STARCH DEGRADATION DURING AND AFTER GERMINATION OF WHEAT SEEDS AND ITS REGULATION BY ETHYLENE

By

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ABSTRACT

Sun, Menghan. M.Sc. The University of Manitoba, Sept, 2017. Starch Degradation During and After Germination of Wheat Seeds and Its Regulation by Ethylene. M.Sc. Supervisor: Dr. Belay T Ayele.

Germination is a crucial process for propagation of most crop plants, and it is regulated by many environmental, and endogenous factors such as plant hormones. However, the germination of seeds before harvest, defined as preharvest sprouting, causes yield and quality losses. Ethylene is one of the plant hormones that act as a positive regulator of seed germination through its interaction with other hormones. Starch degradation is one of the metabolic processes that provides energy for embryo and seedling growth during and after germination. It is also one of the main factors that cause yield and quality losses in grains sprouted due to wet conditions that occur before harvest. To gain insights into the role of ethylene in regulating starch degradation during germination or sprouting, this thesis identified starch degrading genes of wheat and characterized their expression patterns in the endosperm during and after seed germination, and in response to inhibition of ethylene synthesis. The study also examined the activity of starch degradation enzymes, and the levels of starch and products of its degradation, that is, soluble sugars. Furthermore, expression patterns of starch degrading genes were compared between dormant and non-dormant wheat seeds. The results of the study overall suggest that specific gene family members of starch degrading genes play roles in mediating the effect of ethylene on starch degradation during and after wheat seed germination. The findings also indicate that the expression of starch degrading genes is influenced by the level of dormancy.

FOREWORD

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

1.0 GENERAL INTRODUCTION

Wheat is one of the oldest crops in the world and is cultivated in over 520 million acres globally. It serves as a staple food for humans, providing about 30% of the total dietary calories. However, yield and quality of the wheat crop are affected by a number of biotic and abiotic stress factors. One of the major abiotic factors affecting wheat production around the world is pre-harvest sprouting (PHS), which is defined as the germination of mature seed while on the mother plant due to wet and humid field conditions, and it causes significant reduction in yield, end use potential, and seed quality (Olaerts *et al.,* 2016).

Seed dormancy, which is defined as the inability of intact viable seeds to complete germination under favorable conditions (Gao and Ayele, 2014), is closely related with PHS. Seeds with low level of dormancy can easily germinate or sprout under field conditions, while strong dormancy often affects uniformity of germination. Seed dormancy and germination are regulated by extrinsic and intrinsic factors. The extrinsic/environmental factors include moisture, oxygen, light and temperature, while the intrinsic factors mainly refer to phytohormones such as gibberellin (GA), abscisic acid (ABA), ethylene, auxin and brassinosteroids (BR). It is well known that GA and ABA are the two major hormones that influence seed germination and dormancy. ABA is the primary hormone which maintains seed dormancy, whereas GA promotes seed germination by playing an antagonistic role with ABA (Koornneef *et al.*, 2002; Gubler *et al.*, 2005). The role of ethylene in enhancing seed germination is related with its interaction with GA and ABA. The synthesis of ethylene and the transduction of its signaling are regulated by ABA (Beaudoin *et al.*, 2000; Linkies *et al.*, 2009; Linkies and Leubner-Metzger, 2012). For example, previous studies reported that the effect of ABA on germination is related with its suppression of the activity of one of the ethylene synthesis enzymes, namely 1-aminocyclopropane-1-carboxylic

acid oxidase (ACO), and therefore decreasing ethylene production. Furthermore, several studies have reported that promotion of germination by ethylene is related to its effect on altering GA metabolism and signaling (Linkies and Leubner-Metzger, 2012; Corbineau *et al.*, 2014). For example, imbibing seeds with GA causes increased ethylene synthesis via upregulation of *ACO1* and activity of ACO (Calvo *et al.*, 2004).

Starch is the major storage reserve in cereal endosperm, it makes up about 50% to 70% of the seed dry weight in wheat. Since the storage starch acts as the source of energy for embryo growth during seed germination (Smith *et al.*, 2005), its degradation into simple sugars for subsequent use in respiratory pathways to produce energy is an important process. Starch degradation in cereals seeds is mainly catalyzed by four major enzymes, α-amylase (AMY), β-amylase (BAM), α-glucosidase (AGL) and glucan phosphorylase (PHO) (Zeeman *et al.*, 2010). α-amylase directly hydrolyzes starch granules and releases linear and branched glucans. The linear and branched glucans are further degraded into maltose by BAM. The conversion of maltose to glucose is mediated mainly by AGL. In addition to the hydrolysis process, the reversible release of glucose-1-phosphate from non-reducing ends of α-1,4-linked glucan chain by PHO is involved in starch degradation (Zeeman *et al.*, 2010).

Previous studies have analyzed the regulation of starch degradation and/or AMY activity in germinating barley seed (Radchuk *et al.*, 2009), developing wheat seed (Kondhare *et al.*, 2012), and ripening kiwifruit (Hu *et al.*, 2016). However, the role of ethylene in the regulation of starch degradation during wheat seed germination and seedling growth is poorly studied. This thesis investigated the regulation of starch degradation by ethylene during pre-germination and post-germination stages using molecular and biochemical approaches. Furthermore, it compared the

expression pattern of starch degrading genes during imbibition in dormant and non-dormant wheat seeds.

2.0 LITERATURE REVIEW

2.1 Wheat

2.1.1 History of wheat origin and evolution

Wheat, one of the oldest cultivated crops, was domesticated by humans about 10,000 years ago, and this has made a great contribution to the progress of human civilization. Wheat belongs to the family *Gramineae*, tribe *Triticeae* and genus *Triticum*. Species of the *Triticum* genera exhibit different ploidy levels with the basic chromosome number of n=1x=7. Wheat originated from Southwest Asia, which includes Syria, Iraq, Jordan, Turkey and Iran, and it was first cultivated in the Fertile Crescent area, which is also the centre of wheat diversity (Feldman *et al.*, 1995; Gustafson *et al.*, 2009). The earliest forms of wheat to be foraged by hunter-gatherers in Israel were wild einkorn (*Triticum boeoticum*, 2n=2x=14, genome A^bA^b; and *Triticum urartu*, 2n=2x=14, genome A^uA^u) and wild emmer (*Triticum dicoccoides*, 2n=4x=28, genome A^uA^uBB). Wild emmer wheat was produced by hybridization between *Triticum urartu* and *Aegilops speltoides*, (2n=2x=14, genome SS), which is thought to be the ancestor of B genome (Dvorak, 2009). Hunter-gatherers started to practice farming and cultivating wild emmer around 7000 BC, and cultivation of emmer wheat (*Triticum dicoccum*, 2n=4x=28, genome A^uA^uBB) spread from Fertile Crescent area to Mesopotamia around 6000 BC, and then to Egypt, Mediterranean, Europe and middle Asia around 5000 BC. Early spelt (*Triticum spelta*, 2n=6x=42, genome A^uA^uBBDD) was then derived from the hybridization of cultivated emmer (*Triticum tugidum*) with *Triticum tauschii* spp. *strangulate* (2n=2x=14, genome DD), which was the ancestral donor of D genome (Kihara 1944; Peng *et al.*, 2011). Common or bread wheat (*Triticum aestivum*, 2n=6x=42, genome A^uA^uBBDD) resulted from a natural mutation that changed the ear of spelt to

free-threshing ears (Peng *et al.*, 2011). Hexaploid wheat (*Triticum aestivum*) is genetically complex due to the presence of multiple homeologous genomes and its size (15,961 Mb) (Bennett and Leitch, 2010).

2.1.3 Domestication of wheat

Domestication is the achievement of a selection process that results in enhanced adaption of plants to cultivation and use by humans (Fuller *et al.*, 2010). Based on the history of wheat evolution, it is believed that wild einkorn and wild emmer wheats were selected for domestication, and their domestication took place around the same time, that is, about 12,000 years ago. Einkorn wheat (*Triticum monococcum*) was firstly domesticated in southeast Turkey, while wild emmer wheat (*Triticum dicoccoides*) was domesticated in the Fertile Crescent (Gustafson *et al.*, 2009). The hexaploid common or bread wheat, which contributes about 95% of world wheat production, and the tetraploid durum wheat that contributes the remaining 5% (Peng *et al.*, 2011) are domesticated forms of wild emmer wheat. It has been reported that wheat domestication occurred in multiple sites of the Fertile Crescent, and spanned for one thousand years as early farmers focused not only on selection of non-shattering, soft glume and free-threshing mutants in the wild wheat populations, but considered other agronomically important traits such as, grain size, heading time, plant height and grain yield. While non-shattering spikes play important roles in preventing seed loss during maturation before harvest, soft-glume and free-threshing traits enhance harvest efficiency. Large grain size and heading date traits have also been instrumental in improving grain yield (Feldman and Kislev, 2007; Nevo, 2011; Peleg *et al.*, 2011; Peng *et al.*, 2011). Conventionally, domestication of the hexaploid wheat has made successful conversion from primitive wheat with long, thin and small grain to modern wheat

with larger, shorter and wide grain (Eckardt, 2010). Wheat is extensively grown on approximately 17% of all crop areas, in the temperate, Mediterranean-type and subtropical parts of both hemispheres, from 67ºN in Norway, Finland and Russia to 45ºS in Argentina (Gustafson *et al.*, 2009). Canada, India, Russia, China, United States and France are the major wheat producers accounting for approximately 47% of total wheat production world-wide.

2.1.2 Uses of wheat

Wheat grains can be used for several purposes including for food, feed, biofuel and other industrial applications, however, approximately 76% of wheat produced globally is used as food (Gibson and Benson, 2002). Wheat is a major source of carbohydrate and protein for human, in which 20-30% of the calories for humans is provided by wheat (FAOSTAT, 2010). Wheat is also the staple food for about 40% of the world's population, mainly in North America, Europe, and western and northern parts of Asia. The most popular products made from wheat include bread, cookies, cakes, noodles, and pasta. The ratio of starch to protein in wheat flour is an important parameter considered for the production of a wide range of food and industrial products. For example, soft wheat with lower protein level and weak gluten is suitable for making biscuit, whereas hard wheat with higher protein content and strong gluten is suitable for making bread (Ram and Mishra, 2008). Wheat straw, non-feeding proportion, can be used for livestock bedding, newsprint and paper (Graybosch *et al.*, 2009). Another novel usage of wheat is for the production of biofuel, which is considered as a new renewable energy source that can reduce greenhouse gas emission (Ajanovic, 2011). Wheat grain is considered as one of the major feedstocks for bioethanol production worldwide (FAO, 2016). For example, in 2014, wheat accounted for approximately 33% of the bioethanol produced in Europe (European Renewable

Ethanol, 2015). Furthermore, wheat straw can be considered as a lignocellulosic biomass and can be used as a substrate for the production of bioethanol (Lin and Tanaka, 2006).

2.2 Seed

2.2.1 Seed development

Seed development and germination are essential processes for the maintenance of plant species, and furthermore for the determination of crop yield and quality (Ohto *et al.*, 2007). Seeds of cereal crop species develop from a double fertilization event in the ovule, and their developmental process can be characterized by three stages, including early development, differentiation, and maturation (Sabelli and Larkins, 2009). During the early development stage, double fertilization, syncytium formation and endosperm cellularization occur. The occurrence of double fertilization involves the landing of a mature pollen grain on the stigma followed by germination of the pollen grain and growth of a pollen tube down the style (Goldberg *et al.*, 1994). When the pollen tube reaches the embryo sac, two male germ cells are released from cytoplasm of the pollen tube within the ovule. The haploid egg cell and the diploid central cell of the female gametophyte within the ovule each fuse with one sperm cell from the pollen tube to form a diploid zygote and a triploid endosperm cell, respectively (Ohto *et al.*, 2007, Kesavan *et al.*, 2013). The completion of double fertilization is followed by synchronous division of the triploid nucleus, leading to the formation of syncytium (Sabelli and Larkins, 2009). The coenocytic endosperm cell the undergoes cellularization via formation of internuclear radial microtubule system and the endosperm differentiation stage comprises the formation of transfer cells, aleurone cells, starchy endosperm cells, and embryo-surrounding region cells. Completion of the morphogenesis of the embryo and endosperm indicates that seeds enter into the maturation

phase (Sreenivasulu *et al.*, 2010), and a mature seed has three important components: embryo, endosperm and testa (Ohto *et al.*, 2007). The endosperm plays a crucial role in supplying nutrients for embryo development and germination, and thereby controlling embryo growth (Yan *et al.*, 2014). The maturation phase of seeds is associated with induction of dormancy and desiccation, during which seeds lose water and the level of metabolic activity decreases.

2.2.2 Seed dormancy

Seed dormancy is defined as the inability of intact viable seeds to complete germination under favorable conditions (Gao and Ayele, 2014). Based on its onset, seed dormancy can be classified as primary and secondary dormancy (Bewley, 1997). Primary dormancy is initiated during seed development and seeds are harvested as dormant, while secondary dormancy is induced in non-dormant seeds after harvest, mainly due to unfavourable environmental conditions that block germination. Primary dormancy is categorized into five different types: physiological dormancy, morphological dormancy, morphophysiological dormancy, physical dormancy and combinational dormancy (physiological dormancy and physical dormancy) (Baskin and Baskin, 2004).

The most common type of seed dormancy is physiological dormancy, which is characterized by low growth potential of embryos and failure of embryo to penetrate through the seed covering structures such as the testa, endosperm, perisperm and pericarp (Heather, 2010). Physiological dormancy is a trait that is present in many plant species including weeds and crop species. Physiological dormancy can be further divided into non-deep dormancy, intermediate dormancy and deep dormancy. Embryos isolated from non-deep dormant seed are able to germinate and produce healthy seedlings, while embryos excised from deep dormant seed show

retarded growth and produce abnormal seedlings (Koornneef *et al.*, 2002; Baskin and Baskin, 2004). Non-deep and intermediate physiological dormancy can be released by a short period of cold stratification, long time dry storage at room temperature (after-ripening) or treatment with chemical compounds such as potassium nitrate, thiourea, ethylene and gibberellins. However, deep physiological dormancy of intact seeds can only be broken by a long period of cold stratification (Baskin and Baskin, 1998).

Morphological dormancy is caused by an undifferentiated or underdeveloped embryo, however, germination could occur after further development of the embryo (Baskin and Baskin, 2004; Heather, 2010). Morphological dormancy mainly occurs in tropical and temperate species that have rudimentary and linear embryos. Two temperate families, Apiaceae and Ranunculaceae, are reported to have morphological dormancy (Baskin and Baskin, 1998). Morphophysiological dormancy is characterized by an underdeveloped embryo with some physiological dormancy factors (Baskin and Baskin, 2004), and it occurs in the seeds of in many plant families such as the Apiaceae, Aquifoliaceae and Araceae families (Baskin and Baskin, 1998). The other two types of dormancy are physical dormancy and combinational dormancy. Physical dormancy is caused by the seed coat or seed covering layer that is impermeable to water or gas, and thereby blocking germination (Baskin and Baskin, 2004). This type of dormancy occurs in at least 15 families of angiosperms (Baskin and Baskin, 1998), and it can be released by treatment with high temperature, fluctuating temperature or drying. Combinational dormancy is the type of dormancy controlled by the physical and physiological components of the seed (Baskin and Baskin, 2004).

It is important that seeds of crop species exhibit a certain amount of dormancy, especially at the time of physiological and harvest maturity, as it prevents the occurrence of pre-harvest sprouting (Gubler *et al.*, 2005), which is defined as the germination of mature seeds while on the

mother plant. Preharvest sprouting of grains causes significant yield and quality reduction in cereal crops such as wheat, barley and maize, and thereby results in substantial financial loss (Zeeman *et al.*, 2010; Olaerts *et al.,* 2016). However, too much dormancy is not desirable as it can cause some production problems, such as non-uniform germination and poor stand establishment (Gubler *et al.*, 2005; Zeeman *et al.*, 2010).

2.2.3 Seed germination and the associated physiological events

The process of germination starts with imbibition/uptake of water by the dry seed and terminates with radicle penetration through the seed covering layers. (Bewley, 1997; Weitbrecht *et al.*, 2011). Generally, water uptake by seeds exhibits three phases (Bewley, 1997). Phase I is characterized by the initial rapid water uptake by a dry seed that leads to the seed swelling and a change in shape (Bewley, 1997). Membrane structure is disturbed by the fast rehydration, as a result low molecular weight metabolites and cellular solutes start to leak to the surrounding imbibition solution. After a short period of rehydration, the membranes are repaired. During this phase of water uptake, a number of physiological processes are initiated including protein synthesis from existing mRNA and resumption of respiratory activities such as glycolytic and oxidative pentose phosphate respiratory pathways. The resumption of respiratory activities is characterized by a large increase in oxygen consumption and release of carbon dioxide within minutes after imbibition (Bewley, 1997). In addition, repair of existing mitochondria is initiated during phase I of seed imbibition. Since dry seeds contain a low amount of ATP, repair and differentiation of mitochondria during imbibition plays a role in production of more ATP. Phase I of water uptake is also associated with repair of DNA which is damaged during the desiccation

phase of seed development, and the DNA repair involves DNA ligase, which is activated following imbibition (Bewley, 1997; Weitbrecht *et al.*, 2011).

Once the water uptake rate slows down and becomes stable, germinating seeds enter into phase II (Bewley, 1997; Weitbrecht *et al.*, 2011). A number of events take place during this phase, including repair of existing DNA and mitochondria, synthesis of new mitochondria, and synthesis of proteins such as those involved in starch hydrolysis from newly synthesized mRNAs. Furthermore, the initiation of embryo expansion and the weakening of seed covering layers occurs during this phase (Bewley, 1997). The end of phase II marks radicle protrusion through the testa, which is defined as germination, and the start of phase III (post-germination stage). Phase III is characterized mainly by the mobilization of storage reserves in the endosperm, and this triggers further increases in water uptake, leading to seedling growth (Nonogaki *et al.*, 2007). Cell division and DNA synthesis, and elongation of radicle cells occur during the third phase of water uptake.

2.2.4 Regulation of seed dormancy and germination

2.2.4.1 Regulation by plant hormones

Plant hormones such as abscisic acid (ABA) and gibberellin (GA) are among the endogenous factors regulating seed dormancy. ABA content is low during embryogenesis of seed development and increases gradually during the maturation phase, but decreases when seed desiccation is initiated (Vicente-Carbajosa and Carbonero, 2005). The amount of ABA present in the seed is related to the depth of dormancy (Bewley, 1997) in which a high amount of ABA inhibits germination. In dicot plants, such as tobacco, inhibition of seed germination by ABA is correlated with decreased activity of β-1,3-glucanase activity and delayed endosperm rupture

(Leubner-Metzger *et al.*, 1995). The synthesis of ABA is catalyzed by several enzymes, and 9-cis-epoxycarotenoid dioxygenase (NCED) is one of the enzymes that plays an important role in ABA synthesis and inducing seed dormancy during seed development and maturation. For example, the *NCED6* and *NCED9* genes are found to be highly expressed in developing seeds of Arabidopsis, and the *NCED2* of barley has a crucial effect on the regulation of ABA content in developing barley seeds (Chono *et al.*, 2006; Lefebvre *et al.*, 2006). In addition, the ABA8′-hydroxylase (ABA8′OH, encoded by *CYP707A*), which catalyzes ABA catabolism, has been reported to be responsible for reducing ABA level in both Arabidopsis and barley seeds. Seeds of the *cyp707a2* mutant lines of Arabidopsis contain 6-fold more ABA than wild type seeds during imbibition, and this is associated with enhanced seed dormancy (Kushiro *et al.*, 2004). In addition, the expression level of *CYP707A1* was shown to increase dramatically within six hours of imbibition in non-dormant barley seeds as compared to dormant seeds, and this is closely correlated with a decrease in ABA content (Millar *et al.* 2006). The level of ABA is also influenced by factors that affect dormancy such as after-ripening (dry storage) of seeds. After-ripened seeds of Arabidopsis and barley contain lower ABA content levels during seed imbibition as compared to dormant seeds (Millar *et al.*, 2006; Gubler *et al.*, 2008), although similar ABA levels were detected in both dry dormant and after-ripened seeds of Arabidopsis and barley. Previous studies have also shown that seed dormancy and germination can be related to ABA signaling (Walker-Simmons 1987; Liu *et al.*, 2013a). For example, dormancy release in wheat was reported to be associated with a decrease in the expression of ABA signaling elements including SNF1-related protein kinase2 (SnRK2) and ABA insensitive 5 (ABI5) (Liu *et al.*, 2013a).

It is well known that gibberellin (GA) is required for germination and acts as a positive regulator of seed dormancy release and germination initiation (Bewley, 1997). For example, seeds of GA-deficient mutants (*ga1-1* and *ga1-3*) of Arabidopsis cannot germinate without treatment with exogenous GA (Debeaujon and Koornneef, 2000). Bioactive forms of GAs synthesized by the action of GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) accumulated in embryos just before radicle protrusion (Finch-Savage and Leubner-Metzger, 2006; Yamaguchi, 2008). In Arabidopsis, endosperm weakening is a requirement for germination to occur, and this is accomplished by cell wall loosening enzymes such as β-1,3-glucanase and endo-β-mannanase whose activity is induced by GA (Halmer *et al.*, 1976; Leubner-Metzger *et al.*, 1995; Kucera *et al.*, 2005). The expression of genes encoding *GA20ox* and *GA3ox* is reported to be up-regulated during imbibition of after-ripened wheat and barley seeds as compared to the dormant seeds (Gubler *et al.*, 2008; Liu *et al.*, 2013a), and these results suggest that GA plays an important role in the regulation of dormancy release. Furthermore, previous reports indicated that the induction of germination of wheat seeds is correlated with increased expression of genes encoding starch hydrolyzing enzymes including α-amylase and α-glucosidase (Liu *et al.*, 2013a; Gao and Ayele, 2014). The activities of both α-amylase and α-glucosidase, which are induced by GA, showed dramatic increases during barley seed germination (Sun and Henson, 1990). Therefore, GA and ABA play opposite roles in dormancy and germination.

In addition to GA and ABA, other plant hormones such as ethylene, brassinosteroids, cytokinin, auxin and jasmonic acid are involved in the regulation of seed dormancy and germination (Kucera *et al.*, 2005; Subbiah and Reddy., 2010; Rajjou *et al.*, 2012; Chitnis *et al.*, 2014). Ethylene can promote germination by breaking primary and secondary dormancy, and application of exogenous ethephon, a synthetic compound that releases ethylene, stimulates

germination in lettuce, apple, sunflower, and beechnut seeds (Kepczynski *et al.*, 1977; Corbineau *et al.*, 1990; Calvo *et al.*, 2004; Kucera *et al.*, 2005). Brassinosteroid is another plant hormone that can promote seed germination via antagonizing the effects of ABA. Previous studies showed that ABA strongly inhibits the germination of brassinosteroid deficient mutant, *det2-1*, and brassinosteroid signalling mutant, *bri1-1*, of Arabidopsis as compared to that of the wild type (Steber and McCourt, 2001). The role of jasmonic acid and auxin in regulating seed dormancy and germination is still not clear. (Linkies and Leubner-Metzger, 2012; Liu *et al.*, 2013b; Miransari and Smith, 2014).

2.2.4.2 Regulation by environmental factors

Water, oxygen, temperature and light are important environmental factors required for seed germination. Seeds of different plant species have their own requirement of optimum environmental conditions for germination to occur. For example, light is required for the germination of tobacco (Finch-Savage and Leubner-Metzger, 2006) and *Arabidopsis thaliana* (Nelson *et al.*, 2010) seeds, while seeds of other species such as wheat germinate better under dark conditions (Bewley, 1997). With respect to temperature, the ideal temperature for wheat seed germination is 15-20ºC, whereas higher and lower temperature conditions induce seed dormancy (Nyachiro *et al.*, 2002; Izydorczyk *et al.*, 2017). The temperature also has effect on seed dormancy during seed development. A high temperature could induce an earlier ABA production peak in developing barley seed (Goldbach and Michael, 1976). Oxygen is also the other important environmental factor for germination, and seeds germinating under a low level of oxygen exhibit increased sensitivity to ABA and a delayed germination phenotype (Bradford *et al.*, 2008). A previous study also indicated that, although rice seeds can germinate under

anaerobic conditions, the seedlings produced from such seeds were abnormal (Kennedy *et al.*, 1980).

2.3 The role of ethylene during seed dormancy release and germination

2.3.1 Ethylene

Ethylene, the simplest alkene hydrocarbon, is a colorless gaseous hormone involved in seed germination, root initiation, flower opening, sex determination, fruit ripening, plant senescence and stress tolerance (Bleecker and Kende, 2000). Ethylene was discovered as gas generated from coal gas/illumination gas, and in 1886 Neljubow indicated for the first time that ethylene is an active substance that has influence on plant growth and development, as it was found that application of ethylene delayed the growth of pea seedling, leading to a shorter epicotyl (reviewed by Lin *et al.*, 2009). In 1910, Cousins reported that ethylene can be synthesized in plants since banana ripening rate was promoted by the ethylene released by oranges. With the emergence of gas chromatography technology, the detection of ethylene produced in plants was possible and this has made research on ethylene easier (Burg and Stolwijk 1959). The effect of ethylene on promoting fruit ripening and plant senescence is most commonly studied, however, ethylene also plays an important role on seed germination and seedling development by interacting with biosynthesis and signaling pathway of other hormones, such as, GA, ABA, cytokinin and auxin (Ghassemian *et al.*, 2000; Lin *et al.*, 2009; Linkies and Leubner-Metzger, 2012). For example, ethylene interrupts the ABA signalling pathway during seed germination, eliminating seed dormancy and promoting seed germination by playing a synergistic role with GA (Linkies and Leubner-Metzger, 2012). Moreover, ethylene has an inhibitory effect on root elongation due to its stimulation of auxin (Ruzicka *et al.*, 2007).

2.3.2 Ethylene biosynthesis pathway

Methionine acts as the precursor of ethylene biosynthesis (Fig. 1.1), and this was firstly demonstrated by the detection of labeled ethylene synthesized from labeled methionine in apple (Lieberman *et al.*, 1966). Following this discovery, Adams and Yang (1979) indicated that S-adenosyl-L-methionine (SAdoMet), and the cyclic non-protein amino acid, 1-aminocyclopropane-1-carboxylic acid (ACC), were the two major intermediates for ethylene biosynthesis. Adams and Yang (1979) also illustrated the sequence of the ethylene biosynthesis pathway by feeding apple tissue with L- [methyl 14C] methionine or L-[35S] methionine air, and detecting labeled ACC and ethylene, when the tissues were incubated in air. From this study, the authors concluded that the sequence of ethylene biosynthesis pathway involves the conversion of methionine to SAdoMet, and then SAdoMet to ACC, from which ethylene is produced. Conversion of SAdoMet to ACC by ACC synthase (ACS) is the rate-limiting step for producing ethylene (Argueso *et al.*, 2007). ACS is encoded by multigene families. In Arabidopsis, nine genes that encode ACS have been identified to date (Liang *et al.*, 1995; Yamagami *et al.*, 2003), and two *ACS* (*ACS1* and *ASC3*) were identified in apple (Yang *et al.*, 2013). The 5'-methylthioadenosine (MTA) is a by-product of the conversion of SAdoMet to ACC, which goes back to the Yang cycle to produce 5'-methylthioribose (MTR) (Yang and Hoffman, 1984). The last step of the Yang cycle involves the formation of 2-oxo-4-methylthiobutyric (KMB), which is converted to methionine by transamination. The last step of ethylene production involves oxidation of ACC by ACC oxidase (ACO) in which CO_2 and cyanide are co-produced (Yang and Hoffman, 1984; Wang *et al.*, 2002). Arabidopsis has thirteen *ACO* genes (Linkies *et al.*, 2009), while only three *ACO* (*ACO1*, *ACO2* and *ACO3*) genes are characterized in apple (Yang *et al.*, 2013).

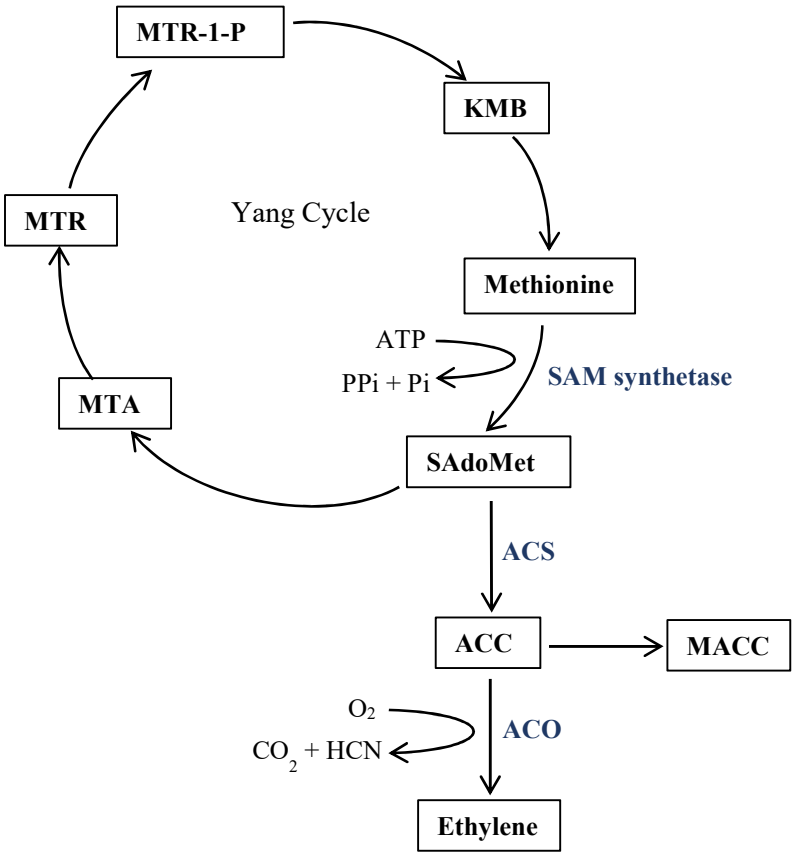

Figure 1.1 A simplified schematic representation of ethylene biosynthesis pathway. SAdoMet, S-adenosyl-L-methionine; MTA, 5'-methylthioadenosine; MTR, 5'-methylthioribose; MTR-1-P, MTR-1-phosphate; KMB, 2-oxo-4-methylthiobutyric; SAM synthetase, S-adenosyl-L-methionine synthetase; ACS, ACC synthase; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; MACC, malonyl ACC. (Adapted from Arc *et al.*, 2013; Hu *et al.*, 2017)

2.3.3 Ethylene signaling pathway

Ethylene is firstly perceived by five receptor proteins including ethylene resistant 1 (ETR1), ETR2, ethylene response sensor 1 (ERS1), ERS2, and ethylene insensitive 4 (EIN4) (Fig. 1.2), all of which predominantly localized in the endoplasmic reticulum (ER) membrane (Hua *et al.*, 1998; Merchante *et al.*, 2013). Ethylene receptors have been identified in many species, including five in rice, four in tobacco, two in maize and one in wheat (Chen *et al.*, 2005). The binding of ethylene with the receptors, which occurs in the hydrophobic N-terminal part of the

receptor dimer, requires copper as a co-factor (Hall *et al.*, 2007). These receptors act as negative regulators of the ethylene signaling pathway. In absence of ethylene, constitutive triple response 1 (CTR1), which is a Ser/Thr protein kinase that negatively regulates ethylene signaling, is activated by receptors and homodimerizes, leading to suppression of ethylene response (Arc *et al.*, 2013; Merchante *et al.*, 2013).

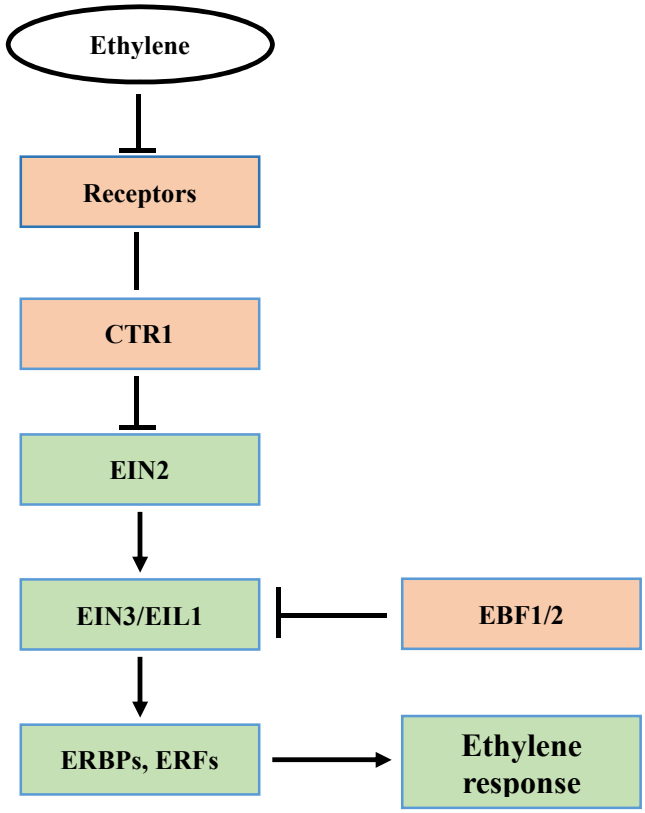

Figure 1.2 Ethylene signaling pathway. CTR1, constitutive triple response 1; EIN2, ethylene insensitive 2; EIN3, ethylene insensitive 3; EIL1, ethylene insensitive 3-like; EBF1/2, ethylene binding F-box protein 1/2; ERBPs, ethylene-responsive element binding proteins; ERFs, ethylene-responsive factors. (Adapted from Merchante *et al.*, 2013)

When ethylene is present, it binds with the receptors and this leads to inactivation of the receptor and thereby CRT1. Consequently, the phosphorylation of EIN2, which acts as a positive

regulator of ethylene signaling pathway, by CTR1 will be abolished. This activates the downstream components such as EIN3/EIN3-like (EIL1), which in turn activates ethylene-responsive element binding proteins (ERBPs) and ethylene-responsive factors (ERFs), and thereby the expression of ethylene regulated genes (Arc *et al.*, 2013; Merchante *et al.*, 2013). In the absence of ethylene, the level of EIN2, and EIN3 and EIL1 proteins is also regulated by degradation via 26S proteasome (Merchante *et al.*, 2013).

2.3.4 Ethylene and seed development

The importance of ethylene for seed development has been reported previously (Matilla, 2000). The maximal amount of ACC and ethylene is detected during the middle stage of embryogenesis, which is correlated with the highest activity of ACS and ACO, for example in chick-pea, and ethylene content declines during the late embryogenesis and seed desiccation stages (Gomez-Jimenez *et al.*, 1998; Matilla, 2000). However, a large amount of ethylene is detected during the early pre-desiccation stages of embryogenesis in crop species such as canola and mustard (Johnson-Flanagan and Spencer, 1994). During cereal seed development, two ethylene accumulation peaks are detected, the first one occurs during programed cell death (PCD) process in the central region of endosperm at about 16 to 20 days after pollination, and the second one is associated with the increase in endonucleolytic activity at about 36 days after pollination (Young *et al.*, 1997). Moreover, PCD has been shown to be promoted by exogenous ethylene in wheat and maize, and inhibition of PCD was observed when seeds were treated with ethylene inhibitor (Young *et al.*, 1997; Young and Gallie, 1999). In rice, ethylene evolution rate was found to be very high during the early and mid-grain-filling stage, but decreased rapidly at about 30 days after anthesis (Yang *et al.*, 2006). Too much ethylene production during seed development can

cause problems. For example, increased ethylene production in wheat resulted in a short grain filling period and induced premature senescence, which in turn caused a decrease in wheat yield (Beltrano *et al.*, 1999). Ethylene production can also be regulated by abiotic stress during seed development. For example, heat stress promoted ethylene production in developing wheat seeds, leading to kernel abortion (Hays *et al.*, 2007).

2.3.5 Ethylene and seed germination

Ethylene is one of the plant hormones that plays an important role in the regulation of seed dormancy and germination (Kucera *et al.*, 2005; Linkies and Leubner-Metzger, 2012). Production of ethylene during seed germination starts with imbibition, and highest level of ethylene was recorded at the time of radicle protrusion in the seeds of lettuce, pea, tomato, Arabidopsis, sunflower (Fu and Yang, 1983; Gorecki *et al.*, 1991; Siriwitayawan *et al.*, 2003; El-Maarouf-Bouteau *et al.*, 2014). The role of ethylene in regulating germination has been reported in tobacco, which is associated with induction in the expression of genes involved in endosperm weakening and rupture, such as, β-1,3-glucanase (*βGlu I*) (Leubner-Metzger *et al.*, 1998). Ethylene also has an effect on germination under abiotic stress condition. It has been shown that thermoinhibition of the germination of sunflower seeds is associated with repression of the conversion of ACC to ethylene (Corbineau *et al.*, 1989). The importance of ethylene during seed germination was further demonstrated by treatment of imbibing seeds with exogenous ethylene or inhibitors of ethylene biosynthesis. Germination of *Amaranthus caudatus* seeds was inhibited by ethylene inhibitor of ethylene action 2,5-norbornadiene and application of ethylene, while ethephon or ACC restored germination (Kepczynski *et al.*, 1977; Kepczynski and Karssen, 1985). Previous studies have shown that ethylene plays an important role in lettuce seed germination

(Matilla, 2000), and aminoethoxyvinylglycine (AVG), which inhibits ethylene biosynthesis via blocking the conversion of SAdoMet to ACC, has been shown to repress the germination of lettuce seeds. Moreover, treating seeds with exogenous ethylene was shown to enhance the rate of seed germination in crop species such as chickpea (Gallardo *et al.*, 1994) and sugar beet (Hermann *et al.*, 2007). This role of ethylene in germination is reported to be associated with its interference with the action of ABA and synergistic action with GA (Beaudoin *et al.*, 2000; Arc *et al.*, 2013; Corbineau *et al.*, 2014) as discussed in the next section.

2.3.6 Crosstalk between ethylene, GA and ABA during germination

The role of ethylene in enhancing seed germination resulted from its complex crosstalk with GA and ABA. Both ethylene production and signaling can be regulated by ABA during germination. For example, mutation in *CTR1* of Arabidopsis, negative regulator of downstream ethylene signaling components, led to seeds that were less-sensitive to ABA and germinated faster as compared to the wild-type (Beaudoin *et al.*, 2000). Ethylene insensitive mutants (*etr*, *ein2* and *ein6*) are hypersensitive to ABA and showed failure to germinate (Corbineau *et al.*, 2014). The role of ABA in inhibiting germination is also related with its effect in supressing ethylene production via repressing the activity of ACO (Matilla, 2000; Linkies *et al.*, 2009). Consistently, the expression level of *ACO1* in germinating seeds of ABA-insensitive mutant (*abi1-1*) is found to be higher than that of wild type seeds, reinforcing that ABA regulates ethylene production by affecting *ACO* expression (Linkies *et al.*, 2009). ABA has also been shown to repress the expression of *ACO1* and *ACO2* in the endosperm cap of *Lepidium sativum*, Arabidopsis and sugar beet seeds (Hermann *et al.*, 2007; Cheng *et al.*, 2009; Linkies *et al.*, 2009). However, ethylene can overcome ABA-induced dormancy without affecting the level of ABA in *Lepidium*

sativum seed, suggesting that the effect of ethylene on dormancy release operates via disruption of ABA signaling (Linkies *et al.*, 2009).

Ethylene also interacts with GA during seed germination. Many studies have indicated that ethylene promotes seed germination through modulating GA metabolic and signaling pathways (Linkies and Leubner-Metzger, 2012; Corbineau *et al.*, 2014). GA can affect ethylene content by regulating the ethylene biosynthesis pathway. For example, imbibing beechnut seeds with exogenous GA_3 led to increased ethylene production and this was associated with enhanced expression of *ACO1* and activity of ACO (Calvo *et al.*, 2004). Similarly, increased *ACO* expression was observed when seeds of the GA deficient *ga1-3* mutant of Arabidopsis were imbibed with exogenous GA_4 (Ogawa *et al.*, 2003). On the other hand, the expression of *ACO2* was inhibited by treatment with GA biosynthesis inhibitor, paclobutrazol (Iglesias-Fernandez and Matilla, 2010). It has been shown previously that the biosynthesis of GA is mediated by ethylene. For example, the expression level of GA biosynthesis genes, *SoGA3ox2* and *SoGA20ox2*, in germinating seeds of *Sisymbrium officinale* was significantly supressed by seed treatment with a mix of ethylene biosynthesis and signaling inhibitor (Iglesias-Fernandez and Matilla, 2010). Furthermore, GA_1, GA_4 and GA_7 have been reported to accumulated in the *etr1-2* mutant of Arabidopsis, and the level GA_4 and GA_7 remained higher than in the wild type during imbibition (Chiwocha *et al.*, 2005).

2.4 Energy source for seed germination

2.4.1 Starch

Storage starch in plants primarily serves as an energy source for plant growth and development (Zeeman *et al.*, 2010). Starch, the most important insoluble glucan biopolymer in higher plants,

mainly consists of amylose and amylopectin. Amylose is a linear chain polymer made up of D-glucose units that are linked by α-1,4 glycosidic bonds, while amylopectin is a highly branched double helical polymer that consists of α-1,4 linked glucose units branched via α-1,6 glycosidic bonds at intervals of 24-30 glucose units (Streb and Zeeman *et al.*, 2012). In contrast to amylose, amylopectin is soluble in water, and it constitutes over 75% of starch granule by weight.

Starch is synthesized in both photosynthetic (leaves) and non-photosynthetic (roots, tubers and seeds) organs. Starch makes up 40-90% of the total dry weight of cereal grains (Shelton and Lee, 2000). The first committed step of starch synthesis in plant tissues involves the synthesis of ADP-glucose, which is catalyzed by AGP-glucose pyrophosphorylase (AGPase), from glucose-1-phosphate and adenosine 5′ triphosphate (ATP). The other enzymes involved in starch synthesis include starch synthases (SS), starch branching enzyme (BE) and debranching enzymes (DBE). ADP-glucose serves as glucosyl donor for SS mediated synthesis of α-1,4 bonded glucan chains, which serve as substrates for the amylose and amylopectin synthesis (Tetlow, 2011; Geigenberger, 2011). Starch synthases are divided into granule bound (GBSS) and soluble starch (SSI, SSII, SSIII, and SSIV) synthases (Tetlow, 2011). The GBSS form of SS mainly catalyzes the formation of amylose, while SSI to SSIV are responsible for amylopectin synthesis (Steup, 1988; Zeeman *et al.*, 2010). Starch branching enzymes are responsible for catalyzing the formation of α-1,6 linkages that induces the branching of amylopectin, and this process involves cleavage of the α-1,4- bond in the glucan chains followed by transfer of six or more glucose units of the cut segment to the C6 position of a glucosyl residue of another glucan chain (Zeeman *et al.*, 2010). Besides of SS and BE, starch DBEs also play an important role in the formation of amylopectin structure by cleaving branch points; isoamylase (ISA) and limit-dextrinase (also called pullulanase) are the two types of DBEs characterized in most higher plants (Zeeman *et al.*,

2010). Storage starch accumulates in the endosperm during cereal seed development, and this starch is rapidly metabolized during germination and post-germination stages by synergetic action of several amylolytic enzymes (Ritte *et al.*, 2002, 2006; Lloyd *et al.*, 2005; Streb and Zeeman, 2012) to provide energy for the growing seedlings.

2.4.2 Starch degradation during seed germination

During the germination of cereal seeds, nutrients and energy required for embryo/seedling growth are supplied by storage reserves, mainly starch, deposited in the endosperm (Smith *et al.*, 2005; Zeeman *et al.*, 2010). The starch degradation pathway was, however, first reported in pea seedlings (Swain *et al.,* 1966). In cereals endosperm, starch granules are directly hydrolyzed by the action of α-amylase (AMY), which produces branched and linear glucans that are further hydrolyzed into glucose by the combined actions of debranching enzyme limit-dextrinase, β-amylase (BAM) and α-glucosidase (AGL) (Zeeman *et al.*, 2010; Fig. 1.3).

α-amylases have been known as the primary enzymes that act on starch granules and produce dextrins, however, a previous study has shown that synergic action of both AMYs and BAMs is important to enhance the rate of starch degradation, rather than that of AMYs alone (Dunn, 1974). Although mature dry seeds contain low levels of AMYs, these enzymes are well synthesized in the aleurone layer during the germination process, and play roles in the mobilization of storage reserves deposited in the endosperm (Rejzek *et al.*, 2011; Andriotis *et al.*, 2016a). Limit-dextrinase converts the branched glucans produced by AMYs to linear glucans, and the BAMs remove the maltose units from non-reducing ends of the linear glucans, but, both limit-dextrinase and BAM play an ambiguous role of starch degradation in the seed. Although maltose has been shown to be produced by BAM in the endosperm during germination (Rejzek

et al., 2011), BAM is considered to have less control on the production of sugars used for embryo/seedling growth. For example, BAM deficient mutants of barley can germinate normally, and some barley cultivars from Tibet, which almost lack endospermic BAM, are able to produce healthy seedlings (Kihara *et al.*, 1999; Kaneko *et al.*, 2000). Furthermore, a study in wheat has shown that the expression of probesets annotated as *BAM* is higher in the dormant seed as compared to non-dormant seed during imbibition (Liu *et al.*, 2013a; Park *et al.*, 2013). The importance of limit-dextrinase for maize seed germination has been reported previously (Dinges *et al.*, 2003), however, the role of this enzyme in germinating seeds of other cereals such as barley is still not clear (Burton *et al.*, 1999; Andriotis *et al.*, 2016a). In addition to these hydrolytic processes, a phosphorylitic mechanism is also involved in endospermic starch degradation, and this mechanism involves the α-glucan phosphorylases (PHO), which catalyzes the reversible release of the glucose-1-phosphate from non-reducing ends of α-1,4-linked glucan chain. The reaction direction is dependent on the relative concentrations of glucose-1-phosphate and orthophosphate (Rathore *et al.*, 2009).

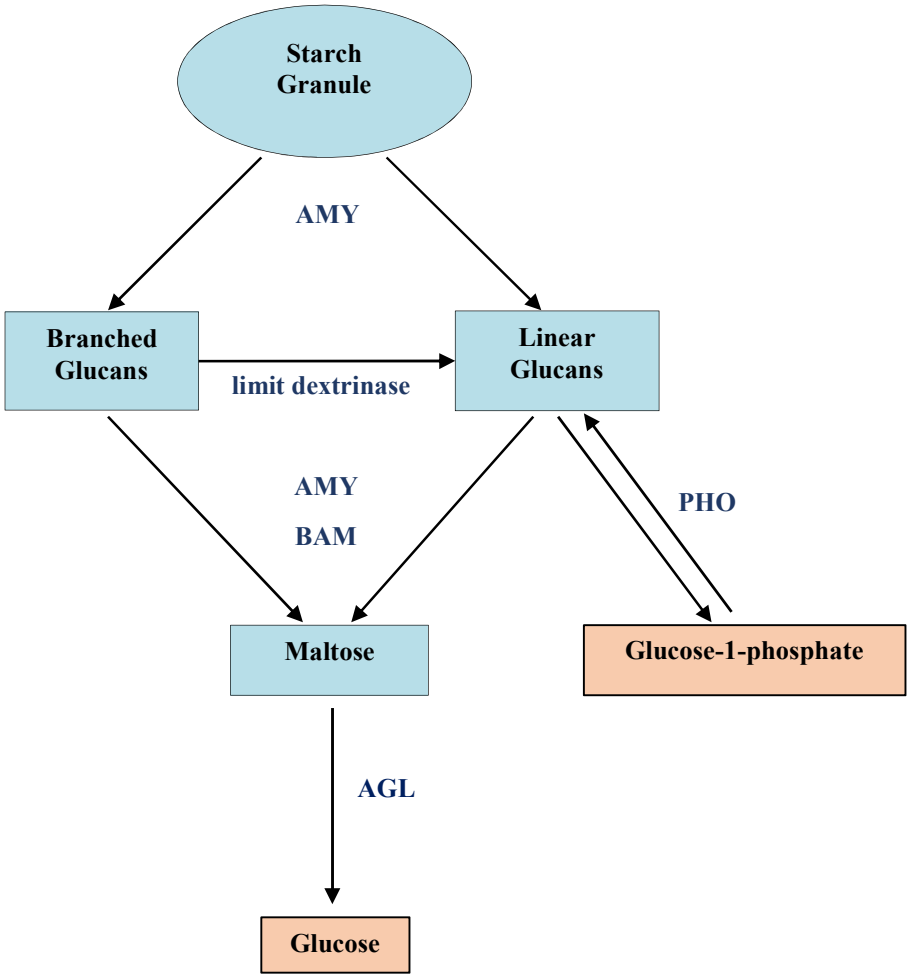

Figure 1.3 Starch degradation pathway in cereal endosperm. AMY, α-amylase; BAM, β-amylase; AGL, α-glucosidase; PHO, glucan phosphorylase. (Adapted from Smith *et al.,* 2005)

Starch degradation in germinating cereal endosperm is different from that observed in other organs of plants, for examples, potato tubers and Arabidopsis leaves (Zeeman *et al.*, 2010; Andriotis *et al.*, 2016a). In the leaves of both Arabidopsis and potato leaves, maltose resulted from starch degradation by β-amylase that is transferred to cytosol, then the maltose metabolized by disproportionating enzyme (DPE). Disproportionating enzymes act as transglucosidase, which transfers α-1,4 bonds from one donor malto-oligosaccharides to an acceptor (polysaccharide or glucose), leading to a release of the other moiety (Lloyd *et al.*, 2005). There is no indication for participation of α-glucosidase in maltose metabolism in Arabidopsis leaves, which is different

from starch degradation that takes place in the endosperm (Niittyla *et al.*, 2004; Zeeman *et al.*, 2010). In Arabidopsis leaves, reversible phosphorylation of glucans, which is mediated by glucan water dikinases and phosphoglucan water dikinases has been shown to be important for determination of starch degradation (Zeeman *et al.*, 2010; Andriotis *et al.*, 2016a). However, there is no report suggesting if glucan phosphorylation occurs in the endosperm of germinating cereal seeds (Zeeman *et al.*, 2010). Many aspects of starch degradation in cereal endosperm have been studied for decades, including the synthesis of hydrolytic enzyme in the aleurone layer and scutellum, and its regulation by plant hormones (reviewed by Andriotis *et al.*, 2016a). However, the molecular aspects of starch degradation during germination and seedling growth requires further investigation in crops such as wheat.

2.5 Enzymes involved in starch degradation

2.5.1 Alpha-amylase

α-amylase (EC 3.2.1.1), which belongs to the glycoside hydrolase family 13, is reported as the predominant enzyme to attack starch granules and hydrolyze α-1,4-glycosidic linkages of starch in cereal endosperm during germination (Smith *et al.*, 2005; Streb and Zeeman, 2012). The AMY synthesized in aleurone layer during seed imbibition is secreted to the starchy endosperm. The synthesis of AMY in the aleurone layer is stimulated by the plant hormone GA, which is produced in embryo (Zeeman *et al.*, 2010). The enzyme AMY is encoded by a multigene family in several species. For example, Arabidopsis has three *AMY* gene family members designated as *AtAMY1*, *AtAMY2* and *AtAMY3*. Genes encoding AMY have also been identified from crop species such as barley and rice. The *AMY* gene family of barley consists of four members designated as *HvAMY1*, *HvAMY2*, *HvAMY3* and *HvAMY4* (Radchuk *et al.*, 2009), while rice has

three *AMY* gene family members that are designated as *OsAMY1*, *OsAMY2* and *OsAMY3* (Huang *et al.*, 1992). In wheat, AMY isoenzymes are grouped into three groups (Kondhare *et al.*, 2015); group 1 consists of the *α-AMY1* gene family encoding high-isoelectric-point (high-pI) AMY that is mainly produced during seed germination, group 2 consists of the *α-AMY2* gene family encoding low-pI AMY that is produced in the endosperm during seed germination and in the embryo during the early stage of seed development, and group 3 consists of the *α-AMY3* gene family encoding very high-pI AMY that is present only in developing seeds (Barrero *et al.*, 2013; Kondhare *et al.*, 2015).

Previous studies showed that the expression of AMY encoding genes such as *AMY1* and *AMY4* and the activity of the corresponding AMY enzymes in the aleurone layer increased rapidly during imbibition of barley seeds (Radchuk *et al.*, 2009). Moreover, the activity of AMY is also related to seed dormancy level, in which high AMY activity was detected in the endosperm of non-dormant/PHS-affected seeds as compared to dormant seeds (Ral *et al.*, 2016). The expression of *AMY* genes is also shown to be significantly higher during imbibition of after-ripened (non-dormant) as compared to dormant seeds of wheat (Gao and Ayele, 2014). The activity of AMY is regulated by GA and ABA; AMY activity is significantly decreased by exogenous ABA while highly induced by GA_3 (Kondhare *et al.*, 2012). Other hormones such as ethylene are reported to induce the expression of *AMY* during fruit ripening in other crop species such as kiwifruit (Hu *et al.*, 2016). The expression of *AMY* is reported to be controlled by demand for sugar; sugar or carbon starvation can activate *AMY* promoter in rice (Lu *et al.*, 1998).

2.5.2 Beta-amylase

β-amylase (EC.3.2.1.2) belongs to glycoside hydrolase family 14, and it is responsible for hydrolyzing the α-1,4 glycosidic bonds of non-reducing ends of dextrin products yielded by AMY to release maltose (Helland *et al.*, 2002). An abundant amount of BAM is synthesised during seed development and maturation, then a large amount of this BAM is stored in the endosperm as insoluble forms in barley (Potokina *et al.*, 2002). β-amylase is synthesized in aleurone cells during germination, and then it is secreted into the starchy endosperm (Wang *et al.*, 1996; He *et al.*, 2015). In barley, Georg-Kraemer *et al.* (2001) found that the highest activity of BAM occurred within two days after imbibition, and this occurrence was earlier than α-amylase, which started to show an increase in activity from the third day after imbibition, during seed germination of barley. The BAM enzyme similar to AMY is encoded by a multigene family. For example, nine *BAM* genes have been identified from Arabidopsis (Steb and Zeeman, 2012). In barley, the *BAM* gene family consists of seven members (*HvBAM1* to *HvBAM7*) (Radchuk *et al.*, 2009), while rice and maize each are reported to have two family members, and three *BAM* gene family members have been identified wheat (Mason-Gamer, 2005). The expressions of *BAM1* and *BAM2* showed increases at eight days after imbibition in barley seeds, while *BAM5*, *BAM6* and *BAM7* showed constant expression during imbibition (Radchuk *et al.*, 2009). Previous studies have shown higher BAM activity in mature, highly dormant, mutant seeds of barley (Swanston and Molina-Cano, 2001). In addition, the expression of *BAM* genes was found to be downregulated in after-ripened (non-dormant) seeds as compared to dormant seeds of wheat (Park *et al.*, 2013; Gao and Ayele, 2014).

Similar to that of α-amylase, the activity of BAM can be regulated by phytohormones such as GA, ABA and ethylene. It has been reported that GA is not needed for the de novo synthesis

of β-amylase in the aleurone layer in rice, however, exogenous application of ABA has been shown to inhibit BAM activity, and the inhibitory effect of ABA can be reversed by GA_3 (Wang *et al.*, 1996). The role of ethylene in regulating BAM activity has been reported in banana in which BAM activity during fruit ripening is significantly decreased by treatment with 1-methylcyclopropene (1-MCP), which is an ethylene biosynthesis inhibitor (Nascimento *et al.*, 2006).

2.5.3 Alpha-glucosidase

The major forms of α-glucosidase (EC 3.2.1.20) found in germinating cereal seeds are exo-acting enzymes that belong to glycoside hydrolase family 31, which can hydrolyze the α-1,4-, α-1,6-, α-1,3- and α-1,2-D-glycosidic bonds (Frandsen and Svensson, 1998; Naested *et al.*, 2006). Glucosidases are synthesized *de novo* in aleurone cells during the early germination stage and are secreted to the endosperm where they act on starch. Previous reports indicated up to six-fold increase in AGL activity during germination (Skadsen and Tibbot, 1998). Three different isoforms of AGL that are formed due to the proteolysis and other post-translational modifications have been reported in barley endosperm, and all the three different forms appeared to be encoded by a single gene, *AGL97* (Andriotis *et al.*, 2016b). Although AGL is capable of attacking starch granules in the absence of AMY, ten-fold increase of starch hydrolysis has been observed due to synergistic action of AMY and AGL as compared to that of AGL alone (Sun and Henson, 1990, 1991; Skadsen and Tibbot, 1998). The AGL however plays a crucial role in the hydrolysis of maltose to glucose. In germinating wheat seed, accumulation of maltose and reduction of glucose content has been observed due to treatment with glucosidase inhibitor (Miglitol, N-hydroxyethl-1-deoxynojirimycin) (Konishi *et al.*, 1994). Consistently, application of

AGL inhibitor resulted in inhibition of glucose production and starch hydrolysis in barley seeds during germination and post-germination stages (Stanley *et al.*, 2011). Regulation of the expression of *AGL* by plant hormones have been reported previously. For example, the expression of *AGL3* was induced by treatment with exogenous ethylene of ripening kiwifruit (Hu *et al.*, 2016) while ABA was shown to downregulate *AGL* in wheat seeds (Liu *et al.*, 2013a).

2.5.4. Starch phosphorylase

There are two types of glucan phosphorylase enzymes (EC 2.4.1.1) identified in plants, plastidial phosphorylase (PHO1, also referred to as PHS1 in Arabidopsis) and cytosolic phosphorylase (PHO2, also referred to as PHS2 in Arabidopsis) (Streb and Zeeman, 2012). The PHO enzyme plays an important role in starch synthesis and elongation of short glucan chains during seed development (Streb and Zeeman, 2012). For example, PHO activity has been detected in developing wheat seed endosperm, and PHO1 plays a role in the synthesis of starch during wheat seed development (Tickle *et al.*, 2009). Moreover, PHO1 has higher catalytic efficiency in synthesis of short-chain malto-oligosaccharides than in the degradation direction in rice (Hwang *et al.*, 2010). Similarly, *pho1* mutant of rice showed abnormal endosperm due to accumulation of small starch granules in developing rice seeds (Satoh *et al.,* 2008). The function of PHO on starch degradation appears to be dispensable. In the leaves of Arabidopsis, the ability of PHS1 to directly attack starch granules *in vivo* and produce glucose-1-phosphate is reported as weak, and mutation in *PHO1* of Arabidopsis causes only a slight decrease of starch degradation in Arabidopsis (Zeeman *et al.*, 2004). In addition, the activity of PHO was shown to slightly increase during banana ripening when starch undergoes degradation (Mota *et al.*, 2002). Recently, Ma *et al.* (2013) reported that *PHO2* is mainly expressed in germinating barley seeds

as compared to other tissue such as root, stem and seedling. The function of PHO during seed germination is, however, still not clear.

3.0 STARCH DEGRADATION DURING AND AFTER GERMINATION OF WHEAT SEEDS AND ITS REGULATION BY ETHYLENE

Abstract

Ethylene is one of the plant hormones that regulates seed germination. Embryo/seedling growth during and after wheat seed germination requires energy, which is supplied mainly by the breakdown of starch stored in the endosperm. This study investigated the regulation of starch degradation in wheat seeds during and after germination by ethylene using different approaches. Expression analysis of starch degrading genes showed that *AMY1, AMY3, BAM2, BAM3, BAM6, BAM9, AGL1* and *AGL2* were highly expressed in the endosperm during and after germination. Activities of AMY and AGL also increased with imbibition, suggesting their significance in the hydrolysis of endospermic starch. Inhibition of ethylene synthesis using a chemical inhibitor did not affect germination but inhibited seedling growth, and this was associated with repression of *AMY1, AMY2, AMY4, AGL1* and *AGL2* genes and the activity of the corresponding enzymes, AMY and AGL. Consequently, accumulation of starch and decreased level of soluble sugars was observed in seeds treated with ethylene biosynthesis inhibitor. These results suggest that ethylene plays an important role in starch degradation in wheat seeds. Comparative analysis of the expression of starch degrading genes between dormant and non-dormant wheat seeds indicated upregulation of *AMY1, AMY2, AGL1, AGL2, PHO1* and *PHO2* genes during imbibition of the non-dormant seeds, and the results imply that these genes contribute to preharvest sprouting.

3.1 Introduction

Seed quality is a vital factor for propagation as most plants or crops start their life cycle with seeds. Seed germination is an essential process for the maintenance of plant species, and also determining crop yield and quality (Ohto *et al.*, 2007). The process of germination starts with imbibition and terminates with protrusion of radicle through seed coat. Generally, seeds exhibit three phases for water absorption, phase I (fast water uptake), phase II (reactivation of most metabolic activities and radicle emergence), and phase III (reserve mobilization and seedling growth) (Bewley, 1997). Seeds also exhibit dormancy, defined as failure to germinate even under favorable condition. Dormancy is considered as an adaptive mechanism to maintain the survival of plant species (Bewley, 1997). In cereals such as wheat, a certain amount of dormancy is important, especially at the time of physiological and harvest maturity, as it prevents the occurrence of pre-harvest sprouting (Gubler *et al.*, 2005). Preharvest sprouting defined as the germination of mature seeds while on the mother plant, causes significant yield and quality reduction, and substantial financial loss (Lukow and Bushuk, 1984; Olaerts *et al.*, 2016). However, a high level of dormancy is not desirable as it can cause some production problems, such as delayed and non-uniform germination.

Seed dormancy and germination are regulated by plant hormones and environmental factors. Among the different plant hormones, abscisic acid (ABA) and gibberellin (GA) are the major regulators of seed dormancy and germination. Abscisic acid is involved in induction and maintenance of dormancy. The amount of ABA present in the seed is related to the depth of dormancy (Bewley, 1997), where a high amount of ABA inhibits germination. Gibberellin is required for germination and acts as a positive regulator of seed dormancy release and germination initiation (Bewley, 1997; Gubler *et al.*, 2005). In addition to ABA and GA, other

hormones such as ethylene are reported to be involved in the regulation of dormancy and germination. Ethylene is extensively studied in relation to the induction of fruit ripening and plant senescence, however, few reports indicate that it plays a role in dormancy release and germination. Ethylene is synthesized during seed development, the maximal amount of ethylene is detected during the middle stage of embryogenesis in species such as chickpea, and the content declines during the late embryogenesis and seed desiccation stages (Gomez-Jimenez *et al.*, 1998; Matilla, 2000). Previous studies showed that ethylene can promote germination by breaking primary and secondary dormancy (Calvo *et al.*, 2004; Kucera *et al.*, 2005). For example, in tobacco, its role is associated with the induction of the expression of genes involved in endosperm weakening and rupture (Leubner-Metzger *et al.*, 1998). Ethylene also plays an important role in the germination of lettuce seeds; inhibition of ethylene synthesis leads to inhibition of lettuce seed germination (Matilla, 2000).

Recent studies reported that the role of ethylene in enhancing seed germination is resulted from its complex crosstalk with GA and ABA. Ethylene interrupts the ABA signalling pathway during seed germination, eliminating seed dormancy and promoting seed germination by playing a syngeneic role with GA (Linkies and Leubner-Metzger, 2012). Mutation in the constitutive triple response *ctr1* gene of Arabidopsis, leads to seeds that are less-sensitive to ABA and germinate faster as compared to the wild-type (Beaudoin *et al.*, 2000). Inhibition of seed germination by ABA is partly caused by its effect on supressing ethylene production via repressing the activity of ACO (Matilla, 2000; Linkies *et al.*, 2009). On the other hand, ethylene promotes seed germination through modulating GA metabolic and signaling pathways (Linkies and Leubner-Metzger, 2012; Corbineau *et al.*, 2014). Gibberellin can induce ethylene synthesis by activating the ethylene biosynthesis pathway. For example, imbibing beechnut seed with

exogenous GA_3 leads to increased ethylene production via enhanced expression of *ACO1* and activity of ACO (Calvo *et al.*, 2004). Moreover, the biosynthesis of GA is also mediated by ethylene. The expression level of GA biosynthesis genes, *GA20ox2* and *GA3ox2* in germinating seeds of *Sisymbrium officinale* is significantly supressed by seed treatment with a mix of ethylene biosynthesis and signaling inhibitor (Iglesias-Fernandez and Matilla, 2010).

Storage starch in endosperm of cereal seeds primarily serves as an energy source for seed germination and seedling establishment (Zeeman *et al.*, 2010). Storage starch accumulates in the endosperm during cereal seed development, and this starch is rapidly metabolized during germination and post-germination stages by synergetic action of several amylolytic enzymes (Ritte *et al.*, 2002, 2006; Lloyd *et al.*, 2005; Streb and Zeeman, 2012) to provide energy for the growing seedlings. In wheat seed endosperm, starch granules are directly hydrolyzed by the action of α-amylase (AMY), which produces branched and linear glucans that are further hydrolyzed into glucose by the combined actions of debranching enzyme limit-dextrinase, β-amylase (BAM) and α-glucosidase (AGL) (Zeeman *et al.*, 2010). In addition to these hydrolytic enzymes, phosphorylases (PHOs) catalyzes the reversible release of the glucose-1-phosphate from non-reducing ends of α-1,4-linked glucan chain (Streb and Zeeman, 2012).

The degradation of starch is also regulated by plant hormones. For example, AMY activity is significantly induced by exogenous GA and suppressed by ABA (Kondhare *et al.*, 2012). The expression of *AMY* and *AGL* genes is also shown to be significantly higher during imbibition of non-dormant, as compared to dormant seeds that fail to complete germination (Gao and Ayele, 2014). A study from ripening kiwifruit has indicated significant induction of the expression of *AMY1, BAM3.1, BAM3L, BAM9* and *AGL3* by treatment with exogenous ethylene (Hu *et al.*, 2016) and this effect on gene expression was shown to be correlated with accelerated

degradation of starch and increased level of soluble sugars (Hu *et al.*, 2016). In contrast, starch degradation was inhibited by application of ethylene biosynthesis inhibitor 1-MCP in ripening banana, and this is associated with decreased content of soluble sugars (Nascimento *et al.*, 2006). However, the role of ethylene in the regulation of starch degradation during and after germination of cereal seeds is much less known. Therefore, the objective of this research was to examine molecular mechanisms regulating starch degradation in wheat seeds by ethylene. To achieve this, candidate starch degrading genes of wheat were identified, and their expression pattern was analyzed during and after germination of wheat seeds in response to inhibition of ethylene synthesis. Furthermore, activity of the enzymes corresponding to the genes, and content of soluble sugars resulting from the starch breakdown was examined. In addition, the expression of starch degrading genes was compared between the dormant, non-dormant and ABA treated non-dormant wheat seeds during germination, to gain a better understanding of the relationship between dormancy and starch degradation.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

The wheat genotypes used for this study are RL4452 and AC Domain. RL4452 is unregistered genotype derived from Glenlea*6/ Kitt backcross. It is characterized by its low level of seed dormancy or susceptibility to preharvest sprouting. AC Domain is a registered cultivar widely grown in Canada and its seeds exhibit high level of dormancy. Plants of the wheat genotype "RL4452" were grown in a growth room at 22/20 ºC (day/night) in a 16/8h photoperiod until harvest. Seeds were germinated in Petri dishes and then transplanted into pots (one seeding per pot) containing Sunshine Mix 4 (Sungro Horticlture, Bellevue, WA, USA). Plants were fertilized with N-P-K (20: 20: 20) at the rate of 20g/gallon water once every two weeks starting from two-week old stage until flowering. Spikes were harvested at maturity, and seeds collected from the spikes of 20 plants were considered as independent replicates with a total of three replicates.

3.2.2 Germination and seedling growth assays

Mature seeds of RL4452 were rinsed with 70% ethanol for 1 min, then surface sterilized with a 5% sodium hypochlorite solution for 20 min, followed by five sterile deionized water rinses. Twenty-five seeds were placed in 9-cm sterile Petri dishes with two layers of sterile Whatman #1 filter paper (GE Healthcare, Little Chalfont, UK) moistened with 7 ml of sterile deionized water. To examine the effect of ethylene on starch degradation, seeds were imbibed with 1 mM of aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor (Cayman Chemicals, Ann Arbor, MI, USA). Three independent biological replicates were performed for each experiment, and each petri dish represent one biological replicate. The petri dishes were then sealed with parafilm and incubated at room temperature. Scoring of seed germination and measurement of

the length of different parts of the seedlings including the lengths of the radicle, seminal root and the coleoptile was performed over a period of seven days.

For expression analysis of starch degrading genes, seeds were harvested at 6, 12, 24, 48, 72, 120 and 168 h after imbibition (HAI). The endosperm (with aleurone layer) was separated from the embryo. For enzyme activity, sugar and starch level analysis, endosperm (including the aleurone) was separated from the embryo of imbibing seeds at 1, 2, 3, 5, and 7 days after imbibition (DAI). The tissues were immediately frozen in liquid nitrogen and then stored at -80°C until further use.

3.2.3 Identification of wheat starch degrading genes and their specific primers

Available sequences of starch degrading genes of barley encoding α-amylase, β-amylase, α-glucosidase and phosphorylase (Table. 3.1) were used to search the corresponding wheat homologs in the National Center for Biotechnology Information's (NCBI) wheat UniGene database using basic local alignment search tool (BLAST).

Table 3.1 List of starch degrading genes of barley used to search for wheat homologs in the NCBI wheat UniGene database.

Name	GenBank ID	Reference
HvAMY1	HZ37L10	Radchuk *et al.* (2009)
HvAMY2	HV02D09	Radchuk *et al.* (2009)
HvAMY3	HV14L21	Radchuk *et al.* (2009)
HvAMY4	HO12I21	Radchuk *et al.* (2009)
HvBAM1	HY09N12	Radchuk *et al.* (2009)
HvBAM2	HZ50O15	Radchuk *et al.* (2009)
HvBAM3	HS08F24	Radchuk *et al.* (2009)
HvBAM4	HI09E01	Radchuk *et al.* (2009)
HvBAM5	HI06F14	Radchuk *et al.* (2009)
HvBAM6	HDP09L01	Radchuk *et al.* (2009)
HvBAM7	AV833737	Radchuk *et al.* (2009)
HvAGL1	AF118226	Tibbot and Skadsen (1996)
HvAGL2	U22450	Matsumoto *et al.* (2011)
HvAGL3	AK374222	Frandsen *et al.* (2000)
HvAGL4	AK375379	Frandsen *et al.* (2000)
HvAGL5	AK375658	Frandsen *et al.* (2000)
HvPHO1	HB21H16	Radchuk *et al.* (2009)
HvPHO2	HO06A20	Radchuk *et al.* (2009)

The resulting sequences of wheat homologs along with starch degrading gene sequences collected from *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, *Hordeum vulgare* were used to generate the phylogenetic trees. The nucleotide sequences of these genes were aligned by ClustalX program and the phylogenetic tree was generated by using Molecular Evolutionary Genetic Analysis (MEGA, version 6) software. The phylogeny analysis was performed using the neighbor-joining method and maximum composite likelihood from 500 bootstrap replicates. Precise primers for each target gene were designed (Table. 3.2) from the respective nucleotide sequences using Primer 3 software, and the specificity of the resulting primers was examined verified by BLAST searching against GenBank database and RT-PCR analysis.

Table 3.2 Primers sequences and PCR efficiency of starch degrading genes in wheat

Name	GenBank ID		Primer Sequence (5'—3')	Amplicon Size (bp)	PCR efficiency (%)	R^2
TaAMY1	CV772865	F	ACCGGCAGAATCTGGTGAAC	100	96.5	0.993
		R	CACCGGGGCGTTCAATATCC			
TaAMY2	CA701040	F	TTACATCGACCGCTCGGCGC	159	90.0	0.994
		R	AAGGTGGTAGCGGGGCCTTTG			
TaAMY4	JV989039	F	GTTTCCGATGCTGTTGTCCAAGA	222	100.4	0.977
		R	GTTCTGTGGCAGATAGCCTTCT			
TaAMY5	AK335373	F	CGCTGGCTGAGAAAGGAAGT	187	90.1	0.952
		R	TTCTCTGCCTGTGAGCATCC			
TaBAM1	X98504	F	ATGATCCTGAAAGCAGCGCA	119	91.6	0.959
		R	TTCTACACAGGGGCAACGAG			
TaBAM2	JP210880	F	AATGGAAGCGAGCGTGCAA	100	95.2	0.950
		R	CTCGTCACCCTTCTCGAACCT			
TaBAM3	BQ804909	F	CTTCACCTACCTCCGCATGG	193	92.5	0.993
		R	TTAGTTGCAGAGGGCGACG			
TaBAM4	BE418955	F	AGTACATGCGAGCTCACCGC	107	107.9	0.962
		R	CACGTGAAGTTGAGCACGGT			
TaBAM6	AK334256	F	TGAGGGCGTAGATTGTCAGC	176	104.4	0.996
		R	TTTACATCTTGGAGCGGCGT			
TaBAM7	CJ908248	F	GACAAAGAGCGAGTCCTTCGA	292	99.3	0.958
		R	CAGCATTATCAGGCCCACGG			
TaBAM9	BQ245790	F	GGACTCGGAGCAAAGCTCAC	181	107.2	0.980
		R	GCCGCTCTTGTTGATGCCATGA			
TaAGL1	DR736759	F	TCGACTACATGGACGGCTTC	111	103.1	0.995
		R	GGACGTATTTCTGGGCGTTG			
TaAGL2	BE516492	F	ACCTTGTAGGTGAGGTAGGACC	132	90. 0	0.997
		R	GGACGTCAACCTCTACGGCT			
TaAGL5	JP210548	F	TCTGTGCAAGCACTTCACCT	106	91.4	0.989
		R	CGGCAGATCAGGGTGTGAAT			
TaAGL7	CK162983	F	GGCCAGGGACCACTTCATTA	159	102.2	0.993
		R	TAGGGTAAGCAGATGGGGCA			
TaPHO1	AK333857	F	TTTTGAGTCGAGGGCTGGTG	234	93.2	0.963
		R	TGCATAATGTCCAAGCTCCACT			
TaPHO2	AY237103	F	GCTGTTTGCAGATTATGTCTCT	161	94.3	0.995
		R	TGAGAAGATCAAGGTTGCTTG			

F, forward primer; R, reverse primer.

3.2.4 RNA extraction and cDNA synthesis

Total RNA samples were extracted from the endosperm of imbibing seeds as described before (Izydorczyk *et al.*, 2017). The RNA samples were treated with DNase (DNA-free kit, Ambion, TX, USA) to be eliminate genomic DNA contamination. The digested total RNA was subjected to cDNA using 5×iScript^{TM} Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturers instruction. The resulting cDNA samples was diluted 20X and used as a template for cDNA synthesis.

3.2.5 Real-time quantitative PCR assay

The real-time qPCR assays were performed on CFX96 real-time system (Bio-Rad) with 20 µl total reaction volume containing 5 µl of the diluted cDNA as template, 10 µl of SsoFast EvaGreen Supermix (Bio-Rad), 1.2 µl of 5 µM forward primer, 1.2 µl of 5 µM reverse primer and 2.6 µl sterile deionized water as described previously (Yao *et al.*, 2012). Samples were subjected to the following thermal cycling conditions: DNA polymerase activation at 95ºC for 5 min followed by 40 cycles of denaturation at 95ºC for 15 s, annealing at 50-66ºC (depending on the melting temperature of the primers of the target genes) for 30 s and extension at 72ºC for 30 s in 96-well optical reaction plates (Bio-Rad). qPCR assay of each sample was performed in duplicate. The wheat β-actin gene (*Taβactin*) was used as a reference gene to normalize transcript levels of target genes. The relative transcript level of the target genes was analyzed as described previously (Livak and Schmittgen, 2001).

3.2.6 Extracting the expression of starch degrading genes from microarray dataset

Microarray experiments were performed with dormant and non-dormant (after-ripened) seed samples of cv. AC Domain, which exhibits a high level of dormancy. The seed samples (both dormant and non-dormant) were imbibed for 12 and 24 h. The non-dormant after-ripened seeds were also imbibed in 50 µM ABA solution for a period of 24 h. Preparation of the mRNA samples from dry (at 0 HAI) and imbibed (at 12 and 24 HAI) seeds for the microarray experiment and subsequent analysis of the resulting dataset were performed exactly as described in Gao *et al*. (2012). After extraction of $\log_2$ transformed signal intensities of probesets corresponding to starch degrading gene families from the microarray dataset, changes in gene expression were presented in $\log_2$ or linear fold changes between seed imbibition stages (0, 12 and 24 HAI) within dormant and non-dormant seed samples, and between the different seed samples/treatments at each stage of imbibition. Generation of heat maps for the probesets identified as starch degrading genes was performed by MultiExperiment Viewer (MeV version 4.6) software using expression values in $\log_2$ fold change.

3.2.7 Enzyme extractions and assays

Frozen endosperm samples of RL4452 (2-6; depending on the imbibition stage) were ground to fine powder in liquid nitrogen by using pre-chilled mortar and pestle. Three independent biological replicates (two technical replicates for each biological replicate) were analyzed for each assay. The extraction and assay for α-amylase and β-amylase were performed using the Ceralpha Method (K-CERA kit, Megazyme International Ltd., Wicklow, Ireland) and Betamyl-3 Method (K-BETA3 kit, Megazyme International Ltd., Wicklow, Ireland). The extraction and assay for α-glucosidase was performed as described previously (Sun and Henson, 1990; Skadsen

et al., 1998) with minor modification. Briefly, the fine powder of each sample was homogenized with extraction buffer containing 50 mM sodium phosphate (pH 9.0), 1 M NaCl, and 1% Triton X-100. The homogenates were centrifuged at 6,000g for 10 min at room temperature. 4-Nitrophenyl α-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50 mM sodium acetate (pH 4.6) for use as substrate. The extract (0.1 ml) was mixed with 1 ml of the substrate and then incubated in a water bath at 37°C for 30 min. The reaction was terminated by adding 0.1 ml of 1 M NaOH and absorbance was determined at 420 nm against water blank (0.1 ml of distilled water instead of 0.1 ml extract). The assay for each sample was performed in duplicate.

3.2.8 Starch content measurements

Freeze dried endosperm samples of RL4452 (10-13; depending on the stage) were ground to fine powder by ball mill. Approximately 100 mg of the powder samples was mixed with 5 ml ethanol (80% v/v) and then incubated in a water bath at ~80-85°C for 5 min, followed by addition of another 5 ml ethanol (80% v/v) and subsequent centrifugation at 1800*g*for 10 min. After discarding the supernatant, the pellet was re-suspended in 10 ml 80% ethanol using a vortex mixer followed by centrifugation at 1800*g* for 10 min. The pellet, after discarding the supernatant, was used for measuring total starch content. The extraction and assay for determination of total starch content were performed according to the amyloglucosidase-α-amylase method (K-TSTA kit, Megazyme International Ltd., Wicklow, Ireland). The assay for each sample was performed in duplicate.

3.2.9 Analysis of sugars with high performance anion exchange chromatography

Approximately 100 mg of the ground freeze dried seed samples of RL4452 was mixed with 1 ml 80% ethanol, and then immediately placed in a water bath at 70~75°C for 10 min with constant mixing. The mixture was centrifuged at 3500*g* for 10 min, and the supernatant transferred to a fresh tube. The pellet remaining after the transfer of the supernatant was re-suspended with 1 ml 80% ethanol, incubated in a water bath at 70~75°C for 10 min and then centrifuged at 3500 *g* for 10 min. The step of washing the sample with ethanol was repeated one more time. The supernatants from the three washes were pooled and completely dried under a nitrogen gas stream. The residue was then mixed with 2 ml deionized water followed by incubation in boiling water for 5 min.

The samples were filtered through a 0.45 μm GH Polypro (GHP) acrodisc syringe filter (PALL Corporation, Mississauga, ON) and analysed with high-performance anion exchange chromatography (HPAEC; Dionex-ICS-5000; Dionex, Sunnyvale, CA, USA) equipped with pulsed amperometric detector (PAD). Separation of sugars (glucose, maltose, sucrose, fructose) in the sample was performed with CarboPac PA-1 column (4*250 mm i.d., Dionex)) and a CarboPac PA-1 guard column (4*50 mm i.d., Dionex) at 30°C using 100 mM sodium hydroxide as eluent A and 100 mM sodium hydroxide containing 400 mM sodium acetate as eluent B. A sample of 10 μl volume was injected and eluted at a flow rate of 1 ml/min with a linear gradient of 50% eluent B for 30 min followed by 100% eluent B for 1 min for washing and 5% eluent B for 15 min for equilibration. Standards of glucose, maltose, maltotriose and maltotetraose (Sigma-Aldrich) were run to determine the retention time. The sugars were identified and quantified relative to known standards. Data collection and peak analysis was performed using the Chromeleon 7.1 (Dionex) software.

3.2.10 Statistical analysis

Significant differences in transcript level, enzyme activities, starch content and sugar level between control and AVG treated samples were determined using Student's t-test ($P<0.05$).

3.3 Results

3.3.1 Identification of starch degrading homologs gene in wheat

Our BLAST search of the GeneBank database for starch degrading genes using available nucleotide sequences from barley identified six *AMY*, nine *BAM*, seven *AGL* and three *PHO* homolog genes in wheat (Table 3.3).

Table 3.3 Starch degrading candidate genes of wheat and their corresponding GenBank IDs.

Name	Unigene ID	GenBank ID	Reference
TaAMY1	Ta.10281	CV772865	Allard *et al.* (2003)
TaAMY2	Ta.58571	CA701040	Unpublished
TaAMY3	Ta.75241	CJ674884	Mochida *et al.* (2006)
TaAMY4	Ta.490	JV989039	Duan *et al.* (2012)
TaAMY5	Ta.47895	AK335373	Kawaura *et al.* (2009)
TaAMY6	Ta.21984	JP208114	Pont *et al.* (2011)
TaBAM1	Ta.3	X98504	Wagner *et al.* (1996)
TaBAM2	Ta.69943	JP210880	Pont *et al.* (2011)
TaBAM3	Ta.4494	BQ804909	Pont *et al.* (2011)
TaBAM4	Ta.92251	BE418955	Anderson *et al.* (2000)
TaBAM5	Ta.32649	AK336127	Kawaura *et al.* (2009)
TaBAM6	Ta.1062	AK334256	Kawaura *et al.* (2009)
TaBAM7	Ta.40299	CJ908248	Manickavelu *et al.* (2010)
TaBAM8	Ta.137	Y16242	Unpublished
TaBAM9	Ta.54269	BQ245790	Unpublished
TaAGL1	Ta.54720	DR736759	Unpublished
TaAGL2	Ta.35523	BE516492	Unpublished
TaAGL3	Ta.38277	CJ566522	Mochida *et al.* (2006)
TaAGL4	Ta.25769	BM136180	Unpublished
TaAGL5	Ta.35088	JP210548	Pont *et al.* (2011)
TaAGL6	Ta.46255	CJ576180	Mochida *et al.* (2006)
TaAGL7	Ta.69833	CK162983	Unpublished
TaPHO1	Ta.1279	AK333857	Kawaura *et al.* (2009)
TaPHO2	Ta.1197	AY237103	Schupp and Ziegler (2004)
TaPHO3	Ta.27525	BT009168	Tingey *et al.* (2002)

3.3.2 Phylogenetic relationship analysis of candidate starch degrading genes

3.3.2.1 Phylogenetic analysis of α-amylase candidate genes

Phylogenetic analysis of the wheat *AMY* genes along with the representative homologs from other species, revealed that the six *AMY* genes of wheat were grouped with more than one homolog derived from the other species (Fig. 3.1).

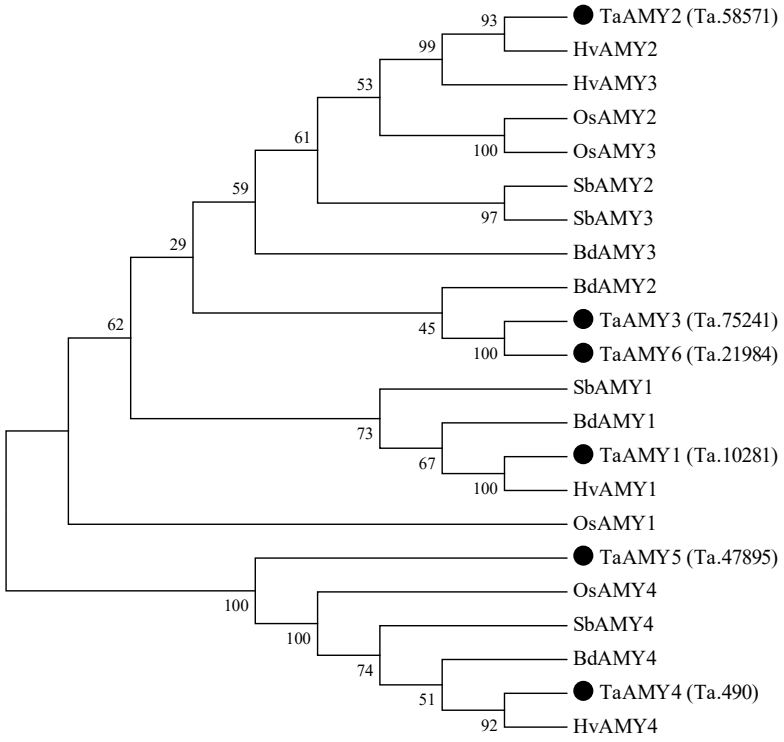

Figure 3.1 Phylogenetic relationships of wheat *AMY* genes with the homologs from other species, including *Hordeum vulgare*, *Oryza sativa*, *Brachypodium distachyon* and *Sorghum bicolor*. Phylogenetic tree of *AMY* genes was generated based on nucleic acid sequence similarity using MEGA program. The trees were inferred using Maximum Likelihood method based on Tamura-nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates is shown next to the branches. ●, wheat *AMY* gene with the corresponding Unigene ID.

The *AMY2* and *AMY3* genes of barley, rice and sorghum showed highly similarity and close phylogenetic relationship between each other. The *TaAMY1*, *TaAMY2* and *TaAMY4* genes

are grouped with the *AMY1*, *AMY2* and *AMY4* genes, respectively, with high bootstrap value. The *TaAMY3* and *TaAMY6* genes are clustered with *BdAMY2* but with a low (45) bootstrap value (Fig. 3.1). Similarly, *HvAMY1* is grouped with *BdAMY1* with lower bootstrap value of 67.

3.3.2.2 Phylogenetic analysis of β-amylase candidate genes

Phylogenetic analysis of the wheat *TaBAM* genes along with the representative homologs from other species (Fig. 3.2) indicated the grouping of *TaBAM1*, *TaBAM2* and *TaBAM9* with *BAM1* and *BAM2* genes of barley and brachypodium. The *TaBAM3* gene was found to be clustered with the *BAM3* of barley with a very high bootstrap value. Likewise, *TaBAM4*, *TaBAM5*, *TaBAM6* and *TaBAM7*were clustered with more than one homolog derived from other species (Fig. 3.2).

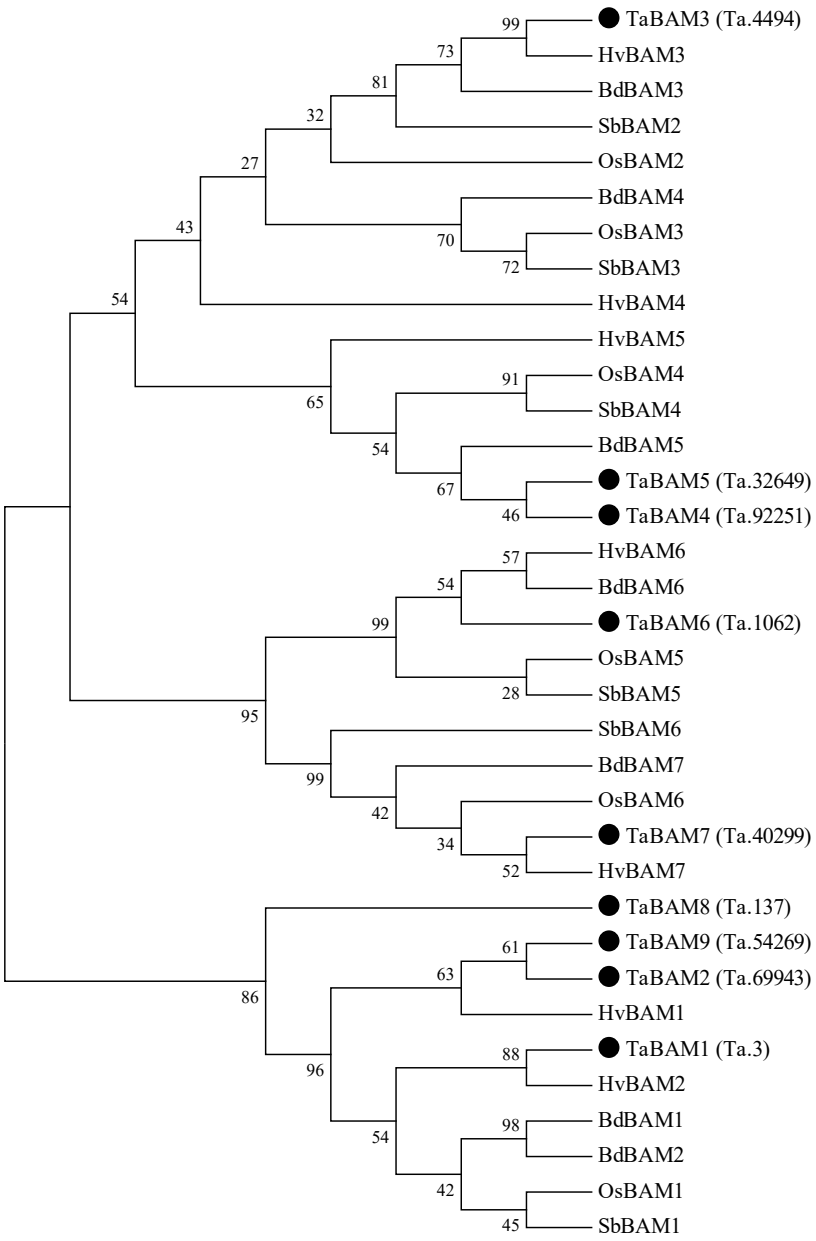

Figure 3.2 Phylogenetic relationships of wheat *BAM* genes with the homologs from other species, including *Hordeum vulgare*, *Oryza sativa*, *Brachypodium distachyon* and *Sorghum bicolor*. Phylogenetic tree of *BAM* genes was generated based on nucleic acid sequence similarity using MEGA program. The trees were inferred using Maximum Likelihood method based on Tamura-nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates is shown next to the branches. ●, wheat candidate *BAM* genes with the corresponding Unigene ID.

3.3.2.3 Phylogenetic analysis of α-glucosidase candidate genes

Of the seven *AGL* genes identified in wheat *TaAGL1* and *TaAGL4* are grouped with *HvAGL1* and *HvAGL4* genes, respectively. Three *AGL* genes of wheat including *TaAGL6*, *TaAGL3* and *TaAGL2* formed their own group, however, they still formed a group with *OsAGL2* and *SbAGL3* (Fig. 3.3). The *TaAGL5* and *TaAGL7* genes were clustered together and showed distant phylogenetic relationship with the other *AGL* homologs.

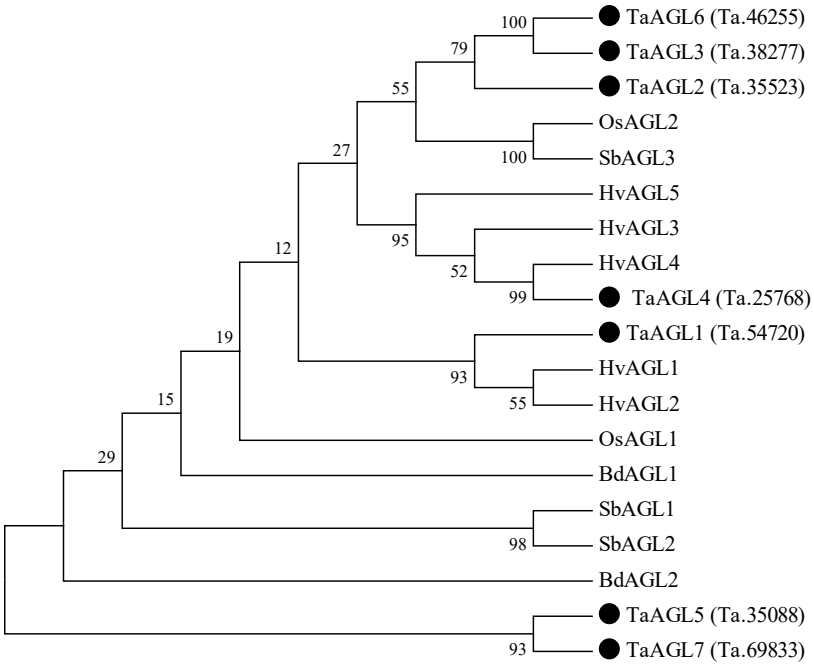

Figure 3.3 Phylogenetic relationships of wheat *AGL* genes with the homologs from other species, including *Hordeum vulgare*, *Oryza sativa*, *Brachypodium distachyon* and *Sorghum bicolor*. Phylogenetic tree of *AGL* genes was generated based on nucleic acid sequence similarity using MEGA program. The trees were inferred using Maximum Likelihood method based on Tamura-nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates is shown next to the branches. ●, wheat candidate *AGL* genes with the corresponding Unigene ID.

3.3.2.4 Phylogenetic analysis of phosphorylase candidate genes

Phylogenetic analysis of the three *PHO* genes of wheat along with the other homologs from other species indicated the clustering of *TaPHO1*, *TaPHO2* and *TaPHO3* with *HvPHO1*, *HvPHO2* and *SbPHO2* genes, respectively, with high bootstrap value. However, *TaPHO1* appeared to have a distant phylogenetic relationship with *TaPHO2*, *TaPHO3*, and the *PHO2* homologs from other species (Fig. 3.4).

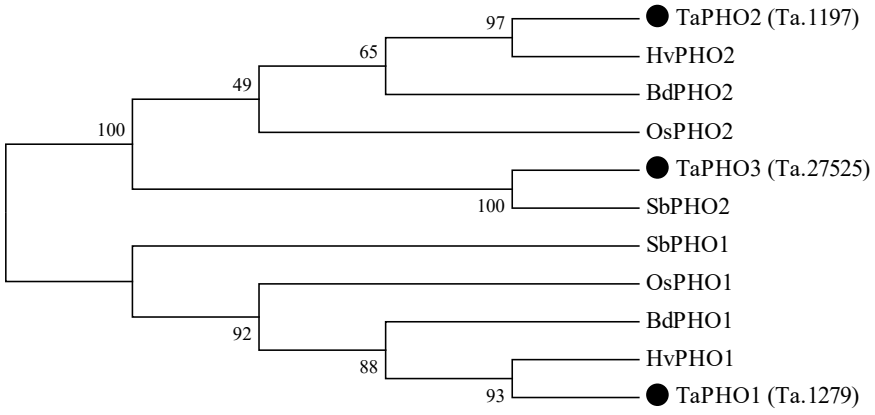

Figure 3.4 Phylogenetic relationships of wheat *PHO* genes with the homologs from other species, including *Hordeum vulgare*, *Oryza sativa*, *Brachypodium distachyon* and *Sorghum bicolor*. Phylogenetic tree of *PHO* genes was generated based on nucleic acid sequence similarity using MEGA program. The trees were inferred using Maximum Likelihood method based on Tamura-nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 500 replicates is shown next to the branches. ●, wheat candidate *PHO* genes with the corresponding Unigene ID.

3.3.3 Seed germination and seedling growth in response to inhibition of ethylene synthesis

Inhibition of ethylene biosynthesis with aminoethoxyvinylglycine (AVG) did not affect the germination percentage of seeds (Figure 3.5). Within 24 hours after imbibition (HAI), 94% and 92% of seeds germinated in the control untreated and AVG treated seeds. Seed germination reached 100% at 48 HAI.

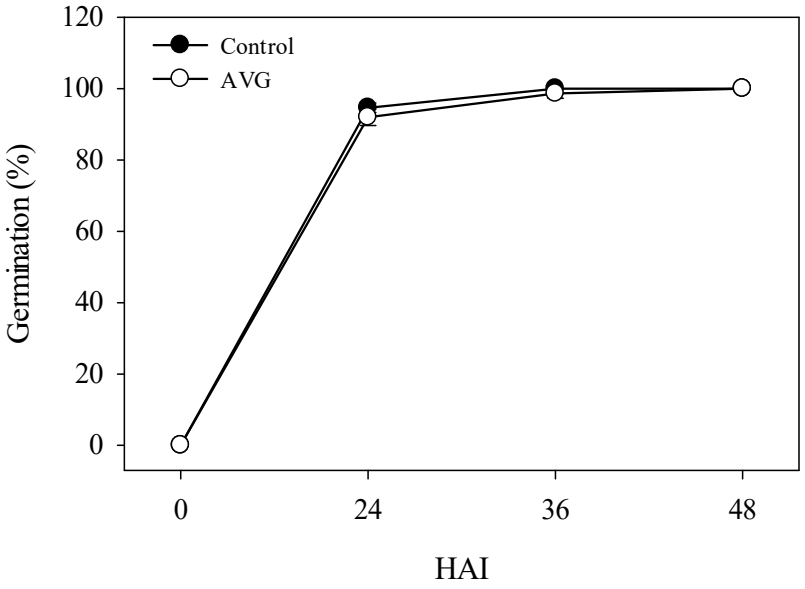

Figure 3.5 Germination percentage of control and AVG treated seeds of RL4452. Data are means ± SE, n =3, where n refers to a batch of 25 seeds. HAI, hours after imbibition.

Monitoring of the seedling growth over a period of seven days showed that inhibition of ethylene synthesis significantly reduced the length of radicle, coleoptile and seminal roots (Fig. 3.6), and the effect was more on the growth of radicle and seminal roots.

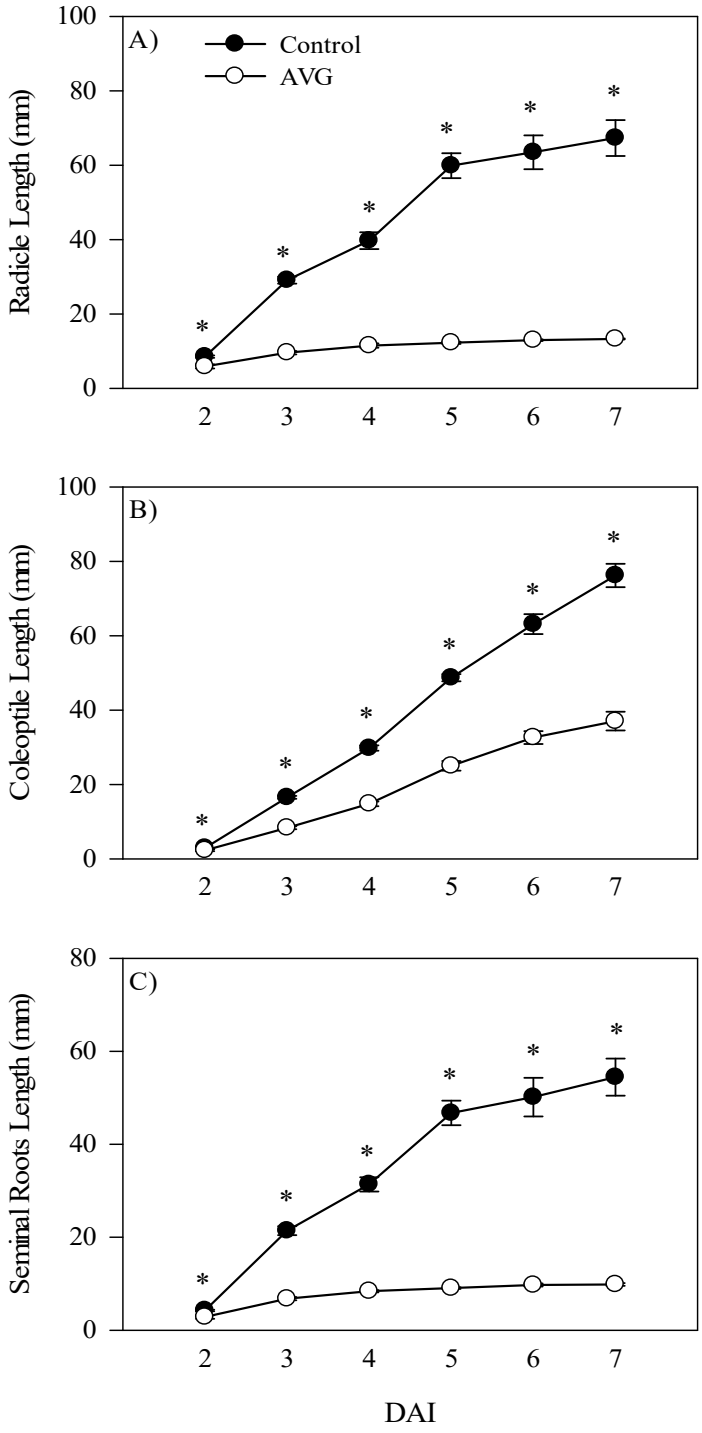

Figure 3.6 Length of radicle (A), coleoptile (B) and seminal roots (C) during seedling growth of the RL4452 genotype with or without aminoethoxyvinylglycine (AVG) treatment. Data are means ± SE, n =3, where n refers to a batch of 25 seeds. Asterisks indicate statistically significant difference in expression between the control and AVG treated samples using Student's t-test, $P<0.05$. DAI, days after imbibition.

3.3.4 Expression of starch degrading genes during germination and seedling growth

3.3.4.1 Expression of alpha-amylase genes

The expression patterns of genes encoding α-amylase (AMY) including *TaAMY1*, *TaAMY2*, *TaAMY4* and *TaAMY5* were examined in the endosperm tissue of dry and imbibed RL4452 seeds.

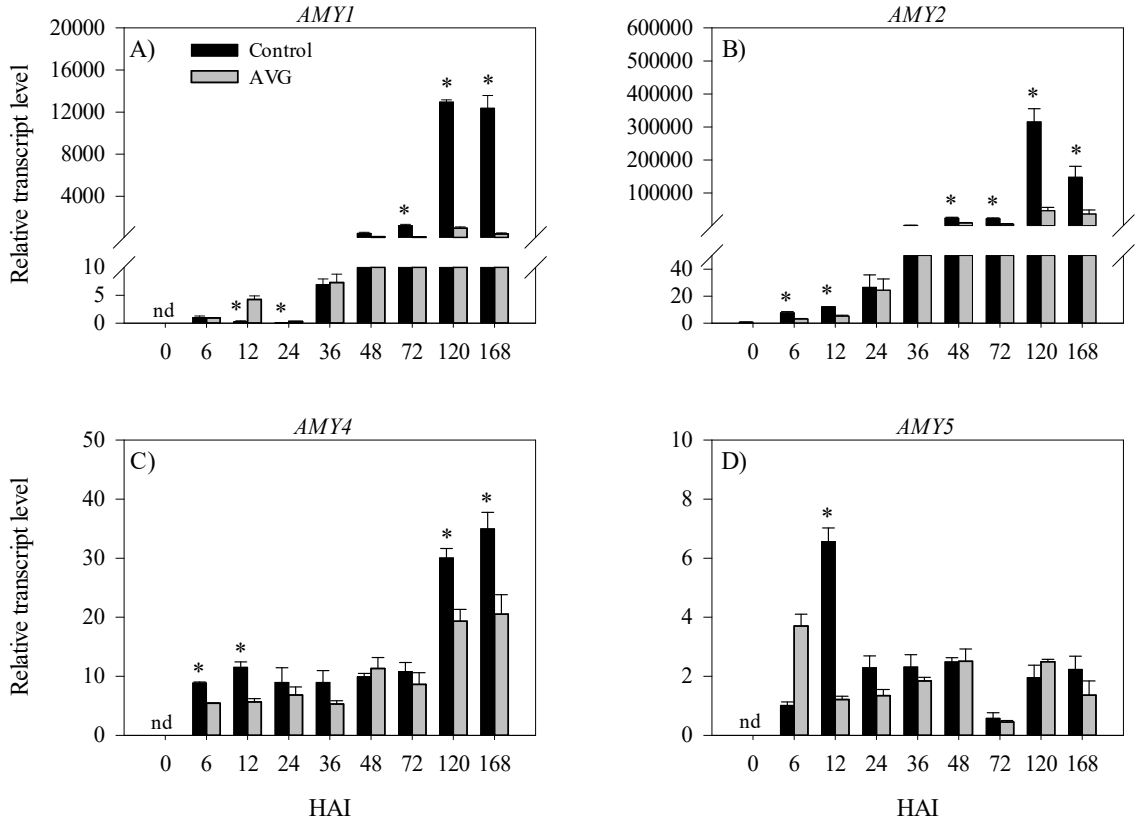

Figure 3.7 Relative transcript levels of *AMY1* (A), *AMY2* (B), *AMY4* (C), *AMY5* (D) in control and aminoethoxyvinylglycine (AVG) treated RL4452 seeds during germination and seedling growth. The transcript level of each gene were determined using β-actin as the reference gene and then expressed relative to that *AMY1* in 6 HAI control seeds, which were set to 1. Data are means of 2 to 3 independent biological replicates ± SE. Asterisks indicate statistically significant difference in expression between the control and AVG treated samples using Student's t-test, $P<0.05$. HAI, hours after imbibition; nd, not detected.

The transcripts of *AMY* genes were either not, or very minimally, detected in the endosperm of dry seeds; however, all the genes appeared to be expressed after imbibition in both

control and AVG treated seeds (Fig. 3.7). The expressions of *AMY1* and *AMY2* exhibited a substantial increase with imbibition in both treated and untreated seed samples. However, AVG treatment caused significant reduction in expression of *AMY1* and *AMY2* especially following 48 or 72 HAI. The expression patterns of *AMY4* were similar to that of *AMY1* and *AMY2* although the magnitude of gene expression was much lower. *AMY5* appeared to have similar level of expression over the entire imbibition period except the induction exhibited at 12 HAI in the control samples.

3.3.4.2 Expression of beta-amylase genes

Unlike the *AMY* genes, the transcripts of all *BAM* genes were detected in the endosperm of dry seeds, and their expression either remained at the same level or increased within 6 HAI. Afterwards, the expressions of all the *BAM* genes except *BAM1* exhibited decreases. The expression of *BAM1*, however, showed constant or increased expression (Fig. 3.8).

AVG treatment led to the repression of *BAM2*, *BAM6* and *BAM9* within 6 HAI (Fig. 3.8B, E, G) while causing upregulation of *BAM1*, *BAM3* and *BAM9* by 12 HAI (Fig. 3.8A, C, G). Following germination, the AVG treatment induced the expression of *BAM1* and *BAM6* by 36 and 72 HAI and that of *BAM7* by 72 HAI, while it inhibited the expression *BAM3* and *BAM7* at 36 HAI. The expression of *BAM2*, *BAM6* and *BAM9* by 120 and 168 HAI were significantly induced (2- to 10-fold) by AVG (Fig. 3.8B, E, G) while the expression of *BAM7* was induced (2-fold) only at 120 HAI (Fig. 3.8F). The expression of *BAM4* appeared not to be affected by AVG treatment except for the repression observed at 120 HAI.

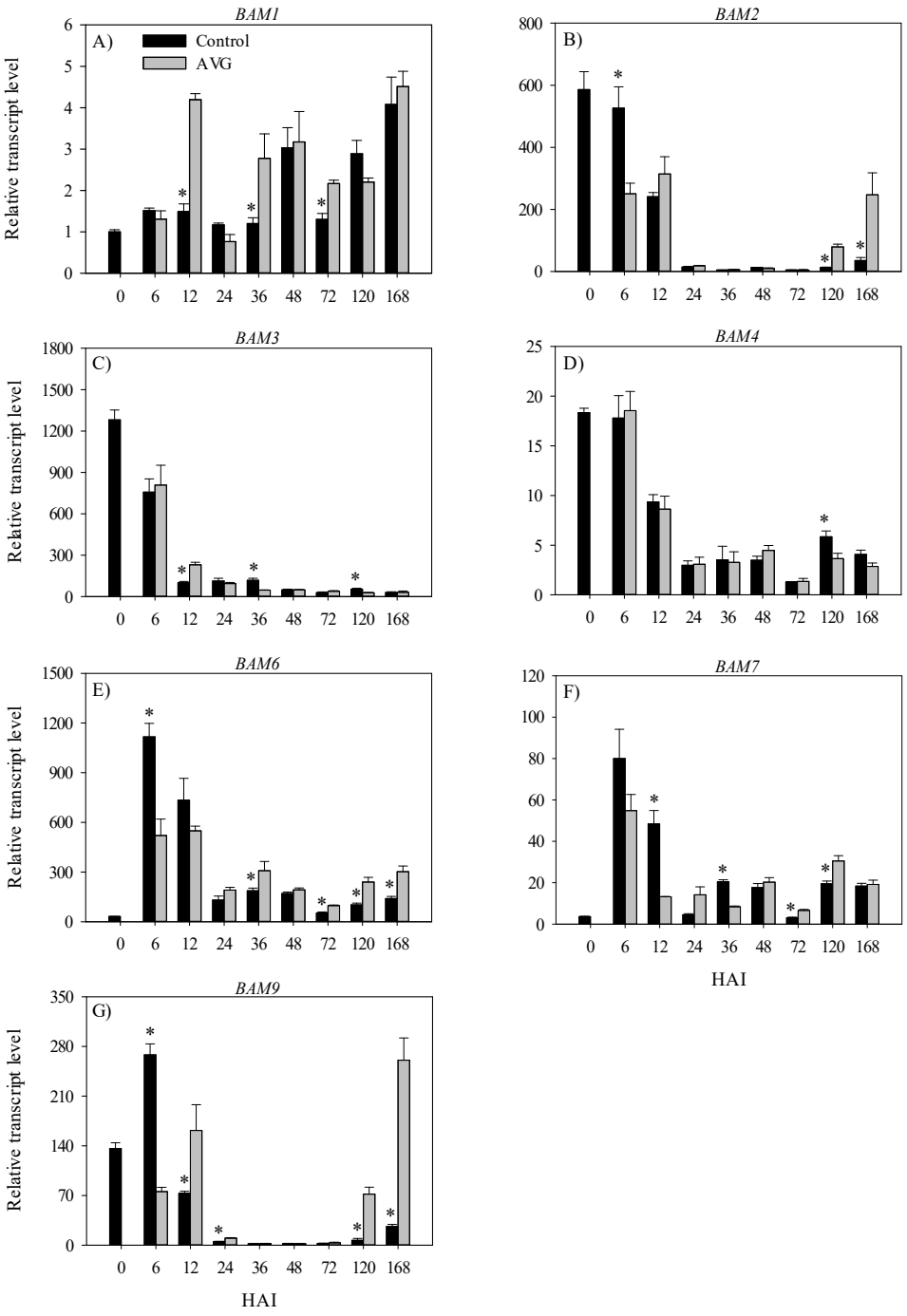

Figure 3.8 Relative transcript levels of *BAM1* (A), *BAM2* (B), *BAM3* (C), *BAM4* (D), *BAM6* (E), *BAM7* (F) and *BAM9* (G) in control and aminoethoxyvinylglycine (AVG) treated RL4452 seeds during germination and seedling growth. The transcript level of each gene were determined using β-actin as the reference gene and then expressed relative to that *BAM1* in 0 HAI control seeds, which were set to 1. Data are means of 2 to 3 independent biological replicates ± SE. Asterisks indicate statistically significant difference in expression between the control and AVG treated samples using Student's t-test, $P<0.05$. HAI, hours after imbibition.

3.3.4.3 Expression of alpha-glucosidase genes

The transcripts of all genes identified as encoding α-glucosidase (AGL) designated as *AGL1, AGL2, AGL5* and *AGL7* were detected in the endosperm of dry seeds (Fig. 3.9).

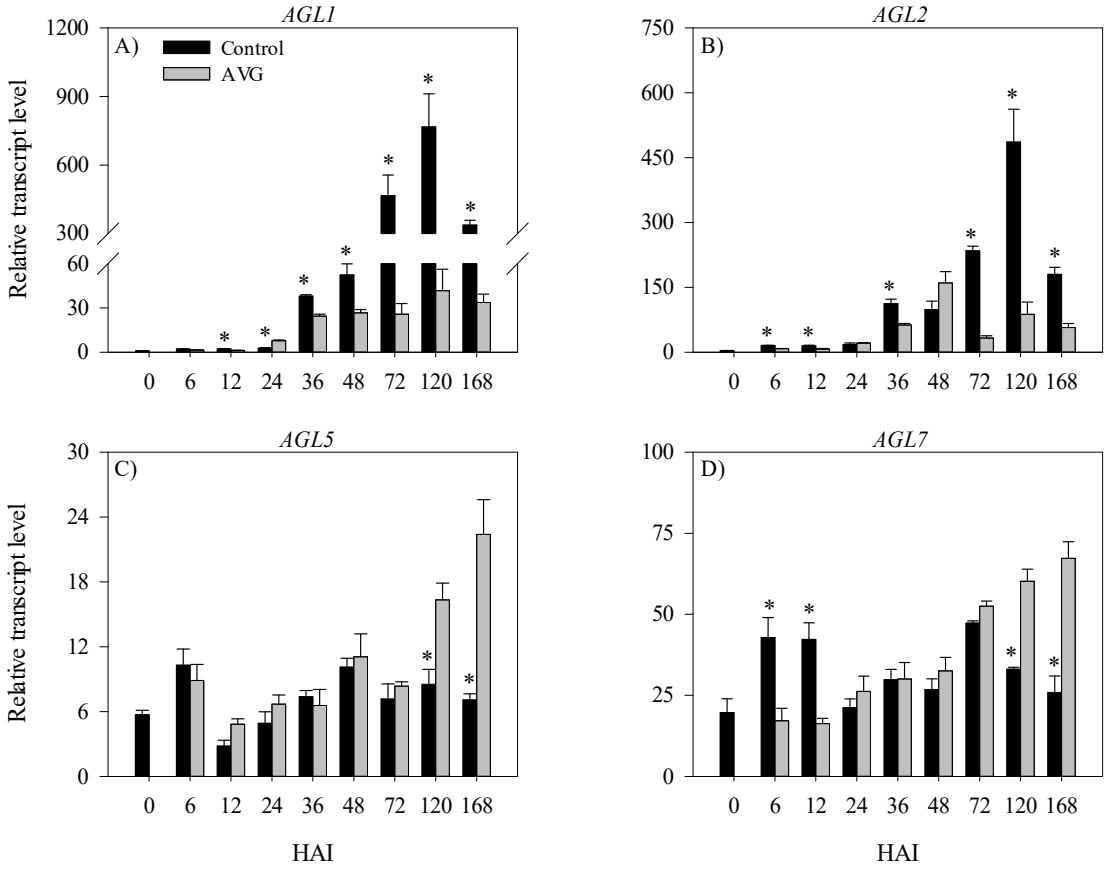

Figure 3.9 Relative transcript levels of *AGL1* (A), *AGL2* (B), *AGL5* (C) and *AGL7* (D) in control and aminoethoxyvinylglycine (AVG) treated RL4452 seeds during germination and seedling growth. The transcript level of each gene were determined using β-actin as the reference gene and then expressed relative to that *AGL1* in 0 HAI control seeds, which were set to 1. Data are means of 2 to 3 independent biological replicates ± SE. Asterisks indicate statistically significant difference in expression between the control and AVG treated samples using Student's t-test, $P<0.05$. HAI, hours after imbibition.

The expressions of *AGL1* and *AGL2* genes in the control seeds showed substantial increases with imbibition with the highest expression occurred at 120 HAI (Fig. 3.9A, B), while that of *AGL5* and *AGL7* appeared to maintain similar expression throughout the imbibition

period (Fig. 3.9C, D). The expressions of *AGL1* and *AGL2* are significantly inhibited (2- to 20-fold) by the AVG treatment almost throughout the entire period of imbibition. The AVG treatment also caused significant decrease in the expression of *AGL7*, but only during the early periods of imbibition (6 to 12 HAI). On the other hand, the expression of *AGL5* and *AGL7* were significantly upregulated (2- to 4-fold) in response to AVG by 120 and 168 HAI (Fig. 3.9C, D).

3.3.4.4 Expression of phosphorylase genes

The transcripts of all genes identified as encoding phosphorylase (PHO) were detected in the endosperm of dry seeds. While the transcript level of *PHO1* showed a slight decrease during imbibition, the expression of *PHO2* appeared to remain constant. The expression of *PHO1* and *PHO2* was not affected by AVG treatment except the AVG mediated inductions observed at 120 and 6 and 12 HAI, respectively (Fig. 3.10).

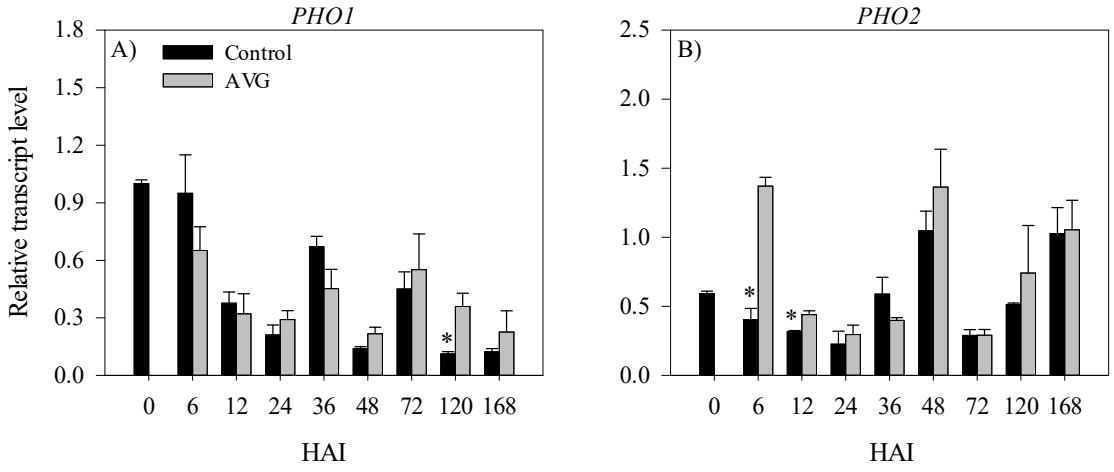

Figure 3.10 Relative transcript levels of *PHO1* (A), *PHO2* (B) in control and aminoethoxyvinylglycine (AVG) treated RL4452 seeds during germination and seedling growth. The transcript level of each gene were determined using β-actin as the reference gene and then expressed relative to that *PHO1* in 0 HAI control seeds, which were set to 1. Data are means of 2 to 3 independent biological replicates ± SE. Asterisks indicate statistically significant difference in expression between the control and AVG treated samples using Student's t-test, $P<0.05$. HAI, hours after imbibition.

3.3.5 Analysis of the activity of starch degrading enzymes during germination and seedling growth

The assays for determining activities of starch degrading enzymes were performed at six imbibition time points. The activities of α-amylase, β-amylase and α-glucosidase were detected in the endosperm of dry seeds (Figure 3.11, Appendix I Figure S1). The activity of α-amylase in the endosperm of control seeds showed a substantial increase with imbibition, reaching the highest level by 7 DAI. (Fig. 3.11A). Treatment with AVG led to significant reduction (2- to 10-fold) in the activity of α-amylase from 2 DAI. (Fig. 3.11A). The activity of β-amylase detected in the endosperm of dry seeds was maintained from 1 to 3 DAI, and showed a slight increase at 5 and 7 DAI. No difference in β-amylase was detected between control and AVG treated seeds (Fig. 3.11B). The activity of α-glucosidase detected in the endosperm of dry seeds was maintained throughout the imbibition period except the 2-fold increases observed by 5 and 7 DAI (Fig. 3.11C). Although no difference in α-glucosidase activity was observed between control and AVG treated seeds from 1 to 5 DAI, the AVG treatment caused significant suppression (1.5-fold) in α-glucosidase activity by 7 DAI (Fig. 3.11C).

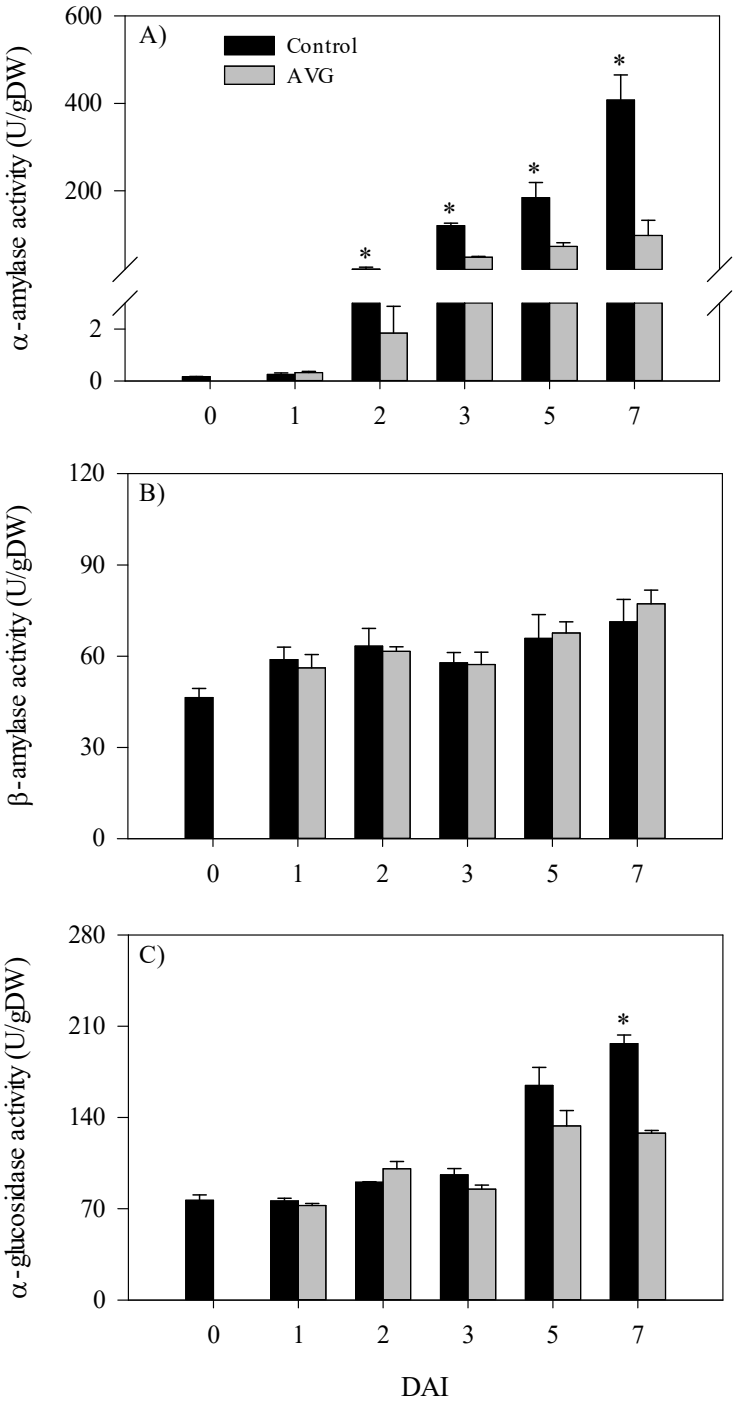

Figure 3.11 Activity of α-amylase (A), β-amylase (B) and α-glucosidase (C) per gram endosperm dry weight during germination and seedling growth of RL4452. Enzyme activities are expressed per gram endosperm. Data are means of three independent biological replicates ± SE. Astrerisks indicate statistically significant difference between the control and aminoethoxyvinylglycine (AVG) treated samples within each imbibition time point using Student's t-test, $P<0.05$. DAI, days after imbibition. U, unit; DW, dry weight.

3.3.6 Analysis of dry weight and starch contents during germination and seedling growth

The dry weight of the endosperm of the control seeds showed only a gradual decrease within the first 3 DAI, but declined drastically afterwards (Fig. 3.12). The decrease in dry weight of the endosperm of AVG treated seeds was similar to that of the control seeds within the first 3 DAI. However, the AVG treated seeds showed a slower rate of decrease in dry weight at 5 and 7 DAI (Fig. 3.12).

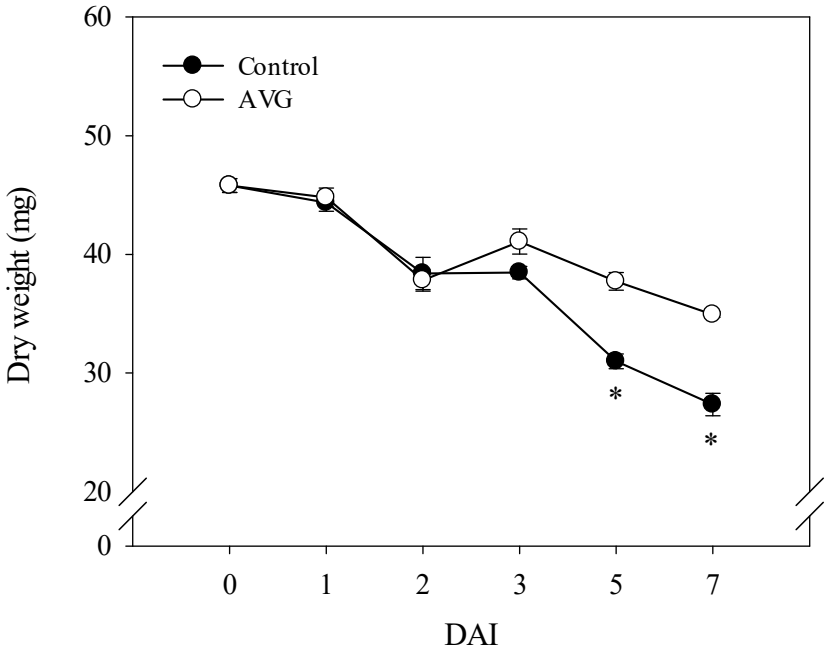

Figure 3.12 Change in dry weight per endosperm (dry weight basis) during germination and seedling growth of RL4452. Data are means ± SE, n =3, where n refers to a batch of 20 endosperms. Astrerisks indicate statistically significant difference between the control and aminoethoxyvinylglycine (AVG) treated samples within each imbibition time point using Student's t-test, $P<0.05$.

The starch content of the endosperm was also measured during germination and seedling growth. Only a slightly decrease of starch content was observed in both control and AVG treated endosperms from 1 to 3 DAI; however, the starch content exhibited a drastic decrease as

imbibition continued through 5 and 7 DAI. (Fig. 3.13, Appendix I Figure S2). Treatment with AVG slowed the decrease of starch content at 5 and 7 DAI markedly, leading to the presence of a higher amount of starch content in AVG treated than control endosperms (Fig. 3.13).

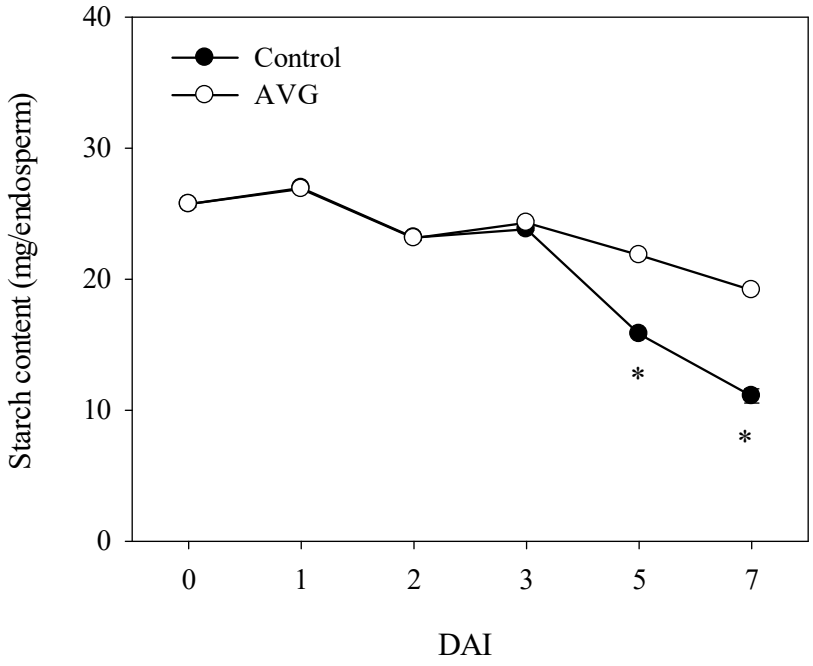

Figure 3.13 Changes in starch content per endosperm (dry weight basis) during germination and seedling growth of RL4452. Data are means of three independent biological replicates ± SE. DAI, days after imbibition. Astrerisks indicate statistically significant difference between the control and aminoethoxyvinylglycine (AVG) treated samples within each imbibition time point using Student's t-test, $P<0.05$.

3.3.7 Analysis of sugar contents during germination and seedling growth

Glucose and maltose were detected in the endosperm of dry seeds and the contents of these sugars showed marked increases (50- to 80-fold) with imbibition (Fig. 3.14A, B, Appendix I Figure S3). The highest increases of glucose and maltose (4- to 7.5-fold) occurred as imbibition continued from 2 to 3 DAI. The effect of AVG treatment on glucose and maltose contents was

evident only after 2 DAI, when it caused significant reduction (1.5- to 2-fold) in the amount of endospermic glucose and maltose.

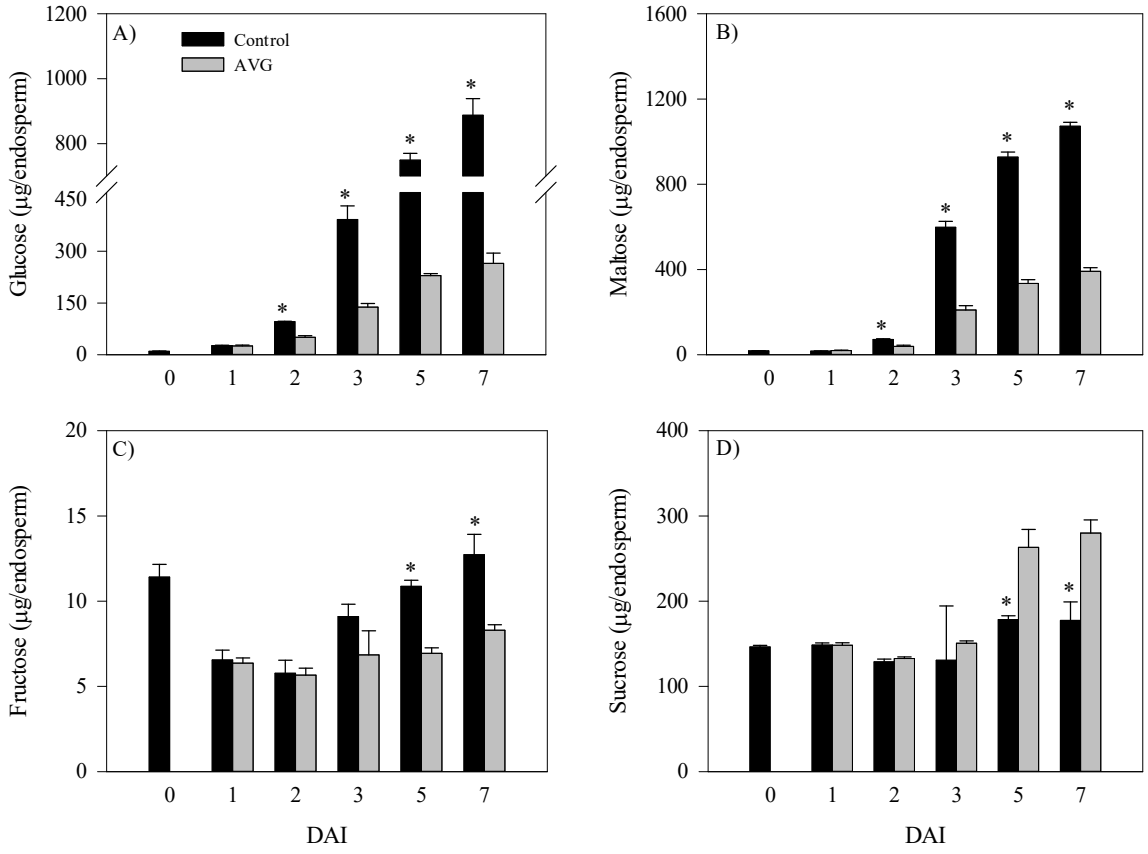

Figure 3.14 Changes in glucose (A), maltose (B), fructose (C) and sucrose (D) contents per endosperm (dry weight basis) during germination and seedling growth of RL4452. Data are means of three independent biological replicates ± SE. Asterisks indicate statistically significant difference between the control and aminoethoxyvinylglycine (AVG) treated samples within each imbibition time point using Student's t-test, $P<0.05$. DAI, days after imbibition.

Fructose and sucrose were also detected in the endosperm of dry seeds and this level was maintained almost during the entire imbibition period. The levels of fructose and sucrose showed no differences between the control and AVG treated endosperm at 1, 2, and 3 DAI; however, the level of fructose was significantly suppressed by AVG at 5 and 7 DAI (Fig. 3.14C), while that of sucrose showed a significant increase (Fig. 3.14D).

3.3.8 Expression pattern analysis of starch degrading genes between dormant and non-dormant wheat seeds

3.3.8.1 α-amylase genes

This part of the study involved the use of dormant and after-ripened seeds of cv. AC Domain, which shows high level of dormancy at the time of harvest. Relative to that observed in the dry seeds, the expression of all probesets annotated as *AMY* did not change during imbibition of the dormant seeds (Fig. 3.15; Appendix II Table S1-S3).

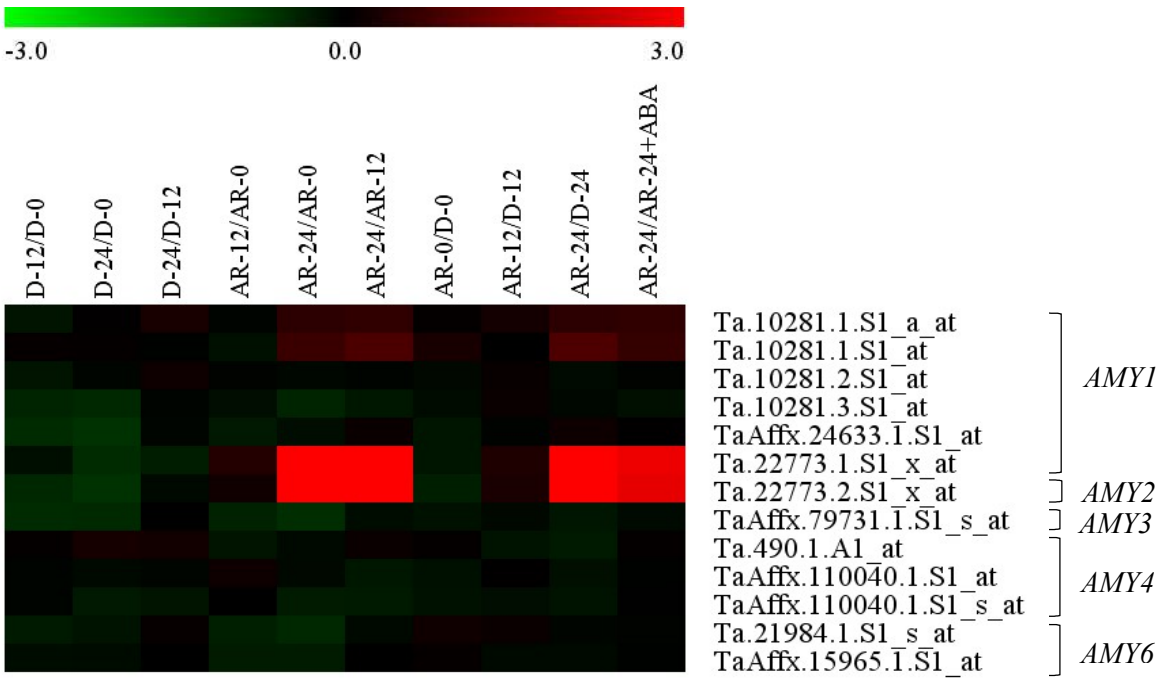

Figure 3.15 Changes in the expression of probesets annotated as *AMY* genes (log_2-scale) during imbibition of cv. AC Domain dormant (D-12/D-0, D-24/D-0 and D-24/D-12) and after-ripened seeds (AR-12/AR-0, AR-24/AR-0, AR-24/AR-12), between dormant and after-ripened seeds in both dry and imbibed states (AR-0/D-0, AR-12/D-12 and AR-24/D-24) and between water and ABA imbibed after-ripened seeds (AR-24/AR-24+ABA) as shown in the heat map. Log_2 transformed signal intensities of the respective probesets were extracted from a microarray dataset and then converted to expression values in log_2 fold changes (the negative and positive numbers on the bar) shown by the color scale at the top of each heat map. Higher and lower expression levels of respective probesets are represented by red and green colors, respectively. Log_2 and linear scaled fold changes in expression of the probesets and the respective *P* values can be found in Appendix II, Table S1, S2 and S3.

The expression of one of the six probesets annotated as *AMY1* and a probeset annotated as *AMY2* were upregulated (26- to 33-fold) following 24 HAI in after-ripened seeds (Fig. 3.15), leading to their 30- to 39-fold higher level of expression in after-ripened seeds than that observed in dormant seeds. Imbibing the after-ripened seeds with ABA led to downregulation (over 4.5-fold) of one of six probesets annotated as *AMY1* and a probeset annotated as *AMY2* (Fig. 3.15).

3.3.8.2 β-amylase genes

The expression of probesets annotated as *BAM1*, *BAM3* and *BAM9* showed downregulation (3.5- to 10.5-fold) during imbibition of the dormant seeds (Fig. 3.16; Appendix II Table S4-S6). Probesets annotated as *BAM1, BAM3, BAM5* and *BAM9* genes were downregulated (3- to 10.5-fold) during imbibition of after-ripened seeds. Comparative analysis of the expression of probesets annotated as *BAM* genes between dormant and after-ripened seeds in both dry and imbibed states revealed no change in expression, except two of the three probesets annotated as *BAM1* and two probesets annotated as *BAM3* exhibited downregulation (~2.5-fold) in the after-ripened seeds by 24 HAI (Fig. 3.16). No difference in the expression of probesets annotated as *BAM* genes was observed due to ABA treatment in the after-ripened seeds (Fig. 3.16).

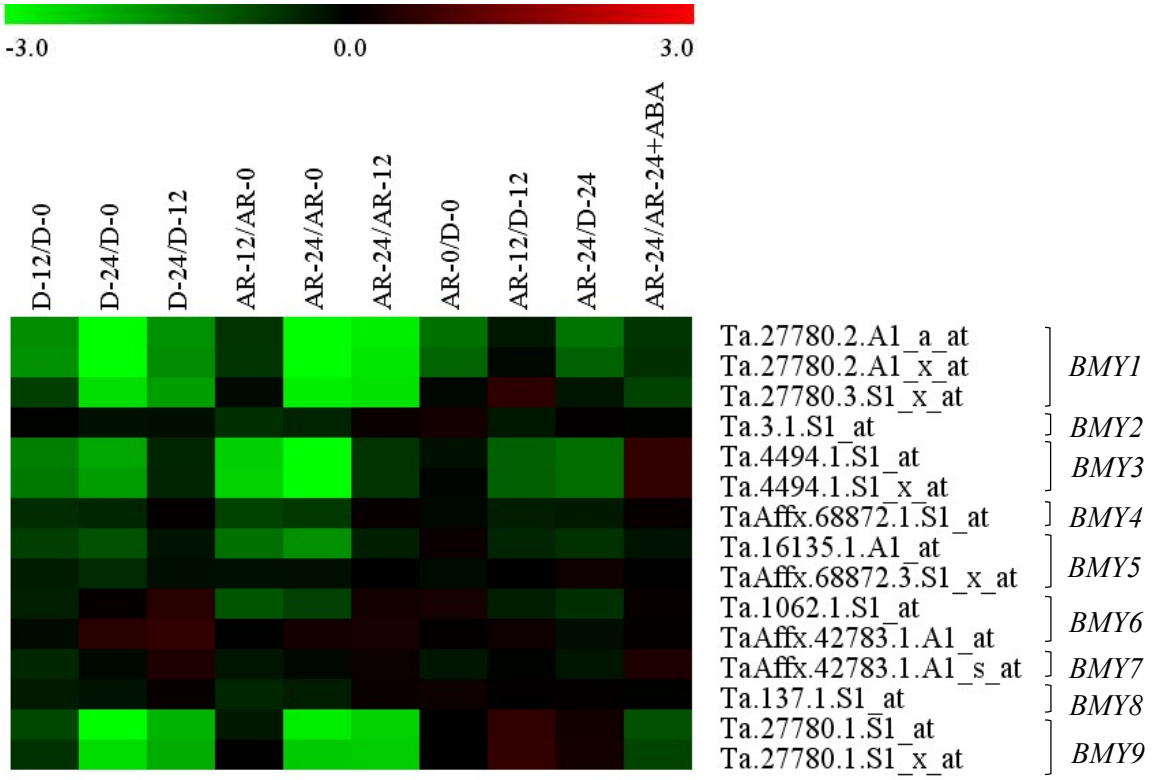

Figure 3.16 Changes in the expression of probesets annotated as *BAM* genes (log2-scale) during imbibition of cv. AC Domain dormant (D-12/D-0, D-24/D-0 and D-24/D-12) and after-ripened seeds (AR-12/AR-0, AR-24/AR-0, AR-24/AR-12), between dormant and after-ripened seeds in both dry and imbibed states (AR-0/D-0, AR-12/D-12 and AR-24/D-24) and between water and ABA imbibed after-ripened seeds (AR-24/AR-24+ABA) as shown in the heat map. Log_2 transformed signal intensities of the respective probesets were extracted from a microarray dataset and then converted to expression values in log2 fold changes (the negative and positive numbers on the bar) shown by the color scale at the top of each heat map. Higher and lower expression levels of respective probesets are represented by red and green colors, respectively. Log_2 and linear scaled fold changes in expression of the probesets and the respective *P* values can be found in Appendix II, Table S4, S5 and S6.

3.3.8.3 α-glucosidase genes

No changes in the expression of probesets annotated as *AGL* genes was detected during imbibition of dormant seeds (Fig. 3.17; Appendix II Table S7-S9). Four probesets annotated as *AGL1* were upregulated (4.5- to 28-fold) in after-ripened seeds by 24 HAI, leading to their higher level of expression (5- to 35-fold) in after-ripened seeds than dormant seeds. Treatment

with ABA of the after-ripened seeds resulted in downregulation (2.4- to 4.9-fold) of four probesets annotated as *AGL1* and a probeset annotated as *AGL5* (Fig. 3.17).

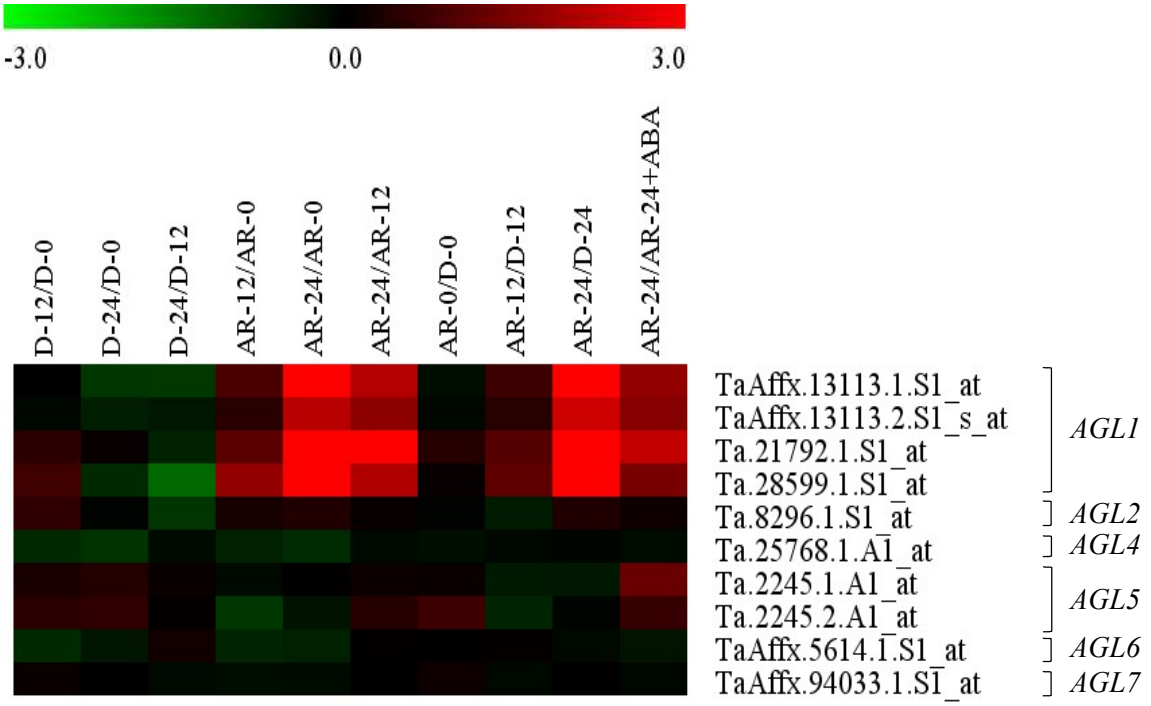

Figure 3.17 Changes in the expression of probesets annotated as *AGL* genes (log_2-scale) during imbibition of cv. AC Domain dormant (D-12/D-0, D-24/D-0 and D-24/D-12) and after-ripened seeds (AR-12/AR-0, AR-24/AR-0, AR-24/AR-12), between dormant and after-ripened seeds in both dry and imbibed states (AR-0/D-0, AR-12/D-12 and AR-24/D-24) and between water and ABA imbibed after-ripened seeds (AR-24/AR-24+ABA) as shown in the heat map. Log_2 transformed signal intensities of the respective probesets were extracted from a microarray dataset and then converted to expression values in log_2 fold changes (the negative and positive numbers on the bar) shown by the color scale at the top of each heat map. Higher and lower expression levels of respective probesets are represented by red and green colors, respectively. Log_2 and linear scaled fold changes in expression of the probesets and the respective *P* values can be found in Appendix II, Table S7, S8 and S9.

3.3.8.4 Phosphorylase genes

The expression of probesets annotated as *PHO1* and *PHO3* did not change during imbibition of dormant seeds, while two of three probesets annotated as *PHO2* were downregulated (2.3- to 3.2-fold) at 12 and 24 HAI (Fig. 3.18; Appendix II Table S10-S12). In after-ripened seeds, two

of three probesets annotated as *PHO2* exhibited ~2-fold upregulation by 24 HAI as compared to that observed in the dry seeds (Fig. 3.18). Following 24 h imbibition, the expression of two probesets annotated as *PHO2* showed upregulation (~5.5- fold) in after-ripened seeds as compared to that observed in dormant seeds, and ABA treatment did not affect the expression of these probesets (Fig. 3.18).

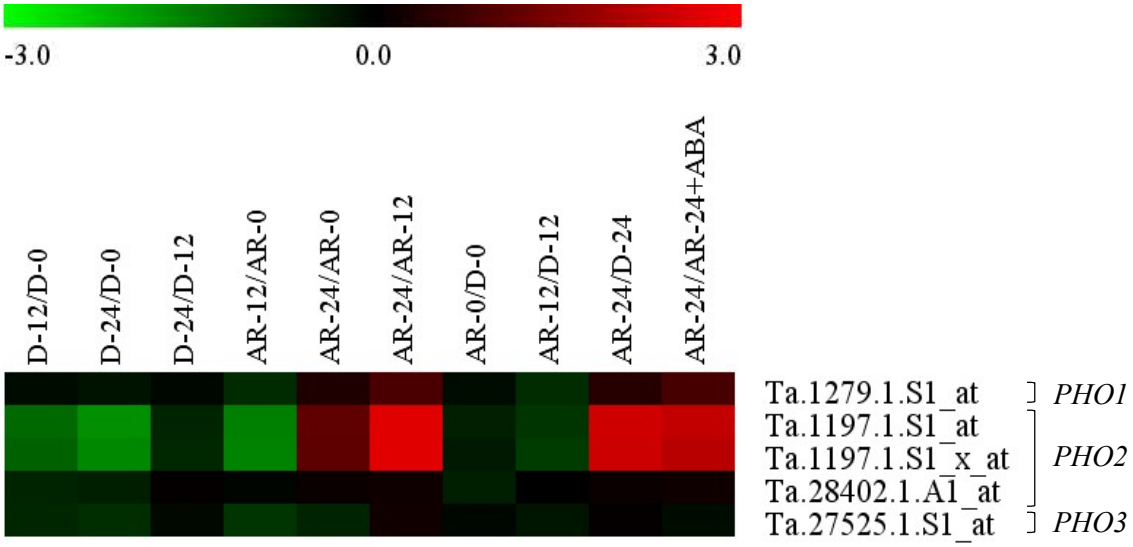

Figure 3.18 Changes in the expression of probesets annotated as *PHO* genes (log_2-scale) during imbibition of cv. AC Domain dormant (D-12/D-0, D-24/D-0 and D-24/D-12) and after-ripened seeds (AR-12/AR-0, AR-24/AR-0, AR-24/AR-12), between dormant and after-ripened seeds in both dry and imbibed states (AR-0/D-0, AR-12/D-12 and AR-24/D-24) and between water and ABA imbibed after-ripened seeds (AR-24/AR-24+ABA) as shown in the heat map. Log_2 transformed signal intensities of the respective probesets were extracted from a microarray dataset and then converted to expression values in log_2 fold changes (the negative and positive numbers on the bar) shown by the color scale at the top of each heat map. Higher and lower expression levels of respective probesets are represented by red and green colors, respectively. Log_2 and linear scaled fold changes in expression of the probesets and the respective *P* values can be found in Appendix II, Table S10, S11 and S12.

3.4 Discussion

This study investigated starch degradation and its regulation by ethylene using different approaches including gene expression and enzyme activity analysis, and measurement of starch and different soluble sugar levels during germination of wheat seeds. To this effect, the study first identified wheat genes encoding different starch degrading enzymes including α-amylase, β-amylase, α-glucosidase and phosphorylase from publicly available GenBank databases based on sequence homology with previously identified starch degrading genes of barley. Phylogenetic analysis of the newly identified genes of wheat encoding the starch degrading genes along with starch degrading genes from other species showed their close relationship.

The results of this study showed that decreasing the level of ethylene production through inhibiting its synthesis during imbibition of non-dormant wheat seeds via AVG treatment does not affect germination (Fig 3.5). The absence of effect of AVG treatment on germination could be due to the presence of endogenous ethylene in the mature seeds of wheat that is sufficient for the completion of the germination process. Consistent with this result, a previous study showed that inhibition of ethylene production through AVG treatment does not inhibit germination of non-dormant *Amaranthus caudatus*, and this is due to the presence of endogenous ethylene that is sufficient to support the germination process (Kepczynski and Karssen, 1985). In contrast, treatment with AVG (2 mM) of lettuce seeds has been shown to lead to 50% reduction of germination within 48 HAI (Matilla, 2000). Although germination was not suppressed, treatment of imbibing seeds with AVG caused 2- to 6-fold repression of post-germination growth of the seedling parts including the radicle, seminal root and coleoptile up to seven days after imbibition (DAI) (Fig. 3.6).

Mobilization of starch, which is the major storage reserve in the endosperm of cereal seeds, during germination is regulated mainly by the activity of α-amylase (Zeeman *et al.*, 2010). Although reserve mobilization mainly takes place after the completion of germination, genes encoding AMY have been shown to exhibit upregulation starting from phase II of seed germination in cereal seeds (He *et al.*, 2011). Our gene expression analysis did not detect the transcripts of *AMY* genes, except that of *AMY2* in the dry seeds. The expression pattern of *AMY* genes significantly increased at 2 and 3 DAI in the aleurone layer of barley seeds (Radchuk *et al.*, 2009). Similarly, the expressions of all the *AMY* genes showed induction after the start of imbibition in this study. The comparison across genes indicated that *AMY2* is the predominant *AMY* gene followed by *AMY1*, and the expression of these genes increased continuously with imbibition, which suggests their important role in regulating α-amylase and starch degradation in the endosperm. And the α-amylase is required to hydrolysis starch into maltose to support the axis/seedling growth. In agreement with this, the activity of α-amylase exhibited continuous increase during imbibition, showing 400-fold increase by 7 DAI (Fig. 3.11A). This was associated with a significant decrease in endosperm dry weight/starch level following 5 DAI (Fig. 3.12) and a significant increase in endosperm maltose content (Fig. 3.14B). Consistent with the increase in starch degradation, radicle, seminal root and coleoptile growth showed significant increases. The activity of α-amylase has also shown an 8-fold increase after ~72 h of imbibition in non-dormant seeds of barley (An and Lin, 2011). The other two *AMY* genes, *AMY4* and *AMY5*, also showed upregulation in response to imbibition, but their expression was relatively low (the expression of *AMY4* is higher than that of *AMY5*), implying their minimal role in starch degradation as compared to *AMY1* and *AMY2*. However, the upregulation of *AMY4* during the

later periods of imbibition, or *AMY5* during the early period of imbibition, might suggest their stage specific complementary role in the regulation of AMY activity.

The role of ethylene in promoting germination has been shown in different species and this is achieved mainly through its antagonistic interaction with ABA, which is known to inhibit germination (Gubler *et al.*, 2005). Such antagonistic interaction between the two hormones has been shown during the germination of Arabidopsis seeds (Ghassemian *et al.*, 2000), tobacco (Kucera *et al.*, 2005) and *Lepidium sativum* (Linkies *et al.*, 2009). Inhibition of ethylene synthesis via AVG treatment caused significant reduction in expression of the predominant *AMY* genes, *AMY1* and *AMY2*, especially following 48 or 72 HAI (Fig 3.7A, B), suggesting a decrease in α-amylase activity. Consistent with this hypothesis, significant inhibition of α-amylase activity was evident following 48 HAI (Fig. 3.11A), and this was associated with a marked decrease in the level of maltose following 3 DAI (Fig. 3.14B). In agreement with the decrease in starch degradation, radicle, seminal root and coleoptile growth was significantly reduced in response to AVG treatment. It has been shown previously that treatment with ethylene of kiwi fruit significantly induces the expression of *AMY1* and this was positively correlated with starch degradation (Hu *et al.*, 2016). Given that inhibition of ethylene biosynthesis by AVG might result in an increase in ABA level (Linkies and Leubner-Metzger, 2012; Hoffmann-Benning and Kende, 1992), which in turn has been shown to repress the synthesis of GA (Gubler *et al.*, 2005; Hoffmann-Benning and Kende, 1992), the repression in the expression of *AMY1* and *AMY2* and activity of α-amylase can be associated with the level of ABA and GA in the AVG treated seeds. Previous studies have shown repression of starch degrading *AMY* genes by ABA treatment during imbibition of wheat seeds (Liu *et al.*, 2013a), while GA treatment caused significant upregulation of *AMY* genes (*α-Amy1-1* and *α-Amy1-2*) (Barrero *et al.*, 2013), or induction of α-

amylase synthesis in the aleurone layer during wheat seed imbibition (Kondhare *et al*., 2012). Since inhibitors of proteases and amylases have been shown to be repressed during germination of non-dormant seeds to facilitate reserve storage proteolysis and hydrolysis (Gao and Ayele, 2014), the inhibition of α-amylase activity and thereby starch degradation and sugar content by AVG treatment can be associated with increased activity of the protease and amylase inhibitors.

β-amylase is involved in the conversion of starch to maltose, either directly, or through straight chain α-dextrins (Zeeman *et al.*, 2004). With respect to the expression patterns of the genes encoding this enzyme, the transcripts of all the seven *BAM* genes were detected in dry seeds, suggesting sequestration of their transcripts in the endosperm during seed maturation. However, *BAM3* and *BAM6* appeared to be the most prominent genes especially during the earlier phase of seed imbibition followed by *BAM2* and *BAM9* (Fig. 3.8). All the *BAM* genes, except the least expressed *BAM1*, either maintained the high amount of transcripts detected in the dry seeds or showed induction within the first 12 HAI, after which their expression decreased to a relatively lower level. The substantial decrease in the expression of the *BAM* genes, especially the predominantly expressed ones, suggest a decrease in BAM activity. However, the high level of BAM activity detected in the dry seeds was maintained at a similar level throughout the entire imbibition period (Figure 3.11B), irrespective of the decrease in seed dry weight or starch level and an increase in the level of maltose (Figure 3.14B) and an increase in radicle, seminal root and coleoptile growth as the duration of imbibition continued (Fig. 3.6). Previous studies on wheat seed germination have also shown a decrease in the expression of *BAM* during imbibition (Park *et al.*, 2013; Gao *et al.*, 2012). The absence of any change in BAM activity during imbibition despite the decrease in seed dry weight or starch level, and an increase in maltose level and growths of the seedling tissues might suggest minimal contribution of BAM in the

mobilization of starch. In line with this hypothesis, β-amylase has been reported to have minor effect on hydrolysis of starch granules during germination of barley seeds, and this was suggested to be due to the fact that it acts only on oligo-dextrins (Sun and Henson, 1991). Furthermore, no difference in starch mobilization was observed between germinating seeds of a high β-amylase activity cultivar and a low β-amylase activity cultivar of soybean (Adams *et al.*, 1981).

Treatment with AVG repressed the expression of *BAM2*, *BAM6*, *BAM7* and *BAM9* during the early period of imbibition (Fig 3.8), when no change in BAM activity (Fig. 3.11B) or starch level (Fig. 3.13) was evident. Whereas AVG treatment caused upregulation of *BAM2*, *BAM6*, *BAM7* and *BAM9* during the later stages of germination but with no significant change in BAM activity. Although a decrease in the rate of starch degradation and seedling growth was evident in response to AVG (Fig. 3.6). Our results might suggest the regulation of *BAM* genes at post-transcriptional level. Previous reports have also shown that ethylene treatment of kiwi fruits causes significant induction in the expression of specific *BAM* such as *BAM3.1*, *BAM3L* and *BAM9*, while repressing the expression of others such as *BAM2L*, *BAM7* and *BAM8* (Hu *et al.*, 2016). Similar to our results, treatment with ethylene biosynthesis inhibitor 1-MCP during banana ripening decreased the rate of starch degradation and thereby level of soluble sugars. In contrast to our result, the reduction in starch degradation rate was shown to be associated with the suppression of BAM activity (Nascimento *et al.*, 2006). It is therefore possible that β-amylase has different roles during seed germination and fruit ripening.

The enzyme α-glucosidase catalyses the final step in starch degradation, that is, the conversion of straight chain α-dextrins or maltose to glucose. It appears from our results that *AGL1* and *AGL2* are the predominantly expressed *AGL* genes and their expression patterns

follow that of *AMY1* and *AMY2* (Fig 3.7 and 3.9), which are continued increase with imbibition, suggesting their important role in regulating AGL and converting α-dextrins or maltose to glucose. The level of AGL activity detected at the start of imbibition showed an increase after 5 DAI (Fig. 3.11C) similar to that observed for imbibing barley seeds in which α-glucosidase activity exhibited marked increase following 3 DAI (Andriotis *et al.*, 2016b). This was associated with an increase in glucose content (Fig. 3.14A) and increases in radicle, seminal root and coleoptile growth.

Treatment of imbibing seeds with AVG caused substantial repression in the expression of both *AGL1* and *AGL2* genes, suggesting a decrease in the activity of AGL. In agreement with this, AVG treatment caused reduction in the activity of α-glucosidase on gDW during the late periods of imbibition (Fig. 3.11C), and this was associated a decrease in glucose content (Fig. 3.14) and thereby inhibition of seedling growth (Fig. 3.6). Treating imbibing seeds of barley with 1-deoxy-nojirimycin (DNJ), inhibitor of α-glucosidase activity has also been shown to strongly decrease glucose level, leading to reduced glucose to maltose ratio, and reduction in the rate of starch degradation and a decrease in root and shoot length (Stanley *et al.*, 2011; Andriotis *et al.*, 2016b). Whereas, the expressions of the other two *AGL* genes, *AGL5* and *AGL7*, appeared to remain almost constant throughout the imbibition period and exhibited upregulation in response to AVG treatment during the later periods of imbibition although the expression of *AGL7* was repressed by AVG within 12 HAI (Fig. 3.9A, B). These results might suggest that the *AGL5* and *AGL7* genes play a minor role with respect to the conversion of maltose to glucose. Previous studies in kiwi fruit has also shown that ethylene induces the expression of specific *AGL* genes such as *AGL3* while repressing others such as *AGL1* (Hu *et al.*, 2016). In addition to the hydrolytic form, starch degradation can take place via phosphorolysis, which is catalyzed by

PHO. The expression of genes encoding phosphorylase, *PHO1* and *PHO2*, exhibited either a decrease or almost constant expression during imbibition, irrespective of a decrease in endosperm dry weight or starch level (Fig. 3.10). Further, treatment with AVG caused an increase in the expression of *PHO1* during the late periods of imbibition and that of *PHO2* early during imbibition despite maintenance of higher endosperm dry weight or starch level (Fig. 3.10). These results suggest the minimal role *PHO1* and *PHO2* in the degradation of starch in imbibing wheat seeds. The expression of phosphorylase genes *PHS1* and *PHS2* of kiwi fruit have been shown to be repressed by ethylene treatment, which enhances starch degradation (Hu *et al.*, 2016).

The absence of any significant change in the levels of fructose and sucrose (Fig. 3.14) with imbibition might suggest that the continuous increase in glucose level is mainly associated with starch degradation, but not hydrolysis of the sucrose present in the seeds. However, the increase of sucrose level by AVG treatment as compared to the control during the later phases of imbibition, along with the decrease in fructose level due to the AVG treatment, suggests that inhibition of ethylene synthesis disrupts not only starch degradation, but also sucrose hydrolysis to fructose and glucose. Therefore, it is possible that hydrolysis of the sucrose present in the seeds may have some contribution to the level of glucose observed. As germination proceeds, increasing energy requirement associated with the level carbohydrate hydrolysis, the level of glucose and fructose can be supplied by breakdown of sucrose. For example, the level of glucose decreased after 24 HAI imbibition, but glucose and fructose were gradually increasing with sucrose hydrolysis after 48 HAI in Arabidopsis seeds (Allen *et al.*, 2010; Rosental *et al.*, 2014). A previous study, however, showed that application of ethylene to kiwi fruit during ripening,

caused an increase in the levels of not only glucose, but also in the levels of fructose and sucrose (Hu *et al.*, 2016), suggesting an increase in the synthesis of sucrose from glucose and fructose.

Comparative analysis of the expression of starch degrading genes between imbibing dormant and non-dormant seeds of AC Domain from a microarray data showed upregulation of genes encoding α-amylase (*AMY1* and *AMY2*) (Fig. 3.15) and α-glucosidase (*AGL1*) in non-dormant seeds (Fig. 3.17). However, the expression of these genes in imbibing non-dormant seeds was repressed by ABA, which is antagonistic to ethylene in its role in regulating germination. On the other hand, expression of *BAM1* and *BAM3* genes exhibited downregulation during imbibition in non-dormant seeds compare to dormant seeds (Fig. 3.16). Our results overall highlight the significance of *AMY1, AMY2* and *AGL1* genes in controlling starch degradation during wheat seed germination. Imbibing wheat seed with ethylene inhibitor AVG downregulated the expression of these genes, and this is associated with decreased activity of AMY and AGL, and accumulation of starch and reduction in the level of soluble sugars, suggesting the role of ethylene in regulating starch hydrolysis during wheat seed germination. This study provides a better understanding of the role of starch degrading genes and gene family members during wheat seed germination.

4.0 GENERAL DISCUSSION AND CONCLUSION

Germination, an important process for the seed to develop into a new plant. It starts with imbibition and ends up with protrusion of radicle through seed coat. Germination can be divided into three phases, the first phase represents the repair of mitochondria and DNA, and synthesis of proteins from existing gene transcripts; the second phase involves synthesis of new mitochondria and synthesis of proteins from newly synthesised gene transcripts, and emergence of the radicle; the third phase is characterized mainly by mobilization of storage reserves and seedling growth. Seed germination is controlled by many factors, including environmental factors such as moisture, oxygen, light and temperature, and endogenous factors such as phytohormones. However, dormant seeds fail to germinate even under favourable condition. Dormancy is also an important factor to regulate germination. An appropriate level of seed dormancy is a desired trait in crop production as it prevents pre-harvest sprouting. Seed dormancy and dormancy release/germination are controlled primarily by GA and ABA. In addition, other hormones such as ethylene control germination. However, there is lack of information on how ethylene regulates processes related to seed germination and seedling growth such as the degradation of storage starch in the endosperm, which serves as energy source for embryo growth. The genes involved in starch degradation have been identified in some higher plants, such as, barley, wheat, Arabidopsis, rice. Therefore, a detailed characterization of regulation of ethylene on starch degradation during germination contributes to our understanding of the mechanisms involved in germination. This in turn will improve genetic manipulation or breeding of wheat for regulating of dormancy and germination.

Using a non-dormant spring wheat genotype, namely RL4452, this thesis studied the role of ethylene on starch degradation during and after germination of wheat seeds. The study first

examined the effect of inhibition of ethylene synthesis on germination, and growth of seedlings, and the findings indicated that inhibition of ethylene synthesis resulted in up to 2- to 6-fold decrease in seedling growth. To examine the effect of ethylene on the expression of starch degrading genes, this thesis identified 25 starch degradation related genes of wheat, including six *AMY* genes, nine *BAM* genes, seven *AGL* genes and three *PHO* genes, and characterized their expression pattern in the endosperm of seeds imbibed for different durations. The gene expression analysis indicated that the expressions of *AMY1, AMY2, BAM1, BAM6, BAM7, AGL1* and *AGL2* genes increased with imbibition, suggesting that these genes play an important role in regulating starch degradation in wheat endosperm. Inhibition of ethylene synthesis by AVG suppressed the expressions of *AMY1, AMY2, AGL1* and *AGL2* genes, which are among the predominant starch degrading genes. AVG treatment also repressed the expression of *BAM2, BAM6,* and *BAM9* early during imbibition (6 HAI), but upregulated late during imbibition (120 and 168 HAI). The expression of *PHO* was consistently low during germination, irrespective of treatment.

In addition to gene expression, the study also measured the activity of the corresponding enzymes, and the enzyme activity studies indicated that the increased expression of *AMY* and *AGL* genes during germination is accompanied by high activity of AMY and AGL. On the other hand, the suppression of *AMY* and *AGL* genes by AVG is related with inhibition on AMY and AGL activity by AVG. Regardless of the changes in the expression of the corresponding genes due to AVG treatment, the activity of BAM remained constant during the whole period of imbibition. Quantification of starch and sugars levels indicated reduction of endosperm dry weight and starch content in the endosperm during imbibition, which is associated with increased expression of *AMY* and *AGL* and activity of AMY and AGL. Higher starch content was detected

in the endosperm of AVG treated samples at 5 and 7 DAI as compared to the control. The amount of starch breakdown products including glucose and maltose greatly increased during the germination and post-germination phase, and this is associated with the induced activity of starch hydrolytic enzymes, AMY and AGL. In contrast, the level of glucose and maltose was significantly suppressed by AVG treatment, in agreement with decreased activity of AMY and AGL by AVG. The thesis also performed comparative expression analysis of starch degrading genes between dormant and non-dormant seeds, and the results showed that the expression of *AMY1, AMY2, AGL1* and *PHO2* was suppressed in dormant as compared to non-dormant seeds, while that of *BAM1* to *BAM3* were upregulated, suggesting the roles of specific *AMY*, *AGL* and *PHO* in influencing the germination of seeds that lack dormancy.

In conclusion, the finding in this study provides important information on the molecular mechanisms underlying starch degradation in response to ethylene during wheat seed germination. However, generating mutants of these genes and studying the mutants is required to better understand the functions of the genes studied here. In addition, analysis of the post-transcriptional regulation of these genes in relation to ethylene is important to extend our knowledge on the topic.

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APPENDIX I. SUPPLEMENTARY FIGURES

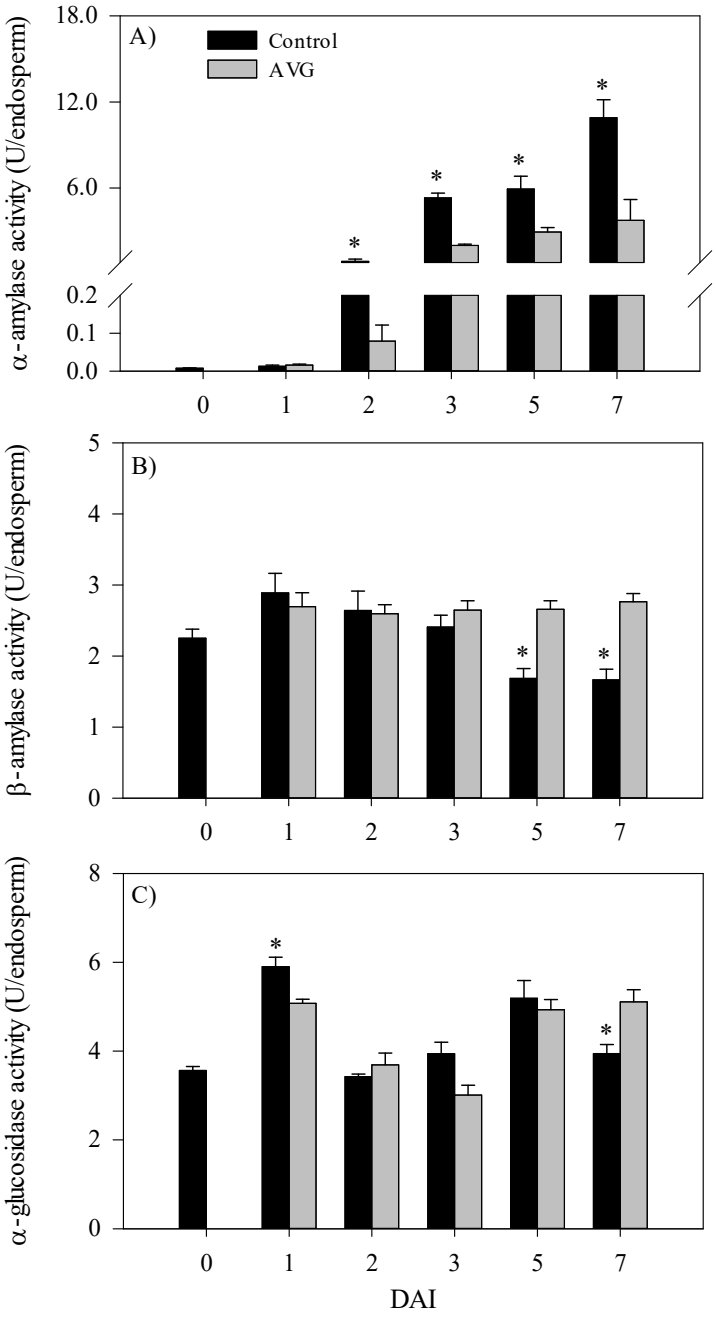

Figure S1. Activity of α-amylase, β-amylase and α-glucosidase per endosperm (dry weight basis) during germination and seedling growth. Enzyme activities are expressed per endosperm. Data are means of 3 independent biological replicates ± SE. Astrerisks indicate statistically significant difference between the control and AVG treated samples within each imbibition time point using Student's t-test, $P<0.05$. DAI, days after imbibition. U, unit.

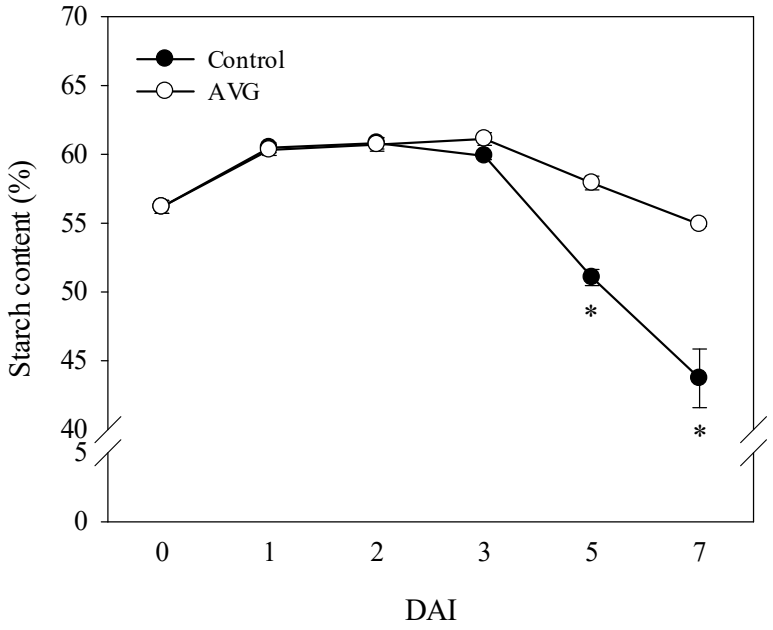

Figure S2. Changes in starch content (percentage) per endosperm gram dry weight during germination and seedling growth. Data are means ± SE, n =3. DAI, days after imbibition. Astrerisks indicate statistically significant difference between the control and AVG treated samples within each imbibition time point using Student's t-test, $P<0.05$.

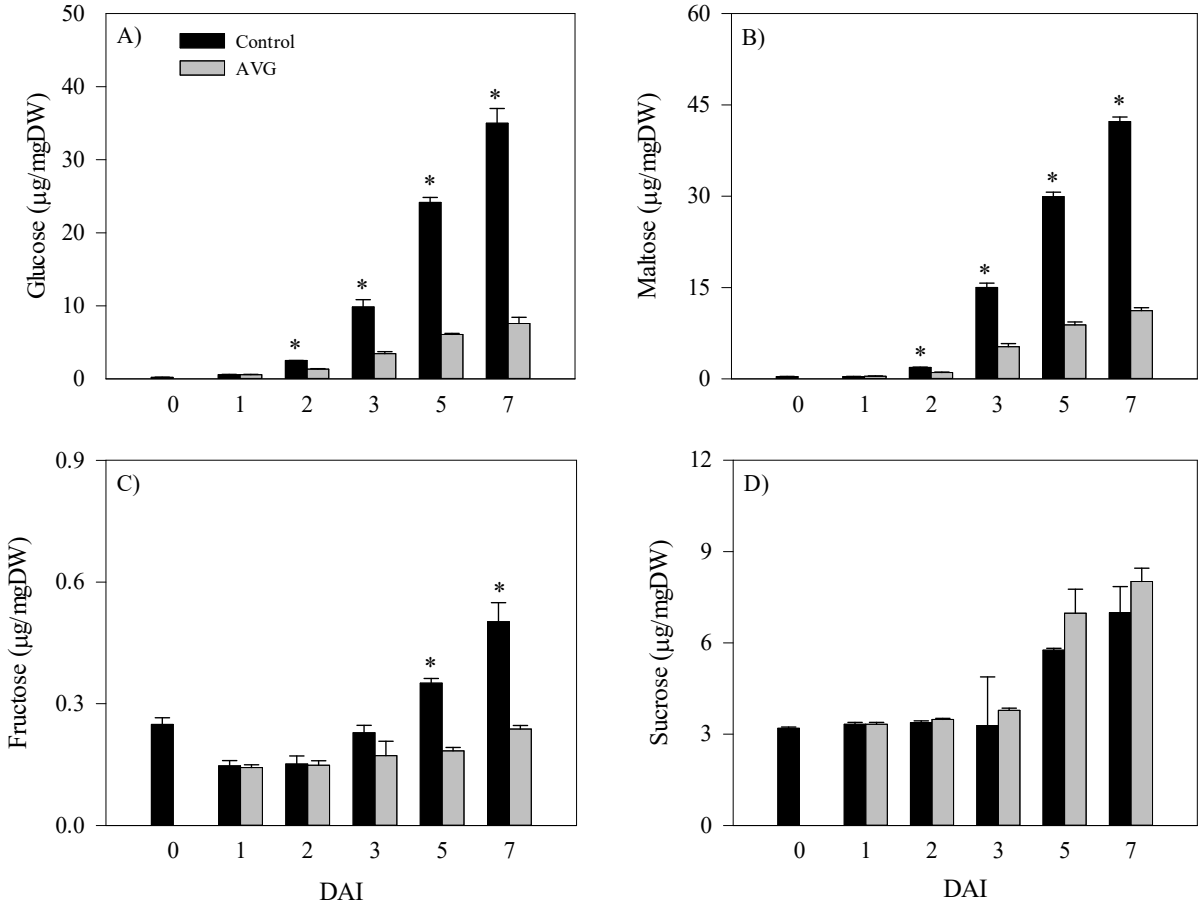

Figure S3. Changes in glucose (A), maltose (B), fructose (C) and sucrose (D) contents per endosperm gram dry weight during germination and seedling growth. Data are means ± SE, n =3. Asterisks indicate statistically significant difference between the control and AVG treated samples within each imbibition time point using Student's t-test, $P<0.05$. DAI, days after imbibition; DW, dry weight.

APPENDIX II. SUPPLEMENTARY TABLES

Table S1. Fold change in the expression of probesets related *α-amylase* genes before and during imbibition of dormant seeds (cv. AC Domain)

	D-12/D-0			D-24/D-0			D-24/D-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.21984.1.S1_s_at	-0.3146	0.0015	-1.2436	-0.2213	0.0071	-1.1658	0.0933	0.1421	1.0668
TaAffx.79731.1.S1_s_at	-0.5057	0.0128	-1.4198	-0.4848	0.0130	-1.3994	0.0209	0.8936	1.0146
TaAffx.15965.1.S1_at	-0.1417	0.4939	-1.1032	-0.1324	0.5321	-1.0961	0.0094	0.9488	1.0065
Ta.22773.2.S1_x_at	-0.4725	0.0155	-1.3875	-0.5862	0.0137	-1.5013	-0.1138	0.2760	-1.0820
TaAffx.79731.1.S1_s_at	-0.5057	0.0128	-1.4198	-0.4848	0.0130	-1.3994	0.0209	0.8936	1.0146
Ta.10281.1.S1_a_at	-0.2465	0.1717	-1.1863	0.0400	0.8172	1.0281	0.2865	0.0166	1.2197
Ta.10281.1.S1_at	0.0783	0.5059	1.0558	0.0480	0.6727	1.0338	-0.0303	0.7780	-1.0212
Ta.10281.2.S1_at	-0.2400	0.1014	-1.1810	-0.0673	0.5394	-1.0477	0.1727	0.0853	1.1272
Ta.10281.3.S1_at	-0.4198	0.0192	-1.3377	-0.4754	0.0167	-1.3903	-0.0556	0.5638	-1.0393
TaAffx.24633.1.S1_at	-0.5141	0.0055	-1.4281	-0.5814	0.0038	-1.4963	-0.0673	0.2222	-1.0478
Ta.22773.1.S1_x_at	-0.1738	0.2079	-1.1280	-0.5176	0.0059	-1.4316	-0.3438	0.0411	-1.2691
Ta.490.1.A1_at	0.0582	0.6057	1.0411	0.2701	0.1023	1.2059	0.2119	0.1213	1.1582
TaAffx.110040.1.S1_at	-0.0475	0.6932	-1.0335	-0.1138	0.3528	-1.0821	-0.0663	0.4363	-1.0470
TaAffx.110040.1.S1_s_at	-0.0489	0.6231	-1.0345	-0.2944	0.0339	-1.2264	-0.2455	0.0428	-1.1855

Table S2. Fold change in the expression of probesets related *α-amylase* genes before and during imbibition of after-ripened seeds (cv. AC Domain)

	AR-12/AR-0			AR-24/AR-0			AR-24/AR-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.21984.1.S1_s_at	-0.3712	0.1164	-1.2934	-0.4799	0.1009	-1.3947	-0.1088	0.4621	-1.0783
TaAffx.79731.1.S1_s_at	-0.4011	0.0179	-1.3205	-0.5431	0.0115	-1.4571	-0.1420	0.3148	-1.1035
TaAffx.15965.1.S1_at	-0.3235	0.0235	-1.2514	-0.3180	0.0289	-1.2466	0.0055	0.9483	1.0038
Ta.22773.2.S1_x_at	0.2078	0.0833	1.1549	4.7208	0.0032	26.3693	4.5130	0.0037	22.8321
TaAffx.79731.1.S1_s_at	-0.4011	0.0179	-1.3205	-0.5431	0.0115	-1.4571	-0.1420	0.3148	-1.1035
Ta.10281.1.S1_a_at	-0.0546	0.7296	-1.0386	0.5183	0.2349	1.4322	0.5729	0.2025	1.4875
Ta.10281.1.S1_at	-0.1940	0.3000	-1.1439	0.7002	0.0582	1.6247	0.8942	0.0162	1.8586
Ta.10281.2.S1_at	-0.0382	0.5740	-1.0268	-0.0924	0.1646	-1.0661	-0.0542	0.2324	-1.0383
Ta.10281.3.S1_at	-0.1623	0.1884	-1.1191	-0.4337	0.0238	-1.3506	-0.2713	0.0422	-1.2069
TaAffx.24633.1.S1_at	-0.2970	0.0755	-1.2286	-0.1591	0.2406	-1.1166	0.1379	0.0694	1.1003
Ta.22773.1.S1_x_at	0.4350	0.0123	1.3520	5.0488	0.0010	33.1010	4.6138	0.0014	24.4838
Ta.490.1.A1_at	-0.2425	0.1708	-1.1830	-0.1062	0.5431	-1.0764	0.1362	0.2408	1.0990
TaAffx.110040.1.S1_at	0.1770	0.0437	1.1305	-0.1051	0.1083	-1.0756	-0.2821	0.0123	-1.2160
TaAffx.110040.1.S1_s_at	-0.0048	0.9637	-1.0033	-0.3208	0.2122	-1.2491	-0.3160	0.2016	-1.2449

Table S3. Fold change in the expression of probesets related *α-amylase* genes before and during imbibition of dormant and after-ripened seeds (cv. AC Domain)

	AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.21984.1.S1_s_at	0.1931	0.3558	1.1432	0.1366	0.0334	1.0993	-0.0655	0.6569	-1.0464	-0.0425	0.7833	-1.0299
TaAffx.79731.1.S1_s_at	-0.2044	0.0834	-1.1522	-0.0998	0.4831	-1.0716	-0.2627	0.1372	-1.1997	-0.1227	0.3998	-1.0888
TaAffx.15965.1.S1_at	0.0723	0.7092	1.0514	-0.1095	0.3580	-1.0788	-0.1133	0.3929	-1.0817	-0.0247	0.7605	-1.0173
Ta.22773.2.S1_x_at	-0.3552	0.0686	-1.2792	0.3250	0.0007	1.2527	4.9518	0.0027	30.9480	2.7247	0.0418	6.6101
TaAffx.79731.1.S1_s_at	-0.2044	0.0834	-1.1522	-0.0998	0.4831	-1.0716	-0.2627	0.1372	-1.1997	-0.1227	0.3998	-1.0888
Ta.10281.1.S1_a_at	0.0472	0.8000	1.0333	0.2391	0.1068	1.1802	0.5255	0.2237	1.4394	0.5864	0.1971	1.5015
Ta.10281.1.S1_at	0.2839	0.1764	1.2175	0.0115	0.9043	1.0080	0.9361	0.0148	1.9133	0.6302	0.0549	1.5478
Ta.10281.2.S1_at	-0.1005	0.3973	-1.0721	0.1013	0.2423	1.0728	-0.1256	0.0410	-1.0909	-0.0416	0.6051	-1.0293
Ta.10281.3.S1_at	-0.1332	0.3759	-1.0967	0.1243	0.1489	1.0900	-0.0914	0.4417	-1.0654	-0.1879	0.2540	-1.1391
TaAffx.24633.1.S1_at	-0.2531	0.1548	-1.1917	-0.0360	0.5774	-1.0252	0.1693	0.0159	1.1245	0.0335	0.4826	1.0235
Ta.22773.1.S1_x_at	-0.2475	0.0768	-1.1872	0.3613	0.0321	1.2846	5.3189	0.0008	39.9157	2.8009	0.0323	6.9687
Ta.490.1.A1_at	0.0664	0.7027	1.0471	-0.2342	0.0365	-1.1763	-0.3099	0.0689	-1.2396	0.0485	0.6135	1.0342
TaAffx.110040.1.S1_at	-0.1902	0.1333	-1.1409	0.0343	0.6808	1.0241	-0.1815	0.0473	-1.1341	-0.0268	0.7266	-1.0187
TaAffx.110040.1.S1_s_at	-0.2033	0.1365	-1.1513	-0.1592	0.1174	-1.1166	-0.2298	0.3324	-1.1726	-0.0191	0.9300	-1.0133

Table S4. Fold change in the expression of probesets related *β-amylase* genes before and during imbibition of dormant seeds (cv. AC Domain)

	D-12/D-0			D-24/D-0			D-24/D-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.3.1.S1_at	-0.0336	0.6320	-1.0236	-0.2087	0.0193	-1.1556	-0.1751	0.0925	-1.1290
Ta.27780.1.S1_at	-0.8506	0.0118	-1.8032	-2.9809	0.0003	-7.8948	-2.1303	0.0007	-4.3782
Ta.27780.1.S1_x_at	-0.5775	0.0119	-1.4923	-2.6216	0.0002	-6.1544	-2.0441	0.0003	-4.1242
Ta.27780.2.A1_a_at	-1.6759	0.0022	-3.1953	-3.3932	0.0001	-10.5065	-1.7173	0.0021	-3.2881
Ta.27780.2.A1_x_at	-1.7212	0.0015	-3.2970	-3.3781	0.0001	-10.3968	-1.6569	0.0022	-3.1534
Ta.4494.1.S1_at	-1.5104	0.0001	-2.8489	-1.9675	0.0002	-3.9110	-0.4571	0.0196	-1.3728
Ta.4494.1.S1_x_at	-1.4236	0.0001	-2.6825	-1.8532	0.0002	-3.6129	-0.4296	0.0291	-1.3469
TaAffx.68872.1.S1_at	-0.5297	0.0085	-1.4436	-0.4486	0.0053	-1.3648	0.0811	0.4546	1.0578
Ta.16135.1.A1_at	-0.7524	0.0005	-1.6846	-0.9655	0.0001	-1.9528	-0.2132	0.0341	-1.1592
TaAffx.68872.3.S1_x_at	-0.3384	0.0186	-1.2643	-0.5097	0.0053	-1.4237	-0.1713	0.2319	-1.1261
Ta.137.1.S1_at	-0.3245	0.0156	-1.2522	-0.2201	0.1024	-1.1648	0.1044	0.3640	1.0750
Ta.1062.1.S1_at	-0.3870	0.0109	-1.3077	0.0904	0.3776	1.0647	0.4774	0.0150	1.3923
TaAffx.42783.1.A1_s_at	-0.4659	0.0813	-1.3812	-0.1011	0.6650	-1.0726	0.3648	0.1125	1.2877
TaAffx.42783.1.A1_at	-0.1279	0.0626	-1.0927	0.4729	0.0067	1.3879	0.6009	0.0022	1.5166
Ta.27780.3.S1_x_at	-0.7526	0.0025	-1.6849	-2.6306	0.0002	-6.1927	-1.8779	0.0007	-3.6755

Table S5. Fold change in the expression of probesets related *β-amylase* genes before and during imbibition of after-ripened seeds (cv. AC Domain)

	AR-12/AR-0			AR-24/AR-0			AR-24/AR-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.3.1.S1_at	-0.5533	0.0054	-1.4674	-0.4156	0.0311	-1.3338	0.1377	0.3104	1.1002
Ta.27780.1.S1_at	-0.2937	0.0089	-1.2258	-2.7822	0.0261	-6.8792	-2.4886	0.0367	-5.6122
Ta.27780.1.S1_x_at	0.0295	0.7415	1.0207	-2.3945	0.0269	-5.2580	-2.4241	0.0253	-5.3668
Ta.27780.2.A1_a_at	-0.6031	0.0689	-1.5190	-3.4183	0.0082	-10.6908	-2.8152	0.0130	-7.0383
Ta.27780.2.A1_x_at	-0.6153	0.0369	-1.5319	-3.3373	0.0058	-10.1074	-2.7220	0.0102	-6.5979
Ta.4494.1.S1_at	-2.4495	0.0000	-5.4622	-3.0602	0.0021	-8.3411	-0.6108	0.2239	-1.5271
Ta.4494.1.S1_x_at	-2.4918	0.0001	-5.6250	-3.1034	0.0014	-8.5945	-0.6116	0.1765	-1.5279
TaAffx.68872.1.S1_at	-0.7886	0.0042	-1.7274	-0.6751	0.0461	-1.5967	0.1135	0.5994	1.0819
Ta.16135.1.A1_at	-1.3370	0.0001	-2.5262	-1.6990	0.0001	-3.2468	-0.3620	0.0323	-1.2852
TaAffx.68872.3.S1_x_at	-0.1987	0.4177	-1.1477	-0.1797	0.4833	-1.1326	0.0190	0.8627	1.0133
Ta.137.1.S1_at	-0.4656	0.0924	-1.3809	-0.3435	0.2657	-1.2688	0.1221	0.5037	1.0883
Ta.1062.1.S1_at	-1.0160	0.0015	-2.0223	-0.7582	0.0072	-1.6914	0.2578	0.0265	1.1957
TaAffx.42783.1.A1_s_at	-0.2484	0.3622	-1.1879	-0.0876	0.7365	-1.0626	0.1608	0.0113	1.1179
TaAffx.42783.1.A1_at	-0.0200	0.8699	-1.0139	0.2508	0.1409	1.1899	0.2708	0.0539	1.2064
Ta.27780.3.S1_x_at	-0.1274	0.5276	-1.0923	-2.8021	0.0184	-6.9745	-2.6747	0.0192	-6.3851

Table S6. Fold change in the expression of probesets related *β-amylase* genes before and during imbibition of dormant and after-ripened seeds (cv. AC Domain)

	AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.3.1.S1_at	0.2482	0.0384	1.1877	-0.2715	0.0370	-1.2070	0.0413	0.7317	1.0291	-0.0428	0.7911	-1.0301
Ta.27780.1.S1_at	0.0156	0.9301	1.0109	0.5725	0.0079	1.4871	0.2143	0.8090	1.1601	-0.9631	0.3277	-1.9495
Ta.27780.1.S1_x_at	0.0128	0.9360	1.0089	0.6198	0.0002	1.5367	0.2399	0.7542	1.1809	-0.8096	0.3346	-1.7527
Ta.27780.2.A1_a_at	-1.3451	0.0087	-2.5404	-0.2722	0.2377	-1.2077	-1.3702	0.1122	-2.5850	-0.6225	0.4345	-1.5395
Ta.27780.2.A1_x_at	-1.1949	0.0061	-2.2893	-0.0891	0.6713	-1.0637	-1.1542	0.1313	-2.2256	-0.5723	0.4451	-1.4869
Ta.4494.1.S1_at	-0.2057	0.1730	-1.1533	-1.1448	0.0001	-2.2112	-1.2984	0.0412	-2.4596	0.5962	0.2647	1.5118
Ta.4494.1.S1_x_at	-0.0629	0.7062	-1.0446	-1.1312	0.0000	-2.1904	-1.3131	0.0287	-2.4848	0.6106	0.2278	1.5269
TaAffx.68872.1.S1_at	-0.0858	0.5910	-1.0613	-0.3447	0.0200	-1.2699	-0.3122	0.1972	-1.2416	0.0956	0.7180	1.0685
Ta.16135.1.A1_at	0.1415	0.1620	1.1031	-0.4431	0.0055	-1.3595	-0.5920	0.0045	-1.5073	-0.2267	0.1889	-1.1702
TaAffx.68872.3.S1_x_at	-0.1406	0.5513	-1.1024	-0.0009	0.9928	-1.0006	0.1894	0.2087	1.1403	-0.0227	0.8180	-1.0158
Ta.137.1.S1_at	0.1841	0.4447	1.1361	0.0431	0.5269	1.0303	0.0608	0.7589	1.0430	-0.0267	0.8841	-1.0186
Ta.1062.1.S1_at	0.2727	0.1126	1.2081	-0.3563	0.0115	-1.2801	-0.5759	0.0070	-1.4906	0.1125	0.3711	1.0811
TaAffx.42783.1.A1_s_at	-0.2691	0.4094	-1.2051	-0.0516	0.6777	-1.0364	-0.2556	0.1479	-1.1938	0.3622	0.0009	1.2854
TaAffx.42783.1.A1_at	0.0667	0.5859	1.0473	0.1746	0.0311	1.1287	-0.1555	0.2656	-1.1138	0.0473	0.6471	1.0333
Ta.27780.3.S1_x_at	-0.0724	0.7311	-1.0515	0.5528	0.0034	1.4669	-0.2440	0.7539	-1.1843	-0.7997	0.3457	-1.7407

Table S7. Fold change in the expression of probesets related *α-glucosidase* genes before and during imbibition of dormant seeds (cv. AC Domain)

	D-12/D-0			D-24/D-0			D-24/D-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
TaAffx.13113.1.S1_at	0.0128	0.9192	1.0089	-0.6485	0.0067	-1.5676	-0.6613	0.0011	-1.5815
TaAffx.13113.2.S1_s_at	-0.0774	0.3952	-1.0551	-0.3533	0.0361	-1.2775	-0.2759	0.0985	-1.2107
Ta.21792.1.S1_at	0.5147	0.0785	1.4287	0.1069	0.7806	1.0769	-0.4078	0.2819	-1.3267
Ta.21792.1.S1_s_at	0.3187	0.0080	1.2472	-0.9149	0.0413	-1.8854	-1.2335	0.0160	-2.3514
Ta.28599.1.S1_at	0.7659	0.0054	1.7005	-0.4838	0.0465	-1.3984	-1.2498	0.0045	-2.3780
Ta.8296.1.S1_at	0.5753	0.0025	1.4900	-0.0498	0.6111	-1.0352	-0.6251	0.0002	-1.5423
Ta.25768.1.A1_at	-0.4845	0.0035	-1.3991	-0.6054	0.0011	-1.5214	-0.1209	0.1304	-1.0874
TaAffx.5614.1.S1_at	-0.4872	0.0062	-1.4017	-0.2661	0.0356	-1.2025	0.2211	0.0921	1.1656
TaAffx.94033.1.S1_at	0.1236	0.1564	1.0895	0.0008	0.9968	1.0005	-0.1228	0.5557	-1.0889
Ta.2245.1.A1_at	0.3153	0.1608	1.2442	0.4350	0.0683	1.3519	0.1197	0.5239	1.0865
Ta.2245.2.A1_at	0.4975	0.1131	1.4118	0.5657	0.0828	1.4801	0.0682	0.7704	1.0484

Table S8. Fold change in the expression of probesets related *α-glucosidase* genes before and during imbibition of after-ripened seeds (cv. AC Domain)

	AR-12/AR-0			AR-24/AR-0			AR-24/AR-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
TaAffx.13113.1.S1_at	0.9015	0.0037	1.8680	3.0195	0.0003	8.1089	2.1180	0.0007	4.3409
TaAffx.13113.2.S1_s_at	0.4773	0.0272	1.3921	2.1698	0.0043	4.4996	1.6925	0.0103	3.2322
Ta.21792.1.S1_at	1.0826	0.0208	2.1179	4.8070	0.0006	27.9922	3.7243	0.0024	13.2172
Ta.21792.1.S1_s_at	1.3891	0.0017	2.6192	4.0343	0.0002	16.3847	2.6451	0.0006	6.2555
Ta.28599.1.S1_at	1.7629	0.0017	3.3937	3.8501	0.0003	14.4207	2.0872	0.0011	4.2492
Ta.8296.1.S1_at	0.2755	0.0204	1.2104	0.3910	0.0146	1.3113	0.1155	0.2571	1.0833
Ta.25768.1.A1_at	-0.3933	0.0245	-1.3134	-0.5053	0.0208	-1.4194	-0.1120	0.3744	-1.0807
TaAffx.5614.1.S1_at	-0.4386	0.0218	-1.3553	-0.3939	0.0243	-1.3139	0.0448	0.4837	1.0315
TaAffx.94033.1.S1_at	-0.1414	0.3087	-1.1030	-0.1404	0.2928	-1.1022	0.0010	0.9917	1.0007
Ta.2245.1.A1_at	-0.1147	0.7808	-1.0828	0.0315	0.9484	1.0221	0.1463	0.5888	1.1067
Ta.2245.2.A1_at	-0.6583	0.0117	-1.5782	-0.2222	0.5804	-1.1665	0.4361	0.2675	1.3530

Table S9. Fold change in the expression of probesets related *α-glucosidase* genes before and during imbibition of dormant and after-ripened seeds (cv. AC Domain)

	AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
TaAffx.13113.1.S1_at	-0.1580	0.3930	-1.1158	0.7307	0.0014	1.6595	3.5100	0.0001	11.3925	1.7431	0.0032	3.3476
TaAffx.13113.2.S1_s_at	-0.0779	0.5071	-1.0555	0.4768	0.0173	1.3916	2.4452	0.0028	5.4459	1.5912	0.0123	3.0130
Ta.21792.1.S1_at	0.4466	0.1172	1.3628	1.0145	0.0245	2.0202	5.1467	0.0008	35.4244	2.2833	0.0094	4.8679
Ta.21792.1.S1_s_at	0.0330	0.8670	1.0232	1.1035	0.0001	2.1488	4.9822	0.0003	31.6070	1.8211	0.0028	3.5334
Ta.28599.1.S1_at	0.1106	0.6627	1.0797	1.1075	0.0013	2.1547	4.4445	0.0001	21.7731	1.4226	0.0069	2.6806
Ta.8296.1.S1_at	-0.0411	0.7029	-1.0289	-0.3408	0.0029	-1.2665	0.3998	0.0091	1.3193	0.1690	0.2233	1.1243
Ta.25768.1.A1_at	-0.1660	0.2176	-1.1220	-0.0748	0.3796	-1.0532	-0.0659	0.5615	-1.0468	-0.1412	0.3645	-1.1028
TaAffx.5614.1.S1_at	0.0288	0.8237	1.0202	0.0773	0.4441	1.0551	-0.0990	0.2381	-1.0710	-0.2397	0.0227	-1.1808
TaAffx.94033.1.S1_at	0.1695	0.1821	1.1247	-0.0955	0.3640	-1.0685	0.0284	0.8893	1.0198	-0.0711	0.4409	-1.0505
Ta.2245.1.A1_at	0.1209	0.7812	1.0874	-0.3091	0.0749	-1.2390	-0.2826	0.3602	-1.2163	1.2342	0.0109	2.3524
Ta.2245.2.A1_at	0.7325	0.0391	1.6615	-0.4233	0.0525	-1.3410	-0.0553	0.8890	-1.0391	0.6594	0.1280	1.5794

Table S10. Fold change in the expression of probesets related *phosphorylase* genes before and during imbibition of dormant seeds (cv. AC Domain)

	D-12/D-0			D-24/D-0			D-24/D-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.1279.1.S1_at	-0.1349	0.5209	-1.0980	-0.2286	0.2887	-1.1717	-0.0937	0.5860	-1.0671
Ta.1197.1.S1_at	-1.2563	0.0063	-2.3888	-1.6943	0.0007	-3.2362	-0.4380	0.0836	-1.3547
Ta.1197.1.S1_x_at	-1.1500	0.0020	-2.2191	-1.6234	0.0001	-3.0809	-0.4734	0.0350	-1.3883
Ta.28402.1.A1_at	-0.4188	0.0001	-1.3368	-0.3547	0.0054	-1.2787	0.0642	0.3695	1.0455
Ta.27525.1.S1_at	-0.4475	0.0900	-1.3637	-0.5443	0.0723	-1.4583	-0.0968	0.5918	-1.0694

Table S11. Fold change in the expression of probesets related *phosphorylase* genes before and during imbibition of after-ripened seeds (cv. AC Domain)

	AR-12/AR-0			AR-24/AR-0			AR-24/AR-12		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.1279.1.S1_at	-0.5175	0.1776	-1.4315	0.3787	0.3893	1.3002	0.8962	0.0186	1.8612
Ta.1197.1.S1_at	-1.5066	0.0015	-2.8414	1.1442	0.0586	2.2103	2.6508	0.0026	6.2802
Ta.1197.1.S1_x_at	-1.5543	0.0002	-2.9370	1.1142	0.0607	2.1648	2.6686	0.0030	6.3579
Ta.28402.1.A1_at	-0.0638	0.7056	-1.0452	0.1248	0.4622	1.0904	0.1887	0.1339	1.1397
Ta.27525.1.S1_at	-0.6171	0.0141	-1.5338	-0.4119	0.0159	-1.3304	0.2052	0.2984	1.1529

Table S12. Fold change in the expression of probesets related *phosphorylase* genes before and during imbibition of dormant and after-ripened seeds (cv. AC Domain)

	AR-0/D-0			AR-12/D-12			AR-24/D-24			AR-24/AR-24+ABA		
Probeset	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC	log_2 FC	P-value	Linear FC
Ta.1279.1.S1_at	-0.1455	0.6998	-1.1061	-0.5280	0.0111	-1.4420	0.4618	0.1463	1.3773	0.8553	0.0238	1.8091
Ta.1197.1.S1_at	-0.3828	0.1979	-1.3038	-0.6331	0.0261	-1.5509	2.4558	0.0036	5.4860	2.3099	0.0047	4.9584
Ta.1197.1.S1_x_at	-0.3031	0.1086	-1.2338	-0.7074	0.0071	-1.6329	2.4345	0.0043	5.4058	2.1701	0.0065	4.5006
Ta.28402.1.A1_at	-0.3592	0.0617	-1.2828	-0.0043	0.9580	-1.0030	0.1203	0.2582	1.0869	0.1983	0.0886	1.1474
Ta.27525.1.S1_at	-0.0781	0.6892	-1.0557	-0.2478	0.2218	-1.1874	0.0543	0.7612	1.0383	-0.1438	0.2313	-1.1048

ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
AGL	α-glucosidase
AGPase	AGP-glucose pyrophosphorylase
AMY	α-amylase
AR	after-ripening
ATP	adenosine 5′ triphosphate
AVG	aminoethoxyvinylglycine
BAM	β-amylase
BE	starch branching enzyme
BR	brassinosteroids
CTR	constitutive triple response
CYP707A	ABA8'-hydroxylase
DAI	days after imbibition
DBE	debranching enzymes
DNJ	1-deoxy-nojirimycin
DPE	disproportionating enzyme
DW	dry weight
ER	endoplasmic reticulum
ERBPs	ethylene-responsive element binding proteins
ERFs	ethylene-responsive factors
ERS	ethylene response sensor
ETR	ethylene resistant
GA	gibberellin
GA20ox	GA 20-oxidase
GA3ox	GA 3-oxidase
GBSS	granule bound synthases
HAI	hours after imbibition
ISA	isoamylase
MTA	5'-methylthioadenosine
NCED	9-cis-epoxycarotenoid dioxygenase
PCD	program cell death
PHO	glucan phosphorylase
PHS	preharvest sprouting
SAdoMet	S-adenosyl-L-methionine
SS	starch synthases
1-MCP	1-methylcyclopropene