The Role of Gibberellin and Abscisic Acid in Regulating Preharvest Sprouting in Barley (*Hordeum vulgare* L.)

By

Lingwei Liu

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Plant Science
Faculty of Agricultural and Food Sciences
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ABSTRACT

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Preharvest sprouting (PHS), the germination of seeds on the maternal plant before harvest, is a big challenge for barley producers worldwide. It is attributed mainly to low seed dormancy. The balance between two classical plant hormones, gibberellin (GA) and abscisic acid (ABA) regulates seed dormancy and germination, and the endogenous level of these two hormones in plants is determined by their biosynthesis and catabolism. This thesis characterized the expression patterns of the major GA and ABA metabolism genes in barley cv. Betzes during seed development, and germination in both dormant and non-dormant seeds. The results indicate that specific gene family members of the two hormones play distinct temporal roles in regulating seed development, dormancy onset and release, and germination. Since only two genes encoding the GA deactivating GA 2-oxidase enzyme have been known so far in barley, this study also identified two new *GA2ox* genes designated as *HvGA2ox1* and *HvGA2ox3*.

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Dedicated to my deeply loved husband

Everything You Do

I appreciate everything that you do,

From the beginning, you've been there for me,

When I was down, you were strong like a tree.

You offer so much, a heart that is kind,

Think to help others, in your beautiful mind.

Everything you do, I respect and praise,

You're a wonderful person,

Wish to say thank you, deep in my heart,

For so many lives, you're a big part.

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ABBREVIATIONS

AAO3	abscisic aldehyde oxidases
ABA	abscisic acid
AR	after-ripened
BLAST	basic local alignment search tool
CCD	conserved domain database
cDNA	complimentary deoxyribonucleic acid
CPS	*ent*-copalyl diphosphate synthase
Cvi	Cape Verde islands
D	dormant
DAA	days after anthesis
dATP	deoxyadenosine triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tags
ExPASy	expert protein analysis system
FAO	Food and Agriculture Organization
GA	gibberellin
GA20ox	GA 20-oxidase
GA3ox	GA 3-oxidase
GA2ox	GA 2-oxidase
GGDP	geranylgeranyl diphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
KAO	*ent*-kaurenoic acid oxidase
KO	*ent*-kaurene oxidase
KS	*ent*-kaurene synthase

LB	luria-Bertani
MEGA	molecular evolutionary genetic analysis
NCBI	national centre for biotechnology information
NCED	nine-cis epoxycarotenoid dioxygenase
OD	optical density
ORF	open reading frame
PA	phaseic acid
P450s	P450 monooxygenases
PHS	preharvest sprouting
RACE	rapid amplification of cDNA ends
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
2ODDs	2-oxoglutarate-dependent dioxygenases
TPS	terpene synthase
U	unit
ZEP	zeaxanthin epoxidase

FORWARD

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

1.0 GENERAL INTRODUCTION

Barley is the fourth important cereal crop in the world; it is widely used for animal feed, malting, and human consumption. Barley also contains many natural compounds beneficial to health, such as tocopherols, phenolics and phytosterols as well as soluble and insoluble fibers. The production of barley is affected by both biotic and abiotic factors. One of the major problems in barley production is preharvest sprouting (PHS), which refers to the germination of seeds while still on the mother plant. PHS in barley is a great concern for producers and seed processers. It causes adverse consequences, including reduction in yield and end use quality, especially for malting, as the malting industry always sets a high seed quality standard.

Seed germination and dormancy are two important traits that are closely related to the PHS. Too high dormancy increases the cost and the potential damage during the seed storage, while too low dormancy favors the occurrence of PHS when cool temperature and high rainfall coincides with harvest maturity (Lin *et al*., 2008). Seed dormancy and germination are known to be regulated by several hormones. It is well understood that two types of plant hormone, gibberellin (GA) and abscisic acid (ABA), regulate seed germination and dormancy. ABA plays an important role in the induction and maintenance of dormancy (Finkelstein, 2004; Gubler *et al*., 2005). Elevated ABA levels are important during mid- and late- embryogenesis to maintain normal seed development, and a relatively high level of ABA in maturing seeds is important to maintain dormancy

and avoid precocious germination. Gibberellin plays antagonistic role to ABA, and promotes seed germination (Koornneef *et al*., 2002). The levels of GA during embryo development are usually high, as it is required for normal seed development (Swain *et al*., 1995). One of the effective methods used to release seed dormancy in many species including barley is after-ripening, which involves a period of dry storage. Seed moisture content and storage temperature play important roles in determining the efficiency of after-ripening. It involves changes in seed GA and ABA metabolism and sensitivity.

The levels of GA and ABA in plant tissues are controlled by their biosynthesis and catabolism (Nambara and Marion-Poll, 2005; Yamauchi, 2008). Genes encoding the major and regulatory enzymes involved in GA and ABA metabolism have been identified. In the GA metabolic pathway, the GA biosynthetic genes, *GA20ox* that encodes GA 20-oxidase and *GA3ox* that encodes GA 3-oxidase, and the GA catabolic gene, *GA2ox* that encodes GA 2-oxidase (Yamauchi, 2008) are considered to play regulatory roles. With respect to ABA, the ABA biosynthetic gene, *NCED* that encodes nine-*cis*-epoxycarotenoid dioxygenases and the ABA catabolic gene, *CYP707A* gene that encodes ABA 8'-hydroxylase play predominant roles in regulating ABA level (Nambara and Marion-Poll, 2005).

This thesis tested the hypothesis that the balance between ABA and GA regulates dormancy induction, maintenance and release in barley seeds. This hypothesis was tested by measuring the expression patterns of major GA and ABA metabolism genes during

seed development, and comparing the transcription of these genes between dormant, after-ripened and ABA treated after-ripened seeds during imbibition. Furthermore, this work identified two new barley *GA2ox* genes (*GA2ox1* and *GA2ox3*). The findings of this study will advance our knowledge about the relationship between GA and ABA in regulating seed dormancy, which is necessary for introducing new strategies to achieve desired level of dormancy in barley seeds, and thus prevent PHS.

2.0 LITERATURE REVIEW

2.1 Barley

2.1.1 Origin of barley

Barley (*Hordeum vulgare* L.) is one of the founder crops of Old World agriculture. Archaeological evidences of barley seeds found in the area of Middle East known as Fertile Crescent (Zohary and Hopf, 1993; Diamond, 1998) have indicated that the crop was domesticated about 10,000 years ago. The wild ancestor of cultivated barley is believed to be *Hordeum spontaneum* C. Koch. (Harlan and Zohary, 1966; Zohary, 1969), and *H. vulgare* comprises both cultivated and wild forms. It is divided into two subspecies: *H. vulgare* L. ssp. *vulgare*, and *H. vulgare* L. ssp. *spontaneum* (C. Koch.). The cultivated barley is referred as *H. vulgare* L. ssp. *vulgare* (Bothmer and Jacobsen, 1985). Research has shown that Central and South western part of Asia, western North America, South America, and the Mediterranean region are the diversity centers of the genus *Hordeum* (Bothmer, 1992). There are 32 species in the genus *Hordeum*, all with a basic chromosome number of x=7. Cultivated barley, *Hordeum vulgare* L. ssp. *vulgare*, is a diploid species with 2n=2x=14 chromosomes. In general, it is a short season and early maturing cereal crop with high yield potential and a wide range of environmental adaptation. It is also more tolerant to drought, saline and alkaline soil conditions than other cereal crops (Poehlman, 1985).

2.1.2 Barley production and its use

Barley ranks fourth in the world cereal crop production, after maize, rice, and wheat, and the world total production for the year 2012 is 130,114 thousand metric tons (USDA, 2012). It is grown globally over 100 countries. According to the Food and Agriculture Organization (FAO) report that takes into account the average barley production from 1992 to 2010, Russian Federation, Germany, Canada, France and Ukraine are the top five barley-producing countries (FAO, 2010).

Barley is mainly used, in order of importance, for animal feed, brewing malts and human food, while about 5% of the world's total barley production is used for seed. In addition, barley has some useful by-products such as its straw can be used for animal bedding and immature barley plants may be collected for forage (Poehlman, 1985; Akar *et al*., 2004). The use of barley seeds for feed is attributed mainly due to its high protein content, which ranges from 10% to 15%. Usage of barley for brewing, as recorded in Egyptian artifacts, is estimated to be 5000 years old (Hardwick, 1977; Katz, 1979). The choice of barley for malting over wheat and rye is due to the fact that its husk protects the acrospires during germination and serves as a filtration aid, and the texture of its kernel is firm (Dickson, 1979). The Canadian brewing industry, which is the end user of the malt from barley, is the largest component of the alcohol beverage sector, and in 2009 alone, sales of goods manufactured by this industry were valued at $4,671.2 million (Statistics Canada, 2009).

With respect to its use for human food, barley is a nutritional powerhouse as it is high in fiber, β-glucan, phenolic antioxidants, and vitamins E and B-complex. For example, among all the cereals crops, barley seeds contain the highest level of β-glucan (3-11%, w/w). These nutritional characteristics are important as they help reduce the risk of heart disease, stroke and cancer, and boost the immunity system. For these reasons, barley has been promoted as a healthy source of soluble and insoluble fiber. For example, in 2009, an application for generic health claims: Barley β-glucan soluble fiber and reduction of blood cholesterol, a risk factor for cardiovascular disease, was submitted to the Health Canada. This application has been approved as scientific evidence exists in support of the therapeutic claim linking barley seed products to a reduction of blood cholesterol (Health Canada, 2012). Furthermore, the unique texture in some barley varieties is suitable to use it in ready-to-eat snacks or as a replacement for nuts in sweet confections, and peeled barley is commonly used to thicken soups. Because of its unique agronomic and nutritional qualities, barley presents huge opportunities to growers, processors, and consumers.

2.1.3 Major constraints of barley production

Like any other crop, barley production is affected by several biotic and abiotic factors. Although barley can grow in a wide range of environment, the occurrence of high temperature during post-anthesis reduces seed weight and affects malting quality (Van

and Vernon, 2006). Besides, barley is very sensitive to soil acidity and deficiency of nutrients such as nitrogen, potassium and phosphorus, which can be major constraints to its growth and productivity. With respect to biotic stresses, barley production is affected by several viral disease, barley yellow dwarf, barley stripe mosaic and oat blue dwarf, which are most important diseases in North America. Barley grown in temperate and humid regions is susceptible to bacterial and fungal foliar pathogens. For example, powdery mildew, caused by the biotrophic fungus *Erysiphe graminis* f. sp. *hordei,* is one of the major diseases of barely on a worldwide basis; especially in northern temperate areas. Other fungal diseases such as leaf rust, stem rust, net blotch and spot blotch also affect barley production (Kiesling, 1985).

Barley seeds used for malting purpose require rapid, uniform and complete germination; at least 50% germination in 1-2 days and 95-100% in 3 days (Briggs, 1978). High or excessive dormancy cause non-uniform germination, poor stand establishment, and low malt extract yield in commercial production and in the malt house. It also increases the cost and potential damage during the storage period (Carn, 1980). Therefore, low dormancy of seeds was selected during domestication; as a result seeds from most of the current barley cultivars exhibit more than 90% germination within 4 days of imbibition (Bothmer, 1992). Low dormancy leads to preharvest sprouting, the germination of seeds on the mother plant, especially when wet condition occurs during the harvest season. It has been reported that a short exposure (less than 24 h) of seeds to

rain water in the field between their physiological and harvest maturity stages may cause embryo growth and thereby preharvest sprouting (Benech-Arnold, 2001).

Preharvest sprouting (PHS) leads to the synthesis of hydrolytic enzymes that promote the degradation of storage reserves (Bewley and Black, 1994), and thereby results in loss of seed weight and reduction in its end use quality. Some dormancy at harvest is favored because it prevents germination of the physiologically mature seed in the head prior to harvest, and thereby improve barley productivity, especially in places where cool and moist conditions are prominent. It is therefore critical to develop barley cultivars that are tolerant to a certain extent to sprouting between physiological maturity and completion of harvest, but not excessively dormant to ensure rapid and uniform germination after harvest to achieve both good-malting grade and good stand establishment in the field.

2.2 Seed

2.2.1 Seed formation and development

Seed development is a crucial process in the lifecycle of higher plants, and it can be divided into two major phases: morphogenesis (embryo and endosperm development) and maturation (Gutierrez *et al*., 2007).

In angiosperm, seed development starts form double fertilization in the ovule. At maturity, the pollen seed is either tricellular (comprised of a vegetative cell and two

sperm cells) or bicellular (comprised of a vegetative cell and a generative cell that undergoes mitosis during pollen tube elongation to produce two sperm cells) (Cheung *et al*., 2000). Pollen tubes elongate by tip growth and the vegetative cell provides many of the activities needed for this process (Cheung *et al*., 2000). Once the pollen tube reaches one of the synergids in the embryo sac, the male gametes are released from the cytoplasm of the tube into the embryo sac, the haploid egg cell and the two polar nuclei of the female gametophyte each fuse with one sperm cell from the pollen tube to form the diploid zygote and triploid endosperm, respectively (Chaudhury *et al*., 2001). After fertilization, early morphogenesis occurs in the embryo sac. The zygote undergoes asymmetric cell division and forms an apical and a basal cell. Most of the embryo originates from the smaller apical cell, while the larger basal cell forms parts of the root and suspensor (Chaudhury *et al*., 2001). The triploid endosperm develops in two steps: first, rapid proliferation and expansion of the endosperm occurs to generate a large and multinucleated cell defined as the syncytial phase, and result in a large increase in seed size. This syncytium is then partitioned into individual cells by a specific type of cytokinesis called cellularization. Following cellularization, endosperm cells enter into differentiation stage (Olsen, 2001). Once the embryo and endosperm have completed the morphogenesis and patterning stages, seeds enter into maturation phase (Wobus and Weber, 1999). During maturation, embryo cells go through a period of expansion and differentiation concomitant with accumulation of storage reserves in the endosperm.

Maturation ends with a desiccation phase, which due to the loss of water results in a gradual reduction in metabolism, leading to an embryo with a quiescent metabolism status. The seed coat, also known as the testa, is derived from the integuments of the ovule, and represents the maternal tissue (Boesewinkel and Bouman, 1984; Bewley and Black, 1995).

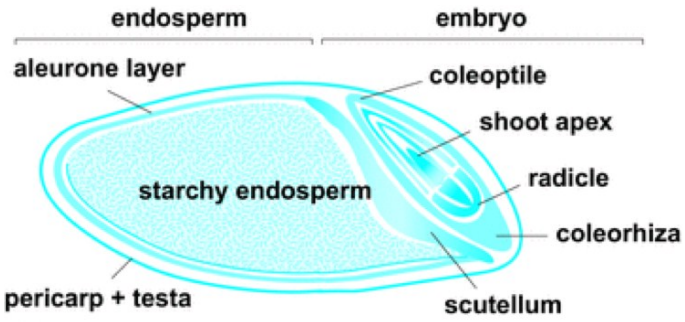


Figure 2.1 Mature barley seed structure
(Source: http://www.accessscience.com/loadBinary.aspx?filename=900110FG0010.GIF; accessed on 5 February 2013)

The seeds of cereal crops including barley contain an embryo with a single cotyledon, called the scutellum (Figure 2.1), which absorbs food from endosperm during germination when the reserves in the endosperm are hydrolyzed. The endosperm of cereal seeds is normally hard and flinty, and it is the organ where storage reserves are laid down. It is enclosed by the aleurone layer, which usually consists of several layers of living cells with high amount of reserve protein. The testa of cereal seeds is fused to the pericarp to give a fruit described as caryopsis.

2.2.2 Seed dormancy

Seed dormancy refers to the failure of viable seeds to germinate under favorable conditions (Harper, 1959; Bewley, 1997). It is an innate property, which is acquired during the evolution of plants to survive in adverse conditions, such as heat, cold and drought (Hilhorst, 2007). There are different classifications of seed dormancy. Based on the timing of its occurrence, dormancy is grouped as primary and secondary dormancy. Primary dormancy is the innate dormancy obtained by seeds when they disperse from mother plants; it is induced by several factors including abscisic acid (ABA) during seed development. Seeds can be released from primary dormancy by a number of factors: after-ripening, chilling, and light. Non-dormant (ND) seeds can enter into a dormant state, which is called secondary dormancy, if they are exposed to conditions that inhibit their germination. As for primary dormancy, Baskin and Baskin (2004) reported a more comprehensive classification system that includes five different types of dormancy: physiological dormancy (PD), morphological dormancy, morphophysiological dormancy, physical dormancy and combinational dormancy (physiological dormancy plus physical dormancy). Of the five types of primary dormancy, physiological dormancy is the most abundant one, and encompasses three different levels: non-deep, intermediate and deep. Non-deep dormancy has been the major focus of contemporary research on seed dormancy induction, maintenance and release. The embryo excised from non-deep physiologically dormant seeds can produce normal seedlings, and gibberellin (GA)

treatment can break this dormancy, but in the case of the deep physiologically dormant seeds, the excised embryo either cannot grow or produce abnormal seedlings. Morphological dormancy occurs in seeds with underdeveloped embryo, thus needs time to germinate and grow; whereas morphophysiological dormancy is exhibited by seeds that possess undeveloped embryos and exhibit physiological component of dormancy. Physical dormancy of seeds is caused by one or more water-impermeable layers of palisade cells in the seed or fruit coat. For the combinational dormancy, not only the seed coat is water impermeable but also the embryo is physiologically dormant.

Two major causes of dormancy are known for non-deep physiological dormancy: embryo dormancy and coat-imposed dormancy. In case of embryo dormancy, the properties of the embryo are vital, immature and underdeveloped embryo or metabolic blocks within the embryo will cause seed dormancy. Whereas in coat-imposed dormancy, the tissues covering the embryo such as the endosperm, perisperm and testa play the role by posing as a mechanical restraint that prevents embryo expansion, decrease leaching of inhibitors from embryo, restrict gaseous exchange and interfere with water uptake. Dormancy in a number of species is imposed by both the embryo and the covering layers. In seeds of most grasses and cereal crops, the glumellae (lemma and palea) adhering to the caryopsis represents a further constraint for embryo germination in addition to that imposed by the endosperm and the surrounding pericarp (Corbineau and Come, 1980; Simpson, 1990; Benech-Arnold *et al*., 1999).

2.2.3 Release of seed dormancy

The release of seed dormancy can be induced by a number of factors including after-ripening (a period of dry storage), chilling (cold stratification), warm stratification, plant hormones such as GA, smoke, light, nitrate and nitric oxide (Bailly, 2004; Kucera *et al*., 2005; Bethke *et al*., 2006).

The periods of dry storage that can induce loss of dormancy vary from species, e.g. few weeks for barley or as long as 60 months for *Rumex crispus* (Bewley and Black, 1994). The efficiency of after-ripening in breaking the dormancy of seeds depends on storage environmental factors such as moisture, temperature, and oxygen. It occurs in non-imbibed viable seeds under very low water potentials (less than 0.1 g water per dry weight; Oracz *et al*., 2007), but not occurs in very dry seeds, as it requires seed moisture above a threshold value. In general, after-ripening takes place at seed moisture contents between 8%-15%, although the value is lower for oil-storing seeds (Leubner-Metzger, 2005; Bazin *et al*., 2011). After-ripening induces changes in the physiological state of the seed such as gene expression. For example, in Arabidopsis after-ripening of dry dormant (D) seeds led to a decrease in the expression of *DELAY OF GERMINATION 1* (*DOG1)* gene, which is known to be associated with the induction of primary dormancy during seed development (Finch-Savage *et al*., 2007). Dry after-ripened (AR) seeds of *Nicotiana tabacum* exhibited low level of transcription and translation of β-1,3-glucanase, which is localized to the inner testa and associated with the after-ripening induced promotion of

testa rupture (Leubner-Metzger, 2005). Besides, Oracz *et al.* (2007) have shown that in dry AR sunflower there is an accumulation of reactive oxygen species in the embryo axes, which leads to carbonylation of specific embryo protein. Previous studies have shown that the role of after-ripening in breaking seed dormancy is modulated by changes in the metabolism of plant hormones mainly that of GA and ABA (see below for detailed discussion).

2.2.4 Seed germination

Seed germination starts with imbibition, and is completed as the embryo radicle emerges through the seed covering layers (Finch-savage and Leubner-Metzger, 2006; Weitbrechet *et al.*, 2011). Germination encompasses three phases of water uptake. Dry seeds have very low water potentials, which cause rapid water uptake in the first phase. During imbibition the seed rapidly swells and changes in size and shape (Robert *et al*., 2008). H-NMR image analysis of imbibed seeds from pea, tobacco, and other species revealed the presence of some major points such as the micropyle for the water to enter into the seed (Manz *et al*., 2005; Wojtyla *et al*., 2006). In Arabidopsis, after imbibition, the mucilage is released from the columellae, and water can enter through the columella quickly (Weitbrecht *et al*., 2011). Structural change of a seed during initial imbibition is accompanied by a massive leakage of solutes and low molecular weight metabolites into the surrounding imbibing solution. Such leakage can accelerate germination by reducing

the concentration of inhibitors in the seed (Matilla *et al*., 2005). However, solute leakage also represents damage to cell membrane and other cellular compartments such as mitochondria, which is important for respiration and energy metabolism, as a result of the fast and inhomogeneous rehydration (Weitbrecht *et al*., 2011). In order to deal with the damages imposed by the fast rehydration process, seeds activate a number of mechanisms that are involved in repairing the membrane, and activating respiration and other processes such as synthesis of proteins and mitochondrial DNA (Bewley, 1997; Weitbrecht *et al*., 2011).

Once the seed size and shape start to stagnate, the seed moves into the second phase of water uptake, which is also called the plateau phase, as the seed water content is reached a constant stage. During this phase, the embryo starts to expand and the seed undergoes a phase change to germination. DNA synthesis associated with cell division accounts for the second phase. At the same time, storage reserves in the endosperm are hydrolyzed and enzymes related to endosperm weakening and embryo growth are synthesized, to induce the essential capacities for the radicle to emerge through the endosperm and the testa (Bewley, 1997). The third phase is characterized by further increase of water uptake by the seed, and is also called the growth phase. The larger water uptake is needed for the mitotic divisions and cell expansion of the growing radicle, and seedling growth to occur (Nonogaki *et al*., 2010).

2.3 Plant hormones regulate seed development and germination

Several plant hormones regulate the process of seed development and germination. However, accumulated evidences indicate that ABA and GA are the major players in controlling these two developmental processes. ABA regulates key events during seed formation such as the deposition of storage reserves, and it is a positive regulator of dormancy induction and maintenance (Kermode, 2005). GA is believed to counteract the roles of ABA, and is involved in the release of dormancy and promotes germination. GA promotes post-germinative growth by triggering the synthesis of hydrolytic enzymes that degrade and mobilize seed storage reserves and stimulate embryo cell expansion (Bewley, 1997). Other plant hormones such as ethylene, cytokinin and auxin regulate seed development and germination in plants. For example, ethylene promotes seed germination by antagonizing ABA signaling (Kucera *et al*., 2005). Cytokinins are present in the liquid endosperm of developing seeds and are used for the promotion of cell division in the embryo (Mok and Mok, 2001), and are also important in embryogenesis, embryonic pattern formation (Dewar *et al.*, 1998). Auxin appears to play a major role in embryogenesis, regulating the correct cellular patterning from the globular stage onwards (Teale *et al.*, 2006). Besides, auxin is involved in the apical-basal pattern formation during embryogenesis (Dewar *et al.*, 1998). Using auxin-responsive promoter fused with GUS reporter gene; Liu *et al.* (2007) suggested that auxin is accumulated in the micropylar region and radicle tip of germinating embryos.

2.3.1 The role of gibberellin during seed development and germination

2.3.1.1 Gibberellin

Gibberellins (GAs), one of the major classes of plant hormones, are a group of diterpenoid compounds that play important roles during plant growth and developmental processes including seed germination, stem elongation, leaf expansion, and floral, fruit, seed development (Yamaguchi, 2008). Gibberellin was first isolated from *Gibberella fujikuroi*, a pathogenic fungus that causes bakanae disease of rice, by a Japanese scientist called Eiichi Kurosawa. Plants infected with the fungus exhibit excessive stem elongation with poorly developed seeds (Crozier *et al.*, 2000). In the 1930s, TeijiroYabuta and Yusuke Sumiki first isolated a crystalline compound from the cultured fungus, and named it gibberellic acid; however, its chemical structure was elucidated in the 1950s.

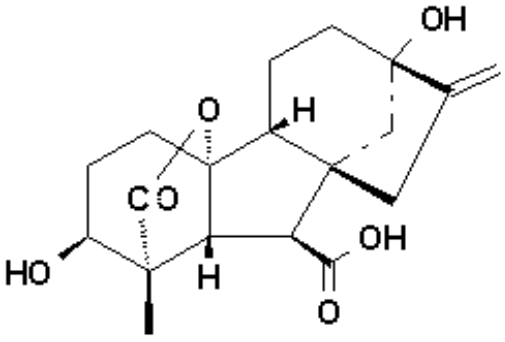


Figure 2.2 Chemical structure of GA_1
(Source: http://www.plant-hormones.info/gibberellins.htm; accessed 10 March 2012)

To date, at least 136 naturally occurring GAs have been identified form plant, fungi and bacteria (http://www.plant-hormones.info/gibberellins.htm; accessed on 5 March 2012). All GAs share a similar chemical structure where they possess either a tetracylic *ent*-gibberellane skeleton containing 20 carbon atoms, C_{20}-GAs, or a 20-nor-*ent*-gillbrellane that lost the C-20 as CO_2 and carry a γ-lactone, named as C_{19}-Gas (Yamaguchi, 2008). Only few of GAs are biologically active including GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7, and the remaining are either metabolic precursors or their deactivation products. Inactive GA precursors might be stored or translocated via the phloem and xylem before their activation and release at the proper time and in the proper tissue (Sponsel, 1995; Arteca, 1996). Of the biologically active GAs, GA_1 (Figure 2.2) has been identified from 86 plants, and is considered as the primary GA important for plants. The other biologically active GA, GA_4 has also been found in plants, mainly in seeds, fruit and leaves.

2.3.1.2 Gibberellin metabolism pathway

The very initial steps of GA biosynthesis involve the formation of isopentenyl diphosphate (IPP). For most plants, the methyl erythritol phosphate (MEP) pathway is considered to be the source of IPP for GA biosynthesis (Yamaguchi, 2008). The IPP (5-carbons) is used to produce intermediate compounds containing 10 carbons (geranyl diphosphate, GPP), 15 carbons (farnesyl diphosphate, FPP), and then 20 carbons

(geranylgeranyl diphosphate, GGDP). All GAs are synthesized from GGDP, which acts as a common precursor for all the diterpenoids.

The GA biosynthetic pathway can be divided into three stages (Figure 2.3); each stage taking place in a different cellular compartment: the plastid, endoplasmic reticulum, and cytosol. Three different classes of enzymes are required for the biosynthesis of bioactive GAs: terpene synthases (TPSs) that contain *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS); cytochrome P450 monooxygenases (P450s) that include *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO); and 2-oxoglutarate-dependent dioxygenases (2ODDs) that consist of GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox).

The first stage of GA biosynthesis involves two cyclization reactions that convert the linear GGPP to *ent*-kaurene, and takes place in the plastid (Helliwell *et al*., 2001; Sun and Kamiya, 1997). The formation of a bicyclic intermediate *ent*-copalyl diphosphate (*ent*-CDP) from GGDP is catalyzed by CPS, and KS catalyzes the production of *ent*-kaurene from *ent*-CDP.

The second stage of GA biosynthesis takes place on the plastid envelope and in the endoplasmic reticulum (Helliwell *et al*., 2001). The C-19 methyl group of kaurene is oxidized in three steps to form *ent*-kaurenoic acid (KA). All the three oxidation steps leading to the formation of KA are catalyzed by KO, a membrane-associated cytochrome P450 monooxygenase. The resulting KA is then oxidized at C-6 and C-7 in two steps to

give GA_{12}-aldehyde, and this involves the contraction of B-ring from 6 to 5 carbons. The newly formed GA_{12}-aldehyde is then oxidized to GA_{12}, which is the first-formed GA and act as a common precursor for all other GAs in higher plants. All the reactions involved in the conversion of KA to GA_{12} are catalyzed by KAO, a multifunctional enzyme.

The final stage of GA biosynthesis takes place in the cytosol and involves the conversion of GA_{12} to bioactive GAs, which is catalyzed by soluble 2-oxoglutrate-dependent dioxygenases. GA_{12} lies at the branch-point in the pathway and undergoes through 13-hydroxylation or non-13-hydroxylation reactions. In some species (pea, maize, rice, barley) GA_{12} is converted to GA_{53} by 13-hydroxylation, which is catalyzed by GA 13-hydroxylase. GA20ox sequentially catalyzes the conversion of GA_{53} to GA_{44}, GA_{19}, and finally to GA_{20}. Then, GA_{20} is oxidized by GA3ox to produce GA_1. While in others such as *Arabidopsis* and members of the Cucurbitaceae family, the non-13-hydroxylation pathway operates. In the non-13-hydroxylation pathway, GA20ox catalyzes the conversion of GA_{12} to GA_{15}, GA_{24} and GA_9. Finally, GA_9 is converted to bioactive GA_4 by GA3ox.

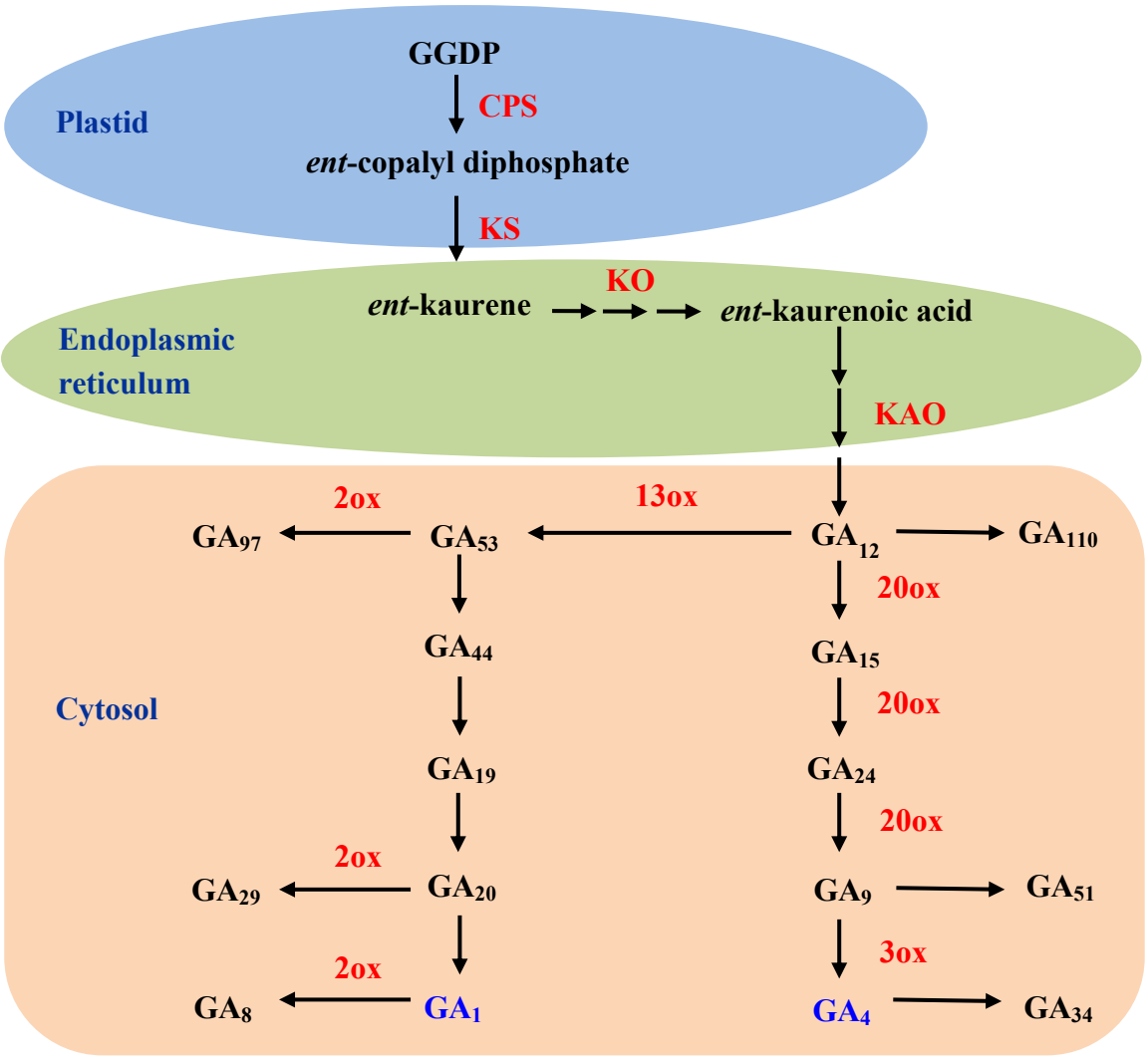


Figure 2.3 Gibberellin metabolism pathways in plants. GA_1 and GA_4 are the biologically active GAs.

In spite of its important roles in plants, overdose of GA can also cause some problems, like excessive growth, and less seed yield as more of the energy is used for stem development. Thus, it's important for plants to produce and maintain optimal levels of bioactive GAs to ensure normal growth and development. Plants employ several ways of GA deactivation. The most universal GA deactivation reaction is 2β-hydroxylation, which is catalyzed by another class of 2ODDs, GA 2-oxidase (GA2ox). The GA2oxs can be divided into three classes: class I GA2oxs directly use bioactive GAs, like GA_1 and GA_4 as substrates and convert them to inactive forms, GA_8 and GA_{34}, respectively; class II enzymes convert the immediate precursors of bioactive GAs such as GA_9 and GA_{20} into another inactive forms, GA_{51} and GA_{29}, respectively. The third class of GA2ox is new discoveries, which only accept the C_{20}-GAs such as GA_{53} and GA_{12} as substrates, converting them to GA_{97} and GA_{110}, respectively.

Recently, two other GA deactivation mechanisms have been identified (Zhu *et al*., 2006; Varbanova *et al*., 2007). The first one involves epoxidation of the 16, 17-double bond of non-13-hydroxylated GAs in rice by ELONGATED UPPERMOST INTERNODE (EUI), which belongs to a P450 class of enzymes (Zhu *et al*., 2006). The second mechanism involves methylation of the C-6 carboxyl group of GAs in *Arabidopsis* (Varbanova *et al*., 2007). This reaction is catalyzed by GA methyl transferases (GAMT) and uses both bioactive GAs and their precursors as their substrates

to produce corresponding methyl esters. Gibberellins can also be deactivated through conjugation, most commonly with glucose. The linkage of the GAs moiety to glucose occurs either via a hydroxyl group to produce GA-*O*-glucosyl ether (GA-*O*-Glc), or via the COOH at C-6 yielding a GA-glucosyl ester (GA-Glc ester). When applied to bioassay plants, the GA-*O*-glucosyl ethers show little-to-no activity, indicating that they are deactivation products. In contrast, the GA-glucosyl esters may be hydrolyzed within a bioassay plant, yielding the free GA. Hence; the formation of glucosyl ester conjugates may be a mechanism for sequestration of bioactive GA that can be released later, as required by the plant.

2.3.1.3 Isolation of genes involved in gibberellin metabolism

Genes involved in GA metabolism have been identified using a number of approaches, and those encoding the enzymes involved in the later part of the pathway including *GA20ox*, *GA3ox* and *GA2ox* are crucial in controlling the level of bioactive GA. The first *GA20ox* gene was identified from pumpkin *(Cucurbita maxima)* by screening a cDNA library derived from developing cotyledons using antibodies raised against a synthetic peptide corresponding to the sequence of a proteolytic fragment of GA20ox purified from pumpkin endosperm (Lange, 1994). After that, a series of *GA20ox* genes has been cloned from different plant species (Phillips *et al*., 1995; Hedden and Phillips, 2000). In Arabidopsis, the first two *GA20ox* genes were identified by PCR from the shoot of

Arabidopsis *ga1-2* mutant using degenerate oligonucleotide primers derived from the pumpkin (*Cucurbita maxima) GA20ox* cDNA sequence. The third one was identified after scrutiny of the database of Expressed Sequence Tags (ESTs; Phillips *et al*., 1995). Later, two more *GA20ox* genes were identified by sequencing of chromosome 1 of Arabidopsis (Hedden and Phillips, 2000).

The first rice *GA20ox* gene (*GA20ox1*) was cloned from seedlings by using degenerate oligonucleotide primers designed based on the amino acid sequences of GA20ox from pumpkin and *Arabidopsis* (Toyomasu *et al*., 1997). While *OsGA20ox2* was obtained from the genome sequence data of rice semi-dwarf (*sd-1*) mutant, and mapping studies showed that this gene is located on chromosome 1 (Spielmeyer *et al*., 2002). *In silico* analysis of all available rice DNA databases with predicted amino acid sequences of genes encoding GA20ox in various plants (including Arabidopsis, lettuce, pea, tomato and rice) revealed the presence of four *GA20ox* (*1* to *4*) genes (Sakamoto *et al.*, 2004). Thus far, two *GA20ox* genes are known in barley; *GA20ox3* was isolated from endosperm cDNA library by using the wheat *GA20ox* as a probe, whereas *GA20ox1* was isolated from the leaf cDNA library by using wheat *GA20ox* and the barley *GA20ox3* as probes (Spielmeyer *et al.*, 2004).

With respect to genes encoding the regulatory GA3ox enzyme, four functional *GA3ox* genes exist in Arabidopsis. The first *GA3ox* gene was isolated from the Arabidopsis *GA4* locus by using the insertional mutagenesis (Chiang *et al.*, 1995), and a

second gene designated as *GA4H* (for *GA4* homolog) was isolated by screening the Arabidopsis genomic DNA library using the *GA4* cDNA as a hybridization probe (Yamaguchi *et al*., 1998). In rice, a fragment related to *GA3ox* gene was first obtained by PCR using degenerate primers and this fragment was further used to screen a genomic DNA library of rice and isolate two *GA3ox* (*1* and *2*) genes (Itoh *et al*., 2001). Similarly, Sakamoto *et al.* (2004) obtained two *GA3ox* like genes (*GA3ox1* and *2*) by screening the rice DNA database. To date, two *GA3ox* genes are known in barley. The barley *GA3ox1* gene was identified by random sequencing of inserts from endosperm cDNA library of cv. Himalaya, while *GA3ox2* is obtained from leaf cDNA library using wheat *GA3ox* gene as a probe (Spielmeyer *et al.*, 2004).

The first GA deactivating *GA2ox* gene was cloned from runner bean (*Phaseolus coccineus* L.) through functional screening of cDNA library derived from developing cotyledons (Thomas *et al*., 1999). Searching the Arabidopsis database using the predicted protein sequence of *PcGA2ox1* produced three related cDNAs, which are designated as *AtGA2ox1*, *AtGA2ox2* and *AtGA2ox3* (Thomas *et al*., 1999). Later two more *AtGA2ox* genes (*4* and *5*) were cloned, and the functionality of these five *GA2ox* genes was confirmed by expressing their cDNAs in *E.coli*. Only *GA2ox5* was found not to be expressed, as it contains a large DNA insert that makes it non-functional (Hedden and Phillips, 2000; Hedden *et al*., 2002). Further, *AtGA2ox6* was identified by Wang *et al.* (2004) using chromatin immunoprecipitation approach, and activation tagging based

mutant screening led to the identification of *AtGA2ox7* and *AtGA2ox8* genes (Schomburg *et al.*, 2003). In the case of rice, the first *GA2ox* gene was cloned by PCR using degenerate oligonucleotide primers designed from the conserved regions of 2ODDs (Sakamoto *et al.*, 2001), and two more *OsGA2oxs* (*2* and *3*) were further obtained by using the same approach (Sakai *et al.*, 2003). The *OsGA2ox4*, *OsGA2ox5* and *OsGA2ox6* genes were identified by searching the rice DNA database against *GA2ox* genes derived from different species (Sakamoto *et al.*, 2004; Lee and Zeevaart, 2005). In barley, two *GA2ox* genes have been identified from leaf cDNA library of cv. Himalaya by using a specific EST related to *GA2ox* (accession number AL505365) as a probe (Spielmeyer *et al*., 2004).

2.3.1.4 Gibberellin and seed development

GA influences a wide range of seed developmental processes including fertilization and embryogenesis. GA biosynthesis in developing seed of many species leads to the accumulation and storage of either the precursors or bioactive GAs (Yamaguchi *et al.*, 2001). The levels of bioactive GAs are usually high during embryo development, but as the seed matures, most of the active GAs are deactivated with the formation of various conjugates or GA catabolites. Treatment of microspore-derived embryos of *Brassica napus* with the GA biosynthesis inhibitor, uniconazole, causes a decrease in bioactive GA level and ~50% reduction in embryo axis elongation (Hays *et al*., 2002). In pea, *KO*,

which encodes the enzyme responsible for the three step oxidation of *ent*-kaurene to produce *ent*-kaurenoic acid, is designated as *LH*. Previous study with *lh-1* and *lh-2* mutants revealed that the *lh-1* mutation has a transient effect on embryo and seed growth that leads to seed abortion, while the *lh-2* mutation results in loss of seed survival by 50% (Swain *et al*., 1997). Another gene, *LE*, which encodes GA3ox, is involved in the pea pericarp and seed development (Ozga *et al*., 2003). The expression of *PsGA3ox1* was very low in pericarps and ovules before pollination. However, after pollination, the mRNA level of *PsGA3ox1* increased dramatically in both tissues. The increases in the transcript abundance of *PsGA3ox1* are associated with rapid expansion of the pericarp and increase in seed size. Moreover, high expression of *GA20ox2, GA2ox1* and *GA2ox2* was found in developing seeds of pea (Ayele *et al.*, 2006). As the embryo matures, a great decrease in the expression of *GA20ox2* and *GA2ox1* occurred, but the *GA2ox2* remained high, suggesting that this gene is important to control the GA level in the later phase of seed development. A study by Singh *et al.* (2002) has also shown that over expression of a GA deactivation gene from pea (p*sGA2ox2*) in Arabidopsis leads to inhibition of pollen tube growth, and seed abortion.

Expression analysis of GA biosynthesis genes during reproductive development in Arabidopsis has indicated that *GA3ox1* expression is initiated under floral apex, becoming stronger as flowers develop and is mainly expressed in stamen filaments and flower receptacles, whereas, the expression of *GA3ox2* was observed in the anther layers

and later in the pollen seeds (Hu *et al.*, 2008). The expressions of *GA3ox3* and *GA3ox4* are localized only in the anthers. Spatial analysis of *GA3ox* genes in developing siliques of Arabidopsis has shown that *GA3ox1* is mainly expressed in the replums, funiculi and silique receptacles; *GA3ox3* only in developing embryos; and *GA3ox4* in the endosperm tissue of developing seeds. Consistently, mutants of *GA3ox1* and *GA3ox3* exhibit a significant fertility defect (Hu *et al*., 2008). In another study, knocking out of the five *GA2ox*s (*GA2ox1*, *-2*, *-3*, *-4* and *-6*) genes, which resulted in the generation of a *ga2ox* quintuple mutant of Arabidopsis, led to over two-fold increase in the levels of GA_1 and GA_4. This enhanced GA level was shown to cause uncoordinated growth of the pistil and petals tissues that leads to reduced efficiency of pollen transfer from the stamen to the pistil, and consequently significant reduction in silique length and seed number (Rieu *et al*., 2008).

2.3.1.5 Gibberellin as a promoter of seed germination

Gibberellin is well known for its role in promoting seed germination. For example, the non-germinating GA-deficient mutant of tomato (*ga-1*) and Arabidopsis (*ga1-3*) can germinate if their seeds are treated with exogenous GA (Groot and Karssen, 1987; Karssen *et al*., 1989). It has been proposed that GA has two roles in the controlling seed germination. The first is to induce the expression of genes encoding enzymes hydrolyzing the endosperm; and the second to stimulate effect on the growth potential of the embryo

(Debeaujon and Koornneef, 2000). Consistent with this, the major increase in GA levels during germination usually occurs just before the radical protrusion, while the ABA content usually decreases during early imbibition (Jacobsen *et al*., 2002; Ogawa *et al*., 2003). A de novo biosynthesis of GAs is required during imbibition, as the application of GA biosynthesis inhibitors, such as paclobutrazo and tetcyclacis, prevent germination (Karssen *et al*., 1989; Nambara *et al*., 1991). The temporal and spatial expression of GA biosynthesis genes during germination is well characterized in Arabidopsis. Expression of early biosynthesis gene *AtCPS1*, which catalyzes the first step of GGDP cyclization, occurs in embryo provasculature in germinating seeds (Yamaguchi *et al*., 2001). Other GA biosynthesis genes such as *AtKO1*, *AtGA3ox1* and *AtGA3ox2* appear to be localized in the cortex and endodermis of embryo axes in germinating seeds. This implies that the intercellular transport of an intermediate of the GA is required for the formation of bioactive GAs (Yamaguchi *et al*., 2001). Besides, the expansion of the cortical cells of the embryonic axis during germination is also well related to the sites of GA biosynthesis (Yamaguchi *et al*., 2001), and a reduced growth rate is observed in GA-deficient embryos (Groot and Karssen, 1987). All these results indicate that GA enhances growth potential of the embryo. In tomato, genes encoding cell wall loosening enzymes such as β-mannanase, expansin, β-1,3-glucanase and chitinase were shown to be expressed specifically in the micropylar endosperm cap region (Chen and Bradford, 2000; Nonogaki *et al*., 2000; Wu *et al*., 2001), suggesting their contribution to the weakening of

this tissue to facilitate radical emergence. Ogawa *et al.* (2003) observed that the expressions of several GA biosynthesis genes, including *AtKO1*, *AtGA20ox3*, and *AtGA3ox1* are upregulated within 8 h of imbibition of Arabidopsis seeds, where as that of *AtGA2ox* transcripts remained at low levels before radical emergence, suggesting that increased synthesis of active GAs plays a major role during seed germination. Moreover, environmental cues such as light and temperature can also alter the expression of GA biosynthetic genes. For example, red light increases the biosynthesis of bioactive GA_1 and GA_4 by upregulating the expression of GA biosynthetic genes in germinating seeds of lettuce and Arabidopsis (Toyomasu *et al*., 1993; Yamaguchi *et al*., 1998). A study in barley has also shown that expression of *GA3ox2* increases following seed imbibition (Gubler *et al.*, 2008).

The dynamics of GA metabolism can also be affected by after-ripening, one of dormancy breaking treatments. For example, the expression of *HvGA3ox2* has been shown to increase rapidly with imbibition in AR seeds, while it expresses at low level in D seeds. The expression of *HvGA2ox3* also increased with imbibition in both AR and D seeds; however, it decreased to low level following imbibition. Thus, the initial increase in the expression of *HvGA2ox3* in AR seeds may suggest its role in balancing the level of GA that may accumulate as inferred by the increased expression of *HvGA3ox2* immediately upon imbibition (Gubler *et al*., 2008).

2.3.2 The role of abscisic acid in controlling seed development, dormancy and germination

2.3.2.1 Abscisic acid

Abscisic acid is a class of isoprenoids, also named terpenoids (Figure 2.4). It contains 15 carbon atoms; in plants, it is formed by cleavage of C_{40} carotenoids synthesized from the methylerythritol phosphate (MEP) pathway, which uses pyruvate and glyceraldehyde 3-phosphate to produce isopentenyl diphosphate in plastids. This class of phytohormone was discovered in the early 1960s, and was named as ABA because the chemical can control the abscission of cotton bolls. As the name indicates, ABA is originally regarded as an inhibitor, but it is now known that this hormone is involved in lots of developmental processes, such as promoting the storage protein synthesis in the seed, increasing the tolerance of plants to stresses, and regulating stomatal conductance (Kermode, 2005).

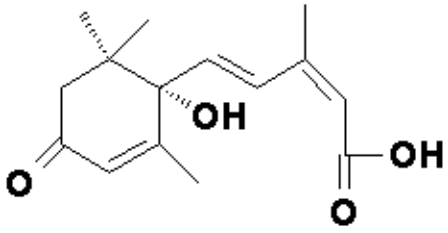


Figure 2.4 Chemical structure of abscisic acid
(Source: http://www.plant-hormones.info/abscisicacid.htm; accessed on 15 August 2012)

2.3.2.2 Abscisic acid metabolism pathway

Abscisic acid is formed by the cleavage of C_{40} carotenoid precursors. Carotenoids, like any other isoprenoids, are synthesized from IPP, which ultimately is converted to GGDP that acts as a precursor for the formation of phytoene, a C_{40} caroteniod. Subsequently, phytoene is converted to carotene, lycopene, β-carotene and then zeaxanthin. The biosynthesis of ABA from zeaxanthin consists of three major stages (Figure 2.5). The first stage involves the synthesis of violaxanthin from zeaxanthin through antheraxanthin, and these reactions are catalyzed by zeaxanthin epoxidase (ZEP). Violaxanthin serves as a substrate for the synthesis of neoxanthin, and this reaction requires two enzymes, a neozanthin synthase (NSY) and an isomerase. The second stage of ABA synthesis is referred as xanthophyll cleavage. Nine-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes cleave the *cis*-isomers of violaxanthin and neoxanthin to a C_{15} product, xanthoxin, and a C_{25} metabolite, C_{25} epoxy apocarotenal or C_{25} allenic apocarotenal (Schwartz *et al*., 2003). The third stage of ABA synthesis is the C_{15} cytosolic pathway which involves the formation of the biologically active form of ABA from *cis*-xanthoxin by two enzymatic reactions. The first reaction is catalyzed by a short-chain alcohol dehydrogenase (ABA2) to form the intermediate abscisic aldehyde, which is then oxidized into ABA by abscisic aldehyde oxidases (AAO3), which requires a molybdenum cofactor (MoCo) for its catalytic activity.

The level of ABA in plants is also controlled by its catabolism (Nambara and

Marion-Poll, 2005). The bioactive form of ABA can be converted into inactive forms either through hydroxylation or conjugation. There are three different ABA hydroxylation pathways that oxidize the methyl groups at three different positions in the ring structure (C-7', C-8', and C-9'), and ultimately forms 7'-hydroxy ABA, 8'-hydroxy ABA and 9'-hydroxy ABA, respectively. The ABA 8'-hydroxylation reaction is thought to be the predominant pathway for ABA catabolism (Cutler and Krochko, 1999), producing phaseic acid (PA). The conversion of ABA to PA begins with the hydroxylation of ABA at the C-8' position, but as the 8' hydroxyl is not stable, it is immediately rearranged to form the PA. About 98% of the 8'-hydroxy ABA exists as PA. ABA 8'-hydroxylation is catalyzed by a cytochrome P450 monooxygenase (Gillard *et al*., 1976; Kushiro *et al*., 2004). The 7'-hydroxy ABA is a minor catabolite, however, can be found in a variety of plant species (Walton *et al*., 1995). The 9'-hydroxy ABA and its isomer neoPA have been found as ABA catabolites in the immature seeds of *Brassica napus* (Zou *et al*., 1995), and also in other plant species such as pea, orange, barley and Arabidopsis (Zhou *et al*., 2004).

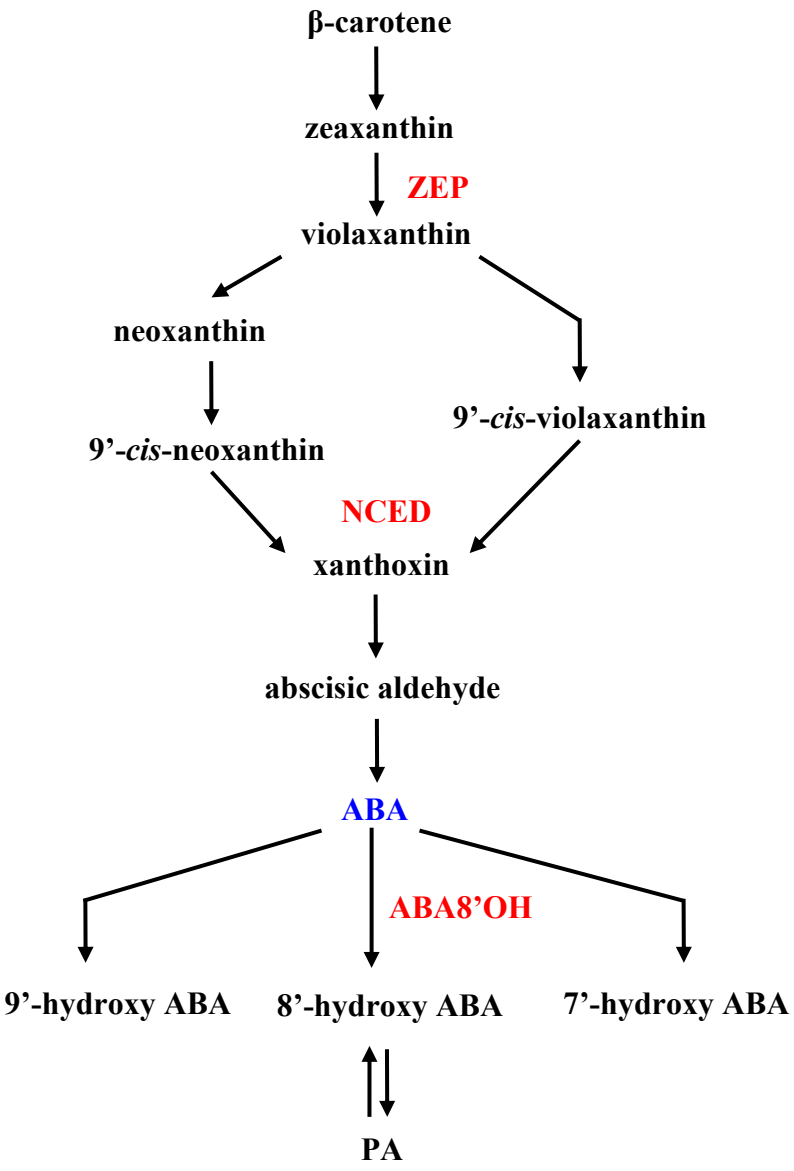


Figure 2.5 Abscisic acid metabolism pathways in plants

In addition to the hydroxylation reactions, the carboxyl group at the C-1 and the hydroxyl groups of ABA and its hydroxylated catabolites can be conjugated to glucose to form inactive forms (Boyer *et al*., 1982; Hirai *et al*., 2003). The ABA glucosyl ester (ABA-GE) appears to be the most widespread conjugate (Boyer *et al*., 1982).

2.3.2.3 Abscisic acid regulates seed development

Abscisic acid regulates key events during seed development, such as deposition of storage reserves (synthesis of storage proteins and lipids), prevention of precocious germination, acquisition of desiccation tolerance, and induction of primary dormancy (Kermode, 2005). ABA helps to maintain embryos development till they are fully formed. Typically, ABA content in seed is low during the early stages of development (histodifferentiation and early pattern formation). However, its level increases as the seed develops, reaching high level during mid-development before declining during the late stage of seed development (Bewley, 1997). It has been shown that the ABA found in seeds of Arabidopsis during the mid-maturation phase is largely synthesized in the maternal tissues, including testa (Karssen *et al*., 1983). This maternally supplied ABA is important for embryo development (Raz *et al*., 2001; Frey *et al*., 2004). The ABA synthesized in the zygotic tissue during late embryo maturation is required for the induction of primary dormancy in Arabidopsis (Karssen *et al*., 1983; Koornneef *et al*., 1989). Recently, Kanno *et al.* (2010) also examined the contribution of ABA synthesis in

the maternal and zygotic tissue to ABA accumulation in developing silique of Arabidopsis. The accumulation of ABA was found to peak first at the middle stage of silique development (9 DAA) but declined by 12 DAA, and then increased again towards the end of development. Measurement of ABA level in the whole silique, seed and seed envelope revealed that the first peak in ABA level is due to ABA accumulation in the seeds, whereas ABA from the silique envelops contributes for ABA accumulation during the later stages of silique development. ABA deficiency during seed development affects normal seed development and dormancy. For example, Cheng *et al*. (2002) reported that severe ABA-deficient *aba2* mutant show an increase in seed abortion when compared to the wild type. Many of the viviparous mutants of maize that exhibit precocious germination of the kernel while it is on the ear are ABA deficient (McCarty, 1995; Suzuki *et al*., 2006).

The expression of the ABA metabolism genes and the ABA content are well correlated during seed development (Chono *et al*., 2006; Lefebvre *et al*., 2006). Of the ABA biosynthetic genes, several *NCED* genes are expressed during seed development; All the *NCED* genes of Arabidopsis, *NCED6* and *NCED9* are the most abundant in seed tissues: *NCED6* is expressed predominantly in endosperm while *NCED9* in both endosperm and embryo tissues (Lefebvre *et al*., 2006). Mutant analysis has shown that only seeds from the *nced6*/*nced9* double mutant have increased germination, while those from the single mutant *nced6* or *nced9* are dormant, revealing that both genes play an important role for ABA accumulation during seed development and onset of dormancy

(Cadman *et al*., 2006; Lefebvre *et al*., 2006). In barley, *NCED2* is mainly expressed during the early stages of seed development, and following the peak expression of *NCED2* (~35 DAA), a maximum level of ABA was observed (Chono *et al*., 2006).

The catabolic activity of ABA is high during mid-maturation stage and reduces maternal ABA level. The *CYP707A1* is the major gene to inactivate ABA during the mid-maturation stage, while *CYP707A2* becomes predominant during late maturation (Okamoto *et al*., 2006). Both immature and mature seeds of *cyp707a1* mutant of Arabidopsis accumulate more ABA than that found in the corresponding tissues of *cyp707a2* mutants (Okamoto *et al*., 2006). Furthermore, constitutive expression of *CYP707A* subfamily in Arabidopsis led to reduced amounts of ABA in seeds at maturity (Okamoto *et al*., 2006). In barley, *CYP707A1* is highly expressed at 40 DAA, and this is followed by rapid decrease in ABA content (Chono *et al*., 2006).

2.3.2.4 Regulation of seed dormancy and germination by ABA

Besides induction of dormancy in developing seeds, ABA is involved in maintaining dormancy during seed imbibition (Debeaujon *et al*., 2000; Grappin *et al*., 2000). The observation that carotenoid and ABA biosynthesis inhibitor, fluridone, can inhibit accumulation of ABA and promote germination of imbibing seeds indicates that de novo ABA synthesis is involved in the maintenance of dormancy (Grappin *et al*., 2000). Dormancy release in seeds is correlated with changes in ABA content during imbibition. Recent studies have shown that the breaking of seed dormancy by after-ripening, stratification and smoke is strongly related to changes in ABA content during imbibition (Ali-Rachedi *et al*., 2004).

After-ripening decreases the ABA content to a lower level in seeds during imbibition. Dry D and AR seeds of Arabidopsis and barely contain similar amount of ABA (Millar *et al.*, 2006). After 6 h imbibition, ABA level drops rapidly to similar level in both D and AR seeds. As the imbibition continues further, by 12 h, the ABA content in AR seeds is only about half of that detected in D seeds. Similar results were also observed by Ali-Rachedi *et al.* (2004) in Arabidopsis and Jacobsen *et al.* (2002) in barley. In both Arabidopsis and barley, the expression of *NCED*s is not well correlated to the change in ABA level. For example, the expression of *AtNCED5* and *AtNCED9*, and *HvNCED1* increased during the first 6 h of imbibition, and higher expression of *HvNCED2* was also observed in AR than D seeds of barley (Millar *et al*., 2006). In contrast, the expression of *CYP707As* in both Arabidopsis and barley was consistent with the changes in ABA content. Among the four *CYP707A* genes in Arabidopsis, *CYP707A2* is the most highly expressed, the expression of which increased rapidly in AR seeds and reached a maximum by 6 h, and decreased thereafter (Millar *et al.*, 2006). However, in D seeds, the *CYP707A2* showed increased expression only in the first 3 h and then declined to four-fold lower by 6 h of imbibition (Millar *et al.*, 2006). The importance of *CY707A2* for the rapid decrease of ABA level during seed imbibition is also verified by Kushiro *et al.* (2004). The *CYP707A2* mRNA predominantly accumulated in dry seed, and was upregulated immediately after imbibition, reaching maximum at 6 h and decreased afterwards. Consistent with this gene expression result, *cyp707a2* dry seeds accumulate

6-fold more ABA than that found in wild type seeds, and this high ABA level is maintained during seed imbibition (Kushiro *et al*., 2004). In barley, the expression of *CYP707A1* is much higher during seed imbibition than *CYP707A2*; and its expression is induced within 6 h of imbibition and show higher expression in AR than D seeds (Millar *et al.*, 2006), suggesting that the amount of ABA in imbibing barley seeds and thereby dormancy release is controlled mainly by *CYP707A1* (Gubler *et al*., 2008).

Similar changes in ABA content have also been found in dormant seeds following stratification. Exposure of imbibing dormant Arabidopsis seeds to 4°C before transferring them to 20°C resulted in a decline in ABA content before germination occurs (Ali-Rachedi *et al*., 2004). Furthermore, ABA content decreased (8-fold) in *Nicotiana attenuate* seeds imbibed in a smoke and water solution than those imbibed in water alone (Schwachtje and Baldwin, 2004). Consistently, over 90% of the smoked-induced seeds germinate within 3 days, whereas all the control seeds failed to germinate. These results indicate that smoke-induced seed dormancy release and germination are mediated by change in seed ABA level. Other signals that promote dormancy release such as darkness and nitrate have also been shown to cause a decline in ABA content in imbibing seeds (Bethke *et al*., 2004). In summary, many studies indicated that changes in seed ABA level is a critical factor that controls seed dormancy maintenance and release in many species.

3.0 Expression analysis of ABA and GA metabolism genes during seed development and germination

Abstract: Abscisic acid (ABA) enhances the onset and maintenance of dormancy, while gibberellin (GA) promotes germination. This section of the thesis characterized the expression patterns of regulatory ABA and GA metabolism genes during the development and germination of barley cv. Betzes seeds. The results of this study revealed that the GA metabolic genes including *HvGA20ox3*, *HvGA3ox1* and *HvGA2ox5*; and the ABA metabolic genes including *HvNCED1* (during the early stage of seed development) and *HvCYP707A1* play important roles in controlling GA and ABA levels in developing seeds. The dormancy of mature seeds is released by after-ripening, and this is associated with changes in the expression patterns of GA and ABA metabolism genes during imbibition. The expressions of *HvGA20ox1*, *HvGA20ox2* and *HvGA3ox2* were induced by imbibition in both dormant (D) and after-ripened (AR) seeds. However, the expressions of *HvGA20ox1*, *HvGA3ox2* and *HvCYP707A1* were more induced specifically in imbibing AR seeds, whereas that of *HvGA2ox1*, *HvGA2ox3*, *HvGA2ox4, HvGA2ox6* and *HvNCED2* in the corresponding D seeds. This result suggests the importance of these genes in regulating GA and ABA to maintain dormancy or induce germination of barley seeds. The role of ABA in repressing the germination process is associated with its negative effect on the expression of GA biosynthesis genes, including *HvGA20ox1* and *HvGA3ox1* during imbibition.

3.1 Introduction

Seed is of great importance to plant life. The first part of seed development consists of embryogenesis and embryo growth. After embryo growth arrest, seed maturation occurs, storage reserves are accumulated, seed dormancy is induced and desiccation tolerance is acquired (Goldberg *et al.*, 1994). Dormancy is initiated early during seed development and increases until the seed is fully developed (Raz *et al*., 2001). Germination commences with the uptake of water by the seed and completes with radicle emergence through the covering layers. In barley, high dormancy is not favorable, since it prevents rapid and uniform germination, which is a requirement for malting process. While little or no dormancy, can easily lead to the germination of seeds on the mother plant if rainy or humidity conditions occur during the maturation and before harvest. Thus, it is critical to develop adequate dormancy for barley cultivars that are resistant to preharvest sprouting, and can generate rapid and consistent germination after harvest.

Seed dormancy and germination are regulated by both developmental and environmental factors. Plant hormones are important factors involved in seed development and its transition to germination. Genetic and physiological studies have shown that barley seed dormancy and germination are regulated mainly by two plant hormones, namely ABA and GA.

Abscisic acid is a well-known participant in the induction and maintenance of dormancy (Kermode, 2005; Nambara *et al*., 2010). In Arabidopsis, genetic studies based

on reciprocal crosses of wild type and ABA-deficient mutant plants showed a dual origin of ABA in developing seeds. Most of the ABA is synthesized during the maturation phase, first in maternal tissues and then at lower levels in the embryo and endosperm. During seed development, the onset of dormancy correlated well with the presence of the embryonic ABA but not with the maternal ABA (Karssen *et al.*, 1983; Groot and Karssen, 1992; Frey *et al.*, 1999). The levels of ABA decrease during seed desiccation and is maintained at low in mature seeds (Karssen *et al.*, 1983). While ABA-deficiency due to mutation or chemical inhibition of its biosynthesis reduces seed dormancy, ABA accumulation due to overexpressing its biosynthetic genes or silencing its deactivating genes can enhance dormancy (Nambara and Marion-Poll, 2003; Kucera *et al.*, 2005). In addition, de novo ABA biosynthesis in imbibing embryos is important to enhance dormancy. Indeed, a transient increase in ABA content has been evident in the embryos of imbibing D seeds (Kucera *et al.*, 2005). The level of ABA in plant tissues is controlled by its synthesis and catabolism (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). Previous studies have demonstrated that two steps in ABA metabolism, one from ABA biosynthesis and one from ABA catabolism, act as the major regulators of ABA level in most plant tissues (Nambara and Marion-Poll, 2005).

Abscisic acid is synthesized from carotenoids via oxidative cleavage, the cleavage of neoxanthin and violaxanthin to produce ABA precursor xanthoxin, which is catalyzed by 9-cis epoxycarotenoid dioxygenase (NCED). This step is one of the rate limiting steps

in ABA biosynthesis pathway (Schwartz *et al.*, 2003). In addition to biosynthesis, ABA level is regulated by its catabolism. The major route for ABA catabolism is hydroxylation of ABA at the C-8' position to produce 8'-hydroxy ABA, and this reaction is catalyzed by ABA 8' hydroxylase (a cytochrome P450 monooxygenase) that is encoded by *CYP707A* genes (Kushiro *et al*., 2004; Saito *et al*., 2004). During Arabidopsis seed development, several *NCED* genes are shown to be expressed; however, *NCED6* and *NCED9* are the most abundant genes in seed tissue (Lefebvre *et al.*, 2006). Both *NCED6* and *NCED9* exhibited seed specific expression; *NCED6* is mainly expressed in the endosperm, and the expression of *NCED9* is observed in the endosperm and the peripheral cells of the embryo at the mid-stage of seed development (Lefebvre *et al.*, 2006). In barley, *NCED1* is particularly important in the regulation of primary dormancy in blue or white light, in which its expression is induced 2- to 3-fold under blue or white light as compared to other light types (Gubler *et al*., 2008). Furthermore, *NCED1* is also involved in inducing the secondary dormancy. The research from Leymarie *et al*. (2008) indicated that its expression remained high at 30°C when secondary dormancy is induced. According to Chono *et al*. (2006), the expression of *NCED2* is regulated in a growth-dependent manner, in which the peak of *NCED2* expression is followed by the peak ABA accumulation during the early to middle stage of seed development. Besides, *NCED2* is also involved in the maintenance of secondary dormancy (Leymarie *et al*., 2008). The *CYP707A1*, an ortholog of the Arabidopsis *CYP707A2* gene, plays the major role in ABA

catabolism required for dormancy release of barley seed dormancy (Chono *et al*., 2006; Millar *et al*., 2006; Gubler *et al*., 2008). This gene is mainly expressed in the coleorhiza, which surrounds the radicle tip (Millar *et al*., 2006).

Conversely, GA plays a role in promoting dormancy release; germination and reserve mobilization in the post germination phases (Mundy, 1984; Bentsink and Koornneef, 2002; Kucera *et al*., 2005). GA has been shown to be important during seed development processes including fertilization, embryo development, embryo axis elongation, and prevention of seed abortion (Swain *et al.*, 1995; Swain *et al.*, 1997; Hays *et al*., 2002; Singh *et al*., 2002; Finkelstein, 2004). What's more, GA also regulates seed germination by weakening the endosperm cells around the radicle tip (Groot and Karssen, 1987).

Gibberellins (GAs) are a group of diterpenoids synthesized from geranylgeranyldiphosphate (GGDP). The conversion of GGDP into bioactive GAs involves three stages that are classified based on the types of enzymes involved and the subcellular compartments they reside in (Yamaguchi, 2008). It has been shown that the enzymes in the final stages of GA biosynthesis, which catalyze the conversion of GA_{12} to bioactive GAs, play regulatory role in controlling GA levels. Two classes of 2-oxoglutartate-dependent dioxygenases (2ODDs) responsible for these conversion processes are GA 20-oxidases (GA20oxs) and GA 3-oxidases (GA3oxs). In addition, enzymes involved in GA deactivation, mainly GA 2-oxidases (GA2oxs), which also

belongs to 2ODDs, are known to regulate GA level in plants (Yamaguchi, 2008). The GA20ox, GA3ox and GA2ox enzymes are encoded by small gene families, and members of these gene families are shown to be differentially regulated during seed development and germination (Yamaguchi, 2008). In developing siliques of Arabidopsis, *GA3ox1* is mainly expressed in the replums, funiculi, silique receptacles and developing embryos (Hu *et al*., 2008). The *GA3ox3* gene is only expressed in anthers and developing embryos, while *GA3ox4* is mainly expressed in the anthers and the endosperm of developing seeds. The expression of *GA3ox2* is shown to be mainly expressed during seed germination and vegetative growth (Mitchum *et al.*, 2006). Imbibition of non-dormant Arabidopsis seeds under continues white light at optimal temperature resulted in up-regulation of several GA biosynthesis genes, including *GA20ox3*, *GA3ox1*, and *GA3ox2*; while the expression of GA2ox genes, including *GA2ox1*, *GA2ox2*, *GA2ox3* and *GA2ox4* remain at low levels (Ogawa *et al.*, 2003).

Abscisic acid and GA are known to act antagonistically in the control of seed germination processes. For example, in the cereal aleurone layer, ABA inhibits the expression of GA inducible genes including those encoding α-amylase and proteases required for post-germinative growth (Bethke *et al*., 1997; Lovegrove and Hooley, 2000). Moreover, it has been reported that an ABA-responsive protein kinase mediates suppression by ABA of GA induction of α-amylase and protease genes in barley aleurone tissue (Gómez-Cardenas *et al*., 1999).

Seeds can be released from dormancy by after-ripening, a period of dry seed storage, which is an effective treatment to facilitate dormancy release (Bewley, 1997; Leubner-Metzger, 2003). This treatment is reported to widen and increase the sensitivity of seeds to factors that promote germination, and at the same time, it narrows and decreases the sensitivity of seeds to conditions/factors that repress germination (Finch-Savage and Leubner-Metzger, 2006). A general property of AR seeds is that it lowered the level of, and decreased sensitivity to ABA but increased GA sensitivity as compared to freshly harvested seeds (Grappin *et al.*, 2000; Romagosa *et al.*, 2001; Koornneef *et al.*, 2002; Ali-Rachedi *et al.*, 2004). In barley, when AR seeds are transferred to germination condition, the ABA content of the embryo declines markedly over the first 12 h, and a low ABA level are maintained till 30 h. While for the D seeds, during the first 12 h, there is some reduction appeared but ABA content gradually increases between 12 and 30 h of imbibition (Jacobsen *et al.*, 2002). A similar reduction in ABA level followed by accumulation also occurs in imbibed dormant Arabidopsis seeds of Cape Verde Islands (Cvi) ecotype (Ali-Rachedi *et al*., 2004). A global transcript analysis of Arabidopsis Cvi seeds by using full-genome microarrays, Cadman *et al*. (2006) showed that the accumulation of the GA biosynthetic gene *GA3ox2* transcripts increased 40-fold in AR seeds, in contrast to this, the GA deactivation gene *GA2ox1* was expressed at a higher level in the D seeds.

In the present study, the expression of GA and ABA metabolism genes were characterized in developing seeds; and also in D and AR seeds before and during imbibition of grain so as to gain insights into the role of GA and ABA in regulating seed development, dormancy and germination. Besides, the expression of major GA and ABA metabolism genes were compared between the D, AR, and ABA treated AR seeds, to gain a better understanding of the effects of after-ripening and ABA treatment on dormancy release.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

Barley (*Hordeum vulgare* L.) cv. Betzes was chosen for this research. It is two-row barley introduced into North America from Kaskaw, Poland, by the USDA. It was released in 1957 by Montana and Idaho Agricultural Experiment Stations.

Seeds germinated in a Petri-plate (see below for details) were then transplanted into 1 gallon-pot (1 seed per pot at a depth of approximately 2.5 cm) containing LA4 sunshine mix (LA4; Sungro Horticulture, Bellevue, WA, USA) and Cornell mixture (1 bag: 100 g calcium carbonate, 150 g osmocote, 120 g superphosphate, 2 g fritted trace elements, 15 g chelated iron [13.2%], 0.7 g chelated zinc [14%]). The ratio for the sunshine mix to solid fertilizer is about 20 gallons sunshine mix to 1 bag of Cornell fertilizer. The plants were grown in greenhouse (Crop Technology Centre, University of Manitoba) at 18-

22°C/14-18°C (day/night) in a 16/8 h photoperiod with high-pressure sodium light (270 $\mu E/m^{-2}sec^{-1}$). Plants were watered every day and fertilized with N-P-K (20:20:20) mix once a week.

3.2.2 Tissue harvesting

Spikes were harvested at 20, 25, 30, 35, 40, 45, 50, 55, and 60 days after anthesis (DAA) from individual primary or secondary tillers (one spike per plant per replication; 3 biological replications). Developing seeds for growth measurements (seed length, diameter, fresh and dry weights), germination test and expression analysis were taken from the middle region of each spike in order to minimize variations between seed samples. The harvested tissues were immediately frozen in liquid nitrogen, and then stored in -80^{o}C freezer until further use. To maintain their dormancy, mature seeds of cv. Betzes were harvested after harvest maturity in liquid nitrogen and stored immediately at -80^{o}C. These seeds were designated as D seeds. To generate AR seeds, mature 'Betzes' seeds were stored at room temperature for ~ 11 months before they are stored at -80^{o}C.

3.2.3 Measurement of different seed parameters

Average length, diameter and fresh weights of the developing seeds harvested at different stages (at 5 days interval from 20-60 DAA) were determined from measurements of 15

individual seeds. Seed dry weights were obtained by drying freshly harvested seeds in an oven at 105°C for 2 days.

3.2.4 Seed germination

Developing, mature D and AR seeds of cv. Betzes were germinated between layers of two Whatman #1 filter papers (GE Healthcare UK Limited, UK) moistened with 7 mL sterile deionized water in a 9-cm Petri-dish (20 seeds per dish). The Petri-dishes were sealed with parafilm and incubated under darkness at room temperature. To examine the germination percentage, seeds whose coleorhiza emerged through the seed coat were counted each day for 7 days. For expression analysis of GA and ABA metabolic genes in germinating seeds, D and AR seeds were harvested at 6, 12, 24 and 30 h after imbibition. The effect of ABA on the expression of these genes was examined by imbibing AR seeds in ABA (50 μM).

3.2.5 RNA isolation

Total RNA samples from seeds were extracted using Aurum^{TM} Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Frozen seeds were ground into a fine powder in liquid nitrogen using pre-chilled pestle and mortar, after which around 100-150 mg seed powder was transferred into 2.0 ml tube and mixed with 1 ml PureZOL. The lysate was incubated at room

temperature for 5 min to allow complete dissociation of nucleoprotein complexes and then centrifuged at 12,000*g* for 10 min at 4°C. Following centrifugation, the supernatant was transferred into a fresh 2.0 ml tube and then mixed with 0.2 ml chloroform. This mixture was incubated at room temperature for 5 min (with periodical mixing by inversion) and then centrifuged at 12,000*g* for 15 min at 4°C. Without disturbing the interphase, the aqueous phase was transferred into a fresh 2 ml tube and mixed thoroughly with an equal volume (approximately 360 µl) of 70% ethanol. The sample was then transferred into the RNA binding mini column (Bio-Rad) and then centrifuged for 1 min at 13,000*g*. After discarding the flow through, 700 µl of low stringency wash solution was added to the RNA binding column and centrifuged for 30 s at 13,000*g*. The flow through low stringency wash solution was discarded and then 80 µl of diluted DNase I (mixture of 5 µl of reconstituted DNase I and 75 µl of DNase dilution) was added to each column to remove any contaminating genomic DNA. Following incubation at room temperature for 15 min to allow the DNase digest, 700 µl of high stringency wash solution was added to the RNA binding column and centrifuged at 13,000*g* for 30 s. The column was then washed again with 700 µl of low stringency wash solution and centrifuged for 2 min at 13,000*g* to remove any residual wash solution. The total RNA sample was precipitated by adding 40 µl of the diethylpyrocarbonate (DEPC) treated water to the center of the binding column, which was placed into a fresh 1.5 ml tube and

subsequent centrifugation for 2 min at 13,000*g* (after 1 min incubation). The eluted total RNA sample was immediately stored at -80°C for later use.

Table 3.1 Sequences of gene specific primers for qPCR analysis

Gene name		Sequence (5' to 3')	Amplicon (bp)	PCR efficiency (%)	r^2
HvGA2ox1	F	AAAGCTAGCCAGGTCATGGA	139	103.9	0.9952
	R	TTGAGTTGCAGGCAATCTGT			
HvGA2ox3	F	GTGGCCAACAGCCTAAAGTC	138	92.9	0.9828
	R	GTACTCGCCCCATGTGAAGT			
HvGA2ox4	F	CGCGTGTCGATGATCTACTT	134	97.4	0.9684
	R	TAGGCCGCCTTCTTGTATTC			
HvGA2ox5	F	GGTGTCCATGATCTTCTTCG	129	91.0	0.9948
	R	TGGGTGCTGCTCTTGTACTC			
HvGA2ox6	F	AGTGGTGGCCAACTACAAGG	129	99.4	0.9878
	R	CTACGGTACTCCCCGAAGGT			
HvGA3ox1	F	AGCACTACCGCCACTTCTCT	89	96.7	0.9911
	R	TACGAGGAACAGCTCCATCA			
HvGA3ox2	F	GCCCATCTCCTCCTTCTTCT	149	101.2	0.9565
	R	CCCTGTGGAACTCCTCCAT			
HvGA20ox1	F	CCCTGGAAGGAGACCCTCT	136	102.7	0.9762
	R	GGCTCATCTCCGAGCAGTAG			
HvGA20ox2	F	AAGCTTCCCTGGAAGGAGAC	131	99.9	0.9985
	R	TCCCCTAGGTGCATGAAGTC			
HvGA20ox3	F	CGCTCACCTTCTTCCTCAAC	113	97.6	0.9946
	R	GAACTCACGCCAAGTGAAGTC			
HvNCED1	F	ACCTCTGGAACTCGTGGGA	96	107.0	0.9769
	R	CGTCCGTGTCGTTGAAGAT			
HvNCED2	F	GCTTCTGCTTCCACCTCTG	113	97.4	0.9738
	R	GCACTCGTCCGACTCGTT			
HvCYP707A1	F	CCATGACCTTCACCCGCAAG	118	91.9	0.9849
	R	GGACACTGACGGATGGAGAAC			
HvCYP707A2	F	TGACGCACAGGGTGATTT	109	108.7	0.9985
	R	CCCTTGGGGATAAGAAACC			
HvβActin	F	CCAGGTATCGCTGACCGTAT	132	92.5	0.9952
	R	GCTGAGTGAGGCTAGGATGG			

3.2.6 cDNA synthesis

Complementary DNA (cDNA) samples for RT-qPCR assay were synthesized from total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad) following the manufacturer's instruction. Briefly, total RNA (1 µg) was mixed with 4µl of 5X iScript reverse transcription supermix and nuclease-free water to a total reaction volume of 20 µl. The reaction mix was incubated at 25°C for 5 min, followed by 30 min reverse transcription at 42°C and inactivation of the reverse transcription reaction by incubation at 85°C for 5 min. The resulting cDNA samples were stored at -80°C for later use.

3.2.7 RT-qPCR Assay

Gene specific primers for GA metabolism genes (*GA20ox*s, *GA3ox*s, *GA2ox*s), ABA metabolism genes (*NCED*s and *CYP707A*s) and *HvβActin* (used as a reference gene for normalization) were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/; Table 3.1) based on reported sequences of *HvGA20ox1* (GenBank ID: AY551428; Spielmeyer *et al*., 2004), *HvGA20ox2* (unpublished), *HvGA20ox3* (GenBank ID: AY551429; Spielmeyer *et al*., 2004), *HvGA3ox1* (GenBank ID: AY551430; Spielmeyer *et al*., 2004), *HvGA3ox2* (GenBank ID: AY551431; Spielmeyer *et al*., 2004), *HvGA2ox1* (unpublished), *HvGA2ox3* (unpublished), *HvGA2ox4* (GenBank ID: AY551432; Dewi, 2006), *HvGA2ox5* (GenBank ID: AY551433; Dewi, 2006), *HvGA2ox6* (unpublished), *HvNCED1* (GenBank ID: AB239297.1; Millar *et al*., 2006), *HvNCED2* (GenBank ID:

AB239298.1; Millar *et al*., 2006), *HvCYP707A1* (GenBank ID: DQ145932; Millar *et al*., 2006), *HvCYP707A2* (GenBank ID: DQ145933; Millar *et al*., 2006). Primer specificity was confirmed by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) based search against the GenBank database, and further by RT-PCR. The RT-qPCR assays were performed using SsoFast^{TM} Eva Green Supermix (Bio-Rad). The reaction mixture contained 5 µl of cDNA (~400 ng, obtained by 8X dilution of the original cDNA from the RT), 10 µl of SsoFast Eva Green Supermix, 0.4 µl of forward primer (5 µM; 100 nM final concentration), 0.4 µl of reverse primer (5 µM; 100 nM final concentration) and 4.2 µl of water, with a total reaction volume of 20 µl. Amplification and fluorescent signal detection was performed on a CFX Connect Real-Time PCR system (Bio-Rad) with the following PCR conditions: initial denaturation and DNA polymerase activation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s in 96-well optical reaction plates covered with optical caps (Bio-Rad). The relative transcript level of each gene was determined by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.2.8 Statistical analysis

Statistical analysis of the gene expression data was carried out by using the mixed procedure model of SAS (Ver. 9.2, SAS Institute Inc, 2008). The analysis of variance

was performed to compare differences in expression of GA and ABA metabolism genes in D and AR (with and with no ABA treatment) seeds during imbibition using Tukey's LSD test ($p<0.05$).

3.3 Results

3.3.1 Seed development

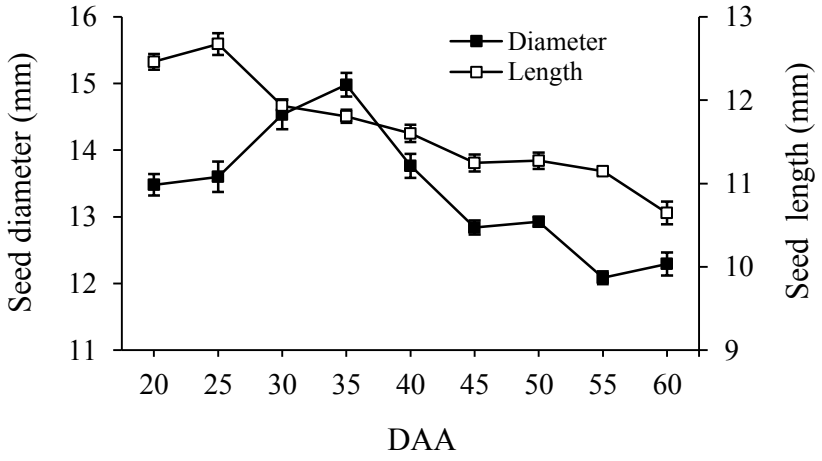
In barley cv. Betzes, anthesis occurs at approximately 40-45 days after planting. Maximum seed length was attained by 20-25 DAA, whereas the highest seed diameter was apparent by 35 DAA (Figure 3.1B). Both seed length and diameter decreased as the seeds start to desiccate, and this decrease continued through maturity. Increase in seed fresh weight was observed from 20 to 35 DAA, after which it decreased (1.4-fold) and remained at a similar level throughout seed maturity. Similarly, seed dry weight increased from 20 to 40 DAA, and was maintained at similar level afterwards. In general, the pattern of changes in dry weight mirrors that of fresh weight (Figure 3.1C).

3.3.2 Seed germination during development

Testing the germination capacity of developing seeds indicated that very minimal or no germination was apparent until after 55 DAA, when ~28% of the seeds exhibited completion of germination after 7 days of imbibition (Figure 3.2).

A)

B)



C)

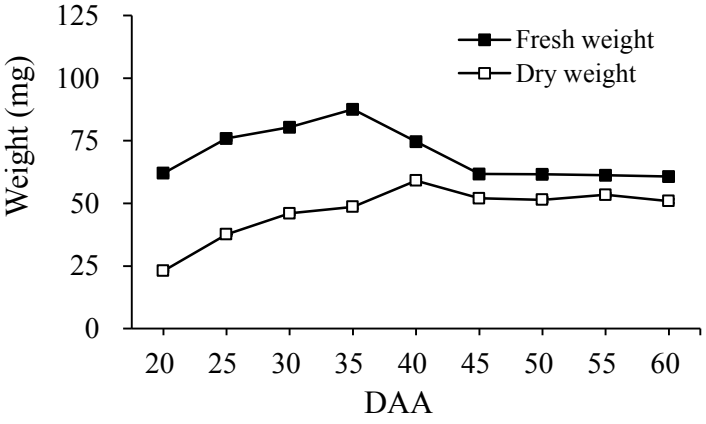


Figure 3.1 Developing seeds of barley cv. Betzes from 20 to 60 DAA (A) with seed length and diameter (B), and seed fresh and dry weights (C). Data are means ± SE, n=15.

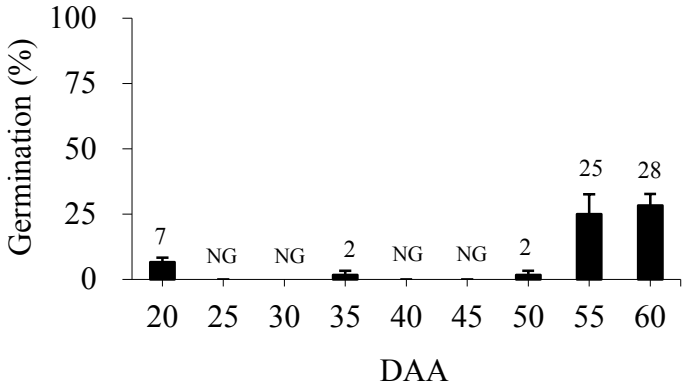


Figure 3.2 Germination percentages of barley cv. Betzes seeds harvested at different developmental stages. NG = no germination recorded.

3.3.3 Comparison of the germination of dormant and after-ripened seeds

Considering the coleorhiza as indicator of germination, freshly harvested dormant seeds of cv. Betzes exhibited only 16% germination within 7 days of imbibition, whereas 97% the AR seeds germinated during the same period (Figure 3.3). Germinated seeds exhibited roots and shoots starting from 3 days after imbibition (3 DAI) (Figure 3.4A). Imbibing the AR seeds with ABA decreased seed germination by 60%. Furthermore, it inhibited shoot and root growth of seedlings (Figure 4.4A; B; C).

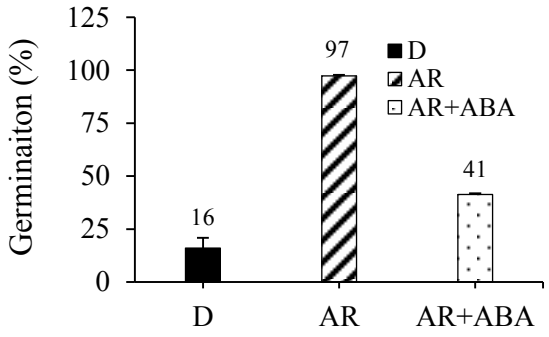


Figure 3.3 Germination of dormant and after-ripened seeds imbibed with and without ABA over a period of 7 days.

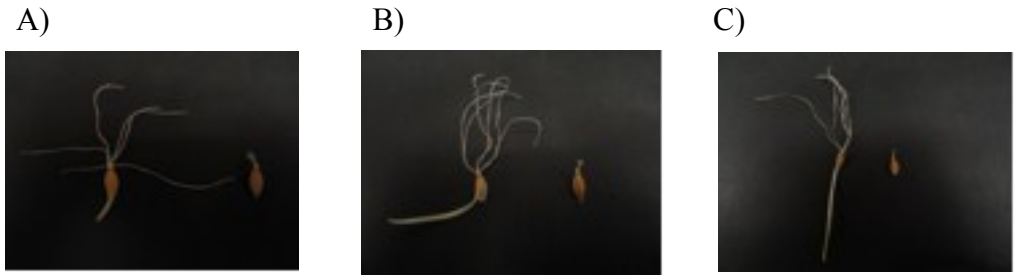


Figure 3.4 Comparison of the growth of seedlings derived from after-ripened seeds imbibed with no (left) and with ABA (right) for 3 (A), 4 (B) and 5 (C) days.

3.3.4 Verification of the specificity of primers for target and reference genes

For real time RT-qPCR based expression analysis of GA and ABA metabolism genes during seed development and germination, gene specific primers were designed from the coding sequence of the target and reference (*HvβActin*) genes. The amplicon sequence of each target gene was BLAST searched against the GenBank database, and the results showed that each amplicon sequence has highest hit with the target gene, and no other

gene from barley was found in the list of genes that share a certain degree of identity with the amplicons representing the target genes. Furthermore, amplifying cDNA sample from barley with the specific forward and reverse primers derived from each of the target and reference genes produced a single PCR product with expected size (Figure 3.5A and B).

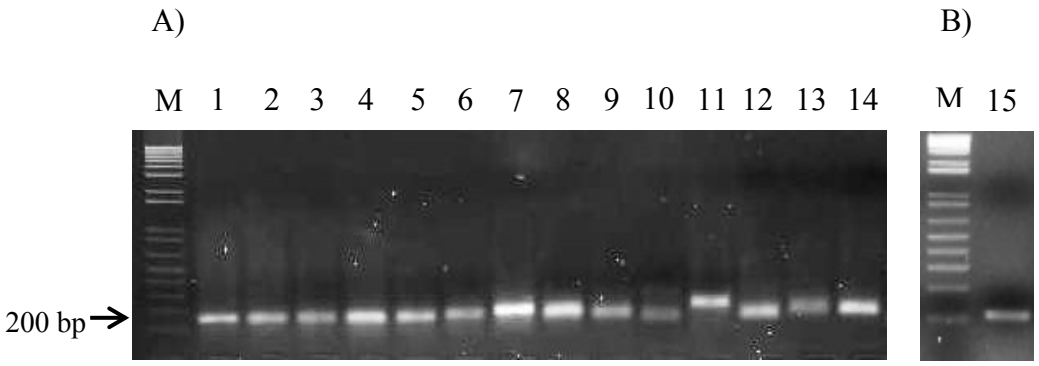


Figure 3.5 PCR analysis of the specificity of primers designed from the target and reference genes with barley cDNA (A and B): *HvGA2ox1* (lane 1), *HvGA2ox3* (lane 2), *HvGA2ox4* (lane 3), *HvGA2ox5* (lane 4), *HvGA2ox6* (lane 5), *HvGA3ox1* (lane 6), *HvGA3ox2* (lane 7), *HvGA20ox1* (lane 8), *HvGA20ox2* (lane 9), *HvGA20ox3* (lane 10), *HvβActin* (lane 11), *HvNCED1* (lane 12), *HvNCED2* (lane 13), *HvCYP707A1* (lane 14) and *HvCYP707A2* (lane 15). Lane M is for 1 Kb plus DNA ladder.

3.3.5 Expression analysis of GA metabolism genes during seed development

Temporal expression patterns of late GA metabolic genes of barley including members of the *GA20ox*s (*HvGA20ox1*, *HvGA20ox2* and *HvGA20ox3*), *GA3ox*s (*HvGA3ox1* and *HvGA3ox2*) and *GA2ox*s (*HvGA2ox1*, *HvGA2ox3*, *HvGA2ox4*, *HvGA2ox5* and *HvGA2ox6*) families were analyzed in developing seeds from 20 to 55 DAA at five days interval.

3.3.5.1 Expression of *GA20ox* genes

The expression of one of the barley *GA20ox* genes, *HvGA20ox3*, was very high at 20 DAA, and increased further by 25 DAA. By 30 DAA, its expression decreased (3.7-fold) and remained at a similar level through 35 DAA. From 35 to 40 DAA, it exhibited an increase (up to 2-fold) and remained at a similar level afterwards. In contrast, the transcripts of *HvGA20ox1* and *HvGA20ox2*, the other members of barley *GA20ox* family, were not detected (Figure 3.6).

3.3.5.2 Expression of *GA3ox* genes

The transcripts of *HvGA3ox1* were detected throughout seed development (Figure 3.7). Its expression showed a 2.3-fold increase from 20 to 25 DAA but decreased by 30 to 35 (2.1- to 2.7-fold) DAA, after which its expression showed almost a linear increase as the seeds mature (Figure 3.7). Its transcript abundance increased 3.3-fold from 35 to 55 DAA. Unlike that of *HvGA3ox1*, the transcripts of *HvGA3ox2* were not detected during seed development (data not shown).

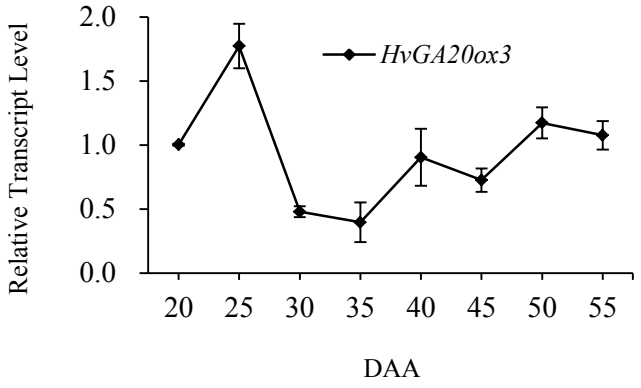


Figure 3.6 Relative transcript abundance of *HvGA20ox3* during seed development. The transcript levels were compared across developmental stages using the mean transcript abundance in 20 DAA seeds, which was set to 1, as a calibrator. Data are means 2-3 independent biological replicates ± SE.

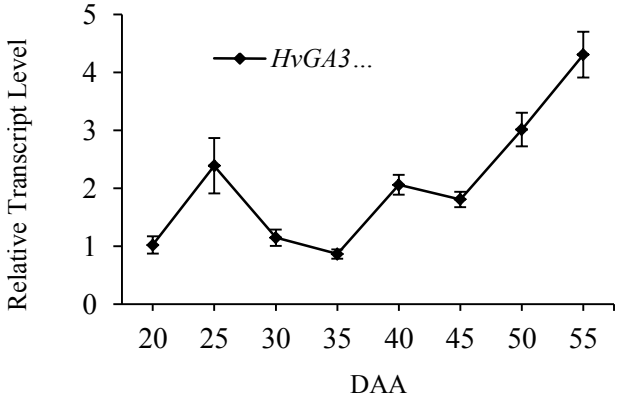


Figure 3.7 Relative transcript abundance of *HvGA3ox1* during seed development. The transcript levels were compared across developmental stages using the average transcript level in 20 DAA seeds, which was set to 1, as a calibrator. Data are means of 2 to 3 independent biological replicates ± SE.

3.3.5.3 Expression of *GA2ox* genes

The expression of all of the five *HvGA2ox* genes analyzed in this study was detected during seed development (Figure 3.8). The *HvGA2ox1* and *HvGA2ox3* genes were

expressed at a relatively higher level during the 20-25 DAA stage of seed development, but their expression declined afterwards and remained at similar level throughout seed maturation (Figure 3.8A and B). In contrast, the *HvGA2ox4* and *HvGA2ox5* genes were expressed at lower level from 20 to 45 DAA, but showed an increase by 50 DAA (3.5-fold for *HvGA2ox4*; 1.8-fold for *HvGA2ox5*). The expression of *HvGA2ox4* declined by 55 DAA (4.5-fold), while that of *HvGA2ox5* was still maintained at a similar level observed at 50 DAA (Figure 3.8C and D). The *HvGA2ox6* gene had an expression pattern different from that exhibited by the other *GA2ox* genes, in which its expression was very minimal at 20 DAA but increased drastically by 25 DAA (12.7-fold). From 25 to 30 DAA, its expression exhibited a marked decline (9-fold), after which it was maintained at very low level through seed maturity (Figure 3.8E).

3.3.5.4 Comparative analysis of the expression of *HvGA2ox* gene family members

Since the transcripts for all members of the *HvGA2ox* gene family were detected in the developing seeds, we compared their expression of all members so as to identify the specific *HvGA2ox* gene with potential importance during barley seed development. The comparative analysis revealed that *HvGA2ox5* is the highly expressed *GA2ox* gene (Figure 3.9). Its transcript abundance during the entire seed developmental stages studied ranged from 603- to 1845-fold higher than that of the other *HvGA2ox* genes.

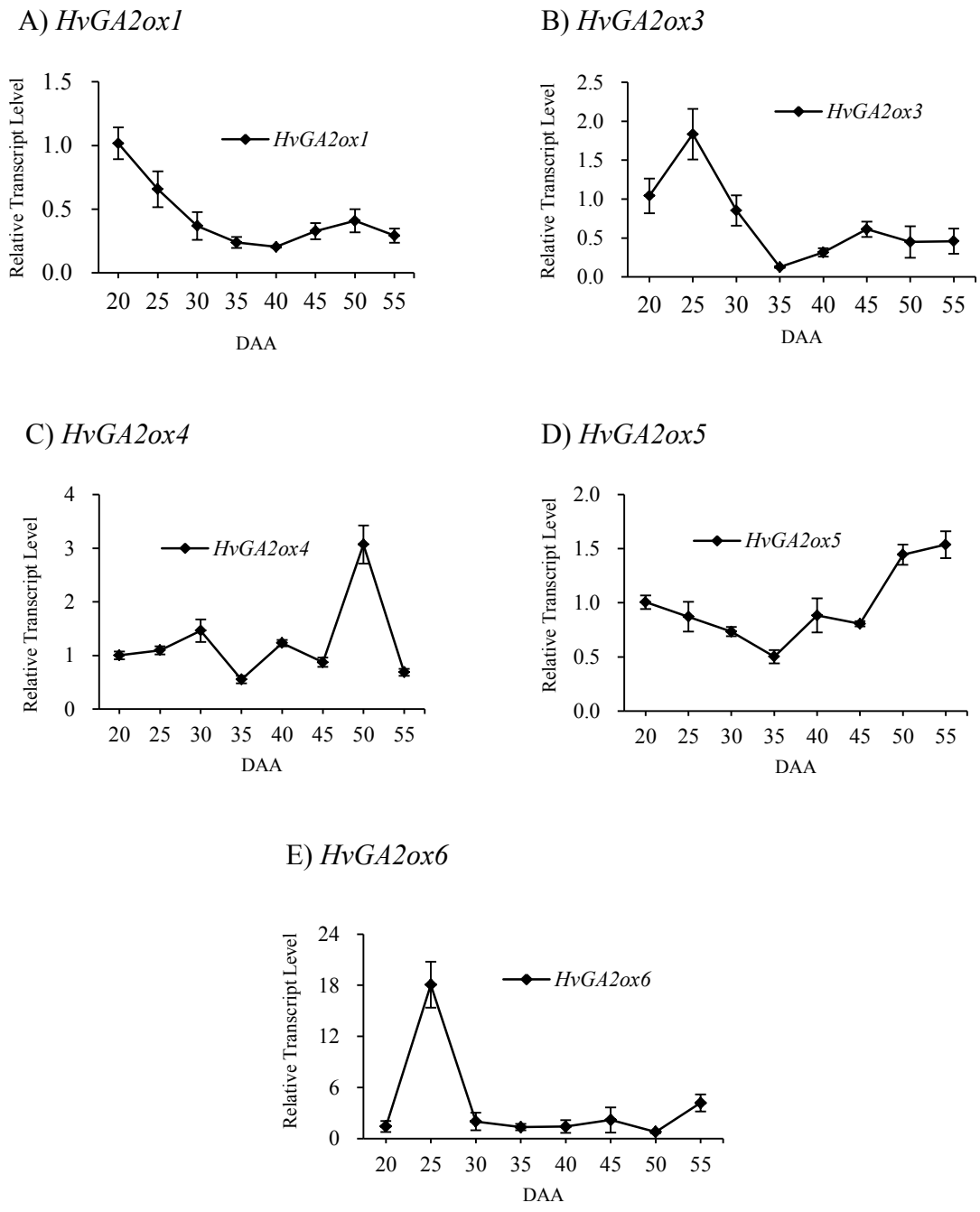


Figure 3.8 Relative transcript abundances of *HvGA2ox1* (A), *HvGA2ox3* (B), *HvGA2ox4* (C), *HvGA2ox5* (D) and *HvGA2ox6* (E) during seed development. The transcript levels for each gene were compared across developmental stages using the respective average transcript level in 20 DAA seeds, which was set to 1, as a calibrator. Data are means of 2 to 3 independent biological replicates ± SE.

The transcript levels of the other members of the family were maintained at low level throughout the seed developmental stages considered (Figure 3.9).

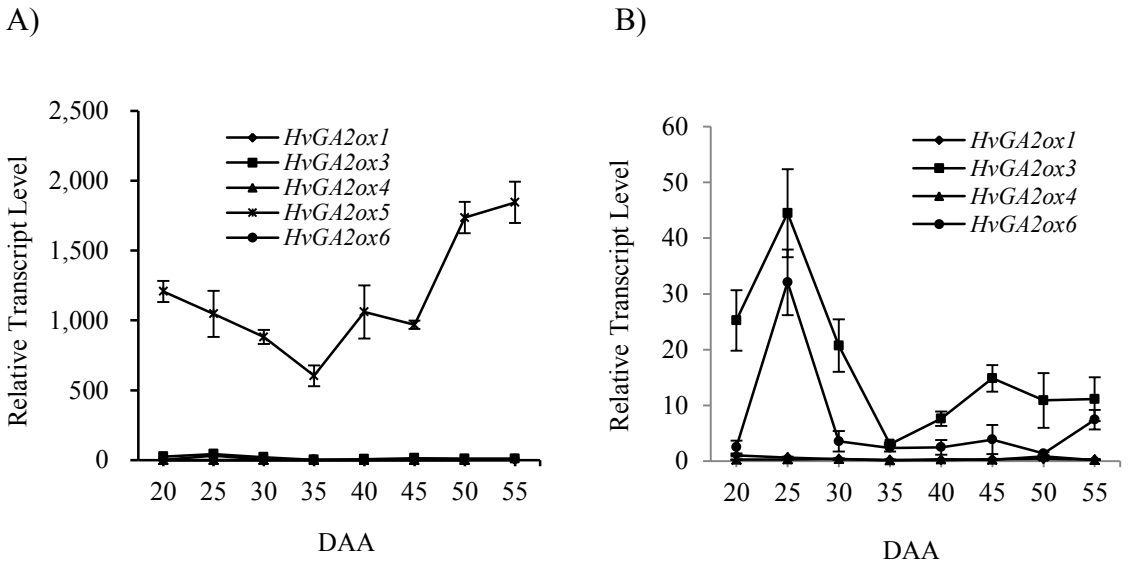


Figure 3.9 Relative transcript abundances of *HvGA2ox1*, *HvGA2ox3*, *HvGA2ox4*, *HvGA2ox5* and *HvGA2ox6* during seed development (A). Magnified representation of the transcript abundances of *HvGA2ox1*, *HvGA2ox3*, *HvGA2ox4* and *HvGA2ox6* (B). The transcript levels were compared across members of the *GA2ox* family members using the mean transcript level of *HvGA2ox1* in 20 DAA seeds, which was set to 1, as a calibrator. Data are means of 2 to 3 independent biological replicates ± SE.

3.3.6 Expression analysis of ABA metabolism genes during seed development

3.3.6.1 Expression of the ABA biosynthetic *NCED* genes

The transcripts of both *HvNCED1* and *HvNCED2* genes were detected in all of the seed developmental stages studied (Figure 3.10). The *HvNCED1* gene was expressed highly at 20 DAA but showed a decline by 25 DAA (2.4-fold). As the seed grew from 25 to 35

DAA, there was further decrease in the expression of *HvNCED1* and maintained at low level through maturity (Figure 3.10A). The *HvNCED2* gene followed a similar expression pattern from 20 to 55 DAA; highly expressed at 20 DAA but showed a 4-fold decline by 25 DAA and remained at similar level until maturity (Figure 3.10B).

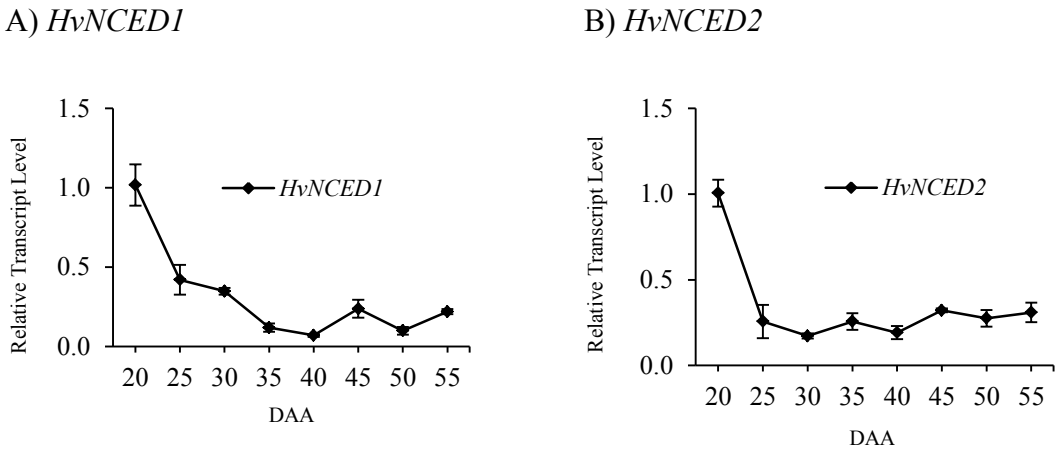


Figure 3.10 Relative transcript abundances of *HvNCED1* (A) and *HvNCED2* (B) during seed development. The transcript levels for each gene were compared across developmental stages using the respective mean transcript level in 20 DAA seeds, which was set to 1, as a calibrator. Data are means of 2 to 3 independent biological replicates ± SE.

Comparative analysis between the expression of *HvNCED1* and *HvNCED2* indicated that the expression of *HvNCED1* was substantially higher (13.4 to 27.8-fold) during the 20 to 30 DAA of seed development. Afterwards, the difference in the level of expression between the two genes was minimal (Figure 3.11).

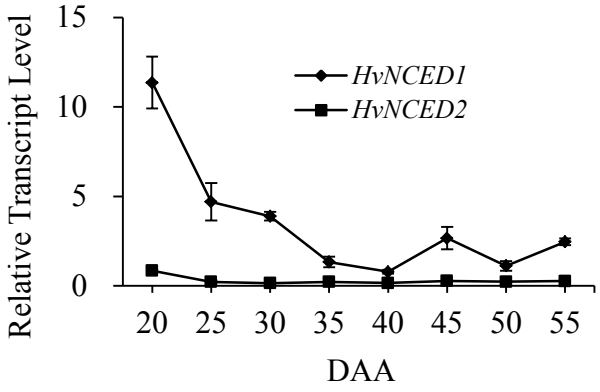


Figure 3.11 Relative transcript abundances of *HvNCED1* and *HvNCED2* during seed development. The transcript levels were compared between the two members of the *NCED* family using the mean transcript level of *HvNCED2* in 20 DAA seeds, which was set to 1, as a calibrator. Data are means of 2 to 3 independent biological replicates ± SE.

3.3.6.2 Expression of the ABA catabolic *CYP707A* genes

Although the expression of *HvCYP707A1* was low at the early stages of seed development (20 to 30 DAA), it showed a 3-fold increase from 30 to 35 DAA and was maintained at this elevated level through maturity. However, the transcripts of *HvCYP707A2* were not detected at any of the seed developmental stages considered in this study (Figure 3.12).

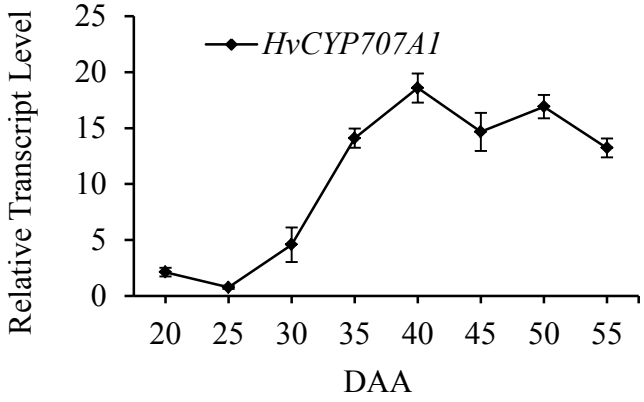


Figure 3.12 Relative transcript abundance of *HvCYP707A1* during seed development. The transcript levels were compared across developmental stages using the mean transcript level in 20 DAA seeds, which was set to 1, as a calibrator. Data are means of 2 to 3 independent biological replicates ± SE.

3.3.7 Expression analysis of GA metabolism genes during seed imbibition

The patterns of expression of GA metabolic genes in D and AR seeds were examined before (0 DAI) and during imbibition (at 12, 24 and 30 hours after imbibition [HAI]). In addition, the effect of ABA on the expression of GA metabolic genes in AR seeds was also examined by imbibing seeds with 50 µM ABA.

3.3.7.1 Expression of *GA20ox* genes

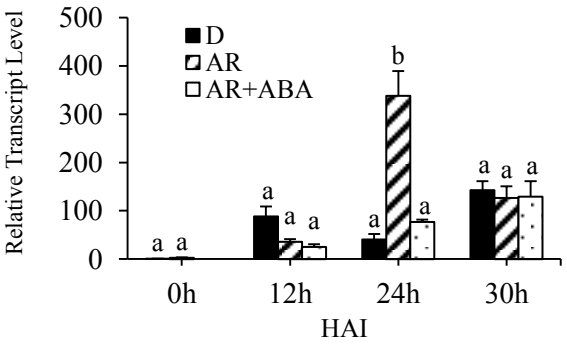
Imbibing the seeds for the first 12 h led to increased expression of *HvGA20ox1* in both D and AR seeds, though its expression in D seeds was higher (2.5-fold) than that detected in AR seeds. However, following 24 h imbibition, the expression of this gene was

significantly higher in AR than that detected in the D seeds (8.3-fold; $p<0.05$), and ABA treatment significantly decreased its expression in AR seeds (4.3-fold; $p<0.05$). At 30 HAI, there was no difference in the mRNA level between the D, AR and ABA imbibed AR seeds (Figure 3.13A).

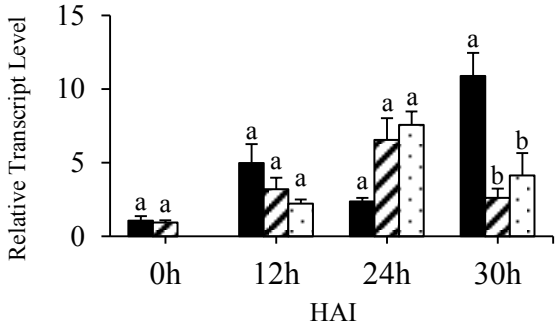
Seed imbibition for 12 h also induced the expression of *HvGA20ox2* genes in both D and AR seeds (3 to 4.6-fold change). Further imbibition of the seeds for 12 h upregulated the expression of *HvGA20ox2* in AR samples (2.7-fold) but downregulated its expression in the corresponding D samples. By 30 HAI, significantly higher ($p<0.05$) accumulation of *HvGA20ox2* transcripts was evident in D than AR seeds, and its expression was 5.5-fold more than that detected in 24 HAI. Throughout the entire imbibition period, ABA treatment did not affect the expression of this gene in AR seeds (Figure 3.13B).

With respect to *HvGA20ox3*, its expression pattern was different from those of *HvGA20ox1* and *HvGA20ox2* in which the dry seeds of both D and AR samples contained high amount of its transcripts. Imbibition of the seeds for 12 h lead to a substantial decrease in the abundance of *HvGA20ox3* transcripts in both D and AR seeds (2.0- to 7.3-fold; Figure 3.13C), however, a significant difference ($p<0.05$) in its expression was evident between the D and AR seed samples.

A) *HvGA20ox1*



B) *HvGA20ox2*



C) *HvGA20ox3*

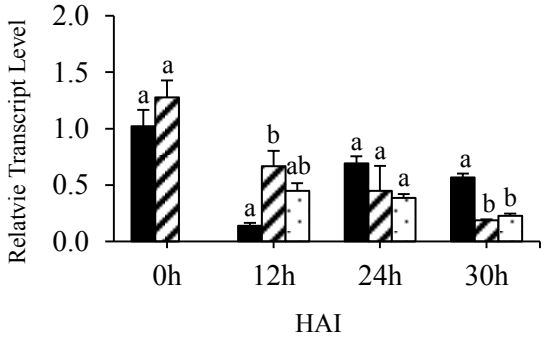


Figure 3.13 Relative transcript levels of *HvGA20ox1* (A), *HvGA20ox2* (B), and *HvGA20ox3* (C) in dormant and after-ripened seeds before and after imbibition. The transcript levels were compared across seed imbibition periods using the mean transcript level in dry (0 HAI) dormant seeds, which was set to 1, as calibrator. Data are means of 2 to 3 independent biological replicates ± SE. Different letters indicate significant difference among different samples within each imbibition period using Tukey's LSD test, $p<0.05$ (Appendix 1; 2; 3).

Imbibing the seeds further increased the expression of *HvGA20ox3* in D seeds (5-fold) and this level was maintained through 30 HAI. However, in AR seeds its expression of *HvGA20ox3* decreased (2.4-fold) with further imbibition (Figure 3.13C). The expression of this gene was not affected by ABA at any stage of imbibition.

3.3.7.2 Expression of *GA3ox* genes

Unimbibed dry AR seeds contained 2-fold more transcripts of *HvGA3ox1* than that found in D seeds. However, its expression level decreased gradually with imbibition. By 30 HAI AR seeds contained 7.6-fold less transcripts of *HvGA3ox1* as compared to that detected in the dry seeds. Similar to that observed in AR seeds, the transcript levels of this gene in D seeds decreased with imbibition, though a slight increase was apparent during the maturation period (Figure 3.14A). Imbibing the AR seeds in ABA showed inconsistent results as it resulted in significant decrease and increase ($p<0.05$) in the expression of *HvGA3ox1* at 12 and 30 h imbibition, respectively (Figure 3.14A).

The pattern of *HvGA3ox2* expression was different from that of *HvGA3ox1* in such a way that very minimal level of *HvGA3ox2* transcripts was detected in dry seeds of both D and AR samples, and its expression level was not affected by imbibition in D seeds. However, in AR seeds its expression increased with imbibition, in which 12 h imbibition led to 30-fold upregulation. Imbibing the seeds further for 12 h resulted in the highest expression of the gene, 4.8-fold increase from that observed at 12 HAI. By 30 HAI,

however, its transcript abundance declined (3.7-fold as compared to that was found at 24 HAI; Figure 3.14B). Overall, a significant difference in the expression of *HvGA3ox2* was observed between D and AR samples at all stage of imbibition ($p<0.05$; Figure 4.14B). It is apparent from the data of this study that ABA did not affect the expression of *HvGA3ox2* during seed imbibition.

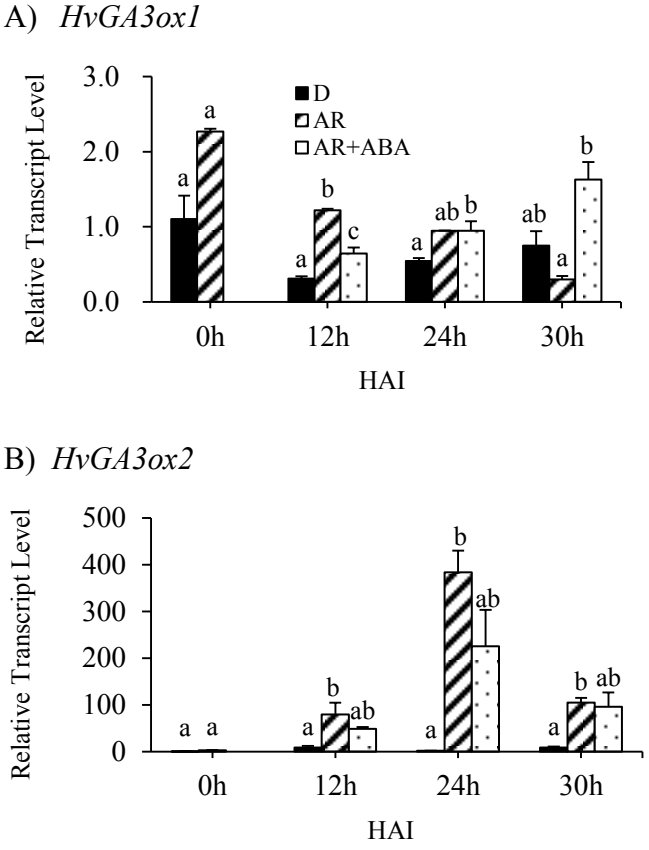


Figure 3.14 Relative transcript levels of *HvGA3ox1* (A), and *HvGA3ox2* (B) in dormant and after-ripened seeds before and after imbibition. The transcript levels were compared across seed imbibition periods using the mean transcript level in dry (0 HAI) dormant seeds, which was set to 1, as calibrator. Data are means of 2 to 3 independent biological replicates ± SE. Different letters indicate significant difference among different samples within each imbibition period using Tukey's LSD test, $p<0.05$ (Appendix 4; 5).

3.3.7.3 Expression of *GA2ox* genes

Imbibition of D seeds for the first 12 h induced 11-fold more expression of *HvGA2ox1* as compared to that detected in the dry seeds. Although there was a slight decline (1.7-fold) in the transcript abundance of *HvGA2ox1* by 24 HAI, it exhibited a 2.6-fold increase at 30 DAI. In contrast, the expression of *HvGA2ox1* in AR seeds was maintained at very low level before and after imbibition. Statistical analysis showed a significant difference ($p<0.05$) in the expression of this gene was evident between D and AR seeds at 12 and 30 HAI (Figure 3.15A).

Similar to that of *HvGA2ox1*, the dry D and AR seeds contained very minimal amount of *HvGA2ox3* transcripts. However, its expression pattern during imbibition was different from that of *HvGA2ox1*. Imbibing the dry AR seeds for the first 12 h led to a 60-fold increase in its expression but only very slight increase was evident in D seeds during the same period of imbibition, resulting in a significant difference ($p<0.05$) in its expression between the D and AR samples. Following 24 HAI, however, there was a drastic increase (10-fold) in the transcript abundance of *HvGA2ox3* in D seeds, and this expression level was maintained through 30 HAI. The AR seeds, however, maintained a similar expression level observed at 12 HAI but exhibited a 3.2-fold decline by 30 HAI (Figure 3.15B). The expression of *HvGA2ox3* in imbibing AR seeds was not affected by ABA.

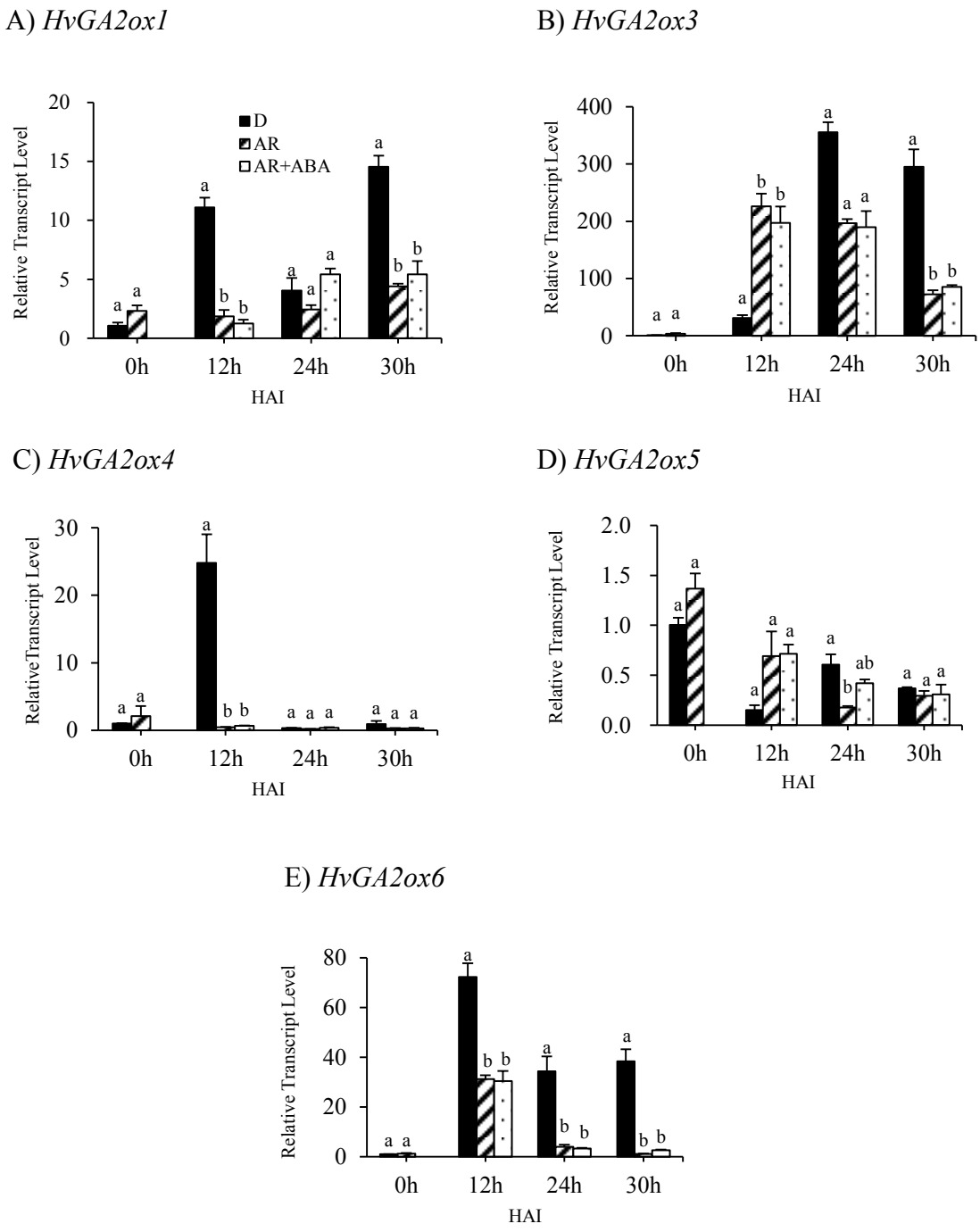


Figure 3.15 Relative transcript levels of *HvGA2ox1* (A), *HvGA2ox3* (B), *HvGA2ox4* (C), *HvGA2ox5* (D) and *HvGA2ox6* (E) genes before and during seed imbibition. The transcript levels were compared cross germination stages using the average transcript level in dormant 0 h seeds, which was set to 1, as calibrator. Data are means of 2 to 3 independent biological replicates ± SE. Different letters indicate significant difference among different samples within each imbibition period using Tukey's LSD test, $p<0.05$ (Appendix 6; 7; 8; 9; 10).

Although its expression remained very low in both D and AR samples before and after imbibition, *HvGA2ox4* exhibited significantly higher expression ($p<0.05$) in D seeds following 12 h imbibition; 60-fold higher than that in the AR seeds, leading to a significant change in gene expression ($p<0.05$; Figure 3.15C). No regulation of this gene by ABA was evident at any of the imbibition periods.

The expression pattern of *HvGA2ox5* was different from the other members of the *GA2ox* family. Higher abundance of *HvGA2ox5* transcripts were detected in the dry seeds of both D and AR samples, which declined as the seeds imbibed. During imbibition, a significant difference in its expression between the D and AR samples was observed only at 24 HAI ($p<0.05$). The expression of *HvGA2ox5* was upregulated (2.3-fold) in response to ABA only at 24 HAI (Figure 3.15D). With respect to the *HvGA2ox6*, a very low level of expression was evident in the dry seeds of both samples. However, its expression was induced by 12 h imbibition in both seed samples (70-fold in D and 30-fold in AR seeds; Figure 3.15E); however, 2.3-fold more transcripts were detected in D than AR seeds. Following 24 h imbibition, the expression of this gene showed a substantial decrease (7-fold) in AR seeds but was still maintained at elevated level in D seeds. Significant difference ($p<0.05$) in its expression was observed between D and AR seeds at all stages of imbibition. No ABA effect was evident in the expression of this gene during imbibition of AR seeds.

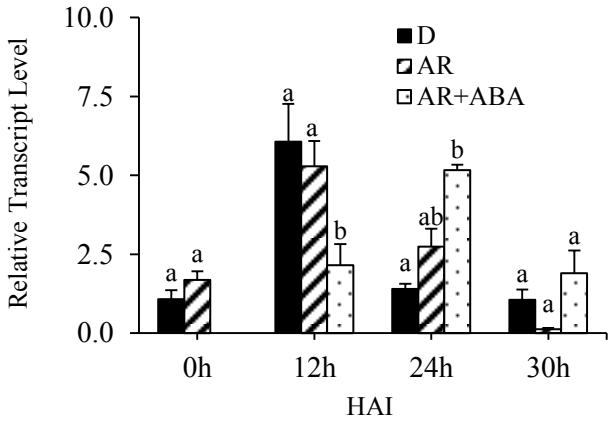
3.3.8 Expression analysis of ABA metabolism genes during seed germination

3.3.8.1 Expression of ABA biosynthetic *NCED* genes

The transcripts of *HvNCED1* were detected in the dry seeds of both D and AR seeds. The expression of this ABA biosynthetic gene increased in both D and AR seeds following 12 h imbibition (approximately 3 to 5-fold) but exhibited a decrease as imbibition continued through 24 HAI (2-fold). By 30 HAI, no transcript of *HvNCED1* was detected in AR seeds while its expression in D seeds was maintained at a level similar to that observed at 24 HAI. Although this gene showed significant downregulation ($p<0.05$) in response to ABA at 12 HAI, its expression was induced by 24 and 30 HAI (Figure 3.16A).

Dry D and AR seeds also contained similar levels of *HvNCED2* transcripts (Figure 3.16 B). Increased expression of this gene was evident in D seeds following 12 h of imbibition (2-fold) while its expression in AR seeds remained at a similar level detected before imbibition, resulting in a significant difference ($p<0.05$) in expression of this gene between D and AR samples. Following 24 h imbibition, very minimal or no expression of *HvNCED2* was detected in both D and AR seeds. Unlike the *HvNCED1* gene, ABA did not affect the expression of this gene at any stage of seed imbibition.

A) *HvNCED1*



B) *HvNCED2*

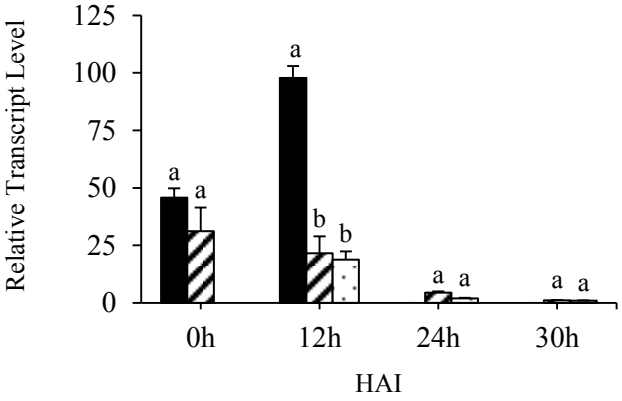
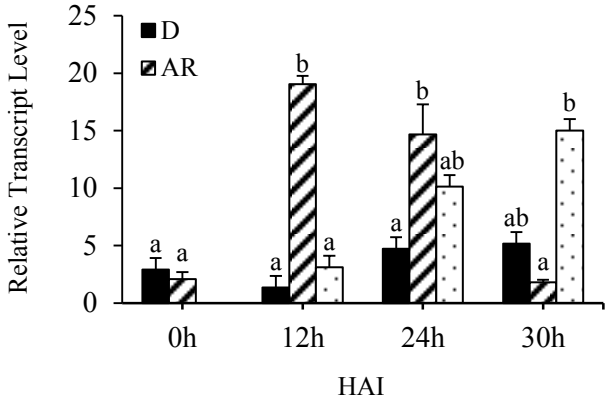


Figure 3.16 Relative transcript levels of *HvNCED1* (A) and *HvNCED2* (B) in dormant and after-ripened seeds before and after imbibition. The transcript levels were compared across imbibition periods using the average transcript level in dry (0 HAI) dormant seeds, which was set to 1, as calibrator. Data are means of 2 to 3 independent biological replicates ± SE. Different letters indicate significant difference among different samples within each imbibition period using Tukey's LSD test, $p<0.05$ (Appendix 11; 12).

3.3.8.2 Expression of the ABA catabolic *CYP707A* genes

The transcripts of *HvCYP707A1* were detected in dry seeds of both D and AR samples. Its expression was upregulated following 12 h imbibition in AR seeds (8-fold) but not in D seeds (Figure 3.17A). The elevated level of *HvCYP707A1* was maintained through 24 HAI, although it declined by 30 HAI (7-fold). From 12 to 24 HAI, there was a slight increase in the expression of this gene in D seeds and remained at the similar level through 30 HAI. At both 12 and 24 HAI, a significant difference in the expression of *HvCYP707A1* was evident between the D and AR seeds. ABA treatment caused a significant downregulation of this gene by 12 HAI (6-fold) but a strong upregulation by 30 HAI (8.4-fold; Figure 3.17A). In contrary to that of *HvCYP707A1*, no *HvCYP707A2* transcript was detected in the dry D and AR, and imbibed D seeds. However, seed imbibition resulted in its upregulation in AR seeds, which was maintained through 30 HAI. ABA treatment appears not to have effect on the expression of this gene during imbibition of AR seeds (Figure 3.17B).

A) *HvCYP707A1*



B) *HvCYP707A2*

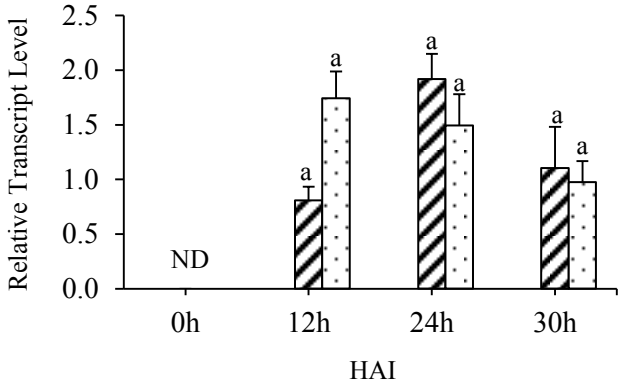


Figure 3.17 Relative transcript levels of *HvCYP707A1* (A) and *HvCYP707A2* (B) in dormant and after-ripened seeds before and after imbibition. The transcript levels were compared across imbibition periods using the average transcript level in dry (0 HAI) dormant (*HvCYP707A1*) and 12 HAI after-ripened (*HvCYP707A2*) seeds, which were set to 1, as calibrators. Data are means of 2 to 3 independent biological replicates ± SE. Different letters indicate significant difference among different samples within each imbibition period using Tukey's LSD test, $p<0.05$ (Appendix 13; 14). ND = not detected.

3.4 Discussion

3.4.1 Seed development and germination

Too low seed dormancy renders the germination of mature seeds on the mother plant before harvesting, which is a recurrent problem for barley in the world, especially those areas that are affected by moist weather before/during harvest (Gubler *et al*., 2005). The results of this study indicated that the seeds of cv. Betzes exhibit very low germination (7%) at early stage of seed development, 20 DAA (Figure 3.2). This is consistent with the very low germination capacity showed by developing embryos harvested from two different barley cultivars with varying degree of dormancy (B1215, low dormancy and Quilmes Palomar, high dormancy; Benech-Arnold *et al.*, 1999). Although the fresh and dry weights and the diameter of the seeds increased as they develop through 40 DAA, there was no change in their germination capacity. Beyond 40 DAA both the fresh and dry weights of the seeds remained constant (Figure 3.1C); however, the diameter of the seeds exhibited a decrease due to desiccation (Figure 3.1B). This along with the low germination percentage (25 %) exhibited by the dry seeds of cv. Betzes freshly harvested after maturity indicate that the seeds of this cultivar are dormant.

Comparative analysis of germination between D, AR and ABA treated AR seeds revealed that after-ripening is an effective methods of breaking the dormancy of barley seeds (Figure 3.2), and ABA inhibits the germination of AR seeds (Figure 3.3). In agreement with this, previous studies have shown that after-ripening causes a loss in seed

dormancy and thereby promoting the germination processes (Carrera *et al*., 2008). Besides, exogenous application of ABA has been shown to retard the germination of AR seeds and inhibit seedling growth (Figure 3.4). This is consistent with the findings of other studies, which showed that ABA inhibited germination and seedling growth (Gubler *et al*., 2005; Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al*., 2008).

3.4.2 Expression of GA metabolism genes during seed development and imbibition

In order to get better understanding of the role of GA in controlling seed development and germination in barley, this study analyzed the temporal expression pattern of the family members of regulatory GA metabolic genes (*HvGA20ox*s, *HvGA3ox*s, and *HvGA2ox*s) in developing and imbibing D and AR seeds. Among family members of these genes, *HvGA20ox3*, *HvGA3ox1* and *HvGA2ox5*, are the most highly expressed ones (Figure 3.6; 3.7; 3.9). As GA is required for normal seed development (Swain *et al*., 1995), the increased expression of the GA biosynthetic genes, *HvGA20ox3* and *HvGA3ox1*, during seed development may suggest that the level of GA required for proper seed development is regulated mainly by these two genes (Figure 3.6; 3.7). The germination data showed that the seeds of cv. Betzes are dormant by the time of harvest maturity, and the transcript abundance of the GA inactivating gene, *HvGA2ox5*, increased towards maturity (Figure 3.9). Since dormancy is expressed during the later stages of seed development, the increased expression of *HvGA2ox5* during these stages may

suggest that this gene is involved in decreasing the active GA pool, thereby contributing to the induction and maintenance of dormancy. In support of this hypothesis, most of the non-13-hydroxylated GAs (GA_{24}, GA_9, GA_4, GA_{51}, and GA_{34}) of Arabidopsis that were detected during the earlier stages of seed development decreased to almost undetectable level during the later stages (Kanno *et al.*, 2010).

Both the *HvGA20ox3* and *HvGA3ox1* genes, which contain high amount transcripts during seed development, exhibited lower expression during imbibition. However, their transcript abundances were still higher in AR seeds than D seeds (Figure 3.13C; 3.14A). Although not detected during seed development, the expressions of *HvGA20ox1*, *HvGA20ox2*, and *HvGA3ox2* are induced during imbibition in both D and AR seed samples. Following 24 h imbibition, *HvGA20ox1* and *HvGA20ox2* were expressed 8- and 3.3-fold higher in AR than D seeds (Figure 3.13A; 3.13B), whereas *HvGA3ox2* exhibited significantly higher expression (8.8- to 189.4-fold) in AR than D samples at all stages of imbibition (Figure 3.14B). The expression of *HvGA3ox2* was also shown to be highly induced in AR seeds during imbibition (Gubler *et al.* 2008). The results suggest that these three genes (*HvGA20ox1*, *HvGA20ox2*, and *HvGA3ox2*) mediate the production of GA, which is necessary for promoting seed germination, in imbibing non-dormant barley seeds. Treatment of seeds with ABA decreased the expression of *HvGA3ox2*, although the effect was not statistically significant (Figure 3.14B). Overall the seed development

and germination studies implied the distinct roles of specific family members of GA biosynthesis genes at different stages of plant development.

With respect to the GA deactivating genes, the expression of *HvGA2ox5*, which was expressed highly in developing seeds, showed a decline during imbibition of both D and AR seeds (Figure 3.15D). In contrast, seed imbibition was accompanied by increases in the expression of *HvGA2ox1*, *HvGA2ox3*, *HvGA2ox4*, and *HvGA2ox6*, which was more induced in D than AR seeds (Figure 3.15), suggesting that these genes are involved in decreasing the GA pool in the non-germinating D seeds. The expressions of *HvGA2ox1*, *HvGA2ox4* and *HvGA2ox6* were induced during the first 12 h imbibition, whereas that of *HvGA2ox3* showed an increase following 24 HAI, indicating the temporally distinct roles of *GA2ox* family members in regulating GA level during barley seed germination. Furthermore, of all the *GA2ox* genes studied here, the *HvGA2ox1* and *HvGA2ox4* genes were found to be suppressed in AR seeds, suggesting the role of after-ripening in decreasing the expression of specific GA catabolism genes. A decrease in the expression of the *GA2ox* genes during imbibition of non-dormant seeds has also been shown in Arabidopsis (Ogawa *et al*., 2003).

3.4.3 Expression of ABA metabolism genes during seed development and imbibition

In addition to the GA metabolic genes, we also characterized the expressions of the family members of regulatory ABA biosynthetic and catabolic genes so as to get better

understanding on the function of ABA in regulating barley seed development, dormancy and germination. This study showed that the ABA biosynthetic genes, *HvNCED1* and *HvNCED2*, are expressed highly at earlier stage of seed development, but decreased afterwards and remained at low level through maturity (Figure 3.10). The expression of *HvNCED1* in developing seeds was higher than that of *HvNCED2* (Figure 3.11). This is in contrast with the results of Chono *et al*. (2006), who showed *HvNCED2* as the major gene contributing to an increase in ABA level, which is associated with accumulation of storage compounds during seed development (Finkelstein *et al*., 2002; Finkelstein, 2004). This could be due to the difference in the experimental plant material and growing condition as they used barley cv. Misato Golden and field grown materials. With respect to the ABA catabolic genes, the highest expression of *HvCYP707A1* was observed during the later stages of seed development (at 40 DAA; Figure 3.12), and this gene expression result is consistent with the low ABA content found in barley embryos derived from the same stage (Chono *et al*. 2006). In contrast to that exhibited by *HvCYP707A1*, the expression of *HvCYP707A2* was not detected during seed development, suggesting that the expression pattern of *HvCYP707A1* in developing seeds is parallel to seed ABA content. However, in Arabidopsis, both *CYP707A1* and *CYP707A2* contribute to the regulation of ABA level in developing seeds; *CYP707A1* playing a role during the early stage whereas *CYP707A2* in the late-maturation stage (Okamoto *et al.*, 2006).

Seed imbibition in barley is associated with a decline in the level of ABA (Miller *et al.*, 2006), and this decline is strongly correlated with the expression level of several key ABA metabolism genes. This study found that dry D and AR seeds contain similar levels of *HvNCED*s transcripts, and this gene expression result is in agreement with the similar ABA level exhibited between D and AR barley embryos (Miller *et al.*, 2006). The expression of *HvNCED2* but not *HvNCED1* increased 2-fold in D seeds after 12 h imbibition, resulting in its significantly higher expression as compared to that in AR seeds. After-ripening did not affect the expression of *NCED1* gene and a similar result has been shown by Gubler *et al.* (2008). In contrast, Barrero *et al.* (2012) reported a strong downregulation of *NCED1* in imbibing AR *Brachypodium* seeds, and this may suggest species specific effect of after-ripening on the expression of *NCEDs*. As the imbibition time continues, the expressions of both *HvNCED1* and *HvNCED2* genes decreased in both D and AR seeds (Figure 3.16). This is in agreement with the decline in ABA level of imbibing embryos of both D and AR seeds (Millar *et al*., 2006; Gubler *et al*., 2008). As the embryo ABA content decreased to a much lower level in AR seeds as compared to D seeds following imbibition (Millar *et al*., 2006; Barrero *et al*., 2009), it is possible that the increased expression of *HvNCED2* shown in imbibing D seeds may contribute to the higher ABA content observed in the D barley embryos.

With respect to the *HvCYP707A* genes, the expression of *HvCYP707A1* during seed imbibition showed a big difference between AR and D seeds. The expression of

HvCYP707A1 remained very low during imbibition of D seeds, but significantly higher expression of this gene was observed in imbibing AR seeds (Figure 3.17A). Consistent with this result, the expression of *HvCYP707A1* showed a decrease during the early imbibition periods of D seeds, but in the AR seeds, the level of *HvCYP707A1* expression greatly increased during the same period (Chono *et al.*, 2006; Gubler *et al*., 2008; Barrero *et al.,* 2009). Furthermore, imbibition of a barley seed is accompanied by rapid conversion of embryo ABA to phaseic acid (Jacobsen *et al*., 2002).

The expression of *HvCPY707A2*, however, was induced in both D and AR seeds, showing no significant difference between the two samples (Figure 3.17B). This result suggests that *HvCPY707A2* does not play a major role in regulating dormancy decay in barley seeds. The data obtained from this study on the expression of *HvCPY707A1* and *HvCYP707A2* agrees with that of Millar *et al.* (2006), who showed that *HvCYP707A1* act as a predominant ABA 8'-hydroxylase gene in controlling dormancy in barley. However, it has been demonstrated by Kushiro *et al.* (2004) that *CYP707A2* of Arabidopsis plays a major role in regulating ABA level during imbibition of Arabidopsis seeds. These results indicate that different members of the *CYP707A* family play important role in regulating ABA level in different species. The findings of this study are consistent with previous reports that ABA catabolism, which is mainly catalyzed by ABA 8'-hydroxylation, controls ABA level and dormancy in imbibing seeds.

Although treating AR seeds with ABA did not have consistent effect on the expression of GA and ABA catabolic genes throughout the entire imbibition period, it significantly reduced the expressions of *HvGA20ox1* and *HvGA3ox1* as compared to that observed in the control AR seeds at 12 HAI and 24 HAI, respectively (Figure 3.13A and 3.14A). Consistent with this result, the expression of a *GA20ox* gene was suppressed in isolated sorghum embryos incubated with ABA (Pérez-Flores *et al*., 2003). In addition, the expressions of *GA3ox1* and *GA3ox2* genes of Arabidopsis were inhibited in the *cyp707a2-1* mutant, which overaccumulate ABA level in seeds and show reduced seed germination, than that found in the wild-type (Seo *et al*., 2006). However, no effect of ABA on the expression of GA deactivating genes was apparent (Figure 3.15). These results suggest that ABA's role in inhibiting germination is mediated by stage specific suppression of GA biosynthetic genes in imbibing barley seeds. The effect of ABA in inhibiting the expression of *HvNCED1* (at 12 HAI; Figure 3.16A) could be explained in terms of feedback inhibition of ABA biosynthetic genes by ABA. Since the level of ABA decreased during the first 24 h imbibition of the AR seeds (Gubler *et al.*, 2008), we anticipated that ABA treatment would increase the expression of the ABA catabolic genes during the early period of imbibition. However, the expression of *HvCYP707A1* was found to be significantly inhibited by ABA treatment (at 12 HAI; Figure 3.17A).

In conclusion, the temporal expression patterns of GA and ABA metabolism genes examined in this study provide better understanding of the roles of these two hormones in

barley seed development and germination. This study showed that the GA metabolic genes *HvGA20ox3* and *HvGA3ox1*, and *HvGA2ox5* are highly expressed during the earlier and later stages of seed development, respectively, suggesting the significance of these genes in maintaining the GA level needed for normal seed development. With respect to the ABA metabolic genes, the *HvNCED1* and *HvNCED2* genes that are highly expressed at the earlier stages of seed development, and the *HvCYP707A1* gene that is induced at the later stages appear to play important roles in regulating barley seed development. The germination capacity of cv. Betzes, which shows low level of germination at fresh harvest stage, is enhanced by after-ripening, and the effect of after-ripening is associated with interaction in the expression of their respective metabolic genes. After-ripening induces the expressions of *HvGA20ox1*, *HvGA3ox2*, and *HvCYP707A1* but represses that of *HvGA2ox1*, *HvGA2ox4* and *HVGA2ox6*, and *HvNCED2* during imbibition, reflecting the changes in GA and ABA levels.

4.0 Molecular Cloning and Characterization of *GA2ox1* and *GA2ox3* in Barley (*Hordeum vulgare* L.)

Abstract: Gibberellin (GA) deactivation is necessary to regulate the GA level in plants. GA 2-oxidases (GA2oxs) are the major GA catabolic enzymes that inactivate GAs by 2β-hydroxylation, and genes encoding these enzymes have been isolated from different plant species. This study reports the cloning of two *GA2ox*s in barley cv. Betzes, *HvGA2ox1* and *HvGA2ox3*. The *HvGA2ox1* encode a protein with 376 amino acids whereas *HvGA2ox3* encodes a 327 amino acid protein. The molecular weight of HvGA2ox1 and HvGA2ox3 are 57kDa and 52 kDa, respectively. Analysis of their amino acid sequences showed that *HvGA2ox1* is identical to *OsGA2ox1* (83% identity), and *HvGA2ox3* to *OsGA2ox3* (90% identity). Phylogenetic analysis of the amino acid sequence of these two genes revealed that they belong to the 2 oxoglutarate (OG) -Fe (II) oxygenase superfamily, a group of enzymes that require ferrous iron and a reducing agent to utilize the 2-OG and oxygen as co-substrates. Furthermore, the expression of the respective cDNAs of *HvGA2ox1* and *HvGA2ox3* in *E.coli* produced the corresponding fusion proteins with expected sizes.

4.1 Introduction

Gibberellin is one of the classic plant hormones that plays important roles in a variety of plant growth and developmental process, including seed germination, stem elongation, flower and seed development (Yamaguchi, 2008). Complex reactions and many enzymes are involved in the biosynthesis and deactivation of GAs. In higher plants, bioactive Gibberellins (GAs) are synthesized from trans-geranylgernayl diphosphate (GGDP) in three major steps. In the first stage, GGDP is transformed into *ent*-karuene in the plastid through two enzymes, copalyl diphosphatesynthase (CPS) and *ent*-kaurene synthesis (KS). In the second stage, *ent*-karuene is further catalyzed into GA_{12} by two membrane-associated P450 mono-oxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO), which are located in the membrane of the plastid and endoplasmic reticulum, respectively. In the third stage, GA_{12} is converted to bioactive GAs by soluble 2-oxoglutarate-dependent dioxygenases (2ODDs).

Deactivation of GA is also important to regulate the concentrations of bioactive GAs in plants. So far three different kinds of GA deactivation reactions have been identified. The 2β-hydroxylation at the C-2 of GA precursors or bioactive GAs, which is catalyzed by GA 2-oxidase (GA2ox), is the major deactivation reaction. Genes encoding GA2ox have been isolated from several plant species. The first *GA2ox* gene was cloned from runner bean (*Phaseolus coccineus* L.) through functional screening of a cDNA library derived from developing cotyledons. The function of this protein is verified by

heterologous expression in *Escherichia coli*, as capable of converting GA_9 to GA_{51} and GA_{51}-catabolite (Thomas *et al*., 1999). Searching the Arabidopsis database against the predicted protein sequence of *PcGA2ox1* revealed three related cDNAs designated as *AtGA2ox1*, *AtGA2ox2* and *AtGA2ox3*, and their functions were verified by in vitro incubation of the labeled GA substrates with the respective protein (Thomas *et al*., 1999). The transcripts of *AtGA2ox1* and *AtGA2ox2* were shown to be abundant in upper stems, flowers and siliques, and less expressed in lower stems and leaves, whereas no *AtGA2ox3* transcripts were detected in these tissues (Thomas *et al*., 1999). In GA-deficient *ga1-2* mutant of Arabidopsis, the transcript levels of *GA2ox1* and *GA2ox2* are reduced, but treatment of the mutants with GA_3 increased their expression (Thomas *et al*., 1999). These gene expression results suggest that GA2oxs are involved in maintenance of the active GA level in plant tissues (Thomas *et al*., 1999). The function of *AtGA2ox4* and *AtGA2ox5* were also tested by expressing their cDNAs in *E.coli*; *AtGA2ox5* was not expressed, thus considered to be a pseudo gene (Hedden *et al*., 2002). Further, Wang *et al.* (2004) isolated a gene corresponded to a portion of the regulatory region of the Downstream Target of AGAMOUS-Like-1 (*DTA1*) gene by chromatin immunoprecipitation approach. Sequence analysis of DTA1 showed that this gene had homology to GA2ox, and the recombinant protein was able to convert GA_4 to GA_{34}, GA_1 to GA_8 and GA_{20} to GA_{29}. Following this analysis, the gene was renamed to *AtGA2ox6*. Whereas, *AtGA2ox7* and *AtGA2ox8* were identified by screening of activation-tagged

mutants (Schomburg *et al.*, 2003), and these two genes are different from previously identified *AtGA2ox*s, as they only recognize C_{20}-GAs as their substrates. Increased expression of either *AtGA2ox7* or *AtGA2ox8* caused a dwarf phenotype in tobacco, and double loss-of-function mutants of these two genes had 2 to 4-fold higher levels of active GAs (Schomburg *et al*., 2003).

Degenerate primers designed from the region conserved among 2ODDs were used to amplify *OsGA2ox1* (Sakamoto *et al.*, 2001), and the recombinant protein prepared by expressing the *OsGA2ox1* cDNA in *E.coli* was able to catalyze the conversion of GA_{20} to GA_{29} and GA_1 to GA_8. Ectopic expression of the *OsGA2ox1* in transgenic rice caused GA deficiency that led to inhibition of stem elongation and development of reproductive organs (Sakamoto *et al*., 2001). Later, *OsGA2ox2* and *OsGA2ox3* were identified by screening of genomic DNA library; however, RT-PCR of *OsGA2ox2* was not successful (Sakai *et al*., 2003). Recombinant OsGA2ox3 protein catalyzed the metabolism of GA_1 to GA_8 and GA_{20} to GA_{29}-catabolite. The transcripts of *OsGA2ox3* are more abundant in stems, flowers, and roots than in the vegetative shoot apices, blades and leaf sheaths (Sakai *et al*., 2003). When treated with uniconazole, an inhibitor of GA biosynthesis, the transcript level of *OsGA2ox3* was low, but increased with GA_3 application. The *OsGA2ox4* was identified by searching the rice database against the sequences of *GA2ox* gene from different species (Sakamoto *et al.*, 2004). Although the *OsGA2ox5* and *OsGA2ox6* have not been characterized, amino acid sequence prediction showed that they

are able to 2β-hydroxylate C_{20}-GAs (Lee and Zeevaart, 2005). The rice GA2ox proteins appear to contain two His residues (eg. at residues 241 and 302 of OsGA2ox1) and one Asp residue (eg. at residue 243 of OsGA2ox1), which are supposed to associate with the catalytic and Fe^{2+} binding sites, respectively (Sakamoto *et al*., 2004).

Two *GA2ox* genes (p*sGA2ox1* and *PsGA2ox2*) have been isolated from pea by using functional screening and reverse transcription (RT)-PCR methods. *PsGA2ox1* expression product efficiently catabolized both GA_{20} and GA_1, and also some GA_{29}, while PsGA2ox2 prefer GA_1 to GA_{20}, and did not metabolize GA_{29} (Lester *et al*., 1999). The transcripts of *PsGA2ox1* were more abundant in maturing seeds than in shoots, while that of *PsGA2ox2* is more abundant in shoots, reflecting their differential roles. A loss-of-function *sln* (slender) mutant, which is due to a base deletion in the *GA2ox1* gene of garden pea, results in a slender or hyper-elongated phenotype (Lester *et al*., 1999; Martin *et al*., 1999). A number of GA biosynthesis and catabolism genes have been isolated from barley using two different cDNA libraries from cv. Himalaya (Spielmeyer *et al*., 2004). Among the *GA2ox* genes, the *HvGA2ox4* and *HvGA2ox5* genes are isolated by screening leaf cDNA library using a specific EST related to *GA2ox* (accession number AL505365) as a probe (Spielmeyer *et al*., 2004).

Phylogenetic analysis of their amino acid sequences revealed that GA2ox proteins are subdivided into three classes. For example, two Arabidopsis GA2ox proteins, AtGA2ox7 and AtGA2ox8, showed less similarity to other GA2ox proteins and belong to

a separate class. Besides, OsGA2ox5, OsGA2ox6 and GA2ox3 of spinach (*Spinacia oleracea*) belong to this class, and all of them use C_{20}-GAs as their substrate (Yamaguchi, 2008). The remaining two groups catabolize C_{19}-GAs, either bioactive GAs or their immediate precursors. The AtGA2ox1, AtGA2ox2 and AtGA2ox3 are grouped with OsGA2ox3 and OsGA2ox4 in class I. Whereas, AtGA2ox4, AtGA2ox5, OsGA2ox1, OsGA2ox2 and PsGA2ox2 form class II (Sakamoto *et al*., 2004; Lee and Zeevaart, 2005).

Previous studies have shown that manipulation of plant GA level and signaling contributes to increased crop production (Peng *et al*., 1999; Spielmeyer *et al*., 2002). As *GA2ox*s are GA deactivating genes, manipulation of plant GA levels and the stature of plants, and thereby enhancement of crop productivity can be achieved by modifying their expression (Sakamoto *et al*., 2003; Appleford *et al*., 2007; Dayan *et al*., 2010). Given that the Arabidopsis genome contains 8 members of *GA2ox* genes, and barley has larger genome size, it is likely that more members of *GA2ox* gene family exist in barley. Thus, this study was aimed at identifying new barley *GA2ox* genes by using the Rapid Amplification of cDNA Ends (RACE)-PCR method.

4.2 Materials and Methods

4.2.1 Plant material and growth condition

Mature dry seeds of barley cv. Betzes were imbibed for 3 days, germinated seeds were transplanted, and the resulting plants grown under conditions described in chapter 3.

4.2.2 Tissue collection

For cloning the full-length cDNAs of *HvGA2ox1* and *HvGA2ox3*, young leaf tissues (from 15 to 20 day-old plants) were harvested. Furthermore, root and shoot tissues were collected from 7 day-old seedlings grown in Petri-dishes. All of the tissues were stored at -80^{o}C until further use.

4.2.3 RNA isolation

Total RNA samples from leaf, root and shoot tissues were extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The frozen leaf, root and shoot tissues were ground to a fine powder in liquid nitrogen using pre-chilled pestle and mortar, and then ~ 100 mg of the fine powder was used to extract the total RNA. To eliminate genomic DNA contamination, ~ 10 µg total RNA was digested with 1 µl DNase I (DNA-*free*^{TM} Kit, Ambion, Austin, TX, USA), 5 µl 10X DNase I buffer in a total of 50 µl reaction volume. The reaction mixture was kept at 37°C for 30 min, and then inactivation buffer was added to stop the reaction.

The integrity of the resulting RNA samples was assessed by gel electrophoresis, and the purity was determined by the average spectrophotometric absorption ratio of 260 nm to 280 nm and 260 nm to 230 nm. After measuring their concentration with Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA), the RNA samples were stored at -80°C until cDNA synthesis.

4.2.4 cDNA synthesis

Complementary DNAs were synthesized from the total RNA samples using Revert Aid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's protocol. Total RNA (1 µg) was mixed with 1 µl of 100 µM random hexamer primer in a total volume of 12 µl, and incubated at 65°C for 5 min. After that, the mixture was incubated on ice for 2 min, then 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTPs, 1 µl RiboLock RNase Inhibitor (20 U/µl) and 1 µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl) were added, with a total of 20 µl reaction volume. The reaction mixture was incubated at 42°C for 1 h, and then the reaction was terminated by incubating the mixture at 70°C for 10 min. The concentration of the cDNA was determined by Nanodrop (Thermo Fisher Scientific) and then stored at -20°C.

4.2.5 BLAST search for the EST sequence of *HvGA2ox1* and *HvGA2ox3*

To obtain the EST sequences of *HvGA2ox1* and *HvGA2ox3*, the barley (taxid: 4512) Expressed Sequence Tags (ESTs) database from the National Center for Biotechnology Information (NCBI) was BLAST searched against the sequences of *OsGA2ox1* (GenBank accession # AB059416; Appendix 15) and *OsGA2ox3* (GenBank accession # AB092485; Appendix 16). This BLAST search produced, EST "CB876549.1" (Appendix 17; 18) and EST "BU972476.1" (Appendix 19) as having the best hits for *HvGA2ox1* and *HvGA2ox3*, respectively (Appendix 20; 21). Specific 5'end RACE and 3'end RACE primers were

designed from these ESTs (Table 4.1) to isolate the 3' end and 5' end fragments of *HvGA2ox1* and *HvGA2ox3* with RACE-PCR using SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA).

4.2.6 Isolation of 3' and 5' end fragments of *HvGA2ox1* and *HvGA2ox3*

4.2.6.1 First strand 3' and 5' RACE ready cDNA synthesis

The first strand 3' and 5'-RACE ready cDNAs were prepared using SMARTer™ RACE cDNA Amplification kit (Clontech) according to the manufacturer's protocol. Briefly, total RNA (1 µg) was mixed with 1 µl of 12 µM 3'-RACE CDS Primer A and 1 µl of 12 µM 5'-RACE CDS Primer A (Clontech) in two separate tubes for synthesizing 3' RACE cDNA and 5' RACE cDNAs, respectively. These two reaction mixtures were incubated first at 72°C for 3 min and then at 42°C for 2 min, followed by centrifugation at 14000 *g* for 10 s at 4°C. Then 1 µl of 12 µM SMARTer IIA oligo (Clontech) was added only to the 5' RACE cDNA. A master mix containing 2 µl of 5X reaction buffer, 1µl of 10 mM dNTPs, 1 µl of 20 mM Dithiothreitol (DTT), 1µl RiboLock RNase inhibitor (20 U/µl) and 1 µl of M-Mulv Reverse Transcriptase (200 U/µl) was prepared and then added to each tubes and mixed very well. The mixture was incubated at 42°C for 90 min followed by further incubation at 70°C for 10 min in a thermal cycler (Bio-Rad). Finally, the synthesized first strand 3' and 5' RACE Ready cDNAs were diluted with 20 µl Tricine-EDTA buffer and stored at -80°C until further use.

4.2.6.2 Isolation of 3' end fragments for *HvGA2ox1* and *HvGA2ox3*

The first strand 3' RACE Ready cDNA samples were used as a template for 3' RACE-PCR. The 3' end fragments of *HvGA2ox1* and *HvGA2ox3* were amplified using outer gene specific (HvGA2ox1-3'outer and HvGA2ox3-3'outer; Table 4.1) and universal (Clontech) primers. The PCR reaction contained 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 mM HvGA2ox1-3'outer or HvGA2ox3-3'outer primer, 2.5 µl 10X Universal Primer A Mix (Clontech), 1.5 µl of cDNA template, 0.2 µl of *Taq* DNA polymerase (5 U/µl) and 17.3 µl distilled water. The thermocycling condition was as follows: initial denaturation at 95^{o}C for 3 min followed by 5 cycles at 95^{o}C for 50 s, 70^{o}C for 2 min; 10 cycles at 95^{o}C for 50 s, 70^{o}C for 55 s and 72^{o}C for 2 min during these 10 cycles the annealing temperature decreased by 1.5^{o}C per each cycle; 26 cycles at 95^{o}C for 50 s, 55^{o}C for 55 s and 72^{o}C for 1.5 min, and a final extension at 72^{o}C for 5 min. Following amplification, the 3' outer RACE products were used as a template for nested PCR, which was performed using inner gene specific primer (HvGA2ox1-3'inner and HvGA2ox3-3'inner; Table 4.1), Nested Universal Primer A (Clontech) and the thermocycling conditions described for first round RACE reaction.

Table 4.1 Sequence of gene specific primers for isolation of 3' and 5' end fragments of *HvGA2ox1* and *HvGA2ox3*

Primer name	3' or 5' RACE	Sequence (5' to 3')
HvGA2ox1-3'outer	3' RACE	AAGGCTGTCCACCATCTACTTCGCAGC
HvGA2ox1-3'inner	3' RACE	TGCAGGCAATCTGTATGGTGGGGATT
HvGA2ox3-3'outer	3' RACE	GGAGGGCCAGCATTGACACAGAGGAT
HvGA2ox3-3'inner	3' RACE	GGGGCGAGTACAAGAAGGCTGCCT
HvGA2ox3-5'outer	5' RACE	TCCCGTTGGTCAGCACCTGCAAG
HvGA2ox3-5'inner	5' RACE	TGGTTCACCCTGAACACCTGGTCGCT

4.2.6.3 Bioinformatic assembly of 5' end fragment of *HvGA2ox1*

The 5' end fragment of *HvGA2ox1* was identified bioinformatically by BLAST searching the assembled sequences (Appendix 22) of EST "CB876549.1" and 3' end fragment of *HvGA2ox1* against the Dana-Farber Cancer Institute (DFCI) Barley Gene Index database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi), and this BLAST searching produced the bioinformatically assembled sequence "TC244240" (Appendix 23; 24) as having the best hit for the search. The sequence of "TC244240" is assembled by the sequences of 4 ESTs ("BU980500"; "FD520421"; "CB876549" and "CB880972"; Appendix 25). Sequence analysis showed "TC244240" still doesn't include the entire coding region of the *HvGA2ox1*. Then, sequence "TC244240" was further BLAST searched against the DFCI barley gene index database, and another EST "CA031963" was obtained (Appendix 26 and 27), assembly of "TC244240" and EST "CA031963" was able to generate the full length coding region of *HvGA2ox1* (Appendix 28).

4.2.6.4 Isolation of 5' end fragment of *HvGA2ox3*

The first strand 5' RACE Ready cDNA sample was used as a template for 5' RACE-PCR. The 5' end fragment of *HvGA2ox3* was amplified using outer gene specific primer (HvGA2ox3-5'outer; Table 4.1) and 10X Universal Primer A Mix (Clontech). The 50 µl PCR reaction mixture contained cDNA (~50 ng) as a template, 10 µl of 5X Phusion High-Fidelity buffer, 1 µl of 10 mM dNTPs, 5 µl of 10X Universal Primer A Mix, 1 µl of 10 µM HvGA2ox3-5'outer primer, 1.5 µl DMSO, 0.5 µl Phusion DNA polymerase (2 U/µl) and sterile distilled water. The PCR reaction mixtures were subjected to touchdown PCR using a thermocycling condition of 5 cycles at 98°C for 30 s and 72°C for 1 min; followed by 5 cycles at 98°C for 30 s, 70°C for 30 s and 72°C for 1 min, then 25 cycles at 98°C for 30 s, 68°C for 30 s and 72°C for 1 min, and final extension at 72°C for 10 min. The 5'outer RACE-PCR product (which produced several unspecific bands) was subjected to nested PCR using inner gene specific primer (HvGA2ox3-5'inner; Table 4.1) and Nested Universal Primer A (Clontech). The template for the nested PCR was prepared by 50X dilution of the outer RACE products with Tricine-EDTA buffer. The 50 µl PCR reaction mixture contained 5µl of the diluted cDNA, 10 µl of 5X Phusion High-Fidelity buffer, 1 µl of 10 mM dNTPs, 1 µl of Nested Universal Primer A, 1 µl of 10 µM HvGA2ox3-5'outer primer, 1.5 µl DMSO, 0.5 µl Phusion DNA polymerase (2 U/µl) and sterile distilled water. The PCR was performed using a thermocycling condition of 98°C

for 30 s followed by 25 cycles at 98°C for 30 s, 68°C for 30 s and 72°C for 1 min, and final extension at 72°C for 10 min.

4.2.6.5 Gel extraction

The products of nested 3' and 5' end RACE-PCR were loaded into a 1% (w/v) agarose gel containing ethidium bromide, electrophoresed and then visualized with Molecular Imager Gel Doc^{TM} XR (Bio-Rad). The size of the PCR product was determined using DNA 1Kb plus marker (Invitrogen, Carlsbad, CA, USA). The bands with expected sizes were excised from the gel, and the DNA fragment purified using GeneJET^{TM} Gel Extraction Kit (Fermentas, Foster City, CA, USA) according to the manufacturer's protocol. The purified DNA was stored at -20^{o}C until further use.

4.2.6.6 A tailing of 5' end DNA fragment of *HvGA2ox3*

The purified 5'end DNA fragment of *HvGA2ox3* amplified with thermostable Phusion High-Fidelity DNA polymerase (Finnzymes) needed to have A tailing before ligation. The A tailing was performed using the following reaction mix: 1 µl of 2 mM dATP, 1 µl of 10X *Taq* buffer, 1 µl of 25 mM $MgCl_2$, 1 µl of *Taq* DNA polymerase (5 U/µl), 6 µl of amplified PCR product, with a total reaction volume of 10 µl, which was incubated at 4^{o}C overnight.

4.2.6.7 Ligation and transformation

The A tailing product was then ligated to pGEM-T Easy Vector (Promega, Madison, WI, USA). The ligation reaction contained the purified DNA fragment, pGEM-T Easy Vector (the molar ratio of the insert DNA to the vector was 3 to 1), 5 µl of 2X rapid ligation buffer, 1 µl of T4 DNA ligase (3 U/µl) and distilled water with a total reaction volume of 10 µl. The reaction mixture was incubated at 16^{o}C overnight, and the product was used to transform *E.coli* DH5α competent cells (Invitrogen). Five µl of the ligation product (~ 10 ng) was mixed with competent DH5α cells (50 µl) and then subjected to incubation on ice for 30 min, and heat shock at 42^{o}C for 90 s. After heat shock, the mixture was incubated on ice for 2 min and then mixed with 600 µl of Luria-Bertani (LB) media (10 g tryptone, 5 g yeast extract and 10 g NaCl per liter; pH 7.0) and then incubated further on a shaker at 225 revolutions per minute (rpm) at 37 ^{o}C for 45 min. The transformation mixture was centrifuged at 12,000 *g* for 5 min, and the pellet resuspended with some of the remaining supernatant was plated on LB agar plates containing 20 µl of 100 mg/ml ampicillin (Sigma, St. Louis, MO, USA), 4µl isopropyl β-D-1-thiogalactopyranoside (IPTG, 200 mg/ml) and 40 µl X-gal (Promega) per plate. The plates were incubated at 37^{o}C overnight.

4.2.6.8 PCR screening, plasmid isolation and DNA sequencing

The white colonies grown on the plate were picked and screened by PCR using reaction mixtures and thermocylcling conditions as described above. PCR products were electrophoresed in agarose gel (1%), and the colonies harboring DNA fragments with predicted band size were grown overnight in LB medium containing Ampicillin (100 mg/ml) on a shaker (225 rpm) at 37^{o}C. Plasmid DNA was isolated from cell cultures of positive colonies using the GeneJET^{TM} Plasmid Miniprep Kit (Fermentas) following the manufacturer's protocol. The purified plasmids were sent out for sequencing (Macrogen, MD, USA) by using pGEM-T Easy specific M13 forward and reverse primers (Table 4.2).

Table 4.2 Sequences of primers used for different purposes and their respective amplicon sizes.

Name	Purpose	Sequence (5' to 3')	Size (bp[c])
HvGA2ox1-F[a]1	PCR assembly	GAGCCAACGCAATGGTGGTG	1364
HvGA2ox1-R[b]1		CGGAAATAGAAGTTGCATCAGG	
HvGA2ox1-F2	PCR assembly	TCCGCCGAGCCAACGCAATG	1170
HvGA2ox1-R2		GCCTGCAACTCAAGCAGCCA	
HvGA2ox1-F3	PCR assembly	GCGGATCCATGGTGGTGCCCTCCACGAC	1146
HvGA2ox1-R3		CGACGTCGACTTATTTTCCTTTGCTGCCAT	
HvGA2ox1-Full-F	End to end PCR	ATGGTGGTGCCCTCCACGAC	1128
HvGA2ox1-Full-R		TTATTTTCCTTTGCTGCCAT	
HvGA2ox3-Full-F	End to end PCR	ATGGTGGTTCTCGCCGGC	981
HvGA2ox3-Full-R		CTACCTGTGGAAGTGAGCCA	
M13 F	Sequencing	GTAAAACGACGGCCAGT	N/A
M13 R		GCGGATAACAATTTCACACAGG	
BamH1-HvGA2ox1-F	Expression	GCGGATCCATGGTGGTGCCCTCCACGAC	1146
HvGA2ox1-R-*Sal1*	vector construct	CGACGTCGACTTATTTTCCTTTGCTGCCAT	
BamH1-HvGA2ox3-F	Expression	GCGGATCCATGGTGGTTCTCGCCGGC	998
HvGA2ox3-R-*HindIII*	vector construct	CCGAAGCTTCTACCTGTGGAAGTGAGCCA	

[a] Forward Primer, [b] Reverse Primer, [c] Base pair

4.2.7 Assembly of the sequences of the fragments for *HvGA2ox1* and *HvGA2ox3*

For the 5' end fragment of *HvGA2ox1*, the sequences of "TC244240" and EST "CA031963" were assembled and the resulting nucleotide sequence was BLAST searched against GenBank database (Appendix 29) using). The resulting sequences of 3' and 5' end fragments of *HvGA2ox3* were also assembled (Appendix 30) with the corresponding EST sequences, and the resulting nucleotide sequence (Appendix 31) BLAST searched against GenBank database. Further, the resulting putative full length nucleotide sequences of *HvGA2ox1* and *HvGA2ox3* were translated into their corresponding protein sequences, which were also BLAST searched against protein databases, to examine whether they contain complete protein structure.

4.2.8 End to end PCR

Based on the assembled sequence of *HvGA2ox1*, six more primers (HvGA2ox1-F1, HvGA2ox1-F2, HvGA2ox1-F3, HvGA2ox1-R1, HvGA2ox1-R2 and HvGA2ox1-R3; Table 4.2) were designed to amplify the coding region of *HvGA2ox1*. PCR amplification was performed by using thermostable Phusion High-Fidelity DNA Polymerase (Finnzymes) and PCR reaction mix containing cDNA (~50 ng) as a template, 10 µl of 5X Phusion High-Fidelity buffer, 1 µl of 10 mM dNTPs, 2.5 µl of 10 µM forward primer, 2.5 µl of 10 µM reverse primer, 0.5 µl Phusion DNA polymerase (2 U/µl) and sterile distilled water to a total reaction volume of 50 µl. The PCR reaction mixtures were subjected to

thermal cycling conditions of initial denaturation at 98^{o}C for 30 s followed by 35 cycles of 98^{o}C for 10 s, 60^{o}C for 30 s and 72^{o}C for 30 s followed by a final extension at 72^{o}C for 10 min using a DNA thermal cycler (Bio-Rad). The first round PCR product was diluted and used as template to do a nested PCR. Primer HvGA2ox1-F3 and HvGA2ox1-R3 were used for the nested PCR, all the nested PCR condition were the same as previously described. For *HvGA2ox3*, the end to end PCR was performed by using forward (HvGA2ox3-Full-F) and reverse (HvGA2ox3-Full-R) primers designed from the respective 5' and 3' ends of the putative full length cDNA sequence of *HvGA2ox3*, respectively (Table 4.2). The PCR reaction and thermal cycling condition was as described above. Following amplification, the PCR products were separated with 1% (w/v) agarose gel. The DNA bands corresponding to the expected sizes of *HvGA2ox1* and *HvGA2ox3* were excised and purified as described previously.

The purified putative full length fragments of *HvGA2ox1* and *HvGA2ox3* were ligated into pGEM-T Easy vector, and transformed with *E.coli* (DH5α) cells. Following PCR screening of the positive colonies, plasmid isolation and subsequent sequencing of the DNA fragment of the putative *HvGA2ox1* and *HvGA2ox3* genes were carried out as described above.

4.2.9 Sequence analysis

The resulting nucleotide sequences of *HvGA2ox1* and *HvGA2ox3* were BLAST searched against GenBank database, and their respective coding regions identified using Open Reading Frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequences of *HvGA2ox1* and *HvGA2ox3* were translated into the corresponding amino acid sequences by using the Expert Protein Analysis System (ExPASy) Translate Tool (http://web.expasy.org/translate/). The translated amino acid sequences were BLAST searched against GenBank Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to annotate the functional units of the putative proteins.

4.2.10 Phylogenetic relationship of HvGA2ox1 and HvGA2ox3 with other known GA 2-oxidases

Protein sequences of 26 known GA2oxs from other species (both monocot and dicot) were collected from GenBank database. Their amino acid sequences were aligned with HvGA2ox1 and HvGA2ox3 using ClustalW alignment tool from Molecular Evolutionary Genetic Analysis (MEGA, version 5). The phylogenetic tree was generated using MEGA5 software with neighbor-joining method and Poisson correction model from 500 bootstrap replicates.

4.2.11 Analysis of *HvGA2ox1* and *HvGA2ox3*

4.2.11.1 Cloning *HvGA2ox1* and *HvGA2ox3* into expression vector

For cloning *HvGA2ox1* and *HvGA2ox3* into the pET32a expression vector, their ORF was amplified by using restriction site linked primers and thermostable Phusion High-Fidelity DNA polymerase. For *HvGA2ox1*, the *BamHI* restriction site was linked with the forward primer (*BamHI*-HvGA2ox1-F; Table 4.2) and *SalI* restriction site with the reverse primer (HvGA2ox1-R-*SalI*; Table 4.2) of *HvGA2ox1*. For *HvGA2ox3*, the *BamHI* restriction site was linked with the forward primer (*BamHI*-HvGA2ox3-F; Table 4.2) and *HindIII* restriction site was linked with the reverse primer (HvGA2ox3-R-*HindIII*; Table 4.2) of *HvGA2ox3*. Restriction analyses of the *HvGA2ox1* and *HvGA2ox3* coding regions were performed to make sure that the restriction enzymes do not cut the gene to be cloned into the expression vector. The respective PCR products were separated by gel electrophoresis and purified as described above. The purified *HvGA2ox1* and *HvGA2ox3* PCR products were first ligated to pGEM-T Easy vector and their identity verified by sequencing. Then, the pGEM-T Easy-*HvGA2ox1* and pGEM-T Easy-*HvGA2ox3* recombinant vectors and the pET32a expression vector were digested with FastDigest *BamHI*, *SalI* and *HindIII* restriction enzymes (Fermentas) prior to ligation. The digestion reaction contained 1µg of plasmid DNA (for each of pET32a, pGEM-T Easy- *HvGA2ox1* and pGEM-T Easy-*HvGA2ox3*), 4 µl of 10X Fast Digest buffer, 2 µl of *BamHI* (1 U/µl) and 2 µl of *SalI* or *HindIII* (1 U/µl) and water to give a total reaction volume of 40 µl.

The reaction mixtures were incubated at 37^{o}C for 3 h, after which the digestion products were separated by gel electrophoresis. The DNA fragments of interest were excised and purified as described above. The purified digested products of *HvGA2ox1*, *HvGA2ox3* and pET32a were ligated using a 10 µl total reaction mix that contained 5 µl of 2X ligation buffer, 50 ng of digested pET32a, 150 ng of each of the digested *HvGA2ox1* and *HvGA2ox3*, 1 µl T4 DNA ligase (3 U/µl) and water. The ligation reaction mixture was incubated at 16^{o}C overnight, and then used to transform *E.coli* (DH5α) cells as described earlier. White colonies were then selected and PCR screened to verify that they contain the *HvGA2ox1* and *HvGA2ox3* inserts. The isolated plasmid DNA was sent out for sequencing. Moreover, plasmid DNA was digested with both *BamHI* and *SalI* or *HindIII* as described previously, and the digestion products were electrophoresed for verifying their sizes.

4.2.11.2 Recombinant protein expression

The molecular weights of the pET32a-HvGA2ox1 and pET32a-HvGA2ox3 fusion proteins were determined using ExPASy ProtParam Tool (http://web.expasy.org/protparam/). The positive clones containing pET32a-*HvGA2ox1* and pET32a-*HvGA2ox3* were transformed with *E.coli* EXPRESS BL21 (DE3) DUOs system (Lucigen, Middleton, WI, USA) using the procedure described previously for transforming DH5α cells.

A single positive colony of each of the pET32a-*HvGA2ox1*, pET32a-*HvGA2ox3* and pET32a was inoculated into 5 ml of LB media containing 100 mg/ml of ampicillin and incubated at 37^{o}C overnight. Subsequently, 0.5 ml of the cell culture was added into 50 ml of fresh LB medium containing 100 mg/ml of Ampicillin and cultured up to an optical density at 600 nm wavelength (OD_{600}) of 0.4-0.6 (which took approximately $2^{1/2}$ h). Following addition of IPTG (final concentration of 1 mM and 0.5 mM), each culture was incubated at 28^{o}C on a shaker at 225 rpm for 4 h. The cell cultures were then harvested by centrifugation at 6000 *g* for 10 min at 4^{o}C and then resuspended with 0.5 ml of lysis buffer (containing 50 mM of Tris-HCl, pH 7.5). Following disruption by sonication (10 cycles of 10 s sonication followed by 10 s incubation on ice), the cells were centrifuged at 12000 *g* for 7 min at 4^{o}C. Both the supernatants and pellets were analyzed for the presence of the respective crude proteins by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.11.3 Extraction of crude recombinant protein

By using a combination of different induction factors (induction time, temperature and IPTG concentrations), the condition for enhanced expression level of the crude recombinant pET32a-HvGA2ox1 and pET32a-HvGA2ox3 proteins in the supernatant was selected. The optimized expression condition was 0.1 mM (for pET32a-*HvGA2ox1*) and 0.05 mM (for pET32a-*HvGA2ox3*) IPTG final concentration and incubation at 12°C

for overnight. All other conditions were as described above. Following induction, the cultures were harvested by centrifugation at 6000*g* for 10 min at 4^{o}C and then resuspended with 0.5 ml of lysis buffer (containing 50 mM of Tris-HCl, pH 7.5). Afterwards, the cells were disrupted by sonication and then centrifuged at 12000*g* for 7 min at 4^{o}C. The supernatant was subjected for analysis of the resulting recombinant proteins by SDS-PAGE.

4.3 Results

4.3.1 Molecular cloning of *HvGA2ox1* and *HvGA2ox3*

4.3.1.1 EST sequence search for *HvGA2ox1* and *HvGA2ox3*

BLAST searching the nucleotide sequences of rice *GA2ox1* (accession # AB059416; Appendix 15) and *GA2ox3* (accession # AB092485; Appendix 16) cDNAs against the barley EST database produced EST "CB876549.1" and EST "BU972476.1" (Appendix 17; 18; 19) as having best hits (Appendix 20; 21), respectively. The amino acid sequences of EST "CB876549.1" showed 78% identity with OsGA2ox1, while the amino acid sequence of EST "BU972476.1" exhibited 87.1% identity with that of OsGA2ox3 (Table 4.3).

4.3.1.2 Isolation of 3' and 5' end fragments of *HvGA2ox1* and *HvGA2ox3*

Amplifying the cDNA samples prepared from different tissues of barley cv. Betzes as a template for RACE-PCR with universal primers and gene specific primers designed from the 3' end of EST "CB876549" and EST "BU972476" (Table 4.1) produced 3' end fragments for *HvGA2ox1* (~227 bp; Figure 4.1A) and *HvGA2ox3* (~268 bp; Figure 4.1B), respectively.

Table 4.3 Amino acid sequence identity of GA2ox1 and GA2ox3 proteins from rice and Arabidopsis with those derived from "BU972476.1" and "CB876549.1" ESTs of barley.

Accession#	Name	Species	Identity (%)	Length
		HvGA2ox1		
BAB40934.1	OsGA2ox1	*O.sativa*	78.0	381
NP_171742.1	AtGA2ox6	*A.thaliana*	51.8	326
		HvGA2ox3		
BAC16752.1	OsGA2ox3	*O.sativa*	87.1	326
NP_174296.1	AtGA2ox2	*A.thaliana*	56.7	335

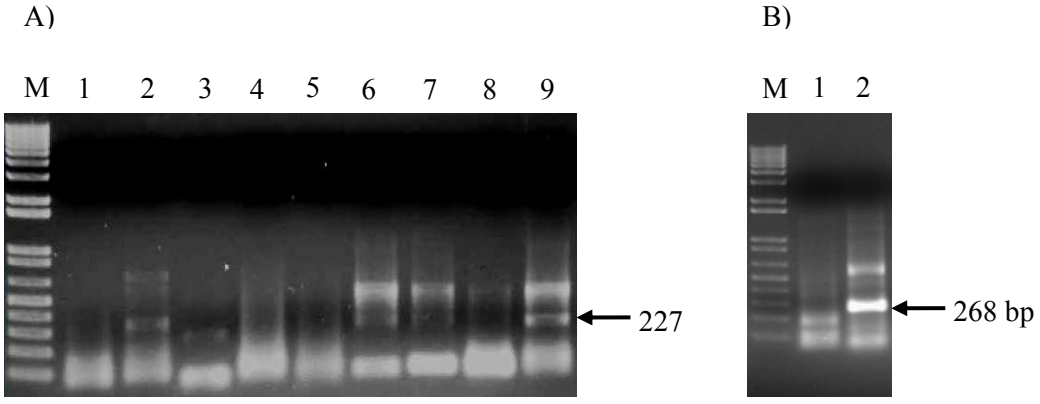


Figure 4.1 RACE-PCR product for 3' end fragments of *HvGA2ox1* (A; lane 9) and *HvGA2ox3* (B; lane 2) amplified from barley cv. Betzes. Lane M is the 1Kb plus DNA ladder.

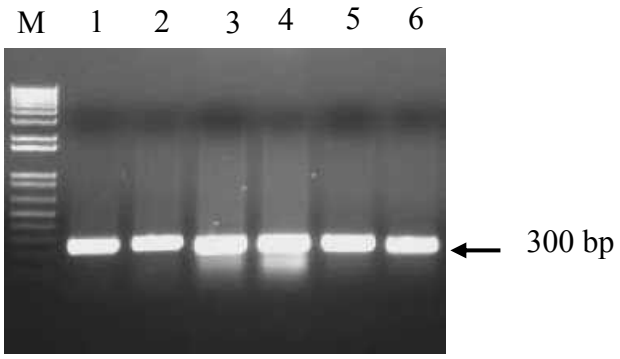


Figure 4.2 PCR screening of 3' RACE end fragments of *HvGA2ox1* (lanes 1-3) and *HvGA2ox3* (lanes 4-6) amplified from barley cv. Betzes. Lane M is the 1kb plus DNA ladder.

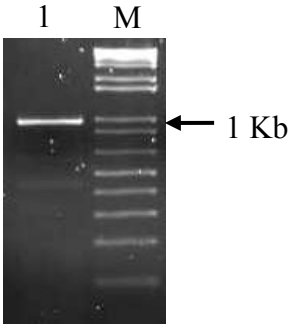


Figure 4.3 RACE-PCR product for 5' end fragment of *HvGA2ox3* (lane 1) amplified from barley cv. Betzes. Lane M is the 1Kb plus DNA ladder.

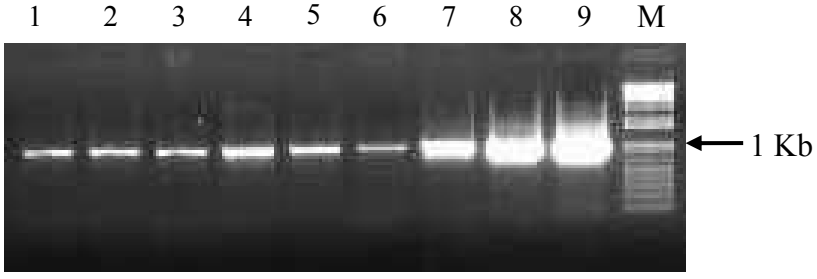


Figure 4.4 PCR screening of 5' RACE end fragment of *HvGA2ox3* (lanes 1-9) amplified from barley cv. Betzes. Lane M is the 1kb plus DNA ladder.

The respective PCR products were gel-purified and cloned into the pGEMT-Easy Vector followed by transformation of each product with *E.coli* (DH5α) cells. PCR screening of the colonies with specific primers designed from the 3' or 5' end of the respective ESTs and universal primer identified positive colonies that carried the inserted DNA fragments (Figure 4.2 and 4.4). Plasmid isolation of the colonies and subsequent DNA sequencing of the fragments showed that the 3' end fragments for *HvGA2ox1* and *HvGA2ox3* are 227 bp and 268 bp, respectively (Figure 4.5).

TGCAGGCAATCTGTATGGTGGGGATTAGTTAGATATAAGAATAGTCCCCAAAATTTTGGC
TCTCTGACAGCTATTGCCAGATATCCCAGTATTATGATGGTGTAGTGTTTGTGTCCATGA
CCTGGCTAGCTTTGTACAGAAGTGATGCTTGTATGATAGTAATTTACCTTCAGCACAAAA
AAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTT

Figure 4.5 Nucleotide sequence of the 3' end fragment of *HvGA2ox1* (227bp). The forward and reverse primers used for amplification of the PCR product are underlined.

GGGGGGAGTACAAGAAGGCTGCCTTCAACTCCAGGCTCGGGGACAACAGGCTGGCTCACT
TCCACAGGTAGCCACGCCTGCCGCCTGGGCCCGCTCAAAGAACAGCCTTTACCAAGACAG
CCAGCCAGCTAGAGCTAGCTTTAGCTAGCTGCTGCGGTTTCCCCCCCGATGAAGATGAAG
ATCAAGATCAAGATAAAGACGAGGAGAGCTACGGAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAGTACTCTGCGTTGATACCACTGCTT

Figure 4.6 Nucleotide sequence of the 3' end fragment of *HvGA2ox3* (268bp). The forward and reverse primers used for amplification of the PCR product are underlined.

The 5' end fragment for *HvGA2ox3* (~969 bp; Figure 4.3) was identified by amplifying the cDNA samples with universal primer and gene specific primer designed

from the 5' end of the EST "BU972476.1" (Table 4.1). For *HvGA2ox1*, BLAST searching the assembled sequences of EST "CB876549.1" and its identified 3' end fragment against the DFCI Barley Gene Index database revealed a contig designated as "TC244240" (Appendix 23; 24), as having the best hit. This contig was further BLAST searched against the DFCI Barley Gene Index database, and this resulted in EST "CA031963" as having a region overlapping to the "TC244240" (Appendix 25; 26). Assembly of the sequences of "TC244240" and EST "CA031963" (Appendix 27) produced a sequence containing the full length coding region of *HvGA2ox1* (Figure 4.7; Appendix 28).

GAGCCAACGCAATGGTGGTGCCCTCCACGACGCCGGCGCGGCAAGAGACGGCGACCCTGC
TTCTTCCACAGGCACAGCCGTCCCCCGGCGGCGGCGCCATACCGACGGTTGACATGTCGG
CGCCCCGCGGCCGCGGCGCGCTGTCCCGGCAGGTGGCGCGTGCGTGCGCGGAGCAGGGCT
TCTTCCGTGCCGTCAACCACGGCGTGCCTCCGGCGGGGCCCCCGGCACGGCTGGACGCGG
CCACCTCGGCGTTCTTCGCGCTCGCGGCACACGACAAGCAGCGCGCCGGCCCGCCGAGCC
CGCTCGGCTACGGCTGCCGCAGCATCGGGTTCAACGGCGACGTGGGCGAGCTGGAGTACC
TTCTCCTCCATGCCAACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGACACCAACG
ATCCCTCACGCTTCAGTTCTGTTGTGAATGAGTATGTGGAAGCAGTGAAGCAGCTTGCAT
GTGACATCCTGGACCTGTTAGGAGAAGGGCTAGGGCTAGAGGACCCCAGGCTCTTCAGCA
AGCTCGTCACAGAAGCTGACAGTGACTCCCTCCTGAGGATCAACCACTACCCTCCATCAT
GCACCGTTCACAAGCTTGACCATGATGACCAATGCAAGCTCAAGGGGATCGCCAGGACCA
AGGCAGGGAATGGTGCGAACCCGGGGGCGGGTGGCCGGATCGGGTTCGGTGAGCACTCCG
ACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGGTGCTTCTGC
CGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGGCCTATTTCG
TCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCATCCGGCACA
GAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAGCCCCACCAC
TGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCACGCCGGTACC
GGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAAGCCACAGCC
GCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAGGAAAATAAG
TCGACGTCG

Figure 4.7 Assembled sequences of “TC244240” and “CA031963” that generated the full length coding region of *HvGA2ox1*.

TCGATCGCACACACTTTACCATACTTGGTAGCAGCAGCCTCTGCAACCATGGTGGTTCTC
GCCGGCACGCCTGCCGTCGATCACATCCCGCTCCTCAGGTCCCCCGACCCCGGGGACAAC
TTCTCCGGCATGCCGGTCGTGGACCTGTCCAGACCTGGCGCGCCGCGGGCCATCGCCGAC
GCGTGCGAGCGCTTCGGCTTCTTCAAGCTCGTCAACCACGGGGTGGCCCTGGACGCGATG
GACCGGCTCGAGTCGGAGGCCGTGAGGTTCTTCTCGCTGCCGCAGGCCGACAAGGACCGC
TCCGGCCCGGCCTACCCGTTCGGCTACGGCAGCAAGCGCATCGGGCTCAATGGCGACATG
GGGTGGCTCGAGTACCTCCTCCTCGCCGTCGACTCCGCCTCGCTCCCCGCCGCCTCCGCC
GTCCCGTCCTGCGCGCTCTTCCGGGCGGCGCTGAACGAGTACATCGCGGCGGTGAGGAAG
GTGGCGGTGCGGGTGATGGAGGCGATGGCGGAGGGGCTGGGCATTGCGCAGGCGGACGCG
CTGAGCGCGATGGTGGCGGCGGAGGGCAGCGACCAGGTGTTCAGGGTGAACCACTACCCG
CCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGCGCCACCGGCTTCGGCGAGCACACCGAC
CCGCAGCTCATCTCCGTGCTGCGCTCCAACGGCACGTCCGGCCTGCAGATCGCGCTCCAG
AGCGGCCACTGGGTGTCCGTGCCCTCCGACCGCGACGCCTTCTTCGTCAACGTCGGCGAC
TCCTTGCAGGTGCTGACCAACGGGAGGTTCAAGAGCGTGAAGCACAGGGTGGTGGCCAAC
AGCCTAAAGTCTAGGGTTTCCATGATCTACTTTGGAGGGCCAGCATTGACACAGAGGATT
GCACCATTGCCGCAGCTGCTGAGAGAGGGAGAGCAGAGCCTGTACAAGGACTTCACATGG
GGCGAGTAC

Figure 4.8 Nucleotide sequence of the 5' end fragment of *HvGA2ox3* (969 bp). The forward and reverse primers used for amplification of the PCR product are underlined.

4.3.1.3 Sequence analysis

Assembling the identified 5' and 3' end sequences of *HvGA2ox3* with EST "BU972476.1" produced a 1226 bp (Appendix 30). BLAST searching the assembled 1226 bp sequence against the NCBI GenBank database and its alignment with *OsGA2ox3* showed that the fragment contained a complete coding region (Appendix 31). Furthermore, BLAST searching the sequence assembled from "TC244240" and 'CA031963' (Figure 4.7) against the NCBI GenBank database also contain a complete coding region, and its alignment with *OsGA2ox1* showed that the fragment contained a complete coding region (Appendix 28).

4.3.1.4 PCR assembly of *HvGA2ox1*

Three sets of gene specific primers were designed from the sequence assembled fromTC244240" and "CA031963" to perform the amplification of the full length cDNA of *HvGA2ox1*. First round PCR assembly for *HvGA2ox1* produced non-specific bands. The products of the first round PCR were diluted 50 times, and used as templates for the nested PCR amplification with HvGA2ox1-F3 and HvGA2ox1-R3 primers, which produced a strong band of about 1100 bp in size (Figure 4.9). The resulting band was gel-purified, cloned into the pGEM-T Easy vector, and then sequenced.

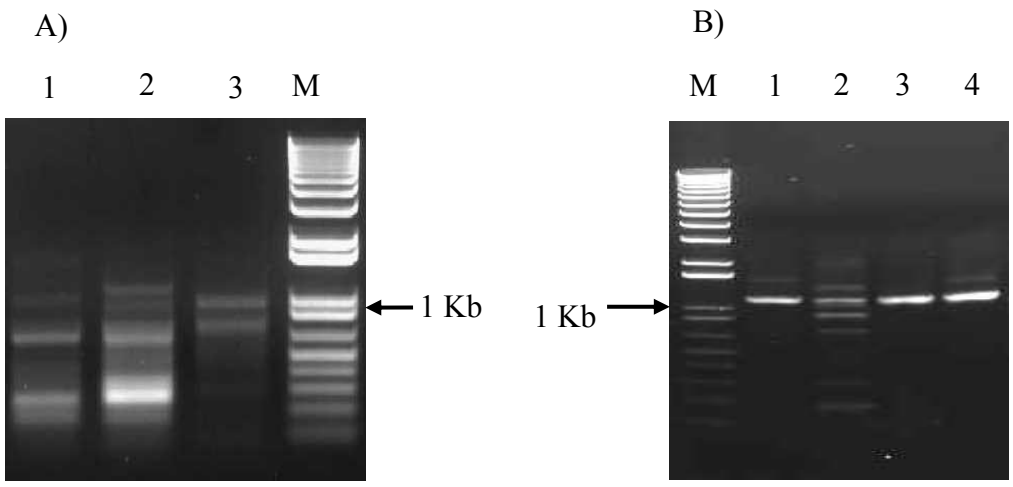


Figure 4.9 PCR products of the full length cDNA for the putative *HvGA2ox1* gene. First round PCR assembly (lanes 1-3; A). Nested PCR assembly (lanes 1-4; B). Lane M is the 1kb plus DNA ladder.

4.3.1.5 End to end PCR of *HvGA2ox1* and *HvGA2ox3*

End to end PCR of *HvGA2ox1* and *HvGA2ox3* were performed with the respective gene specific forward and reverse primers (HvGA2ox1-Full-F, HvGA2ox1-Full-R; HvGA2ox3-Full-F, HvGA2ox3-R; Table 4.2). The bands corresponding to expected sizes from the end to end PCR of *HvGA2ox1* and *HvGA2ox3* were gel-purified and cloned into the pGEM-T Easy vector and then sequenced as described previously. BLAST searching the coding sequence of *HvGA2ox1* against GenBank database exhibited that it has 83% identity with *GA2ox1* genes from rice and sorghum (Table 4.3).

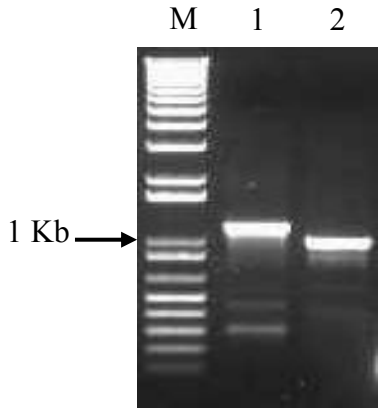


Figure 4.10 End-to-end PCR product for *HvGA2ox1* (lane 1) and *HvGA2ox3* (lane 2). Lane M is for DNA 1Kb plus ladder.

ATGGTGGTGCCCTCCACGACGCCGGCGCGGCAAGAGACGGCGACCCTGCTTCTTCCACAG
GCACAGCCGTCCCCCGGCGGCGGCGCCATACCGACGGTTGACATGTCGGCGCCCCGCGGC
CGCGGCGCGCTGTCCCGGCAGGTGGCGCGTGCGTGCGCGGAGCAGGGCTTCTTCCGTGCC
GTCAACCACGGCGTGCCTCCGGCGGGGCCCCCGGCACGGCTGGACGCGGCCACCTCGGCG
TTCTTCGCGCTCGCGGCACACGACAAGCAGCGCGCCGGCCCGCCGAGCCCGCTCGGCTAC
GGCTGCCGCAGCATCGGGTTCAACGGCGACGTGGGCGAGCTGGAGTACCTTCTCCTCCAT
GCCAACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGACACCAACGATCCCTCACGC
TTCAGTTCTGTTGTGAATGAGTATGTGGAAGCAGTGAAGCAGCTTGCATGTGACATCCTG
GACCTGTTAGGAGAAGGGCTAGGGCTAGAGGACCCCAGGCTCTTCAGCAAGCTCGTCACA
GAAGCTGACAGTGACTCCCTCCTGAGGATCAACCACTACCCTCCATCATGCACCGTTCAC
AAGCTTGACCATGATGACCAATGCAAGCTCAAGGGGATCGCCAGGACCAAGGCAGGGAAT
GGTGCGAACCCGGGGGCGGGTGGCCGGATCGGGTTCGGTGAGCACTCCGACCCACAGCTA
CTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGGTGCTTCTGCCGGACGTCAAT
GGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGGCCTATTTCGTCAATGTTGGT
GATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCATCCGGCACAGAGTAATTGCA
AGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAGCCCCACCACTGCATGCACGA
ATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCACGCCGGTACCGGTCATTCACC
TGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAAGCCACAGCCGCCTGGACCTC
TTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAGGAAAA**TAA**

Figure 4.11 Nucleotide sequence of the coding region of *HvGA2ox1*. The forward and reverse primers used for amplification of the PCR product are underlined.

Table 4.4 Nucleotide sequence identity between the putative full-length coding sequence of *HvGA2ox1* and *GA2ox*s from other species.

Accession#	Description	Max Score	E-value	Max Identity
AB059416.1	*OsGA2ox1*	966	0.0	83%
XM_002439387.1	*Sorghum bicolor* hypothetical protein	785	0.0	83%

ATGGTGGTTCTCGCCGGCACGCCTGCCGTCGATCACATCCCGCTCCTCAGGTCCCCCGAC
CCCGGGGACAACTTCTCCGGCATGCCGGTCGTGGACCTGTCCAGACCTGGCGCGCCGCGG
GCCATCGCCGACGCGTGCGAGCGCTTCGGCTTCTTCAAGCTCGTCAACCACGGGGTGGCC
CTGGACGCGATGGACCGGCTCGAGTCGGAGGCCGTGAGGTTCTTCTCGCTGCCGCAGGCC
GACAAGGACCGCTCCGGCCCGGCCTACCCGTTCGGCTACGGCAGCAAGCGCATCGGGCTC
AATGGCGACATGGGGTGGCTCGAGTACCTCCTCCTCGCCGTCGACTCCGCCTCGCTCCCC
GCCGCCTCCGCCGTCCCGTCCTGCGCGCTCTTCCGGGCGGCGCTGAACGAGTACATCGCG
GCGGTGAGGAAGGTGGCGGTGCGGGTGATGGAGGCGATGGCGGAGGGGCTGGGCATTGCG
CAGGCGGACGCGCTGAGCGCGATGGTGGCGGCGGAGGGCAGCGACCAGGTGTTCAGGGTG
AACCACTACCCGCCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGCGCCACCGGCTTCGGC
GAGCACACCGACCCGCAGCTCATCTCCGTGCTGCGCTCCAACGGCACGTCCGGCCTGCAG
ATCGCGCTCCAGAGCGGCCACTGGGTGTCCGTGCCCTCCGACCGCGACGCCTTCTTCGTC
AACGTCGGCGACTCCTTGCAGGTGCTGACCAACGGGAGGTTCAAGAGCGTGAAGCACAGG
GTGGTGGCCAACAGCCTAAAGTCTAGGGTTTCCATGATCTACTTTGGAGGGCCAGCATTG
ACACAGAGGATTGCACCATTGCCGCAGCTGCTGAGAGAGGGAGAGCAGAGCCTGTACAAG
GACTTCACATGGGGCGAGTACAAGAAGGCTGCCTACAACTCCAGGCTCGGGGACAACAGG
CTGGCTCACTTCCACAGG**TAG**

Figure 4.12 Nucleotide sequence of the coding region of *HvGA2ox3*. The forward and reverse primers used for amplification of the PCR product are underlined.

Table 4.5 Nucleotide sequence identity between the putative full-length coding sequence of *HvGA2ox3* and *GA2ox*s from other species.

Accession#	Gene	Max Score	E-value	Max Identity
AK364775.1	*Hordeum vulgare* mRNA for predicted protein	1807	0.0	99%
AB092485.1	*OsGA2ox3*	1267	0.0	90%
JQ250801.1	*TaGA2ox3*	638	3e-179	97%
AY551432.1	*HvGA2ox4*	568	4e-158	78%

BLAST searching the coding region of *HvGA2ox3* against a protein from GenBank database showed that it has 99% identity with the mRNA of *Hordeum vulgare*, 90%

identity with *GA2ox3* of rice (*OsGA2ox3*), 97% with wheat *GA2ox3* (*TaGA2ox3*), and 78% with barley *GA2ox4* (*HvGA2ox4*) (Table 4.4).

Translation of the putative cDNA sequence of *HvGA2ox1* and *HvGA2ox3* into their respective amino acid sequences with ExPAsy translator tool resulted in HvGA2ox1 and HvGA2ox3 proteins with 376 and 327 amino acids, respectively (Figure 4.13 and 4.14). BLAST search of these proteins against the GenBank database (http://www.ncbi.nlm.nih.gov/) showed that HvGA2ox1 exhibit 75% identity with ZmGA2ox2, 74% with OsGA2ox1 and 69% with OsGA2ox2. The HvGA2ox3 protein showed 99% identity with a protein from *Hordeum vulgare*, 86% identity with OsGA2ox3, 70% with ZmGA2ox1 and 68% with HvGA2ox4.

Furthermore, search for the conserved domains of these proteins demonstrated that both HvGA2ox1 and HvGA2ox3 belong to the 2OG-Fe (II) oxygenase superfamily, which contains the 2OG and Fe (II)-dependent oxygenase family members. Besides, HvGA2ox1 and HvGA2ox3 also contain non-heme dioxygenase in morphine synthesis N-terminal, which is the highly conserved N-terminal region of proteins with 2-OG/ Fe (II)-dependent dioxygenase activity (Figure 4.13 and 4.14).

A)

MVVPSTTPARQETATLLLPQAQPSPGGGAIPTVDMSAPRGRGALSRQVARACAEQGFFRA
VNHGVPPAGPPARLDAATSAFFALAAHDKQRAGPPSPLGYGCRSIGFNGDVGELEYLLLH
ANPAAVAHRASSIDTNDPSRFSSVVNEYVEAVKQLACDILDLLGEGLGLEDPRLFSKLVT
EADSDSLLRINHYPPSCTVHKLDHDDQCKLKGIARTKAGNGANPGAGGRIGFGEHSDPQL
LSLLRANDVDGLQVLLPDVNGKDAWIQVPADSSAYFVNVGDLLQALTNGRLVSIRHRVIA
SACRPRLSTIYFAAPPLHARISALPEMVTASSPRRYRSFTWAEYKTAMYSLRLSHSRLDL
FHVDDDESGNGSKGK

B)

Figure 4.13 Amino acid sequence of the putative HvGA2ox1 (A) and the domains conserved with other members of the 2OG-Fe (II) oxygenase superfamily (B).

A)

MVVLAGTPAVDHIPLLRSPDPGDNFSGMPVVDLSRPGAPRAIADACERFGFFKLVNHGVA
LDAMDRLESEAVRFFSLPQADKDRSGPAYPFGYGSKRIGLNGDMGWLEYLLLAVDSASLP
AASAVPSCALFRAALNEYIAAVRKVAVRVMEAMAEGLGIAQADALSAMVAAEGSDQVFRV
NHYPPCHALQGLGCSATGFGEHTDPQLISVLRSNGTSGLQIALQSGHWVSVPSDRDAFFV
NVGDSLQVLTNGRFKSVKHRVVANSLKSRVSMIYFGGPALTQRIAPLPQLLREGEQSLYK
DFTWGEYKKAAYNSRLGDNRLAHFHR

B)

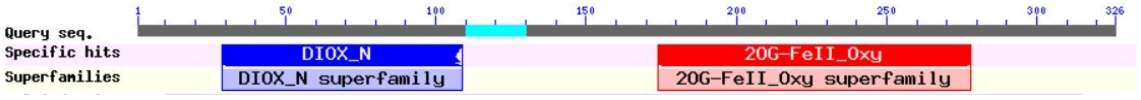


Figure 4.14 Amino acid sequence of the putative HvGA2ox3 (A) and the domains conserved with other members of the 2OG-Fe (II) oxygenase superfamily (B).

4.3.2 Phylogeny of HvGA2ox1 and HvGA2ox3

Phylogenetic analysis of the amino acid sequences of 26 known monocot or dicot GA2ox proteins using the Molecular Evolutionary Genetic Analysis (MEGA, version 5; http://www.megasoftware.net/) software showed that HvGA2ox1 and HvGA2ox3 are closely related to OsGA2ox1 and OsGA2ox3, respectively.

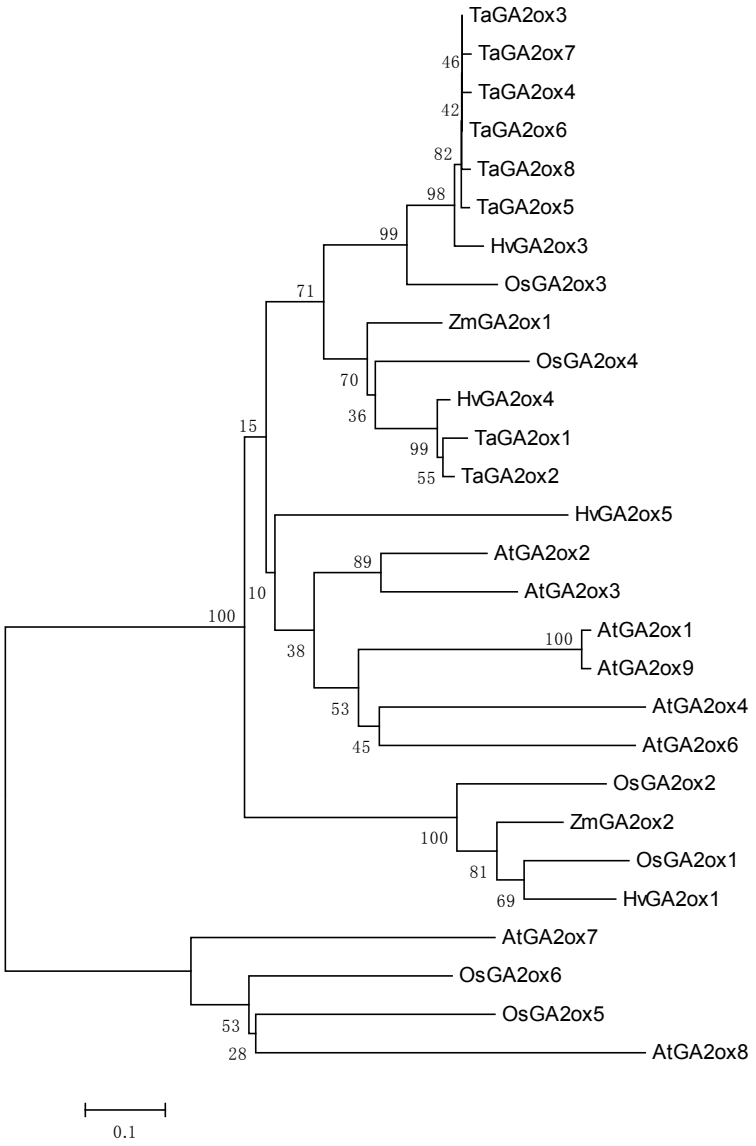


Figure 4.15 Phylogenetic analyses of GA2ox proteins. The amino acid sequences of 26 GA2oxes were aligned as described in the material and methods. The evolutionary history was generated using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distance was computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 28 amino acid sequences. The aligned protein sequences with their accession numbers are *Triticum aestivum*, TaGA2ox1, AEA30106.1; TaGA2ox2, AEA30107.1; TaGA2ox3, AEA30108.1; TaGA2ox4, AEA30109.1; TaGA2ox5, AEA30110.1; TaGA2ox6, AEA30111.1; TaGA2ox7, AEA30112.1; TaGA2ox8, AEA30113.1; *Hordeum vulgare*, HvGA2ox4, AAT49062.1; HvGA2ox5, AAT49063.1; *Oryza sativa*, OsGA2ox1, BAB40934.1; OsGA2ox2, BAC16751.1; OsGA2ox3, BAC16752.1; OsGA2ox4, NP_001056036.1; OsGA2ox5, NP_001058690.1; OsGA2ox6, BAF15255.1; *Arabidopsis thaliana*, AtGA2ox1, AEE36106.1; AtGA2ox2, AEE31171.1; AtGA2ox3, AEC08989.1; AtGA2ox4, AAT49062.1; AtGA2ox6, AEE27425.1; AtGA2ox7, AEE32606.1; AtGA2ox8, AEE84422.1; AtGA2ox9, AAM62763.1; *Zea mays*, ZmGA2ox1, NP_001148268.1; ZmGA2ox2, NP_001152057.1.

4.3.3 Recombinant Protein Expression

The molecular weights of HvGA2ox1 and HvGA2ox3 proteins were predicted by using ExPASy ProtParam tool to be 40041.2 Da and 35159.1 Da, respectively. Amplification of the coding sequences of *HvGA2ox1* and *HvGA2ox3* with gene specific forward and reverse primers engineered to possess the specific restriction sites produced DNA fragments of expected size. Transformation of the cDNA sequences subcloned first into the pGEMT-Easy vector and then into the expression vector pET32a with *E. coli* cells produced colonies. Digestion of the purified recombinant pGEMT-Easy-*HvGA2ox1*, pGEMT-Easy-*HvGA2ox3* and pET32a plasmids with the respective restriction enzymes corresponding to restriction sites introduced into the amplifying primers produced two

fragments, one for the insert and the other one for the vector (Figure 4.16). The fragments with the expected size of *HvGA2ox1* and *HvGA2ox3* fragments were ligated into pET32a vector digested with the respective restriction enzymes. Sequencing of pET32a-*HvGA2ox1* and pET32a-*HvGA2ox3* revealed that ~ 495 bp in the pET32a vector is fused with target gene and formed a recombinant protein (Figure 4.17).

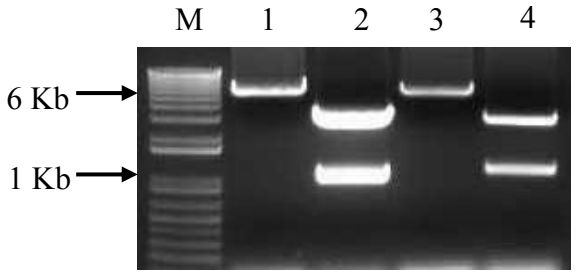


Figure 4.16 Double digestion products of pET32a-*HvGA2ox1* and pET32a-*HvGA2ox3* constructs; pET32a and pGEMT-Easy-*HvGA2ox3* digested with *BamHI* and *HindIII* (lanes 1-2), pET32a and pGEMT-Easy-*HvGA2ox1* digested with *BamHI* and *SalI* (lanes 3-4). Lane M is for DNA 1Kb Plus ladder.

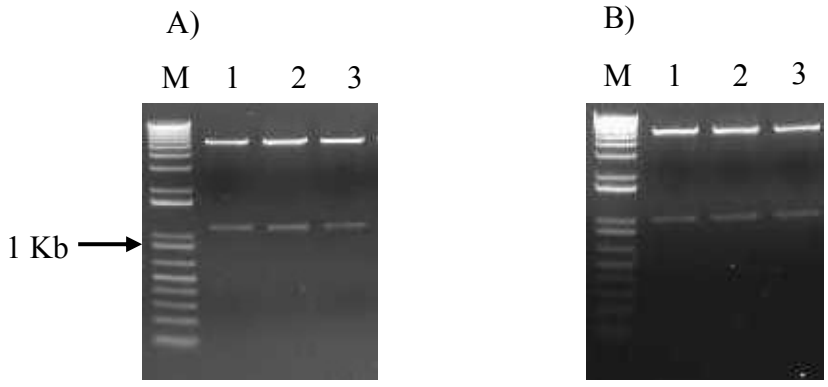


Figure 4.17 Double digestion products of recombinant constructs of pET32a-*HvGA2ox1* (A) and pET32a-*HvGA2ox3* (B).

ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAAGCG
GACGGGGCGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCC
CCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACTGACCGTTGCAAAACTGAAC
ATCGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGACTCTGCTG
CTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCACTGTCTAAAGGTCAGTTG
AAAGAGTTCCTCGACGCTAACCTGGCCGGTTCTGGTTCTGGCCATATGCACCATCATCAT
CATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAAACCGCTGCTGCTAAA
TTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGACGACGACGACAAGGCCATG
GCTGATATCGGATCCATGGTGGTGCCCTCCACGACGCCGGCGCGGCAAGAGACGGCGACC
CTGCTTCTTCCACAGGCACAGCCGTCCCCCGGCGGCGGCGCCATACCGACGGTTGACATG
TCGGCGCCCCGCGGCCGCGGCGCGCTGTCCCGGCAGGTGGCGCGTGCGTGCGCGGAGCAG
GGCTTCTTCCGTGCCGTCAACCACGGCGTGCCTCCGGCGGGGCCCCCGGCACGGCTGGAC
GCGGCCACCTCGGCGTTCTTCGCGCTCGCGGCACACGACAAGCAGCGCGCCGGCCCGCCG
AGCCCGCTCGGCTACGGCTGCCGCAGCATCGGGTTCAACGGCGACGTGGGCGAGCTGGAG
TACCTCCTCCTCCATGCCAACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGACACC
AACGATCCCTCACGCTTCAGTTCTGTTGTGAATGAGTATGTGGAAGCAGTGAAGCAGCTT
GCATGTGACATCCTGGACCTGTTAGGAGAAGGGCTAGGGCTAGAGGACCCCAGGCTCTTC
AGCAAGCTCGTCACAGAAGCTGACAGTGACTCCCTCCTGAGGATCAACCACTACCCTCCA
TCATGCACCGTTCACAAGCTTGACCATGATGACCAATGCAAGCTCAAGGGGATCGCCAGG
ACCAAGGCAGGGAATGGTGCGAACCCGGGGGCGGGTGGCCGGATCGGGTTCGGTGAGCAC
TCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGGTGCTT
CTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGGCCTAT
TTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCATCCGG
CACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAGCCCCA
CCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCACGCCGG
TACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAAGCCAC
AGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAGGAAAA
TAA

Figure 4.18 Nucleotide sequence of pET32a-*HvGA2ox1*. The underlined nucleotide sequence is from pET32a vector, which form the recombinant pET32a-HvGA2ox1 protein along with the cDNA of *HvGA2ox1*.

ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAAGCG
GACGGGGCGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCC
CCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACTGACCGTTGCAAAACTGAAC
ATCGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTATCCCGACTCTGCTG
CTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCACTGTCTAAAGGTCAGTTG
AAAGAGTTCCTCGACGCTAACCTGGCCGGTTCTGGTTCTGGCCATATGCACCATCATCAT
CATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAAACCGCTGCTGCTAAA
TTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGACGACGACGACAAGGCCATG
GCTGATATCGGATCCATGGTGGTTCTCGCCGGCACGCCTGCCGTCGATCACATCCCGCTC
CTCAGGTCCCCCGACCCCGGGGACAACTTCTCCGGCATGCCGGTCGTGGACCTGTCCAGA
CCTGGCGCGCCGCGGGCCATCGCCGACGCGTGCGAGCGCTTCGGCTTCTTCAAGCTCGTC
AACCACGGGGTGGCCCTGGACGCGATGGACCGGCTCGAGTCGGAGGCCGTGAGGTTCTTC
TCGCTGCCGCAGGCCGACAAGGACCGCTCCGGCCCGGCCTACCCGTTCGGCTACGGCAGC
AAGCGCATCGGGCTCAATGGCGACATGGGGTGGCTCGAGTACCTCCTCCTCGCCGTCGAC
TCCGCCTCGCTCCCCGCCGCCTCCGCCGTCCCGTCCTGCGCGCTCTTCCGGGCGGCGCTG
AACGAGTACATCGCGGCGGTGAGGAAGGTGGCGGTGCGGGTGATGGAGGCGATGGCGGAG
GGGCTGGGCATTGCGCAGGCGGACGCGCTGAGCGCGATGGTGGCGGCGGAGGGCAGCGAC
CAGGTGTTCAGGGTGAACCACTACCCGCCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGC
GCCACCGGCTTCGGCGAGCACACCGACCCGCAGCTCATCTCCGTGCTGCGCTCCAACGGC
ACGTCCGGCCTGCAGATCGCGCTCCAGAGCGGCCACTGGGTGTCCGTGCCCTCCGACCGC
GACGCCTTCTTCGTCAACGTCGGCGACTCCTTGCAGGTGCTGACCAACGGGAGGTTCAAG
AGCGTGAAGCACAGGGTGGTGGCCAACAGCCTAAAGTCTAGGGTTTCCATGATCTACTTT
GGAGGGCCAGCATTGACACAGAGGATTGCACCATTGCCGCAGCTGCTGAGAGAGGGAGAG
CAGAGCCTGTACAAGGACTTCACATGGGGCGAGTACAAGAAGGCTGCCTACAACTCCAGG
CTCGGGGACAACAGGCTGGCTCACTTCCACAGGTAG

Figure 4.19 Nucleotide sequence of pET32a-*HvGA2ox3*. The underlined nucleotide sequence is from pET32a vector, which form the recombinant pET32a-HvGA2ox3 protein along with the cDNA of *HvGA2ox3*.

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLN
IDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHH
HHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSMVVPSTTPARQETAT
LLLPQAQPSPGGGAIPTVDMSAPRGRGALSRQVARACAEQGFFRAVNHGVPPAGPPARLD
AATSAFFALAAHDKQRAGPPSPLGYGCRSIGFNGDVGELEYLLLHANPAAVAHRASSIDT
NDPSRFSSVVNEYVEAVKQLACDILDLLGEGLGLEDPRLFSKLVTEADSDSLLRINHYPP
SCTVHKLDHDDQCKLKGIARTKAGNGANPGAGGRIGFGEHSDPQLLSLLRANDVDGLQVL
LPDVNGKDAWIQVPADSSAYFVNVGDLLQALTNGRLVSIRHRVIASACRPRLSTIYFAAP
PLHARISALPEMVTASSPRRYRSFTWAEYKTAMYSLRLSHSRLDLFHVDDDESGNGSKGK

Figure 4.20 Predicted amino acid sequence of the pET32a-HvGA2ox1 recombinant protein. Portion of the recombinant protein derived from the pET32a vector is underlined.

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLN
IDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHH
HHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSMVVLAGTPAVDHIPL
LRSPDPGDNFSGMPVVDLSRPGAPRAIADACERFGFFKLVNHGVALDAMDRLESEAVRFF
SLPQADKDRSGPAYPFGYGSKRIGLNGDMGWLEYLLLAVDSASLPAASAVPSCALFRAAL
NEYIAAVRKVAVRVMEAMAEGLGIAQADALSAMVAAEGSDQVFRVNHYPPCHALQGLGCS
ATGFGEHTDPQLISVLRSNGTSGLQIALQSGHWVSVPSDRDAFFVNVGDSLQVLTNGRFK
SVKHRVVANSLKSRVSMIYFGGPALTQRIAPLPQLLREGEQSLYKDFTWGEYKKAAYNSR
LGDNRLAHFHR

Figure 4.21 Predicted amino acid sequence of the pET32a-HvGA2ox3 recombinant protein. Portion of the recombinant protein derived from the pET32a vector is underlined.

4.3.4 SDS-PAGE analysis

Transformation of the pET32a-*HvGA2ox1* and pET32a-*HvGA2ox3* constructs with *E.coli* (BL21) produced colonies. Isolation of the respective recombinant pET32a-HvGA2ox1 and pET32a-HvGA2ox3 proteins from the corresponding positive colonies followed by the SDS-PAGE analysis produced fusion proteins of pET32a-HvGA2ox1 and pET32a-

HvGA2ox3 with ~57 kDa and ~52 kDa molecular weight (Figure 4.22), respectively, as predicted by the ExPASy ProtParam protein molecular weight calculator tool.

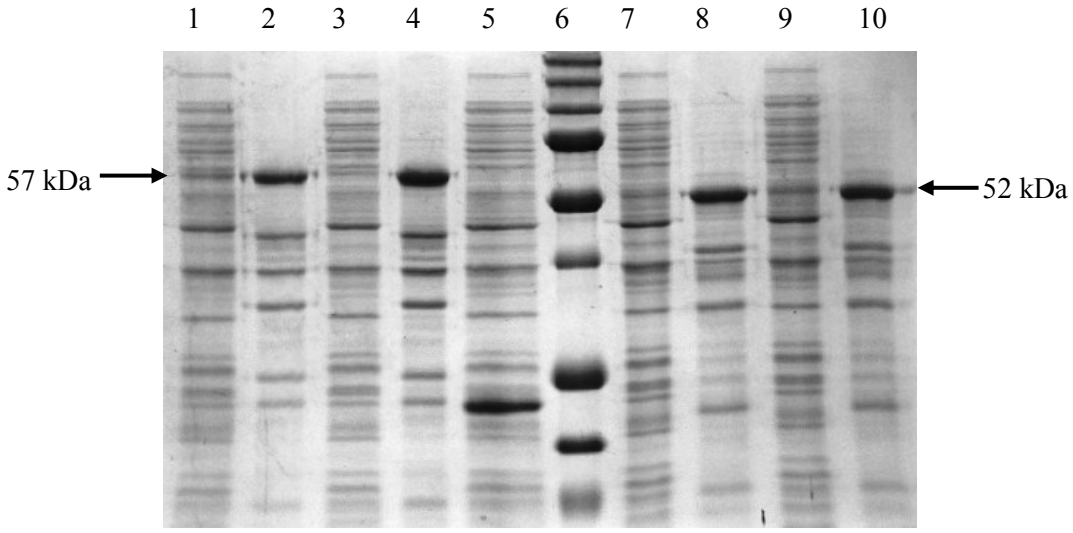


Figure 4.22 SDS-PAGE analysis of the crude extracts of pET32a-HvGA2ox1 and pET32a-HvGA2ox3 recombinant proteins. Crude extracts from pET32a-HvGA2ox1 (lanes 1-4) induced at 28 °C for 4 h with 1 mM IPTG (lanes 1 and 2) and 0.5 mM IPTG (lane 3 and 4); and pET32a-HvGA2ox3 (lanes 7-10) induced at 28 °C for 4 hours with 1 mM IPTG (lane 7-8) and 0.5 mM IPTG (lanes 9-10). Lane 5 is for crude extract derived from empty pET32a vector induced at 28°C for 4 h with 1 mM IPTG. Lane 6 is for a protein marker.

4.4 Discussion

The endogenous level of GA is controlled by its biosynthesis and inactivation (Yamaguchi *et al*., 2008). The most universal GA inactivation reaction is 2β-hydroxylation, which is catalyzed by a class of 2ODDs, GA 2-oxidases (GA2oxs). In barley, only two *GA2ox*s designated as *HvGA2ox4* and *HvGA2ox5* have been identified and characterized (Spielmeyer *et al*., 2004). This chapter of the thesis reports the identification and characterization of cDNAs of two new barley *GA2ox*s, designated as *HvGA2ox1* and *HvGA2ox3*. The *HvGA2ox1* has an ORF of 1128 bp (Figure 4.11), whereas the *HvGA2ox3* an ORF of 981 bp (Figure 4.12), which encode proteins with 376 and 327 amino acids, respectively (Figures 4.13 and 4.14). The molecular mass of *HvGA2ox1* and *HvGA2ox3* correspond to 57 kDa and 52 kDa, respectively. BLAST search of the GenBank database against the nucleotide sequence of the *HvGA2ox1* indicated that it has 83% sequence identity with *GA2ox*s identified from rice and sorghum. The predicted amino acid sequence of HvGA2ox1 showed high similarity with GA2ox proteins derived from other cereals crops including rice (OsGA2ox1; 74% and OsGA2ox2; 69%) and maize (ZmGA2ox1; 75%). The nucleotide sequence of *HvGA2ox3* has high sequence identity with those from rice (*OsGA2ox3*; 90%), wheat (*TaGA2ox3*; 97%) and barley (*HvGA2ox4*; 78%), while its predicted amino acid sequence showed high similarity with those from rice (OsGA2ox3; 86%), maize (ZmGA2ox1; 70%) and barley (protein from barley, 99%; HvGA2ox4; 68%). Analysis of the predicted amino

acid sequences of *HvGA2ox1* and *HvGA2ox3* revealed that their corresponding proteins belong to 2OG-Fe (II) oxygenase superfamily. These 2-oxoglutarate-dependent dioxygenases (2-ODDs) belong to a family of non-heme Fe-containing enzymes. Enzymes in this group catalyze a range of reactions including hydroxylation, desaturation and epoxidation. These enzymes require ferrous iron and a reducing agent for their maximal activity *in vitro*, and utilize 2-oxoglutarate and molecular oxygen as co-substrates (Prescott, 1993). The initial step of the reaction mechanism of 2-ODDs involves the generation of a ferryl oxidant formed by the binding of molecular oxygen to the iron cofactor, and the formation of the oxoferryl species are linked to the oxidative decarboxylation of 2-oxoglutarate to succinate (Blanchard and Englard, 1983). The reaction proceeds by taking a hydrogen atom from the substrate via the ferryl ion, forming a ferric-hydroxyl complex and a carbon-centered structure. Enzymes from this group also catalyze uncoupled reactions, during which 2-oxoglutarate is decarboxylated without subsequent hydroxylation of the substrate; ascorbate is required as an alternative oxygen acceptor in these cases (Myllylä *et al.,* 1984).

Generation of a phylogenetic tree on the basis of amino acid sequence of other known HvGA2oxs (Figure 4.15) showed that *HvGA2ox1* is closely related to *OsGA2ox1*, whereas *HvGA2ox3* to *OsGA2ox3*. Previous studies have shown that OsGA2ox1 belongs to class II of GA2ox proteins while OsGA2ox3 belongs to class I of GA2ox proteins (Lee and Zeevaart, 2005). Phylogenetic analysis along with the previously reported GA2ox

classification suggests that HvGA2ox3 belongs to class II, while HvGA2ox1 to class I. These results suggest the functionality of *HvGA2ox1* and *HvGA2ox3* as GA deactivating genes involved in the deactivation of bioactive GAs and their precursors.

Semi-dwarfism is a desirable trait in crop agriculture, as the semi-dwarf cultivars are more resistant to damage by wind and rain, and also are associated with stable increased yield (Evans, 1993; Hedden, 2003). Besides, dwarf and semidwarf varieties are also preferred in fruit trees, as they allow dense field cultivation, and facilitate maintenance and fruit collection (Busov *et al*., 2003). It has been well demonstrated that dwarfism or semi-dwarfism is associated with deficiencies in GA levels or signaling. Dwarf and semidwarf "Green Revolution" varieties in wheat and rice that contributed to the dramatic increase in cereal crop yield are resulted from mutations in *DELLA*, component of GA signaling, and *GA20ox* genes, respectively (Peng *et al*., 1999; Spielmeyer *et al*., 2002). Since the level of bioactive GAs is regulated by its biosynthesis and catabolism, suppression of its biosynthesis and/or enhancing of its deactivation through increasing the copy number GA deactivating genes such as *GA2ox*s are effective in reducing GA content and elongation growth. For example, ectopic expression of runner bean (*Phaseolus coccineus* L.) *GA2ox* gene (*PcGA2ox1*) in wheat decreased the level of bioactive GA in the transgenic plants and produced a range of dwarf plants with different degrees of severity (Appleford *et al*., 2007). Expressing *PcGA2ox1* in *Solanum*

melanocerasum and *S. nigrum* through *Agrobacterium*-mediated transformation produced transgenic plants with reduced level of GA and dwarf phenotype (Dijkstra *et al*., 2008).

On the other hand, fiber, pulp, wood and biomass product industries prefer to enhance plant height and growth rate, which can be achieved by increasing GA biosynthesis or decreasing its deactivation via suppressing *GA2ox* genes. For example, Dayan *et al*. (2010) reported that silencing *GA2ox* gene in tobacco results in a dramatic increase of growth and fiber production, when compared to the control (wild type) or plants over-expressing the *GA20ox* gene, indicating that silencing of a GA catabolic gene is more effective than over-expressing a GA biosynthetic gene in enhancing the wood and fiber yield. It can be concluded from previous studies that the *GA2ox* genes can be applied to regulate the endogenous bioactive GA level, and in turn to improve crop productivity. Therefore, the identification of two new barley GA deactivating genes (*HvGA2ox1* and *HvGA2ox3*) provides genetic tools for manipulating the GA level and enhancing yield in barley.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Seed dormancy is an adaptive trait developed through evolution enabling the seeds of many species to remain quiescent until conditions become favorable for germination. In important cereal crops such as barley, a certain degree of dormancy at harvest is a desired trait. The lack of seed dormancy at harvest stage in many crops including barley leads to the sprouting of seeds while they are on the mother plant and this causes significant economic loss. On the other hand, the presence of too much dormancy in barley seeds affects rapid and uniform germination after replanting, and thus not acceptable by producers. The goal to achieve optimal dormancy level requires a thorough knowledge of the mechanisms determining dormancy maintenance and release in the barley seeds.

Seed dormancy is regulated by complex interactions between environmental and genetic factors, and previous studies have demonstrated the essential roles of gibberellin (GA) and abscisic acid (ABA) in regulating seed dormancy and germination. High ABA to GA ratio is important for dormancy maintenance, whereas low ABA to GA ratio for dormancy release. In this thesis project, the expression patterns of ten GA metabolic and four ABA metabolic genes were investigated during both seed developmental and germination processes. Only seven of the GA metabolic genes and three of the ABA metabolic genes were detected during seed development, suggesting the importance of these genes during seed development. The detection of high expressions of the GA synthesis genes *HvGA20ox3* and *HvGA3ox1* at the earlier stages of seed development

indicates the requirement of GA for normal seed development, and the increased expression of GA deactivating gene *HvGA2ox5* during the later stages suggests the need for a decrease in GA level for induction of seed dormancy. The increased expression of the ABA biosynthetic genes *HvNCED1* and *HvNCED2* observed during seed development implies increased synthesis of ABA, which is often associated with seed dormancy and accumulation of storage reserves.

The study showed that dormancy in mature cv. Betzes seeds can be broken by after-ripening, the dry storage of seeds, and this process is accompanied by changes in the expression of GA and ABA metabolic genes. Following seed imbibition, the expression levels exhibited by the GA biosynthetic genes, *HvGA20ox1* and *HvGA3ox2* were higher in after-ripened (AR) than dormant (D) seeds. On the other hand, the expressions of GA deactivating genes, *HvGA2ox1*, *HvGA2ox3*, *HvGA2ox4* and *HvGA2ox6* were repressed in imbibing AR seeds. These results suggest that after-ripening promotes germination of AR seeds by enhancing GA synthesis and inhibiting its catabolism during imbibition. With respect to the ABA metabolism genes, the expression was *HvNCED2* gene was decreased, whereas that *HvCYP707A1* significantly increased in imbibing AR seeds. These results suggest after-ripening results in decreased ABA level during imbibition of AR seeds. The results of this study also suggest that treatment of imbibing AR seeds with ABA inhibits their germination mainly by decreasing GA production through repressing the expression of GA biosynthesis genes, mainly *HvGA20ox1* and *HvGA3ox1*.

This study, furthermore, identified two putative GA deactivating genes from barley, designated as *HvGA2ox1* and *HvGA2ox3*. The full length cDNA sequences of the *HvGA2ox1* and *HvGA2ox3* are 1437 bp and 1226 bp with open reading frames of 1128 bp and 981 bp encoding amino acids 376 and 327, respectively. BLAST searching the nucleotide and amino acid sequence of the two genes against GenBank database showed that the two genes have similarity with other *GA2ox* genes in rice and Arabidopsis. Phylogenetic analysis of the two genes indicated that that they are closely related to the previously identified *OsGA2ox1* and *OsGA2ox3*. In vitro expression of *HvGA2ox1* and *HvGA2ox3* in *E.coli* produced proteins with predicted size. Further research is needed to verify their functionality in catabolizing GAs. In summary, the results of this study contributes to further our understanding of the roles of GA and ABA in regulating seed development, onset and release of seed dormancy, and germination of cereal seeds, which is important knowledge for the development of preharvest sprouting resistant varieties.

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APPENDIX

Appendix 1 Analysis of variance of the relative transcript abundance of *HvGA20ox1* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0067	0.6779	A
	AR	2.5367	0.6779	A
12h	D	86.5254	11.0555	A
	AR	35.8967	9.5338	A
	AR+ABA	31.0023	11.0555	A
24h	D	40.77	30.0809	B
	AR	338.49	30.0809	A
	AR+ABA	77.145	30.0809	B
30h	D	142.59	25.3286	A
	AR	127.09	25.3286	A
	AR+ABA	129.28	25.3286	A

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 2 Analysis of variance of the relative transcript abundance of *HvGA20ox2* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0767	0.2394	A
	AR	0.9433	0.2394	A
12h	D	4.9961	0.8629	A
	AR	3.1967	0.7082	A
	AR+ABA	2.2267	0.7082	A
24h	D	0.69	0.1345	A
	AR	0.4467	0.1345	A
	AR+ABA	0.3867	0.1345	A
30h	D	0.57	0.02177	A
	AR	0.1867	0.02177	B
	AR+ABA	0.2267	0.02177	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 3 Analysis of variance of the relative transcript abundance of *HvGA20ox3* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.02	0.1466	A
	AR	1.2767	0.1466	A
12h	D	0.14	0.0893	A
	AR	0.6667	0.0893	B
	AR+ABA	0.4467	0.0893	AB
24h	D	0.69	0.1345	A
	AR	0.4467	0.1345	A
	AR+ABA	0.3867	0.1345	A
30h	D	0.57	0.57	A
	AR	0.1867	0.1867	B
	AR+ABA	0.2267	0.2267	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 4 Analysis of variance of the relative transcript abundance of *HvGA3ox1* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.1033	0.2543	A
	AR	2.2688	0.3109	A
12h	D	0.31	0.05927	A
	AR	1.2621	0.06635	B
	AR+ABA	0.6433	0.05927	C
24h	D	0.5433	0.08642	A
	AR	0.9032	0.1008	AB
	AR+ABA	0.9467	0.08642	B
30h	D	0.75	0.1549	AB
	AR	0.2967	0.1264	A
	AR+ABA	1.63	0.1549	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 5 Analysis of variance of the relative transcript abundance of *HvGA3ox2* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0067	0.6779	A
	AR	2.5367	0.6779	A
12h	D	8.9733	12.0599	A
	AR	79.62	12.0599	B
	AR+ABA	48.6533	12.0599	AB
24h	D	0.5549	70.1088	A
	AR	383.57	57.29	B
	AR+ABA	225.23	57.29	AB
30h	D	8.79	18.8788	A
	AR	104.49	18.8788	B
	AR+ABA	95.5633	18.8788	AB

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 6 Analysis of variance of the relative transcript abundance of *HvGA2ox1* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0067	0.3819	A
	AR	2.3167	0.3819	A
12h	D	11.3951	0.6005	A
	AR	1.8667	0.5381	B
	AR+ABA	1.2567	0.5381	B
24h	D	4.0367	0.7865	A
	AR	2.2241	0.9173	A
	AR+ABA	5.2141	0.9173	A
30h	D	14.8222	1.0062	A
	AR	4.4067	0.8388	B
	AR+ABA	5.42	0.8388	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 7 Analysis of variance of the relative transcript abundance of *HvGA2ox3* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0033	0.7752	A
	AR	3.6267	0.7752	A
12h	D	31.3633	20.9448	A
	AR	226.36	20.9448	B
	AR+ABA	197.32	20.9448	B
24h	D	355.58	18.9326	A
	AR	196.67	23.1876	A
	AR+ABA	190.05	23.1876	A
30h	D	295.02	19.8645	A
	AR	72.81	19.8645	B
	AR+ABA	85.49	24.329	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 8 Analysis of variance of the relative transcript abundance of *HvGA2ox4* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1	0.8466	A
	AR	2.1167	0.8466	A
12h	D	24.795	2.3328	A
	AR	0.44	1.9047	B
	AR+ABA	0.68	1.9047	B
24h	D	0.3133	0.03892	A
	AR	0.1613	0.0425	A
	AR+ABA	0.3893	0.05014	A
30h	D	0.9252	0.2053	A
	AR	0.2667	0.1676	A
	AR+ABA	0.2967	0.1676	A

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 9 Analysis of variance of the relative transcript abundance of *HvGA2ox5* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0033	0.12	A
	AR	1.37	0.12	A
12h	D	0.1533	0.1301	A
	AR	0.69	0.1301	A
	AR+ABA	0.7133	0.1301	A
24h	D	0.6033	0.06409	A
	AR	0.1767	0.06409	B
	AR+ABA	0.42	0.06409	AB
30h	D	0.3667	0.06155	A
	AR	0.2933	0.06155	A
	AR+ABA	0.3033	0.06155	A

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 10 Analysis of variance of the relative transcript abundance of *HvGA2ox6* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0033	0.1225	A
	AR	1.3067	0.1255	A
12h	D	71.9364	4.4709	A
	AR	31.2933	3.6833	B
	AR+ABA	30.4867	3.6833	B
24h	D	34.3667	3.1243	A
	AR	4.0367	3.1243	B
	AR+ABA	3.29	3.8264	B
30h	D	38.38	2.8191	A
	AR	1.1967	2.8191	B
	AR+ABA	2.6633	2.8191	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 11 Analysis of variance of the relative transcript abundance of *HvNCED1* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	1.0833	0.2771	A
	AR	1.6867	0.2711	A
12h	D	6.522	0.7534	A
	AR	5.742	0.7534	A
	AR+ABA	2.1533	0.7309	B
24h	D	1.4067	0.3796	A
	AR	2.74	0.3796	AB
	AR+ABA	5.1651	0.4647	B
30h	D	1.0633	0.4588	A
	AR	0.13	0.4588	A
	AR+ABA	1.8967	0.4588	A

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 12 Analysis of variance of the relative transcript abundance of *HvNCED2* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	45.7633	5.7776	A
	AR	33.0652	6.7066	A
12h	D	97.785	7.1245	A
	AR	21.6	5.8171	B
	AR+ABA	18.805	7.1245	B
24h	D	NA	NA	NA
	AR	4.485	0.4485	A
	AR+ABA	2.005	0.4485	A
30h	D	NA	NA	NA
	AR	1.175	0.1188	A
	AR+ABA	1.01	0.1188	A

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 13 Analysis of variance of the relative transcript abundance of *HvCYP707A1* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	2.9133	0.5512	A
	AR	2.0867	0.5512	A
12h	D	1.3567	0.4432	A
	AR	19.0465	0.5416	B
	AR+ABA	3.0615	0.5416	A
24h	D	4.7267	1.475	A
	AR	14.664	1.8063	B
	AR+ABA	10.1367	1.475	AB
30h	D	5.1667	1.9651	AB
	AR	1.7933	1.9651	A
	AR+ABA	14.8965	2.4012	B

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 14 Analysis of variance of the relative transcript abundance of *HvCYP707A1* between D, AR and AR+ABA seeds at different stages of imbibition.

Hour after imbibition	Sample	Estimate	Error	Group
0h	D	NA	NA	NA
	AR	NA	NA	NA
12h	D	NA	NA	NA
	AR	0.8067	0.1453	A
	AR+ABA	1.7235	0.1771	A
24h	D	NA	NA	NA
	AR	1.9167	0.2605	A
	AR+ABA	1.49	0.2605	A
30h	D	NA	NA	NA
	AR	1.1033	0.2984	A
	AR+ABA	0.9733	0.2984	A

Different letters indicate significant difference among different samples using Tukey's LSD test, $p<0.05$.

Appendix 15 *Oryza sativa* mRNA for *OsGA2ox1*, complete cds (GenBank ID: AB059416.1)

ACGAGCCATTCCGGCCGCGCATTCTCCCGCTCTCGATCGATCGATCGATCATGGTGGTGC
CTTCCGCGACGACGCCAGCGAGGCAGGAGACGGTGGTGGCGGCGGCGCCGCCAGCTGCGG
CGGCGTCCGGTGTCGTCGGCGGCGGCGGCGGCGTGACGATAGCGACGGTGGACATGTCGG
CGGAGCGCGGCGCGGTGGCGAGGCAGGTGGCGACGGCGTGCGCGGCGCACGGGTTCTTCC
GGTGCGTCGGGCACGGCGTGCCGGCGGCGGCGCCCGTCGCGGCGAGGCTGGACGCCGCGA
CGGCGGCGTTCTTCGCGATGGCGCCGGCGGAGAAGCAGCGCGCCGGGCCGGCGAGCCCGC
TCGGGTACGGCTGCCGGAGCATCGGGTTCAACGGCGACGTCGGCGAGCTGGAGTACCTGC
TCCTCCACGCCAACCCCGCCGCCGTCGCGCACCGGGCCAGGACCATCGACGCCATGGACC
CCTCTCGCTTCAGTGCTATTGTGAATGAGTACATTGAAGCCATGAAGAAGCTCGCATGTG
AGATCCTGGACCTGTTAGGAGAGGGGCTAGGTCTCAAGGACCCCAGATACTTCAGCAAGC
TTACCACAAACGCTGACAGTGACTGCCTCCTGAGGATCAACCACTACCCTCCATCATGCA
ACATTCACAAACTTGACCATGATGACCAATGCAATATCAAGAGCCTTGTTAGCACCAAGG
CTAGCAATGGTGGGAATCTGATGGCAGGTGGGCGCATTGGGTTCGGCGAGCACTCTGACC
CGCAGATCCTTAGCTTGCTCCGAGCAAACGATGTGGAAGGGCTACAGGTGTTTGTGCCGG
ACCACGAGGGCAAGGAGATGTGGGTTCAGGTGCCATCGGACCCATCGGCCATTTTCGTCA
ATGTTGGTGATGTCCTCCAGGCTCTGACAAATGGGAGGCTGATAAGTATCCGGCACAGGG
TAATTGCAACCGCCTGCAGGCCAAGGCTGTCCACAATATACTTCGCATCACCACCCCTGC
ATGCACGAATCTCGGCACTCCCAGAGACAATCACAGCCAGCAGCCCACGCCGATACCGAT
CATTCACCTGGGCTGAGTACAAGACGACAATGTACTCACTCCGCCTGAGCCACAGCCGCC
TAGAACTCTTCAAAATTGACGATGATGACAGCGACAATGCCAGTGAGGGAAAAGCATAGG
AATTGCTGGTTAAATTGCAGACGATGCCTATGGACCAGTGGGGATTAGGAAGCTGAAACT
GTCCCCAAAATTTTGGCTCTCTGGCAGTCTGGCTACTATCGTCAGATATCTCACTATTAT
GATGGTGTAGTGCCTAAGTTGACGGGTGTGTAATATCGTTAGCAGTCTACAGAAGCTATG
GTTGTACGGAAGTAATGTACTGTCGCCTTTTCAGCTAACTATCCATGTTCTCTCTTATAT
GTAATGAGTTAGTTGACGGATGTGTAATATTGCTAGCATTGTATATAAGCTATGGTTGTA
TGGAAGTATGTAATATAGCCTTTTCAGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Appendix 16 *Oryza sativa* mRNA for *OsGA2ox3*, complete cds (GenBank ID: AB092485.1)

ATGGTGGTTCTCGCTGGCCCGCCCGCCGTCGATCACATCCCGCTGCTGAGGTCGCCGGAC
CCCGGCGACGTCTTCTCCGGCGTGCCGGTCGTCGACCTCGGCAGCCCCGGCGCGGCGAGG
GCCGTGGTGGACGCCTGCGAGCGGTACGGGTTCTTCAAGGTCGTCAACCACGGCGTGGCC
ACGGACACGATGGACAAGGCCGAGTCGGAGGCCGTCAGGTTCTTCTCCCAGACGCAGCCC
GACAAGGACCGCTCCGGCCCGGCCTACCCGTTCGGGTACGGCAGCAAGCGGATCGGGTTC
AATGGCGACATGGGGTGGCTCGAGTACCTCCTCCTCGCCCTCGACGACGCGTCGCTCGCC
GACGCCTGCACCGTCCCGTCCTGCGCGGTCTTCCGGGCCGCTCTGAACGAGTACATCTCG
GGGGTGCGGAAGGTGGCGGTGCGGGTGATGGAGGCGATGTCGGAGGGGCTGGGCATTGCG
CAGGCGGACGCGCTGAGCGCGCTGGTGACGGCGGAAGGGAGCGACCAGGTGTTCCGCGTG
AACCACTACCCGCCGTGCCGCGCGCTGCAGGGGCTCGGCTGCAGCGTCACCGGCTTCGGC
GAGCACACCGACCCGCAGCTCGTCTCCGTGCTCCGCTCAAACGGCACGTCCGGCCTGCAG
ATCGCGCTCCGCGACGGCCAGTGGGTGTCCGTGCCCTCCGACCGCGACTCCTTCTTCGTC
AACGTCGGCGACTCGTTGCAGGTTCTGACCAATGGGAGGTTCAAGAGCGTGAAGCACAGG
GTGGTGGCCAACAGCCTAAAGTCTAGGGTTTCCTTCATCTACTTTGGAGGGCCACCGTTA
GCACAGAGGATTGCACCATTGCCACAGCTGCTGGGGGAGGGAGAGCAGAGCCTGTACAAG
GAGTTCACATGGGATGAGTACAAGAAGGCTGCCTACAAATCAAGGCTTGGAGACAACAGG
CTGGCCCAGTTTGAGAAGAAGTAG

Appendix 17 mRNA sequence for *Hordeum vulgare* subsp. *vulgare* cDNA clone HX11J23 3-PRIME, (EST for *HvGA2ox1*; GenBank ID: CB876549.1)

GGCTGGAAGGTAAATTACTATCATACAAGCATCACTTCTGTACAAAGCTAGCCAGGTCAT
GGACACAAACACTACACCATCATAATACTGGGATATCTGGCAATAGCTGTCAGAGAGCCA
AAATTTTGGGGACTATTCTTATATCTAACTAATCCCCACCATACAGATTGCCTGCAACTC
AAGCAGCCATTGCCTTATTTTCCTTTGCTGCCATTGCCGCTTTCGTCATCATCAACATGG
AAGAGGTCCAGGCGGCTGTGGCTTAGTCGGAGCGAGTACATTGCCGTCTTGTACTCAGCC
CAGGTGAATGACCGGTACCGGCGTGGTGAGCTGGCTGTGACCATCTCTGGGAGCGCCGAG
ATTCGTGCATGCAGTGGTGGGGCTGCGAAGTAGATGGTGGACAGCCTTGGCCTGCAGGCA
CTTGCAATTACTCTGTGCCGGATGCTGACTAACCTCCCATTTGTCAGAGCCTGAAGGAGA
TCACCAACATTGACGAAATAGGCCGACGAGTCTGCTGGCACCTGAATCCACGCATCCTTG
CCATTGACGTCCGGCAGAAGCACCTGCAAGCCATCAACATCGTTTGCTCGGAGCAAGCTA
AGTAGCTGTGGGTCGGAGTGCT

Appendix 18 The reverse compliment sequence of EST "CB876549.1" (the primers used for 3' RACE PCR of *HvGA2ox1* are underlined).

AGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGG
TGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGG
CCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCA
TCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAG
CCCCACCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCAC
GCCGGTACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAA
GCCACAGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAG
GAAAATAAGGCAATGGCTGCTTGAGTTGCAGGCAATCTGTATGGTGGGGATTAGTTAGAT
ATAAGAATAGTCCCCAAAATTTTGGCTCTCTGACAGCTATTGCCAGATATCCCAGTATTA
TGATGGTGTAGTGTTTGTGTCCATGACCTGGCTAGCTTTGTACAGAAGTGATGCTTGTAT
GATAGTAATTTACCTTCAGCC

Appendix 19 Nucleotide sequence similarity between *OsGA2ox1* (query) and the reverse compliment sequence of EST "CB876549.1" (subject)

Score = 533 (590 bits), Expect = 3e-155, Identities = 459/567(81%)

```
Query  769   AGCACTCTGACCCGCAGATCCTTAGCTTGCTCCGAGCAAACGATGTGGAAGGGCTACAGG  828
             ||||||| ||||| ||| | |||||||||||||||||||||||||| || ||  | ||||
Sbjct  1     AGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGG  60

Query  829   TGTTTGTGCCGGACCACGAGGGCAAGGAGATGTGGGTTCAGGTGCCATCGGACCCATCGG  888
             || || ||||||||  | | ||||||||   |||| |||||||||| | ||| | ||||
Sbjct  61    TGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGG  120

Query  889   CCATTTTCGTCAATGTTGGTGATGTCCTCCAGGCTCTGACAAATGGGAGGCTGATAAGTA  948
             ||  |||||||||||||||||| |||| ||||||||||||||||||||| |  | || |
Sbjct  121   CCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCA  180

Query  949   TCCGGCACAGGGTAATTGCAACCGCCTGCAGGCCAAGGCTGTCCACAATATACTTCGCAT  1008
             |||||||||| ||||||||||  |||||||||||||||||||||| || |||||||||
Sbjct  181   TCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAG  240

Query  1009  CACCACCCCTGCATGCACGAATCTCGGCACTCCCAGAGACAATCACAGCCAGCAGCCCAC  1068
             | ||||| |||||||||||||||||||| ||||||||||   ||||||||||   ||||
Sbjct  241   CCCCACCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCAC  300

Query  1069  GCCGATACCGATCATTCACCTGGGCTGAGTACAAGACGACAATGTACTCACTCCGCCTGA  1128
             |||| ||||| |||||||||||||||||||||||||| ||||||||| ||||| || |
Sbjct  301   GCCGGTACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAA  360

Query  1129  GCCACAGCCGCCTAGAACTCTTCAAAATTGACGATGATGACAGCGACAATGCCAGTGAGG  1188
             |||||||||||| || |||||| |  |||| ||||| || |||| ||||| |||  | |
Sbjct  361   GCCACAGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAG  420
```

Continued

```
Query  1189  GAAAAGCATAGGAATTGCTGGTTAAATTGCAGACGATGCCTATGGACCAGTGGGGATTAG  1248
             |||||  |  | ||| |||| || | |||||| | ||   ||||     ||||||||||
Sbjct  421   GAAAA-TAAGGCAATGGCTGCTTGAGTTGCAGGCAATCTGTATG-----GTGGGGATTAG  474

Query  1249  GAAGCT---GAAACTGTCCCCAAAATTTTGGCTCTCTGGCAGTCTGGCTACTATCGTCAG  1305
               || |     ||  ||||||||||||||||||||||| |||        |||| | |||
Sbjct  475   TTAGATATAAGAATAGTCCCCAAAATTTTGGCTCTCTGACAG--------CTATTGCCAG  526

Query  1306  ATATCTCACTATTATGATGGTGTAGTG  1332
             ||||| || ||||||||||||||||||
Sbjct  527   ATATCCCAGTATTATGATGGTGTAGTG  553
```

Appendix 20 mRNA sequence of *Hordeum vulgare* subsp. *vulgare* cDNA clone HB21M01 5-PRIME (EST for *HvGA2ox3*; GenBank ID: BU972476.1). The primers used for 3' and 5' RACE are underlined with solid and dotted lines, respectively.

CGGCACGAGGGCGCTGAGCGCGATGGTGGCGGCGGAGGGCAGCGACCAGGTGTTCAGGGT
GAACCACTACCCGCCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGCGCCACCGGCTTCGG
CGAGCACACCGACCCGCAGCTCATCTCCGTGCTGCGCTCCAACGGCACGTCCGGCCTGCA
GATCGCGCTCCAGAGCGGCCACTGGGTGTCCGTGCCCTCCGACCGCGACGCCTTCTTCGT
CAACGTCGGCGACTCCTTGCAGGTGCTGACCAACGGGAGGTTCAAGAGCGTGAAGCACAG
GGTGGTGGCCAACAGCCTAAAGTCTAGGGTTTCCATGATCTACTTTGGAGGGCCAGCATT
GACACAGAGGATTGCACCATTGCCGCAGCTGCTGAGAGAGGGAGAGCAGAGCCTGTACAA
GGACTTCACATGGGGCGAGTACAAGAAGGCTGCCTACAACTCCAGGCTCGGNGACAACAG
GCTGGCTCACTTCCACAGGTAGCCACGCC

Appendix 21 Nucleotide sequence similarity between *OsGA2ox3* (query) and the reverse compliment sequence for EST "BU972476.1" (subject)

Score = 699 bits (774), Expect = 0.0, Identities = 444/482(92%)

```
Query  490  GCGCTGAGCGCGCTGGTGACGGCGGAAGGGAGCGACCAGGTGTTCCGCGTGAACCACTAC  549
            |||||||||||| ||||| ||||||| || ||||||||||||||| | ||||||||||||
Sbjct  11   GCGCTGAGCGCGATGGTGGCGGCGGAGGGCAGCGACCAGGTGTTCAGGGTGAACCACTAC  70

Query  550  CCGCCGTGCCGCGCGCTGCAGGGGCTCGGCTGCAGCGTCACCGGCTTCGGCGAGCACACC  609
            |||||||||| |||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  71   CCGCCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGCGCCACCGGCTTCGGCGAGCACACC  130

Query  610  GACCCGCAGCTCGTCTCCGTGCTCCGCTCAAACGGCACGTCCGGCCTGCAGATCGCGCTC  669
            |||||||||||| |||||||||| ||||| ||||||||||||||||||||||||||||||
Sbjct  131  GACCCGCAGCTCATCTCCGTGCTGCGCTCCAACGGCACGTCCGGCCTGCAGATCGCGCTC  190

Query  670  CGCGACGGCCAGTGGGTGTCCGTGCCCTCCGACCGCGACTCCTTCTTCGTCAACGTCGGC  729
            |    |||||| |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  191  CAGAGCGGCCACTGGGTGTCCGTGCCCTCCGACCGCGACGCCTTCTTCGTCAACGTCGGC  250

Query  730  GACTCGTTGCAGGTTCTGACCAATGGGAGGTTCAAGAGCGTGAAGCACAGGGTGGTGGCC  789
            ||||| |||||||| |||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  251  GACTCCTTGCAGGTGCTGACCAACGGGAGGTTCAAGAGCGTGAAGCACAGGGTGGTGGCC  310

Query  790  AACAGCCTAAAGTCTAGGGTTTCCTTCATCTACTTTGGAGGGCCACCGTTAGCACAGAGG  849
            |||||||||||||||||||||||| | |||||||||||||||| | ||  ||||||||
Sbjct  311  AACAGCCTAAAGTCTAGGGTTTCCATGATCTACTTTGGAGGGCCAGCATTGACACAGAGG  370

Query  850  ATTGCACCATTGCCACAGCTGCTGGGGGAGGGAGAGCAGAGCCTGTACAAGGAGTTCACA  909
            |||||||||||||| ||||||||| | |||||||||||||||||||||||| ||||||
Sbjct  371  ATTGCACCATTGCCGCAGCTGCTGAGAGAGGGAGAGCAGAGCCTGTACAAGGACTTCACA  430

Query  910  TGGGATGAGTACAAGAAGGCTGCCTACAAATCAAGGCTTGGAGACAACAGGCTGGCCCAG  969
            ||||  |||||||||||||||||||||| || ||||| || |||||||||||||| ||
Sbjct  431  TGGGGCGAGTACAAGAAGGCTGCCTACAACTCCAGGCTCGGNGACAACAGGCTGGCTCAC  490

Query  970  TT  971
            ||
Sbjct  491  TT  492
```

Appendix 22 Nucleotide sequence of the assembled DNA fragment of *HvGA2ox1* (assembly of EST "CB876549.1" and 3' end fragment).

AGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGG
TGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGG
CCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCA
TCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAG
CCCCACCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCAC
GCCGGTACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAA
GCCACAGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAG
GAAAATAAGGCAATGGCTGCTTGAGTTGCAGGCAATCTGTATGGTGGGGATTAGTTAGAT
ATAAGAATAGTCCCCAAAATTTTGGCTCTCTGACAGCTATTGCCAGATATCCCAGTATTA
TGATGGTGTAGTGTTTGTGTCCATGACCTGGCTAGCTTTGTACAGAAGTGATGCTTGTAT
GATAGTAATTTACCTTCAGCACAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTT
GATACCACTGCTT

Appendix 23 Nucleotide sequence similarity between the assembled DNA fragment of *HvGA2ox1* (query) and "TC244240" (obtained from DFCI, subject)

Score = 3105 (471.9 bits), Expect = 1.3e-135, Identities = 621/621 (100%), Positives = 621/621 (100%)

```
Query:     1 AGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGG 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   368 AGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGG 427

Query:    61 TGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGG 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   428 TGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGG 487

Query:   121 CCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCA 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   488 CCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCA 547

Query:   181 TCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAG 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   548 TCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAG 607

Query:   241 CCCCACCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCAC 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   608 CCCCACCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCAC 667

Query:   301 GCCGGTACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAA 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   668 GCCGGTACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAA 727

Query:   361 GCCACAGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAG 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   728 GCCACAGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAG 787
```

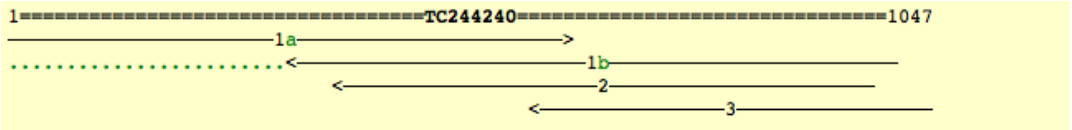
Continued

```
Query:   421 GAAAATAAGGCAATGGCTGCTTGAGTTGCAGGCAATCTGTATGGTGGGGATTAGTTAGAT 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   788 GAAAATAAGGCAATGGCTGCTTGAGTTGCAGGCAATCTGTATGGTGGGGATTAGTTAGAT 847

Query:   481 ATAAGAATAGTCCCCAAAATTTTGGCTCTCTGACAGCTATTGCCAGATATCCCAGTATTA 540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   848 ATAAGAATAGTCCCCAAAATTTTGGCTCTCTGACAGCTATTGCCAGATATCCCAGTATTA 907

Query:   541 TGATGGTGTAGTGTTTGTGTCCATGACCTGGCTAGCTTTGTACAGAAGTGATGCTTGTAT 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   908 TGATGGTGTAGTGTTTGTGTCCATGACCTGGCTAGCTTTGTACAGAAGTGATGCTTGTAT 967

Query:   601 GATAGTAATTTACCTTCAGCA 621
             ||||||||||||||||||||
Sbjct:   968 GATAGTAATTTACCTTCAGCA 988
```

Appendix 24 Nucleotide sequence for bioinformatically assembled DNA fragment of "TC244240" (obtained from DFCI)

CGGCACGAGGAGTACCTCCTCCTCCATGCCAACCCCGCCGCCGTCGCGCACAGGGCCAGC
TCCATCGACACCAACGATCCCTCACGCTTCAGTTCTGTTGTGAATGAGTATGTGGAAGCA
GTGAAGCAGCTTGCATGTGACATCCTGGACCTGTTAGGAGAAGGGCTAGGGCTAGAGGAC
CCCAGGCTCTTCAGCAAGCTCGTCACAGAAGCTGACAGTGACTCCCTCCTGAGGATCAAC
CACTACCCTCCATCATGCACCGTTCACAAGCTTGACCATGATGACCAATGCAAGCTCAAG
GGGATCGCCAGGACCAAGGCAGGGAATGGTGCGAACCCGGGGGCGGGTGGCCGGATCGGG
TTCGGTGAGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGC
TTGCAGGTGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGAC
TCGTCGGCCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTA
GTCAGCATCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTAC
TTCGCAGCCCCACCACTGCATGCACGAATCTCGGCGCTCCCAGAGATGGTCACAGCCAGC
TCACCACGCCGGTACCGGTCATTCACCTGGGCTGAGTACAAGACGGCAATGTACTCGCTC
CGACTAAGCCACAGCCGCCTGGACCTCTTCCATGTTGATGATGACGAAAGCGGCAATGGC
AGCAAAGGAAAATAAGGCAATGGCTGCTTGAGTTGCAGGCAATCTGTATGGTGGGGATTA
GTTAGATATAAGAATAGTCCCCAAAATTTTGGCTCTCTGACAGCTATTGCCAGATATCCC
AGTATTATGATGGTGTAGTGTTTGTGTCCATGACCTGGCTAGCTTTGTACAGAAGTGATG
CTTGTATGATAGTAATTTACCTTCAGCAAACTGATTATCCTGATGCAACTTCTATTTCCG
ATCTATCCCTTACGGTACTTTCCTTGC

Appendix 25 Bioinformatic assembly of "TC244240"



#	Src	EST Id	GB#	Clone/Protein	left	right	Library/Protein name
1a	I015	HA20N12r	BU980500	HA20N12	1	637	HA
1b	I024	RUS17G07w	FD520421	HA20N12	316	1006	HA
2	I015	HX11J23w	CB876549	HX11J23	368	984	HX
3	I015	HM08G07w	CB880972	HM08G07	588	1047	HM

(Source: http://compbio.dfci.harvard.edu/cgi-bin/tgi/ tc_report.pl? tc=TC244240 &species=barley; accessed on 15 January 2013)

1a: Nucleotide sequence information for HA20N12r (EST, GenBank ID: BU980500)

CGGCACGAGGAGTACCTCCTCCTCCATGCCAACCCCGCCGCCGTCGCGCACAGGGCCAGC
TCCATCGACACCAACGATCCCTCACGCTTCAGTTCTGTTGTGAATGAGTATGTGGAAGCA
GTGAAGCAGCTTGCATGTGACATCCTGGACCTGTTAGGAGAAGGGCTAGGGCTAGAGGAC
CCCAGGCTCTTCAGCAAGCTCGTCACAGAAGCTGACAGTGACTCCCTCCTGAGGATCAAC
CACTACCCTCCATCATGCACCGTTCACAAGCTTGACCATGATGACCAATGCAAGCTCAAG
GGGATCGCCAGGACCAAGGCAGGGAATGGTGCGAACCCGGGGGCGGGTGGCCGGATCGGG
TTCGGTGAGCACTCCGACCCACAGCTACTTAGCTTGCTCCGAGCAAACGATGTTGATGGC
TTGCAGGTGCTTCTGCCGGACGTCAATGGCAAGGATGCGTGGATTCAGGTGCCAGCAGAC
TCGTCGGCCTATTTCGTCAATGTTGGTGATCTCCTTCAGGCTCTGACAAATGGGAGGTTA
GTCAGCATCCGGCACAGAGTAATTGCAAGTGCCTGCAGGCCAAGGCTGTCCACCATCTAC
TTCGCAGCCCCACCACTGCATGCACGAATCTCGGCGC

1b: Nucleotide sequence information for RUS17G07W (EST, GenBank ID: FD520421)

GCATCAGGATAATCAGTTTGCTGAAGGTAAATTACTATCATACAAGCATCACTTCTGTAC
AAAGCTAGCCAGGTCATGGACACAAACACTACACCATCATAATACTGGGATATCTGGCAA
TAGCTGTCAGAGAGCCAAAATTTTGGGGACTATTCTTATATCTAACTAATCCCCACCATA
CAGATTGCCTGCAACTCAAGCAGCCATTGCCTTATTTTCCTTTGCTGCCATTGCCGCTTT
CGTCATCATCAACATGGAAGAGGTCCAGGCGGCTGTGGCTTAGTCGGAGCGAGTACATTG
CCGTCTTGTACTCAGCCCAGGTGAATGACCGGTACCGGCGTGGTGAGCTGGCTGTGACCA
TCTCTGGGAGCGCCGAGATTCGTGCATGCAGTGGTGGGGCTGCGAAGTAGATGGTGGACA
GCCTTGGCCTGCAGGCACTTGCAATTACTCTGTGCCGGATGCTGACTAACCTCCCATTTG
TCAGAGCCTGAAGGAGATCACCAACATTGACGAAATAGGCCGACGAGTCTGCTGGCACCT
GAATCCACGCATCCTTGCCATTGACGTCCGGCAGAAGCACCTGCAAGCCATCAACATCGT

TTGCTCGGAGCAAGCTAAGTAGCTGTGGGTCGGAGTGCTCACCGAACCCGATCCGGCCAC
CCGCCCCCGGGTTCGCACCATTCCCTGCCTT

2: Nucleotide sequence information for HX11J23w (EST, GenBank ID: CB876549)

GGCTGGAAGGTAAATTACTATCATACAAGCATCACTTCTGTACAAAGCTAGCCAGGTCAT
GGACACAAACACTACACCATCATAATACTGGGATATCTGGCAATAGCTGTCAGAGAGCCA
AAATTTTGGGGACTATTCTTATATCTAACTAATCCCCACCATACAGATTGCCTGCAACTC
AAGCAGCCATTGCCTTATTTTCCTTTGCTGCCATTGCCGCTTTCGTCATCATCAACATGG
AAGAGGTCCAGGCGGCTGTGGCTTAGTCGGAGCGAGTACATTGCCGTCTTGTACTCAGCC
CAGGTGAATGACCGGTACCGGCGTGGTGAGCTGGCTGTGACCATCTCTGGGAGCGCCGAG
ATTCGTGCATGCAGTGGTGGGGCTGCGAAGTAGATGGTGGACAGCCTTGGCCTGCAGGCA
CTTGCAATTACTCTGTGCCGGATGCTGACTAACCTCCCATTTGTCAGAGCCTGAAGGAGA
TCACCAACATTGACGAAATAGGCCGACGAGTCTGCTGGCACCTGAATCCACGCATCCTTG
CCATTGACGTCCGGCAGAAGCACCTGCAAGCCATCAACATCGTTTGCTCGGAGCAAGCTA
AGTAGCTGTGGGTCGGAGTGCT

3: Nucleotide sequence information for HM08G07w (EST, GenBank ID: CB880972)

GCAAGGAAAGTACCGTAAGGGATAGATCGGAAATAGAAGTTGCATCAGGATAATCAGTTT
GCTGAAGGTAAATTACTATCATACAAGCATCACTTCTGTACAAAGCTAGCCAGGTCATGG
ACACAAACACTACACCATCATAATACTGGGATATCTGGCAATAGCTGTCAAAAAGCCAAA
ATTTTGGGGACTATTCTTATATCTAACTAATCCCCACCATACAGATTGCCTGCAACTCAA
GCAGCCATTGCCTTATTTTCCTTTGCTGCCATTGCCGCTTTCGTCATCATCAACATGGAA
GAGGTCCAGGCGGCTGTGGCTTAGTCGGAGCGAGTACATTGCCGTCTTGTACTCAGCCCA
GGTGAATGACCGGTACCGGCGTGGTGAGCTGGCTGTGACCATCTCTGGGAGCGCCGAAAT
TCGTGCATGCAGTGGTGGGGCTGCAAAGTAGATGGTGGAC

Appendix 26 Nucleotide sequence similarity between the bioinformatically assembled DNA fragment of "TC244240" (query) and EST "CA031963" (subject)

Score = 401 (66.2 bits), Expect = 2.0e-12, Identities = 81/82 (98%), Positives = 81/82 (98%)

```
Query:     9 GGAGTACCTCCTCCTCCATGCCAACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGA 68
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   399 GGAGTACCTTCTCCTCCATGCCAACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGA 458

Query:    69 CACCAACGATCCCTCACGCTTC 90
             ||||||||||||||||||||||
Sbjct:   459 CACCAACGATCCCTCACGCTTC 480
```

Appendix 27 Nucleotide sequence information for HX11J23r (EST, GenBank ID: CA031963)

CGGCACGAGGCACGCATTGCATACGCACGTGAAGCGAGCGTCCGCCGAGCCAACGCAATG
GTGGTGCCCTCCACGACGCCGGCGCGGCAAGAGACGGCGACCCTGCTTCTTCCACAGGCA
CAGCCGTCCCCCGGCGGCGGCGCCATACCGACGGTTGACATGTCGGCGCCCCGCGGCCGC
GGCGCGCTGTCCCGGCAGGTGGCGCGTGCGTGCGCGGAGCAGGGCTTCTTCCGTGCCGTC
AACCACGGCGTGCCTCCGGCGGGGCCCCCGGCACGGCTGGACGCGGCCACCTCGGCGTTC
TTCGCGCTCGCGGCACACGACAAGCAGCGCGCCGGCCCGCCGAGCCCGCTCGGCTACGGC
TGCCGCAGCATCGGGTTCAACGGCGACGTGGGCGAGCTGGAGTACCTTCTCCTCCATGCC
AACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGACACCAACGATCCCTCACGCTTC

Appendix 28 Nucleotide sequence of the full length cDNA of *HvGA2ox1* (assembled from EST "CA031963" and bioinformatically assembled DNA fragment of "TC244240")

CGGCACGAGGCACGCATTGCATACGCACGTGAAGCGAGCGTCCGCCGAGCCAACGCAATG
GTGGTGCCCTCCACGACGCCGGCGCGGCAAGAGACGGCGACCCTGCTTCTTCCACAGGCA
CAGCCGTCCCCCGGCGGCGGCGCCATACCGACGGTTGACATGTCGGCGCCCCGCGGCCGC
GGCGCGCTGTCCCGGCAGGTGGCGCGTGCGTGCGCGGAGCAGGGCTTCTTCCGTGCCGTC
AACCACGGCGTGCCTCCGGCGGGGCCCCCGGCACGGCTGGACGCGGCCACCTCGGCGTTC
TTCGCGCTCGCGGCACACGACAAGCAGCGCGCCGGCCCGCCGAGCCCGCTCGGCTACGGC
TGCCGCAGCATCGGGTTCAACGGCGACGTGGGCGAGCTGGAGTACCTTCTCCTCCATGCC
AACCCCGCCGCCGTCGCGCACAGGGCCAGCTCCATCGACACCAACGATCCCTCACGCTTC
AGTTCTGTTGTGAATGAGTATGTGGAAGCAGTGAAGCAGCTTGCATGTGACATCCTGGAC
CTGTTAGGAGAAGGGCTAGGGCTAGAGGACCCCAGGCTCTTCAGCAAGCTCGTCACAGAA
GCTGACAGTGACTCCCTCCTGAGGATCAACCACTACCCTCCATCATGCACCGTTCACAAG
CTTGACCATGATGACCAATGCAAGCTCAAGGGGATCGCCAGGACCAAGGCAGGGAATGGT
GCGAACCCGGGGGCGGGTGGCCGGATCGGGTTCGGTGAGCACTCCGACCCACAGCTACTT
AGCTTGCTCCGAGCAAACGATGTTGATGGCTTGCAGGTGCTTCTGCCGGACGTCAATGGC
AAGGATGCGTGGATTCAGGTGCCAGCAGACTCGTCGGCCTATTTCGTCAATGTTGGTGAT
CTCCTTCAGGCTCTGACAAATGGGAGGTTAGTCAGCATCCGGCACAGAGTAATTGCAAGT
GCCTGCAGGCCAAGGCTGTCCACCATCTACTTCGCAGCCCCACCACTGCATGCACGAATC
TCGGCGCTCCCAGAGATGGTCACAGCCAGCTCACCACGCCGGTACCGGTCATTCACCTGG
GCTGAGTACAAGACGGCAATGTACTCGCTCCGACTAAGCCACAGCCGCCTGGACCTCTTC
CATGTTGATGATGACGAAAGCGGCAATGGCAGCAAAGGAAAATAAGGCAATGGCTGCTTG
AGTTGCAGGCAATCTGTATGGTGGGGATTAGTTAGATATAAGAATAGTCCCCAAAATTTT
GGCTCTCTGACAGCTATTGCCAGATATCCCAGTATTATGATGGTGTAGTGTTTGTGTCCA
TGACCTGGCTAGCTTTGTACAGAAGTGATGCTTGTATGATAGTAATTTACCTTCAGCAAA
CTGATTATCCTGATGCAACTTCTATTTCCGATCTATCCCTTACGGTACTTTCCTTGC

Appendix 29 Nucleotide sequence similarity between the full-length cDNA of *HvGA2ox1* (query) and *OsGA2ox1* (subject)

Score = 1157 bits (1282), Expect = 0.0, Identities = 977/1199(81%)

```
Query  132   CGGCGGCGGCG---CCATACCGACGGTTGACATGTCGGCGCCCCGCGGCCGCGGCGCGCT  188
             ||||||||||   | ||| ||||||| ||||||||||||      |  ||||||||| |
Sbjct  143   CGGCGGCGGCGTGACGATAGCGACGGTGGACATGTCGGCG------GAGCGCGGCGCGGT  196

Query  189   GTCCCGGCAGGTGGCGCGTGCGTGCGCGGAGCAGGGCTTCTTCCGTGCCGTCAACCACGG  248
             | |  ||||||||||   |||||||||| ||| || ||||||||   ||||   |||||
Sbjct  197   GGCGAGGCAGGTGGCGACGGCGTGCGCGGCGCACGGGTTCTTCCGGTGCGTCGGGCACGG  256

Query  249   CGTGCCTCCGGCGGGGCCCC---CGGCACGGCTGGACGCGGCCACCTCGGCGTTCTTCGC  305
             ||||||  |||||| ||||    ||||  |||||||||| || ||  ||||||||||||
Sbjct  257   CGTGCCGGCGGCGGCGCCCGTCGCGGCGAGGCTGGACGCCGCGACGGCGGCGTTCTTCGC  316

Query  306   GCTCGCGGCACACGACAAGCAGCGCGCCGGCCCGCCGAGCCCGCTCGGCTACGGCTGCCG  365
             | | ||| |    || |||||||||||||| ||| |||||||||||| ||||||||||
Sbjct  317   GATGGCGCCGGCGGAGAAGCAGCGCGCCGGGCCGGCGAGCCCGCTCGGGTACGGCTGCCG  376

Query  366   CAGCATCGGGTTCAACGGCGACGTGGGCGAGCTGGAGTACCTTCTCCTCCATGCCAACCC  425
              ||||||||||||||||||||||| |||||||||||||||| |||||||| ||||||||
Sbjct  377   GAGCATCGGGTTCAACGGCGACGTCGGCGAGCTGGAGTACCTGCTCCTCCACGCCAACCC  436

Query  426   CGCCGCCGTCGCGCACAGGGCCAGCTCCATCGACACCAACGATCCCTCACGCTTCAGTTC  485
             ||||||||||||||| |||||||  |||||||| |||  || ||||| ||||||||| |
Sbjct  437   CGCCGCCGTCGCGCACCGGGCCAGGACCATCGACGCCATGGACCCCTCTCGCTTCAGTGC  496

Query  486   TGTTGTGAATGAGTATGTGGAAGCAGTGAAGCAGCTTGCATGTGACATCCTGGACCTGTT  545
             | ||||||||||||  | |||||  ||||| |||| |||||||| ||||||||||||||
Sbjct  497   TATTGTGAATGAGTACATTGAAGCCATGAAGAAGCTCGCATGTGAGATCCTGGACCTGTT  556

Query  546   AGGAGAAGGGCTAGGGCTAGAGGACCCCAGGCTCTTCAGCAAGCTCGTCACAGAAGCTGA  605
             |||||| |||||||| ||  ||||||||||   ||||||||||||   |||| | |||||
Sbjct  557   AGGAGAGGGGCTAGGTCTCAAGGACCCCAGATACTTCAGCAAGCTTACCACAAACGCTGA  616
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Query  606   CAGTGACTCCCTCCTGAGGATCAACCACTACCCTCCATCATGCACCGTTCACAAGCTTGA  665
             |||||||| ||||||||||||||||||||||||||||||||| | ||||||| |||||
Sbjct  617   CAGTGACTGCCTCCTGAGGATCAACCACTACCCTCCATCATGCAACATTCACAAACTTGA  676

Query  666   CCATGATGACCAATGCAAGCTCAAGGGGATCGCCAGGACCAAGGCAGGGAATGGTGCGAA  725
             ||||||||||||||||  ||||| |  | |  || ||||||||  | ||||||| |||
Sbjct  677   CCATGATGACCAATGCAATATCAAGAGCCTTGTTAGCACCAAGGCTAGCAATGGTGGGAA  736

Query  726   CCCGGGGGCGGGTGGCCGGATCGGGTTCGGTGAGCACTCCGACCCACAGCTACTTAGCTT  785
              | |  ||| ||||| || || |||||||| |||||||| ||||| ||| | ||||||||
Sbjct  737   TCTGATGGCAGGTGGGCGCATTGGGTTCGGCGAGCACTCTGACCCGCAGATCCTTAGCTT  796

Query  786   GCTCCGAGCAAACGATGTTGATGGCTTGCAGGTGCTTCTGCCGGACGTCAATGGCAAGGA  845
             |||||||||||||||||| || ||  | ||||||  || ||||||||  | | ||||||||
Sbjct  797   GCTCCGAGCAAACGATGTGGAAGGGCTACAGGTGTTTGTGCCGGACCACGAGGGCAAGGA  856

Query  846   TGCGTGGATTCAGGTGCCAGCAGACTCGTCGGCCTATTTCGTCAATGTTGGTGATCTCCT  905
                ||||  |||||||||||  | |||  | ||||||  |||||||||||||||||||  ||||
Sbjct  857   GATGTGGGTTCAGGTGCCATCGGACCCATCGGCCATTTTCGTCAATGTTGGTGATGTCCT  916

Query  906   TCAGGCTCTGACAAATGGGAGGTTAGTCAGCATCCGGCACAGAGTAATTGCAAGTGCCTG  965
              ||||||||||||||||||||  |  | ||  |||||||||||  ||||||||||  |||||
Sbjct  917   CCAGGCTCTGACAAATGGGAGGCTGATAAGTATCCGGCACAGGGTAATTGCAACCGCCTG  976

Query  966   CAGGCCAAGGCTGTCCACCATCTACTTCGCAGCCCCACCACTGCATGCACGAATCTCGGC  1025
             |||||||||||||||||  ||  |||||||||  | |||||  ||||||||||||||||||||
Sbjct  977   CAGGCCAAGGCTGTCCACAATATACTTCGCATCACCACCCCTGCATGCACGAATCTCGGC  1036

Query  1026  GCTCCCAGAGATGGTCACAGCCAGCTCACCACGCCGGTACCGGTCATTCACCTGGGCTGA  1085
              ||||||||||   ||||||||||   |||||||| |||||  ||||||||||||||||||
Sbjct  1037  ACTCCCAGAGACAATCACAGCCAGCAGCCCACGCCGATACCGATCATTCACCTGGGCTGA  1096

Query  1086  GTACAAGACGGCAATGTACTCGCTCCGACTAAGCCACAGCCGCCTGGACCTCTTCCATGT  1145
             |||||||||| |||||||||| ||||| || |||||||||||||| || |||||| |  |
Sbjct  1097  GTACAAGACGACAATGTACTCACTCCGCCTGAGCCACAGCCGCCTAGAACTCTTCAAAAT  1156
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Query  1146  TGATGATGACGAAAGCGGCAATGGCAGCAAAGGAAAA-TAAGGCAATGGCTGCTTGAGTT  1204
             ||| ||||| || |||| ||||| |||  | ||||||  |  | ||| |||| || | ||
Sbjct  1157  TGACGATGATGACAGCGACAATGCCAGTGAGGGAAAAGCATAGGAATTGCTGGTTAAATT  1216

Query  1205  GCAGGCAATCTGTATG-----GTGGGGATTAGTTAGATATAAGAATAGTCCCCAAAATTT  1259
             |||| | ||   ||||     ||||||||||  || |     ||  ||||||||||||
Sbjct  1217  GCAGACGATGCCTATGGACCAGTGGGGATTAGGAAGCT---GAAACTGTCCCCAAAATTT  1273

Query  1260  TGGCTCTCTGACAG--------CTATTGCCAGATATCCCAGTATTATGATGGTGTAGTG  1310
             |||||||||| |||        |||| | |||||||| || ||||||||||||||||||
Sbjct  1274  TGGCTCTCTGGCAGTCTGGCTACTATCGTCAGATATCTCACTATTATGATGGTGTAGTG  1332
```

Appendix 30 Nucleotide sequence of the full length cDNA of *HvGA2ox3* (obtained by assembling the 5' end fragment, EST "BU972476.1" and 3' end fragment).

TCGATCGCACACACTTTACCATACTTGGTAGCAGCAGCCTCTGCAACCATGGTGGTTCTC
GCCGGCACGCCTGCCGTCGATCACATCCCGCTCCTCAGGTCCCCCGACCCCGGGGACAAC
TTCTCCGGCATGCCGGTCGTGGACCTGTCCAGACCTGGCGCGCCGCGGGCCATCGCCGAC
GCGTGCGAGCGCTTCGGCTTCTTCAAGCTCGTCAACCACGGGGTGGCCCTGGACGCGATG
GACCGGCTCGAGTCGGAGGCCGTGAGGTTCTTCTCGCTGCCGCAGGCCGACAAGGACCGC
TCCGGCCCGGCCTACCCGTTCGGCTACGGCAGCAAGCGCATCGGGCTCAATGGCGACATG
GGGTGGCTCGAGTACCTCCTCCTCGCCGTCGACTCCGCCTCGCTCCCCGCCGCCTCCGCC
GTCCCGTCCTGCGCGCTCTTCCGGGCGGCGCTGAACGAGTACATCGCGGCGGTGAGGAAG
GTGGCGGTGCGGGTGATGGAGGCGATGGCGGAGGGGCTGGGCATTGCGCAGGCGGACGCG
CTGAGCGCGATGGTGGCGGCGGAGGGCAGCGACCAGGTGTTCAGGGTGAACCACTACCCG
CCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGCGCCACCGGCTTCGGCGAGCACACCGAC
CCGCAGCTCATCTCCGTGCTGCGCTCCAACGGCACGTCCGGCCTGCAGATCGCGCTCCAG
AGCGGCCACTGGGTGTCCGTGCCCTCCGACCGCGACGCCTTCTTCGTCAACGTCGGCGAC
TCCTTGCAGGTGCTGACCAACGGGAGGTTCAAGAGCGTGAAGCACAGGGTGGTGGCCAAC
AGCCTAAAGTCTAGGGTTTCCATGATCTACTTTGGAGGGCCAGCATTGACACAGAGGATT
GCACCATTGCCGCAGCTGCTGAGAGAGGGAGAGCAGAGCCTGTACAAGGACTTCACATGG
GGCGAGTACAAGAAGGCTGCCTACAACTCCAGGCTCGGGGACAACAGGCTGGCTCACTTC
CACAGGTAGCCACGCCTGCCGCCTGGGCCCGCTCAAAGAACAGCCTTTACCAAGACAGCC
AGCCAGCTAGAGCTAGCTTTAGCTAGCTGCTGCGGTTTCCCCCCCGATGAAGATGAAGAT
CAAGATCAAGATAAAGACGAGGAGAGCTACGGAAAAAAAAAAAAAAAAAAAAAAAAAAA
AGTACTCTGCGTTGATACCACTGCTT

Appendix 31 Nucleotide sequence similarity between the full-length cDNA of *HvGA2ox3* (query) and *OsGA2ox3* (subject)

Score = 1267 (686 bits), Expect = 0.0, Identities = 874/967(90%)

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Query  49    ATGGTGGTTCTCGCCGGCACGCCTGCCGTCGATCACATCCCGCTCCTCAGGTCCCCCGAC  108
             ||||||||||||||| ||| |||| |||||||||||||||||||| || ||||| || |||
Sbjct  1     ATGGTGGTTCTCGCTGGCCCGCCCGCCGTCGATCACATCCCGCTGCTGAGGTCGCCGGAC  60

Query  109   CCCGGGGACAACTTCTCCGGCATGCCGGTCGTGGACCTGTCCAGACCTGGCGCGCCGCGG  168
             ||||| |||  |||||||||| |||||||||| |||||   ||| || |||||| || ||
Sbjct  61    CCCGGCGACGTCTTCTCCGGCGTGCCGGTCGTCGACCTCGGCAGCCCCGGCGCGGCGAGG  120

Query  169   GCCATCGCCGACGCGTGCGAGCGCTTCGGCTTCTTCAAGCTCGTCAACCACGGGGGTGGCC  228
             ||| | |  ||||| |||||||| | ||| ||||||||| |||||||||||| ||||||
Sbjct  121   GCCGTGGTGGACGCCTGCGAGCGGTACGGGTTCTTCAAGGTCGTCAACCACGGCGTGGCC  180

Query  229   CTGGACGCGATGGAC-CGGCTCGAGTCGGAGGCCGTGAGGTTCTTCTCGCTGCCGCAGGC  287
               |||| ||||||||  ||| |||||||||||||| |||||||||| | | ||||| |
Sbjct  181   ACGGACACGATGGACAAGGC-CGAGTCGGAGGCCGTCAGGTTCTTCTCCCAGACGCAGCC  239

Query  288   CGACAAGGACCGCTCCGGCCCGGCCTACCCGTTCGGCTACGGCAGCAAGCGCATCGGGCT  347
             |||||||||||||||||||||||||||||||||| |||||||||||||| |||||| |
Sbjct  240   CGACAAGGACCGCTCCGGCCCGGCCTACCCGTTCGGGTACGGCAGCAAGCGGATCGGGTT  299

Query  348   CAATGGCGACATGGGGTGGCTCGAGTAcctcctcctcgccgtcgactccgcctcgctccc  407
             |||||||||||||||||||||||||||||||||||||||| |||||  ||| |||||| |
Sbjct  300   CAATGGCGACATGGGGTGGCTCGAGTACCTCCTCCTCGCCCTCGACGACGCGTCGCTCGC  359

Query  408   cgccgcctccgccgtcccgtcctgcgcgctcTTCCGGGCGGCGCTGAACGAGTACATCGC  467
             || ||||| | |||||||||||||||| ||||||||| || |||||||||||||| |
Sbjct  360   CGACGCCTGCACCGTCCCGTCCTGCGCGGTCTTCCGGGCCGCTCTGAACGAGTACATCTC  419

Query  468   GGCGGTGAGGAAGGTGGCGGTGCGGGTGATGGAGGCGATGGCGGAGGGGCTGGGCATTGC  527
             || |||| |||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  420   GGGGGTGCGGAAGGTGGCGGTGCGGGTGATGGAGGCGATGTCGGAGGGGCTGGGCATTGC  479
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Query  528   GCAGGCGGACGCGCTGAGCGCGATGGTGGCGGCGGAGGGCAGCGACCAGGTGTTCAGGGT  587
             ||||||||||||||||||||| ||||| ||||||| || ||||||||||||||| | ||
Sbjct  480   GCAGGCGGACGCGCTGAGCGCGCTGGTGACGGCGGAAGGGAGCGACCAGGTGTTCCGCGT  539

Query  588   GAACCACTACCCGCCGTGCCACGCGCTGCAGGGGCTCGGCTGCAGCGCCACCGGCTTCGG  647
             |||||||||||||||||| |||||||||||||||||||||||| ||||||||||||
Sbjct  540   GAACCACTACCCGCCGTGCCGCGCGCTGCAGGGGCTCGGCTGCAGCGTCACCGGCTTCGG  599

Query  648   CGAGCACACCGACCCGCAGCTCATCTCCGTGCTGCGCTCCAACGGCACGTCCGGCCTGCA  707
             |||||||||||||||||||||| |||||||||| ||||| ||||||||||||||||||||
Sbjct  600   CGAGCACACCGACCCGCAGCTCGTCTCCGTGCTCCGCTCAAACGGCACGTCCGGCCTGCA  659

Query  708   GATCGCGCTCCAGAG-CGGCCACTGGGTGTCCGTGCCCTCCGACCGCGACGCCTTCTTCG  766
             |||||||||||| | | |||||| |||||||||||||||||||||||| |||||||||
Sbjct  660   GATCGCGCTCC-GCGACGGCCAGTGGGTGTCCGTGCCCTCCGACCGCGACTCCTTCTTCG  718

Query  767   TCAACGTCGGCGACTCCTTGCAGGTGCTGACCAACGGGAGGTTCAAGAGCGTGAAGCACA  826
             ||||||||||||||||| |||||||| |||||||| |||||||||||||||||||||||||
Sbjct  719   TCAACGTCGGCGACTCGTTGCAGGTTCTGACCAATGGGAGGTTCAAGAGCGTGAAGCACA  778

Query  827   GGGTGGTGGCCAACAGCCTAAAGTCTAGGGTTTCCATGATCTACTTTGGAGGGCCAGCAT  886
             |||||||||||||||||||||||||||||||||| | |||||||||||||||| | |
Sbjct  779   GGGTGGTGGCCAACAGCCTAAAGTCTAGGGTTTCCTTCATCTACTTTGGAGGGCCACCGT  838

Query  887   TGACACAGAGGATTGCACCATTGCCGCAGCTGCTGAGAGAGGGAGAGCAGAGCCTGTACA  946
             |  |||||||||||||||||||| ||||||||| | ||||||||||||||||||||||
Sbjct  839   TAGCACAGAGGATTGCACCATTGCCACAGCTGCTGGGGGAGGGAGAGCAGAGCCTGTACA  898

Query  947   AGGACTTCACATGGGGCGAGTACAAGAAGGCTGCCTACAACTCCAGGCTCGGGGACAACA  1006
             |||| ||||||||||  |||||||||||||||||||||| || ||||| || |||||||
Sbjct  899   AGGAGTTCACATGGGATGAGTACAAGAAGGCTGCCTACAAATCAAGGCTTGGAGACAACA  958

Query  1007  GGCTGGC  1013
             |||||||
Sbjct  959   GGCTGGC  965
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