Characterisation of dormancy cycling responses to environmental signals in contrasting *Arabidopsis thaliana* ecotypes

By

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Thesis submitted to the University of Warwick for the

Degree of

Doctor of Philosophy in Plant and Environmental Science

University of Warwick

School of Life Science

April 2013

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ACKNOWLEDGEMENTS

Thanks to the Warwick Postgraduate Research Scholarship, I manage to experience life and work in the UK. During my three and half years' PhD, I owe a lot of gratitude to many people without whom I would not have been able to complete my thesis. First of all, I would like to thank Bill Finch-Savage and Steve Footitt for their excellent supervision of this project. I would like to thank Bill for providing me such a good opportunity to do my PhD in his group. It has been a pleasure to work in this group for the past three years and I have been taught the importance of thinking and how to think scientifically. Thanks also go to Steve for his great supervision and support in the lab and field, and being always there for short discussions. Thank you, Bill and Steve, for teaching me how to express my thoughts and put them into words, thanks for all your patience and not giving up on improving my scientific writing skills. I would also like to thank both the former and current members of the Seed Sciences Group: Karl Morris, Heather Clay, Katherine Dent and Angela Hambidge for their help during this project. Thanks also to Nipurna Jina (UCL Genomics) for help with the Nannostring analysis, Matthew Mitchell (Warwick Crop Science Centre) for the seed nitrate measurements, Andrew Mead and Julie Jones (Quantitative Biology Centre) for the statistical help and advice, and also staff from Horticultural service for assistance in the glasshouse, field and thermal gradient poly-tunnel. It has been such an experience doing PhD in the UK, during which I tried not only to succeed in my studies but also to learn from a different educational system and culture. There has been a whole legion of people who made my stay enjoyable

and even if I cannot name all, I would like to thank them. I would also like to give my special thanks to Peijun Zhang and Limin Xu, for their friendship and encouragement whenever I felt weak. Many thanks also go to all my friends in China for supporting me despite the distance. Finally I would like to dedicate this thesis to my family, without their understanding and support I would not have been able to complete my PhD studies.

AUTHORS DECLARATION

I declare that I am the sole author of the work in this thesis and that it is original except where indicated by special reference in the text. No part of this degree has been submitted for any other degree to any other institution.

Experiments in sections 4.2.2 and 4.2.3 were performed jointly with Steven Footitt as part of a larger project carried out by SF; seed nitrate content (section 6.2.1) was measured by Matthew Mitchell (Warwick Crop Science Centre); some experimental work in section 7.2.2 was performed by Will Rimmington and Valeriya Taylor, two undergraduate project students. I was responsible for maintenance of the experiments and the collection of data in their absence and solely responsible for the remainder of the work.

SUMMARY

Seed dormancy is an important trait refined by evolution, to aid survival in adverse environments and to time germination and thereby select the correct habitat and climate space for subsequent plant growth and reproduction. Depth of dormancy changes continuously in response to the environment surrounding the seed and is therefore a relative rather than an absolute condition. In nature, these changes are triggered by seasonally characteristic environmental signals that are integrated by the seed over time to select the optimum conditions for germination.

The mechanisms by which environmental signals influence this dormancy cycling have been studied in the present work using a combination of eco-physiology and molecular biology. Two contrasting *Arabidopsis thaliana* ecotypes Cape Verdi Isle (Cvi) and Burren (Bur) have been compared. They are adapted to a hot dry (Cvi) and a cool damp (Bur) climate and exhibit winter and summer annual phenotypes respectively. Experimental work in the laboratory, controlled environment and field has focussed on the effect of temperature, light and nitrate during seed maturation and subsequent imbibition. The work was also extended to studying other life cycle events such as the transition from vegetative growth to reproductive growth, flowering and seed maturity. This work has extended our understanding of the responses of life cycle traits to environmental signals. However, climates are changing and further data was collected in a series of experiments in a unique thermal gradient tunnel to provide insight into the impact of predicted global warming scenarios on these traits. The results presented indicate the plasticity of the plant life cycle and the extent to which global warming might affect this in *Arabidopsis*, and how increased temperature is likely to affect different annual phenotypes.

ABBREVIATIONS

ABA: Abscisic acid
ABI: ABA insensitive
ANOVA: Analysis of variance
AR: After-ripening
ATHB: *Arabidopsis thaliana* homeobox protein
bHLH: basic helix-loop-helix
Bur: *Arabidopsis thaliana* accession Burren
CBF: C-repeat binding factor
CHO: Chotto
CTAB: Cetyltrimethylammonium bromide
Col: *Arabidopsis thaliana* accession Columbia
CRY: Crytochrome
Cvi: *Arabidopsis thaliana* accession Cape Verde Island
CYP707A: ABA 8'-hydroxylase
D: Dark
DOG: Delay of germination
EDTA: Ethylenediaminetetraacetic acid
ERH: Equilibrium relative humidity
EXPA: Expansin-A
FLC: Flowering Locus C
FR: Far red light
FRI: Frigida
FT: Flowering Locus T
GA: Gibberellins
GAI: GA insensitive
GA2ox: Gibberellin 2-oxidase
GA3ox: Gibberellin 3-hydroxylase
GID1: Gibberellin insensitive dwarf 1
HDZip: Homeobox-leucine zipper
HI: Harvest index
KNO_3: Potassium nitrate
L: Light
Ler: *Arabidopsis thaliana* accession Landsberg *erecta*
LPP2: lipid phosphate phosphatase 2
LSD: Least Significant Difference
MFT: Mother of FT and TFL1
mRNA: Messenger ribonucleic acid

N: Nitrogen
NaCl: Sodium Chloride
NCED: Nine-cis-epoxycarotenoid deoxygenase
NH_4^+: ammonium
NO_3^-: nitrate ion
NiR: Nitrite reductase
NR: Nitrate reductase
NRT: Nitrate transporter
PHYA-E: Phytochrome A-E
PIF: Phytochrome interacting factor
PIL5: Phytochrome interacting factor 3-like 5
PP2C: Protein Phosphatase 2C
PYL: Pyrabactin-like
PYR: Pyrabactin resistance
QTL: Quantitative trait loci
R: Red light
RGA: Repressor of *ga1-3*
RGL: RGA-like
RIL: Recombinant Inbred Line
Sha: *Arabidopsis thaliana* accession Shahdara
SnRK2: Surose nonfermenting related kinases 2
SPT: Spatula
T_{50}: Time to reach 50% of seed germination
TFL1: Terminal flower 1
Ws: *Arabidopsis thaliana* accession Wassilewskija

CHAPTER 1: INTRODUCTION

1.1 Dormancy in nature

Dormancy is a wide spread phenomenon in biology that can be considered as a period within an organism's life cycle when growth and development is temporarily arrested (Footitt and Cohn, 2001). For example, hibernation is a type of dormancy that is common in mammals and has been considered as a combination of physiological adaptations that give the animal the ability to survive climatic extremes (Andrews, 2007). All three of the deepest branches within the class Mammalia (i.e. placentals, marsupials and monotremes) are suggested to contain hibernators (Carey *et al*., 2003). Many other forms of dormancy occur in the animal kingdom as adaptations to unfavourable environments. For example, diapause is often observed in arthropods, especially insects, and also in the embryo of many of the oviparous species of fish in the order Cyprinodontiformes (Collier and Murphy, 1997), and this enables them to exploit seasonally fluctuating resources, to diversify in the unfavourable habitats and to maintain the population (Koštál, 2006).

Dormancy in plants can also be considered as a trait selected by evolution, for survival in adverse environments (e.g. heat, cold, drought) (Hilhorst, 2007) and it is closely associated with environmental variation. During dormancy the metabolic activity of an organism is reduced to conserve energy, but it remains sensitive to environmental signals (e.g. photoperiod and temperature).

In the plant kingdom, dormancy is an integral part of the plant life cycle and can be found in most of its stages, e.g. it may occur in seeds, bulbs, buds, tubers and

even whole plants (Hilhorst, 2007). Therefore plants need to have mechanisms that monitor and respond to the environment around them. Low temperature, for instance, generally plays an important role in the releasing of bud dormancy. The chilling duration and the range of optimal chilling temperatures required for dormancy release vary between species and also between cultivars within species (Fuchigami and Wisniewski, 1997). For example, raspberries have a chilling requirement of 800-1500 hours below 7.2˚C for bud dormancy release, and this is variable between different cultivars (White *et al*., 1998). Bud dormancy in most tree species is released by exposure to non-freezing chilling temperature ranging from 5 to 7˚C (Rinne *et al*., 1997). However, interestingly, short-term exposure to freezing temperatures (e.g. -8, -16, -24 and -32˚C) was also found to be efficient in releasing bud dormancy in *Betula pubescens* and *B. pendula* (Rinne *et al*., 1997). Similar effects of freezing temperature on bud dormancy have been reported in previous studies: -15˚C was effective for *Ribes nigrum* (Tinklin and Schwabe, 1970), and temperatures ranged from -5 to -10˚C for mulberry (Hasegawa and Tsuboi, 1960). A study of lateral bud dormancy in *Actinidia chinensis* showed that bud dormancy was induced in short days at warm and constant temperatures, while broken by chilling at a temperature of 10˚C (Lionakis and Schwabe, 1984). Thus the expression of bud dormancy was temperature dependent, i.e. the minimum temperature for budburst increases during induction and decreases during the release of dormancy (Junttila and Hänninen, 2012). Therefore temperature is a key aspect of the environment that influences both the induction and release of dormancy throughout the whole plant

life cycle, thus the ability of plants to perceive low and high temperature is important to their survival (Penfield, 2008). However, plants can responds to a much wider range of environmental signals as illustrated for seeds below.

1.2 Seed dormancy

1.2.1 Definition of seed dormancy

Seeds represent the only mobile phase in the life cycle of higher plants. Dormancy during this phase enables seeds to avoid unfavourable conditions and to ensure plant establishment into the correct time of year, habitat and climate space. Consequently, dormancy is an important strategy in the dispersal of plants not only in space but in time and can be termed a mechanism for the control of biological time. Therefore, dormancy is considered as a seed characteristic that provides the best 'schedule' for germination so that the risk of premature death of the resulting plant by catastrophe can be reduced (Hilhorst, 2007). There have been a range of useful definitions of seed dormancy (listed in Table 1.1) and one of the most frequently used definitions of seed dormancy is a block to the completion of germination of an intact viable seed under unfavourable conditions (Bewley, 1997).

However, a broader definition based on eco-physiological studies defines dormancy as 'a dormant seed does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors that are otherwise favourable for its germination' (Baskin and Baskin, 2004), i.e. the degree of dormancy defines what conditions should be met for the seeds to germinate (Vleeshouwers *et al*., 1995).

Table 1.1 Definitions of seed dormancy in the literature

Author and year	Definition
Simpson (1990)	'Temporary failure of a viable seed to germinate…in a particular set of environmental conditions that later evoke germination when the restrictive state has been terminated by either natural or artificial means.'
Bewley (1997)	'Seed dormancy is regarded here as the failure of an intact viable seed to complete germination under favorable conditions.'
Bell (1999)	'Seeds must not be in a state of dormancy and the environmental requirements for germination of that seed must be met before germination can occur.'
Eira and Caldas (2000)	'A state in which the development or germination of a viable seed is blocked by one of many possible limitations located within the seed itself'
Baskin and Baskin (2004)	'a dormant seed does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors that are otherwise favorable for its germination.'
Finch-Savage and Leubner-Metzger (2007)	'Dormancy is a seed characteristic that defines the conditions required for germination, and therefore any cue that widens the environmental requirements for germination should be regarded as a dormancy release factor.'

1.2.2 Seed dormancy and germination

Definitions of dormancy are difficult as it can only be measured by the absence of germination and we can only measure dormancy *post facto* by testing germination. We can observe completion of germination of a single seed as an all-or-nothing event, but seed dormancy is far more than that (Finch-Savage and Leubner-Metzger, 2006) and the absence of germination in viable seeds could be due to limiting factors from the environment or from the seed itself other than dormancy. The transition between dormancy and germination is a crucial process in the life cycle of higher plants and also an important ecological and commercial trait (Holdsworth *et al*., 2008). In general, the range of environmental conditions under

which germination can proceed to completion widens to its maximum as dormancy is released; conversely as dormancy is induced, the range of conditions under which germination can take place narrows until germination is no longer possible (Vegis, 1964). In summary, seed dormancy is an innate seed property that determines the environmental conditions that must be met before germination can be completed (radicle emergence) (Leubner, 2005; Hilhorst, 2007). However, there still remains some controversy concerning the definition of dormancy and Finch-Savage and Footitt (2012) have recently discussed this at length.

1.2.3 Classification of seed dormancy

The most frequently used classification of seed dormancy is that proposed by Baskin and Baskin (1998, 2004), which is based on seed physiology and morphology (Nikolaeva, 1977). Five classes of dormancy are included in this classification system (Table 1.2): Physiological (PD), Morphological (MD), Morpho-physiological (MPD), Physical (PY) and combinational (PY+PD).

Among the five dormancy classes in Table 1.2, physiological dormancy (PD) is caused by a physiological inhibiting mechanism of the embryo that prevents radical emergence (Nikolaeva, 1977). It is regarded as the most prevalent form of dormancy that enables seeds to persist in the seed bank of temperate soils (Finch-Savage and Leubner-Metzger, 2006). It is also the form of dormancy in *Arabidopsis thaliana* — currently the predominant model species used in developing a molecular based understanding of seed biology — PD has therefore attracted most research attention, and can be subdivided into three levels (Table 1.3): deep, intermediate and

non-deep. As the majority of seeds, including *Arabidopsis*, have non-deep PD (Baskin and Baskin, 2004), it is therefore the main focus of this research.

Table 1.2 Simplified classification scheme for seed dormancy types*

Type	Main Cause	Release method
Physiological (PD)	Physiological inhibiting mechanism (PIM) of germination	Warm and/or cold stratification
Morphological (MD)	Immature embryo	Appropriate conditions for embryo growth
Morpho-physiological (MPD)	PIM of germination and undeveloped embryo	Warm and/or cold stratification
Physical (PY)	Seed coats impermeable to water	Opening of specialized structures
Combinational dormancy (PY+PD)	Water-impermeable coats (PY) combined with physiological embryo dormancy (PD non-deep)	cold stratification and after-ripening, the timely order can be species-specific

*Adapted from Nikolaeva (1997)

Table 1.3 Classification scheme of Physiological Dormancy (PD)*

Types	Released by	Examples
Non deep PD	Short period of cold stratification; exposure to high temperature; chemicals (e.g. gibberellic acid, KNO_3); exposure to light.	Common in most weeds, vegetables, garden flowers and some woody plants
Intermediate PD	Cold stratification or application of GA for some species	*Acer negundo, A, pseudoplatanus, A. saccharum, Corylus avellana, Gagus sylvatica, Ferula karatavica, Fraxinus americana, F. pensylvanica, Melampyrum lineare, Polygonum spp.*
Deep PD	Long period of cold stratification (only treatment)	*Impatiens parviflora, Malus domestica, Sorbus aucuparia, Phodotypos kerrioides, Prunus persica, Crataegus sp., Acer tartaricum, Euonymous europaea, Acer platanoides*

*Adapted from Nikolaeva (1997) and Baskin and Baskin (2004)

Embryos excised from seeds with deep PD either do not grow or produce abnormal seedlings; however embryos excised from seeds with intermediate PD and non-deep PD usually grow and the resulting seedlings are normal (Baskin and Baskin, 2004). Freshly matured seeds with non-deep PD either cannot germinate at any temperature or they can only germinate over a narrow range of temperatures. Thus based on patterns of change in physiological responses to temperature, non-deep PD can be sub-distinguished into five types (Fig.1.1).

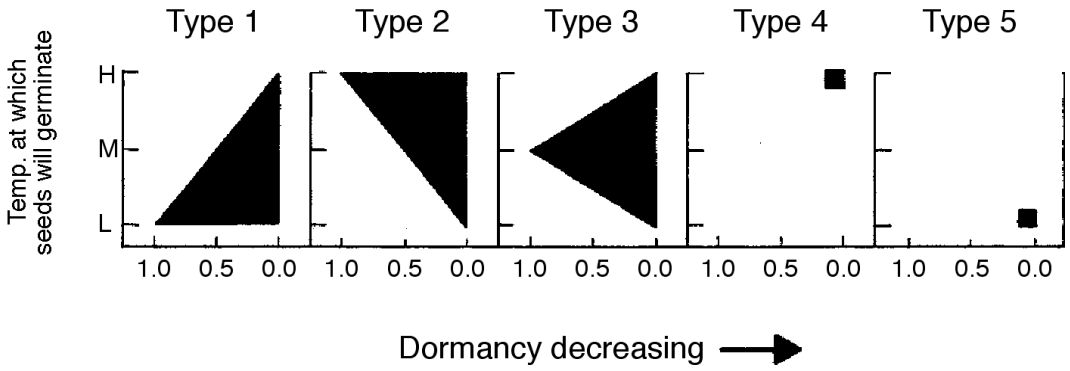

Figure 1.1 Types of non-deep physiological dormancy in seeds based on patterns of change in physiological responses to temperature (from Baskin and Baskin, 2004).

During the progression of non-deep dormancy release, most of the thermal responses of seeds belong to type 1 or 2, in which the temperature range for seed germination widened gradually from low to high temperature (type 1, e.g. *Arabidopsis thaliana*) or from high to low temperature (type 2, e.g. *Helianthus annuus*). Only a few species have type 3 non-deep PD (e.g. *Aster ptarmacoides*). Seeds dormancy of type 4 (e.g. *Callicarpa americana*) can only be released by high temperature while those of type 5 by low temperature (e.g. *Gentianella quinquefolia*) (Baskin and Baskin, 2004). Non-deep PD is thought to be largely regulated by the environment. The induction and loss of non-deep PD can be

triggered by a range of environmental cues via different physiological mechanisms (Finch-Savage and Leubner-Metzger, 2006).

1.2.4 Seed dormancy cycling

The depth of dormancy of imbibed seeds is considered to be continually changing in response to the ambient environment (Finch-Savage and Leubner-Metzger, 2006) (Fig.1.2) and can move between different states, e.g. 'primary dormancy', and 'secondary dormancy' through the process of 'dormancy cycling'. Dormancy cycling is 'the repeated induction and release of dormancy'(Hilhorst, 2007); initially seeds are shed as 'primary dormant', dormancy can then be released and subsequently re-induced (secondary dormant) and continue to cycle between different depths of dormancy in response to seasonal variations, i.e. dormancy is continuously reacting to the environment and adjusting the requirements for germination (Finch-Savage and Leubner-Metzger, 2006). Dormancy cycling is therefore a great advantage in avoiding germination during short spells of unfavourable weather, and for timing germination to occur just before the favourable season that is optimal for plant growth (Hilhorst, 2007).

1.3 Regulation of seed dormancy

1.3.1 Regulation of seed dormancy by environmental factors

Dormancy is regarded as a characteristic of seeds that determines the conditions required for germination, therefore any environmental cue that changes the germination requirements is altering dormancy (Finch-Savage and Leubner-Metzger, 2006).

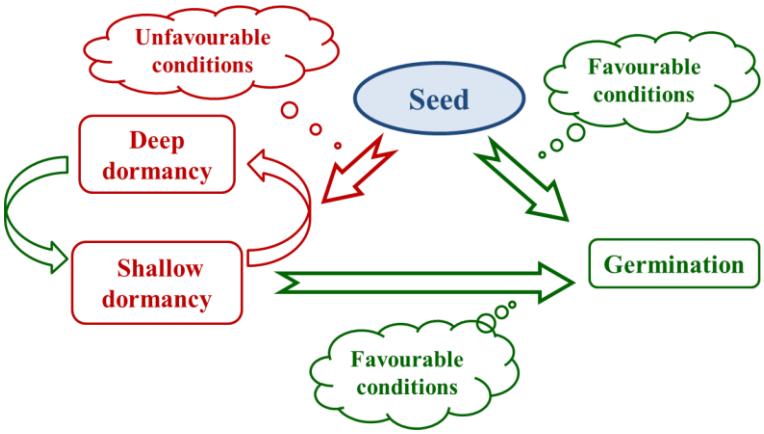

Figure 1.2 Simple description of the relationship between seed dormancy and germination. Dormancy can be released by various environmental factors (favourable conditions). Alternatively, less dormant seeds can be induced into deeper dormancy by some of the same factors (unfavourable conditions). Seeds may cycle between different levels of dormancy with different sensitivity to the environmental factors. The less dormant seeds can progress to germination, again affected by the same environmental factors (favourable conditions). Different colours of the arrows indicate the impacts by favourable conditions (green) and unfavourable conditions (red).

Therefore the cues that widen the environmental requirements for germination should be regarded as dormancy release factors (Leubner, 2005). A range of environmental signals are required for releasing dormancy (e.g. cold or high temperature, alternating temperature, light, nitrate, etc.). However the responses of seeds to these factors are different: (1) there are some factors that are associated with slow seasonal changes, and alter the depth of dormancy and the sensitivity to other factors over time (e.g. temperature); (2) there are also some other factors that indicate the conditions for germination are suitable, and terminate dormancy and induce germination in a more immediate way (e.g. light and nitrate). Both of these factors can remove blocks to germination, but the order in which these factors are perceived is important (Leubner, 2005; Cadman *et al*., 2006; Finch-Savage *et al*., 2007). For example, dry after-ripening or exposure to cold or nitrate is often

required to increase sensitivity to environmental signals such light or alternating temperature, which release the final layer of dormancy inducing germination. If these latter environmental signals are not sensed at the correct time the seed will go increasingly dormant again (Finch-Savage *et al*., 2007).

These latter signals have been considered to be germination cues rather than for dormancy release (Thompson and Ooi, 2010). However, in this thesis these signals are all considered to break dormancy, by removing successive layers until the seed is able to complete germination for the reasons discussed elsewhere (Finch-Savage and Footitt, 2012).

1.3.1.1 Effect of temperature

Temperature is the primary environmental signal regulating both dormancy and germination. Three different effects of temperature on physiological processes in seeds have been identified: (1) temperature together with moisture content determines the rate of deterioration in all seeds; (2) temperature influences the dormancy status (rate of dormancy loss in dry seeds and pattern of dormancy induction and relief in imbibed seeds); (3) temperature determines the rate of progress toward germination in non-dormant seeds (Roberts, 1988). Seasonal variation in temperature is the dominant factor regulating dormancy cycling (Hilhorst, 1998).

- ***Seed development:***

Observations linking seed maturation temperature to seed dormancy and germination have been made in a number of species, such as *Avena fatua* (Sawhney

et al., 1985), *Aegilops ovate* (Datta *et al*., 1972), *Chenopodium album* (Karssen, 1970), soybean (Keigley and Mullen, 1986) and *Arabidopsis* (Kendall *et al*., 2012; Penfield and Springthorpe, 2012). Seed responses to different maternal temperatures were summarized by Baskin and Baskin (1998). They showed that an increase in temperatures during seed development led to a decrease in seed dormancy in a number of species, such as *Aegilops ovate, Anagallis arvensis, Lolium multiflorum, Lolium perenne, Stellaria media, Beta vulgaris* etc. In addition, Fenner (1991) also listed 15 cases where high maturation temperatures led to lower dormancy, while in three species higher maturation temperatures increased seed dormancy. For example, seeds from *Syringa reflexa* plants grown at 18, 21 and 24˚C were more dormant than those harvested from the plants grown at 15˚C (Junttila, 1973); and *Themeda australis* plants grown at 33/28˚C produced seeds that had significantly deeper dormancy than those matured at 21/16˚C (Groves *et al*., 1982). Also seeds of wild oats showed deeper dormancy when developed at 20/10˚C compared to those at constant 20˚C (Sawhney *et al*., 1985).Therefore not only is the environment directly experienced by the developing seed important, but the parent plant is able to transmit signals that provide the seed with vegetative environmental information (Fenner, 1991).

Genetic influences on the induction of strong primary dormancy by low temperature during seed maturation have been demonstrated and roles for both phytochrome and FLOWERING LOCUS C (FLC) have been proposed (Donohue *et al*., 2008; Heschel *et al*., 2008; Chiang *et al*., 2009). Low temperature during seed

maturation was found to induce several genes associated with dormancy, including *DELAY OF GERMINATION1* (*DOG1*), and also influence germination related hormones abscisic acid (ABA) and gibberellins (GA) levels in matured seeds (Kendall *et al*., 2011). Additionally as the key regulatory proteins in cold acclimation, cold-induced C-REPEAT BINDING FACTORS (CBFs) were revealed to play an important role in the regulation of dormancy during seed maturation at low temperature where they regulate expression of *GA2ox6* and *DOG1* (Gery *et al*., 2010; Kendall *et al*., 2011). In seed post-shedding *CBF* expression was less sensitive to low temperature; this inhibition of *CBF* expression was proposed as a critical mechanism allowing cold to promote rather than inhibit germination (Kendall *et al*., 2011).

- ***Post shedding dry seeds (after-ripening)***

After-ripening (AR) can be defined as dry storage of freshly harvested dormant seeds (Bewley, 1997); it is a common method for releasing seed dormancy and a key seed trait in the determination of germination potential (Finch-Savage *et al*., 2007; Carrera *et al*., 2008). AR increases sensitivity of seeds to the environmental signals that release dormancy (Finch-Savage and Leubner-Metzger, 2006). Therefore in the early stages of AR, seeds may not germinate even though the environmental conditions are favourable, but will germinate following an appropriate duration of AR (Carrera *et al*., 2008). Moreover, by comparing the transcriptomes of fresh and AR seeds of ABA mutants that have no dormancy and their wild types, it was found that in both cases fresh seeds had dormant

transcriptomes and following AR both have AR transcriptomes. This demonstrated that the process of AR in seeds operates via a separate pathway to dormancy (Carrera *et al*., 2008). Moreover the dry after-ripening periods that are required to release primary dormancy are also temperature-dependent (Bradford, 2002; Baskin and Baskin, 2004).

The molecular mechanism through which AR reduces dormancy levels is not well understood. However, the progressive effect of AR is thought to be related to the increase in sensitivity to GA and decrease in sensitivity to ABA (Finch-Savage and Leubner-Metzger, 2006). In a study by Cadman *et al*. (2006), the primary dormant seeds were found to have a higher level of expression of genes related to ABA biosynthesis (e.g. *NCED6*, *NCED9* and *ABA1*), but lower expression of the ABA catabolism gene *CYP707A2* compared to AR seeds. The expression of *GA3ox2* (GA biosynthesis gene) was also found to be higher in AR seeds than primary dormant seeds, indicating that AR alters the regulation of GA levels. In addition, in *Arabidopsis* Cvi seed, a microarray approach showed that thirty genes were down-regulated, including the dormancy regulating gene *DOG1* during AR (Finch-Savage *et al*., 2007). However, recent work by Nakabayashi *et al*. (2012) has shown that it was not reduced expression of *DOG1* that led to increased germination potential but changes in the structure of the *DOG1* protein, i.e. DOG1 protein levels appeared to be stable during AR, but this change rendered the protein non-functional.

During a screen of *Arabidopsis* enhancer trap lines for AR and germination phenotypes, a role for the NADPH-oxidase AtrbohB in seed germination and after-

ripening was also identified (Müller *et al*., 2009). It was found that a decrease in *AtrbohB*-transcripts in mature seed embryos led to an alternation in AR; with *atrbohB* mutants failing to after-ripen with respect to their sensitivity to ABA. Levels of protein carbonylation were reduced in the *atrbohB* mutants and this difference became more pronounced during AR. Thus superoxide production by ATRBOHB was suggested to contribute to protein oxidation involved in AR (Müller *et al*., 2009).

A model describing the regulation of AR in seeds, and also the relationship between AR and ABA was suggested by Carrera *et al*. (2008) (Fig.1.3).

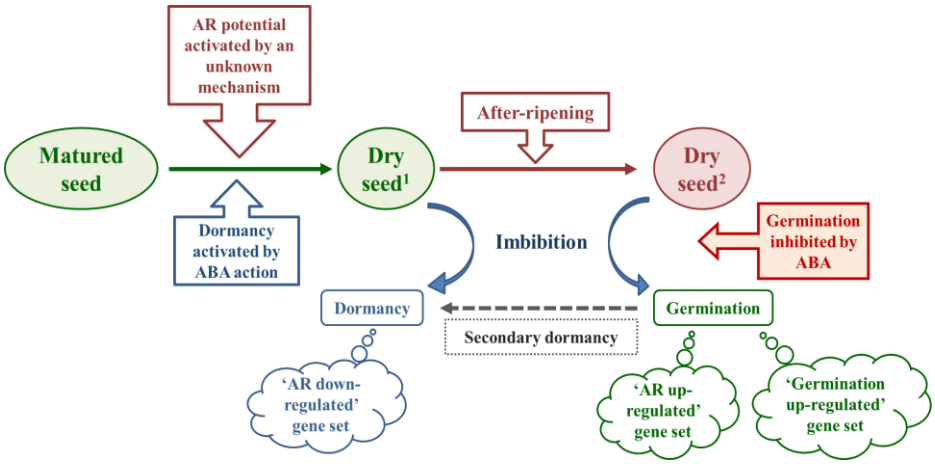

Figure 1.3 A model describing after-ripening (AR) as a distinct developmental process in seeds, and the relationship of AR and ABA in this process. Thin arrows indicate developmental processes; Arrow-shaped boxes indicate the influence of specific factors on developmental pathways. Superscript numbers indicate pre- and post-AR states of unimbibed seeds. A dotted line is used to indicate imbibed seeds induced into a secondary dormancy state (adapted from Carrera *et al*., 2008).

Both dormancy (D) and AR are activated once the seeds have reached maturity, with ABA activating the dormancy pathway and AR operating via an independent unknown mechanism. Freshly harvested seeds (Dry seed^{1}; see Fig.1.3) become dormant once imbibed due to the action of ABA, and meanwhile those 'AR

down-regulated' genes are expressed. However, the seeds following AR (Dry seed^2) express 'AR up-regulated' and 'germination up-regulated' genes when they are imbibed, leading to a less dormant status. Exogenously applied ABA can inhibit germination of those imbibed AR seeds without changing the expression of these gene sets, demonstrating that exogenous ABA cannot be used to reinstate the dormancy programme. Imbibed AR seeds can go into secondary dormancy with up-regulation of the 'AR down-regulated' gene set expression and down-regulation of 'AR up-regulated' gene set expression (Carrera *et al*., 2008).

- ***Post shedding imbibed seeds***

Evidence also shows that low temperature can lead to the induction of dormancy in species adapted for autumn germination (Baskin and Baskin, 1986; Footitt *et al*., 2011; Penfield and Springthorpe, 2012); while in many species, particularly those adapted for spring germination, chilling treatment releases dormancy in imbibed seeds (Probert, 2000; Baskin and Baskin, 2004). For example, seeds of the annual aquatic grass *Zizania palustris* are dormant at the time of dispersal in autumn, but during the winter time, seeds exposed to temperatures close to freezing showed a steady decline in dormancy level (Probert and Brierley, 1989). According to Bewley and Black (1994), the optimum temperatures for the dormancy-releasing effect of low temperature are generally close to 5˚C. However in studies of *Rumex obtusifolius* and *R.crispus*, it is reported that dormancy can be released at a wider range of low temperatures from 1.5˚C to 15˚C, within which the lower the initial temperature, the greater the germination (Totterdell and Roberts,

1979). It was also suggested that there is an optimum period for stratification (i.e. four weeks for *R.obtusifolius* and six weeks for *R.crispus*) as a short-term low temperature treatment can lead to the rapid loss of primary dormancy whereas extended chilling can gradually induce secondary dormancy (Totterdell and Roberts, 1979; Cadman *et al*., 2006; Penfield and Springthorpe, 2012).

The environmental control of germination is a complex process involving a number of factors (e.g. light, alternating temperatures, nitrate etc.), but several studies have shown that in some species low temperature can reduce the requirement of seeds for other environmental factors (Roberts and Benjamin, 1979; Vincent and Roberts, 1979; Probert *et al*., 1989). Low temperature has also been reported to have a direct impact on phytochrome-controlled germination of lettuce seeds (Van der Woude and Toole, 1980). It was also suggested that C-REPREAT BINDING FACTORS (CBFs) are necessary for the regulation of dormancy and of *GA2ox6* and *DOG1* expression caused by low temperature in *Arabidopsis* (Kendall *et al*., 2011). ABA content was reported to decrease before germination following the transfer of imbibed seeds from 4˚C to 20˚C (Ali-Rachedi *et al*., 2004). Additionally a subset of GA biosynthesis genes were found to be up-regulated in response to low temperature, leading to an increase in the level of bioactive GAs and transcript abundance of GA-inducible genes in imbibed *Arabidopsis* seeds (Yamauchi *et al*., 2004).

Warm imbibed temperatures have also been reported to have an impact on germination behaviour in terms of releasing or inducing dormancy. For example, in

general, low temperatures release seed dormancy of summer annuals, whereas high temperatures induce dormancy, and in winter annuals the reverse is true (Baskin and Baskin, 1977; 1978; 1986; 1987). In some desert perennial shrub species, the way pre-incubating seed at 45˚C for 24 hours affected dormancy was dependent on the plants geographic origin and whether they receive winter or summer rain. In the former, germination was negatively influenced while in the latter, germination was stimulated by high temperature pre-treatment (Gutterman, 1990).

1.3.1.2 Effect of light

In addition to temperature, light is one of the most important environmental cues regulating plant development and is also another key regulator of seed dormancy and germination (Franklin and Whitelam, 2004). In nature, white light (e.g. sunlight) releases dormancy but the most effective wavelengths are in the orange/red region of the spectrum (i.e. 660nm); however wavelengths longer than 700nm were reported to be the inhibitory parts of the spectrum for germination, with specially potent wavebands in the far-red (Bewley *et al*., 2013). These wave lengths are detected by phytochromes; a family of plant photoreceptors that perceive red-light and far-red-light wavelengths (Heschel *et al*., 2008) that are involved in the control of seed germination by light (Shinomura *et al*., 1994; Hennig *et al*., 2002). Unlike other pigments, phytochrome has two different chemical structures: they are synthesized in the red-absorbing (Pr) isomer and converted to the far-red absorbing (Pfr) isomer after absorbing red light. Pfr is reported to be the bioactive form which mediates seed germination and other light-induced responses (Donohue *et al*., 2007).

Gene expression analysis has shown that phytochromes (Pfr) can increase the amount of bioactive GAs in seeds by activating the transcription of GA-3-oxidase genes (*GA3ox1* and *GA3ox2*) and also repressing the transcription of the GA degradation gene *GA2ox2* (Bewley *et al*., 2013).

In *Arabidopsis*, five phytochromes have been identified: Phytochrome A-E (*PHYA-PHYE*) (Sharrock and Quail, 1989; Clack *et al*., 1994). All of these phytochromes were reported to have distinct characteristics related to their functions (Koorneef and Kendrick, 1994).

- ***Seed development***

Short vs. long day lengths during seed development influence seed dormancy and induce differences in germination characteristics (Baskin and Baskin, 1998; Munir *et al*., 2001). Baskin and Baskin (1998) listed 21 species exhibiting variation in germination behaviours when the mother plants were grown under different day lengths (e.g. *Aegilops kotschyi*, *Cheiridopsis aurea*, *Diplotaxis harra* etc.). The results showed that seeds matured under the short day conditions (i.e. short light *vs.* long dark period) germinated to lower percentages than those from plants grown under long days. In addition to the day length, light quality during seed development was also shown to influence germination requirement of the progeny (e.g. *Arabidopsis*). Seeds from the plants grown under incandescent lights had a high amount of the inactive, red-absorbing form of phytochrome (Pr) and thus required light for germination; whereas, seeds from the plants grown under fluorescent lights had a relatively high amount of the active far-red-absorbing form of phytochrome

(Pfr), and could germinate in the dark (Hayes and Klein, 1974). The difference in the red: far-red (R: FR) ratio of light during seed maturation was also found to cause variation in the final germination percentage of *Piper auritum* seeds (Orozco-Segovia *et al*., 1993). In *Arabidopsis*, by using single and multiple phytochrome mutants, the contributions to germination by different phytochromes were found to depend on seed-maturation conditions, i.e. PHYB and PHYD were necessary to break cold-induced dormancy, while PHYA contributed to the maintenance of cold-induced dormancy and the suppression of germination on a *phyD* background (Donohue *et al*., 2008; Heschel *et al*., 2008). PHYA and PHYB are the dominant phytochromes in *Arabidopsis*, involved in the regulation of seed germination responses to light, with PHYB being important under red light and PHYA under far-red light (Shinomura *et al*., 1994; Hennig *et al*., 2002; Heschel *et al*., 2008). In addition, the seeds of hy3 (*long hypocotyls 3*) mutant, which lack functional PHYB were not able to germinate in the dark, indicating a role of PHYB in dark regulation of germination (Shinomura *et al*., 1994).

- ***Post shedding seeds***

Light is an important factor for releasing seed dormancy and influencing germination via the influence of the phytochrome system (see above). Germination promoted by exposure to light is partly due to the role of phytochrome in decreasing the ABA content of seeds, through the repression of the ABA biosynthesis genes *NCED6* and *NCED9* and the up-regulation of the ABA catabolism gene *CYP707A2* (Seo *et al*., 2006). In addition, the light activation of phytochrome down regulates the

DELLA genes *RGA*, *GAI*, and *RGL2* that are GA signalling pathway repressors; this in turn leads to an increase in endogenous GA levels and GA responsiveness by releasing repression of the GA biosynthesis genes *GA3ox1*and *GA3ox2*, and repressing the GA catabolism gene *GA2ox2* (Oh *et al*., 2007; Seo *et al*., 2009).

Photoperiodic effects on germination were summarized by Bewley *et al*. (2013): some species require exposure to long days (e.g. *Begonia evansiana*) but others to short days (e.g. *Tsuga Canadensis*). A seeds response to light can also be temperature dependent, for example, lettuce seeds (*Lactuca sativa* 'Grand Rapids') did not require light for germination at low temperature but showed dark dormancy at high temperature (≥ 23˚C) (Kollar, 1962). In *Arabidopsis*, phytochromes were also found to be temperature dependent, i.e. PHYE contributes to germination at low temperatures, whereas PHYA is important for germination at warm temperatures; PHYB, however, is important for germination over a range of temperatures (Heschel *et al*., 2007). This suggests that phytochromes play important roles in regulating germination responses not only to light signals, but also to temperature signals. Additionally sensitivity to light was reported to be enhanced by chilling and various temperature alternations (Bewley *et al*., 2013).

1.3.1.3 Effect of nitrate

Nitrate (N) is an essential inorganic nutrient required for the synthesis of proteins. Plants take up mineral nitrogen (N) directly from the soil in the form of reactive nitrogen, which includes all biologically and chemically active nitrogen

compounds (Ikram *et al*., 2012). The main source of N is nitrate (NO_3^-), sometimes with a contribution from ammonium (NH_4^+) and amino acids (Marín *et al*., 2011).

- ***Effect of soil nitrate***

Several studies have reported an annual variation in soil nitrate and seed nitrate content (Bouwmeester and Karssen, 1993; Derkx and Karssen, 1993). A linear relationship was also seen between seed nitrate content and germination in *Sisymbrium officinale*, a close relative of *Arabidopsis* (Bouwmeester *et al*., 1994). This suggests a link between soil nitrate and seed dormancy. In *Arabidopsis* plants grown with a high level of nitrate (50mM) during seed maturation produced less dormant seeds, compared to those produced under lower nitrate nutrition (10mM and 3mM) (Alboresi *et al*., 2005). In addition, a nitrate reductase (NR) deficient genotype (G'4-3 mutants) was found to accumulate nitrate under higher levels of nitrate supply and produced seeds with lower dormancy compared to wild type. This suggested that seed dormancy level was negatively related to nitrate supply to the mother plants and subsequently with the nitrate content of seeds (Alboresi *et al*., 2005). However, field experiments showed that dormancy patterns remain the same in seeds that were exposed to variation in soil nitrate and where it did not vary (Bouwmeester and Karssen, 1992; Bouwmeester and Karssen, 1993; Derkx and Karssen, 1993; Bouwmeester *et al*., 1994). It was concluded that changes in dormancy were not driven by soil nitrate and most endogenous nitrate was leached from seeds in the first 24h of imbibition on water in the laboratory (Hilhorst, 1990). Thus the endogenous nitrate concentration has little ecological importance and high

nitrate content only temporally release dormancy when seeds are placed in soil (Bouwmeester *et al*., 1994).

- ***Germination sensitivity to nitrate***

Nitrate supply has long been described as one of the important compounds affecting all levels of plant function, especially in releasing seed dormancy and promoting germination in a large number of species (Bouwmeester *et al*., 1994; Yukie Horibe *et al*., 2001; Matakiadis *et al*., 2009). For example, seeds of *Avena fatua* germinated faster in presence of high levels of KNO_3 than in the presence of KCl (McIntyre *et al*., 1996); Saini *et al*. (1985) also observed that nitrate stimulated germination of *Chenopodium album* seeds, and nitrate supplied either exogenously or by the mother (endogenously) increased germination and the response of seeds to ethylene and gibberellins. In *S. officinale*, nitrate was shown to promote germination by increasing GA synthesis in close interaction with light, and this effect was independent of reduction in nitrate, indicating a possible signalling role of nitrate (Hilhorst and Karssen, 1989).

The nitrate effect on seed dormancy in *Arabidopsis* seeds has been studied by several groups: Batak *et al*. (2002) showed that exogenous nitrate could stimulate seed germination in the Ler ecotype by reducing the light requirement. Hilhorst and Karssen (1988) and Alboresi *et al.* (2005) pointed out that nitrate acted as a signal molecule, altering the GA requirement for seed germination. Ali-Rachedi *et al*. (2004) also showed a positive effect of nitrate in releasing dormancy of *Arabidopsis* Cvi seeds, and this effect resulted from a decrease in ABA levels. Transcript profiling in *Arabidopsis* seeds showed that the expression of the ABA catabolic gene *CYP707A2* was positively regulated by exogenous nitrate. The *cyp707a2-1* mutant failed to reduce seed ABA levels in response to both endogenous and exogenous

nitrate; however a reduction in ABA levels was found in the wild type and *cyp707a1-1* mutant, indicating a central role of the *CYP707A2* gene in the nitrate-mediated control of ABA levels during seed development and germination (Matakiadis *et al*., 2009). Moreover the transcript abundance of the nitrate transporter *NRT1.1* was lower in imbibed SD2 seeds (i.e. secondary dormancy re-induced by cold after dormancy loss) of the *Arabidopsis* Cvi ecotype, which is insensitive to nitrate (Finch-Savage *et al*., 2007). Therefore nitrate was capable of releasing dormancy including dormancy induced by warm temperature in the dark, but nitrate was not effective in releasing secondary dormancy induced by prolonged exposure to low temperature in the dark in the laboratory or the field (Cadman *et al*., 2006; Finch-Savage *et al*., 2007; Footitt *et al*., 2011).

Recent work suggests that NRT1.1 is an important component of a sensing system for changes in local external nitrate concentrations (Gojon *et al*., 2011), but NRT1.1 is responsible for the regulation of only a fraction of the nitrate regulated genes and thus additional nitrate sensing and signal transduction mechanisms may exist (Alvarez *et al*., 2012).

1.3.2 Hormonal regulation and genetics of seed dormancy and germination

Abscisic acid (ABA) and gibberellins (GA) are the major hormones influencing germination in *Arabidopsis* seeds, with the ABA/GA balance determining the developmental state of the seed i.e. if it is dormant or non-dormant and therefore able to germinate. A model for the regulation of dormancy and germination by ABA and GA in response to the environment describes how the ambient environmental factors influence the ABA/GA balance (gene expression), the sensitivity to these hormones and the impact on germination sensitivity (Fig.1.4)

(Finch-Savage and Leubner-Metzger, 2006).

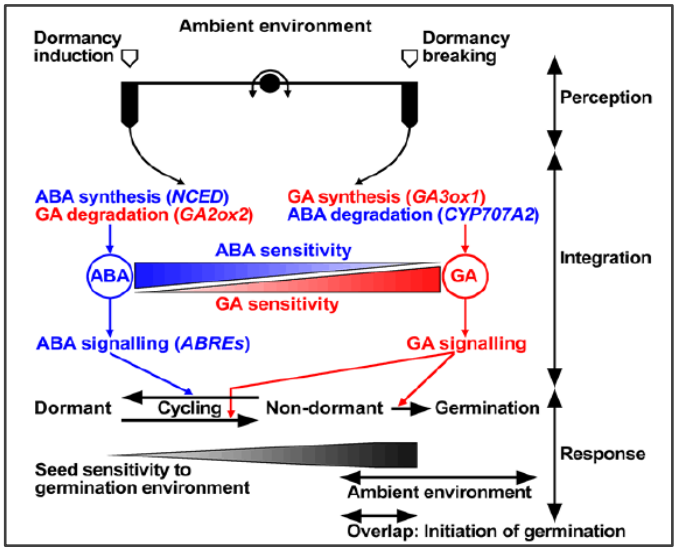

Figure 1.4 Dormancy cycling results from the interplay between hormone synthesis, degradation and sensitivities in response to ambient environmental conditions. According to the model, ABA synthesis and signalling (plus GA catabolism) are dominant during the dormant state, but in the transition to germination, GA synthesis and signalling (plus ABA catabolism) is dominant (Finch-Savage and Leubner-Metzger, 2006).

Regulation by abscisic acid (ABA)

The discovery of ABA as a leaf abscission and seed dormancy promoting sesquiterpenoid was firstly reported in the 1960s (reviewed in Cutler *et al*., 2010 and Raghavendra *et al*., 2010). Since then physiological and genetic evidence has shown that ABA has an important role in the induction and maintenance of primary dormancy (Koornneef *et al*., 1984; Hilhorst and Karssen, 1992; Nambara *et al*., 1992; Bewley, 1997).

Primary dormancy develops at the end of the seed maturation, when the molecular dependence upon the mother plant disappears, water content decreases, storage products are synthesized, and ABA has accumulated (Nambara and Marion-

Poll, 2005; Rodríguez-Gacio *et al*., 2009). The relatively high levels of ABA in maturing seeds are considered to maintain dormancy and prevent early germination during development (pre-harvest sprouting). Following shedding, studies using seeds of *Arabidopsis*, *Striga asiatica* and *Pinus monticola***,** found that dormant seeds treated with fluridone (a compound that promotes germination by inhibiting ABA synthesis) had similar germination responses to non-dormant seeds indicating that the continued synthesis of ABA following imbibition is required to maintain dormancy (Ali-Rachedi *et al*., 2004; Kusumoto *et al*., 2006; Feurtado *et al*., 2007). The ABA content of seeds also decreased rapidly in the early stage of imbibition and then stayed at a low level throughout the process, and thus the germination potential of a given seed was determined primarily by ABA catabolism and the ABA/GA balance (Finch-Savage and Leubner-Metzger, 2006; Liu *et al*., 2009). In addition, dry dormant seeds contained higher levels of ABA than dry non-dormant seeds. During early imbibition, both dormant and non-dormant (after-ripened) *Arabidopsis* seeds had a decrease in ABA content, but the rate of this was slower in dormant seeds. Importantly the ABA content in dormant seeds did not start to increase (i.e. ABA synthesis) until three days after start of imbibition (Ali-Rachedi *et al*., 2004; Millar *et al*., 2006).

Seed ABA levels and the resultant dormancy are controlled by the combined action of differentially expressed enzymes involved in both synthesis and catabolism (Finkelsteine *et al*., 2008). During seed development, the first step in ABA synthesis from carotenoid synthesis involves the NINE-CIS-EPOXYCAROTENOID

DEOXYGENASE (NCED) enzyme family members NCED5, NCED6 and NCED9 which play an important role in ABA synthesis in seeds and are involved in regulating dormancy (Lefebvre *et al*., 2006; Frey *et al*., 2012). The *nced6nced9* double loss-of-function mutants have reduced ABA content and decreased seed dormancy levels (Lefebvre *et al*., 2006). The overexpression of some ABA biosynthesis genes increased seed ABA content, leading to enhanced seed dormancy and subsequently delayed germination (Finkelstein *et al*., 2002; Holdsworth *et al*., 2008; reviewed in Liu *et al*., 2009). The induction of *NCED6* gene expression, and to a lesser degree induction of *NCED9* during imbibition led to higher levels of ABA production, which in turn restricted seed germination. This result indicated that NCED6, as the key rate-limiting enzyme in ABA biosynthesis, was more effective than NCED9 in reducing germination (Martínez-Andújar *et al*., 2011).

In contrast to biosynthetic mutants, ABA catabolism mutants showed increased dormancy with increasing ABA levels; *CYP707A1* and *CYP707A2*, which encode abscisic acid 8'-hydroxylases are the key genes in ABA catabolism (Kushiro *et al*., 2004; Okamoto *et al*., 2006). Mutation in these genes leads to increased seed dormancy corresponding with increased ABA levels (Kushiro *et al*., 2004). Additionally, *cyp707a1* mutants accumulated more ABA at the end of seed maturation as measured in dry seeds compared to *cyp707a2*, which in turn exhibited slower reduction in ABA levels on imbibition. The results indicated that *CYP707A1* plays a role in determining ABA levels during seed maturation, while *CYP707A2* plays a major role in the rapid decrease in ABA levels during early seed imbibition

(Okamoto *et al*., 2006). Additionally, Footitt *et al*. (2011) also found that in the field the expression of *DOG1* in *Arabidopsis* Cvi seeds increased after burial and continued to increase even after ABA content reached a plateau, which suggested that in seeds with deep dormancy like Cvi, *DOG1* expression may be the dominant factor influencing ABA signalling and thus sensitivity rather than ABA synthesis.

The characterization of the *ABA-INSENSITIVE1* (*ABI1*) gene revealed that it encodes a protein composed of a novel N-terminal segment and a C-terminal domain that is an active protein serine/threonine phosphatase 2C (PP2C) (Bertauche *et al*., 1996). Ultimately, it was found that the *ABI2* and *ABI1*genes are homologous to each other, and the encoded proteins share the same molecular architecture composed of a C-terminal domain with PP2C activity. Thus both ABI1 and ABI2 phosphatase have overlapping negative roles in ABA signalling (Leung *et al*., 1997; Merlot *et al*., 2001). Among the ABA sensitivity mutations in *Arabidopsis*, only *abi1-1* and *abi2-1* influence ABA responsiveness both in seeds and in vegetative tissues (Merlot *et al*., 2001).

Three well-characterized transcription factors ABI3 (ABA INSENSITIVE 3), ABI4 (ABA INSENSITIVE 4) and ABI5 (ABA INSENSITIVE 5) were from different transcription factor families: B3-, APETALA2- and bZIP domain, regulating overlapping subsets of ABA-related genes (Giraudat *et al*., 1992; Finkelstein, 1994; Finkelstein *et al*., 1998; Finkelstein and Lynch, 2000). ABI5 is crucial for seed germination and the expression was regulated by the products of ABI3 and ABI4, and activated by its own expression (Bossi *et al*., 2009; Garcia *et*

al., 2008). The *Arabidopsis* RING-type E3 ligase KEEP ON GOING (KEG) is a negative regulator of ABA signalling and KEG E3 ligase activity is required for the regulation of ABI5 abundance (Liu and Stone, 2010). Kinases are important for stress response and some like SUCROSE NONFERMENTING RELATED KINASES 2 (SnRK2) was also important for activating ABI5 (reviewed in Daszkowska-Golec, 2011). All three transcription factors (i.e. *ABI3*, *ABI4* and *ABI5*) are highly expressed in mature seeds, but differ in localization within the seeds (Finkelstein and Lynch, 2000; Penfield *et al*., 2006b). A number of studies using ABA response mutants also revealed that, the ABA signal transduction pathway associated with dormancy induction involve protein kinases (the SnRK2 ABA-activated protein kinase Family), phosphatases (ABI1 and ABI2) and transcription factors (ABI3, ABI4 and ABI5) (Koornneef *et al*., 1984; Finkelstein *et al*., 2002; Himmelbach *et al*., 2003; Liu *et al*., 2009; reviewed in Daszkowska-Golec, 2011).

In addition, there are also two genes involved in ABA sensitivity during seed development and germination, i.e. A RING-finger gene *DESPIERTO* (*DEP*) which regulated the expression of *ABI3*, and the *HDZip* (*ATHB20*) which increased ABA sensitivity and seed dormancy when mutated (Barrero *et al*., 2010). A mutation in the *DEP* gene causes lack of dormancy, whereas the *athb20-1* insertion mutant shows enhanced dormancy compared to the wild type (Barrero *et al*., 2010).

Recent breakthroughs in the field of ABA signalling revealed a unique hormone perception mechanism: i.e. binding of ABA to the ABA receptors RCARs/PYR1/PYLs resulted in the inactivation of PP2C (e.g. ABI1 and ABI2),

which launched SUCROSE NON-FERMENTING PROTEIN-1 (SNF1)-type kinase action targeting ABA-dependent gene expression and ion channels (Cutler *et al*., 2010; Raghavendra *et al*., 2010).

Gibberellic acid (GA)

Gibberellins stimulate germination by inducing hydrolytic enzymes that weaken the barrier tissues such as the endosperm or seed coat, inducing mobilization of seed storage reserves, and promoting expansion of the embryo (Finkelstein *et al*., 2008). Gibberellic acid (GA) plays a key role in promotion of germination, which is antagonistic to ABA during seed development, with respect to dormancy induction (White and Rivin, 2000). GA levels increase during germination just before radicle protrusion (Jacobsen *et al*., 2002). In the GA-deficient mutants (*ga1-3* and *ga2-1*) the failure to germinate without exogenous GA showed that GA is absolutely required for seed germination (Koornneef and Veen, 1980). The transcripts of the key biosynthetic genes *GA3ox1* and *GA3ox2* were accumulated during the early phase of germination and strongly expressed in the axis of the imbibed embryo (Yamaguchi *et al*., 2001; Ogawa *et al*., 2003; Mitchum *et al*., 2006).

GA 2-oxidation by GA 2-oxidases is thought to be the only GA deactivation reaction responsible for GA degradation (Yamaguchi *et al*., 2007). By studing the loss-of-function *ga2ox2* mutant, the GA deactivation gene *GA2ox2* was found to play a key role in lowering GA levels and suppressing germination in dark-imbibed seeds. In addition, the *GA2ox2* transcripts accumulated predominantly in the cortex of embryonic axis in dark-imbibed seeds after FR light irradiation, which was similar to

that observed for *GA3ox1* and *GA3ox2* transcripts in -seeds imbibed in the light. This indicated that phytochrome dependent changes in GA levels are regulated by GA synthesis and degradation that mainly take place in the same cell types (Yamauchi *et al*., 2007).

Arabidopsis CHOTTO1 (CHO1) is thought to be involved in the regulation of *GA3ox1* and *GA3ox2* expression during seed imbibition (Yano *et al*., 2009). The *cho1* mutation was able to restore the delayed seed germination of the *cyp707a2-1* mutant rather than *ga1-3,* indicating that CHO1 acts downstream of ABA to repress GA biosynthesis and plays a role in regulating the endogenous ABA/GA balance in imbibed seeds (Yano *et al*., 2009).

Endogenous GA is perceived by the GA receptors of the GID1 (GIBBERELLIN INSENSITIVE DWARF1) family, which was first identified in rice (Ueguchi-Tanaka *et al*., 2005). Three GID1-type GA receptors, GID1a, GID1b and GID1c are known in *Arabidopsis*, and loss of the three GID1 receptors results in GA insensitivity (Griffiths *et al*., 2006; Willige *et al*., 2007; Voegele *et al*., 2011). The GA-GID1 complex also interacts with DELLA proteins, which are repressors of GA-responsive plant growth and are involved in seed germination (Tyler *et al*., 2004; Cao *et al*., 2006; Penfield *et al*., 2006a). The *Arabidopsis* genome encodes five highly homologous DELLA protein repressors, including GA INSENSITIVE (GAI) and REPRESSOR OF *ga1-3* (RGA) (Richards *et al*., 2001), which have overlapping functions as repressors of elongation growth. Some recent studies have shown that the N-terminal DELLA and VHYNP domains of the DELLA protein RGA are

required for GID1 interactions in *Arabidopsis* (Griffiths *et al*., 2006) and also that GAI DELLA domain is required and sufficient for interaction with the *Arabidopsis* GA receptor protein GID1A (Willige *et al*., 2007).

RGA and GAI play important roles in repressing GA responses during development (Sun and Gubler, 2004; Sánchez-Fernández *et al*., 1998), and light stimulated germination is partly by inactivating GAI and RGA proteins through the regulation of GA metabolic genes (Cao *et al*., 2006). The DELLA genes, *RGA-LIKE1* (*RGL1*) and *RGL2* also play predominant roles in controlling germination and floral development (Lee *et al*., 2002; Cheng *et al*., 2004; Tyler *et al*., 2004). RGL2 function as an integrator of environmental and endogenous cues to control seed germination. The transcript levels of *RGL2* increase rapidly following seed imbibition and then decline rapidly as germination proceeded (Lee *et al*., 2002). The removal of RGL2 function in a *ga1* mutant background restored germination, indicating that RGL2 is a germination repressor in the absence of GA biosynthesis (Lee *et al*., 2002; Tyler *et al*., 2004). Additionally, RGL2 was also suggested to be responsible for promoting ABA synthesis when GA levels were decreased (Piskurewicz *et al*., 2008).

SPATULA (SPT) and PIF3-like 5 (PIL5) are basic helix-loop-helix (bHLH) transcription factors that regulate expression of *GA3ox1* and *GA3ox2* by the interaction of light and cold temperature (Penfield *et al*., 2005). SPT is thought to be stable in the light and regulate germination in response to temperature, whereas PIL5 repress germination and expression of *GA3ox1* and *GA3ox2* in the dark after cold

treatment (Penfield *et al*., 2005). PIL5 inhibits seed germination both by coordinating hormone signalling cascades and by inhibiting cell wall loosening in imbibed seeds (Oh *et al*., 2009). PIL5 was reported to repress germination by activating the expression of GA catabolism gene *GA2ox2* (Oh *et al*., 2006) and increasing the expression of DELLA genes (i.e. *GAI* and *RGA*) in the dark. In addition, PIL5 regulates ABA and GA metabolic genes partly through SOMNUS (SOM) and is involved in the regulation of *SOM* expression, a key negative regulator of seed germination, by interacting with ABI3 at the SOM promoter (Kim *et al*., 2008; Park *et al*., 2011).

However the role of GA in dormancy release is controversial. Although the combination of GA biosynthesis and a high GA sensitivity is essential for germination, both factors do not primarily regulate dormancy, and GA treatment alone did not promote germination in all species or in fully dormant *Arabidopsis* seeds (Derkx and Karssen, 1993; Derkx and Karssen, 1994; Bewley, 1997; Ali-Rachedi *et al*., 2004; Footitt *et al*., 2011). However, the key importance of GA for dormancy release was also supported by observations demonstrating that GA treatment simulated the effect of natural dormancy-releasing factors, such as light and cold stratification (Yamauchi *et al*., 2004; Obroucheva, 2010). For example, an increased level of GA was found to subsequently mediate the dormancy releasing effect of imbibed chilling in *Arabidopsis* seeds (Yamauchi *et al*., 2004). For this reason, the important role of GA in dormancy release is of primary interest. The mechanism of the ABA/ GA balance in response to environmental signals and the

related genes implicated in the regulation of seed dormancy are summarized in Fig.1.5.

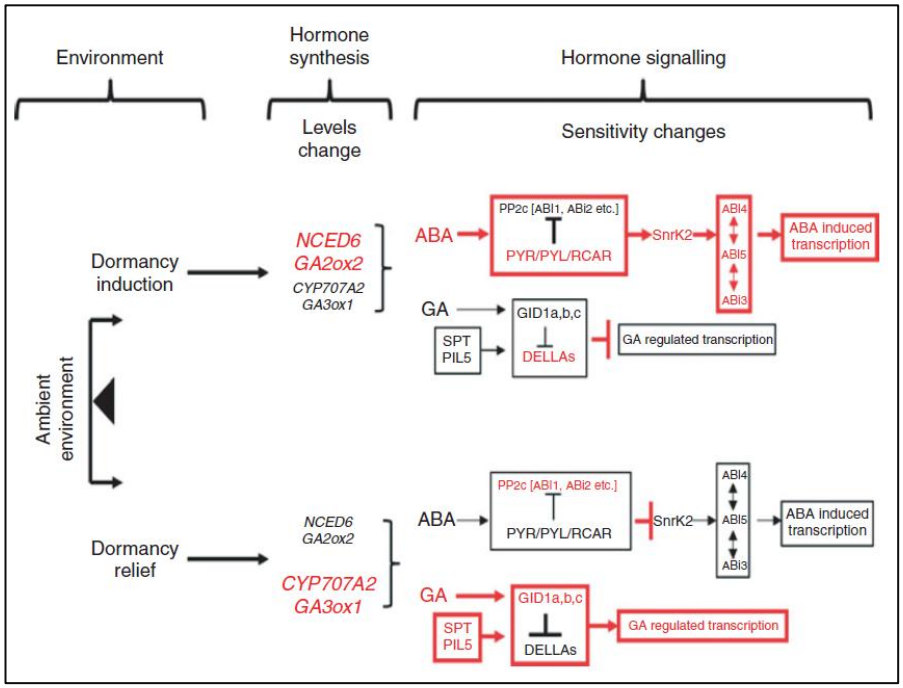

Figure 1.5 A schematic summary of ABA and GA metabolism and signalling genes implicated in the regulation of seed dormancy where environmental signals influence the relative balance of dormancy induction and relief. When induction or relief is induced, the pathways indicated in red dominate (Footitt *et al*., 2011; Finch-Savage and Footitt, 2012).

Other related hormones

Although the contribution of other hormones is not established as well as GA and ABA, roles for hormones such as brassinosteroid (BR), ethylene, auxin and strigolactone have been described.

***Brassinosteroids*:** BR rescues the germination phenotype of severe GA biosynthetic and GA-insensitive mutants, indicating a role of BR in stimulating germination (Steber and McCourt, 2001). The germination of BR signalling mutants were strongly inhibited by ABA (i.e. more sensitive to ABA), and the over-expression of the BR biosynthesis gene *DWARF4* (*DWF4*) overcame inhibition of

germination caused by exogenous ABA, both suggesting that BR signal is needed to overcome inhibition of germination by ABA (Steber and McCourt, 2001; Divi *et al*., 2010). This indicates BR has a positive role in the regulation of germination.

***Ehtylene*:** Ethylene is also a positive regulator of seed germination, i.e. either exogenous ethylene or ethephon (an ethylene-releasing compound) stimulated germination when inhibited by embryo or coat dormancy, unfavourable conditions, or other inhibitors like ABA (KeÇpczyński and KeÇpczyńska, 1997). In addition, analysis of ethylene- and ABA-insensitive mutations showed that endogenous ethylene promoted seed germination by decreasing sensitivity to endogenous ABA (Beaudoin *et al*., 2000). Ethylene also plays a role in counteracting ABA during seed development and promoting the transition from dormancy to germination (Fig.1.6; adapted from Feurrado and Kermode, 2007).

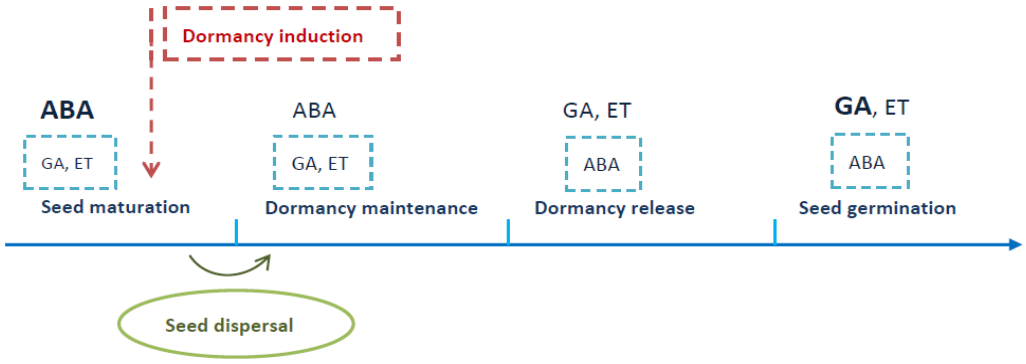

Figure 1.6 The relationship of ethylene (ET), GA and ABA in regulating the processes from seed maturation to germination. Both positive and negative hormones are shown above each process, negative hormones are boxed. The effects of hormones could switch between positive and negative depends on the developmental processes (adapted from Feurrado and Kermode, 2007).

***Strigolactone*:** Strigolactone (SLs) also acts as a positive regulator of germination in *Arabidopsis* during thermo-inhibition in the light by reducing the

ABA: GA ratio, this requires the involvement of HY5, a key transcription factor for light signal transduction (Toh *et al*., 2012). There is also a class of compounds, named Karrikins that are derived from the burnt plant materials. They are structurally similar to strigolactone and stimulate the germination of dormant seeds. Although the role of KARRIKIN INSENSITIVE 2 (KAI2) in karrikin and strigolactone signalling pathways remains unclear, the X-ray crystal structure of KAI2 has been determined and this may provide valuable insight into the possible molecular function of the enzyme (Bythell-Douglas *et al*., 2013).

***Auxin*:** Understanding of the roles of auxin in germination is limited; although recent microarray analysis showed that the expression of several auxin-related genes were modulated by exogenous GA treatment, and GA activity led to changes in auxin level and transport during seed germination (Ogawa *et al*., 2003). Genetic evidence of a role for auxin in germination has also been revealed through an analysis of the regulation of Auxin Response Factor10 (ARF10) by microRNA160, which indicated that interactions between auxin and ABA signalling pathways may have a contribution to the regulation of seed germination potential (Liu *et al*., 2007).

1.3.3 Other genes involved in the regulation of seed dormancy

The induction and release of seed dormancy are controlled by a diverse group of regulators, which act at various levels and show different degrees of specificity (Graeber *et al*., 2012). In addition to those genes that are involved in hormonal action (reviewed above), there are also some important dormancy-associated genes that are worth mentioning:

DELAY OF GERMINATION (DOG1): As reviewed above, most genes identified as involved in seed dormancy have a role in influencing hormone metabolism or perception. However DELAY OF GERMINATION (DOG1) was identified as a major quantitative trait locus (QTL) for seed dormancy in a recombinant inbred line population derived from a cross between low dormant accession Landsberg *erecta* (Ler) and the highly dormant accession Cvi which identified six other *DOG* QTLs (Alonso-Blanco *et al*., 2003). The *DOG1* gene was isolated from a natural variation study using a number of *Arabidopsis* accessions (Bentsink *et al*., 2006). *DOG* QTL analysis was also performed using two novel recombinant inbred line (RIL) populations derived from two *Arabidopsis* genotypes collected at the same site in Kyoto, and this revealed allelic variations at the seed dormancy QTL *DOG1* in one population and at *DOG6* in both populations (Silady *et al*., 2011). Thus the presence of allelic variation at two major seed dormancy loci in two individuals collected from the same location indicated that requirements for germination at the right time may vary within and between seasons (Silady *et al*., 2011).

DOG1 expression was found to be seed-specific, peaking during late seed development, and disappearing after imbibition of the seed. These changes in expression levels coincide with the period during which seed dormancy is initiated and released. Loss-of-function mutant alleles of *DOG1* were also studied, and the *dog1* mutants were non-dormant and did not have any obvious pleiotropic phenotypes, indicating the crucial role of DOG1 in seed dormancy. A major glucose

sensitivity QTL (*GLUCOSE SENSING QTL 5*, *GSQ5*), of which the Cvi allele increases glucose sensitivity was found to be identical to *DOG1* (Bentsink *et al*., 2006), and the expression of the *GSQ5/DOG1* Cvi allele requires the ABA mediated sugar signalling pathway, of which ABI4 is an important component (Teng *et al*., 2008). However recent studies have revealed that DOG1 does not regulate dormancy primarily via changes in hormone levels, i.e. in seeds DOG1 protein functions as a timer for dormancy release largely independent from ABA (Footitt *et al*., 2011; Nakabayashi *et al*., 2012).

The amount of DOG1 protein was highly correlated with dormancy level in freshly harvested seeds rather than the AR seeds, which suggested that the gradual decrease in the amount of functional DOG1 protein may be involved in the mechanism of AR (Nakabayashi *et al*., 2012). Recent studies have also indicated a critical role of DOG1 in temperature-dependent dormancy. The expression of *DOG1* was correlated with the dormancy levels induced by varying temperatures during dormancy cycling in the soil (Footitt *et al*., 2011) and during seed maturation and *DOG1* mutations compromised the ability to enter deep dormancy in response to low seed maturation temperatures (Chiang *et al*., 2011; Kendall *et al*., 2011; Nakabayashi *et al*., 2012). In addition, DOG1 was recently found to influence not only germination, but also flowering time and overall life history, and consequently was the only locus to influence fitness and local adaptation (Chiang *et al*., 2012; Kronholm *et al*., 2012).

Homologues of *DOG1* have been identified in a wide range of species. For example, a comparison between orthologs of *AtDOG1* in *Brassicaceae* relatives suggested a role for *Lepidium sativum LesaDOG1* in the control of germination timing of non-dormant *L.sativum* seeds (Graeber *et al*., 2010).

MOTHER OF FT AND TFL1 (MFT): MFT is a homologue of the Phosphatidyl Ethanolamine-binding proteins (PEBP) FT and TERMINAL FLOWER1 (TFL1), and was initially identified as a regulator of flowering time in *Arabidopsis* because its amino acid sequence is highly similar to those of FT and TFL1 (Yoo *et al*., 2004). However, MFT is thought to be a negative regulator of ABA sensitivity during seed germination in *Arabidopsis* as the *mft* loss-of-function mutant was hypersensitive to ABA; in addition, the expression of *MFT* is down-regulated by ABI3 and up-regulated by ABI5, and by DELLA proteins (Xi *et al*., 2010). This latter observation suggests a negative role in germination. It was also suggested by Nakamura *et al*. (2011) that MFT is a key regulator of seed germination and plays a role in the formation of seed dormancy during seed development in response to temperature in wheat. The repression of germination by MFT was overcome by applying exogenous GA, indicating that GA synthesis can be repressed by MFT (Nakamura *et al*., 2011). Additionally, the high expression of *MFT* was also found to be correlated with increased dormancy levels in Cvi seeds buried in the soil over an annual cycle (Footitt *et al*., 2011).

FLOWERING LOCUS C (FLC): FLC is a major regulator of flowering responses to environmental factors, it was also proposed that FLC-mediated

germination acted through similar pathways to those in FLC-mediated flowering, and subsequently through the pathways of ABA degradation and GA synthesis in imbibed seeds (Chiang *et al*., 2009). *FLC* expression was found to be positively correlated with germination level when seeds were imbibed at low temperature (10˚C), and the increased germination was associated with increasing expression of *CYP707A2* (ABA catabolism); the expression of GA biosynthesis gene *GA3ox1* also increased in high *FLC*-expressing lines after imbibition at 10˚C. These results suggest a strong *FLC* allele promotes the early catabolism of ABA and GA synthesis in seeds imbibed at low temperature (Chiang *et al*., 2009). In the field, *FLC* expression in *Arabidopsis* Cvi seeds was found to increase with falling temperature and increasing dormancy, and then declined with increasing exposure to low temperature (Footitt *et al*., 2011).

1.4 Natural variation in seed dormancy and germination

In nature, seeds are generally released from dormancy prior to the season with favourable conditions for subsequent seedling development and plant growth, whereas dormancy is induced just before the season with unfavourable conditions for plant survival (Karssen *et al*., 1988; Allen *et al.*, 2007). Therefore the seed cycles through dormant and less dormant states until germination can be completed when sensitivity of the seed to environmental signals overlaps with the ambient environment, otherwise the annual cycle repeats itself (Baskin and Baskin, 1983; Allen *et al*., 2007).

Different species respond differently to environmental signals so that they germinate at different times of the year. Natural changes in temperature were suggested to play a key role in controlling the temperature range over which seed germination may occur in the species that exhibit seasonal dormancy patterns (Derkx and Karssen, 1994; Probert, 2000). Therefore two main categories of germination behaviour were suggested: high germinability in late summer and autumn represents winter annual behaviour (Baskin and Baskin, 1983); whereas dormancy release by low winter temperature leading to germination in spring represents summer annual behaviour (Derkx and Karssen, 1994; Probert, 2000). These two patterns of behaviour are described below and summarised in (Fig.1.7):

Summer annuals: during winter, dormancy is gradually released by cold temperature and the minimum temperature permissible for germination extends to lower temperatures (i.e. wider temperature range for germination). When the soil temperature starts to rise during the early spring, the temperature range permissible for germination overlaps with the increasing ambient soil temperature, and germination occurs.

Winter annuals: the dormancy pattern is the reverse of summer annuals. The high temperature during summer time reduces dormancy (e.g. by dry after-ripening or warm stratification of imbibed seeds), and the maximum temperature permissible for germination gradually moves to warmer temperatures (i.e. wider temperature range for germination). When the soil temperature starts to fall in autumn, the temperature range permissible for germination and the decreasing ambient soil

temperature overlap, and germination occurs (Baskin and Baskin, 1988; Probert, 2000; Batlla and Benech-Arnold, 2010).

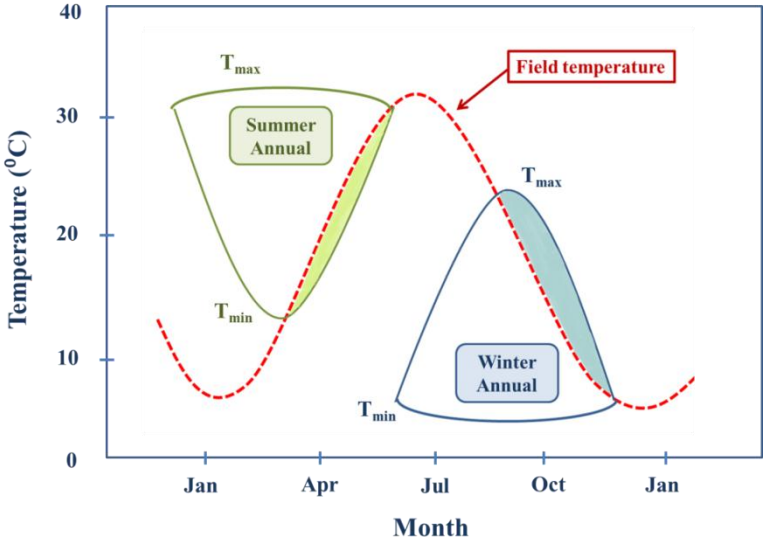

Figure 1.7 Relationship between the field temperature and the changes in the temperature range for germination in summer and winter annuals. The red broken line indicates the mean temperature in the field; green solid line represents the maximum (T_{max}) and minimum (T_{min}) temperature at which germination of summer annuals can occur; blue solid line represents the maximum (T_{max}) and minimum (T_{min}) temperature at which germination of winter annuals can occur; the coloured areas represent the overlaps of field temperature and temperature range for germination (adapted and redrawn from Probert, 2000).

Thus seasonal temperature changes can be considered as a crucial factor in regulating the dormancy status of natural seed populations (Baskin and Baskin, 1983; Karssen *et al*., 1988; Derkx and Karssen, 1994; Probert, 2000). The induction and release of dormancy leads to narrowing or widening of the permissible temperature range for seed germination, coupled with alteration in sensitivity to other germination regulation factors, such as light (Probert, 2000). The different germination timing determines the environment experienced by the developing plants and directly affects subsequent seedling survival, which significantly contributes to plant fitness (Donohue 2002, 2005; Donohue *et al*., 2005a).

1.5 Project objectives

1.5.1 Importance of the project

It is becoming increasingly important to link environmental signals to the mechanisms that control plant population processes due to the raised environmental concerns of climate change and high input farming. Seed dormancy and seed dormancy cycling are key mechanisms that have both agricultural and ecological importance.

***Agricultural importance*:** It has been reported that weed control has higher chemical crop protection costs (herbicides) in the UK than pests or diseases (Oerke, 2006). With increasing pressure from herbicide resistance and increased statutory regulation of herbicide use to protect the environment, more sustainable weed control methods are required. Therefore a greater emphasis will be put on cultural weed management, which attempts to reduce the impact of weeds by limiting weed establishment in crops and thereby reduce chemical and physical weed control interventions. Cultural weed management is a more ecological approach that relies on an improved understanding of weed establishment, which is determined in large part by seed dormancy cycling.

Ecological importance: The analysis of seed traits along climatic gradients was significantly improved by approaches that target both population and community levels simultaneously (Harel *et al*., 2011). A change in the proportions of species recruited from the soil seed bank can result in changes in the balance of plant species in a community. Seed dormancy cycling, which is relevant to theoretical and applied

plant ecology, plays a role in plant community composition because germination timing and rate influence seedling survival and competitive ability (Kaye, 1997). According to ecological theory, populations of annual species from climatically unpredictable arid and semiarid ecosystems should exhibit lower germination rates (or a higher proportion of dormant seeds) and produce larger seeds than species that inhabit more predictable mesic ecosystems (Harel *et al*., 2011). Therefore seed populations should be viewed as a collection of individuals with potentially differing degrees of dormancy. The seed bank that contains dormant seeds consequently plays a key role in maintaining plant populations by buffering populations against temporal variation, avoiding the demographic effects of reproductive failure, and ensuring continuation of a species and long-term survival of plant communities (Evans and Cabin, 1995; Baskin and Baskin, 1998; Clauss and Venable, 2000).

1.5.2 Thesis objectives and structure

The introduction above describes a wide range of environmental and molecular factors have been established as participating in the regulation of seed dormancy and germination. The responses of dormancy in seeds to environmental signals differ between species and also between ecotypes within species through adaptation to the habitat and climate space they inhabit. *Arabidopsis* is widely distributed in the world, thus encountering substantial variation in growth environments. Therefore to start this project several commonly used ecotypes were screened for germination and two ecotypes Cvi and Bur were selected for more detailed analysis (discussed in *Chapter 2*). These two ecotypes naturally inhabit widely different environments (reviewed in

Chapter 2) and have contrasting life cycle patterns. Cvi exhibits the life cycle of a strict winter annual (Finch-Savage *et al*., 2007), and Bur is thought to be a summer annual (Evan and Ratcliffe, 1972),

The **first objective** of my PhD project was therefore: to study the differential responses of dormancy cycling and germination mechanisms by comparing the contrasting *Arabidopsis* ecotypes Bur and Cvi. This investigation was sub-divided to focus on two life history phases:

1) *Phase 1 seed germination*: the environmental signals that influence dormancy release and induction were investigated both in the laboratory (*Chapter 3*) and in the field (*Chapter 4*).

2) *Phase 2 seed maturation*: the maternal effects (i.e. different environmental signals during seed maturation) on seed dormancy are reported in *Chapter 5* and *Chapter 6*.

A major part of the project was therefore to compare in detail contrasting ecotypes of *Arabidopsis* that exhibit summer (Bur) and winter (Cvi) annual phenotypes. Several previous studies have provided a detailed characterisation of Cvi (Finch-savage *et al*., 2007; Ali-Rachedi *et al*., 2004; Footitt *et al*., 2011). However, there has been very little characterization of the Bur ecotype. Thus, the **second objective** of my PhD project was: to characterize dormancy cycling responses of the *Arabidopsis* ecotype Bur to a range of environmental signals both during seed maturation and germination (e.g. temperature, light and nitrate) (*Chapters 2-6*).

Thus the general aim of the project was to apply a unique combination of eco-physiology, molecular biology and model development to get a better understanding

of the mechanism by which environmental signals influence seed dormancy cycling (*Chapters 2-6*). However, our climate is changing and a further aim of the project was to begin an investigation of how global warming will affect plant life cycles and in particular seed dormancy and dormancy cycling. In *Chapter 7* a unique thermo-gradient poly-tunnel was used to address this question and investigate the extent to which predicted global warming would affect the life cycle of *Arabidopsis* and whether this increased temperature will affect different ecotypes in the same way. Therefore the thesis structure can be summarized in the following flowchart (Fig.1.8).

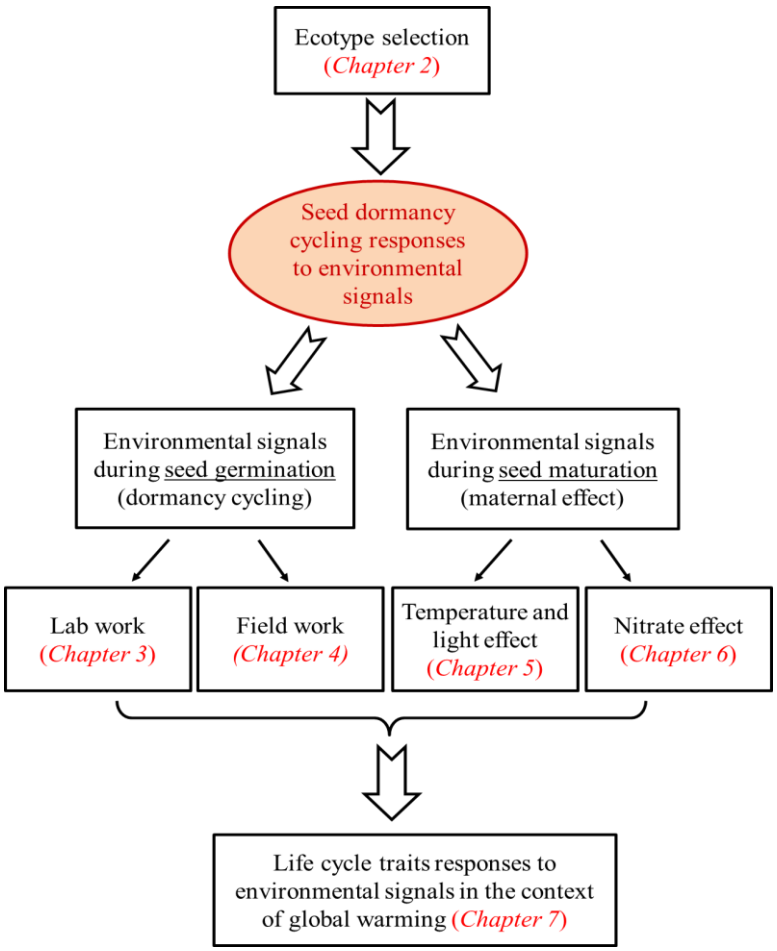

Figure 1.8 Flowchart of thesis structure

CHAPTER 2: USING THE NATURAL VARIATION OF *ARABIDOPSIS THALIANA* ACCESSIONS TO INVESTIGATE THE ADAPTATION OF DORMANCY TRAITS TO CLIMATE

2.1 Introduction

Arabidopsis thaliana has been adopted as a model or reference plant that is especially suitable for genetic and molecular research. This is due to its relatively small genome, and that it is predominantly self-pollinating so that most plants in nature represent inbred lines, which are particularly homozygous (Koornneef *et al*., 2004). *Arabidopsis* is a small annual plant within the *Brassicaceae* family. It is native to Europe and central Asia and is now naturalized in many parts of the world due to its plasticity (Al-Schehbaz and O'Kane, 2002). It has a wide climatic and geographic range from 68°N (North Scandinavia) to 0° (mountains of Tanzania and Kenya), which makes it suitable for analysing variation in adaptive traits (Hoffmann, 2002). There are over 750 natural populations (ecotypes/accessions) that have been collected and each has genetically adapted to a particular habitat (Alonso-Blanco and Koornneef, 2000). Their geographical distribution can be seen in Fig.2.1. The distribution range of *Arabidopsis* is limited by low temperature in spring and autumn and by high temperature with low precipitation during summer (Hoffmann, 2002). There are a number of studies on the adaptive differentiation to environmental conditions among *Arabidopsis* ecotypes (Rutter and Fenster, 2007), and this has shown adaptation has resulted in massive phenotypic variation within the species (Koornneef *et al*., 2004; Table 2.1).

Figure 2.1 The geographical distribution of *Arabidopsis* (green shaded area). The red dots show the location of accessions which have been collected and are available publicly through stock centres (reproduced from Hoffmann, 2002).

Table 2.1 The variable characteristics of *Arabidopsis* ecotypes*

Variable characteristics	**Examples**
Resistance and tolerance to biotic factors	Bacteria
	Fungi
	Virus
	Insect
Resistance to abiotic factors	Freezing temperature
	NaCl and Aluminium tolerance
Developmental traits	Flowering time
	Inflorescence and floral morphology
	Plant and seed size
	Leaf shape
	Leaf trichome density
Physiological traits	Seed dormancy
	Circadian period of leaf movement
	Tissue culture response
	Water and anion content
	Nitrogen use efficiency and nitrogen response
	Temperature response
Chemical contents and enzyme activities	Nitrogen/nitrate/amino acid content
Genetic mechanisms	Cytosine methylation

*Adapted from Koornneef *et al*. (2004)

This variation in all key life cycle traits enables almost every ecotype to be distinguished from other ecotypes collected at different locations. These differences in natural accessions have been used by researchers around the world to study complicated genetic interactions such as the underlying plant responses to environmental signals and evolution of morphological traits. In recent reciprocal transplant experiments conducted by Ågren and Schemske (2012), natural populations of *Arabidopsis* were collected from north-central Sweden and central Italy and grown in each native environment, the results showed a strong adaptive differentiation between these natural populations and also indicated that differences in tolerance to freezing contributed to fitness variation at the northern site (Ågren and Schemske, 2012).

In the investigation reported here the variation in developmental and physiological traits are of most interest. Flowering time and seed dormancy can be considered as two key traits that determine the timing and length of the *Arabidopsis* life cycle (Koornneef *et al*., 2004). In Europe, the majority of *Arabidopsis* ecotypes behave as 'winter annuals'. They flower in spring or early summer, and mature seeds are shed from the mother plant from May to July. Seeds from these early flowering plants subsequently germinate in autumn and overwinter as rosettes (Lawrence, 1976; Baskin and Baskin, 1988; Baskin and Baskin, 1998; reviewed in Donohue, 2009). However, both summer and winter annual races have been reported in Europe, i.e. some *Arabidopsis* were found to flower in late summer up to early autumn, those later flowering plants overwinter as dormant seeds which germinate in spring

(Lawrence, 1976; Baskin and Baskin, 1988; Baskin and Baskin, 1998). In the present study the difference between *Arabidopsis* summer and winter annual life cycle phenotypes has been studied to deepen our understanding of adaptive variation.

Plants have developed mechanisms to ensure seed germination occurs in the optimum season and time of the year for the resulting plant to survive in a particular location. Huge variation of germination and establishment behaviour among natural ecotypes of *Arabidopsis* was demonstrated when the seeds of 73 geographically widely distributed ecotypes was compared. Seeds from mother plants grown at 14 and 22˚C (preconditioning) were screened for germination and seedling establishment at three different temperatures: 10, 18 and 26˚C (Schmuths *et al*., 2006). The broad range of different germination responses was found to be influenced by genotype, preconditioning temperature, germination temperature and their interactions. Positive correlations between germination percentage and seedling establishment and the temperatures and precipitation at the origin of the ecotypes were found to be significant only when seeds were preconditioned at 14˚C, but not at 22˚C. In addition, these correlations were mainly found at germination temperatures of 10 and 18˚C, but not at the higher temperature (26˚C) (Schmuths *et al*., 2006). An appropriate dormancy and germination response to varied environmental conditions is therefore the first requirement for successful establishment in a new location. Consequently many genotypes would be filtered out at this early stage due to the strong impact of natural selection on germination (Donohue *et al*., 2005b; Schmuths *et al*., 2006). Seed dormancy and germination

responses have therefore been considered as important traits to improve understanding of adaptive variation in *Arabidopsis* (Picó, 2012).

As a model plant, *Arabidopsis* has excellent resources available for experimentation and is an appropriate species to study seed dormancy. Within the same species, distinct ecotypes have different depths of dormancy and different seasonal patterns of dormancy cycling (Donohue, 2005a), but to date these differences have not been fully exploited to improve our understanding of dormancy. In *Arabidopsis*, the majority of commonly used accessions such as Landsberg *erecta* (Ler), Columbia (Col) and Wassilewskija (Ws), are considered to have relatively shallow dormancy. In contrast, the ecotype Cape Verde Island (Cvi) has relatively deep dormancy and this makes it particularly suitable for molecular investigations of dormancy (Koornneef *et al*., 2000; Alonso-Blanco *et al*., 2003; Ali-Rachedi *et al*., 2004; Cadman *et al*., 2006; Finch-savage *et al*., 2007; Footitt *et al*., 2011). Another chosen ecotype in our investigation is from the Burren (Bur) on the west coast of Ireland, which has been identified as a notable exception to the winter annual type of behaviour of other studied *Arabidopsis* ecotypes (Evans and Ratcliffe, 1972). In the work presented in this thesis we have further investigated dormancy, germination, flowering time and other key life cycle traits by comparing two *Arabidopsis* ecotypes (Bur and Cvi) that have contrasting life cycle behaviour.

2.2 Environmental conditions in the Burren and the Cape Verdi Islands

The Cvi ecotype is from the Cape Verde Islands, which are located about 600 kilometres off the coast of West Africa, close to the equator (Fig.2.2A). These

islands are volcanic in origin. The climate is milder than that of the African mainland as the temperatures are generally moderated by the surrounding ocean. The islands receive little rainfall annually which makes the local climate semi-desert, but it does rain irregularly between August and October.

The Bur ecotype is native to the Burren region of Ireland, which lies in the northern west corner of Ireland's county Clare (Fig.2.2A). This is a Karst limestone region of about 300 square kilometres. The Burren climate is characterised by cool summer, mild winter, strong winds and year-round rainfall. The climatic differences between the Cape Verde Islands and the Burren are significant, especially in annual temperature and rainfall (Fig.2.2B). The temperature in the Cape Verde Islands is high all the year round, but the Burren has a distinct annual temperature cycle; whereas the rainfall in Burren is constant compared with the Cape Verde Island, which has brief but heavy downpours during the rainy season between August and October. There are also significant differences between the two locations in day length during flower and seed development: average 12h for Cape Verde Islands (ranged from 11.25h to 13h) and 14h for Burren (ranged from 12.5h to 16h) (http://www.timeanddate.com/worldclock/sunrise.html).

2.3 Current knowledge of the germination responses of Bur and Cvi to environmental signals

Several studies have provided a detailed characterisation of Cvi at both physiological and molecular levels (e.g. Ali-Rachedi *et al*., 2004; Cadman *et al*., 2006; Finch-savage *et al*., 2007; Footitt *et al*., 2011).

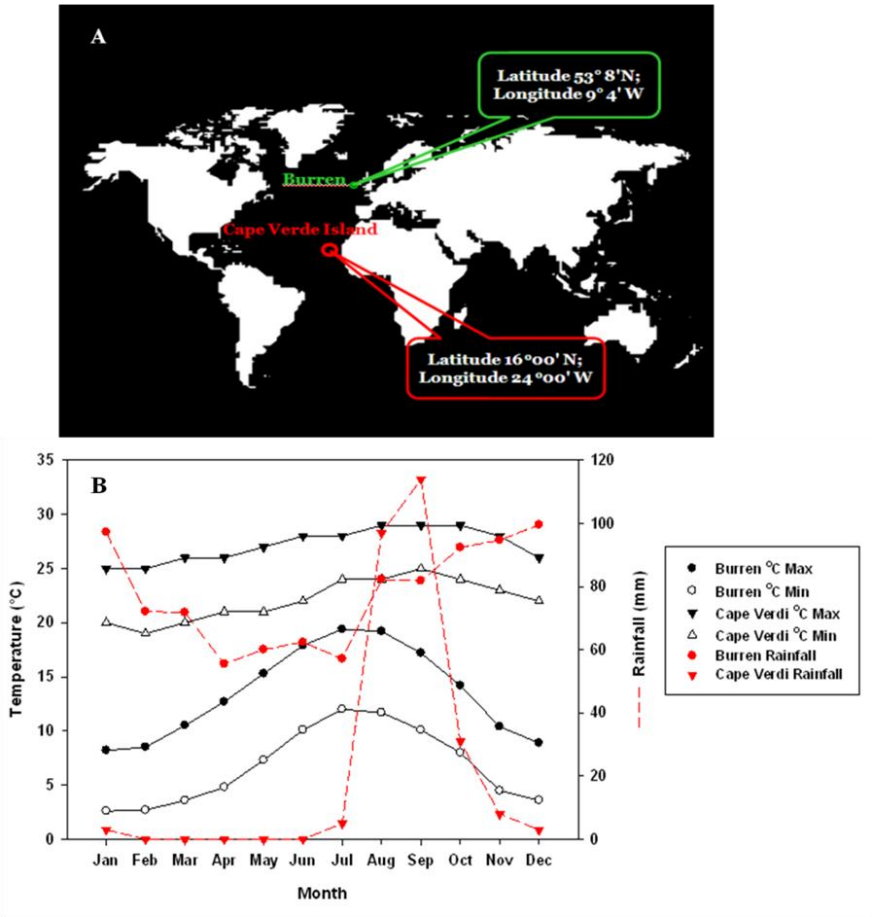

Figure 2.2 Location of the Burren and Cape Verde Islands (A: source http://www.worldatlas.com/aatlas/ latitude_and_longitude_finder.htm); Mean monthly temperature and rainfall for the Burren and Cape Verde Islands (B: Source www.burrenbeo.com/burren-climate-overview and www.bbc.co.uk/weather/world/city_guides/results.shtml?tt=TT004750)

Mature seeds of Cvi, do not germinate at high temperature, but depending on the depth of dormancy can germinate at lower temperatures in the presence of light. This behaviour is consistent with a winter annual phenotype (Baskin and Baskin, 1988, 1998). As dormancy is lost, seeds progressively become able to germinate at higher temperatures. The response of dormant seeds to environmental signals is thought to be integrated through the balance of abscisic acid (ABA) and gibberellic acid (GA) synthesis, catabolism and sensitivity at the level of the transcriptome.

This balance determines the abundance of gene transcripts related to dormancy induction and release (Fig.2.3) (Finch-Savage *et al*., 2007).

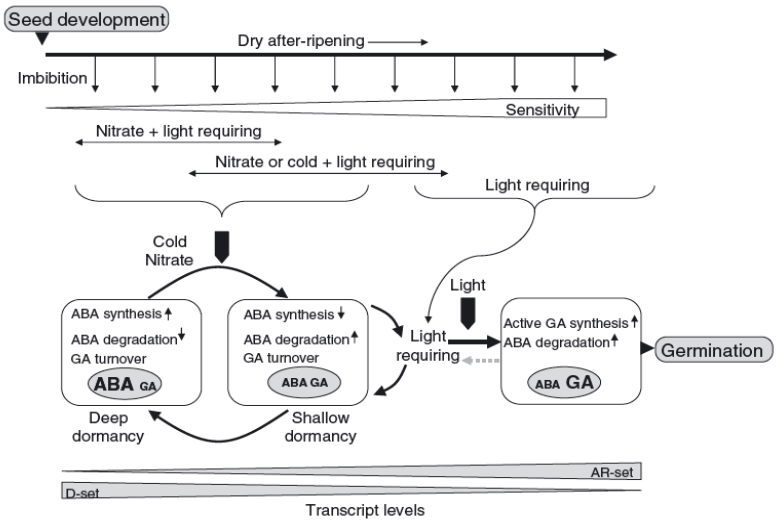

Figure 2.3 Model for the control of dormancy implied by whole genome transcript analysis in *Arabidopsis* Cape Verde Island ecotype (Cvi) (reproduced from Finch-savage *et al*., 2007)

Studies by Evans and Ratcliffe (1972) on an *Arabidopsis* population from the Burren of Ireland, where Ratcliffe had observed plants still flowering in September showed that the Bur seeds had a reversed germination response to temperature compared to other ecotypes they studied. Bur seeds tended to germinate at high temperatures, but not at low temperatures, which is consistent with a summer annual phenotype (Fig.2.4). Bur was also one of the ecotypes used in the study by Schmuths *et al*. (2006) described above. They found that Bur seeds from plants grown at both temperatures (14˚C and 22˚C) showed increased germination with increasing temperature (10, 18 and 26˚C). However, beyond these reports there has been little work on the dormancy phenotype of Bur seeds. A comparison of the contrasting dormancy behaviour of Bur and Cvi seeds should therefore contribute to

our knowledge of the regulation of dormancy cycling responses to environmental signals.

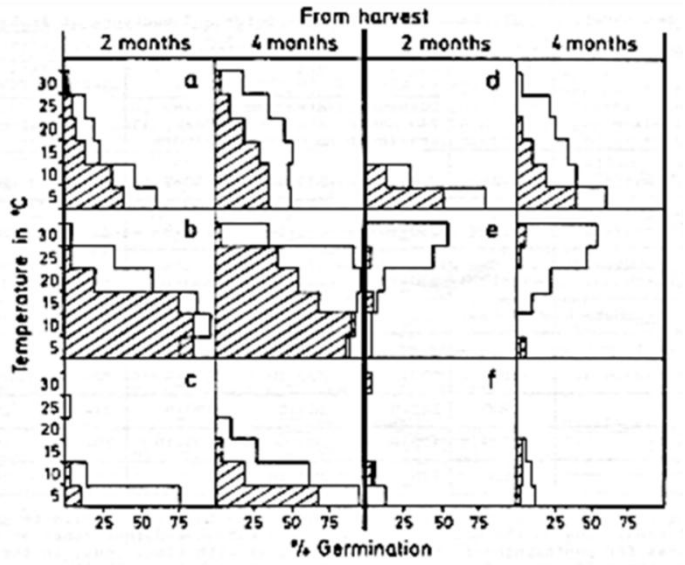

Figure 2.4 Germination of a selection of *Arabidopsis* ecotypes at various temperatures after two and four months dry storage. Hatched area represents germination in constant dark. a: Mean of sixteen races; b: Stockholm population; c: Husband's Bosworth; d: Woebley Castle, Glamorgan; e: Burren, Eire; f: Bush, Midlothian (reproduced from Evans and Ratcliffe, 1972).

2.4 Germination responses of contrasting *Arabidopsis* ecotypes

2.4.1 Materials and Methods

An experiment was set up to compare the germination responses of different *Arabidopsis* ecotypes to light and temperature, and to provide information to aid in the design of subsequent experiments. Seeds of five ecotypes (Table 2.2): Cape Verde Island (Cvi), Shahdara (Sha), Wassileskija-4 (Ws-4), Landsberg (Ler) and Burren (Bur) were produced in July 2009 for comparison. Matured seeds were harvested and dried to an equilibrium relative humidity of 55% above a saturated calcium nitrate solution for six days. Due to the high level of dormancy in Cvi seeds they were dry after-ripened for 240 days at 20°C to increase the germination

potential (Bewley, 1997; Probert, 2000). Before use, seeds of all ecotypes were sealed in aluminium bags and stored at -80˚C.

Seeds were first sterilized in 2.5% bleach (Domestos) for five minutes and washed three times with distilled water, and then incubated in rectangular clear plastic boxes (8×12cm, Stewart Plastics Ltd, UK) containing two layers of 3M chromatography paper (Camlab, UK) and 8ml of distilled water. Two different temperatures were used: a low temperature of 5˚C and a high temperature of 25˚C. Within each temperature, two different light regimes were applied: light and dark. Seeds with light treatment were exposed to constant white fluorescent light. Seeds with dark treatments were sown in the dark under a green safe light (Kodak 7B safelight filter/Green**,** Kodak Limited, London) and germination boxes were wrapped in a double layer of aluminium foil. For each treatment there were three replicates of 40 seeds for each ecotype. Germination was observed and recorded under the green safe light. A seed was considered to have germinated when the radical had emerged through the testa and endosperm.

Table 2.2 Information about the five ecotypes of *Arabidopsis* used in this study

Ecotyps	Geographic origin	Life history[1]	Accession number[2]
Bur-0	Burren, Ireland	SA	N6643
Cvi-0	Cape Verdi Island	WA	N8580
Ler-0	Germany	WA	N24596
Sha	Pamiro-Alay, Tadjikistan	WA	N24597
Ws-4	Wassilewskija, Russia	WA	N66465

Seeds of all ecotypes were produced under glasshouse conditions and harvested in July 2009. Seeds of Cvi were after-ripened for 240 days (for method descriptions refer to *Chapter 3 sec 3.2.1*).

[1] Life history refers to early flowering (winter annuals) versus late flowering (summer annuals) as based on the previous work, hereafter SA and WA.

[2] ID number in the Nottingham *Arabidopsis* Stock Centre (NASC).

2.4.2 Data analysis

All germination data were angular transformed to satisfy the assumption of homogeneity of variance. The transformation is arcsin(sqrt(p/100))*180/pi, where p is a percentage from 0 to 100, and the result is an angle between 0 and 90 degrees. Analysis of variance (ANOVA) was then used to compare the effects of temperatures, light regimes and ecotypes on seed germination. The standard error of the mean was calculated by dividing the standard deviation by the square root of the number of replicates (n) (sd/√n). Statistical analysis was carried out using GenStat software (VSN International, 2012).

2.3.3 Results

Germination responses to light and temperature

A three-way ANOVA indicated significant ($p<0.001$) individual effects of ecotype, temperature, light regime and their interactions on the final germination percentage of the *Arabidopsis* ecotypes compared (Table 2.3).

Table 2.3 Three-way ANOVA of the effects of temperature, ecotype, light regimes and their interactions on final germination percentage

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	4	10104.78	2526.19	49.6	<.001
Light regime	1	7665.94	7665.94	150.51	<.001
Temperature	1	17377.55	17377.55	341.19	<.001
Ecotype × light regime	4	1420.86	355.21	6.97	<.001
Ecotype × temperature	4	15239.02	3809.76	74.8	<.001
Light regime ×temperature	1	2821.57	2821.57	55.4	<.001
Ecotype × light regime × temperature	4	2815.45	703.86	13.82	<.001
Residual	40	2037.3	50.93		
Total	59	59482.47			

The four ecotypes, Sha, Ler, WS-4 and Cvi that were considered to be winter annuals, gave a similar germination response to temperature and light, germinating to a higher percentage at 5°C than at 25°C (Fig.2.5).

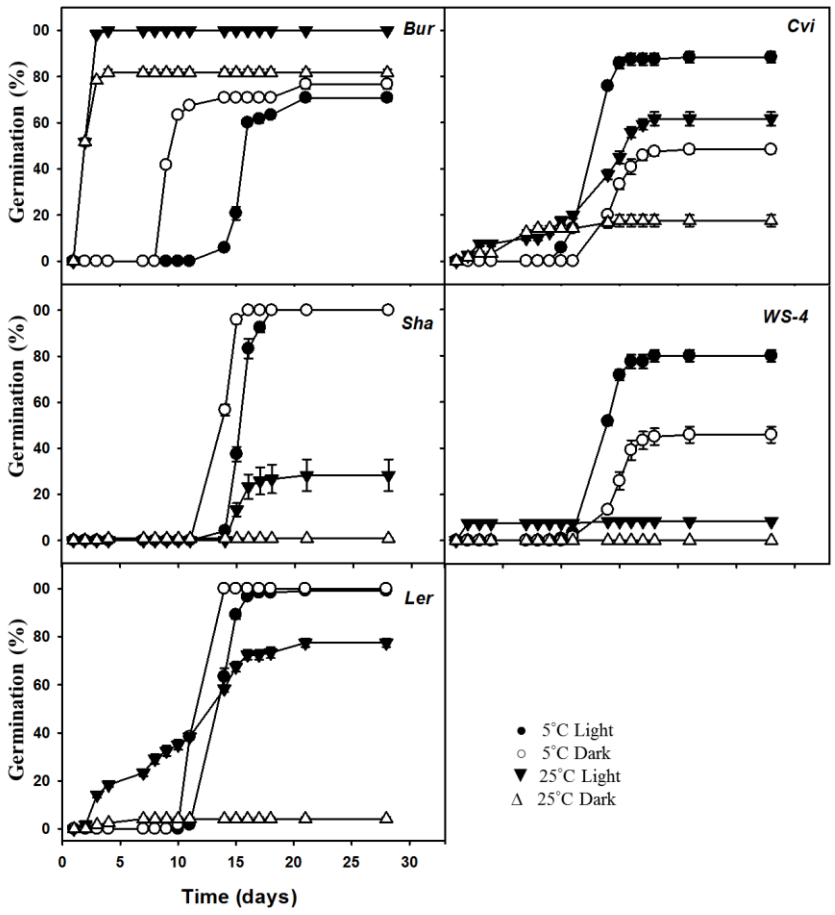

Figure 2.5 Seed germination of different *Arabidopsis* ecotypes under constant temperature with or without light. Data are the mean ± standard error. No error bars indicates symbol is larger than the error.

Light was required to achieve maximum germination when the seeds were at high temperature (25°C). However at 5°C germination was initially higher in the dark than in the light for Bur, Sha, and Ler. The thermal response for the proposed summer annual Bur was opposite to that of these winter annuals (Fig.2.5). Percentage germination of Bur seeds was greater at high temperature (25°C) than at

the lower temperature. Light enhanced germination of Bur seeds at 25°C. In contrast, Fig.2.5 shows that light delayed germination of Bur seeds at 5°C although seeds in both the light and dark achieved a similar final germination percentage at this temperature.

2.3.4 Discussion and conclusions

Arabidopsis ecotypes exhibit germination characteristics of both winter and summer annual phenotypes

Arabidopsis is widely distributed across the world, thus encountering substantial variation in the environments it inhabits. Therefore phenotypic variation is expected to reflect the genetic variation important for adaptation to specific environments (Alonso-Blanco and Koornneef, 2000). The data presented here is in keeping with this and a broad range of different germination responses were shown among the five ecotypes.

The ecotype Ws has a naturally occurring mutantion in the phytochrome D gene (PHYD; Aukerman *et al*., 1997) and its seeds were reported to be light-requiring, and still partially dormant in the light (Debeaujon and Koornneef, 2000). This is consistent with the germination behaviour reported here. The relatively dormant status of Ws seeds is largely due to reduced GA sensitivity (Debeaujon and Koornneef, 2000).

Sha has been considered as one of the ecotypes most tolerant to various seed stresses, thus it has been used to generate a recombinant inbred line (RIL) population for quantitative trait loci (QTL) mapping to identify loci controlling

various aspects of seed longevity during storage and germination (Clerx *et al*., 2004). In the germination experiment here, Sha had high percentage germination at 5˚C in both light and dark, but it exhibited thermal dormancy at 25˚C.

Ler has also been widely used in QTL analysis. Seven QTLs on different chromosomes were found to affect seed dormancy in a RIL population between Ler and Cvi (Alonso-Blanco *et al*., 2003). Two other QTLs were found to influence germination speed in a RIL population between Ler and Sha (Clerx *et al*., 2004; Schmuths *et al*., 2006). The Ler seed here showed a similar germination response to Sha at 5˚C, but it exhibited thermal dormancy at 25˚C only in darkness.

Cvi, one of the most dormant natural ecotypes identified so far, germinated to a high level at 5˚C in the light following AR treatment, but germination was still reduced at 25˚C in the dark. The Cape Verde Islands are characterised by hot, dry summers, the temperature of which is too high for germination in the dormant state, but it is likley that after-ripening will occur in these dormant seeds. As a result, dormancy will gradually be relieved during summer allowing germination in autumn when the temperature decreases. This lower dormancy coincides with the rainy season between August and October in Cape Verde Islands (Fig.2.4B). Thus like most *Arabidopsis* ecotypes, the germination timing of Cvi is consistent with that of a winter annual (Baskin and Baskin, 1983).

Bur seed germinated to a greater extent at 25˚C than at 5˚C in agreement with the response reported by Evans and Ratcliffe (1972). It is likely that germination of the Bur ecotype does not occur in winter because the temperature is too low in the

Burren, but these low temperatures can release dormancy (cold stratification) in the seeds to gradually increase their sensitivity to other environmental signals that promote germination consistent with a summer annual phenotype.

The data presented here show that seeds of Ler, Sha, Ws and Cvi tended to show high temperature thermo-dormancy, this effect became more pronounced when seeds were imbibed in the dark. In contrast, the Bur ecotype exhibited a more shallow dormancy compared to other ecotypes and exhibited low temperature thermo-dormancy. These germination characteristics are consistent with their designation as winter and summer annuals respectively as defined by Baskin and Baskin (1988, 1998). More detailed comparisons of seeds from the deeply dormant Cvi and less dormant Bur at the physiological, ecological and molecular levels are described in the following chapters to gain a greater understanding of their contrasting life cycle behaviours.

CHAPTER 3: THE EFFECT OF TEMPERATURE AND LIGHT ON SEED DORMANCY INDUCTION AND RELIEF

3.1 Introduction

The influence of environmental factors (such as temperature and light) on seed dormancy and germination has been studied over many decades (e.g. Bewley and Black, 1994; Baskin and Baskin, 1998). In the dry state dormancy is progressively relieved at a rate determined by the environment, but in the imbibed state dormancy can be induced and enhanced as well as relieved in response to the environment. In addition, treatments such as after-ripening (the duration of "dry" seed storage) and stratification (period of moist storage) have been investigated as important ways of influencing the level of dormancy (reviewed in Bewley, 1997; Baskin and Baskin, 1978; Finch-Savage and Leubner-Metzger, 2006). The influence of these environmental factors on the depth of seed dormancy are summarised below:

***Temperature*:** It is widely accepted that temperature is one of the most important environmental signals that influence both the induction and loss of seed dormancy (Probert, 2000; Finch-Savage and Leubner-Metzger, 2006; reviewed in *Chapter 1*). This response to temperature differs between species. When the seed is imbibed, low temperature may reduce dormancy in one species, while warm temperatures reduce dormancy in another (Baskin and Baskin, 2004). In general, low temperature (cold stratification) releases seed dormancy of many summer annual plants, reflecting the loss of dormancy over winter so that germination occurs the following spring. Conversely, the germination of many winter annuals often

requires exposure to warm temperatures (warm stratification) over summer to remove dormancy resulting in germination in the autumn (Baskin and Baskin, 1977; 1978; 1986; 1987; 1988; 1998; 2004). However, during stratification treatment two separate processes may occur: a rapid loss of primary dormancy and also a slower induction of secondary dormancy (Totterdell and Roberts, 1979). They found in *Rumex crispus* and *Rumex obtusifolius* that within the stratification temperature ranging from 1.5 to15˚C, the rate of loss of primary dormancy was independent of light and temperature, whereas the rate of induction of secondary dormancy increased with the increasing temperature, and was more rapid in the absence of light. Similarly in oat, the imbibition of primary dormant grains at 30˚C induced a thermo-dormancy that resulted in a loss of the ability to germinate at lower temperature (Corbineau *et al*., 1993). This response can also be seen in barley as the incubation of primary dormant grains at 30˚C resulted in an inability to germinate at 20˚C (Leymarie *et al*., 2008). Interestingly in *Arabidopsis*, extended low temperature exposure of multiple ecotypes leads to secondary dormancy (Finch-Savage *et al*., 2007; Penfield and Springthorpe, 2012). The duration of low temperature for this dormancy induction varied little between ecotypes or in response to seed maturation temperature (Penfield and Springthorpe, 2012).

Temperature plays different roles in distinct ecotypes even within the same species. For example, the two contrasting ecotypes of *Arabidopsis* used in the present work have different germination responses to temperature (*Chapter 2*). This

differential temperature response is investigated further in the work presented below using a range of temperatures across a thermo-gradient table.

***Light*:** Light is another critical environmental signal influencing seed germination, especially in small-seeded species such as lettuce and *Arabidopsis* (Yamauchi *et al*., 2004). In these and similar species, light acting as a signal and not a resource for photosynthesis is involved in the regulation of seed dormancy rather than having a direct effect on germination (Bewley and Black, 1982). In *Arabidopsis*, dormancy release and the completion of germination of Cvi have an absolute light-dependency; dormancy cycling in this ecotype will not end unless exposed to light (Cadman *et al*., 2006; Finch-Savage *et al*., 2007). This influence of light is associated with increasing GA (Hilhorst and Karssen, 1988; Ogawa *et al*., 2003) resulting from an increase in the expression of GA biosynthetic enzymes such as GA 3β-hydroxylase (Yamaguchi *et al*., 1998; Cadman *et al*., 2006). Light also acts by inhibiting the function of PHYTOCHROME INTERACTING FACTOR3-LIKE5 (PIL5), a negative regulator of germination (Oh *et al*., 2006).

In nature, the most effective wavelengths for dormancy release are in the orange/red region of the spectrum (i.e. 660nm); however, wavelengths longer than 700nm are inhibitory for germination, with an especially potent waveband in the far-red region (730 nm) (Bewley *et al*., 2013). As reviewed in *Chapter 1*, phytochromes are involved in the control of seed germination by light and the five phytochromes identified in *Arabidopsis* have overlapping and distinct characteristics related to their functions (Sharrock and Quail, 1989; Clack *et al*., 1994; Koorneef and

Kendrick, 1994; Shinomura *et al*., 1994; Hennig *et al*., 2002). PHYB, PHYD and PHYE are evolutionarily related and separated from PHYA and PHYC (Mathews and Sharrock, 1997). PHYA and PHYB are the best characterized phytochromes in *Arabidopsis* and involved in germination responses to red and far-red light at different fluence rates respectively (Shinomura, 1997). PHYD and PHYE were found to play similar roles to PHYB; however unlike phytochromes B and D, phytochrome E did not inhibit phytochrome A-mediated germination (Hennig *et al*., 2002). Additionally phytochromes were found to play important roles in regulating germination responses not only to light signals, but also to temperature signals, i.e. PHYE contributes to germination at low temperatures, whereas PHYA is important for germination at warm temperatures; and PHYB is important for germination over a range of temperatures (Heschel *et al*., 2007). Thus in this study, the effect of light, and the dual effect of light and temperature on dormancy relief and the completion of germination were investigated by comparing the responses of Bur and Cvi seeds.

***After-ripening*:** After-ripening (AR) is defined as the loss of dormancy during dry storage of freshly harvested dormant seeds (Bewley, 1997). In general, seeds are considered as 'dry' when they have less than 20% water content (dry weight basis) (Bewley *et al*., 2013). AR is a time and environment regulated process occurring in the dry seed that determines the germination potential of seeds (Finch-Savage *et al.*, 2007; Carrera *et al.*, 2008). Thus AR is a common method used to relieve seed dormancy and increase the sensitivity of perception of seeds to environmental signals that allow completion of germination (Finch-Savage and Leubner-Metzger,

2006; Finch-Savage *et al*., 2007). Although AR occurs in dry dormant seeds, it is delayed or prevented in very dry seed, i.e. AR requires seed moisture above a threshold value; however, AR is also prevented during storage at very high air humidity (i.e. high equilibrium seed moisture content) (Leubner-Metzger, 2005b). For example, the AR rate in cereals was reported to be minimal at very low moisture content (< 8%) but greatest between 11-15% moisture content (Roberts, 1962 and 1988; Ellis *et al*., 1983). Similarly in red rice (*Oryza sativa* L.), AR will not occur at moisture content below 5%, and occurs poorly at moisture content above 15% (Leopold and Vertucci, 1989). AR has been modelled in *Cenchrus ciliaris*, during storage at different temperatures and equilibrium relative humidities (ERH) and this showed AR rate increased linearly with the increasing temperature at 40-50% ERH, and that the optimal storage conditions (i.e. highest AR rate) was 40˚C and 50% ERH (Sharif-Zadeh and Murdoch, 2001). However, seeds failed to after-ripen when ERH reached 70%. AR rate is also influenced by temperature and suitable temperatures for AR ranged from 25 to 70˚C in species adapted to warm and seasonally arid regions (Bell, 1993; Mott, 1972). However, temperatures as low as 15˚C are effective in species such as members of the *Asteraceae* (Schütz *et al*., 2002). In this study, seeds of *Arabidopsis* Bur and Cvi ecotypes were after-ripened and the minimum duration of storage for completion of AR in each ecotype was observed.

3.2 Materials and Methods

3.2.1 Seed production of *Arabidopsis* Bur and Cvi ecotypes

Seeds for both ecotypes (Bur and Cvi) were produced in a glasshouse from May to July 2009. The growth medium (Levingtons F1 compost: sand: vermiculite = 6:1:1) was added to P24 cellular trays (36cm × 24cm), then each tray was labelled and placed in a second tray lined with capillary matting to ensure all the plants had a uniform water supply. Approximately five seeds were sown in each cell. The trays were then covered with transparent propagator lids for at least four days, by which time all the seedlings had established. One week after sowing, the seedlings were thinned to one per cell. The glasshouse was vented and heated to control temperature (23/17˚C, light/ dark) and had supplementary lighting to maintain light levels and photoperiod (16/8h, light/ dark). When the seeds were mature (all the siliques had turned brown and dry, but not completely dehisced), the plants were no longer watered. Matured seeds were harvested and threshed by hand. Seeds were dried to an equilibrium relative humidity of 55% above a saturated calcium nitrate solution for six days.

A proportion of the freshly harvested Bur and Cvi seeds were placed in separate sealed moisture-proof containers for AR. The AR conditions in the present work here were 20˚C and 55% ERH resulting in a measured moisture content of 9.9% (dry weight basis). Seeds were after-ripened for eight months matching the requirement for completion of AR in previous studies of Cvi (Cadman *et al*., 2006;

Finch-Savage *et al*., 2007). Both the fresh seeds and after-ripened seeds were sealed in aluminium bags and stored at -80˚C.

3.2.2 Seed germination

Seeds were firstly sterilized in 2.5% bleach (Domestos) for five minutes and then washed three times with distilled water. Germination experiments were either conducted on the thermal gradient table (described in *sec 3.2.2.1* below) or in temperature controlled incubators. Germination conditions in these experiments are described below in the relevant sections. Cumulative germination was recorded by removal of seeds as they germinated. A seed was considered to have germinated when the radical had emerged through the testa and endosperm.

3.2.2.1 *Experiment 1*: The effect of temperature, light and after-ripening on germination: application of a thermal gradient table

A thermal gradient table was used to characterise the different thermal responses of Cvi and Bur seeds. The thermal gradient table has a highly efficient bi-directional temperature gradient system and similar equipment has been used in the investigation of temperature responses of small plants, animals and microorganism (Panagou *et al*., 2005), as well as seeds (Murdoch *et al*., 1989). The temperature gradient along the square aluminium plate is produced by heating one edge with a heating element and cooling the opposite edge with a standard refrigeration evaporator (Siver, 1983). The table was used here to provide a linear range of constant temperatures from 5˚C to 30˚C using a 10×10 matrix of cells each containing a single germination box (7 × 7cm; see below and Fig.3.1). Temperature

is incremented within each column of the matrix and is constant in each row.

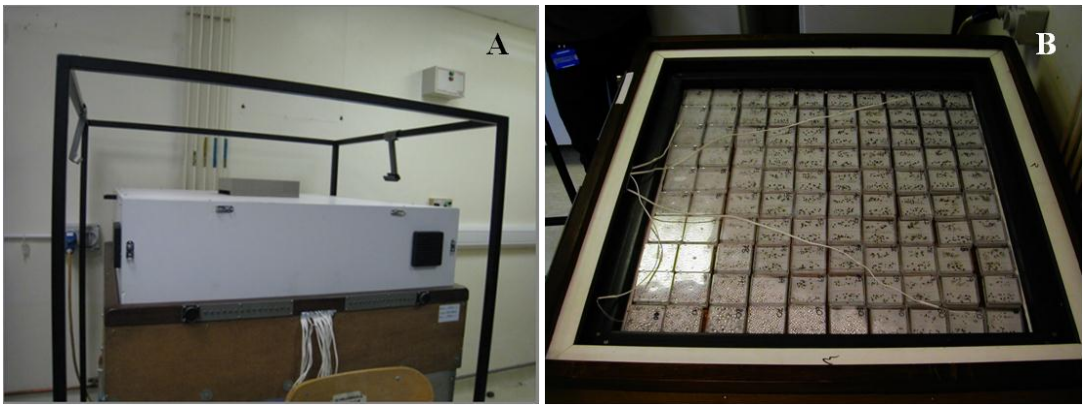

Figure 3.1 Thermo-gradient table for seed germination testing. A: Exterior of thermal gradient table; **B**: Interior of thermal gradient table: the square aluminium plate is suitable to place 100 germination boxes in 10 rows as it provides temperatures from 5˚C to 30˚C.

The thermal responses of Bur and Cvi seeds to constant temperatures ranging from 5-30˚C were investigated in two sub-experiments: one with fresh seeds and the other with after-ripened seeds (AR for 240 days see above *sec 3.2.1*). The experimental conditions were controlled to allow the direct comparison between fresh and AR seeds. In each sub-experiment, the thermal gradient table was divided so that two different light regimes could be applied: constant light (white fluorescent) and dark. Three replicates were set up, each one was a single column of of germination boxes on the thermal gradient table. Each transparent polystyrene germination box (7×7cm; Stewart Plastics Ltd, UK, Fig 3.1) contained 0.7% agarose (30ml/box) to provide a reservoir of water below one sheet of 3M chromatography paper (Camlab, UK). The germination box was split into two parts, each sown with 40 seeds of Bur or Cvi. Germination boxes for dark experiments were wrapped in black sticky back plastic paper (Fablon) and placed under a wooden cover to further exclude light from the lamps. Preliminary experiments had shown this effectively

excluded light. Germinated (radical emerged) seeds were counted and removed at regular intervals throughout the experiment. Germination in boxes kept in the dark was recorded in a dark room under a green safe light (Kodak 7B safelight filter/Green, Kodak Limited, London).

3.2.2.2 *Experiment 2:* The effect of after-ripening on germination responses to temperature and nitrate

Fresh seeds (seed production see above *sec 3.2.1*) were placed in Eppendorf tubes (1.5ml) that were placed into 50ml screw cap tubes sealed with Nescofilm to maintain moisture content of the seeds at 9.9%. Seeds were then stored at 20˚C for 14, 28, 56, 75, 105, 150, 210 days. At each time interval an Eppendorf was removed and the seeds were imbibed at two different temperatures (10˚C and 20˚C) on two layers of 3M chromatography paper with 8ml of distilled water (control treatment) or KNO_3 in clear plastic boxes (8×12cm, Stewart Plastics Ltd, UK) for germination. KNO_3 solution in this experiment was used to relieve dormancy and different concentrations were provided to Bur (1mM) and Cvi (10mM). These nitrate concentrations were in line with their different levels of dormancy, according to preliminary experiments that showed fresh Bur seeds germinated fully in the presence of 1mM nitrate solution and work by Finch-Savage *et al*. (2007) showing 10mM was required for the same response in Cvi seeds. For each treatment, three replicates boxes were set up containing 40 seeds of either Bur or Cvi.

3.2.2.3 *Experiment 3*: The effect of dark incubation and temperature on germination of Bur seeds

This experiment was used to define the role of cold (5˚C and 10˚C) and warm (≥15˚C) temperatures and the effect of duration of pre-incubation in the dark on seed behaviour. Fresh Bur seeds (from *sec 3.2.1*) were incubated on water in the dark at 5˚C, 10˚C, 15˚C, 20˚C, 25˚C and 30˚C for the following periods of time: short periods (1, 2, 3, 5, 7, 14, 21, 35 days) and long periods (56, 75, 105, 150, 200 days). Dark incubation for long periods was set up on 19/12/2011, while the short period incubation was set up on 6/2/2012. Seeds were incubated on two layers of 3M chromatography paper with 4ml of distilled water in transparent polystyrene boxes (7 × 7cm; Stewart Plastics Ltd). All procedures were carried out in a dark room under a green safe light (Kodak 7B safelight filter/Green**,** Kodak Limited, London). Once set up under green light, germination boxes were wrapped with two layers of aluminium foil and then sealed inside a polyethylene freezer bag. Water (2ml) was applied monthly in complete darkness (dark room without safe light) to those seeds in prolonged dark incubation (≥ 35 days) to offset any loss of water. Seeds from different groups of plants were used to provide three replicates containing 40 seeds for each treatment. At the end of the assigned dark incubation period germination was recorded in the light and the boxes were transferred to 20˚C in constant light for another 28 days to record germination.

3.2.4 Data analysis

All percentage germination data was angular transformed for analysis. The transformation is arcsin(sqrt(p/100))*180/pi, where p is a percentage from 0 to 100, and the result is an angle between 0 and 90 degree. Data are presented as the mean ± standard error. The standard error was calculated by dividing the standard deviation by the square root of the number of replicates (n) (sd/√n).

Table 3.1 Summary of ANOVA used in Chapter 3

Analysis of variance	Factors	Variate	Transformation
Three-way ANOVA (*Expt 1. sec 3.2.2.1*)	Germination temperature, Light and dark AR treatment	Final germination%	Angular
One-way ANOVA (*Expt 2. sec 3.2.2.2*)	AR duration	Final germination% for each ecotype at each temperature	Angular
Four-way ANOVA (*Expt 2. sec 3.2.2.2*)	Germination temperature, ecotype, AR duration, Nitrate	T_{50} values*	
Two-way ANOVA (*Expt 3. sec 3.2.2.2*)	Germination temperature, Nitrate	AR_{50} of Cvi	
One-way ANOVA (*Expt 3. sec 3.2.2.2*)	Germination temperature,	AR_{90} of Bur	
Two-way ANOVA (*Expt 3. sec 3.2.2.3*)	Dark incubation time, Dark incubation temperature	Final germination%	Angular
One-way ANOVA (*Expt 3. sec 3.2.2.3*)	Incubation temperature	Final germination% at each incubation day	Angular

* T_{50} is the time required for 50% of viable seeds to germinate, calculated by the software package GERMINATOR (Joosen *et al*., 2010).

Analysis of variance (ANOVA) was used to detect the differences between variates as listed in the Table 3.1. Statistical analysis was carried out using the software package GenStat (VSN International, 2012).

3.3 Results

3.3.1 *Experiment 1*: The effects of light, temperature and after-ripening on germination responses (*sec 3.2.2.1*)

The thermal gradient table was used to characterise the thermal responses of seeds both in the dark and the light. There were significant ($p<0.001$) effects of temperature, light and AR on germination of Bur and Cvi seeds (Appendix Table 3.1). Successive counts are shown in Fig.3.2 to illustrate the pattern of germination at each temperature, and the highest values are the final percentage germination.

Except for fresh seeds in the dark, Bur seeds had higher percentage germination at high temperatures whereas Cvi seeds had higher percentage germination at lower temperatures (Fig 3.2). AR relieved dormancy in both Bur and Cvi, widening the temperature range at which maximal germination occurred. For AR seeds, light had a positive effect on both Cvi and Bur by increasing the final germination percentages. Light had a different effect on germination responses of fresh Cvi and Bur seeds: fresh Cvi seeds did not respond to light as they were too dormant; however light had a positive influence on the germination of fresh Bur seeds at higher temperatures.

Germination increased in Bur seeds after eight days at temperatures below 7˚C, possibly due to the dormancy breaking effect of cold stratification.

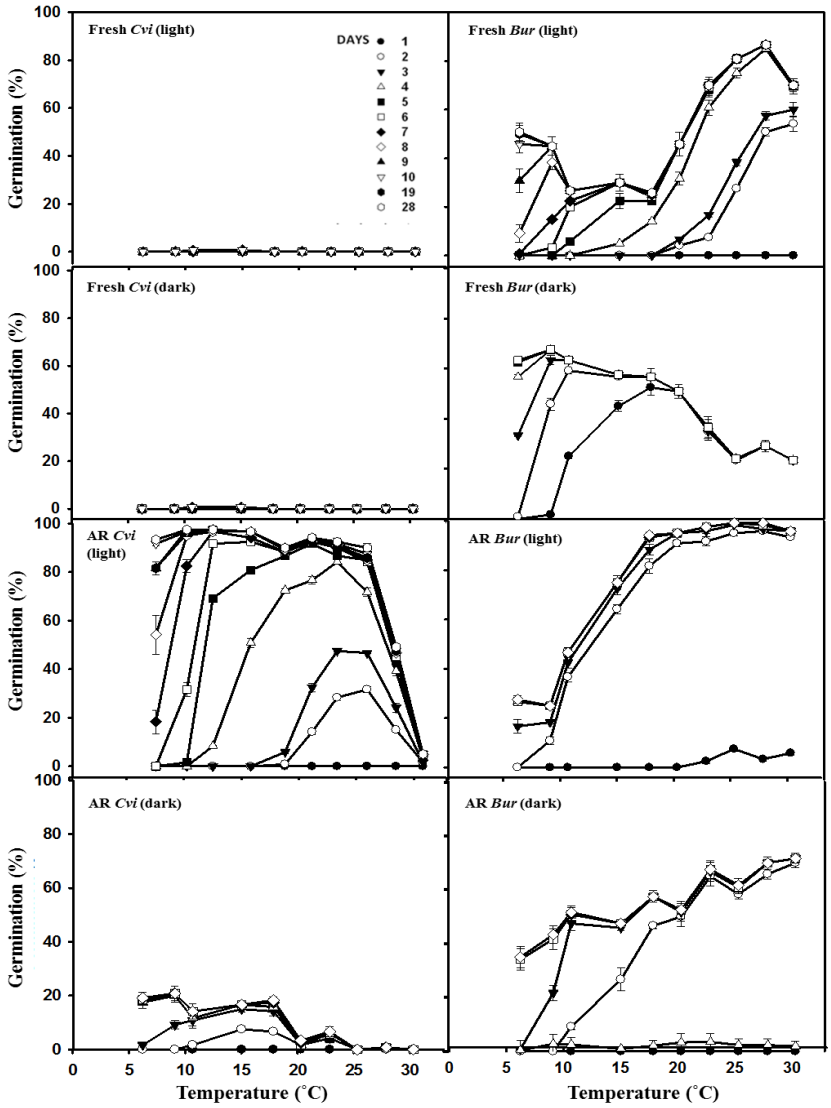

Figure 3.2 Germination responses of fresh and after-ripened seeds along a temperature gradient in the light and the dark. Successive counts are shown to illustrate the pattern of germination at each temperature. Highest values are the final percentage of germination. Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

When final germination percentage is plotted against temperature, it reveals the thermal responses of Cvi and Bur are influenced by light (Fig. 3.3). Seeds of both ecotypes were fully viable as there was 100% germination at optimum temperature following AR. Fresh Cvi seeds did not germinate at any temperature in either the presence or absence of light. However, in the light the AR seeds of Cvi

had high germination percentage at temperatures ranging from 7˚C to 25˚C, but germination was reduced above these temperatures. Fewer seeds germinated in the dark at temperatures up to 20˚C, with no germination above this temperature.

The germination response of Bur seeds was very different. There was a significant interaction ($p<0.001$; Appendix Table 3.1) between light and temperature on final percentage of germination. In fresh Bur seeds light had a positive effect on germination at high temperature with an optimal temperature of 28˚C, in comparison to seed maintained in the dark where increasing temperature had negative effect on germination. The increase in germination at low temperature in the light is possibly the result of cold stratification.

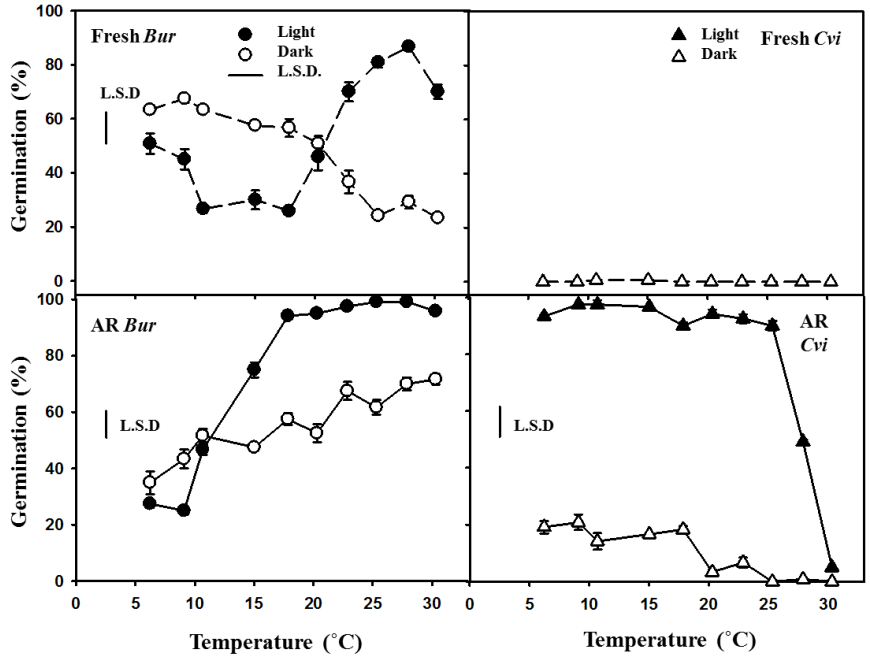

Figure 3.3 Final germination percentages of fresh and after-ripened seeds on the temperature gradient in the light and in the dark. Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The L.S.D bars indicate the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 3.1).

Following AR, the temperature range with high percentage germination in the light increased and extended to lower temperatures. Fewer AR seeds germinated in the dark, but had a similar response to temperature. Germination in the dark was negatively correlated with temperature in fresh Bur seeds but positively correlated in AR seeds. In general, following AR Bur seeds germinated to high percentages at higher temperatures (>18-30˚C), whereas Cvi seeds germinated to high percentages at lower temperatures (5-25˚C).

3.3.2 *Experiment 2*: The effect after-ripening on germination responses to temperature and nitrate (*sec. 3.2.2.2*)

The final germination percentage was significantly ($p<0.001$) affected by the duration of AR (Appendix Table 3.2; Fig.3.4). Bur seeds were less dormant than those of Cvi at the start of the experiment having 83.3% germination compared to 0% in Cvi. In Bur, germination was initially greater at 10˚C than 20˚C on water, but was maximal at both temperatures by 56 days of AR. Nitrate addition resulted in high germination at both temperatures without AR. Germination of Cvi at 10˚C increased significantly for up to 56 days of AR, but thereafter there was no significant increase (Fig.3.4A, Table 3.2).

At 10˚C there was no significant effect of adding nitrate. At 20˚C in the presence of nitrate the response was similar to that at 10˚C. However, unlike at 10˚C the increase in germination with increasing AR was significantly delayed in the absence of nitrate. In the latter case final germination increased up to 210 days, but this increase was no longer significant after 105 days (Fig.3.4B, Table 3.2). At both

germination temperatures germination on nitrate flowed a similar time course indicating that nitrate was able to overcome residual high temperature thermo-dormancy at 20˚C as AR time increased.

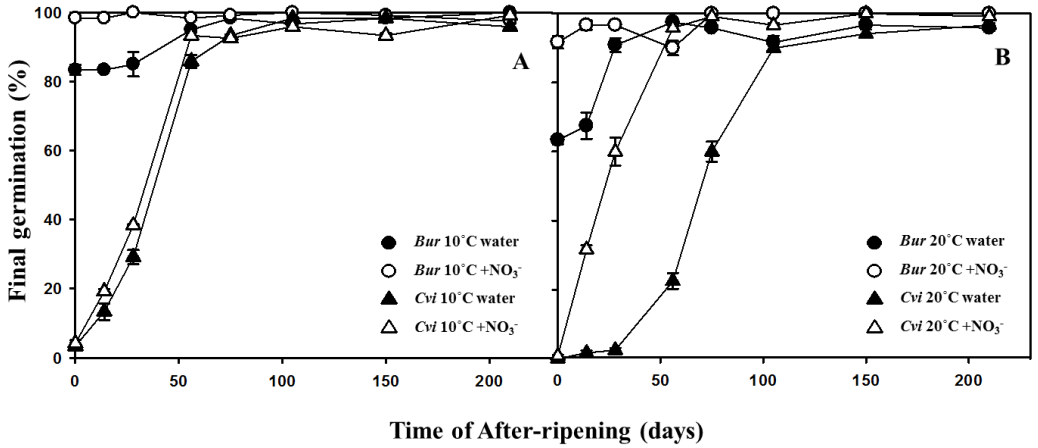

Figure 3.4 Final germination percentages of seeds following dry after-ripening for increasing periods. A: 10˚C ± KNO3; **B**: 20˚C ± KNO3; the concentrations of KNO_3 are: 1mM KNO_3 for Bur seeds and 10mM KNO_3 for Cvi seeds; Data represents the mean ± standard error. No error bar indicates symbol is larger than the error.

Table 3.2 Final germination percentage of Bur and Cvi on water at 10˚C and 20˚C following increasing after-ripening time

		After-ripening in days								LSD*
		0	**14**	**28**	**56**	**75**	**105**	**150**	**210**	**(5%)**
Bur	**10˚C**	83.3 (66.2)	83.3 (65.9)	85 (68.8)	95 (77.4)	98.3 (85.7)	96.7 (83.9)	98.3 (85.7)	100 (90)	**(9.27)**
	20˚C	63.3 (52.8)	67.5 (55.8)	90.8 (75.6)	97.5 (82.7)	95.8 (78.6)	91.7 (74.4)	96.7 (81.4)	95.8 (80.4)	**(11.24)**
Cvi	**10˚C**	3.3 (8.3)	13.3 (20.5)	29.2 (32.5)	85.8 (68.6)	93.3 (75.1)	98.3 (83.9)	98.3 (83.9)	95.8 (80.4)	**(12.38)**
	20˚C	0 (0)	1.7 (4.3)	2.5 (7.3)	22.5 (27.9)	60 (50.9)	90 (72)	94.2 (78.6)	96.7 (81.4)	**(12.84)**

*This table only includes the germination data on water. To correct for non-normality of the germination percentage values, the ANOVA is conducted on angular transformed data. Data in parenthesis are angular transformed. L.S.D. (in parenthesis and bold) indicates significant differences between angular transformed data at the P = 0.05 level (for analysis refer to Appendix Table 3.2).

The number of days to 50% germination (T_{50}) on water was calculated (Table 3.3), this was significantly ($p<0.001$) influenced by ecotype, imbibition temperature, AR duration and KNO_3 application according to a four-way ANOVA (Table 3.3 and Appendix Table 3.3).

In both ecotypes, T_{50} decreased with increasing AR time (i.e. germination rate increased with increasing AR time). Seeds with 210-day AR exhibited the lowest T_{50} values in both ecotypes. Cvi seeds did not reach 50% germination until they had received a period of AR ≥ 28 days. Seeds of both ecotypes at 20˚C germinated significantly ($p<0.001$) faster than those at 10˚C, resulting in significantly lower T_{50} (Table 3.3).

Table 3.3 Germination rate (T_{50} in days) of seeds in response to duration of after-ripening at different temperatures with and without nitrate

	T_{50} values at days of different after-ripening								**LSD**
	0	**14**	**28**	**56**	**75**	**105**	**150**	**210**	**(5%)**
Bur									
10˚C	4.91	4.80	4.78	4.66	4.53	4.47	4.46	4.46	
20˚C	3.42	3.46	2.78	2.71	2.67	2.73	2.66	2.34	
10˚C +KNO_3	4.33	4.27	3.99	3.96	3.90	3.85	3.86	3.83	
20˚C +KNO_3	2.66	2.52	2.39	2.27	2.20	2.20	2.14	2.13	
Cvi									**0.7847**
10˚C	-	-	-	5.19	4.86	4.74	4.70	4.66	
20˚C	-	-	-	-	10.0	3.47	2.81	2.61	
10˚C +KNO_3	-	-	-	5.28	4.92	4.73	4.62	4.39	
20˚C +KNO_3	-	-	18.8	9.65	8.16	3.73	2.78	2.65	

*Missing data indicate final germination percentage is less than 50%. L.S.D. (in bold) indicates significant differences between values at the P = 0.05 level (for analysis refer to Appendix Table 3.3)

Depth of dormancy can be measured in terms of the AR time to reach 50% germination (AR_{50}) (Footitt *et al*., 2011). AR_{50} values of Cvi seeds increased when

the germination temperature increased from 10˚C to 20˚C (Table 3.4). However, if seeds were incubated with 10mM KNO_3 the AR_{50} value decreased at both incubation temperatures, becoming significantly ($p<0.001$) smaller at the higher temperature. As dormancy was shallow in Bur the AR_{90} values were calculated instead of AR_{50}. Less AR was required to achieve 90% germination at 20˚C than at 10˚C. However, Bur seeds treated with KNO_3 germinated to 90% without AR at both10˚C and 20˚C.

Table 3.4 After-ripening time allowing 50% **(**AR_{50}) or 90% (AR_{90}) germination

Ecotype	Treatment	AR_{50} (days)	LSD (5%)
Cvi	10˚C	38.5	**7.18**
	10˚C +KNO_3	34.6	
	20˚C	69.7	
	20˚C +KNO_3	21.4	
	Treatment	**AR_{90} (days)**	**LSD (5%)**
Bur	10˚C	42.3	**6.62**
	10˚C +KNO_3	-*	
	20˚C	27.8	
	20˚C +KNO_3	-	

*Missing data indicate that Bur seeds treated with KNO_3 germinated to 90% at both temperatures without after-ripening. L.S.D. (in bold) indicates significant differences between values at the P = 0.05 level (for analysis refer to Appendix Table 3.4).

3.3.3 *Experiment 3*: The effect of dark incubation at different temperatures on Bur seed germination

In this experiment the effect of dark incubation on seed dormancy was determined by observing subsequent germination in the light. Bur seeds were incubated in the dark at a range of temperatures from 5 to 30˚C for periods of up to 200 days before transferring to the light at 20˚C. Germination was recorded at the end of the dark period and then during time in the light. A two-way ANOVA indicated that final germination (germination in the dark + germination in the light)

of Bur seeds was significantly ($p<0.001$) affected by the duration and temperature of dark incubation (Appendix Table 3.5). The impact of dark incubation temperature was significant at all durations except short incubation periods of two and three days (Appendix Table 3.6).

Short periods of incubation: Bur seeds germinated earlier in the dark, after two days incubation, at 20-30˚C. However dark germination was delayed as temperature decreased. Seed did not germinate in the dark until more than seven days incubation at 5˚C, which was consistent with the data collected from the thermal gradient table where there was an increased germination in Bur seeds after eight days at temperatures below 7˚C (Fig.3.3). The initial rate of release and then induction of dormancy was temperature dependant (Table 3.5).

Table 3.5 The rate of dormancy relief and induction of seeds pre-incubated at different temperatures in the dark

Dark incubation temperature	Time to G_{max} (days)	Rate of dormancy relief*	Rate of dormancy induction*
5˚C	7	1.586	0.279
10˚C	5	0.948	0.382
15˚C	3	0.603	0.455
20˚C	2	0.293	0.574
25˚C	3	0.569	1.711
30˚C	2	1.362	1.34

*The rates of dormancy relief and induction were calculated as the slope of the lines during the initial increase and subsequent decrease in final germination after transferring to the light at 20˚C in Fig.3.5.

The increasing germination potential (dormancy relief) of Bur seeds incubated in the dark at different temperatures initially increased more rapidly at higher

temperature. Once maximal germination had been reached at each temperature, germination potential then decreased (dormancy induction). This decrease was more rapid as the temperature of incubation increased.

On transfer to the light germination responses of seeds incubated for periods up to 14 days showed that after an initial decline in dormancy it then increased (Fig.3.5). Short periods of dark incubation at 15-30˚C for up to three days increased final germination on subsequent transfer to the light (Fig.3.5).

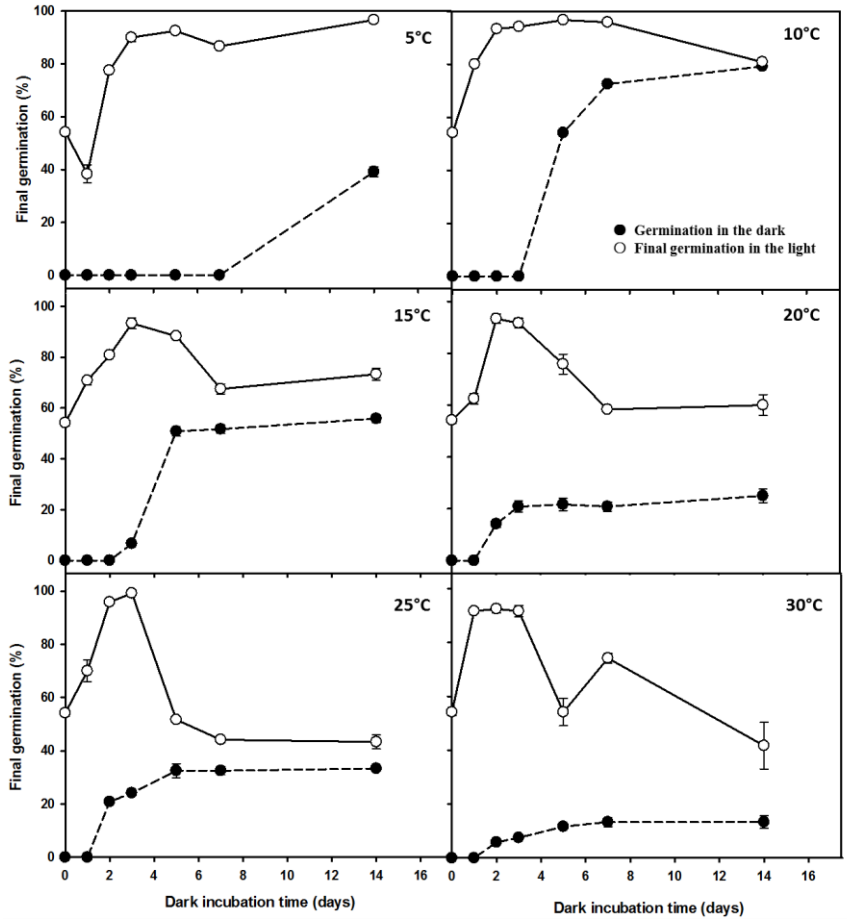

Figure 3.5 Final germination percentages of seeds at different temperatures (5-30˚C) in the dark and at 20˚C in the light following incubation in the dark for up to 14 days at a range of temperatures. Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

However it took a longer time (≥ 7 days) for the seeds pre-incubated at low temperatures (5 and 10˚C) to reach the maximum germination (plateau) (Fig.3.5). In addition, germination of seeds at 5˚C initially dropped after one day and then increased and remained high. At higher temperatures germination declined as incubation time increased. This decline was increasingly rapid as incubation temperature increased (Fig.3.5).

Long periods of incubation: Seeds were exposed for long imbibition times in the dark similar in duration to those overwintering in the soil following shedding in September (Fig 3.6).

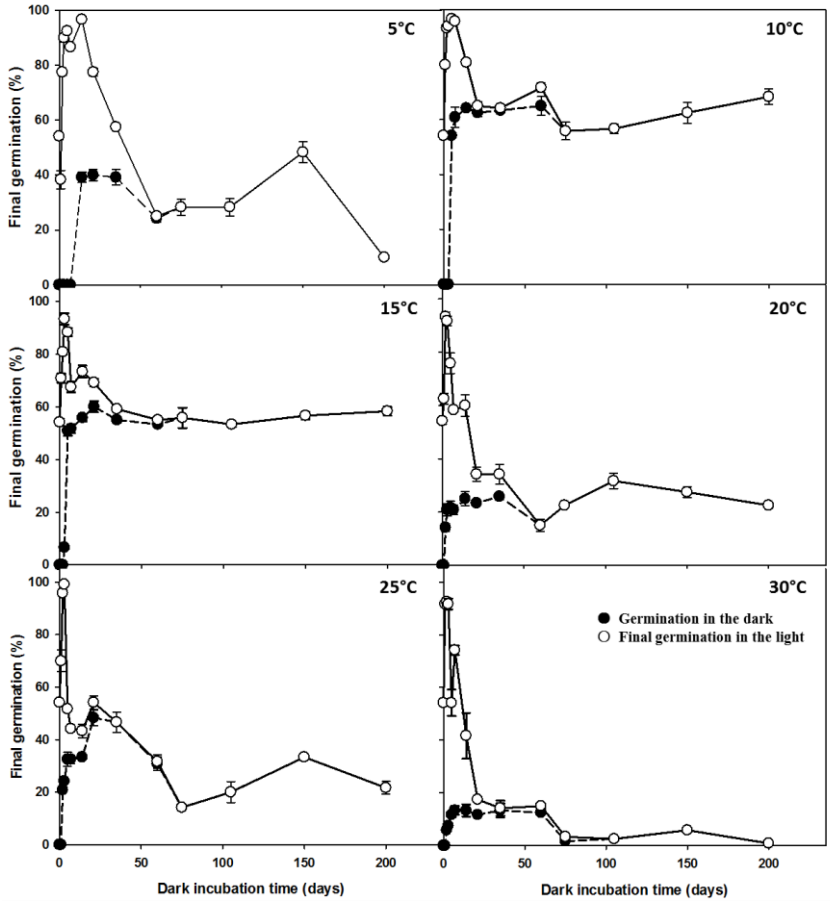

Figure 3.6 Final germination percentages of seeds at different temperatures (5-30˚C) in the dark and at 20˚C in the light following incubation in the dark for increasing periods of time at a range of temperatures. Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

There was a clear pattern to the effect of dark incubation at all temperatures. In general pre-incubation initially increased final germination percentage and then as the length of pre-incubation increased final percentage germination began to decline (Fig.3.5 and Fig.3.6; Table 3.6).

Table 3.6 One-way ANOVA of the effects of dark incubation duration and temperature on final germination of Bur seeds

Dark incubation	**Pre-incubation temperatures in the dark**						**L.S.D**
time	**5˚C**	**10˚C**	**15˚C**	**20˚C**	**25˚C**	**30˚C**	**(5%)**
1d	38.3	80	70.8	62.5	70	91.7	
	(38.1)	(63.5)	(57.4)	(52.3)	(57.7)	(73.5)	**(12.81)**
2d	77.5	93.3	80.8	93.3	95.8	92.5	
	(61.8)	(75.5)	(64.1)	(78.1)	(80.4)	(75.1)	**(13.82)**
3d	90	94.2	93.3	91.7	99.2	91.7	
	(72.2)	(76.4)	(78.7)	(74.4)	(87.0)	(76.5)	**(17.08)**
5d	92.5	96.7	88.3	75.8	51.7	54.2	
	(74.2)	(81.7)	(70.5)	(62.0)	(46.0)	(47.5)	**(13.89)**
7d	87.5	95.8	67.5	58.3	44.2	74.2	
	(69.4)	(78.6)	(55.4)	(49.8)	(41.6)	(59.6)	**(6.90)**
14d	96.7	80.8	73.3	60	43.3	41.7	
	(79.6)	(64.1)	(59.2)	(50.9)	(41.1)	(39.9)	**(16.77)**
21d	77.5	65	69.2	34.2	54.2	17.5	
	(61.9)	(53.8)	(56.3)	(35.6)	(47.4)	(24.7)	**(9.92)**
35d	57.5	64.2	59.2	34.2	46.7	2.5	
	(49.3)	(53.3)	(50.3)	(35.5)	(43.1)	(7.3)	**(12.06)**
60d	25	71.7	55	15	31.7	15.8	
	(29.9)	(58.0)	(47.9)	(21.9)	(34.0)	(23.4)	**(10.69)**
75d	28.3	55.8	55.8	22.5	14.2	3.3	
	(31.8)	(48.4)	(48.4)	(28.3)	(21.9)	(8.6)	**(11.17)**
105d	28.3	56.7	53.3	31.2	20	2.5	
	(31.6)	(48.9)	(46.9)	(33.9)	(25.4)	(7.3)	**(9.96)**
150d	48.3	62.5	56.7	27.5	33.3	5.8	
	(44.1)	(52.5)	(48.9)	(31.5)	(35.2)	(13.6)	**(11.60)**
200d	6.67	68.6	58.3	22.5	21.7	0.8	
	(12.3)	(56.1)	(49.8)	(28.2)	(27.2)	(3.0)	**(13.61)**

*to correct for non-normality of the germination percentage values, the ANOVA was conducted on angular transformed data. Data in parenthesis are angular transform means. L.S.D. (in parenthesis and bold) indicates significant differences between angular transformed data at the P = 0.05 level (for analysis refer to Appendix Table 3.6).

The data suggest that there is a proportion of seeds that can germinate in the dark (Fig.3.2 and 3.3) and then dark pre-incubation initially reduces dormancy by

increasing light sensitivity and then further dark incubation induces secondary dormancy decreasing light sensitivity. Consequently final germination (dark plus light) increases and then decreases with duration time. The timing of this decrease and subsequent increase in dormancy differs at each incubation temperature. The initial increase is fastest at 30˚C and slower at lower temperatures. For example, when seeds were pre-incubated in the dark for only one day, the germination of seeds pre-treated at 30˚C was significantly ($p<0.01$) higher than at other temperatures (Table 3.6). After one day at 5˚C final percentage germination decreased and increased again by day 2. Interestingly, seeds incubated at 10 and 15˚C changed least with increasing dark incubation indicating these temperature are more neutral (Table 3.6; Fig.3.6).

3.4 Discussion

Bur and Cvi ecotypes have similar responses to after-ripening but distinct germination responses to temperature and light

The mechanisms of dormancy-induction and release are sensitive to aspects of the environment, such as light, temperature, after-ripening and some chemicals (Footitt and Cohn, 2001; Cadman *et al*., 2006; Finch-Savage and Leubner-Metzger, 2006; Finch-Savage *et al*., 2007; Tang *et al*., 2008; Footitt *et al*., 2011). The results presented here show that seeds of Bur and Cvi have very different responses to environmental signals, such as light and temperature.

***Temperature*:** Thermal responses of fresh and after-ripened seeds of the two ecotypes were investigated using a thermal gradient. In general, Bur seeds exhibited

low temperature thermo-dormancy having a higher germination potential at higher temperatures, whereas Cvi seeds exhibits high temperature thermo-dormancy and so have higher germination potential at lower temperatures. This is consistent with a summer and a winter annual phenotype respectively (discussed in *chapter 2*). The dark incubation experiment showed that dormancy in Bur seeds is gradually released after short periods of imbibition, reaching minimum dormancy after approximately three days, but then declined (Fig.3.6) This is consistent with previous studies that show ABA content in dormant seeds did not start to increase (i.e. ABA synthesis) until three days after the start of imbibition (Ali-Rachedi *et al*., 2004; Millar *et al*., 2006).

On the thermal gradient Bur seeds had higher germination at higher temperatures, but germination at low temperatures (below 7˚C) continued to increase up to 8 days of imbibition both in the light and dark. This effect of low temperature is likely due to low temperature stratification, which is consistent with a summer annual phenotype, i.e. gradually releasing dormancy during the low temperature of winter. Low temperature can promote the expression of *GA3ox*—the gene encoding the rate-limiting enzyme at the final stage in the biosynthesis of active GA (Yamaguchi *et al*., 1998, 2001; Yamauchi *et al*., 2004). A positive relationship between cold treatment and endogenous GA concentration was found in *Arabidopsis* (Derkx *et al*., 1994). Low temperature may also induce ABA catabolism in *Arabidopsis* seeds (Finch-Savage *et al*., 2007), and the expression of the ABA-inducible protein phosphatase type-2C (*PP2C*) gene increases during the

first week of cold stratification (Bewley *et al*., 2013). Additionally, overexpression of the *PP2C* gene in *Arabidopsis* results in less dormant seeds with reduced ABA sensitivity. This suggests a role of phosphorylation/dephosphorylation in seed dormancy release when seeds are imbibed at low temperature (Bewley *et al*., 2013).

Although low temperature treatment can release dormancy, extended exposure in the dark induced dormancy in both the laboratory and the field (Cadman *et al*., 2006; Finch-Savage *et al*., 2007; Footitt *et al*., 2011; Penfield and Springthorpe, 2012; Footitt *et al*., 2013). Here using Bur, extended low temperature (5˚C) in the dark induced secondary dormancy which has also been observed in the Cvi ecotype (Finch-savage *et al*., 2007).

High temperature can also accelerate dormancy release in many species with physiological dormancy (Baskin and Baskin, 1998). However, the data presented show dormancy can also be induced by high temperatures in Bur. Moreover Bur seeds entered into secondary dormancy more quickly at high temperature than at low temperature. In the Burren, temperatures rarely rise above 20˚C, so temperatures above 20˚C might indicate a future risk of drought for further seedling growth. Adaptation may therefore lead to a response where high temperatures result in a rapid induction of dormancy in Bur seeds, a summer annual, the germination of which does not generally occur during the warmer season.

Therefore it is clear that in addition to loss of dormancy, an induction of secondary dormancy can also occur during stratification (Totterdell and Roberts, 1979; Baskin and Baskin, 1998). The results presented show that prolonged moist

exposure in the absence of light will induce secondary dormancy in Bur seeds at a rate determined by temperature: the rate of induction is greater with higher temperatures.

***Light*:** Light is a critical environmental determinant for seed germination, which has been reported in several species, such as *Chenopodium album* (Tang *et al*., 2008), *Sisymbrium officinale* (Derkx and Karssen, 1993), *Lycopersicon esculentum* (Appenroth *et al*., 2006) and *Arabidopsis* (Shinomura, 1997; Hilhorst and Karssen, 1988). The effect of light on germination is mainly mediated by the red (R)/far-red (FR) light photoreceptor phytochrome (reviewed in *Chapter 1*). The action of light on seeds through phytochrome may lead to the synthesis of GAs that engages a signalling pathway to affect completion of seed germination. During GA biosynthesis, genes encoding GA 3-oxidases are regulated by R and FR light in a photoreversible manner (Yamaguchi *et al*., 1998). Genes involved in GA biosynthesis were also up-regulated by low temperature, leading to an increase in the level of bioactive GAs in imbibed *Arabidopsis* seeds (Yamauchi *et al*., 2004). In addition, by using a loss-of-function *AtGA3ox1*mutant *ga4-2*, the GA biosynthesis gene *GA3ox1* (positively regulated by active phytochrome) was shown to play an important role in mediating the effect of low temperature (Yamauchi *et al*., 2004). Thus the biosynthesis of GA required for germination completion is regulated by both light and low temperature at the level of transcription in the imbibed seed (Yamaguchi *et al*., 1998, 2001; Yamauchi *et al*., 2004).

Data collected from the thermal gradient table in the experiment described above showed that fresh Bur seeds are less dormant than fresh Cvi seeds. The germination of the strongly dormant Cvi seeds had an absolute requirement for light, whatever conditions they were exposed to, but seed dormancy was not released by light without those seeds firstly being exposed to a period of AR. However in fresh Bur, light was not absolutely required for germination of all seeds, but exposure to light led to higher germination in Bur especially at high temperatures. This light response is likely to result from GA synthesis induced by exposure to light (Yamaguchi *et al*., 1998; Cadman *et al*., 2006). However, this light sensitivity decreases after prolonged imbibition in the dark (≥ 60 days). Interestingly when fresh Bur seeds were imbibed in the dark, the requirement for light to promote germination could be offset by low temperature, which may result from up-regulating GA biosynthesis genes and increasing bioactive GAs.

***After-ripening*:** Seed dormancy in both Bur and Cvi seeds was released by dry AR resulting in a widening of the range of permissible germination temperatures. Thus, during AR germination requirements became less specific, and the capacity of seeds to complete germination was enhanced.

Seeds of Cvi are deeply dormant and so the effect of AR was particularly large in this ecotype. At the normally thermo-dormant germination temperature in Cvi of 20˚C, germination did not increase significantly until 75 days of AR (60% germination completed) with maximal germination reached only after 105 days. AR increases sensitivity of dormant Cvi seeds to light to release the final layer of

dormancy (Finch-Savage *et al*., 2007). In the Cape Verdi islands, a long period of AR requirement in Cvi seeds ensures germination coincides with the rainy season, which comes after a hot and dry summer (Fig.2.4 *chapter 2*); ensuring sufficient moisture is provided for subsequent seedling establishment. However, in more temperate soils that do not dry out dormancy is rapidly lost in warm damp soils (warm stratification) and it is likely that dry AR has a limited role in temperate regions (Footitt *et al*., 2011). Hence, for species adapted to regions of dry soil such as the Cvi ecotype of *Arabidopsis* AR may govern the timing of germination under natural conditions. AR can therefore be an important developmental process in low hydrated seeds and can determine the responses of the seeds to environmental cues (Müller *et al*., 2009).

However, in contrast to Cvi, seeds of Bur have shallow dormancy and require shorter periods of AR; 80% were able to germinate at shedding in the absence of AR. In the Burren region of Ireland (Fig.2.4 *chapter 2*) there is rainfall all year round and so there is little chance for long periods of dry AR. Thus the response to AR must be not only species specific but also ecotype specific.

The mechanism by which dormancy is relieved during AR is still not well understood, but transcriptome studies have shown that AR is a process independent of dormancy (Carrera *et al*., 2008). Nevertheless, transcriptome analysis of Cvi seeds also showed that the expression level of 30 genes, including that of the important dormancy related gene *DOG1*, decreased following AR (Finch-Savage, *et al*., 2007). In addition, Ali-Rachedi *et al*. (2004) have shown that AR seeds had

lower ABA levels and decreased sensitivity to ABA compared with fresh seeds. In freshly imbibed seeds, ABA synthesis and accumulation of *ABA1* transcripts (a gene encoding activation of ABA function) increased, leading to the maintenance of dormancy; however in AR seeds, ABA sensitivity was removed by the accumulation of LPP2 (lipid phosphate phosphatase 2), which resulted in the completion of germination (Carrera *et al*., 2008; Katagiri *et al*., 2005).

Nitrate also enhances ABA catabolism and inhibits ABA synthesis (Ali-Rachedi *et al*., 2004) and has been found to play a positive role in the promotion of Cvi seed germination. However, in this highly dormant ecotype a period of AR in the dry or imbibed state was required in the laboratory (dry) and the field (imbibed) before there was a significant effect of nitrate (Finch-Savage *et al*., 2007; Footitt *et al*., 2011). Thus in deeply dormant seeds such as in the Cvi ecotype, nitrate might not be perceived until dormancy has started to decline. In contrast, the shallowly dormant *Bur* seeds were always highly nitrate sensitive.

3.5 Conclusion

Three variables were of great importance in the loss of dormancy in mature *Arabidopsis* seed, i.e. environmental temperature (especially chilling), dry AR and nitrate levels in the soil (Bewley, 1997; Finch-Savage *et al*., 2007; Footitt *et al*., 2011; Penfield and Springthorpe, 2012). This was confirmed by our studies and moreover, the behaviour differed between summer and winter annual ecotypes (Bur and Cvi) that had different depths of dormancy even when the seeds of both ecotypes were produced under the same environmental conditions. Brief chilling can

release this dormancy, but extended low temperature may induce secondary dormancy in both Bur and Cvi. For Bur, dark incubation at high temperatures for more than three days can also result in the induction of secondary dormancy.

Dry after-ripening (AR) improved the final germination in Bur and Cvi by increasing the sensitivity to other environmental signals, such as light, nitrate, and temperature. Seeds of Bur required shorter periods of AR to achieve these changes compared to Cvi, this difference in the rate of the response may be an adaptation of these two ecotypes to their native regions. Under natural conditions the seeds of winter and summer annuals would be produced under widely different environments and this would likely result in greater divergence of responses. This is explored further in subsequent chapters (*Chapters 4-6*).

In the AR experiment above, nitrate played a positive role in the promotion of seed germination. The application of nitrate to both ecotypes could also reduce the time of AR required for the completion of germination. Seeds of Bur were highly sensitive to nitrate even in the absence of AR, whereas the seeds of Cvi have relatively deep dormancy and therefore there is a greater need for AR to increase sensitivity to other environmental signals such as light, nitrate and temperature. The effect of nitrate will be explored further in *Chapter 6* and the change in depth of dormancy and therefore sensitivity to environmental signals over the dormancy cycle in field soils is explored in *Chapter 4*.

CHAPTER 4: CHARACTERIZATION OF DORMANCY CYCLING IN BUR AND CVI SEEDS IN RESPONSE TO ENVIRONMENTAL SIGNALS AT THE MOLECULAR AND ECO-PHYSIOLOGICAL LEVEL

4.1 Introduction

Dormancy is a relative rather than an absolute condition, with the transition between the dormant and non-dormant states continually changing. In nature, the depth of dormancy in seeds cycles in response to a range of environmental signals that inform the seed about the season, its depth in soil and the presence of competing plants. Thus seeds cycle through dormant and less dormant states until dormancy is fully removed to allow completion of germination when the sensitivity of the seed to environmental signals overlaps with the ambient environment, otherwise the annual cycle repeats itself (Baskin and Baskin, 1983; Finch-Savage and Leubner-Metzger, 2006; Allen *et al*., 2007; Footitt *et al*., 2011). Different species or ecotypes within species respond differently to environmental signals through adaptation to the habitat and climate space they inhabit, so that they germinate at different times of the year. There are two main categories of behaviour, winter and summer annuals, which germinate in autumn or spring respectively (Fig.4.1; reviewed in *chapter 2*).

The different seed dormancy cycles and consequent different patterns of seedling emergence are important components of life history in plants, which significantly contributes to plant fitness (Donohue 2002, 2005; Donohue *et al*., 2005b; McNamara *et al*., 2011). Despite the importance of dormancy cycling in

nature, until recently little was known about the regulation of this process at the molecular level. However, recent gene expression studies of laboratory based dormancy cycling and dormancy cycling in field soils has shed light on the involvement, coordination and regulation of this process at the molecular level (Cadman *et al*., 2006; Finch-Savage *et al*., 2007; Footitt *et al*., 2011). These published studies investigated dormancy cycling in the winter annual *Arabidopsis* ecotype Cvi. Here the summer annual ecotype Bur is investigated to further understand the mechanisms by which environmental signals influence seed dormancy cycling using a combination of eco-physiology and molecular biology.

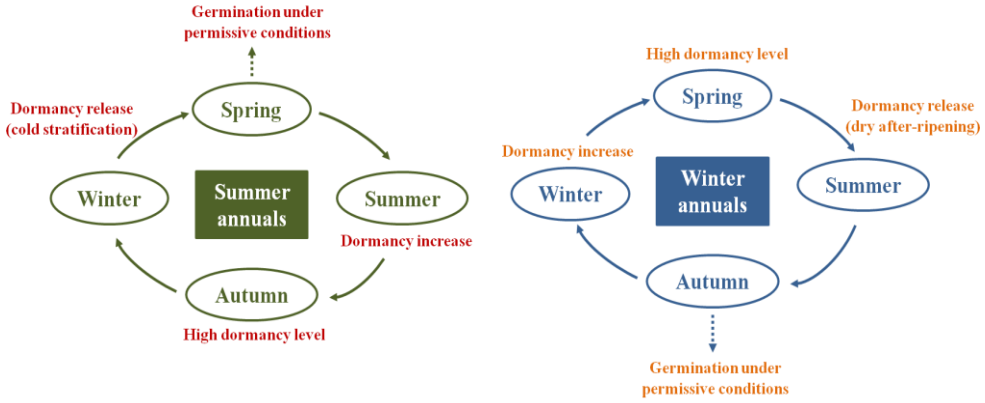

Figure 4.1 Annual dormancy cycles in relation to seasonal variation. Summer and winter annual species show opposite seasonal dormancy patterns resulting in different times of seedling establishment and plant growth.

4.1.1 Molecular regulation of seed dormancy and germination

Abscisic acid (ABA) and Gibberellins (GA) are the major hormones influencing germination in *Arabidopsis* seeds, with the ABA/GA balance determining the developmental state of the seed i.e. if it is dormant or non-dormant and therefore able to germinate (Finch-Savage and Leubner-Metzger, 2006). The molecular regulation of ABA and GA metabolism has been analysed in *Arabidopsis*,

focusing on the three related processes — seed maturation, after-ripening and imbibition. Recent studies of the transcriptome and proteome showed that seeds change and remain responsive at a molecular level in both the imbibed and the dry state (Gallardo *et al*., 2002; Rajjou *et al*., 2004; Cadman *et al*., 2006; Finch-Savage *et al*., 2007; Carrera *et al*., 2008).

The key genes involved in ABA biosynthesis and degradation are *NCED*s and *CYP707A* respectively. The NCED family members NCED5, NCED6 and NCED9 were found to regulate seed dormancy through the first step of ABA synthesis from carotenoid synthesis (Lefebvre *et al*., 2006; Frey *et al*., 2012). The ABA degradation genes *CYP707A1* and *CYP707A2* are expressed abundantly during seed development and after imbibition respectively (Okamoto *et al*., 2006). Studies using ABA response mutants also revealed that, the ABA signal transduction pathway associated with dormancy induction involves protein kinases (the SnRK2 ABA-activated protein kinase Family), phosphatases (ABI1 and ABI2) and transcription factors (ABI3, ABI4 and ABI5) (Finkelstein *et al*., 2002; Himmelbach *et al*., 2003).

In the case of GA, the key biosynthetic genes *GA3ox1* and *GA3ox2* are strongly expressed in the axis of the imbibed embryo (Mitchum *et al*., 2006). The two basic helix-loop-helix (bHLH) transcription factors SPT and PIL5 regulate the expression of *GA3ox1* and *GA3ox2* by their interaction with light and low temperature (Penfield *et al*., 2005). GA 2-oxidation has been reported to be the only GA deactivation reaction so that GA 2-oxidases are responsible for GA degradation (Yamaguchi *et al*., 2007). Previous studies also provided evidence that DELLA

proteins RGL2 and possibly RGL1 control seed germination by repressing GA responses during development (Sun and Gubler, 2004; Tyler *et al*., 2004).

Seed dormancy and germination are determined by the ABA-GA balance, dormancy-releasing environmental signals and internal developmental signals (Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al*., 2008). The network of the genes involved can be seen in Fig.4.2.

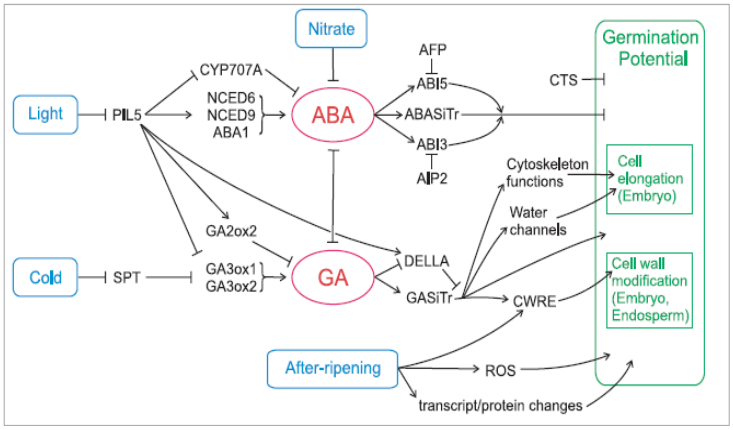

Figure 4.2 Regulation of dormancy status during seeds maturation and imbibition of mature *Arabidopsis* seeds and proposed interactions between some of the genes involved. Factors that influence germination are shown in blue, germination potential is shown in green, and the hormones ABA and GA are shown in red. An arrow indicates a positive effect and a bar a repressive effect (reproduced from Holdsworth *et al*., 2008).

4.1.2 Previous studies using the *Arabidopsis* ecotype Cvi

Recent work has investigated the molecular eco-physiology of dormancy cycling in the field of the *Arabidopsis* ecotype Cvi. The depth of dormancy and gene expression patterns were found to be correlated with seasonal changes in soil temperature (Footitt *et al*., 2011). This work illustrated how molecular mechanisms identified as controlling dormancy in the laboratory could be seasonally coordinated in seeds buried in field soil to fulfil this process. Two forms of environmental

sensing were revealed: (1) temporal sensing which is related to slow seasonal change (e.g. temperature) and appeared to be determined by enhanced sensitivity to ABA. Thus seeds did not respond to signals that inform about germination conditions (e.g. light) but cycled from deep to shallow dormancy in order to select time and space for emergence (Finch-Savage and Leubner-Metzger, 2006); (2) spatial sensing which is concurrent with enhanced DELLA repression of germination and responds rapidly to favourable germination conditions (e.g. light, nitrate) to determine actual time of germination. Therefore when the temporal and spatial sensing overlapped with ambient environmental conditions, seed dormancy could be released fully to result in the completion of germination leading to seedling emergence in the field (Footitt *et al*., 2011).

Bur and Cvi naturally inhabit widely different environments in geographically distant regions and exhibit the contrasting life cycles of summer and winter annuals respectively (discussed in *Chapter 2*). Therefore, these two ecotypes are good subjects ideal for studying the differential adaptation of dormancy cycling and germination mechanisms. Since there is no thorough study of dormancy and germination behaviour reported in the Bur ecotype, an investigation of gene expression combined with a detailed physiological characterization of dormancy cycling behaviour in the field was carried out. The differences in the patterns of temporal and spatial sensing between Bur (shown below) and Cvi (data from Footitt *et al*., 2011) were used to better understand the regulation of summer and winter annual phenotypes respectively (Footitt *et al*., 2013). This paper includes the data

reported below and some data reported in *Chapter 3* and has now been accepted for publication in The Plant Journal.

4.2 Materials and Methods

4.2.1 Field emergence of Bur and Cvi ecotypes of *Arabidopsis*

To investigate the different behaviour of Cvi and Bur in natural variable conditions, seeds were sown in the field in the autumn to monitor seedling emergence following monthly soil disturbance throughout the subsequent year (Fig.4.3). All seeds were dressed with Metalaxyl (Hockley International, UK) at 1 g active fungicide/kg seeds to prevent fungal diseases. 17.5 cm^2 rigid black pots from Fargro (BHGS horticultural code PBQ18) were employed. Pots were sunk into the ground leaving a 2 cm lip above the ground. They were filled with sieved and sterilised sandy loam soil to a depth of 3 cm from the top. A piece of 125µm nylon mesh was placed on the soil surface to stop seeds sinking deeper than 1 cm below the surface and to enable soil disturbance without incorporating seeds into deeper layers. The mesh was lightly covered with a dusting of sterilised soil. There were three replicate pots for Bur and four for Cvi. Each pot had 500 seeds sown over the surface and covered to a depth a 1cm with sterile soil. Pots were then lightly watered to dampen the dry soil. Soil moisture sensors (SM200, Delta-T Devices Ltd, UK) and Thermistore temperature probes (Betatherm, Ireland) linked to a data logger (Delta-T Devices Ltd, UK) recorded soil moisture and temperature at seed depth in dummy pots.

4.2.2 Molecular eco-physiological characterization of Bur seeds

Figure 4.3 Field plots monitoring seedling emergence of Cvi and Bur from November 2009 to September 2010. A: field plots; **B**: weather station and data logger recording soil moisture and temperature.

Seed burial: Bur seeds were produced in a temperature-controlled glasshouse from May to July 2009 (refer to *sec 3.2.1*). Seed mortality in the soil was reduced by dressing with Metalaxyl (Hockley International, UK) as described above. Seeds were dispersed in Ballotini balls (Potters Ballotini Ltd, UK) whose diameter (0.1-0.25mm diameter) was within the range of particle size of the sandy loam soil in the field experimental area. The seed/ballotini ball mixture was placed into nylon-mesh bags and buried in the field at a depth of 5cm in a random plot design. Seeds were dispersed at a density of 40 seeds /g of Ballotini balls to further reduce seed mortality (Van Mourik *et al*., 2005). For physiological analysis 140 mg of seeds were dispersed in 85g of Ballotini balls placed in a nylon bag (10×10 cm) constructed from 125µm nylon mesh Clarcor UK, UK) and sealed with a WeLoc® bag clip (size PA110) (WeLoc - Weland M. AB, Sweden). One bag was prepared for each month of the time course. For molecular analysis of dormancy status four bags were prepared for each month of the time course each with 50mg of seeds

dispersed in 28g of Ballotini balls contained in a nylon bag (7×7 cm) sealed with a PA70 bag clip. Each bag was identified with a plastic label attached to each clip with wire (Fig.4.4). Soil moisture sensors (SM200, Delta-T Devices Ltd, UK) and Thermistore temperature probes (Betatherm, Ireland) linked to a data logger (Delta-T Devices Ltd, UK) recorded soil moisture and temperature at seed depth within a dummy bag.

Figure 4.4 Burial of Bur seed for molecular and physiological characterization.

Seed recovery: Seed bags were recovered from the field monthly. Samples for molecular analysis were recovered from the field in the dark using a light proof box. The box (H × W× L, 300 × 350 × 500 mm) had two circular arm holes 120 mm in diameter located 1/3 of the distance along the length, and 50mm from the edge on the top. Long reach gauntlets were attached to these holes with retaining rings (Marigold Emperor ME106, Buck and Hickman, Coventry, UK). The box was placed over the burial site and the base sealed with soil to exclude light. The four independent molecular samples (replicates) were then exhumed and placed in a laminated foil bag 11 × 24 cm (Moore and Buckle, St. Helens, UK) and sealed with a WeLoc PA150 clip. On return to the laboratory seeds for molecular analyses were

immediately separated from the Ballotini balls in a dark room under a green safe light. Seed bags were removed from the light proof bag and each bag washed in cold water to remove soil. Ballotini balls and seeds from each bag were washed into separate 50ml centrifuge tubes and then washed three times. On the third washing the tubes were gently shaken to migrate seeds over the Ballotini balls to the tube walls where they were removed with a pastette (3ml) (Jencons Ltd, UK) to 2ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80˚C (Footitt *et al.*, 2011; Footitt and Finch-Savage, 2011). Seeds for physiological analysis were separated from Ballotini balls in a glass beaker in the light and immediately used for germination testing. Seeds were first surface sterilized with 2.5% bleach (Domestos) for five minutes, washed three times with distilled water, and then incubated on two layers of 3M chromatography paper in clear plastic boxes (8 × 12cm) (Stewart Plastics) containing 8ml of distilled water. To characterise different thermal responses seed germination was recorded at a range of temperatures (5, 10, 15, 20 and 25˚C) for 28 days. For each treatment there were three replicates of 40 seeds from a bulk collection recovered from the field. Germinated seeds were counted and removed at regular intervals throughout the experiment. A seed was considered to have germinated when the radical had emerged through the testa and endosperm.

4.2.3 RNA extraction and gene expression analysis

Seeds were collected and immediately frozen in liquid nitrogen and stored at -80˚C. RNA was extracted using nuclease free reagents using the methods of Xu *et al*. (2010) and Gehrig *et al*. (2000) adapted for seeds. Seeds were homogenised from

frozen in 0.9 ml preheated extraction buffer (65˚C) (100mM TRIS-HCl pH 8.0, 25mM EDTA, 2 M NaCl, 2% W/V CTAB, 2 % PEG 20,000 and 2% v/v β-mercaptoethanol). Samples were then incubated at 65˚C for 15mins. 0.5 ml of chloroform was added to each sample, briefly vortexed and centrifuged at 12,000 rpm/ 4˚C /10 min. The supernatant was transferred to a new tube and 133 µl of 5M NaCl and 0.4 ml of chloroform added, mixed and re- centrifuged. This last step was then repeated and RNA precipitated for the supernatant by adding ½ volume of isopropanol and ½ volume of 1.2M NaCl/0.8M tri-Na citrate dehydrate mixing gently and incubating at room temperature for 15 minutes. RNA was recovered by centrifugation at 12,000 rpm/ 4˚C /10 min and the pellet washed in 75% ethanol, re-centrifuged and the pellet air dried for 10 minutes and dissolved in 120µl of water and re-precipitated with a ½ volume of 5M NH4oAc and 2.5 volumes of ethanol at -20˚C for at least one hour. The RNA pellet was washed in 75% ethanol and re-centrifuged and the pellet air dried for 10 minutes and dissolved in 40 µl water.

Gene expression was analysed using the Nanostring ncounter gene expression system (Geiss *et al*., 2008) by UCL Genomics, University College London using 100ng of total RNA. Gene family members were selected that exhibited distinct seed expression patterns in a previous laboratory-based microarray analysis of dormancy cycling (Cadman *et al*., 2006; Finch-Savage *et al*., 2007). Genes chosen were those involved in ABA and GA biosynthesis (*NCED6*, *GA3ox1*) and catabolism (*CYP707A2*, *GA2ox2*), ABA signalling (*PYR1*, *PYL7*, *ABI2*, *SnrK2.1* and *-2.4*, *ABI3*, *ABI4*, and *ABI5*) (Nambara *et al*., 2005), the ABA-signalling regulator

MFT (Xi *et al*., 2010), GA signalling (*GID1A*, *RGL2*, *RGA2*), members of the phytochrome interacting factor (PIF) family (*PIL5* and *SPT*), and the dormancy-associated *DOG1* (Bentsink *et al*., 2006). Changes in expression of the germination associated gene *EXP2* and ABA sensitivity related gene *ATHB20* were also measured. The Nanostring probes targeting the genes *ABI2 (*At5g57050), *ABI3 (*At3g24650), *ABI4* (At2g40220), *ABI5* (At2g36270), *CYP707A2* (At2g29090), *DOG1* (At5g45830), *FLC* (At5g10140), *Ga2ox2* (At1g30040), *Ga3ox1* (At1g15550), *GID 1A* (At3g05120), *MFT* (At1g18100), *NCED6* (At3g24220), *NR1* (At1g77760), *NRT 1.1* (At1g12110), *PIL5* (At2g20180), *PYL7* (At4g01026), *PYR1* (At4g17870), *RGA2* (At1g14920), *RGL2* (At3g03450), *Snrk 2.1* (At5g08590), *Snrk 2.4* (At1g10940), *SPT* (At4g36930), *EXPA2* (At5g05290), *ATHB20* (At3g01220) and the internal control gene *TIP41-like* (At4g34270) (Footitt *et al*., 2011) were designed and synthesized by NanoString Technologies (http://www.nanostring.com) (Table 4.1).

4.2.4 Data analysis

The mRNA counts for the genes under investigation were normalized to the Nanostring internal reference and the reference gene *TIP41-like* as described in the nCounter Gene Expression Assay Manual (http://www.nanostring.com/uploads/Manual_Gene_Expression_Assay.pdf/).

Table 4.1: Probe sets for genes of interest used for Nanostring analysis

Gene symbol	Accession number	Targeted Region	Target Sequence
ABI2	NM_125087.2	1154-1254	GCTCATGCTCCGGAAACTGTTGGGTCTACCTCGGTGGTTGCGGTTGTCTTTCCGACTCACATCTTTGTCGCGAATTGCGGCGACTCTAGGGCGGTTTTGT
ABI3	NM_113376.3	801-901	GTCTTCAACATCTCCAGCTCCTGTCAACGCAATCGTCTCCTCAGCCTCTTCTTCTTCGGCAGCTTCTTCCTCCACTTCCTCAGCTGCTTCTTGGGCTATA
ABI4	NM_129580.1	725-825	GCAGGAACAAGGAGGAAGTGGGTGTAATAATAATAGTTCGATGGAGGATTTGAACTCTCTAGCTGGTTCGGTGGGTTCGAGTCTATCAATAACTCATCCA
ABI5	NM_129185.3	513-613	TGGTGTTGGTGTCTTTAGTGGTGGTTCTAGAGGCAACGAAGATGCTAACAATAAGAGAGGGATAGCGAACGAGTCTAGTCTTCCTCGACAAGGCTCTTTG
ATHB20	NM_110988.2	541-641	AGACAGCTCGAGAGAGATTATGATTCACTCAAGAAACAGTTTGAGTCTCTTAAATCCGACAATGCTTCTCTACTTGCCTATAACAAGAAACTCCTTGCTG
CYP707A2	NM_128466.2	255-355	AGAGACACTCCGCCTCTACACAGAAAATCCCAATTCCTTCTTCGCCACTCGCCAAAACAAGTACGGGGATATATTCAAGACGCACATATTAGGATGTCCA
DOG1	NM_123951.2	599-699	CGACAAGCAAGAAGAAGCTATGGCTCGTTTATTGGTCGAGGCCGATAATCTAAGGGTTGATACTTTAGCGAAGATCCTCGGGATTCTATCTCCGGTACAA
EXPA2	NM_120611.2	693-793	AAGCAACACTTACCTCAGAGGTCAAAGCCTTTCTTTCCAAGTCACTGATAGTGATGGTCGGACTGTTGTGAGCTACGATGTTGTGCCTCATGATTGGCAG
FLC	NM_001161231.1	122-222	ATCCGTCGCTCTTCTCGTCGTCTCCGCCTCCGGCAAGCTCTACAGCTTCTCCTCCGGCGATAACCTGGTCAAGATCCTTGATCGATATGGGAAACAGCAT
Ga2ox2	NM_102743.2	464-564	TCAATGCTAATCCTCAGCTCTCCTCTCCTAAAACCTCCGCCGTTTTCCGTCAAACCCCTCAAATTTTCCGTGAGTCGGTGGAGGAGTACATGAAGGAGAT
Ga3ox1	NM_101424.2	320-420	GGGGTGCCTTCCAAATCTCAAACCACGGCGTGCCTTTGGGACTTCTCCAAGACATTGAGTTTCTCACCGGTAGTCTCTTCGGGCTACCTGTCCAACGCAA
GID1A	NM_111384.3	290-390	TCAAAGTAGCCTACAATATCCTTCGTCGCCCTGATGGAACCTTTAACCGACACTTAGCTGAGTATCTAGACCGTAAAGTCACTGCAAACGCCAATCCGGT
MFT	NM_101672.3	309-409	CCGAACATGAGAGAATGGGTCCACTGGATTGTCGTGGATATTCCCGGAGGCACAAATCCCTCAAGAGGAAAAGAGATACTTCCATACATGGAACCAAGGC
NCED6	NM_113327.2	1334-1434	AACCGTAAGGTTATCGTAACCGGAGTGAATTTAGAAGCGGGTCACATAAACCGTAGTTACGTGGGCCGGAAAAGCCAGTTCGTTTACATAGCAATAGCCG
NR1	NM_106425.2	513-613	CTCCATTACGTCCGCAACCACGGTGCAGTTCCCAAAGCGAATTGGTCAGACTGGTCAATCGAAATTACCGGACTCGTTAAACGTCCGGCTAAATTCACCA
NRT 1.1	NM_101083.3	1505-1605	CCCACAATATCTTATTGTCGGTATCGGCGAAGCGTTAATCTACACAGGACAGTTAGATTTCTTCTTGAGAGAGTGCCCTAAAGGTATGAAAGGGATGAGC
PIL5	NM_179665.2	520-620	ATTTCTCGAGGCTGAGAGGGGATTTTAATAACGGTAGAGGTGGTGAATCTGGACCGTTGCTTTCGAAGGCGGTTGTGAGAGAATCTACGCAGGTAAGTCC
PYL7	NM_116332.3	328-428	TATAAGCAGATGCACTGTAAATGGTGATCCTGAGATCGGTTGTCTCAGAGAAGTAAATGTCAAATCTGGTCTTCCAGCAACCACCAGTACAGAGAGATTG
PYR1	NM_117896.2	601-701	TTGTGAAGCTTAATTTGCAGAAACTCGCGACGGTTGCTGAAGCTATGGCTCGTAACTCCGGTGACGGAAGTGGTTCTCAGGTGACGTGAAAATGAAGAAA
RGA2	NM_101361.2	39-139	AGAAGTGGTAGTGGAGTGAAAAAACAAATCCTAAGCAGTCCTAACCGATCCCCGAAGCTAAAGATTCTTCACCTTCCCAAATAAAGCAAAACCTAGATCC
RGL2	NM_111216.2	1068-1168	GTCGTTTCGTCTCACCGGAATCGGACCACCGCAGACGGAGAATTCAGATTCGCTTCAACAGTTAGGTTGGAAATTAGCTCAATTCGCTCAGAACATGGGC
Snrk2.1	NM_120946.4	1315-1415	CACTCCTTGGTGAGTTGGTGACTTGCTTCTGGTAAAAAGAAAAACACATTTGTCAAAGCCAAAGGCTGTAAACTACGTGCTGGTTGTTTCACTTTTAGAT
Snrk2.4	NM_100969.3	1242-1342	AAAGAGAACCCAACCTTCTCCCTTCAGACCGTTGAAGAGATCATGAAGATAGTGGCTGACGCCAAAACACCGCCTCCTGTTTCCCGATCCATCGGAGGTT
SPT	NM_119857.2	1083-1183	TGGTCATTCCAACGCAAACATAACCGGGGAACAAGCTCTGTTTGATGGACAACCTGACCTAAAAGATCGAATTACTTGAACAGTGTCCCAACTTCGGGAT
TIP41like	NM_119592.4	768-868	TATAGGTTTGGCGAAGATGAGGCACCAACTGTTCTTCGTGAAAACTGTTGGAGAGAAGCAACATTTCAGTCTCTATCTGCGAAAGGGTATCCAGTTGACT

The reference gene *Tip41-like* (At4g34270) was selected for normalization of the data due to its stability of expression in the seed dormancy microarray data (Cadman *et al*., 2006; Finch-Savage *et al*., 2007) where it has the lowest fold change in expression (maximum 1.5) and coefficient of variation across 11 different hydrated dormancy states when compared with others evaluated as reference genes in plants (Remans *et al*., 2008). It was also identified as one of the most stable reference genes for use in expression analysis by QPCR in *Arabidopsis* seeds (Dekkers *et al*., 2012). Analysis of correlation coefficients was used to assess the relationship between all pairs of the gene expression data which were considered at 11 time points (Bur) and 12 time points (Cvi) for each ecotype separately. Additionally the relationship between gene expression and measures of the soil temperature prior to the recovery of seeds from the field was also analysed. The significance of results was shown by both R square values from the analysis.

4.3 Results

4.3.1 Seedling emergence responses of Bur and Cvi in the field

Cvi and Bur seeds were sown in the field and emergence was monitored at regular intervals. Soil temperature decreased in winter before increasing from January 2010 onwards, while soil moisture was relatively variable (Fig.4.5A). From June the conditions became warm and dry, which led to a decrease in soil moisture content in the field. However, there was rainfall in late July and August that temporarily increased soil moisture levels. The Cvi and Bur seeds had different emergence responses. Bur seedlings emerged in spring, whereas seedlings emerged

from Cvi seeds in late summer. The steep increase in Cvi seedling emergence is concurrent with the increase of soil moisture in late July and August (Fig.4.5)

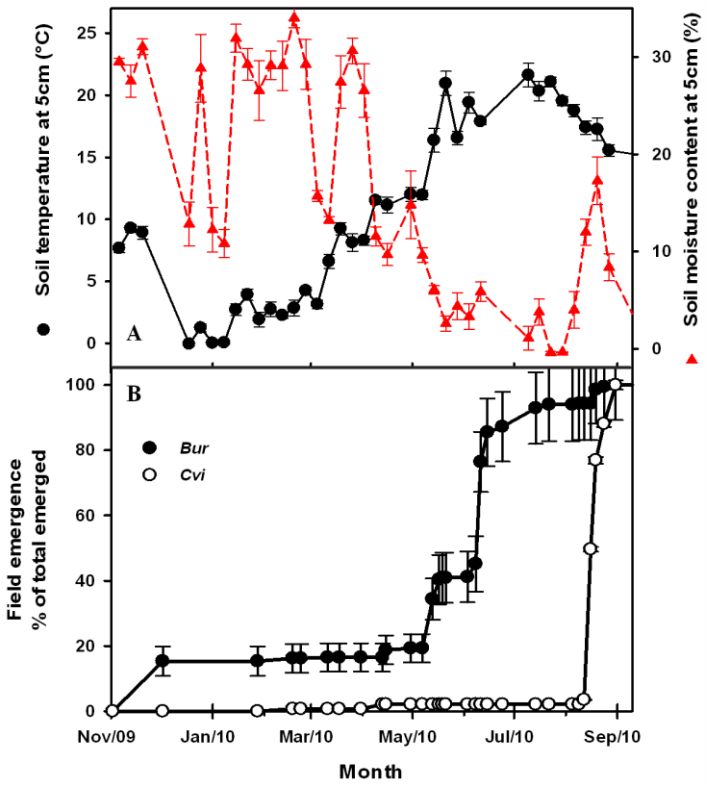

Figure 4.5 Soil conditions and seedling emergence following sowing in November 2009. A: Changes in soil temperature and moisture content measured at seed depth (5cm); and weekly rainfall over 12 months from October 2009. **B**: Seedling emergence in field plots from (● Bur; ○ Cvi). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

4.3.2 Seasonal dormancy patterns in Bur correspond to a summer annual phenotype

The soil temperature and moisture content recorded alongside the buried seeds showed that they were subjected to large seasonal changes in the soil (Fig.4.6). Seeds were exhumed at intervals for one year after sowing. No germination occurred in the soil over this period. The potential for germination was investigated on these exhumed seeds at a range of temperatures both with and without nitrate. At

the start of the experiment germination was greater at lower temperatures and lower at high temperatures indicating the presence of thermo-dormancy. However, germination of exhumed seeds at higher temperatures increased following sowing so that after one month there was near 100% germination at all temperatures and therefore dormancy was minimal (Fig.4.6A).

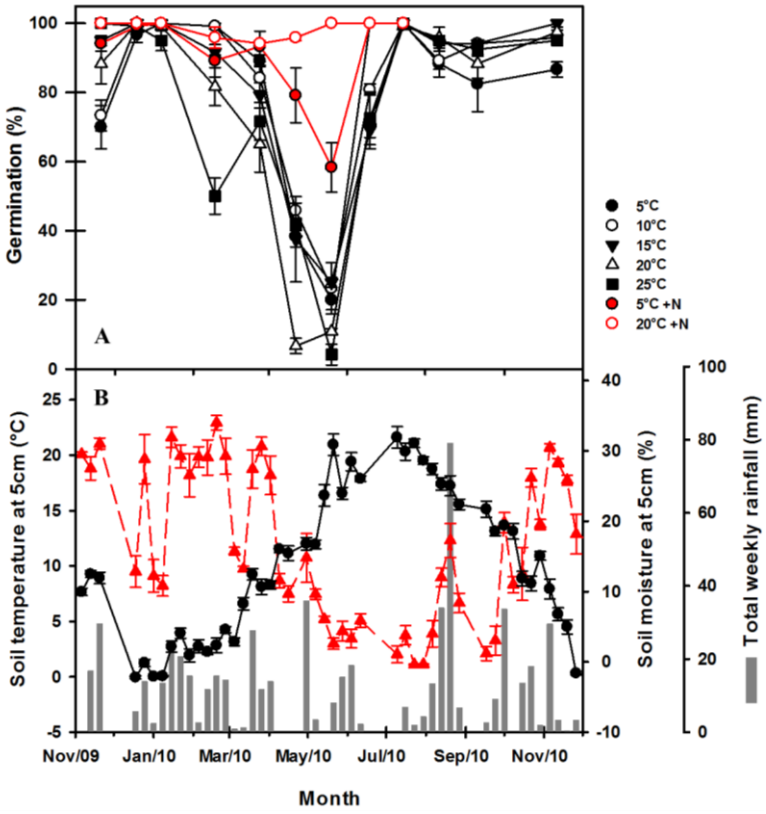

Figure 4.6 Seasonal changes in dormancy status. A: Changing thermo-dormancy in seeds recovered from the field. Following recovery, seeds where incubated in the light at 5-25˚C. **B**: Changes in soil temperature and moisture content measured at seed depth (5cm) and weekly rainfall over 12 months from October 2009 (● soil temperature at 5 cm; ▲ soil moisture content (%). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

As winter progressed thermo-dormancy was induced again, first at higher temperatures, and then germination decreased at all temperatures. Dormancy reached maximum in late April when germination in the light at 20˚C and 25˚C was

the lowest. However, the depth of dormancy then rapidly decreased to a minimum over the next month resulting in high germination at all temperatures. Sensitivity to nitrate was temperature dependent and declined at 5˚C as dormancy increased, but remained level at 20˚C at the relatively high application rate of 10mM (Fig.4.6A). The nitrate response in *Arabidopsis* seeds is dose dependent (Alboresi *et al*., 2005; Finch-Savage *et al*., 2007) and thus lower levels of exposure under natural conditions in the soil would have a smaller effect on dormancy.

4.3.3 Annual gene expression patterns in Bur compared to those for Cvi

Bur seeds were also exhumed from the field to investigate the expression of key genes shown in laboratory studies to be involved in the regulation of seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al*., 2008; Holdsworth *et al.* 2008; Footitt *et al*., 2011). The samples were taken at intervals and used to construct time courses of gene expression over the year. Seeds were not exposed to light on exhumation so the requirement of Bur seeds for light to remove the final layer of dormancy was not fulfilled. Therefore changes in gene expression relate to changes in dormancy level in the soil seed bank and not the downstream germination process. The expression of 24 genes involved in hormone metabolism, hormone signalling and regulation of germination was measured and interpreted in the context of the ecological observation to better understand the coordination of molecular mechanism identified in the laboratory (Table 4.2).

Table 4.2 The correlation coefficients between the expressions of each of 24 genes involved in hormone metabolism, hormone signalling and regulation of germination in *Arabidopsis* Bur seeds, and the correlation coefficients between gene expression and weather data (soil temperature and moisture content)

	Soil Temp	*Soil MC*	*DOG1*	*MFT*	*SnRK2.1*	*Snrk2.4*	*ABI2*	*ABI3*	*ABI4*	*ABI5*	*PYR1*	*PYL7*	*NCED6*	*CYP707A2*	*GA3ox1*	*GA2ox2*	*GID1A*	*PIL5*	*SPT*	*RGA2*	*RGL2*	*NRT1*	*NR1*	*EXPA2*	*ATHB20*
Soil MC	-0.507																								
DOG1	-0.758	0.532																							
MFT	0.631	-0.444	-0.613																						
SnrK2.1	0.754	-0.628	-0.545	0.869																					
Snrk2.4	0.095	-0.145	0.336	0.425	0.548																				
ABI2	0.456	-0.588	-0.407	0.779	0.874	0.607																			
ABI3	0.419	-0.220	-0.096	0.806	0.763	0.830	0.699																		
ABI4	0.744	-0.651	-0.547	0.847	0.946	0.536	0.836	0.748																	
ABI5	0.541	-0.399	-0.139	0.732	0.871	0.850	0.810	0.903	0.876																
PYR1	0.731	-0.577	-0.519	0.863	0.966	0.582	0.887	0.782	0.971	0.908															
PYL7	0.297	-0.477	0.062	0.401	0.651	0.800	0.779	0.659	0.641	0.825	0.699														
NCED6	0.037	-0.299	0.011	0.287	0.247	0.279	0.233	0.384	0.301	0.264	0.149	0.231													
CYP707A2	0.795	-0.627	-0.583	0.866	0.973	0.493	0.838	0.751	0.978	0.866	0.977	0.645	0.275												
GA3ox1	0.309	-0.698	-0.545	0.300	0.331	-0.135	0.370	-0.020	0.530	0.151	0.368	0.130	0.379	0.431											
GA2ox2	0.781	-0.265	-0.359	0.582	0.814	0.505	0.637	0.628	0.700	0.775	0.790	0.617	-0.037	0.781	-0.087										
GID1A	0.728	-0.687	-0.464	0.710	0.915	0.512	0.833	0.671	0.826	0.802	0.876	0.783	0.224	0.896	0.231	0.824									
PIL5	0.558	-0.522	-0.339	0.831	0.855	0.602	0.816	0.843	0.846	0.856	0.863	0.740	0.460	0.892	0.282	0.645	0.865								
SPT	0.532	-0.734	-0.577	0.678	0.702	0.298	0.704	0.455	0.862	0.603	0.764	0.441	0.320	0.767	0.846	0.295	0.547	0.613							
RGA2	0.787	-0.787	-0.691	0.746	0.819	0.201	0.641	0.532	0.842	0.599	0.765	0.452	0.513	0.871	0.605	0.541	0.812	0.802	0.743						
RGL2	0.828	-0.503	-0.448	0.736	0.928	0.578	0.772	0.746	0.894	0.895	0.938	0.730	0.115	0.938	0.188	0.929	0.921	0.825	0.580	0.744					
NRT1	0.626	-0.770	-0.660	0.700	0.799	0.215	0.739	0.402	0.883	0.602	0.805	0.417	0.289	0.836	0.804	0.434	0.652	0.647	0.938	0.811	0.656				
NR1	0.695	-0.756	-0.659	0.832	0.905	0.360	0.863	0.598	0.952	0.745	0.921	0.587	0.299	0.942	0.662	0.584	0.818	0.831	0.900	0.877	0.802	0.949			
EXPA2	0.578	-0.800	-0.567	0.629	0.750	0.323	0.761	0.432	0.878	0.637	0.788	0.556	0.353	0.804	0.840	0.399	0.655	0.651	0.972	0.791	0.653	0.953	0.925		
ATHB20	0.480	-0.829	-0.635	0.312	0.409	-0.141	0.363	0.015	0.560	0.170	0.407	0.191	0.383	0.496	0.924	0.044	0.392	0.324	0.788	0.735	0.304	0.760	0.665	0.815	
FLC	0.688	-0.529	-0.448	0.844	0.958	0.634	0.911	0.812	0.896	0.907	0.967	0.765	0.130	0.937	0.188	0.847	0.932	0.884	0.615	0.714	0.945	0.678	0.849	0.667	0.263

All comparisons have 9 degrees of freedom.

Significant correlation at p>(0.001)

Significant correlation at p>(0.01)

Significant correlation at p>(0.05)

Significant correlation at p>(0.1)

The correlation coefficients were calculated between the expression of each of the 24 genes and also between gene expression and weather data (e.g. soil temperature and moisture content) (Table 4.2).

4.3.3.1 Expression of GA metabolism and signalling genes in Bur during dormancy cycling

The expression of the GA biosynthesis gene GA3ox1 showed a dramatic decrease after burial and stayed at a low level over the winter (Fig.4.7). The expression then increased with increasing dormancy (decreased germination), but kept on rising as dormancy decreased in May.

However the GA catabolism gene *Ga2ox2* showed a relatively constant expression over the winter until May and then increased when the depth of dormancy had already started to drop from its peak (Fig.4.7). After May, *Ga3ox1* expression declined as *Ga2ox2* increased, consistent with inhibition of germination.

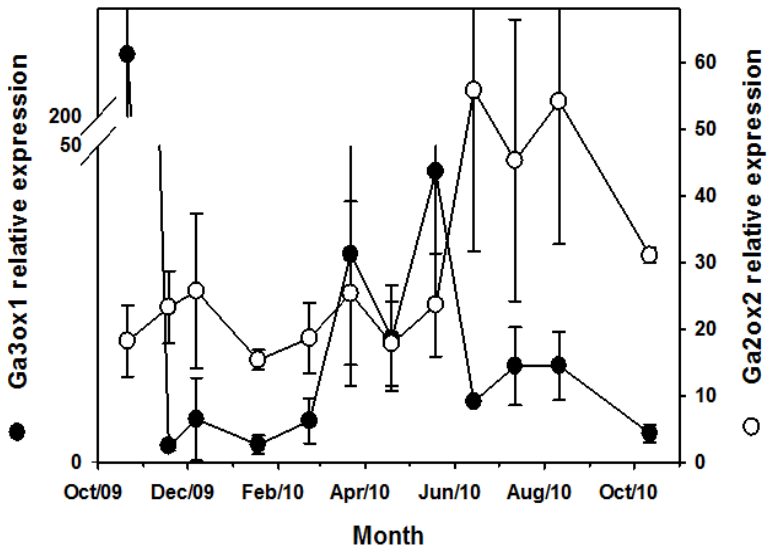

Figure 4.7 Gene expression of *GA3ox1* (GA biosynthesis) and *GA2ox2* (GA catabolism). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

The GA receptor gene *GID1A* enhances germination potential (Griffiths *et al*., 2006), consistent with this function expression increased from April to June as seeds

transitioned from low to high germination potential (dormancy declined) (Fig.4.8) . This pattern was positively correlated ($p<0.01$) with soil temperature. *GID1A* expression then decreased into autumn. The expression was also significantly ($p<0.001$) and positively correlated with the *FLC* gene, which has been recently linked to the regulation of temperature dependent germination in *Arabidopsis* (Chiang *et al*., 2009; Fig.4.8 and Table 4.2).

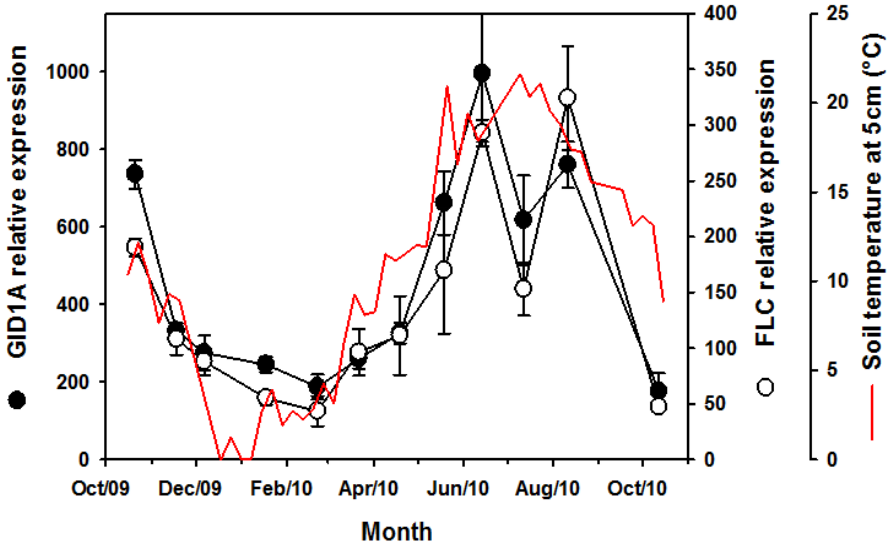

Figure 4.8 The relationship between soil temperature (red line) and gene expression in the GA signalling pathway: *GID1A* (GA receptor) and *FLC* (a flowering time regulator). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

The two negative regulators of germination, *RGA2* and *RGL2* (DELLA genes) both showed a dramatic decrease in expression on burial, but followed a similar pattern to *GID1A* afterwards (Fig.4.9A). The PIF family members, *PIL5* and *SPT*, show decreased expression on burial. Expression then increased with dormancy before declining in the autumn (Fig.4.9B).

Moreover, expression of the early GA-induced transcript *EXP2* was significantly ($p<0.001$) and positively correlated with the *SPT* gene (Fig.4.9B and Table 4.2). Following an initial increase in expression as soil temperature increased,

the expression of all GA signalling associated genes decreased at high soil temperature as soil moisture declined. Rainfall in July (increased soil moisture) positively influenced the expression of these genes.

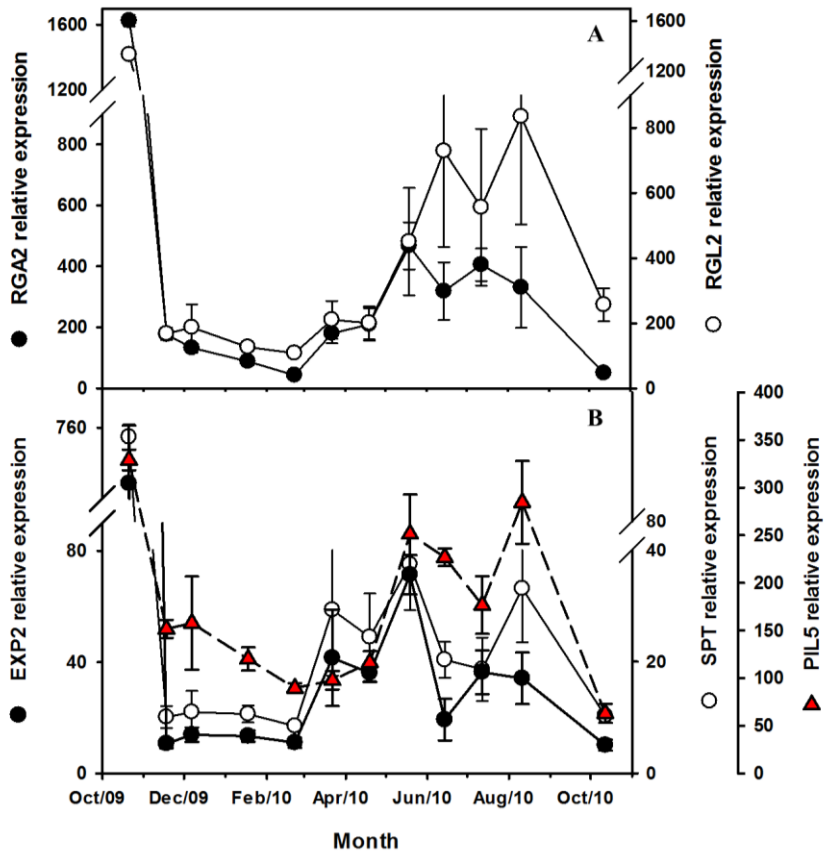

Figure 4.9 Expression of genes involved in the GA signalling pathway. A: expression of *RGA2* and *RGL2* (*DELLA*s -germination repressors); **B**: expression *SPT* and *PIL5* (*bHLH* transcription factors of the PIF family – germination repressors), and *EXP2* (an early GA-induced transcript during seed germination). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

4.3.3.2 Expression of ABA metabolism and signalling genes in Bur during dormancy cycling

Germination potential changed following burial, and decreased to a minimum in April and then had a steep rise to June and stayed at a high level until the end of the year (Fig.4.6). Nevertheless, the expression of the key ABA biosynthesis gene *NCED6* stayed at a relatively constant level throughout these changes (Fig.4.10). In

contrast, the expression of *CYP707A2* (ABA catabolism) showed a dramatic decline in expression following burial, remained low through the winter and then increasing from April to June as germination potential increased (Fig.4.10). Additionally the expression of *CYP707A2* had a similar pattern as the soil temperature though it was not significantly correlated (Table 4.2). The stable level of *NCED6* expression and the changes in *CYP707A2* expression are consistent with the observations in Cvi seeds (Footitt *et al*., 2011) where the depth of dormancy was only related to the endogenous ABA content at low levels of dormancy and not when seeds enter deep dormancy in the field.

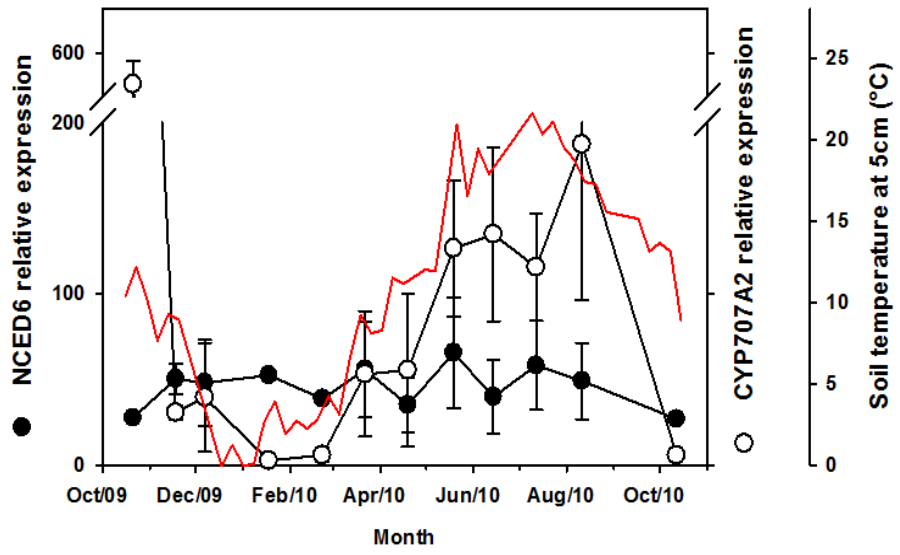

Figure 4.10 The relationship between soil temperature (red line) and gene expression involved in the ABA metabolism: *NCED6* (ABA biosynthesis) and *CYP707A2* (ABA catabolism). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

The expression of *PYR1* (ABA receptor), the SNF1-related protein kinase *Snrk2.1*, and *ABI4* (ABA-induced transcription factor) all showed a rapid decrease to relatively low levels on burial and remained stable over the winter, then gradually increased as dormancy level declined during April to July (Fig.4.11). The expression

of these three genes was significantly ($p<0.001$) and positively correlated with each other (Table 4.2).

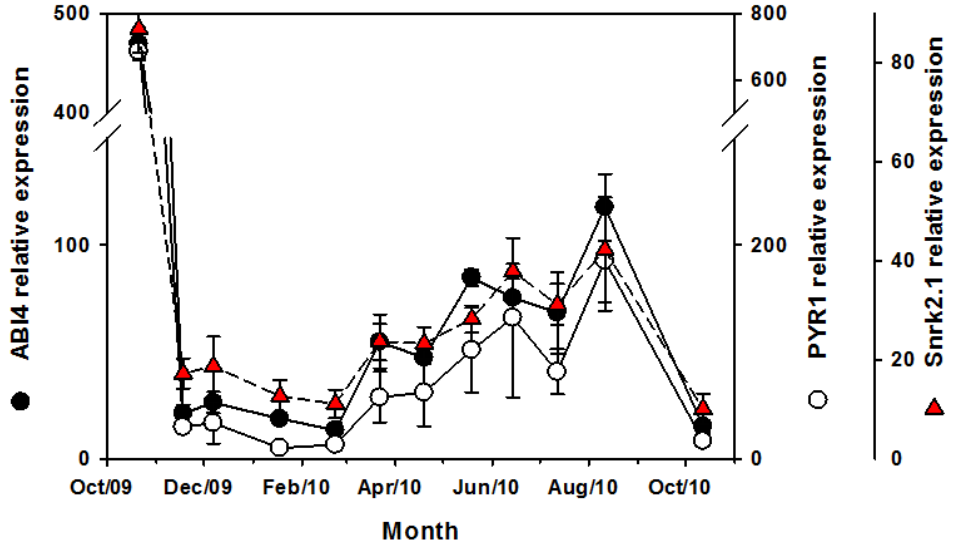

Figure 4.11 Expression of genes involved in the ABA signalling pathway: *SnrK2.1* a SNF1-related protein kinase subfamily member (positive regulator of ABA signalling), *PYR1* (ABA receptor) and *ABI4* (control of energy utilisation). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

Expression of the ABA signalling repressor, *ABI2*, increased as dormancy increased and increased again as dormancy decreased in late spring. After the first month of burial this expression was significantly and positively correlated ($p<0.01$; Table 4.2) to the expression of the ABA catabolism gene *CYP707A2* (Fig.4.10 and 4.12A). The expression of *Snrk2.4* (positive regulator of ABA signalling) and the ABA receptor, *PYL7*, was negatively related to the expression of *ABI2* before the decrease in dormancy level in April, but positively related to the *ABI2* expression afterwards (Fig.4.12). The increase in *ABI2* with the decreasing depth of dormancy may need to be counterbalanced by a promotion of ABA signalling in the dark of the soil seed bank to prevent sensitivity to spatial signals (light). The positive regulator of ABA sensitivity *ATHB20* was positively related to the expression of *ABI2*, but

followed a similar pattern to *EXP2* afterwards (Fig.4.12A and Fig.4.9B). In addition, both *ABI3* and *ABI5* (negative regulators of germination) exhibited a similar expression pattern as ABA receptor (*PYL7*) and *SnRK2.4* (Fig.4.12B).

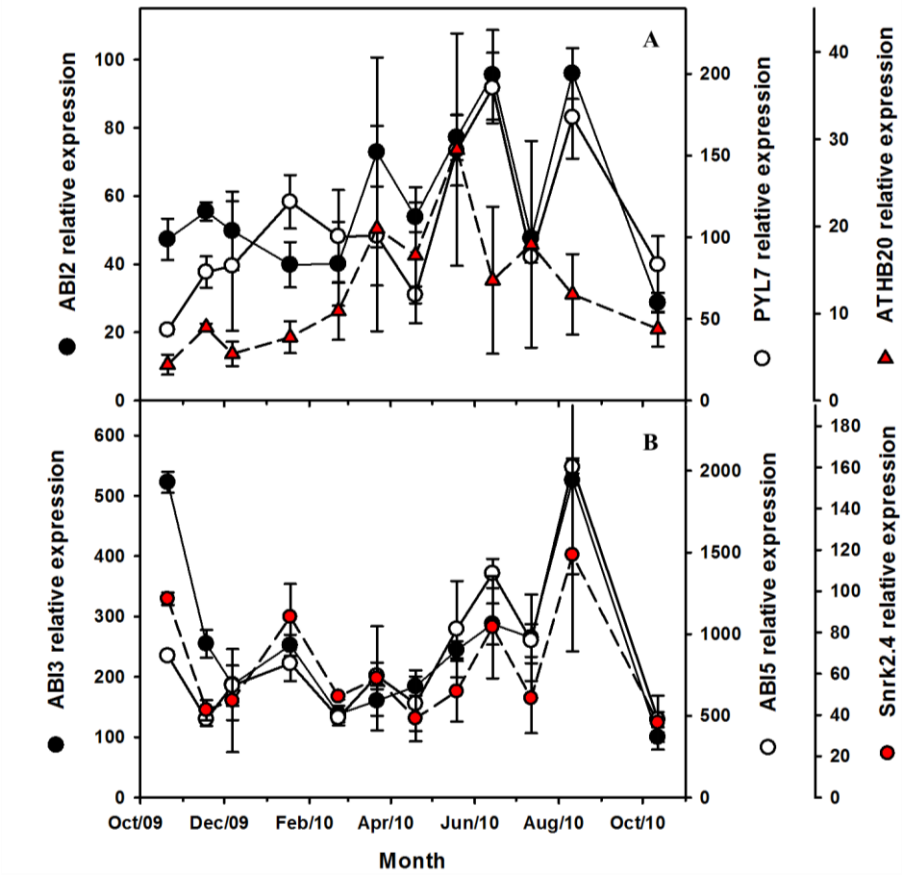

Figure 4.12 Expression of genes involved in the ABA signalling pathway. A: expression of *ABI2* (repressor of ABA signalling), *PYL7* (ABA receptor) and *ATHB20* (positive regulator of ABA signalling); **B**: expression of *ABI3* (dormancy) and *ABI5* (ABRE regulated transcription factor) and *SnrK2.4* (positive regulator of ABA signalling). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

4.3.3.3 Expression of other dormancy-related genes (*DOG1* and *MFT*)

The expression level of the dormancy associated gene *DOG1* was high as germination potential decreased in the months after burial (Fig.4.13). Expression peaked as dormancy level increased most rapidly. The expression level of *DOG1* then remained low when seeds became less dormant until the end of the year. The expression was significantly ($p<0.01$) and negatively related to soil temperature and

also negatively related to most genes, although this relationship was only statistically significant ($p<0.05$) with *MFT* (Fig.4.13), *RGA2*, *NR1*, and *NRT1.1* (refer to Table 4.2).

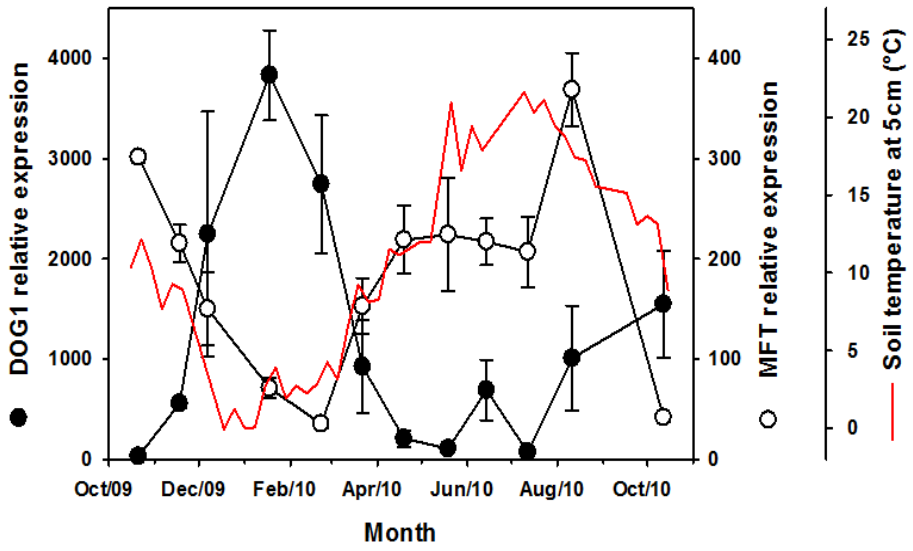

Figure 4.13 The relationship of soil temperature (red line) and expression levels of *DOG1* (dormancy related gene) and *MFT* (ABA-induced germination repressor). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

In addition, *NRT1.1* (nitrate transporter) and *NRI* (nitrate reductase) had similar distinct expression profiles across the seasons, and they were significantly ($p<001$) and positively correlated with each other (Fig.4.14 and Table 4.2). On burial the expression of *NRT1.1* falls to a low level, but the increase in *NRT1.1* expression afterwards is concurrent with increasing *MFT* expression and decreasing *DOG1* expression. Crucially, increased *NRT1.1* expression was followed by increased *CYP707A2* expression and the relevance of this will be discussed below.

4.3.3.4 Comparison of seasonal gene expression patterns in Bur and Cvi

In Bur, there were significant positive linear correlations ($p<0.05$) between the majority of gene expression patterns, especially the annual expression of *CYP707A2* (ABA catabolism) and *GA2ox2* (GA catabolism) were significantly ($p<0.05$) correlated with many genes (Fig.4.7 and Fig.4.10; Table 4.2). This expression was

also positively correlated with soil temperature and negatively correlated with soil moisture content (soil tends to be dry when temperature is high). However in contrast, the expression of *NCED6* (ABA biosynthesis) and *GA3ox1* (GA biosynthesis) appeared to be related with none of the other genes (Fig.4.7 and Fig.4.10).

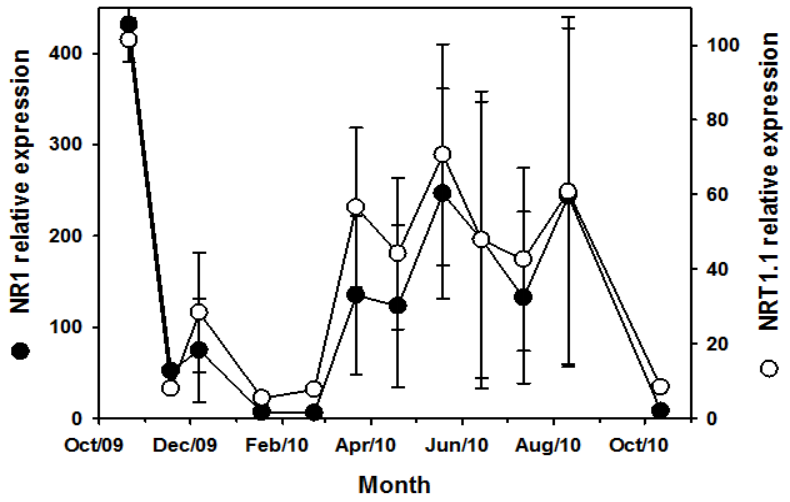

Figure 4.14 Expression of *NR1* (nitrate reductase) and *NRT1.1* (nitrate transporter/sensor). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

DOG1, which is the gene at the *loci* with the strongest dormancy association in QTL analysis (Bentsink *et al*., 2006), had an exceptional expression to most of other genes. The expression of *DOG1* was negatively correlated with soil temperature and thus negatively related to the expression of other genes (Fig.4.13). In a previous study by Footitt *et al*. (2011), seeds of Cvi were buried and exposed to a similar pattern of seasonal temperature as Bur, but the patterns of changing depth of dormancy differed significantly. The expression of ABA synthesis (*NCED6*) and GA catabolism (*GA2ox2*) genes increased with the decreasing soil temperature and increasing dormancy level. Dormancy then declined in spring and summer along with positive ABA signalling as the expression of *ABI2* and *ABI4* increased.

Additionally ABA catabolism (*CYP707A2*) and GA synthesis (*GA3ox1*) genes also showed an increasing expression. However during the summer time when dormancy level was low, the expression of germination repressors *RGA2* and *RGL2* increased. Gene expression patterns in the two ecotypes were therefore compared and this showed that the expression of *DOG1* is significantly negatively correlated with soil temperature in both Bur and Cvi ecotypes (Fig.4.15; Cvi data from Footitt *et al.*, 2011; $p<0.01$ and $p<0.001$ respectively).

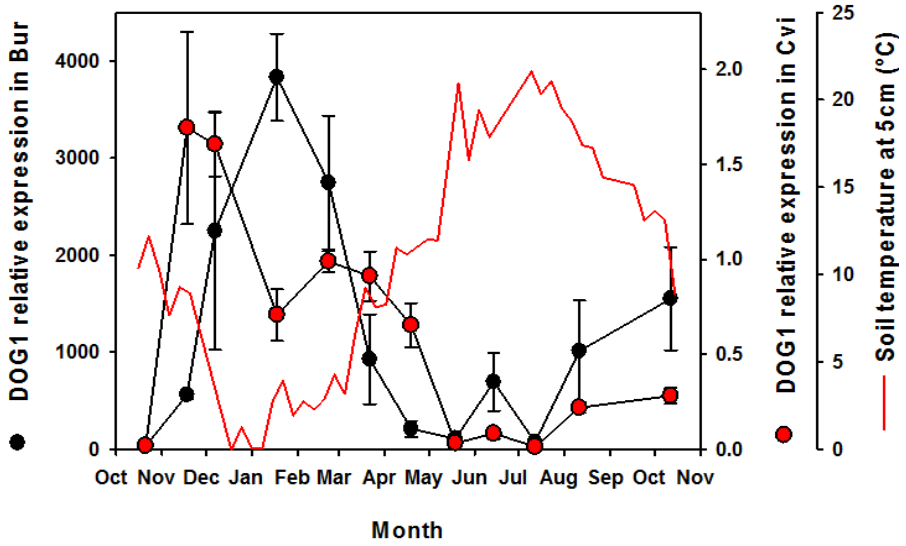

Figure 4.15 The impact of soil temperature (red line) on the expression of *DOG1* in Bur and Cvi. Data represents the mean ± standard error. No error bar indicates symbol is larger than the error.

However the relative expression of several genes showed very different patterns between the two ecotypes, including *MFT* and *Snrk2.1*. The expression of *Snrk2.1* and *MFT* was positively correlated with soil temperature in *Bur*, but negatively correlated in *Cvi* (Fig.4.16 and Table 4.2; *Cvi* data from Footitt *et al.*, 2011). This contrasting relationship with temperature may result from natural variation driven by adaptation of these two ecotypes to widely different habitats.

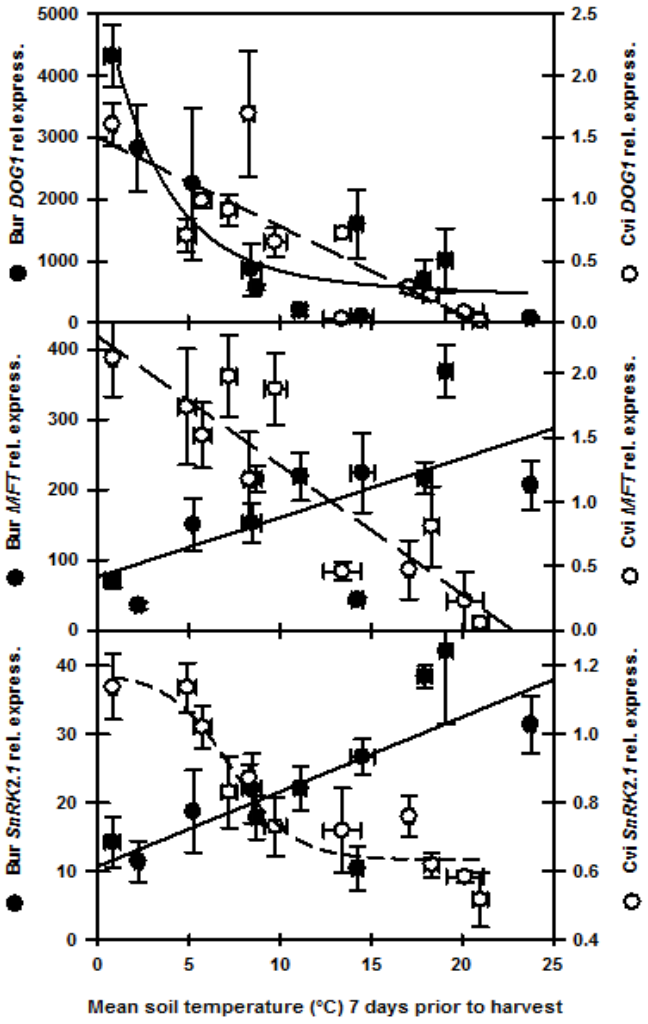

Figure 4.16 The impact of soil temperature on the expression of *DOG1* and *MFT* in Bur and Cvi. (a) The relationship between soil temperature and Bur and *Cvi DOG1* expression fits an exponential decay regression. Bur: (f = 4824.9840*exp(-0.313*x)+775.2525*exp(-0.0207*x) (R^2 = 0.8533). Cvi: (f =0.8695*exp(-0.1023*x)+0.9612*exp(-0.1023*x) (R^2 = 0.6681). (b) The relationship between soil temperature and *MFT* expression fits a linear regression for Bur (f=76.19+8.46(x)) (R = 0.63) and for Cvi (f= 2.28+-0.10(x)) (R = 0.91)). (c) The relationship between soil temperature and *SnrK 2.1* expression fits a linear regression for Bur; (f = 10.697+1.089(x)) (R = 0.75) and for Cvi fits a sigmoidal regression; (f =0.6331+0.5448/(1+exp(-(x-7.2894)/ -1.7427))) (R^2 = 0.8889). Cvi data redrawn from Footitt *et al*. (2011).

4.4 Discussion

The phenology of dormancy and emergence differs in Bur and Cvi to generate summer and winter annual phenotypes

The transition from seed to seedling is very important as the timing of germination determines the environment which is experienced by the subsequent plant and represents one of the most relevant traits to understand adaptive variation in *Arabidopsis* (Donohue, 2009; Picó, 2012). In the laboratory, seeds of Bur and Cvi produced under the same maternal conditions exhibited germination responses to temperature that were characteristic of summer and winter annuals respectively (discussed in *Chapter 3*). The emergence data collected from the field showed that seedlings from Bur seeds emerged in late spring, whereas Cvi seedlings emerged in late summer, which confirmed that Bur behaves as a summer annual while Cvi behaviour is consistent with a winter annual phenotype.

Bur seeds were buried in the field in early October 2009 to mimic the natural time of seed dispersal. Both Bur and Cvi remained light requiring, and thus seeds were dormant throughout the annual cycle and the changes in germination potential and gene expression represent the seasonal variation in dormancy level in the soil seed bank throughout the year. In the laboratory some Bur seeds germinated in the dark, whereas no germination was recorded in the buried seeds. This may be because seeds in the soil are exposed to negative water potentials for most of the time and this mild water stress enhances dormancy (Finch-Savage and Footitt, 2012).The seasonal variation in dormancy status of Bur and Cvi was compared in terms of germination behaviours (Fig.4.17): at burial, Bur seeds showed minimal level of dormancy and after one month the germination at a range of temperature was fully completed in the presence of light, whereas dormancy of Cvi seeds started to increase directly after burial. During the winter period until April, dormancy level of both Bur and Cvi seeds increased and then rapidly decreased during May. The dormancy level of both ecotypes then continued to decline, but more slowly. These results suggest that the

temporal sensing, which is related to slow seasonal change (e.g. soil temperature), is similar in both Bur and Cvi.

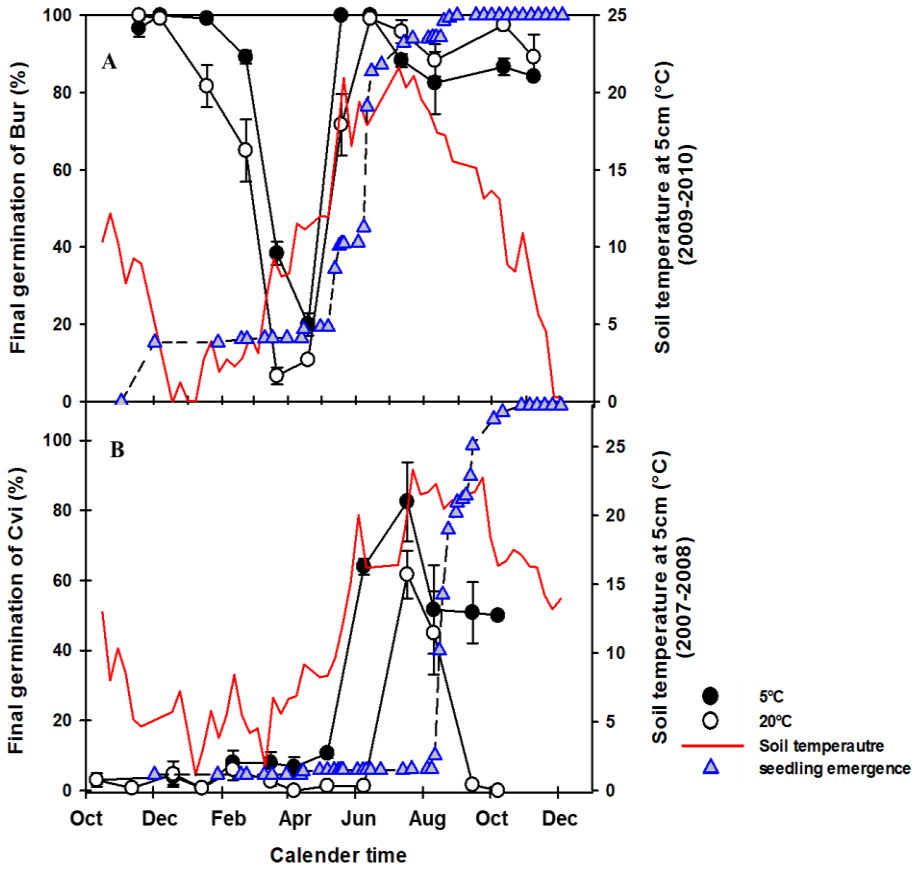

Figure 4.17 Seasonal variations of seed germination and seedling emergence response to soil temperature. A: Changing thermal dormancy and seedling emergence of *Bur* in relation to the annual soil temperature cycle (2009-2010); **B**: Changing thermal dormancy and seedling emergence of *Cvi* in relation to the annual soil temperature cycle (2007-2008). Data are the mean ± standard error. No error bar indicates symbol is larger than the error (*Cvi* data from Footitt *et al*., 2011).

In the presence of light, the increase in germination of Bur seeds at 20˚C was only slightly delayed compared to that at 5˚C (Fig.4.17A). This rapid opening of the temperature window facilitated early germination (following soil disturbance and light exposure) as temperatures rose in spring. In contrast, the change in thermal dormancy of Cvi seeds was found to be slower. Although Cvi seeds had an increase in germination at 5˚C similar to Bur (April), the germination at 20˚C showed a delayed increase until July. Therefore this slower opening of the temperature window

for germination overlapped with the soil temperature in late summer, resulting in later germination and seedling emergence of Cvi compared to Bur (following soil disturbance and light exposure) (Fig.4.17B).

Expression of hormone metabolism and signalling genes during dormancy cycling in buried seeds is consistent with their functions in dormancy regulation proposed in laboratory experiments

The annual dormancy pattern of Cvi in the field has been explained by Footitt *et al*. (2011) using the expression patterns of genes involved in hormone metabolism and signalling over the year. As described above dormancy is regulated via a balance of the hormones ABA and GA and this balance is created by both synthesis and catabolism of the hormones that is regulated by key genes. ABA enhances dormancy and GA is linked to loss of dormancy and the completion of germination.

In Cvi seeds, dormancy increased over the winter with the decreasing soil temperature coincident with increased expression of ABA biosynthesis gene *NCED6* and GA catabolism gene *GA2ox2* (Footitt *et al*., 2011), The expression of SNF1-related protein kinases, *Snrk2.1* and *2.4* also showed an increase consistent with enhanced ABA signalling and sensitivity. When dormancy began to decrease with a decrease in endogenous ABA, there was an increase in the expression of positive ABA signalling genes *ABI2*, *ABI4*, and in the ABA catabolism gene *CYP707A2* and the GA biosynthesis gene *GA3ox1*. However during low dormancy in the summer, the GA3ox repressors *SPT* and *PIL5* are highly expressed as are the germination repressors *RGA2* and *RGL2* (Footitt *et al*., 2011). Therefore slow seasonal changes in dormancy of Cvi seeds (temporal sensing) appeared to be mainly determined by enhanced sensitivity to ABA during deep dormancy, coupled to *DELLA* repression during shallow dormancy (spatial sensing).

The seasonal pattern in Bur was dominated by long periods of low dormancy. The level of dormancy decreased with the increasing expression of GA biosynthesis gene *GA3ox1* (GA catabolism gene *GA2ox2* did not change), which is consistent with an increase in GA and subsequent seedling emergence in May and June. As dormancy declined, the expression of *GA3ox1* decreased and *GA2ox2* increased, as did *GID1A* (GA receptor) and two DELLA genes (*RGA2* and *RGL2*). This expression pattern is consistent with seeds remaining dormant in the dark of the soil determined by high levels of DELLAs to repress germination as dormancy decreased as sensitivity to ABA declined. However, this repression can be removed by exposure to light (soil disturbance or germination test in presence of light) leading to an increase in GA, which can bind with GID1 and DELLAs to remove germination repression. Therefore seeds in shallow dormancy could respond very rapidly to favourable germination conditions (spatial sensing) (Footitt *et al*., 2011).

The expression of *NCED6* changes little over the annual cycle in Bur seeds, but *CYP707A2* showed a pattern that was positively related to soil temperature. This is consistent with stable levels of endogenous ABA as dormancy increased but decreasing levels as dormancy declined, which confirmed the observation in Cvi that the increasing depth of dormancy during cycling was not directly related to the endogenous ABA content in seeds (Footitt *et al*., 2011).

DOG1 expression and dormancy have seasonal patterns determined by temperature (temporal sensing)

The germination timing of *Arabidopsis* has been shown to be under geographically variable natural selection, and is plastic in response to seasonal environmental factors (Donohue *et al*., 2005a, b). Natural variation in the genetic basis of seed dormancy has a strong impact on seasonal germination timing (Chiang

et al., 2011). Natural variation in the dormancy-related gene *DOG1* also plays the largest role in determining dormancy differences between two *Arabidopsis* ecotypes: Cvi and Ler (Alonso-Blanco *et al*., 2003). In this study, the annual pattern of *DOG1* expression was investigated. In Cvi seeds, the expression of *DOG1* increased after burial and continued to increase even after ABA content reached a plateau, which suggested that in these seeds with deep dormancy, *DOG1* expression may be the dominant factor influencing ABA signalling and thus sensitivity rather than the absolute ABA level (Footitt *et al*., 2011). In Bur seeds, the ABA synthesis gene *NCDE6* showed a relatively stable level of expression over the year, whereas the expression of *DOG1* showed a pattern which was negatively related with both soil temperature and the ABA catabolism gene *CYP707A2*. This result agrees with the previous work that increasing dormancy was not directly related to the endogenous ABA content (ABA synthesis), but dormancy decreased with the declining levels of ABA (ABA catabolism). Therefore DOG1 does not regulate seed dormancy via changes in hormone levels and the environment influences ABA and DOG1 independently (Footitt *et al*., 2011; Chiang *et al*., 2011; Nakabayashi *et al*., 2012).

In this study, *DOG1* was the only gene investigated in both Bur and Cvi that exhibited an expression significantly and negatively correlated to the soil temperature throughout the annual cycle. Therefore DOG1 may act as part of a thermal-sensing mechanism to influence dormancy level and drive the slow seasonal response (temporal sensing) by altering ABA sensitivity (Footitt *et al*., 2011).

According to Bentsink *et al* (2006) *DOG1* exhibited the highest level of expression during the mid to late stage of silique development and seed maturation, thus the level of *DOG1* expression was an indicator of the extent of dormancy imposed by the seed maturation environment (Chiang *et al*., 2011). A recent study by

Nakabayashi *et al*. (2012) found that DOG1 protein accumulated during seed maturation and remained stable throughout seed storage and imbibition. The levels of DOG1 protein at maturity correlated with depth of dormancy and further study during after-ripening suggested that DOG1 might act as a timer for seed dormancy release. In this study, both Bur and Cvi dormancy increases as *DOG1* expression increases and declines after *DOG1* expression declines, indicating a seasonal pattern of *DOG1* expression determined by soil temperature (temporal sensing). Before burial the dormancy level of Cvi seeds is higher than that of Bur seeds, which may be the results of greater accumulation of DOG1 protein during seed maturation. This leads to the suggestion that the depth of dormancy and consequently the dormancy cycling behaviour can be altered by changing DOG1 protein levels during seed maturation (Chiang *et al*., 2011). Thus it could be possible for the summer and winter annuals to adjust their germination timing and resultant life cycles dependant on the environment during maturation.

Different expression of some genes in buried Bur and Cvi may account for the natural variation in dormancy cycling and germination behaviour between summer and winter annuals

***MFT*:** *MFT* expression is directly regulated by ABI3 and ABI5 and up-regulated by DELLA proteins in *Arabidopsis* (Xi *et al*., 2010), and in wheat it was thought to promote or maintain dormancy (Nakamura *et al*., 2011). Additionally the repression of germination by MFT could be overcome by the application of exogenous GA, indicating the function of MFT via repressing GA synthesis (Nakamura *et al*., 2011). In the field, high expression of *MFT* was positively correlated with increased dormancy level in Cvi seeds buried in the soil (Footitt *et al*., 2011). In Bur, *MFT* expression also increased with the increasing dormancy

during March to April, and then stayed at a high level when dormancy rapidly decreased after April. This expression is consistent with the function of MFT in seed germination, i.e. negative regulator of germination. The reversed expression patterns of *MFT* in Bur and Cvi (Fig.4.16) may partly account for the germination behaviour of these contrasting ecotypes, i.e. the decreasing expression of *MFT* during later spring in Bur and during late summer in Cvi were in line with the increasing germination potential of Bur and Cvi.

***NRT1.1*:** On burial, the expression of *NRT1.1* falls to low levels in both ecotypes, but unlike the single peak in Cvi, *NRT1.1* expression in Bur followed a different pattern rapidly increasing to five fold higher at maximum dormancy and continuing to rise to eight fold higher as dormancy is relieved to the point where only light is required to complete dormancy loss and progress to germination completion. Thus Bur seeds remain sensitive to nitrate throughout the annual dormancy cycle. This is perhaps not surprising as the ecotype comes from a high rainfall area of limestone pavement characteristic of the Burren in Ireland, in which the highly soluble nitrate may easily be lost through leaching due to the high precipitation. It may also be explained by the high nitrogen use efficiency (NUE) of Bur identified during a comparison of 18 *Arabidopsis* accessions under N-limiting conditions (Chardon *et al*., 2010).

Although the expression patterns in Bur and Cvi were different, an increased *NRT1.1* expression was followed by increased *CYP707A2* expression in both ecotypes, which agrees with the observation that nitrate induced *CYP707A2* expression results in reduced ABA levels when dormancy was broken by nitrate treatment (Ali-Rachedi *et al*., 2004; Matakiadis *et al*., 2009).

***FLC*:** *FLC* has been linked to the regulation of temperature dependent germination in *Arabidopsis* and high expression of *FLC* was found to be significantly associated with increased germination when seeds were imbibed at low temperature (Chiang *et al*., 2009). A high level of *FLC* expression during seed maturation enables seeds to overcome primary dormancy through the pathway of ABA degradation and GA synthesis in imbibed seeds (Chiang *et al*., 2009). In the field, *FLC* expression over the year showed an increase with decreasing soil temperature and increasing dormancy level in Cvi, and then declined with increasing exposure to low temperature. This expression pattern of *FLC* was positively correlated with *DOG1* expression in Cvi seeds buried in the field (Footitt *et al*., 2011).

However in Bur seeds, *FLC* expression decreased over the winter time when the seeds were exposed to low temperature and had increasing level of dormancy. *FLC* expression in Bur was negatively correlated with *DOG1* expression, and positively correlated with the expression of the GA receptor gene *GID1A* (positive germination regulator). The different expression pattern of *FLC* in Bur compared to Cvi may be mainly due to the null mutant at *FLC* allele in Bur (Werner *et al*., 2005), showing little physiological impact on the regulation of seed germination. In addition, the natural variation in *FLC* expression is associated with natural variation in flowering time, which may also contribute to the difference in germination behaviours between the winter and summer annuals of *Arabidopsis* (Chiang *et al*., 2009).

4.5 Conclusion

The natural variation between contrasting *Arabidopsis* ecotypes (Bur and Cvi) has been characterized to investigate the coordination of dormancy regulating mechanisms in the field. It is suggested that there is a seed-specific seasonal

(temporal sensing) sensing of temperature via DOG1. In the contrasting ecotypes shown, germination completion and seedling emergence occurred in response to ambient environmental conditions (e.g. soil temperature and soil moisture). Gene expression in response to environmental signals showed different patterns in the two ecotypes, providing insight into how adaptation to local conditions can generate winter and summer annual phenotype behaviours. However, the initially different dormancy levels set in response to maturation environment are also likely to have an impact on the patterns of the dormancy cycling.

The data in this chapter and *Chapter 3* form a significant component of a publication in The Plant Journal in press

CHAPTER 5: THE EFFECTS OF MATERNAL ENVIRONMENT ON FLOWER DEVELOPMENT, SEED PRODUCTION AND GERMINATION

5.1 Introduction

Arabidopsis ecotypes exhibit adaptive differentiation in response to the environment (Rutter and Fenster, 2007). Such developmental responses to seasonal variation in the environment are considered to be important in understanding the adaption of life histories of plants in nature (Donohue, 2009). For example, seed phenotypes are greatly influenced by the environment in which they develop on the mother plant (Andalo *et al*., 1999; Donohue, 2009). These 'maternal effects' were defined by Roach and Wulff (1987) as 'the contribution of the maternal parent to the phenotype of its offspring beyond the equal chromosomal contribution expected from each parent'. The maternal effects are also regarded as a particular kind of phenotypic plasticity and divided into different classes by Donohue (2005) as shown in Table 5.1. Plant fitness is greatly influenced by the maternal environment, which affects resource allocation and reproductive output (Riginos *et al*., 2007; Zhang *et al*., 2011). The effects of the maternal environment were studied here using the Bur and Cvi ecotypes of *Arabidopsis* that have adapted to very different 'home' environments. Plants of each ecotype were grown in controlled environments that approximate to both their 'home' environments to observe the effects on reproductive development and consequent seed behaviour.

5.1.1 The effect of maternal environment on flower development

The effects of maternal environment on flower development have been reported in a number of species. In the woodland herb *Campanula Americana*, plants grown in high light produced more pollen grains per flower than those in low light; however the size of pollen was neither influenced by the light treatment, nor by the

interaction between genotype and environment (Etterson and Galloway, 2002). Diggle *et al*. (2010) showed that water stress in the maternal environment led to low reproductive success in wild radish (*Raphanus sativus*), impacting on key events during and after pollination (Diggle *et al*., 2010). In *Arabidopsis,* pollen and ovule development were regulated in response to environmental variations due to the resource requirements for reproduction (Sun *et al*., 2004). Salt stress decreased the growth rate of anther filaments, delaying pollination by 12-24 hours, with developing ovules and young embryos more likely to be affected than seeds nearing the end of development (Sun *et al*., 2004).

Table 5.1 The broad definition of maternal effects*

	Maternal effects	**Related reference**
1	Cytoplasmic genetic maternal effects, i.e. the genetic effects caused by maternal inheritance of plastids	Tilney-Bassett (1975); Platenkamp and Shaw (1993)
2	The effects of endosperm, which is triploid with 2/3 of its genotype of maternal origin	Harvey and Oaks (1974)
3	The effect of the seed coat, which is maternal tissue	Roach and Wulff (1987)
4	The effects of maternal provisioning during seed development (nutrient resources, hormones, proteins and transcripts, all capable of being provisioned to seeds by the maternal parent)	Karssen *et al*.(1983); Diggle *et al*. (2010); Finch-Savage and G. Leubner-Metzger (2006)
5	The maternal determination of the progeny environment via dispersal or phenology	Biere (1991); Platenkamp and Shaw (1993)

*Adapted from Donohue, 2005

The stages of flower development of *Arabidopsis* were characterized in detail by Müller in 1961, and re-summarized by Smyth *et al*. (1990). The stages from the time of opening of buds to seed maturation are shown in Table 5.2.

Table 5.2 The landmark events that define each stage of flower development in *Arabidopsis*

Stage*	Landmark events at beginning of stage	Duration (hr)	Age of flower at end of stage (days)
13	Bud opens, petals visible, anthesis	6	0.5
14	Long anthers extend above stigma	18	1
15	Stigma extends above long anthers	24	2
16	Petals and sepals withering	12	2.5
17	All organs fall from green siliques	192	10.5
18	Siliques turn yellow	36	12
19	Valves separate from dry siliques	up to 24	13
20	Seeds fall		

*Landmark events that define the beginning of stage 13 to 20 are summarized from Müller (1961) where they were originally named B3 to B10 (Smyth *et al*., 1990).

5.1.2 The effect of maternal environment on seed production and germination

Seed traits are determined by the genotype and parental environment (Luzuruaga *et al*., 2005). A number of studies have investigated maternal environmental effects on seed development and germination. For example, different temperature and day length during seed maturation can affect seed size in some species such as *Plantago lanceolata* (Alexander and Wulff, 1985), *Desmodium paniculatum* (Wulff, 1986) and *Chenopodium rubrum* (Cook, 1975).

The photoperiod during seed maturation can regulate dormancy levels, whereby short days induce high dormancy and long days induce low levels of dormancy (Munir *et al*., 2001). In *Arabidopsis*, seeds from plants grown in a simulated shade canopy (low red: far red light) were reported to be light-dependant for germination, whereas seeds from plants grown under fluorescent lights can germinate in the dark (Hayes and Klein, 1974). The environment during seed development also influenced progeny germination time, flowering and the weight of seed the progeny produced (Elwell *et al*., 2011). In *Actinobole uliginosum*, seeds produced in a warm environment were more responsive to dry after-ripening than

those from a cool environment (Hoyle *et al*., 2008). The germination percentage of *Amaranthus powellii* was reported to be 40-50% lower for those maturing on plants grown in competition with other plants than without competition (Brainard *et al*., 2005). Interestingly, the position at which seeds matured on the mother plants or the position of seeds in the fruit or maternal branch type can also have an impact on the germinability of seeds depending on the species (Gray and Thomas, 1982; reviewed in Baskin and Baskin, 1998). For example, seeds of *Apium graveolens* and *Daucus carota* from primary umbels germinated to higher percentages than those from lower umbels (Thomas *et al*., 1978, 1979; Jacobsohn and Globerson, 1980); also the seeds of *Mesembryanthemum nodiflorum* formed in the outer portion of the capsules are the first to be dispersed and less dormant than those produced in the interior of the capsule (Gutterman, 1980-1981).

In *Arabidopsis*, low temperature and short-day photoperiod during seed maturation were also reported to increase seed dormancy of two ecotypes (Ler and Col) (Munir *et al*., 2001; Donohue *et al*., 2008). Furthermore, a transcriptomic comparison of dry *Arabidopsis* seeds (Col background) produced in warm and cool seed maturation temperatures was used to identify low-temperature-regulated gene sets (Kendall *et al*., 2011). This revealed that the seed maturation transcriptome was highly temperature sensitive, and that low temperature during seed maturation induced several genes associated with dormancy (e.g. *DOG1*) and affected GA and ABA levels in mature seeds. This study also found that C-REPEAT BINDING FACTORS (CBFs) acted in parallel to a low-temperature signalling pathway in the regulation of seed dormancy. More recently, the response of different ecotypes of *Arabidopsis* to extended chilling were analysed by Penfield and Springthorpe (2012), the results showed that secondary dormancy could be induced by two weeks of

chilling, but it did not occur if seeds were matured at 20˚C. It was further demonstrated that seed dormancy is determined by both genetics and maternal environment; in turn, the maternal environment is also determined by seed dormancy and germination, because germination time determines the time of flowering and seed set. According to Sultan (2004), the application of contrasting environmental conditions rather than a single 'control' environment facilitates the study of ecotype plasticity. Thus in the work reported below plants of *Arabidopsis* ecotypes Bur and Cvi were grown in reciprocal controlled environments that simulated their native environments during reproductive growth to determine the effect of maternal environment on flowering time, seed yield, seed size and germination behaviour.

5.2 Materials and Methods

5.2.1 Plant material and controlled growing environments

Arabidopsis ecotypes Bur and Cvi were grown in two different environmental regimes that represent characteristics of the Burren region of Ireland and the Cape Verde Islands during flower and seed development (Table 5.3). Seeds were sown on compost in P24 cellular trays and later thinned to one plant in each of the 24 cells as described in *Chapter 3* (*sec 3.2.1*). Eight trays of each ecotype were grown in a common environment (23/17˚C, 12/12h, light/dark) until they bolted (bolting was defined as the primary inflorescence stalk reaching 1cm long) and then four trays of each ecotype were placed in each of the two growing regimes (Table 5.3). Rosette diameter and leaf number were also measured at bolting. Watering was stopped when the majority of siliques had turned yellow on the plant. Seeds produced in both regimes were harvested when siliques were fully matured (seeds were considered to be matured when all the siliques had turned yellow and dry on the plant). After harvest seeds were dried to an equilibrium relative humidity of 55% above a

saturated calcium nitrate solution for six days, and then sealed in air tight tubes and stored at -80°C.

Table 5.3 Temperature and light regimes native to the Burren region and Cape Verde Island during flower and seed development

Burren environment		**Cape Verdi Island environment**	
Temperature	Day length	Temperature	Day length
17/10°C (L/D)	14h*	27/22°C (L/D)	12h*

*Day length was determined by 'sun calculator' in the location of Ireland and Cape Verde (source: http://www.timeanddate.com/worldclock/sunrise.html).

5.2.2 Seed yield measurement

Seed was equilibrated at 55% relative humidity/20˚C for six days then seed yield (total seed weight) and seed size (1000-seed weight) were determined.

5.2.3 Dormancy and germination assays

Seeds were surface sterilized with 2.5% bleach (Domestos) for five minutes, washed three times with distilled water, and then incubated on two layers of 3M chromatography paper in clear plastic boxes (8×12cm, Stewart Plastics) containing 8ml of distilled water. Seeds were then incubated at 10˚C and 25˚C in the light for up to 28 days. For each temperature there were three replicates of 40 seeds from a bulk collection of each ecotype produced from the two controlled environments. Germination was recorded at regular intervals. A seed was considered to have germinated when the radical had emerged through the testa and endosperm.

5.2.4 Flower and silique development

A second round of Bur plants were grown using P24 cellular trays (one plant per cell) in the common regime and then transferred at bolting to the respective growth regimes as listed in Table 5.4 (one tray in each regime). Flowers on the primary inflorescence were measured every day after the onset of flowering. Each

day flowering buds were marked by applying different coloured acrylic paint to the pedicel, and flowers collected at each developmental stage (stage 13-15, Smyth, *et al*., 1990) were dissected. Flowering was defined as first appearance of petals from within the enclosing sepals. Plants were separated by perforated plastic bread bags to prevent cross pollination. Sepals and petals were removed to expose the anthers and pistils. The lengths of pistil and stamen were measured to determine the pistil: stamen) ratio (Footitt *et al*., 2007). Siliques were dissected to determine the final seed set; the developed ovules present within each silique were counted to determine the percentage fertilization. In the four growth regimes, each individual flower or silique harvested from the plants in each tray was condisered as a replicate.

Table 5.4 Growth regimes and treatments applied to investigate flower and silique development of the Bur ecotype

	Growth regime	Treatment
A	Bur environment (17/10°C, 14/10h, Light/Dark)	Control
B	Cvi environment (25/20°C, 12/12h, Light/Dark)	Control
C	Cvi environment (25/20°C, 12/12h, Light/Dark)	Hand pollination
D	Cvi environment (25/20°C, 12/12h, Light/Dark)	GA spray

5.2.5 Data analysis

All percentage germination data were angular transformed for analysis. The transformation is arcsin(sqrt(p/100))*180/pi, where p is a percentage from 0 to 100, and the result is an angle between 0 and 90 degree. Data are presented as the mean± standard error. The standard error was calculated by dividing the standard deviation by the square root of the number of replicates (n) (sd/√n). Analysis of variance (ANOVA) was used to detect the differences between variates as listed in Table 5.5.

Statistical analysis was carried out using the software package GenStat (VSN International, 2012). A two-sample unpaired t-test was used to show the significance of the results of cumulative flowering and silique length after flowering on each day, using the cut-off of $p<0.05$ to determine statistical significance.

Table 5.5 Summary of ANOVA used in Chapter 5

Analysis of variance	**Factors**	**Variate**	**Transformation**
One-way ANOVA (*sec 5.2.1*)	Ecotype	1) Bolting time 2) Leaf number	None
Two-way ANOVA (*sec 5.2.2*)	Controlled environment, seed type	1) Seed yield 2) Seed size	None
Two-way ANOVA (*sec 5.2.3*)	Germination temperature, seed type	Final germination%	Angular
Two-way ANOVA (*sec 5.2.4*)	Seed type, floral developmental stage	1) Pistil length 2) Filament length 3) Ratio of stamen/pistil	
One-way ANOVA (*sec 5.2.4*)	Growth conditions	1) Total number of ovule per silique 2) Deveploped ovules %	

5.3 Results

5.3.1 Vegetative phenotypes of Bur and Cvi

Plants were grown in the same common environmental regime (23/18˚C, 12/12h, light /dark) until bolting. There were clear physiological and morphological differences between the two ecotypes throughout growth and development. During the vegetative stage, the rosette leaves of Cvi plants were obovate with an entire margin, whereas the Bur leaves had a slightly serrated margin (Fig.5.1A). The rosette leaves of Bur were less rigid than Cvi at this stage (Fig.5.1B).

Cvi plants had a mean bolting time that was significantly ($p<0.001$) earlier and had fewer leaves in rosettes at bolting than Bur plants (Fig. 5.2; Appendix Table 5.1).

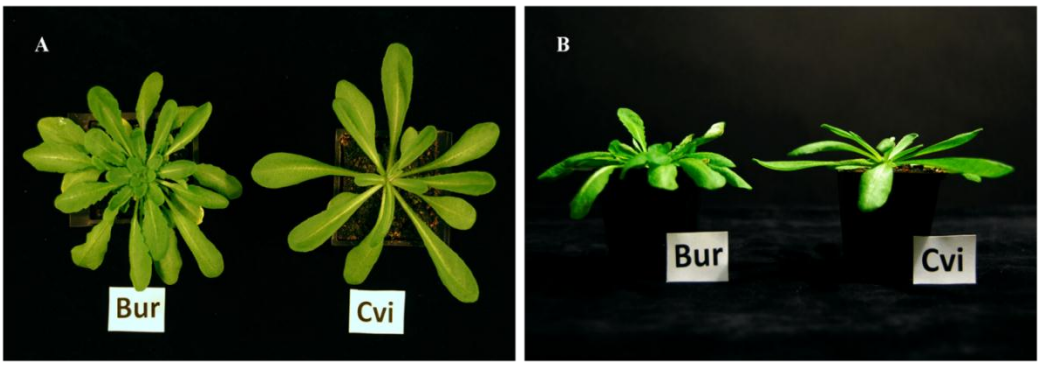

Figure 5.1 Plant phenotypes during their vegetative stage at 28 days after sowing in the common environment: 23/18˚C, 12/12h, light /dark. **A**: Overhead view of the Bur and Cvi rosettes; **B**: side view of the Bur and Cvi rosettes.

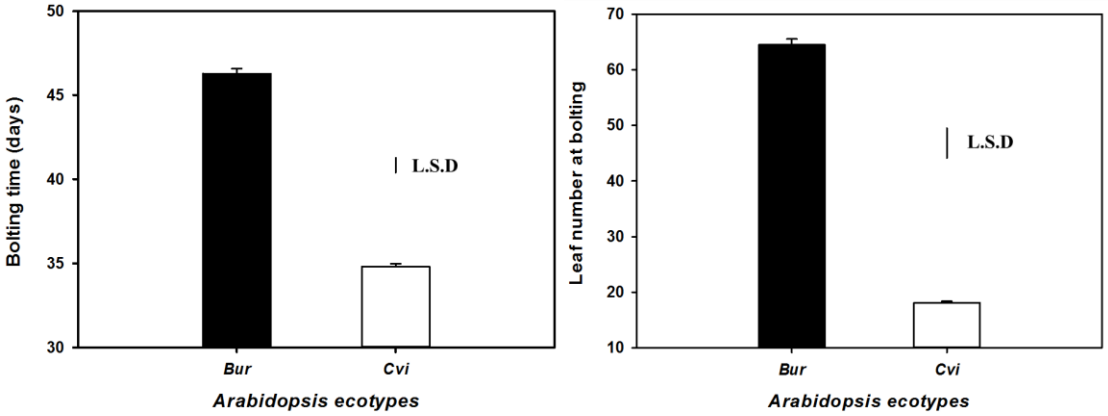

Figure 5.2 Bolting phenotypes in the common environment: 23/18˚C, 12/12h, light /dark. **A**: mean bolting time; **B**: mean rosette leaf number at bolting. Different single bar indicates different bolting time or leaf number at bolting. Data are the mean ± standard error. The L.S.D values are the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 5.1).

Both ecotypes reached a final height of 20-25cm. During reproductive growth, the Bur plants grown in the Cvi environment were bushy compared to the Cvi plants, and few siliques developed fully (Fig.5.3A); but Cvi plants grown in the Bur environment did not show a great difference compared to those grown in their native environment (Fig.5.3B). Most of the siliques on the Bur plants in the Cvi environment were poorly developed, curled with few or no seeds, and even withered (Fig.5.3C).

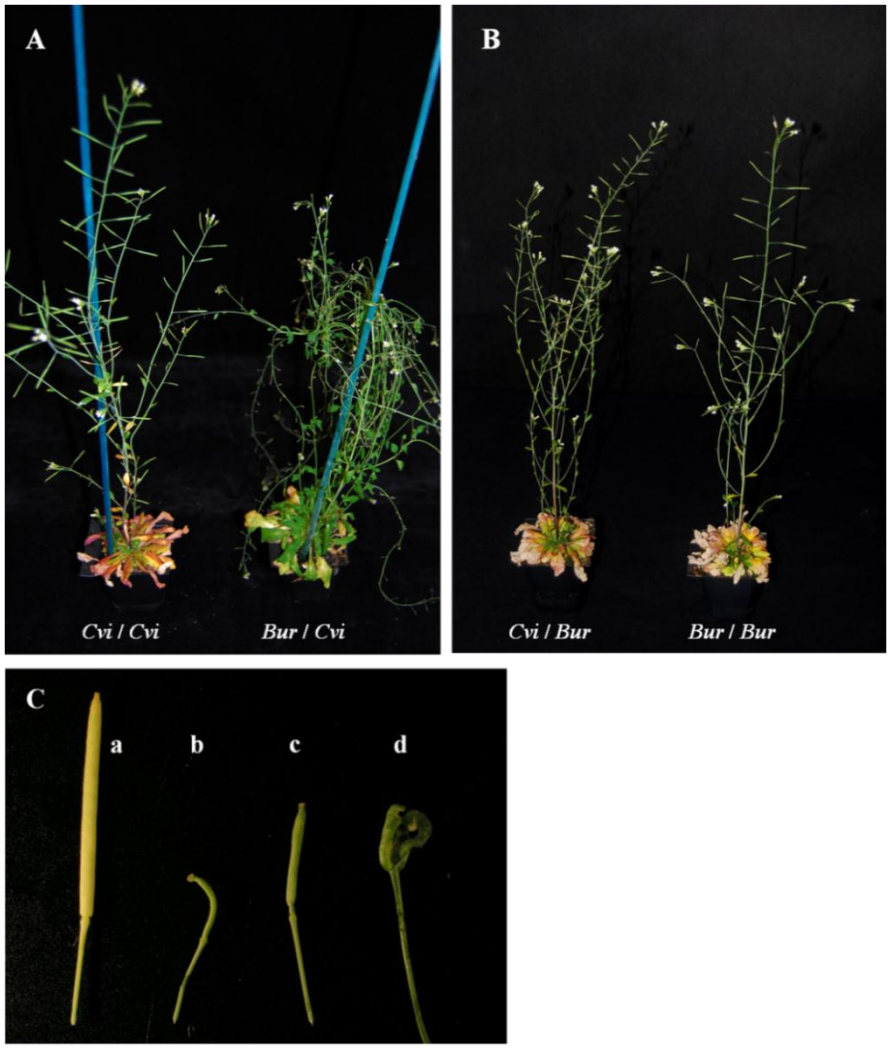

Figure 5.3 Plant phenotypes during reproductive growth. A: Cvi plants grown in its native environment (Cvi/Cvi) and Bur plants grown in the Cvi environment (Bur/Cvi); B: Cvi plants grown in the Bur environment (Cvi/Bur) and its native environment (Bur/Bur); C: siliques of Bur plants developed in the Cvi environment (a: well-developed silique; b-d: curly and withered siliques).

5.3.2 Seed yield and germination responses

Seed yield: Both the environment and ecotype showed significant effects on final seed yield ($p<0.001$ and $p<0.01$ respectively; Appendix Table 5.2). In the warmer Cvi environment, plants of both ecotypes yielded fewer seeds when compared to those in the cooler Bur environment. Seed yield of Cvi in its native environment only reached half of the final yield achieved in the Bur environment

(Fig.5.4). Seed yield of Bur was also significantly ($p<0.001$) different in the two environments.

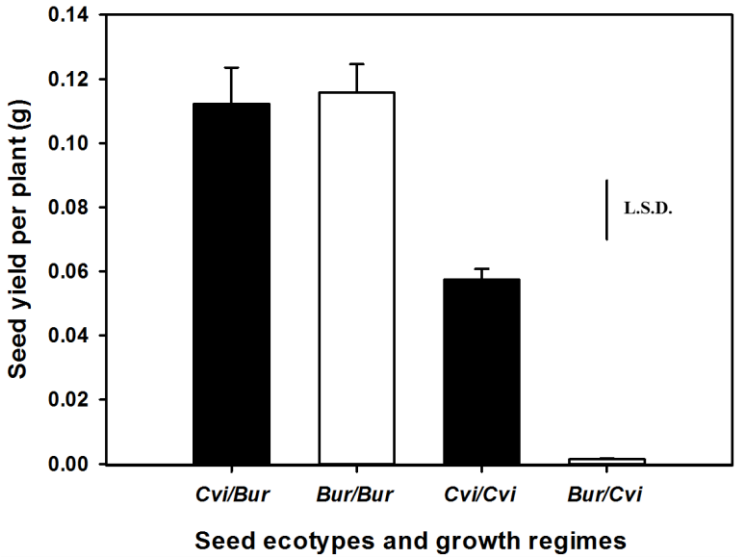

Figure 5.4 Seed yield per plant in simulated Bur and Cvi environments. Cvi/Bur: Cvi seeds produced in Bur environment (17/10˚C, L/D, 14/10h); Bur/Bur: Bur seeds produced in Bur environment (17/10˚C, L/D, 14/10h); Cvi/Cvi: Cvi seeds produced in Cvi environment (25/20˚C, L/D, 12/12h); Bur/Cvi: Bur seeds produced in Cvi environment (25/20˚C, L/D, 12/12h). Data are the mean ± standard error. The L.S.D. bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 5.2)

The 1000-seed weight of seeds produced in controlled environments was measured (Table 5.6). The Bur plants grown in the Cvi environment produced significantly fewer ($p<0.01$), but larger seeds (i.e. higher 1000-seed weight; $p<0.001$) than Cvi or Bur grown in its native environment (Table 5.6). Cvi seed size was not significantly different between the two environments, but produced significantly more seeds in the Bur environment than in the Cvi environment. In contrast, both seed size and number for the Bur ecotype were significantly different ($p<0.01$) in the two environments (Table 5.6).

Table 5.6 Mean 1000-seed weight and number of seeds produced from each plant in controlled environments

Ecotype / environment	1000-seed weight (g ± s.e.)	Seed number ($\times 10^3$ plant^{-1} ± s.e.)
Bur/Bur	0.0301±0.000719	3.843±0.289
Bur/Cvi	0.0373±0.001767	0.039±0.008
Cvi/Bur	0.0326±0.00058	3.434±0.327
Cvi/Cvi	0.0297±0.000171	1.930±0.112
L.S.D (5%)	**0.003083**	**0.6954**

1000-seed weight and seed yield were both recorded as mean value of four trays (biological replicates) ± standard error; The L.S.D. values are the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 5.3 and 5.4).

Germination response to temperature: Thermo-dormancy and germination potential were characterised at different temperatures. A two-way ANOVA indicated that the final seed germination percentage was significantly ($p<0.001$) affected by the temperature during imbibition, also seeds of the two ecotypes produced in the two environments showed significantly ($p<0.001$) different germination behaviours (Appendix Table 5.5). In the Cvi environment Bur plants (Bur/Cvi) yielded few seeds, but they had the highest germination percentage at both 10˚C and 25˚C (Fig.5.5). In contrast, Cvi seeds produced in the Bur environment (Cvi/Bur) did not germinate at either 10˚C or 25˚C. Bur seeds produced in their own environment (Bur/Bur) had higher percentage germination at 10˚C than at 25˚C. Similarly Cvi seeds produced in their own environment (Cvi/Cvi) germinated to higher percentages at 10˚C than at 25˚C.

The T_{50} values were also calculated for seeds that reached more than 50% final germination (Table 5.7). Bur seeds produced in the Cvi environment had the lowest T_{50} (i.e. high germination rate), germinating slightly faster than those produced in the

Bur environment, but the difference was not significant. Cvi seeds produced in their own environment had the highest T_{50} (i.e. low germination rate).

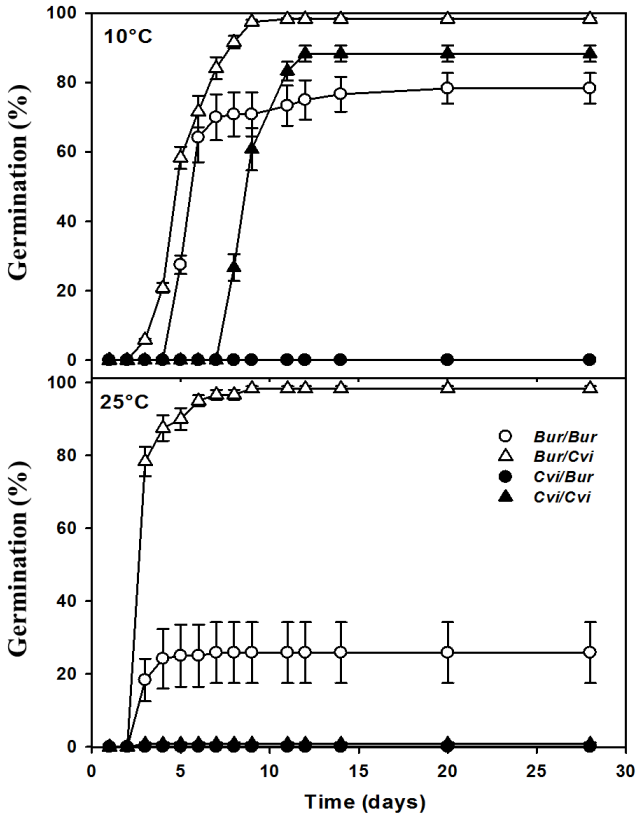

Figure 5.5 Seed germination responses in Bur and Cvi environment (○ Bur/Bur, ● Cvi/Bur, Δ Bur/Cvi, ▲Cvi/Cvi). Bur environment: 17°C/10°C, L/D, 14/10h; Cvi environment: 25°C/20°C, L/D, 12/12h; Data are the mean ± standard error. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 5.5)

Table 5.7 Final germination percentage and T_{50} values of *Arabidopsis* seeds at 10°C and 25°C

Ecotype / Controlled environment	Final Germination (% ± s.e.)		T_{50} values	
	10°C	25°C	10°C	25°C
Bur/Bur	78.3±4.4	25.8±8.35	5.43	-*
Bur/Cvi	98.3±0.33	98.3±0.67	4.93	2.78
Cvi/Bur	0	0	-	-
Cvi/Cvi	88.33±2.33	0.83±0.33	8.60	-

Data are the mean ± standard error; *the missing data indicate that the germination did not reach 50%, so there was no T_{50} value.

5.3.3 Floral and silique development of the Bur ecotype

5.3.3.1 Cumulative flowering on the primary inflorescence

In order to investigate why the production of Bur/Cvi seeds was very low, Bur plants were grown again in the Bur and Cvi environment. The difference in flower number on the primary inflorescence in the two controlled environments gradually increased after day 14 (Fig.5.6).

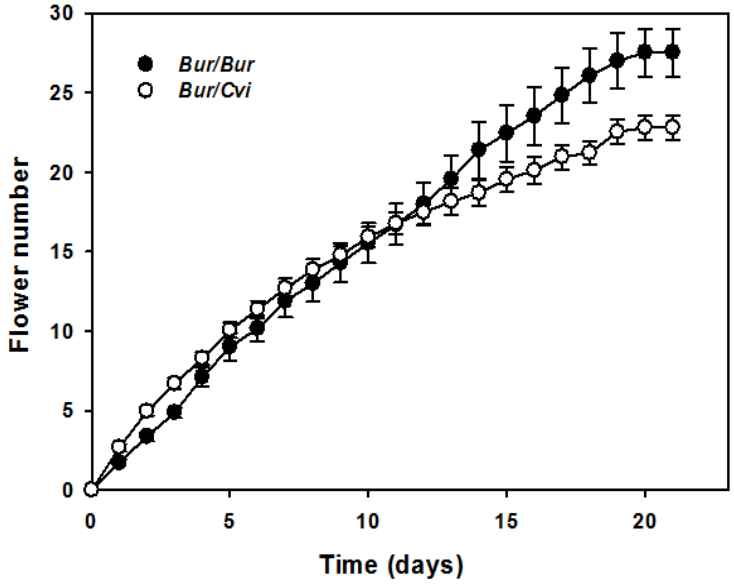

Figure 5.6 Cumulative flowering on the primary inflorescence of Bur plants grown in Bur environment (● Bur/Bur: 17/10°C, L/D, 14/10h) and Cvi environment (○ Bur/Cvi: 25/20°C, L/D, 12/12h). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

5.3.3.2 Flower development

Flowers of Bur plants grown in both environments were dissected to see if flower morphology was altered; in both environments morphology appeared normal, with the exception of stage 14 when the anthers typically extend above the stigma so that pollination can occur (Fig.5.7). During this stage, Bur plants grown in the Cvi environment had pistils longer than the stamens, even during the fertilization period. Therefore flowers failed to be adequately pollinated because of the length difference between pistil and stamens throughout growth and this likely limited the transfer of

pollen (Fig.5.7). Normally (i.e. Bur/Bur) stamens extend past the pistol to facilitate pollination. However, the application of gibberellic acid (GA) to Bur/Cvi flowers stimulated the growth of stamens and this resulted in adequate pollination during the fertilization stage (Fig.5.7).

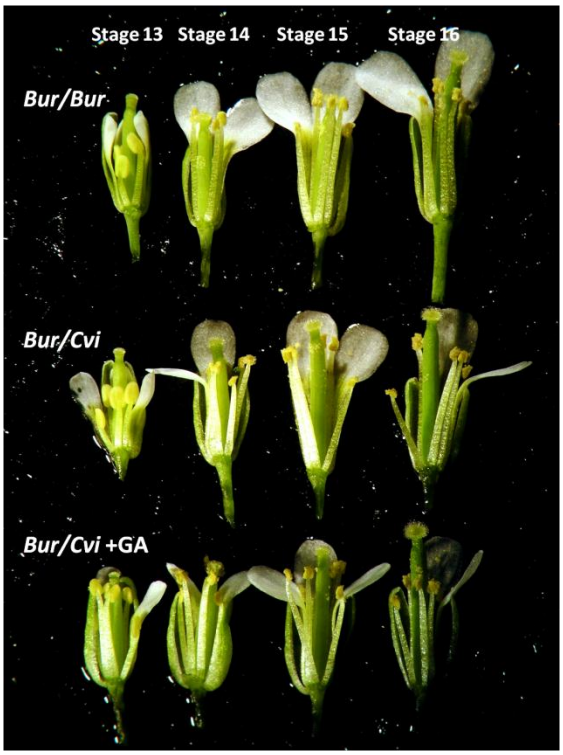

Figure 5.7 Partially dissected Bur flowers (stage 13-16): developed in the Bur (Bur/Bur) and Cvi environments (Bur/Cvi), and treated with a 10μM GA_{4+7} solutions in Cvi environment (Bur/Cvi +GA).

5.3.3.3 Ratio of stamen and pistil lengths

The lengths of pistils and stamens were measured at stages13, 14 and 15 (Smyth *et al*., 1990) and the ratio of stamen / pistil length was calculated at each stage. Pistil lengths at different stages were significantly ($p<0.001$) different regardless of the environment in which Bur plants were grown (Appendix Table 5.6). During the fertilization period (stage14), the mean pistil length in the Cvi environment was longer than that in Bur environment and those applied with GA solution (Fig.5.8A), but the difference was not significant. However, the mean

stamen length in the Cvi environment was significantly shorter ($p<0.001$) than that in the other two environments (Fig.5.8B; Appendix Table 5.7).

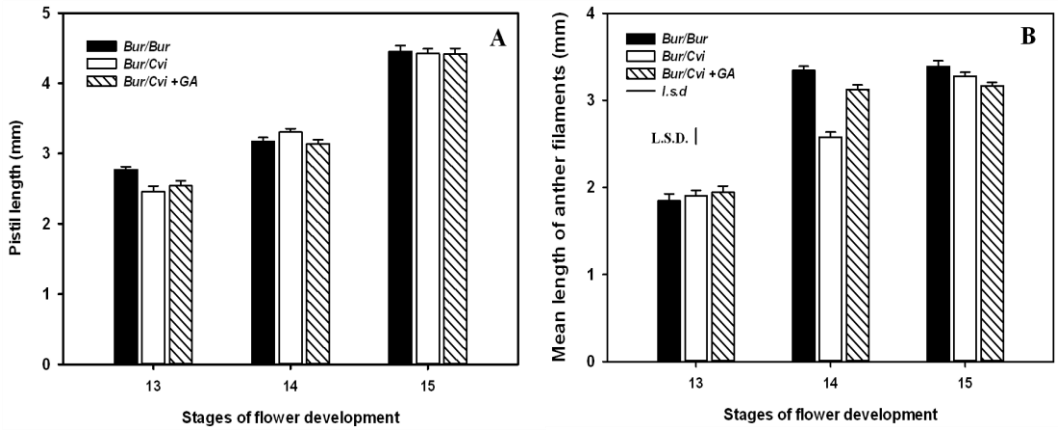

Figure 5.8 Floral phenotype of Bur ecotype in Bur environment (Bur/Bur) and Cvi environment (Bur/Cvi). A: Pistil lengths at different developmental stages; **B**: Stamen lengths at different developmental stages; Bur environment: 17/10°C, L/D, 14/10h; Cvi environment: 25/20°C, L/D, 12/12h; Data are the mean length of pistils and mean length of stamen ± standard error. No error bar indicates symbol is larger than the error. The L.S.D. bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 5.6 and 5.7).

The stamen / pistil length ratio was calculated to show when stamen length surpassed that of the pistil. A two-way ANOVA showed that both stage of flower development and growth environment had significant effects ($p<0.001$) on the ratio of stamen and pistil length (Appendix Table 5.8). The difference in the ratio of stamen/pistil length was significant between the Bur and Cvi environment, but was not significant between the flowers grown in the Bur environment and those treated with GA in Cvi environment. The ratio was greater than 1.0 in flowers developing in the Bur environment and flowers treated with GA, but the ratio recorded from the flowers in the Cvi environment was consistently below 1.0 (Fig.5.9). This result, combined with the data on stamen length (Fig.5.8B), shows that the main cause of poor seed production is abnormal development of stamens in the higher temperature of the Cvi environment, which results in reduced pollination.

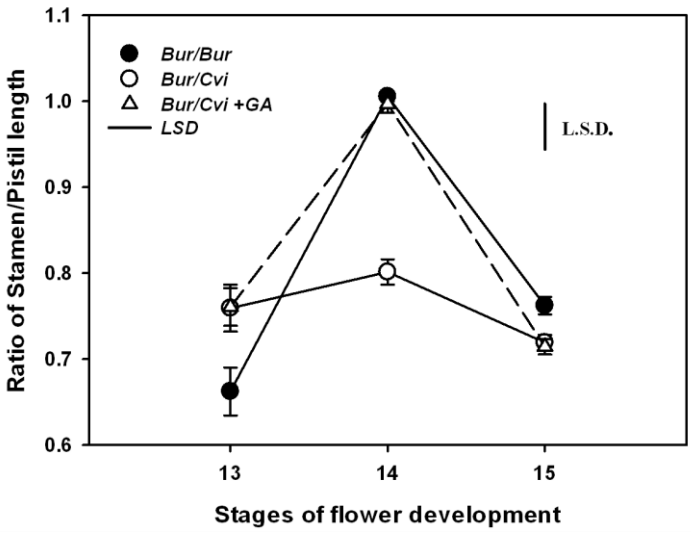

Figure 5.9 Stamen/pistil ratio of Bur ecotype in Bur environment (Bur/Bur) and Cvi environment (Bur/Cvi) (Bur environment: 17/10°C, L/D, 14/10h; Cvi environment: 25/20°C, L/D, 12/12h; Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The L.S.D. bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 5.8).

5.3.3.4 Silique development after flowering

Silique development was monitored after flowering. During the early stage (3 days after flowering) the mean length of Bur siliques was similar to those in the Cvi environment. At later stages (≥ 4 days after flowering) the mean length of Bur siliques developed in the Cvi environment were significantly shorter ($p<0.05$), achieving only half the final length of siliques produced in the Bur environment (Table 5.8 and Fig.5.10).

Table 5.8 Test of significance differences in silique length after flowering of Bur plants in its native and the Cvi environment

Day	t value	Day	t value
3	0.449203	8	4.28E-09*
4	4.15E-05*	9	2.33E-10*
5	2.11E-07*	10	4.14E-12*
6	1.26E-08*	11	6.19E-14*
7	4.35E-12*	12	2.24E-12*

* The two-sample unpaired t-test was used to determine statistical significance at the P<0.05 level.

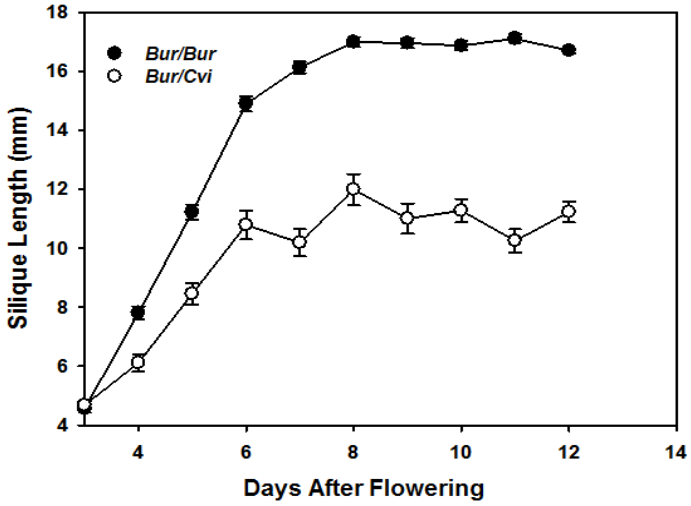

Figure 5.10 Silique length in Bur plants after flowering in the Bur and Cvi environments (● Bur/Bur: 17°C/10°C, L/D, 14/10h; ○ Bur/Cvi: 25°C/20°C, L/D, 12/12h). Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

5.3.3.5 Silique development

The siliques of Bur plants grown in the Bur and Cvi environments were dissected. In Bur siliques developed in the Cvi environment, unfertilized ovules appeared to predominate toward the base of the silique (shown with red arrowhead in Fig.5.11). However, when siliques were treated with GA or hand pollinated, more ovules were fertilized (Fig.5.11).

Both the total number of ovules and percentage of developed ovules within each Bur silique were significantly ($p<0.001$) affected by the growth environment and treatments (Fig.5.12A; Appendix Table 5.9-5.10). In Bur siliques produced in the Bur environment, 98.8% of ovules were fertilized, but in the Cvi environment only 52.9% ovules were fertilized (Fig.5.12B). When flowers were treated with GA or hand pollinated, both total number of ovules and number of fertilized ovules increased significantly in Bur siliques in the Cvi environment (Fig.5.12A), leading to a significant ($p<0.001$) increase in the percentage of fertilized ovules to 83.8% and 93.9% respectively (Fig.5.12B): this showed that the pollen was viable.

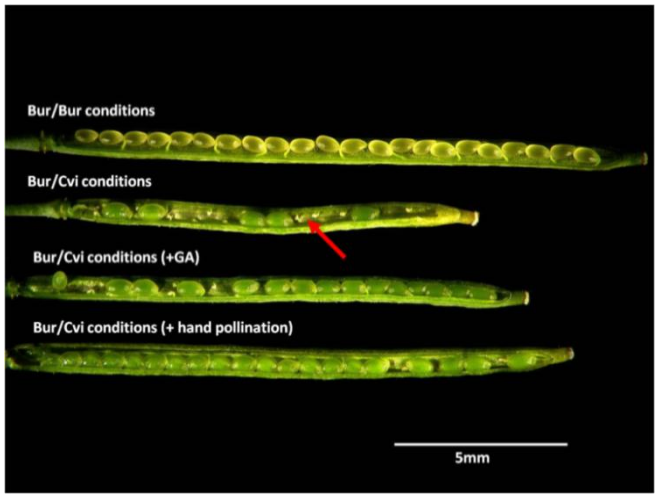

Figure 5.11 Partially dissected siliques of Bur ecotype in different environment: Bur plants grown in Bur environment (Bur/Bur conditions), Cvi environment (Bur/Cvi conditions), treated with 10 µM GA_{4+7} solution in Cvi environment (Bur/Cvi conditions +GA), and hand pollinated (Bur/Cvi conditions +hand pollination). The red arrowhead indicates an unfertilized ovule in Bur/Cvi silique.

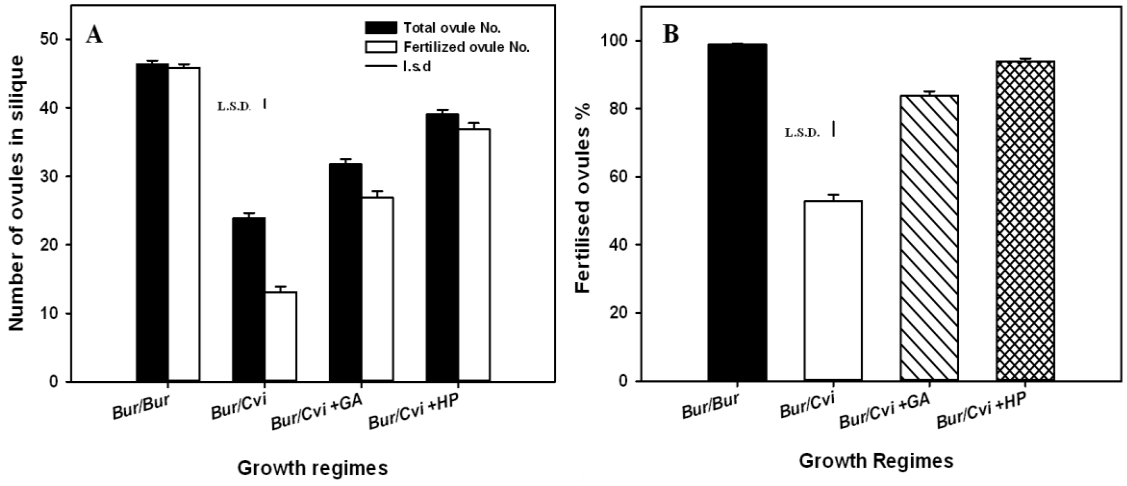

Figure 5.12 The impact of simulated Bur and Cvi environments on ovule development A: total number of ovules and number of fertilized ovules in siliques of Bur plants in different environments. **B**: percentage of ovules fertilized in each environment. Bur plants grown in Bur environment (Bur/Bur), Cvi environment (Bur/Cvi), treated with10µM GA_{4+7} solution in Cvi condition (Bur/Cvi +GA), and hand pollinated (Bur/Cvi +HP). Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The L.S.D. bars indicate the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 5.9-5.10).

5.4 Discussion

Bolting time and leaf number at bolting are ecotype-dependent in Arabidopsis

In *Arabidopsis*, leaves are regarded as the major biomass producing organs and bolting is an important phenotype characteristic marking the transition to the reproductive phase. There is a strong correlation between leaf number and bolting time in *Arabidopsis* (Lee, 2009). In the results presented here Cvi and Bur have significantly different bolting times in the same environment (Cvi is faster), consistent with observations that there is a wide range of natural variation in mean bolting time among *Arabidopsis* ecotypes (Nordborg and Bergelson, 1999; Luquez, *et al*., 2006). Cvi also has fewer leaves at bolting than Bur. During their vegetative phase as rosettes, leaves are initiated through the activities of the shoot apical meristem. Subsequent leaf production is inhibited by floral induction, and the main stem elongates to give rise to the primary inflorescence (Gómez-Mena *et al*., 2001; Lee, 2009). This relationship partly explains why early bolting ecotypes such as Cvi have fewer leaves at bolting compared with late-bolting ecotypes such as Bur.

Maternal temperature has an impact on the final seed yield

Plant behaviour even between populations of the same species can differ greatly when they are established in diverse environments (Donohue *et al*., 2005a). Gan *et al*. (2004) found that seed yield of canola reduced by 15% when high temperature was applied before flowering, while the reduction increased to 58% when high temperature stress was applied during flowering. A similar decrease in seed yield of peanut (*Arachis hypogaea*) at high temperature resulted from poor fertilization, due to both reduced pollen production and viability, and poor pollen tube growth (Prasad *et al*., 1999, 2001, 2003). In the present work, plants of both ecotypes in the Bur environment (low temperature) produced more seeds than those

in Cvi environment (high temperature; Fig.5.4) in agreement with work showing that seed yield of soybean decreased when plants were exposed to high temperature (Gibson and Mullen, 1996). However, temperature did not have a significant effect on the seed size (1000-seed weight; Table 5.5). This is in contrast to the observation in grain legumes that high temperature during reproductive development had a negative effect on cotyledon cell number and seed filling, resulting in smaller seed size and lower seed production (Munier-Jolain and Ney, 1998).

Decreased seed yield results from reduced stamen extension at high temperature

It is widely accepted that plant reproduction is highly sensitive to environmental factors such as temperature (Hedhly *et al*., 2003), and this as discussed above can have an impact on pollen viability (Prasad *et al*., 2003). Polen viability was also considered to be the main reason for decreased seed production in the legume crops, chickpea (*Cicer arietinum*) and cowpea (*Vigna unguiculata*) (Srinivasan *et al*., 1998; Hall, 2004). In *Capsicum* species high temperatures prior to and during the early flower stage affected both pollen germination and pollen tube length and the tolerance to high temperature varied between different species (Reddy and Kakani, 2007). However, in this study, hand pollination was effective in increasing the percentage of fertilized ovules in the Cvi environment from 52.9% to 93.9% (Fig.5.10), indicating that Bur pollen in the Cvi environment was viable. The high temperature Cvi environment had a significant negative impact on stamen growth, but no impact on pistil growth with a resulting reduction in the ratio of stamen to pistil length (Fig.5.8A and Fig.5.9). This reduction in filament growth resulted in anthers not extending above the stigma by stage 14, leading to reduced deposition of pollen on to the receptive pistil to reduce pollination. High temperature had a negative impact on cell expansion during reproductive development in grain

Legumes (Munier-Jolain and Ney, 1998), which could be the cause of the reduced extension of the stamens in Bur flowers developed in the Cvi environment.

The high temperature effect on stamen extension was reversed by applying GA, confirming this as the cause of poor seed production from Bur in the Cvi environment. GA has been considered as a general regulator of floral development and GA-deficient mutants typically have short stamens as a result of reduced cell extension within the filament (Cheng *et al*., 2004). The growth-repressing DELLA proteins RGA, RGL1 and RGL2 work together to repress stamen and anther development in GA-deficient plants (Cheng *et al*., 2004). Therefore GA may have promoted stamen development through cell expansion by stimulating the destruction of DELLAs (Achard *et al*., 2009).

Both the maternal environment and genetics determine seed dormancy

Baskin and Baskin (1998) describe how the environment experienced by the mother plant can influence the germination and dormancy of seeds. An increase in germinability in response to higher maternal temperatures has been reported in several species, e.g. Cotton thistle (*Onopordum acanthium*) (Qaderi *et al*., 2003), ryegrass (*Lolium rigidum*) (Steadman *et al*., 2004), *Goodenia fascicularis* (Hoyle *et al*., 2008). Here seeds of the two ecotypes produced in the same environment exhibit different depths of dormancy showing there is a genetic basis to these differences. However, in both ecotypes seeds produced in different environment also have different depths of dormancy showing that the maternal environment also has an impact. These results are in agreement with work showing that seed dormancy is determined by the interaction between genetics and the environment during seed maturation (Donohue, 2009; Penfield and Springthorpe, 2012). In the experiments reported above, Bur seeds produced in the cooler Bur environment have deeper

dormancy than those produced in the warmer Cvi environment; Cvi seeds produced in the Bur environment did not germinate even at the optimum temperature of 10˚C (Fig.3.5). Thus enhanced primary dormancy in lower production temperatures in the maternal environment may well be a conserved response.

5.5 Conclusion

The results presented show that high temperature in the maternal environment negatively affects the final seed yield. In the case of Bur in the Cvi environment this resulted from failure of pollination due to reduced stamen extension relative to the pistil during the fertilization period. In addition, seed germination was also greatly affected by the maternal environment: seeds produced in the Bur environment (lower temperature) were more dormant than those produced in Cvi environment (higher temperature). Depth of dormancy in seeds produced from the two ecotypes in the same maternal environment also differed illustrating that there is a genetic basis for differences in dormancy between them. Studies on the nitrate supply to the mother plants will be shown in the next chapter illustrating other aspects of the maternal environmental also influence seed characteristics (*Chapter 6*).

CHAPTER 6: THE IMPACT OF NITRATE AND OTHER ENVIRONMENTAL FACTORS ON SEED PRODUCTION AND SUBSEQUENT GERMINATION BEHAVIOUR IN WINTER AND SUMMER ANNUAL *ARABIDOPSIS* ECOTYPES

6.1 Introduction

6.1.1 Nitrate regulation of plant growth

Nitrogen (N) is an essential component for life. For many plant species, nitrate is known as one of the most frequent utilized forms of N from the soil (Nešić *et al*., 2008). N has two major roles in plants, firstly as a nutrient and secondly as a signalling molecule.

Nutrition: N is one of the most important inorganic nutrients for plant growth, which affects plant biomass and crop yield as one of the essential macronutrients (Lam *et al*., 1996). N is a key part of amino acids and nucleic acids that are vital to all the biological processes (Matakiadis, 2008). At the vegetative stage, N limitation leads to a reduced shoot growth and biomass of plants (Khamis *et al*., 2000; Loudet *et al*., 2003), increased remobilization of N from older organs to new organs (Lemaître *et al*., 2008; Masclaux-Daubresse and Chardon, 2011), accumulation of starch and anthocyanins in leaves (Stitt and Krapp, 1999), a decrease in photosynthetic capacity and under severe N deficiency chlorosis of leaves (i.e. reduction in the chlorophyll content) and lack of vigour (Stitt and Krapp, 1999; Khamis *et al*., 2000). Studies at the molecular level have shown that plants experiencing N limitation up-regulate genes involved in protein degradation, anthocyanin and phenylpropanoids production while down-regulating the genes involved in photosynthesis, synthesis of chlorophyll and N compounds (Peng *et al*., 2007). For example, the transcript of *NRT2.1*, a high-affinity nitrate transporter in

Arabidopsis, was significantly increased by N limitation (Filleur *et al*., 2001); also the expression of an MYB-like gene *AtNsr1* was also up-regulated by N deficiency (Todd *et al*., 2004). Diaz *et al*. (2006) identified fifteen QTLs associated with leaf senescence and stress response symptoms such as yellowing and anthocyanin-associated redness in *Arabidopsis* plants grown under low-N conditions.

Signalling: N is also an important signal, N modulates plant development including seed germination, root branching and development, leaf growth, shoot branching and flowering time (Zhang *et al*., 1999; Zhang and Forde, 2000; Marín *et al*., 2011). In *Nicotiana plumbaginifolia* nitrate reductase (NR) mutants, nitrate was found to regulate shoot-root allocation and there was a correlation between the nitrate accumulation in the shoots and an inhibition of root growth (Scheible *et al*., 1997a, b). In *Arabidopsis*, a locally concentrated supply of nitrate increased the mean rate of lateral root elongation and the stimulatory effect was a direct effect of the NO_3^- ion itself rather than a nutritional effect (Zhang *et al*., 1999; Zhang and Forde, 2000). Low nitrate in *Arabidopsis* also led to early flowering via a signalling pathway acting in parallel with known floral induction pathways (e.g. autonomous, photoperiod and gibberellic acid floral induction pathways) (Marín *et al*., 2011). Studies on long-term and short-term effects of N on leaf growth in tobacco plants have also shown a signalling effect of NO_3^- on leaf expansion by supplying the shoot with cytokinins (Walch-Liu *et al*., 2000).

6.1.2 Nitrate effect on seed dormancy and germination

Nitrate has long been known as one of the key variables in the loss of seed dormancy and promotion of germination (Baskin and Baskin, 1998; Alboresi *et al*., 2005; Finch-Savage *et al*., 2007; Matakiadis *et al*., 2009). The promotive effects of nitrate on seed germination can be increased by various environmental factors. For

example, germination of *Sisymbrium officinale*, a species closely related to *Arabidopsis*, showed an absolute requirement for both nitrate and light, and only the combination is successful in dormancy release (Hilhorst and Karssen, 1989). The effect of nitrate on promoting germination in presence of light was also reported in many other species, such as *Chenopodium album*, *Avena fatua*, *Sinapis arvensis* and *Hygrophila auriculata* (reviewed in Baskin and Baskin, 1998). Therefore in general, application of nitrate-containing fertilizers to the soil may not in itself promote large number of seeds to germinate, as many seeds require both nitrate and light to germinate. Thus N fertilizer may be more efficient in promoting germination if they were applied after tillage of the soil (Hilton *et al*., 1984; Goudey *et al*., 1987, 1988).

It was first revealed that nitrate acted as a signal independent of nitrate reduction in *S. officinale*, where nitrate promoted germination when nitrate reductase was completely inhibited (Hilhorst and Karssen, 1989). In the deeply dormant *Arabidopsis* Cvi ecotype, nitrate can substitute for the long period of dry storage (7-12 months after-ripening) or several days of cold stratification required for dormancy release (Finch-Savage *et al*., 2007). Further study of exogenous nitrate on the germination of wild-type and NR-deficient *Arabidopsis* seeds supported the role of nitrate as a signal during germination and revealed that this may involve interaction with the ABA or GA signalling pathways (Ali-Rachedi *et al*., 2004; Alboresi *et al*., 2005). In a study on the interaction of nitrate, ABA and gibberellins on seed germination at high temperatures, nitrate decreased thermo-inhibition of lettuce seed germination (Dong *et al*., 2009). In addition, nitrate feeding experiments in *Arabidopsis* showed that supplying higher nitrate levels to the mother plant (maternal nitrate supply) increased seed nitrate levels, and these were negatively correlated with dormancy levels in the mature seed (Alboresi *et al*., 2005). Nitrate feeding was

subsequently shown to reduce ABA levels in maturing *Arabidopsis* seeds by inducing the ABA catabolic gene *CYP707A2* during seed development and germination (Matakiadis *et al*., 2009).

6.1.3 Nitrate-regulated gene expression

Nitrate, as a signal, modulates nitrogen and carbon metabolism, resource allocation and also plant development (e.g. root branching, leaf expansion) (Zhang and Forde, 2000; Walch-Liu *et al*., 2000). Gene expression analysis revealed rapid and marked changes in gene expression in response to nitrate (Scheible *et al*., 1997b; Wang *et al*., 2000). In addition, nitrate is reported to regulate the expression of nitrate transporters (NRTs), nitrate assimilatory genes and other metabolism genes through a signalling effect (Crawford, 1995; Wang *et al*., 2004).

Two families of nitrate inducible nitrate transporter genes, *NRT1* and *NRT2*, were identified to be involved in the uptake of nitrate by root systems (Crawford and Glass, 1998). *NRT1.1* was the first nitrate transporter gene identified in plants, *NRT1.1* mRNA found predominantly in roots (Tsay *et al*., 1993). Strong *NRT1.1* expression was found in nascent organs of developing roots and shoots, such as root tips, young leaves and developing flower buds, indicating an important role for NRT1.1 in the early stages of organ development (Guo *et al*., 2001). NRT1.1 was also found to repress the nitrate transporter NRT2.1 by a direct signalling role involving N metabolites (Muños *et al* 2004). In the *Arabidopsis* nitrate reductase (NR) double mutant G'4-3 (*nia1-1*/*nia2-5*), nitrate was shown to act as a signal for the induction of the NRT gene *NRT2.1* (Filleur and Daniel-Vedele, 1999).

Following uptake, nitrate is reduced to nitrite by NR, followed by the further reduction of nitrite to ammonium by nitrite reductase (NiR). Ammonia is then incorporated into the amino acid glutamine/glutamate (Crawford, 1995). The

expression of genes encoding NR, NiR, glutamine synthetase, and glutamate synthase were rapidly induced by nitrate; in the case of NR within minutes (Melzer *et al*., 1989; Wang *et al*., 2000). In a comparison of wild type and the NR-null mutant of *Arabidopsis*, genes related to N and C metabolism, energy production, glycolysis/gluconeogenesis and pentose phosphate pathway were found to be overrepresented in the mutants response to nitrate (5mM, 2h), again indicating that nitrate could serve as a signal (Wang *et al*., 2004).

Significant differences in nitrate levels were found in carrot roots depending on soil richness and year of cultivation (Międzobrodzka *et al*., 2006). As discussed in *Chapter 2*, *Arabidopsis* ecotypes have been found in a wide range of habitats, so that weather conditions (e.g. temperature and rainfall), vegetation composition and distribution may lead to a large variation in the soil conditions (e.g. soil type, composition and richness) (Ikram *et al*., 2012). Therefore, distinct *Arabidopsis* ecotypes may have different responses to the nitrate in soil as an adaptation to their native environment. In the experiment reported here, different ecotypes were therefore grown under different levels of nitrate to investigate the effect of maternal nitrate supply on plant growth and seed production and the dormancy and germination behaviour of the seed produced. In addition, the expression of genes with known roles in the regulation of dormancy and germination; and nitrate uptake and reduction were also analysed to gain improved understanding of the relationship between maternal nitrate supply and seed behaviour in summer and winter annual ecotypes.

6.2 Materials and Methods

6.2.1 Plant and seed production in different temperature and nitrate regimes

- Seed production

Different ecotypes of *Arabidopsis* (Bur, Cvi, Ler) were produced under controlled conditions, including a temperature-controlled glasshouse and temperature-controlled incubators in 2009, 2010 and 2012 (Table 6.1). Compost amended with different levels of nitrate (Table 6.2) were added to P24 cellular trays (contains 24 cells).

Table 6.1 Seed production under different conditions

Time		Growth conditions	Ecotype used
2009	July	Temperature-controlled glasshouse (23/17˚C, 16/8h, light /dark)	Bur, Ler
2010	March	Temperature-controlled glasshouse (23/17˚C, 16/8h, light /dark)	Bur, Cvi, Ler
2012	June	Temperature-controlled incubator (15/15˚C, 12/12h, light /dark; 20/20˚C, 12/12h, light /dark)	Bur, Ler

Table 6.2 Composition of compost with different nitrate levels for plant growth and seed production

Compost type	Compost component	Nitrate content (mg/kg dry weight)
High N	Levingtons F1 compost: sand: vermiculite = 6:1:1	421.9
Low N	Levingtons F1 compost: sand: vermiculite = 4:2:2	153.7

Trays were labelled and placed in a second tray lined with capillary matting to ensure all the plants had a uniform water supply. There were two trays for each combination of ecotype, nitrate level and, where relevant, temperature. Approximately five seeds were sown in each cell. The trays were then covered with transparent propagator lids for at least four days, at that stage all the seedlings had established. One week after sowing, seedlings were thinned to leave one per cell. Plants were grown under controlled conditions (Table 6.1) and visually scored each day for bolting. The bolting time was recorded when the inflorescence reached a height of 1cm, the rosette diameter and leaf number were also measured at bolting.

Watering stopped when seeds had reached maturity (all siliques yellow). Seeds were then harvested by hand when all the siliques had turned yellow and were dry on the plant. Seeds were then dried to an equilibrium relative humidity of 55% above a saturated calcium nitrate solution for six days before seed yield and 1000-seed weight was measured. Seeds were then sealed in aluminium foil bags and stored at -80˚C for germination experiments.

- Seed nitrate content measurement

Seed produced on plants with different levels of nitrate supply were collected for nitrate content analysis. Triplicate 150 mg samples of dry seeds were ground using a pestle and mortar, and transferred to a 20ml scintillation vial which has been weighed to 4 decimal places. The vial plus seed sample was then re-weighed before and after drying at 80˚C overnight. Deionised water (10ml) was added to the vial, and the samples were shaken for one hour. The contents of the vial were then transferred to a 50ml centrifuge tube and centrifuged for five minutes at 5000 rpm. The supernatant was filtered using nitrogen free filter paper, and analysed for NO_3-N by a steam distillation method using a FOSS FIAstar 5000 Flow Injection Analyser for end point determination (Bremner and Keeney 1965).

6.2.2 Seed germination experiments

- Dormancy and germination assays

To record germination, seeds were first surface sterilized with 2.5% bleach (Domestos) for five minutes, washed three times with distilled water, and then incubated on two layers of 3M chromatography paper in clear plastic boxes (8×12cm) (Stewart Plastics) containing 8ml of distilled water. For each treatment combination there were three replicates of 40 seeds for each ecotype. To characterise different thermal responses seed germination was recorded at a range of temperatures

(5, 10, 15, 20, 25 and 30˚C) both in the light and in the dark for 28 days. Seeds with dark treatments were sown and germination was recorded in the dark under a green safe light (Kodak 7B safelight filter/Green, Kodak Limited, London) at all other times germination boxes were wrapped in a double layer of aluminium foil. Germinated seeds were counted and removed at regular intervals throughout the experiment. A seed was considered to have germinated when the radical had emerged through the testa and endosperm.

- Nitrate sensitivity experiments

This experiment was designed to test the impact of the maternal environment (temperature and nitrate regimes) on seed dormancy via the sensitivity of seeds to exogenous nitrate and whether that sensitivity was temperature or light dependant. Seeds were sown in three replicates of 40 in clear plastic boxes (8×12cm, Stewart Plastics Ltd, UK) on two layers of 3M chromatography paper with 8ml water or KNO_3 at 1.0mM. Seeds were then incubated in the light or dark at different temperatures (10˚C and 25˚C in *sec 6.3.1* and a range of temperatures from 5 to 30˚C in *sec 6.3.2*). Germination was recorded for 28 days as described above.

6.2.3 RNA extraction and gene expression analysis

Bur seeds produced under high nitrate and low nitrate conditions were collected for gene expression analysis. Triplicate 50 mg samples of dry seeds were imbibed on water for 24h/20˚C in the dark in standard germination boxes as for germination experiments (see above). Seeds were collected and immediately frozen in liquid nitrogen and stored at -80˚C. RNA was extracted using nuclease free reagents using the methods of Xu *et al*. (2010) and Gehrig *et al*. (2000) adapted for seeds (refer to *Chapter 4, sec 4.2.3*). The impact of nitrate supply during seed production on gene expression prior to germination was evaluated using the range of

genes described in *Chapter 4* (Table 4.1). Gene expression was analysed using the NanoString nCounter gene expression system (Geiss *et al.*, 2008) by UCL Genomics, University College London using 100 ng of total RNA (refer to *Chapter 4, sec 4.2.3*).

6.2.4 Data analysis

All percentage germination data was angular transformed for analysis. The transformation is arcsin(sqrt(p/100))*180/pi, where p is a percentage from 0 to 100, and the result is an angle between 0 and 90 degree. Data are presented as the mean ± standard error. The standard error of the mean was calculated by dividing the standard deviation by the square root of the number of replicates (n) (sd/√n). Analysis of variance (ANOVA) was used to detect the differences between variates as listed in Table 6.3. Statistical analysis was carried out using the software package GenStat (VSN International, 2012). The results of individual analyses are given in Appendix tables.

Table 6.3 Summary of ANOVA used in Chapter 6

Analysis of variance	Factors	Variate	Transformation
One-way ANOVA (*sec 6.2.1*)	Treatment (combination of ecotype and maternal nitrate supply)	Bolting time	
Two-way ANOVA (*sec 6.2.1*)	Ecotype, Maternal nitrate supply	1) Leaf number 2) Rosette diameter	None
Three-way ANOVA (*sec 6.2.1*)	Temperature, ecotype, maternal nitrate supply	1) Seed yield 2) Seed size	None
Four-way ANOVA (*sec 6.2.2*)	Germination temperature, nitrate, ecotype, maternal nitrate supply	1) Final germination% 2) T_{50}	Angular
Two-way ANOVA (*sec 6.2.3*)	Maternal nitrate supply, gene	Relative gene expression	

* The T_{50} is the time required for 50% of viable seeds to germinate, calculated by the software package GERMINATOR (Joosen *et al*., 2010).

Nested treatment structures were used in the analysis of T_{50}, which provided both information about ecotype effect (or temperature effect) and other treatment effects nested within ecotype (or temperature). These analysis approaches were necessary to cope with the low germination (missing values of T_{50}) for Ler at 25 ˚C.

6.3 Results

6.3.1 Effect of different nitrate regimes on vegetative growth, seed production and germination of *Arabidopsis* ecotypes

- Vegetative growth

To study the effect of nitrate on plant growth, Bur and Cvi were grown in a temperature-controlled glasshouse (23/17˚C, 16/8h, light /dark) on low N and high N compost (Table 6.2). In both ecotypes, leaves were more developed on high N than on low N, in line with nitrate dependence of plant growth. Cvi seedlings were smaller than those of Bur under both nitrate regimes (Fig.6.1), but did not thrive on low N and eventually died.

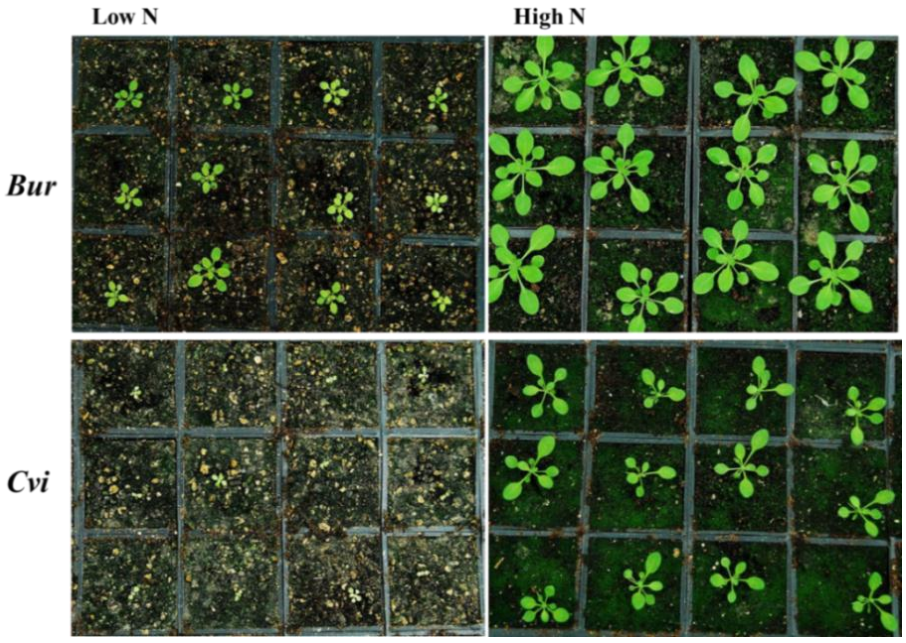

Figure 6.1 Bur and Cvi grown in two nitrate regimes 22 days after sowing in the glasshouse (23/17˚C, 16/8h, light /dark).

Therefore in subsequent glasshouse experiments comparing low with high N to produce seeds for comparisons of germination behaviour Ler, another winter annual ecotype, was substituted for Cvi. Under high N, Cvi plants had a mean bolting time significantly ($p<0.001$) earlier than that of Bur plants (Fig.6.2; Appendix Table 6.1). In Bur a lower N regime led to significantly later bolting (Fig.6.2).

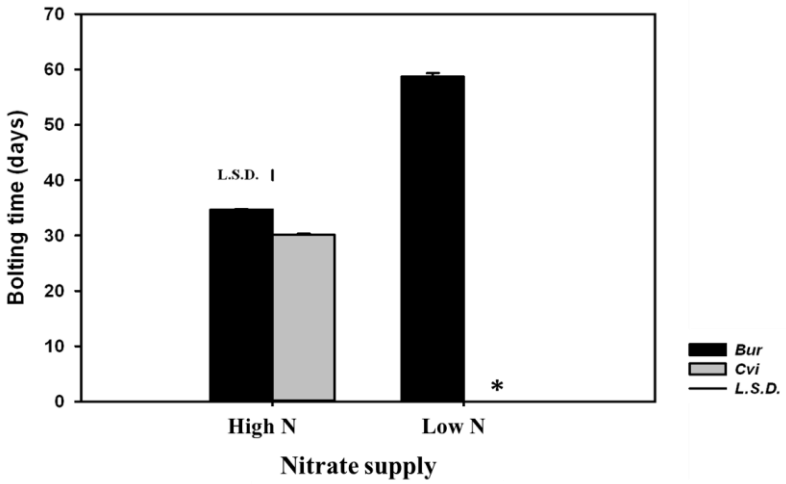

Figure 6.2 Bolting time of Bur and Cvi grown in two nitrate regimes in the glasshouse (23/17˚C, 16/8h, light /dark). Data are mean ± standard error. L.S.D bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 6.1) *Seedlings of Cvi did not survive on compost with low N.

- Seed nitrate content and germination

Nitrate content: Seed nitrate content was measured and found to be significantly ($p<0.001$) affected by both the ecotype and maternal nitrate supply (Appendix Table 6.2). Ler plants grown on high N compost produced seeds with significantly ($p<0.001$) higher nitrate content than those on low N compost (Fig.6.3). However in Bur, seeds nitrate content was not significantly different between the seeds produced in the two N regimes. Thus maternal nitrate supply produced a more significant ($p<0.001$) effect on the nitrate content in Ler seeds than that in Bur seeds.

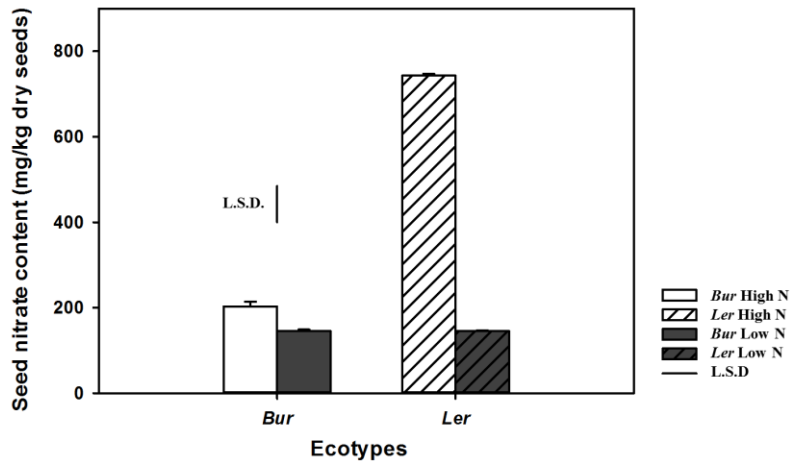

Figure 6.3 Nitrate content in Bur and Ler seeds (mg/kg dry seeds) produced in two nitrate regimes in the glasshouse (23/17˚C, 16/8h, light /dark). Data are mean ± standard error. The L.S.D bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 6.2).

Germination in the light: The depth of dormancy of seeds produced in different growth regimes was characterised by changes in germination sensitivity to exogenous nitrate (KNO_3) in the light (Fig.6.4). The final germination at 25˚C was significantly ($p<0.001$) different between Bur and Ler seeds produced under both nitrate regimes. However at 10˚C, final germination of Bur and Ler seeds was greater than 95% (Fig.6.4). Bur seeds were more sensitive to maternal nitrate and therefore exhibited more shallow dormancy at both temperatures. The maternal nitrate regimes had a greater impact on Ler dormancy than on Bur, i.e. Bur seeds germinated well at 25˚C, although 10mM KNO_3 delayed germination. However, Ler seeds produced in a low N regime did not germinate unless 10mM KNO_3 was applied at 25˚C (Fig.6.4).

The time to 50% germination (T_{50}) of Bur at both temperatures and Ler at 10˚C (germination was too low at 25˚C) were significantly ($p<0.001$) influenced by ecotype, maternal nitrate regime and exogenous KNO_3 at 10˚C (Table 6.4). T_{50} values decreased (i.e. germination rate increased) as KNO_3 concentration increased. The response of seeds treated with KNO_3 was significantly ($p<0.001$) different from the control in both ecotypes at 10˚C; the T_{50} value at 10˚C was also significantly

($p<0.001$) affected by the maternal nitrate regime, ecotype and the interaction between these two factors (Table 6.4). At 25˚C, the T_{50} values for Bur was significantly ($p<0.001$) lower at 10˚C with both maternal N regimes. In the presence of exogenous KNO_3, the T_{50} decreased in the presence of 1mM KNO_3, but increased at 10mM KNO_3, showing that germination was delayed by the higher concentration of exogenous nitrate.

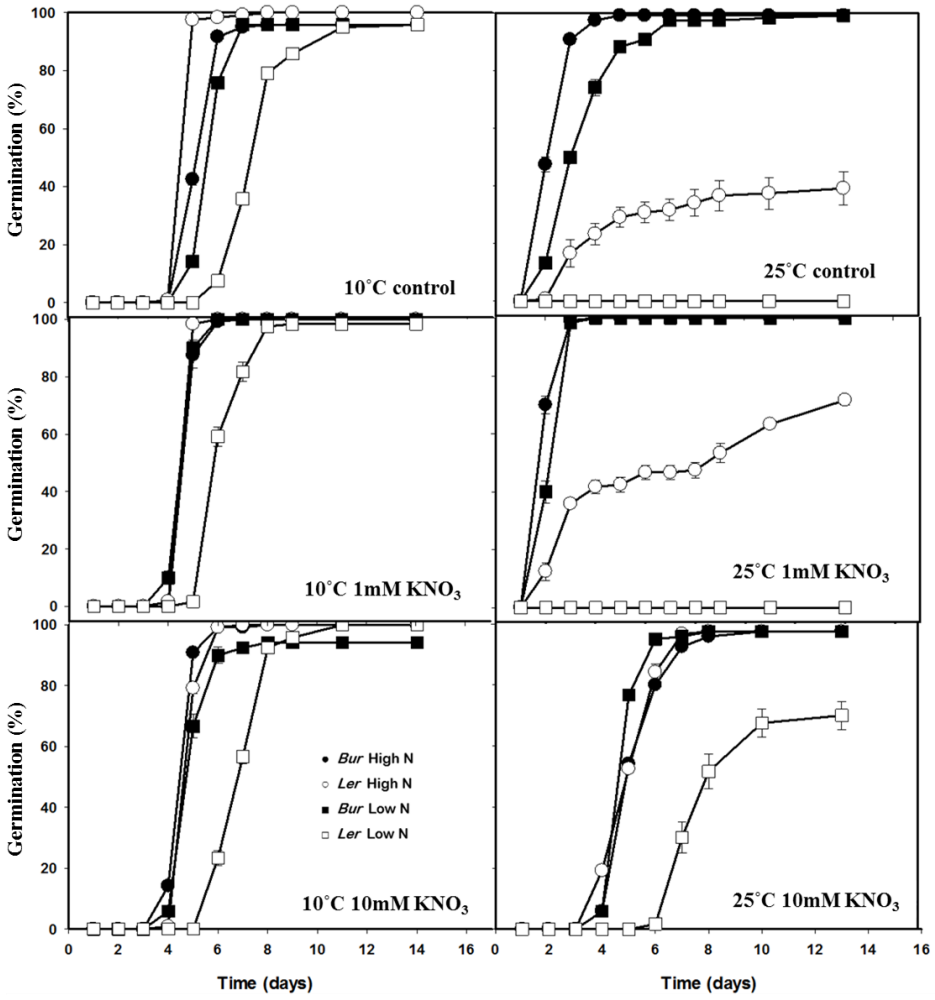

Figure 6.4 Germination responses in the light to temperature (10˚C and 25˚C) and exogenous nitrate. Data represents the mean ± standard error. No error bar indicates symbol is larger than the error (for analysis refere to Appendix Table 6.3).

Table 6.4 Mean T_{50} values for germination in the light of Bur and Ler seeds produced under high and low nitrate regimes

Ecotype	Nitrate supply	10˚C light			25˚C light		
		Water	1 mM KNO_3	10 mM KNO_3	Water	1 mM KNO_3	10 mM KNO_3
Bur T_{50} (d)	High N	5.07	4.68	4.41	2.01	1.91	2.31
	Low N	5.54	4.96	4.77	3.19	2.07	3.47
Ler T_{50} (d)	High N	4.55	4.13	4.44	-*	5.70	10.4
	Low N	7.27	6.46	6.75	-	-	-

*the Ler seeds did not reach 50% germination at 25˚C, so there was no T_{50} value (for analysis see Appendix Table 6.4a and 6.4b)

Germination in the dark: In the dark, Bur percentage germination was not significantly affected by the maternal conditions except for a reduction in germination at 25˚C in seeds from the low N regime, i.e. germination increased significantly ($p<0.001$) on the application of KNO_3 from 55.8% in the control to greater than 98%. Ler seeds from both nitrate regimes exhibited thermal dormancy at 25˚C, which was similar to that in the light. However at 10˚C, germination of Ler seeds produced with low N was significantly ($p<0.001$) lower than those with high N, indicating a positive effect for both light for germination and the maternal nitrate regime. Bur seeds had a higher sensitivity to exogenous KNO_3 and thus a more shallow dormancy than Ler seeds; whereas the nitrate regimes to mother plants had a greater impact on the dormancy of Ler (Fig.6.5). Ler seeds had higher thermal dormancy at 25˚C in the dark, even in the presence of 10mM KNO_3. By comparison, Bur seeds at 25˚C in the dark were less dormant and more sensitive to exogenous KNO_3 (1mM and 10mM), reaching 100% germination. Contrary to the responses shown in the light, Bur seeds produced with high N had significantly ($p<0.001$) higher germination on water than those produced with low N at 25˚C in the dark. Thus light has a positive role in the germination of Bur seeds at 25˚C (Fig.6.5).

At 10˚C, the T_{50} value was significantly ($p<0.001$) affected by ecotype, maternal nitrate regime and exogenous KNO_3 (Table 6.5). The maternal nitrate regime greatly affected germination rate of Ler, i.e. Ler seeds produced under a low N regime had higher T_{50} values than those from a high N regime. However, Bur seeds produced in a low N regime had a lower T_{50} values though the difference was not significant. The T_{50} values of Bur at 25˚C were significantly ($p<0.001$) lower than at 10˚C. The application of KNO_3 (1mM and 10mM) significantly ($p<0.05$) decreased T_{50} values of Bur seeds at 10˚C.

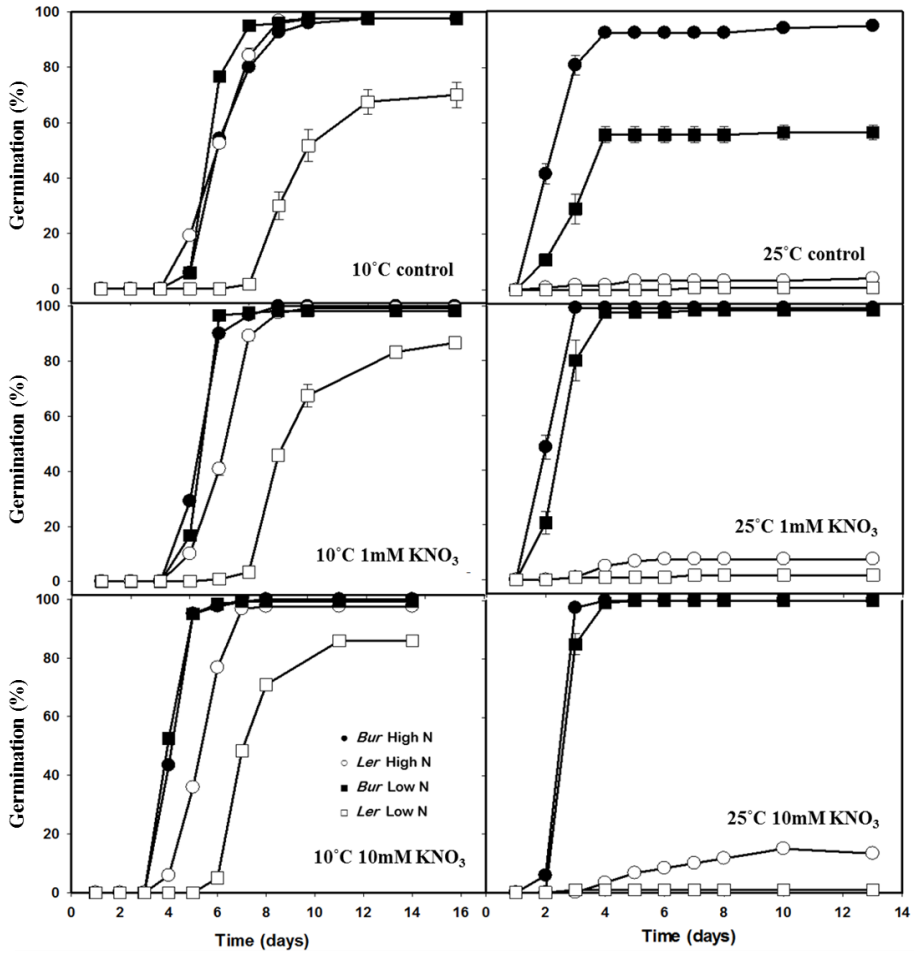

Figure 6.5 Germination responses in the dark to temperature (10˚C and 25˚C) and nitrate of seeds produced under different nitrate regimes in a glasshouse. Data are the mean ± standard error. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 6.5).

Table 6.5 The T_{50} values for germination in the dark of Bur and Ler seeds produced with high and low levels of nitrate.

Ecotype	Nitrate supply	10˚C dark			25˚C dark		
		Water	1 mM KNO_3	10 mM KNO_3	Water	1 mM KNO_3	10 mM KNO_3
Bur T_{50} (day)	High N	4.96	4.23	4.07	2.06	2.01	2.33
	Low N	4.69	4.21	3.97	2.75	2.41	1.89
Ler T_{50} (day)	High N	4.85	5.08	5.25	-	-	-
	Low N	7.33	7.07	6.95	-	-	-

*the Ler seeds did not reach 50% germination at 25˚C, so there was no T_{50} value (for analysis refer to Appendix Table 6.6a and 6.6b)

- Gene expression

The expression (NanoString nCounter gene expression system) of 28 genes involved in hormone metabolism, hormone signalling and the regulation of dormancy and germination was measured after 24h imbibition in Bur seeds produced under low and high N regimes (Fig.6.6).

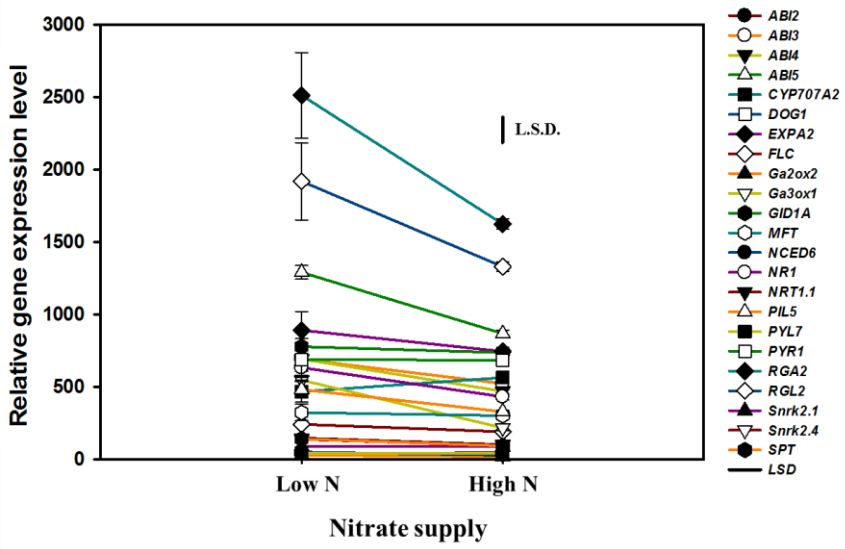

Figure 6.6 Gene expression in Bur seeds produced by plants grown in different nitrate regimes. Data are mean gene expression level ± standard error. Lines indicate increasing or decreasing levels of gene expression between nitrate treatments. L.S.D. bar indicates the significant difference at $P<0.05$ (For analysis refer to Appendix Table 6.7).

Full descriptions of the genes are provided in *Chapter 4*. A two-way ANOVA showed that there was a significant (P<0.001) differernce in expression between genes. Moreover, maternal nitrate supply had a significant (p<0.001) effect on the expression of some genes (Fig.6.6; Appendix Table 6.7).

Genes representative of GA biosynthesis and catabolism show significant (p<0.001) differences with *Ga2ox2* expression (catabolism) significantly lower than that of *Ga3ox1* (biosynthesis) (Fig. 6.7). *Ga3ox1* expression was significantly (p<0.001) higher in the seeds produced in a high N regime. However, expression of *Ga2ox2* and *GID1A* (GA receptor) was not significantly different between the N regimes. The two DELLA genes (negative regulators of germination), *RGA2* and *RGL2*, showed significantly (p<0.001) higher levels of expression in seeds produced in a low N regime (Fig.6.7), which was consistent with their siginificantly slower germination (higher T_{50}; Table 6.4 and 6.5).

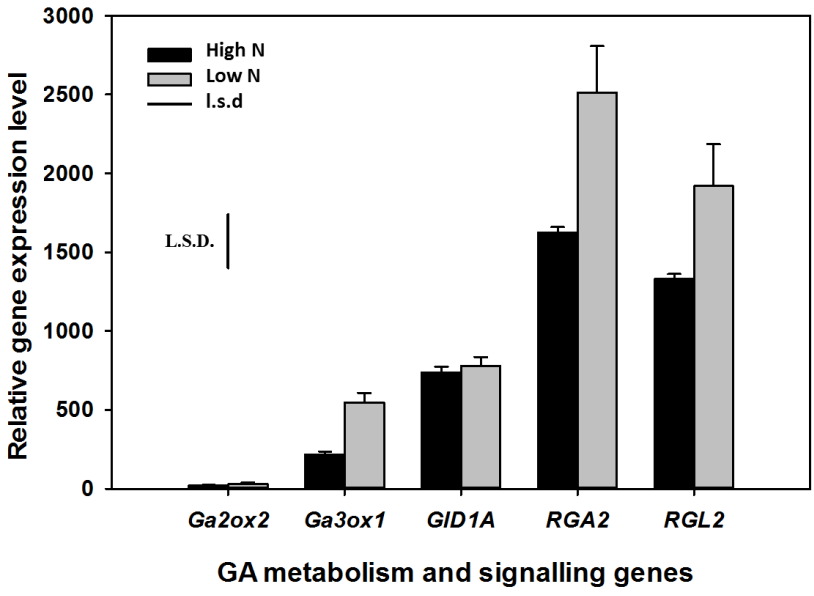

Figure 6.7 Expression of GA metabolism and signalling related genes in seeds produced by plants grown under different nitrate regimes. Data represent the mean ± standard error. L.S.D. bar indicates the least significant difference at the P = 0.05 level. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 6.8).

Genes representative of ABA biosynthesis and catabolism also show significant ($p<0.001$) differences in expression. *CYP707A2* (catabolism) had a significantly ($p<0.001$) higher level of expression than *NCED6* (biosynthesis) (Fig.6.8). However, the difference between the seeds produced in different maternal nitrate regimes were not significant. The ABA-induced transcription factors *ABI3*, *ABI4* and *ABI5* showed high levels of expression and were significantly ($p<0.001$) higher in the seeds from the low N regime (Fig.6.8). This significantly higher level of expression in seeds from the low N over the high N, is again consistent with the slower germination response of the seeds produced with low N, particularly at 25˚C (Table 6.4 and 6.5). The ABA receptor gene, *PYL7*, repressor of ABA signaling *ABI2*, and the two members of SNF1-related protein kinase subfamily, *SnRK2.1* and *SnRK2.4* (positive regulators of ABA signalling) all had low levels of expression, with no significant differences between the seeds produced in different nitrate regimes.

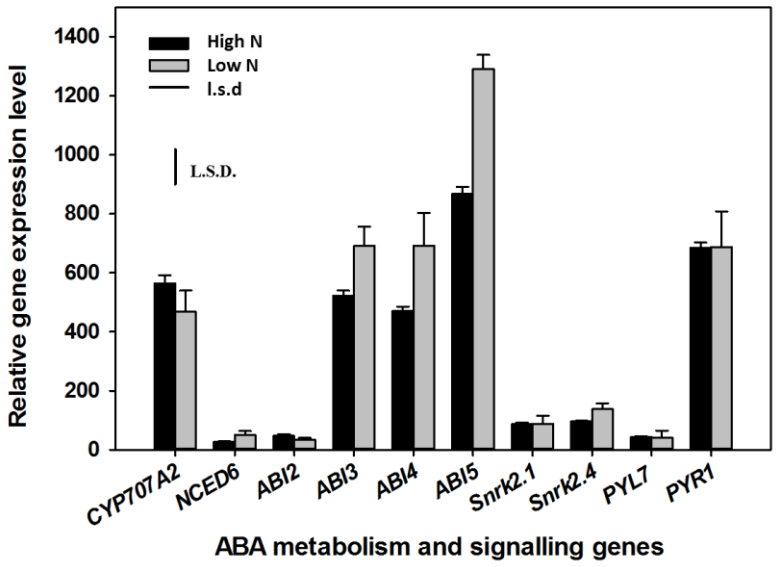

Figure 6.8 Expression of ABA metabolism and signalling related genes in seeds produced by plants grown under different nitrate regimes. Data represents the mean ± standard error. L.S.D. bar indicates the least significant difference at the P = 0.05 level. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 6.9)

There was also significant ($p<0.001$) differences in the expression of the other dormancy/germination related genes studied (Fig.6.9). *DOG1* is the gene at the loci with the strongest dormancy association in QTL analyses (Bentsink *et al*., 2006), thus the relatively low level of expression of *DOG1* is consistent with low level of dormancy of seed produced in both N regimes. *MFT* is a proposed negative regulator of ABA signalling and promotes embryo growth in germinating seeds (Xi *et al*., 2010) but is also proposed to be a positive regualtor of ABA signalling (Nakamura *et al*., 2011). That expression is similar in seeds from both N regimes is again consistent with low doramncy.

Both nitrate reductase 1 (*NR1*) and nitrate transporter 1.1 (*NRT1.1*) expression in the seeds from a high N regime was higher than those from a low N regime (Fig.6.9). This demonstrates that the maternal nitrate supply may have an impact on seed sensitivity to nitrate and thus seed dormancy level.

Expression of the flowering-time regulator *FLC* was positively correlated with seed germination levels at low temperature (Chiang *et al*., 2009). The expression here showed a relatively high level in line with the high germination potential of Bur seeds. However, *FLC* expression was higher in seeds from the low N regime, which is contrary to their germination behaviours (Fig.6.9). This effect may be counterbalanced by the high expression levels of the phytochrome interacting factor (PIF) family members *SPT* and *PIL5* that repress the expression of genes related to increased germination potential (Penfield *et al*., 2005; Oh *et al.*, 2009). Thus the higher expression levels of *SPT* and *PIL5* in seeds from the low N regime is consistent with their lower germination potential.

The *HDZip* (*ATHB20*) gene is involved in ABA sensitivity and acts as a negative regulator of seed germination (Barrero *et al*., 2010). The expression of

ATHB20 was significantly ($p<0.001$) lower than other genes, which is consistent with the shallow dormancy in Bur seeds (Fig.6.9). Again consistent with low dormancy, the germination up-regulator *Expansin A2* (*EXPA2*) showed relatively high levels of expression in Bur seeds produced in both N regimes (Fig.6.9).

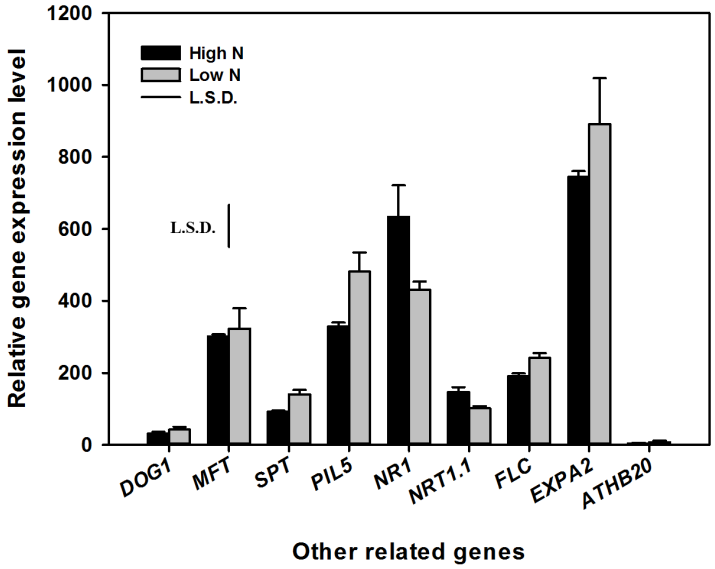

Figure 6.9 Expression of dormancy related genes in seeds produced by plants grown under different nitrate regimes. Data represents the mean ± standard error. The L.S.D. bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 6.10)

6.3.2 Effect of temperature and nitrate supply on vegetative growth, seed production and germination of *Arabidopsis* ecotypes

More Bur and Ler plants were grown to determine the effect of temperature, nitrate supply and the interaction between these two factors on vegetative growth, seed production and germination. Ler was a substitution for Cvi as this ecotype failed to survive on compost with low N. These plants were grown in two temperature-controlled environments, 15/15˚C (12/12h, light /dark) and 20/20˚C (12/12h, light /dark), and supplied with different levels of nitrate (high N and low N). Ler bolted significantly ($p<0.001$) earlier than Bur at both temperatures, which was also significantly ($p<0.01$) influenced by maternal nitrate supply (Table 6.6).

Table 6.6 Summary of ANOVA results to determine the effect of ecotype, temperature and nitrate supply on the transition from vegetative to reproductive growth (bolting)*

Source of variation	F pr of different variates		
	Rosette diameter	**Leaf Number**	**Bolting time**
Ecotype	<.001	<.001	<.001
Temperature	0.035	<.001	<.001
Nitrate supply	<.001	<.001	0.786
Ecotype × Temperature	<.001	<.001	<.001
Ecotype × Nitrate supply	<.001	<.001	0.032
Temperature ×Nitrate supply	<.001	0.654	0.009
Ecotype×Temperature× Nitrate supply	0.163	0.915	0.783

*For analysis refer to Appendix Table 6.11

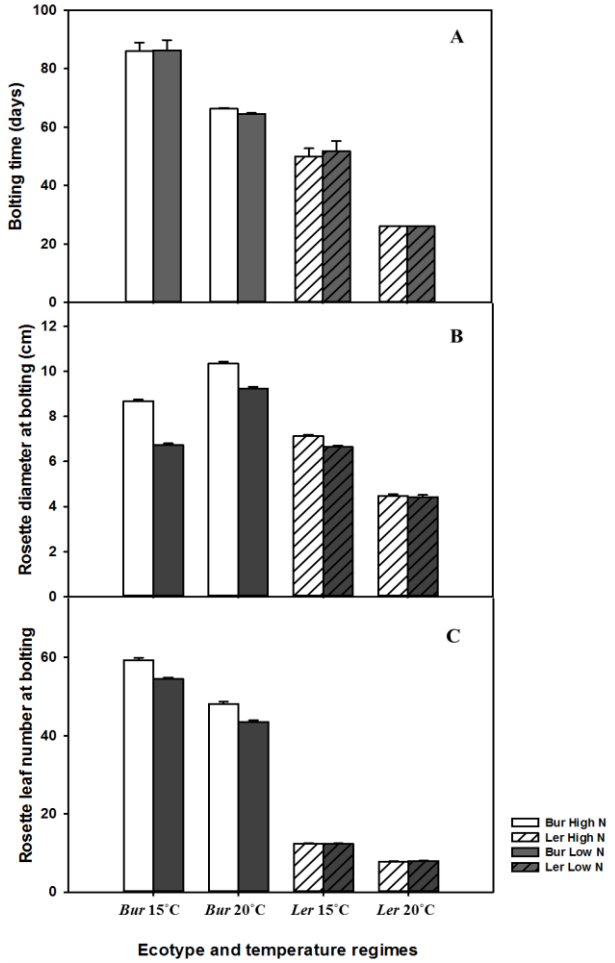

Figure 6.10 Transition from vegetative to reproductive growth (bolting) was affected by temperature and nitrate. A: mean bolting time; **B**: mean diameters of rosette; **C**: mean leaf number at bolting; **15˚C**: 15/15˚C, 12/12h, light /dark; **20˚C**: 20/20˚C, 12/12h, L/D. Data represents the mean ± standard error. No error bar indicates symbol is larger than the error (for analysis see Appendix Table 6.11).

In addition to the differences in bolting time, rosette size and leaf number, the effects of temperature and nitrate supply had a noticeable effect on reproductive growth in both Bur and Ler (Fig.6.11).

Bur and Ler had larger rosettes when supplied with high N than with low N, and in addition the rosette leaves of Bur with low N turned reddish-brown in the 20˚C regime (Fig.6.11C). The effect of temperature can also be seen in both ecotypes, especially in Ler as those plants grown at 15˚C had just started to bolt, whereas those at 20˚C had already finished flowering and begun to develop siliques (Fig.6.11B, D).

Figure 6.11 Bur and Ler plants grown at 15 and 20˚C on high and low N compost 40 days after sowing. A: Bur plants at 15/15˚C (12/12h, L/D); **B**: Ler plants at 15/15˚C (12/12h, L/D); **C**: Bur plants at 20/20˚C (12/12h, L/D; **D**: Ler plants at 20/20˚C (12/12h, L/D).

Interestingly, when compared with the bolting data from *sec 6.3.1*, it is noticeable that Bur plants grown in long-day conditions (16/8h, light /dark) bolted

significantly ($p<0.001$) earlier than those in day neutral conditions (12/12h, light /dark) at the same mean temperature (20˚C) (Appendix Table 6.12). Moreover, this significant effect of long-day photoperiod in accelerating bolting of Bur was more noticeable when plants were grown on high N (Fig.6.12), indicating an interaction between maternal nitrate regime and photoperiod on bolting time.

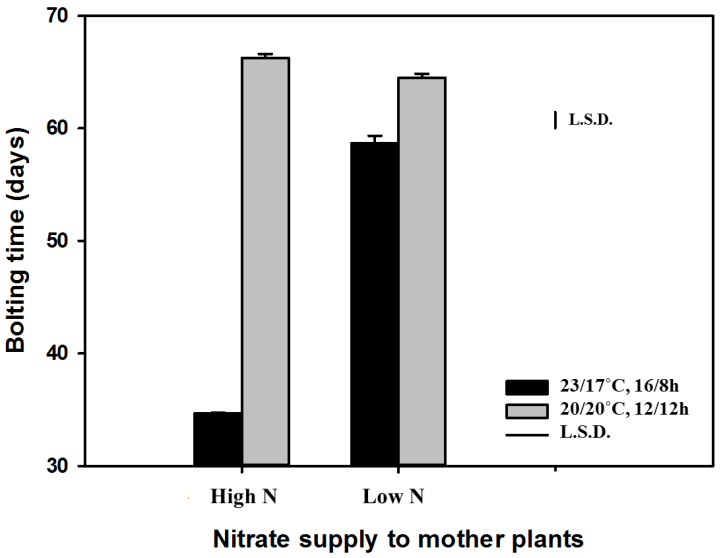

Figure 6.12 Bolting time of Bur plants grown under different temperature and light conditions on high and low N. Data are the mean ± standard error. L.S.D. bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 6.12).

- **Seed production**

At the end of the reproductive phase seeds were harvested from each plant and seed yield was determined (Fig.6.13). In Bur, seed yield per plant was significantly ($p<0.001$) higher at 15˚C than at 20˚C, and moreover at each temperature regime, plants in the high N regime produced significantly ($p<0.001$) higher yields per plant (Appendix Table 6.13). In Ler, however, seed yield per plant did not decline significantly at the higher temperature under high N, but under low N seed yield increased at 20˚C compared to 15˚C (Fig.6.13A). Seed size was calculated in terms of 1000-seed weight, and this also showed an impact of temperature rather than nitrate regime. Overall, Bur plants produced significantly ($p<0.001$) larger seeds than

Ler in both growth regimes (Fig.6.13B). Bur plants produced larger seeds when plants were grown with low N at both 15 and 20˚C, whereas low N led to larger Ler seeds only at 20˚C (Fig.6.13B).

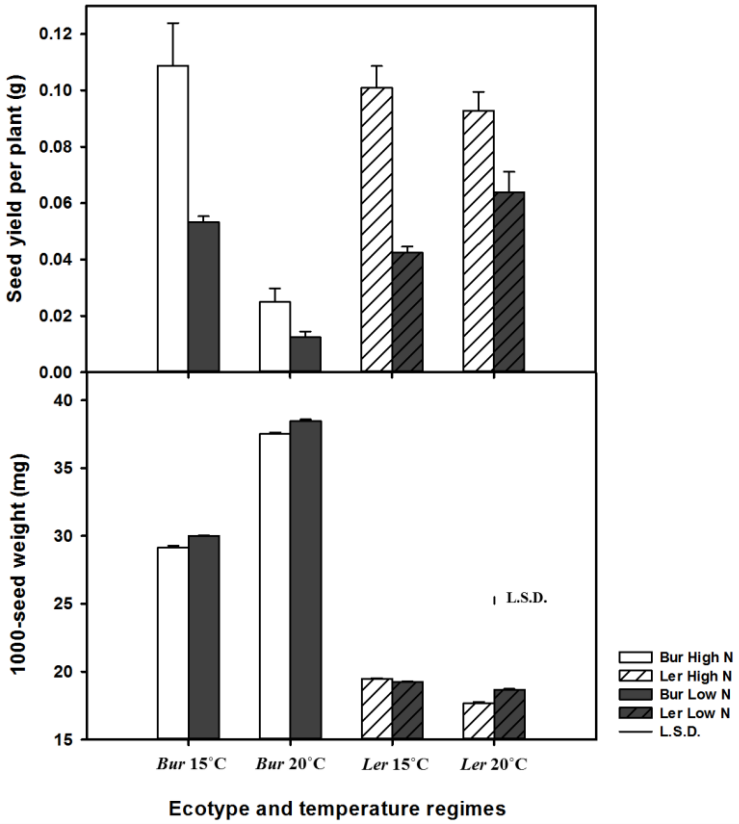

Figure 6.13 Seed yield and seed size of Bur and Ler at 15 and 20˚C on high and low N. A: mean seed weight per plant; **B**: mean 1000-seed weight (**15˚C**: 15/15˚C, 12/12h, L/D; **20˚C**: 20/20˚C, 12/12h, L/D). Data are the mean ± standard error. L.S.D value indicates the least significant difference at the P = 0.05 level. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 6.13).

- Seed nitrate content

Both temperature and N regime had significant effects on seed nitrate content ($p<0.001$ and $p<0.05$ respectively; Appendix Table 6.1). In Ler, seed nitrate content increased dramatically in plants grown at 20˚C compared to 15˚C, especially in the high N regime (Fig.6.14). However, it was a different situation in Bur seeds that had higher nitrate content when produced with high N at 15˚C; in contrast, when grown at 20˚C seeds from low N had higher nitrate content. In general, the maternal nitrate

regime had a more significant ($p<0.01$) effect on the nitrate content in Ler than in Bur seeds (Fig.6.14).

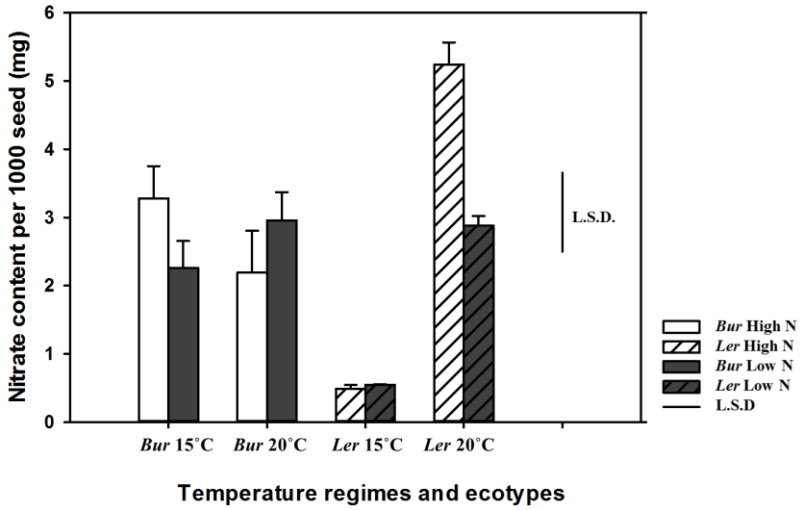

Figure 6.14 Nitrate content per 1000 seeds in Bur and Ler seeds produced at 15 and 20˚C on high and low N (15˚C: 15/15˚C, 12/12h, L/D; **20˚C**: 20/20˚C, 12/12h, L/D). Data are the mean ± standard error of the mean. L.S.D bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 6.14). No error bar indicates symbol is larger than the error.

- Seed germination and nitrate sensitivity

Germination response at a range of temperatures (5 to 30˚C) was also evaluated. Temperature during plant growth (15˚C and 20˚C) had a significant effect ($p<0.001$) on subsequent germination behaviour (Table 6.7). Final germination was significantly different between Bur and Ler except at 15˚C (Table 6.7).

Ler seed produced at 15˚C exhibited increased dormancy with increasing temperatures (Fig.6.15A); seeds produced at 20˚C were less dormant (higher final germination) when imbibed at 15˚C or lower, but also exhibited high temperature thermo-dormancy (Fig.6.15B). Previously, Bur seeds were shown to germinate to high levels at higher temperature (see *Chapter 4*) and the results here for seeds produced at 20˚C confirm this (Fig.6.15D). However, when produced at 15˚C Bur seeds exhibit thermo-dormancy similar to Ler seeds (Fig.6.15C).

Table 6.7 Summary of ANOVA results for the effect of ecotype, temperature, nitrate supply to mother plants and their interaction on final germination of the seed produced

Source of variation	F pr.					
	5˚C	**10˚C**	**15˚C**	**20˚C**	**25˚C**	**30˚C**
Ecotype	<.001	0.016	0.217	<.001	<.001	<.001
Maturation temperature	<.001	<.001	<.001	<.001	<.001	<.001
Nitrate supply	0.028	0.465	0.207	0.699	0.316	0.222
Ecotype × maturation temperature	<.001	0.002	0.579	<.001	<.001	<.001
Ecotype × Nitrate supply	0.122	0.622	0.9	0.096	0.107	0.222
Maturation temperature × Nitrate supply	0.116	0.122	0.083	0.824	0.228	0.222
Ecotype × Maturation temperature × Nitrate supply	0.052	0.301	0.897	0.319	0.812	0.222

Nitrate regime did not have a significant effect on germination of either ecotype, with the exception of Bur seeds germinated at 5˚C, where seeds from the low maternal N regime germinated significantly ($p<0.05$) higher than those from the high N regime (Fig.6.15C). Bur seeds produced at 20˚C germinated to 80% or higher at all temperatures regardless of the nitrate regime during seed production; whereas Bur seeds produced at 15˚C germinated to higher than 50% only at 5 and 10˚C with seeds from the low maternal N regime having higher germination than those from the high N regime (Fig.6.15). The depth of dormancy was characterised by changes in germination sensitivity to exogenous nitrate (1mM KNO_3). Ler seeds matured at 15˚C responded to exogenous KNO_3 with higher germination at all but the highest temperature (Fig.6.15A). In contrast, Ler seeds matured at 20˚C were less dormant and the temperature range for germination was broadened with 100% germination at the temperatures ranging from 5 to 20˚C (Fig.6.15B). However, 1mM KNO_3 was insufficient to release high temperature thermo-dormancy. In contrast, Bur seeds

produced at both temperatures (15 and 20˚C) were more sensitive to KNO_3 and consistent with a more shallow dormancy than Ler seeds.

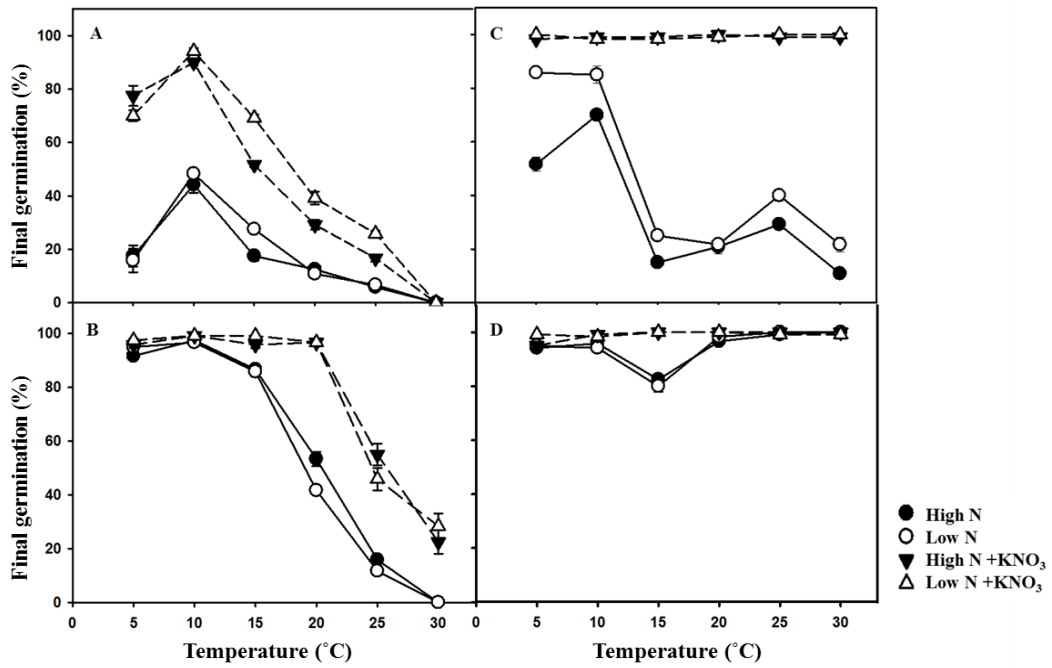

Figure 6.15 Germination responses of Bur and Ler seeds to temperatures (5-30˚C) and exogenous nitrate (1mM KNO_3) produced at 15 and 20˚C on high and low N compost. A: Ler matured at 15˚C; **B**: Ler matured at 20˚C; **C**: Bur matured at 15˚C; **D**: Bur matured at 20˚C; **15˚C**: 15/15˚C, 12/12h, light /dark; **20˚C**: 20/20˚C, 12/12h, light /dark). Data are the mean ± standard error. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 6.15).

In addition, A three-way ANOVA indicated that final germination was significantly ($p<0.001$) affected by ecotype, imbibition temperature and seed maturation conditions (Appendix Table 6.16). For both Bur and Ler ecotypes, lower temperature (15˚C) during their production enhanced seed dormancy (Fig.6.16). Although Ler seeds produced at both growth temperatures exhibited thermal dormancy, long day conditions (16/8h, light /dark) during plant growth reduced seed dormancy levels compared to the day neutral conditions (12/12h, light /dark) (Fig.6.16); whereas day length did not show a significant effect on dormancy level in Bur seeds produced at a mean temperature of 20˚C. This suggests a predominant role

in for temperature during maturation in determining dormancy status in Bur (Fig.6.16).

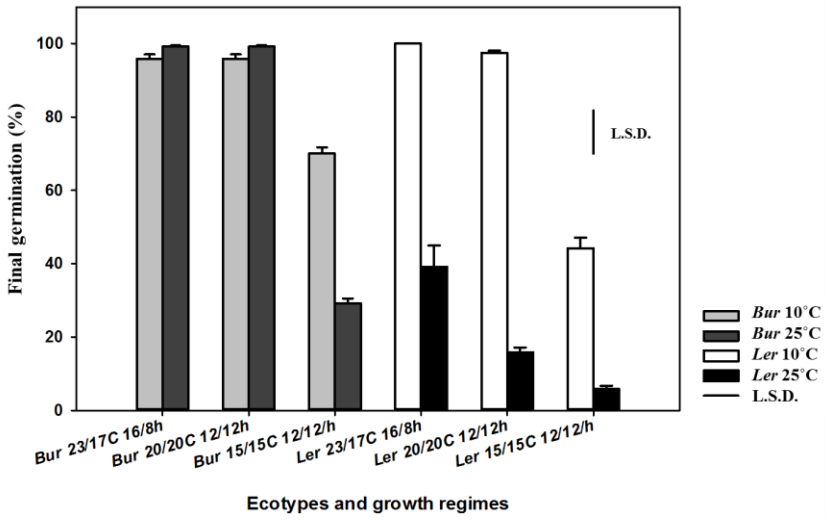

Figure 6.16 Final germination percentages at 10˚C and 25˚C of Bur and Ler seeds produced from different growth regimes on compost with high N. Data are the mean ± standard error. L.S.D bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 6.16).

6.4 Discussion

The timing of the transition from vegetative to reproductive growth (bolting) is dominated by temperature, with temperature and nitrate interacting during seed production to determine subsequent seed behaviour

There are two main phase transitions in the life cycle of plants, the transition from a seed to a seedling (germination) and the transition from the vegetative to reproductive phases of growth (bolting). Here the impact of temperature and nitrate on both phase transitions were investigated in two contrasting *Arabidopsis* ecotypes. The initiation of both germination and bolting is controlled by complex interactions between endogenous genetic programmes and environmental factors such as temperature, light and nutrient availability (Werner *et al*., 2005; Marín *et al*., 2011). There is a wide range of natural variation found for bolting time among *Arabidopsis* ecotypes (Nordborg and Bergelson, 1999). This variation is determined at the

molecular level by allelic variation at the *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) loci. These are major determinants of flowering time in *Arabidopsis* with natural variation arising through the generation of non-functional or weak *FRI* and *FLC* alleles (Gazzani *et al*., 2003; reviewed in Werner *et al*., 2005). In vernalisation requiring ecotypes, FRI functions to up-regulate expression of FLC, which is a floral repressor (Michaels and Amasino, 1999). The results presented here show that the ecotypes Bur, Cvi and Ler, have significantly different bolting times. The Bur ecotype was found to bolt much later than either Cvi or Ler. This is in agreement with earlier reports that both Cvi and Ler could be categorized as early flowering ecotypes with Bur as a late flowering ecotype (Gazzani *et al*., 2003; Werner *et al*., 2005). Both Ler and Cvi have a different molecular basis for early flowering. In Ler the *FRI* allele contains a deletion that disrupts the open reading frame while *FLC* contains a novel non-autonomous *Mutator*-like transposon in the region required for normal *FLC* regulation making a weak *FLC* allele (Gazzani *et al*., 2003; Johanson *et al*., 2000); whereas the *FRI* allele is inactive in Cvi due to an in-frame translation stop codon. In addition, enhanced activity of CRY2 (CRYPTOCHROME2), a blue-light photoreceptor, was found to function by activating downstream floral pathway integrators thus bypassing the floral repression by FLC (Gazzani *et al*., 2003; Simpson and Dean, 2002). The late-flowering phenotype of Bur is independent of FLC due to the null mutant *FLC* allele (Werner *et al*., 2005). Furthermore, delayed flowering QTLs in Bur were located in the region of two circadian rhythm genes suggesting the short circadian period of Bur may be related to its flowering-time phenotype (Werner *et al*., 2005). In Bur, the effect of nitrate supply on bolting time was found to interplay with the environmental factors temperature and photoperiod. Plants bolted in both nitrate regimes earlier at higher temperature (20/20˚C, 12/12h,

L/D) than at lower temperature (15/15˚C, 12/12h, L/D) indicating the predominant role of temperature in bolting time for this ecotype. However, when grown at a mean temperature of 20˚C, Bur plants in long-day conditions (16/8h, light /dark) bolted earlier than in day neutral conditions (12/12h, light /dark). This significant effect of long-day photoperiod in accelerating bolting of Bur was more noticeable when plants were grown on high N compost. Whereas, in the day neutral conditions, Bur plants showed less sensitivity to nitrate levels, indicating an interaction between maternal nitrate supply and photoperiod on bolting time.

In day neutral conditions, there was no significant effect of nitrate on bolting at either production temperature (15˚C and 20˚C). However, nitrate supply had a large effect on vegetative growth up to the point of bolting suggesting that rosette leaf number and bolting time are not directly linked. Rosette leaf number has been positively correlated with the bolting and flowering times in *Arabidopsis* ecotypes from Eurasia (Samis *et al*., 2012). In Bur, day neutral conditions at 20˚C stimulated reproductive growth (i.e. early bolting), leaving shorter time for vegetative growth and thus less leaves in the rosette at bolting; but high temperature also favoured vegetative growth by promoting rosette leaf expansion, resulting in larger rosettes. In Ler, high temperature promoted reproductive growth as in Bur, but both rosette diameters and leaf numbers were smaller. The acceleration of bolting by high temperature is more marked in Bur where although bolting time is reduced vegetative growth is accelerated.

The maturation temperature and nitrate supply to mother plants impacts on seed production in an ecotype-dependent manner

Seed yield and seed size were influenced by nitrate supply and temperature during maturation.

Temperature: seeds produced from Bur plants showed a decrease in seed yield with an increase in temperature from 15˚C to 20˚C, in agreement with data presented in Chapter 3 showing that exposure to higher temperature resulted in decreased seed yield. This reduction was shown to result from poor stamen extension under higher temperature, which reduces pollination. In the present work this decrease in seed yield was seen in Bur plants grown in both N regimes, but not in Ler. Therefore, as in Cvi 20˚C is not a 'detrimental' temperature for Ler during reproductive development as it is for Bur.

Increasing temperature (5-20˚C) during reproductive development in two grain legume species (i.e. pea and soybean),had a negative effect on the cotyledon cell number and seed filling, leading to smaller seed size (Munier-Jolain and Ney, 1998). This was the case in Ler, as smaller seeds were produced at 20˚C compared to 15˚C. However, the negative effect of higher temperature on seed size was reversed in Bur, i.e. significantly larger seeds were produced at 20˚C. Based on a model built by Smith and Fretwell (1974), seed size has been considered to present a fundamental trade-off between producing more small seeds versus fewer large seeds from a given quantity of resource allocated to reproduction. Since higher temperature experienced by the mother plants significantly reduced fertility and subsequent seed yield in Bur, the larger and better-provisioned seeds may be a strategy to increases the chances of successful seedling establishment.

Nitrate supply: environmental conditions especially that of nitrogen in the soil influence seed yield and yield components in rapeseed (Oskouie, 2012). A higher nitrate supply to the mother plant led to higher seed yield per plant in both Bur and Ler, in agreement with previous observations in *Arabidopsis* by Schulze *et al.* (1994). This indicates a positive influence of nitrate supply on seed yield and also an

association between nitrogen remobilization from vegetative tissue to seed and the final seed yield. Limitations to final seed yield are often found in either the source of assimilates (photosynthesis) or in the sink (the site of assimilate utilization). Thus two processes should be considered when studying the final seed yield, i.e. the production of assimilate in the leaves and its utilization during seed development. In the present work, reduced seed yield was found when plants were grown in the low N regime, which was probably due to a source limitation, i.e. smaller rosette and less leaves at bolting under N-limiting conditions resulted in a corresponding decrease in seed number and thus seed yield.

When plants were grown on high N, leaf nitrate concentration was higher in leaves during the vegetative phase (rosette) than during flowering, but these storage pools did not contribute to the bulk flow of resources to seeds (Schulze *et al*., 1994). However, stored resources in rosette leaves were remobilized and made a large contribution to seed biomass under N-limiting conditions. Thus at low N, most of the nitrogen assimilated before and post-flowering was allocated to the seeds, whereas at high N, most of the nitrogen taken up was stored in rosettes and subsequently lost in the dry remains (Masclaux-Daubresse and Chardon, 2011).

The trade-off between seed yield and seed size may contribute to the formation of large Bur seeds seen in the current study. Source-sink alterations are seen to impact on seed size in soybean (Egli and Bruening, 2001). A reduced maternal nitrate supply in Bur led to the production of larger seeds, partially due to high nitrogen remobilization efficiency of Bur at low N rather than at high N (Chardon *et al*., 2010; Masclaux-Daubresse and Chardon, 2011). Nitrogen remobilization is favoured under limiting nitrate supplies, and the increasing nitrogen remobilization has the advantage of re-using nitrogen from the vegetative parts and of lowering

nitrogen loss in the dry remains (Masclaux-Daubresse *et al*., 2010). Therefore the dry plant weight at harvest is also an important component to determine the nitrogen remobilization efficiency and also the impact of source-sink alternations on final seed production. Further studies on the nitrate supply to the mother plants on seed production will also be shown in the next chapter illustrating the relationship between aboveground biomass and seed production components (*Chapter 7*).

Nitrate supply to mother plants affects seed dormancy by interacting with other environmental signals; Bur seeds have higher sensitivity to nitrate than Ler seeds

Seed dormancy is influenced by many factors including maternal growth conditions (McCullough and Shropshire, 1970; Kendal *et al*., 2011), seed storage conditions and environmental/chemical treatments to the seeds (Grey and Thomas, 1982; Hilton, 1984; Bewley, 1997; Finch-Savage *et al*., 2007). Seed germination stimulated by nitrogen compounds (e.g. nitrate) has been documented in several species, such as *Chenopodium album* (Saini *et al*., 1985; Tang, *et al*., 2008), *oryza sativa* (Cohn *et al*., 1983), *Avena fatua* (Mclntyre *et al*., 1996), *Sisymbrium officinale* and *Arabidopsis* (Hilhorst and Karssen, 1988, 1989; Ali-Rachedi *et al*., 2004; Cadman *et al*., 2006; Finch-Savage *et al*., 2007). Nitrate is thought to release seed dormancy by altering the ABA/GA hormone balance through changes in hormone synthesis and signalling (Alboresi *et al*., 2005). The latter reported that nitrate accelerates the decrease in ABA level during seed imbibition and prevents ABA synthesis; nitrate may also enhance GA synthesis or signalling.

Exposure of seeds to exogenous KNO_3 released dormancy of Ler seeds in a dose-dependent manner, especially at 25˚C, the efficacy of this response to nitrate in releasing dormancy was light dependent. In Bur, exposure of seeds to exogenous KNO_3 completely released thermo-dormancy even in the absence of light, indicating

the shallow dormancy of Bur compared to Ler. Interestingly, Bur seeds treated with 10mM KNO_3 germinated 2 days later than those treated with 1mM KNO_3 at 10˚C, consistent with the poor tolerance of Bur under high nitrate nutrition as reported elsewhere (Chardon *et al*., 2010).

Gene expression levels were measured in Bur seeds produced in the glasshouse (23/17˚C, 16/8h, L/D) under both N regimes. *DOG1* was expressed at low levels in seeds from both regimes, consistent with shallow dormancy. *ATHB20*, a gene involved in ABA sensitivity during seed germination also had significantly lower levels than other genes, again consistent with shallow dormancy. In contrast, there was significantly higher expression of five genes in seeds produced under the low N regime: three ABA signalling genes *ABI3*, *ABI4*, *ABI5* and two DELLA genes *RGA2* and *RGL2* (negative regulators of seed germination). This was consistent with the higher dormancy of seeds produced under the low N regime.

Maturation temperature plays a predominant role in nitrate content and germination behaviour of the Arabidopsis seeds

Growth of plants at high nitrate levels is related to higher seed nitrate content (Alboresi *et al*., 2005). However, in this study, only Ler seeds produced under high N at 20˚C had a significantly higher nitrate content. In general, increasing maturation temperature increased the nitrate content of Ler seeds and enhanced the difference in seed nitrate content between seeds produced under different nitrate regimes. This indicates that temperature does play an important role in determining the seed nitrate content in Ler. However, in Bur seeds nitrate content decreased as maturation temperature increased when produced in a high maternal N regime. This may be because at lower temperature plants accumulate more nitrogen from the substrate during their longer life cycle. In seeds produced from the low maternal N regime,

seed nitrate increased with temperature potentially as a result of higher nitrogen assimilation (i.e. larger rosette) of plants at 20˚C than at 15˚C. This reflects the observation that under low maternal N conditions stored resources in the rosette leaves made the major contribution to seed biomass (Masclaux-Daubresse and Chardon, 2011). Moreover, nitrate supply to mother plants did not show a significant effect on nitrate content of Bur seeds. A possible reason for this could be the high nitrogen use efficiency of Bur (Chardon *et al*., 2010).

Lower dormancy of *Arabidopsis* seeds is associated with high nitrate supply to mother plants and consequently a high seed nitrate content (Alboresi *et al*., 2005). However, in this study, seed maturation temperature generally showed a more predominant effect on dormancy in both Bur and Ler seeds. For example, seeds matured at a mean temperature of 20˚C in day neutral conditions (12/12h, light /dark) and long-day conditions (16/8h, light /dark), when imbibed at 10˚C did not differ significantly in the final germination compared to seed matured at 15˚C under day neutral conditions (Fig.6.16). Germination of Ler seeds produced at 20/20˚C had a similar pattern to that of Cvi seeds (results in *Chapter 4*) in response to temperature, with high germination at low temperatures coupled to high temperature thermo-dormancy. In contrast, Bur seeds produced in 20/20˚C showed higher germination at high temperature. Interestingly low maturation temperature (15/15˚C) led to high temperature thermo-dormancy in Bur seeds, but they still remained highly sensitive to exogenous nitrate (Fig.6.15). Therefore in nature, Bur seeds matured at the normally low temperature in the Burren could remain relatively dormant and enter the seed bank, but stay highly sensitive to environment signals that release dormancy. For example, following stratification by cold winter temperatures seeds may fully germinate when exposed to light and nitrate. Sensitivity to nitrate is likely

to be an important spatial indicator for the presence of a suitable substrate for subsequent growth in the limestone pavement of the Burren. If seeds matured at temperatures as high as 20˚C they would lose dormancy quickly and have a reduced ability to overwinter in the soil seed bank under natural conditions. This may result in the risk of seedling establishment into an unfavourable environment.

6.5 Conclusions

The results presented show that Bur seeds have higher sensitivity to nitrate than Ler seeds. Exogenous nitrate provided during imbibition can completely release thermo-dormancy in Bur, leading to full germination even in the absence of light. Nitrate provided exogenously during imbibition releases dormancy of Ler seeds in a dose-dependent manner, and interacts positively with light. The timing of the transition from vegetative to reproductive growth is controlled by interactions between endogenous genetic programmes and environmental conditions. The effect of nitrate supply on bolting time is largely influenced by other environmental factors, such as temperature and photoperiod. Both seed production and seed germination are greatly affected by nitrate supply to the mother plants; however temperature during seed maturation plays the predominant role in determination of subsequent seed behaviour. Picó (2012) provides evidence that the two phenotypes, i.e. winter and summer annual life cycles, can occur in the same population with their proportions able to change systematically along an altitude gradient. The associated changes in maturation temperature along the gradient may alter depth of dormancy and therefore their behaviour from summer to winter annuals. More studies on the impact of a warmer climate on the life history of plants, such as regeneration from seeds, plant growth and reproduction in the context of global warming will be shown in the next chapter (*Chapter 7*).

CHAPTER 7: THE EFFECT OF GLOBAL WARMING ON PLANT LIFE HISTORY AND SUBSEQUENT SEED DORMANCY STATUS

7.1 Introduction

7.1.1 Status of global warming

The global climate is changing as a result of human behaviour and this is likely to have major impacts in the future. However, there is a large and increasing body of evidence that negative changes have already occurred such as increased sea level, increased ice melt, change in precipitation and increased extreme events presented by the Intergovernmental Panel on Climate Change (IPCC). Arguably most importance is the increase in global temperature, the largest change with probably the most severe consequences.

Based on over 29,000 data sets showing the changes in biological and physical systems, the IPCC reported that 89% are consistent with changes caused by global warming (IPCC, 2007). Therefore global warming, which is characterised by increasing atmospheric levels of greenhouse gases such as carbon dioxide coupled with an increase in global mean temperature, has become a worldwide concern according to the Fourth Assessment Report by IPCC (Kipp, 2007). An increase in mean global temperature of 0.6±0.2˚C was observed during the 20th century (IPCC, 2007). The period from 1950 to 2000 was likely the warmest 50-years of the last 1300 years in the Northern Hemisphere and the mean temperature during that time increased twice as fast as in the previous 100 years (IPCC, 2007). The linear trend fitted to the last 25, 50, 100 and 150 years showed greater slopes for the most recent periods demonstrating an acceleration of global warming (Fig.7.1). One of the purposes of the IPCC is to predict the future of climate change. The predicted temperatures varied widely depending on the different climate models and societal

scenarios (Table 7.1). For the best case scenario, i.e. scenario for sustainably developing society (B1), a 1.8˚C rise was predicted (range 1.1-2.9˚C) by 2090 to 2099 compared to 1980 to 1999; whereas for the worst case scenario, i.e. scenario for a fast growing society (A1F1) the temperature was predicted to increase by an average of 4˚C (range 2.4-6.4˚C) (IPCC, 2007). Whatever the scenario, the temperature is likely to increase at a higher rate than that seen in the last century.

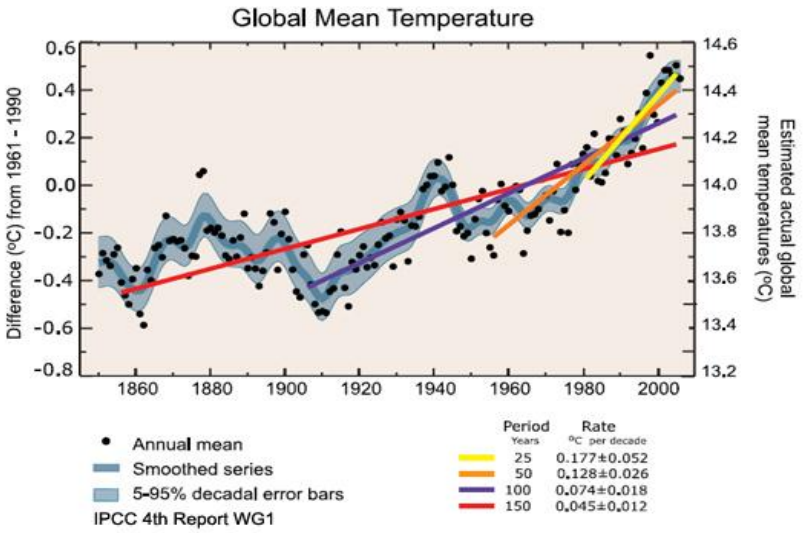

Figure 7.1 Annual global mean temperatures in the last 150 years (Adapted from Fourth Assessment Report IPCC, 2007)

Table 7.1 Temperature increase by 2100 compared to 2000 based on emission scenarios (Figures are adapted from IPCC, 2007)

Emission Scenario*	Average temperature increase (˚C)	Temperature increase range (˚C)
A1FI	4.0	2.4-6.4
A1T	2.4	1.4-3.8
A1B	2.8	1.7-4.4
A2	3.4	2.0-5.4
B1	1.8	1.1-2.9
B2	2.4	1.4-3.8

*A1 refers to a scenario for a fast growing society, which is divided into three sub-scenarios: A1FI with fossil fuels as the world's primary energy sources, A1T with emphasis on non-fossil fuel energy and A1B with a balanced emphasis on all energy sources. A2 refers to a scenario for a diverse society; B1 refers to scenario for a sustainably developping society; B2 refers to scenario for a regional coexistence society.

These predictions are on a global scale, but there are more detailed local predictions made by the UK Climate Prediction (UKCP) project. They predict changes on a scale ranging from the whole of the UK to individual 25 × 25 km grid squares. Taking the medium emission scenario for the West Midlands, the project predicts an increase of 3.7˚C in the average summer temperature by 2100. The predicted change in temperature for the grid box in which Wellesbourne is found can be seen in Fig.7.2. Three 30-year time periods (2020s, 2050s and 2080s) and a range of probability levels (10, 33, 50, 67 and 90%) are provided to show the spread of possible outcomes. For example, it is projected that in the 2080s, there is 90% likelihood that temperatures in Wellesbourne will be equal to or less than 5˚C warmer than temperature within the baseline period (1961-1990) (Fig.7.2).

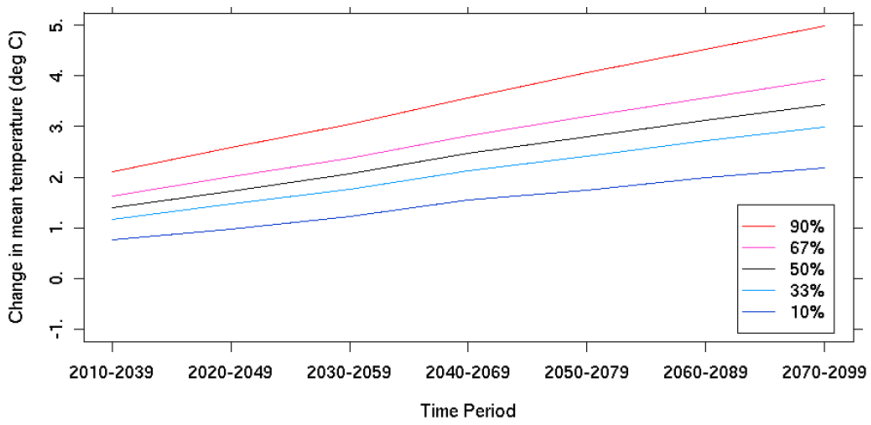

Figure 7.2 The predicted changes in temperature for Wellesbourne (grid box 1469) over the next 90 years. This graph was produced using the UKCIP user interface which allows personalisation of predictions to certain regions and scenarios. The graph shows the medium emissions scenario. The different probability levels (10-90%) are shown as temperature is predicted to increase by up to 5˚C.

7.1.2 Global warming effects on species and ecosystems

Global warming has resulted in serious impacts on plant physiology and development, species diversity, ecosystem structuring, and the composition of plant communities (Kipp, 2007). There are clear examples and evidence for phenological

changes in plants due to global warming. For example, the mean first flowering date of 385 British plant species has advanced by 4.5 days during 1991 to 2000 compared with that during 1954 to 1990. Of these species, 16% flowered significantly earlier and 3% later in the 1990s than previously. These changes in flowering time had a significant impact on the entire ecosystem structure and composition (Fitter and Fitter, 2002). Another way in which global warming could affect plant biology is via changes in the germination requirements of seeds. Shifts in seed germination phenology could strongly influence plant life history traits and may therefore have implications for species migration, species composition and diversity of communities (Walck *et al*., 2011). Global warming influences air and soil temperatures, but also soil water content, and this may have an impact on the distributions and diversity of species, especially terrestrial vegetation, which is most reliant on the combination of temperature and water availability (McCarty, 2001; IPCC, 2001).

Since global warming effects have already occurred, we could assess how natural ecosystems would respond in the future from what has already happened. However, the impact is likely to become even more serious due to the accelerating rate of the climate change (Peters, 1990; IPCC, 2007). Therefore, studies of the impact of global warming using realistic future scenarios on the life history of plants, including regeneration from seeds and plant growth and reproduction is of great importance for the prediction of population dynamics and future species distributions.

7.1.3 Global warming simulation using a thermal gradient tunnel

It is becoming increasingly important to link environmental signals to the mechanisms that control plant population processes due to the raised environmental concerns of climate change and global warming. Consequently there have been many

studies that attempt to simulate climate change to determine the impacts of those changes on plants and ecosystems. For example, the effects of increasing atmospheric CO_2 concentration can be simulated using FACE (Free-air CO_2 enrichment) experiments (Ainsworth and Long, 2005). The CO_2 concentration can be controlled within a large area that is not enclosed by releasing CO_2 through tubes so the investigation is more similar to natural environmental conditions than small enclosed cabinets (Ainsworth and Long, 2005).

The method of simulating climate change (global warming) in this study is the application of a novel thermal gradient tunnel (Fig.7.3). It can be used to assess the potential impact of increasing temperatures associated with global warming on plant growth. The heating and ventilating system creates a linear gradient of temperature along the tunnel. This gradient moves up and down as ambient temperature changes. The temperature at the open end of the tunnel is the same as that outside (ambient). The temperature at the opposite end is maintained 4˚C higher. Plants along the tunnel therefore experience different degrees of temperature elevation that vary with ambient both day by day and over seasons. The thermal gradient tunnel is therefore considered to be an accurate method to simulate global warming and has been previously used to show its impact on the growth of several crops (Wurr *et al*., 1996). We have adopted this apparatus to study the impact of global warming on the phrenology of the two contrasting *Arabidopsis* ecotypes Bur and Cvi.

7.2 Materials and Methods

7.2.1 Monitoring the life history of *Arabidopsis* in the thermal gradient tunnel

The thermal gradient tunnel described in the introduction was used for the majority of this investigation on the *Arabidopsis* ecotypes Bur and Cvi. All seeds were after-ripened before use (AR treatment refer to *Chapter 3 sec 3.2.1*). To

measure the effect of temperature on plant traits throughout the whole life cycle, two separate experiments were sown and then placed at the four locations along the tunnel, i.e. T1 (ambient end), T2, T3 and T4 (warmer end) (Fig.7.3). One experiment was used to monitor seedling emergence, and in another experiment plants were grown to the end of their life cycle so that seeds of the next generation could be harvested and analysed. Plants were watered regularly throughout this investigation to ensure they did not experience water stress.

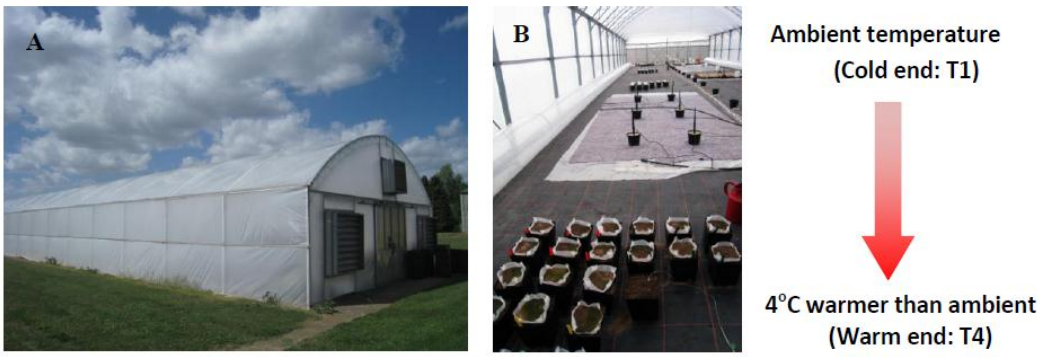

Figure 7.3 The outside and inside of the thermal gradient tunnel. A: the tunnel measures 31.9m long and 8.9m wide. **B**: Pots were placed at four equidistant sites along the tunnel that are referred to as T1 (ambient), T2, T3 and T4 (4˚C warmer than ambient).

***Seedling emergence*:** Compost was mixed with different levels of nitrate as described in Table 7.2. The compost (for composition see Table 7.2) was then added to P24 cellular trays (36cm×24cm) and each tray was labelled and placed in a second tray lined with capillary matting to ensure all the plants had a uniform access to applied water. Twenty five seeds were sown onto the surface of pre-watered compost in each cell and there were four replicate cells for each ecotype (Bur and Cvi). All three levels of nitrate were used with Bur, but only high N compost was used for Cvi as few seedlings survive under N-limiting conditions (data presented in *chapter 6*). The trays were placed at each of four locations along the tunnel, after 24h with exposure to light the seeds were covered with a uniform layer of clean horticultural

sand (0.5cm). Emergence was recorded as days after sowing when the seedling emerged through the sand. The number emerged was recorded each day and once a seedling had emerged it was removed.

Table 7.2 Compost mixes to produce different nitrate contents for plant growth and seed production

Compost type	Compost component	Nitrate content (NO_3-N mg/kg dry weight)
High N	Levingtons F1 compost: sand: vermiculite = 6:1:1	304.3
Medium N	Levingtons F1 compost: sand: vermiculite = 4:1:1	263.5
Low N	Levingtons F1 compost: sand: vermiculite = 4:2:2	127.8

***Bolting time and plant growth to maturity*:** To find the effect of increasing temperature on the growth of different ecotypes, trays were set up as for seedling emergence experiments with different compost mixtures. Approximately five seeds were sown in each cell. There were three replicates of 8 cells for each ecotype grown on compost with each level of N at one of the four locations. After 24 h seeds were covered with sand as above and following emergence seedlings were thinned to one per cell. Trays of plants were visually scored daily for bolting (inflorescence extended 1cm) and the number of plants that had bolted was recorded. At that stage the rosette diameter and leaf number were also recorded. Aracon bases (Arasystem, Belgium) were then placed on plants. When the plant had grown through the bases, Aracon tubes (Arasystem, Belgium) were added to the bases to isolate each plant during pollination and to facilitate seed collection.

***Seed harvesting*:** Plant watering stopped at maturity, i.e. when all the siliques had turned yellow. Seeds were harvested one week later, and then sieved using a 500μm sieve to remove broken siliques and other debris. The cleaned seed sample was then dried to an equilibrium relative humidity of 55% above a saturated calcium

nitrate solution for 6 days. After drying, seed yield (total seed weight) and seed size (1000-seed weight) were recorded. The nitrate content of seeds was determined by a steam distillation method using a FOSS FIAstar 5000 Flow Injection Analyser for end point determination (refer to *Chapter 6 sec 6.2.1*). Following drying the remaining seeds were sealed in aluminium bags (11 × 24 cm) (Moore and Buckle, St. Helens, UK) and stored at -80˚C for germination experiments. When seeds were harvested the plants were cut just above the rosettes, the height was measured and the inflorescence placed in labelled bags. The bags were then left at room temperature to equilibrate for a week, placed in an oven at 80˚C for 24h (overnight) and then plant weight was recorded.

Table 7.3 Dates of seed production in the thermal gradient tunnel *

			2011		2012	
			Set 1	**Set 2**	**Set 3**	**Set 4**
Date planted			11/02/2011	27/07/2011	09/02/2012	01/05/2012
Date transferred	**Bur**		25/03/2011	07/09/2011	23/03/2012	-
to tunnel	**Cvi**		25/03/2011	30/08/2011	23/03/2012	-
Date harvested	**Bur**	**T1**	04/05/2011	25/10/2011	22/05/2012	25/07/2012
		T2	28/04/2011	19/10/2011	22/05/2012	17/07/2012
		T3	26/04/2011	19/10/2011	22/05/2012	16/07/2012
		T4	28/04/2011	19/10/2011	22/05/2012	11/07/2012
	Cvi	**T1**	04/05/2011	18/10/2011	21/05/2012	25/07/2012
		T2	26/04/2011	18/10/2011	16/05/2012	17/07/2012
		T3	26/04/2011	18/10/2011	16/05/2012	16/07/2012
		T4	26/04/2011	18/10/2011	16/05/2012	12/07/2012

*The table shows the dates at which plants were planted, moved to the tunnel and harvested. Plants were grown at four equidistant sites along the tunnel that are referred to as T1 (ambient), T2, T3 and T4 (4˚C warmer than ambient).

Four sets of seeds were produced and harvested from the tunnel; two in 2011 and two in 2012. Set 4 seeds produced in 2012 were produced from seeds sown at four locations in the tunnel to monitor the whole life history. The remaining three

sets were first grown in a glasshouse (glasshouse conditions refer to Chapter 6 Table 6.1) until bolting and then placed at the four locations in the tunnel (Table 7.3).

7.2.2 Germination and nitrate sensitivity experiments

To determine the impact of seed maturation temperature on seed dormancy and germination behaviour, germination experiments were set up using the seeds produced from the thermal gradient tunnel in 2011 and 2012 (described in Table 7.3). Seeds were first surface sterilized with 2.5% bleach for five minutes, washed three times with distilled water, and then sown in three replicates of 40 in rectangular clear plastic boxes (8 × 12cm) on two layers of 3M chromatography paper with 8ml water or KNO_3 at 1.0mM. Seeds were then incubated in the light or dark at 10˚C or 25˚C. Germination was recorded for up to 28 days. A seed was considered to have germinated when the radical had emerged through the testa and endosperm. Germinated seeds were counted and removed at regular intervals throughout the experiment. Germination on boxes kept in the dark was recorded under a green safe light.

7.2.3 Data analysis

All percentage germination and emergence data was angular transformed for analysis. The transformation is arcsin(sqrt(p/100))*180/pi, where p is a percentage from 0 to 100, and the result is an angle between 0 and 90 degree. Data are presented as the mean ± standard error. The standard error of the mean was calculated by dividing the standard deviation by the square root of the number of replicates (n) (sd/√n). Analysis of variance (ANOVA) was used to detect the differences between variates as listed in Table 7.4. Statistical analysis was carried out using the software package GenStat (VSN International, 2012). The results of individual analyses are given in Appendix tables.

Table 7.4 Summary of ANOVA used in Chapter 7

Analysis of variance	Factors	Variate	Transformation
Two-way ANOVA (*sec 7.2.1*)	Temperature location, Maternal nitrate supply	Emergence rate Seed yield Seed nitrate content	None
Three-way ANOVA (*sec 7.2.2*)	Germination temperature, Maternal nitrate supply, seed maturation temperature	1) Emergence 2) Final germination%	Angular
Three-way ANOVA (*sec 7.2.1*)	Temperature location, ecotype, maternal nitrate supply	Bolting time Leaf number at bolting Rosette diameter	None

7.3 Results

In 2011 plants were grown on two occasions in a single nitrate regime (high N), whereas in 2012 Bur was additionally grown in three nitrate regimes (high, medium and low N). In the first section below (*sec.7.3.1*) an investigation of the interaction of temperature and nitrate differences on plant growth and seed production over the life cycle is reported for the second sowing in 2012 (Set 4). In the second section (*sec.7.3.2*), the impact of seed maturation temperature along the thermal gradient tunnel and nitrate regime on subsequent seed performance is investigated at all 4 sowings (Set 1-4). The timing of sowing and protocols used (Table 7.3) to produce the four sets of seeds was adopted for the following reasons:

1) Set 1 in 2011 and Set 3 in 2012 were timed so that seed maturation coincided with the natural time for the winter annual Cvi at ambient temperature at Wellesbourne;
2) Set 2 in 2011 was timed so that seed maturation coincided with the natural time for the summer annual Bur at ambient temperature at Wellesbourne;

3) Set 4 in 2012 was sown when Cvi seeds would be shed at ambient temperature at Wellesbourne. However, the seeds were after-ripened so they germinated immediately following sowing and therefore seed maturation occurred on these plants during higher temperatures in summer, intermediate between summer and winter annual phenotypes.

In this way we could observe the impact of global warming on the two ecotypes at their own and each other's natural times of year, but also have seed maturation under a wide range of temperatures.

7.3.1 Effect of temperature and nitrate regime on *Arabidopsis* life history

In the second sowing of 2012 cellular trays containing compost with three nitrate regimes were placed directly into four locations in the thermal gradient tunnel following sowing to investigate the complete life cycle. As Cvi did not grow successfully in lower N regimes ecotypes are only compared in the high N regime following emergence.

***Seedling emergence*:** A three-way ANOVA indicated a significant ($p<0.001$) difference in final percentage seedling emergence between Bur and Cvi, however the temperature locations and the nitrate level of compost did not show significant effects on seedling emergence (refer to Appendix Table 7.1).

Cumulative mean percentage emergence is presented in Fig.7.4 for seeds sown in three nitrate regimes at four temperature locations along the thermal gradient tunnel. Seedling emergence was significantly higher in Bur than in Cvi in all three nitrate regimes. Interestingly high N compost delayed seedling emergence from seeds of both ecotypes (Fig.7.4).

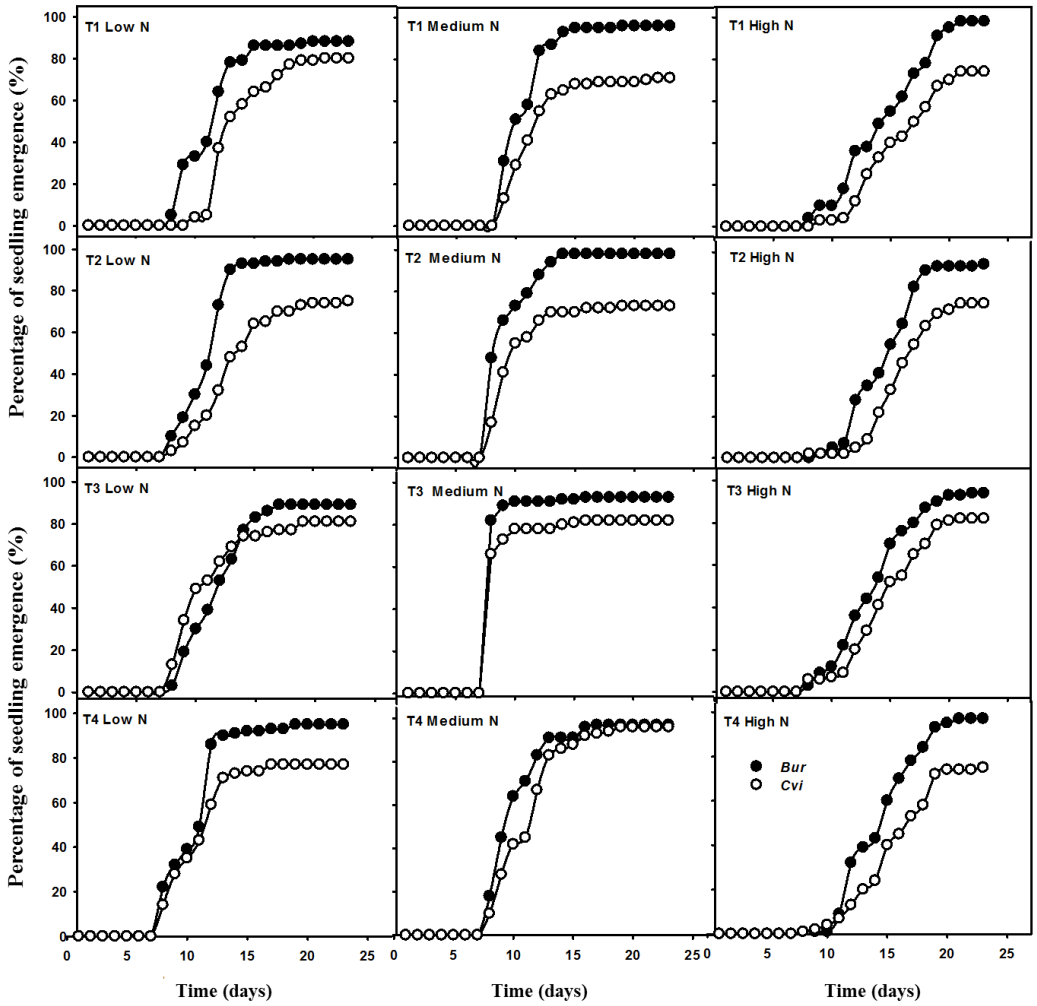

Figure 7.4 Emergence from *Arabidopsis* seeds (Bur and Cvi) in the thermal gradient tunnel (set up in May 2012). Emergence pots were set up at four locations along the tunnel (T1 ambient –T4 warm end) with three levels of nitrate supply (low N, medium N and high N) and at each location there were four replicates of 40 seeds. Data are the mean ± standard error. No error bar indicates symbol is larger than the error (for analysis refer to Appendix Table 7.1).

Seedling emergence rates were calculated as the reciprocal of time to 50% emergence ($1/T_{50}$) and found to be greatest on medium N compost in both ecotypes (Fig.7.5; $p<0.05$ and $p<0.001$ for Bur and Cvi respectively). There was no significant effect of temperature on the emergence rate of Bur in the high N regime, but the effect was significant in the low and medium N regimes (Fig.7.5; $p<0.05$ and $p<0.001$ for Bur and Cvi respectively), indicating that the effect of temperature on seedling emergence depends on nitrate level of the compost.

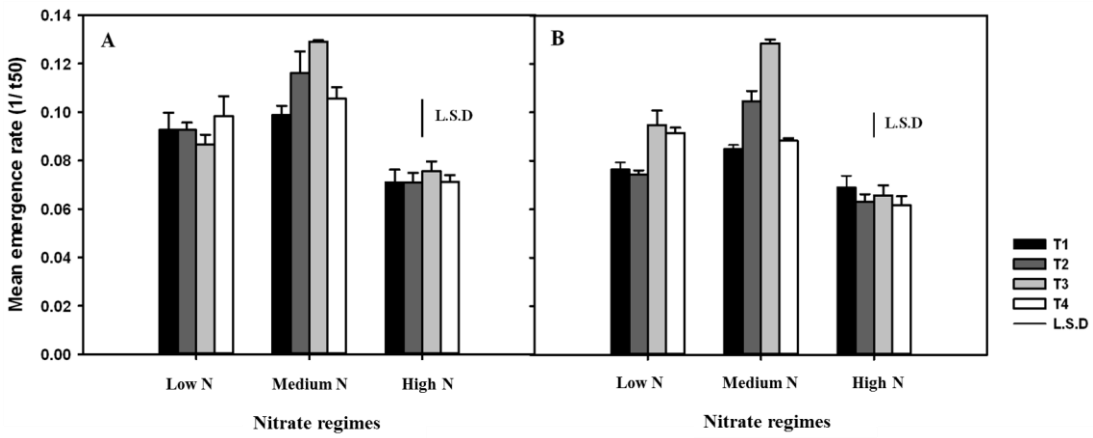

Figure 7.5 Rate of seedling emergence from *Arabidopsis* seeds in the thermal gradient tunnel (set up in May 2012). **A**: Bur; **B**: Cvi. Emergence pots were set up at four temperature sites along the tunnel (T1 ambient –T4 warm end) with three levels of nitrate supply (low N, medium N and high N) and at each location there were four replicates of 40 seeds. Data are the mean ± standard error. L.S.D. bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 7.2).

Bolting time

Ecotypes were compared in the high N regime only as Cvi did not survive in lower N regimes. Bolting time was influenced by both ecotype and temperature (Table 7.5) with bolting significantly ($p<0.001$) earlier at higher temperatures in both ecotypes. Bur plants grown on high N compost were faster to bolt than Cvi at tunnel locations T2-T4, but these differences were not always significantly different (Table 7.4), however bolting time was significantly shortened ($p<0.001$) at the warm end (T4) compared to the ambient (T1) end of the tunnel (Table 7.5).

Table 7.5 Bolting time responses in the thermal gradient tunnel on high N compost in two ecotypes

Temperature location	**Bolting time (days)**		
	Bur	**Cvi**	**L.S.D (5%)**
T1	35.63	34.15	**1.341**
T2	31.29	33.68	
T3	30.58	34.36	
T4	30.83	31.29	

Bolting time (days) were recorded as the mean value of eight replicates ± standard error. Differences between the means are compared by the L.S.D. (in bold) at the P = 0.05 level (for analysis refer to Appendix Table 7.3).

There was little difference in bolting time in Bur in the three nitrate regimes, but the four temperature locations significantly ($p<0.001$) influenced the bolting time (Table 7.6).

Table 7.6 Bolting responses of Bur to different nitrate levels along the thermal gradient tunnel

Temperature location	Bolting time (days)				L.S.D (5%)
	Low N	Medium N	High N	Mean	
T1	36.79	36.56	35.63	36.33	**0.604**
T2	32.72	32.67	31.29	32.23	
T3	30.29	29.71	30.58	30.19	
T4	31.08	31.3	30.83	31.07	

Bolting time (days) was recorded as mean value of eight replicates ± standard error. Differences between the means are compared by the L.S.D. (in bold) at the P = 0.05 level (for analysis refer to Appendix Table 7.4).

At bolting, the leaf number and rosette diameter were measured in both ecotypes and Bur was shown to have significantly ($p<0.001$) larger rosettes and more leaves at bolting than Cvi (Fig.7.6). However, neither the rosette diameter nor the leaf number at bolting was significantly affected by temperature (refer to Appendix Table 7.5).

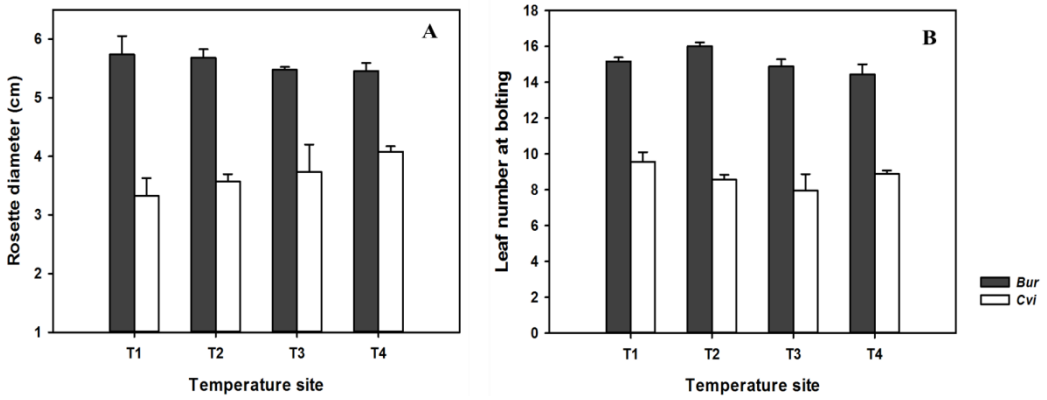

Figure 7.6 Vegetative phenotypes of Bur and Cvi plants along the thermal gradient tunnel (set up in May 2012). **A**: Rosette diameter; **B**: leaf number at bolting. Bolting trays were set up at four temperature sites along the tunnel (T1 ambient-T4 warm end) on high N compost. Data represents the mean rosette diameter/ leaf number ± standard error of the mean.

In Bur both the rosette diameter and leaf number were significantly ($p<0.001$) increased with increasing nitrate. The rosette diameter was much larger when grown on high N than on compost with lower levels of nitrate. These differences were significant ($p<0.05$ and $p<0.001$ respectively) at the temperature sites T1, T2 and T3, but not at T4 (Table 7.7; Appendix Table 7.6). Rosette size was also influenced by temperature, depending on the nitrate supply. Higher temperature led to larger rosettes when plants were grown in medium N and low N regimes, but the reverse occurred in the high N regime (Table 7.7). The increased temperature between T1 and T2 led to an increase in leaf number, but further temperature rise from T3 to T4 appeared to reduce the leaf number resulting in significantly ($p<0.001$) fewer leaves in rosettes at T4 than at T1 (Table 7.7).

Table 7.7 Rosette diameters and leaf number at bolting in Bur

Plant trait	Temperature location	Nitrate regimes			L.S.D
		Low N	Medium N	High N	(5%)
Rosette diameter (cm)	**T1**	5.03	4.52	5.74	**0.42**
	T2	5.26	5.35	5.68	
	T3	4.94	5.18	5.48	
	T4	5.26	5.36	5.45	
Leaf number at bolting	**T1**	14.10	14.88	15.25	**0.93**
	T2	14.68	14.92	16.00	
	T3	13.85	14.67	14.88	
	T4	13.71	13.79	14.20	

Leaf number and rosette diameter were both recorded as mean value of eight replicates ± standard error. Differences between the means are compared by the L.S.D. (in bold) at the P = 0.05 level (for analysis refer to Appendix Table 7.6).

There were also noticeable effects of temperature (Bur and Cvi) and nitrate supply (Bur) on the growth of plants after bolting. Fig.7.7 and Fig.7.8 are photographs of plants taken mid-life cycle to show differences between the two ecotypes at different tunnel locations, and between Bur plants grown in different

nitrate regimes respectively. When grown in the high N regime, temperature had a greater effect on the growth of Cvi than Bur, i.e. Cvi plants showed more obvious differences in height at the four temperature locations along the tunnel compared to Bur plants (Fig.7.7). The effect of nitrate on Bur growth differed between temperature locations. There was a greater effect of nitrate supply on Bur plant growth at T1, T2 and T3 than at T4 (Fig.7.8).

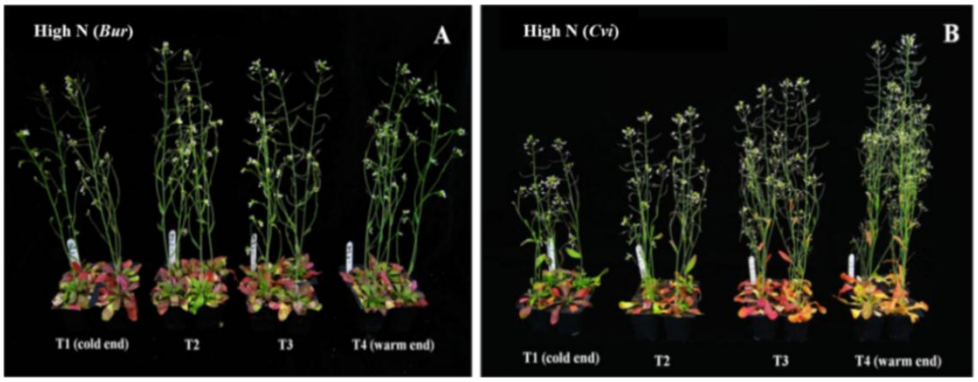

Figure 7.7 Plants of Bur and Cvi grown at different temperature locations along the thermal gradient tunnel on high N compost 56 days after sowing (set up in May 2012; A:Bur; B: Cvi)

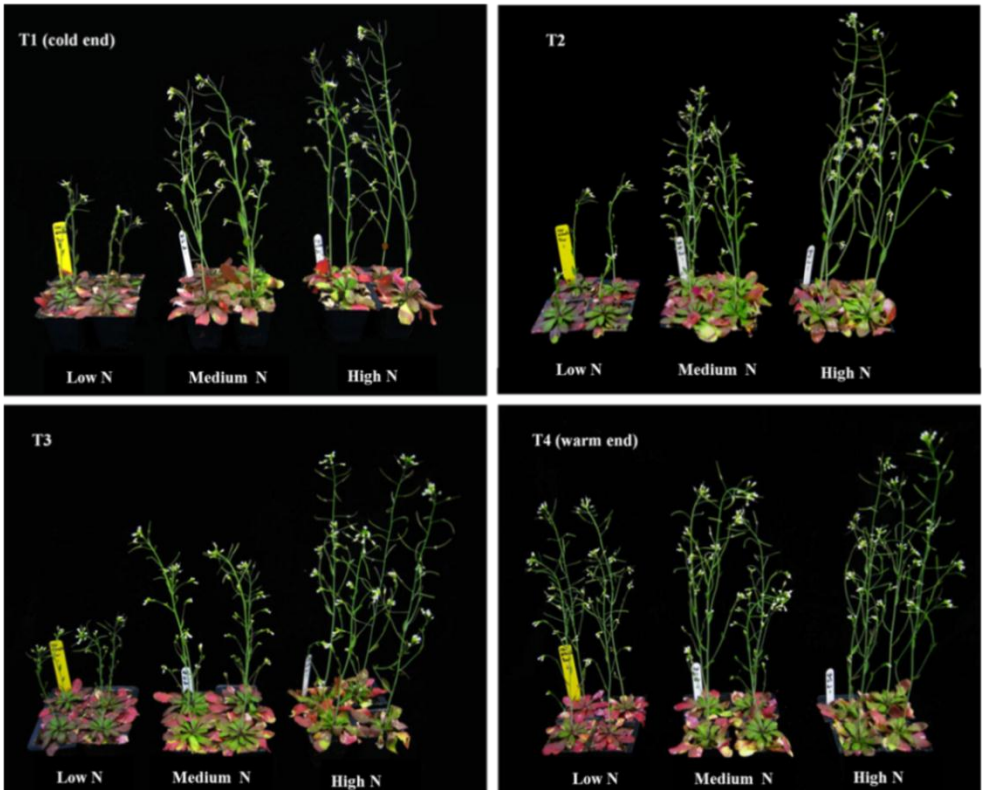

Figure 7.8 Plants of Bur grown at different temperature locations along the thermal gradient tunnel on low N, medium N and high N compost 56 days after sowing (set up in May 2012).

Seed production and yield components

Nitrate regime and increased temperature along the thermal gradient tunnel had a number of effects on the seed produced. Mean seed weight per plant was significantly ($p<0.001$) affected by the interaction between temperature locations along the tunnel and the nitrate supply to the mother plants in Bur and Cvi. In general there was less weight of seed produced per plant for Bur at the warm end (T4) compared to other temperature locations; whereas it was the opposite situation for Cvi (Fig.7.9)

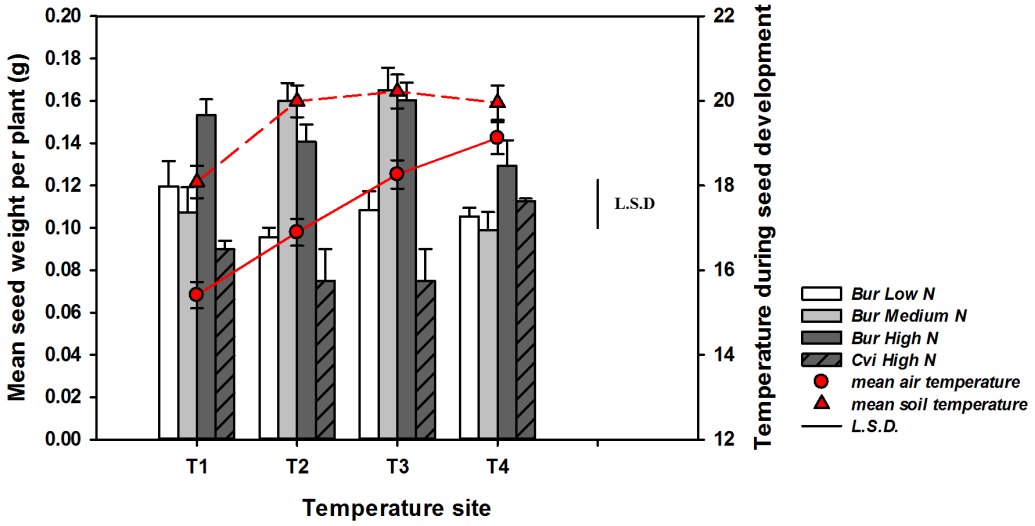

Figure 7.9 Seed yield of *Arabidopsis* from different temperature locations along the thermal gradient tunnel (set up in May 2012). Trays were set up at four temperature sites along the tunnel (T1 ambient –T4 warm end) with three levels of nitrate supply (low N, medium N and high N). Red solid line represents air temperatures for seed maturation; red dash line represents soil temperatures for seed maturation. Data are the mean ± standard error. L.S.D bar indicates the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 7.7)

Seed size was calculated as 1000-seed weight, and the difference between the seeds produced at warm and ambient end of the tunnel was significant ($p<0.01$). In general seed size decreased with increasing temperature, i.e. larger seeds were produced at the ambient temperature than at the warm end of the tunnel (Fig.7.10A). However, there was an exception when Bur plants were supplied with low N, the

1000-seed weight was significantly ($p<0.01$) larger at the warm end than at the ambient end of the tunnel. The number of seeds produced per plant was calculated from the mean seed weight per plant and seed size, which also showed significant ($p<0.05$) difference between the seeds produced from the warm and ambient end of the tunnel (Fig.7.10B). This value was variable, but in general fewer Bur seeds were produced at the warm end (T4) than at ambient end of the tunnel. However, the reverse was found in Cvi as many more seeds were produced per plant at the warm end (Fig.7.10B). Additionally in Bur there were more seeds produced on plants with high N than on those with lower nitrate availability (Fig.7.10B).

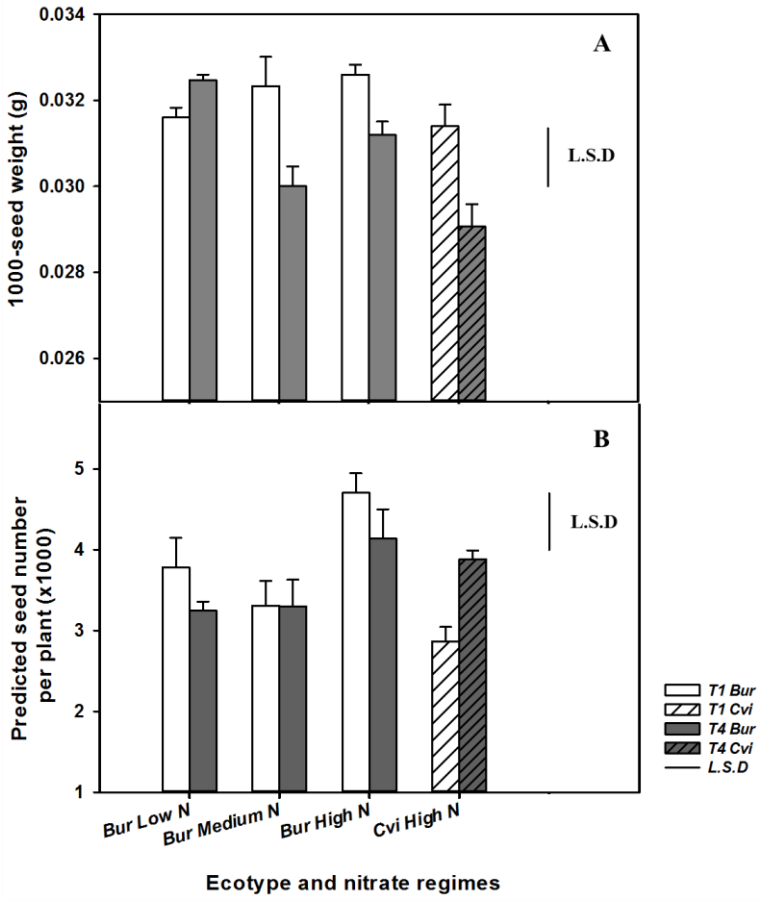

Figure 7.10 Seed production of Bur and Cvi at ambient (T1) and warm end (T4) of the thermal gradient tunnel (set up in May 2012). **A**: seed size (1000-seed weight); **B**: seed number per plant. Data are the mean ± standard error. L.S.D bars indicate the least significant difference at the P = 0.05 level (for analysis refer to Appendix Table 7.8).

The mean seed yield produced from each plant was significantly and positively linearly correlated with the dry weight and height of the plant in Bur (Fig. 7.11).

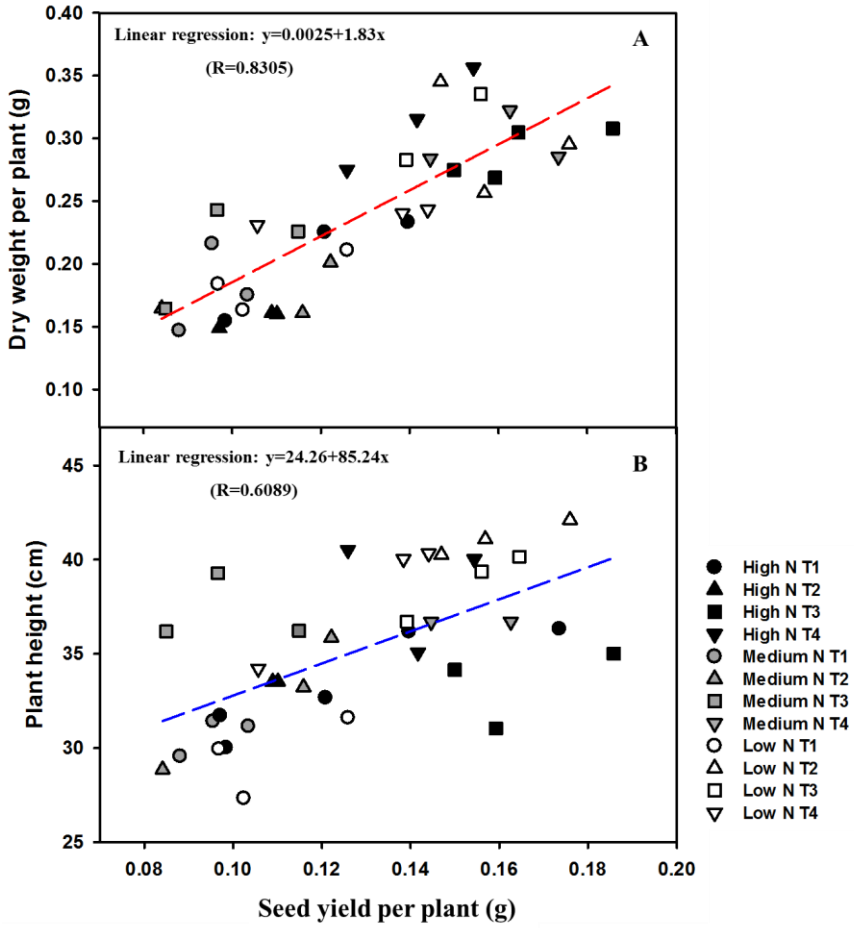

Figure 7.11 The relationship between seed yield per plant and its dry weight or height. A: dry weight per plant; **B**: plant height. Different locations in the tunnel (T1-T4) and nitrate regimes (high, medium, low) are depicted by different symbols shown in the figure legend. Each value is the mean of eight replicates. Linear regressions are fitted **A**: y=0.0025+1.83x (R=0.8305); and **B**: y=24.26+85.24x (R=0.6089).

Seed number per plant, was also found to be significantly and positively linearly correlated with the plant dry weight and height (Fig.7.12).

Harvest index (HI) can be estimated as the (DW_{seeds}) / (DW_{plant}+ DW_{seeds}) ratio, and this is a key indicator of individual plant yield (Masclaux-Daubresse and Chardon, 2011). The HI stayed generally unchanged at different temperature locations along the tunnel in both Bur and Cvi, however, HI varied with nitrate

supply in Bur (Table 7.8). At all the temperature locations (T1-T4), the highest HI value was found in Bur plants with low N.

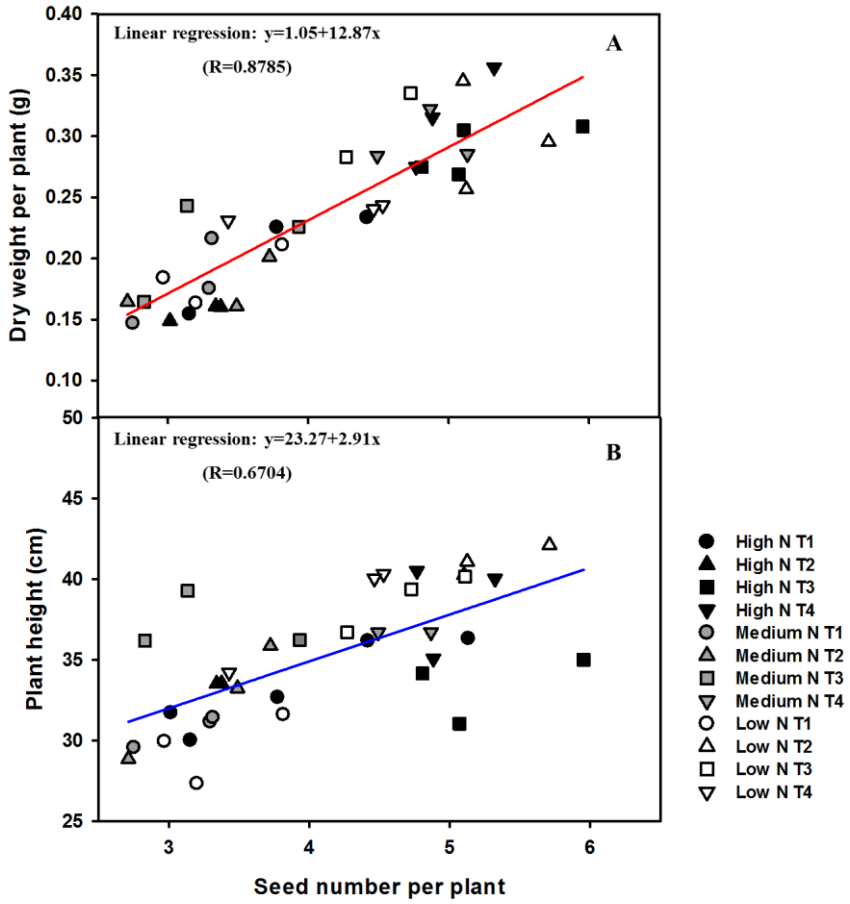

Figure 7.12 The relationship between seed number per plant and its plant dry weight or height. A: dry plant weight; **B**: plant height. Different locations in the tunnel (T1- 4) and nitrate regimes (High, medium, low) are depicted by different symbols shown in the figure legend. Each value is the mean of eight replicates. Linear regressions are fitted A: y=1.05+12.87x (R=0.8785); B: y=23.27+2.91x (R=0.6704).

Table 7.8 Harvest Index (HI) of Bur and Cvi

Ecotype (nitrate supply)	**Temperature site**				**L.S.D (5%)**
	T1	**T2**	**T3**	**T4**	
Bur (High N)	0.3326	0.3087	0.3503	0.3206	**0.05997**
Bur (Medium N)	0.3708	0.3504	0.3671	0.3504	
Bur (Low N)	0.3782	0.3507	0.368	0.4019	
Cvi (High N)	0.3603	0.3196	0.2632	0.3889	

The harvest index (HI) was calculated as (DW_{seeds}) / (DW_{plant}+ DW_{seeds}) ratio. Differences between the means are compared by the L.S.D. (in bold) at the P = 0.05 level (for analysis refer to Appendix Table 7.9).

Correlation coefficients between measured and calculated traits in both ecotypes are shown in Table 7.9. In Bur, three traits were significantly and positively related to seed yield: seed number (per plant), dry plant weight (per plant) and plant height. However, seed size was not significantly related to any measured traits, but there was a positive relationship between seed size and HI. In addition, HI was also significantly correlated with plant dry weight. However, in Cvi, dry plant weight was not significantly related to any traits. All three seed production traits, i.e. seed yield, seed number and seed size, were significantly ($p<0.01$) and positively correlated with HI.

Table 7.9 Correlation coefficients between measured and calculated traits of *Arabidopsis* Bur and Cvi

Traits		Seed yield	Seed size	Seed No.	Dry weight	Plant height	HI
Bur	**Seed yield**	1					
	Seed size	0.081	1				
	Seed No.	0.969**	0.163	1			
	Dry weight	0.831**	0.244	0.878**	1		
	Plant height	0.609*	0.285	0.670*	0.694*	1	
	HI	0.024	0.568*	0.152	0.562*	0.349	1
Cvi	**Seed yield**	1					
	Seed size	0.537	1				
	Seed No.	0.943**	0.234	1			
	Dry weight	0.0003	0.322	0.127	1		
	Plant height	0.613*	0.108	0.640*	0.402	1	
	HI	0.868**	0.623*	0.764**	0.479	0.306	1

*and**: significant at the 5% and 1% levels of probability respectively

A large amount of data has been presented concerning the effect of temperature on seed production of the contrasting ecotypes Bur and Cvi. A summary of the general trends along the thermal gradient tunnel for Bur and Cvi and between nitrate regimes in Bur are presented in Table 7.10.

Table 7.10 Summary table of the effect of ecotype, nitrate supply and temperature location on seed production

Ecotype	**Nitrate regimes**	**Seed yield**	
		Comparison (T1 and T4)	Significant or not?
Bur	**High N**	T1 > T4	No
	Medium N	T1 > T4	Yes*
	Low N	T1 > T4	No
Cvi	**High N**	T1 < T4	Yes*
		Seed size	
		Comparison (T1 and T4)	Significant or not?
Bur	**High N**	T1 > T4	Yes*
	Medium N	T1 > T4	Yes**
	Low N	T1< T4	No
Cvi	**High N**	T1 > T4	Yes**
		Seed number	
		Comparison (T1 and T4)	Significant or not?
Bur	**High N**	T1 > T4	No
	Medium N	T1 > T4	No
	Low N	T1 > T4	No
Cvi	**High N**	T1 < T4	Yes**

*and**: significant at the 5% and 1% levels of probability respectively (T1 is the ambient end and T4 is the warm end of the thermal gradient tunnel; for analysis refer to Appendix Table 7.10).

To illustrate the relative length of components of the life cycle the duration of each phase of plant growth is summarized in Fig.7.13. In general, increased temperature at the warm end resulted in a shorter life cycle by 16.27% in Bur and 15.12% in Cvi compared to ambient end. The increasing temperature delayed seedling emergence from the Bur seeds, and this delay was significant ($p<0.05$) when the seeds were sown on high N compost. However, the increase in temperature

shortened the duration of the vegetative phase of the Bur plants, and this acceleration of bolting time was greater when plants were grown on high N compost (Fig.7.13). The increasing temperature also reduced the time for seed maturation, leading to an earlier seed harvest at the warm end than at the ambient end of the tunnel. In contrast, the duration of all the developmental phases of Cvi tended to be accelerated by the increasing temperature.

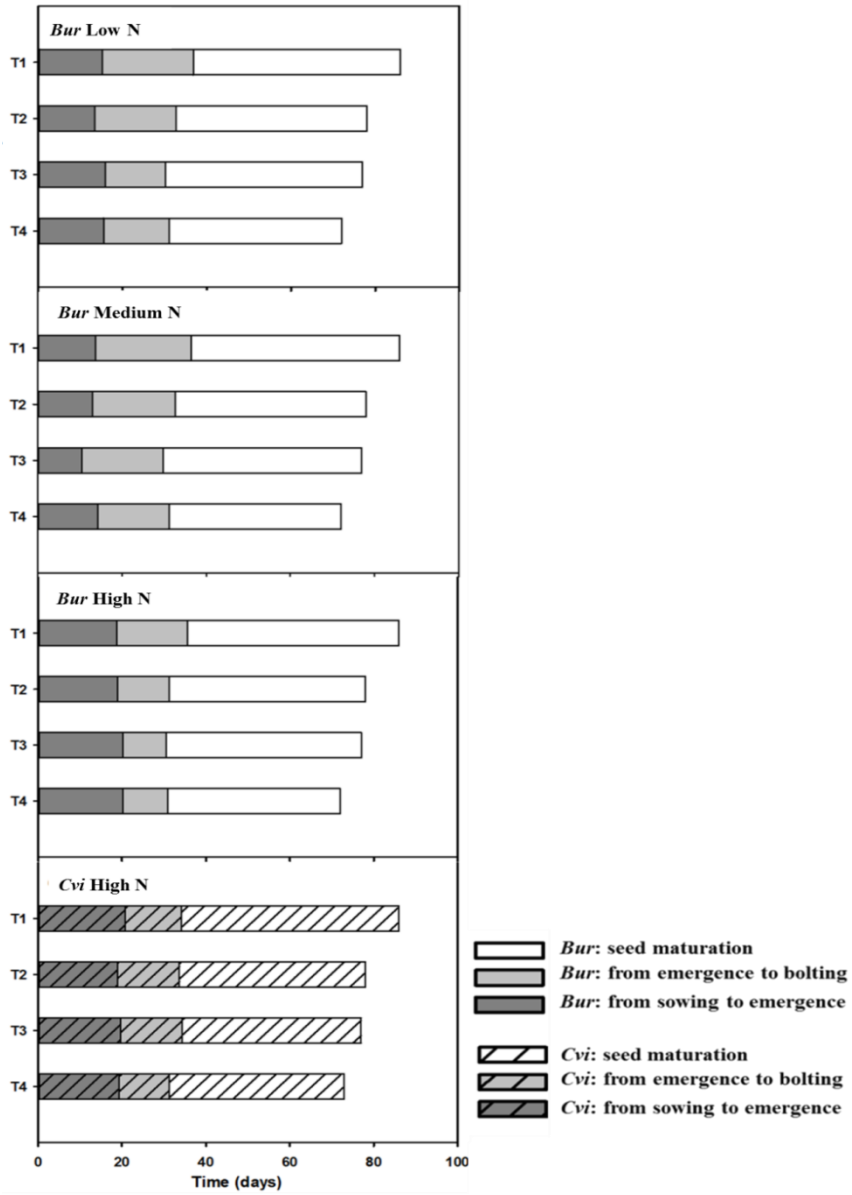

Figure 7.13 Time course of the main life cycle components of Bur and Cvi (set up in May 2012). Trays were set up at four temperature sites along the tunnel (T1 ambient –T4 warm end) with three levels of nitrate supply (low N, medium N and high N).

Seed nitrate content and seed germination

Nitrate content of mature seeds was measured and significantly increased ($p<0.001$) with increased temperature along the tunnel especially in the high N regimes (Fig.7.14; Appendix Table 7.11).

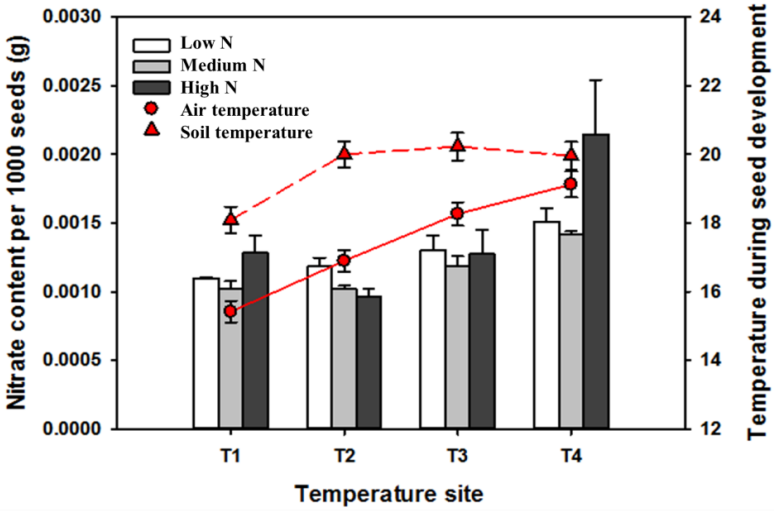

Figure 7.14 Nitrate content per 1000 seeds of *Bur* harvested from different temperature locations along the thermal gradient tunnel (set up in May 2012). Red solid line represents air temperatures for seed maturation; red dashed line represents soil temperatures for seed maturation. Data are the mean ± standard error (for analysis refer to Appendix Table 7.11). No error bar indicates symbol is larger than the error.

Cvi seeds harvested at all positions along the tunnel were too dormant to germinate at either of the temperatures used (10 and 25˚C). Seeds are currently undergoing after-ripening, but the experiment was incomplete at the time of writing this thesis and so is not reported. Germination of Bur seeds occurred at both 10˚C and 25˚C (Fig.7.15). However, final germination was less at the ambient end of the tunnel indicating a proportion of seeds were dormant when produced at this lower temperature. When exogenous nitrate was provided, seeds produced from all the temperature locations germinated to 100% (Table 7.11). There were differences in final germination between nitrate regimes, but there was no consistent pattern and the differences were not significant.

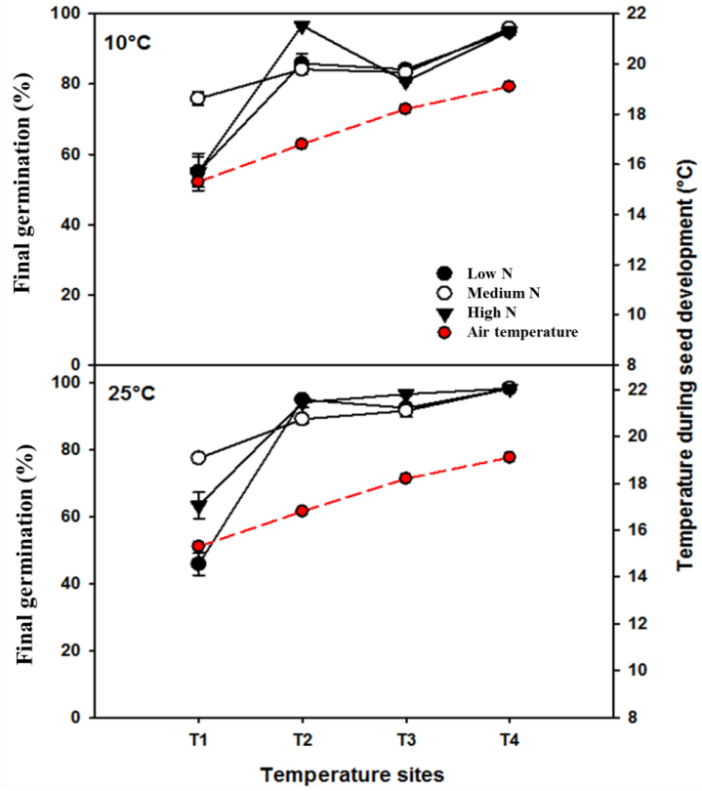

Figure 7.15 Germination responses of Bur seeds produced at different temperature locations along the thermal gradient tunnel in different nitrate regimes (set up in May 2012). ● High N; ○ Medium; ▼ Low N; red line represents temperatures during seed maturation. Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

Table 7.11 Final germination percentage of Bur seeds in the presence of exogenous KNO_3 (1mM)

Temperature location	**Nitrate regime for mother plants**	**Mean germination (%) ± s.e.**	
		10˚C	**25˚C**
T1	**High N**	91.7 ± 1.33	98.3 ± 0.33
	Medium N	98.3 ± 0.33	100
	Low N	91.7 ± 1.76	100
T2	**High N**	95.0 ± 0.58	96.7 ± 0.33
	Medium N	95.8 ± 0.88	96.7 ± 0.88
	Low N	99.2 ± 0.33	100
T3	**High N**	97.5 ± 0.58	100
	Medium N	98.3 ± 0.33	99.2 ± 0.33
	Low N	94.2 ± 1.86	99.2 ± 0.33
T4	**High N**	90.0 ± 0.58	99.2 ± 0.33
	Medium N	98.3 ± 1.33	100
	Low N	99.2 ± 0.33	100

T1 is the ambient end of tunnel and T4 is the warm end. Data were recorded as mean germination percentage of three replicates ± standard error.

7.3.2 The impact of maturation temperature and nitrate regime on germination responses of Bur produced along the thermal gradient tunnel in 2011 and 2012

Air and soil temperatures were recorded over the four experiments over 2011 and 2012 (Fig.7.16 and Fig.7.17). Overall variation in soil temperature had a similar pattern to air temperature, and the 4˚C gradient between the ambient and the warm end was maintained throughout.

There was a strong linear correlation between air temperature and soil temperature over the two years (Fig.7.18).

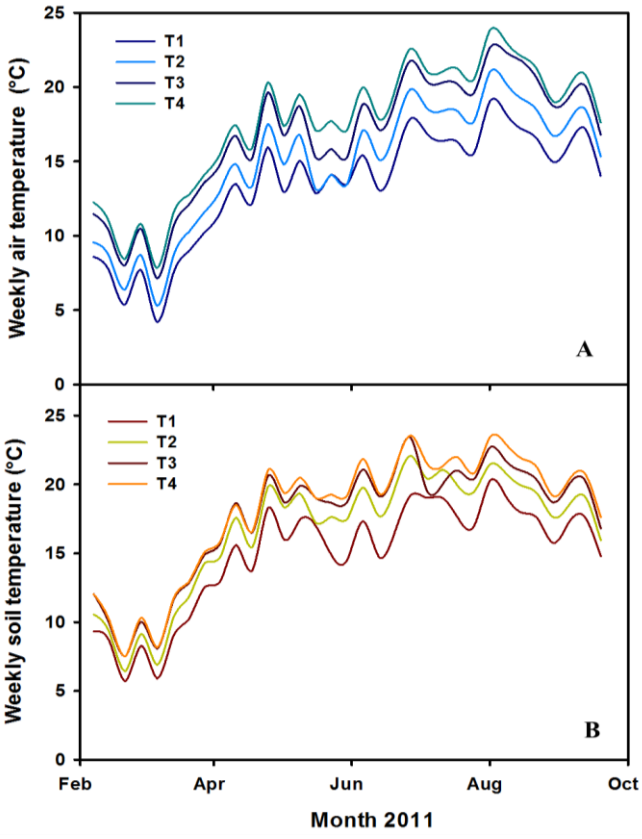

Figure 7.16 The weekly air and soil temperatures in the thermal gradient tunnel during the experiments in 2011. T1 is the ambient and T4 the warm end of the tunnel. **A**: air temperature; **B**: soil temperature.

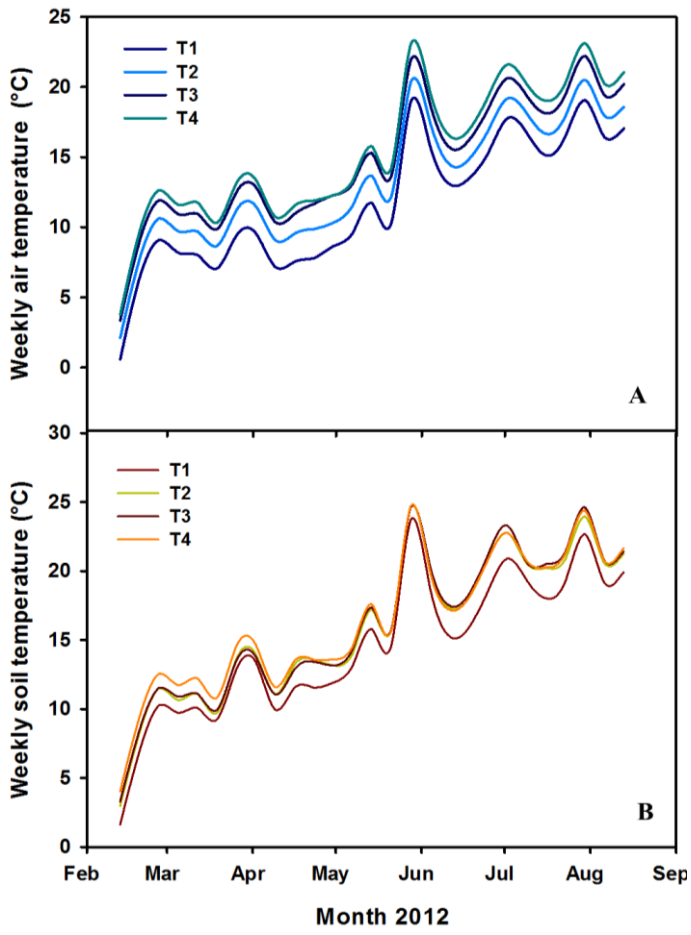

Figure 7.17 The weekly air and soil temperatures in the thermal gradient tunnel during the investigation in 2012. T1 is the ambient and T4 the warm end of the tunnel. **A**: air temperature; **B**: soil temperature.

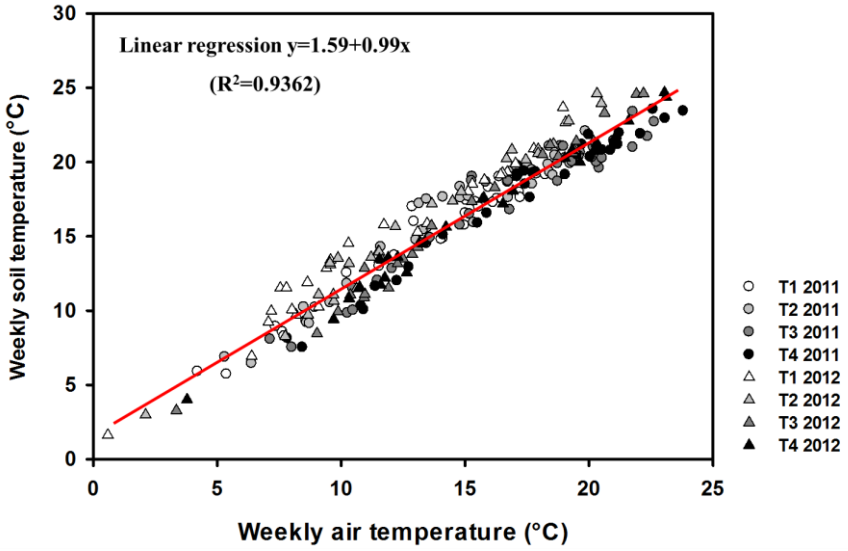

Figure 7.18 Relationship between the weekly mean soil temperatures at 5cm and weekly mean air temperature in 2011 and 2012. Different symbols and colours indicate years and tunnel locations according to the figure legend.

There were four sets of seeds harvested from the tunnel over the two years (Table 7.3). As reported above experiments on Cvi seed are still underway due to their deep dormancy and so are not reported here. We therefore consider only the germination response of Bur below. During 2011, only one level of nitrate (high N) was applied to the Bur plants. The seeds produced showed different germination responses at 10˚C and 25˚C, and importantly, their germination was also greatly influenced by the temperature during seed maturation, i.e. seed dormancy level decreased (i.e. increased germination potential) with the increasing mean maturation temperature (Fig.7.19). Bur seeds produced at low maturation temperature (⩽15˚C) showed a thermal dormancy when imbibed at 25˚C (Fig.7.19B), which was consistent with the germination behaviour of the Bur seeds produced under controlled conditions (15/15˚C, 12/12h, light /dark) presented in *Chapter 6* (Fig.6.13). Interestingly nitrate content in the seeds tended to be lower when seeds were matured at higher temperatures than those matured at low temperatures (Fig 7.19).

The two sets of Bur seeds produced in 2012 on high N showed a similar response to maturation temperature as those in 2011 (Fig.7.20). Also as in 2011, when nitrate content of these seeds is also plotted there is a reverse pattern in relation to temperature to that shown for germination

The mean temperature and day length during vegetative growth and seed maturation for the four occasions investigated are summarized in Table 7.12. The Set 4 seeds produced in 2012 was different from others as it was produced in the tunnel from seed to seed (refer to *sec 7.2.2*).

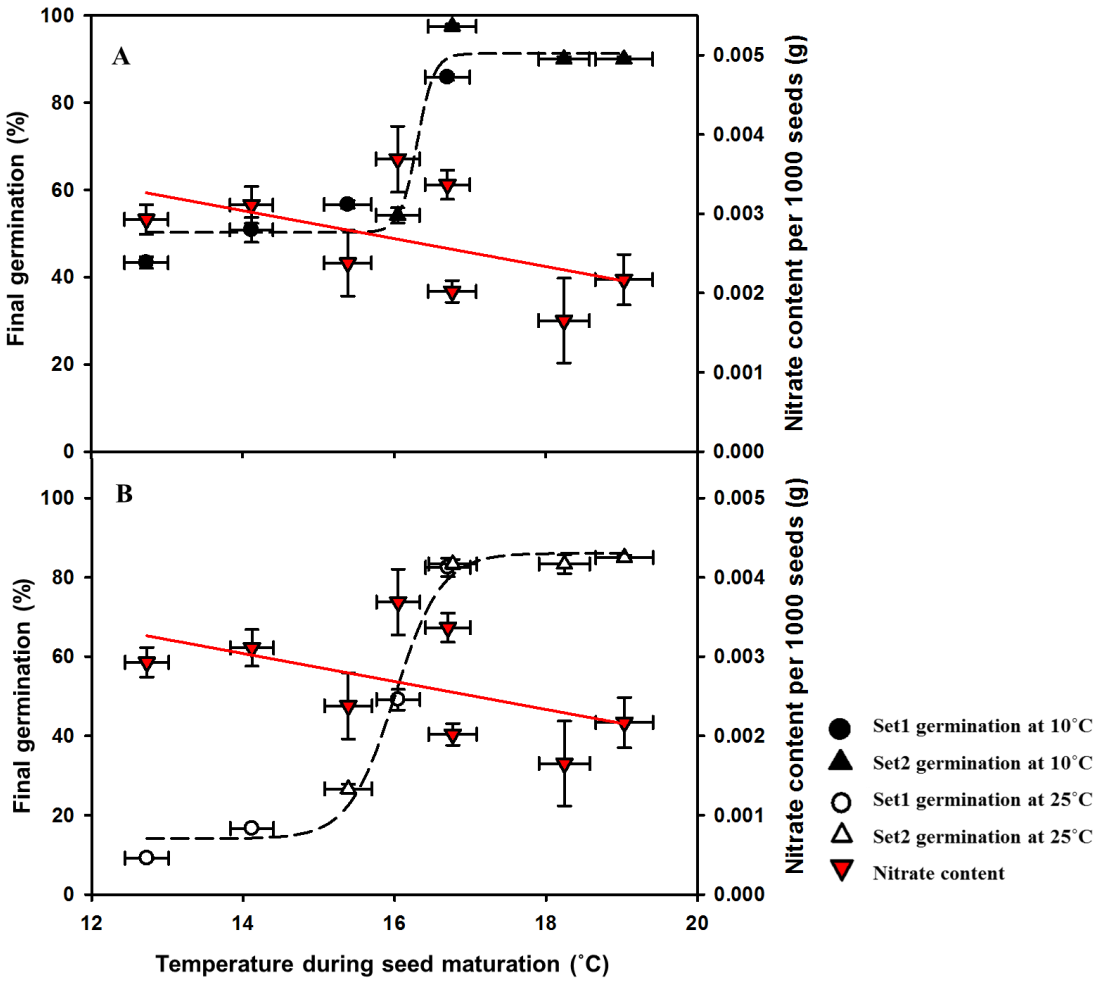

Figure 7.19 The relationship between maturation temperature and final germination of Bur seeds produced in the thermal gradient tunnel during 2011 (**A**: germination at 10˚C; **B**: germination at 25˚C **Set1** and **Set2** seed details are given in table 7.3. The relationship between temperature and germination fits a sigmoidal regression: f=50.29+40.99/(1+exp(-(x-16.3)/0.11)), with R^2=0.9521 for 10˚C; f=14.1+71.99/(1+exp(-(x-16)/0.3)) R^2=0.9890 for 25˚C. The relationship between maturation temperature and nitrate content per 1000 seeds fits a linear regression: f=0.0055-0.0002x (R=0.5089). Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The horizontal error bars show the range of observed temperature during seed maturation, but the regressions are fitted using the mean temperature in each period.

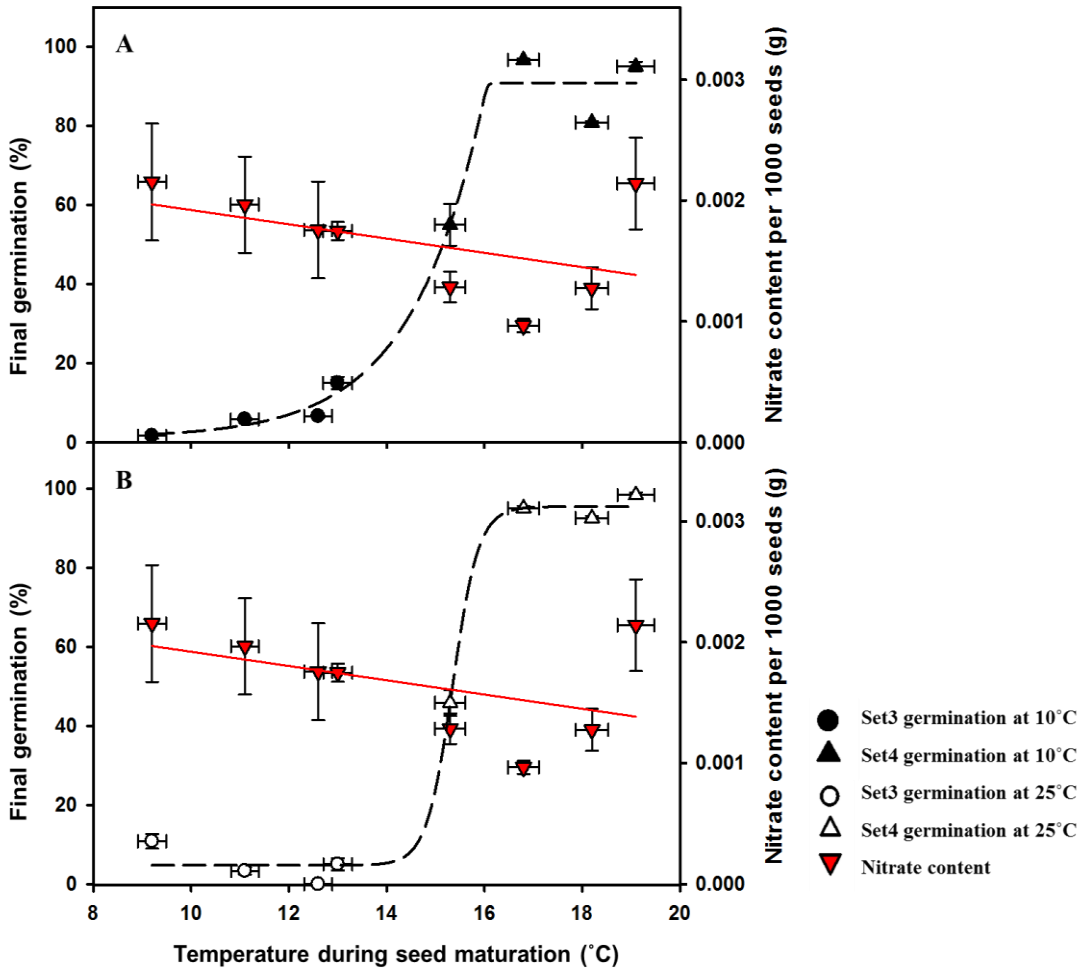

Figure 7.20 The relationship of final germination, nitrate content in seeds and maturation temperature of Bur seeds produced in the thermal gradient tunnel on high N compost in 2012. A: germination at 10˚C; **B**: germination at 25˚C; **Set3** and **Set4** seed details are given in table 7.3. The relationship between maturation temperature and germination of all three replicates fits a sigmoidal regression: f=1.19+89.65/(1+exp(-(x-16.06)/0.03))^0.02 (R^2=0.9862) and f=4.79+90.62/(1+exp(-(x-15.35)/0.26)) (R^2=9944) at 10˚C and 25˚C respectively. The relationship between maturation temperature and nitrate content per 1000 seeds fits a linear regression: f=0.0025-5.8995E-005x (R=0.4701). Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The horizontal error bars show the range of observed temperature during seed maturation, but the regressions are fitted using the mean temperature in each period.

Table 7.12 Growth conditions and final germination percentages for each set of seeds produced in 2011 and 2012

Locations in the tunnel	**Mean temperature and day length during vegetative phase (day/night)[1]**			
	2011		**2012**	
	Set 1	**Set 2**	**Set 3**	**Set 4**
T1	19/15˚C (16/8h)	23/17˚C (16/8h)	23/17˚C (16/8h)	15/10˚C (16/8h)
T2	19/15˚C (16/8h)	23/17˚C (16/8h)	23/17˚C (16/8h)	16/12˚C (16/8h)
T3	19/15˚C (16/8h)	23/17˚C (16/8h)	23/17˚C (16/8h)	18/13˚C (16/8h)
T4	19/15˚C (16/8h)	23/17˚C (16/8h)	23/17˚C (16/8h)	19/14˚C (16/8h)
Locations in the tunnel	**Mean temperature and day length during seed maturation (day/night)[1]**			
	2011		**2012**	
	Set 1	**Set 2**	**Set 3**	**Set 4**
T1	15.3/8.5˚C (14/10h)	16.9/12.1˚C (12/12h)	11.0/6.3˚C (14/10h)	16.5/12.3˚C (16.5/7.5h)
T2	16.7/9.8˚C (14/10h)	18.5/13.3˚C (12/12h)	13.1/7.9˚C (14/10h)	18.0/13.6˚C (16.5/7.5h)
T3	18.6/11.7˚C (14/10h)	19.9/14.9˚C (12/12h)	14.6/9.4˚C (14/10h)	19.5/14.7˚C (16.5/7.5h)
T4	19.1/12.6˚C (14/10h)	20.7/15.7˚C (12/12h)	15.2/9.5˚C (14/10h)	20.3/15.5˚C (16.5/7.5h)
Locations in the tunnel	**Final germination on High N compost (%) [2]**			
	2011		**2012**	
	Set 1	**Set 2**	**Set 3**	**Set 4**
T1	43.3/9.2	56.7/26.7	1.7/10.8	55.0/45.8
T2	50.8/16.7	97.5/83.3	5.8/3.3	96.7/95.0
T3	54.2/49.2	90.0/83.3	6.7/0	85.8/92.0
T4	85.8/82.5	90.0/85.0	15.0/5.0	95.0/98.3

[1] Data are mean temperature during day and night, data in parenthesis are mean hours of day and night;

[2] Data are mean percentage of final germination at different temperature presented as 'germination% at 10˚C /germination% at 25˚C'

There was no significant difference in the average day length during vegetative phase between the four sets of seeds (16h), but the temperatures for Set 4 seeds showed a gradient and also a lower mean value similar to Set 1 (Table 7.12). However, the final germination pattern stayed unchanged compared to that in 2011, which again indicate that the conditions during the vegetative phase of growth had less influence on seed germination potential than those during seed maturation.

During maturation Set 1 and Set 3, seeds had the same mean day length but different temperature; whereas the main difference between the Set 2 and Set 4 seeds was the day length (Table 7.12). The germination results presented show that Set 2 and Set 4 seeds, except for seeds harvested from the ambient end of the tunnel (T1), showed a similarly high germination regardless of the difference in day length (i.e. 12h in 2011 and 16.5h in 2012). The Set 1 seeds harvested from either location in 2011 showed a higher germination than those of Set 3 seeds in 2012 and this can be explained by higher mean maturation temperatures both in the day and night.

Interestingly the *Bur* seeds produced in the regimes with lower nitrate (medium N and low N) showed similar germination patterns against maturation temperature with seeds matured under cooler conditions exhibiting a deeper dormancy (Fig.7.21 and Fig.7.22). However, at the lower nitrate levels the seeds produced at lower temperatures showed an increased percentage of germination, especially when the seeds were imbibed at 10˚C.

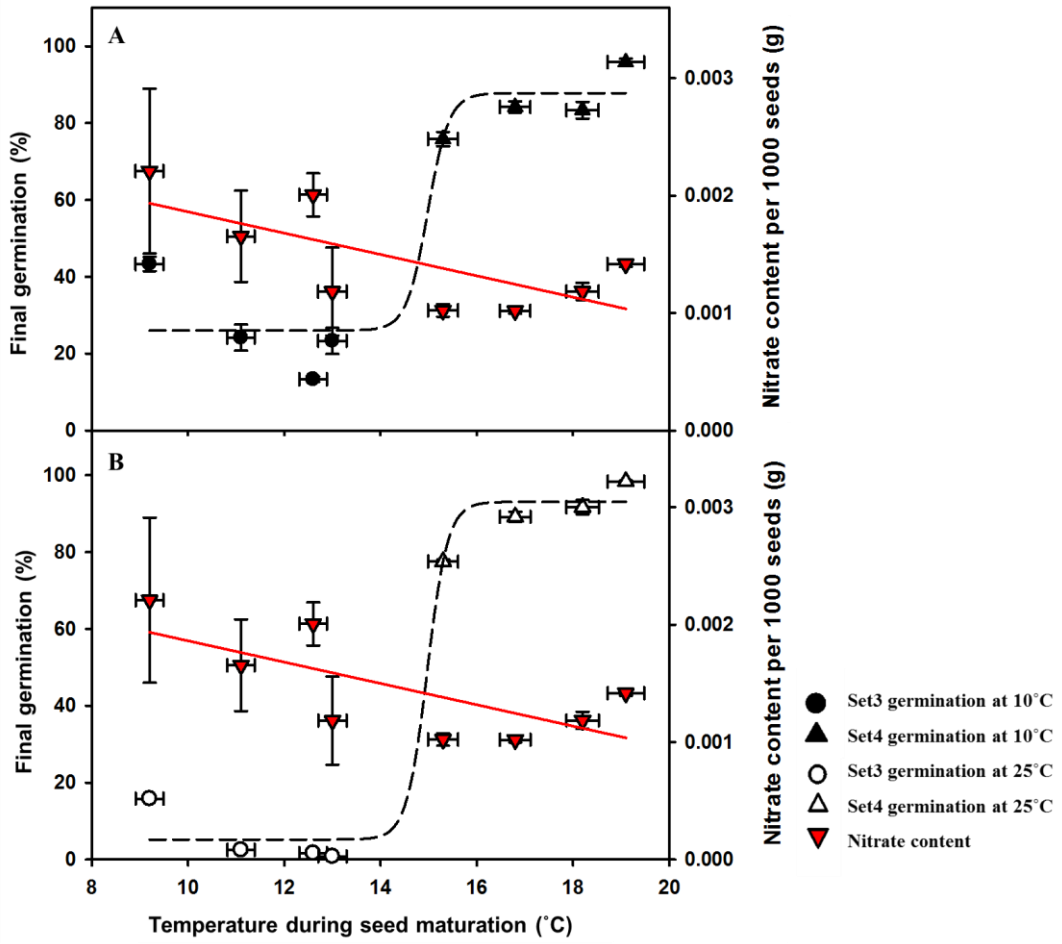

Figure 7.21 The relationship of final germination, nitrate content in seeds and maturation temperature of Bur seeds produced in the thermal gradient tunnel on medium N compost in 2012. A: germination at10˚C; **B**: germination at 25˚C; **Set3** and **Set4** seed details are given in table 7.3. The relationship between temperature and germination of all three replicates fits a sigmoidal regression: f=26.04+61.75/(1+exp(-(x-14.99)/0.22)) (R^2=0.9249) and f=5.2+87.86/(1+exp(-(x-14.97)/0.21)) (R^2=0.9864) at 10˚C and 25˚C respectively. The relationship between maturation temperature and nitrate content per 1000 seeds fits a linear regression: f=0.0028-9.079E-005x (R=0.7034). Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The horizontal error bars show the range of observed temperature during seed maturation, but the regressions are fitted using the mean temperature in each period.

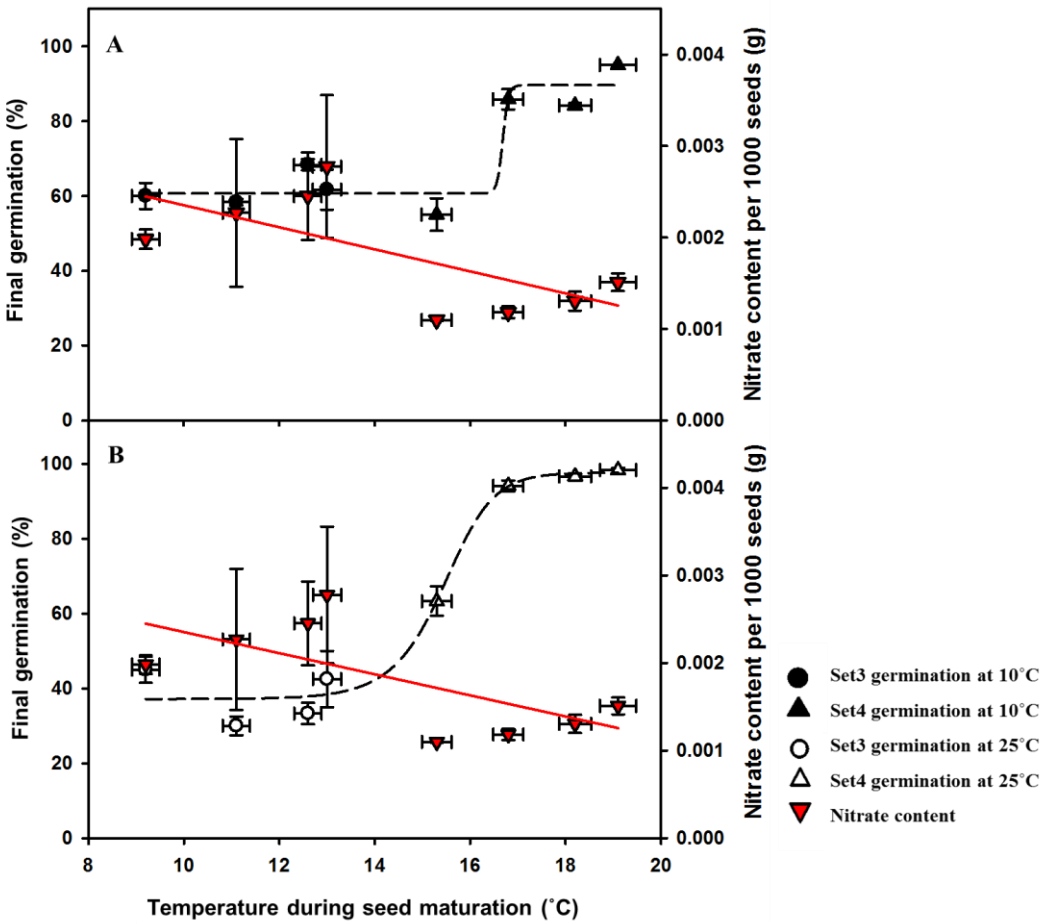

Figure 7.22 The relationship of final germination, nitrate content in seeds and maturation temperature of Bur seeds produced in the thermal gradient tunnel on low N compost in 2012. A: germination at 10˚C; **B**: germination at 25˚C; **Set3** and **Set4** seed details are given in table 7.3. The relationship between temperature and germination of all three replicates fits a sigmoidal regression: f=60.67+28.92/(1+exp(-(x-16.69)/0.06)) (R^2=0.9023) and f=37.48+60.4/(1+exp(-(x-15.44)/0.52)) (R^2=0.9747) at 10˚C and 25˚C respectively. The relationship between maturation temperature and nitrate content per 1000 seeds fits a linear regression: f=0.0036-0.0001x (R=0.6648). Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The horizontal error bars show the range of observed temperature during seed maturation, but the regressions are fitted using the mean temperature in each period.

In 2012 the two sets of seeds were produced at different times of year, with lower mean temperature for Set 3 (11.2˚C) and higher for Set 4 (17.4˚C) in the tunnel. The effect of nitrate content in seeds on germination differed in the two sets, depending on the maturation temperature (Fig.7.23). When the seeds were matured at

low temperature (Set 3), a positive correlation between seed nitrate content and percentage germination can be seen at both imbibition temperatures (Fig.7.23A). However, the seeds that were matured at higher temperature (Set 4) were less dormant and most germinated to high percentages (Fig.7.23B).

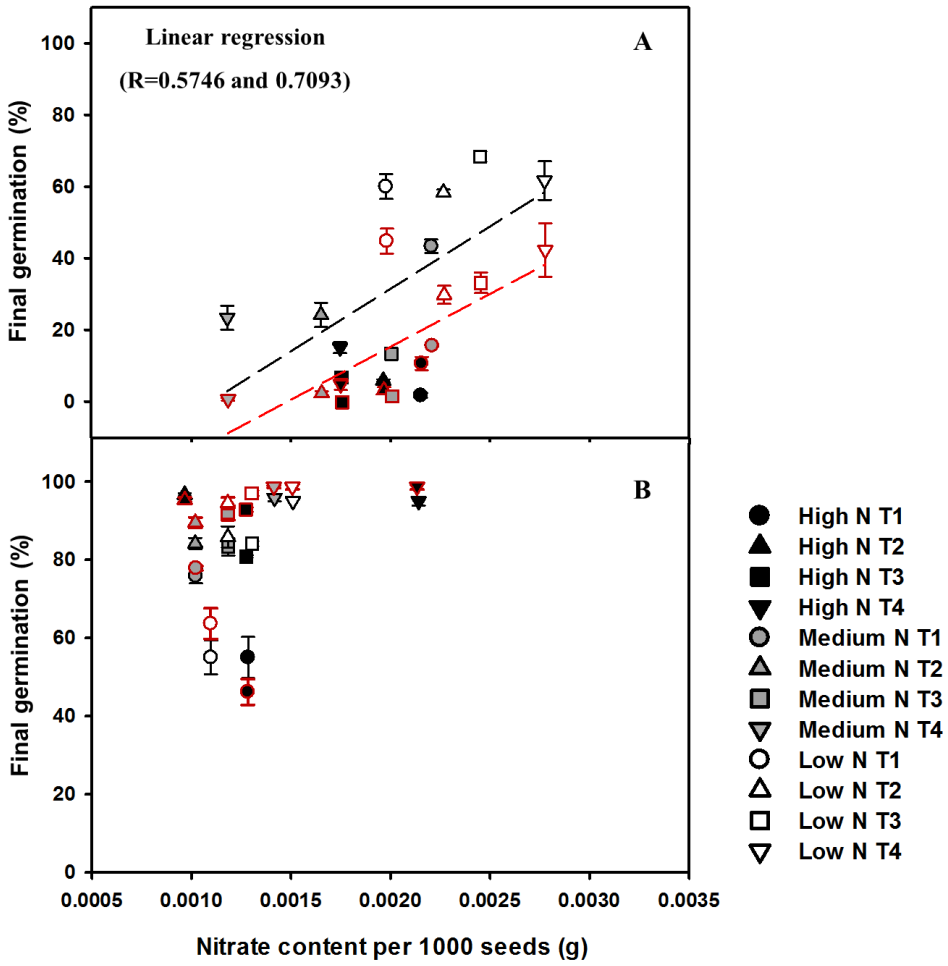

Figure 7.23 The relationship between final germination and nitrate content per 1000 seeds of Bur produced along the thermal gradient tunnel in 2012. A: **Set3** and B: **Set4** seed details are given in Table 7.3. Different locations in the tunnel and nitrate regimes are represented by different symbols shown in the legend. Black symbols are from germination data at 10˚C; red symbols indicate germination at 25˚C. In Set1 a linear regression has been fitted: f=-38.35+34845x (R=0.5746) and f=-43.58+29547x (R=0.7093) at 10˚C and 25˚C respectively. Data are the mean ± standard error. No error bar indicates symbol is larger than the error.

Seed size (1000-seed weight) was also affected by maturation temperature and like percentage germination showed an increase with the increasing maturation temperature (Figure 7.24). However, this increase in seed size as the temperature increased became limited or even reversed above a threshold (Fig.7.24).

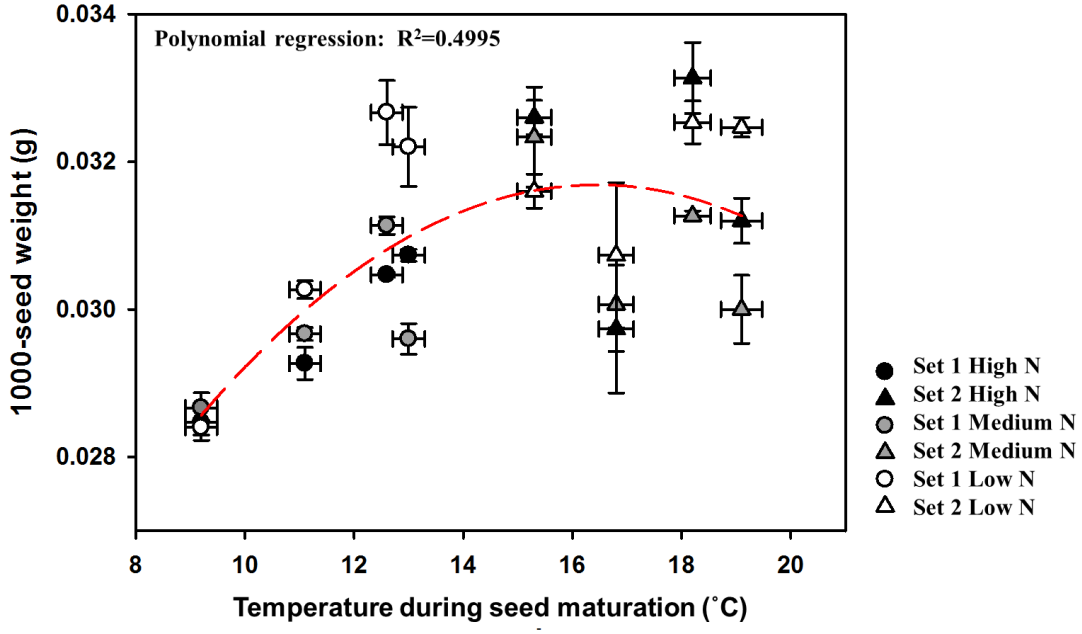

Figure 7.24 The relationship between 1000-seed weight and maturation temperature in Bur seeds produced along the thermal gradient tunnel in 2012. Different sets of seeds and nitrate regimes are indicated by the symbols outlined in the legend. **Set3** and **Set4** seed details are given in table 7.3. A polynomial regression is fitted: f=0.0156+0.002x-5.9522E-005x^2 (R^2=0.4995). Data are the means ± standard error. No error bar indicates symbol is larger than the error. The horizontal error bars show the range of observed temperature during seed maturation, but the regressions are fitted using the mean temperature in each period.

The influence of maturation temperature on final germination is clearly demonstrated when data from both years are presented (Fig. 7.25). When seeds were germinated at both 10 and 25˚C there was a sigmoid relationship. At both germination temperatures, there was a similarly high percentage of germination for seeds matured at warm temperature (≥15˚C). However, seeds matured at lower temperatures resulted in higher percentage germination at 10 than at 25˚C.

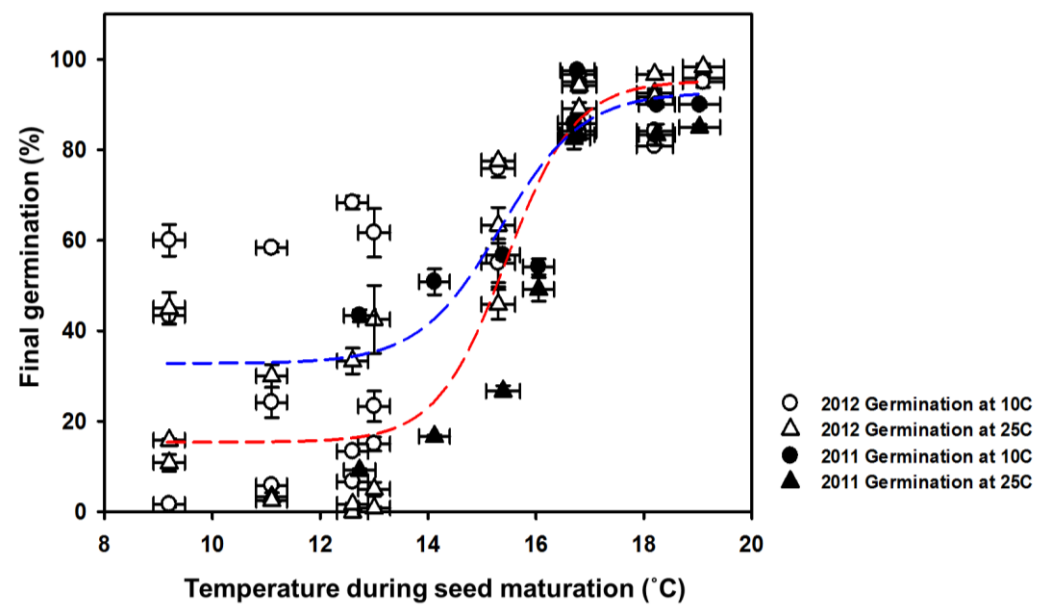

Figure 7.25 The relationship between final germination and maturation temperature of seeds produced along the thermal gradient tunnel in 2011 and 2012. Germination temperature and year are indicated by different symbols explain in the legend. Sigmoidal regressions are fitted. The blue curve is fitted germination data at 10˚C f=32.04+59.97/(1+exp(-(x-15.39)/0.76)) (R^2=0.7098); the red curve is fitted to germination at 25˚C, f=14.65+79.89/(1+exp(-(x-15.49)/0.64)) (R^2=0.8657). Data are the mean ± standard error. No error bar indicates symbol is larger than the error. The horizontal error bars show the range of observed temperature during seed maturation, but the regressions are fitted using the mean temperature in each period.

7.4 Discussion

Simulated global warming has a significant impact on life cycle traits in Arabidopsis that differs between summer and winter annual phenotypes

Increases in mean global temperature and extremes of temperature are predicted for the future and these events are expected to be significantly larger than those that have occurred in recent history. Consequently, large disruptions in the behaviour of wild plants and crops are anticipated, the nature of which is largely unknown (Kumar and Wigge, 2010). It has been predicted that temperature on its own could contribute to the extinction of up to one third of all European plant species (Thuiller *et al*., 2005). Since plants are not able to 'escape' when conditions become unfavourable, it is essential for them to have the capacity to adapt their growth and

developmental processes to the changing environment, i.e. developmental plasticity. For example, the species which are able to adapt their flowering time in response to temperature change are less likely to become extinct than those species which cannot (Willis *et al*., 2008).

The plant life cycle has key stages/traits and these include depth of dormancy, time of seedling emergence, time from seedling emergence to bolting and then to seed maturation. The environment influences the timing of each of these traits (Fig.7.13). This investigation has shown that increased temperature in line with global warming predictions has a large effect on all these key life cycle traits in *Arabidopsis* and the size of the effect is ecotype dependent. The data collected from the thermal gradient tunnel indicates that even a relatively small change in temperature (e.g. 1˚C) can affect *Arabidopsis* life cycle traits. For example in the present study, by comparing the ambient end of the tunnel (T1) and the location that has a mean temperature of 1.5˚C higher than T1 (T2), the mean seedling emergence date has advanced by 4.5 days and the mean bolting date delayed by more than 3 days (Fig.7.13). As temperature affects each life cycle trait this means that each trait has a knock on effect so that the following trait will be exposed to different temperature. In this study, nitrate supply to mother plants was also found to influence this temperature effect on life cycle traits. How temperature influences these traits is not understood, but one possible mechanism of sensing ambient temperature involves changes in the H2A.Z occupancy of nucleosomes, which wrap DNA tightly (Kumar and Wigge, 2010). H2A.Z occupancy increases as temperature decreases and therefore blocks transcription.

Emergence and bolting phenotypes: The large differences in seedling emergence between summer and winter annuals have been discussed in *Chapter 4*.

The seedling emergence experiment was set up in May consistent with the time of year that Bur would emerge in the field (Footitt *et al*., 2013).In nature, Bur as a summer annual would germinate and emerge at this time, whereas it is the time when Cvi seed would be shed from the mother plant. However, all the seeds were after-ripened so emergence from Cvi seeds also occurred immediately following sowing but was less successful than Bur. Seedlings emerged earlier from Bur than from Cvi seeds (low T_{50}, i.e. high emergence rate) at all temperatures. This earlier emergence time in Bur led to slightly earlier bolting. However, Bur has a considerably larger rosette with more leaves in than Cvi, which also indicates a faster growth rate for Bur. Although the increase in temperature did not have a significant effect on leaf number and rosette size at bolting, it did shorten the vegetative phase. Future warming is therefore likely to delay the emergence time but shorten the vegetative growth phase.

Seed production: According to the linear regression analysis (Table 7.8), seed number rather than seed size was the major component that determines seed yield of both Bur and Cvi. Seed yield of both ecotypes was also significantly and positively related to plant height, indicating a phenotypic correlation between seed production components (e.g. seed yield and seed number) and vegetative characters. This correlation was also seen in species such as *Digitaria* and *Sorghum* species (Govil, 1979; Aliero, 2000; Aliero and Morakinyo, 2002).

As discussed in *Chapter 3*, the maternal conditions in which a seed matures has a large effect on the progeny as these conditions determine genetic inheritance, the endosperm and testa composition, seed nutrient content and importantly the dispersal time (Donohue *et al*., 2009). The results presented here confirm this showing that maternal conditions have a large influence on both the weight and size of seeds

produced. Seed size decreased with increasing temperature in both Bur and Cvi ecotypes with the exception of low N conditions (Table 7.10). Seed yield and size are intimately linked as the two are determined by source sink interactions and overall plant fertility. Investing greater resources into seed size could reduce the number of seed produced if plant productivity is compromised by environmental conditions.

In the present work seed yield and seed number were affected by temperature differently in the two ecotypes: i.e. Bur produced more seeds at the ambient end of the tunnel, whereas in Cvi seed yield and seed number were greater at warm end of the tunnel. This may be because Bur is adapted to cooler temperature while Cvi is adapted to warmer temperature. For Bur, cooler temperatures allow plants to have a longer time to accumulate storage compounds and produce a higher mass of seeds, but a higher temperature leads to reduced fertility resulting from stamen extension defects (discussed in *Chapter 3*) and consequently less efficient pollination to reduce seed yield. However, for Cvi, the increased temperature at the warm end of the tunnel during seed maturation is closer to the temperatures Cvi is exposed to in nature so that the seed production mechanism may function better, leading to higher yield and larger seed number. In addition, Cvi plants grown at higher temperature produced more cauline leaves (Fig.7.7), indicating that photosynthate is critical for seed filling. This agrees with the previous work that removal of cauline leaves significantly reduced most measures of fruit and seed production in *Alliaria petiolata* (Susko and Lovett-Doust, 1999).

Therefore although increased temperature reduced seed size in both ecotypes this had a more negative effect on Bur than Cvi. In Cvi there was a fundamental trade-off between seed number and seed size leading to a larger number of small seeds as a result of an increasing sink size *i.e.* more fertilised seeds competing for

resources from a limited sink *i.e.* photosynthetic capacity. This is an example of the plasticity of reproductive fitness with larger seeds in circumstances were seed number is reduced potentially increases the long term survival (longevity) of seeds in the soil seeds bank. Whereas increased yield and small seed size increase the size of the seed generation entering the soil seed bank. However, this 'compensation' effect did not occur in Bur seeds, as a result of the effect of higher temperatures during the reproductive phase on fertility which reduces both seed size and seed number. This suggests that Bur as a summer annual may be less successful than Cvi in the face of global warming.

Bur has a higher individual plant yield and more notably high nitrogen use efficiency under N-limiting conditions

The possibility of lowering N input (fertilizer) and breeding plants with better nitrogen use efficiency (NUE) is an important goal in creating sustainable agricultural systems (Chardon *et al*., 2010; Hirel *et al*., 2007). Due to the large genetic variation in *Arabidopsis* populations, the adaptive responses to N-limiting conditions have been investigated by a quantitative genetic approach, which showed that the response to N availability was influenced by both genotype and a genotype × N fertilization level interaction (Loudet *et al*., 2003). In the study by Chardon *et al*. (2010), 18 *Arabidopsis* ecotypes were grown under ample and limiting nitrate supply, and the natural variation of NUE-related traits was investigated. The ecotype Bur-0 was found to be one of the two notable genotypes, which exhibited high NUE and large biomass, especially under N-limiting conditions. In addition, Bur-0 also appeared poorly tolerant of a high N supply with an increasing N supply mainly resulting in N storage (Chardon *et al*., 2010). Cvi was shown in an earlier chapter to

be less tolerant to reduced nitrogen supply and grew poorly at medium and low N levels and therefore nitrate level is considered here in terms of Bur only.

The work reported above is directed to understanding NUE in an agricultural context. However, under natural conditions *Arabidopsis* occupies habitats with limited soil, such as the limestone pavements of the Burren (reviewed in *Chapter 2*). In the case of Bur regular heavy rain will also tend to reduce available nitrate through leaching. Thus plant response to available nitrate may be a key adaptive growth trait and seed response to nitrate may allow selection of a suitable location for germination and subsequent growth. In the thermal gradient tunnel experiment nitrate supply to the mother plants had a great effect on plant growth during the vegetative phase. Lower nitrate supply accelerated seedling emergence, whereas higher nitrate supply shortened the time from emergence to bolting. In addition, Bur plants grown with higher level of nitrate supply (high N) had larger rosettes and more leaves at bolting than those with low N. However, the nitrate supply to the mother plants did not significantly influence seed dry weight or their nitrate content. This suggests that Bur is able to make use of a higher level of nitrate to increase biomass, but the proportion of their N allocated to seeds is relatively defined. This agrees with the observation that low N conditions increased both nitrogen remobilization and translocation of the nitrogen absorbed after flowering to the seeds, whereas under ample nitrate supply, the nitrogen absorbed post-flowering was mainly driven to the vegetative tissue, which was the limiting factor for seed filling at high N (Masclaux-Daubresse and Chardon, 2011).

NUE was found to be highly correlated to harvest index, suggesting a strong link between nitrogen allocation and yield (Masclaux-Daubresse and Chardon, 2011). The calculated HI values of Bur seeds harvested from the tunnel were

significantly affected by nitrate supply to the mother plants rather than temperature. The HI values increased with decreasing levels of nitrate supply and those grown with low N had the highest HI values. This suggests that the high NUE of Bur is more noticeable under N-limiting conditions.

A linear relationship between endogenous nitrate content in seeds and final germination was found when the seeds were matured at lower temperatures (Fig.7.23). The seeds produced by plants grown on low N compost showed a much higher level of seed nitrate content and resultantly lower level of dormancy than those from the plants grown with higher nitrate supply. This result confirms the suggestion that endogenous nitrate content of *Arabidopsis* seeds is negatively related to depth of dormancy and enhances the effect of exogenous nitrate to relieve dormancy in the light (Alboresi *et al*., 2005; Matakiadis *et al*., 2009).

The effect of temperature during seed maturation can override the influence of factors, such as nitrate supply to the mother plants, to manipulate dormancy level and germination potential of Bur seeds

The investigation of the whole life cycle here has shown differences between Bur and Cvi despite growing conditions and species being the same, demonstrating the developmental plasticity of *Arabidopsis* (Koornneef *et al*., 2004). The predicted patterns of increasing temperature are not the same across the world, and greater warming has occurred over the northern hemisphere (IPCC, 2007). Thus, it is more likely that the change in temperature will be larger for Bur than for Cvi in its natural habitat near the equator. The seed literature shows many effects of temperature during seed maturation. The data collected in the thermal gradient tunnel indicates which of these may occur in realistic global warming scenarios based on future predictions by IPCC and UKCIP described in the introduction to this chapter.

The effect of emperature: in both years of study there was a strong relationship between maturation temperature and germination of Bur seeds, i.e. lower seed maturation temperature resulted in higher levels of dormancy, which agrees with the previous controlled environment work in *Arabidopsis* and other species (Chiang *et al*., 2009; Donohue *et al*., 2009; Schmuths *et al*., 2006; Kendall *et al*., 2011). This result was also shown and discussed in *Chapter 5* and *Chapter 6*. In particular, 15˚C appears to be a pivotal temperature for dormancy relief and induction in Bur seeds, again in agreement with previous work on the summer annual *Polygonum persicaria* (Bouwmeester and Karssen, 1992).

The genetic pathway of dormancy caused by low seed maturation temperature is poorly understood, but low temperature can lead to an increase in ABA levels coupled with a decrease in GA_4 levels (Kendall *et al*., 2011). Low temperature during seed maturation can also induce several dormancy-related genes including *DOG1* (Kendall *et al*., 2011). In addition, FLC plays a role in seed dormancy and may be a part of a dormancy-regulating temperature signal transduction pathway, regulating *CYP707A2* (ABA catabolism) expression (Chiang *et al*., 2009). CBFs (C-REPEAT BINDING FACTORS, reviewed in *Chapter 1*) were also found to act in parallel to a low-temperature signalling pathway in the regulation of seed dormancy (Kendall *et al*., 2011). Furthermore, the expression of *FLC* and *DOG1* exhibited cycling patterns that are linked to dormancy cycling in seeds buried in the field (Footitt *et al*., 2011; Footitt *et al*., 2013; also the results presented in *Chapter 4*).

The promotion of seed germination caused by high maturation temperatures has been reported in many species (Baskin and Baskin, 1998; Fenner, 1991; Hoyle *et al*., 2008; Kozarewa *et al*., 2006). Germination of Bur seeds produced in the tunnel fitted a sigmoidal relationship with temperature, which showed that seeds matured at

high temperature had an increased germination potential, especially when imbibed at 25˚C. This promotional effect of high maturation temperature has been associated with increasing ethylene production (Kozarewa *et al*., 2006), which may counteract ABA during seed development and stimulate the transition from dormancy to germination, for example in *Pinus monticola* seeds (Feurtado *et al*., 2007). Increased activity of endo-β-manannase during imbibition and decreased cell number has also been observed in the lettuce seeds matured at high temperature. Thus warm maturation temperatures may reduce physical resistance of the endosperm by weakening the cell wall and depleting stored reserves leading to cell collapse (Sung *et al*., 2008).

The effect of nitrate: In 2012, plants were grown on compost with different levels of nitrate, which led to larger differences in vegetative phenotypes than in the germination of the next generation. There was little effect of nitrate regime on dormancy levels of the seeds produced. In the thermal gradient tunnel experiment it was temperature rather than nitrate supply to the mother plant that significantly influenced endogenous seed nitrate that subsequently affected germination. Although a positive linear relationship between seed nitrate content and percentage germination was found in seeds matured at lower temperature, germination percentages were low, indicating low temperature induction of dormancy was greater than relief through enhanced endogenous nitrate. This predominant role of maturation temperature persists as temperature increases. Even though endogenous nitrate fell to a low level, seeds matured at high temperature still had very high percentages of germination. These results suggest that neither exogenous nor endogenous nitrate has significant practical impact on seed dormancy of Bur seeds.

The effects of vegetative growth conditions: Both sets of seeds produced in 2011 were first grown in the temperature- and photoperiod- controlled glasshouse (23/17˚C, 16/8h, light /dark) and then transferred to the thermal gradient tunnel once they had bolted. Following transfer they experienced different temperatures at four locations in the tunnel. The seeds developed and matured in the tunnel had different germination behaviours even though they were grown under the same conditions before bolting. This suggests that the conditions after bolting (i.e. flowering and seed maturation) make a larger contribution to dormancy status than the conditions before bolting. In addition, although the Set 4 seed was different from the others as it was produced in the tunnel from seed to seed, and the temperatures for Set 4 seeds showed a gradient and also a lower mean value (Table 7.12), the final germination pattern stayed unchanged compared to that in 2011. Again this indicates that the conditions during the vegetative phase of growth had little influence on seed germination potential.

As mentioned above, Set 1 in 2011 and Set 3 in 2012 were timed so that seed maturation coincided with the natural time for the *Arabidopsis* winter annual ecotype Cvi at ambient temperature at Wellesbourne; whereas Set 2 in 2011 was timed so that seed maturation coincided with the natural time for the *Arabidopsis* summer annual ecotype Bur at ambient temperature at Wellesbourne. Additionally Set4 seed maturation occurred on the plants during higher temperatures in summer, intermediate between summer and winter annual phenotypes. The germination results presented in Table 7.2 show that Set 2 and Set 4 seeds, except for seeds harvested from the ambient end of the tunnel (T1), showed a similarly high germination regardless of the difference in day length. The Set1 seeds harvested from the warm end in 2011 had a significantly higher germination than those of Set3 seeds

harvested from the same location in 2012 which can be explained by a higher maturation temperature. Therefore, conditions during seed maturation showed a greater impact on final seed germination than the conditions during plant vegetative growth.

7.5 Conclusions

In conclusion increasing temperature in line with global warming predictions has a large impact on life cycle traits of *Arabidopsis*, and these differ between the summer and winter annual ecotypes of Bur and Cvi. In particular, temperature during seed maturation plays a paramount role in determining dormancy level (lower temperature enhances dormancy) and germination potential of *Arabidopsis* seeds. This temperature effect can override the influence of other environmental factors, such as nitrate supply to the mother plants, temperature during vegetative growth and day length during seed maturation. In the summer annual Bur, increasing temperature during winter resulting from global warming is likely to limit low temperature stratification to break dormancy. However, the results here show that the higher temperature during seed maturation will tend to reduce dormancy to offset the impact of warmer winter temperatures to reduce the potentially negative impact of global warming. In addition, Bur exhibited higher individual plant yield and NUE especially under N-limiting conditions and appeared poorly tolerant of a high N supply during seedling emergence compared to Cvi. This is likely to have resulted from adaptation to its naturally low N environment/high rainfall environment.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSION

8.1 The influence of environmental signals:

The aims of this study were: (1) to characterize the *Arabidopsis* Bur ecotype, in particular the responses of dormancy cycling and germination to environmental signals; (2) to increase the understanding of the regulation of seed dormancy cycling in response to the environment at an eco-physiological and molecular level using the contrasting *Arabidopsis* ecotypes Bur and Cvi.

8.1.1 Characterisation of dormancy cycling in Bur and Cvi seeds in reponse to environmental signals at the physiological level

The present study confirms that environmental signals including temperature, light and nitrate impact on the subsequent dormancy status of mature *Arabidopsis* seed while they are on the mother plant. These same variables are then also of great importance in determining the dormancy status, the pattern of dormancy cycling and germination following shedding in the soil seed bank. These responses determine the time of seed germination and subsequent key life cycle events, such as flowering and seed production in the next generation. However, there is also a genetic component to these responses, which differed between the ecotypes Bur and Cvi. The combination of genetics and environmental conditions during both seed development and following shedding determine their life cycle phenotypes (summer and winter annuals respectively) and adaptation to their distinct habitats.

8.1.1.1 Effects of temperature:

Seed development: temperature during seed maturation played an important role in manipulating seed dormancy and germination potential of both Bur and Cvi. This temperature effect can override the influence of other environmental factors especially when temperature is high. Lower maturation temperature leads to higher

levels of seed dormancy in both ecotypes. In particular, 15˚C appears to be a pivotal temperature for dormancy release and induction in Bur, which is in agreement with work on the summer annual *Polygonum persicaria* (Bouwmeester and Karssen, 1992). The seeds of *Arabidopsis* Col ecotype matured at 15˚C have also been shown to have decreased germination in response to cold stratification compared to higher temperatures (e.g. 20˚C), and this was mainly due to the lowering of phytochrome expression levels resulting in the imposition of a light requirement (Donohue *et al*., 2008; Kendall *et al*., 2011).

Post shedding dry seeds: The deep dormancy of Cvi seeds was effectively released by after-ripening (AR). At least 105 days of AR (at 20˚C and 55% ERH) is required for sufficient dormancy release to reach the maximum germination potential. In its natural environment, this long period of AR likely ensures germination coincides with the rainy season that arrives after a hot and dry summer in the Cape Verdi Islands. In contrast, dormancy is minimal in Bur and dry AR has a limited role as there is no prolonged dry spells due to the year round rainfall in the Burren.

Post shedding imbibed seeds: fresh Bur seeds are less dormant than fresh Cvi seeds. An opposite thermal response was found in after-ripened Bur and Cvi seeds on the thermal gradient table. Cvi seeds tend to germinate more readily at lower temperatures consistent with a winter annual phenotype, while Bur seeds show the reverse response and germinate more readily at higher temperatures consistent with a summer annual phenotype. In addition, short periods of low temperature (5˚C) appeared to be an efficient way to release dormancy of Bur, but not in Cvi that firstly requires a period of AR to become sensitive to low temperature (Cadman *et al*., 2006; Finch-Savage *et al*., 2007).

Extended low temperature in the absence of light was shown to induce secondary dormancy in both Bur and Cvi. The minimum duration of exposure to low temperature that induces secondary dormancy differs between the two ecotypes (i.e. 4 days in Cvi and 14 days in Bur). The relatively longer period of low temperature required by Bur could pre-adapt the seeds to overwinter in the soil seed bank. Figure 3.6 show that Bur seeds sown in autumn, unlike those of Cvi, initially lose dormancy and then go secondarily dormant to avoid germination during winter.

The time required to induce secondary dormancy in Bur is determined by temperature: the rate of induction is greater with higher temperatures. This response to warm stratification is likely a mechanism to avoid germination before winter in a warm spell following shedding in autumn. Cvi also responds to warm stratification that releases dormancy. Footitt *et al*. (2011) showed in the field that deep dormancy in Cvi was lost in the warm moist soils of the UK summer in less than 30 days, whereas a similar level of dormancy relief would require 200 days of dry after-ripening. In its natural environment rapid loss of dormancy in warm moist conditions may enable rapid completion of dormancy relief by AR to allow germination when rain comes after the dry summer.

8.1.1.2 Effect of light:

Seed development: Long days (16/8h, light /dark) during plant growth reduced seed dormancy levels compared to day neutral conditions (12/12h, light /dark) at a mean temperature of 20˚C. However, day length did not have a significant effect on dormancy level if seeds were matured at higher temperatures (≥ 20˚C).

Post shedding seeds: germination of fresh Cvi was shown to have an absolute requirement for light to remove the final layer of dormancy before gemination can proceed in agreement with previous work by Finch-Savage *et al*. (2007). Whereas,

the less dormant Bur seeds have the capacity for a proportion of the seed population (dependent on temperature) to germinate in darkness after imbibition, indicative of their low level of dormancy; and in one instance Bur dormancy was so low that the light requirement was lost completely. In view of this response it is perhaps surprising that no seed germination was recorded in samples recovered from the soil over a one year cycle. This may be because seeds in the soil are exposed to limiting water availability much of the time, and this mild stress can enhance the tendency of seeds to enter seconary dormancy (Footitt and Finch-Savage pers. comm.). Even in the absense of stress, as summarised above, dark incubation differently influences dormancy status depending on the duration, i.e. short periods of dark incubation cause dormancy release, while prolonged periods of dark incubation lead to the induction of secondary dormancy. In Bur the seeds exhibit high sensitivity to light following short periods of dark incubation, but this sensitivity decreases after prolonged imbibition in the dark (≥ 60 days).

8.1.1.3 Effect of nitrate:

Soil nitrate effect: Cvi was less tolerant to reduced nitrogen content in the growing media and could not survive on compost with low N. Therefore Ler, another winter annual ecotype, was substituted for Cvi in the experiments carried out to determine the impact of nitrate supply to the mother plant on the dormancy of seed produced. High nitrate feeding of the mother plants resulted in less dormant Ler seeds. This impact of nitrate supply on dormancy level was also influenced by other environmental signals. In contrast, nitrate supplied to Bur mother plants had little impact on seed dormancy; seeds produced from both nitrate regimes showed similarly high sensitivity to exogenously supplied nitrate during subsequent imbibition.

Nitrate sensitivity in imbibed seeds: Nitrate was found to play a positive role in the promotion of Cvi seed germination (Alboresi *et al*., 2005), but in this highly dormant ecotype a period of AR is required before there was a significant effect of nitrate (Finch-Savage *et al*., 2007; Footitt *et al*., 2011). Thus nitrate may not be perceived until dormancy has started to decline in deeply dormant Cvi seeds. The freshly harvested, but less dormant Ler seeds exhibit thermo-dormancy at 25˚C like Cvi seeds, however, nitrate provided exogenously during imbibition released dormancy in a dose-dependent manner. In contrast to Ler and Cvi, the shallowly dormant Bur seeds were always highly sensitive to nitrate. Interestingly, higher concentration of nitrate can delay the germination of Bur seeds by two days, which is consistent with previous work showing that Bur plants are poorly tolerant of high nitrate nutrition (Chardon *et al*., 2010).

8.1.2 Characterisation of dormancy cycling in Bur and Cvi seeds in reponse to environmental signals at the ecological and molecular level

Bur and Cvi were grown in a reciprocal environment experiment that approximated to their natural habitats and this clearly illustrated that seed dormancy status at maturity is determined by the interaction between genetics and environment. The different dormancy levels set in response to the maturation environment then impacts on the subsequent dormancy cycling patterns of the seeds. Recently, Pico (2012) have shown that an increasing proportion of *Arabidopsis* populations exhibit winter rather than summer annual behaviour as you progress up an altitude gradient. Thus lower temperature during maturation, shown here to increase depth of dormancy, also increased the likelihood of winter annual behaviour. Further work is required to determine what the relative contributions of genetics and environment play in the adaptation of different ecotypes via the dormancy status of their seeds.

The expression of the genes involved in hormone metabolism and signalling during dormancy cycling of buried Bur and Cvi seeds is consistent with the functions of these genes in dormancy regulation proposed in laboratory experiments. The mechanism of seasonal change in dormancy status appears to be similar in Bur and Cvi seeds and related to the temporal sensing of soil temperature via DOG1 to indicate the correct season (*Chapter 4* and Footitt *et al*., 2013). The expression of *DOG1* was negatively correlated with soil temperature in both ecotypes, but although ABA was required, this change was not correlated with the ABA content in Cvi seeds (Footitt *et al*., 2012). This work also showed that, unlike *DOG1* expression, the level of ABA was not directly related to depth of dormancy in Cvi. ABA was not measured here in Bur, but the expression of *NCDE6* (ABA biosynthesis gene) was not related to dormancy level. Taken together these results indicate that *DOG1* expression might be the dominant factor influencing dormancy via ABA signaling and thus ABA sensitivity rather than ABA synthesis. Further to this, the expression of *DOG1* was negatively related with the ABA catabolism gene *CYP707A2* in both Bur and Cvi seeds, consistent with dormancy level not being directly related to the endogenous ABA content (ABA synthesis), but associated with declining levels of endogenous ABA (ABA catabolism).

Cvi seeds exhibit deep dormancy in winter and low dormancy in the summer (Footitt *et al*., 2011), but the seasonal pattern in Bur was dominated by long periods of low dormancy. During the shallow dormancy in both ecotypes the expression of transcripts for the germination repressor DELLA genes (*RGA* and *RGL2*) increased. This repression, unlike the deep winter dormancy, can be removed by exposure to light. Therefore seeds in shallow dormancy will not germinate, but have the potential to respond rapidly to favourable germination conditions (e.g. light exposure during

disturbance; spatial sensing) when they occur (Footitt *et al*., 2011). Thus seeds germinate under these favourable conditions at the appropriate time of year for plant establishment, rather than when exposed to these same conditions out of season.

The expression of the dormancy-related genes *FLC* and *MFT* showed different patterns in the two ecotypes in response to environmental signals. This suggests they have importance in adaptation to local conditions to generate winter and summer annual phenotype behaviours. Further work is now required to genetically manipulate these genes and determine their impact on dormancy cycling to unravel their true involvement.

With the seasonal cycle set in motion, other gene expression in both ecotypes is consistent with the process of loss and induction of dormancy coordinated by downstream hormone synthesis, catabolism and signalling as postulated from the function of these genes in laboratory experiments.

8.1.3 Characterisation of other life cycle traits of Bur plants responses to environmental signals

Vegetative phase: nitrate supply rather than the temperature mainly influences seedling emergence in Bur following sowing/shedding, i.e. lower levels of nitrate led to earlier seedling emergence from the seeds. However temperature had a more significant impact on the bolting time than nitrate supply, i.e. lowering temperature caused much delayed bolting in Bur. There is also an interaction between the effect of nitrate supply and day length on the bolting time, i.e. longer day length accelerated bolting when Bur plants were grown on compost with higher nitrate. Additionally, both the temperature and nitrate supply have detectable impacts on rosette expansion and leaf number in the rosette. Higher temperature and higher levels of nitrate both positively influence vegetative growth by promoting leaf elongation and

subsequently rosette expansion. However, cooler conditions result in later bolting in Bur plants allowing more leaves to be produced in the rosette.

Reproductive phase: reproductive organs (i.e. stamens) were found to be very sensitive to high temperature during the early flower stage; this significantly limited filament extension in relation to that of the pistils resulting in failure to pollinate. However, high temperature did not influence the viability of Bur pollen as hand pollination successfully increased the percentage of fertilized ovules. Furthermore the decrease in seed yield found in Bur under higher temperatures (≥ 20˚C) could be reversed by applying GA. This confirmed that the lack of filament extension and consequent failure of pollination were the main cause of poor Bur seed production under these conditions.

The seed yield indicator, harvest index (HI) is also greatly influenced by nitrate supply to the mother plants in Bur, and this is related to nitrogen use efficiency (NUE) and nitrogen remobilization efficiency (NRE; Masclaux-Daubresse *et al*., 2010; Masclaux-Daubresse and Chardon, 2011). The highest HI value was found in seeds from mother plants grown with low N, confirming the high NUE and NRE of Bur under N-limiting conditions (Masclaux-Daubresse *et al*., 2010).

8.2 Comparison of Bur and Cvi in context of global warming and the potential impact of increasing temperature on summer and winter annuals

Increases in mean global temperature and extremes of temperature are predicted for the future and these predictions are significantly larger than those that have occurred to date. It is therefore important to understand how and whether plants can adapt their growth and developmental processes to the changing environment. In this thesis the effect of temperature and other environmental signals were investigated and then experiments were conducted in a thermal gradient tunnel to

simulate the impact of predicted future global warming to 2080. Large differences were found between summer and winter annual ecotypes (Bur and Cvi respectively) plant life cycles. Increased temperature in line with global warming predictions had a large impact on all the key life cycle traits observed. These traits included, depth of dormancy, time of seedling emergence, time from seedling emergence to bolting and then to seed maturation.

- Dormancy cycling and germination behaviour

Normally Bur as a summer annual, flowers and sets seeds in late summer up to early autumn, and then overwinters as dormant seeds, which then germinate in spring; the question is will this change as a result of global warming. The work presented show that Bur plants grown at higher temperature during seed maturation produce seeds with more shallow dormancy and high sensitivity to light. As a result of this, Bur instead of entering dormancy and overwintering as a seed could potentially germinate soon after imbibition in early autumn, leading to a shift from spring to autumn emergence. However, it was also shown that seeds rapidly enter secondary dormancy under warm moist conditions and so this scenario may be unlikely for the majority of the seed population.

Another scenario is that increased temperature during winter, as a result of global warming, may cause inadequate cold stratification resulting in fewer seeds having sufficient relief of dormancy to germinate in early spring. However, the lower level of dormancy at maturity due to higher temperature coupled with the rapid loss of dormancy in the presence of low nitrate concentrations in the soil seed bank would offset the reduced potential for low temperature dormancy relief overwinter to limit the impact of global warming on the life cycle of this and likely other summer annuals.

In contrast, reduced dormancy as a result of higher seed maturation temperatures is unlikely to alter germination timing of the winter annual Cvi. There is potentially a consequence, from lower dormancy at maturity and more effective dormancy relief from higher temperatures that seeds may germinate earlier in autumn or late summer, so plants enter winter as a larger rosette. The consequences of this are unknown, but may be beneficial.

Thus in the absence of other climate change influences neither life cycle strategy (summer or winter annual) is likely to be adversely affected. However, it is not clear what the impact will be on recruitment into and maintenance of the seed bank if seeds are generally shed at lower densities as overall temperature increases. It is clear from the data presented that small changes in mean temperature in line with global warming predictions (1-4˚C) around 15˚C during maturation can have a very significant impact on the depth of seed dormancy. This temperature during seed maturation is very relevant to the natural habitats of many *Arabidopsis* ecotypes.

- Plant growth and seed production

Increased temperature had little impact on leaf number and rosette size at bolting in the present study, but the vegetative phase would be shortened by future warming in both Bur and Cvi. Therefore the time to flowering in Bur may be advanced and high summer temperatures, if above the optimum, could cause stamen extension defects and consequently less efficient pollination and reduced seed yield. In contrast, Cvi may produce more cauline leaves as temperature increased with a consequent positive effect on subsequent seed filling.

Seed yield and size are intimately linked as the two are determined by source sink interactions and overall plant fertility. In the present study, Bur produced less seeds whereas Cvi produced more seeds at the warm end than at the ambient end of

the thermal gradient tunnel. Increased temperature reduced seed size in both ecotypes. This is not surprising in Cvi as there was a fundamental trade-off between seed number and seed size leading to a larger number of small seeds. However, this 'compensation' effect did not occur in Bur, possibly due to poor fertility during the reproductive phase, leading to the reduction in both seed size and seed number. This suggests that Bur as a summer annual may be less successful than Cvi in the face of global warming. However, temperatures experienced in the thermal gradient tunnel experiment were closer to the natural environment for Bur than Cvi. The experiments reported here did not extend to temperatures as high as those that may result from global warming in the Cape Verdi Islands. Therefore some caution should be applied when extrapolating these results to the environment into which Cvi has adapted.

8.3 Recommendation for future experimental work

- ***Investigation on light effect***

The results in *Chapter 4* and *Chapter 5* show that a light effect during seed maturation and seed imbibition had an impact on seed dormancy and germination, interplaying with other environmental signals (e.g. temperature and nitrate supply). In this project the effect of day length has only been studied to a limited extent and there is a need for further work on this topic. Long day conditions did not show a significant effect on seed dormancy when the seeds were matured at high temperature (20˚C). However, it would be useful to investigate this long-day effect under low maturation temperature on seed production and subsequent seed germination.

In addition, the original habitats of Bur and Cvi are at different latitudes, leading to not only the difference in day length, but also the proportion of red light relative to the amount of far-red light. The variation in red to far-red (R: FR) ratio of

light either during seed maturation or germination can influence the final germination response (reviewed in *Chapter 1*). Therefore, a comparison between Bur and Cvi in response to different R: FR ratios would be useful to understand how the light requirement for germination differs in summer and winter annuals, and also how the light requirement varies at different temperatures.

- ***Investigations of global warming impacts using the thermal gradient tunnel***

An important investigation would be to carry out further analysis and comparisons of the differences in the seeds produced at different locations in the tunnel to continue the work started in this project. Within the time frame of the project it was only possible to characterise the effect of maturation temperature on dormancy status of Bur seeds collected on four occasions at different points along the temperature gradient in the tunnel. In the experiments described in *Chapter 4* seeds were also collected from Cvi, but their deep dormancy did not enable characterisation in the current project. However, this analysis has now begun in the Finch-Savage and Footitt laboratory. Using the seeds collected from both Bur and Cvi plants the potential exists to follow the entire life cycle in reciprocal experiments that sow seeds produced at one site in the tunnel at all sites. In this way more could be determined about the relative impact of seed maturation conditions and of the conditions following sowing on life cycle patterns.

- ***Thermal time***

Germination occurs at different rates at different temperatures. This property is used in the thermal time concept that can combine temperature and time onto one scale. Development depends on the time spent at a certain temperature and the difference between it and a minimum (T_b) or optimum germination temperature (T_{opt}) (Bradford, 2002). Data collected from the thermal gradient tunnel for Bur and

Cvi could be analysed in terms of thermal time to gain further insight. The use of thermal time could be very effective in comparing different ecotypes in respect of global warming because increasing temperature will shorten the extent of temperature accumulation to alter the timing of life cycle events.

8.4 Project conclusion

In conclusion, the mechanisms by which environmental signals influence dormancy cycling have been studied using a combination of eco-physiology and molecular biology. The two contrasting *Arabidopsis* ecotypes Bur and Cvi, which respectively exhibit summer and winter annual phenotypes, have been compared. This has improved our understanding of the responses of life cycle traits especially dormancy cycling and germination mechanisms to environmental signals (e.g. temperature, light and nitrate). Environmental signals during both seed maturation and imbibition were found to be of great importance in determining depth of dormancy in mature *Arabidopsis* seed and their subsequent dormancy cycling behaviour. This work has centred on characterising Bur, especially the responses of dormancy cycling and germination to the environmental signals as these were previously unknown. This characterisation facilitated an extensive comparison of Bur and Cvi behaviour in the Finch-Savage group that has now been accepted for publication in The Plant Journal (Footitt *et al*., 2013). Further publications are anticipated. In particular, the investigation carried out in the unique thermal gradient tunnel (*Chapter 7*) provided information on the extent to which predicted global warming might affect the life cycle of *Arabidopsis* and how increased temperature is likely to affect different annual phenotypes.

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APPENDIX 1 Analysis of variation (ANOVA) results for each chapter

Appendix table 3.1 Three-way ANOVA of the effects of temperature, ecotype, light regimes and after-ripening (AR) and their interactions on final percentage germination (comparison within boxes and between boxes)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Box stratum					
Treatment of after ripening (AR)	1	40105.97	40105.97	1041.76	<.001
Light regime	1	21944.76	21944.76	570.02	<.001
Temperature	9	2076.07	230.67	5.99	<.001
AR × light regime	1	15321.61	15321.61	397.98	<.001
AR × temperature	9	2331.06	259.01	6.73	<.001
Light regime × temperature	9	4759.64	528.85	13.74	<.001
AR ×light regime × temperature	9	2554.22	283.80	7.37	<.001
Residual	80	3079.85	38.50		
Box.*Units*stratum					
Ecotype	1	53945.72	53945.72	1697.70	<.001
Ecotype × AR	1	11770.03	11770.03	370.42	<.001
Ecotype × light regime	1	4455.85	4455.85	140.23	<.001
Ecotype × temperature	9	10603.37	1178.15	37.08	<.001
AR × Ecotype × light regime	1	5284.16	5284.16	166.30	<.001
AR × Ecotype × temperature	9	9875.84	1097.32	34.53	<.001
Ecotype × light regime × temperature	9	6413.28	712.59	22.43	<.001
AR × Ecotype × light regime × temperature	9	1728.75	192.08	6.05	<.001
Residual	80	2541.96	31.77		
Total	239	198790.15			

Appendix Table 3.2 One-way ANOVA of the effects of AR duration on final percentage germination at each germination temperature

	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
***Bur* 10˚C**	AR days	7	2001.98	286	5.68	0.002
	Residual	16	805.41	50.34		
	Total	23	2807.4			
***Bur* 20˚C**	AR days	7	2891.88	413.13	6.13	0.001
	Residual	16	1077.43	67.34		
	Total	23	3969.32			
***Cvi* 10˚C**	AR days	7	20290.29	2898.61	65.21	<.001
	Residual	16	711.26	44.45		
	Total	23	21001.25			
***Cvi* 20˚C**	AR days	7	23136.8	3305.3	22.15	<.001
	Residual	16	2387.8	149.2		
	Total	23	25524.6			

Appendix Table 3.3 Four-way ANOVA of the effects of ecotype, temperature, nitrate application and duration of AR and their interactions on the time to 50% germination (T_{50})

Source of variation	d.f.	(m.v.)*	s.s.	m.s.	v.r.	F pr.
Ecotype	1		313.2075	313.2075	1333.52	<.001
Temperature	1		9.5384	9.5384	40.61	<.001
nitrate	1		3.4171	3.4171	14.55	<.001
AR days	7		345.7567	49.3938	210.3	<.001
Ecotype × temperature	1		82.8412	82.8412	352.71	<.001
Ecotype × nitrate	1		32.2688	32.2688	137.39	<.001
Temperature × nitrate	1		7.1552	7.1552	30.46	<.001
Ecotype × AR days	5	(2)	315.2593	63.0519	268.45	<.001
Temperature × AR days	7		56.1144	8.0163	34.13	<.001
Nitrate × AR days	7		28.5585	4.0798	17.37	<.001
Ecotype × temperature × nitrate	1		5.7074	5.7074	24.3	<.001
Ecotype × temperature × AR days	4	(3)	55.5584	13.8896	59.14	<.001
Ecotype × nitrate × AR days	4	(3)	30.1361	7.534	32.08	<.001
Temperature × nitrate × AR days	7		10.3336	1.4762	6.29	<.001
Ecotype × temperature × nitrate × AR days	3	(4)	9.5437	3.1812	13.54	<.001
Residual	104	(24)	24.4268	0.2349		
Total	155	(36)	948.4527			

*m.v. stands for missing value

Appendix Table 3.4 Two-way ANOVA of the effects of different treatments (temperature and nitrate application) on the AR_{50} of *Cvi* and AR_{90} of *Bur*

Source of variation		d.f.	s.s.	m.s.	v.r.	F pr.
***Cvi* AR_{50}**	Temperature	1	241.203	241.203	16.59	0.004
	Nitrate	1	2038.413	2038.413	140.16	<.001
	Temperature × nitrate	1	1474.083	1474.083	101.36	<.001
	Residual	8	116.35	14.54		
	Total	11	3870.047			
***Bur* AR_{90}**	Temperature	1	315.375	315.375	36.93	0.004
	Residual	4	34.16	8.54		
	Total	5	349.535			

Appendix Table 3.5 Two-way ANOVA of the effects of dark incubation temperature and duration and their interactions on final germination of Bur

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
rep stratum	2	147.27	73.64	1.51	
Incubation temperature	5	17684.84	3536.97	72.63	<.001
Dark incubation duration (days)	12	56897.13	4741.43	97.37	<.001
Incubation temperature × dark incubation duration	60	21859.67	364.33	7.48	<.001
Residual	154	7499.12	48.70		
Total	233	104088.04			

Appendix Table 3.6 One-way ANOVA of the effects of dark incubation temperature on final germination percentage of Bur at each incubation day

Incubation time (day)	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
1 day	rep stratum	2	94.15	47.08	0.95	
	Temperature	5	2080.57	416.11	8.39	0.002
	Residual	10	495.8	49.58		
	Total	17	2670.53			
2 days	rep stratum	2	12.89	6.45	0.11	
	Temperature	5	884.36	176.87	3.06	0.062
	Residual	10	577.26	57.73		
	Total	17	1474.52			
3 days	rep stratum	2	40.17	20.09	0.23	
	Temperature	5	394.78	78.96	0.9	0.519
	Residual	10	881	88.1		
	Total	17	1315.95			
5 days	rep stratum	2	311.51	155.75	2.67	
	Temperature	5	3188.4	637.68	10.94	<.001
	Residual	10	582.97	58.3		
	Total	17	4082.88			
7 days	rep stratum	2	31.85	15.92	1.11	
	Temperature	5	2677.4	535.48	37.27	<.001
	Residual	10	143.68	14.37		
	Total	17	2852.93			
14 days	rep stratum	2	34.87	17.44	0.21	
	temp	5	3423.9	684.78	8.06	0.003
	Residual	10	850.11	85.01		
	Total	17	4308.89			
21 days	rep stratum	2	2.79	1.4	0.05	
	Temperature	5	2943.95	588.79	19.79	<.001
	Residual	10	297.47	29.75		
	Total	17	3244.21			
35 days	rep stratum	2	59.28	29.64	0.67	
	Temperature	5	4395.23	879.05	20.01	<.001
	Residual	10	439.39	43.94		
	Total	17	4893.9			
60 days	rep stratum	2	3.94	1.97	0.06	
	Temperature	5	3071.36	614.27	17.8	<.001
	Residual	10	345.03	34.5		
	Total	17	3420.33			
75 days	rep stratum	2	226.63	113.32	3	
	Temperature	5	3600.29	720.06	19.09	<.001
	Residual	10	377.29	37.73		
	Total	17	4204.21			
105 days	rep stratum	2	431.7	215.85	7.2	
	Temperature	5	3486.72	697.34	23.25	<.001
	Residual	10	299.91	29.99		
	Total	17	4218.32			
150 days	rep stratum	2	111.73	55.86	1.38	
	Temperature	5	3025.98	605.2	14.9	<.001
	Residual	10	406.26	40.63		
	Total	17	3543.97			

200 days	rep stratum	2	28.92	14.46	0.26	
	Temperature	5	6371.56	1274.31	22.76	<.001
	Residual	10	559.8	55.98		
	Total	17	6960.28			

Appendix Table 5.1 One-way ANOVA of the effects of ecotype on bolting time and leaf number at bolting

Source of variation		d.f.	s.s.	m.s.	v.r.	F pr
Bolting time	Ecotype	1	526.4519	526.4519	635.89	<.001
	Residual	14	11.5905			
	Total	15	538.0424			
Leaf number	Ecotype	1	8590.38	8590.38	436.41	<.001
	Residual	14	275.58	19.68		
	Total	15	8865.96			

Appendix Table 5.2 Two-way ANOVA of the effects of type of controlled environment, ecotype and their interaction on seed yield per plant

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Controlled environment	1	0.028593	0.028594	130.74	<.001
Ecotype	1	0.002762	0.002762	12.63	0.004
Controlled environment × ecotype	1	0.003530	0.003530	16.14	0.002
Residual	12	0.002624	0.000218		
Total	15	0.037510			

Appendix Table 5.3 Two-way ANOVA of the effects of type of controlled environment, ecotype and the interaction on seed size (1000-seed weight)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Controlled environment	1	1.85E-05	1.85E-05	4.62	0.053
Ecotype	1	2.50E-05	2.50E-05	6.24	0.028
Controlled environment × ecotype	1	1.02E-04	1.02E-04	25.47	<.001
Residual	12	4.81E-05	4.01E-06		
Total	15	1.94E-04			

Appendix Table 5.4 Two-way ANOVA of the effects of type of controlled environment, ecotype and their interactions on seed number per plant

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Controlled environment	1	16229.9	16229.9	138.29	<.001
Ecotype	1	1264.5	1264.5	10.77	0.007
Ecotype × controlled environment	1	3044.4	3044.4	25.94	<.001
Residual	12	1408.3	117.4		
Total	15	21947.2			

Appendix Table 5.5 Two-way ANOVA of the effects of seed type*, germination temperature and their interaction on final percentage seed germination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Seed type	3	21785.4	4035.4	31.71	<.001
Temperature	1	4035.4	7261.8	57.07	<.001
Seed type ×temperature	3	5046.4	1682.1	13.22	<.001
Residual	16	2036.1	127.3		
Total	23	32903.3			

*Totally three seed types were included: *Bur/Bur*, *Bur/Cvi* and *Bur/Cvi*+GA

Appendix Table 5.6 Two-way ANOVA of the effects of seed type*, stage of flower development and their interaction on pistil lengths of Bur flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Developmental stage	2	95.51825	47.75912	489.65	<.001
Seed type	2	0.77614	0.38807	3.98	0.021
Developmental stage × seed type	4	0.22982	0.05746	0.59	0.671
Residual	162	15.80105	0.09754		
Total	170	112.3253			

*Totally three seed types were included: *Bur/Bur*, *Bur/Cvi* and *Bur/Cvi*+GA

Appendix Table 5.7 Two-way ANOVA of the effects of seed type*, stage of flower development and their interaction on stamen lengths of Bur flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Developmental stage	2	61.26538	30.63269	383.25	<.001
Seed type	2	2.12573	1.06287	13.3	<.001
Developmental stage × seed type	4	4.3062	1.07655	13.47	<.001
Residual	162	12.94842	0.07993		
Total	170	80.64573			

*Totally three seed types were included: *Bur/Bur*, *Bur/Cvi* and *Bur/Cvi*+GA

Appendix Table 5.8 Two-way ANOVA of the effects of seed type, stage of flower development and their interaction on ratio of pistil and stamen lengths of Bur flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Developmental stage	2	1.286775	0.643387	97.55	<.001
Seed type	2	0.088634	0.044317	6.72	0.002
Developmental stage × seed type	4	0.479829	0.119957	18.19	<.001
Residual	162	1.06842	0.006595		
Total	170	2.923657			

*Totally three seed types were included: *Bur/Bur*, *Bur/Cvi* and *Bur/Cvi*+GA

Appendix Table 5.9 One-way ANOVA analysis of the effects of growth conditions on total number of ovules within each *Bur* silique

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Growth condition	3	21430.37	7143.46	181.97	<.001
Residual	148	5809.84	39.26		
Total	151	27240.21			

Appendix Table 5.10 One-way ANOVA of the effects of growth conditions on the percentage of developed ovules within each *Bur* silique

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Growth condition	3	49101.57	16367.19	191.67	<.001
Residual	148	12638.14	85.39		
Total	151	61739.71			

Appendix Table 6.1 One-way ANOVA of the effects of treatment (ecotype and nitrate regime) on bolting time of Bur and Cvi

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	2787.000	1393.500	760.53	<.001
Residual	15	27.484	1.832		
Total	17	2814.484			

Appendix Table 6.2 Two-way ANOVA of the effects of ecotype, regime (plant growth) and their interaction on seed nitrate content

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	1	322327	322327	92.13	<.001
N regime	1	218905	218905	135.66	<.001
Ecotype × N regime	1	219025	219025	92.18	<.001
Residual	8	19008	2376		
Total	11	779265			

Appendix Table 6.3 Four-way ANOVA of effects of ecotype, nitrate regime (plant growth), temperature, exogenous nitrate (germination) and their interaction on final germination percentage in the light

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	1	15859.18	15859.18	495.68	<.001
N regime	1	4262.52	4262.52	133.23	<.001
Temperature	1	17712.79	17712.79	553.62	<.001
Nitrate	2	701.11	350.55	10.96	<.001
Ecotype × N regime	1	3035.3	3035.3	94.87	<.001
Ecotype × temperature	1	16478.02	16478.02	515.03	<.001
N regime × temperature	1	3359.5	3359.5	105	<.001
Ecotype × nitrate	2	14.16	7.08	0.22	0.808
N regime × nitrate	2	13.25	6.63	0.21	0.819
Temperature × nitrate	2	84.87	42.43	1.33	0.286

Ecotype × N regime × temperature	1	1984.54	1984.54	62.03	<.001
Ecotype × N regime × nitrate	2	152.79	76.4	2.39	0.11
Ecotype × temperature × nitrate	2	34.75	17.38	0.54	0.594
N regime × temperature × nitrate	2	138.11	69.05	2.16	0.135
Ecotype ×N regime × temperature × nitrate	2	274.03	137.01	4.28	0.022
Residual	48	1535.74	31.99		
Total	71	65640.64			

Appendix Table 6.4a Four-way ANOVA of effects of ecotype, nitrate supply to mother plant, temperature, exogenous nitrate and their interaction on T_{50} in the light (treatment effects nested within ecotype)

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Ecotype	1	43.33024	43.33024	522.6	<.001
Ecotype × temperature	1	52.30052	52.30052	630.78	<.001
Ecotype × N regime	1	3.25182	3.25182	39.22	<.001
Ecotype × nitrate	2	1.83704	0.91852	11.08	<.001
Ecotype × Eco_N regime	1	27.1972	27.1972	328.02	<.001
Ecotype × Eco_nitrate	2	1.12944	0.56472	6.81	0.003
Ecotype × temperature × N regime	1	0.48392	0.48392	5.84	0.021
Ecotype × temperature × nitrate	2	2.3042	1.1521	13.9	<.001
Ecotype × N regime × nitrate	2	0.64442	0.32221	3.89	0.03
Ecotype × Eco_N regime × Eco_nitrate	2	0.16388	0.08194	0.99	0.383
Ecotype × temperature ×N regime × nitrate	2	0.3994	0.1997	2.41	0.105
Residual	36	2.81906	0.08291		
Total	53	136.4601			

Appendix Table 6.4b Four-way ANOVA of effects of ecotype, nitrate supply to mother plant, temperature, exogenous nitrate and their interaction on T_{50} in the light (treatment effects nested within temperature)

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	1	91.28471	91.28471	1100.96	<.001
Temperature × ecotype	1	4.34605	4.34605	52.42	<.001
Temperature × N regime	1	17.98986	17.98986	216.97	<.001
Temperature × nitrate	2	2.26479	1.1324	13.66	<.001
Temperature × Temp_N regime	1	3.12232	3.12232	37.66	<.001
Temperature × Temp_nitrate	2	2.54448	1.27224	15.34	<.001
Temperature × ecotype × N regime	1	9.82077	9.82077	118.45	<.001
Temperature × ecotype × nitrate	2	0.46141	0.2307	2.78	0.076
Temperature × N regime × nitrate	2	0.15095	0.07548	0.91	0.412
Temperature × Temp_N regime × Temp_nitrate	2	1.02008	0.51004	6.15	0.005
Temperature × ecotype × N_regime × nitrate	2	0.03666	0.01833	0.22	0.803
Residual	36	2.81906	0.08291		
Total	53	136.4601			

Appendix Table 6.5 Four-way ANOVA of the effects of ecotype, nitrate regime,(plant growth) temperature, exogenous nitrate (germination) and their interaction on final germination percentage in the dark

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	1	28478.3	28478.3	649.36	<.001
N regime	1	2127.56	2127.56	48.51	<.001
Temperature	1	21556.6	21556.6	491.54	<.001
Nitrate	2	1386.07	693.03	15.8	<.001
Ecotype × N regime	1	342.52	342.52	7.81	0.007
Ecotype × temperature	1	15619.33	15619.33	356.15	<.001
N regime × temperature	1	30.82	30.82	0.7	0.406
Ecotype × nitrate	2	268.51	134.26	3.06	0.056
N regime × nitrate	2	253.2	126.6	2.89	0.065
Temperature × nitrate	2	716.75	358.37	8.17	<.001
Ecotype × N regime × temperature	1	221.75	221.75	5.06	0.029
Ecotype × N regime × nitrate	2	54.41	27.21	0.62	0.542
Ecotype × temperature × nitrate	2	360.35	180.18	4.11	0.023
N regime × temperature × nitrate	2	47.83	23.92	0.55	0.583
Ecotype ×N regime × temperature × nitrate	2	577.19	288.6	6.58	0.003
Residual	48	2105.07	43.86		
Total	71	74146.26			

Appendix Table 6.6a Four-way ANOVA of the effects of ecotype, nitrate regime (plant growth), exogenous nitrate (germination), temperature and their interaction on T_{50} in the dark (treatment effects nested within ecotype)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	1	93.3937	93.3937	480.5	<.001
Ecotype × temperature	1	40.1783	40.1783	206.71	<.001
Ecotype × N regime	1	0.0178	0.0178	0.09	0.764
Ecotype × nitrate	2	1.9495	0.9747	5.01	0.012
Ecotype × Eco_N regime	1	19.076	19.076	98.14	<.001
Ecotype × Eco_nitrate	2	0.0024	0.0012	0.01	0.994
Ecotype × temperature × N regime	1	0.2606	0.2606	1.34	0.255
Ecotype × temperature × nitrate	2	0.4362	0.2181	1.12	0.337
Ecotype × N regime × nitrate	2	0.4473	0.2237	1.15	0.328
Ecotype × Eco_N regime × Eco_nitrate	2	0.4649	0.2325	1.2	0.315
Ecotype × temperature ×N regime × nitrate	2	0.639	0.3195	1.64	0.208
Residual	36	6.6085	0.1944		
Total	53	164.1905			

Appendix Table 6.6b Four-way ANOVA of the effects of ecotype, nitrate regime (plant growth), exogenous nitrate (germination), temperature and their interaction on T_{50} in the dark (treatment effects nested within temperature)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Temperature	1	106.5321	106.5321	548.09	<.001
Temperature × ecotype	1	27.0399	27.0399	139.12	<.001
Temperature × N regime	1	8.4095	8.4095	43.27	<.001
Temperature × nitrate	2	1.055	0.5275	2.71	0.081
Temperature × Temp_N regime	1	0.2074	0.2074	1.07	0.309
Temperature × Temp_nitrate	2	0.2733	0.1367	0.7	0.502
Temperature × ecotype × N regime	1	10.7375	10.7375	55.24	<.001
Temperature × ecotype × nitrate	2	1.0599	0.5299	2.73	0.08
Temperature × N regime × nitrate	2	0.1427	0.0713	0.37	0.695
Temperature × Temp_N regime × Temp_nitrate	2	1.0388	0.5194	2.67	0.084
Temperature × ecotype × N_regime × nitrate	2	0.3698	0.1849	0.95	0.396
Residual	36	6.6085	0.1944		
Total	53	164.1905			

Appendix Table 6.7 Two-way ANOVA of the effects of nitrate regime (plant growth), gene type and their interaction on expression levels

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
N regime	1	638376	638376	47.03	<.001
Genes	26	40146810	1544108	113.75	<.001
N regime × genes	26	1779022	68424	5.04	<.001
Residual	108	1466089	13575		
Total	161	44030296			

Appendix Table 6.8 Two-way ANOVA of effect of nitrate regime (plant growth) on expression of GA related genes

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
GA related genes	4	17535646	4383911	86.45	<.001
N regime	1	1036868	1036868	20.45	<.001
GA related genes × N regime	4	832444	208111	4.1	0.014
Residual	20	1014163	50708		
Total	29	20419121			

Appendix Table 6.9 Two-way ANOVA of effect of nitrate regime (plant growth) on expression of ABA related genes

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ABA related genes	9	7156207	795134	123.44	<.001
N regime	1	89164	89164	13.84	<.001
ABA related genes × N regime	9	313579	34842	5.41	<.001
Residual	40	257667	6442		
Total	59	7816617			

Appendix Table 6.10 Two-way ANOVA of effect of nitrate regime (plant growth) on expression of other dormancy related genes

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
N regime	1	76882	76882	14.48	<.001
Genes	8	3350812	418852	78.87	<.001
N regime × genes	8	62471	7809	1.47	0.202
Residual	36	191177	5310		
Total	53	3681342			

Appendix Table 6.11 Three-way ANOVA of the effects of ecotype, temperature and nitrate regime (plant growth) and their interaction on the phenotypes during transition from vegetative to reproductive growth

Variate: rosette diameter					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Ecotype	1	114.3783	114.3783	1564.91	<.001
Temperature	1	0.3467	0.3467	4.74	0.035
N regime	1	9.59537	9.59537	131.28	<.001
Ecotype × temperature	1	61.53008	61.53008	841.85	<.001
Ecotype × N regime	1	4.80452	4.80452	65.73	<.001
Temperature × N regime	1	1.31305	1.31305	17.97	<.001
Ecotype × temperature × N regime	1	0.14784	0.14784	2.02	0.163
Residual	40	2.92357	0.07309		
Total	47	195.0394			
Variate: leaf number					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Ecotype	1	20415.26	20415.26	12833.37	<.001
Temperature	1	736.996	736.996	463.29	<.001
N regime	1	63.155	63.155	39.7	<.001
Ecotype × temperature	1	135.289	135.289	85.04	<.001
Ecotype × N regime	1	67.826	67.826	42.64	<.001
Temperature × N regime	1	0.324	0.324	0.2	0.654
Ecotype × temperature × N regime	1	0.018	0.018	0.01	0.915
Residual	40	63.632	1.591		
Total	47	21482.5			
Variate: bolting time					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Ecotype	1	16710.67	16710.67	10741.65	<.001
Temperature	1	6225.384	6225.384	4001.69	<.001
N regime	1	0.116	0.116	0.07	0.786
Ecotype × temperature	1	47.715	47.715	30.67	<.001
Ecotype × N regime	1	7.634	7.634	4.91	0.032
Temperature × N regime	1	11.893	11.893	7.64	0.009
Ecotype × temperature × N regime	1	0.119	0.119	0.08	0.783
Residual	40	62.228	1.556		
Total	47	23065.76			

Appendix Table 6.12 Three-way ANOVA of the effects of day length during plant growth, nitrate regime (plant growth) plant and their interaction on bolting time of Bur

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
N regime	1	743.945	743.945	427.48	<.001
Day length	1	2092.4	2092.4	1202.31	<.001
N regime × day length	1	1003.073	1003.073	576.37	<.001
Residual	20	34.806	1.74		
Total	23	3874.225			

Appendix Table 6.13 Three-way ANOVA of the effects of ecotype, temperature, nitrate regime (plant growth) and their interaction on seed production components (seed yield and seed size)

Variate: Seed yield per plant					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Ecotype	1	0.00379	0.00379	24.05	<.001
Temperature	1	0.00466	0.00466	29.57	<.001
N regime	1	0.00908	0.00908	57.54	<.001
Ecotype × temperature	1	0.00715	0.00715	45.35	<.001
Ecotype × N regime	1	0.00014	0.00014	0.9	0.356
Temperature × N regime	1	0.00197	0.00197	12.46	0.003
Ecotype × temperature × N regime	1	7.03E-05	7.03E-05	0.45	0.514
Residual	16	0.00252	0.00016		
Total	23	0.02939			
Variate: 1000-seed weight					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Ecotype	1	1.35E-03	1.35E-03	12116.43	<.001
Temperature	1	7.85E-05	7.85E-05	702.82	<.001
N regime	1	2.54E-06	2.54E-06	22.7	<.001
Ecotype × temperature	1	1.39E-04	1.39E-04	1246.58	<.001
Ecotype × N regime	1	3.75E-07	3.75E-07	3.36	0.086
Temperature × N regime	1	6.02E-07	6.02E-07	5.39	0.034
Ecotype × temperature × N regime	1	4.82E-07	4.82E-07	4.31	0.054
Residual	16	1.79E-06	1.12E-07		
Total	23	1.58E-03			

Appendix Table 6.14 Three-way ANOVA of the effects of ecotype, temperature, nitrate regime (plant growth) and their interaction on nitrate content per 1000 seed

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
N regime	1	2.4401	2.4401	6.17	0.024
Ecotype	1	0.878	0.878	2.22	0.156
Temperature	1	16.7495	16.7495	42.36	<.001
N regime × ecotype	1	1.5685	1.5685	3.97	0.064
N regime × temperature	1	0.1527	0.1527	0.39	0.543
Ecotype × temperature	1	20.9314	20.9314	52.94	<.001
N regime × ecotype × temperature	1	6.6089	6.6089	16.71	<.001
Residual	16	6.3264	0.3954		
Total	23	55.6554			

Appendix Table 6.15 Four-way ANOVA of the effects of ecotype, temperature during maturation, temperature during germination, nitrate regime (plant growth) and their interaction on final percentage germination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	1	23827.06	23827.06	721.03	<.001
Maturation temperature	1	43022.81	43022.81	1301.91	<.001
N regime	1	235.07	235.07	7.11	0.009
Germination temperature	5	20557.91	4111.58	124.42	<.001
Ecotype × Maturation temperature	1	1676.27	1676.27	50.73	<.001
Ecotype × N regime	1	210.06	210.06	6.36	0.013
Maturation temperature × N regime	1	350.11	350.11	10.59	0.002
Ecotype × germination temperature	5	14521.99	2904.4	87.89	<.001
Maturation temperature × germination temperature	5	772.41	154.48	4.67	<.001
N regime × germination temperature	5	186.01	37.2	1.13	0.352
Ecotype × Maturation temperature × N regime	1	84.75	84.75	2.56	0.113
Ecotype × Maturation temperature × germination temperature	5	12372.11	2474.42	74.88	<.001
Ecotype × N regime × germination temperature	5	88	17.6	0.53	0.751
Maturation temperature × N regime × germination temperature	5	98.59	19.72	0.6	0.703
Ecotype × Maturation temperature × N regime germination temperature	5	246.26	49.25	1.49	0.2
Residual	96	3172.41	33.05		
Total	143	121421.8			

Appendix Table 6.16 Three-way ANOVA of the effects of ecotype, plant growth conditions (temperature and day length) and their interaction on final percentage germination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Ecotype	1	4743.84	4743.84	98.67	<.001
Growth condition	2	10407.85	5203.92	108.24	<.001
Germination temperature	1	5858.44	5858.44	121.86	<.001
Ecotype × growth condition	2	304.81	152.41	3.17	0.06
Ecotype × germination temperature	1	3782.73	3782.73	78.63	<.001
Growth condition × germination temperature	2	14.72	7.36	0.15	0.859
Ecotype × growth condition × germination temperature	2	1612.51	806.25	16.76	<.001
Residual	24	1153.83	48.08		
Total	35	27878.73			

Appendix Table 7.1 Three-way ANOVA of the effects of ecotype, nitrate regimes, temperature locations in the thermal gradient tunnel and their interaction on final percentage seedling emergence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Temperature	3	58.25	19.42	0.81	0.494
Nitrate regimes	2	27.35	13.68	0.57	0.569
Ecotype	1	809.59	809.59	33.67	<.001
Temperature × Nitrate regimes	6	70.51	11.75	0.49	0.815
Temperature × Ecotype	3	63.93	21.31	0.89	0.452
Nitrate regimes× Ecotype	2	21.72	10.86	0.45	0.638
Temperature × Nitrate regimes× Ecotype	6	115.99	19.33	0.8	0.57
Residual	72	1731.3	24.05		
Total	95	2898.65			

Appendix Table 7.2 Three-way ANOVA of effects of nitrate regimes, temperature locations in the thermal gradient tunnel and their interaction on seedling emergence rate

Source of variation		d.f.	s.s.	m.s.	v.r.	F pr.
Bur	Temperature	3	0.000565	0.000188	1.72	0.181
	N regimes	2	0.012923	0.006462	58.88	<.001
	Temperature × N regimes	6	0.001857	0.00031	2.82	0.024
	Residual	36	0.003951	0.00011		
	Total	47	0.019296			
Cvi	Temperature	3	0.002961	0.000987	24.58	<.001
	N regimes	2	0.011425	0.005712	142.24	<.001
	Temperature × N regimes	6	0.003124	0.000521	12.96	<.001
	Residual	36	0.001446	4.02E-05		
	Total	47	0.018956			

Appendix Table 7.3 Two-way ANOVA of the effects of temperature, ecotype and their interaction on bolting time of Bur and Cvi in the high N regime

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Temperature	3	45.5072	15.1691	26.43	<.001
Ecotype	1	9.9015	9.9015	17.25	<.001
Temperature × Ecotype	3	23.5967	7.8656	13.7	<.001
Residual	16	9.1835	0.574		
Total	23	88.189			

Appendix Table 7.4 Two-way ANOVA of the effects of temperature, nitrate regime and their interaction on bolting time responses of Bur

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Temperature	3	198.4767	66.1589	158.89	<.001
N regimes	2	2.6324	1.3162	3.16	0.06
Temperature × N regimes	6	5.0966	0.8494	2.04	0.099
Residual	24	9.993	0.4164		
Total	35	216.1988			

Appendix Table 7.5 Two-way ANOVA of the effects of temperature, ecotype and their interaction on vegetative phenotypes of Bur and Cvi on high N compost

Variate: Rosette diameter					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	3	0.1748	0.0583	0.33	0.807
Ecotype	1	21.8338	21.8338	121.85	<.001
Temperature × ecotype	3	0.9031	0.301	1.68	0.211
Residual	16	2.8669	0.1792		
Total	23	25.7787			
Variate: leaf number					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	3	3.9043	1.3014	1.91	0.169
Ecotype	1	244.5121	244.5121	358.27	<.001
Temperature × ecotype	3	4.0602	1.3534	1.98	0.157
Residual	16	10.9198	0.6825		
Total	23	263.3965			

Appendix Table 7.6 Two-way ANOVA of the effects of temperature, nitrate regime and their interaction on vegetative phenotypes of *Bur*

Variate: Rosette diameter					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	3	0.63052	0.21017	2.88	0.057
N regime	2	1.80608	0.90304	12.36	<.001
Temperature × N regime	6	1.23006	0.20501	2.81	0.033
Residual	24	1.75408	0.07309		
Total	35	5.42074			

Variate: leaf number					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	3	7.9952	2.6651	8.53	<.001
N regime	2	5.9613	2.9807	9.54	<.001
Temperature × N regime	6	1.2598	0.21	0.67	0.673
Residual	24	7.5008	0.3125		
Total	35	22.7171			

Appendix Table 7.7 Two-way ANOVA of the effects of temperature, seed type* and their interaction on seed yield per plant

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Seed type	3	0.0239848	0.007995	29.19	<.001
Temperature	3	0.0015002	0.0005	1.83	0.162
Seed type × temperature	9	0.0146184	0.001624	5.93	<.001
Residual	32	0.008764	0.0002739		
Total	47	0.048867			

*Totally four seed types were included: *Bur* (high N), *Bur* (medium N), *Bur* (low N) and *Cvi* (high N).

Appendix Table 7.8 Two-way ANOVA of the effects of temperature, seed type* and their interaction on seed size and seed number

Variate: 1000-seed weight					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	1	1.01E-05	1.01E-05	19.13	<.001
Seed type	3	1.23E-05	4.10E-06	7.73	0.002
Temperature × seed type	3	1.03E-05	3.42E-06	6.45	0.005
Residual	16	8.48E-06	5.30E-07		
Total	23	4.12E-05			
Variate: seed number					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	1	0.0035	0.0035	0.02	0.9
Seed type	3	4.8624	1.6208	7.5	0.002
Temperature × seed type	3	2.4393	0.8131	3.76	0.032
Residual	16	3.4593	0.2163		
Total	23	10.7646			

*Totally four seed types were included: Bur (high N), Bur (medium N), Bur (low N) and Cvi (high N).

Appendix Table 7.9 Two-way ANOVA of the effects of seed type*, temperautre and their interaction on Harvest Index (HI)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Seed type	3	0.01241	0.004137	2.74	0.059
Temperature	3	0.009832	0.003277	2.17	0.11
Seed type × temperature	9	0.030188	0.003354	2.22	0.047
Residual	32	0.048258	0.001508		
Total	47	0.100687			

*Totally four seed types were included: Bur (high N), Bur (medium N), Bur (low N) and Cvi (high N).

Appendix Table 7.10 Two-way ANOVA of the effects of temeprature, seed type (ecotype and nitrate supply) and their interaction on seed production components (seed yield per plant, 1000-seed weight and seed number per plant)

Variate: seed yield					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	1	0.000215	0.0002151	0.97	0.339
Seed type	3	0.006171	0.0020569	9.3	<.001
Temperature × seed type	3	0.001834	0.0006114	2.76	0.076
Residual	16	0.00354	0.000221		
Total	23	0.01176			
Variate: seed size					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	1	1.01E-05	1.01E-05	19.13	<.001
Seed type	3	1.23E-05	4.10E-06	7.73	0.002
Temperature × seed type	3	1.03E-05	3.42E-06	6.45	0.005
Residual	16	8.48E-06	5.30E-07		
Total	23	4.12E-05			
Variate: seed number					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Temperature	1	0.0035	0.0035	0.02	0.9
Seed type	3	4.8624	1.6208	7.5	0.002
Temperature × seed type	3	2.4393	0.8131	3.76	0.032
Residual	16	3.4593	0.2163		
Total	23	10.7646			

*Totally four seed types were included: *Bur* (high N), *Bur* (medium N), *Bur* (low N) and *Cvi* (high N).

Appendix Table 7.11 Two-way ANOVA of the effects of temperature, nitrate regime and their interaction on nitrate content of Bur seeds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Temperature	3	2.15E-06	7.17E-07	12.29	<.001
N regime	2	3.98E-07	1.99E-07	3.41	0.05
Temperature × N regime	6	7.39E-07	1.23E-07	2.11	0.089
Residual	24	1.33E-06	6.03E-08		
Total	35	4.69E-06			