GENETIC AND MOLECULAR BASES OF ABSCISIC ACID AND GIBBERELLIN METABOLISM IN REGULATING SEED DORMANCY IN MALTING BARLEY

By

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TABLE OF CONTENTS

ACKNOWLEDGEMENT i

TABLE OF CONTENTS ii

LIST OF TABLES viii

LIST OF FIGURES ix

ABSTRACT xii

FORWARD xiii

1.0 GENERAL INTRODUCTION 1

2.0 LITERATURE REVIEW 4

2.1 Importance of barley 4

2.2 Origin and domestication of barley 4

2.3 Barley production 5

2.4 Physical and chemical characteristics of a barley grain 6

2.5 Uses of barley grains 7

2.6 Constraints in barley production 8

2.7 Seed germination 9

2.8 Preharvest sprouting 10

2.9 Seed dormancy 12

2.10 Types of seed dormancy 12

2.11 Release of seed dormancy 15

2.12 Regulation of seed dormancy and germination 16

2.12.1 Environmental factors 16

2.12.2 Plant hormones 17

2.12.3 Genetic factors 18

2.13 Abscisic acid and its role in plant developmental processes and seed dormancy 19

2.13.1 Abscisic acid metabolism.. 20

2.13.2 ABA metabolism genes and their role in the regulation of seed dormancy ... 24

2.14 Gibberellin and its roles in seed germination 26

2.14.1 Gibberellin metabolism pathway 28

2.14.2 Gibberellin metabolism genes... 30

3.0 GENETIC AND MOLECULAR BASES OF ABSCISIC ACID AND GIBBERELLIN METABOLISM IN REGULATING SEED DORMANCY IN MALTING BARLEY 34

Abstract 34

3.1 Introduction 34

3.2 Materials and Methods 39

3.2.1 Plant materials and growth conditions 39

3.2.2 Seed germination phenotype... 41

3.2.3 Molecular cloning of *ABA8'OH1* and *GA20ox1* from different barley lines 41

3.2.3.1 DNA extraction 41

3.2.3.2 PCR amplification 42

3.2.3.3 Gel extraction 43

3.2.3.4 Ligation and transformation 44

3.2.3.5 Plasmid isolation 44

3.2.3.6 Restriction enzyme digestion 45

3.2.3.7 Sequence Analysis... 45

3.2.4 Gene expression of ABA and GA metabolic genes 46

3.2.4.1 Tissue collection 46
3.2.4.2 RNA extraction 47
3.2.4.3 DNA digestion 47
3.2.4.4 cDNA synthesis 48
3.2.4.5 Primers 48
3.2.4.6 Real-time qPCR 49
3.2.4.7 Statistical analysis 50
3.3 Results 50
3.3.1 Seed germination 50
3.3.2 Molecular cloning of *ABA8'OH1* 52
3.3.2.1 PCR amplification of target gene 52
3.3.2.2 Analysis of *ABA8'OH1* gene sequence for allelic variation 54
3.3.2.3 Analysis of nucleotide sequence for exon-intron structure of *ABA8'OH1* 55
3.3.2.4 Protein sequence analysis of *ABA8'OH1* 57
3.3.2.5 Conserved domain and phylogenetic analysis of *ABA8'OH1* 59
3.3.3 Expression analysis of ABA metabolic genes during imbibition 61
3.3.3.1 Expression patterns of *NCED* genes 61
3.3.3.1.1 Correlation between seed germination and *NCED1* expression 62
3.3.3.2 Expression of *ABA8'OH1* in the embryo 63
3.3.3.2.1 Correlation between seed germination and embryonic *ABA8'OH1* expression 64

3.3.3.2.2 Correlation between embryonic *NCED1* and *ABA8'OH1* expression in the embryo 65

3.3.3.3 Expression of *ABA8'OH1* in the endosperm..... 66

3.3.3.3.1 Correlation between seed germination and endospermic *ABA8'OH1* expression..... 68

3.3.3.4 Expression of *ABA8'OH2* in the embryo tissue..... 69

3.3.3.4.1 Correlation between embryonic *ABA8'OH2* expression and seed germination 70

3.3.3.5 Expression of *ABA8'OH2* in the endosperm..... 71

3.3.3.5.1 Correlation between seed germination and endospermic *ABA8'OH2* expression..... 72

3.3.4 Molecular cloning of *GA20ox1*..... 73

3.3.4.1 Amplification of *GA20ox1* 73

3.3.4.2 Sequence analysis of *GA20ox1*..... 75

3.3.4.3 Analysis of the exon-intron structure of *GA20ox1*..... 76

3.3.4.4 Translation of the coding sequence of *GA20ox1*..... 77

3.3.4.5 Conserved domain and phylogenetic analysis of *GA20ox1* 80

3.3.5 Expression of gibberellin biosynthesis genes 82

3.3.5.1 Expression of *GA20ox2* in the embryo..... 82

3.3.5.1.1 Correlation between seed germination and *GA20ox2* expression..... 83

3.3.5.2 Expression of *GA20ox3* in the endosperm 84

3.3.5.2.1 Correlation between seed germination and *GA20ox3* expression 85

3.4 Discussion 87

4.0 GENERAL DISCUSSION AND CONCLUSIONS 95

LITERATURE CITED 98

APPENDIX 116

Appendix 1. Analysis of variance of seed germination percentage of the different barley lines 116

Appendix 2. Nucleotide substitution/INDELS in the genomic DNA sequence of *ABA8'OH1* 117

Appendix 3. Phylogenetic analysis of the ABA8′OH1 proteins from barley and other species. 117

Appendix 4. Analysis of variance of relative transcript levels of *NCED1* in the embryos of the different barley lines 119

Appendix 5. Correlation between the seed germination percentage and expression levels of embryonic *NCED1* of the different barley lines 120

Appendix 6. Analysis of variance of relative transcript levels of *ABA8'OH1* in the embryos of the different barley lines 121

Appendix 7. Correlation between the seed germination percentage and expression levels of embryonic *ABA8'OH1* of the different barley lines 122

Appendix 8. Analysis of variance of relative transcript levels of *ABA8'OH1* in the endosperm of the different barley lines 123

Appendix 9. Correlation between the seed germination percentage and expression levels of endospermic *ABA8'OH1* of the different barley lines 124

Appendix 10. Analysis of variance of relative transcript levels of *ABA8'OH2* in the embryos of the different barley lines.. 125

Appendix 11. Correlation between the seed germination percentage and expression levels of embryonic *ABA8'OH2* of the different barley lines........................... 126

Appendix 12. Analysis of variance of relative transcript levels of *ABA8'OH2* in the endosperm of the different barley lines.. 127

Appendix 13. Correlation between the seed germination percentage and expression levels of endospermic *ABA8'OH2* of the different barley lines 128

Appendix 14. Phylogenetic tree of GA20ox proteins from barley and other species............... 129

Appendix 15. Analysis of variance of relative transcript levels of *GA20ox2* in the embryo of the different barley lines 130

Appendix 16. Correlation between the seed germination percentage and expression levels of embryonic *GA20ox2* of the different barley lines............................... 131

Appendix 17. Analysis of variance of relative transcript levels of *GA20ox3* in the endosperm of the different barley lines.. 132

Appendix 18. Correlation between the seed germination percentage and expression levels of endospermic *GA20ox3* of the different barley lines 133

ABBREVIATIONS 134

LIST OF TABLES

Table 3.1 List of selected barley lines used in this study 40

Table 3.2 Primer used for amplifying the target genes 43

Table 3.3 Primer used for sequencing of the target genes 46

Table 3.4 Primer sequences used for qPCR 49

Table 3.5 Dormancy phenotype of the different malting barley lines 52

Table 3.6 Nucleotide sequence identity of the sequence of *ABA8'OH1* from the different barley lines with other *ABA8'OH1* gene sequences 54

LIST OF FIGURES

Figure 2.1 ABA biosynthesis and inactivation pathway in plants 23

Figure 2.2 Gibberellin metabolic pathway in plants 29

Figure 3.1 Germination percentages of the mature seeds of the different barley lines over a period of 7 days. 51

Figure 3.2 PCR amplification of the genomic sequence of *ABA8'OH1* from the malting barley genotypes and breeding lines. 53

Figure 3.3 Multiple sequence alignment of *ABA8'OH1* showing nucleotide polymorphisms among different barley lines. 55

Figure 3.4 Genomic DNA sequence of *ABA8'OH1* from start to stop codon............................ 56

Figure 3.5 Amino acid sequence of *ABA8'OH1* aligned with the cDNA nucleotide sequence. 58

Figure 3.6 Amino acid sequence alignment of barley and other ABA8′OH1 proteins. 60

Figure 3.7 Relative transcript level of *NCED1* in the embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype………………..……………...64

Figure 3.8 Correlation between the seed germination percentage and expression levels of *NCED1* in different barley lines 63

Figure 3.9 Relative transcript level of *ABA8'OH1* in the embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype………………………..66

Figure 3.10 Correlation between the seed germination percentages and expression levels of embryonic *ABA8'OH1* of different barley lines 65

Figure 3.11 Correlation between the expression levels of embryonic *NCED1* and *ABA8'OH1* expression in the embryos 66

Figure 3.12 Relative transcript level of *ABA8'OH1* in the endosperm tissues at 24 HAI in different barley lines with a range of dormancy phenotype. 67

Figure 3.13 Correlation between the seed germination percentage and expression levels of endospermic *ABA8'OH1* of different barley lines 68

Figure 3.14 Relative transcript level of *ABA8'OH2* in the embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype.................................... 70

Figure 3.15 Correlation between the expression levels of embryonic *ABA8'OH2* and seed germination percentages of different barley lines 71

Figure 3.16 Relative transcript level of *ABA8'OH2* in the endosperm tissues at 24 HAI in different barley lines with a range of dormancy phenotype…………………………73

Figure 3.17 Correlation between the seed germination percentages and expression levels of endospermic *ABA8'OH2* of different barley lines 73

Figure 3.18 PCR amplification of *GA20ox1* from selected malting barley genotypes and breeding lines 74

Figure 3.19 Multiple alignment of the nucleotide sequence of *GA20ox1* that shows nucleotide polymorphisms among the different barley lines. 76

Figure 3.20 Genomic DNA sequence of *GA20ox1* showing the exon and intron. 77

Figure 3.21 Amino acid sequence of *GA20ox1* with the corresponding nucleotide sequence. 79

Figure 3.22 Amino acid sequence alignment of barley and other GA20ox1 proteins................. 81

Figure 3.23 Relative transcript level of *GA20ox2* in embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype……………………….....84

Figure 3.24 Correlation between the seed germination percentages and expression levels of embryonic *GA20ox2* in different barley lines 84

Figure 3.25 Relative transcript level of *GA20ox3* in the endosperm tissues at 24 HAI in different barley lines with a range of dormancy phenotype................................. 85

Figure 3.26 Correlation between the seed germination percentage and expression levels of endospermic *GA20ox3* of different barley lines 86

ABSTRACT

Toora, Parneet Kaur. M.Sc. The University of Manitoba, November, 2018. Genetic and molecular bases of abscisic acid and gibberellin metabolism in regulating seed dormancy in malting barley. M.Sc. supervisor: Dr. Belay T. Ayele.

Preharvest sprouting (PHS), the germination of the grain prior to harvest under high moisture conditions, is one of the major problems for barley producers and malting industry since it causes reduction in yield and malt quality. PHS is associated with the level of dormancy, a trait that blocks the germination of an intact viable seed under favorable environmental conditions. Reduced seed dormancy allows the seeds to germinate under suitable environmental conditions and causes PHS whereas high dormancy leads to non-uniform germination and problems in the malting process. Therefore, controlling the level of seed dormancy is important in barley to prevent PHS damage and permit uniform germination after harvest during malting. Abscisic acid (ABA), which induces dormancy, and gibberellin (GA), which induces germination, are plant hormones that act antagonistically and control the level of dormancy present in seeds and seed's ability to germinate. The amount of these compounds in seeds is determined by their respective metabolic pathways. Therefore, this thesis project was focused on investigating the molecular bases of ABA and GA metabolism in different malting barley lines with varying level of seed dormancy.

FORWARD

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

1.0 GENERAL INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the major cereal crops cultivated throughout the world, and Canada is the fifth largest producer of barley (USDA, 2017). It is widely adaptable to a range of environmental conditions and grown for various purposes such as malting, animal feed and food processing. However, the production of barley is negatively affected by biotic and abiotic stress factors that can significantly influence its development, growth and productivity. PHS is of a great concern for barley producers and malt industry as it is the main cause for the reduction of yield and crop value (Gubler *et al.,* 2005), as PHS damaged seeds cannot be used for brewing purposes and are often downgraded as animal feed.

The degree of PHS tolerance is closely related to the level of seed dormancy which is defined as a trait that prevents the germination of an intact viable seed under favorable environmental conditions (Gao and Ayele, 2014). Seed dormancy is imposed during the later stages of seed development and prevents premature germination of the seed. In spite of preventing PHS, seed dormancy can also delay the malting process and cause non-uniform germination (Sato *et al.,* 2009). Thus, the control of dormancy is of great practical importance in the production of malting barley. Seed dormancy is influenced by a number of exogenous and endogenous factors; some endogenous factors such as plant hormones are involved in the control of seed dormancy and germination, and abscisic acid (ABA) and gibberellin (GA) are reported to be the major regulators (Finkelstein *et al.,* 2008). The balance between these two hormones determines seeds ability to maintain dormancy or germinate (Tuan *et al.,* 2018).

Abscisic acid controls the onset and maintenance of primary dormancy in several plant species and its level is controlled by the balance between its biosynthesis and catabolism (Fidler *et al.,* 2015). The major regulatory enzyme involved in the ABA biosynthesis is nine-*cis*-epoxy

carotenoid dioxygenases encoded by *NCEDs* (Nambara and Marion-Poll, 2005). In Arabidopsis, the ABA biosynthetic mutants displayed high germination as compared to the wild type whereas overexpression of ABA biosynthetic genes led to high dormancy, suggesting the importance of *NCED* in accumulation of bioactive ABA and seed dormancy (Lefebvre *et al.,* 2006). Imbibition of after-ripened Brachypodium seeds displayed low ABA content due to low expression of *NCED*, suggesting the role of this gene in ABA synthesis and dormancy (Barrero *et al.,* 2012). In cereals including barley and wheat the *NCED* genes have been reported to play roles in regulating the accumulation of bioactive level of ABA and enhanced seed dormancy (Chono *et al.,* 2006; Son *et al.,* 2016). The catabolism of ABA mainly takes place through ABA 8′ hydroxylation, which is catalyzed by ABA8′OH, which is encoded by the cytochrome P450 monooxygenase 707A (*CYP707A*, also referred to as *ABA8'OH*) gene family (Okamoto *et al.,* 2006). Studies in Arabidopsis showed that single and double mutants of *ABA8'OH* family genes have higher level of ABA and increased dormancy whereas overexpression results in decreased ABA content and dormancy indicating the role of ABA catabolism in dormancy (Kushiro *et al.,* 2004; Millar *et al.,* 2006; Okamoto *et al.,* 2006). Previous studies have shown the importance of the wheat homologs of this gene in the regulation of ABA level and dormancy (Chono *et al.,* 2013; Son *et al.,* 2016; Izydorczyk *et al.,* 2018). The barley *ABA8'OH1* has been reported to be involved in lowering the level of ABA and dormancy release (Millar *et al.,* 2006; Leymarie *et al.,* 2008). Further genetic analysis showed the physiological role of *ABA8'OH1* in the control of ABA level and seed dormancy and germination (Gubler *et al.,* 2008).

Gibberellin, which is known to act antagonistic to ABA, positively influences seed germination (Finch-Savage and Leubner-Metzger, 2006). The biosynthesis of GA is catalysed mainly by GA 20-oxidase and GA 3-oxidase enzymes, which are encoded by *GA20ox* and *GA3ox*

gene families, respectively, while the catabolism of GA is catalysed primarily by GA 2-oxidase, encoded by *GA2ox* (Yamaguchi, 2008). Analysis of the *GA20ox1*, *2*, and *3* mutants demonstrated the role of *GA20ox* genes in the production of bioactive GA and regulation of seed germination in Arabidopsis (Plackett *et al.,* 2012). A study in wheat has shown that GA biosynthesis plays a major role in controlling GA level and seed germination rather than its catabolism (Izydorczyk *et al.,* 2018), and the *GA20ox* genes are reported to play regulatory roles with this respect. For example, increased expression of embryonic *GA20ox1* leads to increased accumulation of bioactive GA and enhanced germination in wheat seeds (Izydorczyk *et al.,* 2018). In other cereal crops such as rice, loss of function mutation in *GA20ox2* is also found to be responsible for reduced GA level and enhanced dormancy, suggesting the role of GA in controlling seed germination (Ye *et al.,* 2015). For better understanding of the roles of ABA and GA in dormancy and PHS tolerance, this study investigated the relationship between seed dormancy levels, tissue specific expression patterns of ABA and GA metabolic genes, and allelic variation in ABA catabolic gene *ABA8'OH1* and GA biosynthetic gene *GA20ox1* using different malting barley lines.

2.0 LITERATURE REVIEW

2.1 Importance of barley

Barley (*Hordeum vulgare* L.) is one of the major cereal crops that belongs to the Poaceae or grass family. It is ranked as the fourth most important cereals crop next to maize, rice and wheat (Zohary and Hopf, 2001). Approximately thirty species of the genus *Hordeum* have been domesticated over four continents (Clark, 1967). Barley is not only an important crop (Baik and Ullrich, 2008) but also serves as a model for studying the genetics and genomics of cereal crops (Sreenivasulu *et al.,* 2008). Barley is a widely adapted crop and is used as a major source of food in extreme climates such as Himalayas, Ethiopia and Morocco (OECD, 2004; Baik and Ullrich, 2008; Kremer and Ben-Hammouda, 2009). The genetics of barley is very diverse, and it is divided into winter and spring barley, two or six rowed barley, hulled or hulless barley, and malting or feed barley. Based on grain composition, barley is classified as normal, waxy, high lysine, high β-glucan, high amylose starch or proanthocyanidin free. Its genetically diverse nature provides great opportunities for breeding to increase grain yield and quality. It has immense health benefits, for example, it consists of β-glucans and the antioxidant tocols including tocopherols and tocotrienols that help in lowering the levels of blood cholesterol and serum low density lipoprotein (LDL) cholesterol, respectively (Idehen *et al.,* 2017).

2.2 Origin and domestication of barley

Barley was one of the first crops to be domesticated along with wheat, pea, and lentils. Barley was domesticated from its wild relative (*Hordeum spontaneum* C. Koch) about 8000 B.C. as confirmed by the archaeologists who found some barley grain fractions in the Fertile Crescent. The wild barley was first domesticated in the Israel-Jordan area of Fertile Crescent and then seeds migrated

from the Near East to South Asia. Therefore, the Himalayan-Tibet region is considered a region of domesticated barley diversification (Badr *et al.,* 2000). According to the modern taxonomy, *H. vulgare, H. spontaneum* and *H. agriocrithon* are considered subspecies of *H. vulgare* (Bothmer *et al.,* 2003). The wild barley gave rise to cultivated barley with the same base chromosome number of seven. Wild and domesticated barley are two-row, and the six-row and hulless types of barley are formed from the domesticated two-row types through some mutations (Badr *et al.,* 2000). The selection of cultivated barley from its wild relative during domestication improved the harvest, storage, and utilization of barley. Some of the characters that were selected include reduction/loss of dormancy, rapid germination after sowing, non-brittle rachis and non-shattering of pods, short flowering time and maturity, increased yield per plant, disease resistance and stress tolerance. However, the most important characters for selection are loss of seed dormancy and non-shattering spikes where the seeds stick to the rachis to prevent yield loss (Gross and Olsen, 2010). Initially barley was domesticated for human food but later it was used as feed for animals, malting and brewing purposes because of the preference of consumers for wheat and rice as major food crops. Approximately 65% of the barley produced in western Canada in the year 2017 was used for malting, 34% for feed and about 1% for food (Canadian Grain Commission, 2017).

2.3 Barley production

Barley has a wide geographical distribution from Israel, Turkey, Syria, and Egypt, eastward to Pakistan, India and western China. The selection of the varieties well suited to an end use is very important. Spring barley is planted in the northern hemisphere in April or May when winters are too severe. Winter barleys is planted in September or November in warm regions. The brewing industry uses more than 60 % of the barley produced. As malting and brewing industry is

dependant on barley seed germination therefore barley can be used as a model crop for seed germination research (Mahalingam *et al.,* 2017). In 2017, the worldwide barley production was reported to be 144.26 million tonnes. In the same year, European Union produced the highest amount of barley (59.06 million tonnes) followed by Russia (20.18 million tonnes), Ukraine (8.70 million tonnes), Australia (8.90 million tonnes) and Canada (7.50 million tonnes) (USDA, 2017). The first cultivation of barley in Canada took place in Port Royal in 1606. In western Canada, the total area under barley cultivation in the year 2017 was 2.22 million hectares with the production of about 7.52 million tonnes (Canadian Grain Commission, 2017). Alberta and British Columbia combined produced 3.94 million tonnes of barley in 2017 followed by Saskatchewan (3.13 million tonnes) and Manitoba (0.43 million tonnes) (Canadian Grain Commission, 2017).

2.4 Physical and chemical characteristics of a barley grain

The barley grain consists of the seed, rachilla, palea and lemma. Covered (hulled) barley is characterized by one of these structures attached to the seed. The barley seed consists of the embryo, endosperm and the pericarp. The embryo uses the nutrients derived from the endosperm tissue for its growth and development. The texture of endosperm and color of barley are main traits considered for end use quality. Due to the presence of variable level of anthocyanin, the color of the barley grain varies from light yellow to purple, violet, blue, and black. Barley grain which has clean, bright yellow-white, plump, thin, hulled, medium hard or uniform size is considered good for pearling and food (Pomeranz 1974). White and clean pearled grains and milled flours are preferred by consumers and food manufacturers. Pearled barley can be used as a substitute to polished rice. During the roller milling, barley bran shatters easily making it difficult to produce white flour free from bran particles. The malting barley varieties have soft kernels whereas the

non-malting barley has hard kernels (Fox *et al.,* 2007). The whole grain of barley generally consists of 65-68% of starch, 10-17% protein, 4-9% β-glucan, 2-3% free lipids and 1.5-2.5% minerals (Quinde *et al.,* 2004, Baik *et al.,* 2008). About one-third of the total barley proteins are left in the final beer and a major fraction (80%) of the total proteins is hordeins (storage proteins) (Mahalingam *et al.,* 2017).

2.5 Uses of barley grains

The barley grain is used as animal feed, human food and for beer. The barley grain can be used as feed for poultry, swine, sheep, and cattle, and over 60 percent of the total barley produced worldwide is used for animal feed. The "feed barley" varieties are known to have high protein content and the use of barley as animal feed causes less contamination of the environment with phosphorus from the animal waste since animals can more readily absorb the phosphorus in barley than other feed grains (Bleidere and Gaile, 2012). Barley, mainly hulless barley is used for human consumption due to its ease for processing of food items. A bright white barley kernel obtained after pearling of barley through removal of grain tissues (pericarp, testa, aleurone and germ) is ideal for various food applications (Yeung and Vasanthan, 2001). The layers removed from the grain are called pearl flour and are usually sold as feed, and the remainder part of the grain is called pearled grain (Yeung and Vasanthan, 2001). Pearled grain of barley is used as a rice substitute for the production of soy paste and soy sauce in Korea (Ryu, 1979). Some other food related applications of barley include its use for baking breads and crackers, and making breakfast cereals, noodles and baby foods. One of the major uses of barley is to produce malt, and malting barley is characterized by its high starch content as compared to feed barley. Both two-rowed and six-rowed

malting barley can be used for beer production as long as the grains meet stringent specifications of the malting and brewing industry.

2.6 Constraints in barley production

Grain quality of barley has a direct influence on its end-use quality. While non-sprouted and good quality barley seeds are used for malting purposes, barley with lower quality is used for feed purposes. However, the flavor, texture and taste of barley food products are poor as compared to other cereal crops such as wheat and rice thereby, decreasing its use as a food crop (OECD, 2004). As a result, crops like wheat and rice hold a huge market of food products (Baik and Ullrich, 2008).

Barley seeds require moderate level of dormancy for achieving good yield and quality. However, strong dormancy affects the malting quality as germination of up to 50% within 1-2 days and 95-100% within 3 days of imbibition is required for malting barley seeds. Low level of dormancy on the other hand leads to sprouting of the seeds on the parent plant (defined as pre-harvest sprouting) during maturation even with exposure to very low level of moisture. Thus, pre-harvest sprouting affects barley yield and quality tremendously. Other abiotic stress factors that affect barley production include extreme temperatures and drought. It has been reported that an increase in average temperature by $1^{\circ}C$ leads to a 4.1 to 10.0 % decrease in grain yield of cereals such as barley (Hatfield *et al.,* 2011). Furthermore, barley is very sensitive to elevated soil temperature and acidity (Petra *et al.,* 2013). Increases in drought periods result in a decrease in grain filling period and ultimately reduction in grain yield and quality in barley (Savin *et al.,* 1997).

Apart from the abiotic stress factors, many biotic stresses including diseases and insect-pests affect barley production (Walters *et al.,* 2012). Diseases affect the yield and quality of barley grain every year, leading to a considerable damage to productivity. Some of such diseases include

leaf scald or blotch (causal organism, *Rhynchosporium commune)*, powdery mildew (causal organism, *Blumeria graminis f. sp. Hordei)*, net blotch (causal organism, *Pyrenophora teres f. teres)*, spot blotch (causal organism, *P. teres f. maculata*), brown rust (causal organism, *Puccinia hordei)*, yellow rust (causal organism, *Puccinia striiformis)*, and Ramularia leaf spot (causal organism, *Ramularia collo-cygni)* (Walters *et al.,* 2012).

2.7 Seed germination

Seed germination begins when the quiescent dry seed absorbs water and terminates with the protrusion of the radicle through seed covering layers (Bewley and black, 1994). Further growth of the seedling follows through the depletion of the storage reserves. The process of germination has several requirements such as overcoming the physical barriers to growth by activating the cell wall degradation enzymes and resumption of active growth through cell elongation and division accompanied by mobilization of the storage reserves, which is accumulated during the seed filling/maturation phase (Finkelstein *et al.,* 2008). Seed germination is triphasic in terms of water uptake. It starts with the rapid uptake of water by the seed or imbibition (phase 1), followed by slow and constant rate of water uptake forming a plateau phase (phase 2), and another increase of water up take (phase 3). During the first phase of water uptake, structural changes in cell membranes of seeds occur causing fast leakage of solutes and low molecular weight metabolites into the surrounding imbibition medium. The seed activates the repair of cell membranes, DNA and mitochondria damaged by the desiccation process. After imbibition, the seed starts respiratory activities and protein synthesis using the transcripts already present in the dry seed (Bewley, 1997).

In the second phase of water uptake, the seed resumes its metabolic activity and prepares for subsequent growth. The energy (ATP) for first few hours after imbibition is provided by the

mitochondria present in the mature dry seeds and repaired during imbibition. After that, the energy is provided by the newly synthesized mitochondria in the oil storing seeds, and repaired and revived mitochondria present inside the starchy seeds (Bewley, 1997). Furthermore, enzymes required for overcoming the constraints of seed covering layers and inducing the embryo growth potential are synthesized to trigger the emergence of radicle. The end of the second phase of germination is marked by the emergence of the radicle out of the seed covering layers, and then the seed enters into third phase of water uptake, which is associated with the growth phase.

The third phase is marked by further increase of water uptake by the seed, and during this phase cell division and DNA synthesis that are required for growth of the radicle/seedling occur (Bewley, 1997). Seed storage reserves of the endosperm tissue are also mobilized during this phase to support the growth of the seedling. The growth phase of germination is regulated by diverse signals such as light and water availability, salinity, temperature, and nutrient status, and it is the most critical phase of the life cycle of the plant (Finkelstein, 2010).

2.8 Preharvest sprouting

Preharvest sprouting is defined as the germination of the seed while still on the mother plant under moist conditions (Fang and Chu, 2008; Rodríguez *et al.,* 2015), and it is regulated by a number of genetic and environmental factors (Gao *et al.,* 2013; Gubler *et al.,* 2005; Rodríguez *et al.,* 2015). Wheat, rye and triticale are particularly more affected by PHS (Fidler *et al.,* 2015). PHS is the main cause of yield, productivity and crop value reduction in cereals (Gubler *et al.,* 2005); it is estimated to cause huge economic losses up to $1 billion worldwide (Black *et al.,* 2006). After PHS damage, the grain cannot be used for bread and brewing purposes rather it is often downgraded as animal feed. Moreover, PHS damage may lead to loss of grain viability. PHS of

cereal is tightly related to the extent of seed dormancy, a trait that inhibits seed germination under favorable environmental conditions (Gao and Ayele, 2014). In cereal crops such as barley, high level of dormancy at harvest is not good for the next growing season as it affects rate and uniformity of germination. In addition, it causes the need for longer storage times as dormant barley grains need to be after-ripened before the malting process begins and thereby increased storage costs (Rodriguez *et al.,* 2015). Seed storage is also associated with reduction in germination energy and seed quality, which ultimately causes poor-malt quality possessing high level of wort, β-glucan and low-extract. Contrary, low seed dormancy gives rise to PHS. This entails the need to breed for intermediate level of dormancy to prevent not only PHS but also the problem of non-uniform germination at the time of planting (Chono *et al.,* 2013).

Preharvest sprouting is generally measured more exactly by visually measuring the sprouting score and/or α-amylase activity, or indirectly by falling number test (Ullrich *et al.,* 2008). There are different levels of sprouting damage to the seed. In the case of slightly damaged grains, there is still a possibility of obtaining good quality malt as long as the barley grains are stored under cool and dry conditions that prevent further occurrence of biochemical processes that can lead to the loss of germination potential of the seed (Edney *et al.,* 2013). The major enzyme responsible for the conversion of starch into simple sugars is α-amylase. When humid and moist conditions occur before or at the time of harvest, susceptible mature grains sprout rapidly, triggering increased expression and activity of α-amylases, which causes degradation of the stored starch reserves of the endosperm. One method by which PHS damage in barley grains is estimated is through the rapid viscosity analyser (RVA), which is based mainly on inverse relationship between viscosity of the barley/water slurry, and amount of α-amylase in barley grains (Izydorczyk, 2008). The level of PHS damage can also be assessed by methods such as falling

number test and seed germination percentage. The falling number is estimated based on the activity of α-amylase enzyme; the poor and good quality malting barley grains have high and low falling numbers, respectively. Seed germination percentage is determined based on the number of seeds germinated per the total number of seeds imbibed.

2.9 Seed dormancy

Seed dormancy is an adaptive trait that makes viable seeds unable to germinate under optimal conditions including adequate amount of moisture and oxygen, and optimal temperature (Finch-Savage and Footitt, 2017). It is a quantitative genetic trait involving many genes and influenced by the environment (van der Schaar *et al.,* 1997). The level of dormancy can be measured by seed germination percentage. Wild species of crop plants exhibit seed dormancy to survive unfavorable conditions and decrease competition with other species whereas selection against dormancy has been practiced on domesticated agricultural crops such as barley and wheat, leading to weak dormancy (Nakamura *et al.,* 2016). Dormancy is induced during seed development and maintained through seed maturity. Several endogenous and exogenous factors including hormones, light quality, temperature, and nutrition affect the maintenance of seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.,* 2008).

2.10 Types of seed dormancy

Seed dormancy can be classified into two types based on the time of its occurrence: primary and secondary dormancy. The primary seed dormancy is attained during the seed maturation phase and secondary dormancy is the type of dormancy induced in non-dormant seeds due to the occurrence of unfavorable environmental conditions (Finkelstein *et al.,* 2008). The primarily dormant seeds

have a very low probability of germination under optimal conditions. It is caused mainly by ABA level and sensitivity during seed development (Bewley, 1997). The accumulation of ABA is low during the early stages of seed development, however, its level is highest during the mid-phase of seed development when storage reserves are synthesized, and the level declines as the seed passes through maturation drying.

Primary dormancy is further divided into five types namely, physiological (PD), morphological, morpho-physiological, physical and combinatorial dormancy (Nikolaeva, 1969; Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). Physiological dormancy is the most common type of seed dormancy in model species such as Arabidopsis, Sunflower, tomato and tobacco, and several cereal crop species. Physiological dormancy is further divided into three types; deep, intermediate and non-deep. In case of seeds with deep level of primary dormancy, excised embryos produce abnormal seedling and seeds require a relatively longer period of stratification (7 to 14 weeks) and after-ripening (about 3-4 months) to break dormancy (Nikolaeva, 1969). In addition, their dormancy cannot be broken by the GA treatment. Embryos excised from seeds with intermediate level of primary dormancy produce normal seedling and such seeds require a shorter period of stratification for promoting germination. The most abundant form of dormancy in seeds is the non-deep dormancy; non-deep dormant embryos also produce normal seedlings and this type of dormancy can be broken by GA treatment, scarification, after-ripening, and cold or warm stratification depending on species. Non-deep dormancy is classified into two types: embryo and seed-coat imposed dormancy. Some species have a difficulty of germination due to the inability of the embryo to germinate whereas some seeds with non-dormant embryos are unable to germinate due to structures around its embryo. Hard-seed coat/seed covering layers such as the endosperm in species such as Arabidopsis prevent the uptake of water during seed germination

(waxy or lignified tissue in legume seeds), cause mechanical obstruction due to radicle emergence, interfere with gaseous exchange, and are able to retain or produce germination inhibiting compounds such as ABA. In some species, the glumellae (lemma and palea) also acts as a restriction for embryo germination (Corbineau and Come, 1980; Benech-Arnold *et al.,* 1999). Embryo dormancy is caused by some inherent characteristics of the embryo such as amount of ABA, and embryo sensitivity to this phytohormones (Bewley, 1997). In species such as Arabidopsis and coffee, ABA inhibits germination through suppression of embryo growth potential and the weakening of the endosperm/seed covering layers (Muller *et al.,* 2006; da Silva *et al.,* 2004).

In seeds that show morphological dormancy, the embryos are under developed and are non-dormant physiologically (Baskin and Baskin, 1998). These embryos can germinate once they are fully matured. Morpho-physiological dormancy is caused by under developed embryos along with some physiological dormancy. Seeds with this type of dormancy require dormancy breaking treatments and prolonged period for germination. Physical dormancy is due to the presence of some palisade water-impermeable layers in the seed coat (Baskin *et al.,* 2000), and such type of dormancy can be broken by mechanical or chemical scarification to let water move into the seed to support germination. Combinatorial dormancy is the mixture of physiological and physical dormancy. In this case, the embryo is not able to germinate, and the seed coat is water impermeable. Usually, the physiological component of dormancy is non-deep type and the embryo can germinate very easily after dry storage or some time after seed maturation under field conditions.

2.11 Release of seed dormancy

Seed dormancy can be broken by treatments such as after-ripening, cold stratification, warm stratification, light, nitrate, and treatment with GA (Bewley and Black, 1994; Bethke *et al.,* 2006). The period of dry storage required for breaking dormancy depends on species, and seeds of some barley species can lose dormancy within few weeks while other species can maintain dormancy for extended period of time (Bewley and Black, 1994). For example, a barley cv. Ashkelon was found to lose dormancy after 28 days of after ripening whereas cv. Mehola takes 70 days of after-ripening to lose dormancy and germinate (Vanhala and Stam, 2006). Another study also found that cv. Haruna Nijo losses dormancy after 5 weeks of after-ripening whereas cv. H602 remained completely dormant after 5 weeks of after-ripening (Sato *et al.,* 2009). Seed moisture content, storage temperature and oxygen determine the efficiency of after-ripening in breaking dormancy. Generally, 8-15% of seed moisture is required for after-ripening (Leubner-Metzger, 2005). The loss of seed dormancy in cereals is associated with changes in gene expression, protein expression and epigenetic modifications (Gao *et al.,* 2012; Gao *et al.,* 2013; Gao and Ayele, 2014). Various studies explained the importance of the metabolic and signaling components of different phytohormones in the maintenance and release of seed dormancy (Liu *et al.,* 2013; Shu *et al.,* 2016), mainly the balance of ABA and GA (Baskin and Baskin, 2004). ABA promotes induction and maintenance of seed dormancy whereas GA helps in promotion of seed germination (Kucera *et al.,* 2005; Finkelstein *et al.,* 2008). Therefore, low ABA: GA ratio due to enhanced GA biosynthesis and ABA degradation accounts for the release of seed dormancy (Ali-Rachedi *et al.,* 2004; Shu *et al.,* 2016; Finch-Savage and Footitt, 2017). A decrease in the embryo ABA content plays a role in seed dormancy release whereas GA promotes germination (Fidler *et al.,* 2015). Instead of ABA content, ABA sensitivity is associated with the level of seed dormancy leading to

assumption that changes in ABA signaling are responsible for dormancy release (Gubler *et al.,* 2005).

2.12 Regulation of seed dormancy and germination

2.12.1 Environmental factors

Seed germination and dormancy are regulated by several environmental factors such as oxygen, water, light, temperature and nitrogen (Finch-Savage and Leubner-Metzger, 2006). In barley grain, seed covering layers pose a hindrance to the oxygen availability of the embryo, which cause increased sensitivity to high temperature, ABA and GA (Bradford *et al.,* 2008). Temperature is the other important environmental factor affecting seed dormancy and germination. In some crops species such as wheat and barley, lower temperature enhances seed germination whereas high temperature inhibits germination (Corbineau and Côme, 1996). In mature cereal grains, a growing temperature higher than 15–20˚C deepens the primary dormancy whereas a temperature of 30˚C can induce thermo-dormancy; grain losses its capacity to sprout (Leymarie *et al.,* 2008; Fidler *et al.,* 2015). The seed incubation temperature has also been reported to affect seed dormancy and embryo responsiveness to ABA (Corbineau and Côme, 2000). Light affects ABA and GA metabolism and hence controls germination. For example, blue light promotes dormancy in barley seeds via induction of ABA biosynthetic gene *NCED1* but does not affect the expression of genes encoding the ABA catabolic enzyme, ABA8′OH (Barrero *et al.,* 2009). Previous studies have shown that germination of barley seed is repressed by white light whereas red light stimulates the germination of Brachypodium (*Brachypodium distachyon*) (Fidler *et al.,* 2015). Germination of cereal seeds including barley and wheat is also enhanced by darkness. Other environmental factors such as water and nutrient stress (Matakiadis *et al.,* 2009) also influence the depth of dormancy.

Exogenous application of nitrate or nitrate provided by maternal plant to the growing seeds has been reported to cause a decline seed ABA content and stimulate seed germination in Arabidopsis (Alboresi *et al*., 2005; Matakiadis *et al.,* 2009), and this decline in seed ABA content has been found to occur due to induced expression of *CYP707A2* (Matakiadis *et al.,* 2009).

2.12.2 Plant hormones

Seed dormancy and germination are regulated by internal factors including plant hormones (Koornneef *et al.,* 2002). Hormonal balance between GA and ABA is the most important factor in this regard. The ABA/GA ratio is controlled by synergistic and antagonistic regulation of their metabolic and signalling components, as well as their interaction with other hormones and the environment (Gazzarrini *et al.,* 2015). ABA is considered the most important molecule in dormancy induction and maintenance (Finkelstein *et al.,* 2008; Rodríguez *et al.,* 2015; Shu *et al.,* 2016). Embryonic ABA imposes dormancy during seed development (Nambara and Marion-Poll, 2005), and ensures dormancy maintenance during imbibition (Kucera *et al.,* 2005). The level of ABA in embryos and seeds increases during development until the maturation phase and this increase appears to prevent the precocious germination. Overexpression of ABA biosynthesis genes such as *NCED1* of bean and *NCED3* of Arabidopsis can increase seed ABA content and delay germination (Iuchi *et al.,* 2001; Qin and Zeevaart 2002). GA, which can break dormancy and promote germination in cereals (Tuttle *et al.,* 2015), is mainly synthesized in the embryo of germinating seeds (Jacobsen *et al.,* 2002).

In addition to two major phytohormones ABA and GA, other hormones such as auxin, ethylene, brassinosteriods and cytokinin are also involved in controlling dormancy and germination (Kucera *et al.,* 2005). Auxin is an important regulator in all aspects of plant

development and responses to environment (Zhao, 2010). Recent studies have indicated the participation of auxin in seed dormancy maintenance. For example, the supply of auxin to plants is reported to support the inhibition of seed germination by ABA in Arabidopsis (Liu *et al.,* 2007) and delay seed germination of wheat (Ramaih *et al.,* 2003). The roles of auxin and ABA in seed dormancy are interdependent, increase in auxin biosynthesis or signalling enhances seed dormancy by interacting with ABA signalling genes (Liu *et al.,* 2013). Ethylene and brassinosteroids interact with ABA and GA to promote dormancy release and seed germination by counteracting the effects of ABA (Kucera *et al.,* 2005). Cytokinins are required for the promotion of embryonic cell division and root elongation. They may have roles in formation of embryo, in grain filling of cereals, and in improving sink strength (Kucera *et al.,* 2005). Cytokinins interact with ABA and play a role in controlling the seed germination of sorghum and lettuce (Bewley and Fountain, 1972; Dewar *et al.,* 1998). The seed cytokinin level has been reported to be an important factor for the regulation of wheat seed germination and dormancy (Kucera *et al.,* 2005; Chitnis *et al.,* 2014). The crosstalk of these phytohormones helps in modulation of plant development and stress adaptation, with a direct impact on seed development, germination and dormancy (Finkelstein *et al.,* 2002).

2.12.3 Genetic factors

Many studies have mapped the quantitative trait loci (QTLs) for PHS susceptibility, seed dormancy to the same chromosomal regions and concluded that PHS is predominantly due to the lack of dormancy at harvest (Rodriguez *et al.,* 2015). Four QTLs namely, SD1, SD2, SD3 and SD4 have been identified to be correlated strongly with seed dormancy but the SD2 QTL mapped on chromosome 5HL was found to be the most significant source of seed dormancy (Edney *et al.,* 2013). This QTL was also associated with the malt quality. So, it was not used for breeding against

PHS as it brings some negative effects of not able to produce a good malting type Canadian barley (Li *et al.,* 2003). It is not clear if the difference in malting quality is due to a multiple gene cluster or pleiotropic effect. Many genes with different biochemical functions are affecting PHS resistance or susceptibility particularly ABA and GA metabolism and signaling (Gao *et al.,* 2012; Gao and Ayele, 2014).

2.13 Abscisic acid and its role in plant developmental processes and seed dormancy

Abscisic acid was identified in the early 1960s as a growth inhibiting compound accumulating in abscising cotton fruit "abscisin II" and leaves of sycamore trees photoperiodically induced to become dormant "dormin" (Cutler *et al.,* 2010). It is a sesquiterpenoid compound found in all photosynthetic organisms. ABA plays several roles in plant growth and developmental processes including embryo maturation, storage reserve accumulation, seed dormancy, germination, cell division and elongation, floral induction, and plant responses to abiotic and biotic stress factors such as drought/dehydration, salinity, cold and UV radiation (Finkelstein, 2013).

ABA controls the onset and maintenance of primary dormancy in several plant species including cereals such as wheat and barley (Jacobsen *et al.,* 2002, Finkelstein *et al.,* 2008; Rodriguez-Gacio *et al.,* 2009; Fidler *et al.,* 2016). Various genetic studies show dormancy induction requires de novo synthesis of ABA in embryo and endosperm (Nambara & Marion-Poll, 2003). ABA levels during seed development are low during embryogenesis, increase during the maturation phase, and then decrease during seed desiccation (Bewley, 1997). The first ABA present in the seed is produced by maternal tissues and then by the embryo (Karssen *et al.,* 1983). Maternal ABA is transported through the phloem tissue or synthesised by the seed. Embryonic ABA induces seed dormancy whereas maternal ABA has been shown to increase seed yield and

embryo growth potential in ABA deficient mutants (Frey *et al.,* 2004). ABA synthesised in seed coat and seed capsule positively regulates anthocyanin accumulation and maturation of the testa and capsule (Frey *et al.,* 2004). In species such as Arabidopsis, ABA maintains dormancy in imbibing seeds by blocking endosperm weakening and inhibiting the growth potential of the embryo that is required to overcome the germination constraints imposed by the seed covering layers (Debeaujon and Koornneef, 2000).

2.13.1 Abscisic acid metabolism

ABA is a terpenoid derived from isopentenyl diphosphate (IDP); a common precursor (C5) of ABA (Nambara and Marion-Poll, 2005). ABA synthesis commences in the plastids and utilizes carotenoids as precursors, whereas the later biosynthetic steps take place in the cytosol. IDP is converted to GGPP by two enzymes, IDP isomerase and GGPP synthase. The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP (20 carbon compound) by phytoene synthase to produce a C40 carotenoid known as phytoene. Phytoene is then converted to linear molecule of lycopene by the action of phytoene desaturase and zeta-carotene desaturase, and the lycopene is converted into β-carotene through cyclization reaction, which is catalyzed by lycopene β-25 cyclase. β-carotene hydroxylase catalyzes the hydroxylation of β-carotene to produce zeaxanthin. After this step, ABA specific biosynthesis pathway begins (Cunningham and Gantt, 1998; Hirschberg, 2001).

There are three major regulatory steps in ABA biosynthesis process. The first one is the conversion of zeaxanthin via antheraxanthin to violaxanthin catalyzed by zeaxanthin epoxidase (ZEP). ZEP is the first plant ABA biosynthetic enzyme isolated and characterized (Xiong and Zhu, 2003), and the reaction catalysed by this enzyme can be reversed to produce zeaxanthin catalysed

by violaxanthin de-epoxidase (VDE). The violaxanthin produced in first step is further converted into 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin catalysed by enzymes neoxanthin synthase (NSY) and isomerase, and these enzymes have not been isolated yet (Nambara and Marion-Poll, 2005). The second regulatory step of the ABA biosynthesis is the production of xanthoxin from *cis*-isomers of violaxanthin and neoxanthin, and this reaction is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes (Schwartz *et al.,* 1997). Xanthoxin produced from the cleavage of 9-*cis*-epoxycarotenoid is then converted to abscisic aldehyde catalysed by ABA deficient 2 (ABA2) enzyme. The last regulatory step of ABA biosynthesis is oxidative reaction of abscisic aldehyde to produce biologically active ABA, and this reaction takes place in the cytosol, and is catalyzed by abscisic aldehyde oxidases 3 (AAO3).

The amount of bioactive ABA in plants is also determined by its rate of catabolism, which involves two types of reactions; hydroxylation and conjugation (Davies, 2010). The hydroxylation of carbon atoms at 7´, 8´ and 9´ position of ABA triggers the production of three biologically active forms of hydroxylated ABA namely 7´-hydroxy ABA (7´-OH ABA), 8´-hydroxy ABA (8´-OH ABA), and 9´-hydroxy ABA (9´-OH ABA). The enzyme ABA 8′ hydroxylase catalyses the production of 8′-hydroxyl ABA, which is a predominant ABA catabolic pathway in plants (Kushiro *et al.,* 2004; Saito *et al.,* 2004). The 8´-hydroxy ABA is subsequently isomerized to phaseic acid (PA), a catabolite that is considered not to have biological activity. The PA is occasionally metabolized further to dihydrophaseic acid (DPA), which is also a biologically inactive ABA catabolite (Zhou *et al.,* 2004). The PA and DPA are the most widespread and biologically inactive ABA catabolites whereas the 7´-OH ABA and 8´-OH ABA are reported to have hormonal activity like ABA. In addition to the hydroxylation, conjugation of ABA with glucose also plays an important role in ABA inactivation process. Possible targets for conjugation

with glucose are carboxyl and the hydroxyl group of ABA and its oxidative catabolites. ABA glucosyl ester (ABA-GE), which is a widespread form of ABA conjugate, is inactive and can be stored or hydrolysed in response to stress (Gazzarrini *et al.,* 2015).

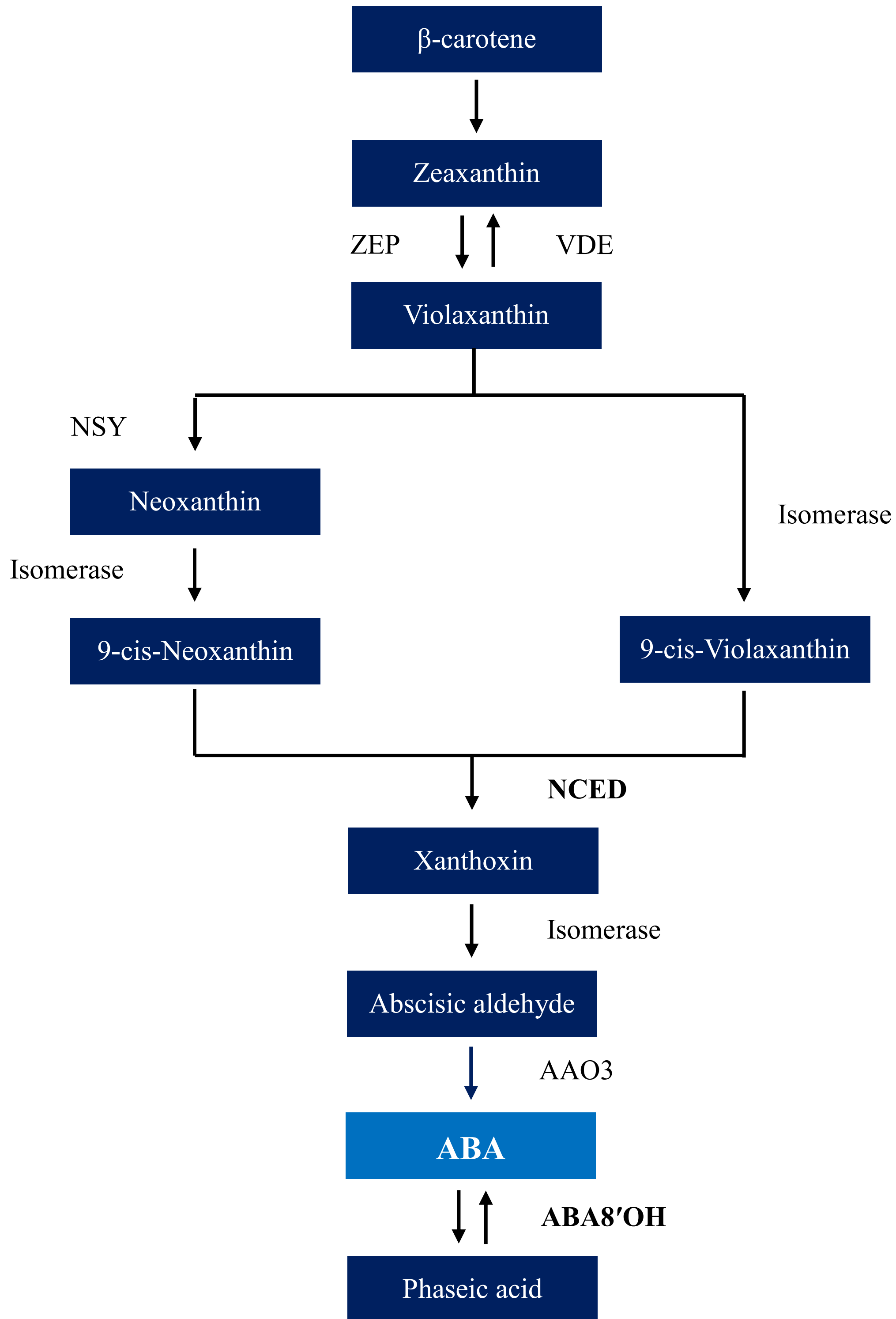


Figure 2.1 ABA biosynthesis and catabolism pathway in plants (adapted from Nambara and Marion-Poll, 2005). ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, Neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; AAO3, abscisic aldehyde oxidases; ABA8′OH, ABA 8′ hydroxylase.

2.13.2 ABA metabolism genes and their role in the regulation of seed dormancy

Both ABA biosynthesis and catabolism control the level of ABA present in plants. Of all the ABA biosynthetic steps, the one catalyzed by NCED is considered as regulatory. The first gene encoding NCED was identified from the maize *viviparous 14* mutant (*vp14*). In the model plant Arabidopsis, nine *NCED* genes have been identified, however, only five (*NCED2*, *3, 5, 6, 9*) are predominantly involved in ABA biosynthesis (Nambara and Marion-Poll, 2005). The *NCED6* and *NCED9* genes are highly expressed in developing seeds; *NCED6* is expressed in the endosperm while *NCED9* is expressed in both endosperm and embryo until late seed maturation (Lefebvre *et al.,* 2006; Frey *et al.,* 2012). Mutant analysis of these genes demonstrated their role in regulating ABA level and seed dormancy. Further study also reported the role of *NCED*5 in fine-tuning seed ABA level and dormancy (Frey *et al.,* 2012). *AtNCED6* and *AtNCED9* mutant analysis revealed their role in seed dormancy by ABA synthesis (Lefebvre *et al.,* 2006).

Genes encoding this enzyme, have also been isolated and characterized from several plant species, and they form a multigene family. For example, rice has three *NCED* genes *NCED1, NCED2* and *NCED3*. A study with dormant, intermediate and non-dormant rice cultivars indicated that the timing for peak expression of the *NCED* genes during seed development varies with the degree of dormancy (Liu *et al.,* 2014). Two *NCED* genes (*NCED1* and *NCED2)* have been identified in wheat (Zhang *et al.,* 2014; Son *et al.,* 2016), and their expression pattern was found to be associated with the level of seed dormancy (Son *et al.,* 2016; Izydorczyk *et al.,* 2018). In barley, there are two *NCED* genes namely *NCED1* and *NCED2*, which are known to play an important role in the synthesis of ABA in seeds through their expression mainly during the late and early-middle phases of seed development, respectively (Chono *et al.,* 2006). Peak of ABA accumulation during the early to middle stages of grain development is caused by high expression

of *NCED2* whereas increased expression of *NCED1* occurs during grain maturation (Chono *et al.,* 2006). A close association between the *NCED* expression and ABA level has been observed in developing seeds of barley (Chono *et al.,* 2006; Lefebvre *et al.,* 2006), and the *NCED1* and *NCED2* genes of barley have been reported to involve in the induction of secondary dormancy at 30˚C due to high embryonic ABA content (Leymarie *et al.,* 2008). The expression level of *NCED* genes is influenced by several environmental factors in different species. For example, the expression of *NCED6* is induced by darkness during imbibition of Arabidopsis seeds while blue light regulates ABA synthesis and dormancy in barley embryos through influencing the expression of *NCED1* (Gubler *et al.,* 2008). Previous reports have shown that high temperature induces the expression of *NCED* genes and causes thermo inhibition of germination in seeds of several species including Arabidopsis, lettuce and wheat (Toh *et al.,* 2008; Huo *et al.,* 2013; Izydorczyk *et al.,* 2018).

ABA catabolism mainly takes place through ABA 8′ hydroxylation, which is catalyzed by ABA8′OH. The ABA8′OH enzyme is encoded by cytochrome P450 monooxygenase 707A (*CYP707A*) gene family (Okamoto *et al.,* 2006). There are four genes encoding ABA8′OH in Arabidopsis namely *CYP707A1, CYP707A2, CYP707A3* and *CYP707A4* (Kushiro *et al.,* 2004). The *CYP707A1* and *CYP707A3* genes of Arabidopsis are expressed during mid-seed development while *CYP707A2* is predominantly expressed in both endosperm and embryo tissues at the end of seed development and during germination (Okamoto *et al.,* 2006). Higher expression of *CYP707A2* is associated with lower ABA levels and germination, and *cyp707a2* mutant have a longer period of dormancy that requires a longer duration of after-ripening to break dormancy (Millar *et al.,* 2006; Okamoto *et al.,* 2006). Studies also showed that single and double mutants of *CYP707A* genes such as *cyp707a1 cyp707a2* have higher level of ABA and hence reduced germination (Kushiro *et al.,* 2004, Okamoto *et al.,* 2006). The rice genome has three *ABA8'OH*

genes, *ABA8'OH1*, *ABA8'OH2* and *ABA8'OH3* (Saika *et al.,* 2007). Studies in rice have reported the role of increased expression of *ABA8'OH1* in causing reduction of ABA content and enhanced seed germination (Yang and Choi, 2006; Saika *et al.,* 2007). For example, two rice gene *ABA8'OH2* and *ABA8'OH3* are found to play roles in glucose induced delay of seed germination (Zhu *et al.,* 2009).

Two *ABA8'OH* genes, *ABA8'OH1* and *ABA8'OH2*, have also been isolated from wheat (Chono *et al.,* 2013; Iehisa *et al.,* 2014), and their expression patterns were found to be associated with seed dormancy (Son *et al.,* 2016; Izydorczyk *et al.,* 2018). Furthermore, a study involving double mutants of *TaABA8'OH1A* and *TaABA8'OH1D* showed increased embryonic ABA level and reduced germination (Chono *et al.,* 2013). In agreement with these results, *TaABA8'OH2* has been shown to be co-located with a major QTL for seed dormancy (Zhang *et al.,* 2009). This dormancy QTL is known to be orthologous to the barley dormancy QTL "SD1", which has been reported to be the most effective seed dormancy QTL in barley (Sato *et al.,* 2009). The barley *ABA8'OH1* gene has been reported to be a key gene for lowering the level of ABA in seeds and breaking primary dormancy (Millar *et al.,* 2006; Leymarie *et al.,* 2008), which is crucial for germination. The expression of *ABA8'OH1* in barley has been shown to increase at maturity, suggesting its role in primary dormancy induction (Chono *et al.,* 2006). Further analysis showed that knocking down of *HvABA8'OH1* results in increased ABA level and decreased germination of barley grain (Gubler *et al.,* 2008).

2.14 Gibberellin and its roles in seed germination

Gibberellins were first isolated by Eiichi Kurosawa from a pathogenic fungus (*Gibberella fujikuroi*) that causes bakanae disease of rice. In 1930s, Teijiro Yabuta and Yusuke Sumiki were

the first to isolate a crystalline compound (*gibberellin A*) by culturing the fungal pathogen. Studies on '*gibberellin A'* reported its role in promoting plant growth as indicated by the excessive growth of the stem leading to lodging of the plant and yield losses (Sun, 2008). This chemical compound '*gibberellin A'* was found to consist of three components, GA_1, GA_2 and GA_3 but the major one was GA_3 (gibberellic acid). The first GA from a plant (designated as GA_1) was extracted using immature vuner bean (*Phaseolus cocineus*) seeds in 1958.

Gibberellins belong to a group of compounds known as diterpenoids, and they act as phytohormones to influence several plant growth and developmental processes such as seed germination, stem elongation, flower induction, seed and pericarp growth and seed development (Davies, 1995; Yamaguchi *et al.,* 2008). GA plays an important role in activating germination by enhancing the embryo growth potential, endosperm weakening and activating α-amylase enzyme to allow the growth of radicle (Kucera *et al.,* 2005). Several mutant studies have explained the importance of GA in completion of the germination process. For example, germination capacity of the GA mutant *ga1-1* of Arabidopsis can be restored with application of exogenous GA (Debeaujon and Koornneef, 2000). Furthermore, seed treatment with GA is important in antagonising the action of ABA and hence seed transition from dormancy to germination (White *et al.,* 2000).

To date 136 GAs have been identified from higher plants and fungi, however, only a small number (GA_1, GA_3, GA_4 and GA_7) are biologically active (Hedden and Phillips, 2000; Sun, 2008; Davies, 2010). All the other GAs are either metabolic precursors or their inactivation products. The bioactive level of GA in plant tissues is maintained through a balance between its biosynthesis regulated by GA 20-oxidase and GA 3-oxidase enzymes and inactivation regulated by GA 2-oxidase enzyme (Yamaguchi, 2008). The genes encoding these enzymes form multigene families

in different plants such as Arabidopsis, wheat, rice and barley (Spielmeyer *et al.,* 2004; Yamaguchi, 2008; Pearce *et al.,* 2015), and the members of the gene families exhibit differential expression patterns (Hedden and Phillips, 2000).

2.14.1 Gibberellin metabolism pathway

The common diterpenoid precursor for GA biosynthesis is a C20 compound geranylgeranyl diphosphate (GGDP), and the biosynthesis of GA is a triphasic process. The first stage occurs inside plastids and commences with the synthesis of *ent*-Kaurene from GGDP by a two-step cyclization reaction, which is the first committed step of GA biosynthesis. This reaction is catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) via *ent*-copalyl diphosphate (*ent*-CDP) as intermediate.

The second stage of GA biosynthesis involves the oxidation of *ent*-kaurene to *ent*-kaurenoic acid, which is catalyzed by *ent*-kaurene oxidase (KO), and subsequently by *ent*-kaurenoic acid oxidase (KAO) to produce GA_{12}. A previous study suggested the presence of early GA biosynthetic enzymes, KO and KAO in the outer plastid membrane and endoplasmic reticulum, respectively (Helliwell *et al.,* 2001). The final step of GA biosynthesis occurs in the cytoplasm, and involves the conversion of GA_{12} to bioactive GAs (GA_1 and GA_4) via 13-hydroxylation pathway catalysed by GA 13-oxidases to produce GA_{53} as intermediate and non-13 hydroxylation pathway, respectively. The final series of oxidation steps in the GA biosynthesis are catalysed by GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), respectively (Sun, 2008).

The level of bioactive GAs in plants is regulated by both its synthesis and conversion of bioactive GAs to inactive forms. The hydroxylation of 2β carbon catalyzed by GA 2-oxidases (GA2ox) is the major form of GA catabolism (Yamaguchi, 2008). These enzymes play a role in

depleting the precursors of GAs (such as GA_{12} and GA_{53}) that are otherwise converted to bioactive forms. Recent studies have reported the presence of GA 16, 17-epoxidation and methylation as two more GA inactivation mechanisms involving non 13-hydroxylated GAs, namely GA_4, GA_9 and GA_{12} (Yamaguchi, 2008).

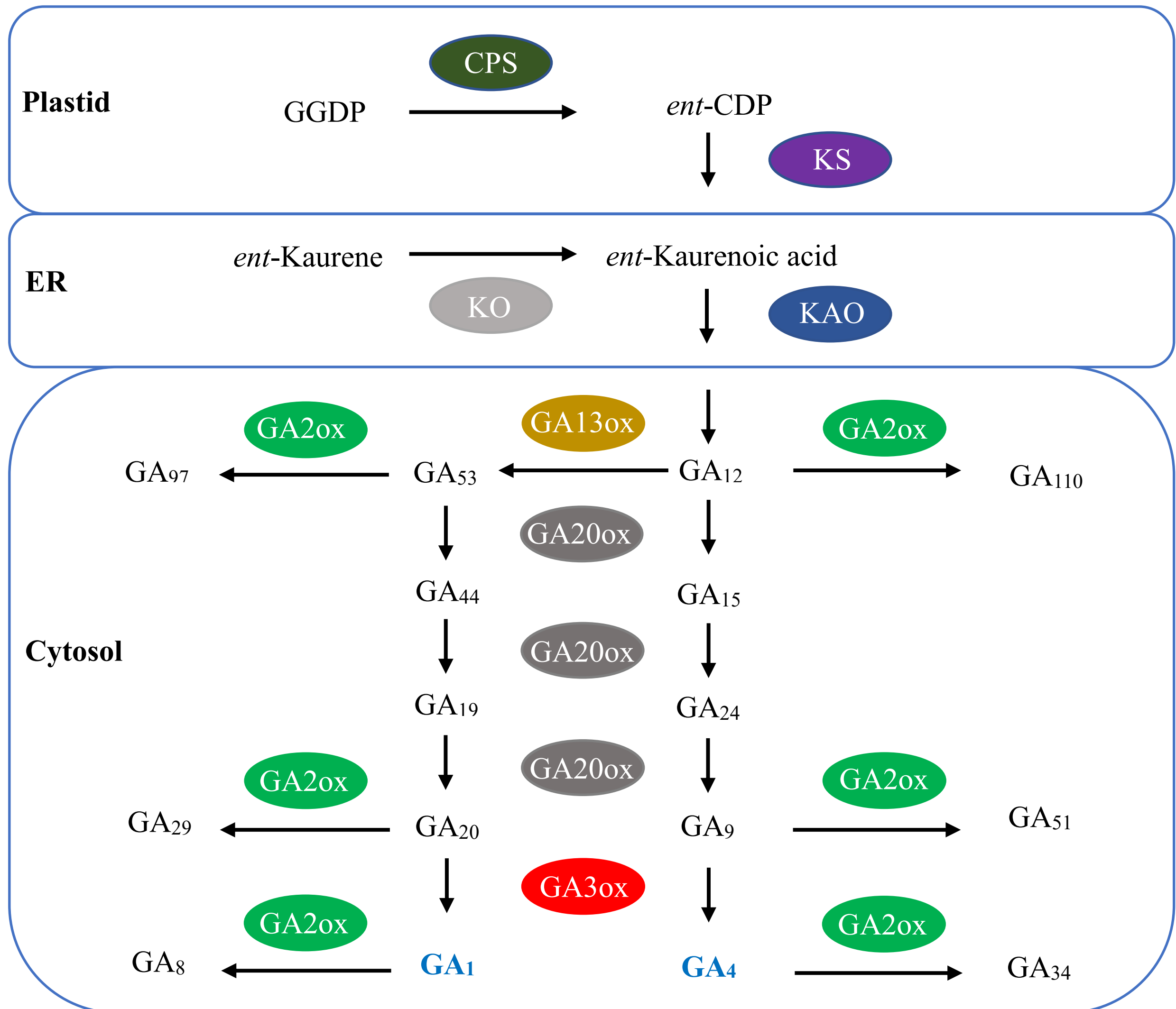


Figure 2.2 Gibberellin metabolic pathway in plants (adapted from Yamaguchi, 2008). GA_1 and GA_4 are bioactive GAs. GGDP, geranylgeranyl diphosphate; ent-CDP, *ent*-copalyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase; GA13ox, GA 13-oxidase; GA20ox, GA 20-oxidase; GA2ox, GA 2-oxidase and GA3ox, GA 3-oxidase. ER, endoplasmic reticulum

2.14.2 Gibberellin metabolism genes

The early GA biosynthesis enzymes including CPS, KS and KO enzymes are encoded by single gene in most of the plant studied (Yamaguchi, 2008). Previous studies in Arabidopsis have reported high amount of *CPS* mRNA in tissues with fast growth such as shoot apices, root tips, developing anthers, and seed (Olszewski *et al.,* 2002). Although the expression patterns of *KS* and *CPS* are similar, the expression level of *CPS* is found to be lower than that of KS (Yamaguchi *et al.,* 1998b), suggesting a primary role of *CPS* expression in the synthesis of *ent*-kaurene. Therefore, *CPS* is hypothesised to act as a check point controlling the activities of GA biosynthetic processes (Olszewski *et al.,* 2002). The KO and KAO enzymes of Arabidopsis are encoded by one (*CYP701A1)*, and two genes (*CYP88A3* and *CYP88A4),* respectively (Helliwell *et al.,* 2000). Both *CYP88A3* and *CYP88A4* genes were found to have higher levels of expression in the inflorescence and silique than the leaf. Furthermore, *CYP88A4* was found to be predominantly expressed in the stem and inflorescence as compared to *CYP88A3* (Helliwell *et al.,* 2000).

In contrast to the early GA biosynthetic enzymes, the late GA biosynthetic enzymes (GA20ox and GA3ox) appear to be encoded by multigene family members (Sun, 2008). There are five *GA20ox* genes reported in Arabidopsis (Hedden *et al.,* 2002), and a loss of function in *GA20ox1*, *GA20ox2* or *GA20ox3* is reported to cause low seed germination, dwarfism and infertility, whereas *GA20ox4* and *GA20ox5* have less effect on these phenotype (Plackett *et al.,* 2012). Rice genome has four *GA20ox* genes (*GA20ox1* to *GA20ox4*), located on chromosomes 3, 1, 7 and 5 respectively (Sakamoto *et al.,* 2004). A recent study has reported *OsGA20ox2* gene as a candidate gene for regulation of seed germination (Magwa *et al.,* 2016), and a loss of function mutant of *OsGA20ox2* produced plants with less seed GA level and strong seed dormancy (Ye *et al.,* 2015), indicating the role of this gene in GA synthesis and seed germination. The *GA20ox1*

and *GA20ox2* genes contribute towards plant height in rice (Pearce *et al.,* 2015). The *sd-1* gene, which led to the green revolution, resulted from the loss-of-function of *GA20ox2* gene of rice and contributed towards dwarf phenotype (Han and Zhu, 2011). Wheat has four *GA20ox* genes (*GA20ox1*, *GA20ox2*, *GA20ox3* and *GA20ox4*) (Pearce *et al.,* 2015). The *TaGA20ox1* is highly expressed during rapidly growing stages of development whereas *TaGA20ox2* appears to be expressed highly after the period of maximal elongation growth in mature tissue, suggesting that *TaGA20ox1* might be more important in determining growth rate (Pearce *et al.,* 2015). It has been shown that *TaGA20ox1* is mainly expressed in embryos and surrounding maternal tissues of developing grain and is assumed to have a major role in grain development and ultimately grain yield and quality (Appleford *et al.,* 2006). The *TaGA20ox3* gene is predominantly expressed in endosperm tissue of wheat throughout seed development suggesting its role in biosynthesis of GA during seed development (Pearce *et al.,* 2015). The *TaGA20ox1* and *TaGA20ox3* genes have been mapped to chromosome 5 and 3 of wheat, respectively (Pearce *et al.,* 2015). With respect to seed germination, a recent study showed that embryonic *GA20ox1* plays important role in control of wheat seed germination and bioactive GA level (Izydorczyk *et al.,* 2018). Barley also contains three *GA20ox* genes, namely *GA20ox1*, *GA20ox2* and *GA20ox3* (Spielmeye *et al.,* 2004). A comparative genomics study involving barley, wheat and rice genomes has identified *GA20ox* as a candidate gene for regulation of PHS in barley (Li *et al.,* 2004). A reduced *HvGA20ox2* expression has been found to contribute towards increased grain yield and poor malting quality (Jia *et al.,* 2009; 2011).

The GA 3-oxidase enzyme is responsible for conversion of inactive GA precursors to bioactive forms of GAs (Davies, 2010). There are four *GA3ox* genes in Arabidopsis, namely *GA3ox1, GA3ox2, GA3ox3* and *GA3ox4* (Hedden *et al.,* 2002), and *GA3ox1* is highly expressed in

most tissues throughout seed development while *GA3ox2* is predominantly expressed during seed germination and vegetative growth (Hu *et al.,* 2008). During seed imbibition, two embryonic *GA3ox* genes of Arabidopsis (*GA3ox1* and *GA3ox2)* are found to regulate GA synthesis and hence germination, vegetative growth, and development (Mitchum *et al.,* 2006; Holdsworth *et al.,* 2008). Another study has reported that double mutants of *GA3ox1* and *GA3ox2* genes in Arabidopsis showed severe reduction in germination potential (Holdsworth *et al.,* 2008), indicating their roles in the regulation of seed germination. Expression patterns of *GA3ox1* and *GA3ox2* of Arabidopsis genes also suggest their role in the regulation of seed germination by light (Seo *et al.,* 2006).

Genes encoding GA3ox genes have also been isolated from cereal crops. There are three *GA3ox* genes in rice (*GA3ox1*, *GA3ox2* and *GA3ox3*), mapped to chromosomes 5S, 1S, and 3S, respectively. Genetic studies have reported that *GA3ox1* and *GA3ox2* genes induce the expression of α-amylase gene which is involved in the hydrolysis of starch and provide energy for germinating cereal such as rice seeds (Itoh *et al.,* 2001; Kaneko *et al.,* 2002). Two *GA3ox* genes (*GA3ox2* and *GA3ox3*) have been reported in wheat (Appleford *et al.,* 2006; Pearce *et al.,* 2015), and *GA3ox2* is predominantly expressed in germinating wheat embryos (Appleford *et al.,* 2006). The imbibition of wheat seeds at 15˚C showed enhanced germination due to an increase in *GA3ox2* expression and bioactive GA level in the embryo (Kashiwakura *et al.,* 2016). Two *GA3ox* genes (*GA3ox1* and *GA3ox2)* have also been reported in barley (Spielmeyer *et al.,* 2004), and *GA3ox2* is the predominantly expressed gene during seed germination (Suzuki *et al.,* 2005; Gubler *et al.,* 2008). Expression analysis of *GA3ox1* and *GA3ox2* genes of barley indicated the role of *GA3ox2* in inducing the expression of alpha-amylase (*Amy1*) gene (Suzuki *et al.,* 2005), and *GA3ox2* is found to be expressed highly in the embryos during imbibition (Gubler *et al.,* 2008). These results demonstrate that *GA3ox2* is a key enzyme in regulating seed germination in barley.

Like the other dioxygenases, GA2ox is encoded by multigene family members. There are seven *GA2ox* genes (*GA2ox1*, *GA2ox2*, *GA2ox3*, *GA20x4*, *GA2ox6*, *GA2ox7* and *GA2ox8*) in Arabidopsis (Yamaguchi, 2008). The Arabidopsis *GA2ox1* and *GA2ox2* genes are expressed in inflorescences and developing siliques, and play roles in reducing bioactive GA level and seed germination (Thomas *et al.,* 1999). Studies on the *GA2ox* of Arabidopsis also suggests the role of *GA2ox2* in the regulation of seed germination in response to light and *GA2ox6* in radicle elongation (Wang *et al.,* 2004; Seo *et al.,* 2006). Ten *GA2ox* genes have been reported in rice (*GA2ox1*, *GA2ox2*, *GA2ox3*, *GA2ox4*, *GA2ox5*, *GA2ox6*, *GA2ox7*, *GA2ox8*, *GA2ox9* and *GA2ox10*) (Sakamoto *et al.,* 2001; Lee and Zeevaart, 2005; Lo *et al.,* 2008). The expressions of *GA2ox5*, *GA2ox6* and *GA2ox9* genes of rice are found to be significantly downregulated within 2 days after imbibition, suggesting their role in regulating bioactive GA accumulation and rapid seed germination (Lo *et al.,* 2008). In addition, overexpression of *GA2ox5* caused severe defects in plant growth and seed development in transgenic rice (Lo *et al.,* 2008). Eight *GA2ox* genes (*GA2ox1*, *GA2ox3*, *GA2ox4*, *GA2ox6*, *GA2ox7*, *GA2ox8*, *GA2ox9* and *GA2ox10*) have been reported in wheat (Pearce *et al.,* 2015). Four *GA2ox* genes namely *GA2ox3*, *GA2ox6*, *GA2ox7* and *GA2ox8* are reported to be responsible for GA catabolism in wheat spike at anthesis, whereas *GA2ox9* is highly expressed in developing wheat spike (Pearce *et al.,* 2015). Although expressed at very low level during imbibition at optimal temperature, the upregulation of *GA2ox3*, *GA2ox6* and *GA2ox9* during imbibition at supraoptimal temperature that inhibits germination may suggest their role in dormancy and germination (Izydorczyk *et al.,* 2018). Four *GA2ox* genes (*GA2ox1, GA2ox2*, *GA2ox3* and *GA2ox4*) have been reported in barley, but only *GA2ox3* was shown to be expressed at high levels in embryos during grain imbibition (Gubler *et al.,* 2008), suggesting its role in barley seed germination.

3.0 GENETIC AND MOLECULAR BASES OF ABSCISIC ACID AND GIBBERELLIN METABOLISM IN REGULATING SEED DORMANCY IN MALTING BARLEY

Abstract

Preharvest sprouting is highly influenced by the level of seed dormancy, which is regulated mainly by the balance between two plant hormones ABA and GA. The levels of these two hormones is maintained by their biosynthesis and catabolism. This thesis investigated genetic variations and expression patterns of ABA and GA metabolic genes in different malting barley lines with varying degrees of seed dormancy. A positive correlation was observed between the levels of *NCED1* (ABA biosynthetic gene) expression in the embryos and seed dormancy in the different barley lines, suggesting the role of ABA biosynthesis in the regulation of ABA level and seed dormancy. Our data also showed a positive correlation between the levels of *ABA8'OH1* (ABA catabolic gene) expression and dormancy, and this could be due to its regulation by ABA level as a strong positive correlation was shown between the expression levels of *NCED1* and *ABA8'OH1*. The detection of a 12 bp deletion in the intron of *ABA8'OH1* is associated with its low level of expression in selected non-dormant lines, and this along with the low expression levels of *NCED1* in the same lines suggest that the deletion forms a genetic mechanism to limit *ABA8'OH1* expression when ABA synthesis is reduced. The observation of a strong negative correlation between the levels of endospermic *GA20ox3* (GA biosynthetic gene) expression and seed dormancy in selected lines suggests the role of this gene in bioactive GA accumulation and seed germination. Sequence analysis of *GA20ox1* showed the presence of four nucleotide substitutions in dormant/intermediate dormant lines. Since *GA20ox1* expression was not detected in seed tissues, these variations could not be associated with gene expression levels.

3.1 Introduction

The life cycle of a plant begins from a seed, which acts as a basic unit of propagation. Seeds of cereal crops play important role in human dietary nutrition by serving as a source of carbohydrates and proteins. They are also used as a source of raw materials for various industrial processes such as bakery, milling, malting and brewery. However, the production of cereal crops is affected by many biotic and abiotic stress factors such as high humidity and wet weather conditions before harvest, which causes PHS, defined as the sprouting of the grain on the spike. PHS have been reported to have a tight association with the degree of seed dormancy, which refers to inhibition of seed germination under favorable environmental conditions (Gao and Ayele, 2014). Barley breeding programs have been aimed at production of new cultivars with appropriate level of seed dormancy to prevent PHS under humid weather conditions. A medium to high amount of seed dormancy is required for protection against PHS, but this may lead to problems in downstream processing of grains since the grains from malting barley varieties should exhibit uniform and fast germination of grains for the production of good quality malt extracts (Hicky *et al.,* 2012; Edney *et al.,* 2013). The barley varieties with high seed dormancy at harvest will have to undergo after-ripening before malting, which leads to increase in extra storage costs.

Grain damage by PHS is generally estimated based on α-amylase activity and the number of field-sprouted grains at harvest (Mares, 1989). During sprouting, the activation of α-amylases and proteases starts, and these enzymes degrade the storage reserves in the starchy endosperm, leading to the deterioration grain quality for beer making. The PHS damage may also cause loss of grain viability (Gualano *et al.,* 2014). One of the strategies used for reducing PHS damage is developing PHS resistant lines through breeding and this approach in most cases involves the use of double haploid or recombinant inbred populations derived from two parental lines having

different dormancy phenotype (Rodrı´guez *et al.,* 2015). Tackling PHS involves controlling the timing of dormancy loss in such a way that dormancy is not lost very early to prevent PHS or very late to prevent strong dormancy that affects re-planting and industrial applications. The level of seed dormancy in barley should be adjusted very precisely as malting industry demands for non sprouted and low dormant genotypes capable of germinating fast for immediate industrialization (Rodrı´guez *et al.,* 2015). Different barley cultivars have genotypic variability with respect to the level of seed dormancy they exhibit; some cultivars can release dormancy very fast, that is, within days, others within weeks, while some varieties likely remain dormant for several months (Benech-Arnold, 2001).

Seed germination begins with water uptake by seed and finishes with emergence of radicle out of the seed covering layers (Bewley and Black, 1994). However, the germination of viable seeds can be blocked by seed dormancy even under optimal conditions (Hilhorst, 2007). Several endogenous and environmental factors regulate seed dormancy and germination, and plant hormones are among the endogenous factors regulating these two developmental processes (Matilla and matilla-Vazquez, 2008). The major plant hormones governing seed dormancy and germination are ABA and GA; ABA induces dormancy while GA enhances germination (Kucera *et al.,* 2005; Finkelstein *et al.,* 2008). The amounts of ABA and GA are controlled by a balance between their biosynthesis and degradation (Nambara *et al.,* 2010). There are two major regulatory steps in ABA metabolism pathway, and these steps are catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) and ABA 8′ hydroxylase (ABA8′OH). ABA controls the onset of dormancy in the grains of cereals like barley (Jacobsen *et al.,* 2002), and inhibits seed germination through delaying radicle expansion and the weakening of endosperm (Muller *et al.,* 2006). Genetic studies show that dormancy is induced by *de-novo* synthesis of ABA in embryo and endosperm (Nambara

and Marion-Poll, 2003). The expression patterns of genes encoding two regulatory ABA metabolic enzymes of barley, NCED (*NCED1* and *NCED2*) and ABA8′OH (*ABA8'OH1* and *ABA8'OH2*) are associated with ABA level and grain dormancy (Chono *et al.,* 2006; Millar *et al.,* 2006; Gubler *et al.,* 2008).

Mutations or chemical treatments that inhibit ABA biosynthesis lead to reduced ABA levels and a decrease in seed dormancy whereas overexpression of the ABA biosynthetic genes or silencing of the ABA catabolic genes leads to accumulation of ABA and high degree of seed dormancy (Nambara and Marion-Poll, 2003; Kucera *et al.,* 2005). Furthermore, dormant wheat cultivar has been reported to exhibit high and low expression of *TaNCED2* and *TaABA8'OH1* genes, respectively, resulting in high ABA level and dormancy as compared to non dormant cultivar (Son *et al.,* 2016). A study involving different temperatures for seed imbibition also showed the role of *NCED* and *ABA8'OH* expression in controlling seed germination at suboptimal, optimal and supraoptimal temperatures (Izydorczyk *et al.,* 2018). In barley, the decrease in ABA content during imbibition of non-dormant seeds is correlated with increased expression of *ABA8'OH1*; however, no such association was observed with the expression of ABA biosynthetic genes (Millar *et al.,* 2006; Barrero *et al.,* 2009). In agreement, suppressing the expression of the *ABA8'OH1* gene has been shown to result in increased ABA level and high grain dormancy (Gubler *et al.,* 2008). These results indicate that *ABA8'OH1* gene of barley is important regulator of seed dormancy and germination in barley.

GA is another important plant hormone known to play a role in promotion of seed germination (Finch-Savage and Leubner-Metzger, 2006), and the level of bioactive GAs is regulated mainly by genes encoding GA 20-oxidase, GA 3-oxidase and GA 2-oxidase, respectively (Yamaguchi, 2008). Several genetic, mutational and physiological studies have indicated the

importance of GA in promoting seed germination and radicle emergence through endosperm weakening, increasing embryo growth potential and activating α-amylase (Chen and Chang, 1972; Koornneef and van der Veen, 1980; Kucera *et al.,* 2005). Increased GA biosynthesis has been reported to play an important role in bioactive GA accumulation and enhancing Arabidopsis and wheat seed germination rather than a reduced GA catabolism (Ogawa *et al.,* 2003; Izydorczyk *et al.,* 2018).

Expression studies indicated the role of *GA20ox* and *GA3ox* genes in regulating imbibition induced accumulation of bioactive GAs and hence enhanced seed germination in Arabidopsis, sorghum and wheat (Ogawa *et al.,* 2003; Rodriguez *et al.,* 2011; Izydorczyk *et al.,* 2018). The low expression of *GA2ox* genes during germination indicated that GA inactivation is not important in regulating GA level and germination (Ogawa *et al.,* 2003; Izydorczyk *et al.,* 2018). The synthesis of bioactive GA during early imbibition of Arabidopsis seeds is mediated through the expression of *GA20ox3* and *GA3ox1*, while during late imbibition GA synthesis is regulated by *GA3ox2* suggesting the role of *GA3ox2* in radicle emergence (Ogawa *et al.,* 2003). A loss-of-function mutant study in rice has indicated that *GA20ox2* cause a high GA level and low seed dormancy, as a result it was selected as a candidate gene for controlling seed germination (Ye *et al.,* 2015). In addition, a report on after-ripened wheat and barley seeds suggested the release of seed dormancy is associated with increased expression of *GA20ox* and *GA3ox* genes and increased bioactive GA_1 during imbibition (Gubler *et al.,* 2008; Liu *et al*., 2013; Kashiwakura *et al.,* 2016). The expression of *GA3ox2* was found to be higher in dormant barley grains imbibed under dark condition that causes germination of the dormant seeds as compared to grains imbibed in light, suggesting the role of GA in promotion of seed germination under darkness (Gubler *et al.,* 2008). A previous quantitative genetic study in barley also reported *GA20ox* as a candidate gene for the

regulating PHS and seed dormancy (Li *et al.,* 2004). All these results indicate *GA20ox* and *GA3ox* genes play important roles in control of seed dormancy and germination in barley.

This thesis tested the hypothesis that allelic variation and differential expression of ABA and GA metabolic genes of barley lead to differences in the level of seed dormancy and PHS tolerance in malting barley. This hypothesis was tested by characterizing the germination phenotype of different barley lines, investigating the expression patterns of ABA and GA metabolic genes in seed imbibing tissues, and analyzing allelic variations in the genomic sequences of selected ABA and GA metabolic (*ABA8'OH1* and *GA20ox1*) genes. In seed of dormant lines, the expression levels of ABA biosynthetic and catabolic genes are expected to be high and low, respectively, to maintain high ABA level and seed dormancy. In addition, the expression levels of GA biosynthetic genes are expected to be low in dormant seeds to contribute to the inhibition of germination.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

Eleven malting barley inbred lines along with the parental lines TR253 (PHS susceptible but with a good malt quality) and Baudin (PHS resistant), and selected malting barley cultivars TR11221, CDC Reserve-01, Morex, Steptoe, Betzes, Himalaya, AC Metcalfe and AAC Synergy were used in this study (Table 3.1). The inbred lines along with the parents and cultivars were obtained from Dr. Ana Badea research group at Agriculture and Agri-Food Canada, Brandon research and Development Center. The inbred lines used in this study have contrasting degree of resistance to PHS.

Table 3.1 List of selected barley lines used in this study

S. No.	Line name	Description
1	TR253	Parent
2	Baudin	Parent
3	BM0253-58	Sister inbred line
4	BM0253-95	Sister inbred line
5	BM0253-103	Sister inbred line
6	BM0253-180	Sister inbred line
7	BM0253-211	Sister inbred line
8	BM0253-217	Sister inbred line
9	BM0253-235	Sister inbred line
10	BM0253-331	Sister inbred line
11	BM0253-402	Sister inbred line
12	BM0253-409	Sister inbred line
13	BM0253-412	Sister inbred line
14	TR11221	Cultivar
15	CDC Reserve-01	Cultivar
16	Morex	Cultivar
17	Steptoe	Cultivar
18	Betzes	Cultivar
19	Himalaya	Cultivar
20	AAC Synergy	Cultivar
21	AC Metcalfe	Cultivar

Mature dry seeds of all the inbred lines and the cultivars were surface sterilized with 70% ethanol for 1 min and 5% sodium hypochlorite solution for 20 min on MaxQ™ 4000 Benchtop orbital shaker (Thermofisher Scientific, Waltham, MA, USA) followed by washing 5 times with sterile water. The seeds were then imbibed with 7 mL sterile water for 3 days in a 9 cm sterile Petri-plate with two layers of Whatman# 1 filter paper under darkness and at room temperature.

After three days of imbibition, germinated seeds were transplanted to the one-gallon plastic pots containing LA4 sunshine mix (Sungro Horticulture, Bellevue, WA, USA) and 18 g fertilizer (ACER®nt 13-12-12 consisting of 13% N, 12% P_2O_5, 12% K_2O and micro elements) in a growth chamber at 22˚C/20˚C (day and night) under a 16/8 hour photoperiod until harvest. Plants were supplied with water every other day until maturity and N-P-K (20:20:20) mix of fertilizer every week. The mature dry seeds were collected and threshed at harvest maturity. These seeds were immediately stored at -80˚C until further use.

3.2.2 Seed germination phenotype

To determine the germination phenotype of seeds derived from the inbred lines along with the parental lines and the malting barley cultivars, seed were surface sterilized and imbibed for 7 days at 22˚C under darkness. Seeds were considered germinated when the coleorhiza penetrates through the seed covering layers.

3.2.3 Molecular cloning of *ABA8'OH1* and *GA20ox1* from different barley lines

3.2.3.1 DNA extraction

Leaf tissues were harvested in liquid nitrogen from each barley line during the vegetative stage of growth and stored immediately at -80˚C until further use. The genomic DNA was extracted from the leaf tissues using CTAB protocol (Doyle and Doyle, 1990). The leaf tissues were first ground to a fine powder in liquid nitrogen using mortar and pestle. Approximately 100-150 mg of the finely ground powder was mixed with 600 µL of CTAB buffer (100 mM Tris-HCL (pH 8), 4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% β-mercaptoethanol) and incubated at 65˚C for 1 hour. After incubation, 200 µL of saturated phenol and 200 µL of chloroform: isoamyl alcohol (24:1) was

added, and then centrifuged at 9000*g* for 10 min. After centrifugation, about 500 µL of the supernatant was transferred to a new microcentrifuge tube to which 3 µL RNase was added. This mixture was incubated at 37˚C for 30 min followed by addition of 400 µL chloroform: isoamyl alcohol (24:1) and then centrifugation at 10000*g* for 10 min. The upper aqueous phase (containing the DNA) was transferred to a fresh microcentrifuge tube, after which ice-cold ethanol was added (800 µL) and mixed gently followed by centrifugation at 16200*g* for 1 min. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol followed by centrifugation at 16200*g* for 1 min. The supernatant was then discarded, and the pellet was allowed to dry for 15 min. The pellet was resuspended in 50 µL of sterile DNase free water and stored at -80˚C until further use. The quality of the DNA samples was verified using gel electrophoresis. The DNA concentration of each sample was determined using Epoch™ Multi-Volume Spectrophotometer System (BioTek Instruments, Winooski, VT, USA).

3.2.3.2 PCR amplification

Amplification of the targeted genes was performed using MyCycler^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) using 100 ng genomic DNA and gene specific primers designed using gene sequences of *ABA8'OH1* (Ensembl ID: HORVU0Hr1G016780) and *GA20ox1* (Ensembl ID: HORVU5Hr1G124120) (Table 3.2) in a 20 µL total reaction mixture. The PCR reaction mixture used for the amplification of the two target genes (*ABA8'OH1* and *GA20ox1*) consisted of DNA template (0.5 µL), 10 mM dNTP mix (0.4 µL), 10X DreamTaq Buffer (2.0 µL), 10 µM Forward primer (0.6 µL), 10 µM reverse primer (0.6 µL), 5U/µL Dream Taq DNA polymerase (0.1 µL) and 15.8 µL sterile water. The thermocycling conditions were initial denaturation at 95˚C for 3 min followed by 37 cycles of denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds

for both *ABA8'OH1* and *GA20ox1* and extension at 72˚C for 2 min followed by final extension at 72˚C for 10 min.

Table 3.2 Primer used for amplifying the target genes

Gene	Type	Sequence (5´ to 3´)	Size (bp)
HvABA8'OH1	Forward	TGCAGGTTGCAGGTAACAGA	2047
	Reverse	CGTTGCCTCTATCGTGGTGT	
GA20ox1	Forward	CTCACTCCACTGAGCTTGAGTC	1453
	Reverse	CTAGTACTAGATCGTCGTCAACC	

3.2.3.3 Gel extraction

The amplified PCR products were separated on a 1% (w/v) agarose gel and visualised under UV light in ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA, USA). The expected bands (2047 bp for *ABA8'OH1* and 1453 bp for *GA20ox1*) were excised from the gel and purified using GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA, United States) according to the manufacturer's protocol. Briefly, the excised gel slice was mixed with binding buffer (1:1; w/w) and then incubated at 55˚C for 10 min or until the gel slice was completely dissolved. The solubilized gel mixture (800 µL) was transferred to the GeneJET purification column, which was centrifuged at 12000*g* for 1 min at room temperature. The flow-through was discarded and the column was placed back into the same collection tube followed by washing the column with 700 µL wash buffer. The column was centrifuged twice at 12000*g* for 1 min to remove any traces of ethanol prior to elution of the purified PCR product with 11 µL of sterile water. The purified DNA was stored at -20˚C until further use. After purification, the quality of the samples was verified by gel electrophoresis.

3.2.3.4 Ligation and transformation

The purified PCR product was ligated to pGEM-T Easy Vector (Promega, Madison, WI, USA). The ligation mixture contained purified DNA (1 µL), pGEM-T Easy Vector (0.5 µL), 2X rapid ligation buffer (2.5 µL), T4 DNA ligase (3 Weiss units/µL) (0.5 µL) and sterile water with a total reaction volume of 5 µL. The ligation mixture was incubated overnight at 16˚C, and used to transform *E.coli* DH5α^{TM} competent cells (Invitrogen, Carlsbad, CA, USA). Transformation was performed by adding 2.5 µL of the ligation product to DH5α competent cells (25 µL) followed by incubation on ice for 30 min, heat shock at 42˚C for 45 sec, and further incubation on ice for 2 min. Subsequently, 600 µL of Luria-Bertani (LB) media (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter; pH 7.0) was added followed by incubation on a shaker at 225 rpm and 37˚C for 1 h. The transformation mixture was then centrifuged at 5000*g* for 5 min after which the supernatant was discarded, and the pellet was suspended and spread on LB plates containing carbenicillin (100 mg/mL, 20 µL) (Sigma, St. Louis, MO, USA), isopropyl β-D-1-thiogalactopyranoside (IPTG) (200 mg/mL, 8 µL) (Invitrogen, Carlsbad, CA, USA) and X-gal (20 µL) (Invitrogen, Carlsbad, CA, USA). The plates were then incubated at 37˚C overnight.

3.2.3.5 Plasmid isolation

The positive colonies grown on the LB plates were selected through blue-white screening, and the white (positive) colonies were first transferred to a fresh LB plates and then used to inoculate LB medium (5 mL) supplemented with Carbenicillin (100 mg/mL) (Sigma, St. Louis, MO, USA). After incubation on benchtop orbital shaker (Thermofisher Scientific, Waltham, MA, USA) at 225 rpm and 37˚C overnight, the plasmid DNA was isolated using the GeneJET^{TM} Plasmid Miniprep Kit (Thermofisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol.

Briefly, the bacterial culture was harvested by centrifugation at 6200*g* for 2 min. The pellet was resuspended in 250 µL of resuspension solution followed by addition of 250 µL lysis solution. Then, 350 µL of neutralization solution was added followed by centrifugation. The supernatant was transferred to GeneJET spin column and then washed with 500 µL of wash solution followed by centrifugation for 1 min (twice) to remove any trace amount of wash solution. The plasmid DNA was eluted using 32 µL sterile water.

3.2.3.6 Restriction enzyme digestion

The fast digest EcoRI restriction enzyme (Thermofisher Scientific, Waltham, MA, USA) was used for screening positive colonies according to the manufacturer's protocol. Briefly, the reaction mixture for the restriction digestion contained 2 µL of 10X FastDigest green buffer, 2 µL Plasmid DNA, 1 µL FastDigest enzyme and 15 µL nuclease free water to make a final reaction volume of 20 µL. The mixture was incubated at 37˚C for 1 hour followed by inactivation of the enzyme at 80˚C for 5 min. The sizes of the digested product and pGEM-T vector were confirmed by gel electrophoresis (1% w/v).

3.2.3.7 Sequence Analysis

The purified plasmids were sequenced (Macrogen, Rockville, MD, USA) with M13 forward, M13 reverse, and gene specific internal primers (Table 3.3). The internal primers were designed using NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The raw sequences data obtained from Macrogen were analyzed using CAP3 software (http://doua.prabi.fr/software/cap3) for assembly of the CONTIG sequences. Alignment of the resulting nucleotide sequences of the target genes from different lines was performed using

MULTIALIN software (http://multalin.toulouse.inra.fr/multalin/) and specificity of the *ABA8'OH1* and *GA20ox1* sequences was confirmed by blasting the respective sequences against GenBank database. The intronic and exonic nucleotide sequences of the target genes were identified using Ensembl Plants (https://plants.ensembl.org/index.html). The amino acid sequence of the genes was identified using ExPASy translate tool (https://web.expasy.org/translate/). To identify conserved protein domains, amino acid sequences of ABA8′OH and GA20ox of other species were obtained from the NCBI database and aligned using DNAMAN software (https://www.lynnon.com/). A phylogenetic tree was constructed for both *ABA8'OH* and *GA20ox* genes using phylogenetic tree generating software (MEGA7, https://www.megasoftware.net/) with neighbor-joining and Poisson correction methods based on 1000 bootstrap replicates.

Table 3.3 Primer used for sequencing of the target genes

Primer name	Type	Sequence (5´ to 3´)
M13	Forward	GTAAAACGACGGCCAG
	Reverse	CAGGAAACAGCTATGAC
ABA8'OH1	Internal-1	TCCATCGAGGCCATCGCC
	Internal-2	GCTGACCCGCTTTGGCAC
GA20ox1	Internal-1	TACGCCAGCAGCTTCACC

3.2.4 Gene expression of ABA and GA metabolic genes

3.2.4.1 Tissue collection

Mature dry seeds of the different malting barley lines were surface sterilized and imbibed for 24 h in three biological replicates as described above. After 24 hour after imbibition (HAI), the embryo was separated from other parts of the seed in liquid nitrogen and stored at -80˚C until RNA isolation.

3.2.4.2 RNA extraction

The total RNA from endosperm tissues was extracted as described previously (Mukherjee *et al.,* 2015). Total RNA from embryo tissues was extracted using a method described in Mornkham (2013) with minor modifications. Tissues were ground into fine powder and then mixed with 950 µL of buffer I (8 M LiCl, 2% PVP) and 50 µL of β-mercaptoethanol. After incubation of the mixture for 5 min at room temperature, 150 µL chloroform was added and then the mixture was centrifuge at 2400*g* for 5 min. The pellet was re-suspended in 588 µL buffer II (1.4% SDS, 0.075 M NaCl and 0.025 M EDTA) and 12 µL β-mercaptoethanol after which 600 µL chloroform was added. The mixture was centrifuged at 2400*g* for 5 min. The RNA was precipitated with 500 µL of isopropanol, incubated on ice for 15 min and centrifuged at 12000*g* for 10 min. The pellets were washed with 1 mL of 75% ethanol and air dried for 15 min before re-suspension in 50 µL diethyl pyrocarbonate (DEPC) water. The pellets dissolved in DEPC water were mixed with 700 µL TRIzol^{TM} (Thermofisher Scientific, Waltham, MA, USA) and incubated for 5 min at room temperature. After adding 140 µL of chloroform, the mixture was incubated for 3 min at room temperature and then centrifuge at 12000*g* for 15 min. The pellets were again washed with 75% ethanol, air dried and re-suspended with 50 µL DEPC water, and then the RNA samples were stored at -80°C.

3.2.4.3 DNA digestion

The total RNA samples were digested with DNase using DNA-*free* Kit (Ambion, Austin, TX, USA) to eliminate genomic DNA contaminants. Briefly, 1 µL rDNase I (2 units/ µL) and 5 µL 10X DNase I buffer were added to 10 µg of total RNA to a total reaction volume of 50 µL. The mixture was incubated for 30 min at 37°C. DNase inactivation reagent (5µL) was subsequently

added to the mixture and incubated for 2 min at room temperature to stop the reaction. Following centrifugation at 10000*g* for 2 min, the supernatant was separated for further use. The integrity and purity of the RNA was determined by analysis with RNA gel electrophoresis and spectrophotometer, respectively.

3.2.4.4 cDNA synthesis

Total RNA (1µg) was used for complementary DNA (cDNA) synthesis using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Briefly, 4 µL of 5X iScript reverse transcription supermix was mixed with 1 µg of total RNA and nuclease-free water to a total reaction volume of 20 µL. The reverse transcription conditions were 5 min at 25˚C for priming, followed by 30 min at 42˚C for reverse transcription, and 5 min at 85˚C for inactivation of the reaction. The cDNA products were diluted 20X and stored at -80°C until further use.

3.2.4.5 Primers

The primers for expression analysis using qPCR were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Table 3.4). The specificity of the primers was determined by BLAST search against GenBank database and RT-PCR analysis.

Table 3.4 Primer sequences used for qPCR

Gene	Type	Sequence (5´ to 3´)
β-Actin	FP	CCAGGTATCGCTGACCGTAT
	RP	GCTGAGTGAGGCTAGGATGG
NCED1	FP	ACCTCTGGAACTCGTGGGA
	RP	CGTCCGTGTCGTTGAAGAT
NCED2	FP	GCTTCTGCTTCCACCTCTG
	RP	GCACTCGTCCGACTCGTT
ABA8'OH1	FP	CCATGACCTTCACCCGCAAG
	RP	GGACACTGACGGATGGAGAAC
ABA8'OH2	FP	TGACGCACAGGGTGATTT
	RP	CCCTTGGGGATAAGAAACC
GA20ox1	FP	CCCTGGAAGGAGACCCTCT
	RP	GGCTCATCTCCGAGCAGTAG
GA20ox2	FP	AAGCTTCCCTGGAAGGAGAC
	RP	TCCCCTAGGTGCATGAAGTC
GA20ox3	FP	CGCTCACCTTCTTCCTCAAC
	RP	GAACTCACGCCAAGTGAAGTC

FP: Forward primer, RP: Reverse primer

3.2.4.6 Real-time qPCR

The qPCR assays were carried out using CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA), and it was performed in duplicate for each sample. The qPCR reaction mixture consisted 5 µL of the diluted cDNA, 1.2 µL of forward primer (5 µM; final concentration 300 nM), 1.2 µL of reverse primer (5 µM; final concentration 300 nM), 10 µL of SsoFast Eva Green Supermix (Bio-Rad, Hercules, CA, USA) and 2.6 µL of water with a total reaction volume of 20 µL. The thermocycling conditions used for the qPCR assays are as follows: initial denaturation and DNA polymerase activation at 95°C for 5 min, and then 40 cycles of denaturation at 95°C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. The relative transcript levels of the

target genes were determined by the Livak and Schmittgen (2001) method. The *β-Actin* gene was used as a reference gene for normalization and the expression level of Steptoe was set to 1 and used as a control.

3.2.4.7 Statistical analysis

The statistically significant difference in seed germination percentages, and relative expression levels of *NCED1*, *ABA8'OH1*, *ABA8'OH2*, *GA20ox2* and *GA20ox3* among different malting barley lines was tested using one-way ANOVA test. The means for germination percentage and gene expression were compared using Fisher least significant difference (LSD) test at $p < 0.05$. The correlation between seed germination and expression levels of different genes in different barley lines was tested using Pearson's coefficient of correlation (r).

3.3 Results

3.3.1 Seed germination

Germination of freshly harvested mature dry seeds of the 21 different malting barley lines was monitored for a period of 7 days. Our results showed that the lines exhibit a range of germination phenotype ranging from no germination observed in Steptoe to 100 percent germination in Himalaya (Figure 3.1).

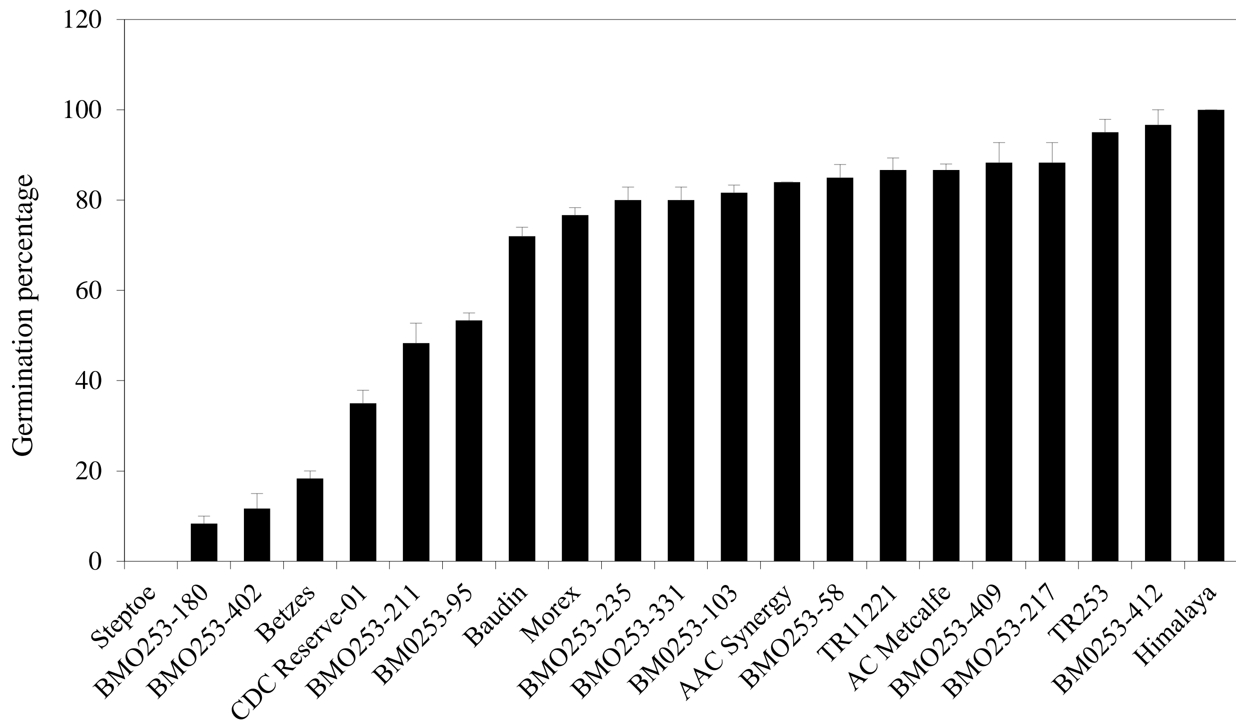


Figure 3.1 Germination percentages of the mature seeds of the different barley lines over a period of 7 days. Data are mean of three independent biological replicates ± SE. Statistically significant difference in the germination percentage of different lines was tested using Fisher LSD test ($p<0.05$; Appendix 1).

Based on their respective germination percentages (Figure 3.1), the different lines are divided into three different levels of dormancy. Lines showing less than 25% of germination are considered as highly dormant while those showing between 25% and 75% are classified as lines with intermediate level of dormancy. Lines that showed over 75% germination are considered as non-dormant lines (Table 3.5).

Table 3.5 Dormancy phenotype of the different malting barley lines

Dormant (<25%)	Intermediate (25-75%)	Non-dormant (>75%)
Steptoe	CDC Reserve-01	Morex
BM0253-180	BM0253-211	BM0253-235
BM0253-402	BM0253-95	BM0253-331
Betzes	Baudin	BM0253-103
		AAC Synergy
		BM0253-58
		TR11221
		AC Metcalfe
		BM0253-409
		BM0253-217
		TR253
		BM0253-412
		Himalaya

3.3.2 Molecular cloning of *ABA8'OH1*

3.3.2.1 PCR amplification of target gene

Amplification of the genomic DNA sequence of *ABA8'OH1* gene from all the 21 malting barley lines with gene specific primers designed from the 5′ and 3′ end of the DNA sequence produced DNA fragment with expected size of 2047 bp (Figure 3.2).

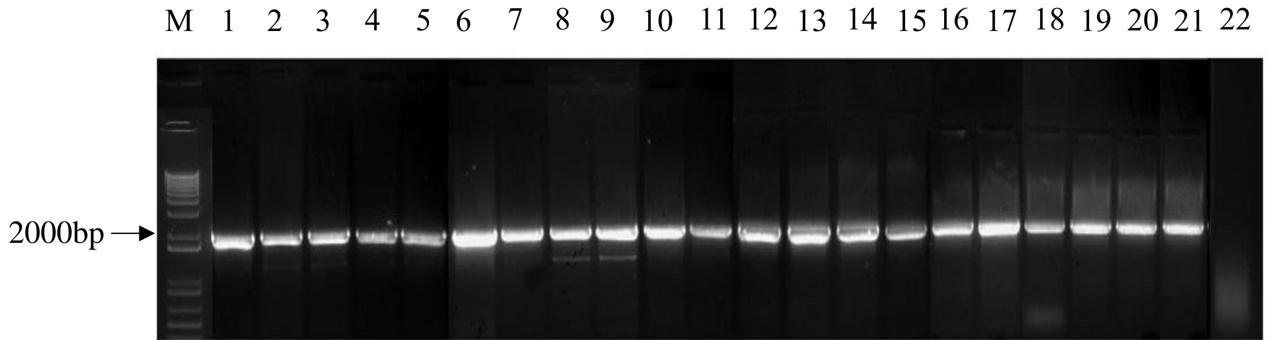


Figure 3.2 PCR amplification of the genomic sequence of *ABA8'OH1* from the malting barley genotypes and breeding lines. Lanes are 1=BM0253-180; 2=BM0253-211; 3=BM0253-217; 4=BM0253-235; 5=BM0253-58; 6=BM0253-331; 7=BM0253-402; 8=BM0253-409; 9=BM0253-95; 10=BM0253-103; 11=BM0253-412; 12=TR253; 13=Baudin; 14=Morex; 15=Steptoe; 16=Betzes; 17=Himalaya; 18=TR11221; 19=AAC Synergy; 20=AC Metcalfe; 21=CDC Reserve-01; 22=Negative control. Lane M is the 1 Kb plus marker.

The sequencing and BLAST searching of the amplified fragments of *ABA8'OH1* gene derived from the different genotypes and lines against the GenBank database confirmed the specificity of the sequenced DNA fragments as its sequence exhibited 100% identity at E-value of 0 with *ABA8'OH1* (GenBank ID: AB239299.1) (Table 3.6). In addition, the sequence of *ABA8'OH1* gene derived from the different genotypes and lines of barley showed over 91% and 90% identity with *TaABA8'OH1-D* (GenBank ID: AB714576.1) and *TaABA8'OH1-B* (GenBank ID: AB714575.1) respectively (Table 3.6).

Table 3.6 Nucleotide sequence identity of the sequence of *ABA8'OH1* from the different barley lines with other *ABA8'OH1* gene sequences

Accession #	Description	Max Score[a]	E-Value[b]	Max Identity[c]
AB239299.1	*ABA8'OH1*	987	0.0	100%
AB714576.1	*TaABA8'OH1-D*	2158	0.0	91%
AB714575.1	*TaABA8'OH1-B*	2126	0.0	90%

[a]Maximum score indicates the amount of sequence homology between *ABA8'OH1* from the different barley lines and other *ABA8'OH1* sequences available in GenBank database.
[b]E-Value determines the significance of the alignment score.
[c]Maximum identity refers to percentage of identical nucleotides between *ABA8'OH1* from the different barley lines and other *ABA8'OH1* sequences available in GenBank database.

3.3.2.2 Analysis of *ABA8'OH1* gene sequence for allelic variation

A number of nucleotide substitutions, addition and deletions (INDELS) were detected among the sequences derived from the 21 different barley lines (Figure 3.3, Appendix 2). Their positions and substitution patterns are C to T at 602th bp (AC Metcalfe, BM0253-402, TR253); C to A at 605th bp (AAC Synergy, CDC-Reserve-01, Himalaya, Morex and TR11221); G to C at 1227th bp (AAC Synergy, CDC-Reserve-01 and TR11221); C to A at 1340th bp (Himalaya, Morex and Steptoe); A to T or deletion of A at 1816th bp (CDC-Reserve-01, Himalaya, Morex, TR11221 and Steptoe). An insertion of 10 bp fragment 'GTGCTGTACC' from 1523th to 1532th bp position was observed in Himalaya and Morex while a deletion of 12 bp fragment 'GATGATGATGAT' from 1838th to 1849th bp position was evident in AAC Synergy, Himalaya, Morex and TR11221. Overall, seven different types of alleles were found in the genomic sequence of the target gene.

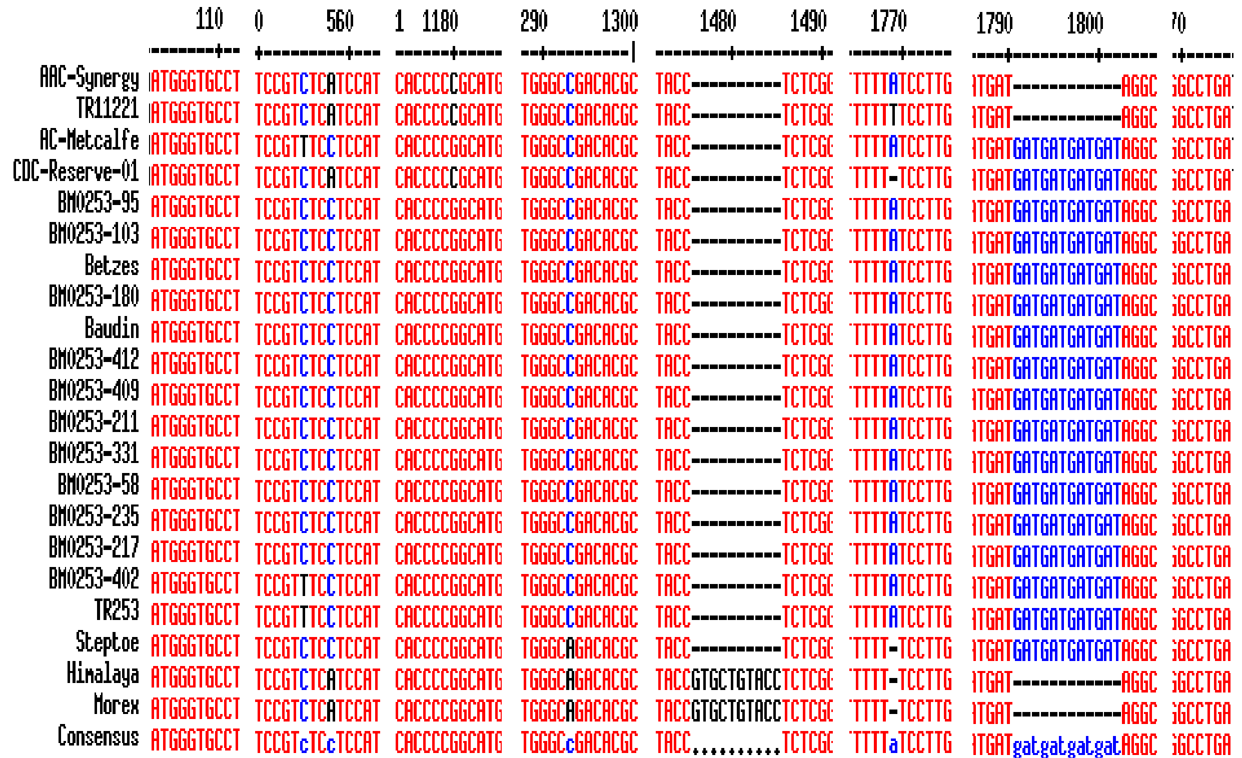


Figure 3.3 Multiple sequence alignment of *ABA8'OH1* gene showing nucleotide polymorphisms among different barley lines.

3.3.2.3 Analysis of nucleotide sequence for exon-intron structure of *ABA8'OH1*

Blast searching of the DNA sequence of *ABA8'OH1* gene from the different barley lines against the Ensembl plants genome database (https://plants.ensembl.org/index.html) indicated that the genomic sequences of the target gene consist of five exons (coding region) and four introns (non-coding region), and the size of the coding sequence (only exons) of the target gene is represented by 1407 bp (Figure 3.4).

ATGGGTGCCTTCATCCTCCTCCTCTGCTTGCTCGTGCCGTTGGTGCTCGTGTGCGCCGTCCGCG
CCAGGAAGGGCGCCGGCGGGCGGTCGTCGTCGGGCGGCGGCAAGAAGGGCAGGCTGCCGCCGGG
GTCCATGGGGTGGCCGTACGTGGGCGAGACCACGCAGCTCTACTCCTCCAAGAACCCCAACGTC
TTCTTCGCCAGGAAGCGTAACAAGTACGGGCCCATCTTCAAGACGCACATCCTCGGGTGCCCCT
GCGTCATGGTGTCCAGCCCGGAGGCCGCCAAGTTCGTGCTCGTCACGCAGGCGCACCTCTTCAA
GCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGCCGCCAGGCCATCTTCTTCCAGCAGGGG
GACTACCACACCCACCTCCGCCGTCTCGTCTCCCGCGCCTTCTCCCCCGAGGCCATCCGCGGCT
CCGT**C**TC**C**TCCATCGAGGCCATCGCCCTCCGCTCCCTCGGCTCATGGGAAGGCCATGAAGTCAA
CACCTTCCAAGAAATGAAGACTGTAAGTTCTTCTTCTTCCATTCCTGCCTCCTCTGTTTTC
ATCTGCTCTGCTCTGCTCTGCGGCTAAATGCTTAGAAATGGTCACTGATGGTTTTGTTGGTGTC
ATTGCGCAGTACGCTCTGAATGTGGCATTGCTGTCCATCTTCGGGGAGGAGGAGATGCAGTACA
TCGAGGAGCTGAAGCAGTGCTACCTGACGCTGGAGAAGGGGTACAACTCGATGCCGGTGAACCT
GCCGGGCACGCTGTTCCACAAGGCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCAC
ATCATCTCAGCCCGGCGCGAGCGGGAGCGCGGGAGCGACCTCCTGGGCTCCTTCATGGACGGCC
GCGAGGCGCTCACCGACGACCAGATCGCCGACAACGCCATCGGCGTCATCTTCGCCGCGCGGGA
CACCACCGCCAGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCCGTCCTCAAA
GCCGTCACCGTAAGTCGCCATCAAACCGACCAGCTGACCCGCTTTGGCACCCC**G**GCATGTCGAA
AGGCAGTGTCTCTGACCCGCGCGCGTGAAACGATTGACAACAGGAAGAGCACGCCGAGATCGCG
AGGGAGAAGGCGTTGTCCGGCGAGCCGCTGTCGTGGGC**C**GACACGCGGCGGATGCGGGTGACGG
GCCGGGTGATCCAGGAGACGATGCGGGTGGCGTCCATCCTCTCCTTCACCTTCAGAGAGGCCGT
CGAGGACGTGGAGTACCAAGGTGAGCACTGAGCTCTGAGCAGAGACATCAATCAACTTCGCTTT
GGTCGTTTGCGGCAGCGCACTGCTGTACC**GTGCTGTACC**TCTCGGAGTACAGCTACAGCAGTGC
GCTGCCTGCGCATGAACTGGCTCGGAAAGGACGTGCTCCTAACCGAACGGACGAAATAGACCAA
CAACTCGAACTCGCAACTCACCTCGGCTCGGCTCGCTCCTCCGTGCAGGGTACCTGATCCCCAA
GGGCTGGAAAGTGCTTCCCCTGTTCCGGAACATCCACCACAACCCCGACCACTTCCCCTCCCCC
GAAAAGTTCGATCCTTCACGATTCGAGGTCAGCATCATCACATCTTCTTCTTCTACTGTTTTTT
TT**AT**CCTTGGATGATGATGATGAT**GATGATGATGAT**AGGCTTGAGAGTCCCCCGTTGTTCATTA
GCTGATTGCGTTTTTGTTCTTGGTGACTGCAGGTGGCCCCCAAGCCCAACACGTTCATGCCGTT
CGGGAACGGGACCCACTCGTGCCCCGGCAACGAGCTGGCCAAGCTGGAGATGCTCGTCCTCTGC
CACCACCTCGCCACCAAGTACAGATGGTCTACCTCCAAGTCCGAGAGCGGCGTGCAGTTCGGCC
CCTTCGCCCTGCCCATCAACGGCCTCCCCATGACCTTCACCCGCAAGGCCTGA

Figure 3.4 Genomic DNA sequence of *ABA8'OH1* gene from start to stop codon. The exonic regions are highlighted in grey while base positions where single nucleotide polymorphisms occur are highlighted in bold yellow. Locations where addition and deletion of bases occur are highlighted in bold red.

We found that the nucleotide substitutions, addition and deletions (INDELS) occurred in both exonic and intronic regions of the gene. The variations in the exonic region of the gene include substitution of C to T and C to A in the 1st exon, and C to A in the 3rd exon (Figure 3.4). Intronic

substitutions include change of nucleotide from G to C in the 2nd intron, A to T/deletion and deletion of T in the 4th intron. An insertion of 10 bp fragment 'GTGCTGTACC' in the 3rd intron and a deletion of 12 bp fragment 'GATGATGATGAT' were also observed in in the 4th intron.

3.3.2.4 Protein sequence analysis of *ABA8'OH1* gene

The translation of the coding nucleotide sequences of the *ABA8'OH1* gene from the different barley lines into amino acids sequences using ExPASy translate tool (https://web.expasy.org/translate/) resulted in putative proteins with 468 amino acids (Figure 3.5). The three nucleotide substitutions in the coding region of the *ABA8'OH1* gene caused a change in codon sequence with no change in amino acid, rendering no change in the protein sequence of the gene. The first substitution of base T to C at 453 bp position led to a change in codon of GTT to GTC at 451 to 453 base pair position in the nucleotide sequence of the gene and both codons code for the same amino acid 'Valine'. The second substitution of base C to A at 456 bp position led to a change in codon of TCC to TCA at 454 to 456 bp position in the nucleotide sequence of the gene and both codons represent the same amino acid 'Serine'. The third substitution of base C to A at 978 position caused a change in codon of GCC to GCA at 976 to 978 bp position in the nucleotide sequence of the gene and both codons stand for the same amino acid 'Alanine'.

```
   1 ATGGGTGCCTTCATCCTCCTCCTCTGCTTGCTCGTGCCGTTGGTG
      M  G  A  F  I  L  L  L  C  L  L  V  P  L  V
  46 CTCGTGTGCGCCGTCCGCGCCAGGAAGGGCGCCGGCGGGCGGTCG
      L  V  C  A  V  R  A  R  K  G  A  G  G  R  S
  91 TCGTCGGGCGGCGGCAAGAAGGGCAGGCTGCCGCCGGGGTCCATG
      S  S  G  G  G  K  K  G  R  L  P  P  G  S  M
 136 GGGTGGCCGTACGTGGGCGAGACCACGCAGCTCTACTCCTCCAAG
      G  W  P  Y  V  G  E  T  T  Q  L  Y  S  S  K
 181 AACCCCAACGTCTTCTTCGCCAGGAAGCGTAACAAGTACGGCCCC
      N  P  N  V  F  F  A  R  K  R  N  K  Y  G  P
 226 ATCTTCAAGACGCACATCCTCGGGTGCCCCTGCGTCATGGTGTCC
      I  F  K  T  H  I  L  G  C  P  C  V  M  V  S
 271 AGCCCGGAGGCCGCCAAGTTCGTGCTCGTCACGCAGGCGCACCTC
      S  P  E  A  A  K  F  V  L  V  T  Q  A  H  L
 316 TTCAAGCCTACCTTCCCGGCCAGCAAGGAGCGGATGCTGGGCCGC
      F  K  P  T  F  P  A  S  K  E  R  M  L  G  R
 361 CAGGCCATCTTCTTCCAGCAGGGGGACTACCACACCCACCTCCGC
      Q  A  I  F  F  Q  Q  G  D  Y  H  T  H  L  R
 406 CGTCTCGTCTCCCGCGCCTTCTCCCCCGAGGCCATCCGCGGCTCC
      R  L  V  S  R  A  F  S  P  E  A  I  R  G  S
 451 GTTTCCTCCATCGAGGCCATCGCCCTCCGCTCCCTCGGCTCATGG
      V  S  S  I  E  A  I  A  L  R  S  L  G  S  W
 496 GAAGGCCATGAAGTCAACACCTTCCAAGAAATGAAGACTTACGCT
      E  G  H  E  V  N  T  F  Q  E  M  K  T  Y  A
 541 CTGAATGTGGCATTGCTGTCCATCTTCGGGGAGGAGGAGATGCAG
      L  N  V  A  L  L  S  I  F  G  E  E  E  M  Q
 586 TACATCGAGGAGCTGAAGCAGTGCTACCTGACGCTGGAGAAGGGG
      Y  I  E  E  L  K  Q  C  Y  L  T  L  E  K  G
 631 TACAACTCGATGCCGGTGAACCTGCCGGGCACGCTGTTCCACAAG
      Y  N  S  M  P  V  N  L  P  G  T  L  F  H  K
 676 GCCATGAAGGCCCGGAAGCGGCTGGGCGCCATTGTGGCCCACATC
      A  M  K  A  R  K  R  L  G  A  I  V  A  H  I
 721 ATCTCAGCCCGGCGCGAGCGGGAGCGCGGGAGCGACCTCCTGGGC
      I  S  A  R  R  E  R  E  R  G  S  D  L  L  G
 766 TCCTTCATGGACGGCCGCGAGGCGCTCACCGACGACCAGATCGCC
      S  F  M  D  G  R  E  A  L  T  D  D  Q  I  A
 811 GACAACGCCATCGGCGTCATCTTCGCCGCGCGGACACCACCGCC
      D  N  A  I  G  V  I  F  A  A  R  D  T  T  A
 856 AGCGTGCTCACGTGGATGGTCAAGTTCCTCGGCGACAACCCCGCC
      S  V  L  T  W  M  V  K  F  L  G  D  N  P  A
 901 GTCCTCAAAGCCGTCACCGAAGAGCACGCCGAGATCGCGAGGGAG
      V  L  K  A  V  T  E  E  H  A  E  I  A  R  E
 946 AAGGCGTTGTCCGGCGAGCCGCTGTCGTGGGCCGACACGCGGCGG
      K  A  L  S  G  E  P  L  S  W  A  D  T  R  R
 991 ATGCGGGTGACGGGCCGGGTGATCCAGGAGACGATGCGGGTGGCG
      M  R  V  T  G  R  V  I  Q  E  T  M  R  V  A
1036 TCCATCCTCTCCTTCACCTTCAGAGAGGCCGTCGAGGACGTGGAG
      S  I  L  S  F  T  F  R  E  A  V  E  D  V  E
1081 TACCAAGGGTACCTGATCCCCAAGGGCTGGAAAGTGCTTCCCCTG
      Y  Q  G  Y  L  I  P  K  G  W  K  V  L  P  L
1126 TTCCGGAACATCCACCACAACCCCGACCACTTCCCCTCCCCCGAA
      F  R  N  I  H  H  N  P  D  H  F  P  S  P  E
1171 AAGTTCGATCCTTCACGATTCGAGGTGGCCCCCAAGCCCAACACG
      K  F  D  P  S  R  F  E  V  A  P  K  P  N  T
1216 TTCATGCCGTTCGGGAACGGACCCACTCGTGCCCCGGCAACGAG
      F  M  P  F  G  N  G  T  H  S  C  P  G  N  E
1261 CTGGCCAAGCTGGAGATGCTCGTCCTCTGCCACCACCTCGCCACC
      L  A  K  L  E  M  L  V  L  C  H  H  L  A  T
1306 AAGTACAGATGGTCTACCTCCAAGTCCGAGAGCGGCGTGCAGTTC
      K  Y  R  W  S  T  S  K  S  E  S  G  V  Q  F
1351 GGCCCCTTCGCCCTGCCCATCAACGGCCTCCCCATGACCTTCACC
      G  P  F  A  L  P  I  N  G  L  P  M  T  F  T
1396 CGCAAGGCCTGA
      R  K  A  *
```

Figure 3.5 Amino acid sequence of *ABA8'OH1* aligned with the cDNA nucleotide sequence. The three nucleotide substitutions and the corresponding codons along with their respective amino acids are highlighted in yellow. '*' represents stop codon.

3.3.2.5 Conserved domain and phylogenetic analysis of *ABA8′OH1*

Multiple sequence alignment of the putative amino acid sequences of ABA8′OH1 translated from the *ABA8′OH1* nucleotide sequences of the different barley lines along with the amino acid sequences of different ABA8′OH1 from different species including *Triticum aestivum, Aegilops taushii, Hordeum vulgare, Brachypodium distachyon, Orzya sativa, Zea mays, Sorghum bicolor, Arabidopsis thaliana, Beta vulgaris, Phaseolus vulgaris* and *Glycine max* showed that all the ABA8′OH1 proteins analyzed share very high sequence identity as 69% of their amino acid residues exhibit ≥ 75% homology in which 254 of the amino acid residues exhibit 100% homology (Figure 3.6). None of the nucleotide polymorphisms occurred in the conserved regions of the ABA8′OH1, which include the active-site cysteine and a putative signal peptide located at the N-terminus of the protein.

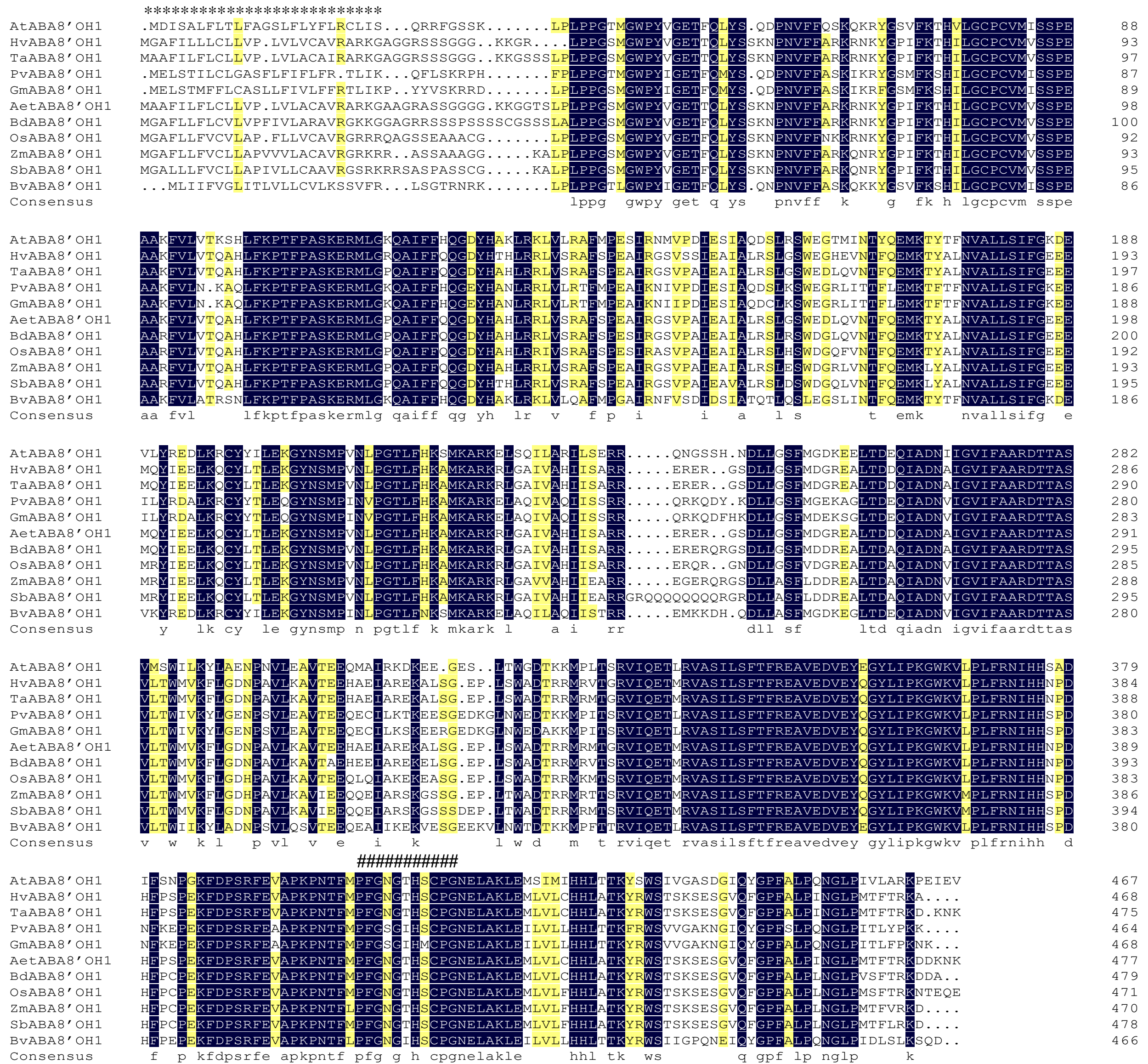


Figure 3.6 Amino acid sequence alignment of barley and other ABA8′OH1 proteins. Amino acid residues with ≥75% homology are shaded in yellow while amino acid residues with 100% homology are represented in dark blue. The '#' symbol represents active-site cysteine of ABA8′OHs and '*' represents a putative signal peptide located at the N-terminus of the proteins. Amino acids were numbered from the start codon representing the amino acid methionine. The species and GenBank IDs of their respective proteins are: *Triticum aestivum*, TaABA8′OH1 (GenBank ID: ACB78189.1); *Aegilops taushii*, AetABA8′OH1 (GenBank ID: F775_16028); *Hordeum vulgare*, *ABA8'OH1* (GenBank ID: ABB71585.1); *Brachypodium distachyon*, BdABA8′OH1 (GenBank ID: BRADI3G52660.1); *Orzya sativa*, OsABA8′OH1 (GenBank ID: Os02g0703700); *Zea mays*, ZmABA8′OH1 (GenBank ID: Zm00001d017762_T002); *Sorghum bicolor*, SbABA8′OH1 (GenBank ID: SORBI_3004G268700); *Arabidopsis thaliana*, AtABA8′OH1 (GenBank ID: AT4G19230.1); *Beta vulgaris*, BvABA8′OH1 (GenBank ID: BVRB_5g102360); *Phaseolus vulgaris*, PvABA8′OH1 (GenBank ID: ABC86558.1) and *Glycine max*, GmABA8′OH1 (GenBank ID: ABQ65856.1).

Phylogenetic analysis of the *ABA8′OH1* protein sequence obtained in this study along with ten known ABA8′OH1 plant proteins separated the proteins into two classes, one class containing all the proteins from monocots and the other class from dicots (Appendix 3). It is evident from the phylogenetic tree that the barley ABA8′OH1 protein is closely related to the ABA8′OH1 proteins of *Triticum aestivum* and *Aegilops taushii*.

3.3.3 Expression analysis of ABA metabolic genes during imbibition

Analysis of the expression patterns of the family members of the ABA metabolic genes, *NCED* and *ABA8′OH* in the mature grains of different barley lines at 24 HAI revealed their differential expression patterns across the lines.

3.3.3.1 Expression patterns of *NCED* genes

The expression data for two known *NCED* genes of barley (*NCED1* and *NCED2*) showed that only *NCED1* is expressed in the embryo of all lines at 24 HAI (Figure 3.7). However, the expression of this gene was either very low or not detected in the endosperm. Embryos of dormant lines including Steptoe, BM0253-180, BM0253-402 and Betzes exhibited high level of *NCED1* expression (Figure 3.7). Embryos of selected lines with intermediate level of dormancy including CDC-Reserve-01 and Baudin showed lower level of *NCED1* expression (over 2.5-fold lower than that observed in the most dormant line Steptoe) while other lines with intermediate dormancy such as BM0253-211 and BM0253-95 showed higher level of *NCED1* expression (over 1.3-fold higher than Steptoe). Embryos of non-dormant lines including Morex, BM0253-235, BM0253-103, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-409, BM0253-217, TR253, BM0253-412 and Himalaya are characterized by low level of *NCED1* expression (from 2-fold up to 10-fold

lower than that observed in the most dormant line Steptoe). Although BM0253-331 is non-dormant, the level of its *NCED1* expression was similar to that shown by the dormant embryos.

The expression of *NCED2* was also very low/not detected in both embryo and endosperm tissues.

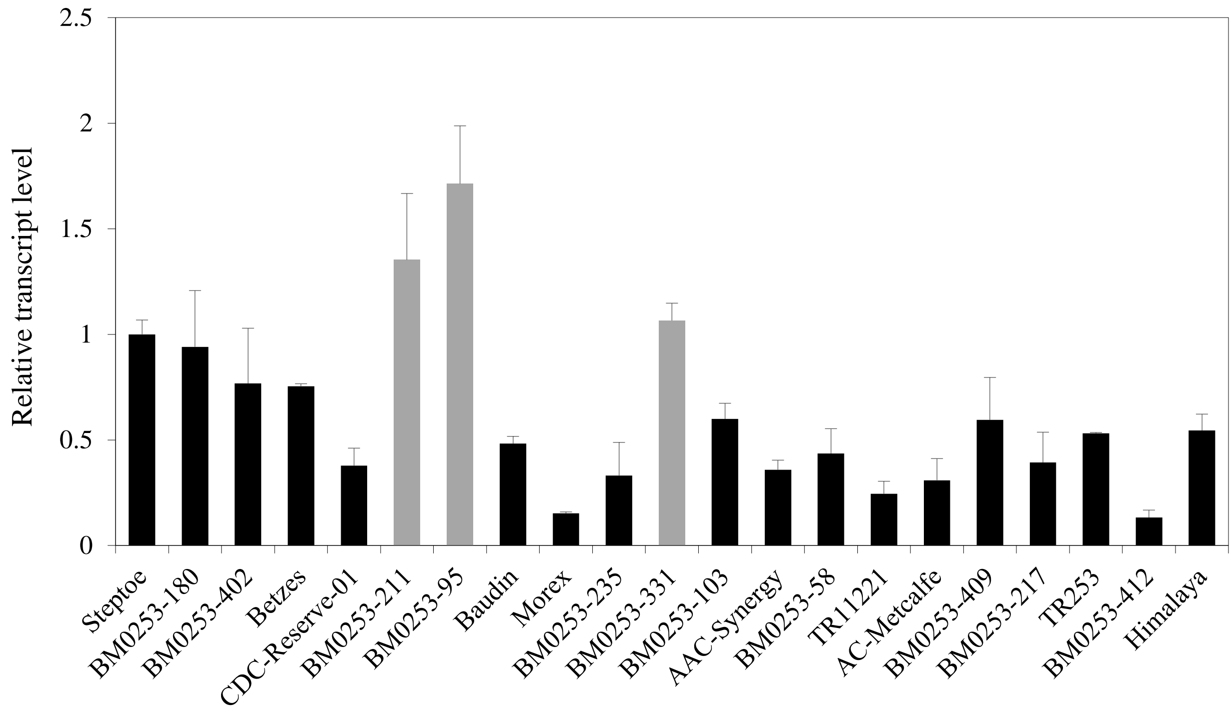


Figure 3.7 Relative transcript level of *NCED1* in the embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype. The transcript level was calculated relative to line Steptoe, which was set a value of 1. The data presented are means of three biological replicates and the error bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test (p<0.05; Appendix 4).

3.3.3.1.1 Correlation between seed germination and *NCED1* expression

Correlation analysis between seed germination percentages and *NCED1* expression of the 21 different barley lines revealed an r value of 0.50 (Appendix 5). Correlation analysis for 18 different barley lines excluding those showing *NCED1* expression patterns that are not consistent with the

germination phenotype, including BM0253-211, BM0253-95 and BM0253-331, produced an *r* value of 0.75 (Figure 3.8).

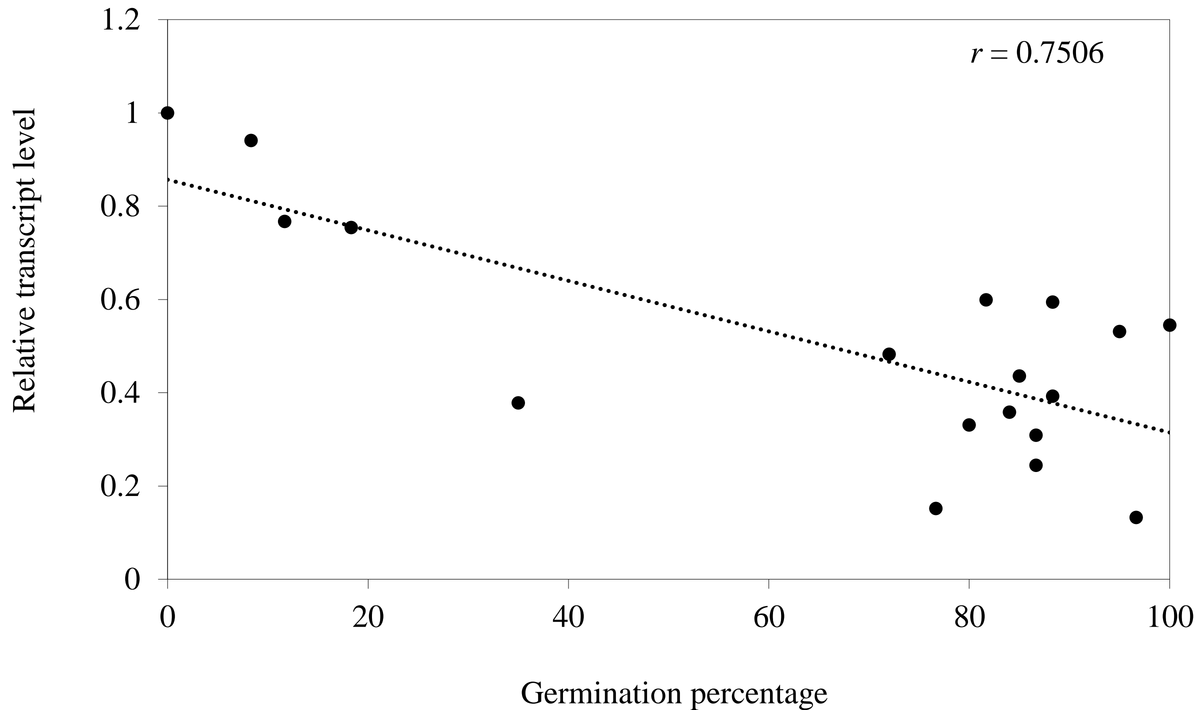


Figure 3.8 Correlation between the seed germination percentage and expression levels of *NCED1* in different barley lines (Steptoe, BM0253-180, BM0253-402, Betzes, CDC Reserve-01, Baudin, Morex, BM0253-235, BM0253-103, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-409, BM0253-217, TR253, BM0253-412 and Himalaya).

3.3.3.2 Expression of *ABA8'OH1* in the embryo

Embryos of dormant lines including BM0253-180, BM0253-402 and Betzes showed expression levels that are either lower (up to 2-fold) or similar to that detected in the most dormant line Steptoe whose expression was set to 1 and was considered as a control (Figure 3.9). Lines with intermediate dormancy, CDC-Reserve-01, BM0253-211, BM0253-95 and Baudin also showed expression levels that are either lower (up to 2.5-fold) or similar to that detected in the most dormant line

Steptoe. The non-dormant lines Morex, BM0253-331, BM0253-103, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-409, BM0253-217, TR253 and Himalaya appeared to have up to 5-fold lower expression than the control whereas two non-dormant lines BM0253-235 and BM0253-412 have upto 2-fold higher expression than the control.

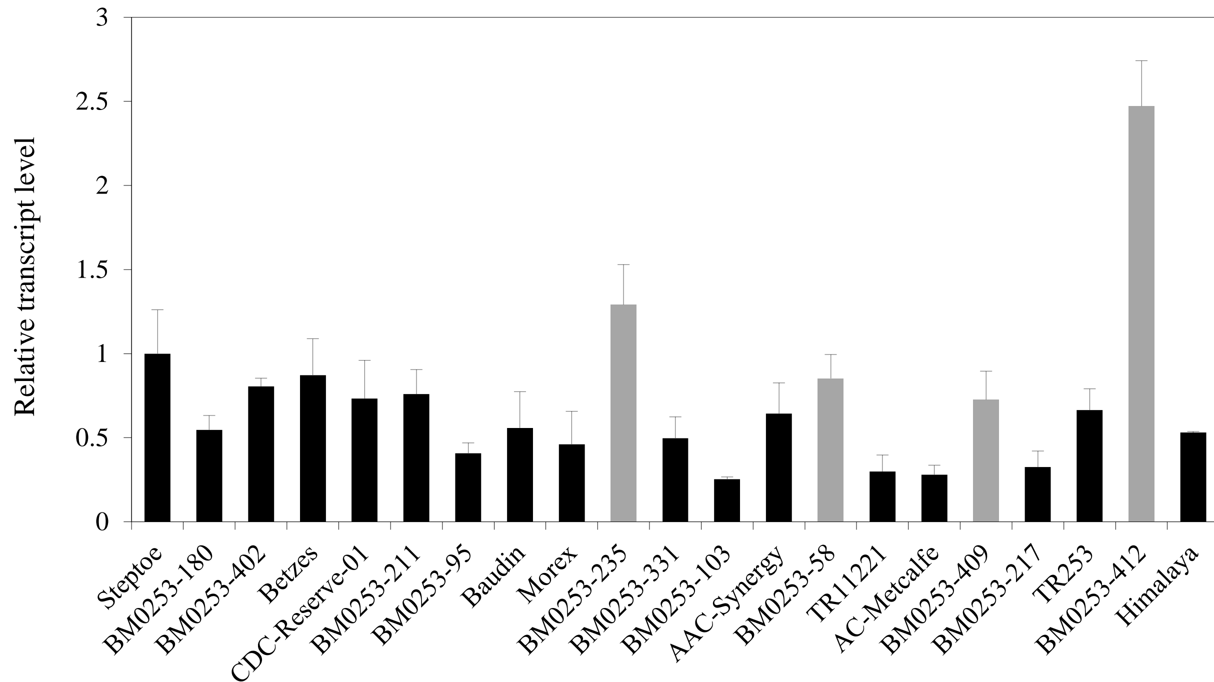


Figure 3.9 Relative transcript level of *ABA8'OH1* in the embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype. Transcript level was calculated relative to line Steptoe, which was set at 1. The data presented are means of three biological replicates and the error bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test (p<0.05; Appendix 6).

3.3.3.2.1 Correlation between seed germination and embryonic *ABA8'OH1* expression

Correlation between seed germination percentages and *ABA8'OH1* expression in the different barley lines revealed an r value of 0.01 (Appendix 7). The correlation analysis of 17 selected barley lines (Steptoe, BM0253-180, BM0253-402, Betzes, CDC-Reserve-01, BM0253-211, BM0253-95,

Baudin, Morex, BM0253-331, BM0253-103, AAC Synergy, TR11221, AC Metcalfe, BM0253-217, TR253 and Himalaya) revealed an *r* value of 0.67 (Figure 3.10).

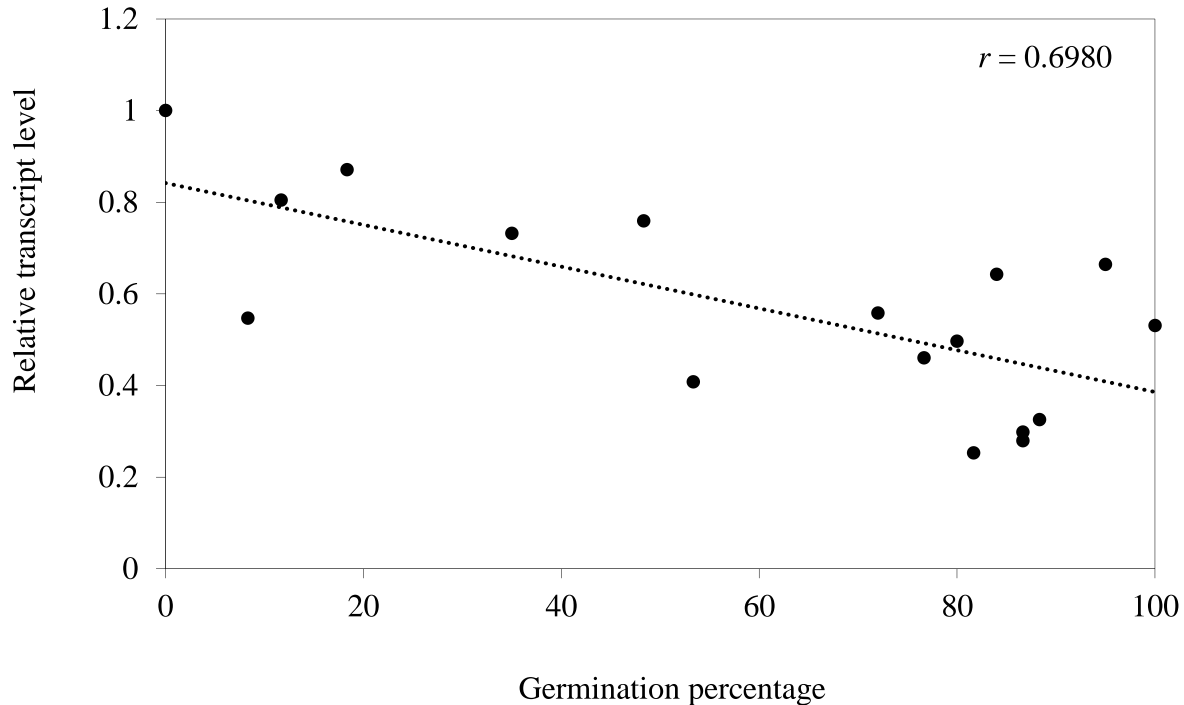


Figure 3.10 Correlation between the seed germination percentages and expression levels of embryonic *ABA8'OH1* of different barley lines (Steptoe, BM0253-180, BM0253-402, Betzes, CDC-Reserve-01, BM0253-211, BM0253-95, Baudin, Morex, BM0253-331, BM0253-103, AAC Synergy, TR11221, AC Metcalfe, BM0253-217, TR253 and Himalaya).

3.3.3.2.2 Correlation between embryonic *NCED1* and *ABA8'OH1* expression in the embryo

Since higher expression levels of both *NCED1* and *ABA8'OH1* were found in the seeds of dormant lines than the non-dormant lines, correlation analysis was conducted between the expression levels of the two genes. Our correlation analysis showed a positive correlation (*r*=0.65) between the expression levels of *NCED1* and *ABA8'OH1* in the embryo of selected lines (Steptoe, BM0253-

402, Betzes, CDC-Reserve-01, Baudin, Morex, BM0253-103, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-409, BM0253-217, TR253 and Himalaya) (Figure 3.11).

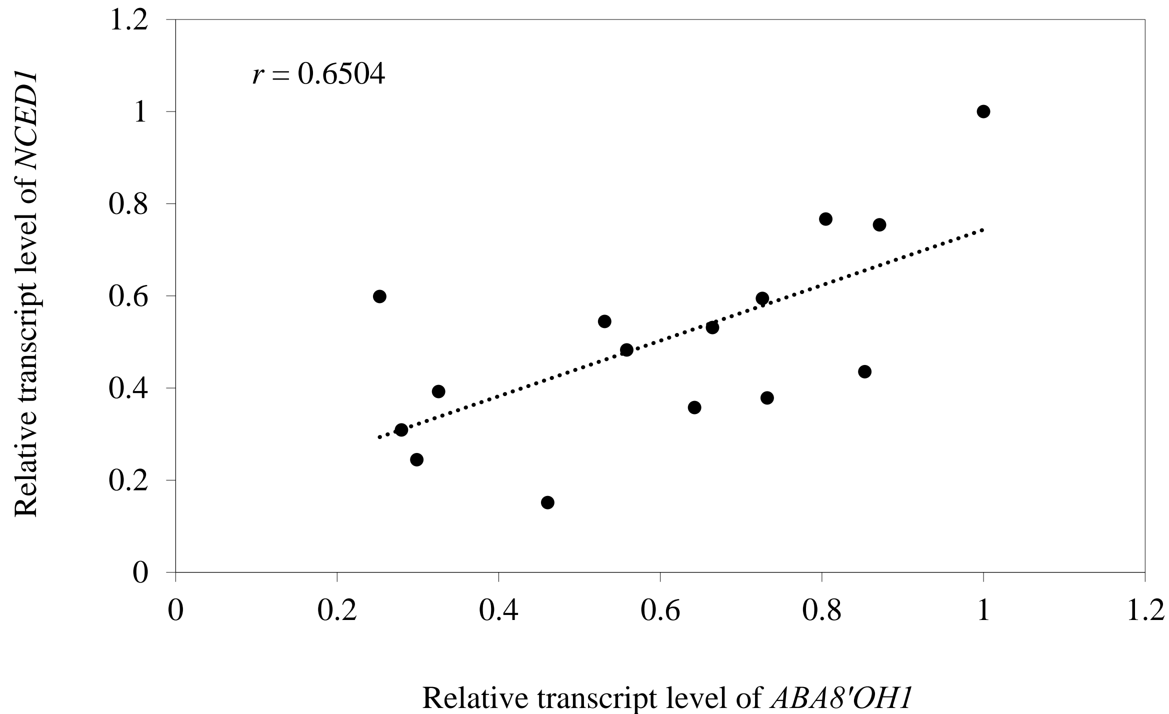


Figure 3.11 Correlation between the expression levels of embryonic *NCED1* and *ABA8'OH1* in different barley lines (Steptoe, BM0253-402, Betzes, CDC-Reserve-01, Baudin, Morex, BM0253-103, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-409, BM0253-217, TR253 and Himalaya).

3.3.3.3 Expression of *ABA8'OH1* in the endosperm

Analysis of the expression of *ABA8'OH1* in the barley lines revealed that its highest expression was observed in non-dormant line BM0253-58 (12.5 fold higher as compared to that detected in the most dormant line Steptoe, which was considered as control) (Figure 3.12). The dormant lines such as Steptoe and Betzes showed low level of *ABA8'OH1* expression, however other dormant lines including BM0253-180 and BM0253-402 showed over 3-fold higher level of expression than

that was detected in the control Steptoe, and this expression pattern is similar to that observed in the lines with intermediate level of dormancy. The intermediate lines including CDC-Reserve-01, BM0253-211, BM0253-95 and Baudin showed over 2-fold higher expression of *ABA8'OH1* as compared to that observed in the dormant lines but over 1.2 fold lower than that detected in the non-dormant lines. The non-dormant lines BM0253-235, BM0253-331, BM0253-58, BM0253-409, BM0253-217 and TR253 showed 5- to 12-fold higher level of *ABA8'OH1* expression than the control. However, non-dormant lines Morex, BM0253-103, AAC Synergy, TR11221, AC Metcalfe, BM0253-412 and Himalaya showed an expression level similar to that observed in dormant and intermediate lines.

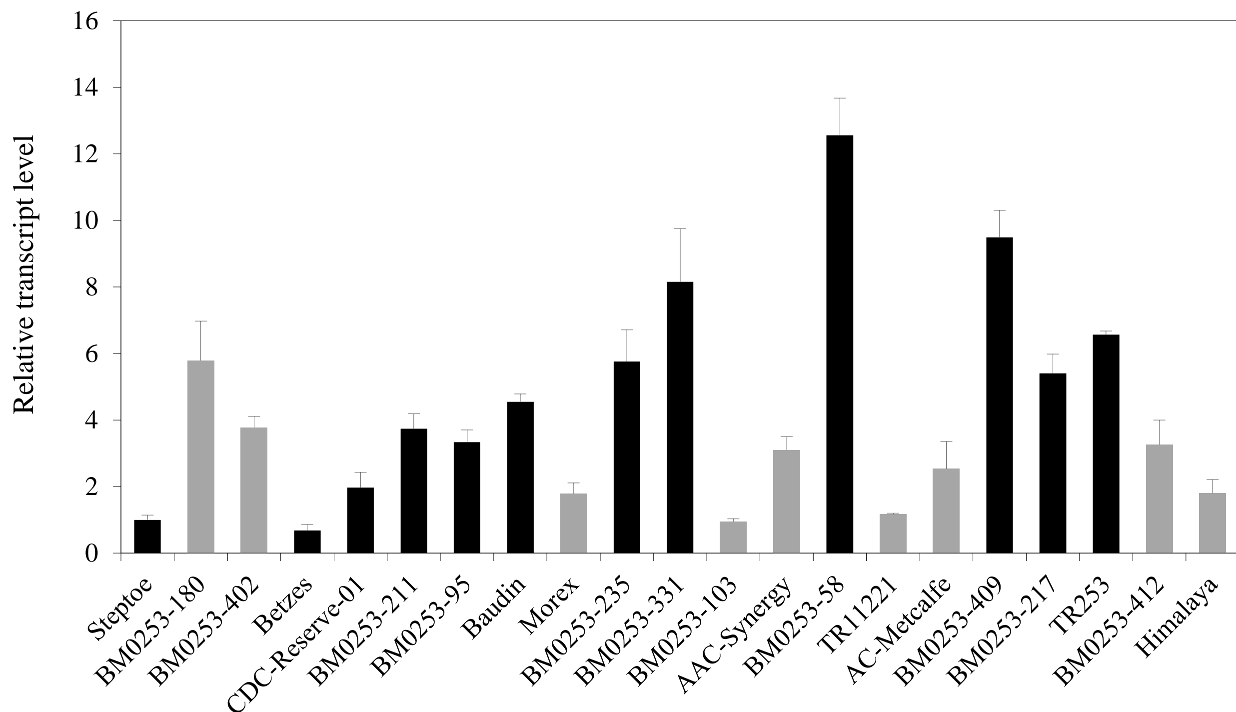


Figure 3.12 Relative transcript level of *ABA8'OH1* in the endosperm tissues at 24 HAI in different barley lines with a range of dormancy phenotype. Transcript level was calculated relative to line Steptoe, which was set at 1. The data presented are means of three biological replicates and the bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test ($p<0.05$; Appendix 8).

3.3.3.3.1 Correlation between seed germination and endospermic *ABA8'OH1* expression

Correlation analysis between germination percentage and *ABA8'OH1* expression revealed an r value of 0.26 (Appendix 9). However, the correlation between the two factors for the selected 12 lines (Steptoe, Betzes, CDC-Reserve-01, BM0253-211, BM0253-95, Baudin, BM0253-235, BM0253-331, BM0253-58, BM0253-409, BM0253-217 and TR253) revealed an r value of 0.81 (Figure 3.13).

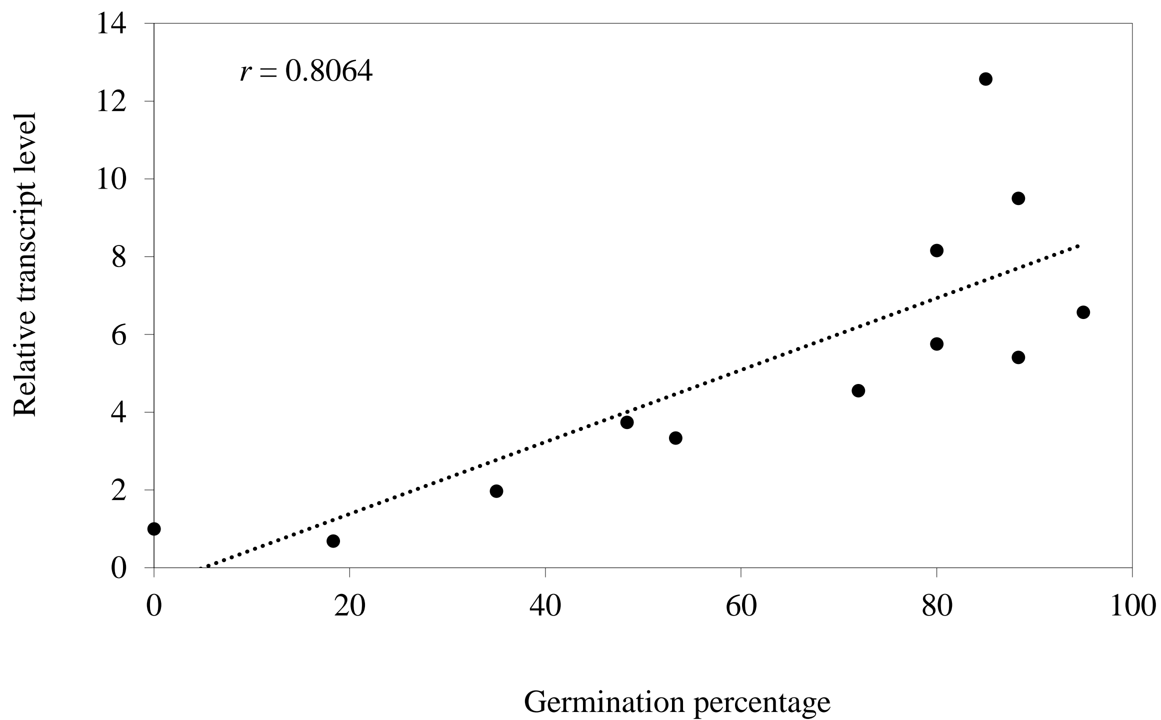


Figure 3.13 Correlation between the seed germination percentage and expression levels of endospermic *ABA8'OH1* of different barley lines (Steptoe, Betzes, CDC-Reserve-01, BM0253-211, BM0253-95, Baudin, BM0253-235, BM0253-331, BM0253-58, BM0253-409, BM0253-217 and TR253).

3.3.3.4 Expression of *ABA8'OH2* in the embryo tissue

The expression of *ABA8'OH2* in embryos of dormant lines Steptoe and Betzes was low while other dormant lines BM0253-180 and BM0253-402 showed over 5-fold more expression as compared to the dormant control line Steptoe (Figure 3.14). Lines with intermediate level of seed dormancy such as BM0253-95 and Baudin had intermediate level of *ABA8'OH2* expression (4- to 8-fold as compared to Steptoe), however, selected lines with intermediate level of dormancy such as CDC-Reserve-01 and BM0253-211 showed exceptionally high level of *ABA8'OH2* expression (over 15 fold as compared to the control Steptoe). The non-dormant lines BM0253-235, BM0253-331, AAC Synergy, BM0253-58, BM0253-409, BM0253-217, TR253, BM0253-412 and Himalaya also had higher (5- to 33-fold) expression as compared to control Steptoe. Selected non-dormant lines Morex, BM0253-103, TR11221 and AC Metcalfe showed only 2- to 4-fold higher expression than the control Steptoe.

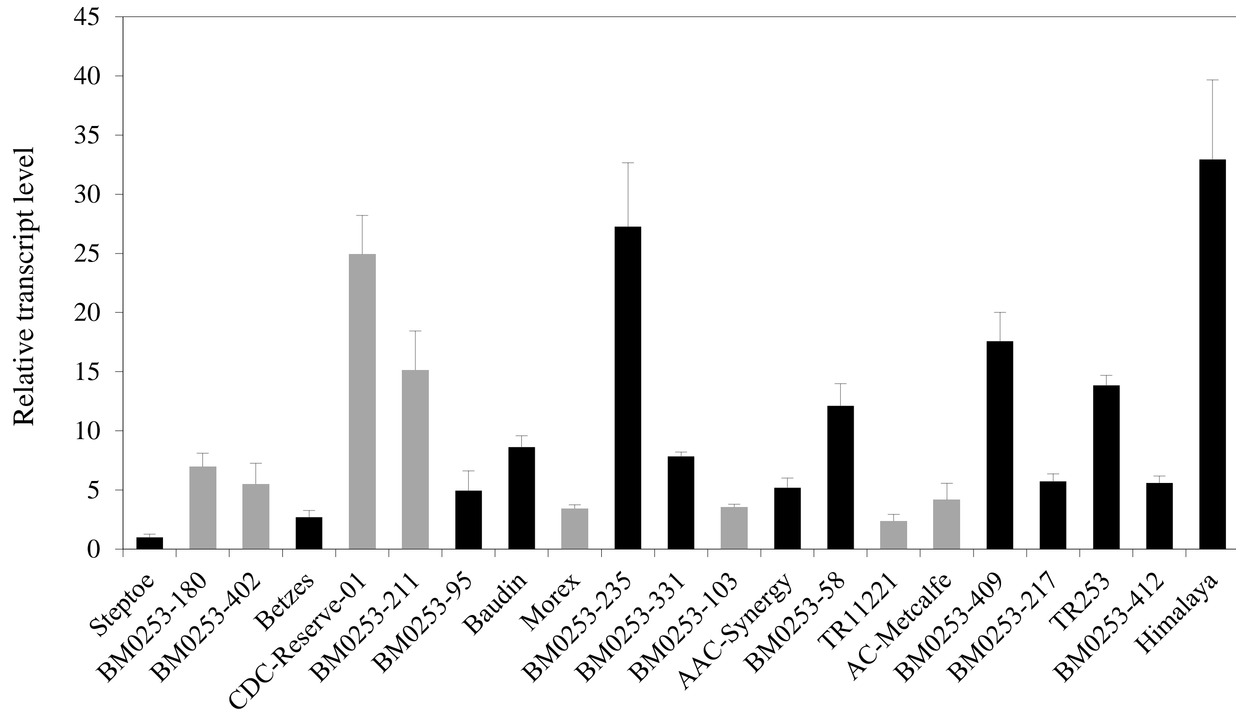


Figure 3.14 Relative transcript level of *ABA8'OH2* in the embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype. Transcript level was calculated relative to line Steptoe, which was set at 1. The data presented are means of three biological replicates and the error bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test ($p<0.05$; Appendix 10).

3.3.3.4.1 Correlation between embryonic *ABA8'OH2* expression and seed germination

Analysis of the correlation between *ABA8'OH2* expression and seed germination levels in 21 different barley lines produced an *r* value of 0.25 (Appendix 11). However, 13 selected lines (Steptoe, Betzes, BM0253-95, Baudin, BM0253-235, BM0253-331, AAC Synergy, BM0253-58, BM0253-409, BM0253-217, TR253, BM0253-412 and Himalaya) appeared to have an expression pattern consistent with the germination phenotype and correlation analysis between the two factors of these lines yield an *r* value of 0.54 (Figure 3.15).

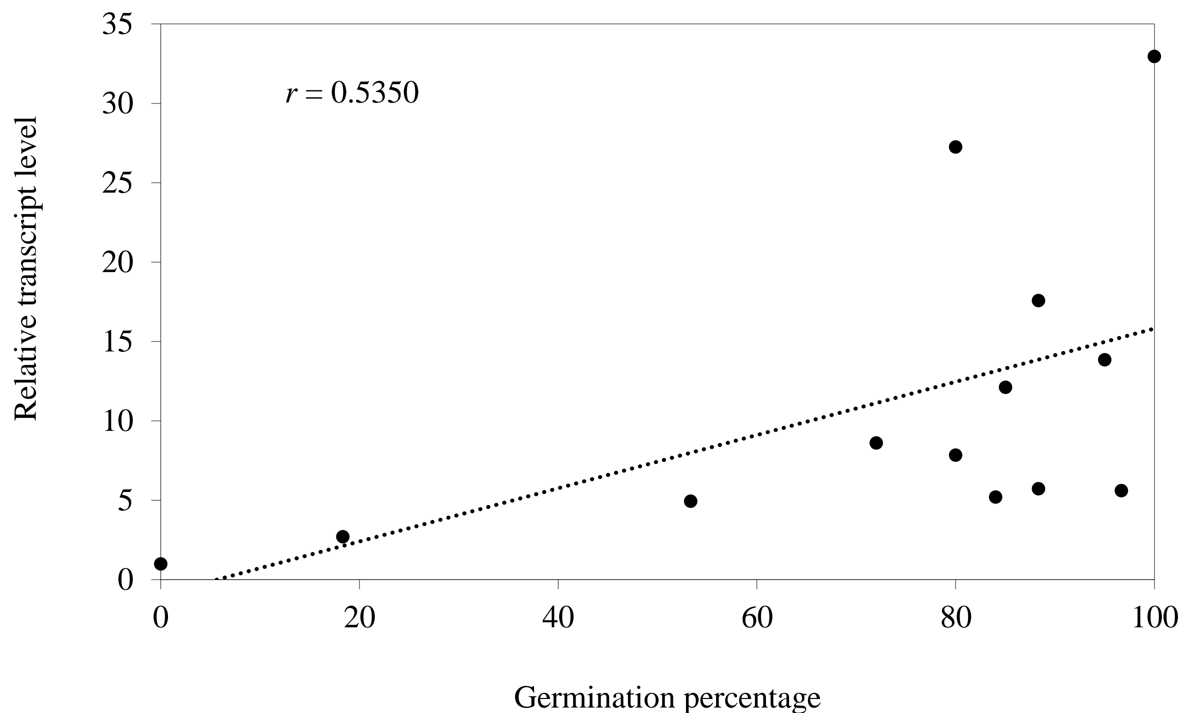


Figure 3.15 Correlation between the expression levels of embryonic *ABA8'OH2* and seed germination percentages of different barley lines (Steptoe, Betzes, BM0253-95, Baudin, BM0253-235, BM0253-331, AAC Synergy, BM0253-58, BM0253-409, BM0253-217, TR253, BM0253-412 and Himalaya).

3.3.3.5 Expression of *ABA8'OH2* in the endosperm

Highest level of *ABA8'OH2* expression was found in the endosperm of non-dormant line BM0253-412 (18-fold as compared to the expression found in the control dormant line Steptoe) (Figure 3.16). The endosperm of dormant lines including BM0253-180 and BM0253-402 showed a level of expression similar to Steptoe except Betzes, which showed ~3-fold higher expression level. The expression level of *ABA8'OH2* in the intermediate dormant lines CDC-Reserve-01, BM0253-211, BM0253-95 and Baudin was found to be intermediate (1.4- to 3-fold as compared to Steptoe). The endosperms of the non-dormant lines BM0253-331, BM0253-103, BM0253-58, TR11221, BM0253-409, TR253 and BM0253-412 exhibited over 3-fold more expression of *ABA8'OH2* (as

compared to that of the control Steptoe). However, selected non-dormant lines such as Morex, BM0253-235, AAC Synergy, AC Metcalfe, BM0253-217 and Himalaya had *ABA8'OH2* expression level that is either similar or slightly higher (up to 2-fold) as compared to the control.

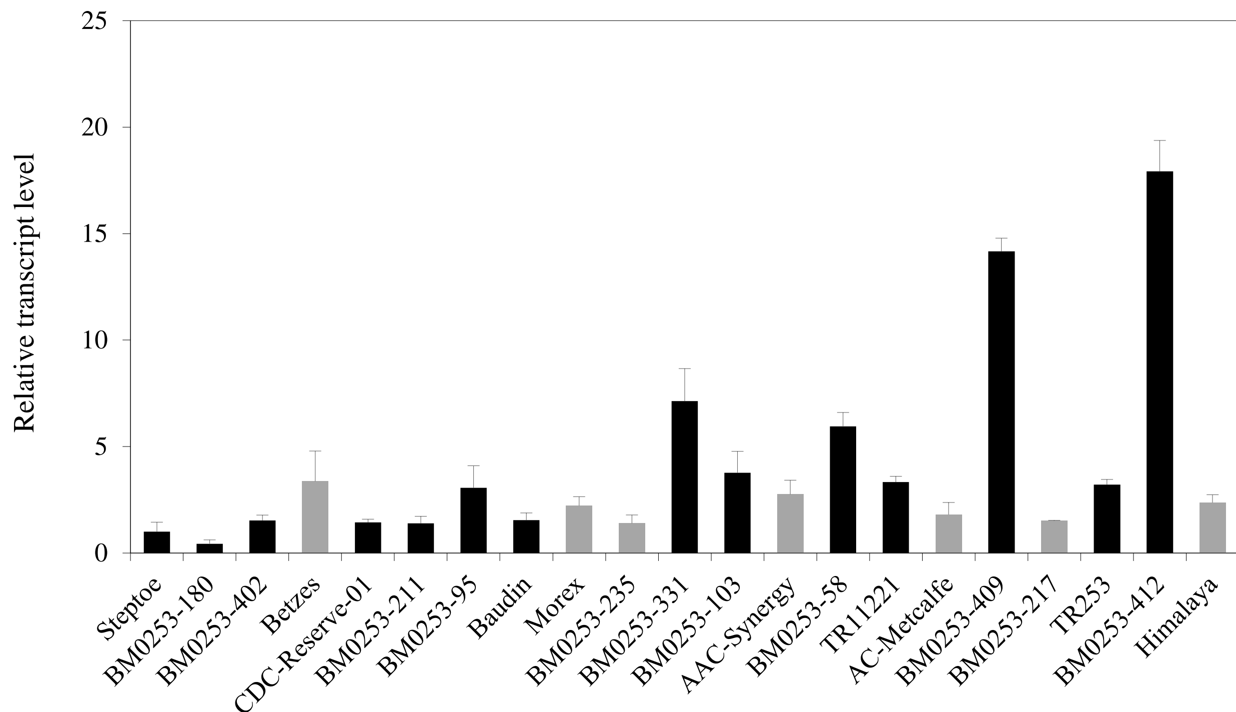


Figure 3.16 Relative transcript level of *ABA8'OH2* in the endosperm tissues at 24 HAI in different barley lines with a range of dormancy phenotype. Transcript level was calculated relative to line Steptoe, which was set at 1. The data presented are means of three biological replicates and the bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test ($p<0.05$; Appendix 12).

3.3.3.5.1 Correlation between seed germination and endospermic *ABA8'OH2* expression

Correlation analysis between seed germination levels and *ABA8'OH2* expression showed an r value of 0.41 (Appendix 13). Correlation analysis of the 13 barley lines (Steptoe, BM0253-180, BM0253-402, CDC-Reserve-01, BM0253-211, Baudin, BM0253-331, BM0253-103, BM0253-

58, TR11221, BM0253-409, TR253 and BM0253-412) that showed a more consistent expression with the germination phenotype revealed an *r* value of 0.61 (Figure 3.17).

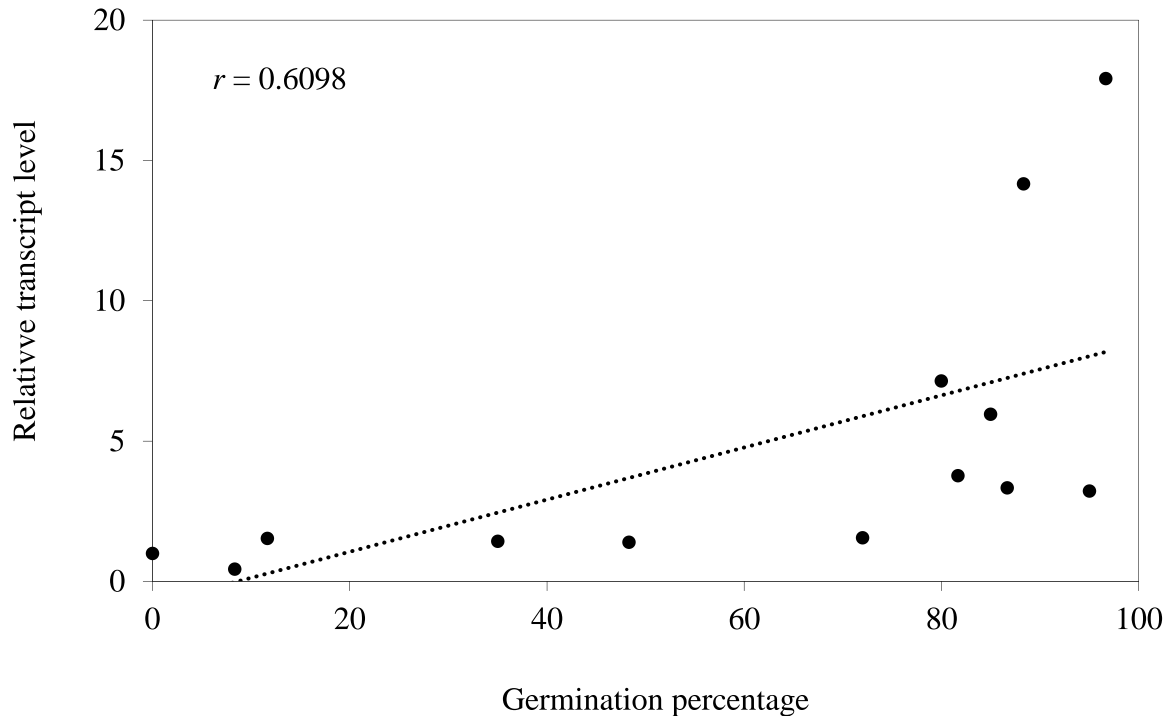


Figure 3.17 Correlation between the seed germination percentages and expression levels of endospermic *ABA8'OH2* of different barley lines (Steptoe, BM0253-180, BM0253-402, CDC-Reserve-01, BM0253-211, Baudin, BM0253-331, BM0253-103, BM0253-58, TR11221, BM0253-409, TR253 and BM0253-412).

3.3.4 Molecular cloning of *GA20ox1*

3.3.4.1 Amplification of *GA20ox1*

A PCR reaction for amplification of the genomic DNA sequence of *GA20ox1* from 21 barley lines with gene specific primers designed from the 5′ and 3′ untranslated regions (UTRs) of the DNA sequence of *GA20ox1* (GenBank ID: AY551428) produced DNA fragment of expected size 1453 bp (Figure 3.18).

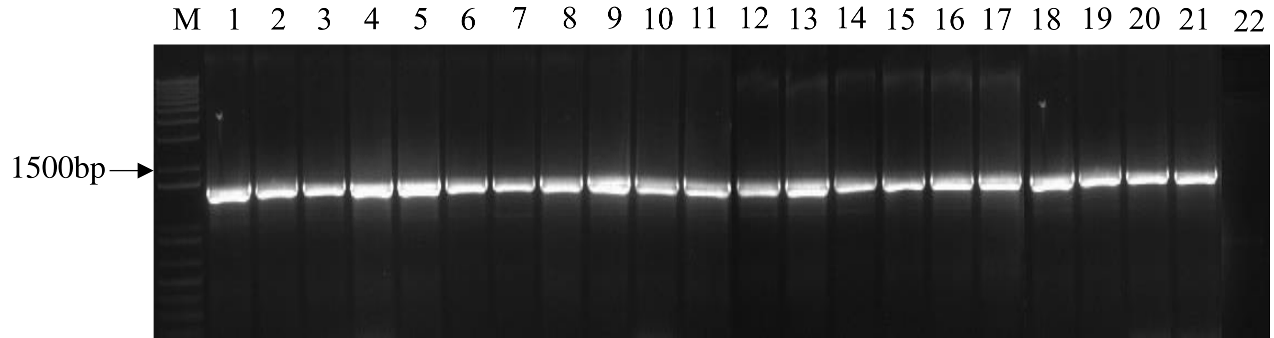


Figure 3.18 PCR amplification of *GA20ox1* from selected malting barley genotypes and breeding lines (Lanes are 1=BM0253-180; 2=BM0253-211; 3=BM0253-217; 4=BM0253-235; 5=BM0253-58; 6=BM0253-331; 7=BM0253-402; 8=BM0253-409; 9=BM0253-95; 10=BM0253-103; 11=BM0253-412; 12=TR253; 13=Baudin; 14=Morex; 15=Steptoe; 16=Betzes; 17=Himalaya; 18=TR11221; 19=AAC Synergy; 20=AC Metcalfe; 21=CDC Reserve-01; 22=Negative control). Lane M is the 1kb plus marker.

Sequencing and subsequent BLAST searching of the resulting *GA20ox1* sequences against the GenBank database confirmed the specificity of the sequenced DNA fragments as the sequences from the different lines showed 100% identity with the *GA20ox1* sequence available in the GenBank database (GenBank ID: AY551428.1) (Table 3.7).

Table 3.7 Nucleotide sequence identity of the *GA20ox1* gene sequences from the different line with other *GA20oxs*

Accession #	Description	Max Score[a]	E-Value[b]	Max Identity[c]
AY551428.1	*GA20ox1*	1330	0.0	100%
XM_020331196.1	*AetGA20ox1*	913	0.0	94%
Y14007.1	*TaGA20ox1B*	913	0.0	94%

[a]Maximum score indicates the amount of sequence homology between *GA20ox1* from the different barley lines and other *GA20ox1* sequences available in GenBank database.
[b]E-Value determines the significance of the alignment score.
[c]Maximum identity refers to percentage of identical nucleotides between *GA20ox1* from the different barley lines and other *GA20ox1* sequences available in GenBank database.

3.3.4.2 Sequence analysis of *GA20ox1*

Multiple alignment of the sequences of *GA20ox1* from the 21 different barley lines for detection of SNPs indicated the presence of nucleotide polymorphisms (Figure 3.19). Two different allelic forms of the gene differing at five positions were found to exist among the different lines. Four base substitutions observed in the gene sequence include substitution from A to C at 339th bp, C to G at 645th bp, C to G at 1262th bp, and a deletion of G at 835th bp. The six lines carrying the mutated allelic form of the gene are the dormant lines BM0253-402 and Betzes, and intermediate dormant lines CDC-Reserve-01, BM0253-211 and Baudin, and the non-dormant line BM0253-217. The two parental lines of the first population, Baudin and TR253 exhibited the allelic variations in the nucleotide sequence of *GA20ox1*. The *GA20ox1* sequence from Baudin consisted of base substitutions from A to C at 339th bp, from C to G at 645th bp, C to G at 1262th bp, and a deletion of G at 835th bp, whereas TR253 displayed the same nucleotide sequence obtained from the GenBank (which is obtained from cv. Himalaya).

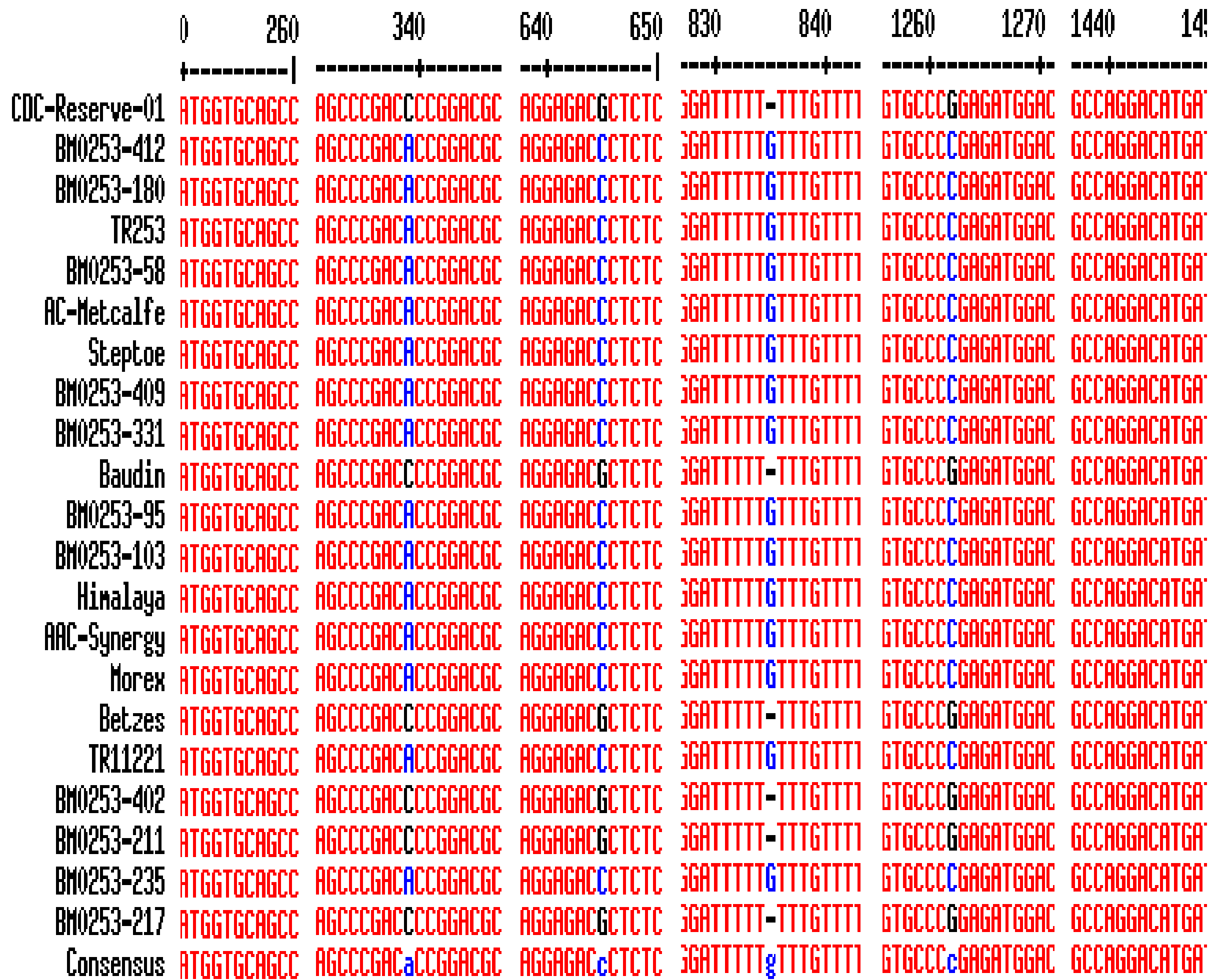


Figure 3.19 Multiple alignment of the nucleotide sequence of *GA20ox1* that shows nucleotide polymorphisms among the different barley lines.

3.3.4.3 Analysis of the exon-intron structure of *GA20ox1*

The *GA20ox1* genomic DNA sequence was analysed for its exonic and intronic regions using the Ensembl plants genome database (https://plants.ensembl.org/index.html), and this analysis indicated the presence of two exonic (coding) and an intronic (non-coding) regions (Figure 3.20). The size of the coding sequence of *GA20ox1* is found to be 1083 bp. Three of the of the four nucleotide polymorphisms identified, which are substitution of A to C, C to G and C to G, occurred

in the exon sequence of the gene. The remaining variation, which is represented by a deletion of G, occurred in the intronic region.

ATGGTGCAGCCGGTGTTCGACGCGGCGGTGCTGAGCGGGCGGACGGACATCCCGTCGCAGTTCA
TCTGGCCGGAGGGGGAGAGCCCGAC**A**CCGGACGCCACCGAGGAGATGCACGTCCCGCTCATCGA
CATCGGCGGCATGCTCTCCGGCGACCCCCGCGCGGCCGCCGAGGTGACGCGCCTCGTCGGCGAG
GCCTGCGAGCGGCACGGCTTCTTCCAGGTCGTCAACCACGGCATCGACGCCCAGCTGCTGGCCG
ACGCGCACCGCTGCGTGGACGCCTTCTTCACCATGCCCCTCCCGGAGAAGCAGCGCGCTCTGCG
CCGCCCCGGCGAGTCGTGCGGCTACGCCAGCAGCTTCACCGGCCGGTTCGCGTCCAAGCTGCCC
TGGAAGGAGAC**C**CTCTCCTTCCGGTCCTGCCCCTCCGACCCCGCCCTCGTCGTCGACTACATCG
TCGCCACCCTCGGCGAGGACCACCGCCGCCTCGGGTAAGCAAAGCAACCTCACCGTCGACTGTC
GATCGACGGGCATGGGATTGATTCATTATTAGAGCAAGCGAAAGCAGAGCATGTCGCTGAGCGT
TGGATTTTT**G**TTTGTTTTCCAGGGAGGTGTACGCTCGCTACTGCTCGGAGATGAGCCGGCTGTC
GCTGGAGATCATGGAGGTGCTCGGGGAGAGCCTGGGGGTCGGCCGCGCCCACTACCGGCGCTTC
TTCGAGGGCAACGAGTCCATCATGCGCCTCAACTACTACCCGCCGTGCCAGCGGCCGTTGGAGA
CGCTGGGCACGGGCCCGCATTGCGACCCGACGTCGCTGACCATCCTCCACCAGGACGACGTGGG
CGGCCTGCAGGTGCATACCGACGGCCGGTGGCGCTCCATCCGGCCGCGGGCCGACGCCTTCGTC
GTCAACATCGGCGACACCTTCATGGCGCTCTCCAACGGGCGGTACAAGAGCTGCCTCCACCGCG
CCGTCGTCAACAGCCGCGTCCCACGCAAGTCGCTGGCCTTCTTCCTGTGCCC**C**GAGATGGACAA
GGTGGTGGCGCCGCCGGGGACGTTGGTGGACGCCGCCAACCCGCGCGCCTACCCGGACTTCACG
TGGCGGTCGCTGCTCGACTTCACGCAGAAGCACTACCGCGCCGACATGAAGACGCTCGAGGTCT
TCTCCTCGTGGGTCGTCCAGCAGCAGCCCGCCAGCGCCAGGACATGA

Figure 3.20 Genomic DNA sequence of *GA20ox1* showing the exon and intron. The exonic region are highlighted in grey, nucleotide polymorphisms occur at nucleotide positions highlighted in bold yellow and deletion of a nucleotide occur at position highlighted in bold red.

3.3.4.4 Translation of the coding sequence of *GA20ox1*

The conversion of the nucleotide sequences of *GA20ox1* from the 21 different barley lines into their respective amino acid sequences with ExPASy translate tool (https://web.expasy.org/translate/) produced putative protein sequences with 360 amino acids. The three nucleotide substitutions caused a change in codons (Figure 3.21). The substitution of A to C at the 90th nucleotide position caused a shift of codon from ACA to ACC at 88th to 90th, however,

both codons code for the same amino acid 'Threonine'. The second nucleotide substitution of G to C occurred at the 654th nucleotide position and this caused a change in the sequence of the respective codon from ACG to ACC at 652th to 654th nucleotide, and the two codons code for same amino acid 'Threonine' irrespective of the change in codon sequence. The third codon nucleotide substitution of C to G at the 897th nucleotide position caused codon sequence change from codon CCC to CCG at 895th to 897th nucleotide positions, but both codons represent the same amino acid 'Proline'. Overall, the three base changes in the coding sequence of *GA20ox1* from the 21 different barley lines did not result in change of amino acid.

```
   1 ATGGTGCAGCCGGTGTTCGACGCGGCGGTGCTGAGCGGGCGGACG
      M  V  Q  P  V  F  D  A  A  V  L  S  G  R  T
  46 GACATCCCGTCGCAGTTCATCTGGCCGGAGGGGGAGAGCCCGACA
      D  I  P  S  Q  F  I  W  P  E  G  E  S  P  T
  91 CCGGACGCCACCGAGGAGATGCACGTCCCGCTCATCGACATCGGC
      P  D  A  T  E  E  M  H  V  P  L  I  D  I  G
 136 GGCATGCTCTCCGGCGACCCCCGCGCGGCCGCCGAGGTGACGCGC
      G  M  L  S  G  D  P  R  A  A  A  E  V  T  R
 181 CTCGTCGGCGAGGCCTGCGAGCGGCACGGCTTCTTCCAGGTCGTC
      L  V  G  E  A  C  E  R  H  G  F  F  Q  V  V
 226 AACCACGGCATCGACGCCCAGCTGCTGGCCGACGCGCACCGCTGC
      N  H  G  I  D  A  Q  L  L  A  D  A  H  R  C
 271 GTGGACGCCTTCTTCACCATGCCCCTCCCGGAGAAGCAGCGCGCT
      V  D  A  F  F  T  M  P  L  P  E  K  Q  R  A
 316 CTGCGCCGCCCCGGCGAGTCGTGCGGCTACGCCAGCAGCTTCACC
      L  R  R  P  G  E  S  C  G  Y  A  S  S  F  T
 361 GGCCGGTTCGCGTCCAAGCTGCCCTGGAAGGAGACCCTCTCCTTC
      G  R  F  A  S  K  L  P  W  K  E  T  L  S  F
 406 CGGTCCTGCCCCTCCGACCCCGCCCTCGTCGTCGACTACATCGTC
      R  S  C  P  S  D  P  A  L  V  V  D  Y  I  V
 451 GCCACCCTCGGCGAGGACCACCGCCGCCTCGGGGAGGTGTACGCT
      A  T  L  G  E  D  H  R  R  L  G  E  V  Y  A
 496 CGCTACTGCTCGGAGATGAGCCGGCTGTCGCTGGAGATCATGGAG
      R  Y  C  S  E  M  S  R  L  S  L  E  I  M  E
 541 GTGCTCGGGGAGAGCCTGGGGGTCGGCCGCGCCCACTACCGGCGC
      V  L  G  E  S  L  G  V  G  R  A  H  Y  R  R
 586 TTCTTCGAGGGCAACGAGTCCATCATGCGCCTCAACTACTACCCG
      F  F  E  G  N  E  S  I  M  R  L  N  Y  Y  P
 631 CCGTGCCAGCGGCCGTTGGAGACGCTGGGCACGGGCCCGCATTGC
      P  C  Q  R  P  L  E  T  L  G  T  G  P  H  C
 676 GACCCGACGTCGCTGACCATCCTCCACCAGGACGACGTGGGCGGC
      D  P  T  S  L  T  I  L  H  Q  D  D  V  G  G
 721 CTGCAGGTGCATACCGACGGCCGGTGGCGCTCCATCCGGCCGCGG
      L  Q  V  H  T  D  G  R  W  R  S  I  R  P  R
 766 GCCGACGCCTTCGTCGTCAACATCGGCGACACCTTCATGGCGCTC
      A  D  A  F  V  V  N  I  G  D  T  F  M  A  L
 811 TCCAACGGGCGGTACAAGAGCTGCCTCCACCGCGCCGTCGTCAAC
      S  N  G  R  Y  K  S  C  L  H  R  A  V  V  N
 856 AGCCGCGTCCCACGCAAGTCGCTGGCCTTCTTCCTGTGCCCCGAG
      S  R  V  P  R  K  S  L  A  F  F  L  C  P  E
 901 ATGGACAAGGTGGTGGCGCCGCCGGGGACGTTGGTGGACGCCGCC
      M  D  K  V  V  A  P  P  G  T  L  V  D  A  A
 946 AACCCGCGCGCCTACCCGGACTTCACGTGGCGGTCGCTGCTCGAC
      N  P  R  A  Y  P  D  F  T  W  R  S  L  L  D
 991 TTCACGCAGAAGCACTACCGCGCCGACATGAAGACGCTCGAGGTC
      F  T  Q  K  H  Y  R  A  D  M  K  T  L  E  V
1036 TTCTCCTCGTGGGTCGTCCAGCAGCAGCCCGCCAGCGCCAGGACA
      F  S  S  W  V  V  Q  Q  Q  P  A  S  A  R  T
1081 TGA
      *
```

Figure 3.21 Amino acid sequence of *GA20ox1* with the corresponding nucleotide sequence. The codon positions where change in codon sequence occurred and the corresponding amino acids are highlighted in yellow. '*' represents stop codon.

3.3.4.5 Conserved domain and phylogenetic analysis of *GA20ox1*

Multiple sequence alignment of the putative amino acid sequences of GA20ox1 from the 21 different barley lines with those identified previously from different plant species indicated 66.1 % of the amino acids have more than 75% similarity of which 114 amino acids (47.8 %) exhibited 100% similarity (Figure 3.22). Furthermore, search for the conserved domains of these proteins demonstrated that the GA20ox1 proteins consisted of three domains which include two substrate binding domains 'LPWKET' (at 127–132 amino acid positions) and 'NYYPPCQRPLETLGTGPH' (at 207-224 amino acid positions), and an iron binding domain with two histidine residues (at 234 and 286 amino acid positions). One of the nucleotide polymorphisms detected, which leads to a change of codon from ACG to ACC (at 652th to 654th nucleotide positions of the coding sequence, Figure 3.20) and that codes for amino acid 'threonine' occurs in the 'NYYPPCQRPLETLGTGPH' 2-oxoglutarate binding domain.

Phylogenetic analysis of the GA20ox1 protein sequences obtained in this study along with 13 other known GA20ox1 plant proteins from both monocot and dicot species separated the proteins into two categories, one of the category exclusively contains all the proteins from monocots while the other category consists of GA20ox1s from dicots (Appendix 14). The phylogenetic tree showed that the GA20ox1 proteins derived from this study are closely related with the wheat GA20ox1 protein from A, B and D genomes with 91% homology.

```
HvGA20ox1    ........................................MVQPVFDAAVLSGRTDIPSQFIWPEGESPTPDATEEMHVPLIDIGGMLS...GD.PRAAA    56
LpGA20ox1    ........................................MVQPVFDAALLSGQSDIPSQFIWPADESPSPDATEELHVPLIDIGGLLS...GD.RVAAA    56
AtGA20OX1    .................MAVSFVTTSPEEEDKPKLGLGNIQTPLIFNPSMLNLQANIPNQFIWPDDEKPSIN.VLELDVPLIDLQNLLS....D.PSSTL    77
BvGA20ox1    .................MAIDCMRNLSSTPKSYQNSKQEQNNPLIFDASFLNHQSHIPQQFIWPDGEKPDFIEPEELAVPLIDLQGFLS...DD.PEETL    79
BdGA20ox1    ......................................MVMQPVLFDAAVLSGRSDIPSQFIWPADESPTPDAAEPLDVPLIDIGGIVASGAGDRAAAVA    62
ZmGA20ox1    MAAHWQAGRAGTSKQAVAVAAASSSASSLLLLTMVLAAHDPPPLVFDAARLSGLSDIPQQFIWPADESPTPDSAEELAVPLIDLS.......G....DAA    89
TaGA20ox1D   ........................................MVQPVFDAAVLSGRADIPSQFIWPEGESPTPDAAEELHVPLIDIGGMLS...GD.PAAAA    56
TaGA20ox1A   ........................................MVRPVFDAAVLSGRSDIPSQFIWPEGESPTPDAAEELHVPLINIGGMLS...GD.AAAAA    56
TaGA20ox1B   ........................................MVQPVFDAAVLSGRADIPSQFIWPEGESPTPDAAEELHVPLIDIGGMLS...GD.AAAAA    56
SbGA20ox1    ..................MVQQASLGEPLLLQ......PPPPSLVFDAARLSGLSDIPQQFLWPADESPTPDAAEELAVPLIDLS.......G....DAA    65
OsGA20ox1    ..........................MSMVVQ.......QEQEVVFDAAVLSGQTEIPSQFIWPAEESPGSVAVEELEVALIDVG.......AG..AERS    58
Consensus                                                f    l     ip qf wp  e p        v li

HvGA20ox1    EVTRLVGEACERHGFFQVVNHGIDAQLLADAHRCVDAFFTMPLPEKQRALRRPGESCGYASSFTGRFASKLPWKETLSFRSCPSD.....PALVVDYIVA   151
LpGA20ox1    EVTRLVGDACERHGFFQVVNHGIDAELLADAHRCVDAFFTMSLQGKQRALRRPGESCGYASSFTGRFASKLPWKETLSFRSCPSE.....PDLVVEYIVA   151
AtGA20OX1    DASRLISEACKKHGFFLVVNHGISEELISDAHEYTSRFFDMPLSEKQRVLRKSGESVGYASSFTGRFSTKLPWKETLSFRFCDDMSR...SKSVQDYFCD   174
BvGA20ox1    KASKLIGEACLKHGFFLVVNHGVERDLIKDAHNLMDDFFEEPLSLKQKALRKLGEHCGYASSFTGRFSCNLPWKETLSFNYSPQESSS.SKTHVQDYFNN   178
BdGA20ox1    SVARLVGDACSRHGFFQVVNHGIDAALLADAHRCVDAFFKLPLAEKQRALRRPGESCGYASSFVGRFSSKLPWKETLSFRSSPSC.....PDLPLDFILS   157
ZmGA20ox1    EVVRQVRRACDLHGFFQVVGHGIDAALTAEAHRCMDAFFTLPLPDKQRAQRRQGDSCGYASSFTGRFASKLPWKETLSFRYTDDDDGDKSKDVVASYFVD   189
TaGA20ox1D   EVTRLVGEACERHGFFQVVNHGIDAELLADAHRCVDNFFTMPLPEKQRALRHPGESCGYASSFTGRFASKLPWKETLSFRSCPSD.....PALVVDYIVA   151
TaGA20ox1A   EVTRLVGEACERHGFFQVVNHGIDAELLADAHRCVDNFFTMPLPEKQRALRRPGESCGYASSFTGRFASKLPWKETLSFRSCPSD.....PALVVDYIVA   151
TaGA20ox1B   EVTRLVGEACERHGFFQVVNHGIDAELLADAHRCADAFFTMPLPEKQRALRRPGESCGYASSFTGRFASKLPWKETLSFRSCPSD.....PALVVDYIVA   151
SbGA20ox1    EVVRQVRRACDLHGFFQVVNHGIDDALLQEAHRCMDAFFTLPMSDKQRAQRRQGDSCGYASSFTGRFASKLPWKETLSFRYSDDQ.GD..GDVVVDYFVD   162
OsGA20ox1    SVVRQVGEACERHGFFLVVNHGIEAALLEEAHRCMDAFFTLPLGEKQRAQRRAGESCGYASSFTGRFASKLPWKETLSFRYSSAGDEE.GEEGVGEYLVR   157
Consensus            ac  hgff vv hg    l   ah       ff       kq   r  g   gyassf grf    lpwketlsf
                                                                       ******************         # #
HvGA20ox1    TLGEDH.RRLGEVYARYCSEMSRLSLEIMEVLGESLGVG...RAHYRRFFEGNESIMRLNYYPPCQRPLETLGTGPHCDPTSLTILHQDDVGGLQVHTDG   247
LpGA20ox1    TLGEDH.RRLGEVYARYCSEMSRLSLEIMEVLGESLGVG...RAHYRRFFEGNESIMRLNYYPPCQRPNETLGTGPHCDPTSLTILHQDDVGGLQVHADG   247
AtGA20OX1    ALGHGF.QPFGKVYQEYCEAMSSLSLKIMELLGLSLGVK...RDYFREFFEENDSIMRLNYYPPCIKPDLTLGTGPHCDPTSLTILHQDHVNGLQVFVEN   270
BvGA20ox1    KFGDSF.NHLGTTYQQYCEAMSTLSLRIMELLGLSLGVG...RRHFREFFEGHESVMRLNYYPPCQRPDLTLGTGPHCDPTSLTILHQDQVGGLQVFADG   274
BdGA20ox1    NLGEEH.RRLGEVYARYCEEMSRVSLEIMEVLGESLGVG...RSHYRSFFEGNDSIMRLNYYPPCQRPYETLGTGPHCDPTSLTILHQDAVGGLQVHVDG   253
ZmGA20ox1    KLGEGY.RHHGEVYGRYCSEMSRLSLELMEVLGESLGVG...RRHFRRFFQGNDSIMRLNYYPPCQRPYDTLGTGPHCDPTSLTILHQDDVGGLQVFDAA   285
TaGA20ox1D   TLGEDH.RRLGEVYARYCSEMSRLSLEIMEVLGESLGVG...RAHYRRFFEGNDSIMRLNYYPPCQRPLETLGTGPHCDPTSLTILHQDNVGGLQVHTEG   247
TaGA20ox1A   TLGEDH.RRLGEVYARYCSEMSRLSLEIMEVLGESLGVG...RAHYRRFFEGNDSIMRLNYYPPCQRPLETLGTGPHCDPTSLTILHQDNVGGLQVHTEG   247
TaGA20ox1B   TLGEDH.RRLREVYARYCSEMSRLSLEIMEVLGESLGVG...RAHYRRFFEGNDSIMRLNYYPPCPRPMETLGTGPHCDPTSLTILHQDNVGGLQVHTEG   247
SbGA20ox1    KLGDAY.RHHGEVYGRYCSEMSRLSLELMEVLGESLGVG...RRHFRRFFQGNDSIMRLNYYPPCQRPYDTLGTGPHCDPTSLTILHQDDVGGLQVFDAA   258
OsGA20ox1    KLGAEHGRRLGEVYSRYCHEMSRLSLELMEVLGESLGIVGDRRHYFRRFFQRNDSIMRLNYYPACQRPLDTLGTGPHCDPTSLTILHQDHVGGLEVWAEG   257
Consensus      g          y  yc  ms  sl  me lg slg     r   r ff    s mrlnyyp c  p  tlgtgphcdptsltilhqd v gl v
                                                  #
HvGA20ox1    R......WRSIRPRADAFVVNIGDTFMALSNGRYKSCLHRAVVNSRVPRKSLAFFLCPEMDKVVAPPGTLVDAAN.PRAYPDFTWRSLLDFTQKHYRADM   340
LpGA20ox1    R......WLSIRPRADAFVVNIGDTFMALSNGRYKSCLHRAVVNSRVPRKSLAFFLCPEMDKVVAPPGTLVDEAN.PRAYPDFTWRALLDFTQKHYRADM   340
AtGA20OX1    Q......WRSIRPNPKAFVVNIGDTFMVTHLDF...................................................................   297
BvGA20ox1    K......WRSISPNFNAFVVNIGDTFMALSNGRYKSCLHRAVVNSEKPRKSLAFFLCPNKDKVVKPPRELVDEENNPRVYPDFTWPLLLEFTQKHYRADM   368
BdGA20ox1    R......WRAIAPRQDAFVVNIGDTFMALSNGRYKSCLHRAVVNSKVPRKSLAFFLCPEMDKTVAPPGKLVDEEN.PRVYPDFTWRALLDFTQKHYRADM   346
ZmGA20ox1    T....LAWRSIRPRPGAFVVNIGDTFMALSNGRYRSCLHRAVVNSRVARRSLAFFLCPEMDKVVRPPKELVDDAN.PRAYPDFTWRTLLDFTMRHYRSDM   380
TaGA20ox1D   R......WRSIRPRADAFVVNIGDTFMALSNGRYKSCLHRAVVNSRVPRKSLAFFLCPEMDKVVAPPGTLVDAAN.PRAYPDFTWRSLLDFTQKHYRADM   340
TaGA20ox1A   R......WRSIRPRADAFVVNIGDTFMALSNGRYKSCLHRAVVNSRVPRKSLAFFLCPEMDKVVAPPGTLVDASN.PRAYPDFTWRSLLDFTQKHYRADM   340
TaGA20ox1B   R......WRSIRPRADAFVVNIGDTFMALSNGRYKSCLHRAVVNSKAPRKSLAFFLCPEMDKVVAPPGTLVDAAN.PRAYPDFTWRSPLDFTQKHYRADM   340
SbGA20ox1    TGPGTGRWRSIRPHPGAFVVNIGDTFMALSNGRYRSCLHRAVVNSRVPRRSLAFFLCPEMDKVVRPPAELVDDAN.PRAYPDFTWRTLLDFTMRHYRSDM   357
OsGA20ox1    R......WRAIRPRPGALVVNVGDTFMALSNARYRSCLHRAVVNSTAPRRSLAFFLCPEMDTVVRPPEELVDDHH.PRVYPDFTWRALLDFTQRHYRADM   350
Consensus           w  i p   a vvn gdtfm

HvGA20ox1    KTLEVFSSWVVQQQPAS.......ART.                                                                          360
LpGA20ox1    KTLEVFSDWIQQGHQP....AATTTTT.                                                                          363
AtGA20OX1    ............................                                                                          297
BvGA20ox1    NTLKVFSQWVQQQRSTQ......QVEK.                                                                          389
BdGA20ox1    KTLEVFSGWVLQQQKQK......QLGS.                                                                          367
ZmGA20ox1    RTLEAFSNWLSTSSNGG.....QHLLEK                                                                          403
TaGA20ox1D   KTLEVFSSWIVQQQQP....QP..ART.                                                                          361
TaGA20ox1A   KTLEVFSSWIVQQQQGQLALQP..AMT.                                                                          365
TaGA20ox1B   KTLEVFSSWIVQQQQGQLALQPQPART.                                                                          367
SbGA20ox1    RTLEAFSNWLNHGGHLS.....SPPPP.                                                                          379
OsGA20ox1    RTLQAFSDWLNHHRHLQ.....PTIYS.                                                                          372
Consensus
```

Figure 3.22 Amino acid sequence alignment of barley and other GA20ox1 proteins. Amino acid residues with ≥75% homology are shaded in yellow while amino acid residues with 100% homology are represented in dark blue. The iron binding domain is represented by # symbol, 2-oxoglutarate binding domain by * symbol, and the GA substrate binding domain by a bar at the top of the residues. The species and GenBank IDs of the aligned proteins are: *Triticum aestivum*, TaGA20ox1-A (GenBank ID: CBX45609.1); TaGA20ox1-B (GenBank ID: CBX45610.1); TaGA20ox1-D (GenBank ID: CBX45611.1); *Hordeum vulgare*, GA20ox1 (GenBank ID: AY551428); *Lolium perenne*, LpGA20ox1 (GenBank ID: AAG43043.1); *Brachypodium distachyon*, BdGA20ox1 (GenBank ID: BRADI1G00950); *Oryza sativa*, OsGA20ox1 (GenBank ID: Os03g0856700); *Zea mays*, ZmGA20ox1 (GenBank ID: Zm00001d034898); *Sorghum bicolor*, SbGA20ox1 (GenBank ID: SORBI_3001G005300); *Phaseolus vulgaris*, PvGA20ox1 (GenBank ID: XP_007152362.1); *Arabidopsis thaliana*, AtGA20ox1 (GenBank ID: AT4G25420).

3.3.5 Expression of gibberellin biosynthesis genes

Expression study of the GA biosynthesis gene *GA20ox* and its family members in in different tissues of the different lines revealed that the expressions of *GA20ox1* in the endosperm and embryo, *GA20ox2* in the endosperm and *GA20ox3* in the embryo was very low/ not detected. However, the expression of *GA20ox2* and *GA20ox3* was detected in both embryo and endosperm tissues imbibed seeds, respectively.

3.3.5.1 Expression of *GA20ox2* in the embryo

The expression of *GA20ox2* in the embryos of dormant line Steptoe was not detected. Relatively higher expression level of this gene was detected in the other dormant lines including BM0253-180, BM0253-402 and Betzes. Lines with intermediate level of dormancy such as CDC-Reserve-01, BM0253-211 and BM0253-95 showed an expression level either slightly lower or similar to that observed in the dormant lines (other than Steptoe). In contrast, Baudin showed the highest level of *GA20ox2* expression (4-fold higher than that observed in the 2nd most dormant line BM0253-180, which was considered as a control for comparing the expression level of this gene in the embryo). Non-dormant lines BM0253-103 and BM0253-409 showed ~2-fold higher expression than the dormant control line while BM0253-235, BM0253-331, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-217, TR253 and BM0253-412 showed either slightly lower or similar level of expression. The expression of *GA20ox2* in the non-dormant lines Morex and Himalaya was either very low or not detected (Figure 3.23).

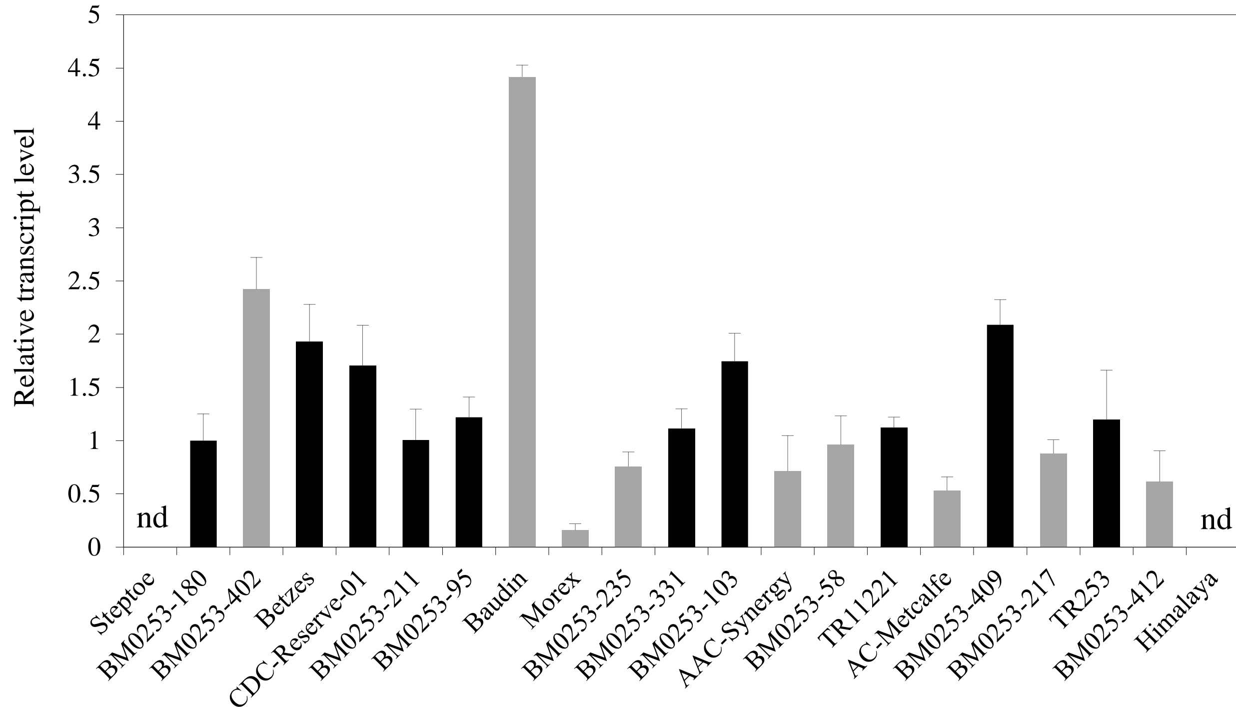


Figure 3.23 Relative transcript level of *GA20ox2* in embryo tissues at 24 HAI in different barley lines with a range of dormancy phenotype. Transcript level was calculated relative to line BM0253-180, which was set at 1. The data presented are means of three biological replicates and the error bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test (p<0.05; Appendix 15).

3.3.5.1.1 Correlation between seed germination and *GA20ox2* expression

The correlation between germination percentages and *GA20ox2* expression levels in the different lines revealed an *r* value of 0.10 (Appendix 16) whereas the correlation analysis in the selected 11 barley lines showing *GA20ox2* expression consistent with the germination phenotype (Steptoe, BM0253-180, Betzes, CDC Reserve-01, BM0253-211, BM0253-95, BM0253-331, BM0253-103, TR11221, BM0253-409 and TR253) revealed an *r* value of 0.53 (Figure 3.24).

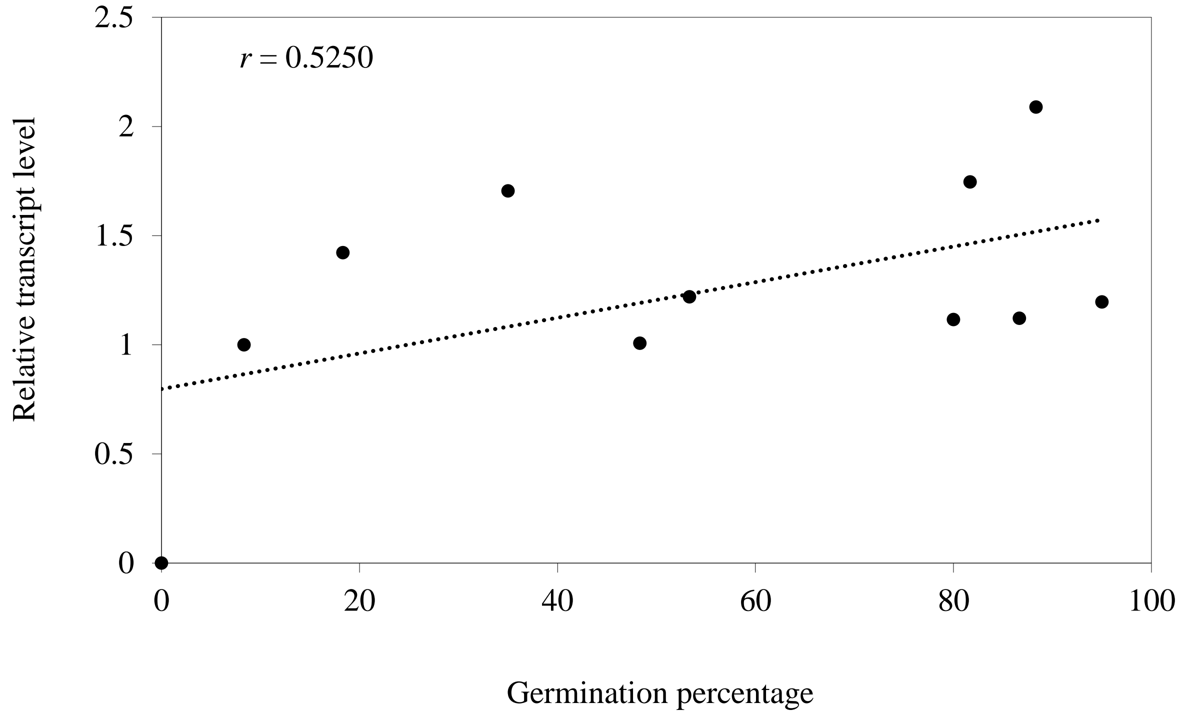


Figure 3.24 Correlation between the seed germination percentages and expression levels of embryonic GA20ox2 in different barley lines (Steptoe, BM0253-180, Betzes, CDC Reserve-01, BM0253-211, BM0253-95, BM0253-331, BM0253-103, TR11221, BM0253-409 and TR253).

3.3.5.2 Expression of *GA20ox3* in the endosperm

The expression of *GA20ox3* in the endosperm of dormant barley lines BM0253-180 and Betzes was either similar or up to 2-fold higher than that observed in the most dormant Steptoe, which is considered as a control in this study. However, dormant line BM0253-402 showed ~2-fold lower expression than the control. Lines with intermediate level of dormancy BM0253-211 and BM0253-95 also showed over 2-fold higher expression than the control, and two intermediate lines CDC-Reserve-01 and Baudin showed up to 3-fold lower expression than the control. Non-dormant lines including BM0253-235, BM0253-58, BM0253-409 and BM0253-217 exhibited up to 4-fold higher level of *GA20ox3* expression than the control Steptoe. Other non-dormant lines such as Morex, AAC Synergy, BM0253-331, BM0253-103 and BM0253-412 showed a similar level of

expression observed in the control whereas TR11221, AC Metcalfe, TR253 and Himalaya showed over 2-fold lower expression than the control (Figure 3.25).

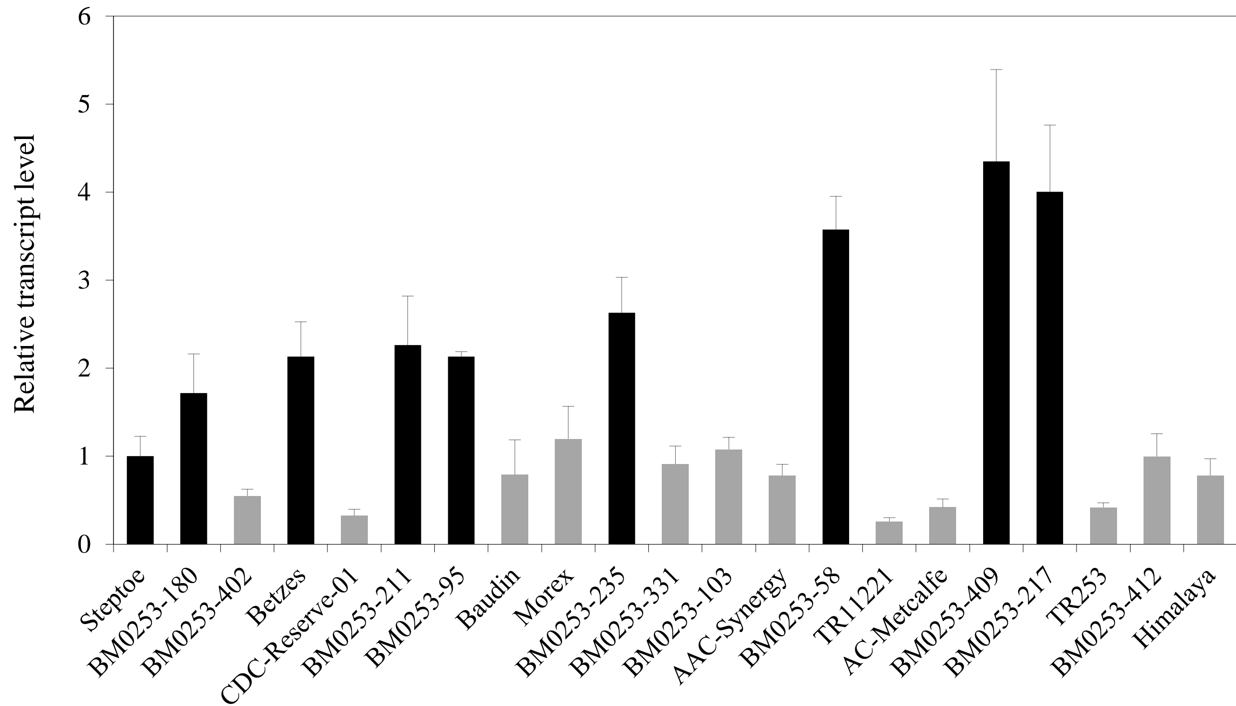


Figure 3.25 Relative transcript level of *GA20ox3* in the endosperm tissues at 24 HAI in different barley lines with a range of dormancy phenotype. Transcript level was calculated relative to line Steptoe, which was set a value of 1. The data presented are means of three biological replicates and the error bars represent standard errors. Statistically significant difference in the relative transcript levels was tested using Fisher LSD test (p<0.05; Appendix 17).

3.3.5.2.1 Correlation between seed germination and *GA20ox3* expression

The correlation between seed germination percentage and *GA20ox3* expression levels in different barley lines revealed an r value of 0.10 (Appendix 18). However, the correlation among the selected 10 barley lines including Steptoe, BM0253-180, BM0253-402, Betzes, BM0253-211, BM0253-95, BM0253-235, BM0253-58, BM0253-409 and BM0253-217 revealed an r value of 0.90 (Figure 3.26).

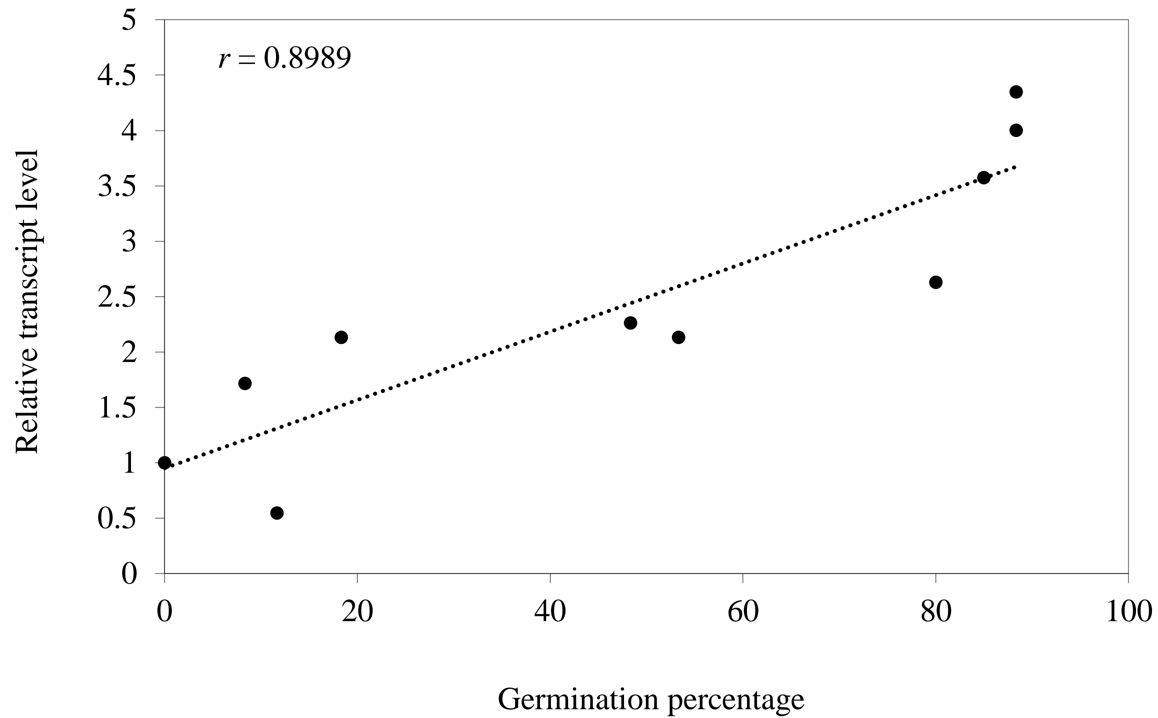


Figure 3.26 Correlation between the seed germination percentage and expression levels of endospermic *GA20ox3* of different barley lines (Steptoe, BM0253-180, BM0253-402, Betzes, BM0253-211, BM0253-95, BM0253-235, BM0253-58, BM0253-409 and BM0253-217).

3.4 Discussion

The antagonistic interaction between ABA and GA regulates seed dormancy and germination (Koornneef *et al.,* 1982). The amount of ABA, which is responsible for induction and maintenance of seed dormancy, is controlled by the balance between its biosynthesis and catabolism, and these processes are controlled mainly by genes encoding the NCED and ABA8′OH enzymes, respectively (Nambara *et al.,* 2010). On the other hand, enzymes and the corresponding genes involving GA biosynthesis (*GA20ox* and *GA3ox*) and catabolism control the production of bioactive GA and promotion of seed germination. In order to examine if genetic variation in seed dormancy and PHS is associated with variation in ABA and GA levels, this study investigated the expression patterns of *NCED*, *ABA8'OH* and *GA20ox* family members in different tissues of imbibing seeds of 21 malting barley lines having different levels of seed dormancy. In addition, the study investigated allelic variations in the sequences of *ABA8'OH1* and *GA20ox1* genes.

Evaluation of the germination percentage of the different malting barley lines studied indicated that the seeds of these lines have different phenotypes of dormancy (high, intermediate and low levels of dormancy), which can be exploited for increasing resistance to PHS. A previous study on the same breeding population also screened breeding lines for PHS tolerance and malt quality (Edney *et al.,* 2013). The report from this study and our data showed similar dormancy phenotype for all the lines included in both studies except for line BM0253-409, which has been shown to exhibit a good malt quality and resistance to PHS (Edney *et al.,* 2013) but displayed a non-dormant phenotype in the present study. Since the growing conditions for the two studies are different, the observation of non-dormant phenotype in this study might be due to the expression of a TR253 parental type allele (PHS susceptible allele) detected in this line by the previous study under our growing condition.

The biosynthesis of ABA determines ABA content and seed dormancy (Nambara *et al.,* 2010). Our study of the expression pattern of the ABA biosynthetic gene *NCED1* in different tissues of the malting barley lines studied showed differential expression patterns of *NCED1* in embryo tissues of imbibed seeds of the malting barley lines displaying contrasting dormancy phenotype. A relatively higher expression of *NCED1* was found in the dormant embryos as compared to the intermediate dormant embryos, and lowest expression of *NCED1* was observed in non-dormant embryos (Figure 3.7), leading to a positive correlation (r=0.75) between the levels of *NCED1* expression and seed dormancy (Figure 3.8). These results suggest high and low ABA synthesis in the dormant and non-dormant lines, respectively. Overall, our findings suggest that *NCED1* plays an important role in regulating the amount of ABA and seed dormancy in the studied malting barley lines. Previous studies have also shown that the important role of ABA biosynthetic gene *NCED1* in ABA content, and the induction and maintenance of seed dormancy either during seed maturation or imbibition in barley, wheat and Brachypodium (Leymarie *et al.,* 2008; Barrero *et al.,* 2012; Son *et al.,* 2016; Izydorczyk *et al.,* 2018). However, the expression patterns of *NCED1* in selected breeding lines (BM0253-211, BM0253-95 and BM0253-331) did not show any correlation with dormancy, and this might be because the breeding lines are still segregating and not fixed yet. The expression of *NCED1* in endosperm tissue was not detected/low expressed.

In addition to its synthesis, ABA catabolism regulates ABA content and dormancy (Kushiro *et al.,* 2004; Millar *et al.,* 2006; Okamoto *et al.,* 2006). Analysis of the expression patterns of ABA catabolic genes (*ABA8'OH1* and *ABA8'OH2)* in embryo and endosperm tissues of different barley lines studied showed differential expression patterns of both genes in imbibed seeds of the barley lines displaying contrasting dormancy phenotype. The comparative expression analysis of *ABA8'OH1* and *ABA8'OH2* in embryonic tissues of different barley lines showed *ABA8'OH1* as a

predominantly expressed ABA catabolic gene. A relatively higher expression of *ABA8'OH1* was found in dormant as compared to intermediate dormant embryos, and lowest expression of *ABA8'OH1* was observed in non-dormant embryos. This led to a positive correlation (*r*=0.70) between the levels of *ABA8'OH1* expression and dormancy in selected barley lines as observed for *NCED1* (Figure 3.10). This might be related with the fact that the expression of *ABA8'OH* genes is regulated by the level of ABA (Qin and Zeevaart, 2002; Saito *et al.,* 2004), which in turn is also controlled by the expression of *NCED1*. In agreement with this, a positive correlation (*r*=0.65) was observed between the expression levels of *NCED1* and *ABA8'OH1* in 15 selected barley lines (Steptoe, BM0253-402, Betzes, CDC-Reserve-01, Baudin, Morex, BM0253-103, AAC Synergy, BM0253-58, TR11221, AC Metcalfe, BM0253-409, BM0253-217, TR253 and Himalaya) (Figure 3.11), indicating the role of ABA content in controlling the expression of *ABA8'OH1* and dormancy. A similar study involving barley embryos explained the importance of *NCED1* and *ABA8'OH1* expression in the accumulation of embryonic ABA during grain development (Chono *et al.,* 2006). The expression of endosperms of dormant barley lines show very low expression of *ABA8'OH1* as compared to intermediate dormant lines, and the highest expression of this gene was observed in the non-dormant barley lines (Figure 3.12). This led to a negative correlation (*r*=0.81) between *ABA8'OH1* expression of endosperm and seed dormancy in selected barley lines (Figure 3.13). These results on *ABA8'OH1* expression pattern suggest the presence of lower ABA catabolism or high level of ABA in dormant lines, and higher ABA catabolism and low level of ABA in the non-dormant lines, respectively, and therefore important role of *ABA8'OH1* in controlling ABA level and dormancy in malting barley. Previous studies in Arabidopsis, barley and wheat have also shown that the reduced expression of *ABA8'OH1* is associated with the increased ABA content and seed dormancy (Okamoto *et al.,* 2006; Gubler *et al.,* 2008; Son *et al.,*

2016; Izydorczyk *et al.,* 2018). However, the expression pattern of *ABA8'OH1* in the endosperm of some of the barley lines studied (BM0253-180, BM0253-402, Morex, BM0253-103, AAC Synergy, TR11221, AC Metcalfe, BM0253-412 and Himalaya) did not correlate with the dormancy phenotype. Since seed dormancy and PHS are very complex traits regulated by multi genes and/or QTLs (Lohwasser *et al.,* 2013), dormancy in these lines might be controlled by non-ABA related factors.

Since *ABA8'OH1* was reported to be a critical player in regulating ABA level and dormancy in barley (Gubler *et al.,* 2008), we also investigated its sequence across the lines studied. Analysis of parts of the *ABA8'OH1* sequences representing the conserved domains (Figure 3.6) and phylogenetic analysis (Appendix 3) of its sequence from the different lines along with those derived from other species showed very high sequence homology among the *ABA8'OH1* of the malting barley lines and other species. Another study on *ABA8'OH1* also revealed conserved nature of the gene, suggesting that it is strongly selected due to the very important function it has in regulating ABA level (Liu *et al.,* 2011). It is also possible that the gene duplication occurred recently and consist of less mutation in its sequence (Liu *et al.,* 2011). This study showed the presence of nucleotide substitutions, and insertion and deletion (INDELS) at seven different positions in both exonic and intronic regions of *ABA8'OH1* (Figure 3.3), suggesting the presence of seven different allelic forms of *ABA8'OH1* in the malting barley lines studied; however, the allelic variations did not cause changes in amino acids, and thus represent a silent mutation. These silent mutations in the exonic region of the gene have no effect on seed dormancy. From this study, it appears that the two parental lines of the first population (TR253 and Baudin) consist of two different allelic forms of *ABA8'OH1* which might contribute to the higher expression of *ABA8'OH1* in TR253 (6.5 fold higher than control) as compared to that found in Baudin (4.5 fold higher than

control) (Figure 3.3). In addition, a 10 bp insertion in 3rd intron of Morex and Himalaya, and a 12 bp deletion in 4th intron of AAC Synergy, Morex, TR11221 and Himalaya were detected in the non-coding sequence of *ABA8'OH1*, and this deletion might be responsible for the low expression of *ABA8'OH1* in the endosperm of the four non-dormant cultivars. This might represent an important factor in the regulation of *ABA8'OH1* expression and seed dormancy. A similar study involving dormant and non-dormant lines of rice showed the presence of INDELS in the coding and non-coding regions of *ABA8'OH5,* and this INDELS are suggested to influence the function of the gene through influencing its binding to the regulatory transcriptional factors (Liu *et al.,* 2013).

The expression pattern of the other ABA catabolic gene *ABA8'OH2* in both endosperm and embryo tissues showed variation in the different barley lines with a diverse dormancy phenotype. Lower expression level of *ABA8'OH2* was observed in the endosperm tissues of dormant lines as compared to that found in the intermediate dormant lines, while the non-dormant lines showed the highest expression levels of *ABA8'OH2* (Figure 3.16). As a result, a negative correlation (r=0.61) was observed between the levels of *ABA8'OH2* expression and dormancy (Figure 3.17). However, no such no correlation was observed in some of the lines studied such as Betzes, Morex, BM0253-235, AAC Synergy, AC Metcalfe, BM0253-217 and Himalaya. Negative correlation (r=0.54) was also observed between the expression level of *ABA8'OH2* in the embryo of the different barley lines excluding BM0253-180, BM0253-402, CDC-Reserve-01, BM0253-211, Morex, BM0253-103, TR11221 and AC Metcalfe, whose expression in the embryo was not consistent with the seed dormancy level (Figure 3.15). Our results in general suggest that *ABA8'OH2* is also involved in determining ABA content and dormancy in some lines. A study in Arabidopsis has also shown association of ABA level and seed dormancy with the expression level of *ABA8'OH2* in both

embryo and endosperm tissues (Okamoto *et al.,* 2006). Several other studies also reported similar expression patterns of *ABA8'OH2* between dormant and non-dormant seeds of barley (Jacobsen *et al.,* 2002; Millar *et al.,* 2006), and wheat (Kashiwakura *et al.,* 2016; Son *et al.,* 2016). The expression patterns of both endospermic *ABA8'OH1* and *ABA8'OH2* in selected barley lines such as Steptoe, Betzes, CDC-Reserve-01, BM0253-211, BM0253-95, Baudin, BM0253-331, BM0253-58, BM0253-409 and TR253 showed strong negative correlation with the dormancy phenotype, indicating the role of ABA catabolism in seed dormancy.

Many studies showed the role of GA biosynthetic gene *GA20ox* in the regulation of GA level, and dormancy and germination. For example, the *ga20ox2* mutants of rice exhibits reduced seed GA level and enhanced dormancy (Ye *et al.,* 2015), and *GA2ox3* of rice has been selected as a candidate gene for controlling seed germination (Magwa *et al.,* 2016). Previous genetic/QTL study that used a population derived from a cross that involved Baudin as one of the parental lines reported a gene encoding the GA biosynthetic enzyme GA20ox as a candidate gene regulating seed dormancy QTL (Li *et al.,* 2004). Since a previous study in rice reported *GA20ox1* as a candidate gene for a major QTL controlling seed dormancy and seedling vigor (Abe *et al.,* 2012), this study investigated allelic variation in *GA20ox1* and expression patterns of the family members of *GA20ox* in barley. Analysis of the sequence of *GA20ox1* in the 21 different lines revealed single nucleotide substitutions at four different positions in both the exon and intron regions of dormant lines (BM0253-402 and Betzes), intermediate dormant lines (CDC-Reserve-01, BM0253-211 and Baudin) and a non-dormant line BM0253-217 (Figure 3.19), indicating the presence of two alleles of the gene among the different lines studied. Previous studies have also reported that *GA20ox1* of barley undergoes alternative splicing and has six splice variants, leading to the formation of six distinct mRNA isoforms (Ensembl plants). Since most of the malting barley lines with the mutated

allelic form showed high or intermediate level of dormancy, it may be possible that the mutation is responsible for induction of dormancy. Due to the absence of any detectable level of *GA20ox1* expression in both embryo and endosperm tissues, we are unable to speculate if the base substitutions/allelic variations we detected in the sequence of *GA20ox1* have any effect on its expression level across the different lines studied.

Although the expression of the other *GA20ox* gene *GA20ox2* was detected in embryos of different barley lines, it did not overall correlate with the level of seed dormancy (Figure 3.23). However, selected lines such as a dormant line Steptoe exhibited not detectable expression of *GA20ox2* whereas non-dormant lines BM0253-103 and BM0253-409 showed relatively higher expression level of *GA20ox2*, suggesting a higher GA biosynthesis in the non-dormant lines. This study also detected the expression of *GA20ox3* only in the endosperm, and its expression level in some non-dormant lines (BM0253-235, BM0253-58, BM0253-409 and BM0253-217) was higher than the intermediate dormant (BM0253-211 and BM0253-95) and dormant (Steptoe, BM0253-180, BM0253-402 and Betzes) lines (Figure 3.25), leading to a negative correlation (r=0.90) between the levels of *GA20ox3* expression and dormancy (Figure 3.26). These results imply the role of *GA20ox3* in regulating GA biosynthesis and seed dormancy level in selected barley lines. Similar studies reported the role of high level of bioactive GA_1 due to enhanced expression of GA biosynthetic genes (*GA20ox* and *GA3ox*) in dormancy release in wheat and barley seeds (Gubler *et al.,* 2008; Liu *et al.,* 2013; Kashiwakura *et al.,* 2016). Further study in sorghum found higher expression of *GA20ox* as well as an accumulation of bioactive GA_4 in imbibed embryos of non-dormant cultivar as compared to a dormant cultivar (Pérez-Flores *et al.,* 2003). No correlation was observed between the levels of expression of any of the *GA20ox* genes and seed dormancy in most

of the barley lines studied. It is possible that dormancy in these lines is regulated by the expression of other GA metabolic genes (*GA3ox* and *GA2ox*) in the seed germination of these lines.

The ratio between ABA and GA levels, which is modulated by their antagonistic interaction, plays important roles in maintenance and release of seed dormancy in wheat and barley (Jacobsen *et al.,* 2002; Izydorczyk *et al.,* 2018). In accordance with this, low expression level of the ABA biosynthetic gene (*NCED1*) and high expression of ABA catabolic genes (*ABA8'OH1* and *ABA8'OH2)* and GA biosynthetic gene (*GA20ox3*) have been observed in some non-dormant lines (BM0253-235, BM0253-58, BM0253-409 and BM0253-217). These expression patterns suggest the presence of low ABA: GA ratio in the seeds of these lines, and this might be responsible for the low level of dormancy observed in the lines. Previous studies with imbibed non-dormant barley seeds has also reported the role of increased expression of the ABA catabolic gene (*ABA8'OH1)* and GA biosynthetic gene (*GA3ox2)* in reducing ABA level and increasing GA level, causing a change in ABA: GA ratio and enhanced germination (Suzuki *et al.,* 2005; Millar *et al.,* 2006). Similarly, imbibition of non-dormant wheat seeds has been shown to induce the expressions of *GA3ox2* and *ABA8'OH1* genes in wheat (Liu *et al.,* 2013; Son *et al.,* 2016).

In summary, the results of this study showed that dormancy phenotype in selected lines is associated with the expression patterns of the ABA biosynthetic gene, *NCED1* and ABA catabolic genes, *ABA8'OH1* and *ABA8'OH2*, and the GA biosynthetic gene, *GA20ox3*. This suggests the contribution of changes in ABA: GA ratio in the maintenance of dormancy in the seeds of malting barley, which is an important factor for improving sprouting tolerance.

4.0 GENERAL DISCUSSION AND CONCLUSIONS

Barley is one of the most important cereal crop used for various purposes such as feed, food and malting but its production is affected by several biotic and abiotic stress factors. PHS, which is defined as the premature germination of seed on the mother plant under humid and rainfall conditions, is one of the factors that affect usage of barley. It causes a decrease in end use quality of the barley seeds as the malting industry requires non-sprouted and good quality seeds. PHS of the seed is dependent on dormancy of the seed, which is defined as the inability of a viable seed to germinate under optimal environmental conditions. Seed dormancy is regulated by a number of environmental and endogenous factors such as phytohormones. ABA and GA are two main plant hormones responsible for the regulation of seed dormancy. ABA plays a role in induction and maintenance of seed ABA level and seed dormancy whereas GA promotes seed germination.

This study investigated the role of ABA and GA metabolic genes in the regulation of seed dormancy and resistance to PHS in 21 different barley genotypes and breeding lines. Based on their germination data, the different barley lines are classified to have high, intermediate and low levels of seed dormancy. Expression analysis of the ABA biosynthetic genes revealed the presence of a positive correlation (r=0.75) between the levels of *NCED1* expression and seed dormancy, indicating the role of this gene in regulating the embryonic ABA level and dormancy level. As the expression patterns of ABA biosynthetic gene *NCED1* is strongly correlated with the seed dormancy, therefore, it is important to study the allelic variation of this gene in different barley lines. A comparative expression analysis between the two ABA catabolic genes showed that *ABA8'OH1* is highly expressed in the embryos during imbibition. Our data indicated a high expression level of *ABA8'OH1* in dormant embryos as compared to that found in the intermediate dormant and non-dormant embryos, and this caused a positive correlation (r=0.70) between the

levels of *ABA8'OH1* expression and seed dormancy at least in in selected lines. This might be due to the fact that the expression of *ABA8'OH1* is subjected to feed forward regulation by the amount of ABA present in the embryos, which is also dependent on the expression of *NCED1*. To verify this, we performed further correlation analysis between the expression of levels of *NCED1* and *ABA8'OH1*, and this analysis showed a positive correlation (r=0.65) between the two factors. Based on our data, we suggest that both ABA biosynthesis and catabolism in the embryo plays a major role in deciding the endogenous ABA content and seed dormancy phenotype in most of the barley lines studied. Therefore, it is important to measure the ABA content in the embryos of different malting barley lines.

Unlike that of *NCED1*, the expression of both *ABA8'OH* genes were detected in the endosperm tissue, however, *ABA8'OH1* was found to be the predominantly expressed gene. The expression level of *ABA8'OH1* in the endosperm of selected barley lines showed a negative correlation (r=0.81) with the dormancy phenotype, suggesting a higher rate of ABA catabolism in the non-dormant lines. Therefore, our results suggest ABA catabolism in the endosperm might play an important role in regulating the amount of seed ABA and dormancy in the different barley lines. However, the expression of *ABA8'OH1* in the endosperm of few lines did not show close correlation with the seed dormancy probably due to the role of some other non-ABA related genetic factors in the control of seed dormancy in these lines. Since comparison of the genomic sequences of *ABA8'OH1* showed insertion and deletion (INDELS) in the non-coding sequence of the gene. Close observation of the correlation between the sequences of *ABA8'OH1* and its expression suggest that the 12 bp deletion observed in *ABA8'OH1* sequence in AAC Synergy, Morex, TR11221 and Himalaya might be responsible for its low level of expression in the endosperm of the non-dormant barley lines. It is important to study the variation in the promoter

sequence of *ABA8'OH1* which might be responsible for controlling expression as there was no variation in the exonic region of gene. As discussed above, it is important to examine the ABA content in endosperm tissues of different barley lines.

The second part of this thesis examined allelic variation in the GA biosynthetic gene *GA20ox1* and the expression patterns of *GA20ox* family members. Our analysis showed a high expression of *GA20ox3* in the endosperm of non-dormant barley lines as compared to that found in the barley lines with high and intermediate levels of dormancy. Strong correlation (r=0.90) was evident between the levels of endospermic *GA20ox3* expression and seed dormancy in selected barley lines. Since the endosperm of the same selected non-dormant lines also show high expression levels of *ABA8'OH1* and *ABA8'OH2*, the ratio of ABA to GA in the endosperm might play a role in regulating dormancy in these lines. Sequence analysis of *GA20ox1* revealed four nucleotide substitutions at different position mainly in the dormant (BM0253-402 and Betzes) and intermediate dormant (CDC-Reserve-01, BM0253-211 and Baudin) lines. Although the expression of this gene was not detected in any of the tissues of the barley lines tested, it is possible that these mutations promote dormancy. Since *GA20ox1* of barley has been reported to undergo alternative splicing, it may be important to study the post transcriptional modification of this gene and the involvement of this in controlling seed dormancy. Overall, the findings of this thesis project provide useful information about the role of ABA and GA metabolic genes in regulating seed dormancy and tolerance to PHS in malting barley lines.

LITERATURE CITED

Abe A, Takagi H, Fujibe T, Aya K, Kojima M, Sakakibara H, Terauchi R (2012) *OsGA20ox1*, a candidate gene for a major QTL controlling seedling vigor in rice. Theor Appl Genet **125:** 647-657.

Alboresi A, Gestin C, Leydecker MT, Bedu M, Meyer C, Truong HN (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant Cell Environ **28:** 500–512.

Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, Grappin P, Jullien M (2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of Arabidopsis thaliana. Planta **219:** 479–488.

Appleford NE, Evans DJ, Lenton JR, Gaskin P, Croker SJ, Devos KM, Hedden P (2006) Function and transcript analysis of gibberellin-biosynthetic enzymes in wheat. Planta **223:** 568-582.

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

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

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

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

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

Baskin JM, Baskin CC (2000) Evolutionary considerations of claims for physical dormancy-break by microbial action and abrasion by soil particles. Seed Sci Res **10:** 409-413.

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

Benech-Arnold RL (2001) Bases of pre-harvest sprouting resistance in barley: physiology, molecular biology and environmental control of dormancy in the barley grain. Barley Sci 481-502.

Benech-Arnold RL, Cristina Giallorenzi M, Frank J, Rodriguez V (1999) Termination of hull-imposed dormancy in developing barley grains is correlated with changes in embryonic ABA levels and sensitivity. Seed Sci **9:** 39-47.

Bethke PC, Libourel IG, Jones RL (2006) Nitric oxide reduces seed dormancy in Arabidopsis. J Exp Bot **57:** 517–26.

Bewley JD (1997) Seed germination and dormancy. Plant Cell **9:** 1055-1066.

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

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

Black M, Bewley JD, Halmer P (2006) The Encyclopedia of Seeds: Science, Technology and Uses. Wallingford: CABI Publishing.

Bleidere M, Gaile Z (2012) Grain quality traits important in feed barley. In Proceedings of the Latvian Academy of Sciences **66:** 1-9.

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

Canadian Grain Commission (CGC) (https://www.grainscanada.gc.ca/index-eng.htm) (Accessed on 15 August 2018).

Chen SS, Chang JL (1972) Does gibberellic acid stimulate seed germination via amylase synthesis?. Plant Physiol **49:** 441.

Chitnis VR, Gao F, Yao Z, Jordan MC, Park S, Ayele BT (2014) After-ripening induced transcriptional changes of hormonal genes in wheat seeds: the cases of brassinosteroids, ethylene, cytokinin and salicylic acid. PloS one **9:** e87543.

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

Chono M, Matsunaka H, Seki M, Fujita M, Kiribuchi-Otobe C, Oda S, Kawakami N (2013) Isolation of a wheat (*Triticum aestivum* L.) mutant in ABA 8′-hydroxylase gene: effect of reduced ABA catabolism on germination inhibition under field condition. Breeding Sci **63:** 104-115.

Clark HH (1967) The origin and early history of the cultivated barleys. Agric Hist Rev **15:** 1.

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

Corbineau F, Côme D (2000) Dormancy of cereal seeds as related to embryo sensitivity to ABA and water potential. Dormancy in Plants Wallingford UK CAB International 183-194.

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol **49:** 557-583.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: Emergence of a core signaling network. Annu Rev Plant Biol **61:** 651-679.

Da Silva EAA, Toorop PE, van Aelst AC, Hilhorst HWM (2004) Abscisic acid controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv Rubi) seed germination. Planta **220:** 251–261.

Davies PJ (2010) The plant hormones: their nature, occurrence and functions. In plant hormones. Springer, Dordrecht, pp 1-15.

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

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

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus **12:** 13–15.

Edney MJ, Legge WG, Izydorczyk MS, Demeke T, Rossnagel BG (2013) Identification of Barley Breeding Lines Combining Preharvest Sprouting Resistance with "Canadian-type" Malting Quality. Crop Sci **53:** 1447-1454.

Fang J, Chu C (2008) Abscisic acid and the pre-harvest sprouting in cereals. Plant Signal Behav **3:** 1046-1048.

Fidler J, Zdunek-Zastocka E, Bielawski W (2015) Regulation of abscisic acid metabolism in relation to the dormancy and germination of cereal grains. Acta Soc Bot Pol **84:** 3.

Fidler J, Zdunek-Zastocka E, Prabucka B, Bielawski W (2016) Abscisic acid content and the expression of genes related to its metabolism during maturation of triticale grains of cultivars differing in pre-harvest sprouting susceptibility. J Plant Physiol **207:** 1-9.

Finch-Savage WE, Footitt S (2017) Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. J Exp Bot **68:** 843-856.

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

Finkelstein R (2010) The role of hormones during seed development and germination. In Plant Hormones. Springer, Dordrecht, pp 549-573.

Finkelstein R (2013) Abscisic acid synthesis and response. The Arabidopsis book **11:** e0166.

Finkelstein R, Gampala S, Rock C (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell **14:** S15-S45.

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

Fox GP, Osborne B, Bowman J, Kelly A, Cakir M, Poulsen D, Inkerman A, Henry R (2007) Measurement of genetic and environmental variation in barley (*Hordeum vulgare*) grain hardness. J Cereal Sci **46:** 82-92.

Frey A, Effroy D, Lefebvre V, Seo M, Perreau F, Berger A, Marion-Poll A (2012) Epoxycarotenoid cleavage by NCED5 fine-tunes ABA accumulation and affects seed dormancy and drought tolerance with other NCED family members. Plant J **70:** 501-512.

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

Gao F, Ayele BT (2014) Functional genomics of seed dormancy in wheat: advances and prospects. Front Plant Sci **5:** 458.

Gao F, Jordan MC, Ayele BT (2012) Transcriptional programs regulating seed dormancy and its release by after-ripening in common wheat (*Triticum aestivum* L). Plant Biotechnol J **10:** 465-476.

Gao X, Hu C, Li H, Yao Y, Meng M, Dong J, Zhao W, Chen Q, Li X (2013) Factors affecting pre-harvest sprouting resistance in wheat (*Triticum aestivum*). J Anim Plant Sci **23:** 556-565.

Gazzarrini S, Tsai AYL (2015) Hormone cross-talk during seed germination. Essays Biochem **58:** 151-164.

Gross BL, Olsen KM (2010) Genetic perspectives on crop domestication. Trends Plant Sci **15:** 529-537.

Gualano NA, Del Fueyo PA, Benech-Arnold RL (2014) Potential longevity (Ki) of malting barley (*Hordeum vulgare* L.) grain lots relates to their degree of pre-germination assessed through different industrial quality parameters. J Cereal Sci **60:** 222-228.

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

Gubler F, Millar AA, Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. Curr Opin Plant Biol **8:** 183-187.

Han F, Zhu B (2011) Evolutionary analysis of three gibberellin oxidase genes in rice, Arabidopsis, and soybean. Gene **473:** 23-35.

Hatfield JL, Boote KJ, Kimball BA, Ziska LH, Izaurralde RC, Ort D, Thomson AM, Wolfe D (2011) Climate impacts on agriculture: implications for crop production. Agron J **103:** 351–370.

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

Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B (2002) Gibberellin biosynthesis in plants and fungi: A case of convergent evolution?. Plant Growth Regul **20:** 319–331.

Helliwell CA, Sullivan JA, Mould RM, Gray JC, Peacock WJ, Dennis ES (2001) A plastid envelope location of Arabidopsis ent-kaurene oxidase links the plastid and endoplasmic reticulum steps of the gibberellin biosynthesis pathway. Plant J **28:** 201-208.

Hickey LT, Lawson W, Arief VN, Fox G, Franckowiak J, Dieters MJ (2012) Grain dormancy QTL identified in a doubled haploid barley population derived from two non-dormant parents. Euphytica **188:** 113-122.

Hilhorst HW (2007) Definitions and hypotheses of seed dormancy. Seed Development, Dormancy and Germination **27:** 50-71.

Hirschberg J (2001) Carotenoid biosynthesis in flowering plants. Curr Opin Plant Biol **4:** 210-218.

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

Hu J, Mitchum MG, Barnaby N, Ayele BT, Ogawa M, Nam E, Swain SM (2008) Potential sites of bioactive gibberellin production during reproductive growth in Arabidopsis. Plant Cell **20:** 320-336.

Huo H, Dahal P, Kunusoth K, McCallum CM, Bradford KJ (2013) Expression of 9-cis-epoxycarotenoid dioxygenase4 is essential for thermo inhibition of lettuce seed germination but not for seed development or stress tolerance. Plant Cell **25:** 884-900.

Idehen E, Tang Y, Sang S (2017) Bioactive phytochemicals in barley. J food drug anal **25:** 148-161.

Iehisa JC, Matsuura T, Mori IC, Takumi S (2014) Identification of quantitative trait locus for abscisic acid responsiveness on chromosome 5A and association with dehydration tolerance in common wheat seedlings. J Plant Physiol **171:** 25-34.

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, Macmillan J (1984) Internode length in Pisum. The Le gene controls the 3β-hydroxylation of gibberellin A20 to gibberellin A1. Planta **160:** 455-463.

Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M, Kobayashi M (2001) Cloning and functional analysis of two gibberellin 3β-hydroxylase genes that are differently expressed during the growth of rice. Proc Natl Acad Sci USA **98:** 8909-8914.

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J **27:** 325-333.

Izydorczyk C, Nguyen TN, Jo S, Son S, Tuan PA, Ayele BT (2018) Spatiotemporal modulation of abscisic acid and gibberellin metabolism and signalling mediates the effects of suboptimal and supra-optimal temperatures on seed germination in wheat (*Triticum aestivum* L). Plant Cell Environ **41:** 1022-1037.

Izydorczyk MS, Dexter JE (2008) Barley β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products. Food Res Int **41:** 850-868.

Jacobsen JV, Pearce DW, Poole AT, Pharis RP, Mander LN (2002) Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. Physiol Plant **115:** 428-441.

Ji X, Dong B, Shiran B, Talbot MJ, Edlington JE, Hughes T, Dolferus R (2011) Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. Plant Physiol **156:** 647-662.

Jia Q, Zhang J, Westcott S, Zhang XQ, Bellgard M, Lance R, Li C (2009) GA-20 oxidase as a candidate for the semidwarf gene sdw1/denso in barley. Funct Integr Genomics **9:** 255-262.

Jia Q, Zhang XQ, Westcott S, Broughton S, Cakir M, Yang J, Lance R, Li C (2011) Expression level of a gibberellin 20-oxidase gene is associated with multiple agronomic and quality traits in barley. Theor Appl Genet **122:** 1451-1460.

Kaneko M, Itoh H, Ueguchi-Tanaka M, Ashikari M, Matsuoka M (2002) The α-amylase induction in endosperm during rice seed germination is caused by gibberellin synthesized in epithelium. Plant Physiol **128:** 1264-1270.

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

Kashiwakura YI, Kobayashi D, Jikumaru Y, Takebayashi Y, Nambara E, Seo M, Kawakami N (2016) Highly sprouting-tolerant wheat grain exhibits extreme dormancy and cold imbibition-resistant accumulation of abscisic acid. Plant Cell Physiol **57:** 715-732.

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. Curr Opin Plant Biol **5:** 33-36.

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

Koornneef M, Van der Veen JH (1980) Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor Appl genet **58:** 257-263.

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

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

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Nambara E (2004) The Arabidopsis cytochrome P450 *CYP707A* encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. EMBO J **23:** 1647-1656.

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

Leubner-Metzger G (2005) β-1, 3-Glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. Plant J **41:** 133-145.

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

Li C, Ni P, Francki M, Hunter A, Zhang Y, Schibeci D, Li H, Tarr A, Wang J, Cakir M, Yu J, Bellgard M, Lance R, Appels R (2004) Genes controlling seed dormancy and pre-harvest sprouting in a rice-wheat-barley comparison. Funct Integr Genomics **4:** 84-93.

Li CD, Tarr A, Lance RCM, Harasymow S, Uhlmann J, Westcot S, Appels R (2003) A major QTL controlling seed dormancy and pre-harvest sprouting/grain α-amylase in two-rowed barley (*Hordeum vulgare* L). Aust J Agric Res **54:** 1303-1313.

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

Liu F, Zhang H, Wu G, Sun J, Hao L, Ge X, Yu J, Wang W (2011) Sequence variation and expression analysis of seed dormancy and germination associated ABA and GA related genes in rice cultivars. Front Plant Sci **2:** 17.

Liu PP, Montgomery TA, Fahlgren N, Kasschau KD, Nonogaki H, Carrington JC (2007) Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. Plant J **52:** 133-146.

Liu S, Sehgal SK, Li J, Lin M, Trick HN, Yu J, Bai G (2013) Cloning and characterization of a critical regulator for preharvest sprouting in wheat. Genetics **195:** 263-273.

Liu X, Zhang H, Zhao Y, Feng Z, Li Q, Yang HQ, He ZH (2013) Auxin controls seed dormancy through stimulation of abscisic acid signaling by inducing ARF-mediated ABI3 activation in Arabidopsis. Proc Natl Acad Sci USA **110:** 15485-15490.

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

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

Lo SF, Yang SY, Chen KT, Hsing YI, Zeevaart JA, Chen LJ, Yu SM (2008) A novel class of gibberellin 2-oxidases control semi dwarfism, tillering, and root development in rice. Plant Cell **20:** 2603-2618.

Lohwasser U, Arif MR, Börner A (2013) Discovery of loci determining pre-harvest sprouting and dormancy in wheat and barley applying segregation and association mapping. Biol Plantarum **57:** 663-674.

Magwa RA, Zhao H, Xing Y (2016) Genome-wide association mapping revealed a diverse genetic basis of seed dormancy across subpopulations in rice (*Oryza sativa* L). BMC Genet **17:** 28.

Mahalingam R (2017) Shotgun proteomics of the barley seed proteome. BMC Genomics **18:** 44.

Mares DJ (1989) Preharvest sprouting damage and sprouting tolerance: assay methods and instrumentation. Preharvest Field Sprouting in Cereals 129-170.

Matakiadis T, Alboresi A, Jikumaru Y, Tatematsu K, Pichon O, Renou JP, Truong HN (2009) The Arabidopsis abscisic acid catabolic gene *CYP707A2* plays a key role in nitrate control of seed dormancy. Plant Physiol **149:** 949-960.

Matilla AJ, Matilla-Vázquez MA (2008) Involvement of ethylene in seed physiology. Plant Sci **175:** 87-97.

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, Scofield G, Reid JB, Gubler F (2006) Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8´-hydroxylase. Plant J **45:** 942-954.

Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, Kato T, Sun TP (2006) Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. Plant J **45:** 804-818.

Mornkham T, Wangsomnuk PP, Fu YB, Wangsomnuk P, Jogloy S, Patanothai A (2013) Extractions of High Quality RNA from the Seeds of Jerusalem Artichoke and Other Plant Species with High Levels of Starch and Lipid. Plants (Basel) **2:** 302-16.

Mukherjee S, Stasolla C, Ayele BT (2015). RNA In situ Hybridization. In Plant Microtechniques and Protocols, Springer, Cham pp. 351-370.

Müller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. Plant Cell Physiol **47:** 864-877.

Nakamura S, Pourkheirandish M, Morishige H, Kubo Y, Nakamura M, Ichimura K, Hensel G (2016) Mitogen-activated protein kinase 3 regulates seed dormancy in barley. Curr Biol **26:** 775-781.

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

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol **56:** 165-85.

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

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

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

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

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

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

Pearce S, Huttly AK, Prosser IM, Li YD, Vaughan SP, Gallova B, Phillips AL (2015) Heterologous expression and transcript analysis of gibberellin biosynthetic genes of grasses reveals novel functionality in the *GA3ox* family. BMC Plant Biol **15:** 130.

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

Pérez-Flores L, Carrari F, Osuna-Fernández R, Rodríguez MV, Enciso S, Stanelloni R, Sanchez RA, Bottini R, Iusem ND, Benech-Arnold RL (2003) Expression analysis of a *GA 20-oxidase* in embryos from two sorghum lines with contrasting dormancy: possible participation of this gene in the hormonal control of germination. J Exp Bot **54:** 2071-2079.

Petra H, Christian P, Sven M, Ellen K, Andreas F (2013) Impacts of temperature increase and change in precipitation pattern on crop yield and yield quality of barley. Food Chem **136:** 1470–1477.

Phinney BO (1956) Growth response of single-gene dwarf mutants in maize to gibberellic acid. Proc Natl Acad Sci USA **42:** 185-189.

Plackett AR, Powers SJ, Fernandez-Garcia N, Urbanova T, Takebayashi Y, Seo M, Jikumaru Y, Benlloch R, Nilsson O, Ruiz-Rivero O, Phillips AL, Wilson ZA, Thomas SG, Hedden P (2012) Analysis of the developmental roles of the Arabidopsis gibberellin 20-oxidases demonstrates that *GA20ox1*, *-2*, and *-3* are the dominant paralogs. Plant Cell **24:** 941-960.

Pomeranz Y (1974) Food uses of barley. Crit Rev Food Technol **4:** 377–94.

Qin X, Zeevaart JA (2002) Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol **128:** 544-551.

Quinde Z, Ullrich SE, Baik BK (2004) Genotypic variation in color and discoloration potential. New York: Marcel Dekker p519–42.

Ramaih S, Guedira M, Paulsen GM (2003) Relationship of indoleacetic acid and tryptophan to dormancy and preharvest sprouting of wheat. Funct Plant Biol **30:** 939-945.

Rodríguez MV, Barrero JM, Corbineau F, Gubler F, Benech-Arnold RL (2015) Dormancy in cereals (not too much, not so little): about the mechanisms behind this trait. Seed Sci Res **25:** 99-119.

Rodríguez MV, Mendiondo GM, Cantoro R, Auge GA, Luna V, Masciarelli O, Benech-Arnold RL (2011) Expression of seed dormancy in grain sorghum lines with contrasting pre-harvest sprouting behavior involves differential regulation of gibberellin metabolism genes. Plant Cell Physiol **53:** 64-80.

Rodríguez-Gacio MDC, Matilla-Vázquez MA, Matilla AJ (2009) Seed dormancy and ABA signaling: the breakthrough goes on. Plant Signal Behav **4:** 1035-1048.

Ryu S (1979) Grain quality of barley for human diet. In Proceedings of Joint Barley Utilization Seminar Korean Science and Engineering Foundation Suweon Korea pp 94-108.

Saika H, Okamoto M, Miyoshi K, Kushiro T, Shinoda S, Jikumaru Y, Arimura SI (2007) Ethylene promotes submergence-induced expression of OsABA8ox1 a gene that encodes ABA 8′-hydroxylase in rice. Plant Cell Physiol **48:** 287-298.

Saika H, Okamoto M, Miyoshi K, Kushiro T, Shinoda S, Jikumaru Y, Arimura SI (2007) Ethylene promotes submergence-induced expression of OsABA8ox1, a gene that encodes ABA 8′-hydroxylase in rice. Plant Cell Physiol **48:** 287-298.

Saito S, Hirai N, Matsumoto C, Ohigashi H, Ohta D, Sakata K, Mizutani M (2004) Arabidopsis CYP707As encode (+)-abscisic acid 8'-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiol **134:** 1439-1449.

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Miyao A (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol **134:** 1642-1653.

Sato K, Takeda K (2009) An application of high-throughput SNP genotyping for barley genome mapping and characterization of recombinant chromosome substitution lines. Theor Appl Genet **119:** 613-619.

Savin R, Stone PJ, Nicolas ME, Wardlaw IF (1997) Effects of heat stress and moderately high temperature on grain growth and malting quality of barley. Aust J Agric Res **48:** 615–624.

Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. Science **276:** 1872–1874.

Seo M, Hanada A, Kuwahara A, Endo A, Okamoto M, Yamauchi Y, North H, Marion-poll A, Sun TP, Koshiba T, Kamiya Y (2006) Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. Plant J **48:** 354-366.

Shu K, Liu XD, Xie Q, He ZH (2016) Two faces of one seed: hormonal regulation of dormancy and germination. Mol Plant **9:** 34-45.

Son S, Chitnis VR, Liu A, Gao F, Nguyen TN, Ayele BT (2016) Abscisic acid metabolic genes of wheat (*Triticum aestivum* L): identification and insights into their functionality in seed dormancy and dehydration tolerance. Planta **244:** 429-447.

Spielmeyer W, Ellis M, Robertson M, Ali S, Lenton JR, Chandler PM (2004) Isolation of gibberellin metabolic pathway genes from barley and comparative mapping in barley, wheat and rice. Theor Appl Genet **109:** 847-855.

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

Sun TP (2008) Gibberellin metabolism, perception and signaling pathways in Arabidopsis. The Arabidopsis Book **6:** e0103.

Suzuki Y, Ito S, Otsuka K, Iwasawa E, Nakajima M, Yamaguchi I (2005) Preparation of functional single-chain antibodies against bioactive gibberellins by utilizing randomly mutagenized phage-display libraries. Biosci Biotechnol Biochem **69:** 610-619.

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA **96:** 4698-4703.

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Iuchi S (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. Plant Physiol **146:** 1368-1385.

Tuan PA, Kumar R, Rehal PK, Toora PK, Ayele BT (2018) Molecular mechanisms underlying abscisic acid/gibberellin balance in the control of seed dormancy and germination in cereals. Front Plant Sci **9:** 668.

Tuttle KM, Martinez SA, Schramm EC, Takebayashi Y, Seo M, Steber CM (2015) Grain dormancy loss is associated with changes in ABA and GA sensitivity and hormone accumulation in bread wheat *Triticum aestivum* (L). Seed Sci Res **25:** 179-193.

Ullrich SE, Clancy JA, del Blanco I, Lee H, Jitkov VA, Han F, Keinhofs A, Matsui K (2008) Genetic analysis of preharvest sprouting in a six-row barley cross. Mol Breed **21:** 249–259.

United States Department of Agriculture (USDA) (https://www.usda.gov/) (Accessed on 15 August 2018)

Van Der Schaar W, Alonso-Blanco C, Léon-Kloosterziel KM, Jansen RC, Van Ooijen JW, Koornneef M (1997) QTL analysis of seed dormancy in Arabidopsis using recombinant inbred lines and MQM mapping. Heredity **79:** 190.

Vanhala TK, Stam P (2006) Quantitative trait loci for seed dormancy in wild barley (*Hordeum spontaneum* C. Koch). Genet Resour Crop EV **53:** 1013-1019.

Von Bothmer R, van Hintum T, Knu¨ pffer H, Sato K (2003) Diversity in Barley (*Hordeum vulgare*). Elsevier, Amsterdam.

Walters DR, Avrova A, Bingham IJ, Burnett FJ, Fountaine J, Havis ND, Hoad SP, Hughes G, Looseley M, Oxley SJP, Renwick A, Topp CFE, Newton AC (2012) Control of foliar diseases in barley: towards an integrated approach. Eur J Plant Pathol **133:** 33-73.

Wang H, Caruso LV, Downie AB, Perry SE (2004) The embryo MADS domain protein AGAMOUS-Like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. Plant Cell **16:** 1206-1219.

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

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

Yamaguchi S (2008) Gibberellin metabolism and its regulation. Annu Rev Plant Biol **59:** 225–51.

Yamaguchi S, Sun TP, Kawaide H, Kamiya Y (1998) The GA2 locus of *Arabidopsis thaliana* encodes *ent*-kaurene synthase of gibberellin biosynthesis. Plant Physiol **116:** 1271-1278.

Yang SH, Choi D (2006) Characterization of genes encoding ABA 8′-hydroxylase in ethylene-induced stem growth of deep water rice (*Oryza sativa* L). Biochem Biophys Res Commun **350:** 685-690.

Ye H, Feng J, Zhang L, Zhang J, Mispan MS, Cao Z, Beighley DH, Yang J, Gu XY (2015) Map-based cloning of seed dormancy1-2 identified a gibberellin synthesis gene regulating the development of endosperm-imposed dormancy in rice. Plant Physiol **169:** 2152-2165.

Yeung J, Vasanthan T (2001) Pearling of hull-less barley: Product composition and gel color of pearled barley flours as affected by the degree of pearling. J Agric Food Chem **49:** 331-335.

Zhang S, Cai Z, Wang X (2009) The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. Proc Natl Acad Sci USA **106:** 4543-4548.

Zhang Y, Miao X, Xia X, He Z (2014) Cloning of seed dormancy genes (*TaSdr*) associated with tolerance to pre-harvest sprouting in common wheat and development of a functional marker. Theor Appl Genet **127:** 855-866.

Zhao Y (2010) Auxin biosynthesis and its role in plant development. Annu Rev Plant Biol **61:** 49-64.

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

Zhu G, Ye N, Zhang J (2009) Glucose-induced delay of seed germination in rice is mediated by the suppression of ABA catabolism rather than an enhancement of ABA biosynthesis. Plant Cell Physiol **50:** 644-651.

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

APPENDIX

Appendix 1. Analysis of variance of seed germination percentage of the different barley lines.

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P Value
Between lines	20	76919.556	3845.978	176.344	<0.001
Within lines	42	916.000	21.810		
Total	62	77835.556			

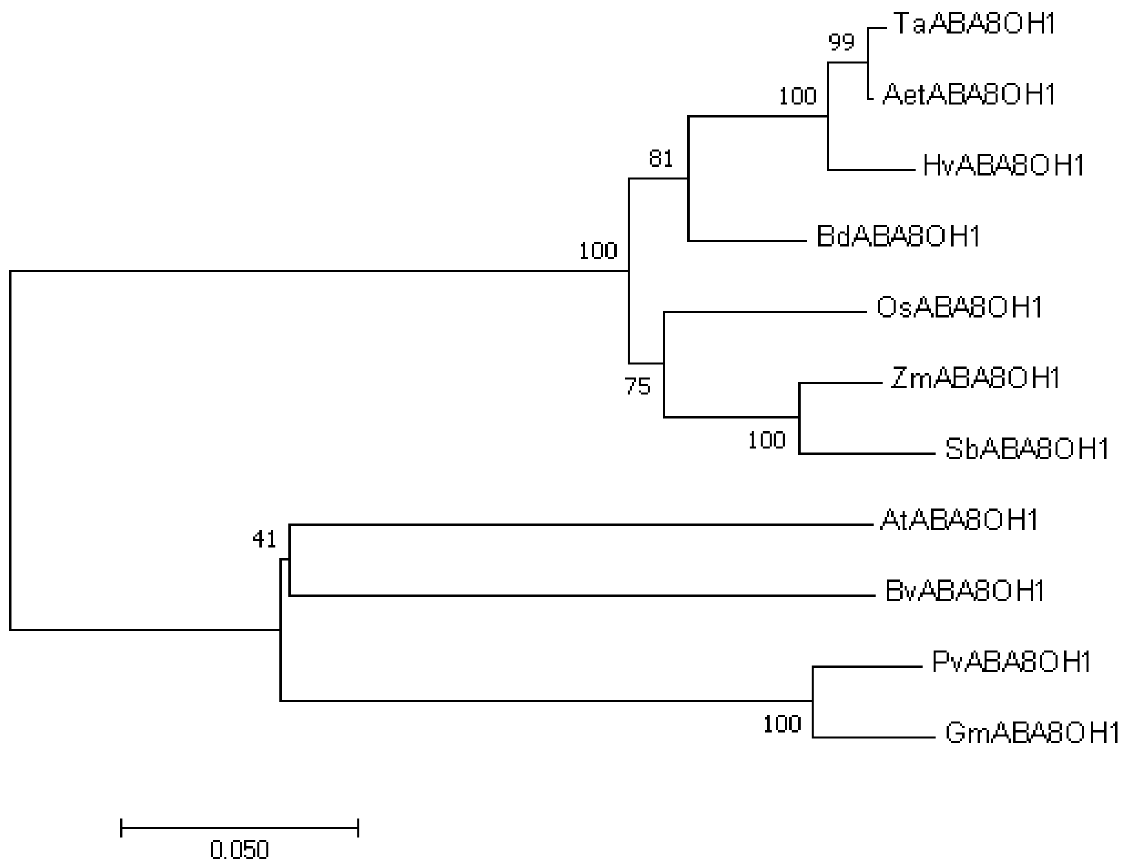
Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 2. Nucleotide substitution/INDELS in the genomic DNA sequence of *ABA8'OH1*

Base substitution/INDELS	Position	Lines exhibiting variation
C to T	602th bp	AC Metcalfe, BM0253-402, TR253
C to A	605th bp	AAC Synergy, CDC-Reserve-01, Himalaya, Morex, TR11221
G to C	1227th bp	AAC Synergy, CDC-Reserve-01, TR11221
C to A	1340th bp	Himalaya, Morex, Steptoe
A to T or deletion of A	1816th bp	CDC-Reserve-01, Himalaya, Morex, Steptoe, TR11221
Addition (GTGCTGTACC)	1523th to 1532th bp	Himalaya, Morex
Deletion (GATGATGATGAT)	1838th to 1849th bp	AAC Synergy, Himalaya, Morex, TR11221

A=Adenine; T=Thymine; G=Guanine; C=Cytosine
INDELS =insertions or deletions

Appendix 3. Phylogenetic analysis of the ABA8′OH1 proteins from barley and other species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Alignment of the sequences was carried out by Clustal W, and the evolutionary history is constructed by the neighbour-joining method using MEGA 7.0 software. The evolutionary distance was computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (0.050). The aligned ABA8′OH protein sequences with their GenBank ID are *Triticum aestivum*, TaABA8′OH1 (GenBank ID: ACB78189.1); *Aegilops taushii*, AetABA8′OH1 (GenBank ID: F775_16028); *Hordeum vulgare*, *ABA8'OH1* (GenBank ID: ABB71585.1); *Brachypodium distachyon*, BdABA8′OH1 (GenBank ID: BRADI3G52660.1); *Orzya sativa*, OsABA8′OH1 (GenBank ID: Os02g0703700); *Zea mays*, ZmABA8′OH1 (GenBank ID: Zm00001d017762_T002); *Sorghum bicolor*, SbABA8′OH1 (GenBank ID: SORBI_3004G268700); *Arabidopsis thaliana*, AtABA8′OH1 (GenBank ID: AT4G19230.1); *Beta vulgaris*, BvABA8′OH1 (GenBank ID: BVRB_5g102360); *Phaseolus vulgaris*, PvABA8′OH1 (GenBank ID: ABC86558.1) and *Glycine max*, GmABA8′OH1 (GenBank ID: ABQ65856.1).

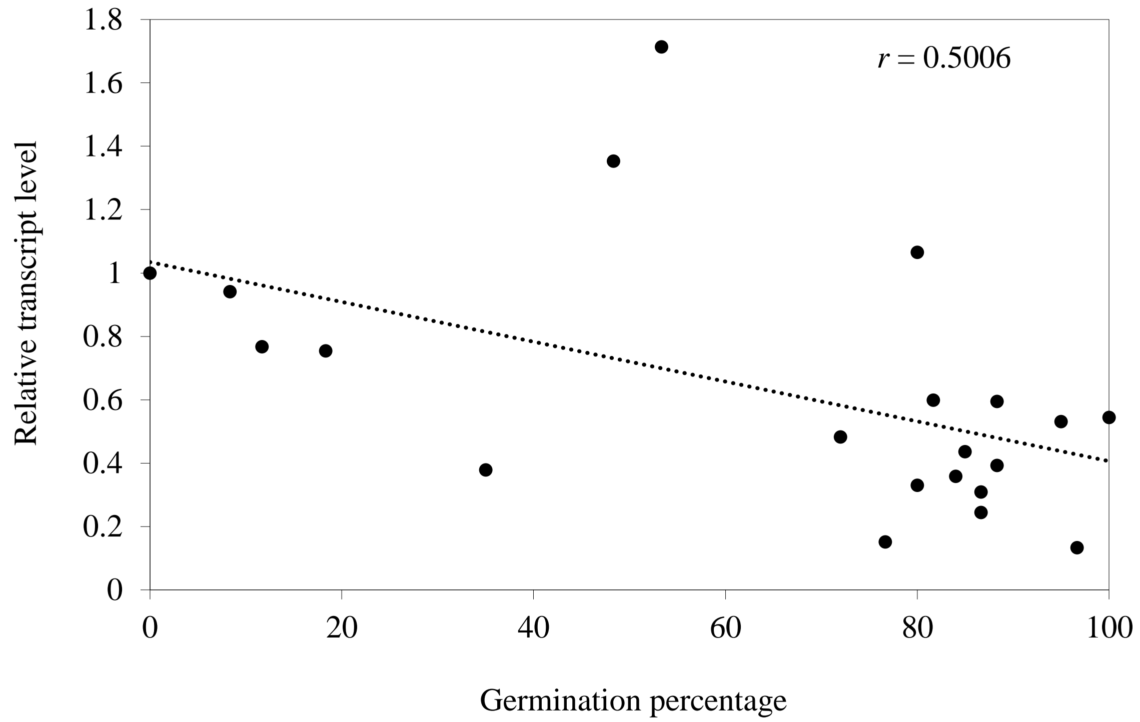


Appendix 4. Analysis of variance of relative transcript levels of *NCED1* in the embryos of the different barley lines.

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between lines	20	9.686	0.484	7.158	<0.001
Within lines	40	2.707	0.0677		
Total	60	12.393			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 5. Correlation between seed germination percentage and expression levels of embryonic *NCED1* of the different barley lines.

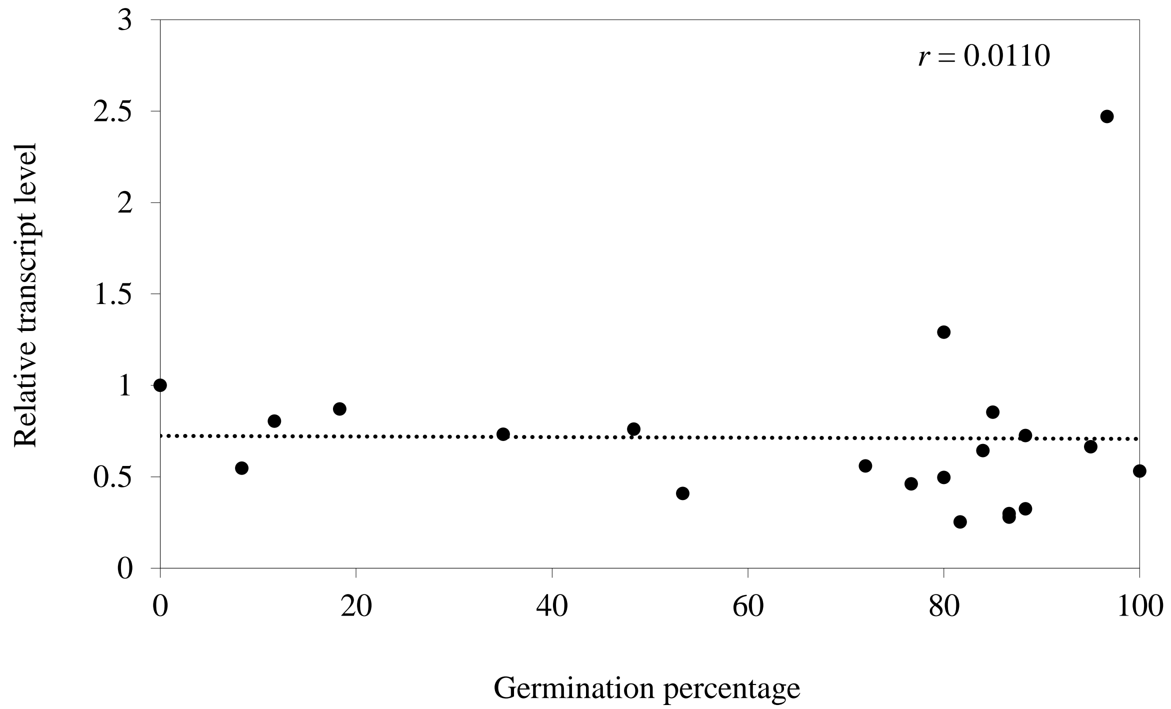


Appendix 6. Analysis of variance of relative transcript levels of *ABA8'OH1* in the embryos of the different barley lines.

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between groups	20	16.059	0.803	10.248	<0.001
Residual	41	3.212	0.0783		
Total	61	19.271			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 7. Correlation between seed germination percentage and expression levels of embryonic *ABA8'OH1* of the different barley lines.

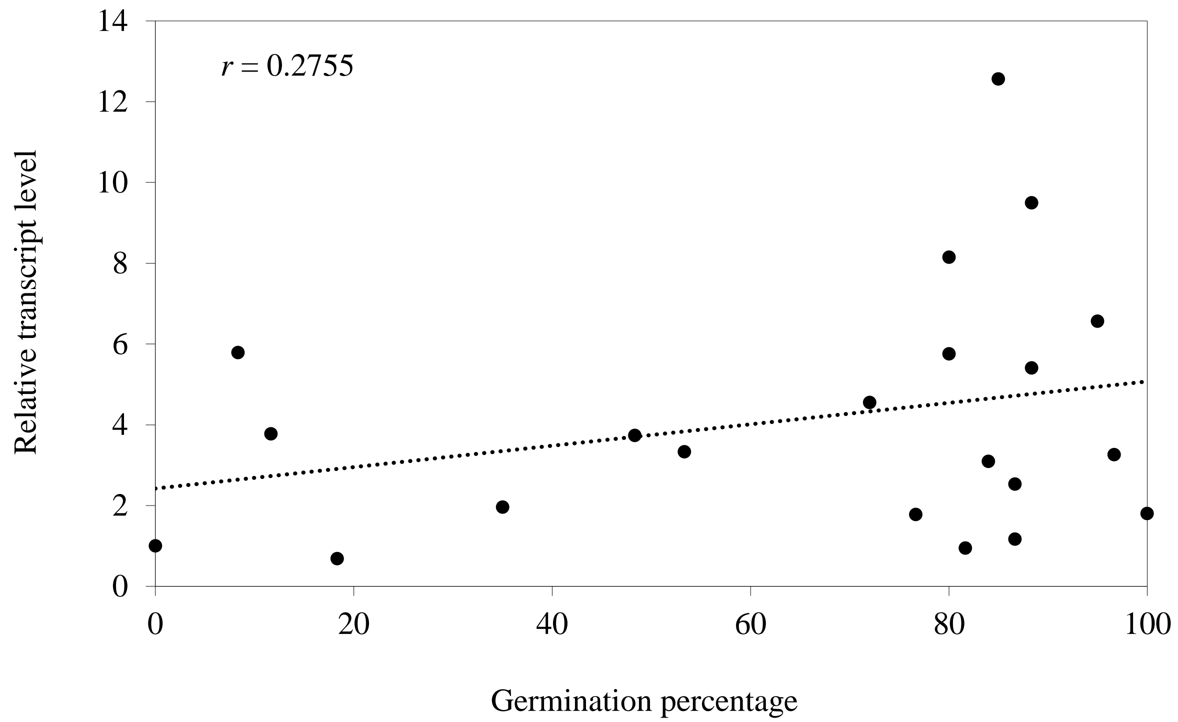


Appendix 8. Analysis of variance of relative transcript levels of *ABA8'OH1* in the endosperm of the different barley lines.

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between groups	20	588.187	29.409	21.983	<0.001
Residual	41	54.852	1.338		
Total	61	643.038			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 9. Correlation between seed germination percentage and expression levels of endospermic *ABA8'OH1* of the different barley lines.

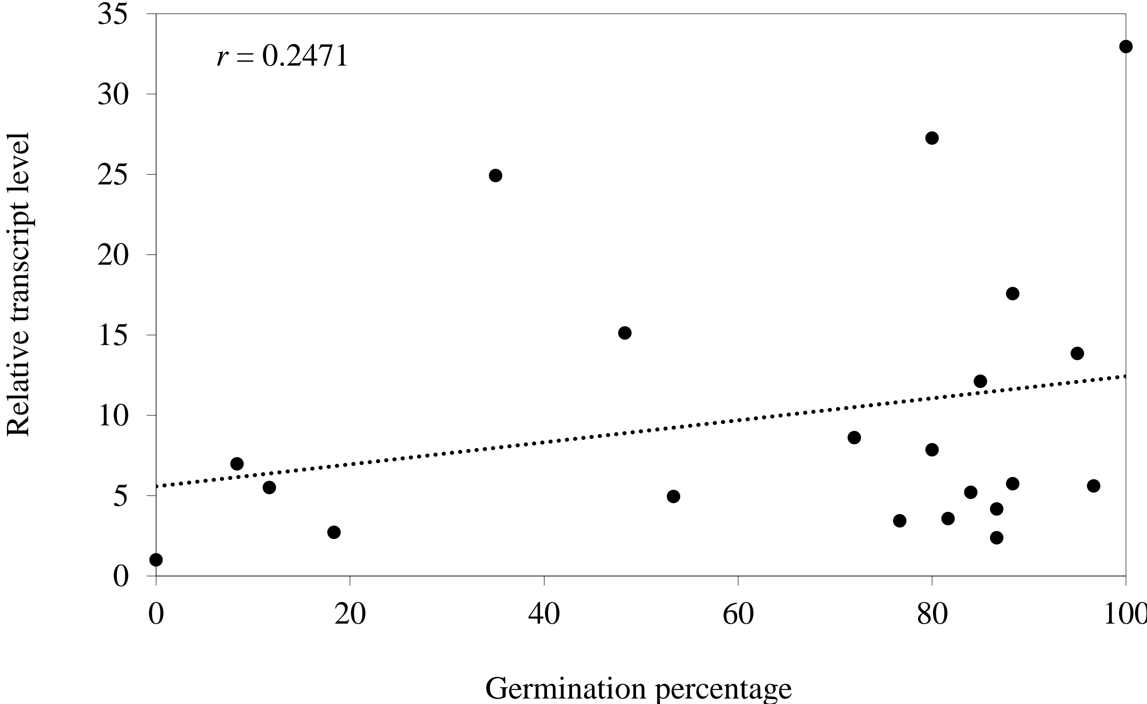


Appendix 10. Analysis of variance of relative transcript levels of *ABA8'OH2* in the embryos of the different barley lines.

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between lines	20	5002.458	250.123	14.417	<0.001
Within lines	41	711.314	17.349		
Total	61	5713.772			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 11. Correlation between seed germination percentage and expression levels of embryonic *ABA8'OH2* of the different barley lines.

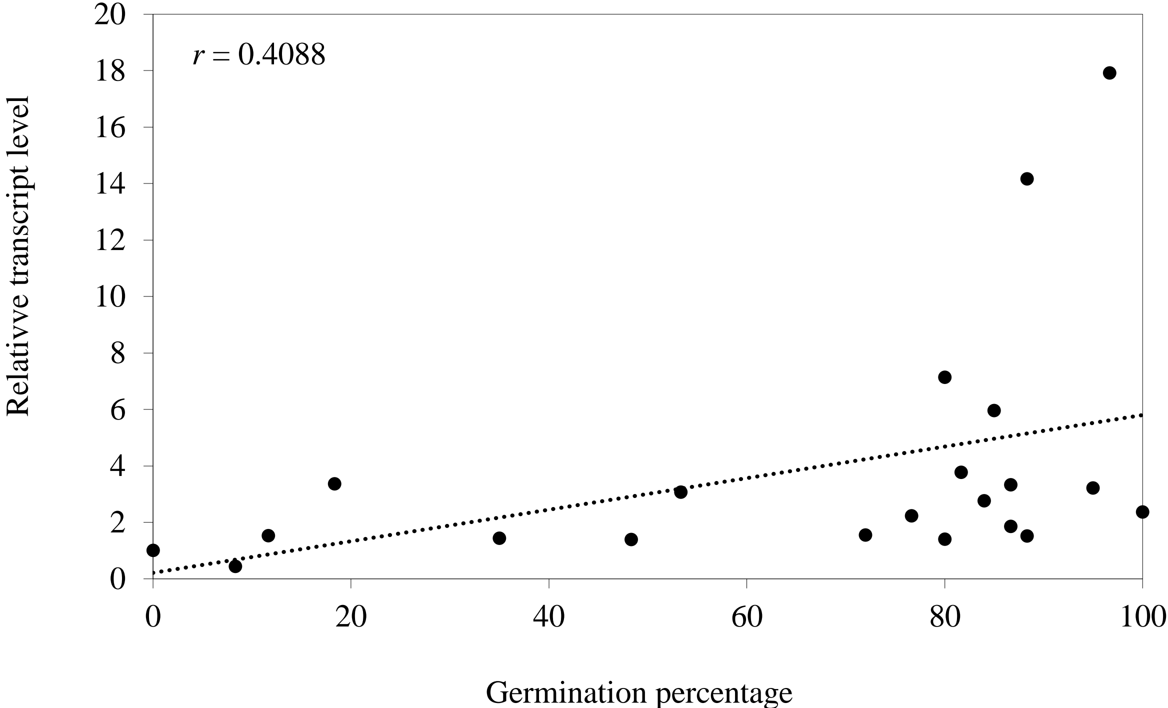


Appendix 12. Analysis of variance of relative transcript levels of *ABA8'OH2* in the endosperm of the different barley lines.

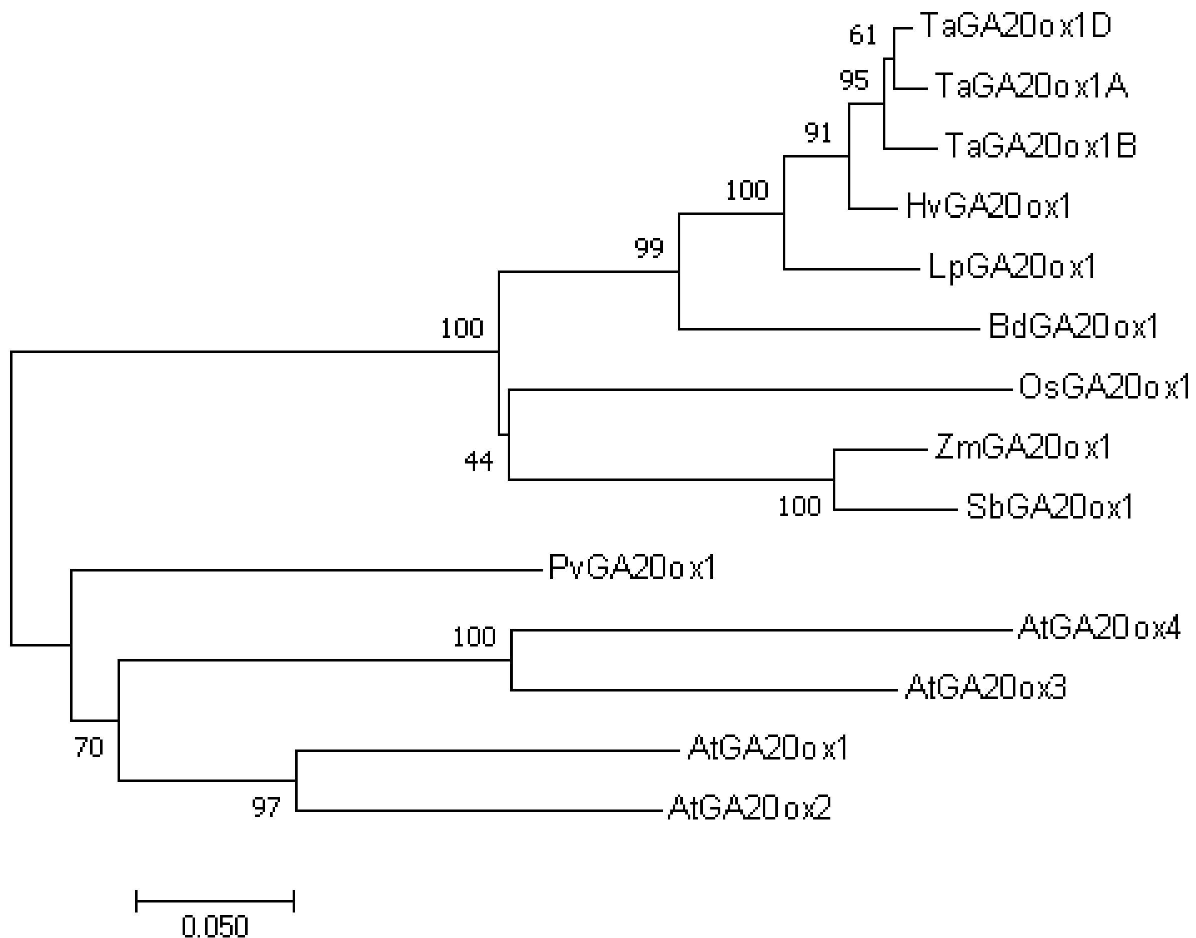
Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between lines	20	1563.495	78.175	48.563	<0.001
Within lines	42	67.610	1.610		
Total	62	1631.104			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 13. Correlation between seed germination percentage and expression levels of endospermic *ABA8'OH2* of the different barley lines.



Appendix 14. Phylogenetic tree of GA20ox proteins from barley and other species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The alignment of the sequences was prepared by Clustal W. This evolutionary history was constructed by the neighbour-joining method in the MEGA 7.0 software. The evolutionary distance was computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (0.050). The aligned protein sequences with their accession numbers are *Triticum aestivum*, TaGA20ox1-A (GenBank ID: CBX45609.1); TaGA20ox1-B (GenBank ID: CBX45610.1); TaGA20ox1-D (GenBank ID: CBX45611.1); *Hordeum vulgare*, GA20ox1 (GenBank ID: AY551428); *Lolium perenne*, LpGA20ox1 (GenBank ID: AAG43043.1); *Brachypodium distachyon*, BdGA20ox1 (GenBank ID: BRADI1G00950); *Oryza sativa*, OsGA20ox1 (GenBank ID: Os03g0856700); *Zea mays*, ZmGA20ox1 (GenBank ID: Zm00001d034898); *Sorghum bicolor*, SbGA20ox1 (GenBank ID: SORBI_3001G005300); *Phaseolus vulgaris*, PvGA20ox1 (GenBank ID: XP_007152362.1); *Arabidopsis thaliana*, AtGA20ox1 (GenBank ID: AT4G25420); AtGA20ox2 (GenBank ID: AT5G51810); At*GA20ox3* (GenBank ID: AT5G07200); AtGA20ox4 (GenBank ID: AT1G60980).

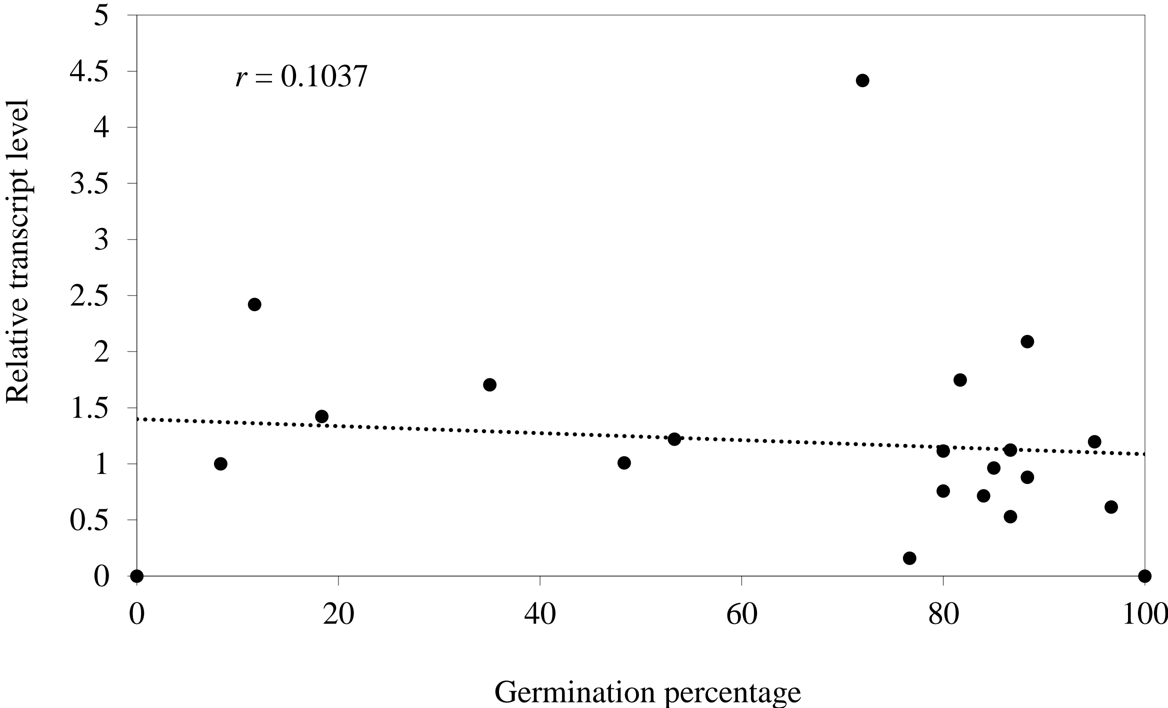


Appendix 15. Analysis of variance of relative transcript levels of *GA20ox2* in the embryo of the different barley lines

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between lines	20	41.375	2.069	3.856	<0.001
Within lines	42	22.534	0.537		
Total	62	63.909			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 16. Correlation between seed germination percentage and expression levels of embryonic *GA20ox2* of the different barley lines.

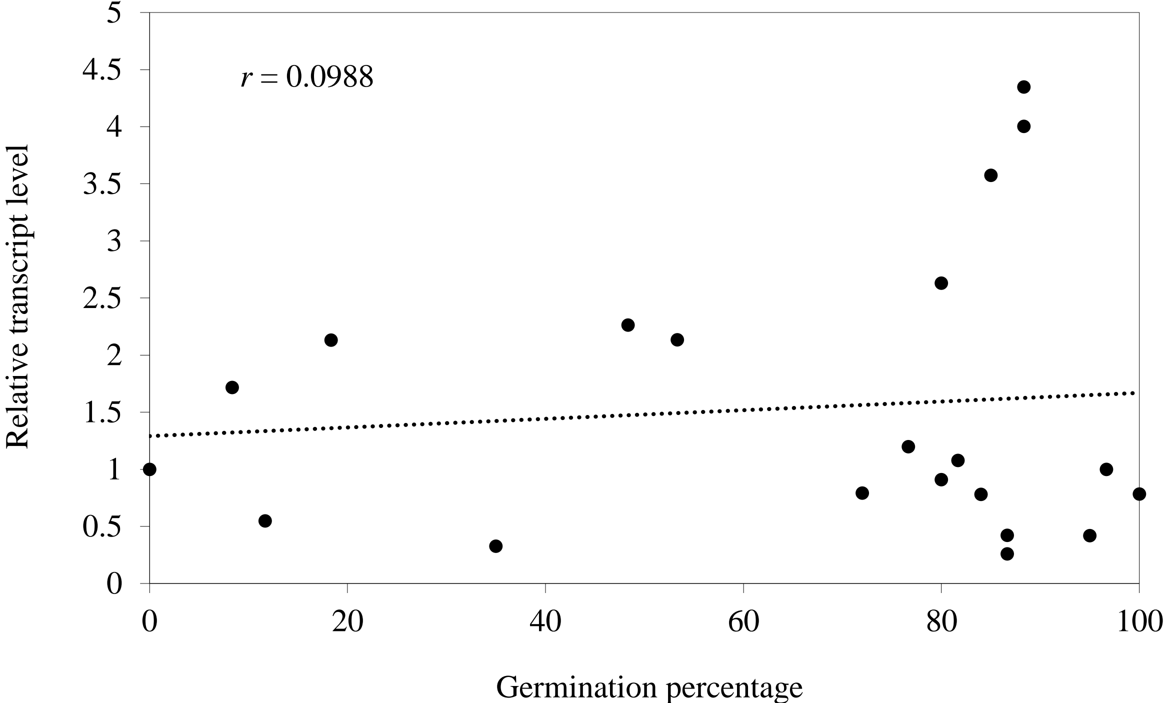


Appendix 17. Analysis of variance of relative transcript levels of *GA20ox3* in the endosperm of the different barley lines.

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of Squares	F value	P value
Between lines	20	84.646	4.232	6.374	<0.001
Within lines	42	27.887	0.664		
Total	62	112.533			

Significant difference was tested with Fisher LSD test at p value of < 0.05.

Appendix 18. Correlation between seed germination percentage and expression levels of endospermic *GA20ox3* of the different barley lines.



ABBREVIATIONS

AAO3	abscisic aldehyde oxidases
ABA	abscisic acid
ABA8′OH	ABA 8′ hydroxylase
ABA-GE	ABA glucosyl ester
ANOVA	analysis of variance
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
cDNA	complimentary deoxyribonucleic acid
CPS	*ent*-copalyl diphosphate synthase
CDP	*ent*-copalyl diphosphate
CTAB	cetyl trimethyl ammonium bromide
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DPA	dihydrophaseic acid
EDTA	ethylene diamine tetraacetic acid
ExPASy	expert protein analysis system
GA	gibberellin
GA20ox	GA 20-oxidase
GA3ox	GA 3-oxidase
GA2ox	GA 2-oxidase

GGDP	geranylgeranyl diphosphate
GGPP	geranylgeranyl pyrophosphate
HCL	hydrochloric acid
HAI	hours after imbibition
INDELS	insertions and deletions
IDP	isopentenyl diphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
KAO	*ent*-kaurenoic acid oxidase
KO	*ent*-kaurene oxidase
KS	*ent*-kaurene synthase
LB	luria-Bertani
LDL	low density lipoprotein
LSD	least significant difference
MEGA	molecular evolutionary genetic analysis
mRNA	messenger RNA
NCBI	national centre for biotechnology information
NCED	nine-*cis*-epoxycarotenoid dioxygenase
NSY	neoxanthin synthase
OECD	organization for economic co-operation and development
PA	phaseic acid
PCR	polymerase chain reaction
PD	physiological dormancy
PHS	preharvest sprouting

PVP	polyvinyl pyrrolidone
qPCR	real time quantitative polymerase chain reaction
QTL	quantitative trait loci
RNA	ribonucleic acid
RVA	rapid visco analysis
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
USDA	united states department of agriculture
UV	ultraviolet
UTRs	untranslated regions
Vp	viviparous
VDE	violaxanthin de-epoxidase
ZEP	zeaxanthin epoxidase