Potential of ABA Antagonists in Promoting Germination of Canola, Chickpea and Soybean Seeds under Low Temperature

A Thesis Submitted to the College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Plant Sciences

Saskatoon

By

Junsheng Zhou

© Copyright Junsheng Zhou, October, 2018. All rights reserved.

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work, or in their absence, by the Head of the Department of Dean of the College in which my thesis was done. It is understood that any copying of publication or use of the thesis, in whole or in part, for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of the material in this thesis in whole or in part should be addressed to:

College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan (S7N 5C9)

Head of the Department of Plant Sciences
University of Saskatchewan
51 Campus Drive
Saskatoon, Saskatchewan (S7N 5A8)

ABSTRACT

Canola (*Brassica napus* L.), soybean (*Glycine max* (L.) Merr.) and chickpea (*Cicer arietinum* L.) are important crops in Canada for their contributions to both the national economy and international markets. However, seed germination of these crops is sensitive to ambient temperature. Cold temperatures in the early spring severely inhibit seed germination, potentially preventing the plant from completing its life cycle within the growing season. One major factor that causes the delay in seed germination is the increased ABA level, which is triggered by cold stress. ABA antagonists, a class of synthetic chemicals, could counteract the effects of ABA and, hence, promote seed germination under low temperature (LT).

The main objective of this study was to identify effective ABA antagonists in promoting germination under LT. ABA 1009 was selected for its significant promoting effect on canola seed germination. ABA 1009 was found to be effective across different canola cultivars and it was able to promote radicle growth. The application of ABA 1009 on canola and soybean seeds during germination counteracted the effects of exogenous ABA application. Hormone analysis was done on canola seeds treated with ABA 1009. The increased amount of ABA metabolites in the seeds indicated up-regulation of ABA catabolism caused by the application of ABA 1009. The increased levels of ABA and ABA 1009 concentrations within the seeds over time indicated that overdosage of ABA 1009 caused an increase in ABA biosynthesis. Hormone analysis of similar experiments in soybean and chickpea seeds suggested that the delay in germination was related to the high ABA levels within the seeds. Gene expression analysis on canola seeds treated with ABA 1009 showed that AAO3, AAO4, NCED5, NCED6, and NCED9 genes were involved in ABA biosynthesis, while CYP707A4 was involved in ABA degradation.

ACKNOWLEDGEMENTS

I would like to thank and express my deepest gratitude to my co-supervisors, Dr. Bunyamin Tar'an, and Dr. Suzanne Abrams and my committee members, Dr. Helen Booker, Dr. Karen Tanino and Dr. Tom Warkentin for their help, support, guidance, and patience. Thank you to Dr. Alison Ferrie of the National Research Council Canada for reviewing this thesis and for serving as the external examiner.

I would like to acknowledge Idralyn Alarcon and Leon Lai for the syntheses of ABA analogs and the preparation of the stock solutions. I am incredibly grateful to Dongyan Song for his assistance and guidance for the experiments. I would like to thank Dr. Sally Vail for providing the canola seeds, and the pulse program of the CDC for providing the chickpea seeds. Finally, I would like to express my appreciation to Dr. Randy Purves for the hormone analysis.

TABLE OF CONTENTS

PERMISSION TO USE i

ABSTRACT ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS....................xii

Chapter 1

1. Introduction....................1

2. General objectives....................4

Chapter 2

2. Literature Review....................5

2.1 Seed Development....................5

2.2 Seed Structure and Composition....................6

2.3 Dormancy....................7

2.4 Genetics of Seed Dormancy....................9

2.5 Germination Process....................10

2.6 GA in the germination process....................11

2.7 GA biosynthesis and catabolism....................12

2.8 ABA in the germination process....................13

2.9 ABA biosynthesis and catabolism....................14

2.10 Agonists and antagonists....................16

2.11 Design of ABA analogs……………………………………………….…………………18

2.12 Hypotheses……….…………………………………………………………...…...….21

Chapter 3

3. Materials and Methods...22

3.1 ABA analogs preparation………………………………….………………...…....22

3.2 Germination assay with ABA antagonists in Canola……………..………………..….....22

3.3 Hormone analysis of Canola treated with ABA antagonists………...……………..….....25

3.4 Gene expression analysis of Canola treated with ABA antagonists……………......28

3.5 Germination assay with ABA 1009 in Soybean………...29

3.6 Hormone analysis of soybean……………...………………………………….………30

3.7 Germination assay with ABA 1009 in chickpea…………...……………….........31

3.8 Hormone analysis of chickpea……………...………………………………….………32

Chapter 4

4. Results and Discussion………………………………………………………….…33

4.1 Germination assay with ABA antagonists in Canola……………………...……....35

4.2 Hormone analysis of Canola treated with ABA antagonists ………………..…...….52

4.3 Gene expression analysis of Canola treated with ABA antagonists………….…...…57

4.4 Germination assay with ABA antagonists in Soybean…………………………...61

4.5 Hormone analysis of soybean………………………………………...………64

4.6 Germination assay with ABA antagonists in chickpea……………...…………….…67

4.7 Hormone analysis of chickpea……………………………...………………..……68

Chapter 5

5. General Discussion ……………………………………………….…………………73

Chapter 6

6. Conclusion…………..………………………………………………..………………81

REFERENCES…………………………………………………………………………..…83

APPENDIX A

Primers information in gene expression analysis…………………………………….…....90

APPENDIX B

Statistical analyses used in germination assays and gene expression study………………....91

LIST OF TABLES

Table 3.1 Transitions and collision energies for MRM analysis……………………………27

Table 3.2 Binary gradient operated at 400 µL/min for UPLC-MRM analysis……..................28

Table 4.1 Standard deviation of abscisic acid (ABA) and its metabolites in the control group (treated with water at 5°C) in hormone analysis of canola……………………….…………53

Table 4.2 Standard deviation of abscisic acid (ABA) and its metabolites in the treatment group (treated with ABA 1009 at 5°C) in hormone analysis of canola………………..………..….55

Table 4.3 Standard deviation of abscisic acid (ABA) and its metabolites in room temperature treated group (22°C) in hormone analysis of chickpea…………………………..…...….70

Table 4.4 Standard deviation of abscisic acid (ABA) and its metabolites in low temperature treated group (7°C) in hormone analysis of chickpea…………………………..………..…72

LIST OF FIGURES

Figure 2.1 The biosynthesis of gibberellins via the terpenoid pathway. GGDP, geranylgeranyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase……………………………….………13

Figure 2.2 The biosynthesis of abscisic acid, starting from the enzymatic cleavage of C40-carotenoid (from Boyd et al. 2009) ………………………………………………14

Figure 2.3 Four abscisic acid degradation pathways and their corresponding metabolites. 7'-OH_ABA,7'-hydroxyabscisic acid; PA, phaseic acid; DPA, dihydrophaseic acid; neo_PA, 9'-hydroxyabscisic acid, ABA_GE, abscisic acid- glucose ester……………………………15

Figure 2.4 ABA antagonists affect the ABA signaling pathway by interfering PYL-PP2C binding. PYL, Pyrabactin resistance1-like ABA receptor; PP2C, Protein phosphatase 2C (from Hayashi and Kinoshita 2014)………………………………………………………………18

Figure 2.5 Synthesis of ASn (the simple ABA analogs that have a chain at C-3') (from Takeuchi et al. 2014) ……………………………………………………………………………19

Figure 2.6 Synthesis of PAOn (The bicyclic tetralone ABA analogs that have two rings fused together) (from Takeuchi et al. 2014)…………………………………….………………...20

Figure 4.1 The structures of ABA analogs screened in this research project ABA 1001, ABA 1002, ABA 1004, ABA 1005, ABA 1006, ABA 1007, ABA 1008, ABA 1009, ABA 1010, ABA 1011 and ABA 1012…………………………….…………………..…………...34

Figure 4.2 Percent germination of canola seeds treated with different ABA antagonists (ABA 1001-ABA 1009) at 100 µM at 5°C (December 2015). ABA 1001 and ABA 1009 had significantly higher percent germination compared to LT control (water with DMSO) ……35

Figure 4.3 Percent germination of stratified canola seeds treated with ABA 1009, ABA 1011 and ABA 1012 at 22°C (November 2016). ABA 1009 was more efficient in counteracting the effect of exogenous ABA……………………………………………….………………39

Figure 4.4 Percent germination of canola seeds treated with ABA 1009 at 300 µM and control (1%DMSO) at 5°C conditions (July 2017). ABA 1009 treated seeds had higher percent germination. Most seeds germinated in 10 days……………………………..………..…...41

Figure 4.5 Percent germination of canola seeds treated with ABA 1009 at 100 µM and control (1%DMSO) at 5°C conditions (January 2017). ABA1009 treated seeds had higher percent germination. Most seeds germinated in 18 days………………………………………..42

Figure 4.6 Percent germination of canola seeds treated with ABA, ABA+ ABA 1009 at 22°C

(February 2017). ABA 1009 was effective in counteracting exogenous AB…………………42

Figure 4.7 Percent germination of canola seeds treated with ABA 1009 at 100 μM, 300 μM and 500 μM at 22°C (April 2017). ABA 1009 at 300 μM had higher percent germination at 24 hours…………..……………………………………………………………………….….44

Figure 4.8 Percent germination of canola seeds treated with ABA and ABA + ABA 1009 in the pouches at 22°C (September 2016). ABA 1009 was effective in counteracting exogenous ABA……………………………………………………………………………………45

Figure 4.9 Radicle growth of control seeds on day 5. Seeds were incubated in pouches under RT (22°C). Eighteen out of twenty-five seedlings had a radicle that was longer than 2.5 cm……………………………………………………..…………………….………..46

Figure 4.10 Radicle growth of ABA 1009-treated seeds on day 5. Seeds were incubated in pouches under RT (22°C). Thirteen out of twenty-five seedlings had a radicle that was longer than 2.5 cm…………………………………………………………………………….47

Figure 4.11 Radicle growth of ABA-treated seeds on day 5. Seeds were incubated in pouches under RT (22°C). Six out of twenty-five seedlings had a radicle that was longer than 2.5 cm…………………………..…………………………………….……………………..47

Figure 4.12 Radicle growth of ABA + ABA 1009 treated seeds on day 5. Seeds were incubated in pouches under RT (22°C). Thirteen out of twenty-five seedlings had a radicle that was longer than 2.5 cm………………………………………………..……………..………48

Figure 4.13 a. Percent germination of NAM- 47 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **b**. Percent germination of NAM- 49 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **c**. Percent germination of NAM- 0 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **d**. Percent germination of NAM- 72 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **e**. Percent germination of NAM- 79 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions (December 2017). For all cultivars listed above, ABA 1009-treated seeds had higher percent germination…..50

Figure 4.14 Changes in ABA metabolite concentrations in the seeds of *Brassica napus* (canola) from day 0 to day 9 in the control group (water at 5°C). ABA and its metabolites decreased over time. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid………………………………………………………………………...……………53

Figure 4.15 The application of ABA 1009 at 300μM to *Brassica napus* (canola) at LT (5°C) caused changes in ABA metabolite concentrations. ABA level increased over time. DPA decreased at first 6 days and increased 3 fold on day 9. ABA, abscisic acid; DPA,

dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.……………………………………54

Figure 4.16 Change in ABA 1009 concentrations from day 0 to day 9 in the treatment group. Seeds were treated with ABA 1009 at 300 µM and incubated at LT (5°C). ABA 1009 concentrations increased linearly over time.……..……………………………………..….56

Figure 4.17 Fold changes of the selected genes from the AAO gene family and NCED gene family in canola seeds treated with ABA 1009. Selected genes had different extent of up-regulation……...…………………………………………………………..…………59

Figure 4.18 Fold changes of selected genes from CYP707A gene family from day 0 to day 9. The expression level of CYP707A4 had a significant increase from day 3…………………..60

Figure 4.19 Percent germination of soybean seeds treated with ABA, ABA+ABA 1009 at 22°C.ABA 1009 was effective in counteracting exogenous ABA…….………………..……..62

Figure 4.20 Percent germination of soybean seeds treated with ABA 1009 at 100 µM, control (1% DMSO) at 7°C. ABA 1009 treated seeds had similar percent germination as control…………………………………………………………………….………………..63

Figure 4.21 Changes in ABA metabolite concentrations of *Glycine max* (soybean) seeds cultured at 22°C. ABA level decreased at day 1 and increased on day 2. All metabolites had different extent of decrease……………………………………...……………………....65

Figure 4.22 Changes in ABA metabolite concentrations of *Glycine max* (soybean) seeds cultured at 7°C. ABA and its metabolites (except for 7OH-ABA) decreased over time………...…………………………………………………………………………………66

Figure 4.23 Percent germination of chickpea seeds treated with ABA, ABA+ ABA 1009 at 22°C. ABA 1009 failed to counteract exogenous ABA since there were similar percent germination for ABA and ABA+ ABA 1009 treated groups…………………...…………...…67

Figure 4.24 Percent germination of chickpea seeds treated with ABA 1009 at 100 µM and control (1% DMSO) at 7°C. The percent germination of ABA 1009-treated seeds was not significantly different from the control group………………………………...…………….68

Figure 4.25 Changes in ABA metabolite concentrations of *Cicer arietinum* (chickpea) seeds cultured at 22°C. ABA and its metabolites (except for ABA_GE) decreased from day 1 to day 2…………………………………………………………………………………………69

Figure 4.26 Changes in ABA metabolite concentrations of *Cicer arietinum* (chickpea) seeds cultured at 7°C. There were sharp decreases from day 0 to day 3 for ABA, PA and DPA. Their

concentrations fluctuated from day 3 to day 12. ABA_GE level increased over time while 7OH_ABA level increase at first 6 days and decreased afterward……………………………71

LIST OF ABBREVIATIONS

ABA	abscisic acid
GA	gibberellic acid
LT	low temperature
RT	room temperature
PYL	pyrabactin resistance1-like ABA receptor
PP2C	protein phosphatase 2C
QTL	quantitative trait locus
NCED	9-cis-epoxycarotenoid dioxygenase
CYPs	Cytochromes P450
AAO	Arabidopsis aldehyde oxidase

Chapter 1. Introduction

In western Canada, long sunny days and suitable soil conditions contribute to the rapid growth of canola (*Brassica napus*), chickpea (*Cicer arietinum*) and soybean (*Glycine max*), while the short growing season may impede their yield and quality. Due to its circumpolar latitude, Canada's growing regions have relatively short growing seasons with low temperatures (LT) in early spring (Morrison 2015). Seed germination and emergence are greatly impeded by LT and, therefore, canola and soybean seeding on average will not start until mid-May when both field conditions and temperature are favorable (Canola Council of Canada, 2013). Despite the longitudinal variations, most of the growing regions in western Canada suffer their first fall frost at the beginning of September (Canola Council of Canada, 2011). Hence, the growing season for most crops including canola, chickpea and soybean is less than four months, which greatly limits the crops to express their full growth potential. By extending the growing season of these crops, there is a potential to gain higher yield and better quality. One possible way to achieve this goal is by shifting the seeding time to an earlier date.

Several studies have pointed out the advantages of early seeding in different crops (Kirkland and Johnson 2000; Jasa 2009; Grassini et al. 2016; Lindsey 2017). In the case of canola, early seeded plants have a higher chance to flower before peak summer heat, preventing damage from flower blast and pod abortion than the normal seeded plants (Canola Council of Canada 2013). Early seeded canola also tends to mature earlier, which could reduce premature senescence and avoid damage from fall frost. However, germination rates in early spring are often significantly reduced due to the potential effects of abscisic acid (ABA). ABA, a major inhibitor associated with seed germination, is upregulated when seeds are subjected to a lower

temperature than ambient (Bewley et al. 2013). The increase of ABA level caused by cold helps to maintain seed dormancy.

ABA antagonists that function against the effect of ABA might be a solution to break the dormancy and to promote seed germination in early spring. An ABA antagonist is a type of ABA analog, which is structurally similar to the ABA molecule, but with slight modifications (Takeuchi et al. 2014). The modifications on ABA analogs interfere with the binding of PYL protein and Type 2C protein phosphatases (PP2C), which subsequently regulate the ABA signaling pathway (Takeuchi et al. 2014).

Understanding the hormonal changes and the gene expression patterns during seed germination at LT is essential since the germination process is mediated by hormones and the underlying genes. Plant hormones are produced in minute amounts. However, they play crucial regulatory roles in all aspects of plant development (Helgi et al. 2005). More than ten types of hormones were identified in recent research and each of them has a unique regulatory role at different stages of development (Dilworth et al. 2017). ABA and GA are the two most important hormones involved in the regulation of seed germination (Black et al. 2007). The GA/ABA ratio determines the fate of a seed. Under favorable conditions, the GA biogenesis is enhanced and ABA biosynthesis is impaired, leading to an increase of GA/ABA ratio and the initiation of seed germination (Black et al. 2007). The GA/ABA ratio also plays a significant role in regulating seed germination under LT. The ABA catabolic pathways, as well as their rates, change under the cold stress (Zhang et al. 2010). The GA accumulation is impaired by ABA under LT condition, resulting in a decrease of GA level. Revealing the activities of ABA/GA metabolism under LT and under ABA antagonist treatment could not only help to distinguish

the regulatory roles of these hormones, but could also improve the designs of future ABA antagonists.

Gene expression variation in response to different conditions can mediate the state of the seeds. For those genes that encode enzymes and proteins involved in catabolism and biosynthesis of ABA and GA, their regulation is affected by the balance of ABA/GA within the seed and, therefore, determines whether the seed can germinate or not. For the model plant, Arabidopsis, the association between its gene expression and seed germination is well documented (Bassel et al. 2011). As for canola, chickpea and soybean, there is still a lot of effort needed to examine the association between gene expression patterns and seed germination (Hatzig et al. 2015). Furthermore, there are limited studies on gene expression during germination under LT. Understanding these gene expression patterns could be beneficial in many aspects. This gene expression study could help to reveal the mechanism of the delay in germination caused by cold stress. It could also help to identify key enzymes and proteins involved in imposed dormancy and delayed germination in canola, chickpea, and soybean. Moreover, information acquired from this study can help to develop an application to promote seed germination under LT, and to develop highly effective new ABA antagonists.

General objectives

The main objectives of this research were:

1. To select the most effective analog from a set of ABA antagonists on canola seed germination, and to test the selected analog on chickpea and soybean seed germination.
2. To identify the optimum concentration of the most potent ABA antagonist on canola seed germination at low temperature.
3. To examine the hormonal levels and the gene expression patterns in canola seeds during germination under low and normal temperatures, and under ABA antagonist treatment.
4. To examine the hormonal levels in soybean and chickpea seeds during germination under low and normal temperatures.

Chapter 2. Literature review

Seed Development

In angiosperms, seed development is initiated by double fertilization. It begins with the release of two male gametes (sperm) from the pollen grain to megagametophyte (female gametophyte) (Gailili and Kigel 1995). This is followed by the joining of the egg cell with sperm nuclei and the fusion of the polar nuclei and the other sperm cell nucleus (Bewley and Black 1994). After double fertilization, embryogenesis occurs, giving rise to two different structures: embryo and endosperm. Development of the embryo and endosperm is regulated by specific genes that are derived from the haploid male, female gamete and the diploid maternal plant.

Based on the types of cell activities, seed development can be divided into three stages: histodifferentiation (phase 1), expansion (phase 2) and maturation drying (phase 3) (Sinniah et al. 1998). In phase 1, cells undergo extensive division and form into different tissue types within the embryo and endosperm. There is a large increase in fresh weight and water content during this stage due to the extensive cell division (Sinniah et al. 1998). In phase 2, the rate of cell division decreases rapidly. Cells begin to enlarge followed by storage reserve deposition. Insoluble stored reserves such as oils, starch and storage protein are synthesized in this stage, replacing the water within the seeds (Galili and Kigel 1995). Hence the increase of dry weight due to the deposition of storage reserves is in proportion to the loss of water in the expansion stage. Maturation drying is the final phase of the seed development in most plants, which results in the loss of water content and fresh weight (Galili and Kigel 1995). Seeds are disconnected from their parent plant in this stage and become desiccation tolerant.

Seed development is regulated by hormones such as ABA and GAs. For many species, their immature seeds have high levels of ABA. ABA concentration in soybean ranged between 2 and 5 mg/kg fresh weight and 0.1 to 1 mg/kg in other crops (Bewley et al. 2013). As seeds develop, the level of ABA increases. In some cases, the increase of ABA content is parallel to the gain in dry weight (Rudall 2007). High level of ABA within the seeds helps to prevent precocious germination and deposit storage reserve. As seeds begin to mature, there is a rapid decrease in ABA. Upon maturation, only a small amount of ABA is left in mature seeds (Rudall 2007). The remaining ABA will then contribute to the acquisition of desiccation tolerance and imposition of dormancy (Bewley et al. 2013). GAs are also involved in the control of seed development. Most of them remain at low concentration during the development process but they play key roles in seed development and mobilization of stored reserves (Camara et al. 2015).

Seed Structure and Composition

The basic composition of angiosperm seeds includes embryo, endosperm and seed coat (Black et al. 2007). The embryo, located at the lower center of the seed, is a small embryonic plant which will grow under suitable environments. The embryo consists of five constituents with distinct functions: (1) the cotyledons are the future seed leaves, (2) epicotyl is the embryonic shoot above the point of the cotyledon attachment, (3) plumule will be the primary bud located on the tip of epicotyl, (4) hypocotyl will be the stem of seedling located in-between the cotyledon and radicle, (5) radicle is the embryonic root found at the base of the hypocotyl (Black et al. 2007). Endosperm is a large nutritive tissue derived from the triple fusion of two polar nuclei and one haploid sperm. It serves as a nutrient storage for the germinating seed (Galili and Kigel 1995). Both embryo and endosperm are enclosed by seed coat, which is

derived from the outer integument (Bewley and Black 1994). The thickness of the seed coat varies among plants. It functions as a protective layer to prevent the embryo from physical injury.

Dormancy

Dormancy is a mechanism to ensure the successful establishment of the seedling by preventing seed germination under unfavorable environments (Black et al. 2007). There are two types of dormancy, innate dormancy and secondary dormancy. Depending on the causes, innate dormancy can be divided into two categories, exogenous dormancy, caused by conditions outside the embryo, and endogenous dormancy, caused by conditions within the embryo (Baskin and Baskin 1998).

There are three subgroups in exogenous dormancy: physical, mechanical and chemical dormancy (Fenner and Thompson 2005). Seeds with physical dormancy have impermeable seed coat that prevents any uptake of water or gases (Baskin et al. 2000). Physical dormancy can be broken by specific abiotic cues such as high temperatures and fire. Mechanical dormancy is caused by a thick seed coat, which inhibits the embryo to grow during germination (Black et al. 2007). Chemical dormancy is caused by the germination inhibitors around the embryo (Fenner and Thompson 2005). As the seeds receive enough water from rainfall or snowmelt, the chemical left within the seeds will be washed out and the chemical dormancy will be broken down.

Endogenous dormancy also has three subgroups: physiological, morphological and combined dormancy (Baskin and Baskin 2004). Physiological dormancy is caused by the inhibiting chemical within the embryo, which prevents embryo growth (Fenner and Thompson

2005). ABA is one of the common germination inhibitors in seeds. The breakdown of physiological dormancy depends on the decrease of inhibiting chemicals. Morphological dormancy is due to the immaturity of the embryo at the time of fruit ripening (Baskin and Baskin 2004). The underdeveloped seeds remain dormant before they mature. Combined dormancy is a combination of both physiological and morphological dormancy.

Some non-dormant and post dormant seeds will undergo secondary dormancy when they face unsuitable environments (Bewley and Black 1994). Secondary dormancy ensures seeds to germinate under favorable conditions and optimizes the survival of the seedling.

Some species exhibit seasonal variation and periodicity in their dormancy status. The presence of periodicity or dormancy cycle not only maintains cell viability for a long period but also synchronizes the dormancy status of the seeds with the environment to optimize their chance to germinate under favorable conditions (Bewley et al. 2013). Spotted lady's thumb (*Polygonum persicaria*) is a summer annual that shows a dormancy cycle (Bewley et al. 2013). In summer, the high temperatures reduce germination potential and trigger secondary dormancy of the seeds. Over the course of the winter, the secondary dormancy is broken gradually due to the release of abiotic stress. Seeds return to the non-dormant state in spring.

As for the crops used in this study, canola seeds (*Brassica napus* L) will undergo non-deep physiological dormancy and exhibit seasonal periodicity in their dormancy states (Baskin and Baskin 1998). Mature soybean seeds (*Glycine max* (L.) Merr.) show physical dormancy and the extent of dormancy is correlated positively with water permeability of its seed coat (Qutob et al. 2008). Chickpea seeds (*Cicer arietinum* L.) exhibit both physical and physiological dormancy (Frisbee et al. 1988).

Genetics of Seed Dormancy

Dormancy is generally considered a quantitative trait controlled by multiple loci (Alonso-Blanco et al. 2003). Genetic investigation of dormancy on the model plant Arabidopsis (*Arabidopsis thaliana*) revealed the quantitative trait loci and the underlying genes (Nakabayashi et al., 2005; Cadman et al., 2006). Alonso-Blanco et al. (2003) conducted a QTL analysis with the use of a recombinant inbred line population derived from Ler (low dormancy)/Cvi (high dormancy) cross. They found seven dormancy QTLs, which account for 61% of Ler/ Cvi phenotypic variation. These QTLs, named as delay of germination 1-7 (DOG 1-7), are closely located in the genetic map of 5 linkage groups. Among these dormancy QTLs, DOG 1 to DOG 3 explain more than 30% of the phenotypic variations. Studies of DOG 1 in different plants suggested that DOG 1 gene is highly conserved (Huo et al. 2016). Mutation in DOG 1 led to complete abolition of seed dormancy since its encoding protein might function as a transcriptional regulator involved in the alteration of GA metabolism (Graeber et al. 2014). The function of DOG 1 is likely to be independent of ABA. However, the maintenance of seed dormancy requires the presence of both ABA and DOG 1 (Kendall et al. 2011). Insufficient amounts of any of these two regulators will result in loss of dormancy, suggesting a convergence of DOG 1 and ABA signaling pathways at downstream step (Kendall et al. 2011).

There are other genes associated with seed dormancy besides DOG. The 9-cis epoxycarotenoid dioxygenase gene family (NCED), the ABA 8′-hydroxylase gene family (CYP707A) and the ABA insensitive (ABI) gene family play critical roles in regulating the dormancy status of Arabidopsis seeds (Millar et al. 2006). Genes from NCED family are involved in ABA biosynthesis during dormant state. They encode protein 9-cis

epoxycarotenoid dioxygenase, key enzymes catalyzing the first committed step of ABA biosynthesis (Schwartz et al. 1997). The expression of NCED genes is directly correlated to the ABA level, indicating a strong regulatory role of NCED. However, ABA level is not only determined by NCED, but also by a gene that encodes zeaxanthin epoxidase (ZEP) (Frey et al. 1999). The overexpression study of ZEP showed an increase of ABA level resulting in imposed dormancy. CYP707A genes also have impacts on seed dormancy since they are involved in the regulation of ABA degradation. A heterologous expression study in yeast showed that CYP707A1- CYP707A4 encode ABA 8′-hydroxylase, a crucial enzyme that catalyzes the hydroxylation at the 8′-position of ABA (Kushiro et al. 2004). ABI genes were identified from genetic screening of ABA insensitive Arabidopsis mutants (Koornneef et al. 1984). These genes are involved in encoding essential components in ABA signal transduction. The encoding protein of ABI 1 and ABI 2 is Group A protein phosphatases type 2C (PP2Cs), which is an important negative regulator in ABA signaling pathway (Leung et al. 1997). Other ABI genes, such as ABI 3, ABI 4 and ABI 5, encode essential transcription factors (Finkelstein and Lynch 2000). Among which, bZIP transcription factor, encoded by ABI 5, functions specifically in seed germination and it plays a key role in monitoring environmental conditions (Lopez-Molina et al. 2001).

Germination Process

Germination is the growth of a plant from a seed after a period of dormancy, which is governed by surrounding environments (Raven et al. 2005). External factors like temperature, water, and light are crucial to determine whether a seed will germinate, and the rate of the germination (Raven et al. 2005). When favorable conditions occur, seed germination begins.

Availability of water is essential for seed germination since the uptake of water in seeds comes along with the activation of the metabolic activity (Bewley et al. 2013). There are two phases of water uptake during germination: imbibition (phase 1) and plateau phase (phase 2) (Rudall 2007). Seeds in imbibition phase continuously absorb water from ambient, resulting in growing seed size. Respiration and protein synthesis with the use of extant mRNAs commence in this phase, followed by mitochondria and DNA repair (Bewley et al. 2013). As the uptake of water increases, the seeds swell to their maximum size and enter to plateau phase. Metabolic activities such as mitochondria synthesis and protein synthesis using new mRNAs occur during this phase (Rudall 2007). The uptake of water up-regulates metabolic level within the seeds, which accelerates the process of radicle extension growth as well as covering layer weakening (Bewley et al. 2013). Eventually, the continuously growing radicle breaks the restraint of the covering layer, indicating the completion of germination.

When ambient conditions are unfavorable, the mobilization of cotyledonary starch will be reduced due to decreased amylase activity, which consequently results in reduced formation of glucose and sucrose (Kaur et al. 1998). The embryo fails to overcome the resistance of the covering layer due to limited energy supplement. Seeds will remain either or both embryo and coat dormant until they sense the suitable conditions.

GA in the germination process

GAs, as a group of promotive hormones, are involved in the process of releasing dormancy and seed germination. The application of GAs to GA-deficient mutant restores full germination of the seeds (Groot and Karssen 1987). GAs push germination forward by inducing the synthesis of α-amylase which initiates starch hydrolysis (Hedden 2014). Glucose from starch

hydrolysis serves as energy resources for the embryo and hence promotes seed germination (Hedden 2014). Meanwhile, GA plays an important role in radicle protrusion and testa rupture by regulating proteins in the later stage of germination (Hilhorst and Karssen 1992). Another study suggested that GA participates in cell cycle activity in early germination (Gallardo et al. 2002).

GA biosynthesis pathway

The biosynthesis of gibberellins is initiated in the plastid, where trans-geranylgeranyl diphosphate (GGDP) is converted into ent-kaurene by two terpene synthases, ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS) as shown in Fig. 2.1 (Gupta and Chakrabarty 2013). Ent-kaurene is subsequently oxidized by ent-Kaurene oxidase (KO) and ent-kaurenoic acid oxidase (KAO) in the outer membrane of the plastid and endoplasmic reticulum (Nelson et al. 2004). After these oxidations, ent-kaurene is converted to GA_{12}. Then, GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) catalyze the oxidation of C-20 and C-3 of GA_{12}. GA_{12} is then processed to become bioactive GA_4.

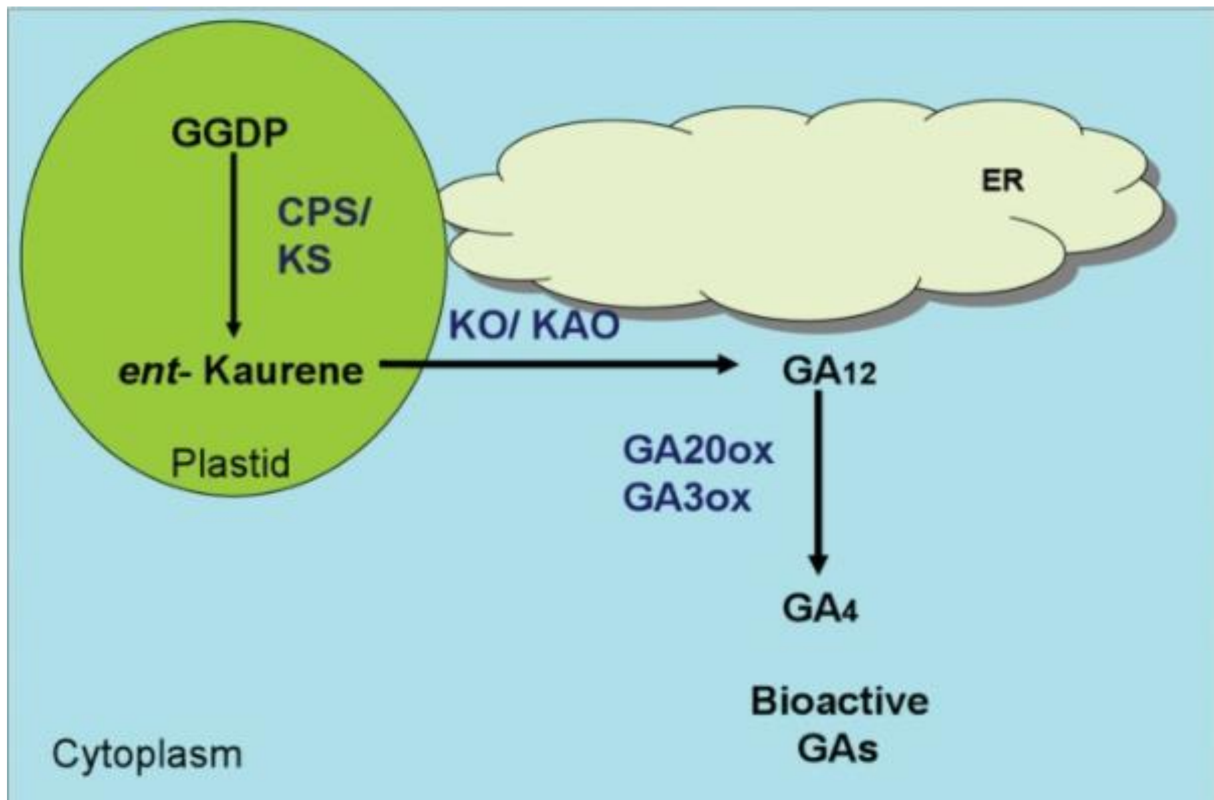


Figure 2.1 The biosynthesis of gibberellins via the terpenoid pathway. GGDP, geranylgeranyl diphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase (from Gupta and Chakrabarty 2013).

GA catabolism pathway

Bioactive GA_s can be converted to inactive forms by 2β-hydroxylation, GA 2-oxidases (GA2ox) is involved in this deactivation pathway. In rice, active GA_s can be deactivated through epoxidation of non-13-hydroxylated GAs (Zhu et al. 2006). Bioactive GA_s is converted to 16α,17-epoxy GA by P450 enzymes. In Arabidopsis, methylation is one of the GA deactivation pathways in germinating seeds, which is catalyzed by GA methyltransferases (GAMT1 and GAMT2) (Varbanova et al. 2007).

ABA in the germination process

ABA, an antagonist to the function of GAs， is an inhibitory hormone produced when plants respond to stress conditions (Bewley and Black 1994; Kucera et al. 2005). Elevated

levels of ABA in seeds will cause the delay in germination or dormancy (Nambara and Marion-Poll 2003). A study of exogenous ABA indicated that applying extra ABA resulted in enhanced dormancy (Nambara and Marion-Poll 2003). ABA suppresses water absorption of seeds in imbibition phase and impedes the embryo from entering the subsequent growth phase (Schopfer and Plachy 1984).

ABA biosynthesis pathway

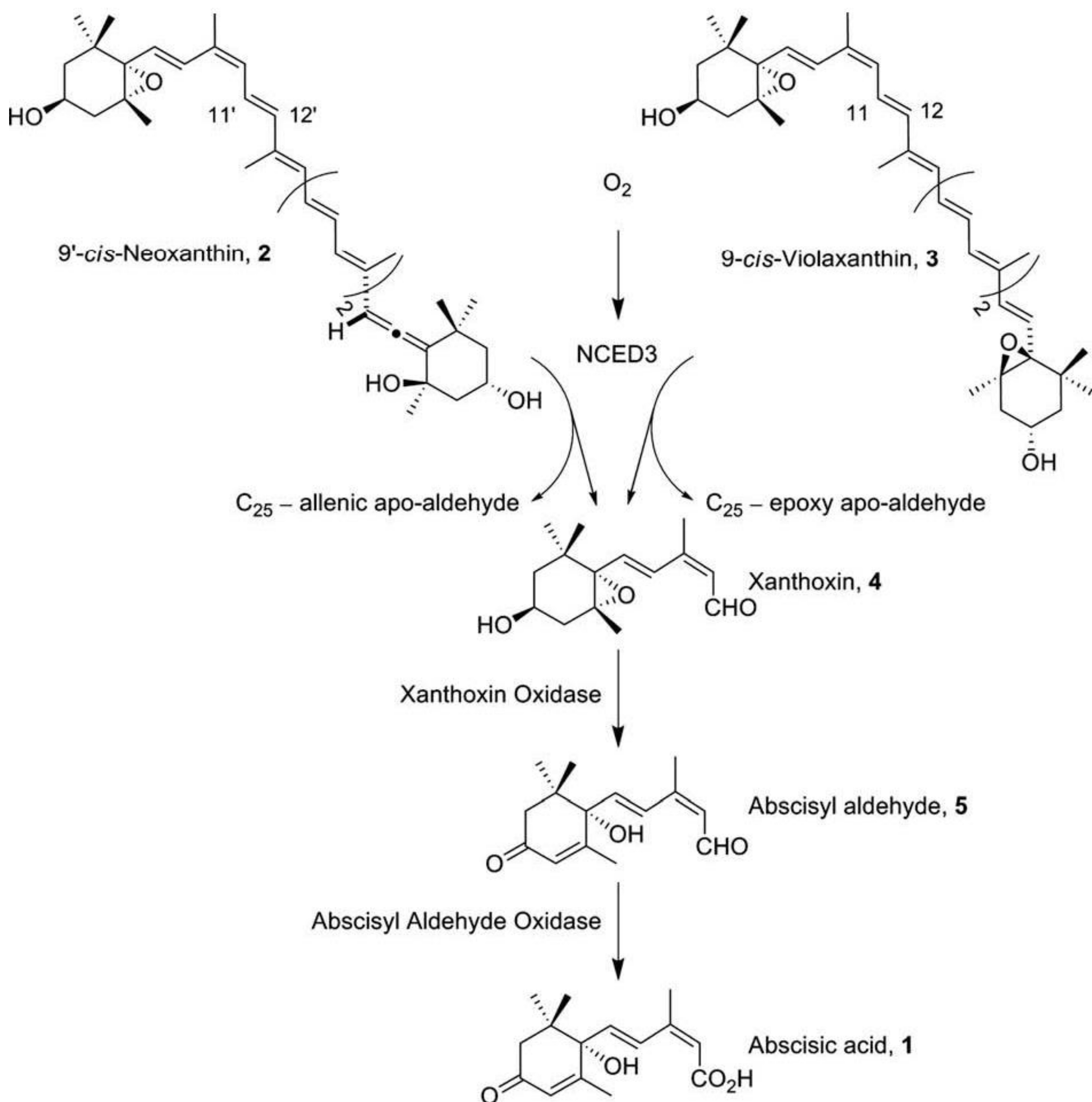


Figure 2.2 The biosynthesis of ABA starting from the enzymatic cleavage of C40-carotenoid (from Boyd et al. 2009).

As shown in Fig.2.2, the biosynthesis of ABA occurs through a series of oxidative steps. 9'-cis-neoxanthin or 9-cis-violaxanthin are converted to xanthoxin by a 9-cis-epoxycarotenoid dioxygenase (NCED) at the first step (Milborrow 2001). Then, an alcohol dehydrogenase (ABA2) catalyzes the oxidation of xanthoxin and converts it to abscisyl aldehyde. Abscisyl aldehyde is subsequently oxidized by abscisic aldehyde oxidase (AAO3) and processed to ABA (Boyd et al. 2009).

ABA catabolism pathway

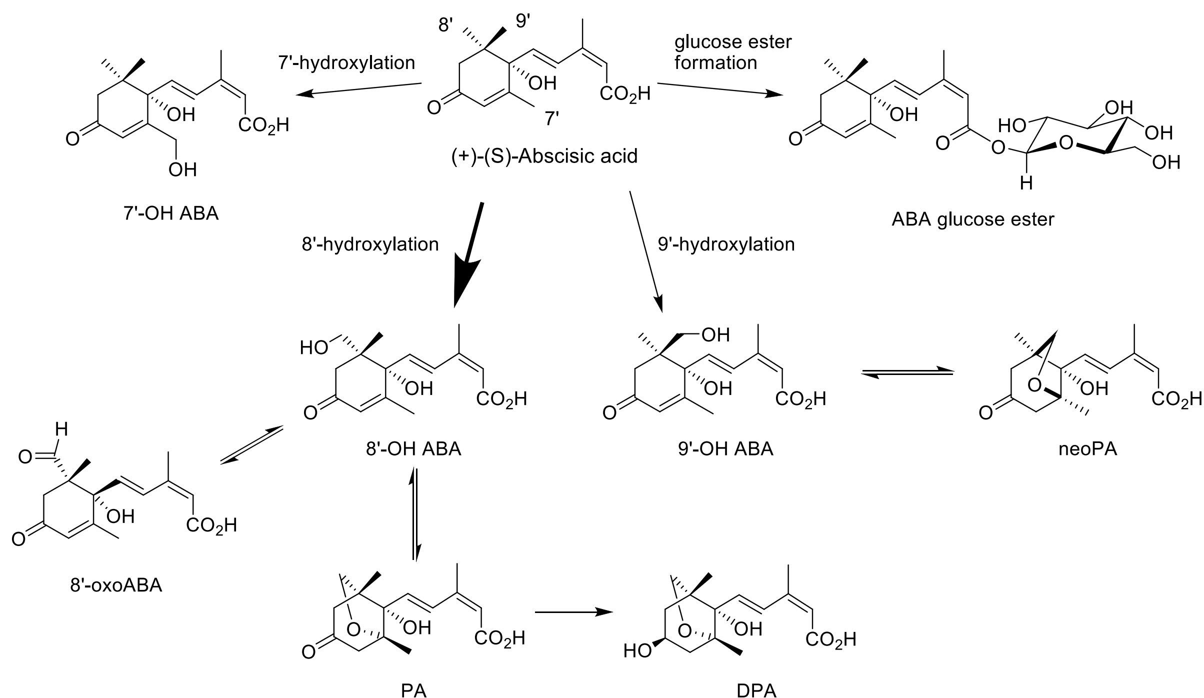


Figure 2.3 Four ABA degradation pathways and their corresponding metabolites. 7OH_ABA,7'-hydroxyabscisic acid; DPA, dihydrophaseic acid; neo_PA, 9'-hydroxyabscisic acid, ABA_GE, abscisic acid- glucose ester.

There are four ABA catabolism pathways in plants (Fig. 2.3). The major pathway is 8-hydroxylation. In this pathway, hydroxylation occurs at the at the 8′-position of ABA. 8'-OH ABA is converted to phaseic acid (PA). The reduction of PA leads to the further conversion of

PA to dihydrophaseic acid (DPA) (Zou et al. 1995). 7’- hydroxylation is another catabolism pathway, where ABA is converted to 7’-OH-ABA (Hampson et al. 1992). An additional pathway occurs through 9-hydroxylation, ABA is first converted to 9’-OH-ABA. The instability of 9’-OH-ABA will cause a structural change, resulting in the formation of neo-PA (Zhou et al. 2004). The inactivation of ABA can be achieved by glucose ester formation, where ABA is conjugated with glucose to form ABA-glucose ester (Xu et al. 2002).

Agonists and antagonists

ABA analogs are synthetic chemicals having similar structures to ABA (Huang et al. 2007). Based on the different functions, ABA analogs can be divided into two categories: agonists and antagonists. The most potent ABA agonists have similar functions as ABA but with higher efficacy and long-lasting effect due to the structural modifications. Application of agonists during seed germination will cause more severe inhibition compared to ABA. ABA antagonists, on the other hand, function against ABA. Applying antagonists could accelerate the release of seed dormancy (Takeuchi et al. 2015).

The functions of ABA analogs mainly come from their interaction with PYR/PYL/RCAR (PYL) proteins (Cutler et al. 2010). PYL proteins are ABA receptors, which undergo a conformational change upon binding ABA and create an interaction surface for the binding to Type 2C protein phosphatases (PP2Cs) (Takeuchi et al. 2014). In the absence of ABA, there is an open hydrophobic pocket formed inside of the PYL receptor with gate and latch attached in the flank of the entrance. ABA fits in the pocket when it is present and the interaction between ABA and the conserved residues within PYL proteins causes conformational change (Figure 2.4), which gate and latch are closed to form a stable closed conformation (Bai et al. 2013).

The rearrangement of PYL protein due to PYL-ABA binding allow binding to the active site of PP2Cs. PP2Cs, as crucial negative regulators of ABA signaling, are inhibited upon binding PYL proteins, resulting in an enhancement of ABA signaling.

During the interaction of PYL with ABA and PP2Cs, there is a formation of a solvent-exposed tunnel in PYL proteins that faces directly toward the PP2Cs interaction surfaces (Takeuchi et al. 2014). This tunnel is crucial for ABA antagonists to achieve their functions. When ABA antagonists were applied, they can be recognized by PYL receptors and fit in the hydrophobic pocket since they have similar structures to ABA. Their molecular modification, for instance, an addition of a chain of greater than five non-hydrogen atoms at the C-3'-carbon of ABA allows the chain to protrude into the channel and interfere the binding between PYL and PP2Cs (Takeuchi et al. 2014). The long chain of ABA antagonists interferes with the PYL-ABA-PP2C binding by occupying a position originally taken up by Val393, a highly conserved PP2C residue (Takeuchi et al. 2014). PP2Cs are then free from binding to PYL protein and negatively regulate the ABA signaling pathway (Figure 2.4). ABA agonists, on the contrary, reinforce the binding between PYL and PP2Cs, leading to an increased ABA signaling. ABA agonists normally have a tether that is shorter than three (Takeuchi et al. 2014). Their agonisms come from the stabilization of the gate closure by the short tether. ABA agonists can be more resistant to the ABA-degrading enzymes due to their structural modifications at the 8'-carbon atom of ABA (Abrams et al. 1997).

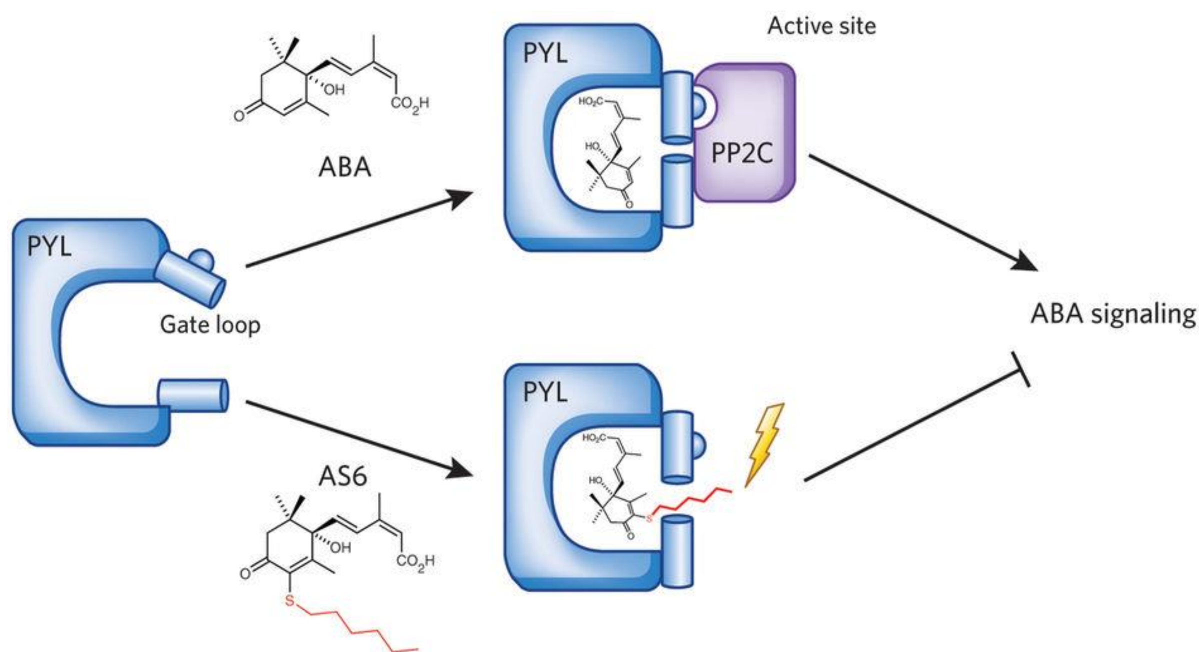


Figure 2.4 ABA antagonists affect the ABA signaling pathway by interfering PYL-PP2C binding. PYL, Pyrabactin resistance1-like ABA receptor; PP2C, Protein phosphatase 2C (from Hayashi and Kinoshita 2014).

Design of ABA analogs

During the binding with ABA receptors, ABA's 3' and 4' positions are involved in conformational change via hydrophobic contacts. The results of multiple PYL-ABA X-ray structures study suggest hydrophobic tunnels are formed closely to ABA's 3'-CH and 4′-C=O, which are connected to the interface that contacted PP2C (Takeuchi et al. 2014). If the modification occurs on 3' position of ABA, for example, a substitution with alkyl chain at C-3'. The tunnel will accept alkyl substituents and form hydrophobic contacts with it. Depending on the type and length of the substituents, analogs could either stabilize the closured loop or interfere the binding with PP2C through the tunnel (Takeuchi et al. 2015).

ABA antagonists can be divided into several categories based on their synthetic approaches. Among which, two types of ABA antagonists are commonly synthesized: the simple analogs, which have a chain at their 3' position, and the bicyclic tetralone analogs that have two rings

fused together.

The simple analogs, such as 3′-hexylsulfanyl-ABA (AS6) developed by Takeuchi et al., have long flexible chains at C-3' to prevent the binding to PP2C (Takeuchi et al. 2014). The syntheses of these analogs are simpler than that of the bicyclic tetralone analogs, which makes them ideal for large-scale productions. However, the high flexibility derived from the long thin chains impair their abilities in binding with ABA receptor (Takeuchi et al. 2015). The synthesis of 3'- thio ABA analogs is shown below (Fig. 2.5).

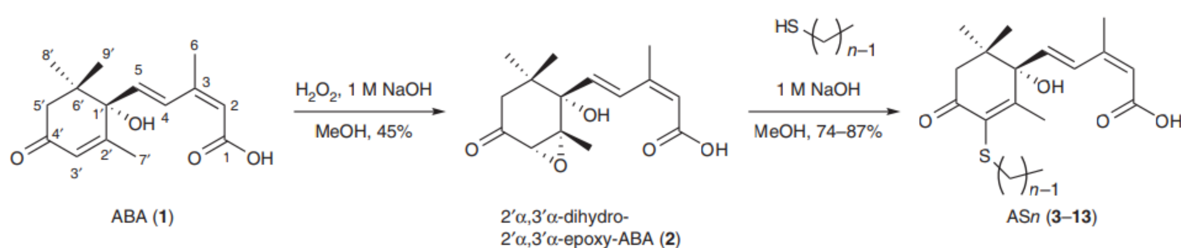


Figure 2.5 Synthesis of ASn (the simple ABA analogs that have a chain at C-3') (from Takeuchi et al. 2014).

The core for designing bicyclic tetralone analogs is the fusion of cyclohexenone ring and the benzene ring. The fused rings can effectively reduce the flexibility and therefore, increases their abilities in binding with ABA receptors (Nyangulu et al. 2005). The study on propenyl-ABA with O-butyl (4 carbons) chain (PAO4), one of the bicyclic tetralone analogs designed by Takeuchi et al. (2014) have proved that PAO4 (bicyclic tetralone analog) is more active as antagonists than AS6 (simple analog). PBI-664, reported by Rajagopalan et al. (2016) has almost an identical molecular structure as PAO4 except for hydroxyl terminus. Yet, PBI-664 exhibited more universal antagonisms and greater efficacy than PAO 4 (Rajagopalan et al. 2016). PAO4 only showed antagonist activities on monomeric receptors while PBI-664 exhibit

antagonisms on both monomeric and dimeric receptors. The polar terminus of PPI-664 might have an impact on the interference of homodimers from acquiring the binding site, resulting in the acquisition of antagonisms on dimeric receptors (Rajagopalan et al. 2016). However, the high complexity of their structures makes them hard to synthesize and undesirable for mass production. The synthesis of bicyclic tetralone analogs is shown below (Fig. 2. 6).

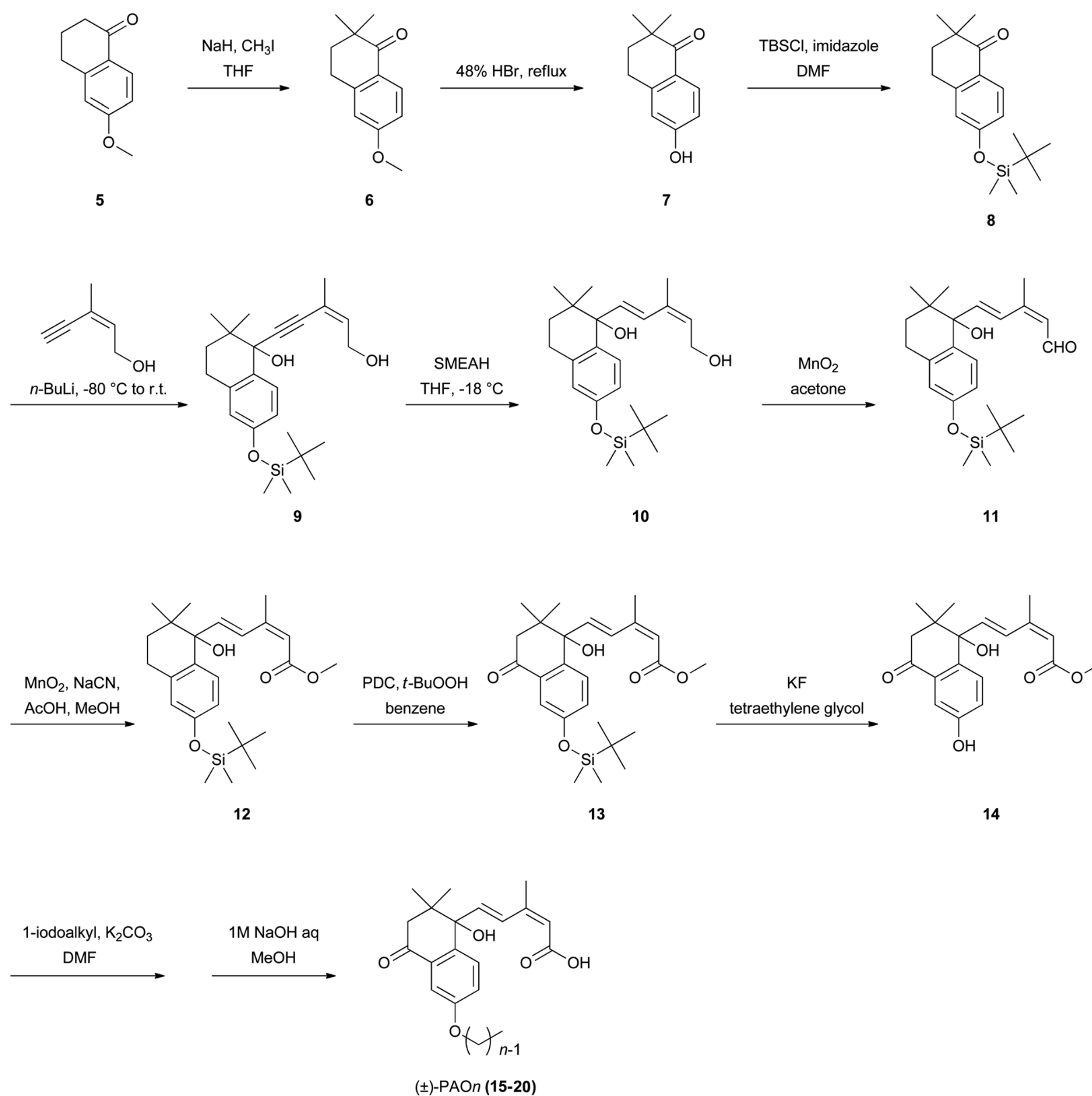


Figure 2.6 Synthesis of PAOn (The bicyclic tetralone ABA analogs that have two rings fused together) (from Takeuchi et al. 2015).

Hypotheses

The research was designed to test the following hypotheses:

1. ABA antagonists can be applied to canola, chickpea and soybean seeds to promote germination under low temperature.
2. ABA antagonists can be applied to canola, chickpea and soybean seeds to promote germination in the presence of exogenous ABA.
3. Simple 3'- linked ABA analogs may perform better with a carbon atom linker rather than sulfur.
4. The application of an effective ABA antagonist inhibits the ABA signaling pathway, leading to fluctuations of ABA concentration within seeds.
5. ABA concentration in low temperature-treated seeds is higher than in room temperature-treated seeds during germination.
6. NCED and CYP707A gene families are involved in the regulation of ABA level during low-temperature canola germination.

Chapter 3: Materials and Methods

3.1 ABA analogs preparation

The synthesis of the analogs and the preparation of the stock solutions were carried out by Mrs. Idralyn Alarcon and Mr. Leon Lai at the Chemistry Department, University of Saskatchewan. Stock solutions were kept in narrow-mouth amber glass bottles and stored at 5°C in the refrigerator until use. Solutions for germination assays were diluted from stock solutions. Stock solutions were brought to RT and shaken to become homogenous before dilution. DMSO was added to one percent of each diluted solution to ensure solubility of the ABA analogs. Distilled water was used for dilution. Diluted solutions were put in amber glass bottles and kept at 5°C.

3.2 Germination assay with ABA antagonists in canola

Seeds of canola (*Brassica napus* L.) cultivar Excel were used to assess the effects of ABA antagonists on seed germination. The seeds were kindly provided by Dr. Sally Vail from the Saskatoon Research Centre of the Agriculture and Agri-Food Canada. The cultivar was grown in 2015 at plot 94 at the Agriculture and Agri-Food Canada research station in Saskatoon. After harvest, the seeds were kept in -80°C freezer since 2015.

The ABA analogs (Fig 4.1) used for the germination test at 5°C were as follows: ABA 1001, ABA 1002, ABA 1004, ABA 1005, ABA 1006, ABA 1007, ABA 1008, ABA 1009, each at 100 µM concentration plus control treatment of 1% DMSO.

The ABA analogs (Fig 4.2) used for the germination test at 22°C were as follows: ABA 1009, ABA 1011 and ABA 1012, each at 100 µM concentration. 10 µM exogenous ABA was applied in each treatment.

Confirmation test of the effects of ABA 1009

The same seed source was used as described in 3.2. The germination test at 5℃ included ABA 1009 at 100 μM and control (1% DMSO). The germination test at 22℃ included ABA 1009 at 100 μM, ABA at 10 μM + ABA 1009 at 100 μM, and control (1% DMSO).

Concentration test of ABA 1009

The same seed source as described in 3.2 was used. The test was conducted at RT (22℃). ABA analog treatments included: ABA at 10 μM, ABA 1009 at 100 μM, ABA 1009 at 300 μM, ABA 1009 at 500 μM, ABA at 10 μM + ABA 1009 at 100 μM, ABA at 10 μM + ABA 1009 at 300 μM, and ABA at 10 μM + ABA 1009 at 500 μM.

Germination test of canola seeds with dormancy variation using ABA 1009

Seeds of the following canola varieties were used in this part of the study: NAM-47, NAM-49, NAM-0, NAM-72, and NAM-79. All varieties were grown at the Agriculture and Agri-Food Canada research station in Saskatoon in 2017. The seeds were provided by Dr. Sally Vail from the Saskatoon Research Centre, Agriculture and Agri-Food Canada. The seeds were kept in -20℃ freezer since 2017. NAM-47, NAM-49 are the cultivars with high dormancy harvested from tent 7 and 9 isolation. NAM-0, NAM-72, NAM-79 are the cultivars with low dormancy harvested from tent 3, 4, and 6. The ABA analog treatments included ABA 1009 at 100 μM and control (1% DMSO). The test was conducted at LT (5℃).

Germination Protocols

Germination test at 5℃

Forty seeds of uniform size were sown on each Petri dish (100mm in diameter) with two layers of filter paper and 5 ml of control or treatment solution. Eight Petri dishes were prepared

for each treatment and served as the replications. All Petri dishes were wrapped in two sheets of aluminum foil to avoid the confounding effects of light. One set of the control group was incubated at RT (22°C). Another set of the control group and the rest of the treatment groups were incubated at 5°C in the refrigerator (VWR series under counter free-standing refrigerator). The number of germinating seeds in each Petri dish was recorded every 24 hours. A seed was considered germinated after its radicle had broken through the seed coat.

Germination test at 22°C

Forty 40 seeds of uniform size were sown on each Petri dish (100mm in diameter) with two layers of filter paper and 5 ml of control or treatment solution. Seeds in stratified groups were sown two days earlier than the non-stratified and kept at 5℃ in the refrigerator before commencing the germination assays. The non-stratified seeds were sown in Petri dishes and the stratified seeds were withdrew from the refrigerator as the germination assay began. Each control or treatment group had 8 Petri dishes which served as the replications. All Petri dishes were wrapped in two sheets of aluminum foil and incubated at 22°C. The number of germinating seeds in each Petri dish was recorded every 4 hours from the eighth hour of the experiment.

Germination test with ABA 1009 in pouches

The experiment used canola seeds of cultivar Excel from the same seed lot as used in the previous experiment. The ABA analogs included ABA at 10 μM, ABA at 10 μM + ABA 1009 at 100 μM.

Twenty-five seeds of approximately the same size were sown in each pouch with 18 ml treatment solution. Four pouches were prepared for each treatment. All pouches were held

vertically in a holder and were covered with two sheets of aluminum foil. All pouches were incubated at 22°C. The number of germinating seeds in each pouch was recorded every 24 hours. Photographs of radicle growth were taken at day 5 (Fig 4.9, Fig 4.10, Fig 4.11 and Fig 4.12).

3.3 Hormone analysis of canola seeds treated with ABA antagonists

Seeds of canola cultivar Excel were used for hormone analysis. The analysis was done using the same seed lot as for confirmation test for ABA 1009 at 300 μM and control (1% DMSO).

Forty seeds of approximately the same size were sown on per Petri dish with two layers of filter paper and 5 ml of control or treatment solution. Six grams of canola seeds (around 2,400 seeds) were prepared for each treatment. The Petri dish was covered with aluminum foil and was incubated in a 5°C refrigerator.

Seeds used for the germination test, hormone analysis and gene expression study were incubated at the same time and the same conditions so that the results of the hormone analysis and gene expression study could be directly correlated with the difference in the percent germination between the control and treatment groups.

A half grams of canola seeds (around 200 seeds) from the control or treated samples with ABA 1009 solutions were collected daily after the start of incubation and immediately stored at -80°C. Samples were freeze-dried before use. Samples collected at day 3, 6, and 9 were selected for hormone analysis based on the results of the germination test.

The sample preparation and liquid chromatography-multiple reaction monitoring (LC-MRM) analyses were carried out at the core mass spectrometry facility within the Health

Sciences building at the University of Saskatchewan by Haixia Zhang. Powdered samples (50 mg) were weighed and transferred into 15 mL glass tubes. One mL of extraction solvent consisting of 80:19:1 methanol: water: formic acid containing deuterated internal standards (namely d_6-ABA, d_3–PA, d_3–DPA, d_4–7-OH-ABA, d_2-GA_7, d_2-GA_1 and d_5-ABA GE) was added to each tube to extract the plant hormones. Meanwhile, 1 mL of hexane was added to extract lipids out of the powder. After vortexing the tube for 5-10s (Vortex Maxi Mix II, Thermo Scientific), the samples were placed onto a mixer (IKA-VIBRAX-VXR, Terochem Scientific, Toronto) at RT for 30 min at 1000 rpm. Samples were then centrifuged (Sorvall ST 16R, ThermoFisher Scientific, Germany) for 10 min at 4900 rpm, the hexane layer was removed, and another 1 mL of hexane was added to repeat the lipid extraction. After hexane was removed, an 800 µL aliquot of the aqueous supernatant was transferred into a 2-mL microtube. A second extraction from the remaining pellet was carried out by adding 500 µL of the extraction solvent (80:19:1 methanol: water: formic acid) with no internal standard. After vortexing, mixing, and centrifuging as described above, 500 µL from the second extraction was combined with the 800 µL from the first extraction to give 1300 µL of extract. This combined extract was vortexed and then centrifuged for 5 min at 12,000 rpm on a Thermo Legend Micro 17 (ThermoFisher Scientific, Germany). From this combined extract 450 µL were transferred to a new microtube and evaporated to dryness using a speed vac (Labconco Corp., Kansas City, MO). The dried sample was then reconstituted in 150 µL of 79:20:1 water: methanol: formic acid, vortexed vigorously for 15 s and then placed into a Thermo mixer C (Eppendorf AG, Germany) at RT for 30 min at 1400 rpm. A volume of 120 µL was transferred into an HPLC vial containing a 150 µL insert.

The instrumentation consisted of an Agilent 1290 UPLC coupled to a Q-Trap 6500 (Sciex, Concord, ON). A volume of 5 µL was injected from the vial and analytes were separated using an Agilent Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm) along with a 5 mm guard column prior to quantification using multiple reaction monitoring (MRM). The transitions and collision energies for the compounds are shown in Table 3.1, and the binary gradient is shown in Table 3.2.

Table 3.1 Transitions and collision energies for MRM analysis.

Compound	Transition	CE (V)
ABA	263 → 153	-16
PA	279 → 139	-18
DPA	281 → 171	-24
7’-OH-ABA	279 → 151	-22
ABA-GE	425 → 263	-16
neo-PA	279 → 205	-19
8’-OH-ABA glut ester	423 → 279	-20
GA_1	347 → 273	-32
GA_3	345 → 143	-34
GA_4	331 → 257	-32
GA_7	329 → 223	-24
1009	347 → 237	-18
d_6-ABA	269 → 159	-16
d_3-PA	282 → 142	-22
d_5-ABA-GE	430 → 268	-18
d_4-7’-OH-ABA	283 → 221	-16

d_3-DPA	284 → 240	-18
d_2-GA7	331 → 225	-26
d_2-GA1	349 → 261	-26

* 8'-OH-ABA glut ester is 8'-OH-ABA hydroxyl methyl glutaryl ester.

Table 3.2 Binary gradient operated at 400 µL/min for UPLC-MRM analysis*

Time (min)	%A	%B
0	90	10
5	80	20
7	70	30
11	58	42
12	10	90
14	10	90
14.1	90	10
17	90	10

* A=1% formic acid in water, B=1% formic acid in 90:10 acetonitrile: water.

Area ratios for each analyte were determined from the peak area of the analyte divided by the peak area of the labeled standard. The concentrations were determined from the area ratios of the samples compared with the calibration curve and based on the weight, the values were reported as ng g^{-1} dry weight. For each sample, three replicates were analyzed and the average values are reported.

3.4 Gene expression analysis of canola seeds treated with ABA antagonists

Seeds of canola cultivar Excel were used for gene expression analysis. The same seed

source and protocol as of germination test with ABA 1009 were used for gene expression analysis.

Forty seeds of approximately the same size were sown on per Petri dish with two layers of filter paper and 5 ml of control or treatment solution. Six grams of canola seeds (around 2,400 seeds) were prepared for each treatment. The Petri dish was covered with two sheets of aluminum foil and incubated in a 5°C refrigerator.

A half gram of canola seeds (around 200 seeds) treated with control or ABA 1009 solutions was collected daily after the start of incubation. Samples were immediately frozen in liquid nitrogen and kept in -80°C until further analysis. Samples of day 3, 6, and 9 were selected for gene expression analysis. Three biological replications (10 seeds per replicate) were chosen randomly from each selected sample. Each replicate was immersed in liquid nitrogen and ground to homogenous powder. Total RNA was isolated with the use of the SV total RNA isolation system. The RNA concentrations were measured using nanodrop. SensiFAST™ cDNA synthesis kit was applied for the conversion from mRNA to cDNA. Quantitative RT-PCR was used in sub-sequential steps to assay the expression patterns of several germination-related genes (Appendix Table 1). cDNA was treated with SensiFAST™ SYBR® No-ROX kit and analyzed by Bio-Rad's qPCR system. The extent of expression was calculated based on the Cq values of reference and germination related genes. Four genes (TIPS-41, ACT 7, SAND, and PP2A) were used as the internal controls in canola.

3.5 Germination assay with ABA 1009 in soybean

Seeds of soybean (*Glycine max* L. Merr.) cultivar TH33003R2Y were used for the germination tests. Seeds were purchased from Elmy's seed farm located at Box 477, Saltcoats,

Saskatchewan. Seeds were stored in a drawer without exposure to light and kept at RT (22℃) since 2015. The ABA analogs used for germination test at 22℃ were as follow: ABA at 10 μM, ABA 1009 at 100 μM, ABA at 10 μM + ABA 1009 at 100 μM and control treatment (1% DMSO).

Germination protocol for soybean

Twenty seeds of uniform size were sown on each Petri dish with two layers of filter paper and 10 ml of control or treatment solution. Three Petri dishes were prepared for each treatment and served as the replications. All Petri dishes were wrapped in sheets of aluminum foil. All Petri dishes were cultured at RT (22℃). The number of germinating seeds in each Petri dish was recorded every 24 hours. A seed was considered germinated after its radicle had broken through the seed coat.

3.6 Hormone analysis of soybean

Seeds of soybean (*Glycine max* L. Merr.) cultivar TH33003R2Y were used for hormone analysis. The analysis was done on the same seed lot as for germination tests.

Twenty seeds of uniform size were sown on each Petri dish with two layers of filter paper and 10 ml of water. Eighteen grams of soybean seeds (around 120 seeds) were prepared. Each Petri dish was covered with aluminum foil. Nine grams of soybean seeds (around 60 seeds) were incubated at RT (22℃) and the other 9 grams of seeds were incubated under 7℃ in the refrigerator (VWR series under counter freestanding refrigerator). For both low (7℃) and room (22℃) temperature treatments, three gram of soybean seeds (around 20 seeds) was collected at day 0, 1, 2 after the start of incubation and immediately stored at -80 °C freezer. Samples were freeze-dried before analysis. The protocols for sample preparation and HPLC/MS/MS

analysis were the same as for hormone analysis of canola.

3.7 Germination assay with ABA 1009 in chickpea

Seeds of kabuli chickpea (*Cicer arietinum* L.) cultivar CDC Leader were used for germination tests. The seeds were provided by the pulse breeding program of the Crop Development Centre, University of Saskatchewan. The cultivar was grown at plot 7643 at the Lucky Lake research field in 2015. Seeds were stored in a drawer without exposure to light and were kept at RT (22°C) since 2015. The ABA analogs used for the germination test at 7°C included: ABA 1009 at 100 µM and control treatment (1%DMSO). The ABA analogs used for the germination test at 22°C were as follow: ABA at 10 µM, ABA at 10 µM + ABA 1009 at 100 µM and control treatment (1%DMSO).

Germination test at 7°C and 22°C.

Twelve seeds of uniform size were sown on each Petri dish with two layers of filter paper and 10 ml of control or treatment solution. Eight Petri dishes were prepared for each treatment and served as the replications. All Petri dishes were wrapped in two sheets of aluminum foil. All seeds were incubated under 7°C in the refrigerator. The number of germinating seeds in each Petri dish was recorded every 24 hours. A seed was considered germinated after its radicle had broken through the seed coat.

Twelve seeds of uniform size were sown on each Petri dish with two layers of filter paper and 10 ml of control or treatment solution. Each group consisted of 10 Petri dishes which served as the replication of the experiment. All Petri dishes were wrapped in two sheets of aluminum foil and incubated at 22°C. The number of germinating seeds in each Petri dish was recorded every 24 hours.

3.8 Hormone analysis of chickpea

Seeds of chickpea (*Cicer arietinum* L.) cultivar CDC Leader were also used for hormone analysis. The analysis was done on the same seed lot as for germination tests.

Twelve seeds of uniform size were sown on per Petri dish with two layers of filter paper and 10 ml of water. Twenty-one grams of chickpea seeds (around 72 seeds) were prepared. Each Petri dish was covered with aluminum foil. Six grams of chickpea seeds were incubated at RT (22°C) and the other 15 grams of seeds were incubated under 7°C in the refrigerator. For RT treatment, three grams of seeds were collected on day 0 and day 3. For LT treatment, three grams of seeds (around 10 seeds) were collected on day 0, 3, 6, 9, 12. Samples were immediately stored at -80°C after collection. Samples were freeze-dried before use. The methods of sample preparation and HPLC/MS/MS analysis were the same as for the hormone analysis of canola.

Chapter 4: Results and Discussion

The effects of ABA antagonists ABA 1001, ABA 1002, ABA 1004, ABA 1005, ABA 1006, ABA 1007, ABA 1008, ABA 1009, ABA 1010, ABA 1011and ABA 1012 on seed germination were assessed in this study. ABA 1001, which is structurally identical to PBI664, is a bicyclic tetralone analog, which has two rings fused together (Rajagopalan et al. 2016). ABA 1002 is a simple analog with an alkylsulfanyl chain attached on 3' position. It was reported as AS6 by Takeuchi et al. (2014). ABA1004, ABA 1005, ABA 1006, ABA 1007, ABA 1008, ABA 1009, ABA 1010, ABA 1011 and ABA 1012 are simple analogs with different 3' modifications. Among these simple analogs, ABA 1004, 1005, 1006 and 1007 have a sulfur atom linker at their C-3'. The linker for ABA 1008, ABA 1009, ABA 1010, ABA 1011 and ABA 1012 is a methyl group (Figure 4.1).

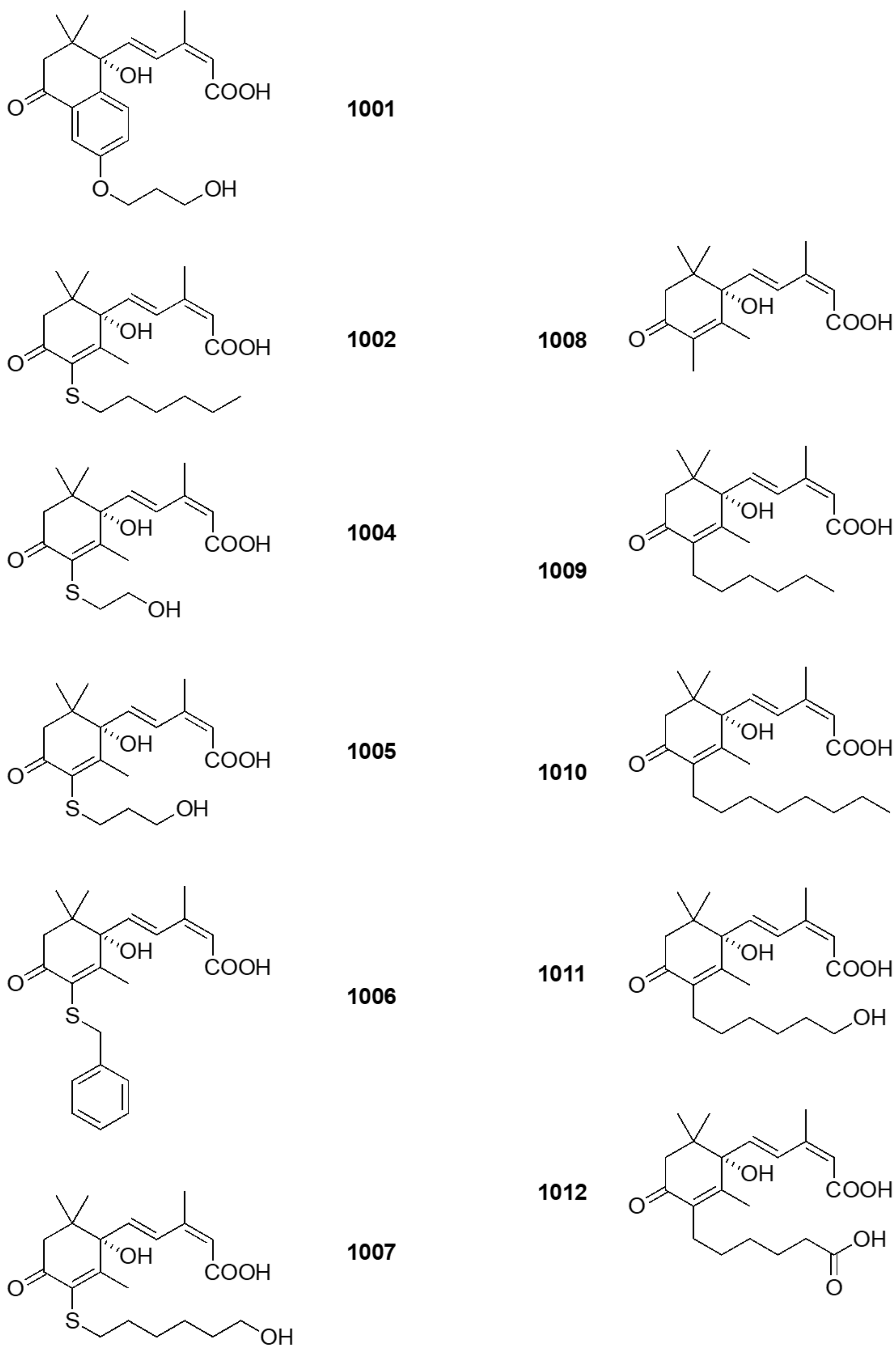


Figure 4.1 The structure of ABA analogs screened in this study: ABA 1001, ABA 1002, ABA

1004, ABA 1005, ABA 1006, ABA 1007, ABA 1008, ABA 1009, ABA 1010, ABA 1011 and ABA 1012 (All ABA analogs were prepared and provided by Idralyn Alarcon and Leon Lai at the Chemistry Department, University of Saskatchewan).

LT Germination assay with ABA antagonists in Canola

Two sets of controls were prepared in the LT germination assay, one was incubated at RT (22°C) and the other was incubated at LT (5°C). Canola seeds from RT control reached 100 percent germination at day 2; however, LT control seeds only began to germinate at day 9, 15% of which remained ungerminated at day 18. Compared to RT control, LT seeds treated with ABA antagonists required a longer time to complete their germinations (Figure 4.2), but some of them exhibited significantly higher percent germination than LT control, indicating the potential of the ABA antagonists in promoting LT canola germination.

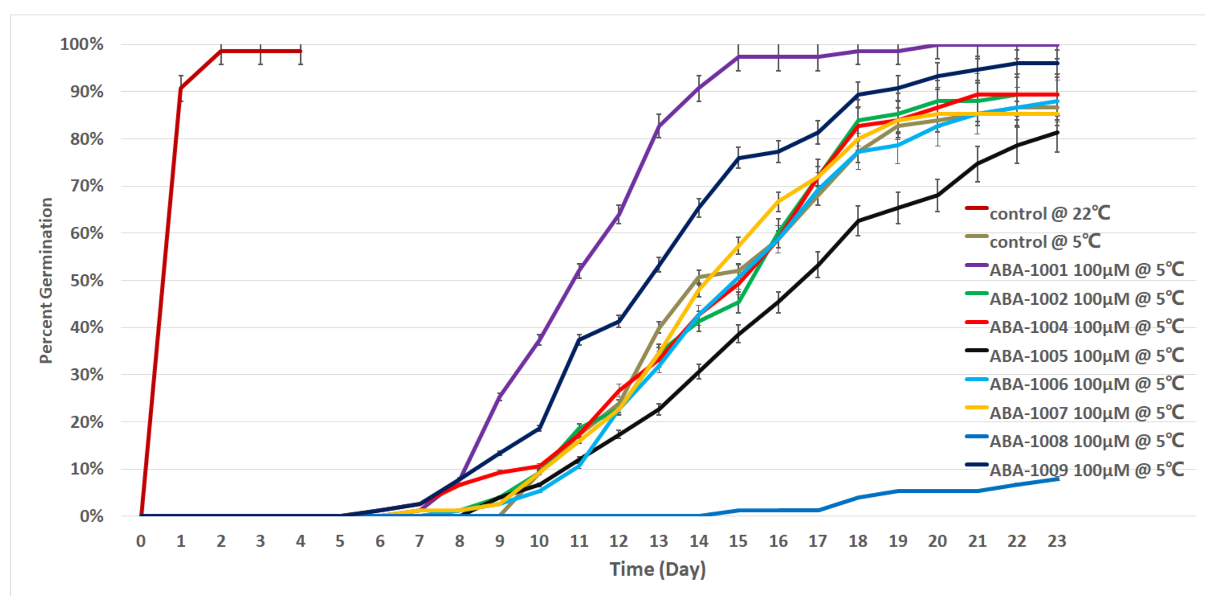


Figure 4.2 Percent germination of canola seeds treated with different ABA antagonists (ABA 1001-ABA 1009) at 100 μM at 5°C (Conducted by Dongyan Song in December 2015). ABA 1001 and ABA 1009 had significantly higher percent germination compared to LT control (water with DMSO).

Of the compounds tested in the LT experiment, ABA 1001 and ABA 1009 treated seeds

had significantly higher percent germination compared to LT control and other treatments (Figure 4.2). This result supports the hypothesis that ABA antagonists can be applied to canola seeds to promote germination under LT. ABA 1001-treated seed reached 87% germination on day 14, which was 43% higher than the LT control. ABA 1009-treated seed achieved 78% germination on day 16, while LT control reached only 54% germination. Together, these results indicated that ABA 1001 and ABA 1009 were effective in promoting canola seed germination at 5°C condition.

In comparison to ABA 1009, ABA 1001 showed a better promotive effect on LT canola germination. ABA 1001 is a tetralone analog with a fusion of cyclohexenone and benzene ring at its base portion. The modification of ABA 1001 reduces the flexibility of the base while the long chain at the tip could still protrude through the tunnel and interfere with the PYL-PP2C binding. The tetralone ABA 1001 increased the rigidity of the chain and led to a decrease of entropy resulting in a higher affinity for the binding to PYL receptors.

ABA 1001 has an almost identical structure as PAO4, but with a hydroxyl terminus. Yet, ABA 1001 exhibited a better and more universal antagonism than PAO4 (Rajagopalan et al. 2016). PAO4, with a methyl terminus, was effective in monomeric ABA receptors but lack of antagonism in dimeric receptors like PYR1 (Rajagopalan et al. 2016). ABA 1001 showed antagonist activities on both monomeric and dimeric receptors. The hydroxyl terminus on ABA 1001 is polar, which might interfere with the homodimer from gaining access to the binding site and results in the acquisition of antagonism to dimeric receptors (Rajagopalan et al. 2016). Moreover, the germination assays on Arabidopsis suggested that ABA 1001 had a better promotive effect than PAO4 (Rajagopalan et al. 2016). The results of canola germination

assays also indicated that ABA 1001 had a high efficacy in promoting LT germination. However, the synthesis of ABA 1001 is complex and time-intensive, which makes it less appealing for practical application.

ABA 1009, a simple analog with an addition of a hexyl group at 3' position, could effectively promote seed germination. Different from tetralone analogs, such as ABA 1001 and PAO4, the modification of ABA 1009 consists of six sp^3 atoms, resulting in high flexibility at its base and low affinity for binding to PYL. Yet, ABA 1009 was almost as effective as ABA 1001 in promoting seed germination in canola. ABA 1002, identical to AS6 as reported by Takeuchi et al. (2014), is a $3'$-alkylsulfanyl ABA analog. ABA 1002 had a sulfur atom linker at the base followed by a hexyl group, which is highly similar to the modification of ABA 1009. Yet, ABA 1002 did not show antagonism activities in canola germination. Rajagopalan's study also suggested that AS6 (ABA 1002) failed to promote germination of dormant Arabidopsis seeds (Rajagopalan et al. 2016). Moreover, the modification of ABA 1004, 1005, 1006 and 1007 involved a link by a sulfur atom. None of them showed promotive effects on canola seed germination under LT. The lack of function in sulfur linked ABA analogs compared to the promotive effects in carbon linked ABA analogs like ABA 1009 supported the hypothesis that simple 3'- linked ABA analogs may perform better with a carbon atom linker rather than sulfur. The lack of function might be attributed to the instability of the sulfur atom within the plant. ABA analogs with sulfur linker might be more susceptible to oxidation.

ABA 1008 is a simple analog with an addition of a methyl group on its 3' position. Unlike other ABA analogs tested in this study, the chain added to ABA 1008 was too short to interfere with the binding of PYL and PP2C. ABA 1008 showed a strong inhibition of seed germination

since the percent germination of ABA 1008-treated seeds is much lower than LT control. The study of chain length on tetralone and 3' linked analogs suggested that an analog tends to exhibit agonism when its tether is shorter than three (Takeuchi et al. 2015). Analog with short chain behaves as an agonist since its chain helps to stabilize the gate closure (Takeuchi et al. 2014). The result of ABA 1008 germination assay is consistent with Takeuchi's finding.

In conclusion, ABA 1001 was the most effective analog in promoting canola seed germination but it is not readily available. ABA 1009 showed promotive effects in the same assay. The latter compound requires fewer steps to synthesize, making it more practical for large-scale application.

RT Germination assay with ABA antagonists in canola

Ten µM exogenous ABA was added together with treatment or control solution to assess the effects of different ABA antagonists on seed germination. Compared to the LT germination test, experiments conducted at RT with the addition of ABA not only shortened the duration of the experiment but also removed the variability caused by the changes in seed dormancy after storage. LT canola germination tests usually lasted for one to three weeks, varied significantly by the dormancy status of the seeds. RT canola germination could achieve the same task in four days, which greatly increased the efficiency of selecting effective ABA analogs. Germination assays at RT were relatively easy to accomplish as the temperature would be less variable. Data from numerous germination assays suggested that the dormancy of canola seeds changed over time. However, the dormancy variation shown under RT germination test is less significant. Therefore, the results acquired from RT germination assays were more consistent.

Treatment of ABA+ ABA 1009, ABA+ ABA 1011 and ABA+ ABA 1012 produced higher

percent germination than ABA-treated seeds on day 2. The results supported the hypothesis that the application of ABA antagonist (ABA 1009, ABA 1011 and ABA 1012 in this case) was effective in promoting canola seed germination in the presence of exogenous ABA (Figure 4.3). These three analogs were simple analogs with a long chain substituted at 3' position. The promotive effects were presumed to come from the interference of PYL-PP2C binding by these chains. A similar study was reported by Takeuchi et al. (2014).

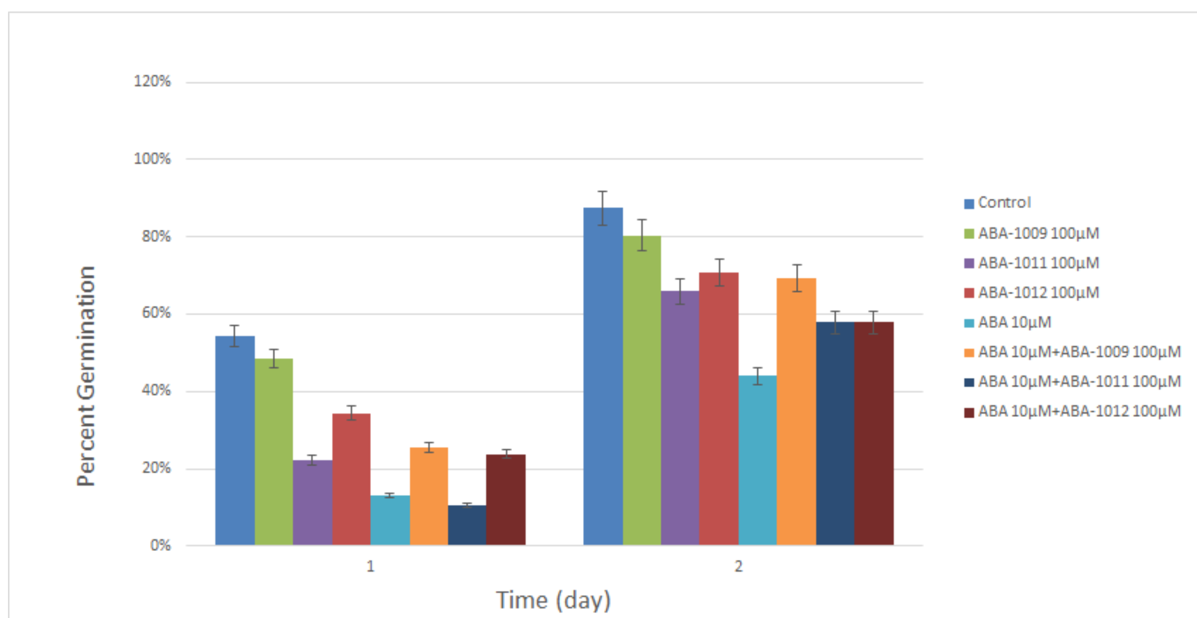


Figure 4.3 Percent germination of canola seeds treated with ABA 1009, ABA 1011 and ABA 1012 at 22℃ (November 2016). ABA 1009 was more efficient in counteracting the effect of exogenous ABA.

ABA 1009 showed consistent antagonism from day1 to day 2 while ABA 1011 and ABA 1012 exhibited different extents of agonism at day 1. Both ABA 1011-treated and ABA-1012 treated groups had significantly lower percent germination than control at day 1. Moreover, the percent germination of ABA+ABA 1011-treated seeds was close to the ABA-treated group. The modifications on ABA 1011 and ABA 1012 might be the causes of the early agonist

activities. ABA 1009, 1011 and 1012 were modified by a substitution with a hexyl, hexanol and hexanoic acid on their C-3’, respectively. Compared to ABA 1009, the hydroxyl and the carboxylic acid functional groups on ABA 1011 and 1012 had greater polarity. The polar terminus of ABA 1011 and 1012 might be less efficient in occupying the highly conserved positions that originally taken up by PP2C residues. Therefore, ABA 1011and 1012 exhibited agonism at the early phase.

Among the treatments with exogenous ABA applications, ABA+ABA 1009 treated seeds had highest percent germination, which indicated that ABA 1009 was more effective than both ABA 1011 and ABA 1012. The application of the ABA 1009 at 100 μM could only partially overcome the effects of exogenous ABA as the percent germination of ABA+ ABA 1009 treated group was in between ABA-treated group and the control group.

Confirmation test of the effects of ABA 1009

ABA 1009-treated seeds had higher percent germination than control in two independent experiments, which confirmed ABA 1009 was an effective antagonist in promoting seed germination under LT. For the experiment conducted in July 2017, it took 10 days for the control seeds to achieve more than 85% germination (Figure 4.4). As for the experiment conducted in January 2017, the control seeds did not reach 85% germination until day 18 (Figure 4.5). The materials used in these two experiments including the solutions and the seed sources were the same. The setting of the fridge temperature was identical. Yet, there were big variations in germination response, which indicated that the canola seeds used in this study might have a dormancy cycle. The fluctuation of the dormancy status had a great impact on the speed of germination. Data from the experiments that were conducted at different time points

was used as a comparison. It appeared that the dormancy status of the canola seeds had a seasonal periodicity. Seeds tend to have higher dormancy in winter resulting in slow responses of germination. The dormancy is released in summer which leads to fast germination under the same condition. Yet, the performance of ABA 1009 in this experiment was consistent despite the potential change of seed dormancy.

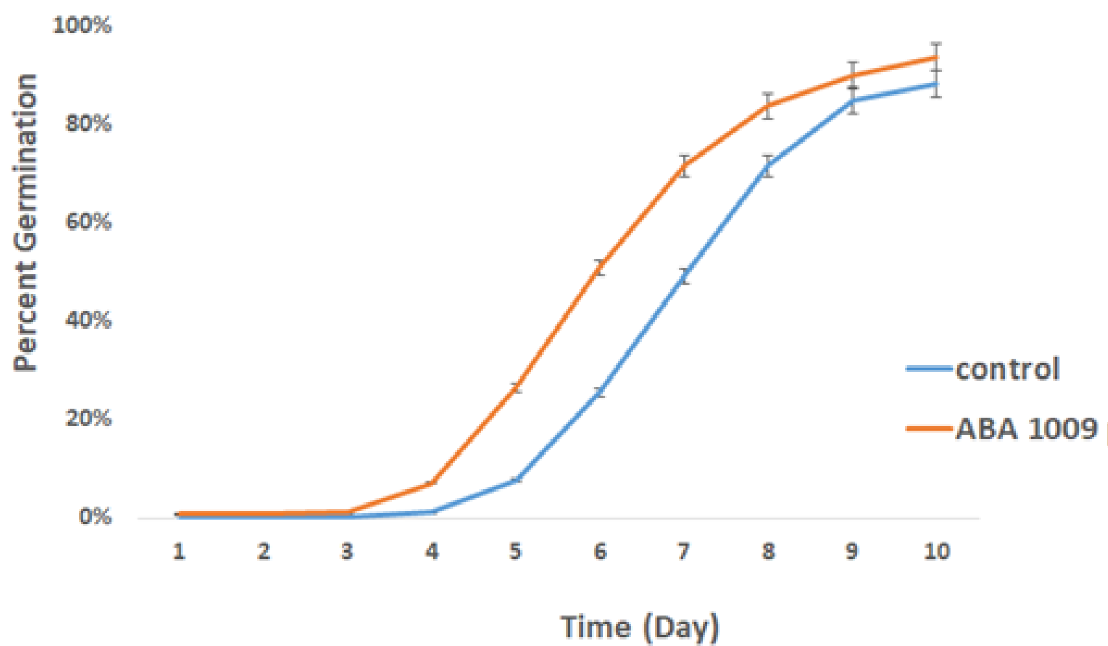


Figure 4.4 Percent germination of canola seeds treated with ABA 1009 at 300 μM and control (1%DMSO) at 5°C conditions (July 2017). ABA 1009 treated seeds had higher percent germination. Most seeds germinated in 10 days.

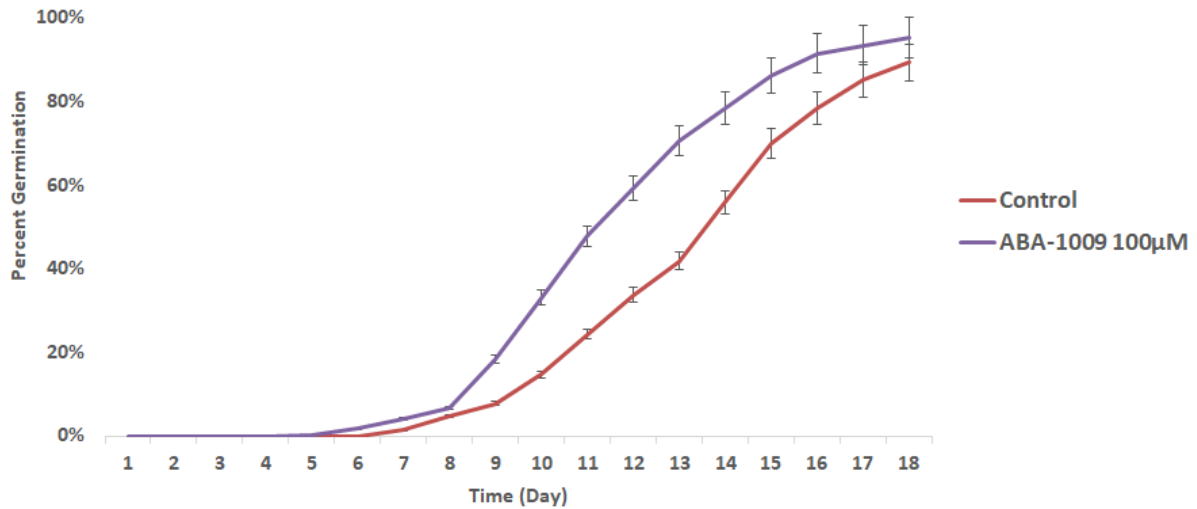


Figure 4.5 Percent germination of canola seeds treated with ABA 1009 at 100 µM and control (1%DMSO) at 5℃ conditions (January 2017). ABA1009 treated seeds had higher percent germination. Most seeds germinated in 18 days.

ABA +ABA 1009-treated group had higher percent germination than the ABA-treated group, but it was lower than the control group (1% DMSO) (Figure 4.6). This result indicated that ABA 1009 was effective in promoting canola seed germination in the presence of exogenous ABA, however, ABA 1009 at 300 µM could only partially overcome 10 µM ABA, especially in the early hours.

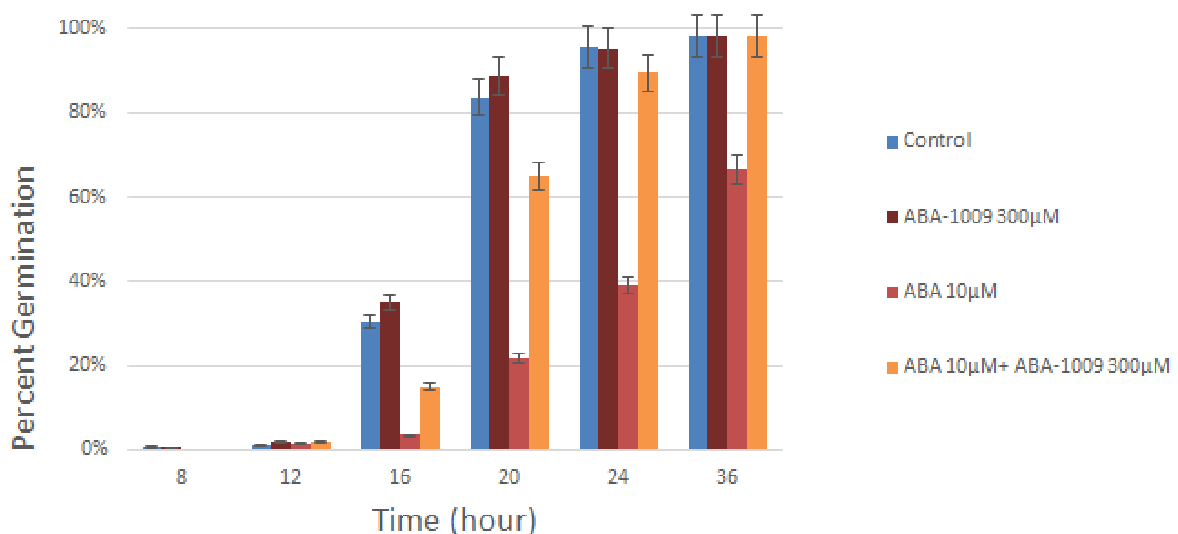


Figure 4.6 Percent germination of canola seeds treated with ABA, ABA+ ABA 1009 at 22℃

(February 2017). ABA 1009 was effective in counteracting exogenous ABA.

The results acquired from RT germination assay were consistent with the results from LT germination test. Yet, RT germination assay was more efficient for their abilities in producing reliable results within three days. The high efficiency of the RT germination test makes it ideal for quick screen of future ABA analogs. Even though LT germination assay is time-intensive, its experimental condition is a better approximation to the natural conditions in the early spring. Seeds have complex mechanisms in response to cold temperature more than just producing ABA. LT germination assay would provide better assessments on the effects of ABA analogs and provides more accurate and convincing results. Therefore, LT germination assay is necessary for the confirmation of the agonism or antagonism. Future experiments can be conducted as a follow up to achieve fast and reliable results. RT germination test will be conducted as the first step for the large-scale screening of the available ABA analogs. Analogs with significant agonism or antagonism will be selected and used in LT germination test to confirm their effects.

Test of different concentrations of ABA 1009

For the first 12 hours, seeds treated with ABA 1009 at 100 μM, 300 μM and 500 μM did not germinate in the presence of exogenous ABA. Most of the seeds germinated between 12 to 24 hours. At 24 hours, seeds treated with 300 μM ABA 1009 had the highest percent germination of 89%. ABA-treated group had 58% germinated seeds, 100 μM ABA 1009 treated group had 79% and 500 μM ABA 1009 treated group had 80% at 24 hours. ABA 1009 at 300 μM had a better promotive effect since it achieved the highest percent germination among all tested concentrations. They all reached 100% germination at 48 hours except ABA-treated

group. ABA 1009 at 300 µM was chosen at this time point as an intermediate concentration since it was more effective in promoting germination.

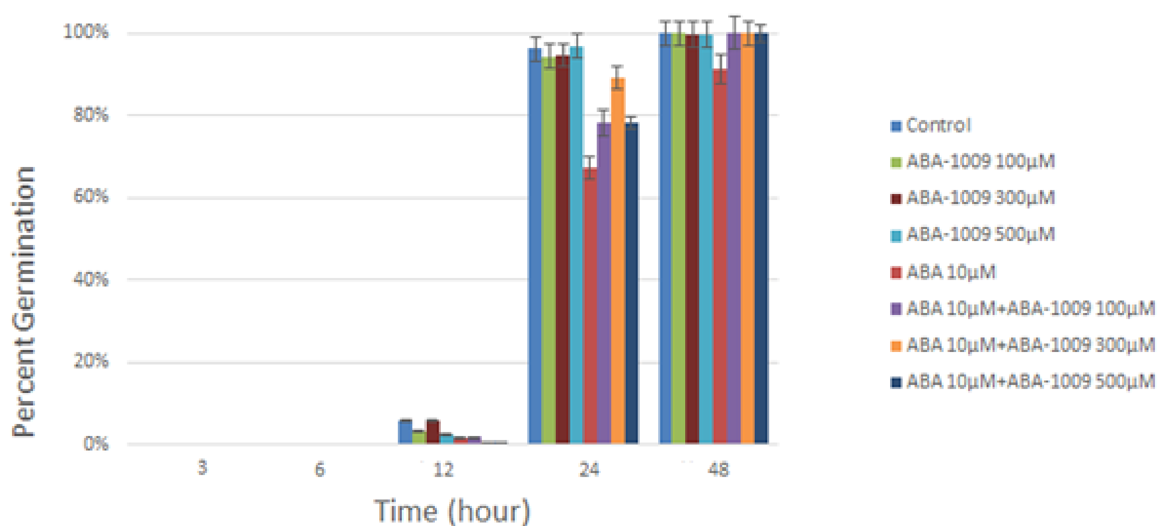


Figure 4.7 Percent germination of canola seeds treated with ABA 1009 at 100 µM, 300 µM and 500 µM at 22°C (April 2017). ABA 1009 at 300 µM had higher percent germination at 24 hours.

ABA 1009 at 300 µM was used in subsequent canola tests, including LT germination assays and hormone analysis. Although ABA 1009 at 300 µM exhibited promotive effects on LT germination, the results of hormone analysis suggested that ABA 1009 at 300 µM might act as an inhibitor in the later stage. Within ABA 1009-treated seeds, the ABA level increased greatly as ABA 1009 levels rose over time. An over-dosage effect occurred in LT germination when ABA 1009 at 300 µM was applied. The increased ABA level, caused by the application of ABA 1009 at high concentration, might mask the promotive effect of the analog. Therefore, 300 µM is not necessary the optimal concentration for all conditions. This result (Figure 4.7) only proved that 300 µM was better in counteracting the effect of 10 µM exogenous ABA at RT. One of the future foci could be to find out the optimal concentration for canola germination at

LT, which could significantly promote seed germination without inducing ABA biosynthesis.

Germination test with ABA 1009 in pouches

This experiment aims at finding out the effect of ABA 1009 on radicle development. An effective ABA antagonist should not only promote seed germination but also promote or, at least, not inhibit the latter development processes. Therefore, it is important to know the effects of ABA 1009 on radicle growth. In this experiment, seeds were cultured in vertically placed pouches with the addition of exogenous ABA and treatment control solutions. Compared to radicle development in Petri dishes in the former germination assays, the radicles grown in pouches are straighter and more linear due to gravity. Hence, it is easier and more accurate to compare their radicle lengths.

ABA+ ABA 1009 treated group had higher percent germination than ABA-treated group when they were treated in pouches at RT (Figure 4.8). This result proved again that ABA 1009 was effective in counteracting the effects of exogenous ABA.

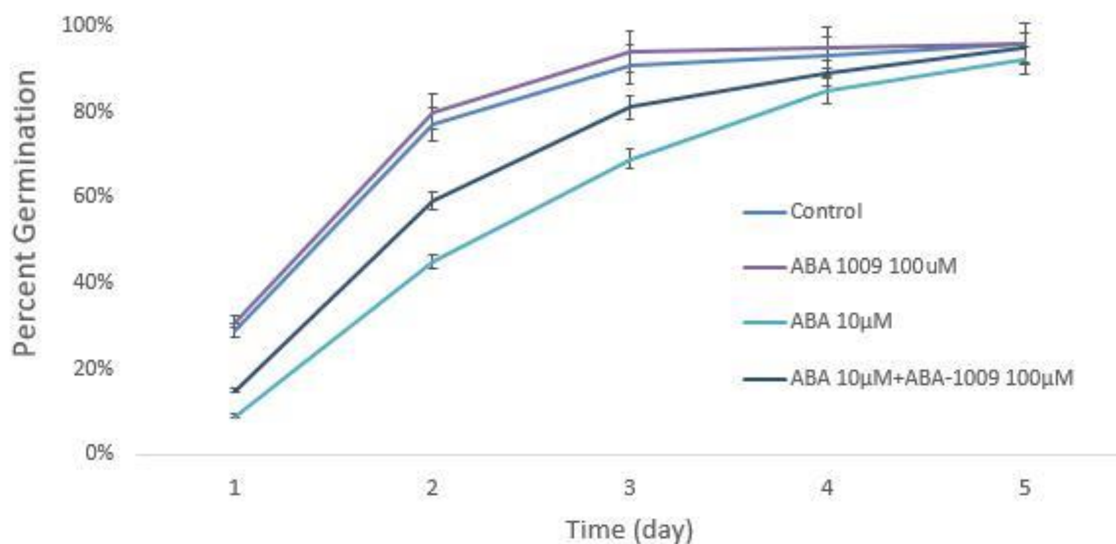


Figure 4.8 Percent germination of canola seeds treated with ABA and ABA + ABA 1009 in pouches at 22°C (September 2016). ABA 1009 was effective in counteracting exogenous ABA.

The upper, middle and bottom lines shown in the Figures 4.9, 4.10, 4.11, 4.12 were 1.25, 2.5 and 5cm away from starting point, respectively. In the control group, eighteen of twenty-five seedlings had a radicle that was longer than 2.5 cm and nine of them grew longer than 5 cm (Figure 4.9). Thirteen of ABA 1009 treated seedlings grew more than 2.5 cm and eight of them grew to 5 cm (Figure 4.10). In ABA-treated group (Figure 4.11), only six seedlings had radicles that reached the middle line (2.5 cm). In ABA + ABA 1009 treated group (Figure 4.12), thirteen seedlings grew to 2.5 cm while two of them reached 5 cm.

Figure 4.9 Radicle growth of control seeds on day 5. Seeds were incubated in pouches under RT (22°C). Eighteen out of twenty-five seedlings had a radicle that was longer than 2.5 cm.

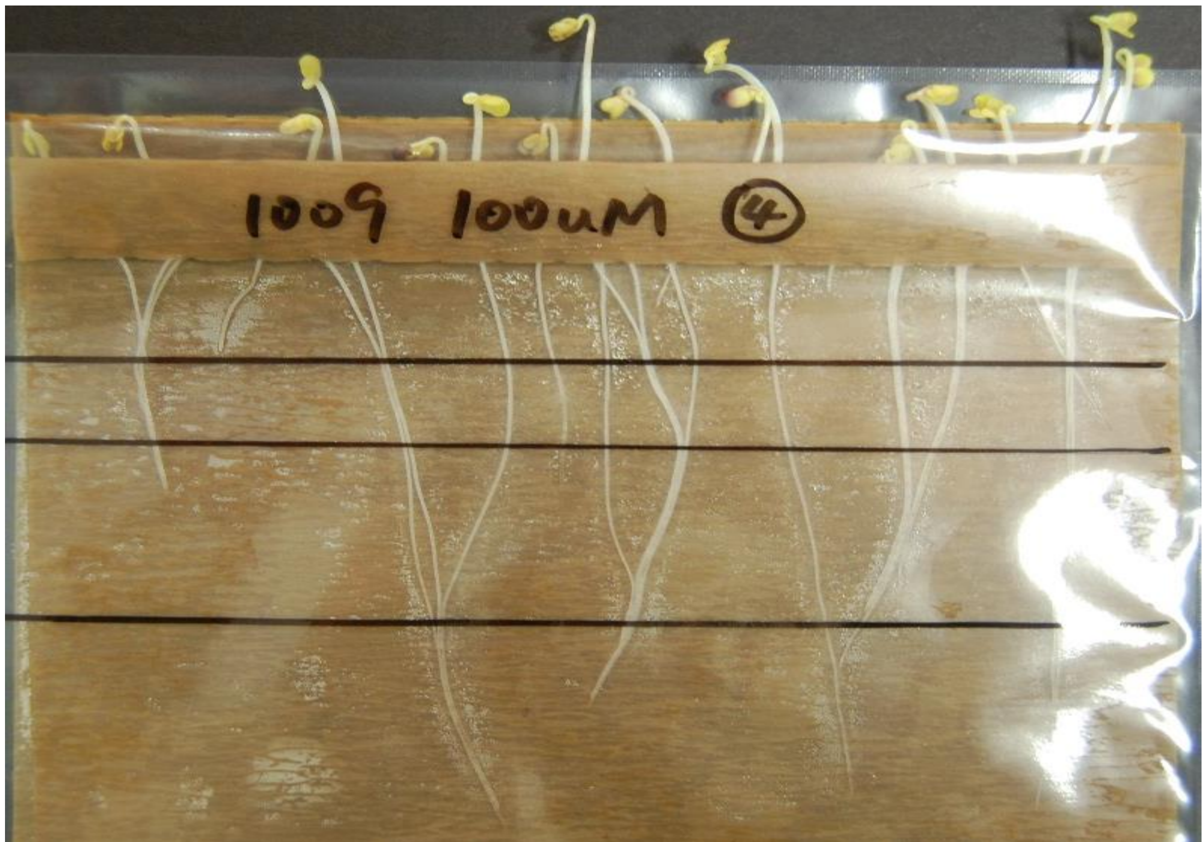


Figure 4.10 Radicle growth of ABA 1009-treated seeds on day 5. Seeds were incubated in pouches under RT (22°C). Thirteen out of twenty-five seedlings had a radicle that was longer than 2.5 cm.

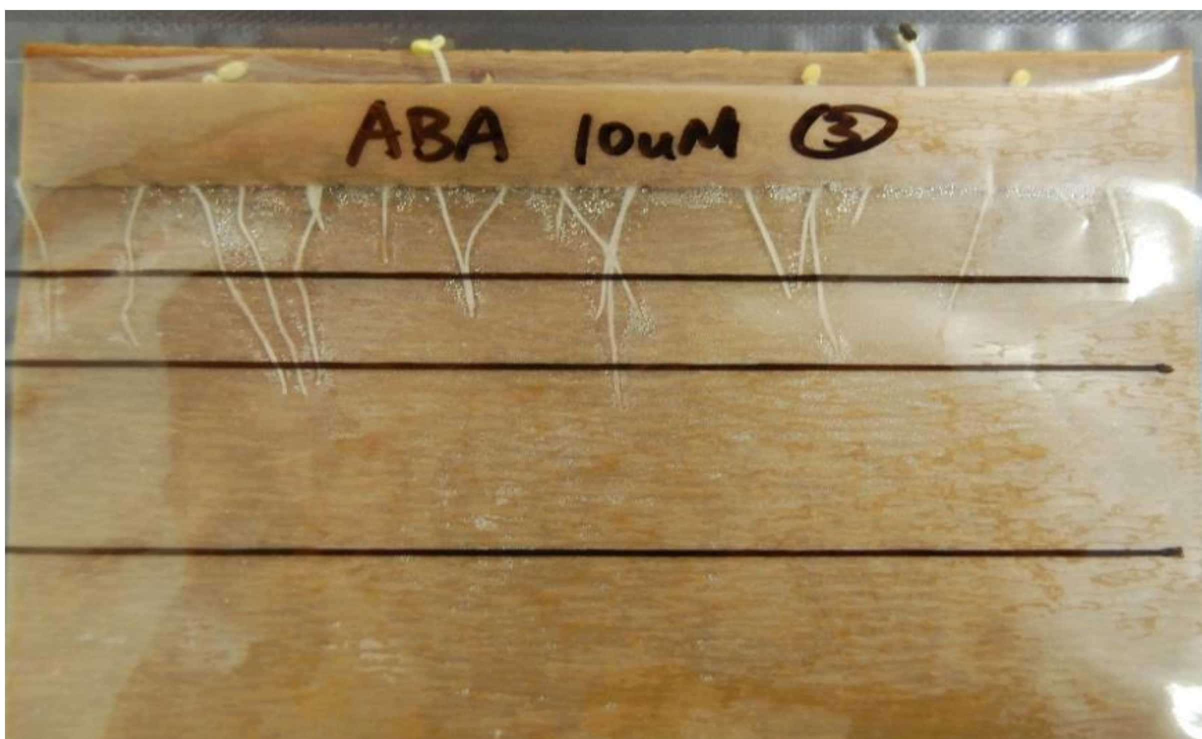


Figure 4.11 Radicle growth of ABA-treated seeds on day 5. Seeds were incubated in pouches under RT (22°C). Six out of twenty-five seedlings had a radicle that was longer than 2.5 cm.

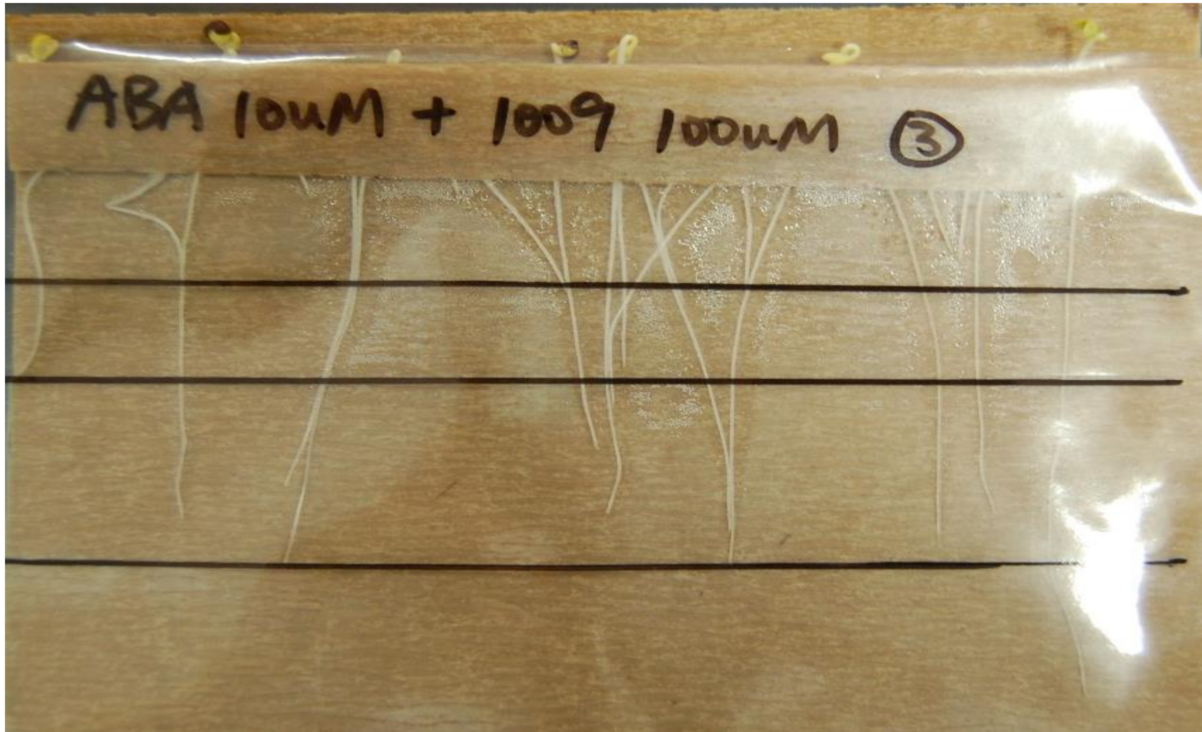


Figure 4.12 Radicle growth of ABA + ABA 1009 treated seeds on day 5. Seeds were incubated in pouches under RT (22°C). Thirteen out of twenty-five seedlings had a radicle that was longer than 2.5 cm.

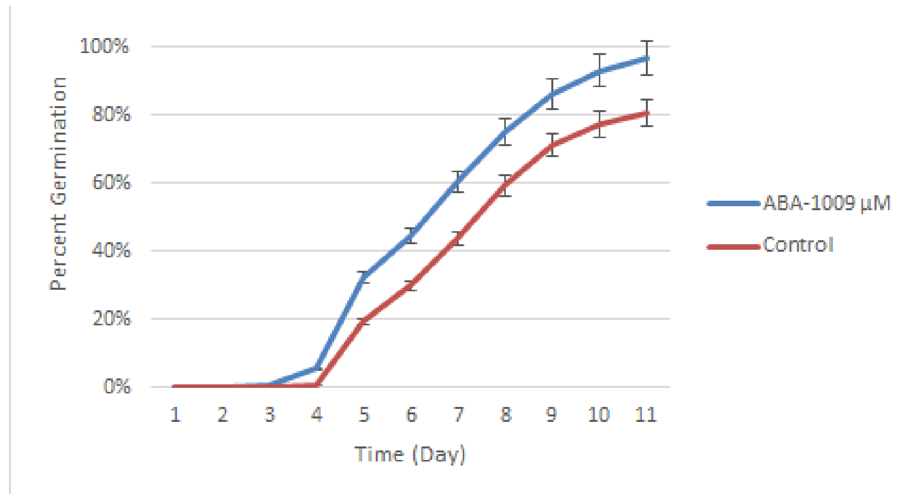
Compared to ABA-treated seeds, ABA+ ABA 1009 treated seeds were better for the average radicle growth. Meanwhile, ABA+ ABA 1009 treated group had higher percent germination than the ABA-treated group. These results indicated that the application of ABA 1009 not only accelerated the germination, but also promoted the radicle growth in the presence of exogenous ABA. The efficacy of ABA 1009 included, but not limited to the germination stage. ABA 1009 was effective in promoting radicle elongation during seedling establishment stage. The long-lasting effect of ABA 1009 has significant meaning in field application. The goal of this study is to design ABA antagonists, which can promote seed germination in early spring. An effective ABA antagonist should not only promote seed germination, but also ensures the survival of the seedlings under LT. The results suggested that ABA 1009 had a long-

lasting effect in promoting germination as well as radicle elongation when exogenous ABA was present. One of the future foci could be to conduct this experiment at LT and to confirm the effect of ABA 1009 in promoting radicle growth.

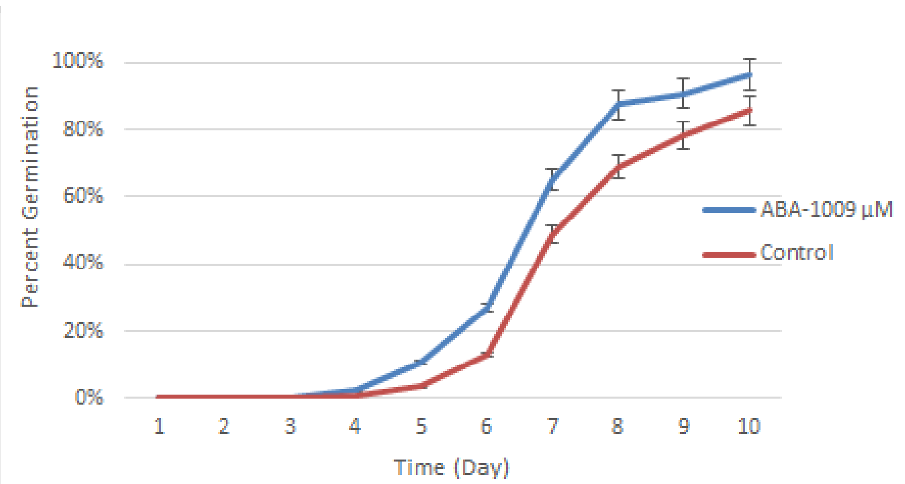
Germination test with ABA 1009 on canola seeds with dormancy variations

Five different canola varieties, which were expected to have variations in dormancy, were tested in this experiment. Among which, NAM 47 and NAM 49 are high-dormancy seeds while NAM 0, NAM 72 and NAM 79 were low-dormancy seeds. Although there were variations in dormancy among the tested cultivars, all ABA 1009 treated seeds had higher percent germination compared to control, which indicated that ABA 1009 was able to exhibit antagonist activities on different canola cultivars (Figure 4.13 a-e). However, the extents of antagonism were different among the cultivars. For NAM-47, the biggest difference of percent germination between the ABA 1009-treated group and the control group was 17%. The biggest difference for NAM-49 was 19%. It was 26% for NAM-0, 11% for NAM-72 and 23% for NAM-79.

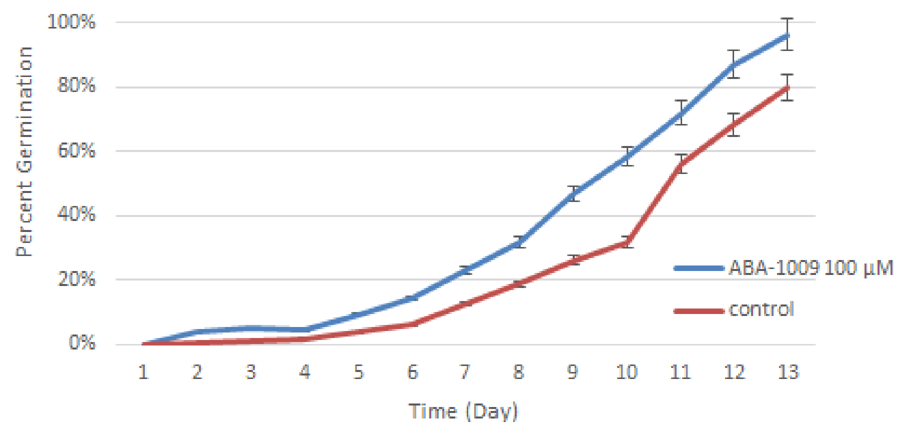
a.



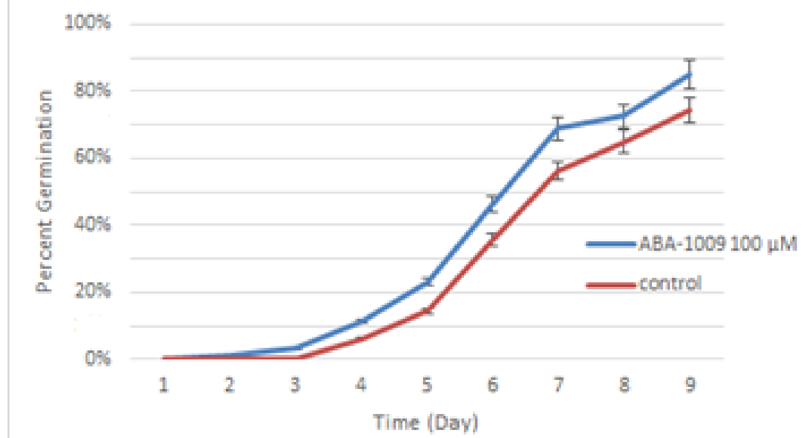
b.



b.



d.



e.

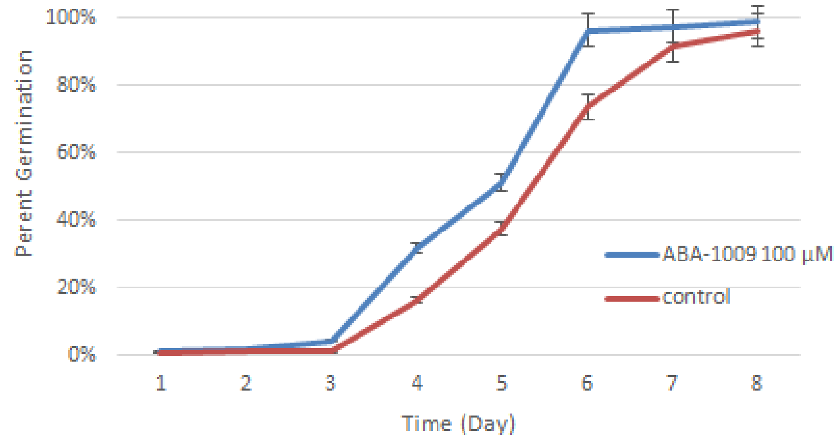


Figure 4.13 a. Percent germination of NAM- 47 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **b**. Percent germination of NAM- 49 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **c**. Percent germination of NAM- 0 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **d**. Percent germination of NAM- 72 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions. **e**. Percent germination of NAM- 79 canola seeds (high dormancy) treated with ABA 1009 at 5°C conditions (December 2017). For all cultivars listed above, ABA 1009-treated seeds had higher percent germination.

The dormancy variation among these canola cultivars was not as had been predicted. Most seeds germinated within 10 days despite the cultivars. The lack of dormancy variation might be attributed to the independent dormancy cycle of each cultivar. Each of these cultivars might undergo an independent dormancy cycle, which had their own periodicity that is different from others. Therefore, they might exhibit disparate dormancy status at a specific time point, leading to an indistinctive dormancy variation. In this case, the lack of dormancy variation could be the result of low-dormancy seeds happened to be at imposed dormancy phase and high-dormancy seeds were at dormancy release phase. One of the future foci could be to conduct a series of germination assays with these canola cultivars at different time points. It will help to reveal their dormancy cycles and contribute to a better assessment of the antagonism of ABA 1009. Overall, ABA 1009 was effective on different canola cultivars, but its ability to improve seed germination varies among cultivars.

Hormone analysis of canola treated with ABA antagonists

This hormone analysis measured the levels of ABA, its catabolites and four GAs. The result only showed the changes in ABA and its metabolite concentrations since the change of GAs were minor and below the detection limit.

In the control group, the ABA concentration decreased as the germination began, which indicated the biosynthesis of ABA is slower than its catabolism in the control seeds. DPA, PA, 7OH-ABA, and neo-PA decreased over time. Only ABA-GE remained constant, which indicated the glucose conjugation might not be involved in seed germination processes. There was only a small amount of PA left in the seeds over time due to its metabolism to DPA. Most of ABA was catabolized and turned into DPA in this case since the amount of DPA was greater

than other metabolite and its trend was correlated to the decreases of ABA. Therefore, the main pathway of ABA degradation is hydroxylation of 8'-carbon atom of ABA. 7-hydroxylation and 9-hydroxylation were also involved in ABA degradation processes since they had the same trend as ABA. Only a small amount of ABA went through these two pathways (Figure 4.14).

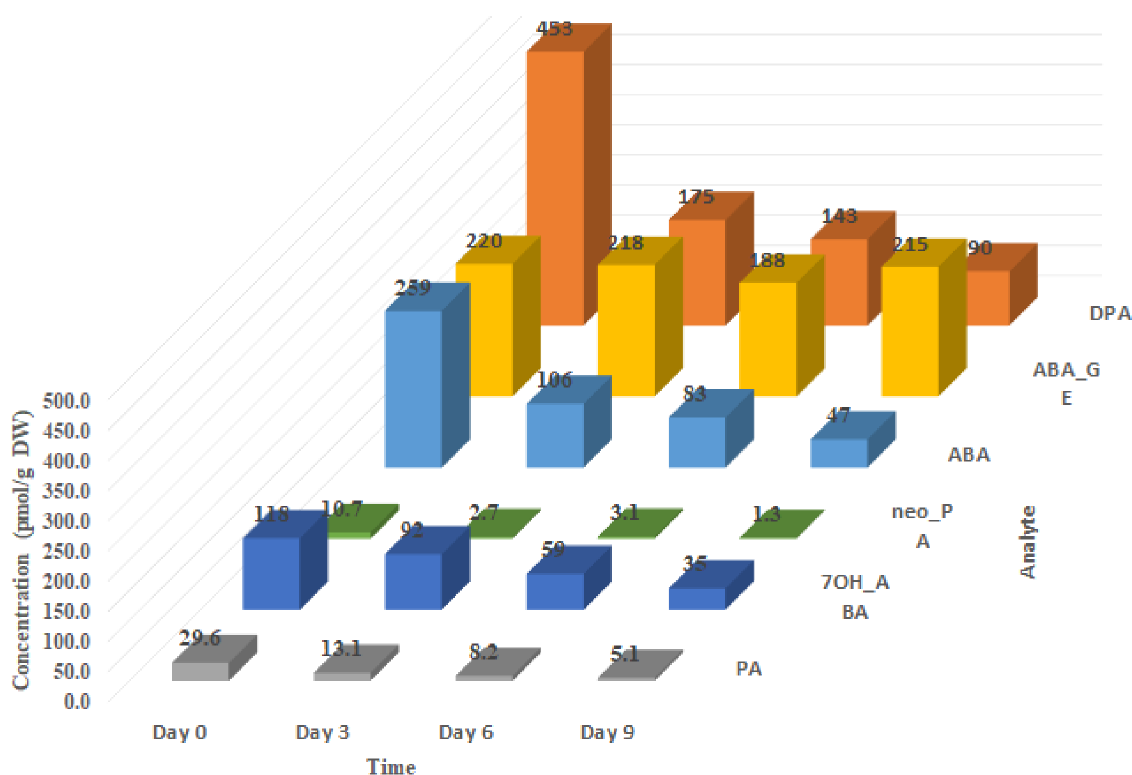


Figure 4.14 Changes in ABA metabolite concentrations in the seeds of *Brassica napus* (canola) from day 0 to day 9 in the LT control group (water at 5°C). ABA and its metabolites decreased over time. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.

Table 4.1 Standard deviation of abscisic acid (ABA) and its metabolites in the control group (treated with water at 5°C) in hormone analysis of canola.

Time (Day)	ABA	DPA	PA	ABA_GE	7OH_ABA	Neo-PA
0	259±1.53	453±13.0	29.6±2.13	220±6.03	118±2.71	10.7±0.10
3	106±1.00	175±6.66	13.1±0.81	218±4.00	92±2.51	2.7±0.15
6	83±1.00	143±5.69	8.2±0.44	188±7.02	59±2.70	3.1±0.31
9	47±2.31	90±4.51	5.1±0.21	215±9.02	35±0.97	1.3±0.15

ABA and GA play crucial roles in regulating seed germination under favorable conditions. Seeds undergo up-regulation of GA level and down-regulation of ABA content upon germination (Shu et al. 2016). The regulatory role of ABA is even more important under stress conditions such as LT and high salinity. Under stress conditions, the GA biogenesis is impaired, seed germination is regulated by ABA (Zhang et al. 2010). The results showed a significant decrease of ABA level over time, which indicated that ABA was down-regulated during LT germination. GA was not detected in this study, which is in agreement with Zhang et al. (2010) finding in LT canola germination. In LT seeds germination, the GA biogenesis is inhibited, the initiation of seed germination might solely rely on the down-regulation of ABA. Therefore, seeds germinated much slower in LT.

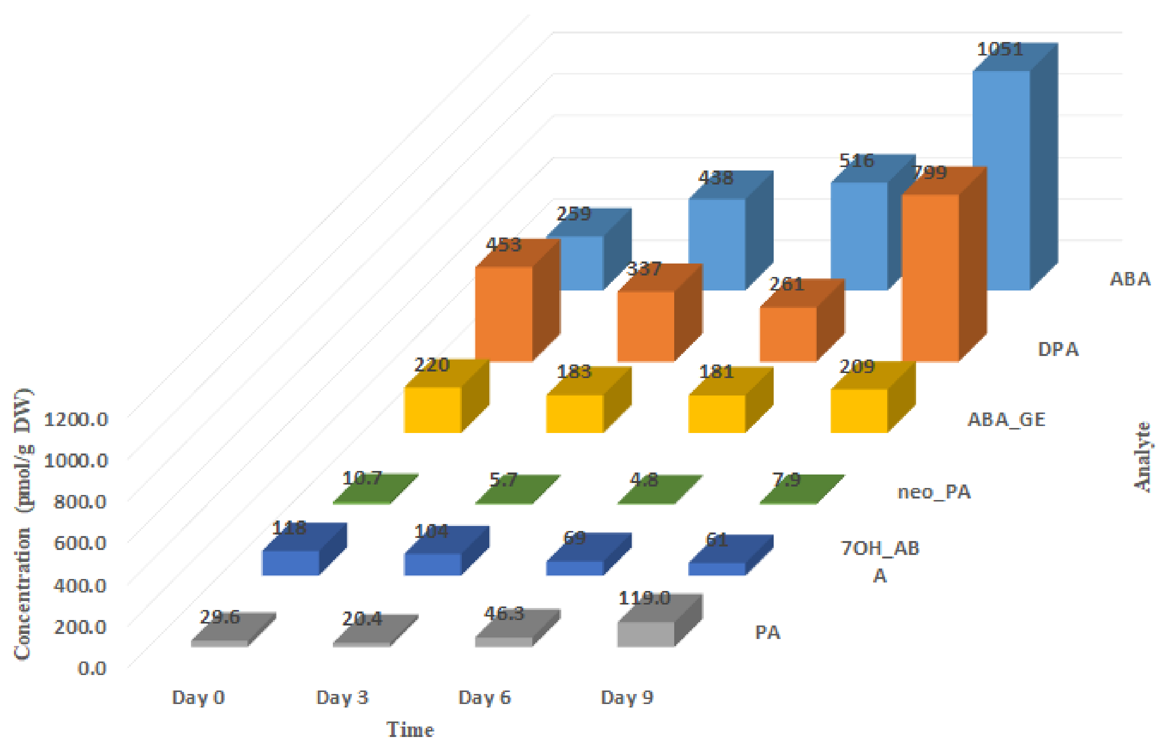


Figure 4.15 The application of ABA 1009 at 300µM to *Brassica napus* (canola) at LT (5°C) caused changes in ABA metabolite concentrations. ABA level increased over time. DPA

decreased at first 6 days and increased 3-fold on day 9. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.

Table 4.2 Standard deviation of abscisic acid (ABA) and its metabolites in the treatment group (treated with ABA 1009 at 5°C) in hormone analysis of canola.

Time (Day)	ABA	DPA	PA	ABA_GE	7OH_ABA	Neo-PA
0	259±1.53	453±13.0	29.6±2.13	220±6.03	118±2.71	10.7±0.10
3	438±2.00	337±1.73	20.4±0.72	183±7.81	104±3.52	5.7±0.46
6	516±13.65	261±2.89	46.3±1.79	181±4.16	69±1.16	4.8±0.23
9	1051±6.43	799±22.19	119.0±1.95	209±9.17	61±1.52	7.9±0.46

In comparison to the result shown in Figure 4.14, the level of ABA increased over time, which suggests the application of ABA 1009 caused the up-regulation of ABA biosynthesis processes. The amount of DPA is larger than other metabolites, suggesting that most of ABA were catabolized via 8-hydroxylation and was turned into DPA. The level of DPA in the treatment group dropped on day 3 and day 6, but at a much slower rate than the control group. The trend of DPA indicated that the application of ABA 1009 increased the ABA degradation activities. More DPA might be produced due to the up-regulated ABA degradation, which results in higher DPA accumulations in seeds. At day 9, the level of DPA increased to 799 pmol/g, which was a 3-fold increase compared to day 6. This might be due to the accelerated ABA degradation that produced more DPA than before, and the downstream reactions were unable to catabolize them in time. So, the increased level of DPA at day 9 also suggests the up-regulation of ABA degradation activities. The increases of ABA and the metabolite levels supported the hypothesis that the application of effective ABA antagonist affects the ABA

concentration by altering the rate of ABA biosynthesis and degradation pathways. No change in GAs was detected in treated seeds, which suggests ABA 1009 did not act on GA related pathways.

When ABA 1009 was applied to canola seeds, ABA degradation activities were up-regulated, accelerating the pace to achieve proper ABA/GA ratio and hence resulting in a fast germination. ABA content was continuously accumulated, probably owing to the excess amount of ABA 1009. However, the increased ABA level did not impact the germination. It can be explained as follows. Proper ABA/GA ratio might be achieved in the early phase when ABA accumulation was not significant leading to seed germination. The impact of the excess ABA 1009 was limited at that time since there was only a small uptake of ABA 1009 within the seeds. By the time ABA accumulation became significant, most of the seeds have already undergone the germination.

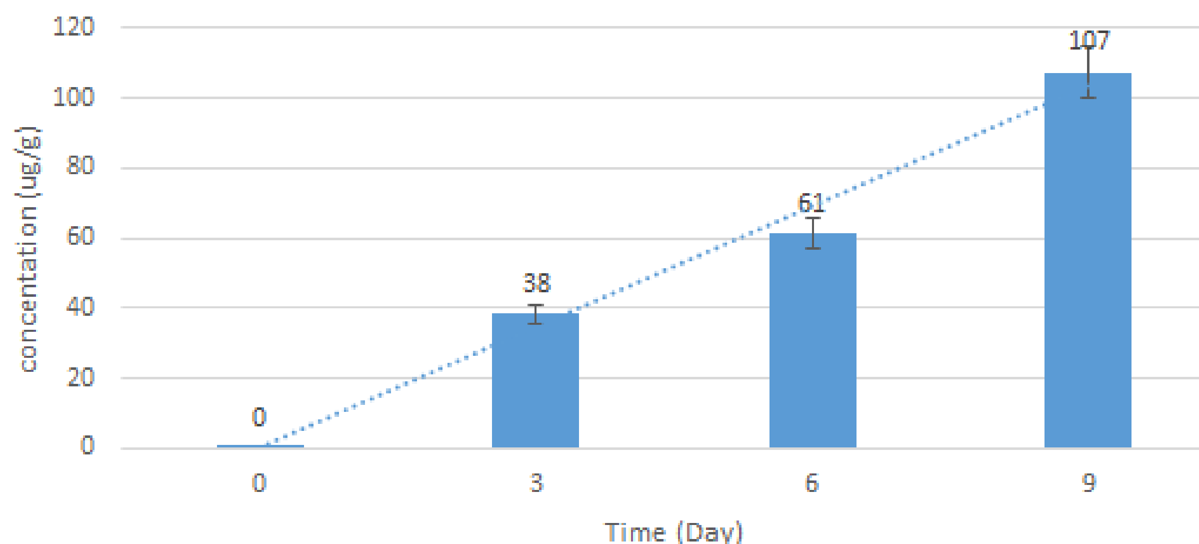


Figure 4.16 Change in ABA 1009 concentrations from day 0 to day 9 in the treatment group. Seeds were treated with ABA 1009 at 300 μM and incubated at LT (5°C). ABA 1009 concentrations increased linearly over time.

Seeds in the treatment group were constantly absorbing ABA 1009 from the treatment solution since there was a linear correlation of ABA 1009 concentrations over time (Figure 4.16). In combination to the result shown in Figure 4.15, the continuous absorption of ABA 1009 was correlated with the increases in the ABA generation activities. The application of ABA 1009 promoted seed germination by accelerating the rate of ABA degradation. Too much ABA 1009 (the amount of ABA 1009 is at least 400-fold larger than the amount of ABA and its metabolites on day 9) could also cause the increases of ABA biosynthesis activities. The overdosage effect could hinder the germination process since seeds produce more ABA. Reducing exposure to ABA 1009 at the later stage might help to avoid the situation of overdose effect.

Gene expression analysis of canola treated with ABA 1009

9-cis epoxycarotenoid dioxygenase (NCED), encoded by genes from NCED family, is a key enzyme involved in the committed step of ABA biosynthesis, catalyzing the cleavage of C40-carotenoid cis-xanthophylls (Schwartz et al., 1997). Genes from CYP707A family encode ABA 8'-hydroxylases, essential enzymes in ABA catabolism (Kushiro et al., 2004). Both NCED and CYP707A gene families have proved to be essential in regulating dormancy status in Arabidopsis (Millar et al., 2006). However, their roles remain unknown in canola. Therefore, NCED5, NCED6, and NCED9 from NCED family and CYP707A1-4 from CYP707A gene family were selected for the gene expression study to reveal their roles and their response to ABA 1009. The results of hormone analysis suggested that large amounts of ABA were synthesized in the presence of 300μM ABA 1009. Genes from AAO family encode aldehyde oxidase, an essential enzyme involved in the last step of ABA biosynthesis (Seo et al. 2000).

This gene family might be involved in the up-regulation of the ABA level. Hence, AAO3 and AAO4 were selected for this study.

The expression levels of AAO3, AAO4 and NCED5, NCED6, and NCED9 were associated with ABA biosynthesis activities. The fold change from day 0 to day 3 was not the same for all the tested genes. However, the ABA content within the seeds continuously increased over time. Together, these data suggested that the selected genes might not be attributed to ABA biosynthesis in the first 3 days (Figure 4.17).

From day 3 to day 6, the expression levels of NCED5, NCED6, and NCED9 had 1.69, 2.27, 2.67- and 1.64-folds increase, respectively. The cold stress, as well as the excess ABA 1009 within the seeds, enhanced the biogenesis of ABA. The expression of AAO3, NCED5, NCED6, and NCED9 were induced and more aldehyde oxidase and 9-cis epoxycarotenoid dioxygenase were encoded to catalyze the reactions in ABA biosynthesis.

From day 6 to day 9, the levels of AAO3, NCED5, NCED9 went up to 2.82, 3.69 and 2.71 folds. The level of NCED6 dropped to 1.87 folds. Meanwhile, AAO4 increased from 1 to 2.72 folds. As ABA 1009 was consistently absorbed, the levels of ABA 1009 accumulation within the seeds was 107 μg/g at day 9. The overdosage effect of ABA 1009 was more severe. The redundant ABA 1009 left in seeds caused changes in ABA biosynthesis pathways. The expression of AAO4 was induced while NCED 6 was impaired. The up-regulation of AAO3 as well as the activation of AAO4 lead to an increase of aldehyde oxidases. This enzyme is involved in the oxidation of abscisic aldehyde, the final step of the ABA biosynthesis.

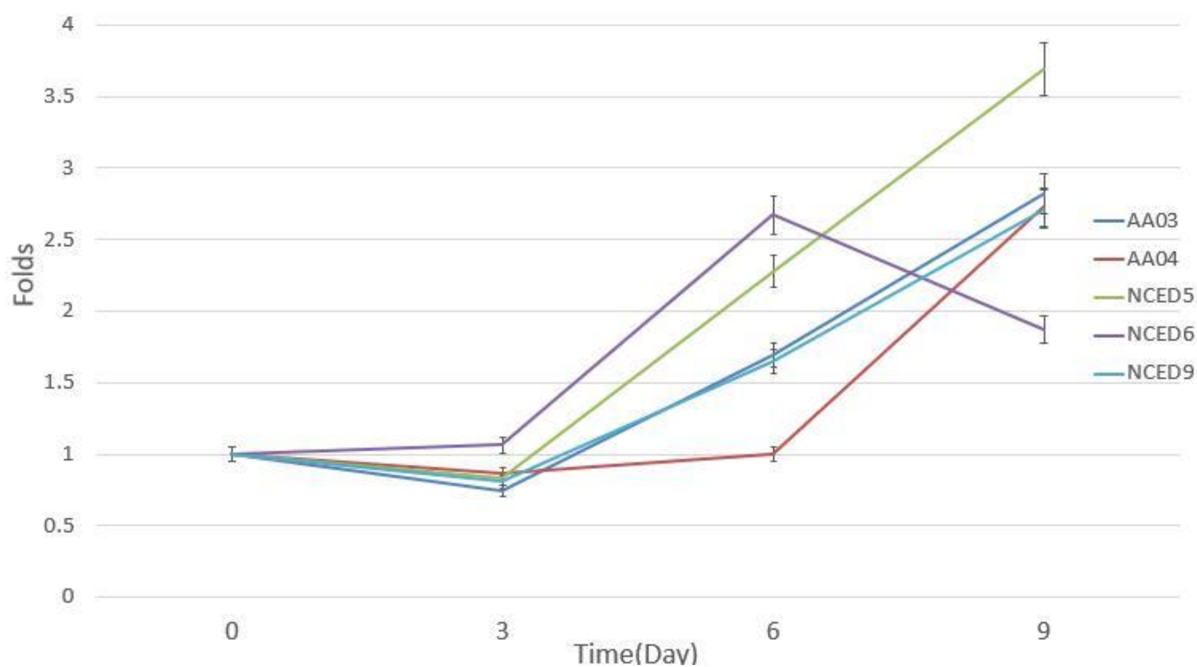


Figure 4.17 Fold changes of the selected genes from the AAO gene family and NCED gene family in canola seeds treated with ABA 1009. Selected genes had different extent of up-regulation.

CYP707A1-4 were selected for gene expression analysis related to ABA degradation. Among which, CYP707A1-3 did not have distinct fold changes over time. For CYP707A4, its expression slightly decreased from day 0 to day 3, but then, its expression level had 3 folds increases from day 3 to day 6 and it reached a 3.5-fold increase at day 9. The trends of CYP707A4 was correlated with the change of dihydrophaseic acid (DPA) in the treatment group (Figure 4.18), which indicated that CYP707A4 might be the candidate genes for encoding 8'-hydroxylase.

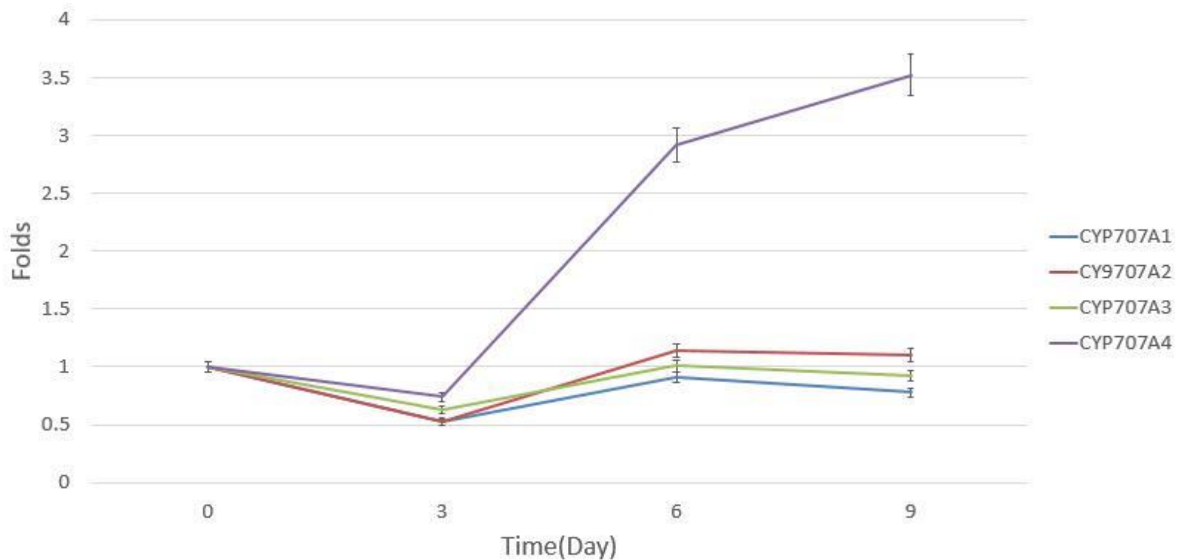


Figure 4.18 Fold changes of selected genes from CYP707A gene family from day 0 to day 9. The expression level of CYP707A4 had a significant increase from day 3.

The increased expression of NCED 5,6,9 and CYP707A4 supported the hypothesis that genes from NCED and CYP707A family are involved in the regulation of ABA levels in ABA antagonist-treated canola seeds. A study on Arabidopsis suggested that the expression of NCED 5 and NCED 6 genes increased in the first few hours and declined afterward, while NCED 9 expression continuously declined (Millar et al. 2006). In this study, NCED 5, 6, 9 exhibited completely different expression patterns, which might be attributed to the cold stress and the presence of ABA 1009. The expression of CYP707A4 corresponded to the dynamics of ABA metabolites, which suggested that CYP707A4 was involved in controlling ABA degradation pathway. CYP707A1, CYP707A2 and CYP707A3 consistently expressed at the same levels over time, which is in agreement with Millar et al. (2006) study in Arabidopsis. In conclusion, genes from AAO, NCED and CYP707A families were involved in canola germination, but their expression patterns were different from Arabidopsis.

Germination assay with ABA antagonists in soybean

ABA 1009 was effective in promoting canola germination either at LT or in the presence of exogenous ABA. However, its effect on other crops remains unknown. Similar to canola, ABA signaling pathways in many plants are triggered by the activation of ABA receptors. ABA 1009 was designed to bind to ABA receptor and to interfere with the interaction between ABA receptors and PP2C. It is possible that ABA 1009 shows antagonisms on other crops as long as it could effectively inhibit the PYL-PP2C interaction. Moreover, the activities of ABA analogs vary among different types of ABA receptors. For example, PAO4 reported by Takeuchi et al. (2015), exhibited better antagonist activities on monomeric ABA receptors over dimeric receptors. ABA 1009 might have different extents of antagonism on other crops.

Soybean and chickpea were selected for further study of ABA 1009. Both crops are economically important for the agricultural industry in Canada. Like canola, their germinations are strictly limited by the cold temperature. Soybean and chickpea are less cold-resistant than canola as they have higher temperature requirements for germination. The minimum threshold temperature for soybean and chickpea germination is 7°C. It might provide a better assessment of the effects of ABA 1009 if it is applied on less cold-resistant crops. Hormone analysis was conducted on soybean and chickpea without the application of ABA 1009, seeking the differences in hormonal change between the optimal and the LT conditions.

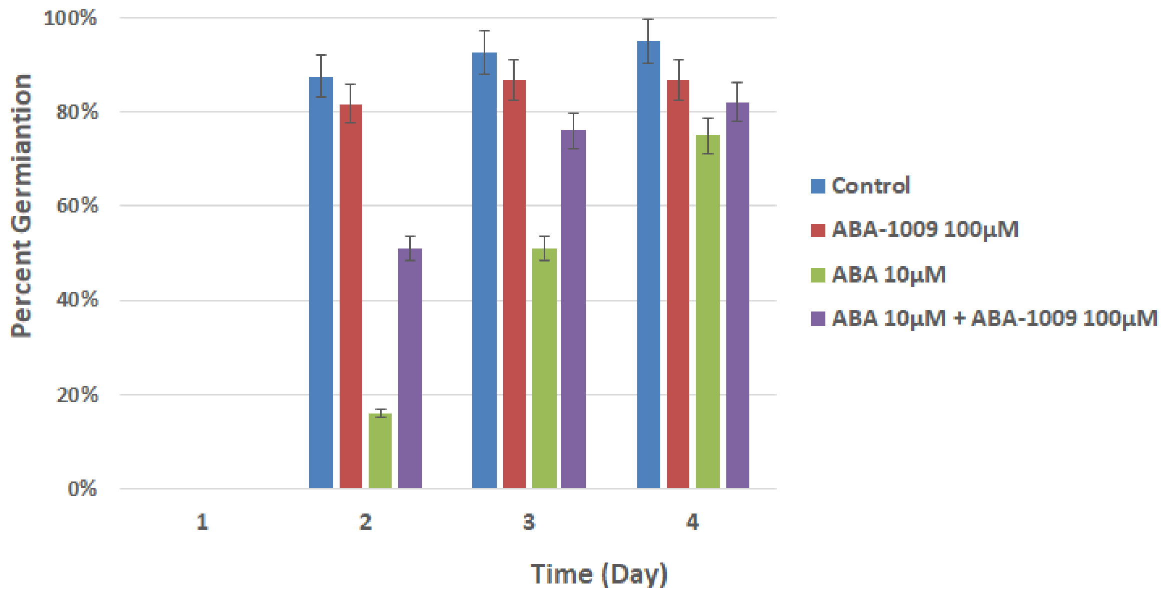


Figure 4.19 Percent germination of soybean seeds treated with ABA, ABA+ABA 1009 at 22°C. ABA 1009 was effective in counteracting exogenous ABA.

The percent germination of ABA + ABA 1009 treated soybean seeds was significantly higher than ABA-treated seeds in the first 3 days. ABA 1009 was effective in counteracting the effect of exogenous ABA in soybean, which supported the hypothesis that ABA antagonists can be applied to seeds to promote soybean germination in the presence of ABA. The effect of ABA 1009 was most distinctive at day 2. More than 50% of seeds in ABA + ABA 1009 treated group germinated on day 2, while there were only 16% germinated seeds in ABA-treated groups. Control groups had higher percent germination than ABA + ABA 1009 treated seeds, which indicated the application of ABA 1009 did not completely overcome the exogenous ABA (Figure 4.19). ABA 1009 at 100 µM were used in this study. This might not the optimal concentration for soybean so that the application of ABA 1009 could only partially counteract the effect of ABA.

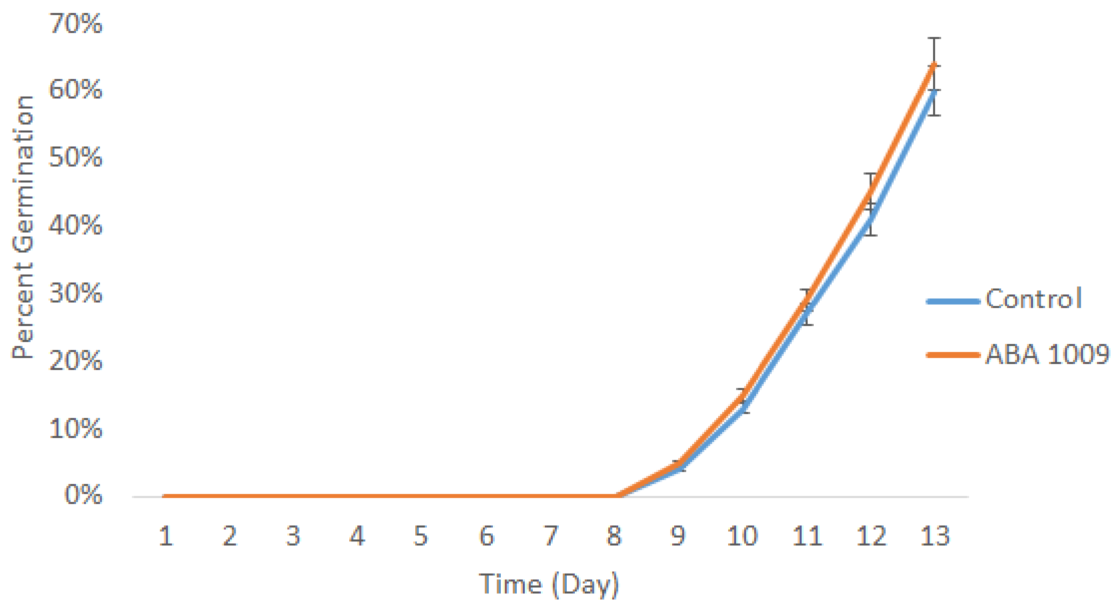


Figure 4.20 Percent germination of soybean seeds treated with ABA 1009 at 100 µM, control (1% DMSO) at 7°C. ABA 1009 treated seeds had similar percent germination as control.

Soybean seeds did not germinate until day 9 in both control and ABA 1009-treated groups at 7°C (Figure 4.20). In comparison to canola and chickpea, soybean seeds had a longer imbibition phase before the commencement of germination. Soybean seeds were incubated at 7°C in LT germination assays, however, the germination rate of soybean was slower than canola (treated at 5°C) and chickpea (treated at 7°C). Together, these data suggested that soybean seeds were less cold-tolerant and their germinations were severely suppressed by LT.

The percent germination of ABA 1009-treated seeds was similar to that of control seeds indicating that ABA 1009 at 100 µM did not exhibit antagonism on soybean under LT. High level of ABA in soybean seeds might be attributed to the induced ABA biosynthesis and impaired ABA catabolism, triggered by cold stress. ABA 1009 at 100 µM might be insufficient to counteract such large amount of ABA, resulting in similar percent germination between ABA 1009 -treated and control seeds. The lack of effect of ABA 1009 could also be explained by a

possibility that ABA might not be the limiting factors in LT soybean germination. Soybean exhibits more physical and less physiological dormancy compared to canola (Qutob et al. 2008). Therefore, the impact of ABA on LT soybean germination might be less significant. The result of LT soybean germination test failed to support the hypothesis that ABA antagonists can be applied to seeds to promote soybean germination at LT. However, with improvement in the designs, some of the future ABA antagonists might exhibit promoting effect on LT soybean germination.

Hormone analysis of soybean seeds

The GA level was below the detection limit during soybean germination, which indicated that the germination process of soybean seeds was mainly decided by the change in ABA concentration. The concentration of ABA had sharp decreases from day 0 to day 1 as the germination began (Figure 4.21). Other metabolites (PA, DPA, ABA-GE, 7OH- ABA) decreased largely along with the change of ABA. Among which, PA had the highest concentration compared to others, which suggests that the main pathway for ABA degradation in soybean seeds was 8- hydroxylation. Unlike canola and chickpea, only a small portion of PA in soybean was further reduced to DPA. Therefore, there were large amounts of PA but small amounts of DPA in soybean. 7- hydroxylation and glucose conjugation were involved in ABA degradation but their effects were minor since the 7OH- ABA and ABA-GE level was significantly lower than the level of PA. From day 1 to day 2, the decreases of PA, DPA, ABA-GE, 7OH- ABA slowed down. The ABA level increased. From the results of soybean germination test, most of the seeds germinated on day 2 (Figure 4.19). High GA/ABA ratio is required for the commencement of the germination. However, the majority of seeds had

completed the germination process at day 2. There was no need to maintain the high GA/ABA ratio. Therefore, the increase of ABA level at the later phase did not delay germination. Furthermore, hormone levels, as well as their regulatory roles, change across different developmental activities. An increase of ABA level might be required for the regulations of the later development. Therefore, increased amounts of ABA and slower rates of ABA degradation were observed on day 2.

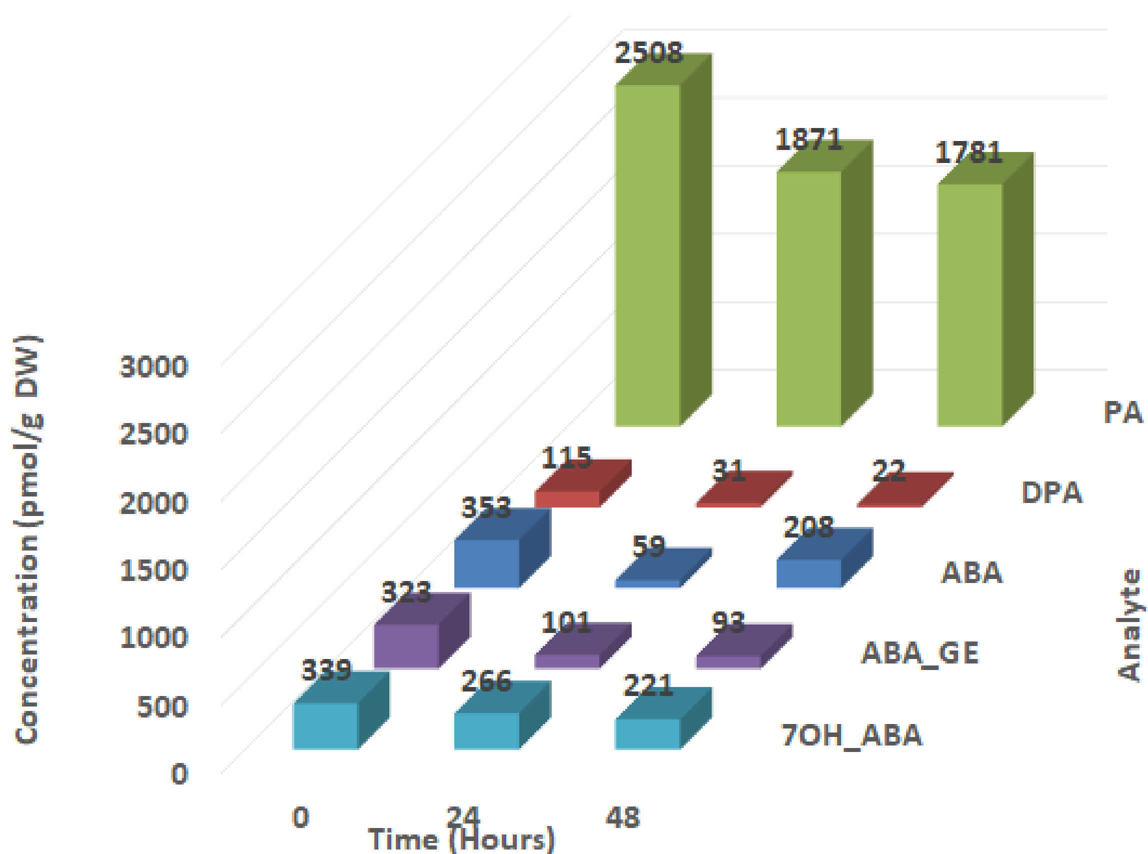


Figure 4.21 Changes in ABA metabolite concentrations of *Glycine max* (soybean) seeds cultured at 22°C. ABA level decreased at day 1 and increased on day 2. All metabolites had different extents of decrease. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.

GAs were not detected in the seeds during the germination of soybean at LT. The concentration of PA was the highest, which suggests that the major degradation pathway for

LT treated soybean was 8- hydroxylation. Different temperature treatments did not alter the degradation pathway. The levels of ABA, PA, ABA-GE dropped down over time, while the level of 7OH-ABA did not have significant fluctuation (Figure 4.22). The decrease rates of these metabolites were much slower in LT treated seeds. Slow decreases of ABA metabolites followed by a slow decrease of ABA. The ABA level of LT treated seeds at day 2 were still higher than the ABA level of RT treated seeds at day 1, which supported the hypothesis that ABA concentration in the LT treated seeds is higher than in the RT treated seeds during germination. Under optimal concentration, the ABA degradation activities increased largely, followed by a sharp decrease of ABA, resulting in seeds germinated rapidly. When seeds were cultured at LT, the ABA degradation was slow. It took a longer time for ABA levels to drop down to the threshold. Therefore, LT treated seeds took longer to germinate.

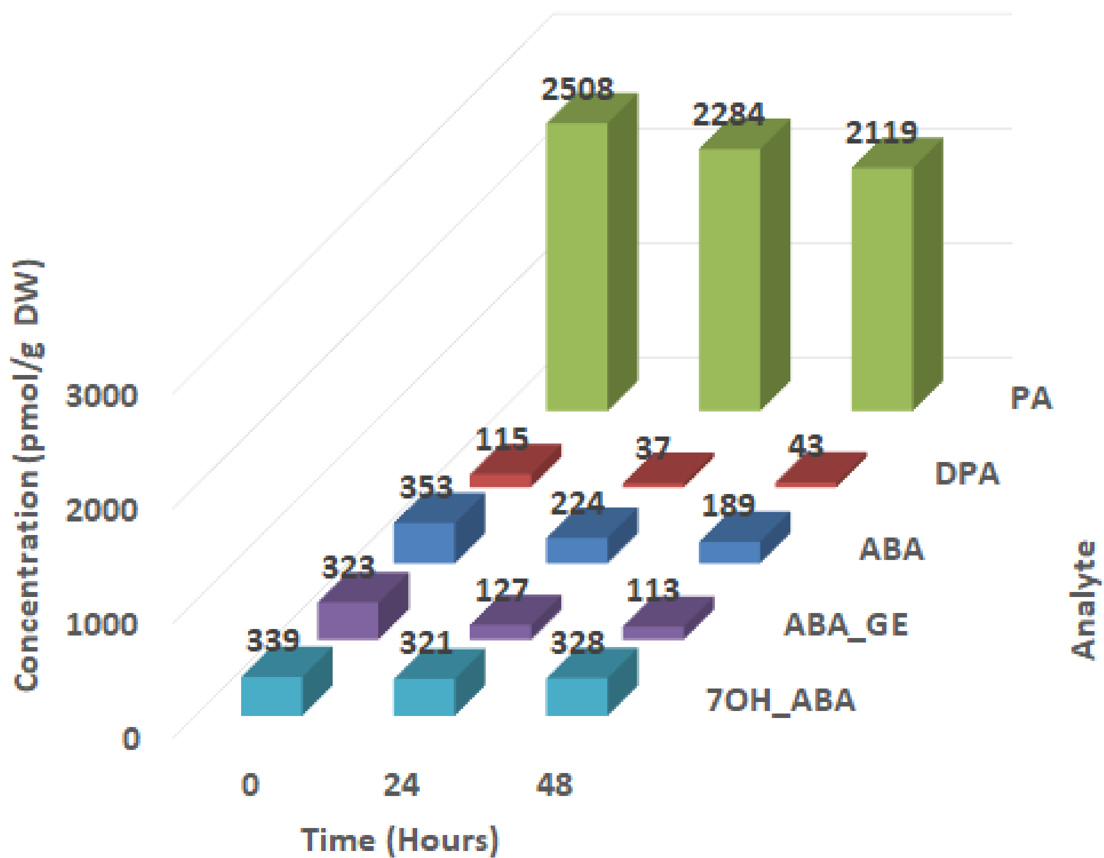


Figure 4.22 Changes in ABA metabolite concentrations of *Glycine max* (soybean) seeds

cultured at 7°C. ABA and its metabolites (except for 7OH-ABA) decreased over time. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.

Germination assay with ABA antagonists in chickpea

Control group had significantly higher percent germination compared to ABA-treated and ABA 1009 + ABA-treated groups. Except for the control group, the other three groups could not be differentiated from each other in terms of percent germination, which means the application of ABA 1009 had no effects in this case (Figure 4.23). It might be the results of improper concentrations. Additionally, ABA 1009 might not be effective to promote chickpea seed germination. This result failed to support the hypothesis that ABA antagonists can be applied to seeds to promote chickpea germination in the presence of ABA. There might be better ABA antagonists in the future, which exhibit promoting effect in counteracting the exogenous ABA.

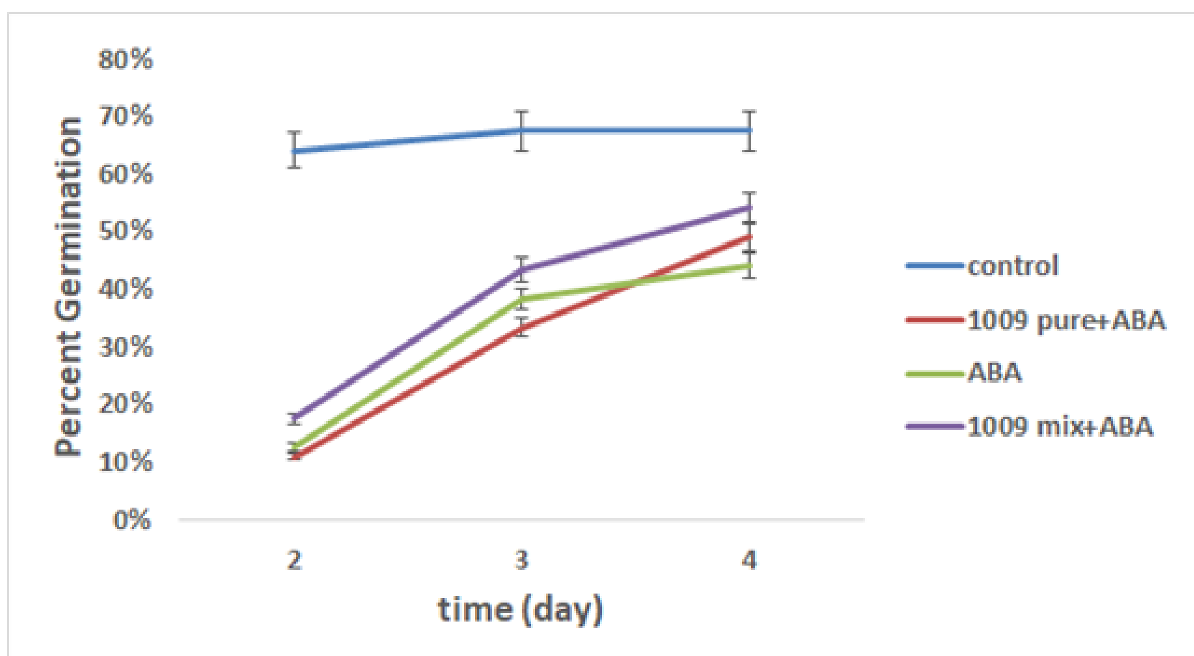


Figure 4.23 Percent germination of chickpea seeds treated with ABA, ABA+ ABA 1009 at

22°C. ABA 1009 failed to counteract exogenous ABA since there was similar percent germination for ABA and ABA+ ABA 1009 treated groups.

The percent germination of ABA 1009 treated seeds was not significantly different from control seeds under 7°C conditions, which failed to support the hypothesis that ABA antagonists can promote seed germination at LT (Figure 4.24). The results of RT and LT germination test on chickpea were consistent, where control and ABA 1009 treated seeds had similar germination percentage overtime. ABA 1009 might not be effective on chickpea, or the concentration applied in these two studies was not in the range.

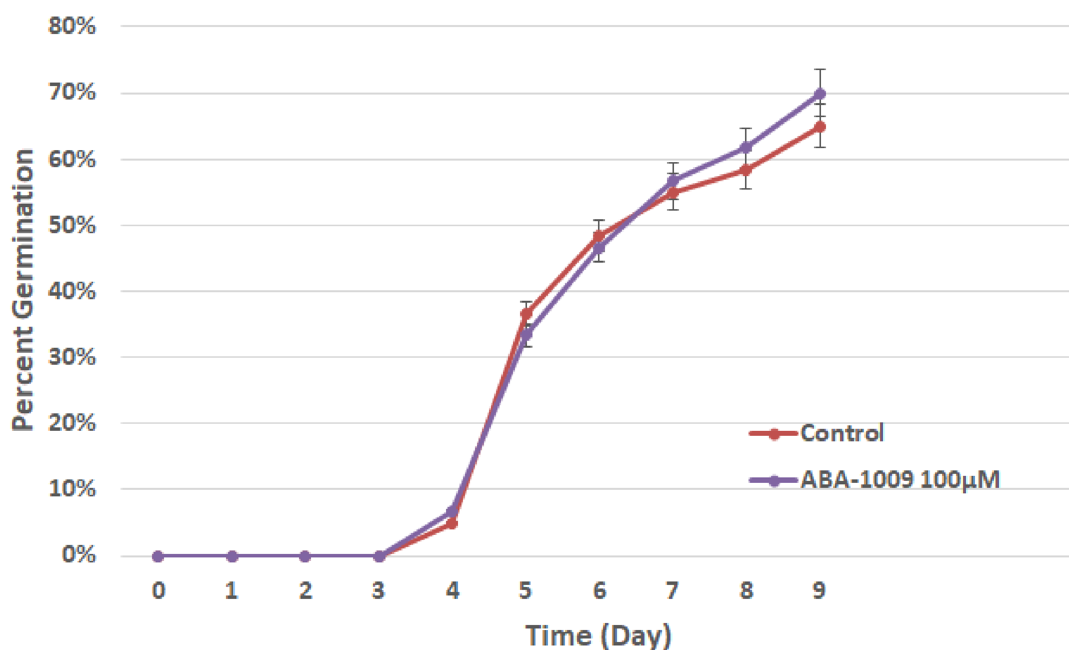


Figure 4.24 Percent germination of chickpea seeds treated with ABA 1009 at 100 μM and control (1% DMSO) at 7°C. The percent germination of ABA 1009-treated seeds was not significantly different from the control group.

Hormone analysis of chickpea

GAs were not detected during the chickpea seed germination at RT. Among all ABA metabolites, DPA had the highest concentration, therefore, 8- hydroxylation was the major

ABA degradation pathway. PA in chickpea was further reduced to DPA so there was only a small amount of PA left in the seeds. ABA level decreased largely from day 0 to day 3 (Figure 4.25). The decreased level of ABA allowed for the germination of chickpea seeds. 7-hydroxylation and glucose conjugation were involved in ABA degradation. The role of 7-hydroxylation was minor since its concentration was small and it changed within a narrow range over time.

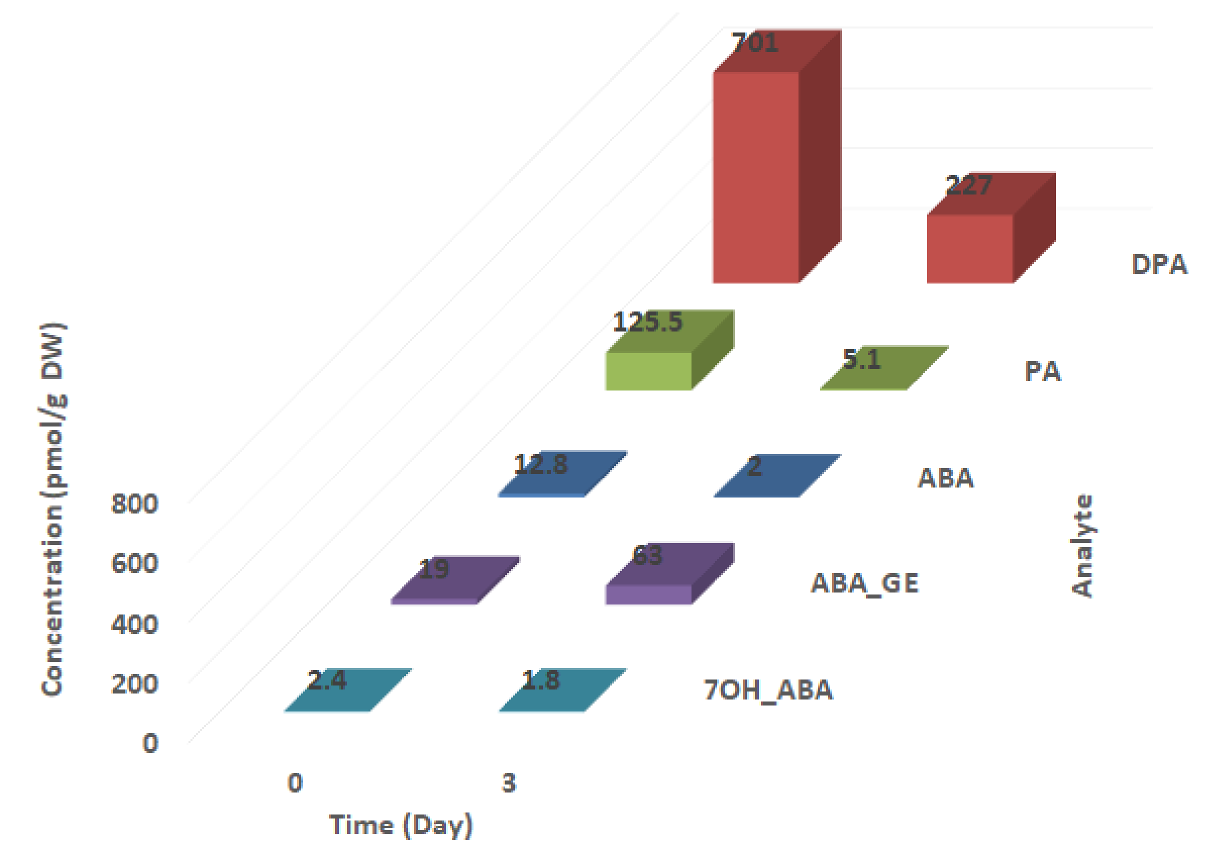


Figure 4.25 Changes in ABA metabolite concentrations of *Cicer arietinum* (chickpea) seeds cultured at 22°C. ABA and its metabolites (except for ABA_GE) decreased from day 1 to day 2. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid-glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.

Table 4.3 Standard deviation of abscisic acid (ABA) and its metabolites in room temperature treated group (22°C) in hormone analysis of chickpea.

Time (Day)	ABA	DPA	PA	ABA_GE	7OH_ABA
0	12.8±2	701±38	125.5±5.2	19±1	2.4±0.7
3	2±0.2	227±5	5.1±1.0	63±7	1.8±0.5

A small amount of GA3 and GA 4, each at 0.6 pmol/g, were detected at day 12 in LT treated seeds, which suggests the GAs were involved in chickpea germination under LT, however, they acted at the later phase. The amount of ABA largely decreased from day 0 to day 3, however, the ABA levels in LT treated seeds were still twice larger than RT treated seeds, which supported the hypothesis that ABA concentration in the LT treated seeds is higher than in the RT treated seeds during germination. The ABA levels fluctuated in a small range from 1.6 to 5 since day 3. Seeds received sufficient water from day 0 to day 3, which initiated the germination process after the amount of ABA decreased (Figure 4.26). The seeds treated under LT, the cold stress from ambient slowed down the germination process by increasing the ABA production. The combination of these two abiotic factors, sufficient water condition and LT, acted on chickpea seeds, contributed to fluctuation of ABA levels over time. This cycle was only observed in chickpea hormone analysis. It might due to the unique biology of chickpea itself, or it might be caused by different time point selection among canola, soybean and chickpea hormone analysis.

The major ABA degradation pathway of chickpea was 8-hydroxylation since DPA, the metabolites of 8-hydroxylation, had the highest concentration and it changed along with ABA.

The levels of ABA-GE increased over time, which suggests that the glucose conjugation was involved in inhibiting ABA and the metabolite of this pathway, ABA-GE, had a slow rate to catabolize to downstream products. Therefore, ABA- GE accumulated over time. The amount of 7OH- ABA increased from day 0 to day 6, which suggested the 7-hydroxylation activities increased before day 6. The decrease of 7OH- ABA might be due to the decrease of 7-hydroxylation activities. Or the accumulation of 7OH- ABA might alter its catabolic rate. Therefore, the amount of 7OH- ABA decreased after day 6.

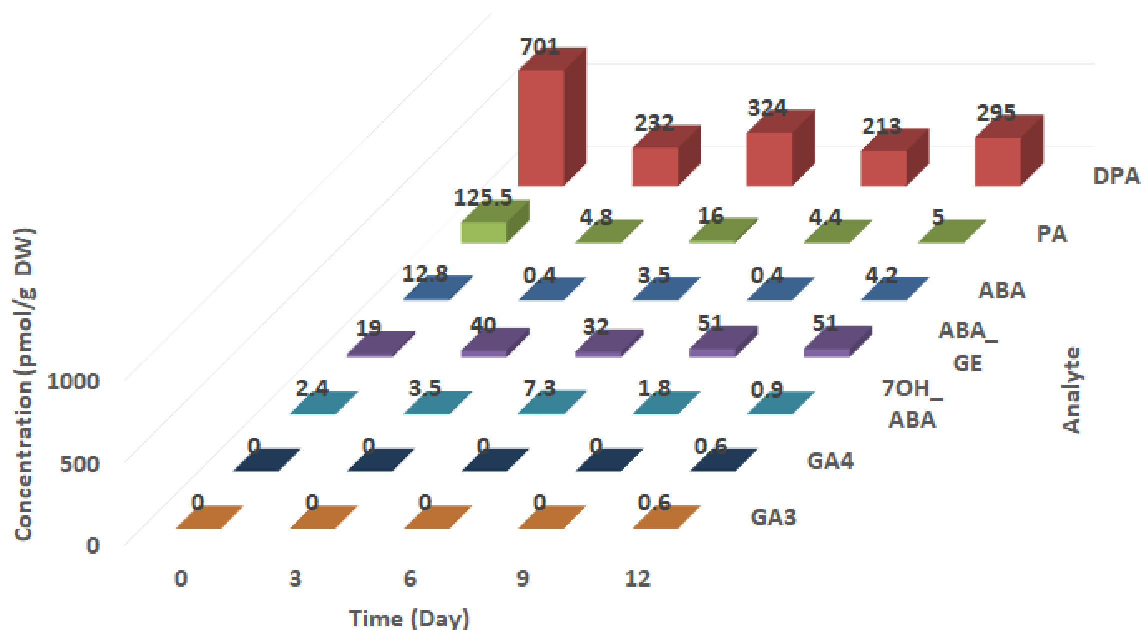


Figure 4.26 Changes in ABA metabolite concentrations of *Cicer arietinum* (chickpea) seeds cultured at 7°C. There were sharp decreases from day 0 to day 3 for ABA, PA and DPA. Their concentrations fluctuated from day 3 to day 12. ABA_GE level increased over time while 7OH_ABA level increased at the first 6 days and decreased afterward. ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid; ABA_GE, abscisic acid- glucose ester; neo_PA, 9'-hydroxyabscisic acid; 7OH_ABA, 7'-hydroxyabscisic acid.

Table 4.4 Standard deviation of abscisic acid (ABA) and its metabolites in low temperature treated group (7°C) in hormone analysis of chickpea.

Time (Day)	ABA	DPA	PA	ABA_GE	7OH_ABA
0	12.8±2	701±38	125.5±5.2	19±1	2.4±0.7
3	0.4±0.1	232±8	4.8±0.7	40±2	3.5±0.9
6	3.5±1.4	324±11	16±5.6	32±1	7.3±1.8
9	0.4±0.2	213±31	4.4±0.6	51±10	1.8±0.4
12	4.2±0.7	295±23	5±0.8	51±10	0.9±0.5

Chapter 5: General Discussion

ABA 1009 is an effective and readily accessible analog for canola

Eleven ABA analogs were assessed in this study. ABA 1001 was the most effective analog in promoting seed germination under LT, followed by ABA 1009. ABA 1001 is the only bicyclic tetralone analog in this study, which has a benzene ring attached to its cyclohexenone ring. This structural modification results in less flexibility of the chain, leading to a higher affinity for binding to PYL. Hence, ABA 1001 had better performance than other ABA analogs tested in this study. The synthesis of ABA 1001, however, is costly and time-consuming since this structural modification requires more than 10 steps to achieve. Though ABA 1001 has the best promotive effect among the tested analogs, the high cost of its synthesis making it unfavorable for large-scale application. ABA 1009 was the second most effective analog. It is a simple ABA analog with an addition of a hexyl group on its 3' position. The thin long chain at C-3' ensures it to fit in the 3' tunnel while the high flexibility of the base decreases its affinity for binding to PYL; therefore, its promotive effect is slightly lower compared to ABA 1001. Yet, ABA 1009 is readily accessible since its synthesis can be achieved within 2 steps. It has a significant promotive effect while it is relatively easy to synthesis, making it ideal for large-scale application.

ABA 1002 has an almost identical molecular structure as ABA 1009. The only difference between ABA 1002 and ABA 1009 is that the linker for ABA 1002 is a sulfur atom. Yet, ABA 1009 exhibits a great promotive effect for LT germination while the application of ABA 1002 did not exhibit any antagonist activities. Besides, other simple analogs with a sulfur linker, such as ABA 1004, ABA 1005 ABA 1006 and ABA 1007, are proved to be non-effective under LT

germination. The lack of promotive effects for sulfur linked analog might be attributed to the susceptibility of sulfur atom to oxidation in the plant.

ABA 1011 and ABA 1012 also have a similar modification to ABA 1009. Their C-3' is substituted with hexanol and hexanoic acid respectively. Unlike the alkyl group on the terminus of ABA 1009, the hydroxyl and carboxylic acid functional groups on ABA 1011 and 1012 are highly polar. Their polarity might interact with the conversed residues in the binding pocket, decreasing their affinity for the binding to the ABA receptors. Therefore, ABA 1011 and 1012 were less effective than ABA 1009 in counteracting the exogenous ABA.

ABA 1008 is modified by a substitution with a methyl group at the 3' position. The short tether might help to stabilize the PYL-PP2C interaction upon conformational change. Hence, ABA 1008 exhibited agonism in LT germination assays. The activity of ABA 1008 was consistent with Takeuchi's finding (2014). His study on ASn, a series of 3′-alkylsulfanyl ABA analogs, suggested that an ABA analog tends to behave as an agonist when its tether is shorter than 3. Despite the difference in their linkers, both ABA 1008 and AS2 showed agonist activities since they all had a short tether.

The impact of ABA 1009 on hormone and gene analysis

ABA 1009 promotes LT germination of canola by accelerating the rate of ABA degradation as shown by the increased concentrations of the ABA metabolites in ABA 1009 treated group. Meanwhile, the application of ABA 1009 did not alter the major ABA degradation pathway since the majority of ABA were degraded by 8-hydroxylation in both control and 1009 treated groups. Increased level of ABA was observed in 1009 treated group in canola, which is in parallel with the consistent uptake of ABA 1009 into the seeds. The

increased ABA concentration can be explained as the result of the high ABA 1009 content accumulated in the seeds. Canola seeds used in hormone analysis were treated with ABA 1009 at 300 μM. As ABA 1009 was continuously absorbed over time, an excess amount of ABA 1009 was accumulated within the seeds, causing overdosage effect in the later stage. The up-regulated ABA levels observed during hormone analysis is not contradictory to the results of germination assays. In germination assays, seeds are considered germinating after their radicle break the seed coat. This happened in the early phase of the entire germination and establishment process. There is only a small amount of ABA 1009 accumulated in the seeds when they are exposed to the ABA 1009 in the early stage. At this stage, the concentration of ABA 1009 within the seeds is too low to cause ABA biosynthesis. As the ABA 1009 is continuously taken up, ABA 1009 becomes an inhibitor gradually. Yet, the promotive effect of ABA 1009 offset the inhibitory effect caused by high ABA 1009 content. Therefore, seeds treated with ABA 1009 had higher percent germination as seen in the germination assay. If seeds are continued to be exposed to ABA 1009 in the later stage, the stress caused by increasing ABA 1009 becomes more severe, which might exceed the compensation of its promotive effect and caused depression on their seedling growth and establishment. In conclusion, the increase in ABA level in ABA 1009-treated seeds indicated that the overdosage occurs at the later stage, which might hinder the growth of the germinated seeds. One of the future foci could be the optimization of ABA 1009 usage by altering the concentration applied to the system and the exposure time to ABA 1009.

AAO3 and AAO4 genes from AAO family, and NCED5, NCED6, and NCED9 genes from NCED family were attributed to the up-regulation of ABA biosynthesis. Their expression

levels changed with the increasing over-dosage effects of ABA 1009. None of these selected genes had significant fold changes in the first 3 days; therefore, these genes were not involved in the regulation of ABA biosynthesis before day 3. As a significant amount of ABA 1009 accumulated within the seeds on day 6, the expression of AAO3, NCED5, NCED6, and NCED9 increased, leading to increased synthesis of aldehyde oxidase and 9-cis epoxycarotenoid dioxygenase. These two proteins catalyzed the committed step and the last step of ABA biosynthesis. Hence, ABA biosynthesis was enhanced. At day 9, the excess ABA 1009 caused severe inhibition, which might alter the ABA biosynthesis pathway. Therefore, AAO4 gene was induced and NCED6 was impaired at day 9.

CYP707A4 was involved in the regulation of ABA degradation. The fold change of CYP707A4 was correlated with the change of ABA metabolites in treatment groups indicating a positive regulation of CYP707A4 on ABA degradation. As the germination process began, the expression of CYP707A4 increased. More 8'-hydroxylases were synthesized, accelerating the rate of 8-hydroxylation. Hence, ABA degradation was enhanced.

ABA 1009 activities on soybean and chickpea

ABA 1009 was effective in counteracting the effect of exogenous ABA in the RT soybean germination test. However, it failed to promote germination when soybean seeds were treated with LT. Based on the results of the hormone analysis, soybean had the highest initial ABA level compared to canola and chickpea. As the germination process began, the ABA level in soybean seeds decreased drastically from 353 to 59 nmol g^{-1}. In the case of canola, seeds started to germinate as their ABA level dropped from 259 to 106 nmol g^{-1}. Together, these data

suggest that a high GA/ABA ratio is required for the commencement of soybean germination. In the LT germination test, GA biogenesis is impaired under stress conditions, only the decrease of ABA level within the seeds could change the GA/ABA ratio and initiate germination. Moreover, the cold stress induces ABA biosynthesis, while slow down the rate of ABA degradation, leading to a high ABA level within the seeds. There might be an insufficient amount of ABA 1009 to counteract such high level of ABA. Therefore, ABA 1009 at 100 μM did not exhibit antagonism in LT soybean germination test. The lack of effect of ABA 1009 could also be explained by a possibility that ABA is not the major limiting factor in LT soybean germination. Since soybean undergoes different types of dormancy compared to canola, the impact of ABA might be less important in LT soybean germination.

In RT soybean germination test, seeds were incubated in a condition that provides favorable temperature and sufficient water for the germination. Although 10 μM of exogenous ABA was added into the system, the favorable temperature, and water conditions accelerated the rate of ABA degradation. The ABA level within RT treated seeds might be lower than that of LT treated seeds. ABA 1009 at 100 μM are sufficient to counteract the ABA level resided in RT treated seeds. Hence ABA 1009 was effective in promoting seed germination at RT.

One of the future foci could be to assess whether ABA 1009 could exhibit promotive effects on LT soybean germination. Assessments could be achieved by conducting another set of germination test, either using different concentrations of ABA 1009 or adjusting experimental temperature to a less severe level for soybean germination.

ABA 1009 was effective in promoting seed germination of canola under LT or in the presence of ABA. It also counteracted the effect of exogenous ABA in RT soybean germination,

but it did not exhibit any antagonist activities on chickpea. The various responses to ABA 1009 might be attributed to their difference in ABA receptors. A small difference on the residue among these ABA receptors might cause a big impact on the interaction between ABA 1009 and the ABA binding pocket. ABA 1009 might have higher affinities for the binding to the ABA receptors of canola and soybean, leading to more efficient regulations of the ABA signaling pathway. Hence ABA 1009 showed antagonisms on canola and soybean, but not on chickpea. The effect of ABA 1009 is not universal. It is interesting to know how other crops would respond to ABA 1009.

One of the suggestions for the future work is to conduct a series of germination test on different crops and assess the effect of ABA 1009 on their LT germination.

Hormone analysis on soybean and chickpea

Seeds of soybean and chickpea treated under LT had slow ABA catabolism since LT treated seeds contained more ABA but less ABA metabolites compared to RT treated seeds. Delayed germination at LT can be explained as the result of slow ABA metabolism caused by cold stress.

Canola seeds have a dormancy cycle

Canola seeds used in this study exhibit a dormancy cycle. Multiple germination assays were done over a period of 2 years. Yet, there were variations in percent germination of the control groups assessed at different times. This variation indicated the fluctuation of the seed dormancy status. Canola seeds appear to have a seasonal periodicity of their dormancy. Their dormancy is released in summer and imposed in winter, synchronizing with environmental potential for germination (Black et al. 2007). The future focus for this part is to test the

dormancy profiles at different times and to achieve the accurate dormancy cycle for the canola seeds. Overall, the effects of ABA 1009 are consistently significant despite the periodic changes of the seed dormancy.

Comparison between RT and LT germination test

Germination assays conducted at RT (22℃) with exogenous ABA accelerated the pace of screening effective ABA antagonists. LT germination test usually lasted for one to three weeks depending on the dormancy status of the seeds. RT germination test can be accomplished within 4 days, which is ideal for the fast screen of large numbers of ABA analogs. The effects of ABA analogs can be scored by the extent of counteracting the exogenous ABA. Furthermore, RT germination test can be conducted in a more controlled way since it has less temperature fluctuation. RT in the laboratory normally fluctuates within 3 degrees Celsius. The fridge temperature, on the other hand, could change in the range of 5 degree Celsius. It must be closely monitored to keep the average temperature steady. Therefore, the results acquired from RT germination test were more consistent. Moreover, the canola seeds exhibited a seasonal periodicity on their dormancy. Seeds under different dormancy status show disparate responses to the cold stress, resulting in different levels of ABA accumulations. The dynamics of dormancy status in combination with cold stress caused significant variations in ABA levels within the seeds, leading to various germination responses at different time points. Therefore, the results of LT germination assays fluctuated over time. RT germination test, on the other hand, provided more consistent results despite the dynamics of the dormancy status. In RT germination test, a fixed amount of exogenous ABA was added into the system, resulting in less variation of ABA level and more consistent results.

Although LT germination test is time-intensive, it has an irreplaceable role in testing ABA analogs. Compared to RT germination test, LT test provides a better approximation to the conditions in the early spring, contributing to a better assessment to the effect of ABA analogs. The results of soybean germination test indicated that ABA analogs, that are effective in RT germination test, might not exhibit the same activities in LT germination test. Therefore, it is necessary to conduct LT germination tests to confirm the effects of the candidate ABA analogs.

For the future screening of the available ABA analogs, RT germination test could be conducted at the first step to increase the efficiency. LT germination test with candidate analogs could be conducted at the last step to ensure the accuracy.

Stratified seeds had better performance

Stratified seeds had higher germination rate compared to non-stratified seeds at RT germination assays. Stratified seeds were sown in solution and kept at LT for 2 days prior to the beginning of the germination test. During this period, seed germination might be suppressed by the LT. Yet, seeds were continuously absorbing water during stratification followed by gradual uplifted metabolic activities. By the end of stratification, seeds have already entered the imbibition or plateau phase, resulting in higher germination rate than non-stratified seeds.

Chapter 6: Conclusions

Six hypotheses were proposed at the beginning of the research. With the results acquired from germination tests, hormone and gene expression analyses, conclusions were made as follow. ABA 1009 was effective in promoting canola germination under LT (5°C) or in the presence of exogenous ABA, which partially supported hypothesis 1 and hypothesis 2. The success of ABA 1009 on canola proved that ABA antagonists can be applied to promote LT germination. ABA 1009 failed to exhibit antagonism in LT soybean, RT or LT chickpea germinations. The results suggested that the effect of an ABA antagonist is not universal on all crops. For soybean and chickpea, the future available ABA antagonists that are designed specifically for these crops might be able to promote their germination. Therefore, hypothesis 1 and hypothesis 2 were accepted. Sulfur linked simple analogs (ABA 1004, ABA 1005, ABA 1006, ABA 1007) were tested in this research and none of them exhibited any promotive effects. As for carbon linked simple analogs, ABA 1009, ABA 1011 and ABA 1012, exhibited different extents of antagonisms during germination tests. These results supported hypothesis 3. Hormone analysis of canola seeds suggested that the application of ABA 1009 at 300 μM increased both ABA biosynthesis and ABA degradation, hence, ABA level in ABA 1009-treated seeds was significantly different from control, which supported hypothesis 4. The hormone analyses on soybean and chickpea indicated higher ABA concentrations in LT treated seeds compared to RT treated seeds, hence, hypothesis 5 was accepted. The results of gene expression analysis of canola seeds suggested that the application of ABA 1009 caused the up-regulation of ABA biosynthesis related genes: AAO3, AAO4, NCED5, NCED6, and NCED9. Gene expression study also suggested that Gene CYP707A4 was involved in the ABA degradation

process. Therefore, hypothesis 6 was accepted.

The cost of ABA 1009 synthesis is much lower than the other effective ABA analogs (such as ABA 1001), making it desirable for large-scale application. Germination tests on canola seeds after different storage periods suggested that the seed dormancy might change over time, yet, the effects of ABA 1009 were relatively consistent. The application of ABA 1009 could also promote radicle growth in the later stage of germination. Germination tests conducted on several canola cultivars with known variations in dormancy showed that ABA 1009 was effective in promoting seed germination on all the cultivars. Among all the tested concentrations, ABA 1009 at 300 μM exhibited a better antagonist activity against 10 μM exogenous ABA at RT. Hormone analysis of canola seeds indicates that the overdosage effect occurred at the later phase when seeds were treated with ABA 1009 at 300 μM.

REFERENCES

Abrams, S.R., Rose, P.A., Cutler, A.J., Balsevich, J.J., Lei, B., and Walker-Simmons, M.K. 1997. 8[prime]-Methylene Abscisic Acid (An Effective and Persistent Analog of Abscisic Acid). Plant Physiology, **114**: 89–97.

Alonso-Blanco, C., Bentsink, L., Hanhart, C.J., and Vries, H.B. 2003. Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics, **164**: 711-729.

Agriculture and Agri-Food Canada. 2016. Canada: outlook for principal field crops. Available from http://www.agr.gc.ca/eng/industry-markets-and-trade/statistics-and-market-information/by-product-sector/crops/crops-market-information-canadian-industry/canada-outlook-for-principal-field-crops/canada-outlook-for-principal-field-crops-2016-09-01/?id=1472772767455 [accessed 12 February 2018].

Baskin, J.M., Baskin, C.C. and Li, X. 2000. Taxonomy, anatomy and evolution of physical dormancy in seeds. Plant Species Biology, **15**: 139-152.

Baskin, C.C., and Baskin, J.M. 1998. Seeds: Ecology, biogeography, and evolution of dormancy and germination. San Diego, Academic Press.

Baskin, C.C.., and Baskin, J.M. 2004. A classification system for seed dormancy. Seed Science Research, **14**:1–16.

Bassel, G.W., Lan, H., Glaab, E., Gibbs, D.J., Gerjets, T., Krasnogor, N., Bonner, A.J., Holdsworth, M.J., and Provart, N.J. 2011. Genome-wide network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions. PNAS, **108** (23): 9709-9714. Available from https://doi.org/10.1073/pnas.1100958108 [accessed 18 February 2018].

Bai, G., Yang, D., Zhao, Y., Ha, S., Yang, F., Ma, J., Gao, X., Wang, Z., and Zhu., J. 2013. Interactions between soybean ABA receptors and type 2C protein phosphatases. Plant molecular biology, **83**: 651-664.

Bewley, J.D., Bradford, K.J., Hilhorst, J.W.M, and Nonogaki, H. 2013. Seed: Physiology of development, germination and dormancy,3rd ed. Springer, New York.

Bewley, J.D., and Black, M. 1994. Seeds physiology of development and germination. *In* The language of science. Plenum Press, New York. pp. 230.

Black, M., Halmer, P., and Bewley, J. D. 2007. The Encyclopedia of Seeds: Science, Technology and Uses. Oxford University Press, Oxford.

Boyd, J., Gai, Y., Nelson, K.M., Lukiwski, E., Talbot, J., Loewen, M.K., Owen, S., Zaharia, L.I., Cutler, A.J., Abrams, S.R., and Loewen, M.C. 2009. Sesquiterpene-like inhibitors of a 9-cis-epoxycarotenoid dioxygenase regulating abscisic acid biosynthesis in higher plants. Bioorg Med Chem. **17**:2902-12.doi: 10.1016/j.bmc.2009.01.076.

Cadman, C.S.C., Toorop, P.E., Hilhorst, H.W.M. and Finch-Savage, W.E. 2006. Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism Plant J. **46**: 805.

Camara, M. C., Cristine, R., Silva, L.D., Vandenberghe, A.L., and Carlos, L.S. 2015. General Aspects and Applications of Gibberelins and Gibberellic Acid in Plants. *In* Gibberellins and Gibberellic Acid: Biosynthesis, Regulation and Physiological Effects. 1st ed. Edited by J.Hardy. Nova Science Publishers, Hauppauge. pp. 1-21.

Canola council of Canada. 2011. When is too late to seed canola? Available from http://www.canolawatch.org/2011/05/26/when-is-too-late-to-seed-canola-2/ [accessed 31 January 2018].

Canola Council of Canada. 2013. Time of seeding. Available from http://www.canolacouncil.org/canola-encyclopedia/crop-establishment/time-of seeding/#references [accessed 31 January 2018].

Cutler, S.R., Rodriguez, P.L., Finkelstein, P.R., and Abrams, S.R. 2010. Abscisic Acid: Emergence of a Core Signaling Network. Annu. Rev. Plant Bio. **61**: 651-679.

Dilworth, L.L., Riley, C.K., and Stennett, D.K. 2017. Plant constituent: carbohydrates, oils, resins, balsams, and plant hormones. *In* Pharmacognosy. Edited by S. Badal and R. Delgoda. Acadamic Press, Waltham. pp.61-80.

Fenner, M., and Thompson, K. 2005. The ecology of seeds. Cambridge University Press, Cambridge.

Finkelstein, R.R. and Lynch, T.J. 2000. The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell. **12**: 599–609.

Frey, A., Audran, C., Marin, E., Sotta, B. and Marion-Poll, A. 1999. Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. Plant Mol Biol. **39**: 1267-1274.

Frisbee, C.C., Smith, C.W., Wiesner, L.E., and Lockerman, R.H. 1988. Short term storage effects on dormancy and germination of chickpea (*Cicer Arietinum*). Journal of Seed Technology. **12**: 16-23.

Galili, G., and Kigel, J. 1995. Seed development and germination. M. Dekker, New York.

Gallardo, K., Job, C., Groot, S. P., Puype, M., Demol, H., Vandekerckhove, J., and Job, D. 2002. Proteomics of Arabidopsis seed germination. A comparative study of wild-type and gibberellin-deficient seeds. *Plant physiology*, ***129*****(2)**: 823-37.

Grassini, P., Jennifer, R., Nicolas, C.L.M., and Specht, J. 2016. What does it take to produce 80+ bu/ac soybean? Available from http://extensionpublications.unl.edu/assets/pdf/ec3000.pdf [accessed 15 February 2018].

Graeber, K., Linkies, A., Steinbrecher, T., Mummenhoff, K., Tarkowská, D., Turečková, V., Ignatz, M., Sperber, K., Voegele, A., Jong, H., Urbanová, T., Strnad, M., and Leubner-Metzger, G. 2014. *DELAY OF GERMINATION 1* mediates a conserved coat-dormancy mechanism for the temperature- and gibberellin-dependent control of seed germination. Proc. Natl Acad. Sci. **111**: 3571–3580.

Groot, S.P. and Karssen, C.M. 1987. Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. Planta. **171**:525-31.

Gupta, R., and Chakrabarty, S.K. 2013. Gibberellic acid in plant Still a mystery unresolved. Plant Signal Behav. **8**: e25504. doi: 10.4161/psb.25504

Hampson, C. R., Reaney, M. J. T., Abrams, G. D., Abrams, S. R., and Gusta, L.V. 1992. Metabolism of (+)-abscisic acid to (+)-7′-hydroxyabscisic acid by bromegrass cell cultures. Phytochemistry. **31**: 2645-2648.

Hatzig, S.V., Frisch, M., Breuer, F., Nesi, N., Ducournau, S., Wagner, M.H., Leckband, G., Abbadi, A. and Snowdon, R.J. 2015. Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. Front. Plant. Sci. Doi: https://doi.org/10.3389/fpls.2015.00221.

Hayashi, K., and Kinoshita, T. 2014. Plant signaling: Abscisic acid receptor hole-in-one. Nat Chem Biol. **10**: 414–415. doi:10.1038/nchembio.1529

Hedden, P. (2014). Gibberellins. *In* AccessScience. McGraw-Hill Education, New York. doi: http://dx.doi.org/10.1036/1097-8542.289000.

Helgi, O., Stephen, R.A., Willis, A.J., and Street, H.E. 2005. The physiology of flowering plants, 4th ed. Cambridge University Press, Cambridge.

Hilhorst, H.W.M. & Karssen, C.M. 1992. Seed dormancy and germination: the role of abscisic acid and gibberellins and the importance of hormone mutants. Plant. Growth. Regul. **11**: 225-238.

Huang, D., Jaradat, M.R., Wu, W., Ambrose, S.J., Ross, A.R., Abrams, S.R., and Cutler, A.J. 2007. Structural analogs of ABA reveal novel features of ABA perception and signaling in

Arabidopsis. Plant J. **50**:414-28.

Huo, H., Wei, S. and Bradford, K. J. 2016. *DELAY OF GERMINATION 1* (*DOG1*) regulates both seed dormancy and flowering time through microRNA pathways. Proc. Natl Acad. Sci. **113**: 2199–2206.

Jasa, Lisa. 2009. Adjust Maturity with Early Planted, Rainfed Soybeans. Available from https://cropwatch.unl.edu/adjust-maturity-early-planted-rainfed-soybeans [accessed 08 February 2018].

Kaur, S., Gupta, A. K., and Kaur, N. 1998. Gibberellin A3 reverses the effect of salt stress in chickpea (*Cicer arietinum* L.) seedlings by enhancing amylase activity and mobilization of starch in cotyledons. Plant. Growth. Regul. **26**: 85–90.

Kendall, S.L., Hellwege, A., Marriot, P., Whalley, C., Graham, I.A., and Penfield, S. 2011. Induction of dormancy in *Arabidopsis* summer annuals requires parallel regulation of *DOG1* and hormone metabolism by low temperature and CBF transcription factors. Plant Cell**. 23**: 2568–2580.

Kirkland, K.J., and Johnson, E.N., 2000. Alternative seeding dates (fall and April) affect *Brassica napus* canola yield and quality. CJPS. **80**: 713-719.

Koornneef, M., Reuling, G., and Karssen, C.M. 1984. The isolation and characterization of abscisic acid-insensitive mutants of Arabidopsis thaliana. Physiol Plant. **61**: 377–383.

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

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

Leung, J., Merlot, S., Giraudat, J. 1997. The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell. **9**: 759–771

Lindsey, L. 2017. Soybean planting date, row width, and seeding rate recommendations. Available from https://agcrops.osu.edu/newsletter/corn-newsletter/2017-09/soybean-planting-date-row-width-and-seeding-rate-recommendations [accessed from 15 February 2018].

Lopez-Molina, L., Mongrand, S., and Chua, N.H. 2001. A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci USA. **98**: 4782–4787.

Milborrow, B.V. 2001. The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. J Exp Bot. **52**:1145-64.

Millar, A.A., Jacobsen, J.V., Ross, J.J., Helliwell, C.A., Poole, A.T., Scofield, G.,Reid, J.B. and Gubler, F. 2006. Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8′-hydroxylase. *Plant J.* **45**: 942–954.

Morrison, J.W. 2015. Crops. Available from http://www.thecanadianencyclopedia.ca/en/article/crops/ [accessed 31 January 2018].

Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y., and Nambara, E. 2005. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. Plant J. **41**: 697–709.

Nambara, E., and Marion-Poll, A. 2003. ABA action and interactions in seeds. Trends Plant Sci. **8**: 213–217. doi: http://dx.doi.org/10.1016/S1360-1385(03)00060-8.

Nelson, D.R., Schuler, M.A., Paquette, S.M., and Werck-Reichhart, D. 2004. Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. Plant Physiol.**135**:756–72.doi: 10.1104/pp.104.039826

Nyangulu, J.M., Galka, M.M., Jadhav, A., Gai, Y., Graham, C.M., Nelson, K.M., Cutler, A.J., Taylor, D.C., Banowetz, G.M., and Abrams, S.R. 2005. An affinity probe for isolation of Abscisic acid- binding proteins. J. Amer. Chem. Soc. **127**: 1662-1664.

Qutob, D., Ma, F., Peterson, C.A., Bernards, M.A., Gijzen, M. 2008. Structure and permeability properties of the soybean seed coat. Botany. **86**: 219-227.

Rajagopalan, N., Nelson, K.N., Douglas, A.F., Jheengut, V., Alarcon, I.Q., McKenna, S., Surpin, M., Loewen, M.C., and Abrams, S.R. 2016. Abscisic Acid Analogues That Act as Universal or Selective Antagonists of Phytohormone Receptors. Biochem. **55**: 5155-5164.

Raven, P. H., Evert, R.F., and Eichhorn, S.E. 2005. Biology of Plants, 7th ed. W.H. New York: Freeman and Company Publishers.

Rudall, P.J. 2007. Anatomy of Flowering Plants: An Introduction to Structure and Development. Cambridge University Press, Cambridge.

Schopfer, P, and Plachy, C. 1984. Control of Seed Germination by Abscisic Acid: II. Effect on Embryo Water Uptake in Brassica napus L. Plant Physiology. **76**: 155-60.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A. and McCarty, D.R. 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. Science. **276**: 1872–1874.

Shu, K., Liu, X., Xie, Q., and He, Z. 2016. Two Faces of One Seed: Hormonal Regulation of Dormancy and Germination. Molecular Plant. **9**: 34-45.

Sinniah, U.R., Ellis, R.H., and John, P. 1998. Irrigation and Seed Quality Development in Rapid-cycling Brassica: Seed Germination and Longevity. Annals of Botany. **82**: 309-314. Doi: https://doi.org/10.1006/anbo.1998.0748

Seo, M., Peeters, J.M., Oritani, K., Marion-Poll, A., Zeevaart, J., Koornneef, M., Kamiya, Y., and Koshiba, T. 2000. The *Arabidopsis* aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Natl Acad of Sci. **97**:12908-12913.

Takeuchi, J., Okamoto, M., Akiyama, T., Muto, T., Yajima, S., Sue, M., Seo, M., Kanno, Y., Kamo, T., Endo, A., Nambara, E., Hirai, N., Ohnishi, T., Cutler, S.R., Todoroki, Y. 2014. Designed abscisic acid analogs as antagonists of PYL-PP2C receptor interactions. Nat Chem Biol. **10**:477-82. doi: 10.1038/nchembio.1524.

Takeuchi, J., Ohnishi, T., Okamoto, M., and Todoroki, Y. 2015. Conformationally restricted 3'-modified ABA analogs for controlling ABA receptors. Org Biomol Chem. **13**:4278-88. doi: 10.1039/c4ob02662d.

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

Xu, Z.J., Nakajima, M., Suzuki, Y., and Yamaguchi, I. 2002. Cloning and characterization of the abscisic acid-specific glucosyltransferase gene from adzuki bean seedlings. Plant Physiol. **129**: 1285-1295.

Zhang, W.T., Chiwocha, S.D.S., Trischuk, R., and Gusta, L.V. 2010. Profile of Plant Hormones and their Metabolites in Germinated and Ungerminated Canola (Brassica napus) Seeds Imbibed at 8°C in either GA4+7, ABA, or a Saline Solution. Journal of Plant Physiology. **29**: 91–105. doi: 10.1007/s00344-009-9117-4.

Zhou, R., Cutler, A. J., Ambrose, S. J., Galka, M. M., Nelson, K. M., Squires, T. M., and Abrams, S. R. 2004. A New Abscisic Acid Catabolic Pathway. Plant Physiol. **134**: 361–369. http://doi.org/10.1104/pp.103.030734.

Zhu, Y., Nomura, T., Xu, Y., Zhang, Y., Peng, Y., Mao, B., Hanada, A., Zhou, H., Wang, R., Li, P., Zhu, X., Mander, L.N., Kamiya, Y., Yamaguchi, S., and He, Z. 2006. Elongated uppermost internode encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. Plant Cell. **18**:442-56.

Zou, J., Abrams, G. D., Barton, D. L., Taylor, D. C., Pemeroy, M. K., Abrams, S. R. 1995.

Induction of lipid and oleosin biosynthesis by (+)-abscisic acid and its metabolites in microspore-derived embryos of Brassica napus L. cv Reston. Plant Physiol. **108**: 563-571.

APPENDIX A

PRIMERS INFORMATION IN GENE EXPRESSION ANALYSIS

Function	Gene	Primers (F/R) Brassica napus (5'-3')
ABA biosynthesis	AAO3	CGACGAGGGAAGGTTTGATAG
		GACGGATGCTCCAATCTCAATA
	AAO4	GAGGACTGTGTTGTGATGAGAG
		GGTATTGGGACGCTGATTGA
	NCED5	CACAGACTCTCCGGCTTTATT
		CGTTGGAGAGGATTCCAGTTAG
	NCED6	TACCCGACTGCTTCTGTTTC
		CGACCCAATGACGACGATAA
	NCED9	TTTCCCATCTCCTCGTCTCT
		GGTTCGTCGTGTTAGGAATCA
ABA degradation	CYP707A1	CACCAAGTACAGGTGGTCAATA
		CCTACTTCCGAGCAAGCATAA
	CYP707A2	TGAAGGCAAGGAGGGAATTAAG
		CGCCAAGAAGTACTCCCAATAG
	CYP707A3	GCTACAACTCGATGCCGATTA
		CGTTGATGGGTTCTGTCTTCT
	CYP707A4	GAGGAAGCGGCTAAAGACAATA
		GGTTAGCACCCGACCTTTATC
Reference gene	TIP 41	AAAGGGGAGACACCCACAGT
		TCTGCTTAATCACCGGAAGC
	ACT7	TTCAATGTCCCTGCCATGTA
		GAGACGGAGGATAGCGTGAG
	SAND	GGGACCCCACAAGACTCAAT
		TTTTACCCTCTGGCACACCT
	PP2A	AGATCTGCATTGGCCTCAGT
		TCACTTGGTCAAGTTTGCTGA

APPENDIX B
STATISTICAL ANALYSES USED IN GERMINATION ASSAYS AND GENE EXPRESSION STUDY

Data analysis for germination assays

The number of germinated seeds in each Petri dish was recorded daily. Percent germination for each group at day i was calculated as Gi= $\frac{(ni1+ni2+\cdots+nix)}{x*N}$, where i is i^{th} day, x is the number of Petri dishes in the group, nix is the number of germinated seeds in x^{th} Petri dish at day i and N is the total number of seeds within a Petri dish. Standard deviations were calculated for each group. All data analyses were done by Excel 2016 program.

Data analysis for gene expression study

The fold changes of the selected gene were calculated as follow:

$$\Delta Cq_x = Cq_{GOI} - Cq_{RG}$$

$$\Delta\Delta \mathrm{Cq} = \Delta Cq_{treatment} - \Delta Cq_{control}$$

$$F_i = 2^{-\Delta\Delta Cq}$$

$$\mathrm{F} = \frac{F_1+F_2+F_3}{3}$$

Where x is the control or treatment group, Cq_{GOI} is the Cq value of the gene of interest, Cq_{RG} is the Cq value of the reference gene, i is the ith biological replicate, F_i is the fold change of the selected gene in i^{th} biological replicate and F is the average fold change among three biological replicates. Standard deviations were calculated for each group. All data analyses were done by Excel 2016 program.