of after-ripening but caused a reduction in that of *TaCYP707A1*. Extending the period of imbibition to 24 h did not have effect on the expression

level of embryonic *TaNCED1* in the different seed samples except the down-regulation it caused in the seed samples after-ripened for 8 weeks. In contrast, the expression level of *TaCYP707A1* in all seed samples decreased as seed imbibition continued from 12 to 24 h (over 3.5-fold). No difference in the transcript level of *TaNCED1* and *TaCYP707A1* was detected amongst the seed

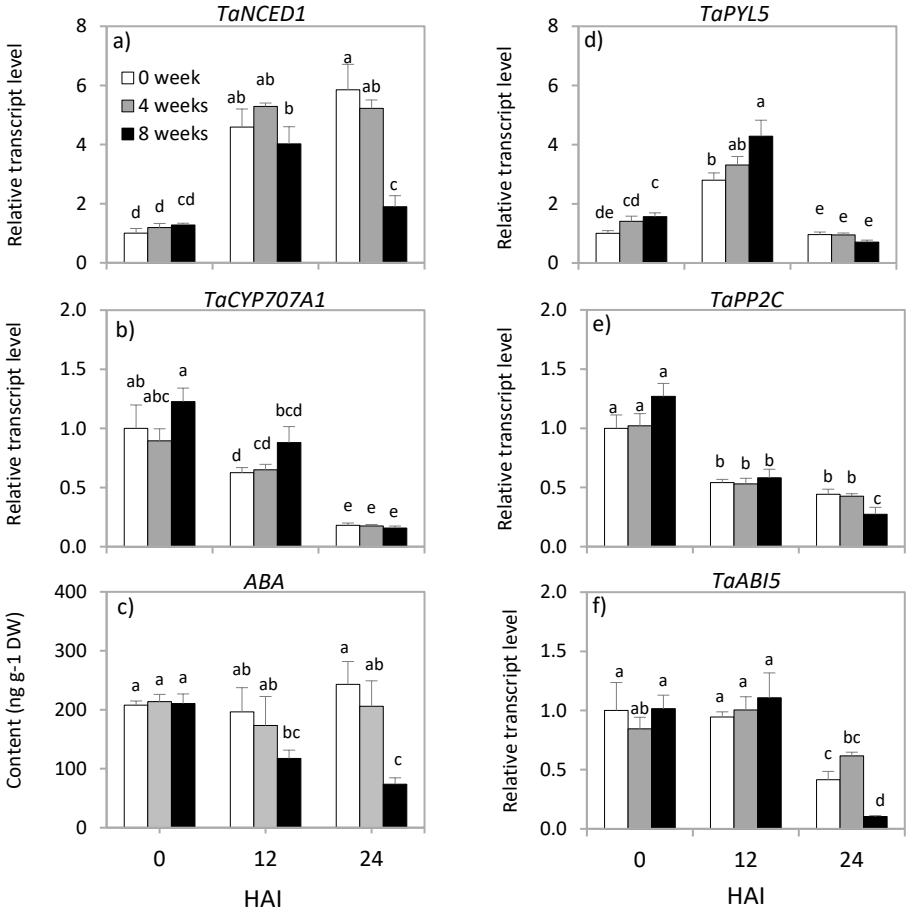

Figure 4.9. Expression of abscisic acid (ABA) metabolic and signaling genes and content of ABA in response to after-ripening. Relative transcript levels of *TaNCED1* (a) and *TaCYP707A1* (b), content of ABA (e), and relative transcript levels of *TaPYL5* (d), *TaPP2C* (e) and *TaABI5* (f) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior the imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples of seed after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

samples after-ripened for different duration at each imbibition time point except that seeds after-ripened for 8 weeks exhibited a substantial decrease in the expression level of *TaNCED1* as compared to those after-ripened for 0 and 4 weeks (over 2.7-fold). After-ripening did not have any effect on expression levels of *TaNCED2* while caused changes in those of *TaCYP707A2* (Fig. 7S).

A substantial amount of embryonic ABA was detected in the dry seeds, and the level was not affected by the duration of after-ripening (Fig. 4.9). The ABA level detected in the dry seeds was maintained during imbibition of seed samples after-ripened for 0 and 4 weeks. In seed samples after-ripened for 8 weeks, however, the ABA level showed a gradual decrease with imbibition, leading to lower embryonic ABA level than that observed in seeds after ripened for 0 and 4 weeks at 24 HAI.

The transcripts of the ABA signaling genes *TaPYL5*, *TaPP2C* and *TaABI5* were detected in the dry seeds and after-ripening did not have substantial effect on their transcript levels (Fig. 4.9). Overall, seed imbibition for 12 and 24 h led to decreases in the transcript levels of these ABA signaling genes except that it causes a transient increase in the expression level of *TaPYL5* at 12 HAI, and the transcript level of *TaABI5* detected in the embryos of dry seeds was not affected by 12 h imbibition. Duration of after-ripening did not affect the expression levels of the three ABA signaling genes in the embryo of both dry and imbibed seeds except that after-ripening for 8 weeks led to marked repression in the expression levels of *TaABI5* at 24 HAI as compared to that observed in seed samples after-ripened for 0 and 4 weeks. After-ripening for 8 weeks also caused slight reduction in the expression level of *TaPP2C*. Similar effects of after-ripening were observed for the expression level of ABA signaling genes *TaSnRK2* and *TaABI3* (Fig. 7S).

4.4.5 Expression patterns of GA metabolic and signaling genes and content of GA

The expression patterns of genes encoding the GA metabolic enzymes (Fig. 4.10a) – GA20ox, GA3ox and GA2ox – and GA signaling components (Fig. 4.10e) – GID1, GID2, DELLA (*RHT1*) and GAMyb, and the content of bioactive GA_1 were analyzed in this study.

4.4.5.1 Hormone treatment

Relative to the control, treatment with MeJA increased the expression level of *TaGA20ox1* and *TaGA3ox2* at 24 HAI (over 1.8-fold; Fig. 4.10). Whereas treatment with GA_3 alone decreased the expression levels of the *TaGA20ox1* and *TaGA3ox2* at 6, 12 and/or 24 HAI (over 2.7-fold) while it increased that of *TaGA3ox2* at 24 HAI (2.9-fold). When compared to treatment with MeJA alone, treatment with a combination of MeJA and GA_3 reduced the expression levels of *TaGA20ox1* at 12 and 24 HAI and that of *TaGA3ox2* at 12 HAI (over 2.3-fold) while treatment with MeJA plus PAC reversed the effects MeJA and GA_3 on the expression levels of *TaGA20ox1* at 24 HAI. The effect of treatment with MeJA plus PAC on the expression level of *TaGA3ox2* was similar to that of MeJA plus GA_3 except that it also led to a decrease in the expression of *TaGA3ox2* at 24 HAI. Treatment with MeJA increased the expression level of *TaGA2ox3* at 12 and 24 HAI (over 2.5-fold; Fig. 4.10) while treatment with GA_3 alone almost decreased the expression level of this gene at 6 HAI (1.5-fold). Relative to that observed for treatment with MeJA alone, combining MeJA with GA_3 increased the expression levels of *TaGA2ox3* at 6 and 12 HAI (over 1.5-fold) but this effect was reversed by treatment with MeJA plus PAC.

In comparison to the control, the treatment of MeJA alone increased transcript level of *TaGID1* during imbibition (over 1.5-fold; Fig. 4.10). It also increased that *TaGAMyb* at 24 HAI (1.9-fold; Fig. 4.10). By contrast, treatment with GA_3 alone decreased the transcript level of *TaGID1* at 24 HAI (4.6-fold) and that of *TaGAMyb* at 6 and 12 HAI (over 1.4-fold). Relative to

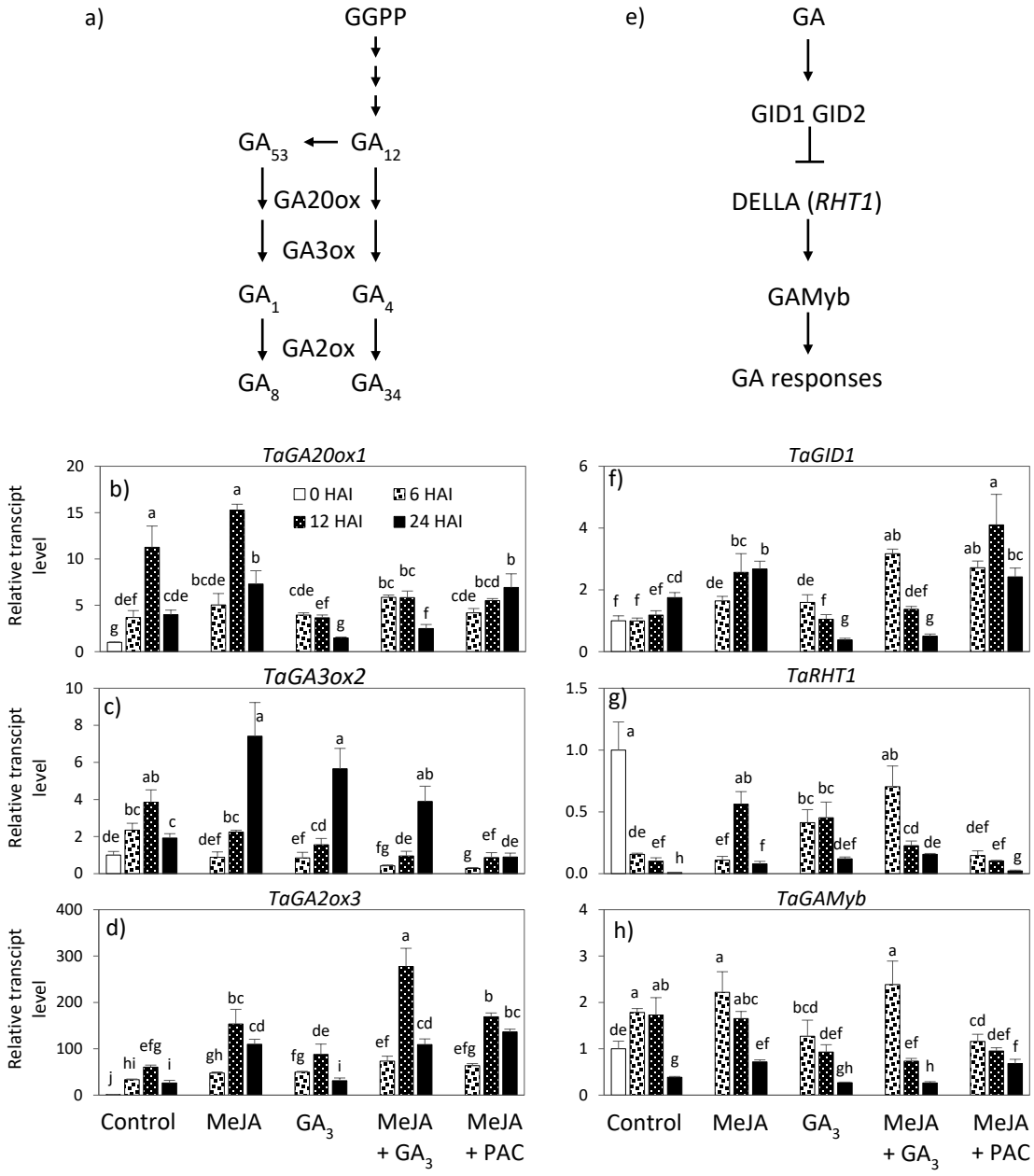

Figure 4.10. Expression of gibberellins (GA) metabolic and signaling genes in response to hormone treatment. Simplified pathways of GA metabolism (a) and signaling (e); and relative transcript levels of *TaGA20ox1* (b), *TaGA3ox2* (c), *TaGA2ox3* (d), *TaGID1* (f), *TaRHT1* (g) and *TaGAMyb* (h) in embryos of dormant seeds collected at different hours after imbibition (HAI). GA_1 and GA_4 are bioactive in plant. The seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

that observed for treatment with MeJA alone, combining MeJA with GA_3 increased the expression levels of *TaGID1* at 6 HAI (1.9-fold) but led to decreases at 12 and 24 HAI (over 1.9-fold) while combining MeJA with PAC either increased the expression level of *TaGID1* (over 1.6-fold) or reversed the effect of MeJA plus GA_3. Treatments with MeJA or GA_3 increased the expression level of *TaRHT1* at 12 and 24 HAI (over 4-fold; Fig. 4.10), and GA_3 alone also increased its expression level at 6 HAI (2.6-fold). Relative to treatment with MeJA alone, combining MeJA with GA_3 increased the transcript level of *TaRHT1* at 6 and 24 HAI (over 2-fold) although a transient decrease was observed at 12 HAI (2.5-fold) while combining MeJA with PAC led to a decrease in *TaRHT1* expression level with imbibition for 12 and 24 HAI (over 3.6-fold). Combining MeJA with GA_3 or PAC reduced the expression level of *TaGAMyb* during imbibition (over 1.7-fold).

The express pattern of *TaGA20ox2*, *TaGA2ox6* and *TaGID2* also showed changes in response to the hormone treatments (Fig. 8S).

4.4.5.2 After-ripening treatment

Embryos of dry seeds contained low transcripts levels of the GA metabolic genes *TaGA20ox1*, *TaGA3ox2* and *TaGA2ox3*, and after-ripening increased only those of *TaGA3ox2* (over 2.4-fold; Fig. 4.11). Seed imbibition for 12 or 24 h led to an increase in the expression levels of all the genes studied irrespective the after-ripening periods. The expression levels of *TaGA20ox1* and *TaGA3ox2* in 12 and 24 h imbibed seeds increased with progression of after-ripening except that of *TaGA20ox1* which was not affected by duration of after-ripening by 24 HAI. After-ripening for 8 weeks, in particular, led to marked increases (over 2-fold) and decreases (over 3.3-fold) in the expression level *TaGA3ox2*, and *TaGA2ox3* genes, respectively, following imbibition for 12 and/or 24 h as compared to that observed in corresponding seeds after-ripened for 0 and 4 weeks.

The effects of after-ripening on the expression level of *TaGA20ox2* and *TaGA2ox6* (Fig. 9S) were similar to those of *TaGA20ox1* and *TaGA2ox3*, respectively.

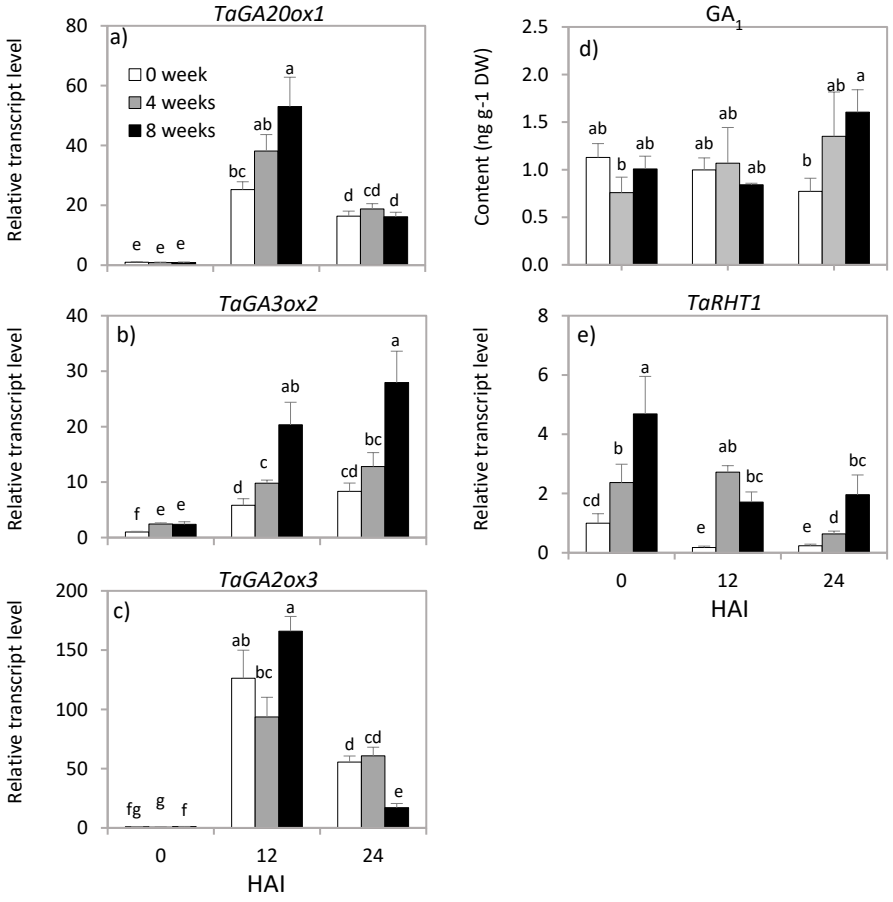

Figure 4.11. Expression of gibberellins (GA) metabolic and signaling genes and content of GA in response to after-ripening. Relative transcript levels of *TaGA20ox1* (a), *TaGA3ox2* (b) and *TaGA2ox3* (c), content of GA_1 (d), and relative transcript level of *TaRHT1* (e) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior the imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples of seed after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

The amount of GA_1 detected in the embryo of dry seeds was not affected by the period of after-ripening and almost similar GA_1 level was maintained throughout the imbibition periods with no apparent variation amongst the seed samples after-ripened for different durations (Fig. 4.11d). However, after-ripening for 4 and 8 weeks triggered induction in the level of GA_1 (1.7-fold) by 24 HAI as compared that observed in seeds after-ripened for 0 weeks. No GA_4 was detected in all samples analyzed.

After-ripening for 4 and 8 weeks increased the expression level of GA signaling gene *TaRHT1* in both dry and imbibed seeds (over 2-fold; Fig. 4.11e). In all seed samples, the expression level of *TaRHT1* showed gradual decrease with imbibition. After-ripening almost did not have any effect on expression level of GA signaling genes *TaGID1*, *TaGID2* and *TaGAMyb* (Fig. 9S).

4.5 Discussion

Jasmonates have been shown to have roles in regulating seed germination and dormancy. Our data show that MeJA was able to release seeds from dormancy (Fig. 4.1), and this in agreement with a previous report (Jacobsen et al., 2013). The inability of control/untreated imbibing seeds to complete germination might indicate that imbibition-induced synthesis of embryonic bioactive JAs did not increase to the level that is sufficient to break dormancy. Regulation of the transcription of hormonal metabolic genes has been reported to act as an effective mechanism for regulating the level of endogenous hormones (Kaufmann et al., 2010). The biosynthesis of JAs is catalyzed by several enzymes including AOS and AOC that act in plastid and OPR, ACX and KAT which act in the peroxisome, and AOS, AOC and OPR catalyze reactions that are committed to the pathway (Schaller and Stintzi, 2009). Under control conditions, the expression levels of *TaAOS2* and *TaAOC1* increased after 12 and 24 hours of imbibition. In contrast, the expression levels of *TaOPR2, TaACX1* and *TaKAT2* exhibited gradual decreases with imbibition (Figs. 4.3, 2S). These

results suggest the significance of the expression of genes encoding peroxisomal enzymes, particularly *TaOPR2*, in controlling the level of embryonic JAs and dormancy. Consistently, seeds of the *opr3-1* mutant of Arabidopsis do not produce JA and JA-Ile at detectable levels and exhibit high level of dormancy (Dave et al., 2016). Furthermore, the *opr3-1* seeds were found to be highly sensitive to ABA mediated inhibition of germination, and ABA represses the expression level of *TaOPR2* (Wang et al., 2016).

Previous studies have shown that jasmonate biosynthetic and signaling genes including *AOS, AOC, OPR*, *ACX, JAZ* and *MYC* are up-regulated by increased levels of JA-Ile, the most biologically active form of JAs (Wasternack and Hause, 2013). Consistently, treatment with MeJA led to up-regulation of the jasmonate biosynthetic genes, particularly *TaAOS2* and *TaAOC1* (Figs. 4.3, 2S), and the jasmonate signaling genes, *TaCOI2*, *TaJAZs* and *TaMYC2* (Figs. 4.5, 4S) in imbibing seeds. The up-regulation of the genes encoding proteins acting as positive regulators of jasmonate signaling, *TaCOI2* and *TaMYC2* imply that jasmonate content in imbibing wheat seeds is regulated mainly by these two genes (Lee et al., 2013; Yan et al., 2013). The observation of enhanced expression of the *JAZ* genes that act as negative regulators of jasmonate signaling along with those encoding the positive regulators might suggest its regulation at the post transcription level. The involvement of JA-Ile signaling pathway in the regulation of germination by exogenous JA or MeJA has also been reported in Arabidopsis seeds (Dave et al. 2011; Oh et al. 2009). In addition, up-regulation of *TaAOS2* (Fig. 4.4), *TaJAZ3*, *7* (Figs. 4.7, 5S) and *TaMYC2* (Fig. 4.7) was observed in response to imbibition of after-ripening seeds that are characterized by increased synthesis of JA-Ile (Fig. 4.4).

It has been shown previously that the MeJA can release wheat seeds from blue light-induced dormancy by reducing embryo ABA content mainly through repression of *TaNCED1*

(Jacobsen et al., 2013), indicating the role of JAs in suppressing ABA formation rather than enhancing its catabolism. Results of the present study showed that the MeJA treatment, which releases seeds from dormancy, caused up-regulation of *TaCYP707As* rather than down-regulating *TaNCED*s in imbibing seeds (Figs. 4.8, 6S), suggesting the significance of ABA catabolism in reducing ABA level. However, the MeJA treatment either had no effect (*TaSnRK2* and *TaABI3*) or increased (*TaPYL5* and *TaABI5*) the expression levels of genes encoding proteins that act as positive regulators of ABA signaling during imbibition (Figs. 4.8, 6S), which suggests an increase or no alteration in seed sensitivity to ABA due to MeJA treatment. In contrast, the MeJA treatment, which releases seeds from dormancy, increased the expression levels of GA biosynthetic genes especially that of the regulatory *TaGA3ox2* during imbibition (Fig. 4.10), suggesting MeJA-induces increase in GA synthesis. The observation of increased expression of the GA catabolic *TaGA2ox* genes (Figs. 4.10, 8S) along with the biosynthetic genes in the same samples might be due to the positive feedforward regulation of their expression by the elevated level of GA (Sponsel and Hedden, 2010). Furthermore, the MeJA treatment increased the expression levels of *TaGID1, TaGID2* and *TaGAMyb* that encode positive regulators of GA signaling (Figs. 4.10, 8S), and this implies MeJA-induced increase in the sensitivity of our seed samples to GA.

GA response is induced mainly by the degradation of DELLA, which acts as a negative regulator of GA signaling (Hauvermale et al., 2012), and the binding of JAZ to DELLA has been reported to prevent DELLA from negatively regulating the downstream transcription factors of GA signaling (Song et al., 2014). Therefore, the up-regulation of *TaJAZ*s in MeJA treated seed samples (Figs. 4.5, 4S) likely reflects the role JAZ proteins in repressing DELLA and thereby inducing embryo GA response and dormancy release. Our results overall indicate the role of MeJA-induced increases in seed GA level and seed GA sensitivity as the underlying causes for

changes in ABA–GA balance and therefore dormancy release in response to exogenous MeJA. However, since JAZ–DELLA interaction occurs at a protein level and *JAZ* transcripts could be subjected to post-transcriptional regulations, elucidating this role of MeJA requires quantitative analysis of the JAZ proteins and performing binding affinity assays to determine the JAZ–DELLA interaction.

Consistent with the above results, treatments with exogenous GA_3 and after-ripening, which induced a more enhanced release of dormancy in our seed samples, led to repression of the expression levels of the ABA biosynthetic genes *TaNCED1* and *TaNCED2* (Figs. 4.8, 6S, 4.9), suggesting a decrease in ABA production. The observation of down-regulation of the ABA catabolic genes *TaCYP707A2* in the samples treated with exogenous GA_3 (Figs. 6S) might explain the feedforward regulation of the two genes by ABA level present in the seed samples (Saito et al., 2004). In addition, treatment with exogenous GA_3 and after-ripening led to repressions in the expression levels of genes encoding proteins that acts as positive regulators of ABA signaling including *TaSnRK2*, *TaABI3* and *TaABI5* (Figs. 4.8, 4.9, 6S, 7S), reflecting a decrease in seed sensitivity to ABA. However, our data showed that treatments with GA_3 and after-ripening effectively released seeds from dormancy (Figs. 4.1, 4.2) without remarkable inductions in the expression levels of most of the regulatory jasmonate biosynthesis genes (*TaAOS2, TaOPR2, TaACX1, TaKAT2, TaJAR*s; Figs. 4.3, 4.4, 2S, 3S), JA-Ile content (Fig. 4.4) and expression levels of jasmonate signaling genes (*TaCOIs, TaJAZs, TaMYC2, TabHLH13* and *TaMED25*; Figs. 4.5, 4.7, 4S, 5S). These results imply that the role of GA and after-ripening in breaking dormancy is independent of changes in jasmonate synthesis and seed response to JAs. This is consistent with previous reports, which showed that dormancy release in jasmonate biosynthesis and signaling mutants of Arabidopsis including *aos, opr3-1* (Dave et al., 2011; Dave et al., 2016), *jar1* (Staswick

et al., 1992), *coi1-16* (Ellis et al., 2002), *myc2* (Berger et al., 1996; Abe et al., 2003; Gangappa et al., 2010), and *med25* (Chen et al., 2012) by after-ripening or cold-stratification occurred at a similar rate to that observed in the wild-type. By contrast, the repression of germination in response to seed treatment with MeJA plus PAC as compared to treatment with MeJA alone (Fig. 4.1) suggests that MeJA-induced release of dormancy in wheat seeds is partly dependent on GA synthesis. The down-regulation of the GA biosynthetic genes *TaGA20ox*s and *TaGA3ox*2 (Fig. 4.10, 8S), and the GA signaling genes *TaGID1* and *TaGAMyb* (Fig. 4.10), and up-regulation of *TaRHT1* (Fig. 4.10) that acts as a negative regulator of GA signaling in response to seed imbibition with exogenous GA_3 implies GA-induced transcriptional feedback in GA signaling (Middleton et al., 2012).

Treatment with a combination of MeJA plus GA_3 led to a higher degree of decay in dormancy, suggesting the synergistic effects of MeJA and GA_3. Indeed, maintenance of both MeJA-mediated up-regulations of *TaGID1, TaGID2* and *TaGAMyb* genes encoding proteins that acts as positive regulators of GA signaling at early stage of imbibition (Fig. 4.10, 8S), and that of *TaJAZ*s encoding a protein that is able to bind with DELLA and thereby represses its activity (Fig. 4.5, 4S), which altogether might lead to increased seed sensitivity to GA, was apparent in seed samples treated with MeJA plus GA_3. Furthermore, the GA-induced down-regulations of the ABA biosynthetic genes *TaNCED1* and *TaNCED2* and ABA signaling genes *TaSnRK2*, *TaABI3* and *TaABI5* (Fig. 4.8, 6S), which might lead to suppression of ABA production and seed ABA sensitivity, were observed in seed samples treated with MeJA plus GA_3. Unlike that observed in response to MeJA treatment, the MeJA plus GA3 treatment led to down-regulation of *TaGID1* and *TaGAMyb* at later stages of imbibition and up-regulation of *TaRHT1* (Fig. 4.10), indicating GA-induced negative feedback regulation of its own signaling as discussed above.

The repression of germination by treatment with MeJA plus PAC might be associated with inhibition of GA synthesis as evidenced by the down-regulation of *TaGA3ox2* (Fig. 4.10). Furthermore, combining MeJA with PAC, also caused an increase in the expression level of one of the genes representing JA-Ile receptor, *TaCOI2*, at 24 HAI to the level that observed in the control samples that also showed no germination by that time (Fig. 4.5). Given that the expression level of *TaCOI2* at 24 HAI exhibited a negative relationship with germination in all the samples studied (Fig. 4.6), our data might suggest the release of dormancy requires decreased response to JAs during imbibition. In support of this hypothesis, the induction of germination by GA_3 when applied alone or in combination with MeJA was associated with decreases in the expression levels of *TaJARs* and *TaCOI2* genes (Figs. 4.5, 2S). In addition, the expression levels of *TaCOI*s in 24 HAI seeds decreased with increasing duration of after-ripening (Fig. 4.7), suggesting that dormancy release in wheat seeds requires decreases in jasmonate synthesis and response. Consistently, JA and JA-Ile contents have been shown to decrease during germination of non-dormant Arabidopsis (Preston et al., 2009; Dave et al., 2011) and wheat (Liu et al., 2013; Martinez et al., 2016) seeds. Treatment with MeJA plus PAC did not have any effect on the expression patterns of ABA metabolism and signaling genes (Figs. 4.8, 6S). It is likely that the concentration of PAC used in this study was not sufficient to reduce GA to a level that affects the expression of these genes. In support of this hypothesis, the MeJA plus PAC treatment inhibited germination only partially (Fig. 4.1).

In summary, our results highlight that wheat seeds could be released from dormancy by increases in jasmonate level and/or sensitivity in seed tissues, particularly embryos, because elevated jasmonate response could increase embryonic GA level and sensitivity and decrease embryonic ABA level, creating an ABA–GA imbalance in favor of GA (Fig. 4.12). However,

manipulation of jasmonate levels and sensitivity might not be involved in regulation of seed dormancy as an essential mechanism during after-ripening, which is the most common treatment that induces dormancy release. In addition to after-ripening, other treatments such as cold stratification release seeds from dormancy. Since the mechanisms of seed dormancy release by different treatments vary, the next chapter of the thesis investigated interactions between JAs, and GA and ABA during dormancy release by cold treatment.

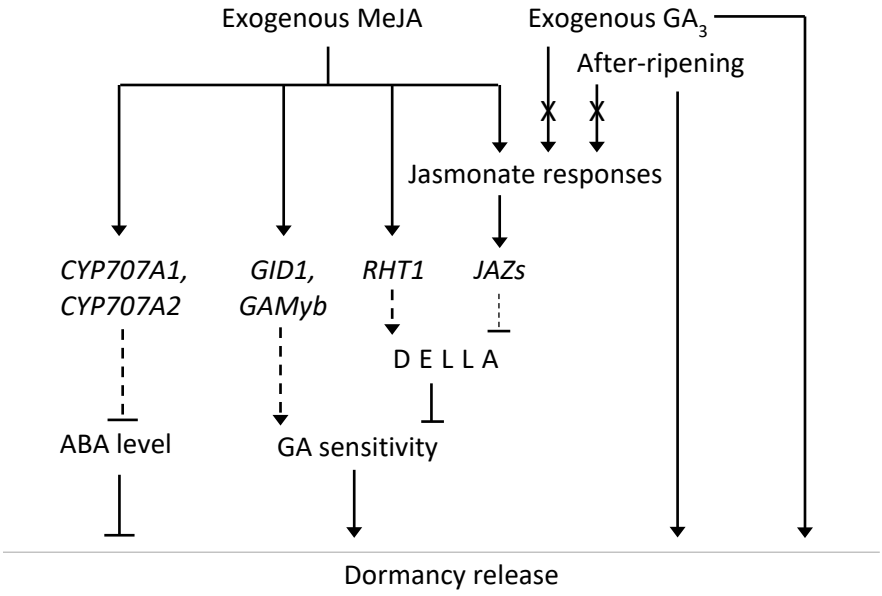

Figure 4.12. A schematic diagram of dormancy release by jasmonates. Exogenous methyl jasmonate (MeJA)-induced up-regulation of *JAZ* expression can increase tissue sensitivity to GA. Dormancy release by exogenous GA and after-ripening is not associated with induction of *JAZ* expressions. Dashed lines indicate expected effects; arrows with "X" indicate absence of positive effect.

5 THE ROLE OF COLD-INDUCED JASMONATES RESPONSE IN BREAKING SEED DORMANCY IN WHEAT

5.1 Abstract

Seed dormancy can be released by cold treatment. To understand the influence of JAs on ABA–GA balance during cold treatment, the present study examined changes in the contents of the three hormones and expression of their metabolic and signaling genes in dormant seeds during cold treatment and imbibition. Cold treatment for a day was sufficient to release dormancy in 90% of seeds. Cold treatment increased the expression levels of jasmonate responsive genes including *TaAOS2*, *TaAOC1*, *TaOPR2*, *TaACX1*, *TaJAZs* and *TaMYC2*, and these increases were accompanied by increased expression level of *TaCOIs* and reduced level of JA-Ile, suggesting that cold treatment induced jasmonate response by up-regulation of the JA-Ile receptor COI. Cold treatment however did not affect ABA level and ABA signaling probably due to its simultaneous inductions of the expression of levels *TaCYP707A*s and *TaNCED*s, and genes that act as both positive and negative regulators of ABA signaling. The cold treatment on the other hand increased GA_1 level, which is associated with increases and decreases in expression levels of *TaGA20ox*s and *TaGA2ox*s, respectively, and GA signaling through inducing the expressions of *TaGID1*, *TaGID2* and *TaGMyb*, which positively regulate GA signaling. Although the expression of *TaRHT1*, which encodes DELLA and acts as a negative regulator of GA signaling was up-regulated, the cold-induced up-regulation of *TaJAZ*s might enhance JAZ–DELLA interaction making the tissue more sensitive to GA. These results highlight the involvement of jasmonate signaling, via COIs and JAZs, rather than JA-Ile level in dormancy release by cold treatment; and the cold-induced increase in jasmonate-response might enhance cold-induced increase in GA level, which appear to be the key factor in dormancy release by cold treatment.

5.2 Introduction

Seed dormancy in many plant species can be broken by several environmental signals including after-ripening, temperature, light and nitrate (Finkelstein et al., 2008; Bazin et al., 2011; Gao and Ayele, 2014; Rodríguez et al., 2015). Among the temperature treatments that break dormancy is cold treatment, which is defined as imbibing seeds at low temperature, typically at 2 to 5ºC, for a period of time depending on the level of dormancy present in seeds (Finkelstein et al., 2008). However, in some crop species such as wheat, seed treatment temperature can go up to 15ºC depending on the level of dormancy in which the treatment temperature decreases with an increasing level of dormancy (Cone and Spruit, 1983; Mares, 1984). Cold treatment can also cancel the germination inhibition effects of some environmental factors such as darkness and blue light in different plant species such as Arabidopsis, barley and wheat (Yamauchi et al., 2004; Gubler et al., 2008; Xu et al., 2016). The role of cold treatment in breaking seed dormancy is associated with regulation of ABA and GA level and sensitivity. Cold treatment broke dark-induced dormancy in Arabidopsis seeds via increasing GA level and sensitivity (Yamauchi et al., 2004). Moreover, it simultaneously induced increases in level of embryo GA and decreases in level of embryo ABA to release dormancy in wheat seeds (Xu et al., 2016).

Jasmonates have also been reported to have a link with cold treatment/cold stress in several plant species previously. The level of JAs in plants is regulated by its metabolism which involves more than ten enzymes (Fig. 2.6). Exposure of Arabidopsis and tomato seedlings to cold temperature (4ºC) leads to an increase in jasmonate contents as compared to the seedlings that are exposed to the control temperature (22ºC), and this increase in jasmonate contents in the Arabidopsis seedlings was found to accompanied by up-regulations of jasmonate biosynthetic genes including *LOX*, *AOS*, *AOC* and *JAR* (Hu et al., 2013). Given that specific jasmonate

biosynthetic genes including *LOX*, *AOS* and *AOC* are jasmonate-responsive, these results might also imply the presence of enhanced jasmonate signaling in the seedling tissues. In support of these results, exogenous JA alleviates cold stress in synthetic seeds of *Taraxacum pieninicum* due to their storage at low temperature (4ºC) (Kamińska et al., 2018). However, treatment of imbibing wheat seed with cold temperature (4ºC) that breaks dormancy has been shown not to alter embryonic jasmonate level as compared to those imbibed at control temperature (20ºC) (Xu et al., 2016). Given that cold treatment of seeds increases the level of bioactive GA (Yamauchi et al., 2004), and bioactive GA can upregulate the expression of gene encoding MYC2 which acts as an activator of jasmonate signaling (Hong et al., 2012; Qi et al., 2014), it is likely that cold treatment enhances jasmonate signaling. Therefore, it is important to investigate the regulation of jasmonate signaling in seeds in response to cold treatment.

Jasmonates signaling involves several components including COI, JAZ, MYC2 and MED25 (Fig. 2.5). Jasmonate response can be regulated by COI1 that functions as JA-Ile receptor and is stabilized by SCF^{COI1} complex which contains CUL1 and ARABIDOPSIS SPIKE1-LIKE1 (ASK1), and then degraded through 26S proteasome pathway (Yan et al., 2013). It has been reported previously that the *coi1-16* mutant of Arabidopsis is much less sensitive to MeJA-induced effects such as inhibitions of root and leaf growths, leaf deformation, expression of luciferase and accumulation of anthocyanin in the leaves. Moreover, *coi1-16* seeds is less sensitive to MeJA-induced inhibition of germination (in the presence of exogenous ABA) as compared to wild-type seeds (Ellis and Turner, 2002). The level of COI1 protein in *ask1* mutant is much lower than that in wild-type (Yang et al., 1999); this decrease in COI1 level is accompanied by reduction in the expression level of jasmonate-responsive gene *VSP1* in response to exogenous MeJA (Yan et al., 2013). Similarly, a decrease in the expression level of *VSP1* in response to MeJA is associated with decrease in COI1 level in the heterozygous CUL1 mutants *axr6-1*/*AXR6* and *axr6-2*/*AXR6*

(Hobbie et al., 2000; Yan et al., 2013). By contrast, the level of COI1 protein in *rpt5a-4*, the 26S proteasome mutant, is much higher than that observed in the wild-type, and this increase in COI1 level in the mutant is accompanied by a significant increase in the expression level of jasmonate-responsive gene *VSP1* in response to exogenous MeJA (Yan et al., 2013). Moreover, enhanced sensitivity to JAs has been observed in plants exhibiting increases in the expression levels of the *COI* genes. For example, expressing *COI* genes of Arabidopsis and rice in *coi1* mutants leads to increases in the expression levels of jasmonate-responsive genes including *AOS* and *JASMONIC ACID RESPONSIVE2* (*JR2*) in response to exogenous MeJA (Lee et al., 2013). However, COIs might not act as a simple positive regulator of jasmonate response since they are also implicated in the negative feedback regulation of JA-Ile turnover. For example, silencing of *COI1* leads to increased and prolonged accumulation of JA-Ile due to a decrease in the rate of JA-Ile turnover, not because of an increase in level of JA and/or Ile (Paschold et al., 2008). Nevertheless, cold treatment is equally effective in releasing dormancy in both *coi1-16* mutant and wild-type seeds (Ellis and Turner, 2002). Similarly, *myc2* and *med25* mutants of Arabidopsis are less sensitive to JAs in terms of root growth while the response of their seeds to cold treatment that breaks dormancy are similar to that of wild-type (Abe et al., 2003; Cerdán and Chory, 2003; Kidd et al., 2009; Gangappa et al., 2010; Chen et al., 2012).

Previous studies have shown that enhanced jasmonate response can influence GA synthesis and signaling. For example, inhibition of germination by GA biosynthesis inhibitor (PAC) is found to be much lower in seeds overexpressing *JAZ9* than wild-type, and the reverse is true for comparison between *JAZ9* silenced mutant and wild-type seeds (Yang et al., 2012). The MYC2 protein acts as an activator of GA signaling (Qi et al., 2014), and JAZs, which binds to DELLA and prevents it from negatively regulating its target downstream transcription factors in GA signaling (Hou et al., 2010; Yang et al., 2012; Song et al., 2014), indicating their roles in influencing GA signaling positively. Genes encoding these two proteins of jasmonate signaling,

MYC2 and *JAZs*, are reported to be among the early jasmonate-responsive genes (Wasternack and Hause, 2013). Therefore, GA signaling might form one of the early jasmonate-responsive physiological processes. However, genes encoding DELLA such as *RGL3* of Arabidopsis are direct target of MYC2, leading to jasmonate-mediated induction in the expression of *DELLA* gene, which in turn represses JAZ activity and thereby activates the transcription of jasmonate-responsive genes (Wild et al., 2012). With a specific hypothesis that JAs–GA synergistic interactions are involved in dormancy release by cold treatment, this chapter of the thesis investigated transcriptional regulations of the metabolism and signaling genes of JAs, ABA and GA during dormancy release by cold treatment.

5.3 Materials and Methods

5.3.1 Plant material and cold treatments

Mature dormant seeds of wheat cultivar AC Domain (Townley-Smith and Czarnecki, 2008) were harvested from plants grown under growth conditions described in chapter 3. Seeds were immediately stored at -80ºC. Preparation of seeds for imbibition (25 seeds per Petri-dish as a replicate with a total of four biological replicates) was performed as described in chapter 3. One set of imbibing seeds were incubated at 22ºC (referred as RT) under darkness while two other sets of imbibing seeds were subjected to imbibition at cold (4ºC) under darkness for 1 day and 2 days before performing further imbibition at RT (Fig. 5.1a). Seed germination was monitored daily for 7 d. For gene expression and hormone level analysis, embryos samples were collected before and after the start of imbibition under cold and RT, at 12, 24, 48, 40 and 72 HAI.

5.3.2 Gene Expression and hormone level analyses

Gene expression and hormone level analysis were performed as described in chapter 3. List of genes studied in this chapter and their respective primers are described in Tables 1S-4S.

5.3.3 Statistical analysis

Significant differences among samples were tested by two-way ANOVA analysis at a probability of $P<0.05$ while difference between two samples was examined using LSD test at the same probability. Logarithm- or square root-transformation was used when needed to satisfy the statistical assumptions of normality and homogeneity of variances.

5.4 Results

5.4.1 Seed Germination

Under control conditions, only 4% and 15% of seeds germinated at 1 and 2 DAI, respectively, and the germination percentage increased gradually with imbibition and reached 40% at 7 DAI (Fig. 5.1). In contrast, seed samples cold treated for 1 day before the start of imbibition exhibited approximately 75% and 85% of germination at 1 DAI and 2 DAI, respectively. Over 90% of seeds cold treated for 1 day completed their germination by 5 DAI. Cold treatment for 2 days before the start of imbibition at RT led to the germination of 93% and 97% of the seeds at 1 DAI and 2 DAI, respectively, and all the seeds germinated by 3 DAI.

5.4.2 Expression patterns of jasmonate metabolic genes and contents of JA-Ile

The expression patterns of jasmonate metabolic genes, including *AOS*, *AOC*, *OPR*, *ACX*, *KAT* and *JAR*, and the content of JA-Ile were measured in this study (Fig. 5.2).

The transcripts of all the jasmonate metabolic genes analyzed in this study were detected in the embryo of dry seeds (Fig. 5.2). Seed imbibition in the cold for 12 and/or 24 h increased (over 1.3-fold) the expression levels of jasmonate metabolic genes except that it either did not have effect or caused a slight reduction in the expression levels of *TaACX1* and *TaKAT2*, respectively.

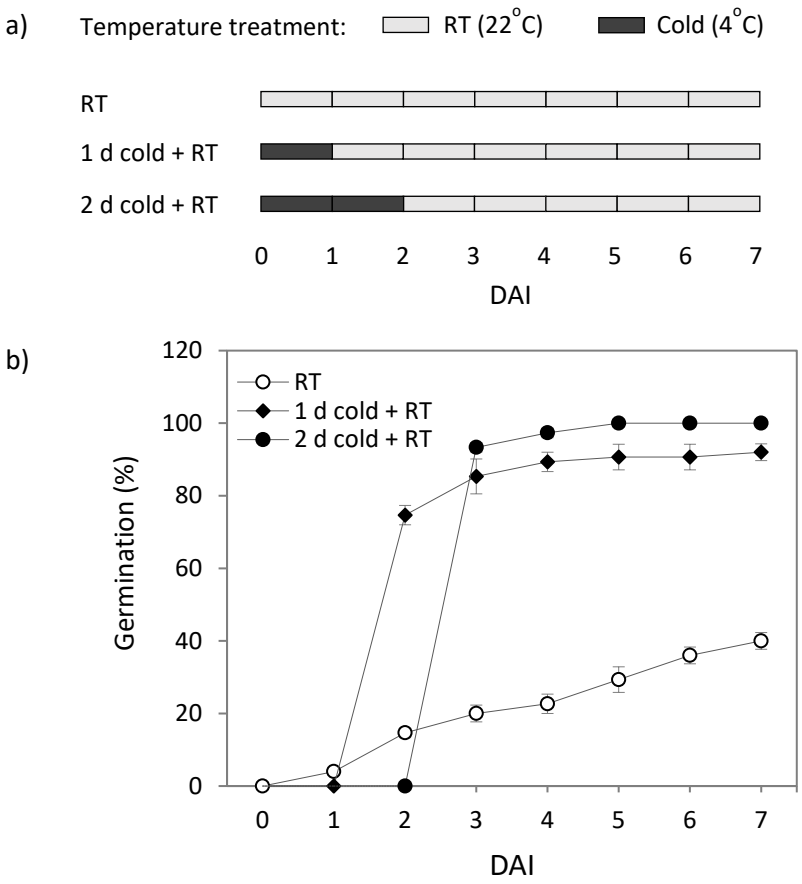

Figure 5.1. Germination of dormant seeds in response to cold treatment. Seeds were imbibed at cold temperature for 0, 1 or 2 days prior to imbibition at room temperature (a). Germination percentage (b) was calculated based on number of seeds germinated over seven days after imbibition (DAI). Data are mean ± SE of four biological replicates.

Further imbibition in the cold led to marked decreases in the expression levels of all the genes (over 1.5-fold). Seed imbibition at RT for 12 and/or 24 h also increased the expression levels of *TaAOS2-D* and *TaAOC1* genes (over 2-fold) but led to decreases in the expression levels of the other jasmonate metabolic genes (over 1.5-fold). Extending the duration of imbibition at RT to 48 h, however, increased the expression level of all the genes as compared to that observed at 24 HAI (over 1.3-fold). Overall, all the jasmonate metabolic genes exhibited higher levels of expression during 12 and/or 24 h imbibition in the cold than at RT (over 1.5-fold) while seeds imbibed at RT

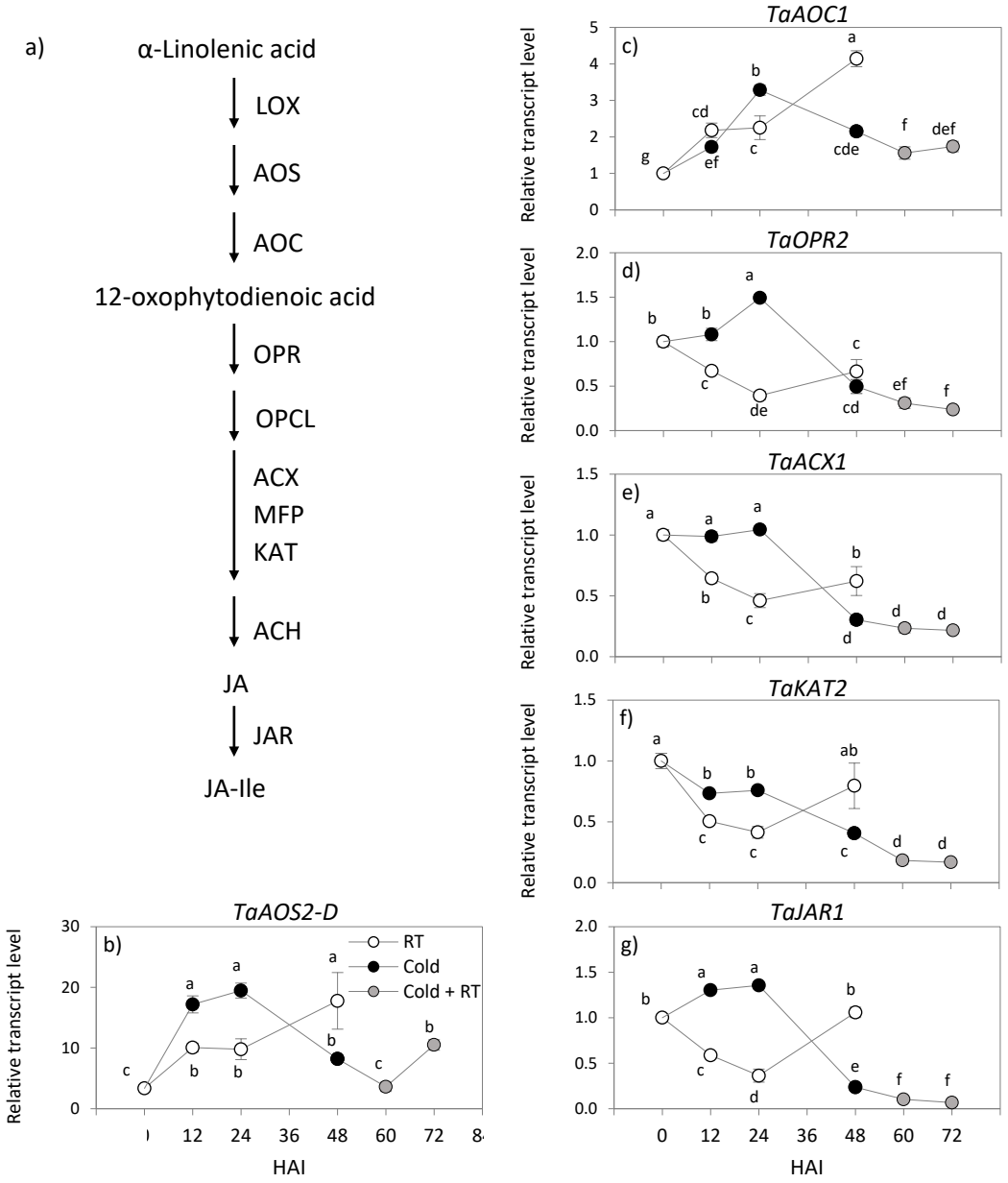

Figure 5.2. Expression of jasmonate biosynthesis genes in response to cold treatment.A simplified pathway of jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile) biosynthesis (a); and relative transcript levels of *TaAOS2-D* (b), *TaAOC1* (c)*, TaOPR2* (d), *TaACX1* (e), *TaKAT2* (f) and *TaJAR1* (g) in embryos of control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaAOS2-D*, *TaAOC1, TaOPR2, TaACX1, TaKAT2* and *TaJAR1* were expressed relative to the transcript levels of *TaAOS2-A* (Fig. 10Sa), *TaAOC1, TaOPR2, TaKAT2, TaACX1,* and *TaJAR1* in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

for 48 h exhibited higher expression levels of the jasmonate metabolic genes than that observed in the corresponding seeds imbibed in the cold (over 1.9-fold) except for *TaOPR2* which showed similar expression levels between the two imbibition temperatures. Further imbibition at RT for 12 and 24 h of the seeds first imbibed in the cold for 48 h led to decreases in the expression levels of all jasmonate metabolic genes to levels lower than that observed in dry seeds or seeds imbibed continuously in cold or at RT. However, the expression levels of *TaAOS2-D*, *TaAOC1* and *TaACX1* were maintained at levels similar to that found in 48 h cold imbibed seeds, leading to the observation of higher/similar expression levels of these three genes as compared to that observed in dry seeds or seeds imbibed for 12 and 24 h at RT only. The cold treatment also affected the expression of *TaAOS2-A, TaAOS2-B* and *TaJAR2s* (Fig. S10). The expression patterns of *TaAOS2-A* and *TaAOS2-B* were similar to that of *TaAOS2-D*; however, *TaJAR2-A* and *TaJAR2-B* showed expression patterns which were different from that of *TaJAR1*.

Certain amount of JA-Ile was detected in the embryos of dry seeds (Fig. 5.3), and its levels either remained unaffected or showed decline during imbibition in the cold while exhibiting increases (over 3-fold) during imbibition at RT, leading to the prevalence of higher level of JA-Ile

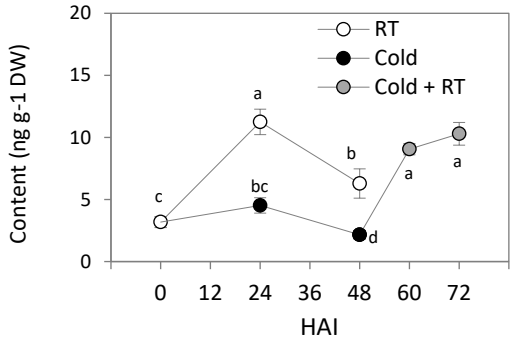

Figure 5.3. Content of jasmonoyl-isoleucine (JA-Ile) in response to cold treatment. JA-Ile content in embryos of control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

in embryos imbibed at RT than those imbibed in the cold. Further imbibition at RT for 12 and 24 h of the seeds first imbibed in the cold for 48 h caused substantial increases (over 4-fold) in the level of JA-Ile to levels similar to that found in samples continuously imbibed at RT.

5.4.3 Expression patterns of jasmonate signaling genes

The expression patterns of jasmonate signaling genes including *TaCOI2, TaJAZ4*, TaJAZ7, *TaMYC2* and *TaMED25* were also analyzed (Fig.5.4). The transcripts of these genes were detected in the dry embryos, and their expression levels either showed reductions/increases or maintenance at similar levels during imbibition at RT. In contrast, imbibition in the cold for 12 and 24 h maintained or increased their expression levels, leading to the observation of higher expression levels of these genes in samples imbibed for 12 and/or 24 h in the cold than the corresponding samples imbibed at RT (over 2-fold). However, as imbibition in the cold continued from 24 to 48 h, the expression levels of all the jasmonate signaling genes decreased to a level similar to or lower than that observed in seeds imbibed at RT for 48 h (over 1.3-fold) except for *TaMED25* whose expression level remained unaffected during the entire period of imbibition in the cold. Further imbibition at RT for 12 and 24 h of the cold treated seeds led to decreases in the expression levels of *TaCOI2*, *TaMYC2* and *TaMED25* to levels lower than that observed in dry seeds or seeds imbibed continuously in cold or at RT (over 1.3-fold). However, the same imbibition at RT of the cold treated seeds either caused very slight increases or did not affect the expression levels of *TaJZ4* and *TaJAZ7*, leading to the observation of their expression levels similar to or slightly higher than that observed in dry seed samples and those imbibed continuously at RT. The cold treatment also had similar effects on the expression of *TaCOI1*, *TaCOI3*, *TaJAZ1*, *TaJAZ10* and *TabHLH13* (Fig. 11S).

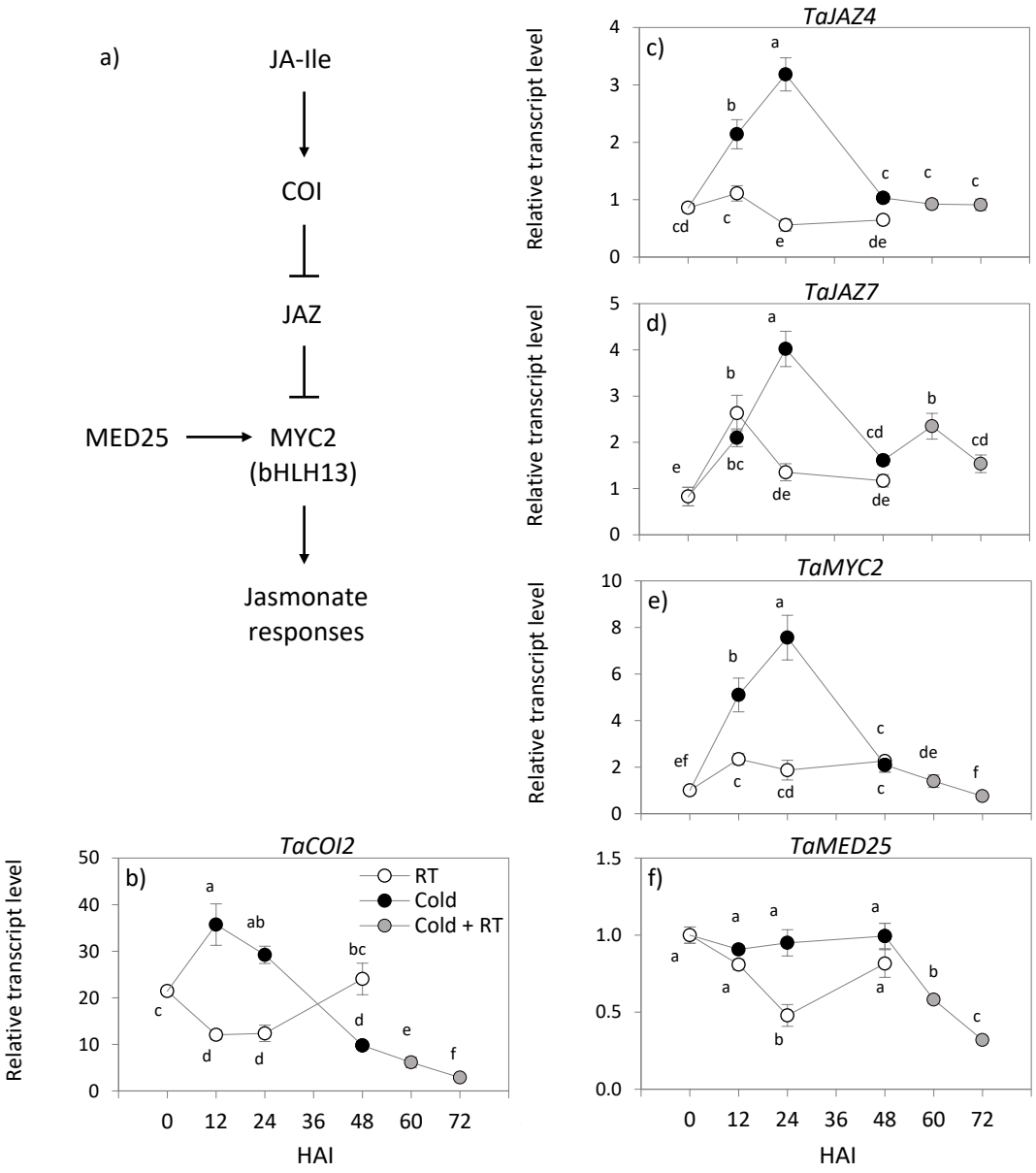

Figure 5.4. Expression of jasmonate signaling genes in response to cold treatment. A simplified pathway of jasmonoyl-isoleucine (JA-Ile) signaling (a); and relative transcript levels of *TaCOI2* (b), *TaJAZ4* (c), *TaJAZ7* (d), *TaMYC2* (e), and *TaMED25* (f) in embryos of control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOI2*, *TaJAZ*s, *TaMYC2* and *TaMED25* were expressed relative to the transcript levels of *TaCOI1* (Fig. 11Sa), *TaJAZ1* (11Sc), *TaMYC2* and *TaMED25* in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

5.4.4 Expression patterns of ABA metabolic genes and contents of ABA

This study analyzed the expression level of genes encoding the ABA biosynthetic enzyme NCED and those encoding the ABA catabolic enzyme CYP707A, and changes in ABA contents in embryos (Fig. 5.5). Low transcript levels of the *TaNCED1* and *TaCYP707A2* genes were detected in embryos of dry seeds, and the expression of *TaNCED1* was increased (over 3-fold) while that of *TaCYP707A2* was decreased (over 1.7-fold) during imbibition at RT. Imbibition in the cold for 12 and 24 h led to increases in the expression levels of the two ABA metabolic genes (over 1.9-fold). Further imbibition from 24 to 48 h led to decreases in their expression levels (over 1.8-fold). In general, our analysis revealed that the expression levels of the ABA metabolic genes were found to be higher (over 4-fold) in embryos of seeds imbibed in the cold than those imbibed at RT. Further imbibition at RT for 12 and 24 h of the cold imbibed seeds caused reductions in the expression of the ABA metabolic genes to levels lower than that observed in seeds imbibed continuously at RT. The effects of cold treatment on the expression of *TaNCED2* and *TaCYP707A1* (Fig. 12S) were almost similar to those observed for *TaNCED1* and *TaCYP707A2*, respectively (Fig. 5.5).

Certain amount of ABA was detected in the embryos of dry seeds and the level decreased during imbibition either in the cold or at RT (over 1.2-fold), and no difference in ABA level was found between the two imbibition temperatures (Fig. 5.5). However, further imbibition for 12 and 24 h at RT of the seeds imbibed in the cold for 48 h caused a substantial decrease in ABA level, leading to the observation of a much lower level of ABA than that observed in seeds continuously imbibed in the cold or at RT (over 3-fold)

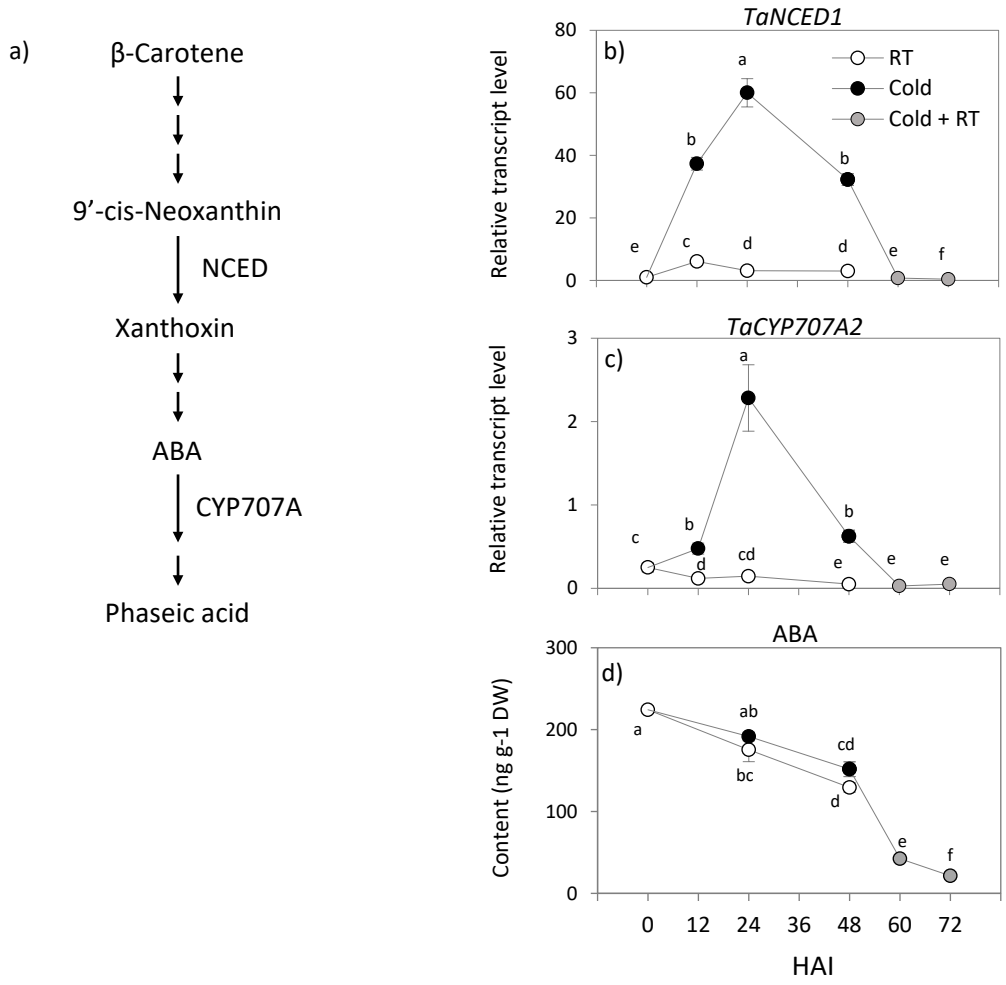

Figure 5.5. Expression of abscisic acid (ABA) metabolic genes and content of ABA in response to cold treatment. A simplified pathway of ABA metabolism (a); and relative transcript levels of *TaNCED1* (b) and *TaCYP707A2* (c), and content of ABA (d) in embryos of control (RT) and cold-treated seeds (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaNCED1* and *TaCYP707A2* were expressed relative to the transcript levels of *TaNCED1* and *TaCYP707A1* (Fig. 12Sb) in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

5.4.5 Expression patterns of ABA signaling genes

To examine changes in ABA sensitivity, we examined the expression levels of ABA signaling genes including *PYL5*, *PP2C*, *SnRK2*, *ABI3* and *ABI5* (Fig. 5.6). Transcripts of *TaPYL5, TaPP2C,*

TaSnRK2 and *TaABI5* were present in embryos of dry seeds. Seed imbibition for 12 and/or 24 at RT led to decreases in the expression levels of these ABA signaling genes (over 1.6-fold) except for that of *TaPYL5*, which showed slight increases as compared that observed in the dry embryos.

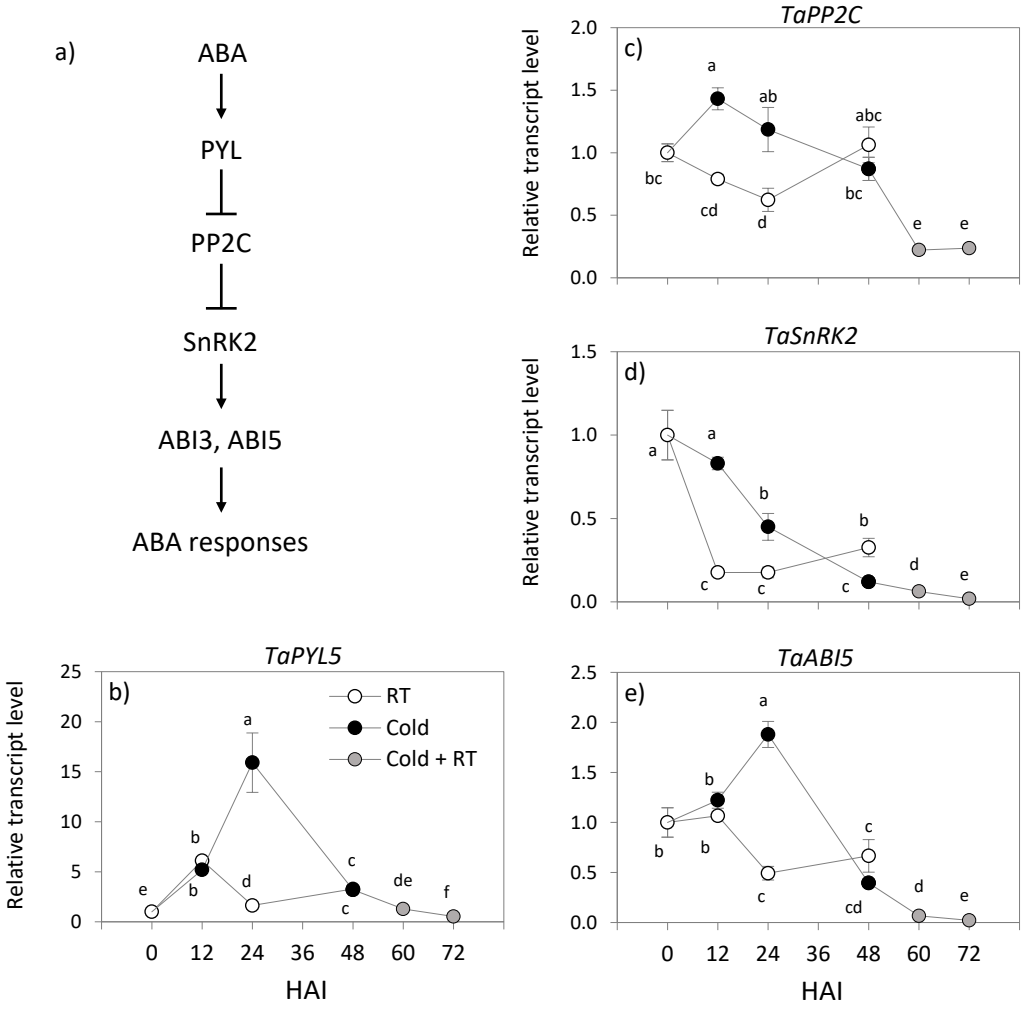

Figure 5.6. Expression of abscisic acid (ABA) signaling genes in response to cold treatment A simplified pathway of ABA signaling (a); and relative transcript levels of *TaPYL5* (b), *TaPP2C* (c), *TaSnRK2* (d) and *TaBAI5* (e) in embryos of the control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

Increasing the period of imbibition from 24 to 48 h caused increases in the expression levels of the ABA signaling genes (over 1.8-fold) except that the expression level of *TaABI5* remained at a similar level. Imbibition in the cold for 12 h, in contrast, increased the expression levels of *TaPYL5* and *TaPP2C* (over 1.4-fold) and maintained the expression levels of *TaSnRK2* and *TaABI5* at a similar level observed in the dry embryos. Further imbibition in the cold caused decreases in the expression levels of all genes except the transient increases occurred at 24 HAI for *TaPYL5* and *TaABI5* (over 1.5-fold). When compared to seeds imbibed at RT, the expression levels of all the ABA signaling genes were higher in seeds imbibed in the cold for 12 and/or 24 h (over 1.8-fold). However, by 48 HAI, seed samples imbibed in the cold exhibited either similar (*TaPYL5*, *TaPP2C* and *TaABI5*) or lower (*TaSnRK2;* 2.7-fold) expression levels of the ABA signaling genes as compared to that found in seeds imbibed at RT. Imbibition of the cold imbibed seeds further at RT for 12 and 24 h led to substantial decreases in the expression of all the genes to very low levels. The effects of cold treatment on the expression of *TaABI3* (Fig. 12S) were similar to those observed for *TABI5.*

5.4.6 Expression patterns of GA metabolic genes and contents of GA

The expression patterns of GA metabolic genes *GA20ox*, *GA3ox* and *GA2ox*, and the content of bioactive GA_1 were analyzed (Fig. 5.7). Transcripts of *TaGA20ox1*, *TaGA3ox2* and *TaGA2ox3* were also detected in the embryos of dry seeds. The expression levels of *TaGA20ox1* were increased by imbibition at RT (over 7-fold). Imbibition in the cold for 12 and/or 24 h also increased the expression levels of *TaGA20ox1* (over 15-fold) and their expression showed a decrease as imbibition continued from 24 to 48 h (over 6-fold). In spite of the decrease, the expression levels of *TaGA201* were higher during imbibition in the cold than at RT (18-fold at 24 HAI). Imbibition

of the seeds imbibed in the cold further for 12 and 24 h at RT led to reduction in the expression levels of *TaGA20ox1* to very low levels. The expression pattern of *TaGA20ox2* (Fig. 13S) was similar to that of *TaGA20ox1*. In regard to the *TaGA3ox2* gene, its expression level was almost

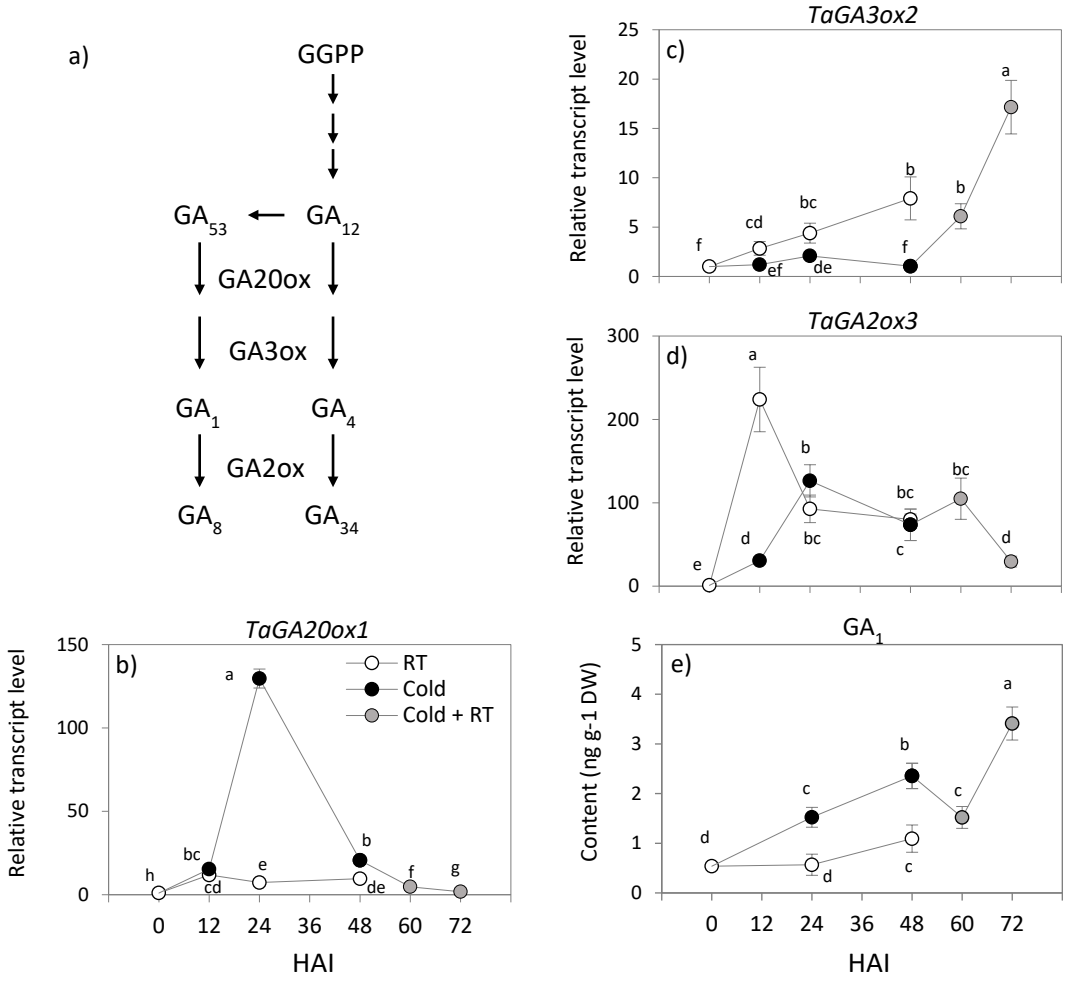

Figure 5.7. Expression of gibberellins (GA) metabolic genes and content of GA_1 in response to cold treatment. A simplified pathway of GA metabolism (a); and relative transcript levels of *TaGA20ox1* (b), *TaGA3ox2* (c) and *TaGA2ox3* (d) and content of GA_1 (f) in embryos of control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). GGPP, geranylgeranyl diphosphate; HAI, hour(s) after the start of imbibition; RT, room temperature.

unaffected by imbibition in the cold but increased gradually during imbibition at RT (over 1.6-fold). As a result of this, embryonic *TaGA3ox2* had higher level of expression during imbibition at RT than in the cold (over 2-fold). Imbibition at RT for 12 h of seeds pre-imbibed for 48 h in the cold led to an increase (over 2.8-fold) in the expression of *TaGA3ox2* to a level similar to that observed in seeds continually imbibed at RT for 48 h. Further imbibition for another 12 h of the same seed samples caused over 3-fold further increase in the expression level of *TaGA3ox2*.

The expression levels of embryonic GA catabolic gene *TaGA2ox3* showed substantial increases within 12 h imbibition at RT (224-fold) but decreased to lower levels by 24 HAI (over 2-fold) and remained at similar levels thereafter (Fig. 5.7). Seeds imbibing in the cold also showed gradual increases in the expression level of *TaGA2ox3* and its expression levels peaked at 24 HAI (126-fold). Afterwards, the expression level was maintained at a similar level. Comparison between the two temperatures showed lower expression level of *TaGA2ox3* in embryos imbibed in the cold for 12 h than the corresponding samples imbibed at RT (7.3-fold) while seeds imbibed in the cold for 24 and 48 h appeared to have similar expression level as compared to the respective samples imbibed at RT. Imbibition of the seeds pre-imbibed for 48 h in the cold further for 12 h at RT did not affect the expression level of *TaGA2ox3*; its expression levels remained at a level similar to that observed in seed imbibed in the cold for 48 h. However, extending imbibition of the same seed samples at RT for another 12 h caused reduction in the expression level of the gene to a very low level. Another GA catabolic gene, *TaGA2ox6* (Fig. 13S), showed an expression pattern similar to that observed for *TaGA2ox3*.

A minimal amount of GA_1 was detected in the embryos of dry seeds (Fig. 5.7). Imbibition for 24 h in the cold but not at RT caused an increase (2.8-fold) in the level of GA_1. Increases in GA_1 level (over 1.6-fold) were observed in response to further imbibition for 24 h at both

temperatures. However, a higher level of embryonic GA_1 was detected in seeds imbibed continually in the cold than those imbibed at RT (over 2-fold). Imbibition at RT of the seeds pre-imbibed for 48 h in the cold caused a marked increase in GA_1 level after 24 h imbibition although a transient decline was evident at 12 HAI as compared to that found in seeds imbibed in cold for 48 h.

5.4.7 Expression patterns of GA signaling genes

To examine changes in GA sensitivity we examined the expression levels of GA signaling genes including *GID*, *RHT* and *GAMyb* (Fig. 5.8). Transcripts of the *TaGID2*, *TaRHT1* and *TaGAMyb* were detected in the embryos of dry seeds. The expression levels of *TaGAMyb* increased during imbibition at RT (over 1.5-fold) while that of *TaGID2* decreased (over 1.6-fold). The expression levels of *TaGID2* and *TaGAMyb* genes, however, increased in embryos of seeds imbibed for 12 and/or 24 h in the cold (over 1.5-fold), leading to the observation of higher expression levels of these genes than that in seeds imbibed at RT for the same duration (over 1.9-fold at 24 HAI). Further imbibition in the cold for another 24 h reduced the expression of these genes to levels similar to (*TaGID2*) or lower (*TaGAMyb*, over 2-fold) than found in seeds imbibed for the same duration at RT. Imbibition at RT of the seeds pre-imbibed for 48 h in the cold caused decreases in the expression levels of *TaGID2* and *TaGAMyb* (over 1.8-fold). The effects of cold treatment on the expression of *TaGID1* (Fig 13S) was similar to those observed in *TaGID2*. While the expression level of *TaRHT1* was maintained at similar level during imbibition at RT (Fig. 5.8), imbibition in the cold for 24 h increased its expression level (2.9-fold). By 48 HAI in the cold, the expression level of *TaRHT1* was similar to that detected in seeds imbibed for the same duration at RT. Further imbibition at RT for additional 24 h of seeds imbibed in the cold for 48 h led to decreases in the expression of *TaRHT1* to very low levels.

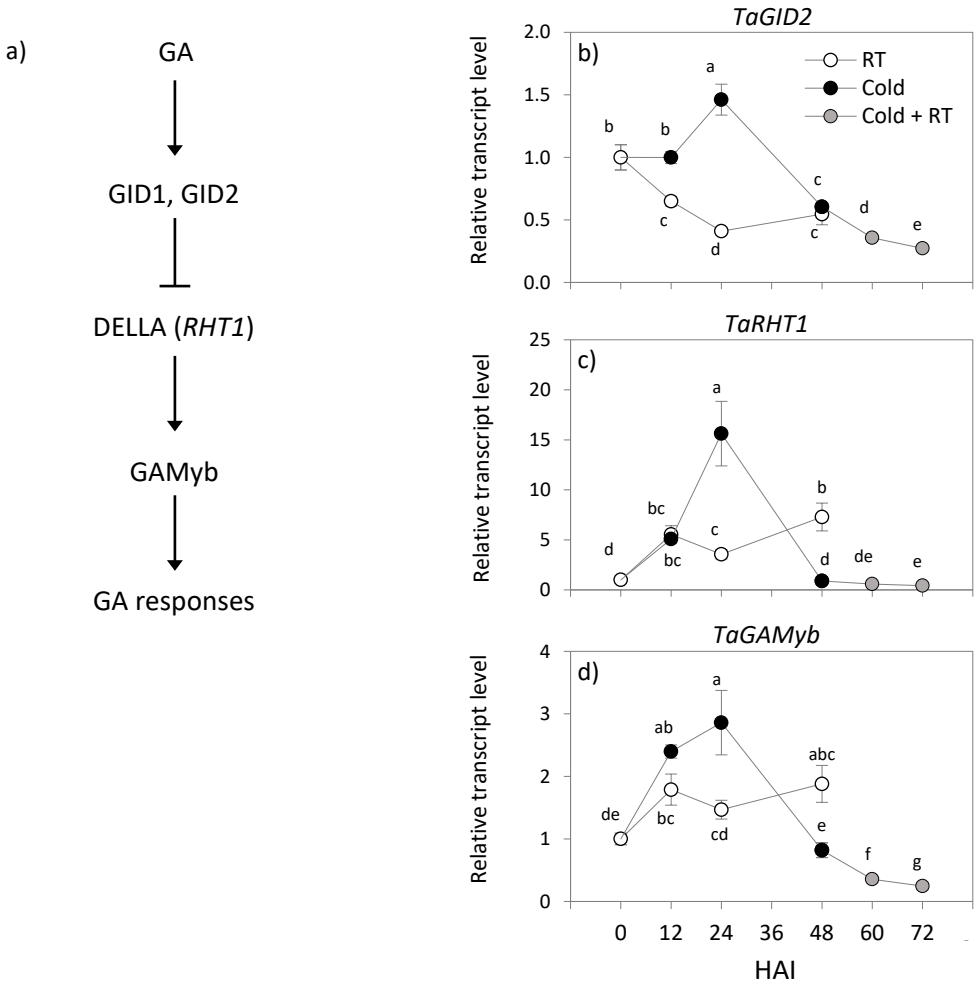

Figure 5.8. Expression of gibberellins (GA) signaling genes in response to cold treatment. A simplified pathway of GA signaling (a); and relative transcript levels of *TaGID2* (b), *TaRHT1* (c) and *TaGAMyb* (d) in embryos of the control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of each gene was expressed relative to their transcript levels in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

5.5 Discussion

Cold treatment is among the treatments that release seeds of different plant species from dormancy (Finkelstein et al., 2008). Imbibing dormant wheat seeds, which showed very low percentage of

germination during imbibition at RT, in the cold did not induce germination. However, imbibing at RT after the cold treatment was able to break the dormancy and induced their germination (Fig. 5.1). These data indicate that the effect of cold treatment in releasing dormancy in wheat seeds is expressed during imbibition at optimal temperature condition such as at RT after the cold treatment. Similarly, cold treatment followed by imbibition at optimal conditions was able to break dormancy and promote germination in blue light treated dormant seeds of wheat (Xu et al., 2016).

This study showed that seed imbibition in the cold for 12 and/or 24 h as compared to that in RT alone caused a substantial decrease in the level of JA-Ile in the embryo. However, the same cold treatment induced (over 1.5-fold) the expression levels of the jasmonate biosynthetic (*AOS2*, *AOC1*, *OPR2, ACX1*, *KAT2* and *JAR1;* Figs. 5.2, 10S) and signaling genes (*COI1, 2, 3*, *JAZ1, 4, 7, 10, MYC2, bHLH13* and *MED25*; Figs. 5.4, 11S). Since most of these genes are jasmonate-responsive, especially *JAZ* and *MYC* are reported to be early jasmonate-responsive (Wasternack and Hause, 2013), our result suggests an increase in jasmonate response. Cold temperature increased jasmonate content and jasmonate response in Arabidopsis and tomato seedlings (Hu et al., 2013) and exogenous JA alleviates cold stress in synthetic seeds of *Taraxacum pieninicum* (Kamińska et al., 2018). Therefore, it is likely that the increased jasmonate response observed in our study is originally important as cold stress response. However, the expression levels of all of the jasmonate-responsive genes measured were significantly reduced by 48 h of imbibition in the cold, implying that the cold treatment caused only a transient increase in jasmonate response. It has also been shown previously that jasmonate response in plants, in most cases, is characterized by a transient burst (Miersch et al., 2008; Heitz et al., 2012; Widemann et al., 2013). The increase in jasmonate response shown in cold treated seeds was not accompanied by an increase in JA-Ile level (Fig. 5.3), rather by increases in the expression levels of *TaCOI*s (Figs. 5.4, 11S). This result

is consistent with previous reports that suggest the importance of COIs as important players not only in enhancing jasmonate response but also in fine-tuning it. For example, when the Arabidopsis plants were treated with MeJA, the expression level of jasmonate-responsive genes such *AOS* and *JR2* were undetectable in *coi1-1* mutant, but their expression level was up-regulated in *coi1-1* mutants expressing *COI* genes of Arabidopsis (*AtCOI1*) and rice (*OsCOI1a*, *OsCOI1b* and *OsCOI2*) as compared to the wild-type which exhibits lower expression level of *COI1* (Lee et al., 2013).

Although JAs breaks dormancy (Chapter 4), there was no germination of seeds imbibed in the cold in which a transient burst in jasmonate response was evident. Our results thus imply that induction of jasmonate response in the embryo due to seed imbibition in the cold is an important factor for the release of dormancy during subsequent imbibition of seeds at RT. Induction in the expression levels of *JAZ*s in particular might have a positive influence on GA signaling since JAZs can bind to DELLA and prevent it from negatively regulating the downstream GA signaling transcription factors (Song et al., 2014). Consistent with this, *JAZ*-overexpressing Arabidopsis plants were shown to be more sensitive to GA during their growth than the wild-type plants (Yang et al., 2012). Moreover, both MeJA-induced dormancy release in wheat seeds and enhancement of the GA-induced dormancy release by MeJA are shown to be accompanied by marked increase in jasmonate responses including increase in the expression levels of *JAZ*s (Chapter 4). Taken together, these results suggest that an increase in jasmonate response due to cold treatment might contribute to a change in seed GA response to favor dormancy release during imbibition at optimal conditions following cold treatment (Fig. 5.1).

Only minor differences in the effectiveness of dormancy release were observed between 1 and 2 d of cold treatment (Fig. 5.1), suggesting that the biological events occurring during the first

24 h of cold treatment might be critical for inducing dormancy release. This is well supported by the temporal expression patterns of the genes investigated in this study. Decreases in ABA level and sensitivity and/or increase in GA level and sensitivity, which are regulated by the expression patterns of ABA metabolic and signaling genes, are among the crucial events that are important for dormancy release (Shu et al., 2016; Tuan et al., 2018). However, no difference in ABA level of the embryo was evident between seeds imbibed in the cold for 24 h as compared to those imbibed at RT alone. This can be explained by the fact that up-regulations of the ABA biosynthesis genes *TaNCED1* and *TaNCED2* in cold imbibed seeds were accompanied by increased expression levels of the ABA catabolism genes *TaCYP707A1* and *TaCYP707A2* (Figs. 5.5, 12S). In contrast, embryos of cold imbibed seeds exhibited up-regulation in the expression levels of ABA signaling genes including *TaPYL5*, *TaSnRK2*, *TaABI3*, *TaABI5* (Figs. 5.6, 12S), suggesting an increase in ABA response is responsible for inhibition of germination during cold imbibition. Imbibing seeds in the cold for 24 h, on the other hand, caused an increase in GA_1 level through expression of GA biosynthetic genes, *TaGA20ox1* and *TaGA20ox2* (Fig. 5.7, 13S), and up-regulation of the GA signaling genes *TaGID1*, *TaGID2*, *TaGAMYb* (Fig. 5.8, 13S) as compared to imbibition at RT alone, indicating that cold treatment induces both embryo GA levels and GA sensitivity. These results are consistent with previous reports on Arabidopsis and wheat which showed that cold treatment increases seed GA level and response, leading to germination during imbibition at optimal conditions (Yamauchi et al., 2004; Pearce et al., 2015; Xu et al., 2016).

Imbibing cold treated seeds at RT reduced the expression of all jasmonate biosynthesis and signaling genes in embryos to levels that are similar to/below that observed in seeds imbibed in cold or at RT alone. However, we observed an increase in JA-Ile level, and this might suggest post-transcriptional regulation of the jasmonate biosynthetic and signaling genes. Alternatively,

the increase in JA-Ile level in response to the cold + RT treatment as compared to treatment with cold or RT alone could be attributed to the down-regulation of *TaCOI*s (Figs. 5.4, 11S), which are implicated in the negative feedback regulation of JA-Ile level through enhancing JA-Ile turnover (Paschold et al., 2008; Howe et al., 2018). It has been shown previously that *Nicotiana attenuata* plants with silenced *COI1* gene (*ir-coi1*) exhibit increased and prolonged accumulation of JA-Ile as compared to wild-type plants due to a decrease in the rate of JA-Ile turnover in the mutant (Paschold et al., 2008).

Imbibition of cold treated seeds at RT, which induced dormancy release and germination, decreased the expression level of ABA biosynthetic and catabolic genes, and the amount of ABA below that observed in seeds imbibed in cold/RT alone (Fig. 5.5). These results suggest the importance of ABA biosynthesis in regulating ABA level. In addition, the same cold + RT treatment substantially suppressed the expression levels of ABA signaling genes (Figs. 5.6, 12S), suggesting a decrease in tissue response to ABA. Altogether, our data suggest that both ABA level and response play important roles in dormancy release/germination during imbibition at RT of the cold imbibed seeds (Xu et al., 2016; Tuan et al., 2018). Imbibition at RT following cold treatment induced germination and caused further increase in GA_1 level (Fig. 5.7), and this was associated mainly with the up-regulation of the GA biosynthetic gene *TaGA3ox2* (Fig. 5.7). However, the expression of all GA signaling genes were down-regulated in the same samples (Figs. 5.8, 13S). Since cold treated dormant seeds were able to germinate following imbibition at RT, our results might suggest that the GA signaling genes are subjected to post-transcriptional regulation. A previous study in Arabidopsis has also shown that dark-induced dormancy can be broken by cold treatment mainly through cold-enhanced increases in bioactive GA level as well as GA response (Yamauchi et al. 2004a).

The present study in general showed a decrease in the ratio of ABA/GA_1 contents (Figs. 5.5d, 5.7f), and increases in ABA-, GA- and jasmonate-response, as evidenced by the expression patterns of their signaling genes, during the first 24 h of cold treatment (Figs. 5.4, 5.6, 5.8). It is thus likely that the increased sensitivity to ABA is responsible to inhibit germination during imbibition in the cold while the increased GA content, and GA and jasmonate content contributes to the breakage of dormancy during imbibition at RT after the cold treatment. Since cold treatment induced the expression level of jasmonate-responsive *TaJAZ*s which down-regulate DELLA, it is possible that the increased jasmonate response contribute to an increase of embryo response to GA. In agreement with this, cold treatment enhanced expression of *TaGAMyb* (Fig. 5.8), a downstream transcription factor that is key to GA signaling in plants. The result of this study also showed that seed imbibition at RT following the cold treatment caused reduction in ABA level (Fig. 5.5) and ABA response (Fig. 5.6), but increased in GA and jasmonate levels (Figs. 5.3, 5.7). Although the expression levels of GA and jasmonate signaling genes were down-regulated during imbibition at RT following cold treatment (Figs. 5.4, 5.8), the breakage of dormancy by the same treatment implies increased responses to GA and JAs through posttranscriptional regulation of the expression of genes involved in their respective signaling pathways. In summary, this study implies the role of synergistic interaction between JAs and GA in decaying dormancy by cold treatment, and this synergistic interaction between the two hormones could create an ABA–GA imbalance in favor of GA, leading to the release of dormancy (Fig. 5.9).

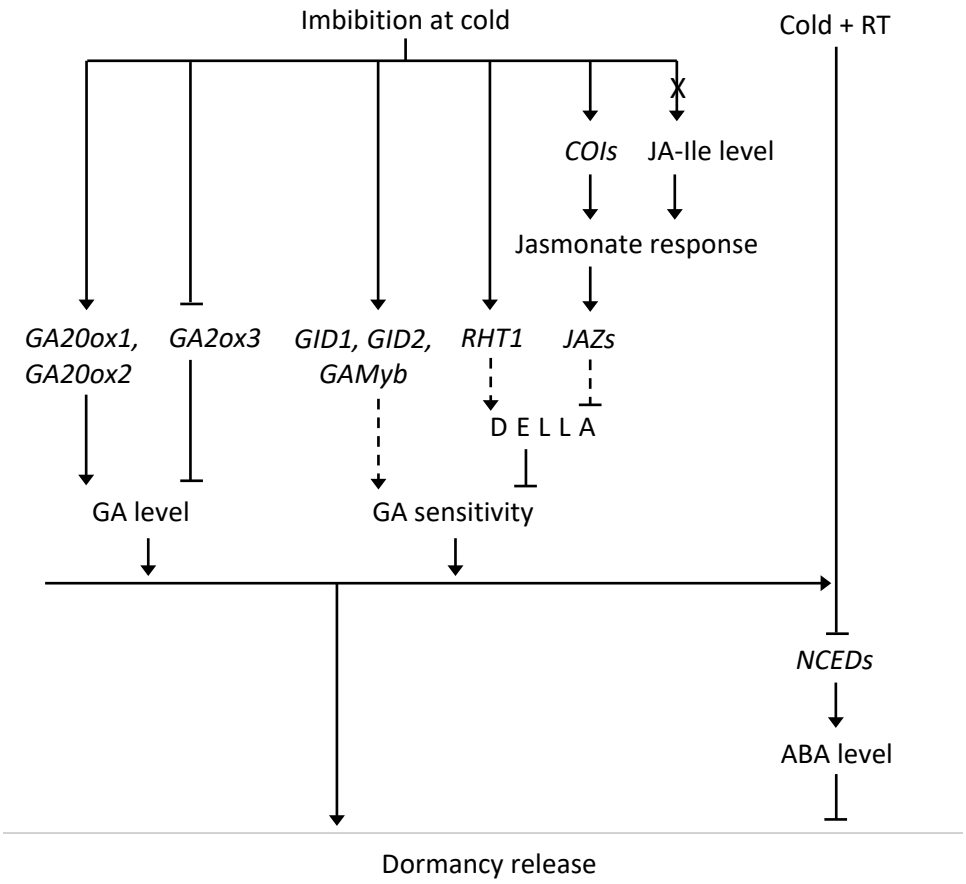

Figure 5.9. A schematic diagram of dormancy release by cold treatment Imbibition in cold up-regulates the expression of *JAZ*s, which in turn can enhance GA sensitivity. Increases in GA level and sensitivity during imbibition in cold along with decrease in ABA level due to repression of *NCED* genes during subsequent imbibition of cold treated seeds at room temperature lead to seed dormancy release. Dashed lines indicate expected effects; an arrow with "X" indicates absence of positive effect.

6 GENERAL DISCUSSION AND CONCLUSION

The balance between ABA and GA is a core factor in the regulation of seed dormancy and germination. While induction and maintenance of dormancy require an imbalance in favor of ABA, release of dormancy require an imbalance in favor of GA. Changes in level of ABA and/or GA as well as sensitivity of seeds to these hormones can create such imbalances. The balance between these two hormones can also be influenced by their synergistic and antagonistic interaction with other hormones such as JAs. Treatments that effectively break seed dormancy, including after-ripening and cold-stratification, can alter the ABA–GA balance via directly regulating ABA and/or GA content and/or sensitivity or indirectly through influencing the contents and sensitivity of other hormones. To gain insights into the interaction of JAs with ABA and GA in regulation of seed dormancy in wheat, this study has investigated the changes in the temporal expression patterns of genes involved in the metabolism and signaling of the three hormones and their level in the embryos of seeds during seed maturation in dormant and non-dormant cultivars (Chapter 3), and in response to dormancy decay by exogenous hormones, after-ripening (Chapter 4) or cold treatment (Chapter 5).

Our results of the third chapter showed that transition of seeds from 40 to 50 DAA during their maturation reduces dormancy level in seeds of less dormant genotype but not in seeds of highly dormant genotype. The decrease in embryo ABA level, which was associated with down-regulation of the ABA metabolic gene *TaNCED2* and up-regulation of the ABA catabolic genes *TaCYP707A*s, appears to be the key factor explaining the reduction in dormancy level in the seeds of less dormant genotype during their transition from 40 to 50 DAA (Fig. 6.1). In addition, increases in expression levels of genes encoding positive regulators of GA signaling, GID2 and GAMyb, observed in the embryos of less dormant genotype during transition from 40 to 50 DAA

suggest that the transition might also enhance sensitivity of the tissue to GA, participating in alteration of the ABA–GA balance.

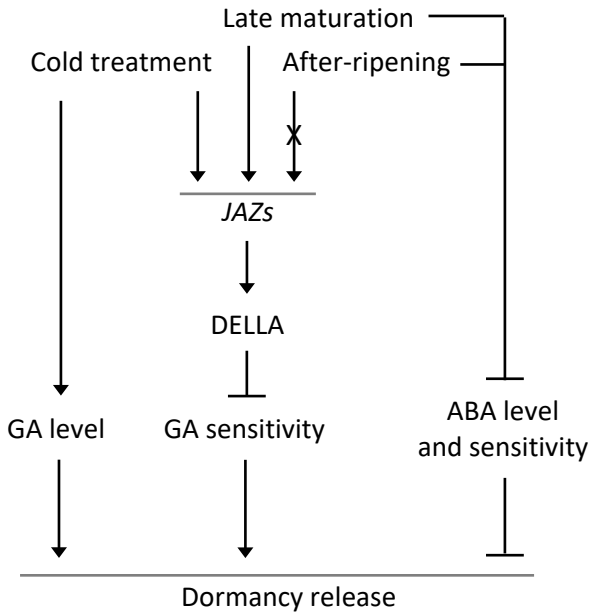

Figure 6.1. Involvement of jasmonates (JAs), and gibberellins (GA) and abscisic acid (ABA) in dormancy release. Reductions in ABA level and sensitivity are the main regulators of dormancy release during late phase of seed maturation and after-ripening. An increase in GA level is the main cause of cold treatment-induced dormancy released. Jasmonates are involved in dormancy release during the late phase of seed maturation and cold treatment, but JAs are not involved in the release of dormancy by after-ripening. Up-regulation of the jasmonate signaling genes *JAZs* during late phase of seed maturation or imbibition in the cold can lead to an increase in GA sensitivity. An arrow with "X" indicates absence of positive effect.

Consistent with results presented in chapter 3 of this thesis in which the differential ABA level accounts for differences in dormancy status between the two genotypes, results of chapter 4 showed that loss of dormancy in the wheat seeds by after-ripening appears to be associated with decreases in ABA level during imbibition (Fig. 6.1). Moreover, after-ripening of the dormant seeds

might decrease ABA sensitivity through down-regulation of genes encoding positive regulators of ABA signaling, namely SnRK2, ABI3 and ABI5. Results from the hormone treatment experiments indicated that exogenous JAs alone can create an ABA–GA imbalance in favor of GA and thereby induces dormancy decay in dormant wheat seeds. This jasmonate-induced ABA–GA imbalance was accompanied by increased expression levels of jasmonate synthesis genes including *TaAOS2*, *TaAOC1*, *TaOPR2* and *TaACX1*, and jasmonate signaling genes including *TaJAZ1, 4, 7, 10* and *TaMYC2* in the embryos, implying that these jasmonate-responsive genes function in embryos of wheat seeds. Particularly, *JAZs* are fast jasmonate-responsive genes and JAZs can down-regulate activity of DELLA, a negative regulator of GA signaling, suggesting that the response to exogenous JAs can enhance sensitivity of embryos to GA during breaking the dormancy. In addition, this suggestion is supported by the detection of higher level of dormancy release in seeds treated with combination of MeJA and GA_3 as compared to seeds treated with GA_3 alone being accompanied by higher induction in the expression level of *TaJAZ*s.

Previous studies demonstrated that elevated jasmonate level or response can cause a decrease in ABA level, and the findings of our study showed evidence for the involvement of enhanced sensitivity of embryos to GA (Fig. 6.1). These influences of JAs are in the same direction of creating an ABA–GA imbalance required for dormancy release. However, no considerable elevation of embryo JA-Ile content was observed during after-ripening treatment which effectively release seeds from dormancy, and the decrease in dormancy level found during the last stage of seed maturation (40-50 DAA) in the less dormant genotype was accompanied by a decrease in embryo JA-Ile (Fig. 6.1). These results suggest that although, like treatment with exogenous JAs, elevation of endogenous jasmonate level and/or sensitivity would be able to release dormancy, or at least would have positive effects on dormancy release, plants do not prefer this approach to

release dormancy during treatment with after-ripening and during seed maturation under normal condition.

In contrast to that observed in response to after-ripening in which ABA–GA imbalance was mainly caused by decreases in ABA level during imbibition, results from chapter 5 of this thesis revealed that imbibition of dormant seeds under cold temperature induced ABA–GA imbalance, which leads to dormancy decay, via increases in GA level whose effect might be amplified by cold-induced JA-mediated enhancement of GA signaling as discussed below (Fig. 6.1). Up-regulation of JA synthesis and signaling genes during imbibition of dormant seeds in the cold as compared to the corresponding seeds imbibed at RT indicates cold-induced increase in jasmonate response in wheat embryos. The up-regulation of jasmonate-responsive genes encoding JAZs in seeds imbibed under cold implies an increase in seed sensitivity to GA because JAZs can bind and negatively regulate DELLA proteins which functions as a negative regulator of GA signaling. This would serve as a potential explanation for the role of JAs in regulation of wheat seed dormancy via its effect on ABA–GA balance.

In conclusion, the finding in this study provides insights into our understanding of the molecular mechanisms underlying the role of JAs in the regulation of seed dormancy by affecting the ABA–GA balance, particularly the potential of JAZ–DELLA-mediated induction of JAs–GA synergism. However, there are number of gaps to be addressed in future studies. Firstly, the *JAZ* gene family is a big family, consisting of 13 and 15 family members in Arabidopsis and rice, respectively. It is therefore necessary to identify all *JAZ* genes in wheat, and study their expression dynamic with respect to induction and release of seed dormancy to identify the *JAZ* genes that are important in regulation of wheat seed dormancy. Secondly, JAZs are subject to JA-Ile-mediated degradation, and each JAZ, even each alternative spliced isoform of a JAZ, would have different

level of JA-Ile-mediated degradation because of differences in their abilities to interact with COI. Therefore, to precisely elucidate the regulatory role of specific wheat JAZs, it is crucial to study their stability through analyzing their ability and strength in forming association with COI. Finally, previous studies on Arabidopsis have shown that that not every JAZ can bind to DELLA, and those JAZs that bind to DELLA exhibit different binding strengths, implying that the level of JAZ-mediated repression of DELLA varies among JAZs. It is therefore important to study the binding affinity between a specific wheat JAZ and DELLA to gain insights into the role of a JAZ protein in mediating the JAs–GA synergism in breaking wheat seed dormancy. Furthermore, further studies are required to clarify the contradictory roles of JAs in regulation of seed dormancy and germination in different plant species.

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APPENDIX 1: ABBREVIATIONS

10,11-EHT	10,11(S)-epoxy hexadecatrienoic acid
11-HPHT	11(S)-hydroperoxy-hexadecatrienoic acid
12,13-EOT	12,13(S)-epoxy-octadecatrienoic acid
13-HPOT	13(S)-hydroperoxy-octadecatrienoic acid
ABA	Abscisic acid
ABI	ABSCISIC ACID INSENSITIVE
ACH	ACYL-THIOESTERASES
ACX/acx	ACYL CoA-OXIDASE
AIP	ABA-INTERACTING PROTEIN
AOC	ALLENE OXIDE CYCLASE
AOS/aos	ALLENE OXIDE SYNTHASE
ASA	Acetylsalicylic acid
ASK/ask	ARABIDOPSIS SPIKE1-LIKE
AXR/axr	AUXIN RESISTANT
bHLH	Basic helix-loop-helix
COI/coi	CORONATINE INSENSITIVE
CoR	Co-repressor
CTS/cts	COMATOSE
CUL1	CULLIN1
cv.	Cultivar
CYP707A	ABA 8'-HYDROXYLASE
CYP94B3	JA-Ile-12-HYDROXYLASE
CYP94C1	12-OH-JA-Ile CARBOXYLASE
DAA	Day(s) after anthesis
DAD/dad	DELAYED ANTHER DEHISCENCE
DAI	Day(s) after the start of imbibition
DGL/dgl	DONGLE
DMSO	Dimethylsunfoxide
dnOPDA	Dinor-12-oxophytodienoic acid
E2	Ubiquitin-conjugating enzyme
ERA	ENHANCED RESPONSE TO ABA
ET	Ethylene
FAO	Food and Agriculture Organization
FLU	Fluridone
GA	Gibberellin(s)
GA_1	Gibberellin A1
GA20ox	GA 20-OXIDASE
GA2ox	GA 2-OXIDASE
GA_3	Gibberellin A_3 (gibberellic acid)

GA3ox	GA 3-OXIDASE
GA_4	Gibberellin A_4
G-box	CACGTG (G-box motif)
GI	Germination index
GID	GIBBERELLIN INSENSITIVE DWARF
GL	GLABRA
HAC1	(Transcription factor name)
HAI	Hour(s) after the start of imbibition
IAR	IAA-ALA RESISTANT
ILL	IAA-LEU RESISTANCE1-like
IWGSC	International Wheat Genome Sequencing Consortium
JAs	Jasmonates
JA	Jasmonic acid
JA-CoA	Jasmonoyl-CoA
JA-Ile	Jasmonoyl-isoleucine
JAM	JASMONATE-ASSOCIATED MYC2-LIKE
JAR/jar	JASMONATE RESISTANT
JAT	JASMONATE TRANSPORTER
JAZ	JASMONATE ZIM DOMAIN
JMT	JASMONIC ACID CARBOXYL METHYL TRANSFERASE
JOX/JAO	JASMONATE-INDUCED OXYGENASE
JR	JASMONIC ACID RESPONSIVE
KAT	L-3-KETOACYL-COA-THIOLASE
KEG	KEEP ON GOING
LACS/lacs	LONG-CHAIN ACYL-COA SYNTHETASE
LOX	13-LIPOXYGENASE
MED25	MEDIATOR25 (a subunit of MEDIATOR complex)
MEDs	(Mediator complex subunits other than MED25)
MeJA	Methyl jasmonate
MFP	MULTIFUNCTIONAL PROTEIN
MJE	MeJA ESTERASE
MYB	(Transcription factor name)
MYC	(Transcription factor name)
NCED	9-CIS-EPOXYCAROTENOID DIOXYGENASE
NINJA	NOVEL INTERACTOR OF JAZ
NO	Nitric oxide
OE	Over expressing
OPC4	3-oxo-2-(20(Z)-pentenyl)-cyclopentane-1-tetranoic
OPC6	3-oxo-2-(20(Z)-pentenyl)-cyclopentane-1-hexanoic
OPC8	3-oxo-2-(20(Z)-pentenyl)-cyclopentane-1-octanoic
OPCL/opcl	OPC-8:CoA LIGASE
OPDA	12-oxophytodienoic acid
OPDA-Ile	Isoleucine-conjugate of 12-oxophytodienoic acid

OPR/opr	OPDA ACID REDUCTASE
PAC	Paclobutrazol
phyB	Phytochrome B
PIF	PHYTOCHROME-INTERACTING FACTOR
PIL/pil	PHYTOCHROME INTERACTING FACTOR 3-LIKE
PLA_1	PHOSPHOLIPASE A_1
Pol II	RNA POLYMERASE II
PP2C	PROTEIN PHOSPHATASE
PXA/pxa	PEROXISOMAL ADENOSINE TRIPHOSPHATE-BINDING CASSETTE-TRANSPORTER
PYL	PYRABACTIN RESISTANCE-LIKE
RBX1	RING-H2 PROTEIN1
RGL	REPRESSOR OF GIBBERELLIC ACID -LIKE
RPT5/rpt5	Proteasome RPT5 subunit
RHT	REDUCED HIGHT
RT	Room temperature
SCF	SKP1–CUL1–F-BOX PROTEIN (complex)
SCF^{COI1}	(SCF E3 ubiquitin ligase complex containing SKP1, CUL1, F-box protein COI1, RBX1, and E2 loaded with ubiquitin)
Sdr	SEED DORMANCY
SnRK2	SNF1-RELATED PROTEIN KINASE2
SPK1	S-PHASE KINASE-ASSOCIATED PROTEIN1
SPL	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
spr8	SUPPRESSOR OF PROSYSTEMIN-MEDIATED RESPONSES8
ST2A	12-OH-JA SULFOTRANSFERASE
tnOPDA	Tetranor-12-oxophytodienoic acid
TPL	TOPLESS
Ub	Ubiquitin
VP	VIVIPAROUS
VSP	VEGETATIVE STORAGE PROTEIN
WD-Repeat	TRYPTOPHAN-ASPARTIC ACID (WD)-REPEAT PROTEIN

APPENDIX 2: SUPPLEMENTARY TABLES

Table 1S. Accession numbers and qPCR primers of jasmonates metabolic genes

Name	IWGSC accession # (Version 1.1)	Forward primer	Reverse primer	PCR efficiency (%)	References
LOX6	TraesCS4A02G009400.1	TGGTCAGATATCTTTCGAACCCC	GCCGTATTTACCGTCTCAAACAC	109.4	Geer et al. (2010) (XP_015630875.1)
	TraesCS4B02G295200.1				
	TraesCS4D02G294100.1				
AOS1	TraesCS4A02G061800.1	GTTCGGCCTCCCTTTGATCC	ACTTGTAGAGCGCCTTGTAGTC	102.8	Xu et al. (2016)
	TraesCS4B02G237500.1				
	TraesCS4D02G238700.1				
AOS2-A	TraesCS4A02G061900.1	CATGTAGGTGCCGGTGAAGAG	GAGTCCCGCGTCGAGAG	100.2	Xu et al. (2016)
AOS2-B	TraesCS4B02G237600.1	GCTGGAATATGAGCCACTTTGC	CGTGGAGTCGCAGATCGTG	94.5	Xu et al. (2016)
AOS2-D	TraesCS4D02G238800.1	TGGCCCTGGAAGTAGTAGAAGTC	CGCGAGATCCAGAGTTCCAAAC	90.1	Xu et al. (2016)
AOC1	TraesCS6A02G334800.1	AGCCTGGACAAGCGGATC	TAGTCGCCGAAGTAGATGCTG	103.1	Xu et al. (2016)
	TraesCS6B02G365200.1				
	TraesCS6D02G314300.1				
OPR2	TraesCS7A02G412400.1	CACCAACCGTTGCAAGTTTC	GACACCCTCACCGCAGTTC	102.5	Wang et al. (2016)
	TraesCS7B02G311600.1				
	TraesCS7D02G405500.1				
ACX1	TraesCS4A02G494700.1	CGGATAGCACTATACCTTACAGC	CCAAGTTACAAGGGAGGGAAAG	93.9	Geer et al. (2010) (XP_015643031.1)
	TraesCS7A02G003900.1				
	TraesCS7D02G004400.1				
KAT2	TraesCS6A02G392400.1	CAATGCTCATCGGGGCTACA	GGGTTTACTTGGCCTTCCCA	102.9	Geer et al. (2010) (XP_015625435.1)
	TraesCS6A02G392400.2				
	TraesCS6B02G432600.1				
	TraesCS6D02G493900LC.1				
JAR1	TraesCS1A02G425100.1	TGGCCCTGACTTCAACCAATC	GTGAGCAAACGTCGAGAACAC	102.1	Wakuta et al. (2011)
	TraesCS1A02G425100.2				
	TraesCS1B02G459500.1				
	TraesCS1B02G459500.2				
	TraesCS1D02G434100.1				
	TraesCS1D02G434100.2				
JAR2-A	TraesCS3A02G145300.1	GGAGGTCCGGTATATCTGCATG	GTCACCTCCATCTCCCTAAGC	99.3	Wakuta et al. (2011)
JAR2-B	TraesCS3B02G172400.1	TACGTACAGAGTTGTCCGCATG	TTTGCAATATCACGCACCGC	93.6	Wakuta et al. (2011)

Table 2S. Accession numbers and qPCR primers of jasmonates signaling genes

Name	IWGSC accession # (Version 1.1)	Forward primer	Reverse primer	PCR efficiency (%)	References
COI1	TraesCS1A02G279100.1 TraesCS1B02G288100.1 TraesCS1D02G278400.1	GCTCCACTCTGAGGTAAGTCAAG	TCCATGACCTTGCTGCCAAC	103.5	Liu et al. (2016)
COI2	TraesCS3A02G367500.1 TraesCS3B02G399200.1 TraesCS3B02G399200.2 TraesCS3D02G360400.1 TraesCS3D02G360400.2 TraesCS3D02G360400.3	AGAGCAAGGAGGAGTTTCTCAG	ACCTGCTTTTGTCTGTCGAG	103.0	Geer et al. (2010) (ADK66974.1)
COI3	TraesCS4A02G091200.1 TraesCS4B02G213500.1 TraesCS4D02G214200.1	TGATGGACTGCTGGCATTTG	TGCACGTTCACTAAAGCAGC	104.3	Lee et al. (2013)
JAZ1	TraesCS2A02G506500.1 TraesCS2B02G534800.1 TraesCS2D02G507200.1	AGACATCGCTGGAGGCTTAAC	TGTGTGTATGACTCAGTGCCAC	98.3	Toda et al. (2013)
JAZ3	TraesCS5A02G533000.1 TraesCS5A02G533100.1 TraesCS4B02G364700.1 TraesCS4B02G575500LC.1 TraesCSU02G139000.1 TraesCSU02G139100.1	TGCCAACATAGCGTCATTGC	ATACCAGTTGTCAAAGAGCCCG	98.2	Toda et al. (2013)
JAZ4	TraesCS5A02G204900.1 TraesCS5B02G203400.1 TraesCS5B02G203400.2 TraesCS5D02G211200.1 TraesCS5D02G211200.2	TGAAGATTTTGGCACTGCGG	ATACCACAGCGTGTTTCACCTC	95.2	Toda et al. (2013)
JAZ7	TraesCS2A02G169300.1 TraesCS2B02G195600.1 TraesCS2D02G176800.1	ACGCCTTTCTAGCAATTGGC	AACGTCTCTGCGACCACAAC	95.7	Toda et al. (2013)
JAZ10	TraesCS4A02G007900.1 TraesCS4B02G297000.1 TraesCS4B02G297000.2 TraesCS4D02G295900.1	CTGACGCAGCCAATTAACGG	TCACTGCTTCTCCACGTAGTAC	100.6	Toda et al. (2013)
MYC2	TraesCS1A02G193200.1 TraesCS1B02G208000.1 TraesCS1D02G196900.1	TCTCAGATTTCGCGTCCAAC	GCGCCAAAGTTTAGGATCTCAC	105.6	Cai et al. (2014)
bHLH13	TraesCS3A02G158600.1 TraesCS3B02G185400.1 TraesCS3D02G166300.1 TraesCS3D02G166300.2	GAACAGAGGACTGCTGGTGG	GGGCCTGACTCCAAGTGAAA	102.8	Geer et al. (2010) (XP_015621308.1)
bHLH13-like	TraesCS4A02G028900.1 TraesCS4B02G276900.1 TraesCS4D02G275500.1	CCTCCCGTTTGCTGGTTTTC	CTCAGTCACAGACACCGAGG	108.3	Geer et al. (2010) (XP_015621308.1)
MED25	TraesCS5A02G179400.1 TraesCS5A02G179400.2 TraesCS5A02G179400.3 TraesCS5B02G177300.1 TraesCS5B02G177300.2 TraesCS5B02G177300.3 TraesCS5D02G184100.1 TraesCS5D02G184100.2 TraesCS5D02G184100.3	CCAGCAACCATCGAAGTATGTC	ACGCTGTTCCACTCCTATAACC	97.6	Liu et al. (2016)

Table 3S. Accession numbers and qPCR primers of abscisic acid metabolic and signaling genes

Name	IWGSC accession # (Version 1.1)	Forward primer	Reverse primer	PCR efficiency (%)	References
NCED1	TraesCS5B01G029300.1	AGCAGAGGACGAGCAGAAATTTG	AGTAACCGCCGCTAACTGTATC	101.7	Izydorczyk et al. (2017)
	TraesCS5D01G038800.1				
NCED2	TraesCS5A01G374000.1	CGCCTTGCTCTGTTCTACG	AGGTGGCCGTTGAAGTAGAC	101.5	Izydorczyk et al. (2017)
	TraesCS5B01G375900.1				
	TraesCS5D01G383500.1				
CYP707A1	TraesCS6A01G271300.1	AGACTTACGCTCTGAATGTGGC	CTTCAGCTCCTCGATGTACTGC	107.0	Izydorczyk et al. (2017)
	TraesCS6B01G298500.1				
	TraesCS6D01G243800.1				
CYP707A2	TraesCS5A01G238000.1	AAGGGTCGAACTTCTGTGGATC	GACGTGGAGTACAAAGGGTTTC	102.4	Izydorczyk et al. (2017)
	TraesCS5B01G236500.1				
	TraesCS5D01G244900.1				
PYL5	TraesCS2A02G089400.1	TACTCCCAAACCAAGCCAAACG	GCTAGCTGTTTGTTTGGTGGG	96.8	Izydorczyk et al. (2017)
	TraesCS2B02G105300.1				
	TraesCS2D02G087500.1				
PP2C	TraesCS3A02G209200.1	GGGGGTTTACATCAATTTGCGG	ACCATATTGTTGCCCGTGAG	93.9	Izydorczyk et al. (2017)
	TraesCS3A02G209200.2				
	TraesCS3B02G240000.1				
	TraesCS3D02G212100.1				
SnRK2	TraesCS2A02G493800.1	TTCCGGACTACGTTCGAGTC	ATGGATGGTTCTTGATCTCCGG	102.1	Izydorczyk et al. (2017)
	TraesCS2B02G521800.1				
	TraesCS2D02G493700.1				
ABI3	TraesCS3A02G417300.1	ACCGATTTTGGCCCAACAAC	GCCGCGTATCAGATATTTGCC	95.4	Izydorczyk et al. (2017)
	TraesCS3A02G417300.2				
	TraesCS3B02G452200.1				
	TraesCS3B02G452200.2				
	TraesCS3D02G412800.1				
	TraesCS3D02G412800.2				
ABI5	TraesCS3A02G371800.1	AGGAGGAAGCAACCTAGCCC	CAGGAGGCTGCTGTGAGG	96.2	Izydorczyk et al. (2017)
	TraesCS3B02G404200.1				
	TraesCS3B02G404300.1				
	TraesCS3B02G404400.1				
	TraesCS3B02G404500.1				
	TraesCS3B02G404600.1				
	TraesCS3D02G364900.1				

Table 4S. Accession numbers and qPCR primers of gibberellins metabolic and signaling genes

Name	IWGSC accession # (Version 1.1)	Forward primer	Reverse primer	PCR efficiency (%)	References
GA20ox1	TraesCS4A01G319100.1	CGAAGAAGCGCCGGTAGTG	CTGTCGCTGGAGATCATGGAG	100.8	Izydorczyk et al. (2017)
	TraesCS5B01G560300.1				
	TraesCS5D01G566200.1				
GA20ox2	TraesCS3A01G406200.1	CCCTGGAAGGAGACCCTCT*	GGGTGCTGGTGAAGTAGTCC*	93.5	Pearce et al. (2015)
	TraesCS3B01G439900.1				
	TraesCS3D01G401400.1				
GA3ox2	TraesCS3A01G122600.1	AGGTCGCCGCCGTCGAGTCC	CAGTTGAGGTGCATTGTGGC	103.8	Izydorczyk et al. (2017)
	TraesCS3B01G141800.1				
	TraesCS3D01G124500.1				
GA2ox3	TraesCS3A02G294000.1	GACACAGAGGATTGCACCATTG	TTGTACTCGCCCCATGTGAAG	97.4	Izydorczyk et al. (2017)
	TraesCS3B02G328700.1				
	TraesCS3D02G293800.1				
GA2ox6	TraesCS2A02G379000.1				
	TraesCS2B02G396000.1	TACTTCCTGTGCCCGTCGTAC	TGACATCGTCCTGCACCTTC	101.8	Izydorczyk et al. (2017)
	TraesCS2D02G375300.1				
GID1	TraesCS1A02G255100.1	CACACATGGGTGCTCATCTC*	GATGAAGTGGTCGAAGGAGG*	93.3	Pearce et al. (2015)
	TraesCS1B02G265900.1				
	TraesCS1D02G254500.1				
GID2	TraesCS3A02G056000.1	AAGGCCTGCAGTGTGGTC	ACAGCAAGGGCCATATTACAC	98.7	Pearce et al. (2015)
	TraesCS3B02G068800.1				
	TraesCS3D02G056100.1				
RHT1	TraesCS4A02G271000.1	GAGATGGCCATGGGGATGG	CGCGTTGAGCTCCGACAG	93.2	Izydorczyk et al. (2017)
	TraesCS4B02G043100.1				
	TraesCS4D02G040400.1				
GAMyb	TraesCS3A02G336500.1	AGCTGGCTCAAGTATACCGTG	TCTTCCAAGAGACCGCTGTTC	98.6	Haseneyer et al. (2008)
	TraesCS3B02G367500.1				
	TraesCS3B02G367500.2				
	TraesCS3D02G329400.1				
	TraesCS3D02G329400.2				
	TraesCS3D02G329400.3				

* denotes that primer sequence is from the reference paper

APPENDIX 3: SUPPLEMENTARY FIGURES

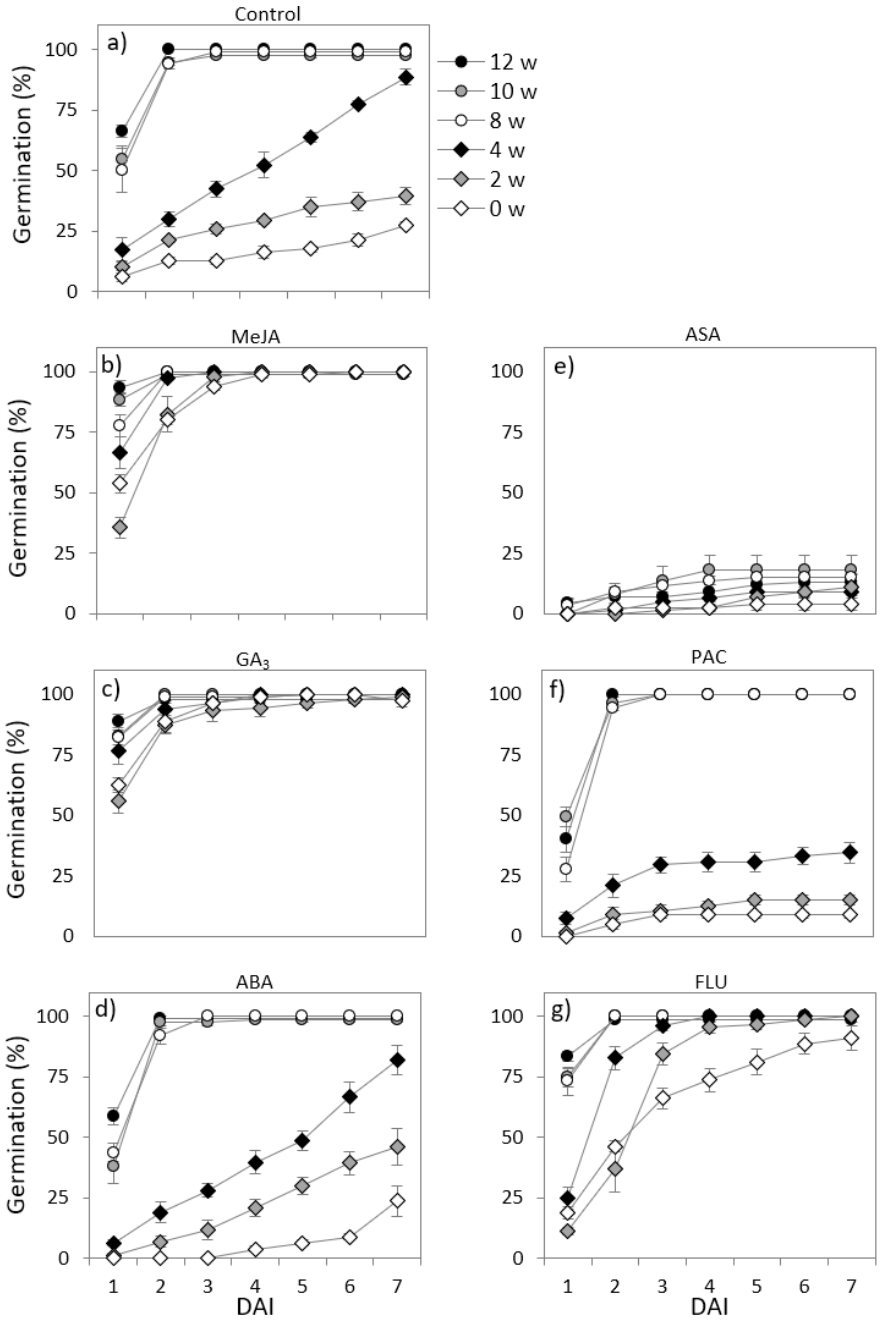

Figure 1S. Germination of dormant seeds in response to after-ripening and hormone treatment. The dormant seeds were after-ripened for different periods of time and then imbibed without (control; a) or with methyl jasmonate (MeJA; b), gibberellic acid (GA_3; c), abscisic acid (ABA; d), acetylsalicylic acid (ASA; e), paclobutrazol (PAC; f), fluridone (FLU; g). Data were means and SE of four replicates; DAI, day(s) after the start of imbibition.

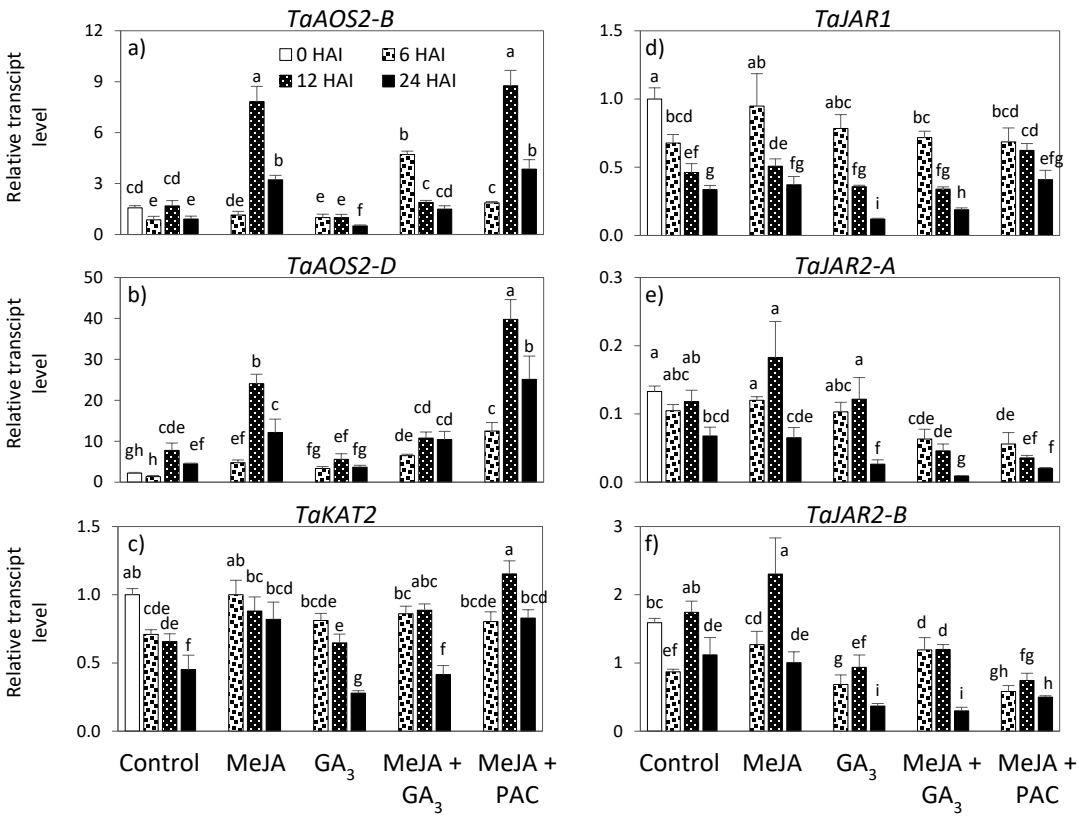

Figure 2S. Expression of jasmonates biosynthetic genes in response to hormone treatment. Relative transcript levels of *TaAOS2-B* (a), *TaAOS2-D* (b)*, TaKAT2* (c), *TaJAR1* (d), *TaJAR2-A* (e) and *TaJAR2-B* (f) in embryos of dormant seeds after different hours of imbibition (HAI). Seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaAOS2*s, *TaKAT2* and *TaJAR*s were expressed relative to the transcript levels of *TaAOS2-A* (Fig. 4.3a), *TaKAT2* and *TaJAR1* in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

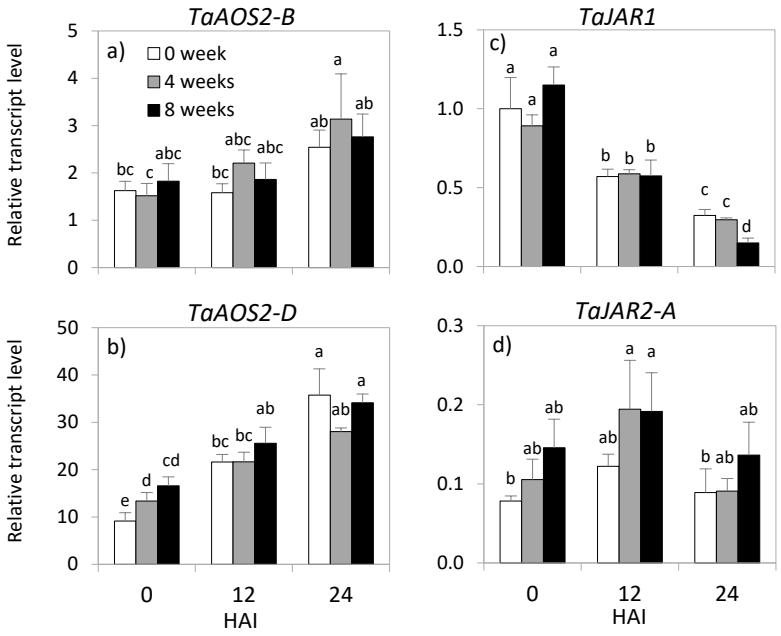

Figure 3S. Expression of jasmonates biosynthetic genes in response to after-ripening. Relative transcript levels of *TaAOS2-B* (a), *TaAOS2 D* (b), *TaJAR1* (c) and *TaJAR2-A* (d) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior to imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaAOS2*s and *TaJAR*s were expressed relative to the transcript levels of *TaAOS2-A* (Fig. 4.4a) and *TaJAR1* in 0 HAI samples of seeds after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

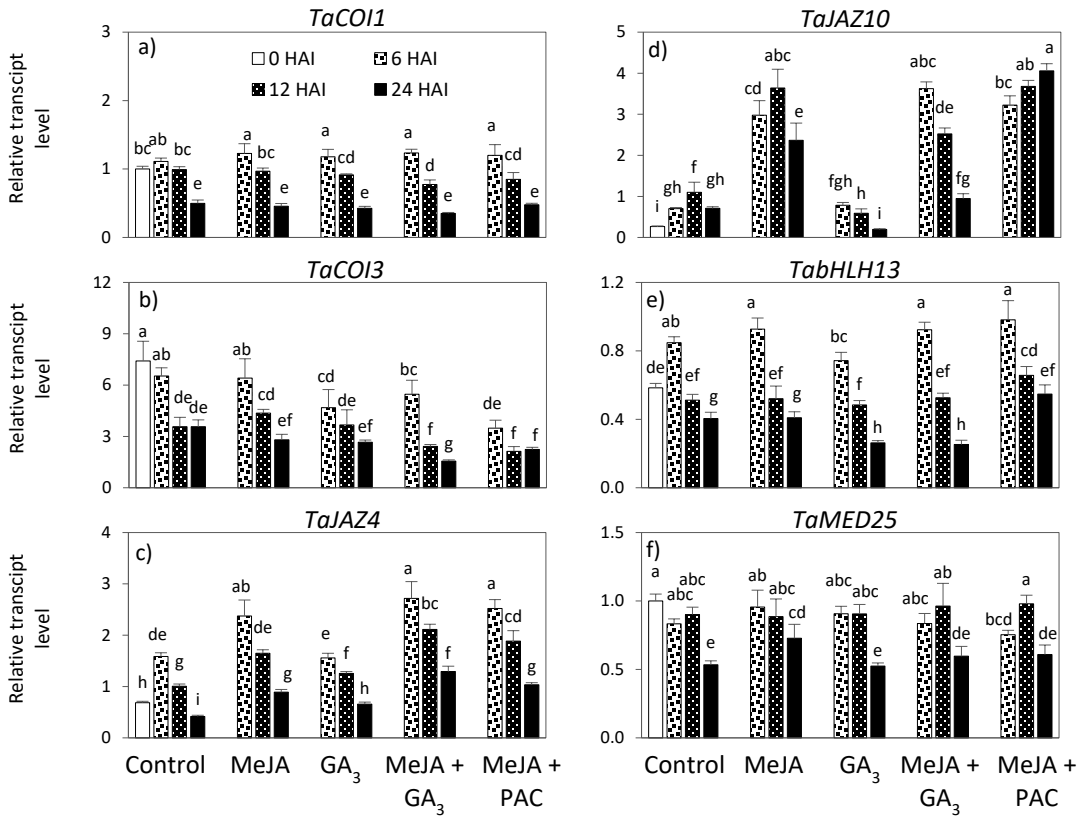

Figure 4S. Expression of jasmonates signaling genes in response to hormone treatment. Relative transcript levels of *TaCOI1* (a), *TaCOI3* (b)*, TaJAZ4* (c), *TaJAZ10* (d), *TabHLH13* (e) and *TaMED25* (f) in embryos of dormant seeds after different hours of imbibition (HAI). Seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOI*s, *TaJAZ*s*, TabHLH13* and T*aMED25* were expressed relative to the transcript levels of *TaCOI1*, *TaJAZ1* (Fig. 4.5c), *TaMYC2* (Fig. 4.5e) and *TaMED25* in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at *P<0.05* (LSD test).

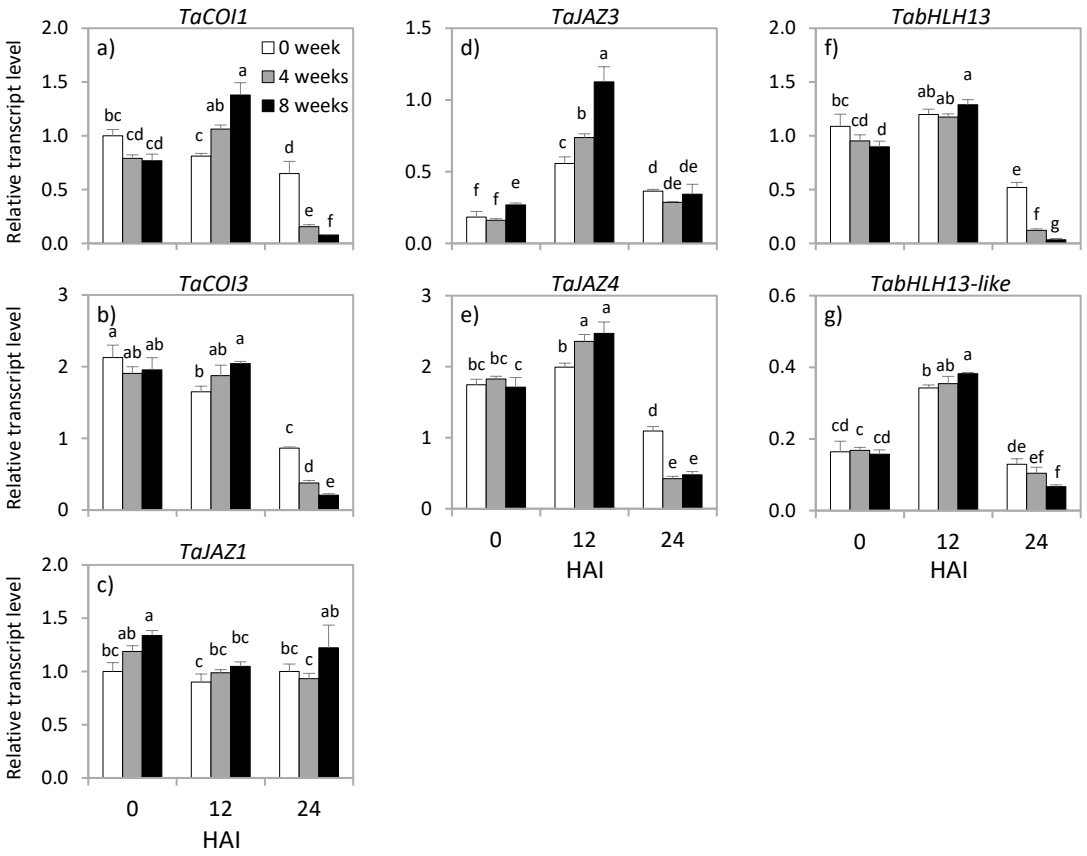

Figure 5S. Expression of jasmonates signaling genes in response to after-ripening. Relative transcript levels of *TaCOI1* (a), *TaCOI3* (b), *TaJAZ1* (c), *TaJAZ3* (d), *TaJAZ4* (e), *TabHLH13* (f) and *TabHLH13-like* (g) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior to imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOIs*, *TaJAZ*s and *TabHLH13* (*TabHLH13* and *TabHLH13-like*) were expressed relative to the transcript levels of *TaCOI1*, *TaJAZ1* and *TaMYC2* (Fig. 4.5e) in 0 HAI samples of seeds after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

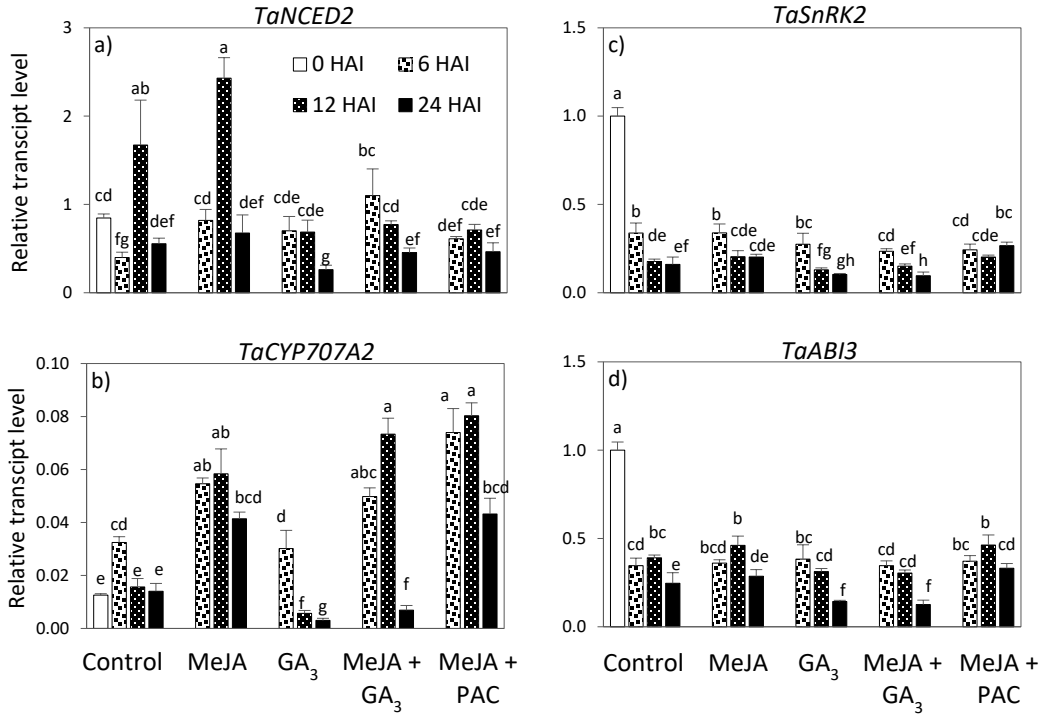

Figure 6S. Expression of abscisic acid metabolic and signaling genes in response to hormone treatment. Relative transcript levels of *TaNCED2* (a), *TaCYP707A2* (b)*, TaSnRK2* (c) and *TaABI3* (d) in embryos of dormant seeds after different hours of imbibition (HAI). Seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaNCED2*, *TaCYP707A2, TaSnRK2* and *TaABI3* were expressed relative to the transcript levels of *TaNCED1* (Fig. 4.8c), *TaCYP707A1* (Fig. 4.8d)*, TaSnRK2* and *TaABI3* in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

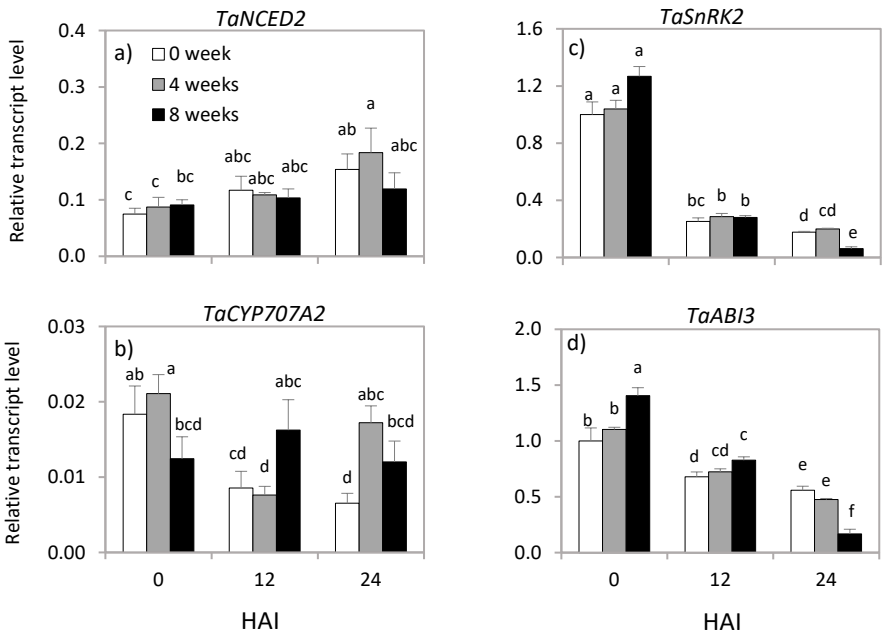

Figure 7S. Expression of abscisic acid metabolic and signaling genes in response to after-ripening. Relative transcript levels of *TaNCED2* (a), *TaCYP707A2* (b)*, TaSnRK2* (c) and *TaABI3* (d) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior to imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaNCED2*, *TaCYP707A2, TaSnRK2* and *TaABI3* were expressed relative to the transcript levels of *TaNCED1* (Fig. 4.9a), *TaCYP707A1* (Fig. 4.9b)*, TaSnRK2* and *TaABI3* in 0 HAI samples of seeds after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

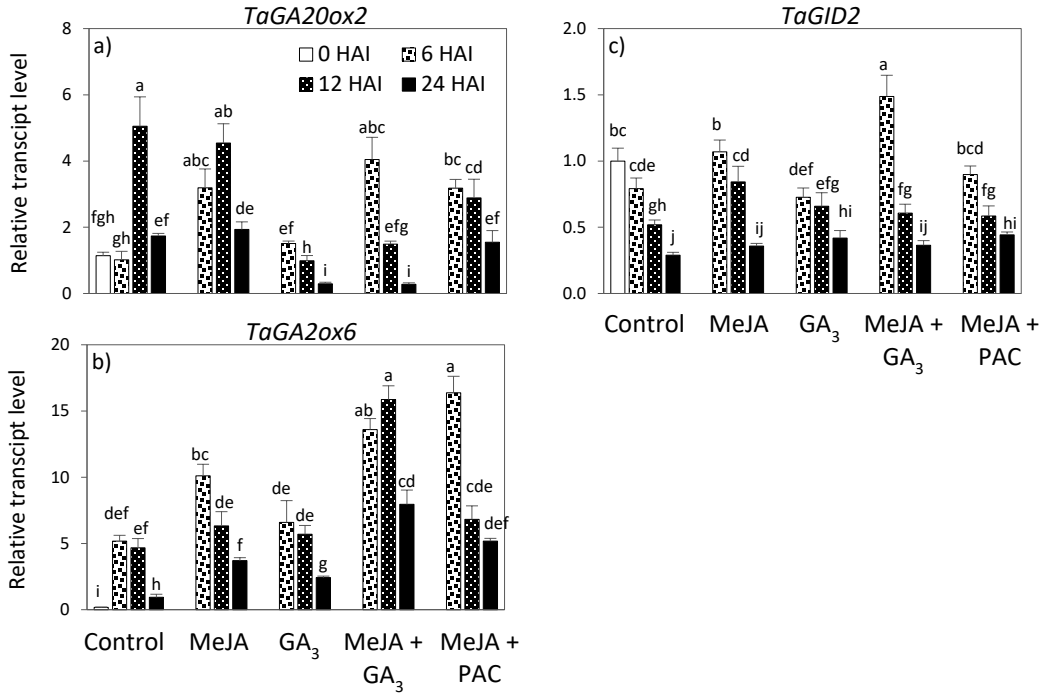

Figure 8S. Expression of gibberellins metabolic and signaling genes in response to hormone treatment. Relative transcript levels of *TaGA20ox2* (a), *TaGA2ox6* (b), and *TaGID2* (c) in embryos of dormant seeds after different hours of imbibition (HAI). Seeds were imbibed without or with methyl jasmonate (MeJA), gibberellic acid (GA_3) and/or paclobutrazol (PAC). Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaGA20ox2*, *TaGA2ox6,* and *TaGID2* were expressed relative to the transcript levels of *TaGA20ox1* (Fig. 4.10b), *TaGA2ox3* (Fig. 4.10d), and *TaGID2* in 0 HAI samples of the control, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of three biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

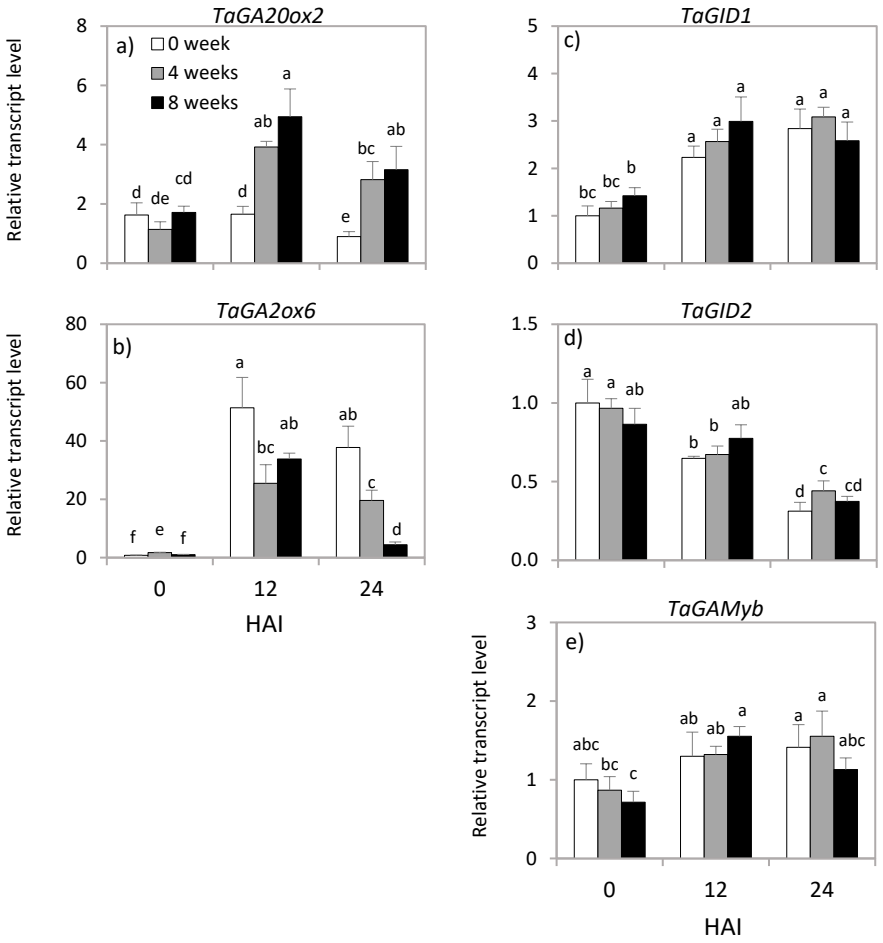

Figure 9S. Expression of gibberellins metabolic and signaling genes in response to after-ripening. Relative transcript levels of *TaGA20ox2* (a), *TaGA2ox6* (b)*, TaGID1* (c), *TaGID2* (d) and *TaGAMyb* (e) in embryos of dormant seeds collected at different hours after imbibition (HAI). Seeds were after-ripened for 0, 4 or 8 weeks prior to imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaGA20ox2*, *TaGA2ox6, TaGID1*, *TaGID2* and *TaGAMyb* were expressed relative to the transcript levels of *TaGA20ox1* (Fig. 4.11a), *TaGA2ox3* (Fig. 4.11c)*, TaGID1*, *TaGID2* and *TaGAMyb* in 0 HAI samples of seeds after-ripened for 0 week, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show statistically significant difference at $P<0.05$ (LSD test).

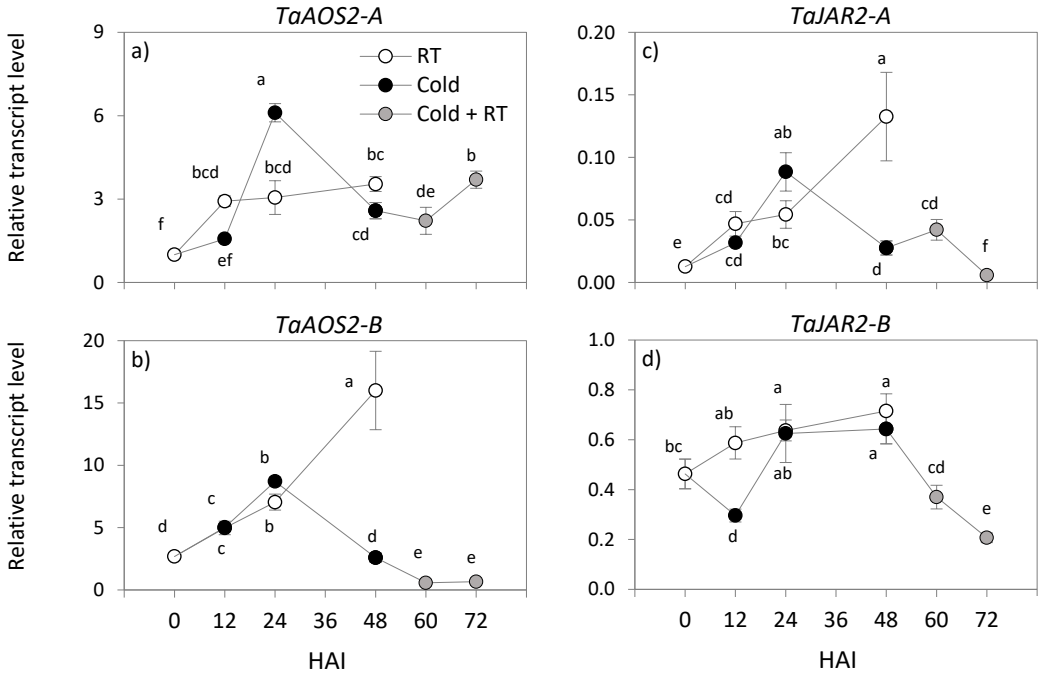

Figure 10S. Expression of jasmonates biosynthesis genes in response to cold treatment.Relative transcript levels of *TaAOS2-A* (a), *TaAOS2-B* (b), *TaJAR2-A* (c) and *TaJAR2-B* (d) in embryos of control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaAOS2*s and *TaJAR2*s were expressed relative to the transcript levels of *TaAOS2-A* and *TaJAR1* (Fig. 5.2g) in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

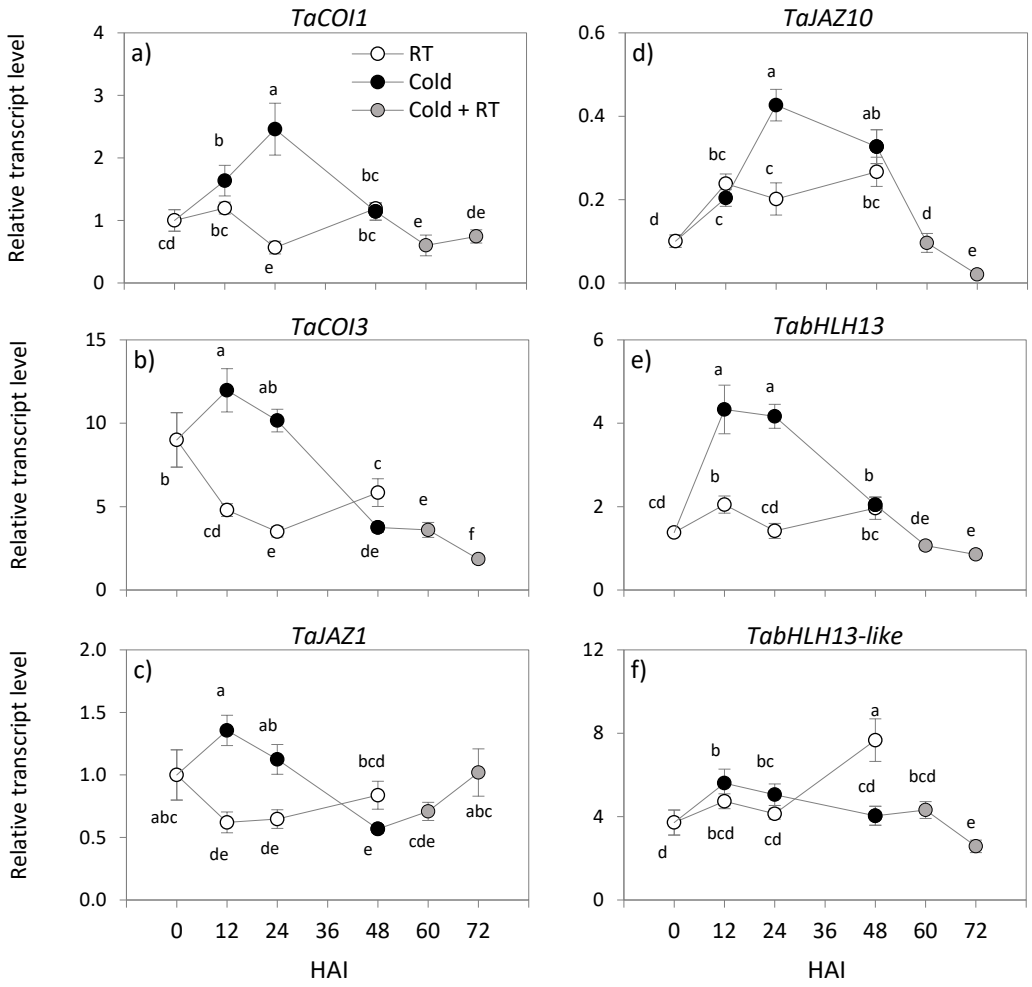

Figure 11S. Expression of jasmonates signaling genes in response to cold treatment. Relative transcript levels of *TaCOI1* (a) *TaCOI3* (b), *TaJAZ1* (c), *TaJAZ10* (d), *TabHLH13* (e), and *TabHLH13-like* (f) in embryos of control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaCOI*s, *TaJAZ*s, *TabHLH13*s (*TabHLH13* and *TabHLH13-like*) were expressed relative to the transcript levels of *TaCOI1*, *TaJAZ1* and *TaMYC2* (Fig. 5.4e) in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

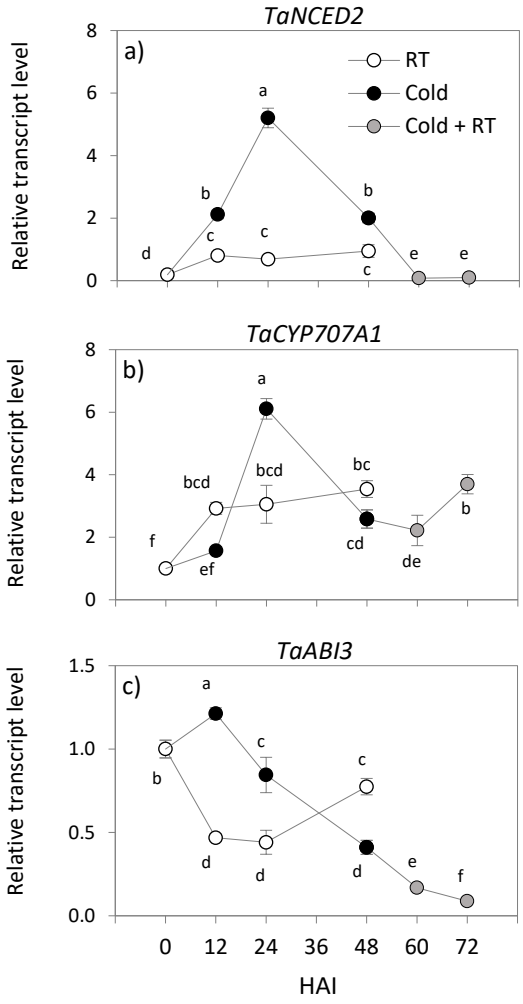

Figure 12S. Expression of abscisic acid metabolic and signaling genes in response to cold treatment. Relative transcript levels of *TaNCED2* (a), *TaCYP707A1* (b) and *TaABI3* (c) in embryos of control (RT) and cold-treated seeds (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaNCED2*, *TaCYP707A1* and *TaABI3* were expressed relative to the transcript levels of *TaNCED1* (Fig 5.5b), *TaCYP707A1* and *TaABI3* in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.

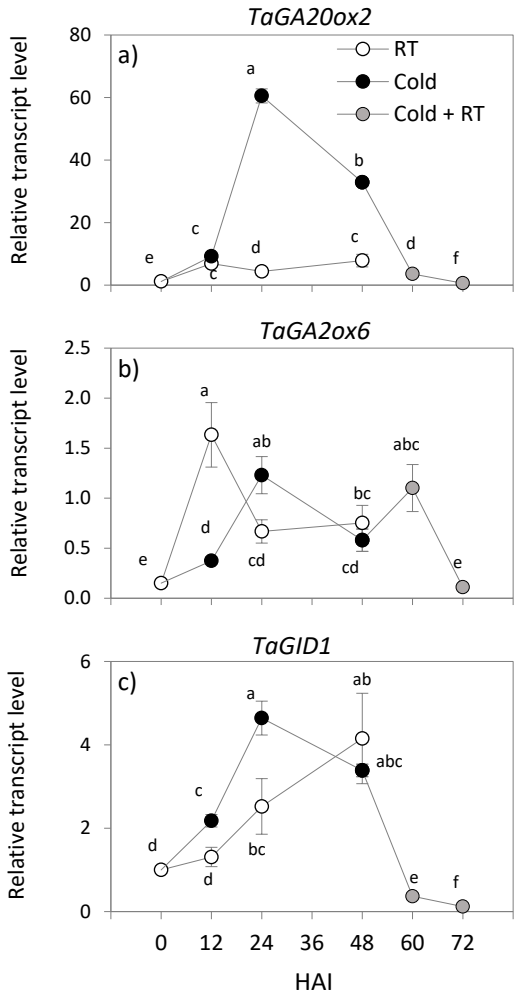

Figure 13S. Expression of gibberellins metabolic and signaling genes in response to cold treatment. Relative transcript levels of *TaGA20ox2* (a), *TaGA2ox6* (b) and *TaGID1* (c) in embryos of the control (RT) and cold-treated (Cold and Cold + RT) seeds at different time points of imbibition. Gene transcript levels were determined using *Taβ-actin* as reference gene, and the transcript levels of *TaGA20ox2*, *TaGA2ox6* and *TaGID1* were expressed relative to the transcript levels of *TaGA20ox1* (Fig. 5.7b), *TaGA2ox3* (Fig.5.7d) and *TaGID1* in 0 HAI samples, respectively, which were arbitrarily set a value of 1. Data are mean ± SE of four biological replicates. Different letters show significant difference at $P<0.05$ (LSD test). HAI, hour(s) after the start of imbibition; RT, room temperature.