Chemical Genetics of Seed Germination

Modulation of a key step in abscisic acid biosynthesis

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I. Contents

II. List of Figures

III. List of Tables

IV. List of Equations

V. Acknowledgements

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VI. Author's Declaration

I declare that this thesis and the work presented herein is my own, unless otherwise stated, and has not been submitted for another degree. Acknowledgements of significant contributions from other people are made below:

Figure 3.3 was based on data provided by Syngenta Seeds B.V., Enkhuizen, NL.

Maize cold sand tests, cold soaking tests, and cold paper experiments in Chapter 3 (Figure 3.4, Figure 3.5, Figure 3.6 and Figure 3.16) were carried out by myself at Syngenta Seeds B.V., Enkhuizen, NL, with design and practical assistance (Barbara Westland). Cold sand test, submergence and wet cellulose/ filter paper experiments were designed based on internal protocols at Syngenta. All analyses and representations were solely my own work.

The *in vitro* assays of NCED inhibition (Chapter 4, Figure 4.10) were carried out by Peter J. Harrison (Department of Chemistry, University of Warwick) using *E. coli* harbouring pGEX-4T-1: *ZmNCED* generated by me. The analysis and representation of inhibition data provided by Peter J. Harrison were performed by me.

Quantification of abscisic acid in Chapter 6 (Figure 6.3, Figure 6.7 and Figure 6.8) was carried out by the lab of David Brocklehurst, Syngenta, Jealott's Hill, UK. Samples were collected from experiments designed and executed me. Analysis and representation of concentration data was performed by me.

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Jake Chandler

VII. Summary

Cold conditions during imbibition can result in slow or no germination in some maize seed, leading to sub-optimal crop density and uniformity and loss of yield. A novel seed treatment is required that restores germination in seed batches that perform poorly under cold conditions.

Germination of seed batches from different varieties was characterised following imbibition under cold conditions which permit no or slow germination. Hydroxamic acid inhibitors of 9-*cis*-epoxycarotenoid dioxygenase (NCED) stimulate germination through ABA biosynthesis inhibition in other species and had a small significant effect in increasing the proportion of normal seedlings after cold imbibition. This indicated that normal germination of maize may be inhibited by dormancy-related mechanisms during or after imbibition in cold conditions.

The maize *NCED* (*ZmNCED*) family was characterised. D2 and D4 inhibit other enzymes in the carotenoid cleavage dioxygenase family and exhibit relatively weak inhibition of NCED. *ZmNCED*s were cloned for *in vitro* enzyme inhibition studies to aid identification of NCED-specific inhibitors.

An RT-qPCR assay for measuring *ZmNCED* expression was developed. Seed *ZmNCED* expression and ABA concentration was elevated under cold conditions, compared to optimal germination conditions.

An assay was developed to screen for germination stimulating compounds. 965 of a diverse library of 5074 compounds were identified as potential germination stimulators. Germination stimulating activity was replicated in 171 of these compounds, with some more efficacious than D4. Germination stimulating activity of 88 compounds related to the current lead compound, D4, was assessed at concentrations of 10 ppb to 10 ppm. Compounds were identified that, at less than 10 ppm stimulated germination more than D4 at 312 ppm. The mode-of-action of these compounds will need to be determined and may yield novel targets for germination stimulation.

Thus novel seed treatments for improving germination of low vigour maize seed lots under cold conditions could be based on NCED inhibition or the action of the newly identified compounds.

VIII. List of Abbreviations

Abbreviation	Meaning
A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
AA	Amino acid
AAO	Abscisic aldehyde oxidase
ABA	Abscisic acid
ABA-Gtase	ABA glucosyl transferase
abi	ABA insensitive
ABRE	ABA responsive element
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analysis
AP2s	Apetala 2
Arabidopsis	*Arabidopsis thaliana*
At	*Arabidopsis thaliana*
B73	*Zea mays* near-isogenic line B73
BCH	β-carotene hydroxylase
BG	ß-glucosidase
BLAST	Basic local alignment search tool
Bur	Arabidopsis ecotype Burren
C426	A new hydroxamic acid-related compound developed by Syngenta
CCD	Carotenoid cleavage dioxygenase
CE	Cis-element
Chr	Chromosome
CPK	Creatine phosphokinase
$\mathbf{C_q}$	Cycle of quantification (threshold cycle value)
CrtISO	Carotenoid isomerase
cv	Cultivar
Cvi	Arabidopsis ecotype Cape Verdi Islands
CYP707A	ABA 8'-hydroxylase
D2	Hydroxamic acid D2
D27	Carotenoid isomerase D27
D4	Hydroxamic acid D4
DCM	Dichloromethane
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOG	Delay of germination
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GA	Gibberellic acid
GM	Genetic modification; genetically modified

GST	Glutathione S-transferase
Hv	*Hordeum vulgare*
ISTA	International Seed Testing Association
KNO_3	Potassium nitrate
LCY-B	lycopene-ß-cyclase
Le	*Solanum lycopersicum*
Ls	*Lactuca sativa*
LSD	Least significant difference
Maize	*Zea mays*
MCS	Molybdenum co-factor sulfurase
MD	Morphological dormancy
MES	2-(*N*-morpholino)ethanesulfonic acid
MGT	Mean germination time
MPD	Morphophysiological dormancy
mRNA	Messenger ribonucleic acid
MTT	Tetrazolium salt
NCED	9-*cis*-epoxycarotenoid dioxygenase
NCEI	9-*cis*-epoxycarotenoid isomerase
NDGA	Nordihydroguaiaretic acid
ORF	Open reading frame
PD	Physiological dormancy
PD30	A deep primary dormant state
PDS	Phytoene desaturase
PP2A	Protein phosphatase 2A
PP2C	Protein phosphatase 2C
PY	Physical dormancy
QTL	Quantitative trait loci
RCAR/PYL	Regulatory component of ABA receptor 11/ pyrabactine resistance 1 like
SD	Secondary dormancy
SD2	A deep secondary dormant state
SDR	Short-chain alcohol dehydrogenase
SLCCD	Sesquiterpene-like carotenoid cleavage dioxygenase inhibitors
SnRK2	Sucrose nonfermenting related kinase
T_{10}	Time to reach 10% germination
T_{50}	Time to reach 50% germination
T_{90}	Time to reach 90% germination
T_{90-10}	Time between 10% and 90% germination
T_b	Base temperature
Tomato	*Solanum lycopersicum*
VDE	Vioxanthin de-epoxidase
vp	Viviparous
ZDS	ζ-carotene desaturase
ZEP	Zeaxanthin epoxidase
ZISO	ζ-carotene isomerase
Zm	*Zea mays*
Ψ_b	Base water potential

1 Introduction

1.1 Project Brief

Requirement for novel seed treatment technology: Cold conditions during imbibition can result in slow or no germination in some maize seed, leading to suboptimal crop density and uniformity and loss of yield. A novel seed treatment is required which restores germination in seed batches which perform poorly under cold conditions, and which would otherwise fail quality control tests.

Dormancy: Dormancy can prevent seed germination under potentially germination-permissive conditions. *De novo* biosynthesis of the phytohormone abscisic acid (ABA) is often crucial for maintenance of dormancy in imbibed seed. Inhibition of ABA biosynthesis can release dormancy, allowing germination in some seed, however there is a requirement for more specific inhibitors of ABA biosynthesis, as current inhibitors are not specific, or do not positively affect seed germination.

Novel carotenoid cleavage dioxygenase (CCD) inhibitors: ABA biosynthesis is dependent on 9-*cis*-epoxycarotenoid cleavage dioxygenases (NCEDs) which belong to the CCD family. Novel hydroxamic inhibitors inhibit NCED *in vitro,* reduce ABA accumulation *in vivo* and stimulate germination of some dormant seed. However these inhibitors also inhibit other members of the CCD family, and so there is a requirement for more specific NCED inhibitors. Many plants have multiple *NCED* genes and it is unknown if these CCD inhibitors preferentially inhibit particular NCEDs.

Summary: It is hypothesised that up-regulation of maize NCEDs during imbibition at suboptimal temperatures leads to ABA accumulation which may induce dormancy in some seed in low-vigour maize seed lots.

Overall aim: To investigate the roles of *NCED* genes and ABA in maize germination under suboptimal temperatures and to develop chemical control strategies for enhancing seed performance, which could be based on inhibition of NCED.

1.2 The Importance of Seeds

The importance of seeds is difficult to overstate. Around a quarter of a million plant species, approximately 90% of all extant plant species, sexually reproduce through seed production (Crepet and Niklas, 2009; Linkies, *et al.*, 2010). This 'seed habit' involves the production of a zygotic embryo encapsulated inside a seed coat, which facilitates dispersal and propagation of the plant species. Seed production is not only important for survival of plant species in nature; it is also a means through which humans have exploited plants for the provision of materials and most importantly, food.

Many of the top worldwide agricultural or food commodities are harvested for their seed or fruit (FAO, 2013). Three of the top five commodities (weight produced per year) are seed of the cereals maize, rice and wheat, and represent vastly important worldwide human food sources (FAO, 2013). Around 36% of all cereal seed is also used as feed for livestock, representing the indirect use of seeds as food (Alexandratos and Bruinsma, 2012). Seeds not only have use as a food, but can also be used to produce chemical feedstocks such as through ethanol and seed oil production.

The production of seed is also required for propagation of plants utilised for purposes other than seed production. For example, many plants grown for their edible (e.g. carrots, lettuce, and *Brassica* spp.) or otherwise useful vegetative organs are grown from seed. Even in important crop species that are often propagated asexually (e.g. sugarcane, potato, cassava), the breeding and production of new varieties can be achieved through seed production. Thus it is no surprise that seed production is an important element of the agricultural industry.

This literature review outlines one of the key problems associated with the use of seeds, seed dormancy, and the underlying mechanism which may be a target for future seed improvement strategies. Understanding dormancy depends on an understanding of seed germination and its relation to seed structure.

1.3 Seed Structure and Germination

1.3.1 Seed structure

In angiosperms, seed fertilisation and development results in three main seed components. Two components, the embryo and the endosperm tissues, form as a result of double-fertilisation (Berger, *et al.*, 2008). The embryo tissues develop from the zygote (diploid) formed by fusion of a single male gamete (sperm) and female gamete (egg). The endosperm tissues (triploid) develop from the fusion of two maternal polar nuclei and a sperm cell in the embryo sac. The third component, the outermost seed coat (testa and pericarp) is derived from (diploid) maternal tissues.

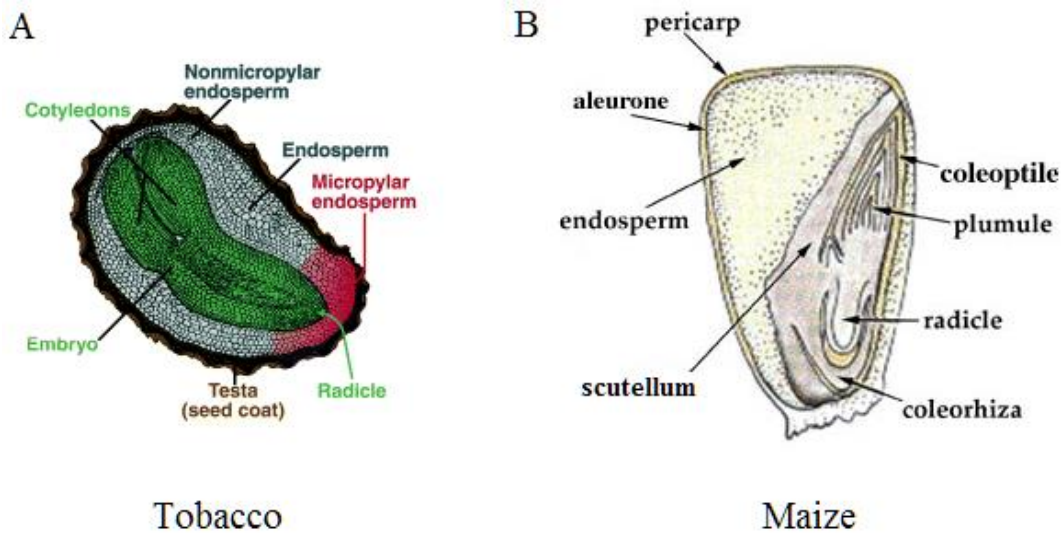

Figure 1.1 Seed Structures. Diagrams of the internal seed structures of tobacco (A), taken from 'The Seed Biology Place' (http://www.seedbiology.de/) (Leubner, 2012b) and maize (B), adapted from http://www.hobart.k12.in.us/jkousen/Biology/seed_c_2.jpg

Mature seed vary widely in structure. Dicot seeds, like the tobacco seed shown in Figure 1.1 A, contain a plant embryo, which has cotyledons (leaf-like structures), hypocotyl (embryonic stem) and radicle (embryonic root) within the protective testa. In some monocot seeds (e.g. *Poaceae*), like the maize seed shown in Figure 1.1 B, the embryo structure is quite different. The embryo axis is composed of a radicle and plumule (embryonic root and shoot) and enclosed in the protective sheath of the coleorhiza and coleoptile, respectively. The scutellum sits between the embryo axis and endosperm, and transfers nutrients from the endosperm to the seedling after germination (Leubner, 2012b).

Seeds vary in the amount of the reserve-storing endosperm tissue. Some "non-endospermic" seeds contain little or no endosperm tissue and instead contain nutrient reserves within the cotyledons themselves, transferred from the seed coats during development, such as in Arabidopsis (Baud, *et al.*, 2002; Mansfield and Briarty, 1992) and pea (*Pisum sativum*) (Marinos, 1970; Murray, 1979). In mature *Poaceae* seed, the endosperm is composed of dead tissue with a high starch concentration, surrounded by a thin layer of living cells known as the aleurone layer (Leubner, 2012b). The outermost layer, the testa, is composed of dead tissue of maternal origin. In *Poaceae*, the fruit tissue, known as the pericarp, is fused to the outside of the testa, which together with the endosperm and embryo forms the caryopsis.

1.3.2 Seed germination

Germination is the process by which a plant embryo emerges from the seed coat (endosperm and testa). Germination begins with the accumulation of water by the seed (imbibition) and ends in most cases in the emergence of the radicle from the seed coat. Non-dormant seed can germinate over the widest range of conditions possible for the given genotype. Seed germination has a basic requirement for water, oxygen and an appropriate temperature, and sometimes particular requirements for light or nitrate (Finch-Savage and Leubner-Metzger, 2006).

Water uptake during imbibition generally occurs in three phases (Manz, *et al.*, 2005; Schopfer and Plachy, 1984; Stiles, 1948). Imbibition begins with an initial rapid uptake of water (phase I), followed by a plateau (phase II). During these two phases, seed metabolism is initiated and transcription and protein synthesis occurs. DNA damage and other structural damage resulting from desiccation and imbibition is repaired (Bewley, 1997). In non-dormant seed, a further uptake of water occurs as the seed germinates (phase III), leading to the penetration of the embryo through the seed coat by hydraulic growth of the embryo (radicle cell elongation), and water uptake continues as the seedling emerges from the seed coat (Finch-Savage and Leubner-Metzger, 2006; Manz, *et al.*, 2005). In endospermic seed, like tobacco (Figure 1.1 A), the testa ruptures, and the radicle then penetrates through the endosperm; whereas in non-endospermic seed such as pea, the radicle has to penetrate only through the testa (Leubner, 2012b). In caryopses of *Poaceae* (Figure 1.1 B) the radicle has to penetrate through the coleorhiza, which may be analogous to the endosperm penetration in tobacco seed (Barrero, *et al.*, 2009). Following radicle

emergence, seed storage reserves are mobilised, cell division occurs and the seedling continues to grow (Bewley, 1997). The rate of water uptake (controlled by the water potential surrounding the seed) and proximity of the temperature to its optimum for that genotype, determine the speed of germination (Alvarado and Bradford, 2002).

However, viable seed do not always germinate under conditions that would potentially allow germination for the given genotype because of dormancy.

1.4 Seed Dormancy and the involvement of ABA

1.4.1 Seed dormancy

Dormancy restricts the range of environmental conditions under which a seed is able to germinate. This can lead to the absence of germination under conditions that would otherwise allow completion of germination. Dormancy mechanisms are adaptations that regulate germination so that it occurs when prevailing conditions are likely to allow plant growth and successful reproduction (Finch-Savage and Leubner-Metzger, 2006). The timing of dormancy induction or release, and its depth, can be related to environmental cues experienced by both the maternal plant and the seed. This timing can be based on the life-history of the species/ecotype; and as a product of acclimation to variable environmental conditions (Footitt, *et al.*, 2011; Footitt, *et al.*, 2013; Springthorpe and Penfield, 2015). Dormancy mechanisms can also spread germination spatially or temporally as a bet-hedging strategy, ensuring that at least some offspring survive to reproduction (Gremer and Venable, 2014; Philippi and Seger, 1989; Springthorpe and Penfield, 2015).

Whilst diverse and species-specific, there are distinct classes of dormancy mechanisms (summarised in Table 1.1). For the purposes of this thesis, physiological dormancy (PD) is most relevant and so is discussed in most detail.

Table 1.1. Classes of dormancy mechanisms.

Dormancy Class	Mechanism Basis	Dormancy release
Physiological (PD)	Sub-cellular signalling mechanisms, often resulting in reduced embryo growth potential and increased seed coat resistance of radicle emergence	Cold or warm stratification. May be released by hormone application, after-ripening, and sometimes scarification (see PY).
Morphological (MD)	Embryo immaturity	Conditions which allow further embryo development
Morphophysiological (MPD)	A combination of MD and PD	Cold or warm stratification with cold or warm embryo growth conditions. Hormone application relieve PD.
Physical (PY)	Water impermeable seed coat	Mechanical or chemical scarification (making the coat permeable to water)
Combinatorial (PY + PD)	A combination of PY and PD	As with PD and PY, but order of treatment species specific.

Based on (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006)

1.4.2 Physiological seed dormancy

Physiologically dormant seeds unlike morphologically dormant seeds, contain fully mature embryos; and unlike physically dormant seeds, are able to imbibe water from their environment (Table 1.1). Yet physiologically dormant seeds still do not germinate owing to molecular mechanisms that will be described later in this section. Physiological dormancy is also the most abundant dormancy class 'in the field' in temperate seed banks and has been studied extensively in the laboratory due to its prevalence amongst model species such as Arabidopsis, tomato and tobacco (Finch-Savage and Leubner-Metzger, 2006). This class of dormancy, which probably evolved from an ancestral morphophysiological dormancy, has acted as an 'evolutionary hub' from which the other dormancy classes and types (described below) have emerged. The diversity in PD types may be due to the degree of plasticity that PD can accommodate compared to other dormancy types (Willis, *et al.*, 2014).

1.4.2.1 Dormancy release

Whilst dormancy is sometimes defined as the absence of germination of viable seed under otherwise favourable conditions for a non-dormant seed (Baskin and Baskin, 2004; Bewley, 1997), unlike germination which has a binary outcome, the level of dormancy may be relative (i.e. a seed can become more or less dormant) (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). This means that during

dormancy release a seed may be able to germinate over an increasingly wider range of environmental conditions. There are various conditions that can stimulate the release of dormancy (summarised in Table 1.1).

Physiological seed dormancy is commonly lost through after-ripening (Finch-Savage and Leubner-Metzger, 2006). This involves dry storage of fresh mature seed: e.g. tobacco (Leubner-Metzger, 2002; Leubner-Metzger, 2005); Arabidopsis (Ali-Rachedi, *et al.*, 2004); and barley (Jacobsen, *et al.*, 2002) and typically requires a few months. Stratification also promotes loss of seed dormancy in some species. This involves the imbibition of seed at cold temperatures [e.g. Arabidopsis ecotype Cvi (Ali-Rachedi, *et al.*, 2004)] or warm temperatures [e.g. *Leptecophylla tameiameiae* (Baskin, *et al.*, 2005)] for prolonged periods of time depending on the species or ecotype (from days to months).

Some seeds also require more complicated stratification treatments, such as *Empetrum nigrum* spp. *hermaphroditum* (exhibits PD) and *Fraxinus excelsior* (exhibits MPD) in which dormancy is broken by 3-4 months of warm stratification followed by 3-4 months cold stratification (Baskin, *et al.*, 2002; Finch-Savage and Clay, 1997; Finch-Savage and Leubner-Metzger, 2006).

These conditions likely reflect the seasonal variations in temperatures and moisture contents that the seeds may encounter in their natural environments, which act as cues for dormancy release. Whilst temperature appears to be a widely utilised cue for dormancy release, chemicals released following wild fires (van Staden, *et al.*, 2000), chemicals in the leachate of leaf litter (Krock, *et al.*, 2002), nitrate and light quality (Batak, *et al.*, 2002; de Castro, *et al.*, 2001; Finch-Savage, *et al.*, 2007) are also amongst important environmental cues regulating dormancy release. Dormancy is often also released, in the lab, by the addition of chemicals, which modulate the underlying molecular mechanisms of PD, such as addition of gibberellins (GA), and this will be discussed later. Types of PD are based on their requirement for different dormancy release treatments.

1.4.2.2 Types of physiological dormancy

Within physiological dormancy, there are deep, intermediate and non-deep dormancy levels. Deep physiological dormancy is typified by the requirement for long periods of cold stratification [e.g. *Acer platanoides* (Finch-Savage, *et al.*, 1998)]

or warm stratification [e.g. *Leptecophylla tameiameiae* (Baskin, *et al.*, 2005)] to break dormancy, and the inability of embryos to grow normally when extracted from the seed coat (Baskin and Baskin, 2004). This deep physiological dormancy also appears to be prevalent amongst alpine species (Schwienbacher, *et al.*, 2011).

Intermediate physiological seed dormancy is characterised by the ability of excised embryos to produce normal seedlings, and a requirement for shorter periods of stratification compared to deep PD, which may be further shortened by after-ripening, to break dormancy. Additionally, treatment with GA may promote germination (Baskin and Baskin, 2004). Seeds of *Acer psuedoplatanus* (Finch-Savage, *et al.*, 1998), *Geum reptans*, *Oxyria digyna* (Schwienbacher, *et al.*, 2011) and some seeds, dependent on achene positions, of *Heteracia szovitsii* and *Garhadiolus papposus* (Baskin, *et al.*, 2014) exhibit this type of dormancy. It is evident amongst these studies that seeds of plants within the same genus, the same species, or even seeds from the same plants may exhibit different PD types.

However, the most prevalent type of physiological dormancy is non-deep physiological dormancy. Non-deep PD is characterised by the ability of an excised embryo to produce a normal seedling; the promotion of germination by GA or sometimes scarification; breaking of dormancy by cold or warm stratification; and dormancy loss through after-ripening (Baskin and Baskin, 2004). Non-deep PD also appears to be the most common of PD (Baskin and Baskin, 2004). Non-deep PD has further sub-types based on the relationship between dormancy depth and the range of environmental conditions (usually temperature) under which germination will proceed (Figure 1.2) (Baskin and Baskin, 2004; Nur, *et al.*, 2014).

1.4.2.3 Primary dormancy, secondary dormancy and dormancy cycling

The release of physiological seed dormancy, unlike morphological or physical dormancy, is a reversible process. Primary dormancy of a seed is induced on the parent plant during development and typically lasts until after the seed is fully developed and mature (Finch-Savage and Leubner-Metzger, 2006). Lack of primary dormancy leads to vivipary (germination whilst still attached to the parent plant) and this can result from mutations that perturb the induction of primary dormancy (e.g. *vp14*, outlined in section 1.4.3).

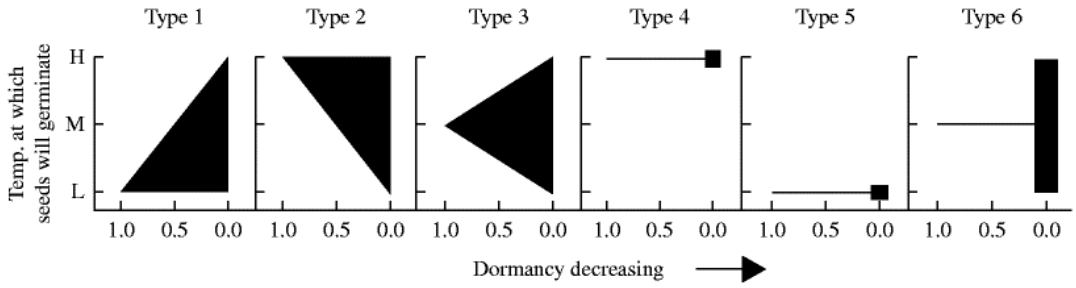

Figure 1.2. Six types of physiological dormancy. The temperature range at which seeds will germinate at different levels of dormancy. For example, deeply dormant seeds to Type 1 will only germinate at low temperatures. As dormancy is released in Type 1 dormancy, seeds are able to germinate at increasingly higher temperatures. Type 4 seeds require a high temperature to germinate, but aren't able to germinate at any temperature until dormancy is released. Type 6 seeds will germinate at any permissive temperature once dormancy is released (Figure taken from Nur, et al., 2014).

Following the loss of primary dormancy (e.g. by after-ripening or stratification), if conditions that permit germination are not present, then secondary dormancy may be induced. Secondary dormancy can be repeatedly released and induced and this results in the phenomenon of annual seed dormancy cycling, which is partly responsible for the maintenance of the soil seed bank (Finch-Savage and Leubner-Metzger, 2006).

Arabidopsis thaliana ecotype Cape Verdi Islands (Cvi) has proved to be a valuable model for the study of primary and secondary dormancy. Seeds of Cvi have a requirement for light to germinate (the final step in dormancy release/ initiation of germination), and will cycle through different depths of dormancy unless light is provided (Cadman, *et al.*, 2006). The state of primary dormancy (which occurs in seed that have not been after-ripening) deepens over time in Cvi if conditions which permit germination are not provided (light), for example after thirty days 98% of seeds can become insensitive to light, indicating an increase in dormancy depth ('PD30'). This dormancy can be broken by combined application of light and potassium nitrate (KNO_3). Imbibition of after-ripened seeds in the dark results in a secondary dormant state (can be released by KNO_3 + light), and if these seeds are further imbibed under prolonged cold conditions, secondary dormancy deepens further (cannot be released by KNO_3 + light) (Cadman, *et al.*, 2006). The molecular mechanisms involved here are discussed later (Section 1.4.3).

Similar behaviour of dormancy is witnessed in other species. For example incubation of primary dormant seeds of oat (Corbineau, *et al.*, 1993) and barley (Hoang, *et al.*, 2013; Leymarie, *et al.*, 2008) at supraoptimal temperatures (30°C) results in an inability of the seeds to germinate (referred to as secondary dormancy in those studies) at optimal temperatures, similar to the deepening of the primary dormant state ('PD30') in Cvi (Cadman, *et al.*, 2006). Indeed the secondary dormant and deep primary dormancy states appear to be mechanistically similar (Cadman, *et al.*, 2006). Secondary dormancy is also induced in response to other stimuli, such as low water potential [e.g. oilseed rape (Gulden, *et al.*, 2004)] and hypoxia [e.g. barley (Hoang, *et al.*, 2013)].

1.4.2.4 Relation of physiological dormancy to seed structure

Regarding seed structure, there are two main components of physiological seed dormancy: the seed coat (endosperm, and maternal tissues including the testa) and the embryo. These are often described as exogenous and endogenous dormancy, respectively (Hilhorst, 2007). In physiological dormancy, germination under potentially suitable conditions is prevented because the growth potential of the embryo is too weak to penetrate the surrounding tissues, either due to a reduction of embryo growth potential, a maintenance of seed coat strength, or a mixture of both (Finch-Savage and Leubner-Metzger, 2006; Hilhorst, 2007).

In seeds expressing non-deep physiological dormancy, excision of the embryo from the seed coat allows growth (Baskin and Baskin, 2004). Prior to germination in tomato (Toorop, *et al.*, 2000) and coffee (da Silva, *et al.*, 2004), a decrease in the amount of force required to puncture the endosperm is observed, sufficient to allow radicle penetration and germination; in this case dormancy is maintained primarily by a maintenance of endosperm strength. Testa properties (e.g. thickness, composition) are determined during seed development and can result in varied dormancy depths (Debeaujon, *et al.*, 2000; Hilhorst and Downie, 1996). For example, in Arabidopsis, the tannin content of the testa, and in turn the dormancy depth, is varied depending on the vegetative growth temperature of the parent plant (Chen, *et al.*, 2014).

Dormancy may also regulate growth potential of the embryo in order to prevent radicle emergence. Whilst the endosperm in Arabidopsis was the primary element of

dormancy, growth potential was also reduced in dormant seeds (Bethke, *et al.*, 2007). Coffee seeds treated with the germination inhibitor, ABA, also exhibited reduced growth potential (da Silva, *et al.*, 2004), and in *Datura ferox* embryo growth is promoted by red light (dormancy breaking); although far-red light (dormancy promoting) does not inhibit embryo growth (de Miguel, *et al.*, 2000).

Thus physiological dormancy is a sum of the embryo and coat dormancy components and interactions between them. The molecular mechanisms that govern these dormancy mechanisms in the embryo and seed coat are discussed in the following section.

1.4.3 Control of physiological seed dormancy by ABA

ABA biosynthesis, catabolism and signalling is reviewed in Section 1.7, and may aid with the understanding of this section. The specific steps of ABA biosynthesis, catabolism and signalling are discussed in more detail in that section.

1.4.3.1 Role in primary dormancy – during maturation and prevention of vivipary

Many mutants which are deficient in ABA biosynthesis, catabolism or have impaired signalling exhibit altered dormancy phenotypes. Many viviparous maize mutants (Robertson, 1955), unable to properly express primary dormancy, are deficient in ABA biosynthesis [e.g. *vp10* (Giulini, *et al.*, 2011); *vp12* (Maluf, *et al.*, 1997); *vp14* (Schwartz, *et al.*, 1997; Tan, *et al.*, 1997)]. At least one of these maize viviparous mutants are deficient in ABA perception and downstream signalling [e.g. *vp1* (McCarty, *et al.*, 1989)]. Some seeds of ABA deficient mutants still express some primary dormancy owing to either ABA biosynthesis contributed from heterozygous maternal tissues (Maluf, *et al.*, 1997), due to genetic redundancy, or contribution from maternal expression of functionally related genes (Tan, *et al.*, 1997). Thus in maize, ABA biosynthesis in maternal and zygotic tissues appears to be important in establishing primary dormancy. Reciprocal crossing or grafting studies indicate that only ABA produced by zygotic tissues is able to establish lasting dormancy (Kucera, *et al.*, 2005), although maternal ABA can increase dormancy in the tomato mutant *sitiens* (Groot and Karssen, 1992). Mutations in genes involved in ABA biosynthesis also result in vivipary in other species, including tomato [e.g. *sitiens* (Groot and Karssen, 1992; Harrison, *et al.*, 2011; Taylor, *et al.*, 1988)]. ABA produced by

maternal tissues also has a role in seed development that generally appears to be separate to the role of ABA in dormancy induction (Kucera, *et al.*, 2005).

Although many mutations resulting in ABA biosynthesis deficiency or ABA insensitivity do not result in vivipary in Arabidopsis, they do result in reduced primary dormancy in fresh seed [e.g. *abi3* (Nambara, *et al.*, 1994), *nced5, nced6, nced9* and crosses (Frey, *et al.*, 2012; Lefebvre, *et al.*, 2006)], such as increased sensitivity to germination stimulation by GA. Thus a role of ABA in dormancy induction during maturation appears to be conserved.

1.4.3.2 ABA during after-ripening

Dormancy levels are sometimes associated with the decrease in ABA concentration and ABA sensitivity during seed after-ripening (Finch-Savage and Leubner-Metzger, 2006). ABA concentration was much lower in non-dormant after-ripened seed of tobacco compared to mature, dormant seeds (Grappin, *et al.*, 2000), although ABA concentration was only marginally higher in dry dormant Arabidopsis Cvi seed compared to after-ripened seed (Ali-Rachedi, *et al.*, 2004). Similar trends of higher ABA concentrations in dormant seed are seen in other species, including tomato (Groot and Karssen, 1992).

However, the role of ABA in the dry seed in maintaining dormancy is disputed, since dormancy does not always correlate to dry seed ABA levels [e.g. Arabidopsis ecotype C24 and barley (Millar, *et al.*, 2006)]. Arabidopsis mutants that are unable to synthesise (*aba1-1*) or perceive (*abi1-1*) ABA are still able to undergo the after-ripening processes, resulting in distinguishable changes in the transcriptome upon imbibition. Furthermore, in the presence of exogenous ABA, the induced transcriptome in Arabidopsis does not reflect that of dormant seeds (Carrera, *et al.*, 2008).

Nonetheless, after-ripening does alter the expression of genes involved in ABA perception and signalling when seed are imbibed (i.e. ABA signalling is likely to be reduced in imbibed after-ripened seeds) (Carrera, *et al.*, 2008; Finch-Savage, *et al.*, 2007). Moreover, in some cases, changes in sensitivity rather than metabolism may determine the role of ABA in dormant and after-ripened seeds, such as in wheat (Gao, *et al.*, 2012).

Thus whilst ABA biosynthesis and perception appear to play a crucial role in inhibiting germination in hydrated dormant seeds, it does not appear to control the after-ripening process in dry seeds.

1.4.3.3 Role of ABA in maintaining primary dormancy upon seed imbibition

ABA biosynthesis is widely required in order for dormancy to be expressed upon imbibition of dry, mature, dormant seed. Inhibition of *de novo* ABA biosynthesis upon seed imbibition, using fluridone, led to increased germination in dormant seed of Arabidopsis ecotype Cvi (Ali-Rachedi, *et al.*, 2004), barley (Leymarie, *et al.*, 2009), potato (Alvarado and Bradford, 2005), sunflower (Lepagedegivry and Garello, 1992) and tobacco (Grappin, *et al.*, 2000).

Indeed, an increase in ABA biosynthesis and/or accumulation is often seen upon imbibition of primary dormant seeds [e.g. barley (Benech-Arnold, *et al.*, 2006); Cvi (Ali-Rachedi, *et al.*, 2004); and tobacco (Grappin, *et al.*, 2000)]. Addition of exogenous ABA during seed imbibition, or over-expression of genes involved in ABA biosynthesis can also lead to a non-germinating ('dormant') phenotype (Hai Ha, *et al.*, 2014; Martinez-Andujar, *et al.*, 2011; Qin and Zeevaart, 2002; Thompson, *et al.*, 2000). An increase in ABA biosynthesis upon imbibition is also observed, and required, for the expression of the thermoinhibition of lettuce seed germination at supraoptimal temperatures (Argyris, *et al.*, 2008; Huo, *et al.*, 2013).

Additionally, seed expressing primary dormancy are often more sensitive to ABA, often due to differential expression of regulators of downstream ABA signalling [e.g. Arabidopsis; barley (Barrero, *et al.*, 2009) and wheat (Liu, *et al.*, 2013a)] or due to a reduction in ABA catabolic activity [e.g. Arabidopsis (Cadman, *et al.*, 2006; Millar, *et al.*, 2006) and barley (Hai Ha, *et al.*, 2014; Millar, *et al.*, 2006)].

However, although accumulation and maintenance of ABA concentrations generally appears to be required for the expression of dormancy, application of exogenous ABA does not necessarily induce dormancy. Application of exogenous ABA fails to induce: lasting dormancy in sunflower or tobacco (Grappin, *et al.*, 2000; Lepagedegivry and Garello, 1992); or dormancy in the absence of endogenous ABA biosynthesis in rice (Gianinetti and Vernieri, 2007). Additionally, Arabidopsis mutants unable to synthesise (*aba1-1*) or perceive (*abi1-1*) ABA were still able to exhibit transcriptomes similar to dormant (not after-ripened) seed of the wild-type;

and ABA application to ABA deficient (*aba1-1*) or ABA insensitive (*abi1-1*) does not induce a transcriptome similar to the dormant seed (Carrera, *et al.*, 2008). Thus, although ABA signalling appears to be necessary for the correct expression of physiological dormancy, much of the mechanism functions upstream (or somewhat independently) of ABA signalling. A similar conclusion was made in a study of dormancy release in *Lolium rigidum* (Goggin, *et al.*, 2009).

1.4.3.4 Role of ABA in secondary dormancy and dormancy cycling

Treatment of Cvi seed expressing secondary dormancy ('SD2') with fluridone, an inhibitor of ABA biosynthesis, allowed 50% germination suggesting that ABA was at least in part necessary for maintaining secondary dormancy (Cadman, *et al.*, 2006). Furthermore, genes containing ABA responsive elements were significantly over-represented in a dormancy up-regulated gene set (Cadman, *et al.*, 2006). The expression of genes encoding enzymes catalysing ABA biosynthesis (e.g. *AtNCED6*, *AtNCED9*) and enabling ABA signal transduction (e.g. *ABI3*) were generally up-regulated in dormant seed compared to non-dormant seed. *CYP707A2*, which encodes and enzyme that catalyses ABA catabolism, was up-regulated in imbibed non-dormant seed treated with light compared to dormant seed. Based on expression information and effect of fluridone, ABA accumulation and sensitivity appear to be crucial in secondary dormancy. These trends were generally reflected during dormancy cycling of Cvi in the field in *AtNCED6* (ABA biosynthesis); *CYP707A2* (ABA catabolism); *SnrK 2.1*, *SnrK 2.4* (ABA perception), and ABA accumulation occurring during periods of dormancy (Footitt, *et al.*, 2011). However in cycling of Arabidopsis ecotype Bur, *CYP707A2* rather than *AtNCED6* appears to be regulating ABA accumulation, although the patterns of increased ABA signalling during dormancy are still evident (Footitt, *et al.*, 2013).

In both Arabidopsis Cvi and Bur, dormancy depth does not appear to be directly related to ABA content. In Cvi the levels of ABA plateau as dormancy depth continues to increase (Footitt, *et al.*, 2011). The expression of genes involved in ABA metabolism appear to reflect this trend in Bur too (Footitt, *et al.*, 2013). Thus ABA, whilst seemingly very important in the expression of dormancy, is not the key regulator of dormancy depth or cycling. Rather, the gene *DELAY OF GERMINATION 1* (*DOG1*) appeared to correlate best with dormancy cycling.

The deepening of dormancy in barley seeds imbibed at high temperature or under blue light is dependent on the maintenance of high ABA concentration, resulting from increased ABA biosynthesis and decreased ABA catabolism (Hai Ha, *et al.*, 2014; Leymarie, *et al.*, 2008). However, the increase in dormancy depth in barley seed imbibed under hypoxic conditions which do not permit germination is not dependent on ABA biosynthesis as fluridone does not prevent or alleviate the increase in dormancy (Hoang, *et al.*, 2013). Hypoxia does however appear to stall the decrease in ABA concentration that is seen in seeds imbibed in air (Hoang, *et al.*, 2013). Thus ABA also appears to facilitate changes in dormancy status in barley.

1.4.3.5 Mechanism of prevention of germination by ABA

Studies in which ABA accumulation is increased by exogenous ABA application, or by over-expression of ABA biosynthetic genes, have provided some insight into how ABA can prevent germination in imbibed seed. Exogenously applied ABA inhibits endosperm weakening in tomato (Toorop, *et al.*, 2000), coffee (da Silva, *et al.*, 2004), *Lepidium sativum* and Arabidopsis (Mueller, *et al.*, 2006), increasing the puncture force (embryo growth potential, increased by increase in turgor) required in order for the radicle to penetrate the endosperm. ABA also reduces the embryo growth potential (da Silva, *et al.*, 2004; Toorop, *et al.*, 2000). During germination, endosperm is, in part, weakened through the action of cell wall hydrolases. ß-1,3-glucanases. ABA prevents endosperm weakening, at least in part, by preventing the accumulation of ß-1,3-glucanases (Leubner-Metzger, 2003). Exactly how ABA is able to mediate all of these downstream effects (e.g. change in growth potential) in seeds is not yet fully elucidated.

The radicle emergence inhibition function of the endosperm in dicot species may be replaced by the coleorhiza, which encloses the radicle prior to germination, in *Poaceae* spp. In barley, the coleorhiza showed reduced ABA accumulation in non-dormant (after-ripened) seed after only 8 hours of imbibition compared to dormant seed, whereas differences between dormant and non-dormant seed ABA concentration were only apparent in root and other embryo tissue after 24 hours (Barrero, *et al.*, 2009). Consistent with this, *HvABA8'OH-1* (ABA catabolism) and genes conferring ABA sensitivity were up-regulated particularly in the coleorhiza of non-dormant barley seeds; although there was less evidence for changes in genes encoding members of the ABA biosynthetic pathway (Barrero, *et al.*, 2009; Millar, *et*

al., 2006). After-ripening also led to up-regulation of genes encoding *ß-1,3-glucanases* and other potential cell-wall modifying enzymes which were highly expressed in the coleorhiza (Barrero, *et al.*, 2009). Similar potential roles for ß-mannanases in coleorhiza-limited germination of the *Poaceae* model species *Brachypodium distachyon* have been uncovered (Gonzalez-Calle, *et al.*, 2015).

Thus it is possible in *Poaceae* spp. that the coleorhiza assumes a similar role to the micropylar endosperm in dicot species in mediating dormancy and may also be regulated by ABA.

1.4.3.6 The balance between ABA and gibberellins

As for ABA, the biosynthesis, catabolism and downstream signalling of the phytohormone gibberellic acid (GA) are also regulated in relation to dormancy. However, the presence of GA tends to release dormancy or promote germination, and acts antagonistically to ABA (Finch-Savage and Leubner-Metzger, 2006).

A theory of hormone balance controlling dormancy/ germination (Karssen and Lacka, 1986) proposed that ABA induced dormancy during maturation, whilst GA was responsible for breaking dormancy after this; however there is now a plethora of evidence to suggest that ABA and GA act simultaneously during maturation, and beyond, to maintain or release dormancy (Finch-Savage and Leubner-Metzger, 2006). Actually, during maize development, GA is required to stimulate germination, even in the absence of ABA: ABA deficient mutants germinate because the germination stimulation activity of GA in the developing embryo promotes germination in the absence of ABA (White, *et al.*, 2000; White and Rivin, 2000). In Cvi, although GA was unable to fully overcome the ABA-dependent dormancy, addition of GA did result in an increase in ABA, perhaps suggesting a feedback mechanism regulating the ABA/GA ratio during dormancy (Ali-Rachedi, *et al.*, 2004). Dormancy release by chilling is indeed characterised by an increase in GA sensitivity (Yamauchi, *et al.*, 2004). Consistent with this, dormancy states in Cvi are associated with transcriptional up-regulation of ABA signalling and down-regulation of GA signalling (e.g. reduction in biosynthesis and perception/ increase in catabolism) (Cadman, *et al.*, 2006; Finch-Savage, *et al.*, 2007).

Both ABA and GA hormonal signalling pathways are regulated to determine seasonal dormancy cycling in Cvi, (Footitt, *et al.*, 2011; Footitt, *et al.*, 2013),

generally with high ABA:GA ratio during deep dormancy and low ABA:GA ratio during more shallow dormancy. ABA and GA are also antagonistic in barley dormancy induced by blue light, high temperature or hypoxia (Hai Ha, *et al.*, 2014; Hoang, *et al.*, 2013; Leymarie, *et al.*, 2008). Hypoxia-induced secondary dormancy appears to be regulated more by changes in GA signalling than ABA signalling; whereas high temperature-induced secondary dormancy appears to be regulated relatively more by changes in ABA signalling (Hoang, *et al.*, 2013; Leymarie, *et al.*, 2008). The effects of ABA are generally antagonised by GA signalling; and vice versa. For example, GA promotes ß-1,3-glucanase activity (which is inhibited by ABA), leading to endosperm weakening, and ABA catabolism (Kucera, *et al.*, 2005).

1.4.3.7 The role of other phytohormones in dormancy and germination

Seed dormancy is not controlled only through the action of the hormones GA and ABA. For example ethylene appears to be a key regulator of endosperm weakening in Arabidopsis, *Lepidium sativum* and tobacco and counteracts the effect of ABA (e.g. in the inhibition of ß-1,3-glucanase mediated endosperm weakening) (Leubner-Metzger, 2003; Linkies, *et al.*, 2009). Indeed ethylene production is commonly associated with the onset of germination, and inhibition of its biosynthesis commonly associated with dormancy; and ethylene is typically antagonistic to the role of ABA (Arc, *et al.*, 2013b; Kucera, *et al.*, 2005; Linkies and Leubner-Metzger, 2012).

Strigolactones, prominent for their roles in stimulating germination in *Striga* spp. and *Orobanche* spp and plant architecture; and karrikins known as a smoke-derived germination promoting signal, are dormancy breaking or germination stimulating compounds which share common signalling pathways (Smith and Li, 2014). In plants, strigolactones, like ABA, are derived from carotenoid precursors (Alder, *et al.*, 2012). Strigolactones are able to break Cvi secondary dormancy, have an endogenous function in germination thermoinhibition in Arabidopsis and function in part by modulating the ABA/ GA ratio (Toh, *et al.*, 2012).

Cytokinins typically known for their growth promoting properties may also stimulate germination or relieve dormancy. In some cases cytokinins can antagonise the ABA-inhibition of germination (Guan, *et al.*, 2014), however cytokinin loss-of-function *Arabidopsis* mutants appear to germinate more quickly than wild-type (Riefler, *et al.*,

2006). Brassinosteroids also promote germination in some cases, and may function in reducing sensitivity to ABA in germination inhibition; and application can restore germination where it is inhibited by lack of GA (Kucera, *et al.*, 2005; Steber and McCourt, 2001). Nitric oxide, which promotes germination/ breaks dormancy, tends to act antagonistically to ABA. Nitric oxide is produced as a product of nitrate (which also stimulates dormancy release) assimilation and may be part of the nitrate perception mechanism (Arc, *et al.*, 2013a; Manoli, *et al.*, 2014).

Roles of auxins in seed germination do not appear to be as clear as those of ABA and GA. However, there does appear to be some evidence that auxin functions to promote primary dormancy in Arabidopsis with involvement of an ABA signalling-related transcription factor, *ABI3* (Liu, *et al.*, 2013b). Jasmonates may also have a role in dormancy and germination, but the roles are unclear and insensitivity and hypersensitivity to ABA have both been reported, although jasmonates typically tend towards inhibition of germination (Linkies and Leubner-Metzger, 2012).

Whilst some of the most prominent mechanisms of primary dormancy are discussed here, other hormones and second messengers (such as cyanide, reactive oxygen species), and molecular pathways are already implicated in seed dormancy and germination, they are not discussed here as the primary focus is the role of ABA.

1.4.3.8 Other important modulators of seed germination and dormancy and the integration of environmental signals

Perception of the environment and integration of multiple environmental cues is key to the regulation of dormancy – i.e. the way in which ABA and other signals are regulated in order to determine if dormancy is induced. For example, in Arabidopsis, low temperature and light control of germination is mediated through the regulation of GA biosynthesis by a transcription factor, SPATULA (Penfield, *et al.*, 2005), and this transcription factor also acts on GA and ABA signalling pathways to control dormancy during maturation (Vaistij, *et al.*, 2013).

Another important mediator of dormancy in Arabidopsis is *DELAY OF GERMINATION 1* (*DOG1*), identified as the principle quantitative trait locus (QTL) associated with primary dormancy (Bentsink, *et al.*, 2006). *DOG1* expression correlates with secondary dormancy and seasonal soil temperature during annual dormancy cycling in Arabidopsis and it may regulate sensitivity to ABA (Footitt, *et*

al., 2011; Footitt, *et al.*, 2013). Light and nitrate sensitivity were also seasonally regulated to control dormancy (Footitt, *et al.*, 2013). *DOG1* and ABA are also both involved in the establishment of different dormancy depths dependant on the maternal and seed maturation temperatures (Dekkers and Bentsink, 2015); and there are links to the regulation of flowering time in controlling dormancy and life-history traits in Arabidopsis (Chen, *et al.*, 2014; Springthorpe and Penfield, 2015). Both *DOG1* and ABA appeared to be required for proper expression of dormancy.

There is evidence that such mechanisms are also conserved in domesticated cereal species, since ectopic expression of wheat and barley *DOG1*-like genes in Arabidopsis induces dormancy (Ashikawa, *et al.*, 2010; Ashikawa, *et al.*, 2013). Furthermore, ectopic expression of wheat and barley *DOG1*-like genes in wheat enhanced seed dormancy; and RNAi mediated knock-down of endogenous wheat *DOG1*-like genes resulted in reduced dormancy (Ashikawa, *et al.*, 2014). Thus whilst ABA appears to be very important in dormancy, and may be manipulated for alteration of dormancy, it acts in the context of many other factors and is not the sole determinant of dormancy.

1.4.3.9 Summary

ABA is an inhibitor of germination and has critical functions in the induction of primary dormancy during maturation, maintenance of dormancy subsequent to imbibition, and in dormancy cycling. ABA also acts downstream of some other regulators to prevent germination or maintain dormancy under conditions that do not favour survival of the plant to reproduction. Seeds incorporate and integrate environmental cues and a regulating range of molecular process (e.g. ABA:GA ratio) that determine the range of conditions under which seed germination will occur.

1.5 Vigour

1.5.1 Definition of vigour

Vigour may be defined as the "***the sum of properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments***" (ISTA, 2015b); based on Perry, 1978. This means that seed batches (lots) that germinate equally well under optimal conditions may perform differently if assessed for germination and emergence (penetration of the shoot upwards though the soil) under suboptimal conditions. Seed batches of different vigour may also vary

in germination uniformity, seedling growth, and the ability to germinate following storage.

Thus ideally, a vigorous seed lot will show high-rate and uniformity of germination and growth (ISTA, 2015b). Seed are often tested for viability (i.e. germination under optimal conditions) and vigour before sale, with high seed vigour being a desirable trait. The results of vigour tests are generally regarded as a better prediction of field performance, since field conditions are often suboptimal (e.g. during the early sowing of maize at high latitudes). Vigour testing, particularly regarding maize, is discussed in more detail at the beginning of Chapter 3.

1.5.2 Environmental and genetic factors influencing vigour

Whilst vigour reflects the ability of seed to perform in varied environments, this ability can be pre-determined by both environmental and genetic factors, and the interaction between them. For example, differences in seed maturity at harvest can affect vigour (Tekrony and Egli, 1997), and sub-optimal conditions during seed maturation, such as frost, supraoptimal temperatures, defoliation or drought (DeVries, *et al.*, 2007; Tekrony and Egli, 1997; Tekrony and Hunter, 1995; Woltz, *et al.*, 2006) can have a negative impact on seed vigour. Beyond seed harvest, storage conditions [e.g. humidity and seed moisture content (Ellis, *et al.*, 1991; Ellis, *et al.*, 1995)] and seed selection [e.g. by size and shape, traits which themselves can be linked to vigour (Elliott, *et al.*, 2007; Graven and Carter, 1990; Msuya and Stefano, 2010; Muchena and Grogan, 1977)] can also impact vigour of seed lots.

Seed vigour, which is comprised of complex germination and seedling growth traits, has been linked to numerous genes, particularly though quantitative trait loci (QTL) analysis. For example, multiple QTL were linked to mean germination rate, initial downward growth rate, and upward growth in strong soil in *Brassica oleracea* (Finch-Savage, *et al.*, 2010). In rice, QTLs were identified for vigour (germination rate, root and shoot length, and dry weight) at three temperatures. Interactions between genotype and temperature were identified (Zhang, *et al.*, 2005), suggesting that aspects of seed vigour may be influenced differently by particular genetic loci depending on temperature. Some of the loci identified were also linked to seed vigour in the drained and flooded field conditions, whereas some QTL were specific to environment (Zhou, *et al.*, 2007). Thus vigour traits are polygenic, and some

genes appear to be pleiotropic with regards to vigour traits. For example, selection of progeny from an interspecific cross (*Solanum lycopersicum* x *S. pimpinellifolium*) based on rapid germination under either salt, cold or drought stresses, individually, resulted in progeny that exhibited rapid germination under all three stresses, suggesting that germination speed under salt, cold or drought is controlled by a similar set of genes (Foolad, *et al.*, 2003). Selection of progeny under stress conditions, but not under non-stress, also resulted in seed that germinated quicker under non-stress conditions, perhaps illustrating the usefulness of vigour as a selection trait for seed improvement (Foolad, *et al.*, 2003). Indeed common QTL for germination under stressed and non-stress conditions were later identified (Foolad, *et al.*, 2007). QTL controlling vigour under different conditions have been identified in many species, for example maize under suboptimal temperatures (Hund, *et al.*, 2004); wheat under osmotic stress (Landjeva, *et al.*, 2008); and *Medicago truncatula* at suboptimal and supraoptimal temperatures (Dias, *et al.*, 2011).

1.5.3 Molecular determination of vigour

Whilst many QTL controlling vigour have been identified, a lot of these have not yet been resolved to the function of individual genes. Rather a lot of information has been gained from '-omics' (e.g. transcriptomics, proteomics and metabolimics) studies (Rajjou, *et al.*, 2012). Because both the presence and rate of germination under suboptimal factors is important in high vigour seed, some of the QTL identified are likely to be involved in seed germination and dormancy, related to the mechanisms discussed in section 1.4. Indeed QTL analyses have resulted in the identification of *DOG1* (Bentsink, *et al.*, 2006) controlling dormancy in Arabidopsis, and *LsNCED4*, responsible for ABA mediated inhibition of germination of lettuce at high temperatures (Argyris, *et al.*, 2008; Huo, *et al.*, 2013). Genes encoding ABA signalling components were also identified in supraoptimal germination QTL intervals of *M. truncatula* (Dias, *et al.*, 2011).

In the absence of dormancy-related mechanisms, there are still many factors on which seed vigour is dependent. The speed of germination is highly dependent on the ability of the dry, quiescent seed to resume metabolic activity. This is therefore dependent on the integrity of stored proteins, mRNA, and DNA, which enable the seed to restore metabolic activity through *de novo* transcription and translation and then mobilisation of seed storage reserves, enabling the embryo to grow (reviewed in

Rajjou, *et al.*, 2012). So the accumulation of appropriate storage elements during maturation, influenced by the maturation conditions, and maintenance of integrity, influenced by storage conditions, is required for seed vigour. For example, a proteomics study looking at the difference in the proteomes of sugar beet seed which were aged artificially, and/or primed (a vigour enhancement treatment) identified that the following were linked to increased seed vigour: increases in voltage-dependent anion channels (involved in ATP production); increases in enzymes involved in mobilisation of lipid (via glyoxylate cycle) and starch (α-glucosidase) reserve mobilisation; regulation of amino acid synthesis and translation ability (Catusse, *et al.*, 2011). Additionally, accumulation of protein phosphatase 2A (PP2A) a negative regulator of ABA signalling and 14-3-3 protein, also involved in downstream ABA signalling, were increased in primed seed (high vigour) and decreased in artificially aged (low vigour) seed (Catusse, *et al.*, 2011). Thus treatments that affect seed vigour can also be related to changes in ABA signalling. Although the role of ABA in abiotic stress avoidance/tolerance (particularly drought and osmotic stress) is well known, roles of ABA in vigour subsequent to germination (i.e. 'seedling vigour') have not been extensively studied. However, it has been found that ABA signalling mediates the establishment of a postgermination arrest checkpoint which enables seedling desiccation tolerance (Lopez-Molina, *et al.*, 2001). Recently, it was shown that partial reduction of ABA by constitutive over-expression of *OsABA8ox* (an ABA 8'-hydroxylases involved in ABA catabolism) resulted in increased vigour in rice seedlings grown under cold conditions (Mega, *et al.*, 2015), indicating that ABA plays a role (in this case, inhibitory) in seed vigour determination beyond germination.

1.6 Improving Seed Germination and Vigour

1.6.1 Agricultural importance of dormancy and vigour

Establishment of an optimally dense and uniform crop population can be key to maximising yield, and this inevitably depends on the rate and uniformity of germination and seedling growth (i.e. seed vigour).

Lack of uniformity in a crop stand can be related to the spread in time of seedling emergence in a number of species (Benjamin, 1990). Lack of uniformity and a decrease from optimal crop density can result in loss-of-yield in some crops, such as

maize, which do not exhibit the ability to compensate for temporal or spatial variations in uniformity (Andrade and Abbate, 2005; Liu, *et al.*, 2004). However, some crops, such as soybean are able to 'fill the gaps' created by lack of uniformity during vegetative growth (Andrade and Abbate, 2005; Egli, 1993). Indeed, low seed vigour does seem to have the most impact on crops harvested at a vegetative or early reproductive stage (Tekrony and Egli, 1991). Since dormancy can act to spread germination over time, or result in non-germination, it too could result in sub-optimal population densities and uniformity. In many crop species prevention of germination in the field by dormancy is not considered to be such an issue. However, in some species, such as wheat, barley and rice, moderate seed dormancy may be maintained (by breeding) in order to prevent pre-harvest sprouting, but this can also result in dormancy following sowing due to the linkage between the two traits (Gao, *et al.*, 2003; Nakamura, *et al.*, 2007; Shu, *et al.*, 2015). Inhibition of lettuce germination by high temperatures (controlled by ABA) is also a dormancy issue relevant to crop production (Huo, *et al.*, 2013). Thus treatments that improve crop seed germination and vigour are a valuable resource, some of which are discussed in the following sections.

1.6.2 Enhancement of Seed Germination and Vigour

Various methods have been employed to improve seed germination and vigour, including treatments based on seed hydration (pregermination and priming), seed coating with chemicals (i.e. hormonal modulation, nutrient addition, fungicide treatment), and mechanical seed conditioning (e.g. selection of vigorous or uniform seed) (Leubner, 2012a; Taylor, *et al.*, 1998). This section focuses on priming, and chemical seed treatments that modulate hormonal pathways.

1.6.2.1 Pregermination

There are multiple methods of improving seed germination based on seed hydration. The most basic method is to soak seeds in water (sometimes allowing germination – which can be referred to as 'pregermination') under optimal conditions, before sowing in the less than optimal field environment, potentially reducing the delay in emergence. In this treatment, water uptake is not controlled, and seed are able to enter phase III of imbibition (Taylor, *et al.*, 1998). This type of method is exemplified in the fluid drilling techniques that have improved field emergence in a number of crops including carrots, celery and lettuce (Gray, 1981). However, control

of emergence uniformity in this method may be limited by the inherent asynchronous germination of the used seed, unless it is otherwise controlled (e.g. by priming), such as in carrot seed (Finch-Savage, 1986).

1.6.2.2 Priming

Another method to improve germination is priming. In priming, seed are imbibed, but radicle emergence is prevented, either by low water potential (osmopriming), controlled addition of water (hydropriming), or by limiting seed water uptake using a material with a low matrix potential (matrixpriming). In this case water uptake is controlled, and seeds are prevented from entering phase III of imbibition, and maintain desiccation tolerance (Taylor, *et al.*, 1998). Seed are usually dried back to their original moisture content for storage, before being re-sown in the field (Leubner, 2012a). This treatment allows the initialisation of seed repair mechanisms (e.g. DNA repair), *de novo* transcription and translation, and mobilisation of seed storage reserves (i.e. the early stages of seed germination) whilst maintaining the desiccation tolerance, allowing subsequent seed drying and storage. Once re-imbibed, seed exhibit more rapid and uniform germination as some of the steps that might otherwise delay germination in less vigorous seed have already been achieved (Rajjou, *et al.*, 2012). In some species, pregerminated seedlings are also desiccation tolerant and can be dried back, stored, and re-imbibed with similar germination improvements as seen in primed seed (Leubner, 2012a).

There are numerous examples of improvements in germination achieved through priming techniques [e.g. in lettuce thermoinhibition (Valdes, *et al.*, 1985); and maize (Afzal, *et al.*, 2008); many others (Parera and Cantliffe, 1994)]. However, the advantages of priming are dependent on optimisation of conditions for the seed species and seed lot, and sometimes yield inconsistent results (Parera and Cantliffe, 1994), thus determination of efficacy of priming can sometimes only be determined retrospectively. This, in part, has led to an interest in identifying biomarkers to monitor priming treatments to identify exactly how priming can improve vigour (Catusse, *et al.*, 2011; Gallardo, *et al.*, 2001; Rajjou, *et al.*, 2012). It has also been noted that the drying of primed seeds (for increasing storage life) can sometimes lead to partial loss of the benefits gained by the priming treatment (Weges and Karssen, 1990).

A disadvantage commonly associated with priming is the negative effect on seed longevity, as vigour of primed seeds can deteriorate more quickly than unprimed seeds during storage (Parera and Cantliffe, 1994), for example in sweetcorn (Chiu, *et al.*, 2002), lettuce (Tarquis and Bradford, 1992) and tomato (Argerich, *et al.*, 1989). Additionally primed lettuce seed is more sensitive to seed moisture content and controlled deterioration which indicates reduced longevity during storage (Hill, *et al.*, 2007). Longevity of primed sweetcorn seeds was increased by storage at 10°C or -80°C and deterioration of primed seed at 25°C was dependent on the priming method (Chiu, *et al.*, 2002). Thus primed seed have more stringent storage requirements.

1.6.3 Chemical treatment strategies

Molecular signalling pathways that regulate germination and dormancy (such as GA and ABA signalling, see Section 1.4) have been targets for the improvement of seed germination and vigour (Villedieu-Percheron, *et al.*, 2014). However, typically fungicides and insecticides appear to be the most prevalent chemical seed treatments, and physiological seed treatments (e.g. priming) are used to manipulate vigour (Black and Bewley, 2000; Syngenta, 2015). Specialised coating (e.g. film-coating) methods have been developed for the deployment of chemicals, and for the control of seed germination (Black and Bewley, 2000; Leubner, 2012a; Taylor, *et al.*, 1998), but there appears to be developing interest in identifying novel chemical treatments which can enhance seed germination and vigour. Because of ABA's prominent role in seed dormancy, chemicals which modulate ABA signalling are regarded as an interesting avenue of research (Villedieu-Percheron, *et al.*, 2014). Specific seed treatments involving maize are discussed in Chapter 3.

1.7 Regulation of ABA Signalling

1.7.1 Overview

As highlighted in Sections 1.4 and 1.5, ABA has important roles in seed germination, dormancy and vigour. ABA also has many other roles, including abiotic stress avoidance and tolerance (particular response to water-deficit), response to biotic stress and developmental functions (Zhang, 2014). In this section, the biosynthesis, catabolism and downstream signalling are reviewed and the most

significant or relevant points are discussed. Very detailed discussions of this topic can be found elsewhere (particularly in Zhang, 2014).

1.7.2 Biosynthesis

1.7.2.1 Overview

ABA biosynthesis can occur by two main pathways. In fungi, ABA is synthesised through the 'direct' pathway from sesquiterpenes (C_{15}) generated in the mevalonate pathway. However, in plants, ABA is synthesised via cleavage of carotenoids which are derived from tetraterpenes (C_{40}) generated through the non-mevalonate pathway in plastids (Taylor, *et al.*, 2005). In brief, pyruvate and glyceraldehyde 3-phosphate are metabolised to produce isopentenyl pyrophosphate and dimethylallyl pyrophosphate (both C_5), which are then metabolised to produce the diterpene (C_{20}) geranylgeranyl pyrophosphate. Two geranylgeranyl pyrophosphates are converted to phytoene (C_{40}) by phytoene synthase (reveiwed in Taylor, *et al.*, 2005).

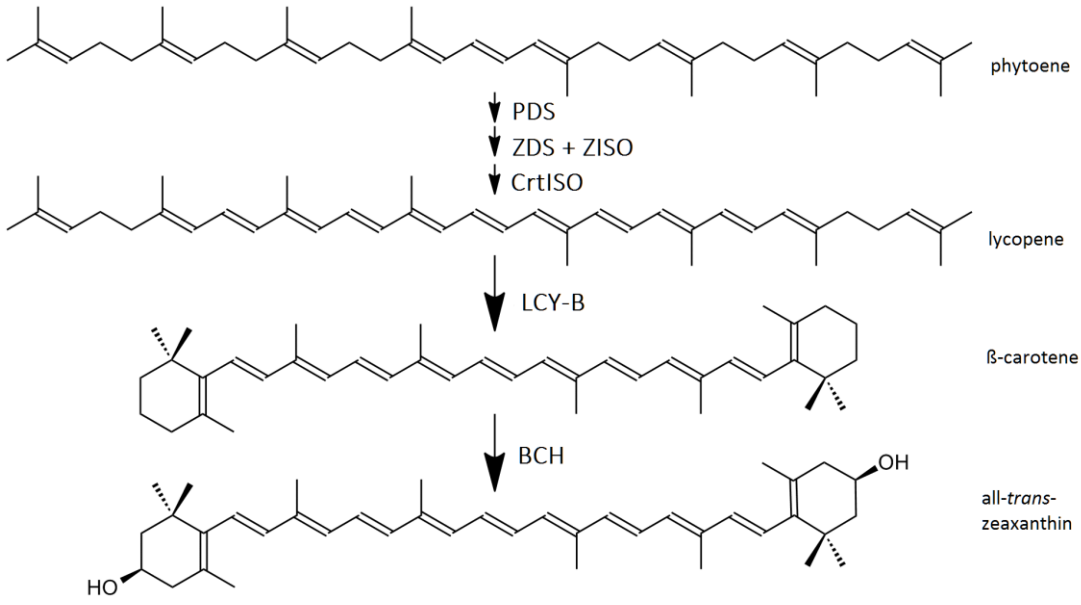

Figure 1.3 Synthesis of zeaxanthin from phytoene (simplified). PDS, phytoene desaturase; **ZDS**, ζ-carotene desaturase; **ZISO**, ζ-carotene isomerase; **CrtISO**, carotenoid isomerase; **LCY-B**, lycopene-β-cyclase; **BCH**, β-carotene hydroxylase (based on Taylor, *et al.*, 2005).

Phytoene desaturation, catalysed by phytoene desaturase (PDS), further desaturation by ζ-carotene desaturase (ZDS) and *cis-trans* isomerisation steps catalysed by ζ-carotene isomerase (ZISO) and carotenoid isomerase (CrtISO) are required for the formation of lycopene, which forms an important carotenoid precursor for a number of carotenoid products (Figure 1.3) (biosynthesis and roles of many carotenoids is reviewed in Cazzonelli, 2011). The isomerisation steps may in part be mediated by

photoisomerisation by light, though the isomerase activities of CrtISO and ZISO both appear to be important for lycopene synthesis in light and dark (Chen, *et al.*, 2010; Isaacson, *et al.*, 2002; Park, *et al.*, 2002).

The conversion of lycopene to ß-carotene and then to *trans*-zeaxanthin is catalysed by lycopene-ß-cyclase (LCY-B) and then β-carotene hydroxylase (BCH), respectively (Figure 1.3). ß-carotene also serves as a precursor for the biosynthesis of: strigolactone, a branching and germination signal (Alder, *et al.*, 2012); ß-ionone, a volatile aroma compound which has roles in insect attraction (Hammack, 2001; Rubio, *et al.*, 2008) and fruit flavour (Paterson, *et al.*, 2013); and other potential ß-carotene derived stress signals (Havaux, 2014). Zeaxanthin epoxidase (ZEP) catalyses the conversion of zeaxanthin to all-*trans*-violaxanthin (Figure 1.4).

1.7.2.2 Neoxanthin synthesis and 9-cis-isomerisation

Exactly how all-*trans*-violaxanthin is converted to xanthoxin is not yet known. Xanthoxin can definitely be produced via cleavage of 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) *in vitro* (Schwartz, *et al.*, 1997) (Figure 1.4). The enzymes responsible for 9-*cis*-isomerisation (putative 9-*cis*-epoxycarotenoid isomerases, or NCEIs), and for neoxanthin synthesis, have not yet been identified. However, neoxanthin-deficient mutants have been identified: *aba4* (North, *et al.*, 2007) and *nxd1* (Neuman, *et al.*, 2014). Neither of the genes affected by these mutations encode enzymes which catalyse neoxanthin synthesis when expressed in *E. coli*. The neoxanthin deficient mutants do however indicate that ABA can be synthesised from 9-*cis*-violaxanthin in Arabidopsis and tomato.

However the *aba4* neoxanthin-deficient mutant of Arabidopsis is also ABA deficient, and this affects its ability to acclimate to water deprivation (North, *et al.*, 2007), suggesting that neoxanthin is the primary stress-induced ABA biosynthesis precursor in Arabidopsis. On the other hand, the *nxd1* neoxanthin-deficient mutant of tomato is not ABA deficient, and actually indicates increased acclimation to water-deprivation (Neuman, *et al.*, 2014) suggesting that 9-*cis*-violaxanthin is the primary precursor for stress-induced ABA biosynthesis in tomato.

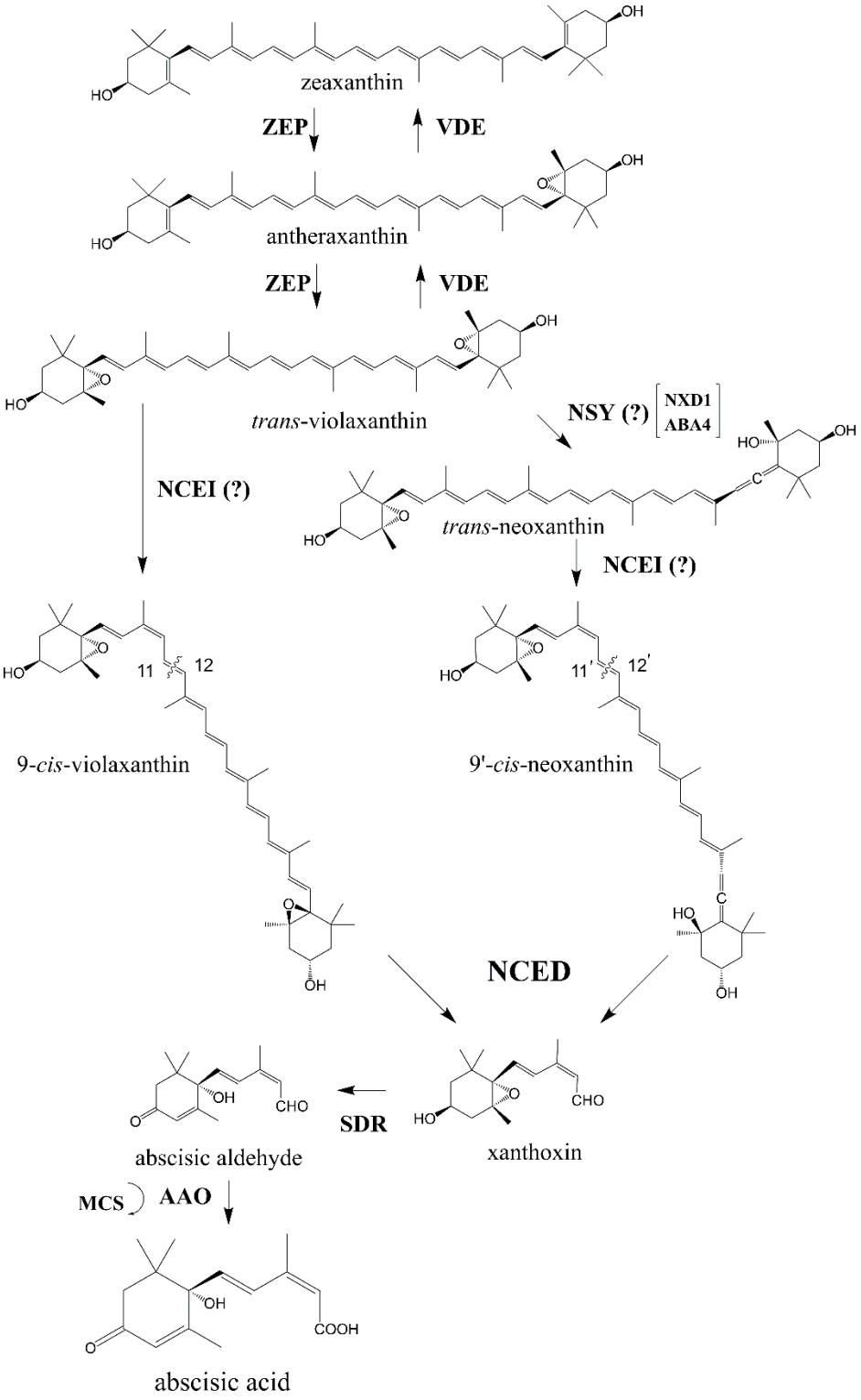

Figure 1.4. Abscisic acid biosynthesis. Abscisic acid biosynthesis pathway from zeaxanthin. **ZEP**, zeaxanthin epoxidase; **VDE**, vioxanthin de-epoxidase; **NSY**, neoxanthin synthase; **NXD1** and **ABA4**, required for NSY activity (see text); **NCEI**, 9-*cis*-epoxycarotenoid isomerase; **NCED**, 9-*cis*-epoxycarotenoid dioxygenase; **SDR**, short-chain alcohol dehydrogenase; **AAO**, abscisic aldehyde oxidase; **MCS**, Molybdenum co-factor sulfurase, required for AAO activity. Wavy lines indicate 11,12 or 11′,12′ cleavage sites of NCED on 9-*cis*-vioxanthin and 9′-*cis*-neoxanthin, respectively.

Furthermore, *Cuscuta reflexa*, a parasitic plant which lacks neoxanthin, is still able to synthesise ABA via the indirect pathway (Qin, *et al.*, 2008). Thus the preferred 9-*cis*-epoxycarotenoid substrate for NCED may depend on the species. An isomerase which catalyses the conversion of *trans*-ß-carotene to 9-*cis*-ß-carotene, D27, was recently identified (Alder, *et al.*, 2012). It is tempting to speculate that a related enzyme might catalyse the *cis-trans* isomerisation of the structurally similar violaxanthin and neoxanthin.

The xanthophylls zeaxanthin, violaxanthin and neoxanthin also have important roles in the photoprotection of photosystem II (reviewed in Jahns and Holzwarth, 2012) and zeaxanthin may also be cleaved to form compounds involved in the colour, flavour and aroma of saffron and citrus fruits (Frusciante, *et al.*, 2014; Ma, *et al.*, 2013).

1.7.2.3 Rate-limiting steps and first committed step of ABA biosynthesis

Prior to cleavage by NCED (Figure 1.4), the epoxycarotenoids and their precursors appear to have multiple products. The first committed step in ABA biosynthesis is the cleavage of 9-*cis*-epoxycarotenoids by NCED (Schwartz, *et al.*, 2003). This step also appears to be a rate-limiting step in ABA biosynthesis as over-expressing *NCED* usually results in an increase in ABA accumulation. For example, overexpression of *LeNCED1* increased ABA accumulation in leaves and seeds of tomato and tobacco, and enhances drought stress tolerance (Qin and Zeevaart, 2002; Thompson, *et al.*, 2000), indicating that the step catalysed by NCED is rate limiting in leaves and seeds. Thus NCED is a likely regulatory step in abscisic acid biosynthesis, and this is supported by many studies in which *NCED* expression is increased in situations where ABA accumulation occurs such as in dormancy, (mentioned in Section 1.4), and under water-deficit tolerance (Iuchi, *et al.*, 2001; Iuchi, *et al.*, 2000). However, other steps in the biosynthetic pathway may also be modulated under such conditions, and overexpression of genes such as *ZEP* can also facilitate increased ABA biosynthesis and related phenotypes (Frey, *et al.*, 1999; Park, *et al.*, 2008). This may especially be the case in tissues where xanthophylls are not as abundant, and may be limiting [e.g. roots (Thompson, *et al.*, 2007)].

1.7.2.4 From xanthoxin to ABA

Xanthoxin is converted to ABA through two enzymatic steps. Firstly conversion to abscisic aldehyde by a xanthoxin-specific short chain alcohol reductase, then conversion to ABA by abscisic aldehyde oxygenase (Figure 1.4). Other minor pathways may also exist, since loss-of-function mutants can still accumulate small amounts of ABA (Taylor, *et al.*, 2005).

1.7.3 Catabolism

Accumulation of ABA is also regulated by its catabolism (Figure 1.5). The ABA-8′-hydroxylase activity of CYP707A enzymes generally appears to be the primary step in ABA catabolism, forming phaseic acid which is enzymatically reduced to dihydrophaseic acid (Saito, *et al.*, 2004). 9′-hydroxylation is also catalysed, but less readily, by CYP707A (Okamoto, *et al.*, 2011), and the enzyme that catalyses the 7′-hydroxylation is not yet known (Endo, *et al.*, 2014). The hydroxylation of ABA is important in regulation of seed dormancy in Arabidopsis and barley (Millar, *et al.*, 2006; Okamoto, *et al.*, 2006).

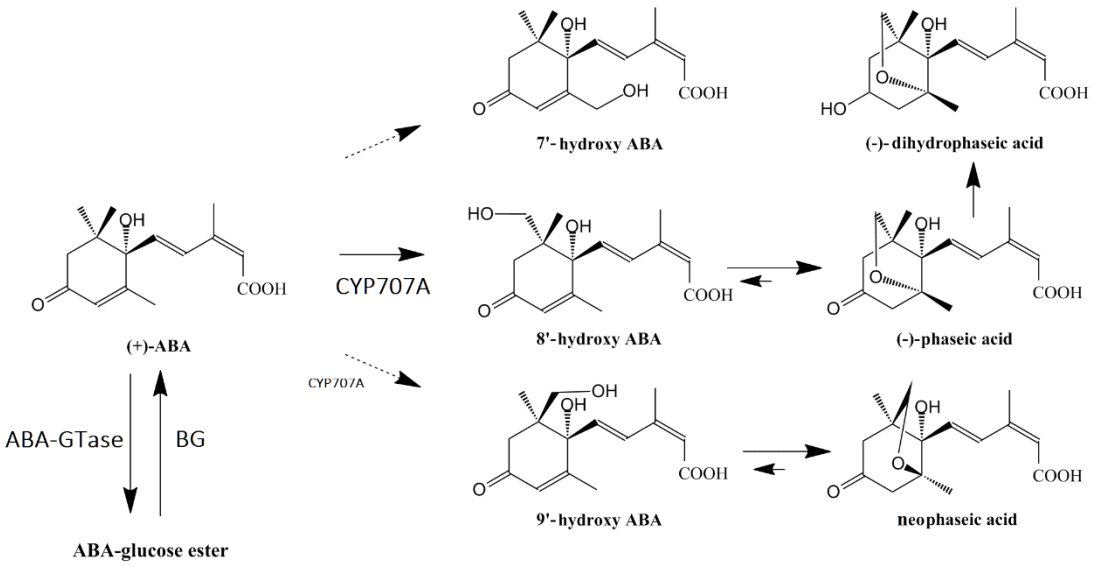

Figure 1.5. Catabolism of ABA. ABA levels are reduced through two main pathways – hydroxylation, such as 8′-hydroxylation by CYP707A (Kushiro et al., 2004) or conjugation, such ABA – glucose conjugation, which is catalysed by an ABA-glucosyl transferase (**ABA-GTase**) [e.g. *Vigna angularis* (Xu, *et al.*, 2002); Arabidopsis (Lim, *et al.*, 2005; Priest, *et al.*, 2006)] CYP707A produces 9′-Hydroxy ABA as a side reaction (Okamoto, *et al.*, 2011). **BG**, ß-glucosidase. Diagram adapted from figure 2 in Nambara and Marion-Poll (2005).

ABA may be conjugated to glucose (ABA-glucosyl ester) which may act as a storage or transport form for ABA (reviewed in Endo, *et al.*, 2014). ABA glucosylation also represents another ABA inactivation route, which has been observed in germinating lettuce seeds (Chiwocha, *et al.*, 2003). It is worth noting that hydrolysis of the ABA-glucosyl ester by ß-glucosidase also represents a potential source of ABA other than through its biosynthesis (Figure 1.5).

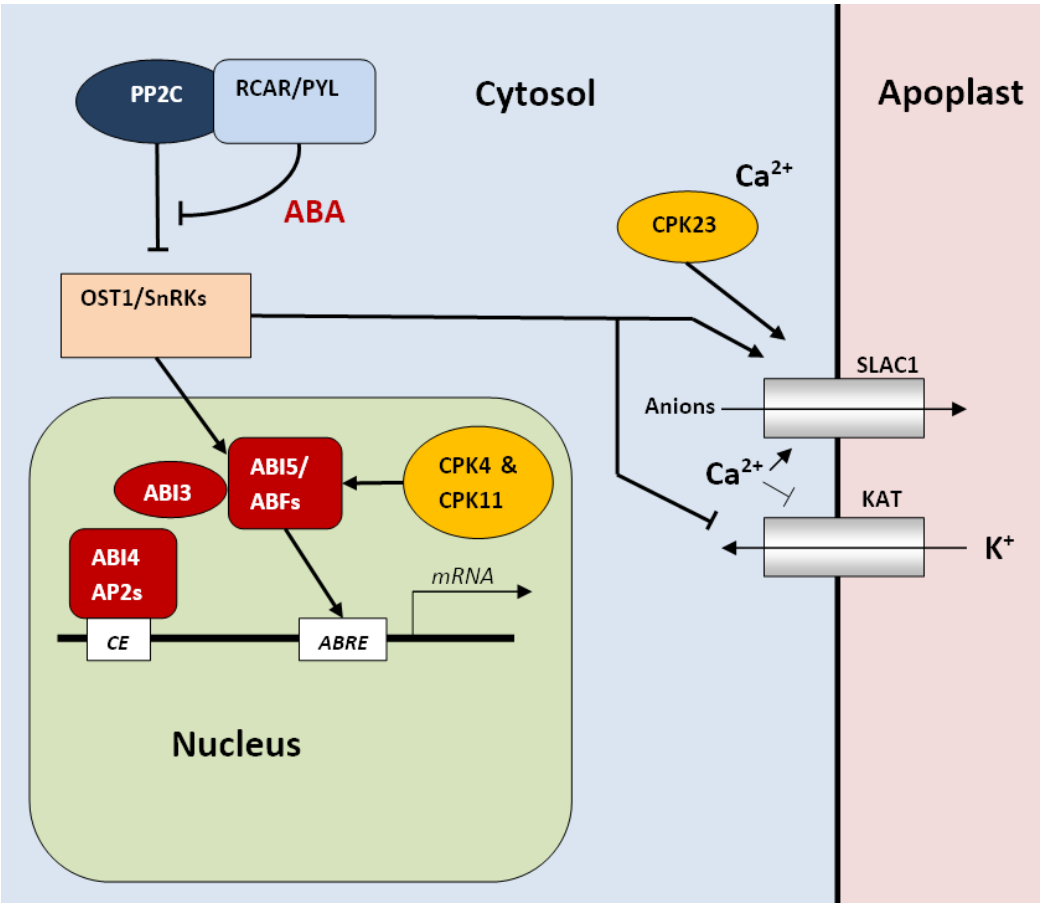

Figure 1.6. ABA signal reception and signal transduction. PP2C, Type 2C Protein Phosphatases; **RCAR/PYL**, Regulatory Components of ABA Receptor/Pyrabactin Resistance Protein1 – Like; **OST**, Open Stomata (a SnRK); **SnRK**, (Sucrose-non-fermenting 1)-Related Protein Kinases; **CPK**, Calcium-dependent Protein Kinase; **SLAC1**, Slow Anion Channel; **KAT**, Inwardly rectifying potassium transport channel; **ABI**, Abscisic acid Insensitive; **AP2s/ ABF**, *ABRE* binding factors - are involved in transcriptional regulation in response to ABA signal; ***ABRE***, ABA Responsive Promoter Element; ***CE***, coupling element. The PP2C/ PYL components behave as co-receptors in a heteromeric complex with high ABA binding affinity. (Adapted from Figure 2 in Raghavendra, *et al.*, 2010).

1.7.4 Downstream signalling

Although the downstream events of ABA signalling have been investigated in some depth, only fairly recently was a receptor with very convincing experimental evidence been discovered (Raghavendra *et al.*, 2010). The pathway illustrated in Figure 1.6 outlines a basic model of ABA signal perception and signal transduction. Components of the ABA signaling pathway represent potential targets for chemical

modulators of ABA signalling. For example inhibition of Ca^{2+} signalling mechanisms by DFPM was found to prevent ABA signal transduction (Kim, *et al.*, 2011). Recently, agonists and antagonists of ABA receptors have been designed (Hayashi and Kinoshita, 2014; Takeuchi, *et al.*, 2015).

1.8 Inhibition of ABA Biosynthesis – A Novel Seed Treatment?

1.8.1 Inhibition of ABA signalling hastens germination

ABA accumulation is linked to dormancy induction in seeds, and may reduce seedling vigour during germination under sub-optimal conditions (discussed in sections 1.4 and 1.5). Genetic inhibition of ABA signalling in seeds, by knocking out ABA biosynthetic genes [e.g. *NCED* (Lefebvre, *et al.*, 2006), *AAO* (Groot and Karssen, 1992; Harrison, *et al.*, 2011)], by up regulating ABA catabolic genes [e.g. *CYP707A* (Millar, *et al.*, 2006)], or by genetically modulating ABA signalling [e.g. *PP2C* (Wu, *et al.*, 2003)], can result in prevention of dormancy induction and hastening of germination.

However, agricultural use of such genetic modifications (GM) may be limited for a number of reasons. Firstly, a GM approach may be confounded by the multiple roles of ABA, such as in drought response. Whilst this can be somewhat overcome by targeted strategies (e.g. knocking out seed-specific *NCED*s; or inducible expression strategies), it may be complex to implement. Secondly, there may not exist the resources (e.g. GM protocol, genome) for the target crop. Furthermore, use of genetically modified organisms may be prohibited, or extensive regulation may impede their development and use. This may be overcome somewhat by the use of breeding strategies, but sufficient genetic diversity (e.g. natural mutations, alternative alleles in wild relatives) must exist and this can still be confounded by pleiotropic effects (e.g. seed dormancy and post-harvest sprouting). Such techniques are likely to require a lot of resources in each species in which they are pursued.

Chemical treatment strategies may be an alternative approach. Whilst chemical treatment strategies may also have complex regulatory issues regarding agricultural application, they may be more easily applied to a wide range of species in which a target mechanism is conserved. The role of ABA in dormancy and germination inhibition appears to be widely conserved (Section 1.4, and Graeber, *et al.*, 2012). Thus chemical inhibition of ABA signalling may represent a broadly useful tool for

improvement of germination in a wide range of target species under problematic environmental conditions.

1.8.2 Chemical inhibition of ABA biosynthesis

1.8.2.1 Carotenoid biosynthesis inhibitors

Inhibition of ABA biosynthesis by herbicides norflurazon and fluridone during imbibition often results in breaking of dormancy [e.g. Annual ryegrass (Goggin, *et al.*, 2009); Arabidopsis (Ali-Rachedi, *et al.*, 2004); barley (Leymarie, *et al.*, 2008); lettuce (Yoshioka, *et al.*, 1998); sunflower (Grappin, *et al.*, 2000)]. However, fluridone and norflurazon inhibit ABA biosynthesis by inhibiting phytoene desaturase, preventing the biosynthesis of carotenoids (Bartels and Watson, 1978). This inhibition of carotenoid biosynthesis typically results in photodestruction of chlorophyll in seedlings, and seedling death, limiting its use as germination stimulator for crop seed (Goggin, *et al.*, 2009; Han, *et al.*, 2004; Sebastian, *et al.*, 2014; Villedieu-Percheron, *et al.*, 2014; Yoshioka, *et al.*, 1998). The effect of fluridone, in modulating dormancy (Gianinetti and Vernieri, 2007), and in bleaching (Goggin, *et al.*, 2009) can persist even when seed imbibed in fluridone are transferred to media not containing fluridone. This may make norflurazon and fluridone useful as a method to stimulate germination of weed species in the soil seed bank, and simultaneously eliminate the species (Goggin and Powles, 2014; Yoshioka, *et al.*, 1998). Fluridone also inhibits the production of the germination stimulating hormone strigalactone, which may also have roles in germination promotion under sub-optimal conditions (Alder, *et al.*, 2012; Toh, *et al.*, 2012). Thus carotenoid biosynthesis inhibition may not be the best method for inhibiting ABA biosynthesis with a view to stimulating crop seed germination or for studying ABA roles. Specific inhibition of ABA biosynthesis would be desirable.

1.8.2.2 NCED inhibitors

As the first committed and rate-limiting step in ABA biosynthesis, inhibition of the cleavage of 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin by NCED may be an ideal target for specific inhibition of ABA biosynthesis. A number of NCED inhibitors already exist. Nordihydroguaiaretic acid (NDGA) (Figure 1.7), naproxen and other known inhibitors of lipoxygenase inhibited ABA accumulation *in vivo* (Creelman, *et al.*, 1992). NDGA inhibited NCED *in vitro*, however it exerts phytotoxic effects

associated with lipoxygenase inhibition, at concentrations (1 µM) lower than required for even 50% NCED inhibition *in vitro* (~50 µM) (Creelman, *et al.*, 1992; Elakovich and Stevens, 1985; Han, *et al.*, 2004). Development of more specific NCED inhibitors, based on NDGA was pursued, and resulted in the development of abamine (Han, *et al.*, 2004). Abamine (Figure 1.7), which appeared to exhibit similar *in vitro* and *in vivo* inhibition of NCED to NDGA, was not as phytotoxic as NDGA and actually stimulated cress radicle elongation, whilst fluridone and NDGA inhibited radicle elongation (Han, *et al.*, 2004). However, further compound development was pursued due to severe inhibition of growth of Arabidopsis by abamine, and its relatively weak inhibition of ABA biosynthesis *in vivo* (< 40% under osmotic stress) (Kitahata, *et al.*, 2006). A compound derived from abamine, abamineSG (Figure 1.7), inhibited ABA accumulation under osmotic stress by 77%, and was found to be a more potent NCED inhibitor than abamine *in vitro* (Kitahata, *et al.*, 2006). Abamine and abamineSG were found to be competitive inhibitors with K_is of 38.8 µM and 18.5 µM, respectively against cowpea NCED (Han, *et al.*, 2004; Kitahata, *et al.*, 2006). Whilst abamine promoted germination in Arabidopsis (Li, *et al.*, 2012) and barley (Yoshida and Asami, 2006) there does yet not appear to be any evidence of germination promoting effects of abamineSG in the literature.

A further set of inhibitors were designed based on the sesquiterpenoid segment of the 9-*cis*-epoxycarotenoids which cleaved by NCED, called sesquiterpene-like carotenoid cleavage dioxygenase inhibitors (SLCCDs) (Boyd, *et al.*, 2009). Some of these SLCCD compounds inhibited *At*NCED3 *in vitro* with K_is ranging from 57 µM to 87 µM, and were effective at inhibiting ABA accumulation in osmotically stressed plants. Two of these compounds (13 and 18) appeared to have slight germination-stimulating effects at low concentrations (0.1 µM), but one of these compounds (18) became moderately inhibitory to germination at higher concentrations (1 µM), whereas compound 13 (Figure 1.7) was only slightly inhibitory to germination at 3 µM (Boyd, *et al.*, 2009). The significance of these results is questionable as no replication was used due to the limitations in compound availability. The inhibitory activity of the compounds on germination may be due to their molecular resemblance to ABA, allowing their interaction with ABA receptors (Boyd, *et al.*, 2009).

Thus there exists a range of NCED inhibitors, although only modest evidence that they stimulate germination without unwanted side-effects. In the next section, a novel class of NCED inhibitors that stimulate germination without phytotoxic side-effects are described.

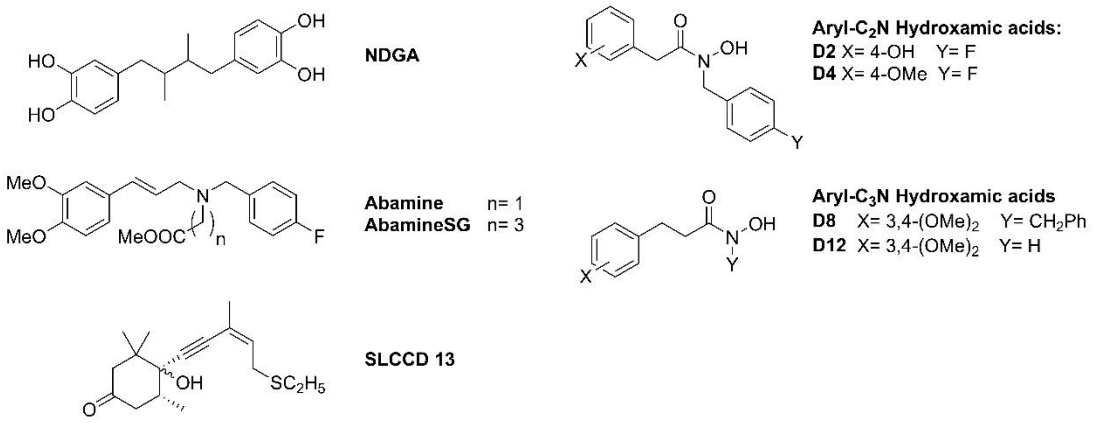

Figure 1.7. Inhibitors of NCED. Compounds shown are nordihydroguaiaretic acid (NDGA); abamine (Han, *et al.*, 2004), abamineSG (Kitahata, *et al.*, 2006), sesquiterpene-like CCD inhibitor (SLCCD) 13 (Boyd, *et al.*, 2009), and hydroxamic acid CCD inhibitors (Sergeant, *et al.*, 2009).

1.8.3 Hydroxamic acid CCD inhibitors that promote germination

1.8.3.1 Hydroxamic acid CCD inhibitors

NCEDs belong to a family of non-heme iron containing enzymes known as CCDs. CCDs cleave a variety of carotenoid substrates forming apocarotenoid products, and have a variety of roles in plants (Table 1.2). CCDs may be categorised by the cleavage position on the polyene backbone and substrate preference. For example, NCED cleaves 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin between the 11/12 or 11′/12′ carbons respectively (Figure 1.4). Typically plants contain at least one member of the five CCD families, CCD1, CCD4, CCD7, CCD8 and NCED (often multiple NCEDs – see Chapter 3) (Table 1.2), although some plants also appear to have CCDs with specialised roles, such as CCD2 in *Crocus sativus* (Frusciante, *et al.*, 2014). Different to the other CCDs, CCD1 does not appear to be plastid-localised. CCD1 and CCD4 appear to exhibit loose specificity to substrate and cleavage position *in vitro*, and work still appears to be ongoing to confirm *in vivo* activities. However, clear roles and *in vivo* substrate specificity for CCD7 and CCD8 in strigolactone biosynthesis, and NCEDs in ABA biosynthesis have been described (Alder, *et al.*, 2012; Neuman, *et al.*, 2014).

Table 1.2. CCDs cleave different substrates at different positions yielding many apocarotenoid products with different functions (brief summary).

CCD subfamily	Substrates	Cleavage sites	Products	Function(s) References	Refs[a]
CCD1	Many e.g. ß-carotene	9,10 (9′,10′) 7,8 (7′,8′) 5,6 (5′,6′)[a]	Many e.g. ß-ionone	Flavour Aroma volatiles More?	1
CCD4	Many carotenoids	9,10 (9′,10′) 7,8 (7′,8′) 5,6 (5′,6′)[a]	Many	Pigmentation Aroma volatiles More?	1, 2
CCD7	9-*cis*-ß-carotene	9′,10′	9-*cis*-ß-apo-10′-carotenal, ß-ionone	Strigolactone biosynthesis	3
CCD8	9-*cis*-ß-apo-10′-carotenal	Complex[b]	Carlactone	Strigolactone biosynthesis	3
NCED	9-*cis*-violaxanthin 9′-*cis*-neoxanthin	11,12 11′, 12′	Xanthoxin, C_{25}	ABA biosynthesis	4

[a]References: 1 (reviewed in Ohmiya, 2009); 2 (Lashbrooke, *et al.*, 2013; Ma, *et al.*, 2013); 3 (Alder, *et al.*, 2012); 4 (Neuman, *et al.*, 2014; Schwartz, *et al.*, 1997). [b]CCD8 catalyses a molecular rearrangement where three oxygens atoms are incorporated rather than cleavage (Alder, *et al.*, 2012).

Sergeant *et al.*, (2009), designed a set of arylalkyl hydroxamic acid compounds to inhibit CCDs (Figure 1.7; Table 1.3). The length of the alkyl chain between the hydroxamic acid moiety (aimed to chelate the non-heme iron) and the aromatic ring (mimicking the carotenoid substrate) was varied to gain compounds with specificity to the different CCD families, and functional groups were also varied (Sergeant, *et al.*, 2009). Some compounds did indeed exhibit some degree of specificity to inhibition of a particular CCD. For example 100 µM D3 (Table 1.3) inhibited CCD1 activity by more than 95%, but NCED activity by only 4%. Many of these compounds also appeared to inhibit CCD7 or CCD8 *in vivo* (assayed through the strigolactone-related shoot branching phenotype) (Sergeant, *et al.*, 2009). The compounds have since been confirmed to induce this phenotype through CCD8 inhibition (Harrison, *et al.*, 2015). However, no compounds that preferentially inhibited NCED compared to CCD1 (and presumably CCD8) were identified (Sergeant, *et al.*, 2009). However, D8 (Table 1.3) showed the best NCED-specificity and multiple compounds were more effective *Le*NCED1 inhibitors than abamine (abamine also inhibited *Le*CCD1a more than *Le*NCED1) (Sergeant, *et al.*, 2009).

'*Le'*, i.e. in *Le*NCED1, refers to *Lycopersicon esculentum*, a now outdated synonym for *Solanum lycopersicum* or tomato.

1.8.3.2 The effects of hydroxamic acid CCD inhibitors on seed germination

Despite their ability to preferentially inhibit CCDs other than NCED, many of the hydroxamic acid compounds exhibited equivalent, or better, *in vitro* inhibition of *Le*NCED1 than abamine (Sergeant, *et al.*, 2009). Thus the ability of the inhibitors to promote seed germination was investigated (Awan *et al.*, in preparation). Tomato seed constitutively overexpressing *LeNCED1* (Thompson, *et al.*, 2000), sp12, were imbibed in solutions of the different CCD inhibitors (Table 1.3) (Awan *et al.*, in preparation). Two of the inhibitors, D4 and D7, significantly reduced the mean germination time (MGT) of sp12 compared to control, whilst two compounds, abamine and D8, significantly increased MGT. This was somewhat surprising since abamine has previously been shown to promote germination, although 100 µM abamine may have been phytotoxic. In addition D8, which was found to be more specific to NCED than other inhibitors, increased MGT, presumably due to non-specific effects (Awan *et al.*, in preparation).

Table 1.3. In vitro CCD inhibition and germination effects of hydroxamic acid inhibitors.

Inhibitor				*In vitro* inhibition @ 100 µM		MGT (days)[a]
Class	Name	X	Y	*Le*CCD1a*	*Le*NCED1*	
None				N/A	N/A	8.9
Fluridone		N/A		N/A	N/A	2.7
Abamine				35	20	11.9^{+}
Aryl-C_2N	**D1**	4-OH	H	>95	27	8.1
	D2	4-OH	F	>95	29	8.8
	D3	3,4-(OH)2	F	>95	4	9.7
	D4	4-OMe	F	>95	33	**6.7**$^{-}$
	D5	3,4-(OMe)2	H	>95	8	8.0
	D6	3,4-(OMe)2	F	>95	18	9.4
	D7	3,4-OCH2O	F	>95	33	**7.3**$^{-}$
Aryl-C_3N	**D8**	3,4-(OMe)2	Ch2Ph	61	40	11.4^{+}
	D9	4-OMe	Ch2Ph	>95	27	-
	D10	3,4-(OMe)2	n-octyl	65	14	-
	D11	4-OMe	n-octyl	53	15	-
	D12	3,4-(OMe)2	H	26	11	10.7
	D13	4-OMe	H	46	13	-

*data from Sergeant *et al.*, 2009; [a]Awan *et al.*, in preparation – mean germination time (MGT) of sp12 seed imbibed on 1 mM of inhibitor compound; $^{+}$significantly larger MGT than no inhibitor; $^{-}$ significantly lower MGT than no inhibitor. General structures indicated in Figure 1.6.

D4 was selected for further *in vivo* germination studies and was found to promote germination in a number of situations: in tobacco seed overexpressing *LeNCED1*, in thermodormant lettuce seed [which is dependent on NCED (Huo, *et al.*, 2013)], and in tomato seed imbibed at low water potentials (Awan *et al.*, in preparation). D4 also caused a modest reduction in ABA accumulation in the sp12 tomato (ca. 20%) seed and wild-type tomato seed (ca. 30%) imbibed at low potentials (Awan *et al.*, in preparation). Moreover, D4 was found not to inhibit plant growth at concentrations at which it stimulates germination (1 mM). Thus D4 represents a novel NCED inhibitor which stimulates seed germination, apparently without phytotoxic effects. D2, a related hydroxamic acid that exhibits *in vitro* NCED inhibition (Table 1.3), was also reported to stimulate germination in *Brassica* spp. and thermodormant lettuce seed (Paul van den Wijngaard, personal communication, December 1, 2011).

Hydroxamic acids D2 and D4 therefore represented potential tools to investigate the effects of NCED on germination of other species, and also as positive controls for developing a screen for new, novel, inhibitors. However, increased specificity to particular NCEDs was desired since D2 and D4 also inhibit other CCD enzymes. Their mode of action in germination stimulation was presumed to be through NCED inhibition (and reduction of ABA biosynthesis) as inhibition of CCD7 or CCD8 would actually lead to depletion of the germination-stimulating hormone strigolactone (Alder, *et al.*, 2012). Interestingly the germination stimulating effect of D4 was substantially less than fluridone (Table 1.3), indicating there may be a lot of scope for improvement of ABA biosynthesis inhibition by hydroxamic acid inhibitors (Awan *et al.*, in preparation).

1.9 Chemical Genetics of Seed Germination

Chemical genetics is the study of biological systems using small molecule tools (O'Connor, *et al.*, 2011). Parallels can be drawn between classical genetics and chemical genetics (Figure 1.8). Typically, in forward genetics, phenotypes would be identified from a population of individuals that contain random mutations (Figure 1.8 A). Further study would then be required to identify the gene associated with the chosen phenotype. Similarly, in forward chemical genetics, phenotypes may be identified amongst a population of individuals each of which are exposed to different compounds of a diverse library (Figure 1.8 B). Further study is then required to

identify the protein target which the chemical is modulating. In both cases, the phenotype is selected first, and the genetic factor controlling that phenotype is subsequently identified. In reverse genetics (Figure 1.8 C) and reverse chemical genetics (Figure 1.8 B) a specific gene is mutated or a protein is chemically modulated, respectively, and the function of the gene or protein is revealed through the resulting alterations to phenotype.

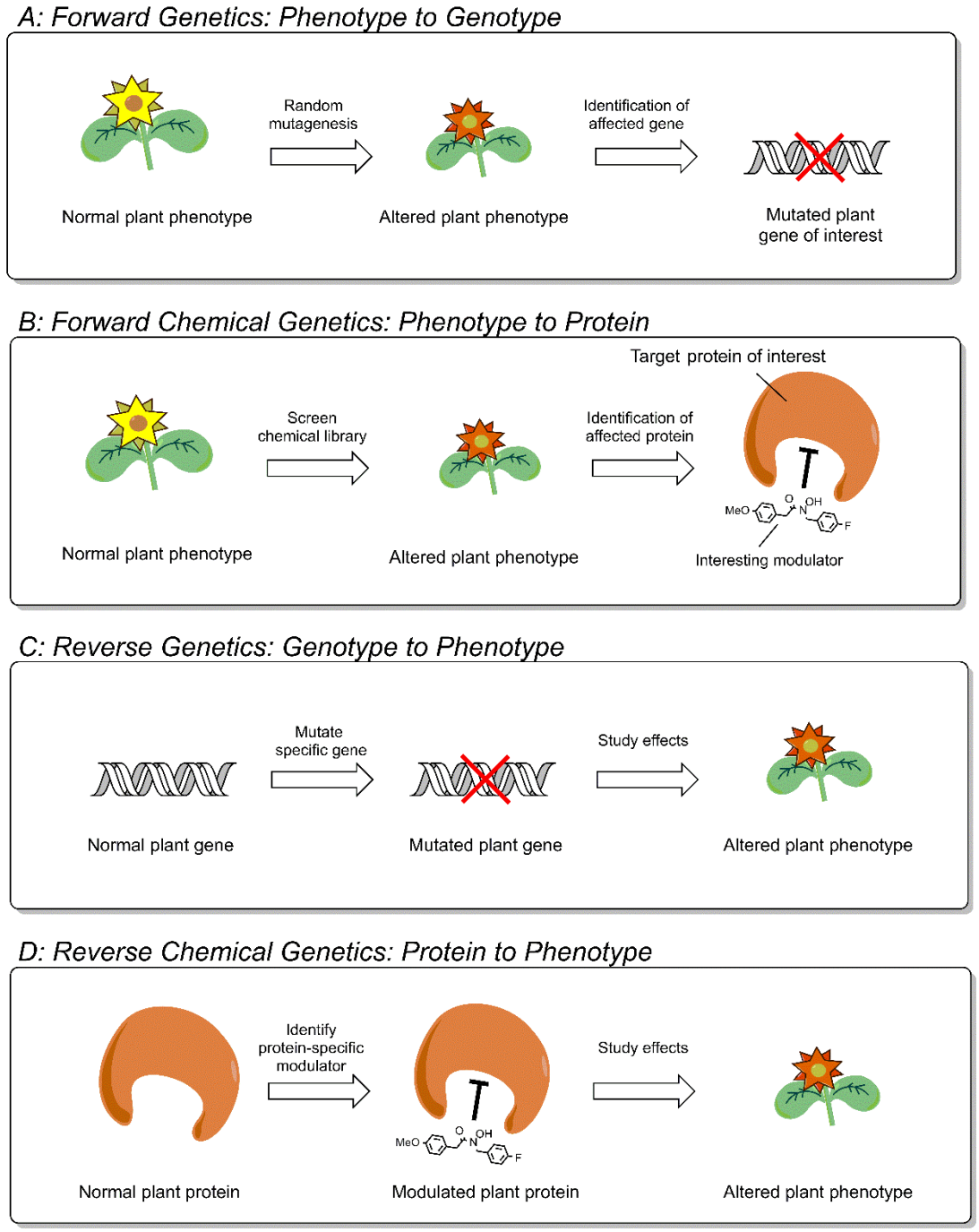

Figure 1.8. Parallels between chemical genetics and classical genetics. See text for further discussion of the schematic (based on scheme 1 from O'Connor, *et al.*, 2011).

The chemical genetics approach can be utilised to screen for chemical modulators which cause a desired phenotype, for example in *in vitro* assays using selected protein targets to identify active compounds from large libraries. This approach has been used with great success in the pharmaceutical industry and has also been adopted by agrochemical companies (Drewes, *et al.*, 2012; Mayr and Bojanic, 2009).

Chemical genetics can sometimes have advantages over genetics approaches. This is particularly true when genetic redundancy exists. In this case, knocking out a single gene (by random or directed mutagenesis) may not result in a change in phenotype. However a chemical modulator which act against the common function of the genes will, allowing the identification of the function of a related gene family (Toth and van der Hoorn, 2010). Conversely, the use of a specific agonist of a particular ABA receptor, pyrabactin, facilitated the forward genetics approach to identify mutants with 'pyrabactin resistance' (Park, *et al.*, 2009; Toth and van der Hoorn, 2010). Chemical genetics studies are becoming increasingly popular in plant science (Toth and van der Hoorn, 2010).

A chemical genetics approach could be used to identify new chemicals that modulate seed germination, which may elucidate novel targets (e.g. forward chemical genetics). The CCD inhibitors described in section 1.8.3 may also be used to investigate the function of CCDs (in particular, NCEDs) in germination by side-stepping the genetic redundancy that can occur in *NCED* gene families (Priya and Siva, 2015; Tan, *et al.*, 2003). However, NCED-specific inhibitors would be more desirable for this task.

1.10 Aims and Objectives

1.10.1 Outline

Cold conditions during imbibition can result in slow or no germination in some maize seed, leading to suboptimal crop density and uniformity and loss of yield (discussed further in Chapter 3). A novel seed treatment is required which restores germination in seed batches which would otherwise perform poorly under cold conditions and fail quality control tests.

Overall hypotheses: a) Germination of low vigour maize seed lots at suboptimal temperatures is inhibited by accumulation of ABA, and could be related to the induction of physiological dormancy mechanisms. **b)** Germination of low vigour maize under cold can be restored by small biologically active molecules.

1.10.2 Aims and objectives of the project

Aim 1: To determine which, if any, maize *NCED*s control germination, vigour and ABA biosynthesis at suboptimal temperatures.

> **Objective 1a:** Based on seed vigour tests, identify conditions under which maize germination and vigour is inhibited by cold.
>
> **Objective 1b:** Probe the role of ABA biosynthesis under the identified conditions using NCED inhibitors.
>
> **Objective 1c:** Identify or confirm putative maize *NCED*s using a bioinformatics approach.
>
> **Objective 1d:** Measure the expression of maize *NCED*s and ABA accumulation during imbibition under suboptimal conditions.

Importance: Identifying the role of maize NCEDs and ABA biosynthesis under suboptimal conditions may aid in the development of new seed treatment strategies (e.g. application of ABA signalling inhibitors to improve germination under suboptimal conditions). For example, a target NCED which inhibits germination may be identified, and this may allow specific inhibition strategies or breeding programs to be developed. This may improve the success of maize cultivation in less than optimal environments.

Aim 2: To develop novel germination stimulating compounds and NCED-specific inhibitors.

Objective 2a: Clone maize *NCED*s for expression in *Escherichia coli* to aid the *in vitro* identification of maize NCED-specific inhibitors.

Objective 2b: Develop a high-throughput assay to screen chemical libraries for germination stimulating compounds.

Objective 2c: Screen a diverse chemical library for germination stimulating compounds

Objective 2d: Direct compound design by testing designed and modified ABA biosynthesis /NCED inhibitors for germination stimulating properties.

Importance: The current range of NCED inhibitors do not only inhibit NCEDs, but also other targets, such as other CCDs. A specific NCED inhibitor, that can inhibit ABA biosynthesis in seeds, would be useful for probing the roles of NCED and ABA biosynthesis in seeds. Furthermore, since plants generally have multiple NCEDs, inhibitors which target specific NCEDs (e.g. maize NCEDs) could be identified through *in vitro* chemical screening.

Additionally, many NCED inhibitors do not appear to have very strong effects on germination stimulation. An *in vivo* germination screen may identify novel NCED inhibitors which promote germination, or chemicals which stimulate germination through action on novel target.

This chemical genetics approach may yield new targets for germination improvement, or new chemicals which act on known targets (NCED). This may yield novel seed treatment technologies.

1.10.3 Thesis structure

The thesis structure and the relation of the chapters to the objectives is outlined in Figure 1.9.

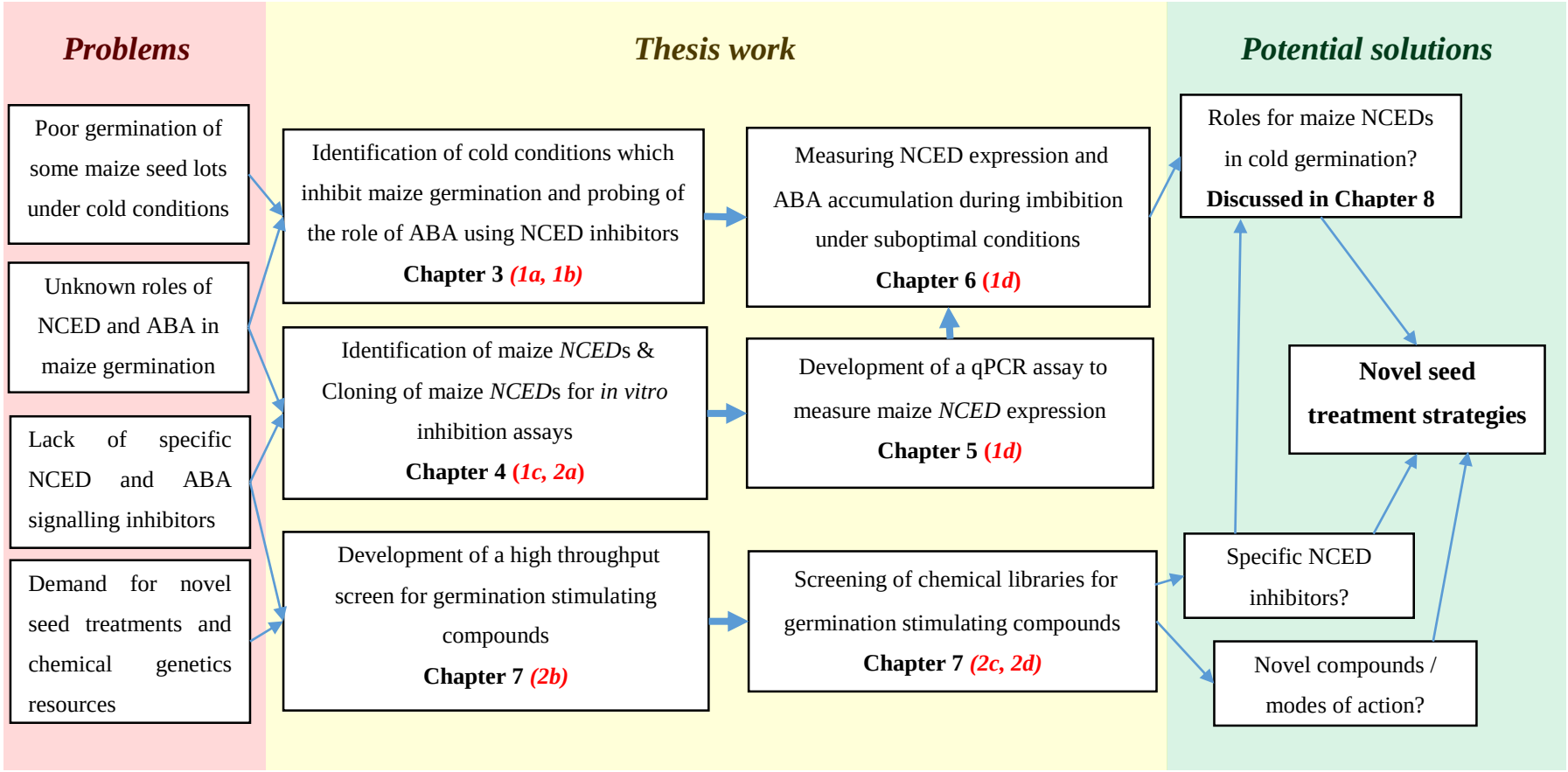

Figure 1.9. Flowchart of thesis structure. Problems the research addresses and potential outcomes of the project. Objectives are highlighted in red italics.

2 Materials and Methods

2.1 General

2.1.1 Reagents and chemicals

Reagents and chemicals were typically obtained from Sigma-Aldrich Company Ltd., Dorset, UK (Sigma); Fisher Scientific UK Ltd., Loughborough, UK (Fisher); and Melford Laboratories Ltd., Ipswich, UK (Melford). Enzyme related reagents and purification kits were obtained from QIAGEN Ltd., Manchester, UK (QIAGEN); Bio-Rad Laboratories B.V., Veenendaal, NL (Bio-Rad); or New England Biolabs (UK) Ltd., Hitchin., UK (NEB). Catalogue numbers and exceptions are noted in the text.

2.1.2 Water

Unless otherwise specified, such as in cases where tap water was used, 'water' refers to water purified using the Direct-Q 3 water purification system (Millipore) which has a resistivity of >18.2 MΩ•cm and from which particulates > 0.22 µM were removed by filtration. This water was used to produce different types of water, below:

Sterile water: Refers to autoclaved water.

Diethylpyrocarbonate (DEPC)-treated water: DEPC-treated water was produced by adding DEPC (Melford D1378) to a final concentration of 0.1% (v/v) in water, which was shaken vigorously, incubated at 37°C overnight and then autoclaved.

2.2 Plant Material Used

2.2.1 Maize seed

Details of the maize seed used in the thesis experimental work are given in Table 2.1

2.2.2 Tomato seed

Tomato lines which constitutively overexpress *LeNCED1* ('Sp5') are described in Thompson et al., 2000 and seed of this line was used from accession AT2456 provided by Prof. Andrew J. Thompson.

Table 2.1. Maize seed used. Details of the origin and nomenclature of the maize seed lots used in the experimental work in this thesis are indicated below.

Supplier	Variety	Variety name	Seed lot	Accession[d]	Thesis name
Syngenta Seeds B.V., Enkhuizen, NL[a]	1	NK Octet	A	12CN002	**1A**
			B	12CN005	**1B**
	2	NK Galactic	A	12CN004	**2A**
			B	12CN006	**2B**
	3	SY Multitop	A	12CN003	**3A**
			B	12CN008	**3B**
	4	NK Magitop	A	12CN009	**4A**
			B	12CN011	**4B**
	5	NK Famous	A	12CN007	**5A**
			B	12CN010	**5B**
Syngenta , Jealott's Hill, UK[b]	-	NK Falkone	1	-	**NK Falkone**
University of Warwick[c]	-	B73	1	-	**B73**

[a] 10 maize seed lots were provided by Paul van den Wijngaard. [b] Provided by Dr. David Brocklehurst. [c] Provided by David Schafer.[d] Syngenta accessions for seed lots; may be used in some data presented (e.g. appendices).

2.2.3 Tobacco seed (and bulking)

Tobacco seed harbouring the *tetR* gene encoding the tetracycline repressor protein and/ or tetracycline-inducible *LeNCED1* (pBHT-NCED) have been previously described (Thompson, *et al.*, 2000). Tobacco seed were sown on filter paper from stocks of 'pBHT-F2-1 (2010)' (pBHT-NCED) and 'TetR7 (2006)' (TetR) in November 2011 and transferred to compost and sand (Levington F2+S) approximately 10 days after sowing and grown in a partially environmentally controlled glasshouse at 22/18^{o}C day (16 hr light) night set-point temperatures, and with supplementary lighting. Plants were transferred to Levington M2 peat-based compost in 9 inch diameter pots after approximately 1 month. Paper bags were placed over flowers to prevent cross-pollination and seed pods were allowed to dry. Seed were harvested in August 2012, separately from 10 plants of pBHT-NCED and 10 plants of TetR.

2.3 Germination Assays

2.3.1 Maize germination assays

Where indicated, maize were scored daily for radicle emergence. Germination and vigour were also scored in vigour tests as outlined in sections 2.3.2 to 2.3.6.

2.3.2 Maize cold sand / profile green test

Silver sand was obtained from Stoel van Klaveren Bouwstoffen B.V., Zaandam, NL. 'Profile Green' refers to Profile Porous Ceramic Greens Grade (PROFILE Products LLC, Buffalo Grove, IL, US). 1 kg of sand mix was prepared from 813 g silver sand and 187 g tap water. 994 g of profile green mix was prepared from 211 g profile green, 500 g sand and 283 g water. Both substrates were cooled to 5°C before use.

Plastic food trays (18 x 14 x 6 cm, bamibak 137040, Buurman Facilitaire Producten, Oldenzaal, NL) were filled with 800 g sand or 750 g profile green mix. 50 maize seed were sown (pressed gently into the substrate) and covered with 700 g sand or 600 g of profile green mix. Lids (deksel 137048, Buurman) were placed on the trays, they were then incubated at 5°C for 7 days and then the lids were removed. The trays were then placed under 16 h light per day at 100% humidity for 4 days at 25°C, and seeds or seedlings were scored using the classification criteria outlined in Table 2.2. Humidity was maintained by placing multiple trays in a larger tray container (kweekbak favorite 46x31x8 cm) with a taller, transparent lid (doorz.kap favorite 46x31x8 cm) obtained from A. Mauritz & Zn. B.V., Bussum, NL. Temperature was maintained in a controlled environment room at Enkhuizen. A light intensity of approximately 3000 – 5000 lux was provided by multiple fluorescent lights (specification: 36 W or 58 W Cool White, colour temperature of 4000 K, colour rendering index $R_a \geq 80$).

Table 2.2. Classification of seedlings in cold sand tests or cold profile green mix tests. Seedlings were classified by the sub-classification criteria. Figures indicate the pooled classification unless sub-classifications are represented. Germinated seeds includes normal and abnormal classes.

Pooled-Classification	Sub-classification	Short name	Criteria
Normal	Normal	N	First leaf unrolled, second leaf visible
	Normal, small	S	First leaf partially unrolled, but second leaf not visible
Abnormal	Abnormally small	AbnS	First leaf not unrolled
	Abnormal else	AbnE	No root or shoot; deformity
Non-germinated	Non-germinated	NG	No radicle emergence

2.3.3 Maize cold paper test

43 cm x 27 cm cellulose paper (Item no.900914, Papierwerk Sundern GmBH, Germany) was wet with 500 ml of water or appropriate solution and cooled to 5°C or warmed to 25°C. 100 maize seed per tray (kweekbak favorite 46x31x8 cm) were sown embryo face up or embryo face down to wet parts of the paper (so that the side of the seed face down is partially submerged). Trays were incubated for the time periods and temperatures specified in the results section; germination was scored after 72 hours as outlined in Table 2.3. Trays were placed in polyethylene bags to maintain humidity and temperature was maintained in an incubator (12010-KK12006, IVL-van den Berg Klimaattechniek B.V., Wijk bij Duurstede, NL.)

2.3.4 Maize cold submergence test

4 l of tap water was added to a cylinder and temperature was maintained at 5°C or 25°C (within ± 1°C) using a liquid heated/cooled element submerged in the water. The water in the cylinder was aerated from the bottom of the cylinder with compressed air. An overview of this system is given in Figure 2.1. 50 maize seed were placed in nylon mesh bags and submerged in the water for time periods specified in the results section. Seeds were subsequently transferred to wet filter paper for germination. The trays were prepared with paper (same as cold paper test) with additional filter paper (TA-3, 41 x 27 cm, 25.1 g, F5.100.265, AllPaper B.V., Didam, NL.) placed on top and 650 ml tap water was used. Trays were warmed to 25°C. Seed were either dried (described below) before transfer, or transferred directly to trays. Germination was scored 72 hours after transfer as outlined in Table 2.3.

2.3.5 Maize seed drying

Seed were dried in nylon mesh bags for 24 hours at ambient temperature using an air drying cabinet built for Syngenta Seeds B.V., Enkhuizen, NL by Selecta Machinefabriek B.V., Enkhuizen, NL (Droogtafel 80-80, Serial No. 960801, 1996).

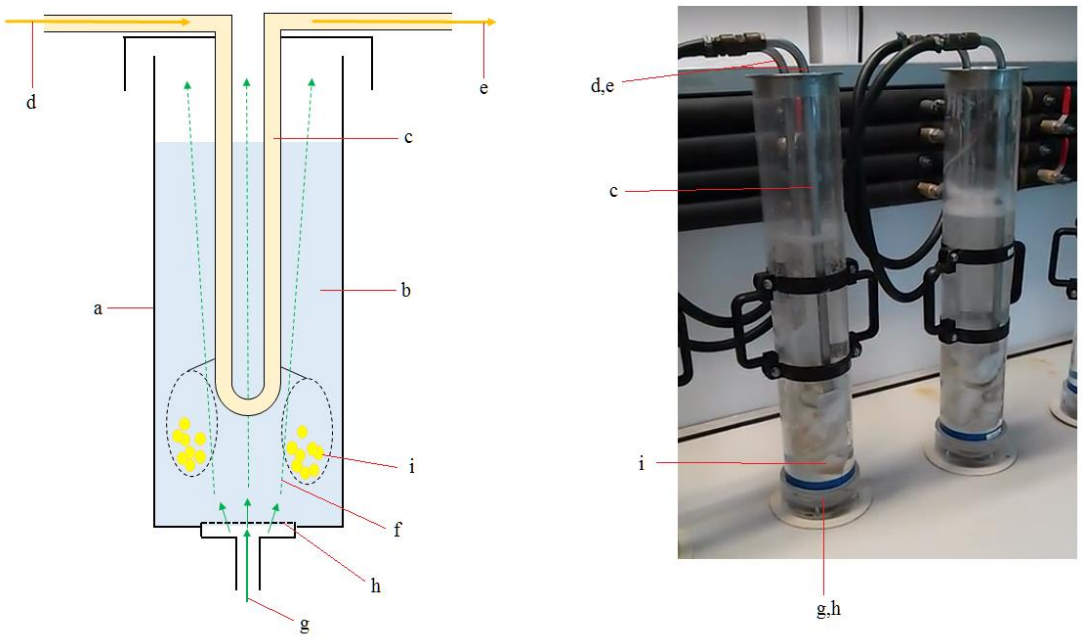

Figure 2.1. Maize submergence test system. In this system, a cylinder (a) of tap water (b) is cooled or heated by an element (c) whose temperature is regulated by a coolant liquid which is input from (d) and output to (e) a temperature regulator (K/W bad, B0205-15090-124, Boekens koeltechniek B.V., Enkhuizen, NL). Water is aerated with bubbles (f) of compressed air (g) via a sparger (h). 50 maize seed were placed in nylon mesh bags and attached to the cooling element with string to prevent movement (i). A schematic is shown on the left and a photograph on the right.

2.3.6 Maize cold vermiculite test

For 30 seeds per tray, mini seed trays (17 x 10 x 5 cm, G35CASE, LBS Worldwide Ltd.) were filled with 20 g of medium grade vermiculite (100 LTR VERMB, LBS Worldwide Ltd.) and 80 g of water or appropriate solution was added (20°C to 25°C) and mixed thoroughly. Alternatively, for 50 seed per tray, small seed trays (23 x 17 x 6 cm, G18BCASE, LBS Worldwide Ltd.) were used with 40 g of vermiculite and 160 g of water or solution. Humidity was maintained by placing trays in 9″ x 13″ medium duty re-sealable bags (Growtivation, Lancing, UK). Temperature was maintained in a Sanyo MLR 350 HT at the specified temperature.

Seed were classified to the criteria outlined in Table 2.3.

Table 2.3. Classification of germination in cold vermiculite and cold paper tests. Seeds or seedlings were classified to the criteria outlined below. Germinated includes both normal and abnormal classes.

Classification	Criteria
Normal	Radicle and shoot emerge and showed evidence of post-germination radicle growth
Abnormal	Shoot emergence and growth without radicle emergence; or radicle abortion (no growth) following emergence
Non-germinated	No radicle emergence or shoot growth was observed

2.3.7 Filter paper

For germination of tobacco and tomato on filter paper, 90 mm triple vented Petri dishes were used with 85 mm diameter Whatman Grade 1 filter paper (Sigma) wetted with 1.5 ml of the appropriate solution. Petri dishes were placed in an airtight box and humidity maintained by adding wet tissue paper to the box. Seeds were incubated at 25 ± 1°C in darkness. Radicle emergence was scored daily.

2.3.8 Imbibition in hydroxamic acid solutions

In experiments not containing hydroxamic acid C426, hydroxamic acid solutions of D2 and D4 were prepared from a 1 M stock in dimethylsulfoxide (DMSO, Sigma 472301), and diluted with water to a final concentration of 1 mM and 0.1% (v/v) DMSO. In experiments containing C426, C426 and D2 were prepared at 150 mM in DMSO and diluted to 150 or 40 µM with a final DMSO concentration of 0.1% (v/v). 0.1% (v/v) DMSO was used as a control.

2.3.9 Norflurazon

Agrochemical grade (80% w/w) norflurazon was obtained from Syngenta via Dr Ian Taylor (University of Nottingham). A stock solution was prepared at 5 mg ml^{-1}, which was subsequently diluted to the appropriate working concentration in the aqueous assay solution.

2.3.10 Seed treatment with solutions in acetone or dichloromethane

For soaking of maize seed, D2 or D4 were dissolved in acetone (Fisher A949) at 1 mM. 50 seeds were soaked in 15 ml of acetone with or without 1 mM D2 or D4 for 2 hours after which seed and solution were poured on to a 125 mm diameter Whatman Grade 1 filter paper and the acetone was allowed to evaporate overnight in a fume hood, before sowing.

For soaking of tomato seed, D4 was dissolved at 0 mM, 1 mM or 15 mM in acetone or dichloromethane. Tomato seed were soaked for 2 hours in 1.5 ml of the solution and then the solution and seeds were poured on to an 85 mm Whatman No.1 filter paper and allowed to dry overnight in a fume hood before sowing.

For application of ABA to maize seed, (±)-abscisic acid (Sigma A1049) was dissolved at 0 mM, 1 mM, 5 mM or 10 mM in acetone. The seed pericarp was pierced with a scalpel blade once on either side of the embryo axis (into the

scutellum). For each seed, 7.5 µl of ABA solution in acetone was pipetted on to the embryo face and acetone allowed to evaporate, and repeated once again for each seed. Seed were stored at ambient temperature (approximately 20°C) overnight before sowing.

2.3.11 *Seed treatment using Proseed coating with hydroxamic acid*

0 mg, 6.25 mg, 25 mg or 100 mg solid hydroxamic acid D2 was added to 4.430 mL water and 570 µl Proseed® (Syngenta), sonicated for 15 minutes, mixed by pipetting and then 250 µl of the mix was added to 50 seed. Seed were shaken by hand in a small container for 1 minute to provide an even coating.

2.3.12 Seed sterilisation

Tomato and tobacco seed were sterilised before sowing. 0.5% sodium hypochlorite was prepared by diluting 10% sodium hypochlorite (Sigma 71696) with water (v/v), and Tween 20 (Melford P1362) was added to final concentration of 0.1% (v/v). Seed were soaked in the 0.5% sodium hypochlorite and 0.1% Tween 20 solution for 20 minutes. Seed were then washed with sterile water.

2.4 Compound Screening Assay

2.4.1 Medium preparation

'½ MS' was prepared by dissolving 2.2 g Murashige and Skoog medium (Melford M0221) per litre of water and pH was adjusted to 5.7 using 1 M potassium hydroxide (KOH) (Fisher P250). Agar (Sigma A1296) was added to 0.2% or 0.6% (w/v) and the mixture was autoclaved. 0.2% agar was allowed to cool overnight with mixing and a predetermined mass of sterilised tobacco seed were suspended in a predetermined volume of 0.2% agar (see 2.3.12) to obtain a particular seed density in agar. Tobacco seed density in agar was based on the average seed mass and the mass of the total added seed. 1 M MES [2-(N-moprholino)ethanesulfonic acid] (Sigma 5287) was prepared in water and filtered through a 0.22 µM cellulose acetate syringe filter and added to the medium to a final concentration of 3.6 g l^{-1} (18.45 mM) in the 96-well plate assay.

2.4.2 Tetracycline

Tetracycline (Sigma T3383) was dissolved in DMSO at 10 mg l^{-1} and diluted in sterile water such that the lowest possible DMSO concentration was used in the assay. For example, in assays using a maximum tetracycline concentration of 0.8 mg

l^{-1}, a minimum additional final concentration of 0.008% DMSO was added to the assay. Control treatments were always designed to have the same DMSO concentration as test treatments.

2.4.3 Hydroxamic acid D4 addition

1 mM D4 in 0.1% DMSO was prepared from a 1 M D4 stock in DMSO. 1 mM D4 in combination with 0.8 mg l^{-1} tetracycline, resulted in a final DMSO concentration in of 0.108%. Control wells (0 mg l^{-1} tetracycline and/ or 0 mM D4) therefore also contained 0.108% DMSO. When MES supplemented medium was used to neutralise hydroxamic acids diluted in alkali, 1 M D4 was diluted to 10 mM in 50 mM KOH resulting in a final KOH concentration of 5 mM, which was also added to negative control (0 mM D4) assay wells.

2.4.4 Compound libraries

The 88 compound library was received from Syngenta Crop Protection Münchwilen AG (Stein), Switzerland. The 5 K compound library was received from Syngenta, Jealott's Hill, Berkshire, UK. All compounds were dissolved at 10,000 ppm in DMSO.

2.4.5 Growth conditions

The 5K compound library screen was performed under 1 Cool White fluorescent lamp per shelf providing 50 µmol m^{-2} s^{-1} photosynthetic photon flux density in a controlled temperature room (25±1°C), with 16 hours light per day.

2.4.6 Photography and Image analysis

Photographs were taken using a Nikon COOLPIX S8000 digital camera. For the 5K compound library screen, 96-well plates were placed on white paper and illuminated by ambient laboratory fluorescent light in a room without windows. The camera was in 'auto' mode with flash set to "off", exposure bias set to +1.0 step, and an ISO sensitivity of 100. Exposure time was ⅓ second or ¼ second and the f-stop was 4.7 with the camera 1 metre from the plate. Images were cropped to the top edges of the outermost wells on the microtitre plate and resized to 1200 pixels by 800 pixels. Image J (1.4.3.67 64-bit) (Schneider, *et al.*, 2012) was used to analyse leaf area. Green leaf area was identified using threshold colour (Hue 32 – 255, pass; Saturation 40 – 255, pass; Brightness 0 – 255 pass; invert, threshold). Leaf areas of each well were measured in pixels by using microarray profiler (http://www.optinav.com/

MicroArray_Profile.htm) using a grid of 8 rows and 12 columns of regions of interest, each with a diameter of 80 pixels.

2.5 Bioinformatics

2.5.1 Basic Local Alignment Search Tools

MaizeGDB BLAST tool was used to query the Maize B73 genome (B73 RefGen_v3) for genomic DNA sequences and to identify the position of sequence loci, and identify related annotated genome models using protein sequences (tblastn default values for 'highly similar sequences', E-value cutoff 1e-4). NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to query wider databases including genomic DNA, cDNA, expressed sequence tags (ESTs) (using default values).

2.5.2 Identification of Open Reading Frames (ORFs)

ORFs were identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequences were input in FASTA format and ORFs were predicted using standard genetic codes. ORF output sequences were saved in FASTA format.

2.5.3 Alignment of sequences

Sequences were aligned in Geneious version 6.12 (http://www.geneious.com) (Kearse, *et al.*, 2012) using Geneious or MUSCLE alignment with default values.

2.5.4 Production of phylogenetic trees

Phylogenetic trees were produced using Jukes-Cantor genetic distance model (Jukes and Cantor, 1969) and Neighbour-Joining tree method (Saitou and Nei, 1987) in Geneious using the default parameters.

2.5.5 Prediction of restriction sites, in silico cloning

Plasmid constructs were designed in Geneious using *in silico* prediction of primer products, restrictions sites and ligations.

2.5.6 Primer design

Primers were designed in Geneious using Primer3 (Untergasser, *et al.*, 2012). Appropriate maize *NCED* sequences were input as a custom mismatch library when designing primers for a specific maize *NCED*. The primers used for cloning *NCED*s (Chapter 4) and generating qPCR standards (Chapter 5) are given in their respective chapters. The sequences of primers used for measurement of *ZmNCED* expression

(Chapter 5 and Chapter 6) are given in Table 2.4. Other primer sequences are given in their respective results chapters.

Table 2.4. Primers used for qPCR. The following primers were used for qPCR in Chapter 5 and Chapter 6.

Target	Pair name	Direction	Sequence
ZmNCED1	ZmNCED1-1	F	CCGGCACGTCGGAGCTACTT
		R	GAGCTCCTGGCCCGTGATGA
	ZmNCED1-2	F	TTTCTCGGAGGAGGAACAGAG
		R	CAAAGCAAAGCAACTACCAACTG
	ZmNCED2-1	F	CAGCGGCCAACTCGGTCTTC
		R	TGCAGGAAGCTCAGGCCTTTCTC
ZmNCED2	ZmNCED2-2	F	CCTGATGATTCCTTGGTTGTTCT
		R	CTCAGGCTCCCTCTGGTAAC
	ZmNCED2-3	F	GGAAGAAGGCTGTTACCAGAG
		R	CAGAGATTGCATTGCATATGTTATGA
ZmNCED3a	ZmNCED3a-1	F	CCAGCTCGACACCGCCATGAT
		R	GCCGTCGGCGGTGAAGTAGA
	ZmNCED3a-2	F	CACCGTCGACAAGTTCATCTAC
		R	GCTAGCTACTGTATTGTGTGTGTA
ZmNCED3b	ZmNCED3b-1	F	GCAGCTGCTGGGCAGGAAGA
		R	TACCGGCCCTCGCCGTAGA
	ZmNCED3b-2	F	ACCTCACGTACATACACAACAGA
		R	TGTACTACGCAACACTAGCTGTA
ZmNCED9	ZmNCED9-1	F	CCGACCCAGCCGTGCAGAT
		R	CGCGTAGACGCCGCTGATGA
	ZmNCED9-2	F	GACTCAGCTCCACGTTTCTTG
		R	AAAGCAACTGTACAGCCTGTG
β-actin	β-actin	F	CGAACAACTGGTATTGTGATGGA
		R	GATCCAAACGGAGAATAGCATGA

2.6 Molecular Biology

2.6.1 Purification of DNA from plants

Leaves or roots of Maize B73 were frozen in liquid nitrogen and pulverised with a mortar and pestle. DNA was extracted from the powdered tissue using DNeasy plant mini kit (QIAGEN 69104) according to manufacturer's instructions.

2.6.2 Purification of DNA from bacterial cultures

Ampicillin resistant *E. coli* cultures were isolated on 1.2% agar containing 25 g l^{-1} LB broth (Melford GL1704), adjusted to pH 7.2 using 5 M sodium hydroxide

(NaOH) (Melford S8045) and 100 mg l^{-1} ampicillin (Sigma a9518). Liquid cultures (same as solid media above, but without addition of the 1.2% agar) were inoculated with *E. coli* and grown overnight at 37°C. DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN 27104) according to manufacturer's instructions.

2.6.3 Purification of DNA from agarose gel and PCR reactions

DNA fragments separated by agarose gel electrophoresis were purified using QIAquick Gel Extraction Kit (QIAGEN 28704) according to manufacturer's instructions. DNA was purified from PCR reactions using QIAquick PCR Purification Kit (QIAGEN 28104) according to manufacturer's instructions.

2.6.4 Purification of RNA from maize kernels

Maize embryo tissue was isolated from the kernel and frozen in liquid nitrogen, before pulverisation with pestle and mortar. RNA was purified from approximately 25 mg of tissue using RNeasy Plant Mini Kit (QIAGEN 74904). For one experiment (Figure 5.8) the NucleoSpin® RNA Plant kit (740949, Machery-Nagel GmBH & Co. KG, Düren, Germany) was used according to manufacturer's instructions with on-column DNase I digestion.

Alternatively a modified version of the hot-borate extraction protocol (Wan and Wilkins, 1994) was used. Whole maize kernel tissue was frozen in liquid nitrogen, before homogenisation in a coffee grinder (Bodum Bistro Electric Blade Coffee Grinder, obtained from Amazon EU SARL, London, UK) chilled with liquid nitrogen. Powder was further homogenised under liquid nitrogen using pestle and mortar. 160 mg polyvinyllpyrrolidone (Sigma PVP40) and 1.2 g tissue were added to 8 ml XT Buffer [0.2 M sodium tetraborate decahydrate (Sigma B3545); 30 mM EGTA (Melford E1102); 1% w/v sodium dodecyl sulphate (Melford B2008); 1% w/v sodium deoxycholate (Sigma D6750), adjusted to pH 9.0 using 5 M NaOH)] at 80°C and homogenised using an ultra-turrax (IKA-Werke GmBH & Co. KG, Staufen, Germany). 0.7 ml homogenate was added to 20 µl 20 mg ml^{-1} proteinase K (Melford MB2005) and incubated for 90 minutes at 42°C. 56 µl 2 M KCl was added and mixture incubated for 2 hours on ice; the material was then centrifuged at a relative centrifugal force (RCF) of 18,625 at 4°C, for 20 minutes. All subsequent centrifugation steps used the same parameters. 12 M lithium chloride (LiCl) (Melford L1351) was added to the supernatant at a final concentration of 2 M, and the mixture was incubated overnight at 4°C. Supernatant was removed and the pellet

washed with 250 µl 2 M LiCl and centrifuged, twice. The pellet was dissolved in 300 µl water and centrifuged. 30 µl 2M potassium acetate (Melford P0575) (pH 5.5 with acetic acid) was added and the mixture centrifuged. 990 µl ethanol was added and mixture incubated at -80°C for 1 hour. The mixture was centrifuged and the pellet was washed with 1 mL 70% ethanol. The pellet was dissolved in 40 µl water. 4 µl 3 M sodium acetate (Melford B4017) and 132 µl ethanol was added and the mixture was incubated for 1 hour at -80°C. The mixture was centrifuged and washed with 70% ethanol. The pellet was dried at ambient temperature and suspended in 50 µl water. All aqueous salt solutions used after the homogenisation step were treated with DEPC to destroy RNases.

2.6.5 DNase digestion of RNA preparations

RNA sample concentrations were adjusted to 180 ng μl^{-1} and any co-purifying DNA was digested using Recombinant DNase I (RNase-free) (AM2235, Life Technologies Ltd. Paisley, UK). The heat inactivation protocol was used, according to manufacturer's instructions resulting in 100 ng μl^{-1} RNA with 4 mM $MgCl_2$ and 4 mM EDTA. DEPC-H_2O was used for these steps.

2.6.6 Reverse-transcription quantitative PCR (RT-qPCR)

RT-qPCR was carried out with a one-step protocol (reverse transcription and qPCR performed in the same tube) using Rotor-Gene SYBR® Green PCR Kit (QIAGEN 204074), according to manufacturer's instructions with the cycling conditions outlined in Table 2.5.

RT-qPCR was also carried out with a two-step protocol. cDNA was synthesised using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (4368813, Life Technologies Ltd.) according to manufacturer's instructions with 1 µg of RNA per 20 µl reaction. The cDNA product was diluted ten-fold. qPCR was performed using QuantiTect SYBR® Green PCR Kit (QIAGEN 204143) with 2 µl diluted cDNA per 10 µl reaction.

For the above two qPCR methods, reaction master-mixes were prepared by hand and added and mixed with template using the QIAgility (QIAGEN) and Rotor-Disc 100 (QIAGEN 9001603). Temperature cycling and real-time measurements were performed using the Rotor-Gene Q (QIAGEN 9001580) and analysed using the Rotor-Gene Q Software (2.3.1.49).

Table 2.5. PCR cycling conditions. Temperatures (*T*) and times (*t*) for initial denaturation (ID), denaturation (D), annealing (A), extension (E) and final extension (FE) and the number of D-A-E cycles (C). a, was used for cloning (Chapter 4); b was used for generation of qPCR standards (Chapter 5); c and d were used for qPCR (Chapter 5 and Chapter 6). Temperature gradients for annealing are indicated e.g. 55/72 corresponds to a gradient of 55°C to 72°C. M indicates a melt curve (0.5°C steps from 65°C to 95°C). N/A in annealing temperature indicates two-step protocol with combined annealing and extension step.

	Enzyme	*T*(°C)					*t*(s)					C
		ID	D	A	E	FE	ID	D	A	E	FE	
a	Hotstar HF	95	94	55/72	72	72	300	15	60	120	600	41
b	Hotstar HF	95	94	50/68	72	72	300	15	60	60	600	41
c	Rotorgene SYBR	95	95	N/A	60	M	300	5	N/A	10	M	40
d	Quantitect SYBR	95	94	50	72	M	900	15	30	30	M	40

2.6.7 End-point polymerase chain reaction (PCR)

PCR using HotStar HiFidelity Polymerase Kit (QIAGEN 202602) was carried out according to manufacturers' instructions unless stated otherwise (e.g. alterations to primer or template concentration). Cycling conditions, based on the manufacturers' recommendations, with varied annealing temperatures, are outlined in Table 2.5. Primers were diluted in TE buffer (Sambrook, *et al.*, 1989).

2.6.8 Transcription

Transcription of *ZmNCED* PCR products was performed using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB E2040S) according to manufacturer's instructions, with each 20 µl reaction containing 2 µl of undiluted PCR product. Transcription products were precipitated by addition of LiCl to a final concentration of 2.2 M and cooled at -80°C for 30 minutes. The sample was centrifuged (RCF of 18625 at 4°C for 20 minutes) and the pellet was dissolved in 50 µl of DEPC-treated water.

2.6.9 Restriction digestion

Restriction digestions were performed with *Bam*HI (NEB R0136), *Eco*RI (NEB R0101), *Pst*I (NEB R0140) and *Xho*I (NEB R0146) according to manufacturer's instructions.

2.6.10 Ligation and transformation

Ligation of *ZmNCED* fragments and pGEX-4T-1 (GE Healthcare UK Ltd., Little Chalfont, UK) were performed using T4 DNA Ligase (NEB M0202) according to manufacturer's instructions. NEB Turbo Competent *E. coli* (High Efficiency) (NEB

C2984) were transformed with ligation products according to manufacturer's instructions.

2.6.11 Sequencing of DNA

Purified plasmid DNA was sequenced by Source BioScience, Nottingham, UK, (sanger-sequencing service) using pGEX 5′ (GGGCTGGCAAGCCACGTTTGGTG) and pGEX 3′ (CCGGGAGCTGCATGTGTCAGAGG) primers. Chromatograms were aligned and compared with *in silico* constructs in Geneious.

2.6.12 Determination of nucleic acid concentration

Concentrations of double stranded-DNA (dsDNA) and single stranded RNA (ssRNA) were measured using Picodrop Pico100 (Picodrop Ltd., Cambridge, UK).

2.6.13 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out according to the recommendations of Sambrook et al., 1989. Staining was performed with SafeView Nucleic Acid Stain (NBS-SV1, NBS Biologicals, Huntingdon, UK) (5 µl / 100 ml agarose gel). 1 kb DNA Ladder (NEB N3232), 100 bp DNA Ladder (NEB N3231), or GeneRuler 1kb DNA ladder (Fisher SM0331) were used for size comparison.

2.6.14 Formaldehyde – agarose gel electrophoresis

Running buffer contained 5.23 g l^{-1} MOPS (Melford B2003) and 0.186 g l^{-1} EDTA (Melford E0511) (pH 7.0 with 5 M NaOH). A 1.4% w/v agarose (Melford MB1200) gel was prepared in running buffer plus 6.67% formaldehyde (Sigma F8775). Samples were denatured in running buffer plus 47.5% formamide (Sigma F9037), 6.8% formaldehyde, 33 g l^{-1} sucrose, 4 g l^{-1} bromocresol blue and 4 g l^{-1} xylene cyanol. Samples were denatured at 70°C for 10 minutes. Gel was equilibrated with running buffer for 30 minutes before samples were loaded. Gel was stained with 2.5 mg l^{-1} ethidium bromide and washed with water overnight at 4°C before visualisation. ssRNA Ladder (NEB N0362) was used for fragment size comparison.

2.7 Statistical Analyses

Statistical analysis of experiments was performed using GenStat 13.3 (VSN International Ltd.), or SPSS Statistics Version 22 (IBM) primarily using analysis of variance (ANOVA) procedures. Experimental design, treatment structure and replication are indicated in the respective chapters and appendices. Untransformed data for tobacco leaf area did not have a normal distribution, and square-root

transformation of data prior to analysis was used to increase data normality and reduce the mean-variance relationship. Seed germination percentage data was also angular transformed (arcsin(√x)) to increase normality and decrease mean-variance relationship. Multiple comparisons were performed for Chapters 3 and 6 using Tukey's honest significant difference test (Tukey's HSD) in SPSS. Multiple Student's *t*-test comparisons in Chapter 7 were performed in Microsoft Excel and adjusted to control the false discovery rate (FDR) at 5% using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3 Germination of Maize in Cold Conditions

3.1 Introduction

3.1.1 A tropical plant cultivated at high latitudes

Cultivated maize (*Zea mays* subsp. *mays* L.) likely resulted from domestication of a wild relative, *Zea mays* subsp. *parviglumis*, in Mexico circa 9000 years ago, based on biological and archaeological studies. However, its origin is still discussed (Doebley, 1990; Matsuoka, *et al.*, 2002; Piperno, *et al.*, 2009; van Heerwaarden, *et al.*, 2011). Nonetheless maize is a crop with tropical origins and is now cultivated at much higher latitudes in temperate climates, and thus is exposed to diverse environments (Figure 3.1).

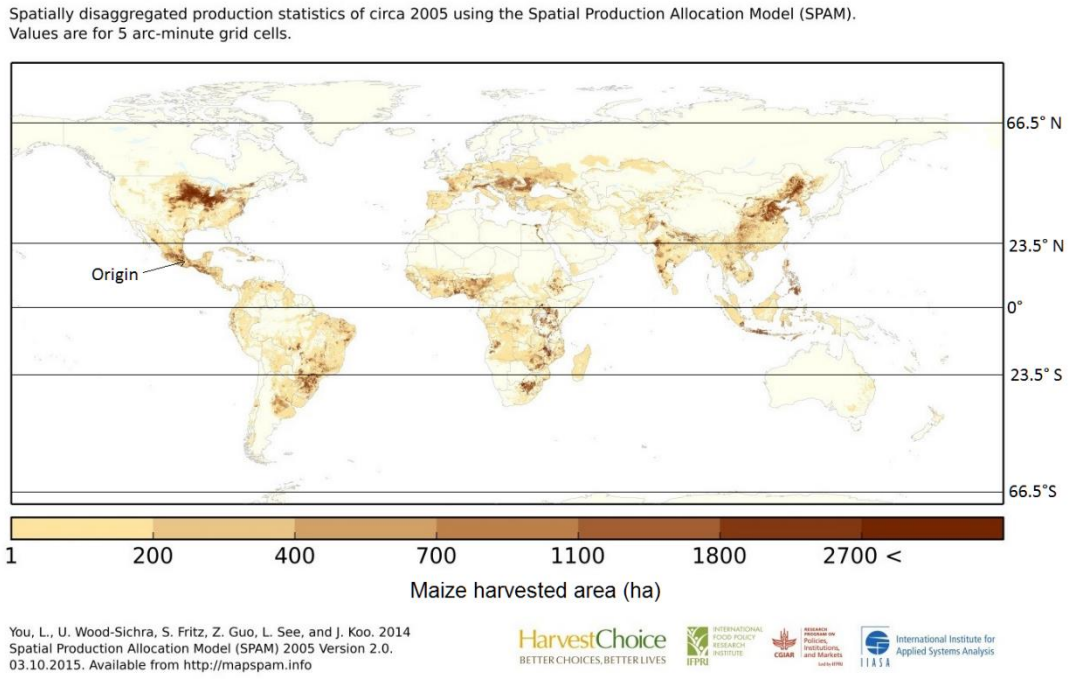

Figure 3.1. Worldwide cultivation of *Zea mays*. Map represents the harvested area of maize (indicated by color code in hectares) in different parts of the world – from the tropical origin to subtropical and temperate latitudes (You L., *et al.* 2005).

Since the growing season is shorter at higher latitudes, sowing early in the season can be more favourable. Early sowing will mean that plants are harvested before the end of the season, when cold (i.e. frosts) and wet weather is more likely. This can avoid damage to the crop and may also reduce the costs of drying the harvested seed (Lauer, *et al.*, 1999). Additionally if sowing occurs earlier, grain filling can occur during longer days, which may increase yield (Cirilo and Andrade, 1994; Otegui, *et al.*, 1996). It is also possible that early sowing may avoid the sensitive pollination

stage coinciding with drought conditions in summer that can reduce yield in rain-fed crops (Campos, *et al.*, 2004; Grassini, *et al.*, 2009; Otegui, *et al.*, 1996). Indeed adoption of earlier sowing and long-season hybrids is occurring and appears to be increasing maize yield (Kucharik, 2006; Kucharik, 2008). Despite potentially increasing yields, earlier sowing does however mean that maize is more likely to encounter suboptimal temperatures (and frosts) in temperate climates during germination and emergence leading to crop loss and unpredictable yields (Otegui, *et al.*, 1996). It is possible for farmers to re-sow maize if plant loss occurs early in the season, but only as a damage limitation strategy as replanting inevitably reduces the profit margin and reduces the growing season for the re-sown crop (Nafziger, 2009).

3.1.2 Germination and emergence of maize in cold conditions

Suboptimal temperatures can prevent or retard germination and emergence leading to both a reduction in crop population and a decrease in uniformity. The effect of temperature on *Zea mays* germination is outlined in Figure 3.2. It is apparent that for this cultivar the minimum temperature required for germination to occur is above 6°C, and germination rate increases to the optimum of between 32°C and 38°C and the maximum temperature that allows germination is above 40°C. However, the minimum temperature for root elongation is above 8°C, with an optimum temperature of 32°C. Increasing temperature above this optimum leads to a large decrease in pre-emergence shoot elongation which is halted at 40°C. Rate of root elongation also reflects the pattern seen in shoot elongation (Blacklow, 1972; Erickson, 1959). Low temperature therefore can slow emergence, which is upwards penetration of the coleoptile through the soil, at the germination and post-germination stages. Because seeds within a lot may vary in the thermal time required to complete germination, this can subsequently lead to a spread of emergence times, which will result in a lack of uniformity in the crop stand (Alvarado and Bradford, 2005; Bradford, 2002). Time to emergence, which is increased by low temperatures, correlates with a lack of uniformity in corn seedling emergence. Low vigour seed lots that exhibit lower germination and emergence rates (i.e. time to 10% emergence) have an additional lack of germination uniformity (Egli and Rucker, 2012). As hybrid maize yield is dependent on establishing dense and uniform crop populations, lack of germination and varying rates of emergence are detrimental (Andrade and Abbate, 2005; Liu, *et al.*, 2004; Tokatlidis and Koutroubas, 2004). This is because

the maize plants that do germinate are not able to 'fill the gaps' of those that do not (i.e. maize does not tiller as much as other grasses (Doebley, *et al.*, 1997)) and larger plants do not fill in the yield gap caused by outcompeting smaller plants. Therefore there is great interest in maize cultivars that, in combination with seed technologies, exhibit high percentage and uniform emergence even under cold conditions.

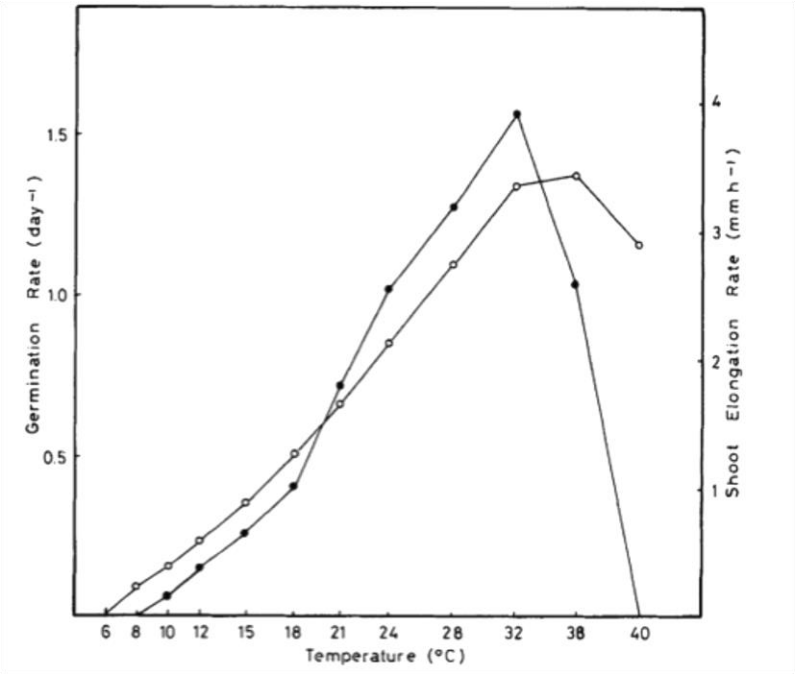

Figure 3.2. *Zea mays* germination at low temperatures. Effect of temperature on rate of germination (○) and shoot elongation (●) (before emergence) in *Zea mays* cv. Fronica (taken from Miedema, 1982).

3.1.3 Tests of seed germination and seedling vigour

To be fit for sale, seed lots must be capable of adequate germination and seedling establishment (e.g. in terms of uniformity). Minimum germination requirements are sometimes enforced by national governments requiring seed to be submitted to authorised testing stations and approved before seed are marketed. For example in the UK, maize seed lots generally must be able to exhibit at least 90% germination according The Seed Marketing Regulations 2011 SI 2011/643 which enforces EU regulations (European Commission Council Directive 66/402/EEC). Meanwhile sometimes it is only necessary to accurately indicate the tested germination capacity, as is federal law in the U.S. for agricultural seeds according the Federal Seed Act of 1939 (amended 1998), although state law may add to these minimum requirements. For this purpose, tests of germination under optimal conditions are used. For example a standard (warm) germination test in maize may be performed by imbibing

seed on top of paper, between paper sheets or in sand at 20°C, 25°C or alternating between 20°C and 30°C. Seeds and seedlings are classified after 4 and 7 days (ISTA, 2015c). The test reflects the viability of seed in a seed lot, i.e. the proportion of seeds that are able to germinate under optimal conditions. Viability may also be determined through the tetrazolium test in which tetrazolium chloride is reduced to a red-staining compound by the dehydrogenase activity associated with living tissue (ISTA, 2015b).

However such tests may not reflect field performance as conditions are often not optimal during sowing. Thus alternative tests (i.e. 'vigour tests') are often employed in industrial practice to better estimate the field performance of maize seed lots. Such tests often impose a stress to the seed prior to or during germination. Standardisation of such tests has been sought after by organisations such as the Association of Official Seed Analysis (AOSA) or the International Seed Testing Association (ISTA), who run accreditation and certification schemes which are aimed to guarantee rigour in labs and their analyses. Nonetheless alternative and varied methods and procedures may be used for 'in-house' testing by seed companies for research purposes.

The general test types used to assess maize germination and vigour are as follows:

Cool test: Maize seed are imbibed continuously at suboptimal temperatures (e.g. between 10°C and 20°C) to reflect the soil temperature during spring-sowing. An ISTA approved test for maize germination involves imbibition of seed on paper towels at 20°C or 13°C and counting radicle emergence at 66 hours or 144 hours, respectively (ISTA, 2015a).

Cold test: Maize seed are imbibed for a period of time in suboptimal (or non-permissive) conditions and then transferred to optimal temperatures after which germination is assessed. Typically seeds are imbibed for 7 days at 10°C and then transferred to 25°C (sometimes under light) and germination or emergence is assessed. Seed may be imbibed on paper, or sown in a moist substrate such as soil or sand. The latter substrate allows for emergence to be scored. Germination is often compared to seed imbibed under optimal conditions without the prior cold imbibition (i.e. a warm test). A variation of this test is the 'saturated cold test' in which the moisture content of the medium is increased. These conditions reflect the cold and

wet conditions which could occur during spring sowing. ISTA does not have a validated test for this (ISTA, 2015a). AOSA have protocols for cold tests (AOSA, 2015) and AOSA member seed testing labs may offer different cold test types (ISU-Seedlab, 2015; OSU-Seedlab, 2015). Cold tests may simulate spring filed conditions by imbibing seed in wet soils, above 70% field capacity, with seven days of cold (5°C to 10°C) for a period (e.g. 7 to 14 days); seed are subsequently transferred to favourable germination conditions (25°C) (OSU-Seedlab, 2015). Various cold test like methods are evident in the literature, discussed below.

Accelerated ageing test: Seed are typically subject to a high temperature and high relative humidity for a short period (e.g. 45°C, 100% relative humidity for 3 days) before being tested for germination (in a warm test). Low-vigour seed lots are more likely to result in reduced warm test germination following this treatment. ISTA has a validated test for *Glycine max* but not maize (ISTA, 2015a)

Soak test: Seed are soaked in water for a duration of time, usually 24 to 48 hours, at 25°C, before being transferred to optimal germination conditions (e.g. Maree, *et al.*, 2007). Variations in soak time and temperature may be used (Saab, 2014). This may reflect the cold, saturated conditions that occur in the field following early spring soils (Saab, 2014).

Conductivity: Seed are soaked in water for a short duration (typically for 24 hours at 25°C) and the conductivity of the soak water is subsequently measured to estimate the electrolyte leakage from seed. Low-vigour seed / seed lots generally exhibit higher electrolyte leakage and therefore higher conductivity is measured in the soak water. A validated ISTA conductivity test protocol is available for multiple species, but not maize (ISTA, 2015a).

Many studies have examined the correlation between germination or vigour scores in vigour tests and the actual field performance of seed lots. It is often found that the standard germination test (i.e. warm test) is not a strong indicator of field emergence, especially if field conditions are suboptimal and if seed lots with high standard germination (<90%) are used (Ilbi, *et al.*, 2009; Lovato, *et al.*, 2005; Noli, *et al.*, 2010; Noli, *et al.*, 2008; Woltz and TeKrony, 2001). However other attributes measured in warm test conditions, such as germination rate, root and shoot length can sometimes correlate to cool, wet field conditions in the case of artificially aged

seed and seed lots with below 90% standard germination scores (Van De Venter and Lock, 1991).

The cold test, and its variants, have been used extensively to estimate the vigour of maize seed lots. The cold test is a good predictor of field emergence, especially if field conditions are stressful, and is usually better than the standard germination test in predicting field emergence (Ilbi, *et al.*, 2009; Lovato, *et al.*, 2005; Martin, *et al.*, 1988; Noli, *et al.*, 2010; Noli, *et al.*, 2008; Woltz and TeKrony, 2001). Variations in the cold test can significantly influence the result, for example, altering the temperature or duration of the cold period, and the substrate and moisture content of the germination medium (Lovato, *et al.*, 2005; Nijenstein and Kruse, 2000; Woltz, *et al.*, 1998). The water potential of a soil medium affects the result of the cold test, with lower water potentials (-1000 kPa) resulting in increased germination compared to high water potentials (-30 kPa) during the cold test. Treatment of the seed with fungicide or sterilisation of the soil medium reduced, or eliminated the effect of water potential, respectively, suggesting that the effect of moisture was due to pathogen activity (Woltz, *et al.*, 1998). In this study, cold period temperatures of 4.5°C and 13°C increased subsequent germination compared to 9°C or 10°C, and this effect was also negated when sterilised soil was used, indicating that the negative effect of pathogens on corn germination in the cold test is dependent on temperature and water potential (Woltz, *et al.*, 1998). However, in sterile sand, reducing the temperature during the cold period can reduce germination, and potentially allow for the discrimination of vigour in more cold-tolerant seed lots (Lovato, *et al.*, 2005). Indeed, inconsistencies in cold test practices can lead to variability in results between different labs (Nijenstein and Kruse, 2000). It has been suggested that multiple standard cold tests should be devised in order to have appropriate for multiple climates (Nijenstein and Kruse, 2000).

Accelerated ageing is also an extensively used vigour test, and appears to give a better indication of field performance than the standard germination tests, again perhaps more so when field conditions are stressful (Lovato, *et al.*, 2005; Noli, *et al.*, 2008; Van De Venter and Lock, 1991; Woltz and TeKrony, 2001). However, the vigour of seed lots may not be ranked the same by the accelerated ageing and the cold test. This may be because the accelerated ageing test imposes a different stress than that of the cold test. Whilst both tests may correlate well to field conditions,

they may correlate less to each other. In addition aged seed seem more susceptible to reduced germination following the accelerated ageing test compared to the cold test (Noli, *et al.*, 2008; Woltz and TeKrony, 2001).

The cool germination test can also be a good predictor of field emergence, and like the cold test, variations of this protocol exist. In one study cool germination was concluded to be the best overall predictor of field emergence (Van De Venter and Lock, 1991), although in other studies the cold test was deemed slightly more superior in terms of predicting field emergence (Ilbi, *et al.*, 2009; Noli, *et al.*, 2010). However, the temperatures used vary between studies, for example in Ibli *et al*., 2009 the best temperature was found to be 18°C (compared to 12°C and 15°C), but in Noli *et al.*, 2010 only 13°C is used. However, this method may be favoured as it could be less time consuming and labour intensive than the cold test.

Measurements of radicle emergence were found to correlate to cold test emergence and emergence time. As such calculation of mean germination times on moist towels at 13°C was proposed as an alternative indicator of vigour (Matthews and Hosseini, 2006). This may be generally less labour intensive than the cold test, although frequent seed counts need to be made. However single, early counts were proposed as an estimation of mean germination time, and as an alternative cool germination vigour test (Matthews, *et al.*, 2010), and this has recently been accepted as an ISTA protocol (ISTA, 2015b). The protocol involves measurement of radicle emergence on a paper towel medium at 20°C, 66 hours after sowing, and at 13°C, 144 hours after sowing. There is also interest in automating this procedure using imaging techniques (Matthews and Powell, 2011).

Such tests are useful for both identifying maize varieties that produce high vigour seed lots and seed technologies which may improve germination and vigour in cold germination conditions. For example, a study of reciprocal maize hybrids of inbred lines divergent in cold test performance was used to identify genes with potential roles in maize cold germination (Kollipara, *et al.*, 2002). Indeed such treatments have long been used to identify lines with cold hardiness and used for breeding programmes (Haskell and Singleton, 1949; McConnell and Gardner, 1979). A better understanding of the mode of action of cold stress in maize germination, and reproducible laboratory tests which reflect field performance will aid in future

selection of seed varieties, seed lot testing, and development of seed technologies that will improve maize germination in the cold.

3.1.4 The need for novel seed treatment technology in maize

Whilst many modern maize varieties have been bred for cold tolerance and the ability to germinate and emerge in suboptimal conditions, seed quality can still be affected during production. For example, suboptimal environmental conditions during the seed maturation period, such as frost can adversely affect maize seed germinability and vigour (DeVries, *et al.*, 2007; Rossman, 1949; Woltz, *et al.*, 2006). Other environmental variations such as high temperatures and drought may also affect germinability and vigour of maize seed (Goggi, *et al.*, 2007; Tekrony and Hunter, 1995). Indeed, there are a plethora of criteria (from the growth of the parent plants through to harvesting and storage) which may affect production of high vigour maize seed, and it is probably impossible to guarantee only high vigour seed are produced. Even within one ear, seed characteristics can be heterogeneous, for example, each maize ear contains seed with different shapes and sizes which can determine germinability and vigour (Graven and Carter, 1990; Msuya and Stefano, 2010; Muchena and Grogan, 1977). Seed can be separated based on such physical properties to obtain seed lots with more uniform germination characteristics, potentially leading to pools of higher quality seed and lower quality seed. Therefore there is interest in seed treatments which increase the germinability and vigour of poorly performing seed lots which might not otherwise be marketable.

3.1.4.1 Priming

Priming, as previously discussed in the main introduction (Section 1.6.2), involves controlled imbibition, allowing activation of the seed metabolism, but halting imbibition before radicle emergence occurs and often drying the seed whilst desiccation tolerance is still present. This leads to a reduced germination time and increased germination uniformity upon full imbibition. Primed seed also often exhibit reduced storability and have stricter storage requirements (e.g. refrigeration). For example in maize, soaking seed prior to sowing can increase the speed and final percentage germination under certain conditions, but can perturb the optimum conditions and reduce germination rate and performance under more extreme conditions (e.g. high temperatures, hypoxia) (Finch-Savage, *et al.*, 2004). Hydropriming did not improve maize germination under cool conditions, but did

yield some improvements in vigour characteristics (such as increasing final percentage emergence and decreasing the time to 50% emergence), and can improve germination characteristics at optimum temperatures (Dezfuli, *et al.*, 2008). Halopriming (seed imbibed in salt solutions, in this case $CaCl_2$) further increased the effectiveness of the priming treatment and increased the final percentage germination and emergence. In addition this treatment decreased germination and emergence time amongst other positive effects under cool conditions (Afzal, *et al.*, 2008). Similar treatments can also improve germination under salt stress (Ashraf and Rauf, 2001). Maize seed primed by soaking in the presence of chitosan exhibited slightly decreased mean germination times and produced more cold tolerant seedlings (Guan, *et al.*, 2009), although the lack of a large effect on germination may be due to germination at relatively optimal temperatures (25°C). Primed maize seed can be stored in the dried state, however seed longevity may still be reduced and have requirements for refrigeration (Chiu, *et al.*, 2002).

3.1.4.2 Chemical seed treatments

Maize seed are commonly treated with fungicides and insecticides, the former of which can particularly improve germination and emergence in field conditions and cool temperatures where maize is susceptible to fungal attack (Falloon, 1982; Woltz, *et al.*, 1998). Plant hormone treatments, and other biologically active compounds, aimed at increasing the growth potential can also be utilised, and in maize can be applied during seed priming. For example treatment of seed with GA_3, indole acetic acid or ascorbate can lead to additional acceleration of germination in combination with hydropriming (Afzal, *et al.*, 2008). Potassium nitrate priming was also able to alleviate the effects of drought and salinity stress on germination (Anosheh, *et al.*, 2011). GA_3 was found to be a more effective than kinetin in accelerating seedling emergence at 10°C with seedlings exhibiting greatly increased shoot length (Wang, *et al.*, 1996). However this elongated, 'spindly', phenotype is often an undesirable result of GA_3 stimulated germination particularly as it may lead to lodging (Stowe and Yamaki, 1959). In many of these seed treatment cases, seed have been imbibed in chemical solutions meaning seed would require drying for storage (i.e. a priming method would be required) or on-farm treatment would be required before sowing. Other methods of chemical application are often used, such as film coating and seed pelleting (Leubner, 2005). These can be used to allow the release of chemical

treatments upon imbibition of the seed, such that the seed treatment has little effect on seed storability. For example, a film coating which allows quicker release of salicylic acid under cold conditions, which allowed quicker germination and seedling emergence under cold conditions (Guan, *et al.*, 2015), although salicylic acid is not always associated with increased germination and seedling emergence in maize (Guan and Scandalios, 1995).

3.1.5 Roles of ABA in maize germination

As previously discussed in the introduction (Section 1.4) ABA has roles in inhibiting germination during dormancy establishment and maintenance. The role of ABA in maintaining primary dormancy is well studied in maize and many viviparous mutants are ABA deficient, such as *vp14*, which has mutation in an *NCED* gene required for ABA biosynthesis (Schwartz, *et al.*, 1997; Tan, *et al.*, 1997). Whilst not studied in depth in maize, it is known that ABA signalling can inhibit germination of barley under hypoxia and high-temperature, although hypoxia is regulated more so by gibberellic acid (Benech-Arnold, *et al.*, 2006; Hoang, *et al.*, 2013; Leymarie, *et al.*, 2008). As outlined in the main introduction, inhibition of ABA biosynthesis can accelerate germination, particularly in physiologically dormant seeds or seeds germinating under suboptimal conditions. Inhibition of ABA biosynthesis using hydroxamic acid inhibitors of NCED may uncover roles for abscisic acid in maize germination.

3.1.6 Aim and Objectives of this Chapter

The aim of this chapter was to investigate cold conditions that inhibit maize germination and emergence and the role ABA in these conditions.

Objectives

- Investigate the effect of cold tests, on germination of seed lots varying in vigour
- Investigate the role of abscisic acid on maize seed germination through seed treatment with hydroxamic acid CCD inhibitors
- Identify conditions under which *NCED* expression and ABA concentration can be measured

3.2 Cold Inhibition of Germination and Emergence

3.2.1 Cold imbibition on paper

10 seed lots from 5 varieties were imbibed on wet cellulose for 7days at 10°C and then 3 days at 25°C ('10°C cold test', used as an indicator of vigour) (Figure 3.3). ANOVA revealed normal germination (p = 0.002), abnormal germination (p = 0.001) and non-germination (p = 0.025) percentages were significantly affected by variety (Appendix 3.1). The seed lots varied significantly in normal germination (p < 0.001), abnormal germination (p < 0.001) and non-germination (p = 0.003) percentages. For example, Variety 1 seed lot A (1A) had significantly lower (p < 0.05) germination than Variety 1 seed lot B (1B) (Figure 3.3). This may have been due to the significant increase in abnormal seedling percentage observed in 1A. Similar within-variety differences in germination percentage were observed in Variety 4 and Variety 5, the latter also exhibiting within-variety differences in non-germination percentage (Figure 3.3). Thus the 10°C cold test indicated that there may be vigour differences amongst seed lots, including within-variety differences.

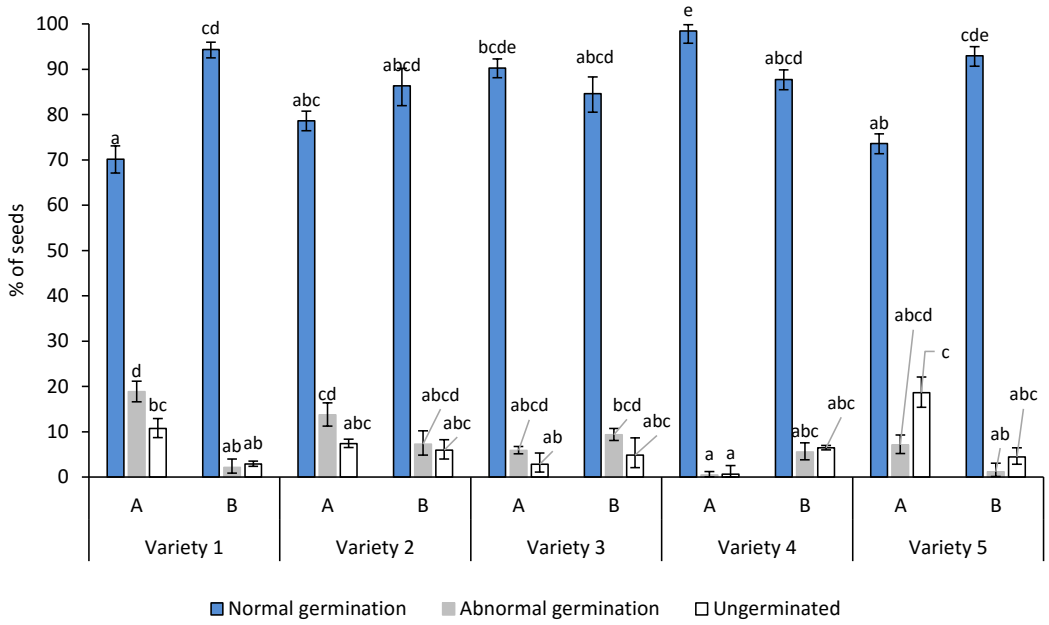

Figure 3.3. Performance of 10 maize seed lots subsequent to 7 days at 10°C. 5 varieties of maize seed, each with two seed lots, were germinated at 25°C following 7 days at 10°C on wet paper. Seeds were scored as having germinated, as abnormal, or non-germinated after 3 days at 25°C. Error bars indicate standard error (n = 4 * 50 seeds) (Data provided by Syngenta). Bars with no matching letters have significantly mean percentages for each category (Tukey's HSD, p < 0.05).It was hypothesised that reducing the temperature of the cold imbibition step to 5°C may further reduce germination in low vigour seed lots, and allow discrimination between high and low vigour seed lots. Furthermore, test duration was considered, as it was

though that a short duration, 2 days, at 5°C might allow quick discrimination between high and low vigour seed lots. Seed orientation was considered, as the orientation of the embryo (down, or up) may affect germination (Finch-Savage, *et al.*, 2004). The effect of 2 or 7 days imbibition at 5°C, and embryo orientation on subsequent germination of four seed lots (two varieties) at 25°C was tested on paper (Figure 3.4).

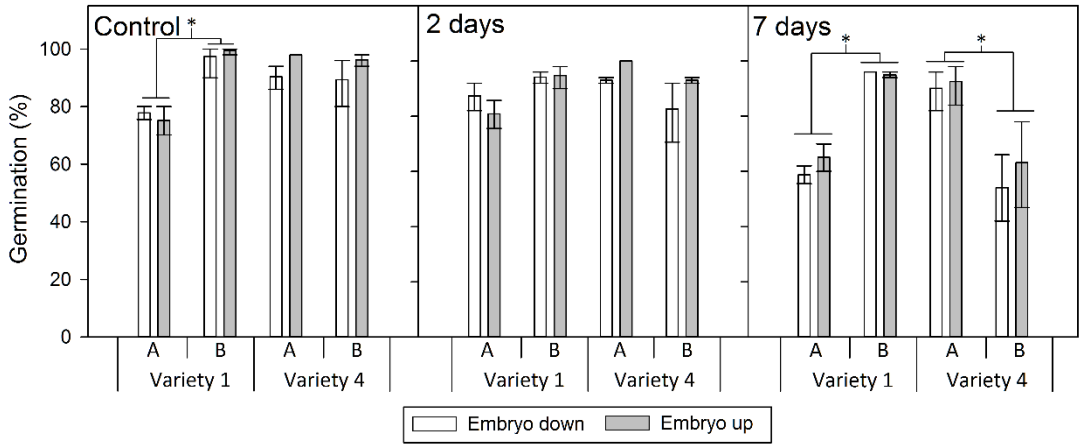

Figure 3.4. Germination of maize seed lots at 25°C subsequent to 2 and 7 days at 5°C. 2 varieties of maize seed, each with two seed lots, were germinated at 25°C following 2 or 7 days at 5°C on wet paper, or without cold pre-treatment (control). Germination was scored after 3 days at 25°C. Back-transformed means of transformed data (arcsin(√x)) are represented. Error bars indicate standard error (n=2 * 50 seeds for each treatment combination).*indicate significance between two seed lots according to Tukey's HSD, $p < 0.05$ (Appendix 3.2).

ANOVA indicated a large effect of seed lot ($p < 0.001$), effects of cold-pre-treatment duration ($p = 0.020$), as well as potential interactions between variety and embryo orientation ($p = 0.034$) (Appendix 3.2). Compared to direct germination at 25°C (control), germination at 25°C following 7 days at 5°C significantly reduced (Tukey's HSD, $p < 0.05$) germination percentage of Variety 4 lot B (4B) by 35% but not Variety 4 lot A. Following 7 days at 5°C, lot 4B germinated significantly less (36%) than Variety 4, lot A (4A) (Figure 3.4). Taken together these results indicated that lot 4A had higher vigour than lot 4B. After 7 days at 5°C, 1A also germinated less than 1B. However the same trend is present without a prior cold treatment (Figure 3.4). This suggested that lot 1A may have had low seed viability, i.e. inability to germinate even under favourable conditions, rather than low vigour. The germination percentages of seed lots 1B and 4A did not differ significantly between temperature regimes indicating 1B was a seed lot with similar vigour to 4A. This experiment indicated that there are two high vigour seed lots (1B and 4A), a potentially low viability seed lot (1A) and a low vigour seed lot (4B).

Due to potential embryo orientation effects, i.e. the variety-orientation interaction (p = 0.034) which showed that the embryo down orientation decreased germination in Variety 4 by 11% compared to embryo up (Appendix 3.2), embryo orientation was controlled in further experiments. The cold test duration of seven days at 5°C was adequate for discriminating between the vigour of Variety 4 seed lots.

3.2.2 Cold soaking

An alternative method of cold imbibition, soaking seed in cold, aerated water was investigated. Seed lots from varieties 1 and 4, as used in Figure 3.4, were soaked in aerated water at 5°C (or at 25°C) for up to seven days before being transferred to wet filter paper at 25°C and scored for germination three days later (Figure 3.5).

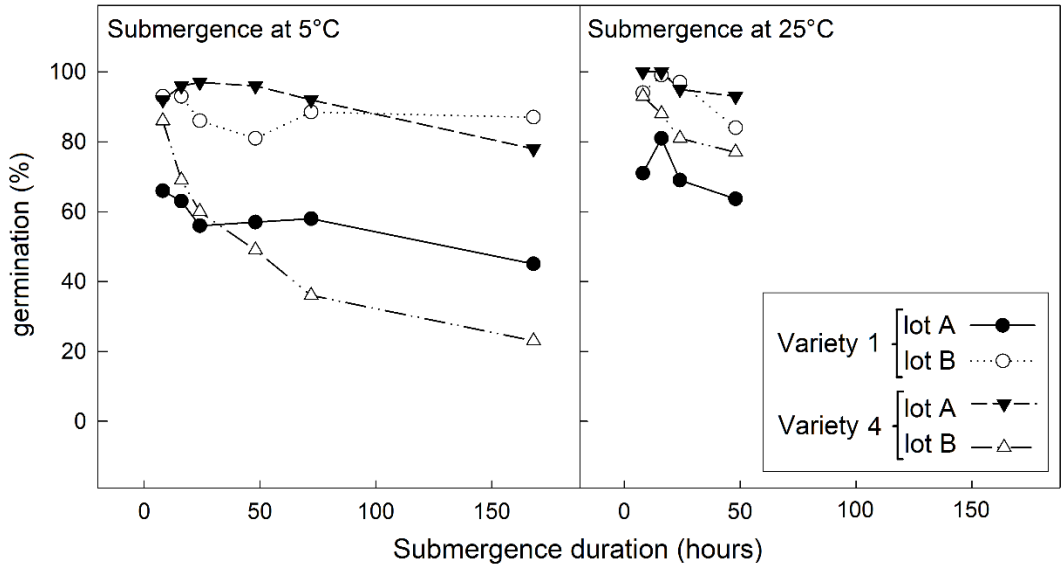

Figure 3.5. Germination of maize seed lots at 25°C subsequent to soaking at 5°C and 25°C for varied periods of time. Seed were submerged in aerated water at 5°C or 25°C for varied amounts of time before being transferred to wet paper at 25°C. Germination was scored 3 days after transfer to wet paper. Data is plotted from germination percentage of 100 seeds. Seeds were soaked at shorter times at 25°C for two reasons: sterility of pots became a problem after 48 hours; and germination began occurring whilst seed were submerged.

Replicates were not used in this experiment, but 100 seed per treatment were used. Soaking the high vigour seed lots 1B and 4A at 5°C for up to one week did not reduce subsequent germination at 5°C.

Seed lot 1A showed reduced germination at 25°C following any duration of soaking at 5°C, compared to seed lot 1B. Germination of lot 1A may have been slightly further reduced by longer periods of cold soaking. This indicated that again, lot 1A may be of low viability, though it may exhibit lower vigour also, i.e. it is affected by

cold soaking. Lot 4B showed reduced germination subsequent to soaking at 5°C, and this appeared to be dependent on the duration of the cold soak period. The 25°C-germinability of 4B fell from above 90% to below 40% to between 0 and 72 hours of soaking at 5°C. Thus 72 hours submergence at 5°C showed that lot 4B was a lower vigour line than lots 4A and 1B, and that 1A may be a low quality seed lot.

Soaking seed at 25°C for more than 2 days was problematic due to problems with sterility; and at least some seed germinated during the soak treatment. This interfered with transfer of seedlings as partially germinated seed could be damaged during this process. However, it is apparent that germination of seed lot 1A exhibits < 80% germination subsequent to any period of soaking. The low vigour seed lot 4B does not seem to be as affected by warm soaking as cold soaking, in terms of subsequent germination at 25°C on filter paper, for equivalent durations of soaking. The high vigour seed lots again appear be relatively unaffected by soaking.

The low vigour seed lot 4B may be sensitive to soaking in a time-dependant manner. Indeed it also seems that the cold soak duration required to reduce subsequent germination at 25°C is shorter than the duration required than by cold imbibition on paper (48 hours imbibition at 5°C on paper has little effect on subsequent germination of low-vigour seed lots).

3.2.3 Cold sand test

Cold sand tests, where seed are imbibed in cold wet sand before being transferred to warm conditions are often used to discriminate between low vigour and high vigour seed lots. Seed are then classified in to different groups such as normal, small, abnormally small, abnormal else, and non-germinated (Table 2.2), giving an indication of germination and seedling vigour. The use of sand occurs because it is more uniform and easier to control than soil, although issues may arise due use of sand as the water may drain to the bottom of trays. A porous substrate, Profile green, may be mixed to the sand to prevent this.

The seed lots 1A, 1B, 4A and 4B were assessed by their germination and emergence abilities at 25°C under light subsequent to 5°C for 7 days in cold wet sand or profile green mix (Figure 3.6). ANOVA showed that the percentages of useable (normal plus small), abnormal (abnormal small plus abnormal else) and non-germinated seed were significantly affected by seed lot (all $p < 0.001$), but not variety or choice of

medium (Appendix 3.3, Figure 3.6 A and B). Seed lots 1A and 4B again performed worse than the high vigour seed lots 1B and 4A, as seed lots 1A and 4B had significantly fewer usable seedlings (Tukey's HSD $p < 0.05$). Seed lots 1A and 1B also had significantly more abnormal seedlings and non-germinated seeds than the lots 1B and 4A (Tukey's HSD $p < 0.05$). Whilst the poorly performing seed lots 1A and 4B had similar proportions of abnormal seedlings, lot 4B had a significantly increased proportion of non-germinated seeds and significantly fewer useable seedlings than lot 1A (Tukey's HSD $p < 0.05$). The effect of a period of cold imbibition on emergence of Variety 4 is particularly evident in the photograph in Figure 3.6 C, as lot 4A formed a dense and more uniform seedling population, whereas lot 4B formed a non-uniform and sparse seedling population.

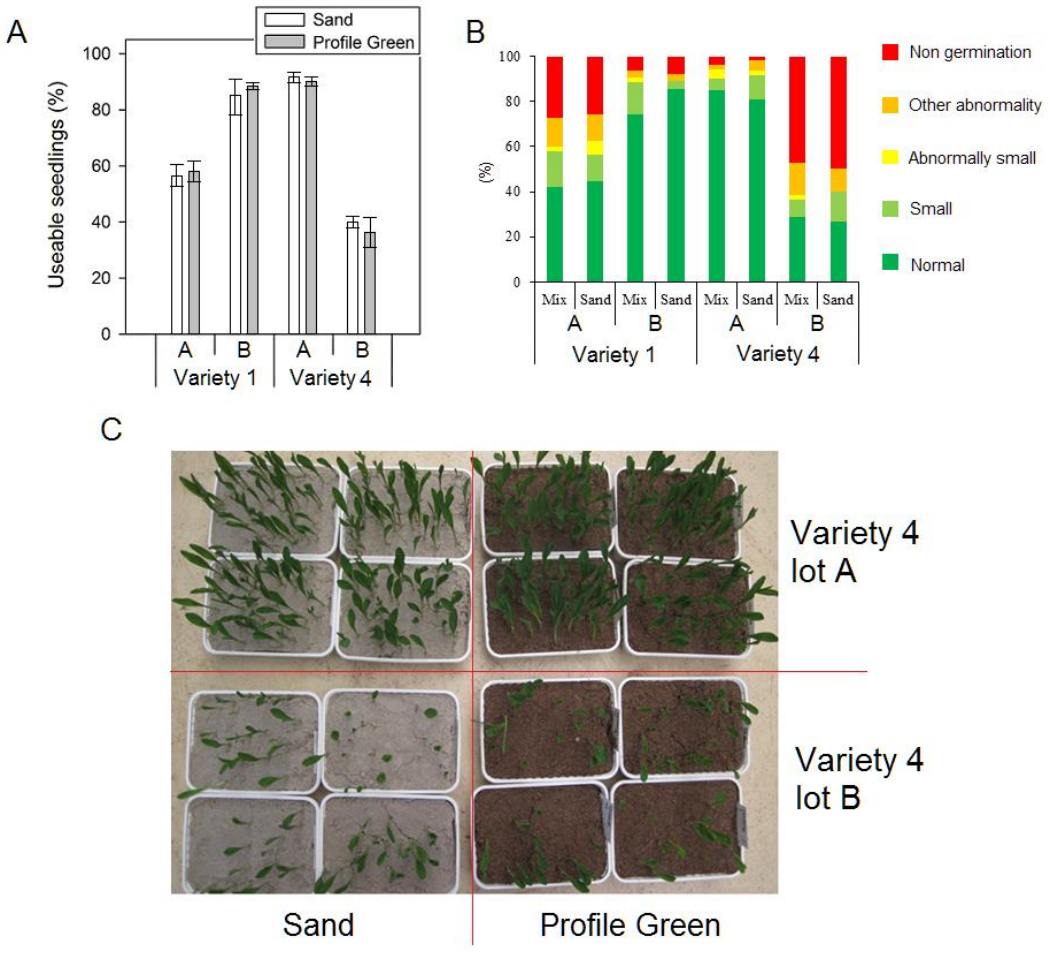

Figure 3.6. Effect of 7 days at 5°C on subsequent germination and emergence at 25°C (sand and profile green mix). Maize seed were imbibed in pre-chilled (5°C) wet sand or profile green mix at 5°C for 7 days in the dark and then transferred to light conditions at 25°C. Seeds and seedlings were classified 4 days after transfer. Analysis was carried out on transformed data (arcsin(√x)) (back-transformed means represented) Appendix 3.3. Error bars indicate standard error (n = 4 * 50 seeds for each treatment combination). B: Indicates the proportion of seeds falling into different classifications. C: A photograph of emerged seedlings of variety D in the cold sand and profile green cold tests.

3.2.4 Cold vermiculite

Two days cold soaking of seed appeared to have an equivalent negative effect on subsequent germination at 25°C (Figure 3.5) to one week cold imbibition on paper, and placing seed embryo side down (so as to have the embryo submerged) also slightly decreased germination overall (Figure 3.4). In the cold sand test (Figure 3.6) the seed are also covered by 2 cm of sand; which may mean the seed are also somewhat 'submerged' in water. Therefore it was deemed possible that the low-vigour lines are not just showing sensitivity to cold, but also to being submerged (i.e a combination of submergence and cold).

To test the effect of the cold, rather than cold and submergence, seed were imbibed on the surface of vermiculite. Seed were depressed lightly embryo face down, so as to allow uniform imbibition (which is problematic on flat filter paper, for example), but not to 'submerge' the seed in water. Seed were imbibed on vermiculite at 5°C for seven days before transfer to 25°C, or directly at 25°C and assessed after 3 days at 25°C (Figure 3.7).

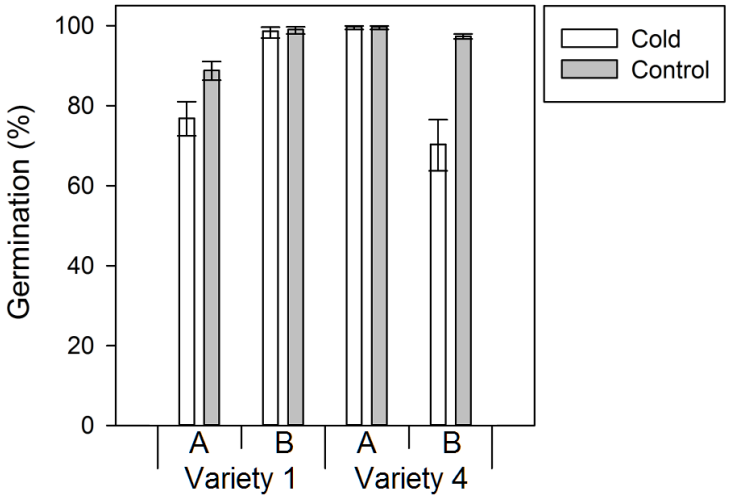

Figure 3.7. Germination of maize seed lots at 25°C subsequent to 7 days at 5°C in vermiculite. Seed were imbibed in vermiculite wet to field capacity at 5°C for 7 days then transferred to 25°C (Cold) or imbibed directly at 25°C (Control). Germination was scored after 3 days at 25°C. Back-transformed means of transformed data (arcsin(√x)) are represented. Error bars indicate standard error (n = 3 * 50 seeds for each treatment combination).

ANOVA indicated that there were significant main effects of conditions and seed lot on normal germination, abnormal and non-germination percentages (Appendix 3.4), and a significant interaction between conditions and seed lot on germination was observed (p = 0.002).

The germination of the high vigour seed lots 1B and 4A was not significantly affected by the cold treatment compared to control. Germination in seed lot 4B was not significantly different to the high vigour seed lots under control conditions, but was significantly reduced compared the high vigour seed lots under cold conditions (Tukey's HSD $p < 0.05$) (Figure 3.7). In contrast, germination of seed lot 1A was significantly lower than the high vigour seed lots under both conditions (Tukey's HSD $p < 0.05$). These results again suggest that lots 1B and 4A are high vigour, that lot 1A may have low viability and that lot 4B has low vigour. Seed lot 1A also had a significant increase (5%) in abnormal seedlings, only under cold conditions (Tukey's HSD $p < 0.05$) indicating that lot 1A may also exhibit low vigour.

3.2.5 Summary of cold test results

A collection of 10 seed lots from 5 maize varieties varied in germination performance at 25°C following imbibition for 7 days at 10°C. Four of these seed lots, from two varieties were characterised under further cold test and control conditions to determine vigour characteristics. Seed lot 1A was shown to have low viability, as it germinated poorly (< 80%) even under optimal conditions i.e. direct imbibition at 25°C. Seed lot 4B showed good germination under optimal conditions (>90%), but germination was reduced under cold test regimes, i.e. incubation for 7 days at 5°C before being transferred to 25°C to germinate. Thus seed lot 4B had low vigour. Seed lots 1B and 4A indicated good germination (>90%) under optimal and suboptimal conditions, indicating these seed lots had high vigour. Cold tests based on imbibing seed at 7 days at 5°C and transferring to 25°C were adequate to discriminate between high and low vigour seed lots. These cold test setups therefore provided a basis for testing the effects of hydroxamic acids on vigour (Section 3.3.4), and for sampling to measure gene expression and ABA measurement in Chapter 6.

3.2.6 Germination of NK Falkone at low temperatures

Germination of NK Falkone was reported to be accelerated at low, but germination permissive, temperatures by hydroxamic acid CCD inhibitors (David Brocklehurst, personal communication, September 22, 2014). I tested a seed lot NK Falkone in the cold vermiculite test (outlined in Section 3.2.4), and germination percentage was 99% under the control and cold temperature regimes, indicating that NK Falkone is a high-vigour seed lot (data not shown). Germination of this seed lot at low temperatures was characterised because acceleration of germination of high vigour

seed lots under suboptimal conditions is also of interest. Seed were imbibed at 10°C, 15°C and 20°C on wet vermiculite. Germination was delayed by reducing the temperature from 20°C to 15°C, and further again by reducing it to 10°C (Figure 3.8). Mean germination increased from 83±3 hours (± standard error) to 117±2 hours at 15°C and to 238±2 hours at 10°C. Almost all seeds germinated in any case. This system also provided a basis for testing the effects of hydroxamic acid CCD inhibitors on germination acceleration (Section 3.3.4) and for gene expression and ABA measurement in Chapter 6.

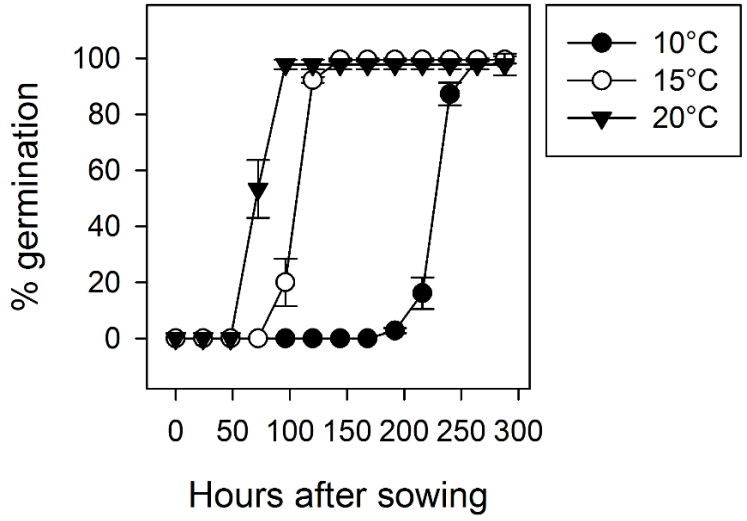

Figure 3.8. Effect of temperature on germination of a high vigour seed lot (NK Falkone). Seed were imbibed in vermiculite wet to field capacity at 10°C, 15°C (n = 5 * 30) and 20°C (n = 3 * 30). Germination was scored daily. Error bars indicate standard error.

3.3 Mitigation of the Effects of Cold Imbibition

3.3.1 Seed drying

Based on the alleviation of dormancy seen by seed desiccation other species (e.g. Bouwmeester and Karssen, 1993a), it was hypothesised that drying the seed subsequent to a cold imbibition treatment would improve germination at 25°C compared to directly transferring the seed from the cold imbibition to optimal conditions (Figure 3.10).

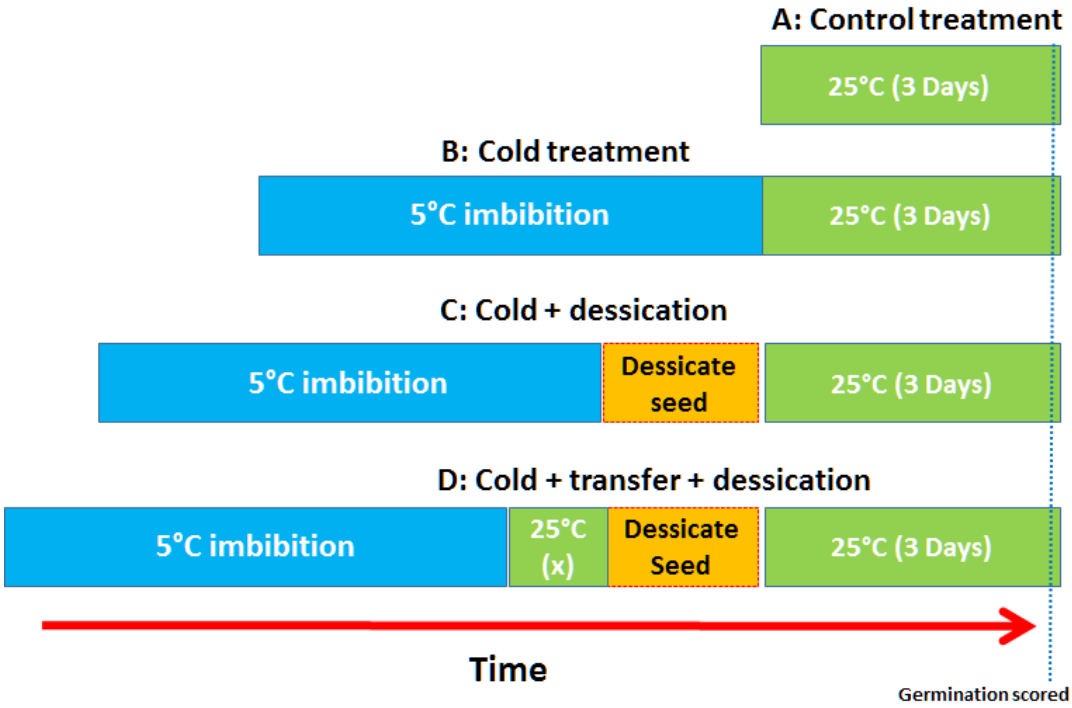

Figure 3.9. Schematic of seed drying treatments. I hypothesised that drying seed subsequent to cold imbibition may restore the ability of some seed to germinate at 25°C A: Imbibition of seed at 25°C shows the maximum germination percentage of a seed lot. B: A cold imbibition treatment prior to transfer to 25°C leads to a reduction of germination in low vigour seed lots. C: Drying the seed after the cold imbibition, then sowing at 25°C allows more seed to germinate than in B (Figure 3.10 A, B). D: Germination may still be restored in non-germinating seeds by drying seed that have already experienced cold imbibition and transfer to 25°C (Figure 3.10 C).

Imbibition on filter paper allows seed lot 4B to germinate to >95% if sown directly at 25°C (Figure 3.10 A). However, if lot 4B is imbibed at 5°C for seven days prior to transfer to 25°C, then germination is reduced by ca. 50%. Seed of 4B that are imbibed at 5°C for seven days, then dried before being sown at 25°C, germinate to a higher percentage (ca. 75%) indicating that desiccation has reversed the negative effects of cold imbibition in some of these seed. A similar but less pronounced effect is seen seed lot 1A. This is because cold imbibition on paper only reduced germination by ca. 10% in this seed lot.

Again, imbibition at 25°C on the cellulose diaper (very wet, thick, tissue paper) allows ca. 80% germination of 4B (the cellulose itself reduces germination slightly). 5°C imbibition for seven days then transfer to 25°C allows only ca. 25% germination in 4B. Drying the seed after the seven days 5°C imbibition before re-sowing the seed at 25°C allows ca. 80% germination, an effect reflected in seed lot A1. Therefore it drying the seed after cold imbibition restores the ability of seed to germinate at 25°C.

This effect can also be seen if seed are dried after soaking at 5°C for two days and sown on filter paper at 25°C (Figure 3.10 B). Therefore drying can reverse the effect of a period of cold imbibition if drying occurs before transfer to optimal conditions.

However, it was unknown if drying seed that had been already been transferred to 25°C, after cold imbibition, could restore germination (Figure 3.9 D). One hypothesis was that the transfer from 5°C to 25°C could 'shock' seed resulting in seed death. This shock may have been avoided in the desiccation treatment in Figure 3.9 C, and this may be why the desiccation has a positive effect on germination. To test this, seed of lot 4B were soaked at 5°C for two days and transferred to filter paper at 25°C. Seed of lot 4B were dried after being transferred to 25°C after 1.5 h at various time points (i.e. x = 1.5 to 24 h in Figure 3.9D) and then sown again at 25°C.

4B seeds that were not soaked indicated 100% final germination after 3 days at 25°C (Figure 3.10 C). Germination of seeds that were soaked at 5°C for two days was reduced by ca. 50%, similar to the results seen in Figure 3.10 B. Seed that were soaked at 5°C for two days and transferred to filter paper at 25°C for 1.5 hours before being dried showed increased germination (ca. 90%) compared to seed that were not dried following the soak treatment. Similar increases were seen by drying seed three and six hours following transfer from the 5°C soak treatment. 24 hours after transfer, some seed had already germinated (ca. 35%) and drying seed that had not yet germinated and re-sowing these at 25°C also increased final germination percentage.

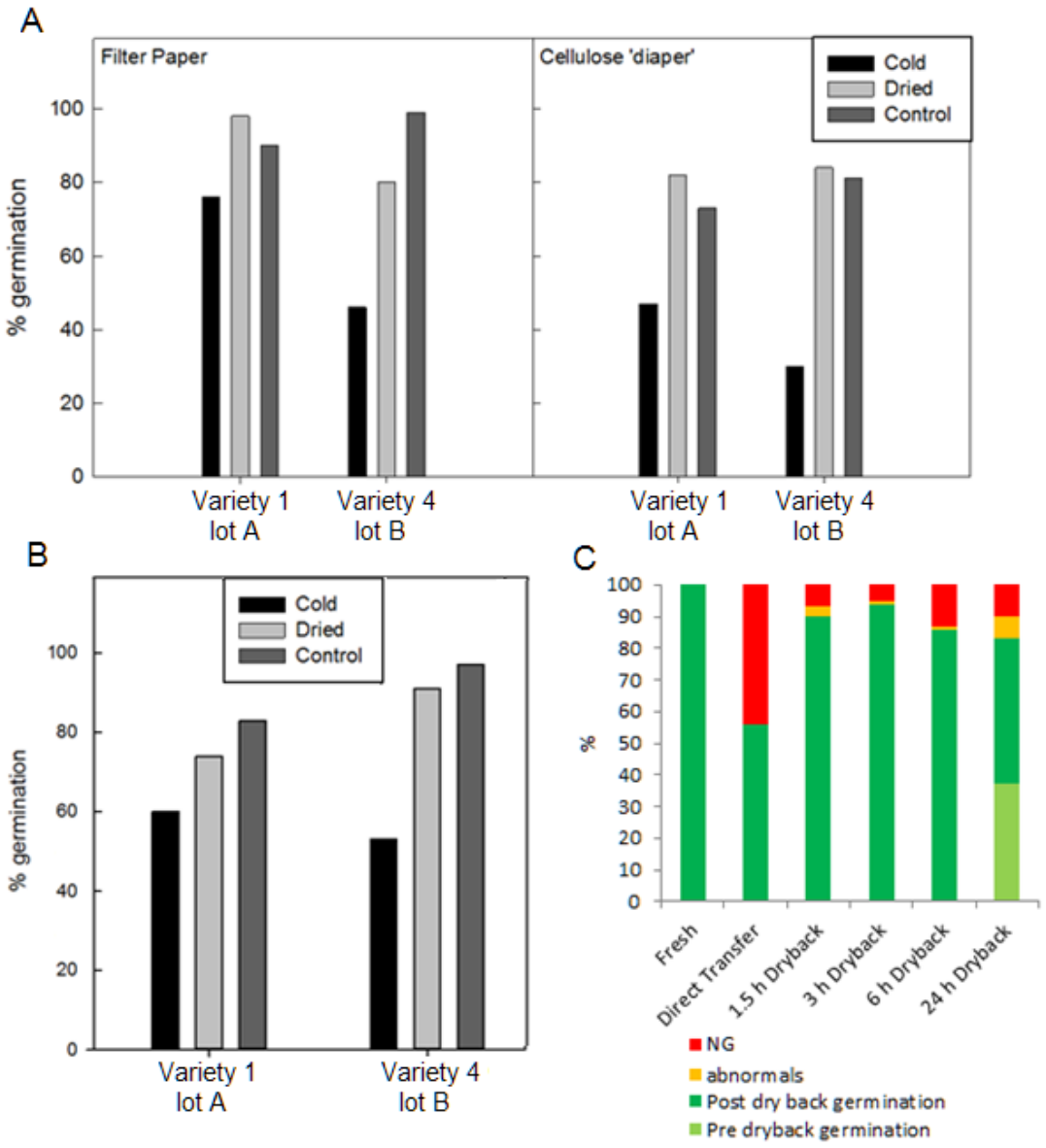

Figure 3.10. Reversibility of effect of cold pre-treatment by seed drying.A: Seed were: imbibed at 5°C for 7 days on wet cellulose 'diaper' and transferred directly to wet cellulose diaper or filter paper at 25°C (Cold); imbibed at 5°C for 7 days on wet cellulose 'diaper', dried for 1 day then imbibed on wet cellulose diaper or filter paper at 25°C (Dried); or sown directly to filter paper or wet diaper at 25°C (Control). **B:** Seed were: submerged at 5°C for 2 days and transferred directly to filter paper at 25°C (Cold); submerged at 5°C for 2 days, dried for 1 day then imbibed on wet filter paper at 25°C (Dried); or sown directly to filter paper at 25°C (Control). **C:** Seed of seed lot Variety 4 lot B were submerged for 48h at 5°C then: transferred to wet filter paper at 25°C (direct transfer); or transferred to wet filter paper at 25°C for 1.5 hours to 24 hours and dried for 1 day before being re-sown on wet filter paper at 25°C (Cold + Dried (x) h after transfer). Seed were sown directly to wet filter paper at 25°C as a control (control).

Germination was not restored by drying seed that had not germinated following two days 5°C soak and three days at 25°C (data not shown). Therefore the state of non-germination induced by a period of cold imbibition could be reversible by drying, up to at least 24 hours after transfer to optimal conditions, but further studies with

replication are required to confirm this finding. Furthermore the non-germination state may not be caused by temperature shock or change as hypothesised, but something else. If the non-germinating state is reversible it would suggest that the cold imbibition period may have induced secondary dormancy in the low-vigour seed. It was hypothesised that alteration of hormone signalling may be able to stimulate or alleviate the non-germinating state. This hypothesis was tested in the following sections.

3.3.2 Altering hormone signalling through seed treatment (development of a protocol)

Hydroxamic acid CCD inhibitors are able to stimulate germination in tomato, tobacco and lettuce, particularly in situations where ABA biosynthesis is likely to be elevated, probably owing to NCED inhibition (Awan *et al.*, in preparation). Thus it was hypothesised that cold imbibition halted maize germination, even on transfer to optimal conditions, due to stimulation of ABA biosynthesis. Furthermore it was hypothesised and that prevention of ABA biosynthesis, such as by application of hydroxamic acid inhibitors, may restore germination.

Due to a limited availability of hydroxamic acid inhibitor compound (i.e. due to the expense of synthesis), and the large volume of water required for imbibition of maize seed imbibition, an efficient method of compound delivery was sought so that this hypothesis could be tested. Application of compound dissolved in a solvent such as acetone, to dry seed, which is evaporated prior to seed imbibition allows localised seed treatment with a compound without dissolving the compound in the imbibition medium (Khan, *et al.*, 1973; Tao and Khan, 1974). To test this technique, tomato seed constitutively overexpressing *LeNCED1* (seed described as 'sp5' in Thompson, *et al.*, 2000), were treated with acetone or dichloromethane (DCM) solutions containing the hydroxamic acid D4 or norflurazon. Acetone or DCM was allowed to dry and seed were subsequently germinated on filter paper and compared to seed imbibed on equivalent water solutions. Germination time-courses are indicated in Figure 3.11 and MGT is indicated in Figure 3.12.

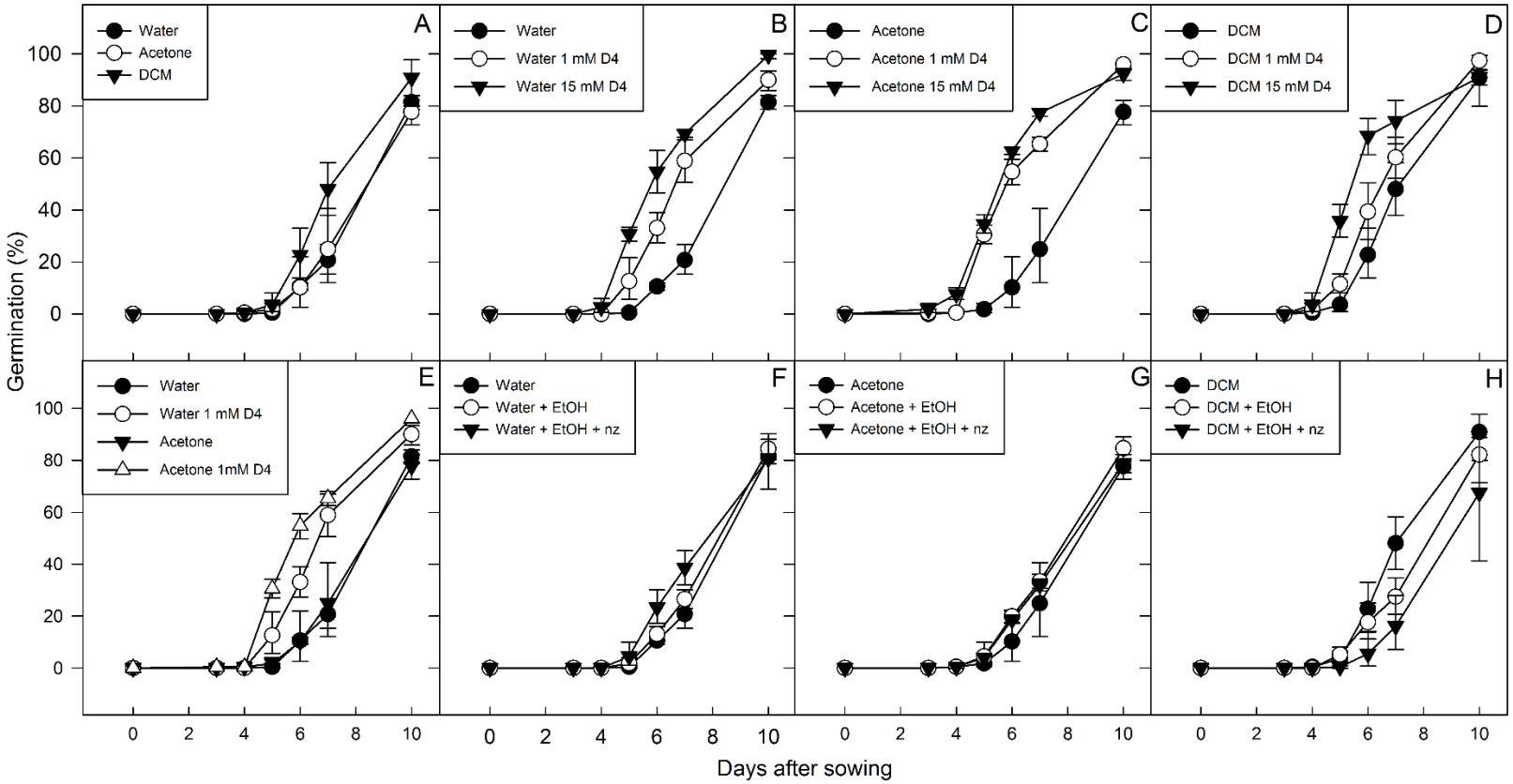

Figure 3.11. Application of germination stimulating compounds to tomato seed – germination time courses Tomato seed overexpressing *LeNCED1* ('Sp5') were soaked with acetone or dichloromethane (DCM) solution containing 0 mM, 1 mM, 15 mM hydroxamic acid D4, 0.0025% ethanol (EtOH) or 0.5 mg l^{-1} norflurazon (nz) in 0.0025% ethanol, and imbibed on water, or not pre-treated with solvent and imbibed on water containing equivalent solutions (outlined in figure legends). Germination (radicle emergence) was scored daily. Germination data was transformed (arcsin(√x)) prior to analysis. Error bars indicate standard error (n = 3 replicates of 25 seed).

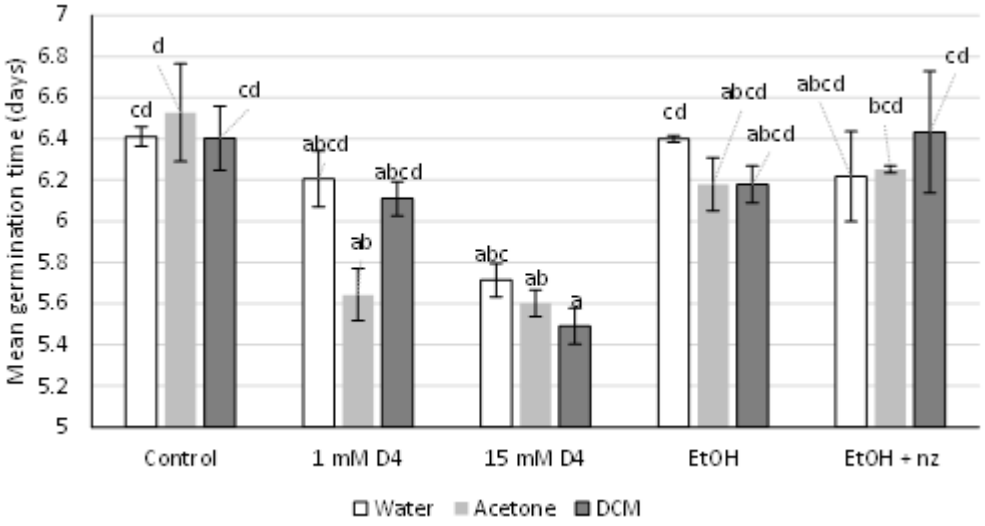

Figure 3.12. Application of germination stimulating compounds to tomato seed – mean germination time. Tomato seed overexpressing *LeNCED1* ('Sp5') were soaked with acetone or dichloromethane (DCM) solution containing 0 mM, 1 mM, 15 mM hydroxamic acid D4, 0.0025% ethanol (EtOH) or 0.5 mg l^{-1} norflurazon (nz) in 0.0025% ethanol, and imbibed on water, or solvent not pre-treated and imbibed on water containing equivalent solutions. Germination (radicle emergence) was scored daily. Mean germination time was calculated and analysed using ANOVA (Appendix 3.5). Error bars indicate standard error (n = 3 replicates of 25 seed). Bars with no common letters are significantly different (Tukey's HSD, $p < 0.05$).

ANOVA indicated that there was a significant effect of seed treatment ($p < 0.001$), and seed treatment concentration ($p = 0.002$) on MGT. The choice of delivery method (acetone, DCM solvent or imbibition in water solution) had no significant effect on MGT (Appendix 3.5). Pre-treatment of Sp5 seed with acetone or DCM solutions did not appear to affect the germination time-course Sp5 seed compared to direct imbibition on water (Figure 3.11 A). This was reflected in the similarity in MGT (Figure 3.12). However, addition of D4 to the water medium, or pre-treatment of seed with 1 mM D4 acetone or DCM accelerated germination compared to the solvent only controls (Figure 3.11 B, C and D). This showed up as a significant reduction in MGT seed treated with 15 mM D4 in DCM and acetone and 1 mM D4 in acetone (Figure 3.12). When directly compared using Tukey's HSD, imbibition of sp5 seed directly on 1 mM or 15 mM D4 in water did not significantly affect MGT compared to imbibition on water without D4 ($p = 0.999$ an $p = 0.082$, respectively). The percentage germination of seeds imbibed on 1 mM D4 and 15 mM D4 was significantly higher on days 5, compared to water (Tukey's HSD, $p < 0.05$), but not on days 6 or 7, perhaps indicating a minor effect of D4 in water on sp5 seed. Thus D4 may be more effective in reducing MGT when applied in acetone or DCM prior to imbibition of seed, which is evident in the germination time course (Figure 3.11 E).

There did not appear to be an effect of 0.5 mg L^{-1} norflurazon on the germination time course of Sp5 seed (Figure 3.11 F, G and H) via any of the application methods, nor any significant effect on MGT (Figure 3.12).

Soaking seed in solvent containing hydroxamic acids therefore appears to be an effective method of targeted compound delivery in the model species tomato. This method of delivery was therefore used to test the effect of hydroxamic acids (and ABA) on maize seed germination.

3.3.3 The effect of exogenous ABA on maize germination

To see if ABA delayed seed germination in mature maize seed, ABA was applied to seed before germination. Preliminary tests indicated that piercing the seed pericarp close to the embryo, and applying ABA via acetone prior to imbibition delayed germination of lot 4B compared to application of acetone alone (or application of ABA in the water imbibition medium). However, complete inhibition of germination was not observed by application of ABA at 25°C, rather a short delay in germination was observed. It was hypothesised that the delay in germination may only be temporary due to an optimal temperature being used (25°C) which may accelerate ABA degradation or reduce ABA sensitivity. Additionally it was hypothesised that the effect of ABA on high and low vigour seed lots may differ due to differences in ABA sensitivity.

Therefore, the effect of ABA treatment on germination of low vigour and high vigour seed lots was tested at 15°C (a less optimal germination temperature) and 25°C (Figure 3.13). Seed were imbibed on the vermiculite medium to avoid other germination inhibitory effects. ANOVA indicated that MGT was significantly affected by temperature ($p < 0.001$); and seed treatment ($p < 0.001$), but no interactions were observed, or effects of seed lot (Appendix 3.6). Final germination percentage was significantly affected by seed treatment ($p < 0.001$), and there was a weakly significant three-way interaction between seed treatment, seed lot and temperature ($p = 0.034$) (Appendix 3.6). MGT was significantly increased by 34 hours at 15°C compared to 25°C in both lines and all treatments. ABA seed treatment significantly increased MGT by approximately 40 hours regardless of seed lot or temperature (Figure 3.13).

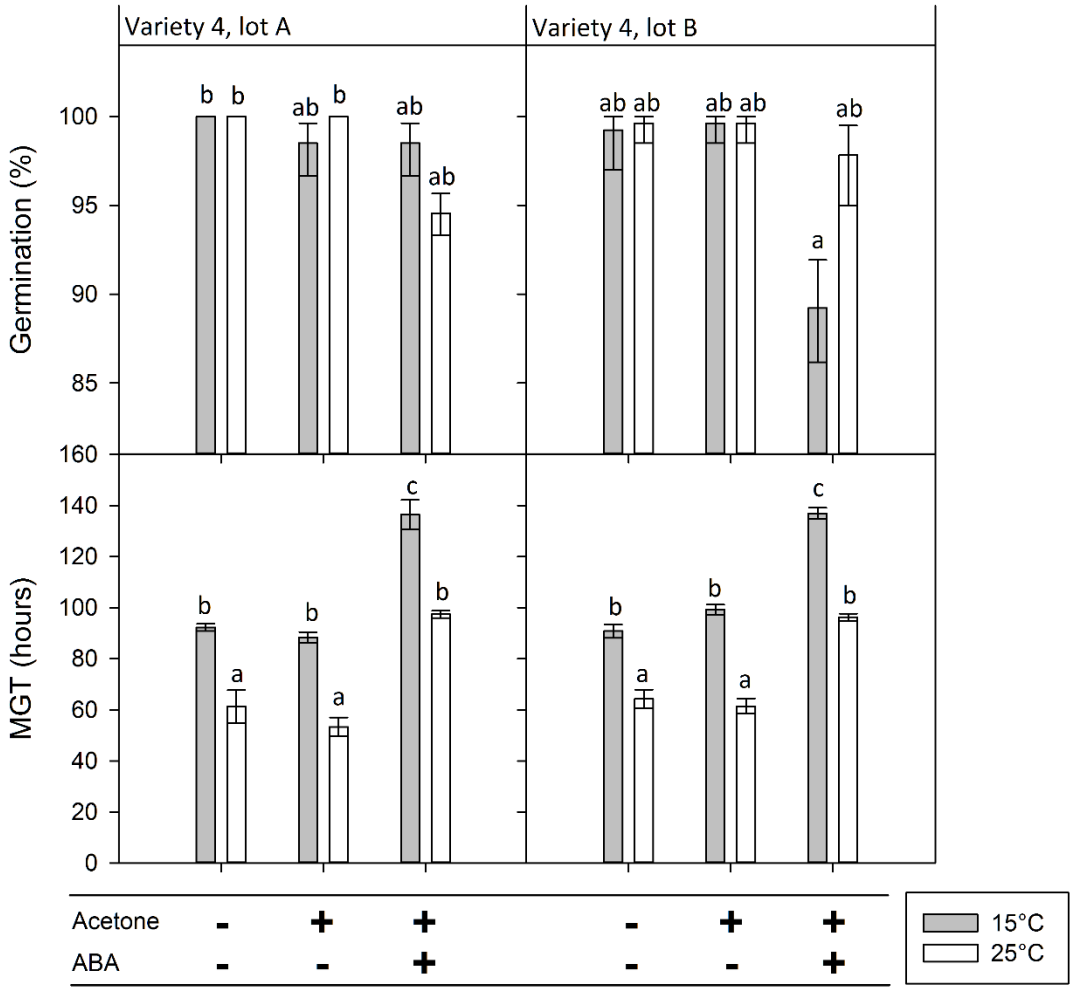

Figure 3.13. The effect of exogenous abscisic acid on germination of low-vigour and high-vigour seed lots at two temperatures. Maize seed of Variety 4 lot A or Variety 4 lot B were pierced, pierced and treated with 15 µl acetone, or pierced and treated with 15 µl 10 mM (±)-abscisic acid in acetone and imbibed in vermiculite wet to field capacity at 15°C or 25°C. Germination (radicle emergence) was scored daily. Final percentage germination after 1 week was calculated from transformed data (arcsin(√x)). And back-transformed means are plotted. Error bars indicate standard error (n = 3 replicates of 30 seed per treatment combination). Bars with no common letters are significantly different (Tukey's HSD, $p < 0.05$) (Appendix 3.6).

ABA may have had an overall effect of reducing final percent germination by 4%. However in the presence of ABA, final germination percentage of lot 4A is higher at 15°C than at 25°C; but final germination percentage of lot 4B is higher at 25°C than at 15°C. This effect is likely the main reason for the weakly significant lot*temperature*treatment interaction.

Thus application of ABA delayed germination in a manner that is mostly independent of seed lot or temperature. There is some evidence that ABA may also have a small effect on final percentage germination, and for the most part, ABA did not induce a persistent non-germinating state.

3.3.4 The effect of hydroxamic acids on maize germination

Hydroxamic acid D2 and D4 inhibit a key step of ABA biosynthesis *in vitro* and D4 reduces ABA accumulation *in vivo* (see section 1.8.3). To see if inhibition of ABA biosynthesis mitigated the effects of cold imbibition in maize, seed were treated with hydroxamic acid CCD inhibitors D2 and D4, and the ability to germinate at 25°C after 7 days imbibition at 5°C was assessed (Figure 3.15). As previously seen, 7 days imbibition at 5°C inhibits subsequent germination at 25°C, in this case reducing the final germination percentage to approximately 60%. None of the seed treatments had a significant effect on germination under control or cold test conditions; although cold test conditions significantly reduced germination by about 40% compared to control (Figure 3.15 A; Appendix 3.7).

In addition to germination, the masses of the roots and shoots of germinated seedlings were measured to see if the seed treatment had any post-germination effects. Overall there was no significant effect of the acetone or hydroxamic acid seed treatments on root or shoot growth following cold test (Figure 3.15 B) or control (Figure 3.15 C) conditions (Appendix 3.8). No significant effect of the seed treatments on root:shoot ratio were found (Appendix 3.8).

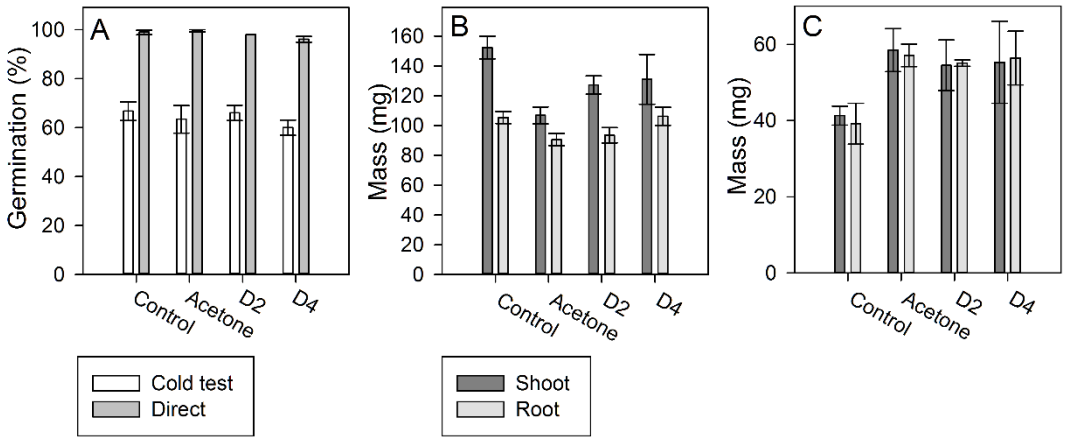

Figure 3.14. The effect of D2 and D4 applied by acetone on low-vigour maize germination and seedling growth subsequent to 7 days at 5°C in vermiculite. Seed of Variety 4 lot B were soaked with acetone solutions containing 1 mM D2 or 1 mM D4, or acetone alone, or without pre-treatment (control). Seed were imbibed in vermiculite wet to field capacity at 5°C for 7 days then transferred to 25°C (Cold test) or imbibed directly at 25°C (Direct). (A) Germination was scored after three days at 25°C (n = 3 replicates of 50 seeds), means were calculated from transformed (arcsin(√x)). Shoot and root masses were measured after 4 days of growth at 25°C for fifteen seedlings per treatment replicate (n=3) from the cold test (B) or direct germination (C).

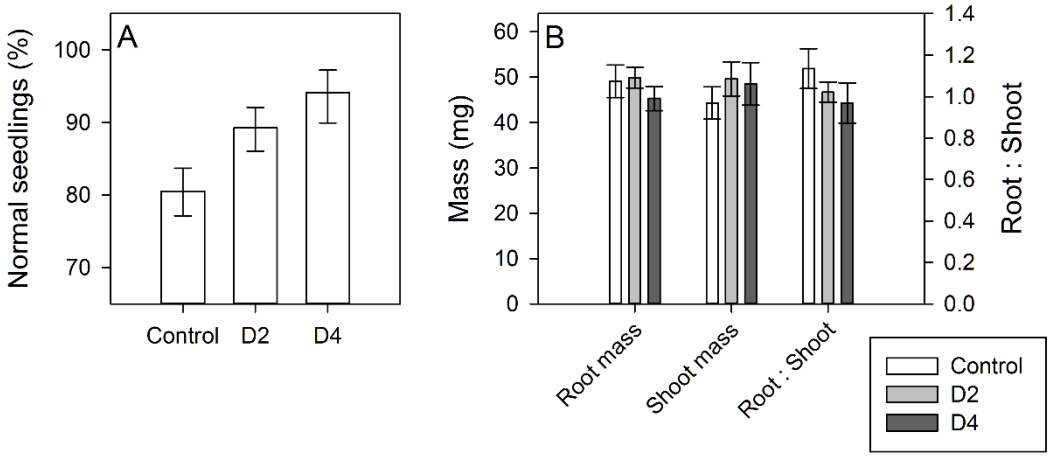

Figure 3.15. The effect of D2 and D4 applied in imbibition medium on low-vigour maize germination and seedling growth subsequent to 7 days at 5°C in vermiculite. Seed of Variety 4 lot B were imbibed in 1 mM D2 or 1 mM D4 or Control (0.1% DMSO) solutions for 7 days at 5°C before being transferred to 25°C. **A**: Germination was scored after 3 days at 25°C, means and standard error were calculated on transformed data (arcsin(√x)). Back-transformed means and errors are represented. **B** Fresh root and shoot mass was measured 3 days after transfer and root:shoot ratio by mass was calculated (n = 6 replicates of 30 seed).

The difference in shoot mass under the cold test is almost significant overall (p = 0.055) with the acetone treatment perhaps reducing shoot mass compared to control (Figure 3.15 B; Appendix 3.8).

A similar experiment was performed, looking at the effect of hydroxamic acids D2 and D4 dissolved in the water in the vermiculite imbibition medium on the germination and seedling growth of lot 4B after seven days at 5°C (Figure 3.15). Overall, the effects of D2 or D4 treatment on germination and seedling growth were not significant (Appendix 3.9); although there is an almost significant effect on the proportion of normal seedlings (p = 0.061). However, pooling the data for D2 and D4 ('hydroxamic acids') and comparing them to the control results in an overall significant effect of hydroxamic acids increasing the percentage of normal seedlings by 11% (p = 0.032). Additionally the effect of hydroxamic acids on the percentage of non-germinating seeds then becomes almost significant (p = 0.061) (Appendix 3.9). This may not be significant as the increase in normal seeds is partially as a result of a reduction in both non-germinating (-8% in hydroxamic acid vs. control) and abnormal (-3% in hydroxamic acid vs. control) seedlings.

Therefore hydroxamic acid treatment may have had a weak effect in increasing the percentage of normal seedlings in the low vigour maize seed lot 4B under cold test conditions. Confidence in this effect is not very strong. There appears to be no

significant effect of the hydroxamic acids on root or shoot growth at 25°C subsequent to germination (Figure 3.15 B; Appendix 3.9).

Hydroxamic acids were also tested for effects on germination of NK Falkone at 10°C and 15°C (Figure 3.16). In any case the final germination percentage was almost 100% and the germination time-plots indicated little effect, if any, of the treatments on germination (Figure 3.16); although temperature did have a clear effect in delaying germination. The lack of treatment effect and the germination delaying effect of low temperature was also evident in the MGT (Table 3.1).

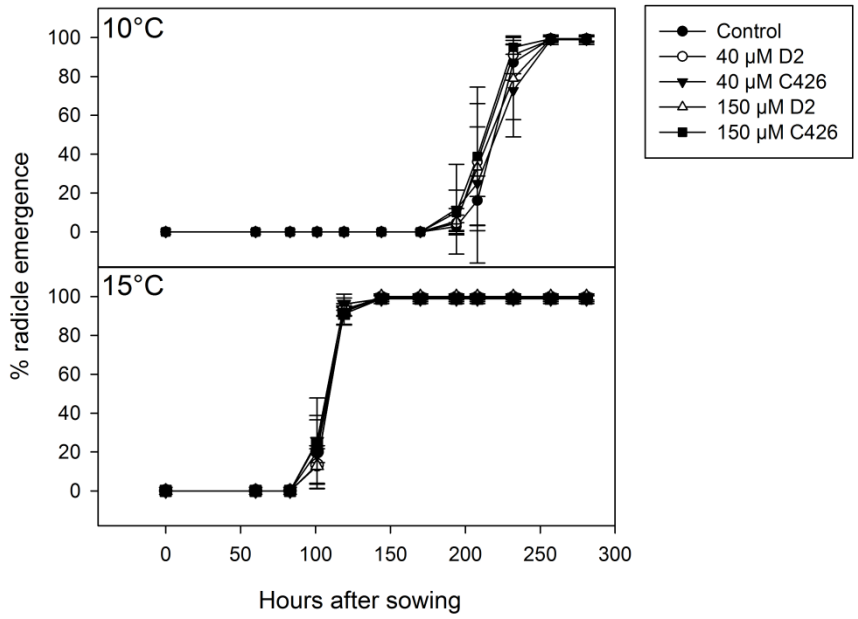

Figure 3.16. The effect of D2 (and C426) on germination at low temperatures. Seeds of NK Falkone were imbibed in vermiculite wet to field capacity with solutions of D2 or C426 at 40 µM and 150 µM in 0.1% DMSO. 0.1% DMSO was used as a control. Germination (radicle emergence) was scored daily, errors bars indicate standard deviation (n=5). *Many data points are obscured as very little effect of the treatments is apparent.

Table 3.1. Curve-fitting for germination of NK Falkone at low temperatures in the presence of D2 and C426.

Parameter	t_{50}		t_{10}		t_{90}		t_{90-10}		Mean germination time*	
Temperature	**10°C**	**15°C**	**10°C**	**15°C**	**10°C**	**15°C**	**10°C**	**15°C**	**10°C**	**15°C**
Control	218.6	106.99	206	97.42	224.6	111.79	18.55	14.37	230.4	116.92
40 µM D2	212.7	107.69	200.5	98.87	218.6	112.09	18.06	13.22	224.5	117.84
40 µM 426	218.4	105.1	206	98.26	224.3	108.47	18.28	10.22	229.8	114.95
150 µM D2	216.4	107.86	201.7	99.01	223.4	112.21	21.75	13.2	228.1	118.33
150 µM 426	209.3	105.7	199.8	96.91	213.9	110.17	14.15	13.26	222.3	116.41
LSD (5%)	11.03	2.746	8.84	*3.668*†	12.42	3.368	*6.596*†	4.67	10.86	2.926

Germination characteristics derived from fitting a 4 parameter logistic curve [(F(*x*) = ((A-D)/(1+((*x*/C)^B))) + D] to germination data. Mean values and 5% least significant difference (LSD) values are represented based on Genstat ANOVA.*Mean germination time was calculated without the curve fitting approach. †Data distribution was not normal according to Shapiro-Wilk test and Q-Q plot therefore LSDs may not be valid.

$$y = \frac{a - d}{\left(1 + \left(\frac{x}{c}\right)^{d}\right)} + d$$

Equation 3.1. Four parameter logistic equation Minimisation of sums of squares was used to fit curves by adjusting parameters a, b, c and d. *x* is equal to time (hours after sowing) and *y* is equal to the percentage of germination. Parameters were then used to calculate the time to 10%, 50%, 90% (t10, t50, t90, respectively) germination and the time between 90% and 10% germination (t90-10) used as an indicator of germination uniformity.

To investigate further if the treatments had an effect on the speed and uniformity of germination curves were fitted using a 4 parameter logistic curve (Equation 3.1) to each treatment replicate. ANOVA was used to test for treatment effects (Table 3.1). No overall significant effects of the treatment on time to 10%, 50%, and 90% germination and time between 10% and 90% germination were observed at either temperature. The effect of temperature was again clearly evident. The least significant difference (5%) was not exceeded compared to control in any case; although the difference was exceeded between some treatments (e.g. 40 µM 426 resulted in a smaller t90 compared to 40 µM and 150 µM D2). Given the number of treatment comparisons this could be expected (i.e. there is approximately 1/20 chance of the differences equalling or exceeding the LSD), and there is no evidence that the treatments had an effect compared to control.

Hydroxamic acids were applied as a coating to low-vigour maize seed before imbibition and growth in cold sand test and control conditions (Figure 3.17). There were no significant effects of the seed coating or hydroxamic compound treatment on seedling classification in cold test and control conditions. This was measured in terms of useable seedlings (normal + small) abnormal seedlings (abnormal small + abnormal else) or non-germinated seeds. There were some small effects of seed lot (i.e normal seedlings and abnormal seedlings in control conditions), and a clear effect of the temperature regime on lot 4B, as expected, but no effect of the seed treatments (*statistics not indicated in appendices*).

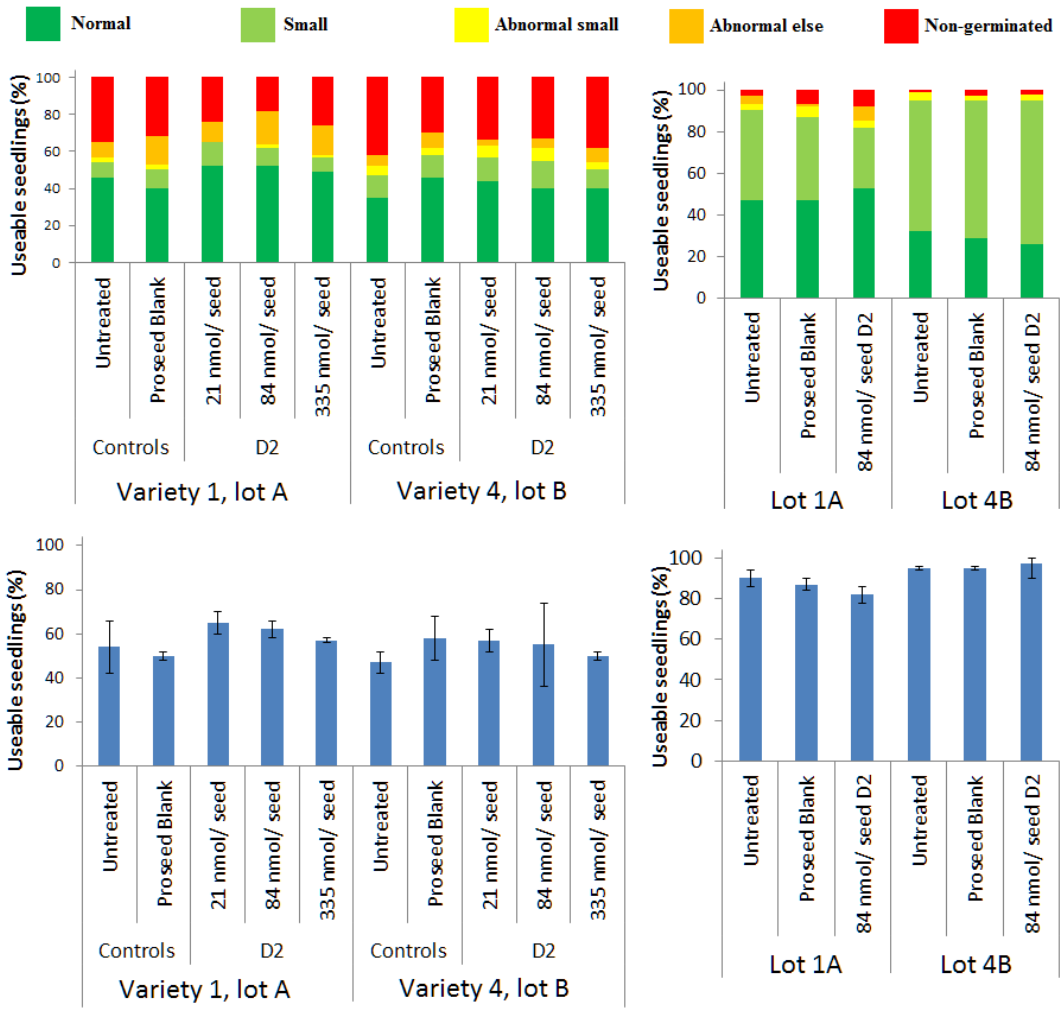

Figure 3.17. The effect of D2 on germination in sand subsequent to 7 days at 5°C. Seed of Variety 1 lot A and Variety 4 lot B were coated with proseed containing different concentrations of D2. Proseed-only and untreated seeds were used as controls. Seed were imbibed in cold, wet sand for 7 days at 5°C (dark) before being transferred to 25°C (light) (cold test), or imbibed directly at 25°C (Direct). Seedlings were classified as Normal, Small, Abnormally Small, Abnormal Else or Non-germinated after 4 days at 25°C. Usable seedlings (%) is calculated from the number of normal and small seedlings. The back transformed means and errors of transformed data (arcsin(√x)) are represented. Error bars indicate standard error (n = 2 replicates of 50 seed per treatment combination).

3.4 Discussion

3.4.1 Conditions that distinguish between high-vigour and low-vigour seed lots

It is common practice to imbibe maize seed at 10°C for seven days in a cold test to determine seed lot vigour. 10 seed lots imbibed on cellulose exhibited variations in germination, seedling abnormality and non-germination under such a test (Figure 3.3). Although not directly compared, treatment of 5°C for seven days on cellulose appeared to be more effective in discriminating between some of the high and low vigour seed lots (Figure 3.4). The 10°C cold test did not show significant differences between the proportion of non-germinating seeds of lots 4A and 4B; however the 5°C cold test on paper induced 33% more non-germination in lot 4B than 4A. Whilst both 5°C and 10°C tests resulted in a significant difference in the percentage of normal seedlings, the magnitude of the difference was larger in the 5°C test (36%) than in the 10°C test (10%). The effectiveness of the 5°C test could be proven by directly comparing the 10°C and 5°C treatments. Indeed, lower cold test temperatures than 10°C have been previously recommended for cold tolerant varieties to distinguish differences in seed lot vigour (Lovato, *et al.*, 2005).

More than 90% of seed in the high vigour seed lots tested (1B and 4A) are able to germinate following seven days at 5°C, indicating Varieties 1 and 4 are cold hardy varieties. However, germination of a seed lot of Variety 4, 4B, was reduced by imbibition for seven days at 5°C, indicating this seed lot had lower vigour than 4A. Similarly lot 1A exhibited lower germination than lot 1B following seven days at 5°C, although 1A also exhibited lower germination when sown in favourable conditions. This indicated 1A has a lower proportion of viable seed than 1B. However, 1A did show further reductions in germination under some conditions indicating this variety may also have lower vigour in addition to lower viability compared to lot 1B. Seed lot 4B was particularly interesting for further study because it showed a clear response to the cold treatments: i.e. it was the low temperature imbibition that clearly induced the non-germinating state.

Soaking the seed at 5°C in aerated water appeared to induce the non-germinating state in low vigour seed more quickly than other methods: 48 hours at 5°C had little effect on paper, but a large effect in the aerated soak treatment. It is unknown why

this might be the case. There may be a link between the availability or excess, of water and the induction of the non-germinating state in the cold, as germination of Variety 4 is reduced by embryo-down orientation on paper. It is possible that the excess water is exerting hypoxia on the seed (although the soak water is aerated) or perhaps that it facilitates faster imbibition. The sand and profile green mix media also appeared to inflict a larger reduction of germination in lot 4B compared to the vermiculite medium in combination with the cold. In the sand a profile green mix the seeds are covered with a wet medium, whereas in the vermiculite the seed are placed embryo face down, but are not covered. The difference between these media could also be a result of the availability of air (hypoxia) or water. The effects of hypoxia could be tested by using equipment to control the partial pressure of oxygen during imbibition. The effects of water availability may be tested by changing the water potential of the imbibition medium.

The combinations of seed lots and cold conditions appeared to be valuable studying the potential role of ABA in determining seed vigour under cold conditions. The vermiculite, soak and paper imbibition methods allow for easier quantification of germination (i.e. radicles can be observed during germination) and may allow easier sampling of seed (for RNA or hormone extraction) than the sand and profile green media.

3.4.2 Mechanism of action of the effect of cold

Two main hypotheses for the reason of non-germination following a cold treatment were considered. It is possible that the cold inflicted injury to the seed, such as imbibitional chilling injury. Imbibitional chilling injury can typically occur in dry seeds, including maize, that are imbibed at cold temperatures and can lead to non-germination, radicle abortion and seminal root proliferation, which was observed in some of the abnormal seedlings after cold treatment (Bedi and Basra, 1993). This may also explain the additional stress inflicted when there is a larger excess of water (such as in the soak treatment). The fact that seed are able to germinate in the same media at optimum temperatures indicate that the injury isn't caused by the hydration alone. However it is also possible that the injury is a non-imbibitional chilling injury and this could be checked by increasing the seed moisture content before imbibition

in the cold (increased germination on transfer to 25°C would be found if injury is imbibitional chilling injury).

An alternative hypothesis is that the cold period induced secondary dormancy, resulting in the non-germination of the imbibed seed even when transferred to optimal conditions. The induction of seed dormancy is a reversible process (Finch-Savage and Leubner-Metzger, 2006) and whilst imbibitional chilling injury can be circumvented by increasing the moisture content of the seed prior to cold imbibition, it is not known to be a reversible process (Bedi and Basra, 1993). It may also be expected that other chilling injuries are not readily reversible. However evidence for the reversibility of the non-germinating state in cold imbibed maize seeds was found. It was hypothesised that if maize seed were dried after cold imbibition before being re-sown under optimal conditions, that the germination may be improved compared to seed directly transferred from being imbibed in the cold to warm conditions. Secondary dormancy has been alleviated by seed desiccation treatments in other species, such as *Chenopodium albium*, *Orobanche* spp, *Sisymbrium officinale* and *Spergula arvensis* (Bouwmeester and Karssen, 1993a; Bouwmeester and Karssen, 1993b; Karssen, *et al.*, 1988; Kebreab and Murdoch, 1999), although secondary dormancy has not been reported in maize.

Preliminary data shown here indicated that the 24 hour drying treatment used here may have restored the ability of maize to germinate at 25°C, although it varied in the extent of germination restoration depending on the combination of conditions and seed lot used. This finding would support the idea that the non-germinating state resulted from the induction of dormancy, rather than a chilling injury. Although replication was not used; that similar findings were observed amongst similar treatments does indicate the drying treatment might restore germination. Preliminary data also indicated the drying treatment might not just allow seeds to avert a shock of the transfer from cold to warm conditions as drying non-germinating seed subsequent to transfer to the warm conditions appeared to restore germination upon re-sowing, up to 24 hours after transfer. Further testing with replication will be necessary to confirm this hypothesis.

3.4.3 The role of ABA in maize germination inhibition following a period of cold

Synthesis and perception of ABA are often required for establishing and maintaining physiological dormancy (Finch-Savage and Leubner-Metzger, 2006), including high-temperature induced secondary dormancy in barely and dormancy cycling in Arabidopsis, and dormancy induction and maintenance can be interrupted by inhibition of ABA biosynthesis (Cadman, *et al.*, 2006; Leymarie, *et al.*, 2008). Similarly, application of exogenous ABA can result in a dormancy phenotype, as can transgenically induced endogenous ABA biosynthesis (Leymarie, *et al.*, 2008; Martinez-Andujar, *et al.*, 2011; Thompson, *et al.*, 2000). Maize seed that were treated with ABA exhibited a 40 hour delay in germination but still exhibited nearly 100% final germination at 25°C and 15°C. Thus application of ABA did not mimic the effect of the cold pre-treatment on germination at 25°C, and highlighted that both lines had a similar sensitivity to ABA. It is possible that the non-germinating state induced by cold is also mediated by an increase in ABA sensitivity or a reduction in ABA catabolism which is not replicated in this experiment allowing the exogenously applied ABA to be metabolised. Regulation at the catabolism and perception level is also observed in barley seed (Hoang, *et al.*, 2013). Inhibition of ABA catabolism, application of a catabolism resistant ABA analogue or measurement of ABA catabolites and related gene expression could be employed in future experiments to confirm this.

Hydroxamic acids D2 and D4 inhibit tomato NCEDs *in vitro* (Sergeant, *et al.*, 2009), and can stimulate germination in tomato, tobacco and lettuce where germination is inhibited by ABA accumulation (Awan *et al.*, in preparation). These inhibitors can also reduce ABA accumulation *in vivo* in tomato seed (Awan *et al.*, in preparation).

Here, hydroxamic acid NCED inhibitors were used to inhibit ABA biosynthesis during maize imbibition in the cold. Whilst the hydroxamic acids delivered via acetone application were effective in stimulating germination in the model system of tomato overexpressing *NCED* they did not appear to restore the germination of maize seed under vermiculite cold test conditions. However hydroxamic acids dissolved in the imbibition medium slightly restored germination by 11%, in terms of the percentage of normal seedlings, in lot 4B under vermiculite cold test conditions. Neither mode of application indicated an effect of the hydroxamic acids on root or shoot growth.

Hydroxamic acid D2 applied by seed coating also had no significant effect on germination or seedling classification under cold sand test conditions. Thus there is some evidence that the inhibition of maize germination following a period of cold is somewhat dependent on ABA biosynthesis, but further work will be necessary to determine the extent of the role of ABA.

Therefore, if ABA signalling is required for the induction of the non-germinating state in maize, an effect of D2 or D4 may not be seen either because the inhibition is not strong enough, or there is an issue with compound delivery (i.e. method of application). Indeed, D2 and D4 inhibition of NCEDs *in vitro* is indeed relatively weak (Sergeant, *et al.*, 2009), and at least small effects were observed here on maize germination.

It is possible the non-germinating state is only partially dependant on ABA (i.e. the mechanism is composed of ABA dependent and ABA independent elements). If this is true, the small effect of exogenously applied ABA in preventing germination in Variety 4B is consistent with the small increase in germination in seed imbibed in hydroxamic acid solution under cold test conditions. It would be desirable to identify stronger NCED inhibitors in order to further investigate the role of ABA under cold conditions (discussed further in Chapters 4, 7 and 8). To investigate the role of ABA without the aid of alternative inhibitors, quantification of ABA and the expression of *NCED* was pursued (Chapters 5 and 6).

3.4.4 Germination of maize in suboptimal, but permissive temperatures

Our collaborators at Syngenta reported that germination of NK Falkone seed was accelerated at low, but germination permissive, temperatures by hydroxamic acid CCD inhibitors (David Brocklehurst, personal communication, September 22, 2014). Additionally, application of ABA to maize seed led to an increase in MGT in a similar to what might be expected if seed were imbibed at lower, but permissive, conditions and so it was hypothesised that induction of ABA biosynthesis might contribute to the delay in germination at lower temperatures. Contrarily, priming in the presence of ABA appears to improve germination performance (Basra, *et al.*, 1989), although this could still be through desensitising seed to ABA produced on imbibition.

A seed lot of NK Falkone was provided by Syngenta for the purposes of this study. It was found that NK Falkone was a high vigour seed lot (i.e. compared to 4B) as it exhibited almost total germination under vermiculite cold test conditions. Germination this NK Falkone seed lot was characterised at 10°C, 15°C and 20°C. Temperatures of 10°C and above were permissive of the germination of NK Falkone, although mean germination time increased with each decrease in temperature. Imbibition in solutions containing hydroxamic acids had no effect on germination speed or uniformity. However, low concentrations of hydroxamic acids were used due to a limited supply compared the other experiments (40 µM and 200 µM), and D4 was not used at all. With the small effect of 1 mM hydroxamic acids seen the vermiculite cold test it may be that a combination of lower concentrations and inefficacy of the compounds resulted in a lack of effect. Quantification of NCED expression and ABA was also pursued in this system as an alternative approach (Chapter 6).

3.4.5 The importance of compound delivery methods

Exogenous ABA is reported to delay or inhibit germination of many species of seed [e.g. tobacco (Manz, *et al.*, 2005), Arabidopsis and barley (Millar, *et al.*, 2006)] and the requirement for ABA in developing maize seed to prevent viviparous germination is well established (Tan, *et al.*, 1997). However there appears to be a lack of literature documenting the inhibition of germination of mature maize kernels by ABA. It was found here that piercing the embryo and applying ABA solution in acetone resulted in a germination delay, much like the effect seen in seed of other species when imbibed on ABA. Conversely, acetone application of hydroxamic acid compounds in maize did not yield any effects on germination even though applying the compound in the imbibition medium had a small effect. This may be due to the relatively small amount of compound applied by acetone (i.e. 15 µl of 1 mM hydroxamic acid in acetone as a seed treatment compared to 40 ml of 1 mM hydroxamic acid dissolved in the imbibition medium). The compound may have leached from acetone treated seeds during imbibition resulting in very low concentrations of the compound.

However acetone delivery of D4 in tomato was more efficient in stimulating germination than delivery in the imbibition medium. In this case 30 seed were soaked in 1.5 ml of 1 mM D4 in acetone (a maximum average of 50 µl 1 mM D4 per

seed) compared to 2 ml of 1 mM D4 in water for wetting the filter paper for imbibition (a maximum average of 67 µl 1 mM D4 per seed). Since the acetone imbibed tomato seed were imbibed on filter paper wetted with 2 ml of water, even if the compound leached there could still be a 0.75 mM D4 concentration (although not all the acetone seed treatment was absorbed by the seeds). However the potential for loss of compound concentration due to leaching was much higher for the maize seed, so further investigation of the acetone compound application method may be required. Tomato seed treated with D4 in acetone could be imbibed increasingly sparsely on filter paper medium to test the effect of dilution of the compound by leaching.

Nonetheless, an indication that the hydroxamic acids improve maize germination under cold test conditions was identified by imbibing seed in high hydroxamic acid concentrations (1 mM).

3.4.6 Future investigations

In this chapter, and thesis as a whole, there is a focus on testing the hypothesis that ABA (and thus *NCED* genes) are involved in the germination of maize under suboptimal conditions. There are many other avenues of investigation that could be pursued. In particular, the potential for reversing the effects of 7 days of cold imbibition on subsequent germination (in this case by a drying treatment) is very interesting, as it suggests a dormant like condition in mature maize seed which may have been previously regarded as seed mortality, and this needs further study for confirmation. If this condition is caused by dormancy then investigations of the roles of many other factors (particularly the role of gibberellins for example) may also explain the condition. Alternatively there may be another explanation, such as the cold allowing imbibition but not the onset of normal physiological activity seen in germination which may then be prevented by an excess of water when seed are transferred to optimal conditions. Thus drying the seed may then resynchronise the imbibition with the physiological state of the seed. This could perhaps be tested with cold imbibition of media containing different levels of osmoticum (e.g. polyethylene glycol). The reason for the difference in germination between different vigour seed lots after cold treatment may then be simply due to the physics of imbibition (i.e. seed permeability). However, the identification of a small effect of the hydroxamic acid inhibitors may be indicative of an NCED and ABA biosynthesis dependent

dormancy mechanism. Thus probing the roles of NCED remains the main focus for the rest of this thesis.

Additionally it would be interesting to investigate other conditions relevant to spring sowing of maize seed. The focus of this chapter was cold, however spring sowing conditions in the field at high latitudes can bring frosts, flooding or drought and a mix of soil characteristics. For example wet clay soil, or dry capped soil could exert hypoxia and physical resistance against emergence. In addition other natural phenomena such as day and night cycling of temperatures were not tested, and low vigour maize could be more susceptible to such conditions. Thus smaller differences between seed vigour could be much more crucial under such conditions.

3.4.7 Conclusion

Work in this chapter provided potentially useful conditions for further study of a role of ABA in maize seed germination under suboptimal temperatures. One experiment suggested that inhibition of ABA biosynthesis may improve germination of low vigour seed lots under cold test conditions. Further studies are required to determine if seed drying can restore germination in seed that have been imbibed under cold test conditions. The need for further investigation into the mechanisms of the effect of cold and the requirement for use of a more effective seed germination promoting compound is highlighted.

4 Identification and Inhibition of the 9-*cis*-*epoxycarotenoid dioxygenase* (*NCED*) Gene Family in *Zea mays*

4.1 Introduction

The identification of a viviparous, ABA deficient maize mutant, *viviparous14* (*vp14)*, led to the cloning of the first known *NCED* gene, *Vp14* (Schwartz, *et al.*, 1997; Tan, *et al.*, 1997). The sequence of *Vp14* was subsequently used to identify *NCEDs* in other species: such as in tomato (Burbidge, *et al.*, 1999), where a mutation of an *NCED* gene, *notabilis*, results in a wilty phenotype; and in avocado (Chernys and Zeevaart, 2000), bean (Qin and Zeevaart, 1999) and cowpea (Iuchi, *et al.*, 2000). Indeed, the phylogenetically related *carotenoid cleavage dioxygenase* (CCDs) genes (of which *NCEDs* are a subset) were also identified due to their ability to cleave different carotenoids at different positions such as the 9, 10 / 9′, 10′ cleavage activity exhibited by *AtCCD1* (Schwartz, *et al.*, 2001).

Subsequent to sequencing of the *Arabidopsis thaliana* genome, five *NCED*s and four *CCD*s were identified, namely *AtNCED2*, *AtNCED3*, *AtNCED5*, *AtNCED6*, *AtNCED9*, *CCD1*, *CCD4*, *CCD7* and *CCD8* (Tan, *et al.*, 2003). The Arabidopsis *NCED*s were each found to have unique expression patterns, and a variety of different roles have now been attributed to each of the Arabidopsis *NCED*s, allowing ABA control over a variety of plant processes (Frey, *et al.*, 2012; Lefebvre, *et al.*, 2006; Seo, *et al.*, 2006; Tan, *et al.*, 2003; Toh, *et al.*, 2008). It is also now apparent that plant species often have multiple *NCED* genes.

Evidence of multiple *NCED* genes in maize was detected during the characterisation of *Vp14* (Tan, *et al.*, 1997), and multiple sequences homologous to *NCED* have been identified in maize (Capelle, *et al.*, 2010; Vallabhaneni, *et al.*, 2010), with five reliably predicted to be *NCED*s (Vallabhaneni, *et al.*, 2010). However, only cleavage activity of one maize NCED, VP14 ('ZmNCED1'), has so far been confirmed. The genome of the inbred maize cultivar B73 has been sequenced (Schnable, *et al.*, 2009), and has been made accessible online, along with other resources and tools (for example at www.maizegdb.org or www.maizesequence.org). Therefore this

chapter uses the available resources, in combination with the literature, to identify and describe the maize *NCED* gene family. The main aim of this Chapter was to identify the specificity of hydroxamic acid CCD inhibitors to different NCEDs in the target crop, maize.

Objectives:

- Identify and clone all maize *NCED*s (*ZmNCED*s)
- Express the *ZmNCED*s in *E. coli* and purify maize *Zm*NCEDs
- Assay the inhibition of each NCED by the hydroxamic acid inhibitors

The identification of *ZmNCED*s will also facilitate the design of primers for measuring *ZmNCED* expression (discussed in Chapter 5).

4.2 Identification of Putative Maize *NCED*s

4.2.1 Putative NCEDs in the maize B73 genome

To identify putative *NCED*s in the maize B73 genome the VP14 amino acid sequence from maize inbred line W22 (GenBank accession AAB62181) (Tan, *et al.*, 1997) was used as a query to identify nucleotide sequences with highly similar translated sequences in the B73 RefGen_v3 dataset (E-value cutoff: 1E-4) using BLAST program tblastn (http://blast.ncbi.nlm.nih.gov/blast.cgi). 65 matching translated sequences were found. 12 results contained more than 150 matching residues (Table 4.1). Since all known *NCED*s do not contain introns, results with fewer than 150 matching amino acid (AA) residues would have less than 25% similarity to VP14 which has 604 amino acid residues. To identify open reading frames (ORFs) in the genomic sequences, target sequences were extracted, including flanking sequences (± 6 kb) and used as a query in ORF finder (http://www.ncbi .nlm.nih.gov/gorf/gorf.html). Translated sequences of ORFs longer than 1000 bp were aligned with the VP14 sequence on NCBI BLAST (http://blast. ncbi.nlm.nih.gov/blast.cgi) using blastp. Nine translated ORFs had both over 40% identities and query coverage when aligned with VP14. The nine translated ORFs were aligned with carotenoid cleavage dioxygenases from *Arabidopsis thaliana*, *Crocus sativus*, *Hordeum vulgare*, *Pisum sativum*, *Solanum lycopersicum* and VP14 using Geneious (Geneious MUSCLE alignment, default values) to identify if the sequences belonged to the NCED family, or another CCD family (Appendix 4.1).

Table 4.1. BLAST with VP14 - results with more than 150 matching amino acid (AA) residues

GenBank BAC Accession	**B73 RefGen_v3 dataset** Chromosome (match position)	**Associated Gramene Gene model**	**Identities**	**ORFs >1000 bp in flanking sequences**	**ORFs >1000 bp similarity[c] to VP14 (**AA length**)**	**Predicted product based on NCBI BLAST**	**cTP** (predicted AA length)
AC201886	1 (250953970 - 250954573)	GRMZM2G014392 'vp14'	600 / 604	3	1 (605) "Chr1#1"	NCED	Yes (23)
AC217286	1 (174551708 – 174552236)	GRMZM2G407181 'nced2'	442 / 529	3	1 **(431)** "Chr1#2"	NCED	No *(8)*
AC205109	2 (235245347- 235245869)	GRMZM2G408158 'nced4'	391 / 523	3	1 (576) "Chr2#1"	NCED	Yes (52)
AC199036	3 (87358843 - 87359322)	GRMZM5G858748 'nced3'	405 / 488	1	1 **(431)** "Chr3#1"	NCED	No *(8)*
AC209376	3 (148416489 - 148416746)	GRMZM2G361459*	182/258	1	0	-	-
AC190588	4 (159751171 – 15975168)	GRMZM2G110192 'nced6'	213/514	3	1 (639) "Chr4#1"	CCD4	Yes (50)
AC195932	5 (16869346 – 16869950)	GRMZM5G838285 'nced9'	564 / 605	1	1 (602) "Chr5#1"	NCED	Yes (23)
AC194862	5 (200743351 - 200743867)	GRMZM2G150363 'nced8'	218 / 517	1	1 (718) "Chr5#2"	CCD4	No *(76)*
AC205347	6 (41884182 – 41884518)	GRMZM2G362993*	257 / 337	0	0	-	-
AC212820	7 (5981551 – 5982074)	GRMZM2G417954 'nced5' / 'nced10'	391 / 524	1	1 (574) "Chr7#1"	NCED	Yes (46)
AC194135	7 (175907867 – 175908150)	GRMZM2G330848 'nced7[a]'	198 / 284	2	1 **(361)** "Chr7#2"	NCED	No *(52)*
AC188718	7 (125606400 - 125606683)	GRMZM2G012410[b]	196 / 284	1	0	-	-

*Red text indicates working gene models [a] Gene model contains an intron [b] Transposable element [c] >100 AA identity.

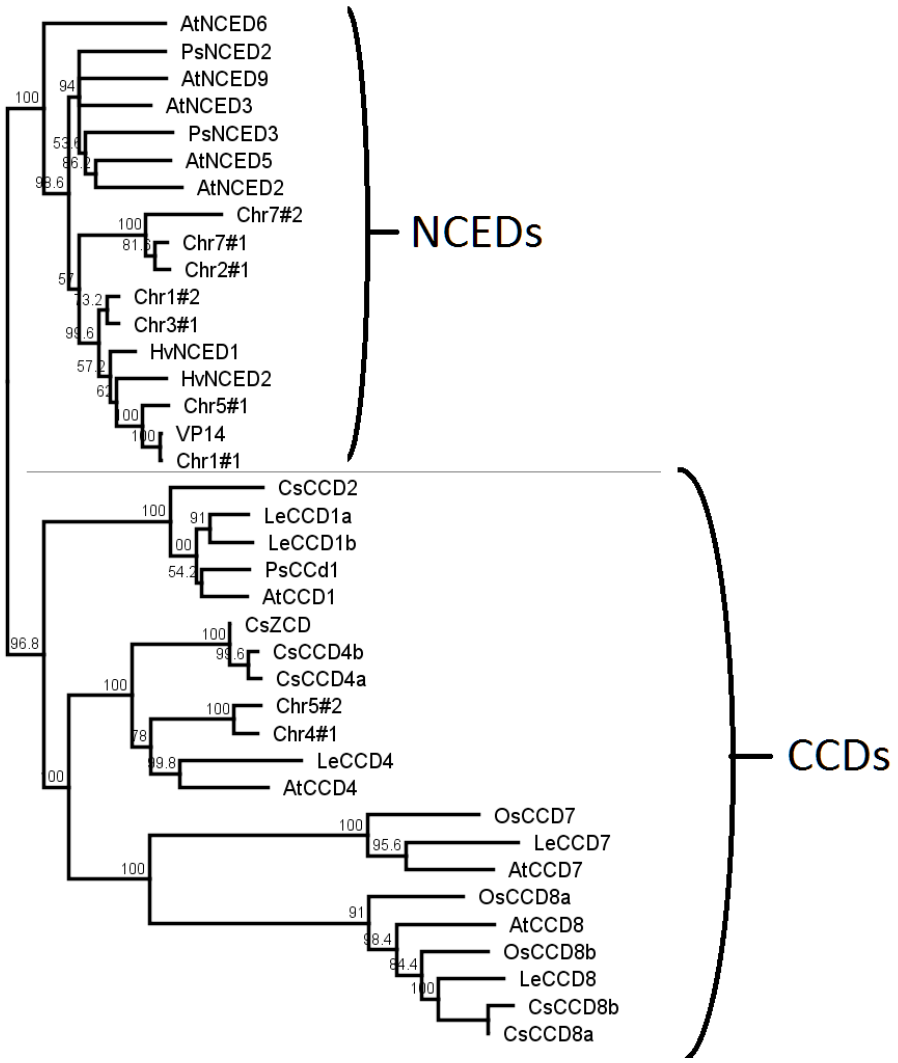

Figure 4.1. Phylogenetic analysis of putative maize NCEDs. Protein sequences were aligned using Geneious MUSCLE alignment (Appendix 4.1). Jukes-Cantor genetic distance model and Neighbour-Joining tree build method were used with 500 bootstrap replicates. Bootstrap support above 40% is indicated at nodes. Accessions used: *Arabidopsis thaliana* (*At*CCD1, O65572; *At*CCD4, O49674; *At*CCD7, Q7XJM2; *At*CCD8, Q8VY26; *At*NCED2, O49505; *At*NCED3, Q9LRR7; *At*NCED5, Q9C6Z1; *At*NCED6, Q9LRM7; *At*NCED9, Q9M9F5), *Crocus sativus* (*Cs*CCD2, ACD62475; *Cs*CCD4a, ACD62476; *Cs*CCD4b, ACD62477; *Cs*CCD8a, AIF27229; *Cs*CCD8b, AIF27230; *Cs*ZCD, CAD33262), *Hordeum vulgare* (*Hv*NCED1, ABB71582; *Hv*NCED2, ABB71584), *Solanum lycopersicum* (*Le*CCD1a, NP_001234542; *Le*CCD1b, NP_001233838; *Le*CCD4, XP_004246005; *Le*CCD7, NP_0123433; *Le*CCD8, NP01266276), *Oryza sativa* (*Os*CCD7, Q7XU29, *Os*CCD8a, Q93VD5; *Os*CCD8b, Q8LIY8); *Pisum sativum* (*Ps*CCD1, Q8LP17; *Ps*NCED1, BAC10550; *Ps*NCED2, BAC10551), *Zea mays* (VP14, AAB62181) and putative maize NCED ORFs identified in Table 4.1 ('Chr*x*#*y*').

Seven of the sequences shared most similarity with NCEDs, and the remaining two sequences shared similarity with CCD4 (Figure 4.1). When aligned with VP14, it was apparent that three of the seven amino acid sequences were too short (Chr1#2, Chr3#1 and Chr7#2), missing some of the conserved N-terminus of the protein, including a conserved α-helix 1 (Figure 4.2) and a chloroplast targeting peptide (Table 4.1).

Figure 4.2. Alignment of putative maize NCEDs with VP14. Sequences of translated putative maize *NCED* ORFs (see table 4.1) were aligned with annotated VP14 (Messing, *et al.*, 2010) using Geneious MUSCLE alignment. Arrows (a) indicate start of short maize NCED ORFs, missing the conserved alpha helix domain (b). Chr7#2 translated ORF has extended and non-NCED homologous C-terminus (c). Purple cylinders indicate alpha helices; blue arrows indicate turns, and yellow arrows indicate beta-sheets. Consensus agreements are highlighted.

It was thought, therefore, that either the maize B73 reference sequence was erroneous (i.e. the ORF N-terminus was truncated due to poor sequence and missing start codon), or that these loci did not contain functional *NCED* genes. Therefore more evidence was sought to verify the reference, by using the sequences at the identified loci as query in NCBI BLAST to find homologous nucleotide sequences

from *Zea mays* (Appendix 4.2). For Chr3#1, there was an obvious gap in the sequence upstream of the truncated ORF, however using NCBI BLAST, a homologous full-length cDNA was found for this sequence (NM_01154055), which contained an ORF coding for a full length NCED protein including the conserved α-helix 1 and chloroplast targeting peptide (Table 4.2).

Table 4.2. Identity and nomenclature of maize *NCED*s identified in this and previous studies.

Putative NCED ID	**Location** Chromosome (position, Mb)	**Vallabhaneni *et al*., 2010**	**Capelle *et al*., 2010**	**Here**	**cTP length** (AA)
Chr1#1	1 (251)	***NCED1***	***NCED1***	***ZmNCED1***	23
Chr1#2	1 (175)	-	***NCED2***	*ZmNCEDx*	N/A
Chr2#1	2 (235)	***NCED3b***	-	***ZmNCED3b***	52
Chr3#1	3 (87.4)	***NCED2***	***NCED3***	***ZmNCED2***	65
Chr5#1	5 (16.9)	***NCED9***		***ZmNCED9***	23
Chr7#1	7 (5.98)	***NCED3a***	-	***ZmNCED3a***	46
Chr7#2	7 (176)	-	-	*ZmNCEDy*	N/A

Chr1#2 was also contained a truncated NCED ORF, but there was no obvious error in the B73 reference sequence. Translating the reference sequence upstream of the ORF produces an amino acid sequence homologous to VP14, including the α-helix 1 domain, but an upstream start codon is missing. BLAST results provided no additional coverage of this N-terminus-coding region, but expressed sequence tags (ESTs) confirmed the sequence within the identified, truncated, ORF. Additionally one EST (EB816224) and full-length cDNA (NM_001196156) were almost identical to the reference except containing a 147 bp gap, perhaps indicated the presence of an intron. Whilst the translated ORF would remain in-frame, 49 amino acids containing conserved regions in NCED would be missing. Therefore it seems unlikely that this locus is a functional *NCED* gene. Chr7#2 also exhibits a further truncated N-terminus and a slightly longer C-terminus not homologous to other NCEDs. There is no obvious error in the reference. Translation of upstream sequence does not produce amino acid sequence homologous to NCED in any frame, and all frames contain stop codons in this region. BLAST results provided no additional coverage of the upstream region, and no cDNAs homologous to the identified ORF were found, indicating that the Chr7#2 locus is unlikely to contain a functional NCED gene.

4.2.2 Comparison to previously identified maize NCEDs

Two previous studies claim to identify five maize *NCED* genes (Capelle, *et al.*, 2010; Vallabhaneni, *et al.*, 2010). However, the study by Capelle, *et al.* 2010

identifies three *NCED*s (one of which is *Vp14*) similar to *NCED*s from other monocot species, and closely related to other dicot species; and two *NCED*s which appear to be highly divergent, but that have homologs in rice. In contrast, the study by Vallabhaneni, *et al.*, 2010 identifies five putative maize *NCED*s (one of which is *Vp14*), which are all very similar to *NCED*s in other monocots, and closely related to *NCED*s in dicot species. A closer look at the GenBank or EMBL database accessions used by both studies reveals that Capelle, *et al.*, 2010 may have incorrectly identified *CCDs* as *NCED*s (Table 4.2). Indeed the putative *NCED* loci identified here are the same as previously identified by Vallabhaneni *et al.*, 2010. However, here a full *ZmNCED2* sequence was identified (Table 4.2).

4.2.3 Expression of identified NCEDs in previous studies and available RNA-seq or microarray data

Because putative maize NCED loci were identified it was possible to query existing expression data-sets, particularly the RNA-seq data processed at qTeller (http://qteller.com) and the microarray data processed on the maize eFP browser (bar.utoronto.ca/maizeefp/). The gene models used to query expression for each are shown in Table 4.2. A summary of the expression data seen in the eFP browser (Appendix 4.3), qTeller (Appendix 4.4), and two previous studies is provided in Table 4.3. Although the expression data does not include treatments directly relevant to the study of maize cold germination, the data do show that the maize *NCED*s identified are expressed in a wide range of tissues. Particularly noteworthy are that: *ZmNCED1* appears to be the most abundant *NCED, ZmNCED2* is specifically expressed in drought-stressed ovaries and expression of *ZmNCED3a* is induced in drought-stressed leaf meristems. All five of the *ZmNCED*s that were predicted to be functional seem to be expressed in some tissue. *ZmNCEDx* which was not predicted to be functional appears to exhibit low level and variable expression. Similarly *ZmNCEDy* was not predicted to be functional and shows no expression in the data used by qTeller except some variable expression for in bundle sheath cells and mesophyll cells at very low levels compared to the other *NCED*s. Data are not available for *NCEDy* on the eFP browser. These data support the argument that the five identified *ZmNCED*s may have roles in maize whereas *ZmNCEDx* and *ZmNCEDy* may not be functional as *NCEDs*.

Table 4.3. Summary of expression of identified *NCED* genes in previous data.

Gene	Vallabhaneni (2010) (B73)	Capelle (2010)	qTeller (RNA-Seq data)	eFP Browser	Notes
***ZmNCED1* (*VP14*) *ZmNCED1* (Capelle)**	Most prevalent in most tissues The most expressed *NCED* in root, leaves, embryo, endosperm (20 DAP) and second most in endosperm 25 DAP.	The most expressed *NCED* in developing seed at 40-60 DAP. *Colocates with leaf ABA and water QTLs, and kernel DW,FW and water QTLs*	Expressed **widely** at relatively high levels. Increases in drought stressed leaf meristems Notable expression in silk	Expressed widely at relatively high levels. High in germinating **seed/ root** 6 DAS. High in 13th leaf (VT, R2). High 24DAP in embryo.	Appears to be the most prevalent NCED
***ZmNCED2* *ZmNCED3* (Capelle)**	Minor expression in leaves, roots and developing seed,	Second most expressed *NCED* in embryo and endosperm 40 – 60 DAP *Colocates with leaf ABA QTL*	Appears to be key in ovaries, especially in **drought stressed ovaries**	All tissues **low expression** with **less root/shoot specificity** than NCED1.	Probably has at least one specific role in ovaries and drought stress
***ZmNCED3a* *ZmNCED4* (Capelle)**	Second most expressed in leaves (50% of *NCED1* level).Joint most expressed in roots.	*Couldn't design primers of sufficient quality	Induced in drought stressed **leaf meristems** similar to NCED1 Notably high expression in **seedling roots**.	Higher expression in **primary root (V1)** and some root 6DAS. Otherwise all round moderate-low expression.	May have a role in roots
ZmNCED3b	Minor expression in root and embryo 20 DAP	- Not studied	Relatively low expression with wide deviations. Increased expression in seedling roots Induced in drought stressed ovaries	All round low expression with some preference to root over shoot 6 DAS and V1.	No obvious major roles
ZmNCED9	Most expressed *NCED* in endosperm 25 DAP, second most expressed in seed at 20 DAP and expressed in roots and leaves.	- Not studied	Widely expressed like NCED1 but at lower levels. High in embryo 14DAP compared to other tissues	All round v.low expression with minor root preference V1.	Perhaps roles in seed development.
***ZmNCEDx* *ZmNCED2* (Capelle)**	- Not studied	Minor expression in developing seed. *Colocates w/ kernel and leaf water and kernal ABA QTLs, but near Aquaporin.*	***Has relatively very low expression with large variation***	All round low expression. Some shoot specificity V1. Highest expression in tip of leaf V5, V7 and in Seed pericarp 18DAS.	Low levels in all currently studied tissues and large errors may suggest this is not a fully functional gene.

DAP: Days after pollination; DAS: Days after sowing; DW: Dry weight; FW: Fresh weight; VT, R2, V1, V5, V7: Stages of maize development (Sekhon, *et al.*, 2011)

4.3 Cloning of Maize *NCED*s

4.3.1 Construct design for heterologous expression of ZmNCEDs

For *in vitro* enzyme inhibition assays, maize *NCED*s were cloned and expressed as glutathione S-transferase (GST)-fusion proteins in *E. coli*. VP14 is targeted to the chloroplast membranes by an N-terminal chloroplast targeting peptide (cTP) sequence, which is then cleaved to yield the mature form of the protein (Tan, *et al.*, 2001). The mature form of the NCED protein, lacking the cTP exhibits more efficient cleavage of 9-*cis*-violaxanthin than the protein retaining the cTP (Qin and Zeevaart, 1999), therefore it was decided to clone *ZmNCED*s lacking the N-terminal cTP coding sequence. ChloroP was used to predict the length of the chloroplast targeting peptide from the *Zm*NCED amino acid sequences (Table 4.2). Primers were designed to amplify from the coding sequence immediately adjacent to the N-terminus cTP coding sequence. Primers (Table 4.4) had 5′ extensions including a restriction enzyme recognition site (*Eco*RI and *Bam*H1 restriction sites for forward and reverse primers respectively) and an additional 4-5 nucleotides to increase efficiency of cleavage (recommended by the manufacturer of the restriction enzymes). This was to allow for insertion into pGEX-4T-1, linearized using the same enzymes. The final designed constructs allowed for lactose-inducible expression of NCED ORFs lacking cTP and fused to GST (with a thrombin cleavage site) at the NCED N-terminus (GST-*Zm*NCED). The expression cassettes were flanked by universal primers for sequencing, and the plasmid contained the *AmpR* ampicillin resistance gene for selection of transformed bacteria (Figure 4.3).

4.3.2 Amplification and cloning of ZmNCED2, ZmNCED3a and ZmNCED9

A high fidelity DNA polymerase was used to amplify DNA to decrease the chance of cloning mutated DNA fragments. B73 genomic DNA was amplified with QIAGEN HotStar High-Fidelity DNA polymerase with a range of annealing temperatures (55°C to 72°C) yielding bands consistent with the size of fragments of *ZmNCED2*, *ZmNCED3a*, *ZmNCED3b* and *ZmNCED9* but not *ZmNCED1*, and also low molecular weight non-specific products. A 69.4°C annealing temperature was optimal for the four amplified fragments, although the band for *ZmNCED3a* fragment was weaker than the rest (Figure 4.4).

Table 4.4. Primers used to amplify *ZmNCED*s for cloning purposes.

Target (expected size, kb)	Direction	Primer sequences	Predicted T_m (°C)[a]
ZmNCED1 **(1.77)**	Forward	**ACGT**GGATCCGCCTCCAATTCCGTCAGGTTC	55.5
	Forward (2)	**ATAT**GGATCCGCCTCCAATTCCGTCAGGTTCTCGCC	63.3
	Reverse	**ATCAT**GAATTCTCAGGCCGCCTGGGCCTCGAG	64.9
	Reverse (2)	**GAGTC**GAATTCTCAGGCCGCCTGGGCC	64.6
ZmNCED2 **(1.72)**	Forward	**ATTA**GGATCCGCAGCGGCAGCGGCCAACTCG	65.9
	Reverse	**AGTCG**GAATTCTCAGGCCTGGGCCTCTAGCTC	59.4
ZmNCED3a **(1.61)**	Forward	**ATTA**GGATCCGCCGCCGCTCCCAAGTGGAACCCG	68.0
	Forward (2)	**ATCT**GGATCCGCCGCCGCTCCCAAGTGGAAC	68.7
	Reverse	**ACGTA**GAATTCCTAGGCCTGCCGCTGCAGCTC	62.1
ZmNCED3b **(1.59)**	Forward	**ATCT**GGATCCGCGGCCGCCAAGTGGAACCC	63.3
	Reverse	**ATCAT**GAATTCCTAGGCCTGCCGCTGCAGCTC	62.1
ZmNCED9 **(1.76)**	Forward	**ACGT**GGATCCGCCTCCAACTCCGTCAGGTTC	57.1
	Reverse	**ACTG**GAATTCTCAGGCCTGGGCCTCGAGCTC	62.3

5′ extensions in **bold underlined**. ***BamHI*** and ***EcoRI*** restriction sites highlighted in blue and red, respectively. [a]Predicted melting temperature of primer (without 5′ extenstion) calculated by Primer3 in Geneious. (2) Indicates redesigned, second set, of primers.

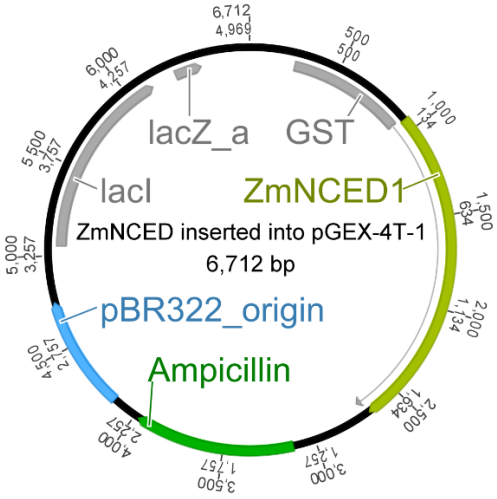

Figure 4.3. *ZmNCED* inserted in to pGEX-4T-1. The diagram illustrates *ZmNCED1* inserted into pGEX-4T-1 using *Bam*HI and *Eco*RI restriction sites present in the vector and added by primers to the *NCED* fragment. The plasmid harbours a gene coding for resistance to ampicillin and a lactose inducible expression system for expression of the GST-fusion protein.

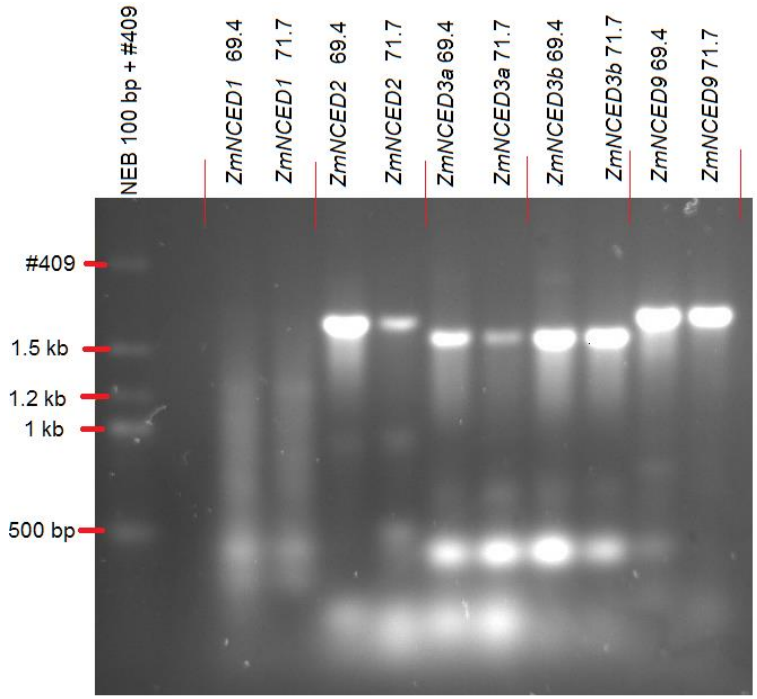

Figure 4.4. Amplification of *ZmNCED* fragments. DNA was extracted from maize B73 and amplified in a two-step reaction with Hotstar high-fidelity DNA polymerase (QIAGEN) using primers designed to amplify *ZmNCEDs 1, 2, 3a, 3b,* and *9*. NEB 100 bp ladder and plasmid #409 were used as markers. #409 represents the uncut pGEX-4T-1 plasmid (smallest band is at ~2.5 kb, shown). Fragments were separated by agarose gel electrophoresis and stained with Safe view (NBSBio) and imaged under UV trans-illumination. Annealing temperatures are indicated at the top of the lanes.

The reaction was repeated at larger scale (50 µl), and was purified (Section 2.6.3), before digestion with *Bam*HI and *Eco*RI (NEB). Plasmid DNA was purified (Section 2.6.2) from *E.coli* harbouring pGEX-4T-1 and also digested with *Bam*HI and *Eco*RI. Plasmid and PCR fragments were separated by agarose gel electrophoresis and visualised by UV trans-illumination (photographs were not taken to minimise exposure of DNA to UV). The pGEX-4T-1, *ZmNCED2, ZmNCED3b* and *ZmNCED9* fragments were observed, but *ZmNCED3a* was not visible. The digested *ZmNCED* and pGEX-4T-1 fragments were excised from the gel and purified (Section 2.6.3). Fragments of *ZmNCED2, ZmNCED3b* and *ZmNCED9* were ligated, separately, with the pGEX-4T-1 fragment and used to transform *E.coli* (Section 2.6.10). Transformation of *E.coli* with the ligation reactions of *ZmNCED2*, *ZmNCED3b* and *ZmNCED9* yielded 12, 4 and 8 ampicillin resistant colony forming units (CFU), respectively whereas transformation of *E.coli* with *Eco*RI and *Bam*HI digested pGEX-4T-1 yielded 1 CFU. Colonies of presumed pGEX-4T-1:*ZmNCED* transformed *E.coli* were cultured in liquid medium from which plasmid DNA was purified.

4.3.3 Verification of ZmNCED2, ZmNCED3b and ZmNCED9 clones

To verify that the purified plasmids harboured *ZmNCED* fragments and the *ZmNCED* identity before samples were sent for sequencing, an analytical digestion using enzymes *Pst*I and *Xho*I was designed to discriminate between plasmids harbouring the different *ZmNCED* fragments upon agarose gel electrophoresis (Table 4.5; figure 4.5); the results are shown in Figure 4.6.

Plasmid DNAs were sequenced using pGEX 5′ and pGEX 3′ universal primers. In pGEX-4T-1:*ZmNCED2* from colony '2-5', a synonymous substitution (a G instead of a C) was found at a position equivalent to base 648 in NM_001154055, with both codons coding for alanine. Therefore plasmid DNA extracted from colony '2-2' was also sequenced with the same primers. The sequence had the same synonymous mutation. Plasmid DNA extracted from a third colony, '2-8,' also had the same substitution, suggesting that NM_001154055 contains a sequencing error, or that it is due to a cultivar-specific polymorphism.

Nonetheless the substitution is synonymous and has no predicted effect on translated sequence, meaning the protein product should be usable for *in vitro* enzyme studies. The sequence of the *ZmNCED3b* fragment insert in pGEX-4T-1: *ZmNCED3b* extracted from colonies '3b-2' and '3b-4' matched the MaizeGDB B73 reference sequence. The sequence of the *ZmNCED9* fragment insert in pGEX-4T-1: *ZmNCED9* extracted from colonies '9-2' and '9-6' also matched the MaizeGDB B73 reference sequence. Therefore cloning of *ZmNCED2*, *ZmNCED3b* and *ZmNCED9* fragments was successful, however optimisation of PCR of *ZmNCED1* and *ZmNCED3a* fragments was required for successful cloning.

Table 4.5. Predicted fragments produced by digesting pGEX-4T-1:*ZmNCED*s with *Pst*I and *Xho*I. Calculated fragment product sizes (base pairs) from restriction digestions.

	No digest	***Pst*I**	***Xho*I**	***Pst*I + *Xho*I**
pGEX-4T-1	4969	4973	4974	4009, 968
pGEX-4T-1-*ZmNCED1*	6712	6716	5720, 964, 40	4756, 948, 964, 40
pGEX-4T-1-*ZmNCED2*	6664	4129, 2543	6668	4129, 1579, 968
pGEX-4T-1-*ZmNCED3a*	6550	5557, 1001	5039, 712, 520, 295	4075, 968, 712, 520, 262, 37
pGEX-4T-1-*ZmNCED3b*	6538	4951, 1001, 598	5552, 709, 289	4588, 968, 367, 346, 256, 37
pGEX-4T-1-*ZmNCED9*	6703	5014, 1697	5198, 520, 505, 463, 37	4234, 968, 520, 463, 268, 241, 37

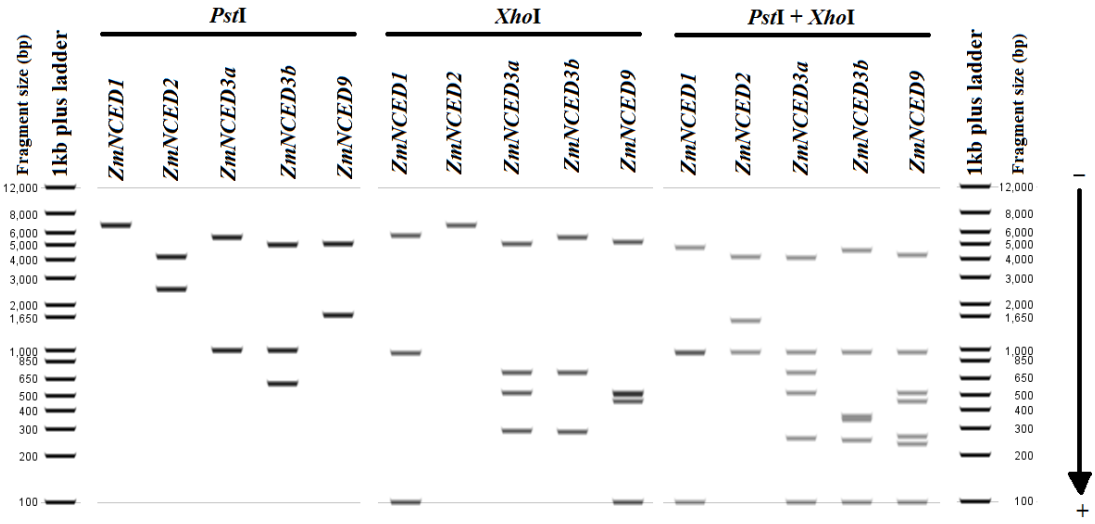

Figure 4.5. Predicted fragments of PstI and XhoI digestion of pGEX-4T-1: *ZmNCED* constructs separated by gel electrophoresis. Constructs were assembled and digested with *Pst*I, *Xho*I or *Pst*I and *Xho*I *in silico* using Geneious. Fragment sizes are indicated in table 4.5.

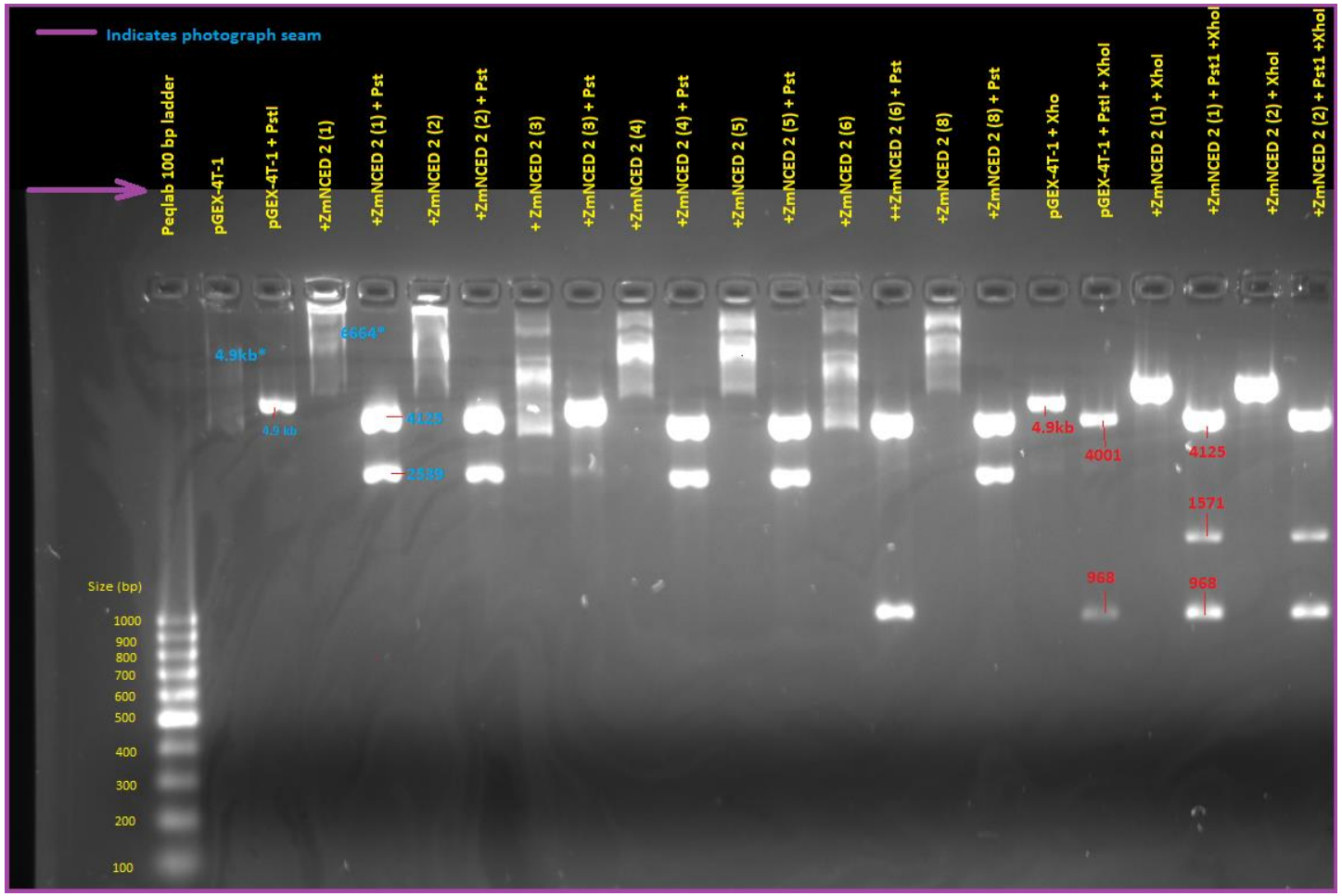

Figure 4.6. DNA fragments produced by digestion of purified plasmid DNA with *Pst*I and *Xho*I.
Plasmid DNA purified from transformed *E. coli* was digested with PstI and/ or XhoI. DNA fragments in the reactions were separated by gel electrophoresis. Peqlab 100 bp ladder sizes are indicated in yellow. Linearized pGEX-4T-1 has a known size of 4.9 kb. Deduced fragment sizes (based on predictions in Table 4.5 and Figure 4.5.) are annotated. (Part 1 of 3). Purple line represents where photo was cropped or spliced.

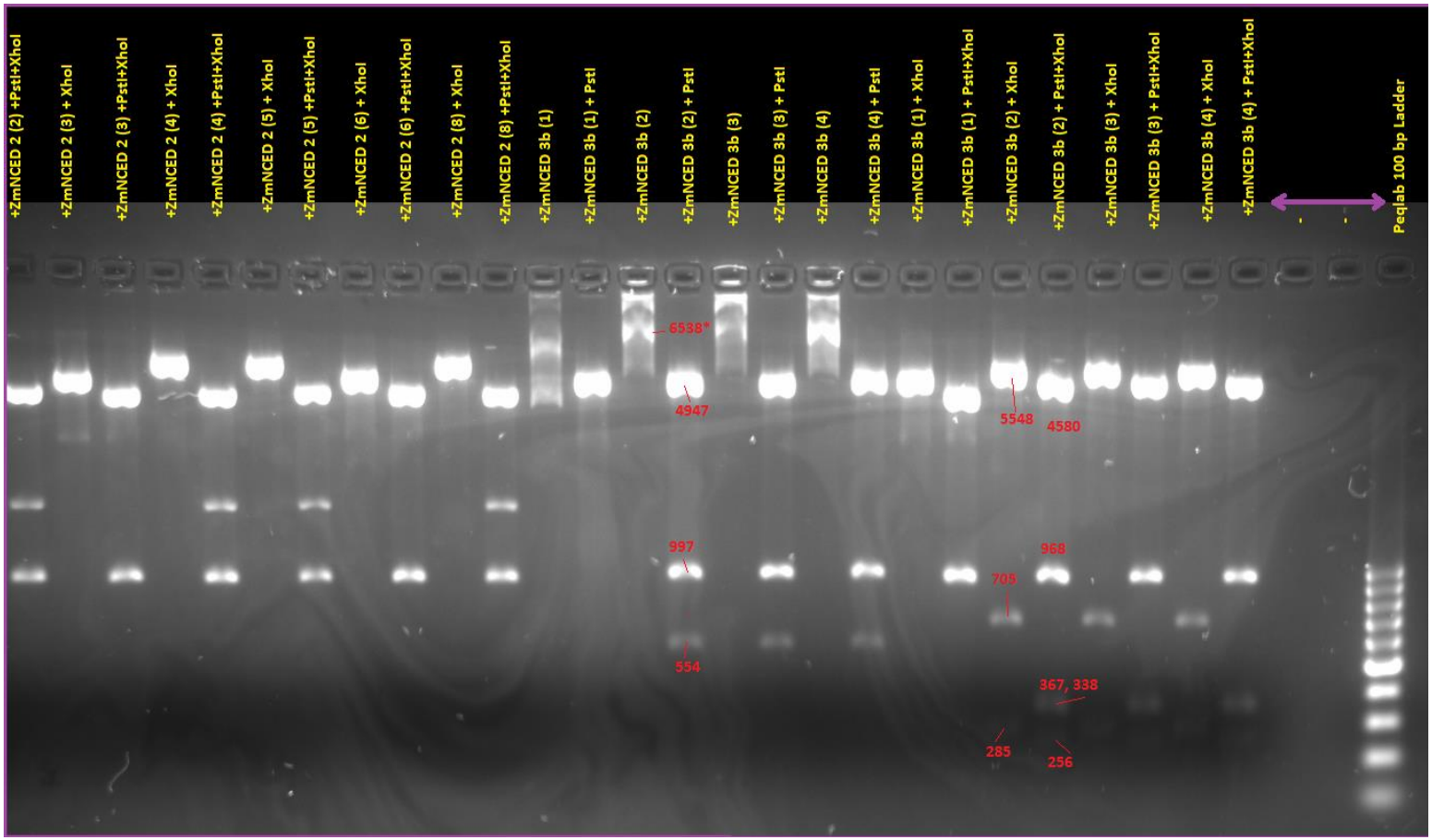

Figure 4.6. (Part 2 of 3).

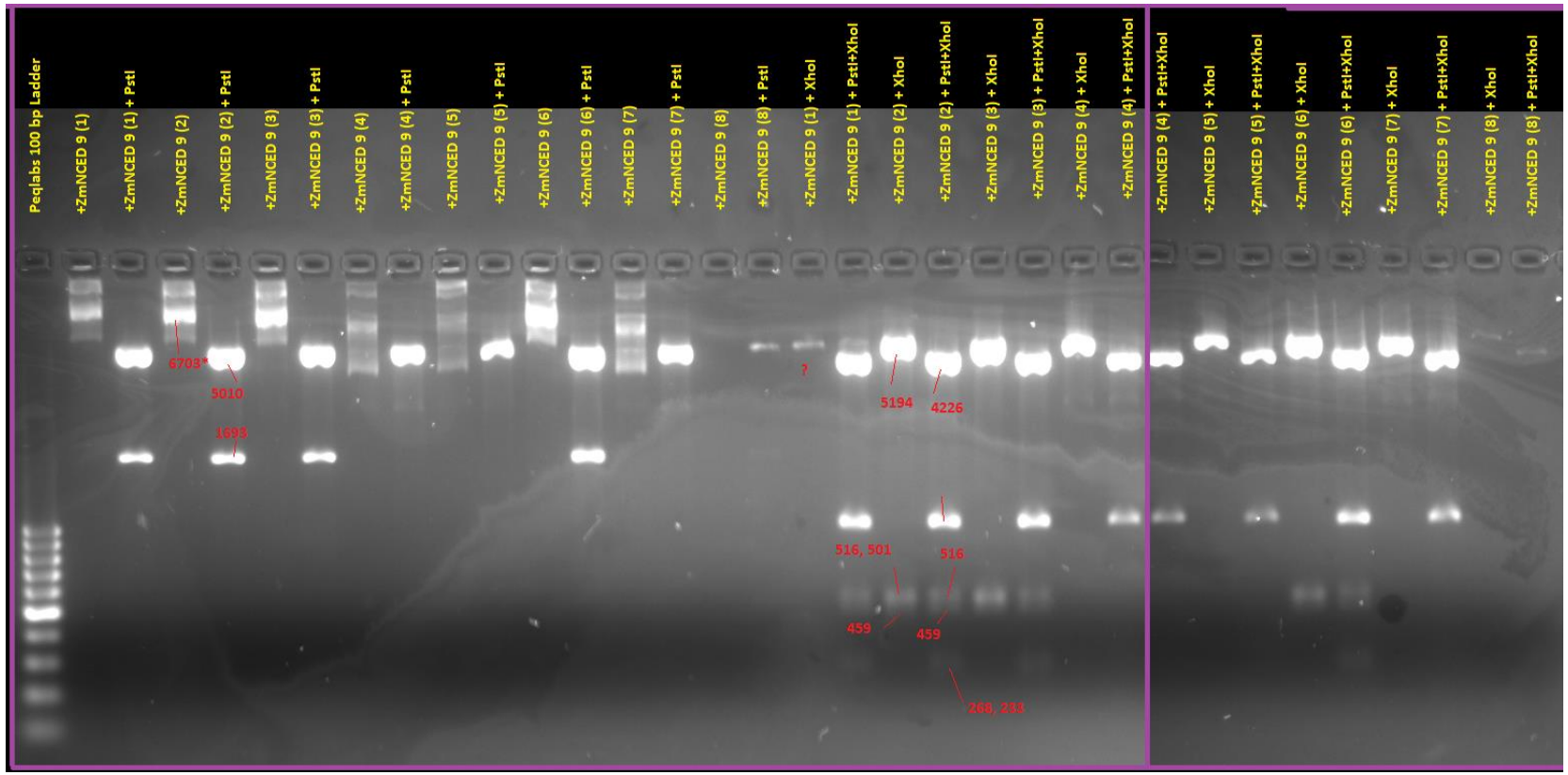

Figure 4.6. (Part 3 of 3).

4.3.4 *Amplification and cloning of ZmNCED1 and ZmNCED3a*

Since PCR was previously successful for *ZmNCED2*, *ZmNCED3b* and *ZmNCED9*, but only sometimes for *ZmNCED3a* and not at all for *ZmNCED1*, new primers were designed to amplify *ZmNCED1* and *ZmNCED3a* fragments (Table 4.4). PCR using the previously optimised conditions for amplification of *ZmNCED* fragments with HotStar High-Fidelity DNA polymerase were used. However, combinations of the new and old primers for the *ZmNCED1* and *ZmNCED3a* targets were used, with annealing temperatures of 55°C to 72°C. A combination of the new forward primer and old reverse primer for *ZmNCED1* produced a fragment of the expected size, however the band was still faint amongst the non-specific products. Use of either the old or new forward primer with the old reverse primer to amplify *ZmNCED3a* produced a fragment of the expected size (Figure 4.7).

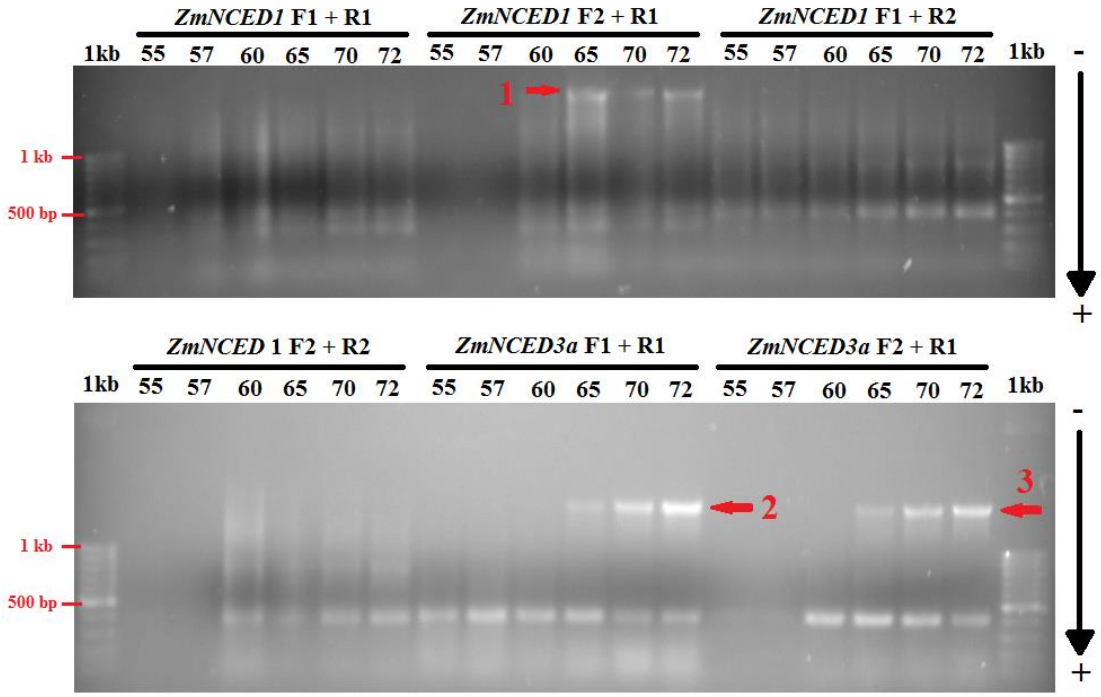

Figure 4.7. Gel electrophoresis of PCR products using new primers targeting *ZmNCED1* and ZmNCED3a. PCR was carried out with combinations of new and old primers (Table 4.4) using the default HotStar High-Fidelity DNA polymerase conditions with annealing temperatures of 55°C to 72°C (indicated above lanes). 1. Predicted *ZmNCED1* fragment; 2,3. Predicted *ZmNCED3a* fragment.

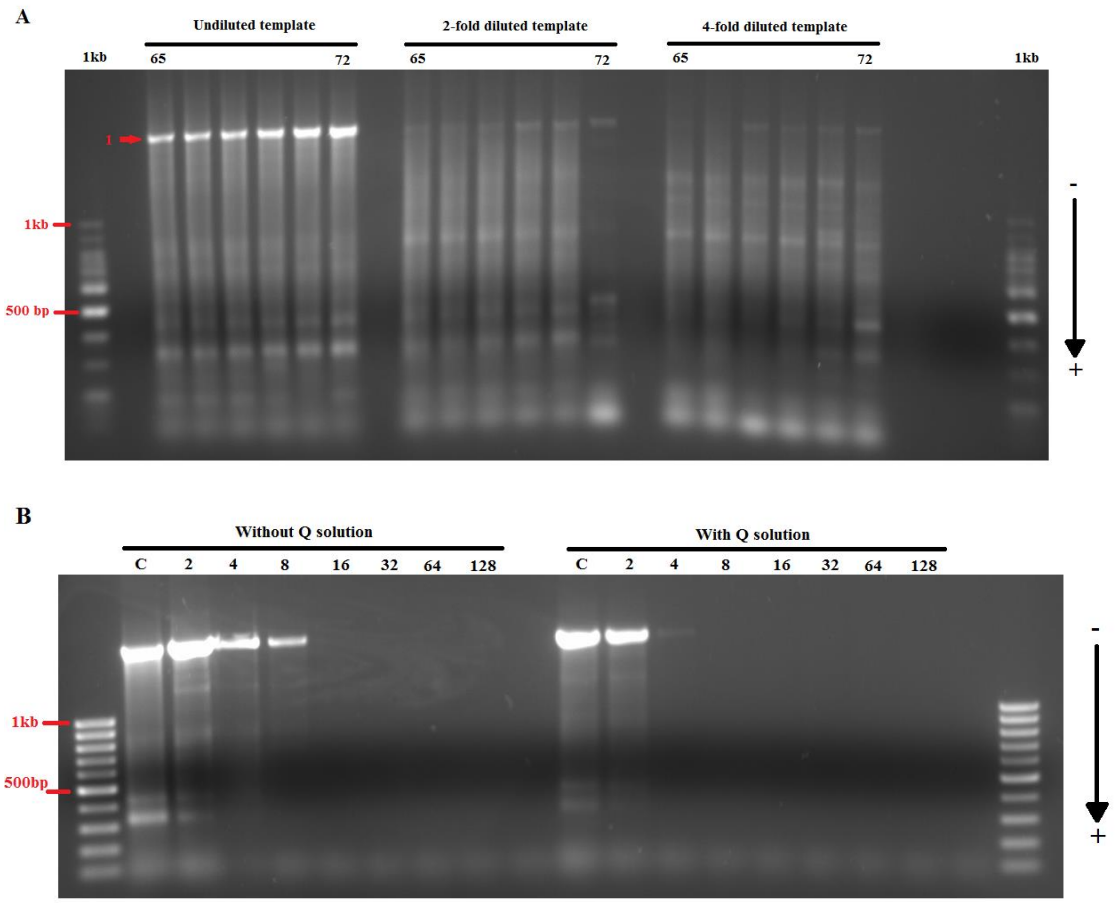

Figure 4.8. Gel electrophoresis of PCR targeting *ZmNCED1* with varied template and primer concentrations, and with Q-solution. A. PCR was performed using the default HotStar High-Fidelity conditions except template DNA (initially ~2 ng μl^{-1}) was diluted up to 4-fold before addition to the reaction and annealing temperatures of 65°C to 72°C were used (indicated above lanes) 1. Predicted *ZmNCED1* fragment. **B.** PCR was performed using the default HotStar High-Fidelity conditions except primers were diluted up to 128 fold (2 – 128, C – undiluted, 1 µM each of each primer) before addition to the reaction and an annealing temperature of 72°C was used. Also Q-solution was added as recommended by the manufacturer to some reactions (indicated above lanes).

PCR of *ZmNCED1* fragments was further optimised using the new forward primer and old reverse primer. Firstly, the concentration of template was altered by diluting the purified B73 genomic DNA, using annealing temperatures of 65°C – 72°C (Figure 4.8 A). Diluting the template reduced the amount of the *ZmNCED1*-sized fragment being produced, although 2-fold diluted template appeared to reduce the low molecular weight smear at 72°C annealing temperature. Undiluted template with a 72°C annealing temperature was considered optimal.

These conditions were further modified by altering primer concentrations and by adding Q-solution to the reaction (Figure 4.8 A). Addition of Q-solution reduced the amount of product in reactions with 4-fold or more diluted primers, but with 2-fold

diluted primers, the amount of specific product was maintained, and the non-specific products were greatly reduced. A halved primer concentration (0.5 µM) in the presence of Q-solution with an annealing temperature of 72°C appeared to be optimal PCR conditions for amplification of the *ZmNCED1* fragment.

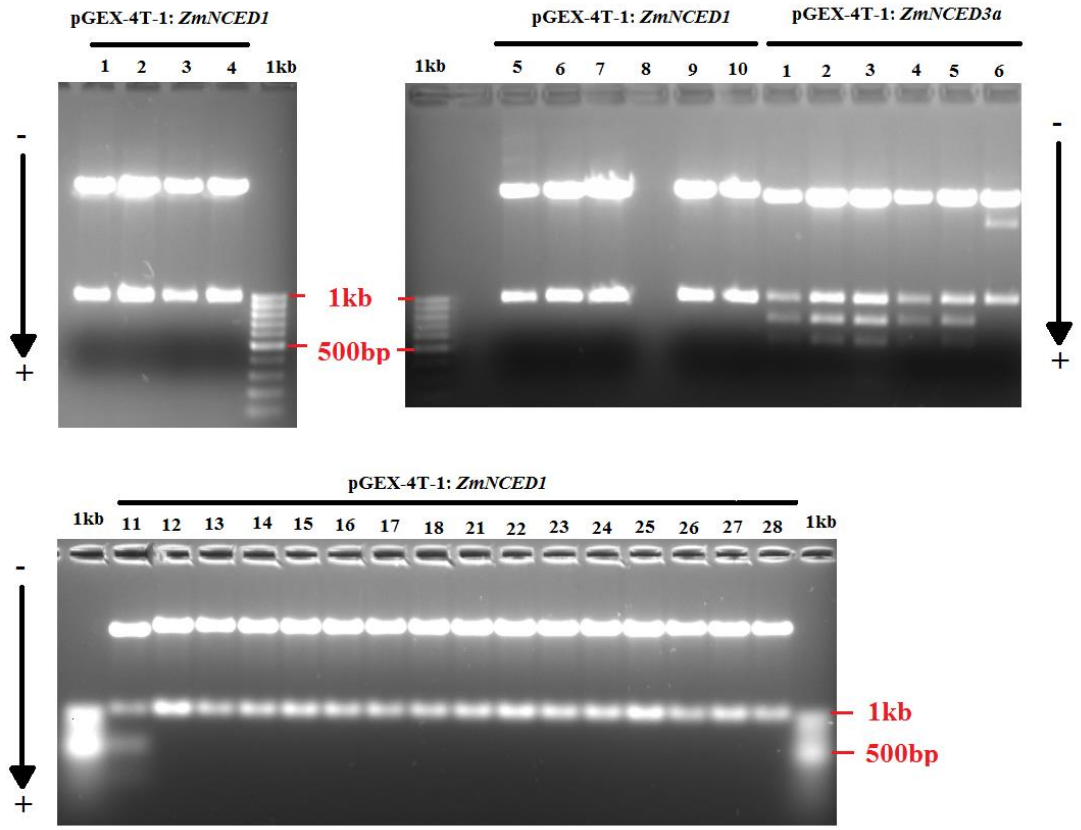

Figure 4.9. DNA fragments produced by digestion of purified plasmid DNA expected to harbour *ZmNCED1* with *Pst*I and *Xho*I DNA was extracted from maize B73 and amplified in a two-step reaction with Hotstar High-Fidelity DNA Polymerase (QIAGEN) using primers designed to amplify *ZmNCEDs 1, 2, 3a, 3b,* and *9.ZmNCED* PCR fragments and pGEX-4T-1 were digested with *Bam*HI and *Eco*RI, gel purified and ligated. NEB Turbo competent *E.coli* were transformed with the ligation products. Plasmids were isolated from ampicillin resistant colonies and were single or double digested with *Pst*I and *Xho*I. The gel is annotated to highlight the appearance of predicted band sizes, which indicate some cultures harbour the desired plasmid.

Thus fragments were purified, inserted in to pGEX-4T-1 and used to transform *E.coli* as previously described for *ZmNCED2*, *ZmNCED3b* and *ZmNCED9*. Transformation of 100 µl of NEB Turbo-competent *E.coli* with pUC19 yielded 3900 CFUs, whereas transformation with *ZmNCED3a* yielded six CFUs. *ZmNCED1* fragments from isolated different PCR reactions were inserted in to pGEX-4T-1 and used to transform *E.coli* during different stages the PCR optimisation procedure. *ZmNCED1* fragments isolated from the PCR conditions outlined in Figure 4.7, when used, yielded 17 CFU. *ZmNCED1* fragments isolated from the optimised PCR

conditions outlined in Figure 4.7 B, yielded 126 CFUs and 78 CFUs in two separate transformations of 100 µl NEB Turbo competent *E.coli*. A total of 6 colonies expected to harbour pGEX-4T-1: *ZmNCED3a* and a total of 26 colonies expected to harbour pGEX-4T-1: *ZmNCED1* were used to inoculate liquid cultures from which plasmid DNA was purified. Five of the six cultures expected to harbour pGEX-4T-1: *ZmNCED3a* and 24 of the 26 cultures expected to harbour pGEX-4T-1: *ZmNCED1* were confirmed by restriction digestion with *Pst*I and *Xho*I (Figure 4.9).

Plasmid DNA extracted from cultures *ZmNCED1* 12 and 13 were sequenced. Plasmid DNA from culture 12 had two mutations in the *ZmNCED1* fragment compared to the B73 reference, the first being a synonymous mutation, and the second being a nonsynonymous mutation changing a lysine to an isoleucine at *ZmNCED1* amino acid 581 (alignments with the predicted construct are shown in appendix 4.8). The sequence of plasmid DNA from culture 13 did not contain either of these mutations, and the *ZmNCED1* fragment was identical to the reference. Plasmid DNA extracted from cultures *ZmNCED3a* 4 and 5 were sequenced. Plasmid DNA from *ZmNCED3a* 4 contained a nonsynonymous mutation at *ZmNCED3a* amino acid 181, changing a highly conserved lysine to a methionine (AAG to ATG). Plasmid DNA from *ZmNCED3a* 5 did not contain this mutation and the sequence matched the B73 reference. Therefore all five identified *ZmNCED*s had been cloned in the pGEX-4T-1 expression vector and used to transform *E. coli*. Cultures *ZmNCED1* 13, *ZmNCED2* 2, *ZmNCED3a* 5, *ZmNCED3b* 4 and *ZmNCED9* 2 were sent to Peter Harrison (Department of Chemistry, University of Warwick) for use in *in vitro* ZmNCED inhibition assays.

4.4 *In vitro* ZmNCED Inhibition Assay Results

To test the effect of CCD inhibitors on the cleavage of 9′-*cis*-violaxanthin *in vitro*, *ZmNCED*s were expressed as a GST-fusion protein in *E. coli*, purified and assayed *in vitro*. *ZmNCED1*, ZmNCED2, ZmNCED3a, ZmNCED3b were purified by Peter Harrison, but ZmNCED9 could not be solubilised (Harrison *et al*., 2014). All four purified ZmNCEDs catalysed the cleavage of 9′-*cis*-neoxanthin to produce xanthoxin. Hydroxamic acid CCD inhibitors D2 and D4 were added to the reaction to observe inhibition of ZmNCED1, ZmNCED2, ZmNCED3a and ZmNCED3b (Figure 4.10).

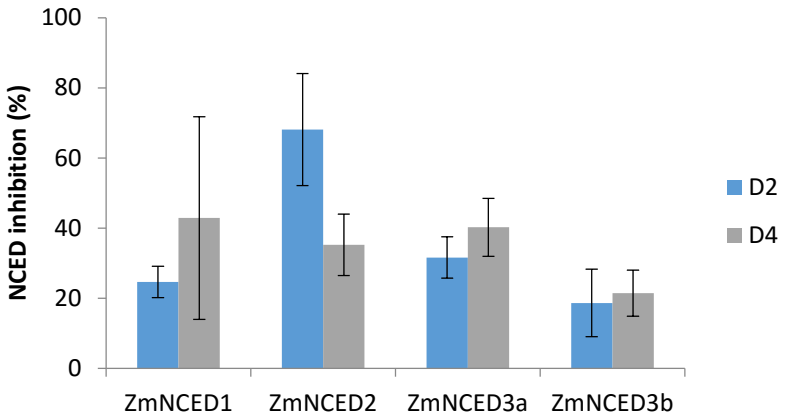

Figure 4.10. Hydroxamic acid inhibition of maize NCEDs *in vitro*. Percentage inhibition of activity (compared to control without inhibitor present) of *Zm*NCEDs *in vitro* by hydroxamic acid CCD inhibitors D2 and D4. Error bars indicate standard error. Expression, purification and *in vitro* inhibition assay of NCEDs was carried out by Peter Harrison. *ZmNCED*s were identified and cloned by Jake Chandler for this purpose.

D2 and D4 were found to moderately inhibit *ZmNCED*s (combined average ± standard error of 35±4%). Two-way ANOVA revealed no significant differences in the inhibition of the different *Zm*NCEDs (p = 0.143) or differences in the effect of hydroxamic acid compounds D2 and D4 (p = 0.928) (Appendix 6.4). Thus D2 and D4 have similar potency inhibition of *Zm*NCEDs *in vitro*, and do not inhibit a particular *Zm*NCED preferentially.

4.5 Discussion

4.5.1 Identification and cloning of ZmNCEDs

In this chapter, five maize *NCED* genes were identified using the maize B73 reference genome and other tools and datasets available online. The five maize genes identified here were the same as those identified by Vallabhaneni *et al*., 2010. Previously only a truncated genomic DNA sequence for *ZmNCED2* was identified (Vallabhaneni, *et al.*, 2010), whereas here a full length cDNA matching *ZmNCED2* was identified and used to clone the sequence coding for a mature protein. Additionally it was noticed that some of the five maize *NCED*s identified by Capelle *et al*., 2010 are not actually *NCED*s, but *CCD*s, and one was the truncated *NCEDx* identified here as a non-functional *NCED*. Two loci identified contained the truncated *NCEDx* and *NCEDy*, which are likely to be non-functional *NCED*s because they lack some of the N-terminus of the expected coding sequence, including a chloroplast transit peptide required for import of the NCED in to chloroplasts where the 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin substrates are present; and the

conserved α-helix which is required for the membrane-binding hydrophobic patch which would allow the enzyme access to its substrates (Messing, *et al.*, 2010). Of course, this was initially based on the assumption that the B73 reference is correct. However, unlike *ZmNCED2*, there did not appear to be any matching transcripts or expressed sequences tags, or extra coverage of the genomic DNA that contradicted the B73 reference sequences, in fact transcripts of *NCEDx* appear to have an intron. Known *NCED*s do not contain introns, and this intron would have also removed some of the conserved coding regions. *NCEDx* and *NCEDy* also seem to have low or no measured expression in the eFP browser and qTeller data (Appendix 4.4 and 4.5), and for this combination of reasons they were considered not to be functional *NCED* genes and were not studied further.

Review of the expression profiles of the other *ZmNCED*s revealed potential functions and roles, with expression in seed development and drought stress seen, which agrees with known *NCED* functions, although data was not available for cold imbibed seeds. Consistent with previous studies, it was found that the *NCED*s cluster in monocot and dicot clades, meaning it is not possible to infer roles of the five maize *NCED*s based on roles discovered for *NCED*s in dicots, particularly Arabidopsis (Vallabhaneni, *et al.*, 2010). However, some roles of *NCED*s in monocots have been studied, therefore it may be possible to infer roles based on these. Barley NCEDs have roles in hypoxia, high-temperature and blue-light induced dormancy (Hai Ha, *et al.*, 2014; Hoang, *et al.*, 2013; Leymarie, *et al.*, 2008); however further alignments may be necessary to determine NCED families within monocots, as Figure 4.1 does not appear to show clear homologs of *HvNCED1* and *HvNCED2* in maize. Roles probably diverge even with monocots / dicots as many species appear to have different numbers of NCEDs (e.g. barley appears to have two, maize five), indicating not every NCED has an equivalent homolog in another species.

The successful cloning of the *ZmNCED*s for expression as a GST-fusion protein was not straightforward, and particularly the amplification of *ZmNCED1* and *ZmNCED3a* fragments required several steps of PCR optimisation. This is probably, in part, due to the inability to alter primer locations because the amplification needed to begin immediately after the cTP coding region and to end at the stop codon. Additionally, not enough *Zm*NCED9 protein has yet been purified for *in vitro* assays

of enzyme inhibition by the hydroxamic acids. However, four out of five of the *Zm*NCEDs were successfully purified and assayed *in vitro*.

4.5.2 In vitro hydroxamic acid inhibition of ZmNCEDs

One of the main purposes of cloning and expressing *ZmNCED*s was to determine if hydroxamic acids specifically inhibited particular ZmNCEDs. This could allow the phenotypic effects of hydroxamic acids, e.g. the effect on germination observed in Chapter 3, to be attributed to inhibition of a particular NCED. In effect, the roles of particular NCEDs could be probed by identifying an inhibitor specific to that particular NCED. It was found here that hydroxamic acids D2 and D4 do moderately inhibit *Zm*NCEDs *in vitro* (ca. 35% at 100 µM). Thus effects of hydroxamic acids on maize germination could be through inhibition of *Zm*NCEDs. However, hydroxamic acids D2 and D4 did not specifically inhibit a particular *Zm*NCED, thus phenotypic effects cannot be attributed to a particular *Zm*NCED.

D2 and D4 also inhibit *Le*NCED1 *in vitro* (also by ca. 35% at 100 µM), and lack of specificity of inhibition of particular NCED homologues is not surprising because D2 and D4 also inhibit (>95% at 100 µM) other members of the CCD family including *Le*CCD1 (Sergeant, *et al.*, 2009); and it was more recently found that they also inhibit *At*CCD8 (Harrison, *et al.*, 2015). Nonetheless, Harrison, *et al.*, 2015 identified hydroxamic acid inhibitors that selectively inhibit CCD8 rather than other CCDs. Thus D2 and D4 exhibit unspecific inhibition within NCEDs, and perhaps preferentially inhibit other CCDs. D2 and D4 also do not inhibit *At*CCD7 (Harrison, *et al.*, 2015). Thus current hydroxamic acids do exhibit some selectivity between different enzymes of the CCD family, so there may be scope to identify NCED-specific inhibitors. Targeting of specific NCED homologues, however, could be limited due to the high conservation of amino acid sequences within NCEDs (usually more than 60%, even including the less conserved cTP sequence).

4.6 Future Work

During the identification of *NCED*s, some loci were found to contain partial, truncated *NCED* ORFs. Although, based on available sequence coverage of the loci, it is likely that *NCEDx* and *NCEDy* are not functional *NCED*s, it could be worth re-sequencing these regions (and cDNA product, if possible) to verify the reference sequence, and conclude that these do not code for functional *NCED*s. It may be

possible to verify this *in silico* by examining the same loci in sequences of different maize accessions. In addition to the qTeller and eFP Browser data already queried (where results are readily accessible), there are many other microarray and RNA-seq datasets in which *NCED* expression could potentially be analysed (although these are often raw sequence data which would require significant bioinformatics resources to analyse). An atlas of *NCED* expression data in maize could be created from which more functions of *NCED* could be hypothesised.

Identification, or design, of inhibitors (e.g. next generation hydroxamic acid compounds) that specifically target NCED much more than other CCDs would be a desirable accomplishment. The cloning and expression of *Zm*NCEDs, shown here, is a step towards achieving this objective in maize and it has shown that current hydroxamic acids do inhibit maize NCEDs. A next step, cloning and expression of *Zm*CCDs, would be complementary to the work done here, in order to determine the specificity of future CCD inhibitors in the target crop maize.

5 Development of a qPCR Assay to Measure Maize *NCED* Expression

5.1 Introduction

To continue investigating the role of ABA in maize germination under cold conditions quantification of the expression of the *ZmNCED*s was pursued. As is discussed in more detail in the main introduction (Section 1.7), NCED catalyses the rate limiting step in ABA biosynthesis and its expression is one of the key regulators of ABA signalling. In addition, it is the target of the hydroxamic acid CCD inhibitors, so a more complete understanding of its role may be gained through combined expression and chemical inhibition studies. In Chapter 3, application of CCD inhibitors suggested a role of NCED in germination inhibition of maize seed imbibed in cold conditions. Chapter 4 revealed that maize NCEDs are encoded by a multigene family with at least 5 different members.

5.1.1 Introduction to qPCR

The quantitative polymerase chain reaction (qPCR) method allows the measurement of the starting concentration of DNA template in a PCR reaction by measuring the number of cycles required before the DNA concentration in the reaction reaches a particular threshold. The higher the initial concentration of the template, the fewer the cycles that are required for the DNA concentration to surpass the threshold. Each cycle, assuming 100% efficiency, results in duplication of all target DNA and doubling of the concentration; for example, if sample A is half the concentration of sample B, it will require one extra cycle to reach the threshold. A sample that takes three more cycles to reach the threshold than sample A would be 8 (2^3) times less concentrated than sample A, and so on. The concentration of target DNA in the reaction can be measured by dyes which fluoresce only when bound to dsDNA (nucleotides and primers are not measured), such as SYBR Green, or by sequence specific probes which exhibit a quantifiable change in fluorescence characteristics when bound to the target (Higuchi, *et al.*, 1992; Higuchi, *et al.*, 1993; Wittwer, *et al.*, 1997). In this chapter, reverse transcription-qPCR (RT-qPCR) will be used to quantify the abundance of *ZmNCED* mRNA in maize seed, in which reverse transcription is used to generate *ZmNCED* DNA copies from which to perform qPCR (Bustin, 2000). Whilst this technique has become commonplace in many

modern biology laboratories there are a plethora of concerns that must be addressed (Bustin, *et al.*, 2009).

5.1.2 Concerns in RT-qPCR

5.1.2.1 RNA sample quality

The first concern after producing biological samples for analysis by RT-qPCR is the purification of intact RNA from the samples. RNA is very sensitive to the presence of RNases which are abundant in the sample, environment, and on the researcher (Ambion, 2012). In particular the endogenous RNases are a particular risk because lysis of cells, which typically occurs in the first step of extraction or upon freezing of the sample, exposes mRNA to these RNases. Therefore the integrity of RNA must be checked before further use. This can be done using more automated methods [e.g. Bioanalyzer (Agilent Technologies) or Experion (Bio-Rad)] or by denaturing gel electrophoresis (e.g. formaldehyde gel electrophoresis) (Fleige and Pfaffl, 2006). The RNA is assessed on the discrete electrophoresis of two dominant RNA species in eukaryotic RNA – the 25S and 18S ribosomal RNA (rRNA), which, due to the length of the rRNAs, should have an approximate mass ratio of 2:1, respectively. A high mass band indicating genomic DNA should generally be absent. Furthermore, the RNA purity is also important because contaminants such as guanidinium salts or phenol, which are used to denature proteins during extraction can inhibit downstream processes (such as cDNA synthesis). Nucleic acids exhibit a typical absorbance spectrum that peaks at approximately 260 nm. The presence of some contaminants can be observed as deviations in this absorption spectrum. For example, absorbance at 320 nm and above indicates the presence of particulates, absorbance at 280 nm can indicate the presence of proteins and absorbance at 230 nm can indicate both protein and phenol, for example (Gallagher and Desjardins, 2006). Typically the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is calculated – very pure RNA has a ratio of approximately 2.0 but is somewhat dependent on the base composition (Gallagher and Desjardins, 2006; Manchester, 1996). The ratio of absorbance at 260 nm and 230 nm (A_{260}/A_{230}) is typically 1.8 – 2.2. Ratios may vary by 0.2 -0.3 due to differences in buffer pH and ionic strength. The overall spectral quality may also be assessed to determine sample quality (Desjardins and Conklin, 2010). Prior to cDNA synthesis, the RNA must be free of genomic DNA (which could be measured in

qPCR) and thus digestion with DNase is usually used. It is important that the DNase be inactivated prior to cDNA synthesis.

5.1.2.2 Methods of quantification

There are two main methods for quantification of gene expression (i.e. the abundance of the target mRNA): relative quantification and absolute quantification. In the former the abundance of the target mRNA is compared to one or more endogenous reference RNAs by the difference in the threshold cycle value (C_q). Thus the relative abundance of the mRNA compared to the endogenous reference gene can be calculated. In absolute quantification, standards of known concentrations (or more simply standards with known factors of dilution) are used to draw a standard curve to which the C_q value of the samples can be compared (Pfaffl, 2004). Relative quantification is dependent on comparing the target gene abundance to that of an endogenous reference gene (often a 'housekeeping' gene) that is constitutively expressed at an equal level in all sample tissues – and usually that is expressed at somewhat similar levels to the target gene. Whilst many studies have used only one reference gene (such as *ß-actin* or 18S RNA) it is advised that a panel of genes be used and that the expression of these genes is checked across samples (Bustin, *et al.*, 2009). Absolute quantification is not dependent on a housekeeping control, but requires appropriate standards from which to produce a standard curve, and standards should appropriately mimic samples containing different target mRNA concentrations. For example a plasmid standard would not represent changes in quantification arising from the reverse-transcription step, whereas a recombinant RNA produced from in *vitro* transcription would. In addition, inclusion of 'background RNA' i.e. RNA extracted from unrelated species, may allow the standard sample to more accurately represent a real sample from which unknown mRNA abundances are to be determined (Pfaffl, 2004). The endogenous control used in relative quantification also acts to normalise data to variations in the amount of starting material added to each reaction; in absolute quantification, normalisation to the total cellular RNA amount has been found to be "the least unreliable method" (Bustin, 2000; Bustin, 2002; Pfaffl, 2004).

5.1.2.3 Amplification efficiency

Quantification usually relies on the assumption that every PCR cycle (at least during the exponential phase of amplification) leads to a doubling in concentration of the

target DNA – i.e. that the reaction has 100% efficiency. In relative quantification the target and endogenous gene must amplify with equal efficiencies, or mathematical correction must be applied based on accurate quantification of amplification efficiencies (Pfaffl, 2001). Otherwise, small differences in efficiency can lead to large errors in estimation of target mRNA abundance and ratios of abundance between samples. Likewise, in absolute quantification, when samples are compared to a standard curve, the sample and standards should exhibit the same amplification efficiency. The efficiencies can generally be tested by diluting the template and measuring the C_q across the different concentrations. Plotting the C_q against log[template] should result in a slope of -3.322 assuming 100% efficiency. The co-efficient of determination (R^2) is also an important factor, as almost all of the variability in C_q values should be explained by the template concentration.

5.1.2.4 Sensitivity

The limits of detection of the assay are also important. Low and high abundance mRNAs may not be detected reliably by the qPCR reaction as the relationship between C_q value and concentration may not be linear. The range of target concentrations over which the standard curve is linear (C_q inversely proportional to Log target concentration) is known as the linear dynamic range (Bustin, *et al.*, 2009). The C_q versus concentration plot allows the assay detection limit to be determined; if the target is not detected by the reaction it can be said that its concentration must be below the detection limit. The plot may also be useful for determining low concentration RNA even if the relationship between Log concentration and C_q becomes non-linear.

5.1.2.5 Specificity

It must be ensured that only quantification of the intended target is made. For example, if due to lack of specificity in the primer sequences, a reaction leads to amplification of two targets, then in a SYBR green reaction, the sum of both targets will be quantified; the same is true in the formation of primer dimers. Specific primers or the measurement of inclusion of sequence specific probes are ways to avert such problems (Wittwer, *et al.*, 1997). Here the issue of specificity is pertinent as closely related genes (the five *ZmNCEDs*) are being quantified. Specificity can be determined by electrophoresis of the PCR product(s) (only one product of the correct size should be present), melting curves determined by SYBR Green fluorescence

(only one melting point should be identified; two melting points indicates the presence of two DNA species), and by sequencing of the PCR product. Primers can also be tested against templates of potentially cross-specific targets, as was done in the characterisation of the five Arabidopsis *NCED*s (Tan, *et al.*, 2003).

5.1.3 Aims and Objectives

The aim of the work outlined in this chapter was to create a qPCR assay that could be used to quantify maize *ZmNCED* expression, mainly in imbibing seeds. The objectives were:

- Extract high quality RNA (intact, pure) from maize seeds
- Develop a method of qPCR quantification for each *ZmNCED,* such that quantification of each *ZmNCED* is specific and exhibits reasonable sensitivity and reproducibility to detect seed *ZmNCED* expression.

5.2 RNA Extraction from Maize Seeds

RNA from whole maize kernels imbibed for 24 hours was not successfully extracted using the RNeasy Mini kit (QIAGEN). Extraction may have been prevented by the formation of a viscous lysate produced when combining the guanidinium hydrochloride-based buffer and maize kernel tissue. Continuation of the extraction led to failure to extract RNA (very low concentration (A_{260}), low $A_{260/280}$ and $A_{260/230}$ values – data not shown). However, the same extraction protocol performed on embryos isolated from imbibed maize seeds proved successful, yielding intact RNA with acceptable absorbance spectra (Figure 5.1; Table 5.1). Due to potential interest in gene expression in the rest of the seed (endosperm, aleurone, pericarp), and the requirement for embryos to be isolated before processing, an alternative extraction method was investigated. A modified version of the hot-borate protocol that has been previously used to extract RNA from Arabidopsis seeds for use in microarrays was used (Maia, *et al.*, 2011; Wan and Wilkins, 1994), with some further modifications to suit the purposes of extracting RNA from maize. This also produced pure RNA which appeared to be intact on a denaturing gel Figure 5.1 B; Table 5.1).

Table 5.1. Purity of extracted maize RNA. RNA was purified from imbibed maize embryos of Variety D lot 2 (36 replicates of 20 embryos) (The experiment outlined in Chapter 6, Figure 6.2) using the RNeasy Mini Kit. RNA was purified from imbibed maize kernels of Variety E lot 1 using the hot borate method (12 replicates of the powder from 20 whole kernels from maize Variety E lot 1). RNA concentration, A_{260}, A_{230} and A_{280} of RNA samples were determined using the picodrop spectrophotometer. The table here is to illustrate the potential of both methods to produce pure RNA, rather than a conclusive determination of the better method.

Attribute	Method	Mean ± SD	Min	Max
Concentration (ng µl^{-1})	RNeasy embryo	693 ± 158	362	1150
	Hot borate kernel	258 ± 87.0	135	425
$A_{260/230}$	RNeasy embryo	2.16 ± 0.19	1.71	2.49
	Hot borate kernel	2.37 ± 0.07	2.22	2.46
$A_{260/280}$	RNeasy embryo	2.15 ± 0.09	1.98	2.27
	Hot borate kernel	1.99 ± 0.07	1.92	2.18

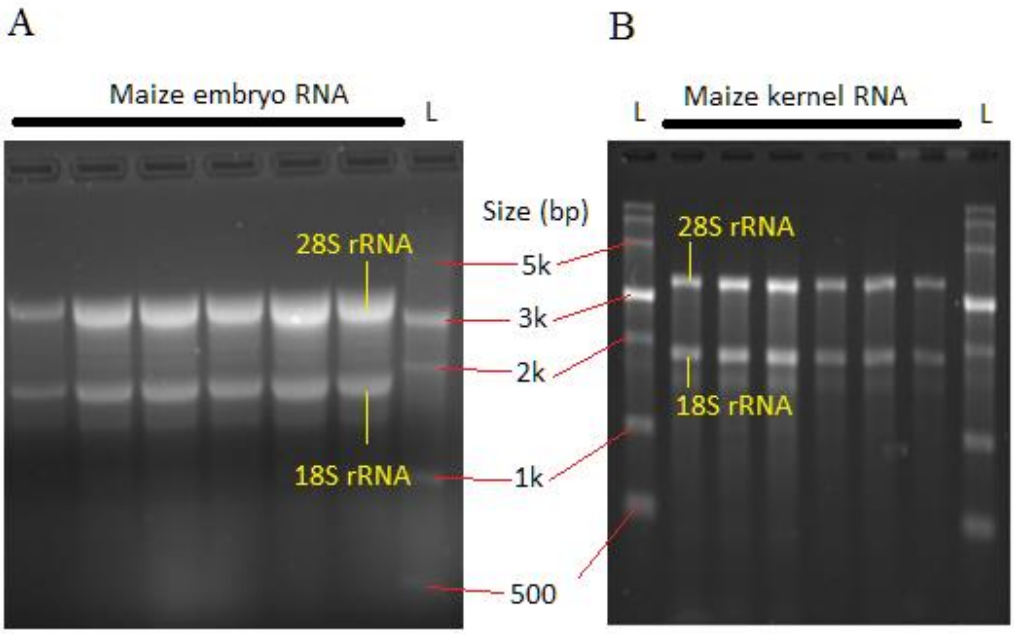

Figure 5.1. Extraction of intact maize seed RNA. A. RNA was extracted from excised maize embryos (from imbibed seed of Variety D lot 2) using the RNeasy Mini Kit (QIAGEN), denatured and separated by formaldehyde agarose gel electrophoresis (stained with Safeview). B. RNA was extracted from excised maize embryos (from imbibed seed of Variety E lot 1) using the modified hot borate method, denatured, and separated by formaldehyde gel electrophoresis and stained with ethidium bromide. The sizes of the ssRNA ladder (L) are indicated in base pairs, and the 25S rRNA and 18S rRNA fragments are indicated.

5.3 Assay Development

5.3.1 *Primer specificity*

Five pairs of primers were designed to amplify sequences within the cloned regions of the *ZmNCED* genes (Chapter 4), to allow the plasmid constructs to be used for testing the primer pairs (specificity, efficiency) or potentially as standards. To test the specificity of primers within the maize *NCED* gene family, each primer pair was tested for amplification of DNA of each *ZmNCED* clone. Initially, the Rotor-Gene SYBR® Green RT-PCR Kit (QIAGEN) was used (Table 5.2), which indicated that cross-specificity of some primer pairs was occurring: primers intended to target *ZmNCED2* amplified similarly from the *NCED3a* template; primers intended to target *NCED3a* amplified from an *NCED3b* template, although at a rate 33 times less than the intended *NCED3a* template; primers intended to target *NCED3b* amplified from an *NCED3a* template at 18 times less than the intended *NCED3b* target.

A similar result was seen when using the QuantiTect SYBR® Green PCR Kit (QIAGEN) (Table 5.3). This kit appeared to provide better meltcurves (i.e. less non-specific low temperature melting product), for example when amplifying *ZmNCED1,* and exhibited efficiencies closer to 100% with sample dilutions (Figure 5.2 A,B). In contrast, the Rotor-Gene SYBR® Green RT-PCR Kit (QIAGEN) often indicated anomalous efficiencies of above 110%, at least in preliminary tests (data not shown). It was also observed that primers designed to amplify *ZmNCED2* did not produce a specific product (by melt curve analysis), even when using the QuantiTect SYBR® Green PCR Kit (QIAGEN) (Figure 5.2 C), which indicated the production of two distinct products. Thus only two of the primer pairs, *ZmNCED1-1* and *ZmNCED9-1* appeared to exhibit suitable specificity.

Five new primer pairs were designed to amplify from each of the *ZmNCED*s, including from the 3′- untranslated region (UTR) and the 5′-UTR because it was otherwise difficult to design primers that adhered to the recommendations for the QuantiTect SYBR® Green PCR Kit (QIAGEN) and to maintain specificity within the *ZmNCED*s.

Table 5.2. Specificity of the first primer set – One-step RT-qPCR. Primer pairs were tested for amplification of *ZmNCED* clones in factorial combinations. Approximately 0.8 ng of plasmids harbouring *ZmNCED* were used as a template. Reactions were performed in triplicate. Values expressed are the fold detection of the template *ZmNCED* relative to the intended target ($2^{\Delta cq}$). Values highlighted in red indicate cross-specificity.

		Primer pair				
		NCED1-1	**NCED2-1**	**NCED3a-1**	**NCED3b-1**	**NCED9-1**
Plasmid template	***NCED1***	1	$1.5x10^{-7}$	$8.0x10^{-5}$	$3.7x10^{-5}$	$1.6x10^{-4}$
	NCED2	$1.4x10^{-7}$	1	$4.9x10^{-6}$	$6.4x10^{-5}$	$8.6x10^{-4}$
	NCED3a	$1.5x10^{-5}$	**1.16**	1	**$5.5x10^{-2}$**	$1.7x10^{-7}$
	NCED3b	$2.4x10^{-8}$	$3.6x10^{-8}$	**$3.0x10^{-2}$**	1	$1.8x10^{-5}$
	NCED9	$9.6x10^{-7}$	$2.0x10^{-4}$	$2.7x10^{-5}$	$4.1x10^{-6}$	1

Table 5.3. Specificity of the first primer set – Two-step RT-qPCR. Primer pairs were tested for amplification of *ZmNCED* clones in factorial combinations. Approximately 0.16 ng of plasmids harbouring *ZmNCED* were used as a template. Reactions were performed in triplicate. Values expressed are the fold detection of the template *ZmNCED* relative to the intended target ($2^{\Delta cq}$). Values highlighted in red indicate cross-specificity.

		Primer pair				
		NCED1-1	**NCED2-1**	**NCED3a-1**	**NCED3b-1**	**NCED9-1**
Plasmid template	***NCED1***	1	N/A	$1.40x10^{-6}$	N/A	$1.45x10^{-6}$
	NCED2	$1.76x10^{-8}$	1	$8.14x10^{-8}$	$2.30x10^{-7}$	$1.75x10^{-5}$
	NCED3a	$4.83x10^{-4}$	$3.26x10^{-4}$	1	**$8.76x10^{-2}$**	$2.75x10^{-9}$
	NCED3b	N/A	N/A	**$5.64x10^{-3}$**	1	$4.82x10^{-7}$
	NCED9	$1.82x10^{-4}$	N/A	$3.00x10^{-5}$	$1.31x10^{-8}$	1

Table 5.4. Specificity of the second primer set – Two-step RT-qPCR. Primer pairs were tested for amplification of *ZmNCED* clones in factorial combinations. Approximately 0.16 ng of *ZmNCED* fragments including partial 3'UTR and 5'UTR were used as a template. Reactions were performed in triplicate. Values expressed are the fold detection of the template *ZmNCED* relative to the intended target ($2^{\Delta cq}$). Values highlighted in red indicate cross-specificity.

		Primer pair					
		NCED1-2	**NCED2-2**	**NCED2-3**	**NCED3a-2**	**NCED3b-2**	**NCED9-2**
Template	***NCED1***	1	$1.43\ x10^{-5}$	$1.17x10^{-4}$	$8.65x10^{-8}$	$4.73x10^{-8}$	$1.24x10^{-7}$
	NCED2	**$3.20x10^{-2}$**	1	1	$1.20x10^{-5}$	$1.61x10^{-7}$	$2.15x10^{-7}$
	NCED3a	$2.63x10^{-4}$	$1.79\ x10^{-4}$	$8.68x10^{-4}$	1	$2.12x10^{-5}$	$2.96x10^{-7}$
	NCED3b	$3.71x10^{-5}$	$2.77\ x10^{-5}$	$1.28x10^{-5}$	$1.42x10^{-4}$	1	$6.47x10^{-5}$
	NCED9	$2.08x10^{-5}$	$7.32\ x10^{-6}$	$1.84x10^{-5}$	$2.41x10^{-5}$	$1.73x10^{-4}$	1

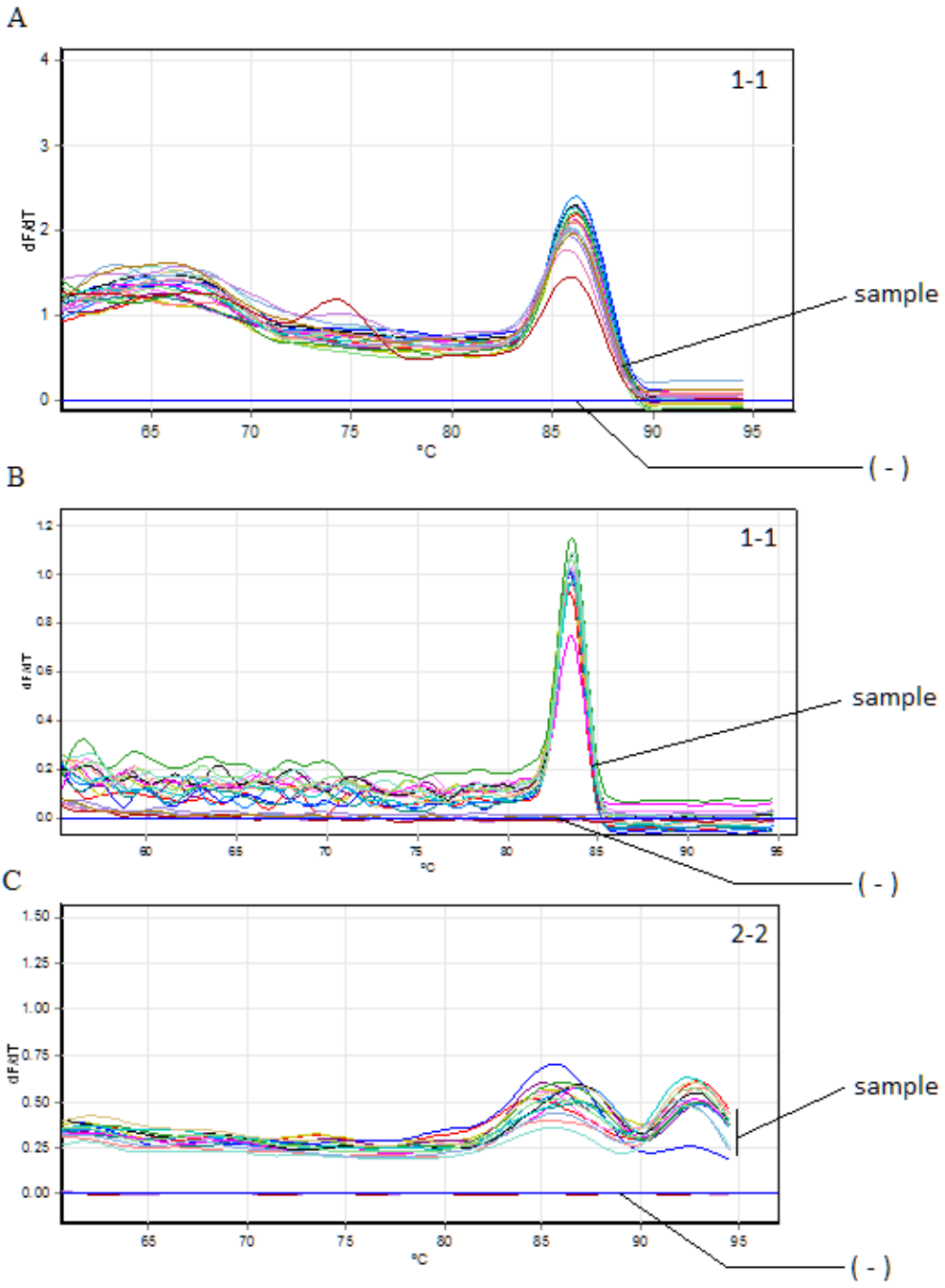

Figure 5.2. Melt curve analyses. Melt curve obtained after amplification of *ZmNCED1* from gDNA using Rotor-Gene SYBR® Green RT-PCR Kit (QIAGEN) (A) and QuantiTect SYBR® Green PCR Kit (QIAGEN) (B) with the ZmNCED1-1 primer pair. Melt curve obtained after amplification of *ZmNCED2* using QuantiTect SYBR® Green PCR Kit (QIAGEN) using the with the ZmNCED2-2 primer pair. Negative control (-) and sample amplifications are indicated.

Fragments were amplified from B73 genomic DNA using primers flanking the coding region of the *ZmNCED*s, separated by gel electrophoresis and purified. The second set of primers were tested for specificity based on their amplification of DNA from the *ZmNCED* fragments containing the 3′-UTR and 5′-UTR regions (Table 5.4). All of the primer pairs produced were specific except for one pair (*ZmNCED1-2*) which amplified also from *ZmNCED2* although 31 times less than the intended *ZmNCED1* target. A third pair of primers to amplify from *ZmNCED2* were therefore designed (*ZmNCED2-3*) because *ZmNCED2-2* primers were later found to have a very poor efficiency and melt curve. These *ZmNCED2-3* primers also amplified specifically from the *ZmNCED2* template.

5.3.2 *Primer efficiencies*

Primer efficiencies and coefficients of determination (R^2) were determined by measuring C_q values across 5-fold serial dilutions of B73 genomic DNA, summarised in Table 5.5. A very strong correlation between log template concentration and C_q value ($R^2 > 0.98$) was seen for all primer pairs tested except for ZmNCED2-2.

Table 5.5. Measurement of primer characteristics. RT-qPCR reactions (QuantiTect SYBR® Green PCR Kit) amplifying 5-fold serial dilutions of maize B73 genomic DNA were used to asses primer characteristics. *Dilutions of amplified *ZmNCED2* fragments containing the 3'-UTR and 5'-UTR were used instead.

			Minimum Linear Dynamic range	
Primer pair	**Efficiency (%)**	**R^2**	**Fold dilution**	**min. C_q - max. C_q**
ZmNCED1-1	93	0.985	3125	17.41 – 27.49
ZmNCED1-2	111	0.998	3125	18.48 – 26.97
ZmNCED2-1	*Not tested - unspecific*			
ZmNCED2-2	N/A	N/A	N/A	N/A
ZmNCED2-3*	86	0.998	15,625	12.61 – 25.62
ZmNCED3a-1	*Not tested - unspecific*			
ZmNCED3a-2	89	0.984	3125	20.73 – 31.03
ZmNCED3b-1	*Not tested - unspecific*			
ZmNCED3b-2	94	0.993	3125	23.03 – 32.68
ZmNCED9-1	84	0.991	3125	26.81 – 37.26
ZmNCED9-2	115	0.989	3125	21.88 – 30.14

The ZmNCED2-2 primers appeared to produce sporadic C_q values that were not predicted by template concentration. However a third set of specific primers intended to amplify *ZmNCED2* (ZmNCED2-3) exhibited characteristics similar to the other primer pairs. Primer efficiencies (except ZmNCED2-2) ranged from between 84% and 115%. The primer pairs chosen for measuring *ZmNCED* expression were as follows: ZmNCED1-1, ZmNCED2-3, ZmNCED3a-2, ZmNCED3b-2, and ZmNCED9-2 (Table 2.4).

5.4 Generation of RNA Standards for Absolute Quantification of *ZmNCED*s

To generate an assay that was not dependent on characterising further primers for endogenous control genes, a method of absolute quantification was pursued in which the starting total RNA levels would be normalised by measurement of A_{260} values using the picodrop spectrophotometer (i.e. 'the least unreliable method' of normalisation (Bustin, 2000; Bustin, 2002; Pfaffl, 2004)). The standard curve would be prepared from dilutions of recombinant RNA (containing the primer target sequences) in a background of total tomato RNA to mimic real samples.

Table 5.6. Primers used for generating transcription templates for RNA RT-qPCR standards. The minimal T7 promoter sequence is indicated in underlined text and the complementary sequence for the 5' extensions is indicated in **bold text**.

Target	Forward primer	Reverse primer	Product size (bp)	
Forward primer 5' extension	GAGAATTCTAATACGACTC ACTATA**GGGCAGCAGAC CAG**	N/A	(Below, plus 24 bp)	**Generic primer**
ZmNCED1	**AGGGCAGCAGACCAG**AA CGAGTCCGACGAGCGC	TGTAACTCTGGT GTGCGTGG	623	**Specific primers**
ZmNCED2	**AGGGCAGCAGACCAG**TG ATCGATCCATCCATCCGC	GGAGTAGTGGT GACTGGTGC	192	
ZmNCED3a	**AGGGCAGCAGACCAG**GG TCGTGGTGATCGGGTC	ACATGAAGATG TAGGGCCGG	766	
ZmNCED3b	**AGGGCAGCAGACCAG**GC ACCGTGGAGAAGTTCG	AGCAGGAGCAT GATAACTCGG	681	
ZmNCED9	**AGGGCAGCAGACCAG**TC CGAGCTCCTGGTGGTC	AGATGGAGCTG GAGGTGGAT	545	

5.4.1 Generation of RNA standard solutions

The target amplicons of the RT-qPCR primers and flanking regions were amplified using the specific primer pairs indicated in Table 5.6, which added a 5' extension to the sequence to add a primer binding site for the generic primer. An annealing temperature gradient was used to quickly identify the optimal annealing temperature for the reaction. DNA fragments were separated by gel electrophoresis (Figure 5.3) and fragments of the predicted sizes were purified from the gel.

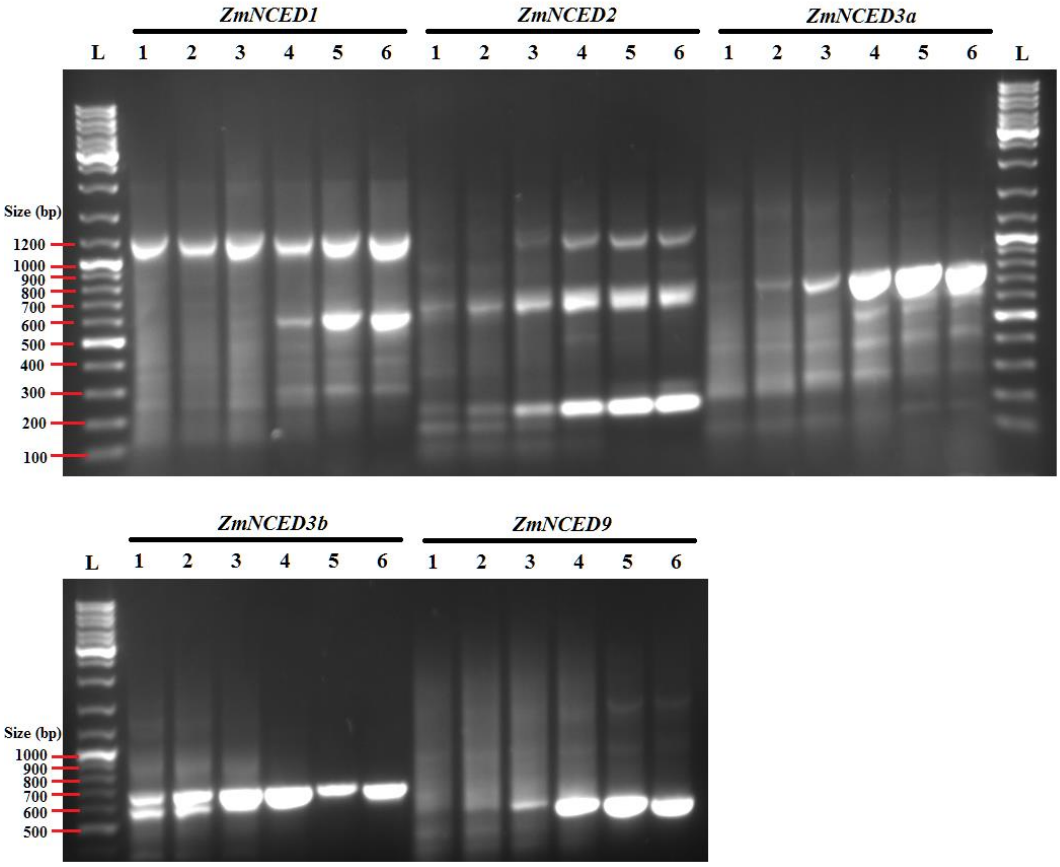

Figure 5.3. Amplification of the qPCR primer target amplicons for generation of qPCR standards. The target amplicons for the chosen qPCR primers and the flanking regions were amplified from maize B73 genomic DNA with HotStar HiFidelity Polymerase Kit (QIAGEN) using the primer pairs specified in Table 5.6. Six different annealing temperatures were used, 55°C, 57°C, 59°C, 63°C, 66°C and 68°C, indicated by lanes marked 1,2,3,4,5 and 6, respectively. Fragments were separated by electrophoresis along with GeneRuler DNA ladder mix (Thermo); fragment sizes are indicated.

These purified fragments were then used as a template in subsequent PCR reactions using the generic primers in Table 5.6 and the specific reverse primers to add the T7 promoter sequence to the fragments (Figure 5.4). It was noticed that amplification of the *ZmNCED1* fragment produced an non-specific band (~300 bp), but at relatively lower abundance to the predicted band (623 bp). The optimal annealing temperature

was identified as 63°C for all reactions apart from *ZmNCED3b* (66°C). Apart from *ZmNCED1*, the amplification produced a single visible band. The reactions were scaled up, using the 63°C annealing temperature to produce enough transcription template for a transcription reaction. The templates were transcribed using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB) and RNA was subsequently purified from the reactions. The RNA product was denatured and visualised on an agarose gel to confirm the transcription of the templates (Figure 5.5).

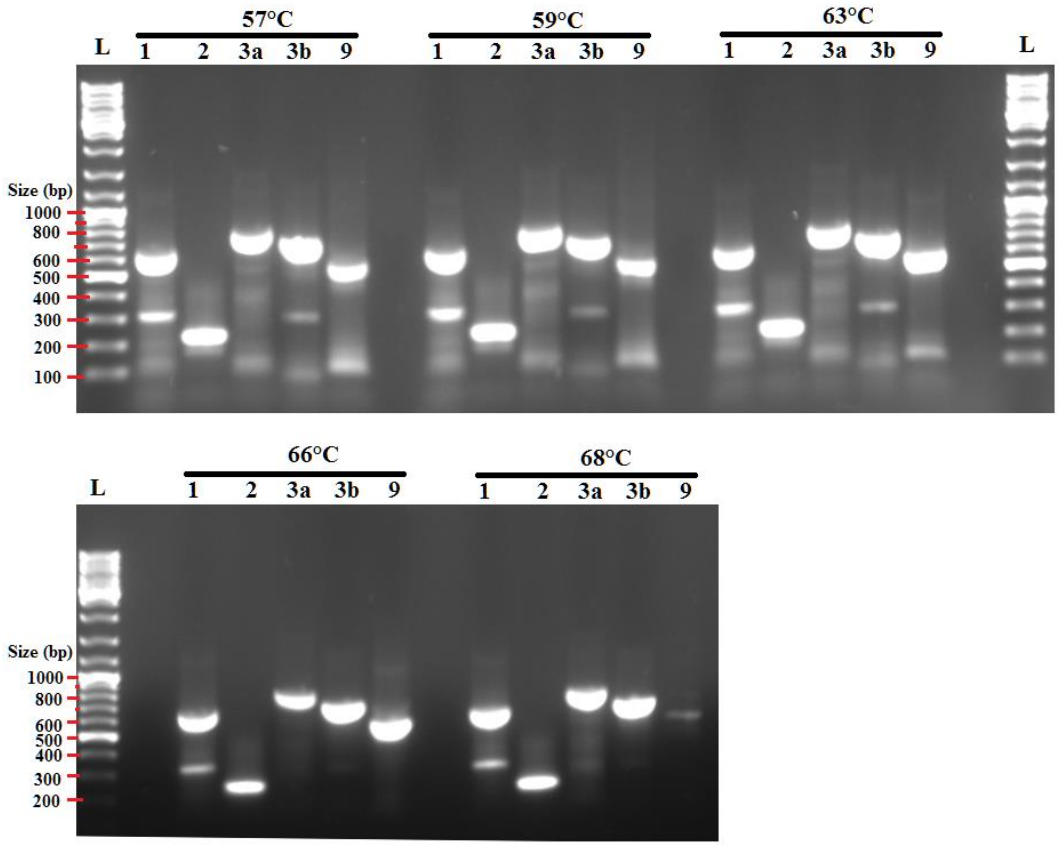

Figure 5.4. Amplification of transcription template for *ZmNCED* RT-qPCR standards. Purified fragments of the qPCR primer target amplicon and flanking sequences were amplified with the generic forward primer and *ZmNCED*-specific reverse primers (indicated above lanes) in Table 5.6. Five different annealing temperatures were used for amplification with with HotStar HiFidelity Polymerase Kit (QIAGEN). Fragments were separated by electrophoresis along with GeneRuler DNA ladder mix (Thermo), fragment sizes are indicated.

Similar to the DNA template, the *ZmNCED1* fragment transcription product also contained the non-specific band, however it was in much lower in abundance compared to the desired fragment. The concentration of the purified RNA fragments was measured using the picodrop spectrophotometer and the number of copies of the standards predicted (Table 5.7). The RNA standard molecule solutions were all diluted to 10^{12} copies per microliter. The solutions were then combined in equal

ratios to obtain a solution of all *ZmNCED* standard molecules at 2 x 10^{11} copies μl^{-1}. The solution was diluted in a 10 fold series to 2 copies μl^{-1} (producing 11 solutions plus the original). The diluted series, from 2 x 10^{7} copies μl^{-1} to 2 copies μl^{-1} were then combined 50:50 with purified total RNA from tomato leaf (360 ng μl^{-1}). This resulted in concentrations of *ZmNCED* standard molecules ranging from 2 x 10^{7} copies μl^{-1} to 2 copies μl^{-1} in a background of 180 ng μl^{-1} total RNA.

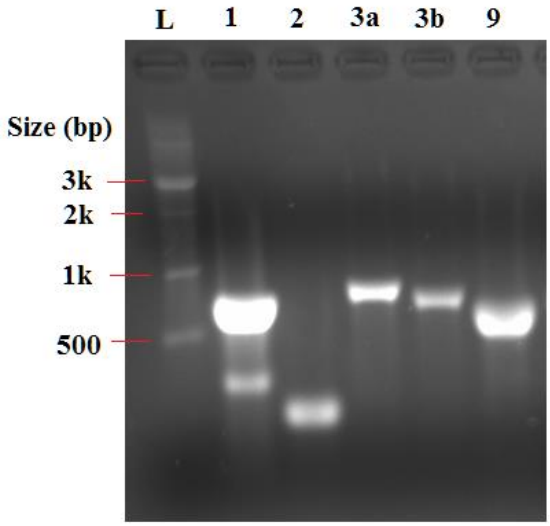

Figure 5.5. Transcription of target amplicons of selected RT-qPCR primer pairs for use as standards. *ZmNCED1* (1), *ZmNCED2* (2), *ZmNCED3a* (3a), *ZmNCED3b* (3b) and *ZmNCED9* (9) RT-qpCR primer target amplicons was transcribed using HiScribe™ T7 High Yield RNA Synthesis Kit. Products were denatured and separated by agarose gel electrophoresis alongside an ssRNA Ladder (NEB) (fragment sizes are indicated).

Table 5.7. Normalisation of concentration of RNA standard molecules. The concentration of RNA standard molecule RNA was measured using the picodrop spectrophotometer. The concentration of molecules μl^{-1} was calculated in order to find out the dilution factor required to obtain 10^{12} molecules μl^{-1}. Molecular weight (M.W.) was calculated *in silico* using Geneious.

		Concentration			
RNA standard molecule	**M.W. (Daltons)**	**ng μl^{-1}**	**pmol μl^{-1}**	**molecules μl^{-1} x 10^{12}**	**Dilution factor (To obtain 10^{12} molecules μl^{-1})**
ZmNCED1-S	200803.4	1329	6.618	3.99	3.99
ZmNCED2-S	65736.2	1708	25.98	15.6	15.6
ZmNCED3a-S	247004	1628	6.591	3.97	3.97
ZmNCED3b-S	223508	867.0	3.879	2.34	2.34
ZmNCED9-S	179146.8	2278	12.71	7.66	7.66

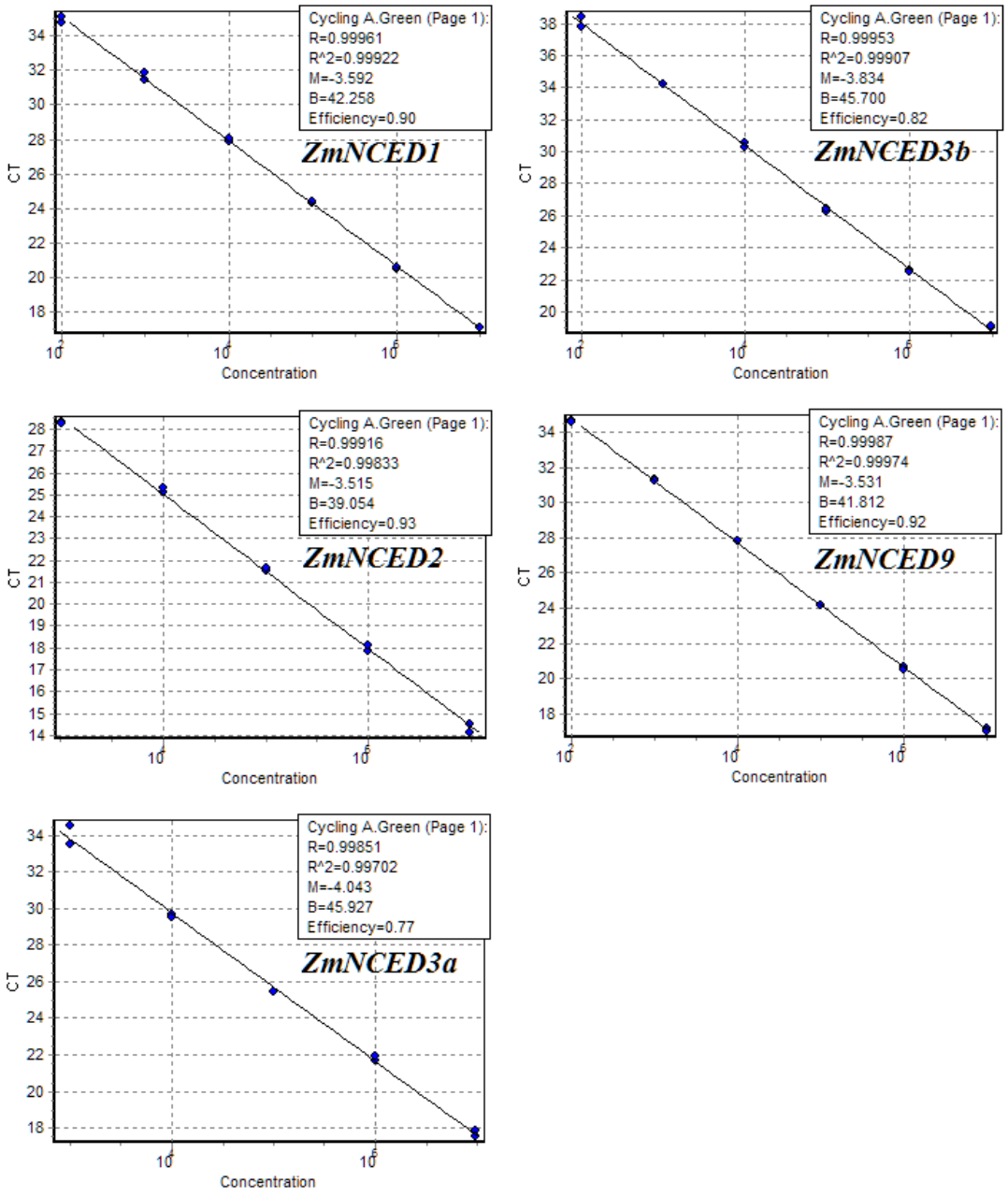

Figure 5.6. Standard curve plots for *ZmNCED* RNA standards. Recombinant RNAs were used to prepare standard curves for quantifying *ZmNCED* expression. Standards were processed as normal samples and C_q values measured by RT-qPCR using primers targeting *ZmNCED*s and plotted against the concentration (copy number) of the RNA standard. Plots and curve attributes rendered in Rotor-Gene Q series software (n = 2 per concentration).

5.4.2 Testing of RNA standard solutions

To test the RNA standards, they were treated as ordinary RNA samples. The samples were treated with DNaseI (Ambion) and cDNA was synthesised using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was used as a template for RT-qPCR reactions with the five pairs of *ZmNCED* primers, and standard curves were drawn, using concentrations that allowed a linear relationship (Figure 5.6). The linear range, efficiency and co-efficient of determination of the primer pairs are indicated in Table 5.8. C_q was dynamic between 100 and 10^7 copies for all primer pairs, however the linearity of the relationship was lost below 1000 copies for the ZmNCED2-3 and ZmNCED3a-2 primer pairs. The linear range measured for all primer pairs (between 100 or 1000 and 10^7) showed very high coefficients of determination indicating that the number of copies of the *ZmNCED* targets can be deduced accurately. Three of the primer pairs showed good efficiencies (90 – 93%) whereas the ZmNCED3a-2 and ZmNCED3b primer pairs showed 77% and 82% efficiencies, respectively. However as the quantification is drawn from a standard curve, the efficiency is accounted for, assuming that the amplification efficiency from the standards is equal to the amplification efficiency from the samples. The products of amplification with all primer pairs appeared to be specific, each resulting in one distinct product as determined by melt curve analysis (Appendix 5.1). Thus the assay appeared to be ready for use in quantification of *ZmNCED* expression in samples.

Table 5.8. Characteristics of primer pairs when tested with the RNA standards. Recombinant RNAs were used to prepare standard curves for quantifying *ZmNCED* expression. Standards were processed as normal samples and C_q values measured by RT-qPCR using primers targeting *ZmNCED*s and plotted against the concentration (copy number) of the RNA standard. Curve attributes calculated in Rotor-Gene Q series software.

Primer pair	Efficiency (%)	R^2	Linear dynamic range minimum (copies μl^{-1})
ZmNCED1-1	**90**	**0.999**	**100**
ZmNCED2-3	**93**	**0.998**	**1000**
ZmNCED3a-2	**77**	**0.997**	**1000**
ZmNCED3b-2	**82**	**0.999**	**100**
ZmNCED9-2	**92**	**1.000**	**100**

5.5 Discussion

5.5.1 Extraction of intact, pure, RNA from maize kernels

Purification of RNA from maize and other cereal seed, and seed in general is often reported to require specific extraction techniques in order obtain high yield and high quality RNA. In maize, it is the abundance of polysaccharides (starch) that appears to be particularly problematic, although many protocols have now been reported that allow for adequate RNA extraction from maize and cereal seed (Li and Trick, 2005; Messias, *et al.*, 2014; Wang, *et al.*, 2012). Here, the use of a modified hot-borate protocol allowed the extraction of pure and intact RNA from whole mature maize kernels. Whilst the hot-borate method has previously been reported to be ineffective due to poor $A_{260/280}$ and $A_{260/230}$ values and RNA degradation (Messias, *et al.*, 2014; Wang, *et al.*, 2012), here pure and intact RNA was extracted. The successful method involved immediate homogenisation of the borate extraction buffer and pulverised maize tissue with an ultra-turrax, and it is likely that this modification was essential for high yield and quality of RNA. Additionally, extraction with protocols using guanidinium thiocyanate (GITC), which is widely found in commercial RNA extraction kits, is often unsuccessful and results in the formation of a viscous lysate from which pure, intact RNA is not extracted. Some kits, such as the RNeasy Plant Mini Kit (QIAgen) provide an alternative lysis buffer that uses guanidinium hydrochloride instead as the chaotropic agent and is supposed to aid in the RNA extraction from starchy tissues. This did not appear to be successful with mature maize kernel tissue, and led to similar results to those seen in Messias et al., 2014. The RNeasy Plant Mini Kit (QIAgen) extraction did however work with excised embryos, which reduces the amount of starch processed. Embryo excision and purification using the RNeasy Plant Mini Kit (QIAgen) was chosen as the preferred method for gene expression studies because embryo excision during sampling and processing was found to be more high throughput (two extractions of 24 samples could be performed in a day) compared to the hot-borate method (extraction of 24 samples takes at least two days due to overnight LiCl precipitation). Additionally the hot-borate protocol yielded lower concentration RNA. Furthermore homogenisation of whole seeds in the hot borate protocol was difficult as it required three homogenisation steps (coffee-grinder, mortar and pestle, then ultra-turrax when added to the extraction buffer). This did mean, however, that the expression of

tissues in the maize endosperm or aleurone would be largely ignored, although most of this tissue is dead in mature seed and may not contribute a large amount to overall gene expression.

5.5.2 Development of an assay to measure ZmNCED expression

Primer pairs that were specific to each the *ZmNCED*s were designed and validated that allowed adequately specific amplification and detection of *ZmNCED* abundance using SYBRgreen-based non-specific amplicon detection without the need to use of gene-specific fluorescent probes (which may have added additional expense and optimisation steps). Primer pairs were designed to accommodate probes if necessary, and a multiplex assay could be developed from this basis in the future which would increase the throughput of expression measurement. Assays of maize *ZmNCED* expression, at least in developing seed, have been previously made; although not always with complete knowledge of the maize *ZmNCED* family, as discussed in chapter 4 (Capelle, *et al.*, 2010; Vallabhaneni, *et al.*, 2010). The study by Vallabhaneni et al., 2010 does however identify the five *ZmNCED*s and confirmed the PCR products of the primers by sequencing. Here, the specificity of primers was tested against DNA fragments or plasmids harbouring sequences of other *ZmNCED*s confirming the specificity of primer pairs within the *ZmNCED*s. A method of absolute quantification was also produced, allowing the abundances of the *ZmNCED*s (i.e. copy number) to be determined. This allows a more precise determination of the relative abundance of each of the *ZmNCED*s to be made (the relationship between C_q and starting *ZmNCED* RNA concentration is quantified).

Both relative and absolute quantification rely on some assumptions. One of the most important assumptions in relative quantification is the primer efficiency which can be and needs to be accounted for. This is often tested using dilutions of sample cDNA which depends on a high-enough initial concentration of the target and assumes that dilutions of cDNA accurately reflect the varied target abundance following reverse-transcription. These assumption may not be met, as the background cDNA is not accounted for, and reverse-transcription efficiency may vary for the target at different abundances). In the method used here, the efficiency of the reverse-transcription step and background are accounted for as the standards are prepared in a background of tomato total RNA. There are still assumptions made however, such as that the tomato total RNA reflects the normal maize total RNA

background and that the recombinant RNA target behaves like the real mRNA. It also assumes the PCR reaction efficiency in the standards is the same as that in samples. Another assumption often made is that an endogenous control gene is stably expressed throughout all treatments and reflects the total amount of mRNA in a given sample. It was found in a preliminary experiment that *ß-actin* was stably expressed in the cold and control treatments, however *ß-actin* levels varied somewhat in another experiment (data not shown) and in some experiments the assumption about non-responsiveness of references genes may not be correct. This may be counteracted by the use of multiple reference genes. Similar to the use of standard curves, this can increase the number of reactions required in ordered to analyse expression in a sample. Thus there are many variables that can influence the outcome of a qPCR assay and these must be kept in mind when interpreting the data and comparing to other studies, and all efforts should be made to confirm assumptions so that interpretation of experiments is justified (Bustin, *et al.*, 2009).

5.5.3 Conclusion

A method for measuring *ZmNCED* expression in maize seed is presented in this chapter which allowed *ZmNCED* expression to be investigated further in Chapter 6, along with ABA quantification.

6 NCED Control of ABA Biosynthesis in Maize Germination

6.1 Introduction

6.1.1 A key regulatory step in ABA biosynthesis is controlled by multiple NCEDs

In green tissues where the xanthophyll precursors of ABA are abundant, the enzymatic cleavage of the 9-*cis*-epoxycarotenoids, 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin, by NCED limits the rate of ABA biosynthesis. Elevation of *NCED* expression is correlated to the accumulation of ABA during water-stress and its overexpression can lead to large increases in ABA accumulation (Qin and Zeevaart, 1999; Qin and Zeevaart, 2002; Taylor, *et al.*, 2005; Thompson, *et al.*, 2000). Overexpression of *zeaxanthin epoxidase*, whose product catalyses the conversion of zeaxanthin into 9-*cis*-violaxanthin, can also result in elevated accumulation of ABA (Audran, *et al.*, 1998; Frey, *et al.*, 1999). However this is not the last committed step in ABA biosynthesis and its expression does not always correlate to ABA accumulation as it is also involved in regulation of the xanthophyll cycle (Audran, *et al.*, 1998; Taylor and Harman, 1990; Wang, *et al.*, 2008). The biosynthesis steps downstream of NCED appear to be limited by the availability of xanthoxin (Sindhu and Walton, 1987; Taylor, *et al.*, 2005). Thus *NCED* represents a key regulatory step on the ABA biosynthesis pathway (see Section 1.7.2 for more information).

Many plants appear to have multiple *NCED*s, including maize, sorghum, rice, grape vine, avocado, and tomato amongst others (Chernys and Zeevaart, 2000; Priya and Siva, 2015; Vallabhaneni, *et al.*, 2010; Zhang, *et al.*, 2009). Arabidopsis has five *NCED*s which are expressed in different spatiotemporal patterns, e.g. seed specific *AtNCED6* and *AtNCED9* expression (Frey, *et al.*, 2012; Lefebvre, *et al.*, 2006; Tan, *et al.*, 2003). *AtNCED*s are also differentially expressed in response to various environmental conditions, e.g. *AtNCED3* in response to drought (Tan, *et al.*, 2003). Each therefore is likely to regulate the accumulation of ABA in a unique way, and seed specific roles have been identified (discussed in 6.1.2).

Maize also has five *NCED*s, however roles have not been extensively studied, apart from *VP14* (*ZmNCED1*). Furthermore roles cannot necessarily be deduced from

homology to Arabidopsis *NCED*s since the duplication of *NCED*s likely occurred following the evolutionary separation of monocots and dicots as shown in Chapter 4.

6.1.2 NCED control of seed germination

As outlined in the main introduction, ABA is involved in the induction and maintenance of physiological dormancy in many species. In maize, primary dormancy is dependent on ABA biosynthesis, and disruption leads to a viviparous phenotype. An example of this is observed in the maize mutant *viviparous 14* which lacks a functional copy of *VP14* (*ZmNCED1*) (Schwartz, *et al.*, 1997; Tan, *et al.*, 1997). Disruption of ABA biosynthesis in the tomato mutant *sitiens*, which contains a mutation in the gene encoding an ABA-aldehyde oxidase also leads to a viviparous phenotype, even though the *notabilis* mutant which contains a mutation in the gene encoding *LeNCED1* does not (Harrison, *et al.*, 2011; Taylor, *et al.*, 2005). This could be as a result of the low levels of dormancy observed in cultivated tomato, or because of the action of a redundant gene. Similarly the *vp14* mutant phenotype (more viviparous germination; germination of some seeds on the parent plant) only exhibits weak penetrance, i.e. it is not always viviparous, owing to residual ABA accumulation and still exhibits stress-induced ABA accumulation in vegetative tissues (Tan, *et al.*, 1997). Thus both primary dormancy and stress-induced ABA accumulation in maize appears to be dependent on more than one *ZmNCED*. Both *ZmNCED1* and *ZmNCED9* appear to be expressed in developing maize embryo and endosperm, and *ZmNCED3a* is expressed in vegetative tissues (Capelle, *et al.*, 2010; Vallabhaneni, *et al.*, 2010).

ABA produced in the developing seed is often required for the initiation of dormancy during development on the parent plant and dormancy loss is sometimes associated with a decline in ABA content of the developing seed (Ali-Rachedi, *et al.*, 2004; Frey, *et al.*, 2012; Groot and Karssen, 1992; Koornneef, *et al.*, 1982; Lefebvre, *et al.*, 2006; Marin, *et al.*, 1996; Tan, *et al.*, 1997). However *de novo* ABA biosynthesis upon imbibition appears to be important in maintaining seed dormancy. *De novo* ABA biosynthesis is observed during the imbibition of dormant seeds, but less so in non-dormant seeds (Ali-Rachedi, *et al.*, 2004; da Silva, *et al.*, 2004; Grappin, *et al.*, 2000; Lepagedegivry and Garello, 1992). Overexpression of *NCED* in imbibing seeds also leads to ABA accumulation which can induce a dormant phenotype (Martinez-Andujar, *et al.*, 2011; Qin and Zeevaart, 2002; Thompson, *et*

al., 2000). Thus *NCED* expression may control ABA biosynthesis upon imbibition in response to the environmental conditions, influencing the decision of seed whether or not to germinate.

6.1.3 The role of NCED in germination under varied and suboptimal conditions

In Arabidopsis, the *NCED* expression is regulated in response to imbibition under different environmental conditions. *AtNCED9* has a major role in ABA biosynthesis and germination inhibition during imbibition at high temperatures, whilst *AtNCED2* and *AtNCED5* have minor roles (Toh, *et al.*, 2008). *AtNCED6* has a role in mediating the phytochrome regulation of germination in response to red and far red light (Seo, *et al.*, 2006). *AtNCED*9, *AtNCED6* and *AtNCED2* are also regulated in different dormancy states of *Arabidopsis thaliana* cvi and in response to nitrate, with the gene regulation also correlating with ABA biosynthesis and seasonal dormancy cycling in the field, although this regulation is ecotype dependent (Cadman, *et al.*, 2006; Finch-Savage, *et al.*, 2007; Footitt, *et al.*, 2011; Footitt, *et al.*, 2013). Similarly, in lettuce, expression of *LsNCED4* is required for the inhibition of germination at high temperatures, but is not essential for seed development of stress tolerance (Huo, *et al.*, 2013). In barley, *HvNCED1* is expressed at non-permissive high temperatures, maintaining ABA concentration during primary dormancy, and *HvNCED2* expressed after the transfer to permissive conditions following the induction of secondary dormancy (Leymarie, *et al.*, 2008). Both *HvNCED1* and *HvNCED2* expression is increased under blue light, which results in extra ABA accumulation and dormancy (Hai Ha, *et al.*, 2014). Thus the *NCED* families in plants may have a variety of roles in seeds, from inhibition of germination during development, imbibition under different conditions and under secondary dormancy.

6.1.4 A potential role for ZmNCEDs in germination under suboptimal conditions

In Chapter 3, seven days imbibition at 5°C induced a inability to germinate at 25°C in low-vigour maize seed lot 4B. There was evidence that this was partially reversed by treatment with CCD inhibitors, perhaps indicating the non-germinating state is an ABA biosynthesis dependent secondary dormancy. Thus it may be expected that expression of one, or more, of the *ZmNCED*s increases during the cold test treatment. Not all seed lots exhibit the non-germinating phenotype following treatment at 5°C, therefore it may be possible to correlate cold test germination to *ZmNCED* expression. Seed imbibed on the CCD inhibitors at low temperatures did

not show increased germination, although low concentrations were used due to limitations in the compound availability. Therefore there may still be a role of NCED in delaying germination at these temperatures. This chapter investigates the potential roles of *ZmNCED*s in germination under suboptimal (low-temperature) conditions through measurement of *ZmNCED* expression and ABA concentrations.

6.1.5 Aims and objectives

The aim of this chapter was to investigate the potential roles of each of the *ZmNCED*s in ABA biosynthesis during germination of maize under suboptimal conditions. The objectives were as follows:

- Investigate the trends of *ZmNCED* expression during maize imbibition under cold test and low temperature conditions
- Determine if ABA accumulation also results as a consequence of imbibition under cold test and low temperature conditions
- Determine the effects of CCD inhibitors on ABA accumulation in maize

6.2 *ZmNCED* Expression and ABA Accumulation in a Low Vigour Seed Lot under Vermiculite Cold Test Conditions

6.2.1 Collection of samples

In Chapter 3, it was found that seven days imbibition at 5°C induced a reversible non-germinating state at 25°C in some seed of low-vigour maize seed lot 4B (Figure 6.1 A). Furthermore, small but significant germination promoting effects of hydroxamic acid CCD inhibitors were found. It was therefore hypothesised that this non-germinating state may result from the induction of dormancy that is ABA dependent. Thus the expression of *ZmNCED*s and the accumulation of abscisic acid under cold test conditions was quantified to investigate their potential roles in germination inhibition.

Maize seed of the low vigour lot 4B, were imbibed in vermiculite under cold test (7 days at 5°C and transfer to 25°C) and control conditions (direct imbibition at 25°C) and samples were taken at the intervals outlined in Figure 6.1 B. Samples of dry seed were taken to determine the initial *ZmNCED* and ABA concentrations. Samples were taken at 2 hours, 6 hours and 24 hours after both control and cold test imbibition.

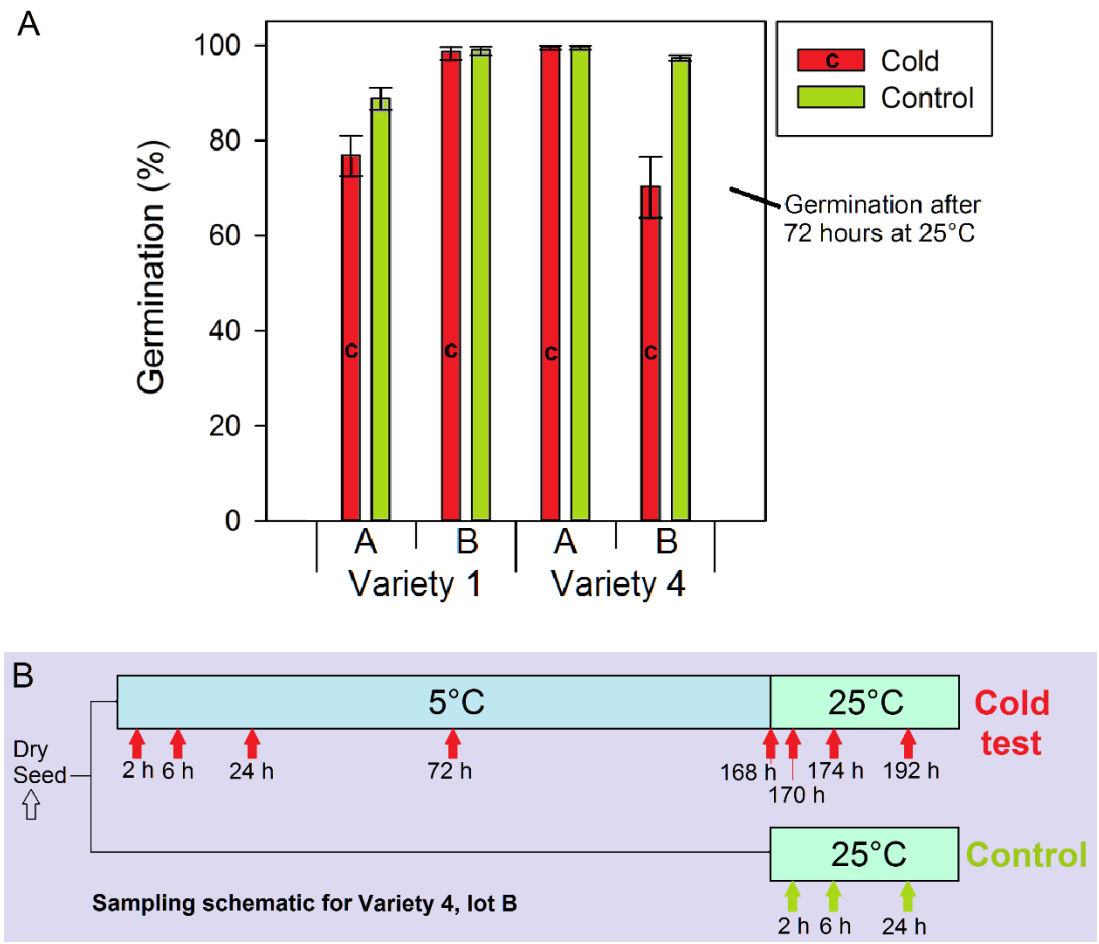

Figure 6.1. Cold test sample collection for RT-qPCR and ABA quantification.

A: ***Germination of 4 seed lots after 72 hours at 25°C.*** Maize seed were imbibed on wet vermiculite for 7 days at 5°C then transferred to 25°C (Cold test) or imbibed directly at 25°C (Control). Germination was scored 72 hours after imbibition at 25°C. Error bars indicate standard error (further detail in Section 3.2.4).

B: ***Schematic for collections of samples of Variety 4 lot B under cold test conditions****:* Maize seed of Variety 4 lot B were imbibed on wet vermiculite for 7 days at 5°C then transferred to 25°C (Cold test) or imbibed directly at 25°C (Control). 20 embryos were taken at 2 h, 6 h, 24 h, 72 h and 168 h, 170 h, 174 h and 192 h after sowing in the cold test and 2 h, 6 h and 24 h after sowing in the control, with three replicates per time point. Three samples of 20 dry seed embryos were also taken. Samples were processed for *ZmNCED* expression quantification (Figure 6.2 and Figure 6.3) and ABA quantification (Figure 6.3). Seed for Variety 1 seed lot A through Variety 5 seed lot B were sampled at the 168 h time point under cold test conditions Figure 6.4.

Samples were also taken at 72 hours and 168 hours after cold imbibition to determine the effect of a long period of cold on *ZmNCED* expression and ABA accumulation. Samples were taken 170 hours, 172 hours, 174 hours and 192 hours in to the cold treatment, which were equivalent to 2 hours, 6 hours and 24 hours after transfer to 25°C, respectively. This was to allow for direct comparison to the control treatment. 20 embryos were isolated for each replicate, with 3 replicates per treatment time point. RT-qPCR was performed using the assay developed in Chapter

5 (comparing expression of *ZmNCED*s relative to the recombinant RNA standards) and a fraction of the pulverised sample, used for both RT-qPCR and ABA quantification, was sent to Syngenta, Jealott's Hill, UK for ABA quantification.

6.2.2 *Expression of ZmNCED*s under cold test conditions

The RT-qPCR measurement of *ZmNCED* expression revealed gene specific, time and treatment dependent expression of *ZmNCED*s (Figure 6.2). There were significant differences in all *ZmNCED*s between each of the treatments ($p < 0.001$) (Appendix 6.1). Further comparisons of data were made using Tukey's HSD. Expression of all *ZmNCED*s did not change significantly ($p > 0.05$) in the first 24 hours of imbibition at 5°C.

ZmNCED1 expression increased significantly ($p < 0.05$) after 72 hours and again after 168 hours during imbibition at 5°C. *ZmNCED1* expression did not change significantly until 6 hours after transfer to 25°C when expression was largely reduced ($p < 0.05$) to levels not significantly different to the 72 hour time point. Direct imbibition at 25°C lead to a small, but significant ($p < 0.05$) increase in *ZmNCED1* expression between 6 and 24 hours.

ZmNCED2 expression was not largely influenced by cold imbibition with an significant ($p < 0.05$) increase observed at 72 hours, and a significant ($p < 0.05$) decrease in expression 6 hours after transfer to 25°C. A decrease in *ZmNCED2* expression was observed 24 hours after direct imbibition at 25°C. However *ZmNCED2* was expressed overall at much lower levels than *ZmNCED1*.

ZmNCED3a expression appeared to have even lower levels than that of *ZmNCED2* (always 100 fold less than *ZmNCED1*). There was however a significant ($p < 0.05$) increase in expression observed between 24 hours and 168 hours imbibition in the cold, with a significant ($p < 0.05$) reduction 6 hours after transfer to 25°C, similar to the trend seen in *ZmNCED1*. *ZmNCED3a* expression did not change significantly after direct imbibition at 25°C.

Copies of *ZmNCED3b* were very abundant in dry seed, compared to the other *ZmNCEDs* and expression of *ZmNCED3b* dropped significantly ($p < 0.05$) after 168 hours imbibition at 5°C, but then increased significantly ($p < 0.05$) 2 hours after transfer to 25°C to levels not significantly different to dry seed. *ZmNCED3b*

expression then decreased significantly to the lowest levels ($p < 0.05$) between 6 and 24 hours after transfer. Similarly to the transfer to 25°C, when seed were imbibed directly at 25°C, ZmNCED3b expression increased significantly ($p < 0.05$) 2 hours after sowing and decreased significantly ($p < 0.05$) to low levels after 24 hours. The highest expression of *ZmNCED3b* was observed in the control treatment between 2 hours and 6 hours.

ZmNCED9 expression increased significantly ($p < 0.05$) after 72 hours imbibition at 5°C and increased significantly ($p < 0.05$) further after 168 hours then dropped significantly ($p < 0.05$) to low levels 6 hours after transfer to 25°C. This was similar to the trend seen in *ZmNCED1*, although *ZmNCED9* expression was greater than *ZmNCED1* after 72 hours, but both displayed equal expression after 168 hours. *ZmNCED1* and *ZmNCED9* were very abundant after 168 hours cold imbibition. Again, similar to *ZmNCED1, ZmNCED9* was expressed at relatively low levels after direct imbibition at 25°C.

The sum expression of *ZmNCED* varied significantly between the collected samples ($p < 0.001$) (Appendix 6.1). The sum expression of *ZmNCED*s increased significantly ($p < 0.05$) during imbibition at 5°C (Figure 6.3). Sum *ZmNCED* expression did not change in the first 24 hours of imbibition at 5°C, but increased significantly up to 72 hours, and increased again by a further ca. 2 fold by 168 hours. This high level of expression was maintained for at least 2 hours after transfer to 25°C, and was much higher ($p < 0.05$) than in seeds imbibed directly at 25°C; but sum *ZmNCED* expression then dropped greatly within 6 hours after transfer, to levels lower than that seen in seed imbibed directly at 25°C. However, at the end of the cold test, 168 hours at 5°C and 24 hours at 25°C, and at the end of the control treatment, 24 hours at 25°C, sum *ZmNCED* expression was still significantly 1.6 fold higher in cold test treated seed than control treated seed ($p < 0.05$).

Cold treated seeds also had relatively high levels of *ZmNCED* maintained for long periods of time (for at least 96 hours, between 72 and 168 hours imbibition), which could result in increased ABA biosynthesis.

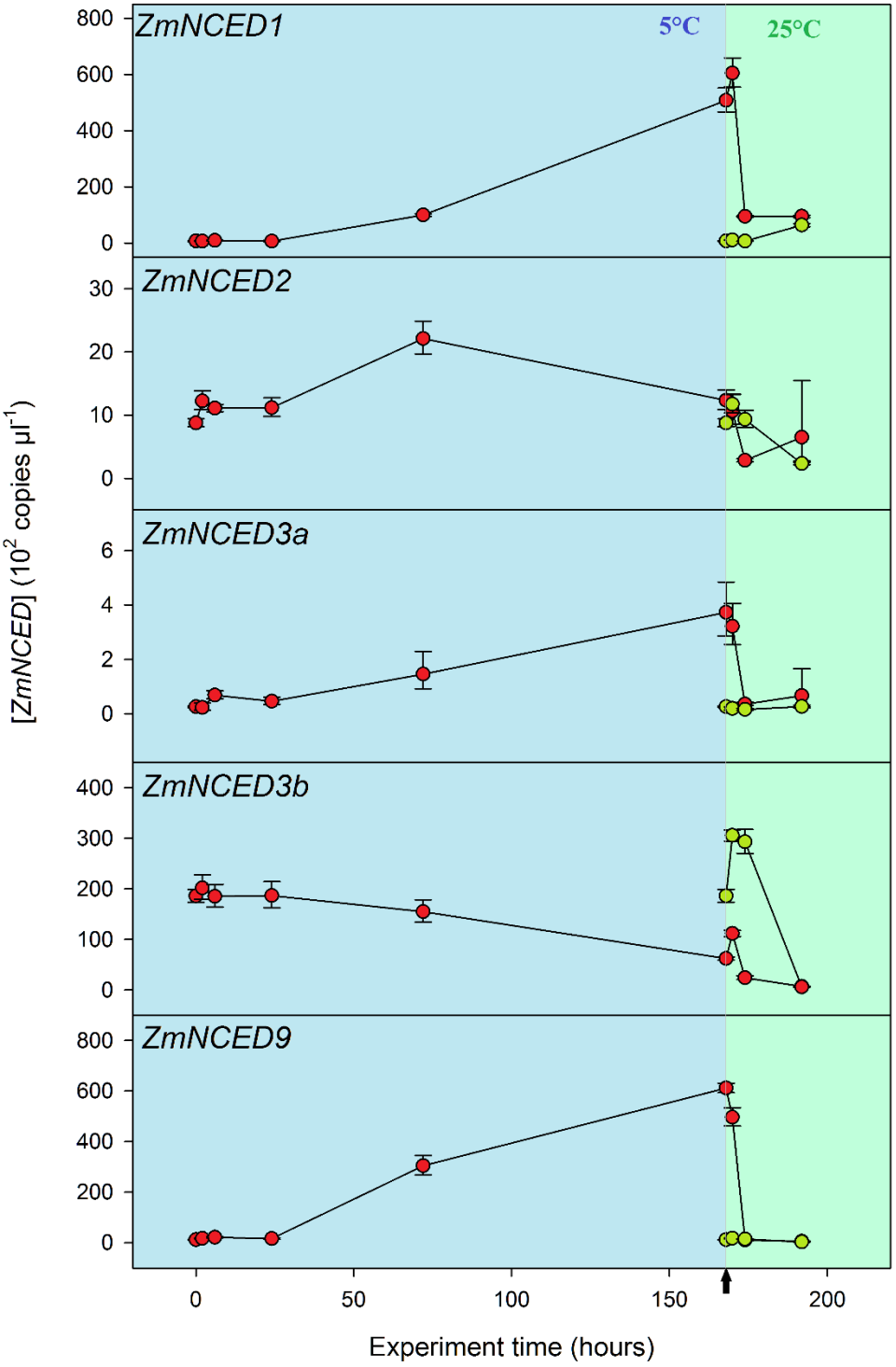

Figure 6.2. Expression of *ZmNCED*s in Variety 4 lot B under vermiculite cold test conditions.Samples were collected as outlined in Figure 6.1. RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5. Seeds were subject to cold test conditions (●) or control conditions (●). The black arrow indicates the moment of transfer of Cold test treated seeds to 25°C. The first point for each series is from the dry seed sample. Error bars indicate standard error (n = 3). Means and error bars were calculated on log-transformed data, back-transformed data is indicated. Please note that the y-axis scale is different for each plot. Back transformed data is plotted.

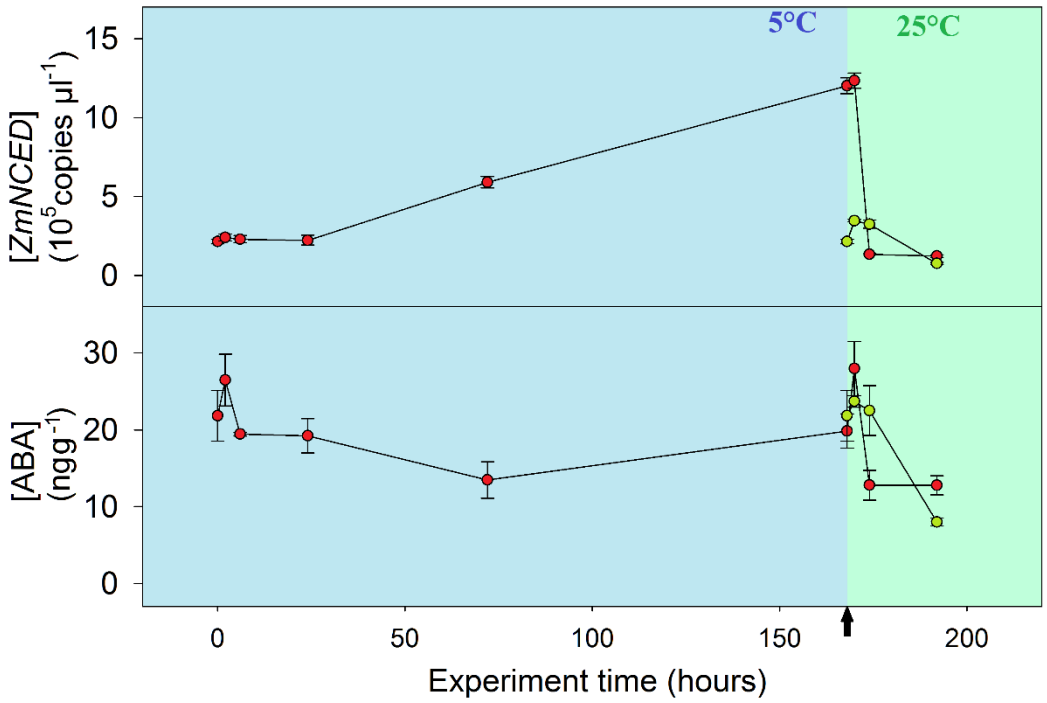

Figure 6.3. Sum of *ZmNCED* expression and ABA accumulation in Variety 4 lot B under vermiculite cold test conditions. Samples were collected as outlined in Figure 6.1. The sum of the number of copies measured in all *ZmNCED*s is indicated here (see Figure 6.2 for individual measurements). Seeds were subject to cold test conditions (●) or control conditions (●). The black arrow indicates the moment of transfer of cold test treated seed to 25°C. The first point for each series is from the dry seed sample. Error bars indicate standard error calculated by on log-transformed data in for [*ZmNCED*] and untransformed data for [ABA] (n = 3) (Appendix 6.1). ABA data is represented in nanograms ABA per gram of embryo (ng g^{-1}). Back transformed data is plotted for [*ZmNCED*].

6.2.3 Accumulation of ABA under cold test conditions

ABA was quantified from the samples outlined in Figure 6.1 to find out if the increased *ZmNCED* expression under cold test conditions also resulted in increased ABA accumulation compared to the control conditions (Figure 6.3). ABA concentration differed significantly between collected samples (p <0.001) (Appendix 6.1) Comparisons between means were made using Tukey's HSD test. The concentration of ABA in the seed dropped significantly (p < 0.05) from 26 ng g^{-1} to 13 ng g^{-1} between 2 and 72 hours imbibition at 5°C. ABA concentration significantly increased 2 hours after transfer to 25°C compared to the 72 hour time point, and decreased again significantly (p < 0.05) to the lowest concentration seen in the cold test treatment 6 hours after transfer. In seed directly imbibed at 25°C ABA concentration was maintained for 6 hours, but then dropped significantly (p < 0.05) at 24 hours, to concentrations not significantly different to cold test treated seed that had been transferred to 25°C for 24 hours.

Overall, the cold test prevented a significant decline in ABA concentration (compared to dry seed levels) until 6 hours after seed were transferred to 25°C conditions (174 hours); whereas direct imbibition at 25°C resulted in a significant decrease in ABA levels compared to dry seed by 24 hours. Thus seed imbibed under cold conditions are exposed to relatively high ABA levels for longer periods of time. Furthermore the prior cold treatment altered the pattern of ABA accumulation at 25°C: A short increase in ABA concentration was observed up to 2 hours after transfer from 5°C to 25°C and the ABA concentration in cold treated seed then decreased more rapidly than in seed directly sown at 25°C. The final ABA content tended to be higher in cold treated seed than in control treated seed, although not significantly.

6.2.4 Summary

Expression of *ZmNCED*s was induced by the cold test treatment but the increase was almost completely reversed on transfer to 25°C; though it remained 1.6 fold higher in seeds that had experienced a prior cold treatment. *ZmNCED* expression in seed sown directly at 25°C increased by a smaller amount and is reduced to lower than starting levels after 24 hours. *ZmNCED3b* expression is high in dry seed and declines slowly in cold imbibed seed, whereas expression initially increases in seed sown at 25°C, but declines to lower than starting levels after 24 hours. An increase in *ZmNCED1* and *ZmNCED9* expression drives the increase in overall *ZmNCED* expression during cold imbibition. *ZmNCED2* and *ZmNCED3a* appear to have relatively low levels of expression. The decline in ABA accumulation is slowed by imbibition at 5°C compared to imbibition at 25°C. ABA levels decline and have similar levels after 24 hours at 25°C in seeds that have or have not had prior cold treatment.

6.3 Expression of *ZmNCED*s after 7 days Cold in Different Seed Lots

To aid in the investigation of the role of Zm*NCED*s in maize seed vigour, *ZmNCED* expression was measured in the 10 seed lots of five different varieties introduced in Chapter 3. In seed lot 4B the highest level of *ZmNCED* expression occurred 168 hours after imbibition at 5°C (Figure 6.3), so samples from all seed lots were taken at this point. The expression of the *ZmNCED*s after 168 hours at 5°C in vermiculite in

the 10 seed lots is shown in Figure 6.4. One-way ANOVA revealed that there were significant differences in expression of *ZmNCED1* ($p < 0.001$), *ZmNCED3a* ($p = 0.007$), *ZmNCED3b* ($p < 0.001$), and *ZmNCED9* ($p < 0.001$), and total *ZmNCED* ($p < 0.001$) but not *ZmNCED2* ($p = 0.136$) between seed lots (Appendix 6.2). Comparisons between means were made using Tukey's HSD ($p < 0.05$).

ZmNCED1 was expressed at relatively high levels in all seed, with lot 3B indicating significantly ($p < 0.05$) lower *ZmNCED1* expression that the other seed lots (including 3A, also of variety 3). Lot 5A also expressed *ZmNCED1* significantly ($p < 0.05$) more than most other seed lots, including 5B which belongs to the same variety.

ZmNCED2 was again expressed at relatively low levels, and no significant differences in *ZmNCED2* expression were observed between seed lots.

ZmNCED3a appeared to be expressed at similar levels (sometimes higher), to *ZmNCED2*. Lots 2A, 3A, and 5A showed significantly higher levels of *ZmNCED3a* expression than one other seed lot, 4B ($p < 0.05$).

ZmNCED3b was generally expressed at higher levels than seen with *ZmNCED2* and *ZmNCED3a* but less than *ZmNCED1*. *ZmNCED3b* expression was particularly high in Variety 4 (significant compared to 4 other seed lots) and particularly low in Variety 5 (significant compared to 6 other seed lots). No significant within-variety differences of *ZmNCED3b* expression were observed.

ZmNCED9 was expressed at relatively lower levels than *ZmNCED1* in all seed lots except for seed lots of Variety 4, whose *ZmNCED9* expression matched that of the *ZmNCED1* expression. The difference between *ZmNCED9* expression lots of variety 4 was not significant.

The total *ZmNCED* expression was highest in Variety 4. A significant ($p < 0.05$) within-variety difference in total *ZmNCED* expression was only seen in variety 3, with 3A having higher overall *ZmNCED* expression than 3B. Thus expression patterns of *ZmNCED*s in the cold test can be seed lot and variety dependent.

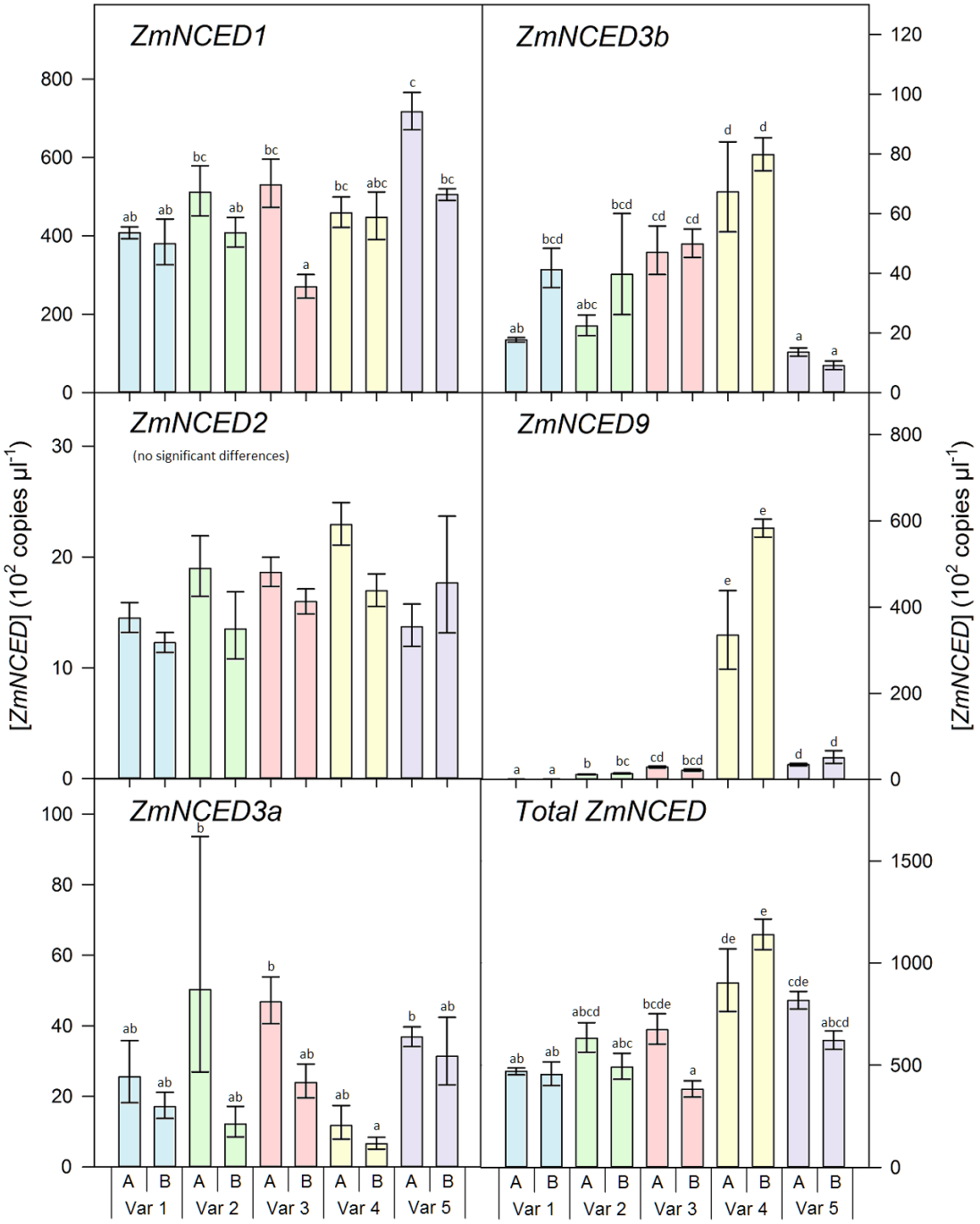

Figure 6.4. Expression of *ZmNCED*s in different seed lots under vermiculite cold test conditions. Maize seeds from different seed lots (from Variety 1 lot A to Variety 5 lot B, left to right) were imbibed at 5°C for seven days in vermiculite (168 hour time point indicated in Figure 6.1). Embryos were isolated, RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5. Error bars indicate standard errors calculated from log-transformed data (n = 3). Please note that the y-axis scale is different for each plot. Bars that do not share common letters within each plot are significantly different according to Tukey's HSD (Appendix 6.2). Back transformed data is plotted.

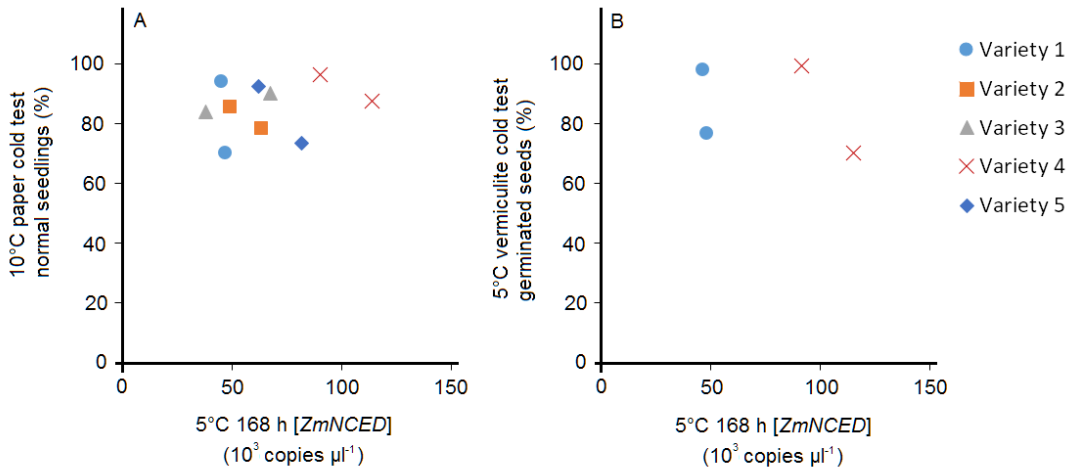

Figure 6.5. Relationship between *ZmNCED* expression and vigour indicators. A: The relationship between *ZmNCED* expression after 168 hours imbibition at 5°C in vermiculite and 10°C paper cold test results (provided by Syngenta, Figure 3.3, used as an indicator of vigour for all seed lots). **B**: The relationship between *ZmNCED* expression after 168 hours imbibition at 5°C in cold vermiculite and 5°C vermiculite cold test germination. Data for 5°C vermiculite cold test germination only available for four seed lots from two varieties.

Whilst only four of the 10 seed lots have been characterised under the 5°C vermiculite cold test (used for sampling for expression measurements), vigour has been approximated in the 10°C cold test (Figure 3.3). To compare *ZmNCED* expression and vigour, the sum number of *ZmNCED* transcripts was plotted against the 10°C paper cold test germination percentage (Figure 6.5 A). There was no obvious overall trend evident; but in four out of five varieties, the low vigour seed lot of the variety, determined by the 10°C cold test, tends to have a higher number of total *ZmNCED* transcripts, Variety 3 was the exception (Figure 6.5 A). The same trend is evident in two varieties, 1 and 4, tested in the 5°C vermiculite cold test (Figure 6.5 B), although it is worth noting that the within variety differences in *ZmNCED* expression were not significant for these two varieties (Figure 6.4.). Thus whilst expression patterns of *ZmNCED*s in the cold test can be seed lot and variety dependent, there is not yet a clear relationship between these patterns and vigour.

6.4 *ZmNCED* Expression and ABA Accumulation during Imbibition at Different Temperatures

Germination of NK Falkone was reported to be accelerated at suboptimal (but germination permissive) temperatures by hydroxamic acid CCD inhibitors (David Brocklehurst, Syngenta, personal communication, September 22, 2014), suggesting a possible role of ABA delaying germination at low temperatures.

In Chapter 3 (Section 3.3.4), no effect of the hydroxamic acids on germination time was found when seed were imbibed at 10°C, 15°C and 20°C on vermiculite. It is possible that the particular experimental setup differed to the proprietary test used by Syngenta. For example, here it could be that vermiculite may adsorb the compounds, or that the delivery method or concentration of the compounds was not optimal.

So to further investigate a potential role of ABA biosynthesis in delaying maize germination at low, germination permissive temperatures, *ZmNCED* expression and ABA concentration were measured. Samples were obtained from NK Falkone seed imbibed for 6 hours, 24 hours, 48 hours and 72 hours at 10°C, 15°C and 20°C. Dry seed were also sampled.

6.4.1 ZmNCED expression during imbibition at different temperatures

The expression of the *ZmNCED*s during imbibition at different temperatures was quantified (Figure 6.6). ANOVAs revealed that there were significant effects of temperature at some time-points and the effects differed between *ZmNCED*s (Appendix 6.3). It is worth noting that radicles had emerged (> 95%) from kernels at the 72 hour time point at 20°C (see Figure 6.7 for germination plot). Tukey's HSD was used to identify significant comparisons between means.

Whilst *ZmNCED1* expression was relatively low in dry seed and remained so throughout the course of 72 hours at 20°C, however *ZmNCED1* expression at 15°C was slightly induced at some time points, and at 10°C *ZmNCED1* was considerably induced. *ZmNCED1* expression at 15°C was significantly ($p < 0.05$) higher at 15°C than 20°C at 6 and 24 hours after sowing. *ZmNCED1* expression increased significantly ($p < 0.05$) 6 hours after sowing at 10°C and 15°C, but a very large, significant ($p < 0.05$), induction of *ZmNCED1*was seen between 24 and 48 hours, and this level was maintained at 72 hours.

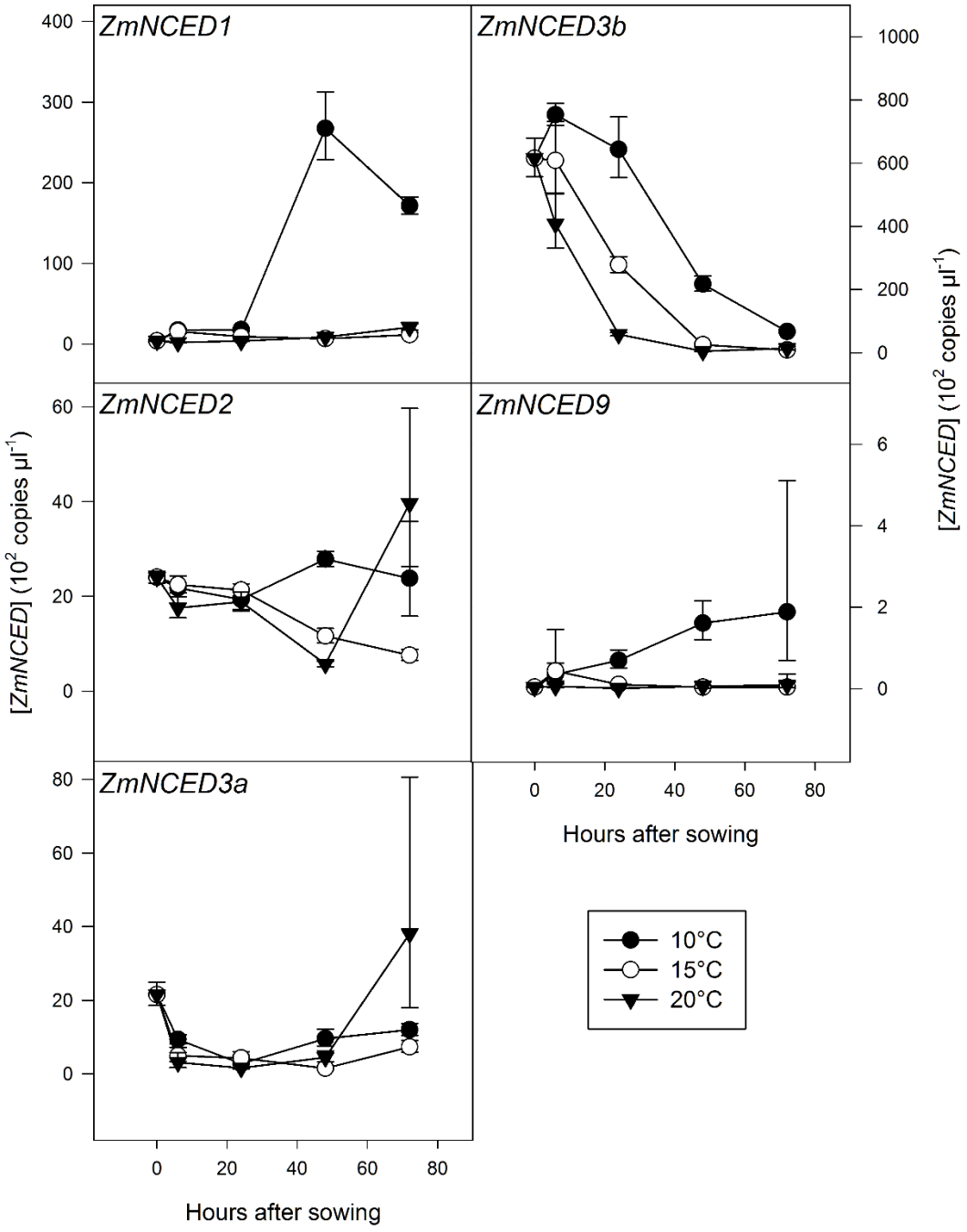

Figure 6.6. Expression of *ZmNCED*s during maize imbibition at different temperatures. Seed of NK Falkone were imbibed at 10°C, 15°C or 20°C. Samples (3 replicates of 20 embryos) were taken at 6 hours, 24 hours, 48 hours and 72 hours after sowing. Dry seed were also sampled. RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5. Error bars for [*ZmNCED*] indicate standard error as calculated on log-transformed data (n = 3). Back transformed data is plotted. Please note that the y-axis scale is different for each plot.

No significant change in *ZmNCED2* expression was seen in the first 24 hours of imbibition at any temperature and *ZmNCED2* expression was higher than expression of *ZmNCED1* during this time period. *ZmNCED2* expression significantly ($p < 0.05$) lowered between 24 hours and 48 hours of imbibition at 15°C, whilst the higher

levels of expression were observed at 10°C. Indeed the expression of *ZmNCED2* did not change significantly at any time point at 10°C. *ZmNCED2* expression increased significantly ($p < 0.05$) at 20°C after 72 hours compared to 15°C, rising to levels not significantly different to those observed at 10°C. This rise in expression occurred after radicles had emerged at this temperature (20°C).

ZmNCED3a expression was similar to *ZmNCED2* expression in dry seed. *ZmNCED3a* expression dropped after 6 hours, and low levels were maintained throughout the 72 hours at 10° and 15°C. Similar to the change seen in *ZmNCED2*, *ZmNCED3a* expression increased significantly ($p < 0.05$) between 48 hours and 72 hours at 20°C (again after radicles had emerged at this temperature). *ZmNCED3a* expression was significantly higher at 10°C than at 15°C and 20°C at the 48 hour time point. No significant differences in *ZmNCED3a* expression were seen between the temperatures at the 72 hour time point.

ZmNCED3b levels were very high in dry seed – the highest level of any *ZmNCED3b* expression seen. Expression of *ZmNCED3b* did not significantly change after 6 hours of imbibition at any temperature. After 24 hours, *ZmNCED3b* expression decreased significantly ($p < 0.05$) in seed imbibed at 20°C. However *ZmNCED3b* expression remained at the high levels in seed imbibed at 10°C which were not significantly more than *ZmNCED3b* expression at 15°C at 24 hours. A further significant ($p < 0.05$) reduction in *ZmNCED3b* expression was seen after 48 hours at all temperatures, but expression at 10°C was significantly ($p < 0.05$) higher than *ZmNCED3b* expression at 15°C, which was significantly ($p < 0.05$) higher than *ZmNCED3b* expression at 20°C. After 72 hours, a further reduction in *ZmNCED3b* expression was seen at 10°C, but expression was still significantly ($p < 0.05$) higher than at 15°C and 20°C. At this point, similar to seen with *ZmNCED2* and *ZmNCED3a*, *ZmNCED3b* expression increased slightly, but significantly, in seed imbibed at 20°C.

ZmNCED9 expression was only seen at very low levels (lower than all other *ZmNCED*s), and expression of *ZmNCED9* at 10°C appeared was not significantly ($p < 0.05$) higher than expression at 15°C and 20°C at 48 hours and 72 hours.

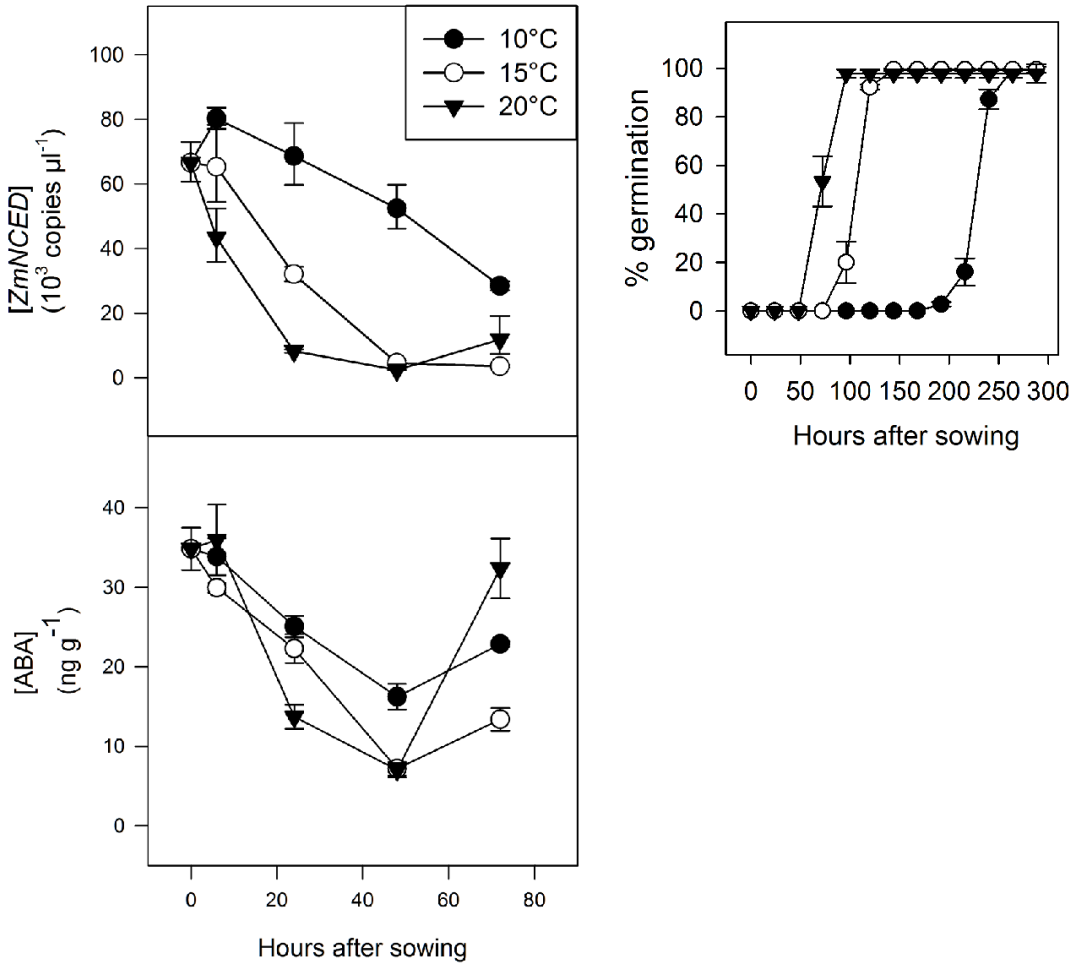

Figure 6.7. Sum of *ZmNCED* expression and ABA accumulation during maize imbibition at different temperatures. Seed of NK Falkone were imbibed at 10°C, 15°C or 20°C. Samples (3 replicates of 20 embryos) were taken at 6 hours, 24 hours, 48 hours and 72 hours after sowing. Dry seed were also sampled. RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5, and pulverised sample was sent for ABA quantification. The sum of the number of copies measured in all *ZmNCED*s (see Figure 6.5 for individual measurements) was log-transformed prior to analysis. Back-transformed means and standard errors are indicated. [ABA] data was untransformed for analysis. Data is represented in nanograms ABA per gram of embryo (ng g^{-1}). Error bars indicate standard error (n = 3).

Thus the largest absolute changes in *ZmNCED* expression occurred with the induction of *ZmNCED1* at 10°C after 48 hours, and the reduction of *ZmNCED3b* over time. The slower reduction in *ZmNCED3b* expression and the induction of *ZmNCED1* expression contributed to the overall high levels of *ZmNCED* in maize seed imbibed at 10°C (Figure 6.7). Over the course of the 72 hours imbibition, *ZmNCED* expression generally declined, but the decline took longer at lower temperature. The decline in *ZmNCED* expression was significant ($p < 0.05$) at 24 hours at 20°C and 15°C but the decline was only significant ($p < 0.05$) at 72 hours in at 10°C. At 24 hours, the *ZmNCED* expression was significantly ($p < 0.05$) higher at 10°C than at 15°C and significantly ($p < 0.05$) higher at 15°C than 20°C. Between 48

and 72 hours, the total *ZmNCED* expression increased significantly ($p < 0.05$) at 20°C (after radicle emergence had occurred) and expression at 10°C decreased, whilst expression at 15°C remained the same. Between 6 hours and 72 hours, the total *ZmNCED* expression was always significantly ($p < 0.05$) higher in seed imbibed at 10°C. Between 6 hours and 48 hours, *ZmNCED* expression was significantly ($p < 0.05$) higher at 15°C than at 20°C, however at 72 hours *ZmNCED* expression was higher at 20°C than at 15°C; although at this point radicle emergence was observed in the seed imbibing at 20°C but seed imbibed at 15°C remained ungerminated.

6.4.2 ABA accumulation during imbibition at different temperatures

Samples from the same experiment were also sent for ABA measurement at Syngenta, Jealott's Hill, UK (Figure 6.7). Overall effects of time ($p < 0.001$) and temperature ($p = 0.003$), and an interaction between time and temperature ($p < 0.001$) were observed (Appendix 6.3).

ABA concentration did not significantly ($p > 0.05$) change after 6 hours of imbibition at any temperature. At the 24 hour time point, ABA concentration was significantly ($p < 0.05$) lower at 20°C than at 10°C or 15°C. At the 48 hour time point, ABA concentration was significantly ($p < 0.05$) lower at 20°C and 15°C than at 10°C. At 72 hours, the ABA concentration at 15°C was significantly ($p < 0.05$) lower than 10°C and 20°C. At 10°C, the ABA concentration reduced significantly at 24 hours and again at 48 hours ($p < 0.05$). However the concentration at 72 hours at 10°C was not significantly ($p > 0.05$) different to the 24 hour time point. In similarity, At 15°C, the ABA concentration reduced significantly at 24 hours and again at 48 hours ($p < 0.05$), but remained significantly lower than the 24 hour time point at 72 hour time point. Again at 20°C, ABA concentration dropped significantly ($p > 0.05$) by 24 hours compared to the dry seed, but no further significant reduction was seed at 48 hours; but a significant increase in ABA concentration was seen at 72 hours compared to the 48 hour time point (i.e. when radicles had emerged at this temperature). ABA concentration at this time point was not significantly ($p > 0.05$) lower than in dry seed.

Thus the overall trend was that ABA concentration reduced in seed over time at any temperature, but that the decrease in ABA was faster at 20°C than at 15°C, and the

decrease was larger at 15°C and 20°C than at 10°C, resulting in generally elevated. ABA concentration at 10°C compared to the other two temperatures. An increase in ABA concentration was seen after radicle emergence at 20°C.

6.4.3 *Summary*

The induction of *ZmNCED1* and the slower decline of *ZmNCED3b* expression at 10°C leads to a maintenance of higher *ZmNCED* expression during imbibition at 10°C compared to 15°C and 20°C. At 15°C, the rate of *ZmNCED3b* reduction is slower than at 10°C, leading to an overall higher expression of *ZmNCED*, but only a very small induction of *ZmNCED1* occurs (compared to that seen at 10°C). At 25°C the decline in *ZmNCED3b* expression happens quicker leading to more rapid reduction in overall *ZmNCED* expression compared to the two higher temperatures. At 72 hours, there is an increase in *ZmNCED* expression at 20°C with *ZmNCED1*, *ZmNCED3a* and *ZmNCED3b* induction contributing most to this trend, which occurs subsequent to radicle emergence. The overall trend of ABA accumulation is similar to the trend observed in total *ZmNCED* expression, with the most rapid decline in ABA observed at 20°C, followed by 15°C and 10°C. ABA accumulation is observed post-radicle emergence at 20°C, and at the 72 hour time point ABA also increased in seed imbibed at 15°C and 10°C. Thus ABA concentration and *ZmNCED* expression are maintained at higher levels in seed imbibed at lower temperatures.

6.5 The Effect of NCED Inhibitors on ABA Accumulation during Imbibition at Different Temperatures

In a proprietry test at Sygenta, germination of NK Falkone was reported to be accelerated by hydroxamic acid CCD inhibitors at low temperatures (David Brocklehurst, Syngenta, personal communication, September 22, 2014). An effect of low temperatures on inducing *ZmNCED* expression and elevating ABA concentration was observed in vermiculite (Section 6.4), but no effect of the hydroxamic acid CCD inhibitors on germination was observed (Chapter 3 Section 3.3.4).

To test if NCED inhibitors affected ABA accumulation in this system, seed of NK Falkone were imbibed on media containing NCED inhibitor solutions at 10°C and 15°C. ABA was quantified from samples taken 24 hours and 72 hours after sowing (Figure 6.8). There was no significant effect of inhibitor treatment at either

temperature at 24 or 72 hours (p = 0.971 and 0.687, respectively), but temperature had a significant effect at both time points (p < 0.001 for both). At 24 hours, ABA concentration was 25% lower at 15°C than at 10°C. At 72 hours, the ABA concentration at 15°C was 38% lower than at 10°C. The ABA concentration at both temperatures reduced between 24 hours and 72 hours (by 55% reduction at 10°C and a 63% reduction at 15°C). Although no effect of inhibitors was observed, these results reinforced the previous finding (Figure 6.6) that the decline in ABA concentration is faster at 15°C than at 10°C.

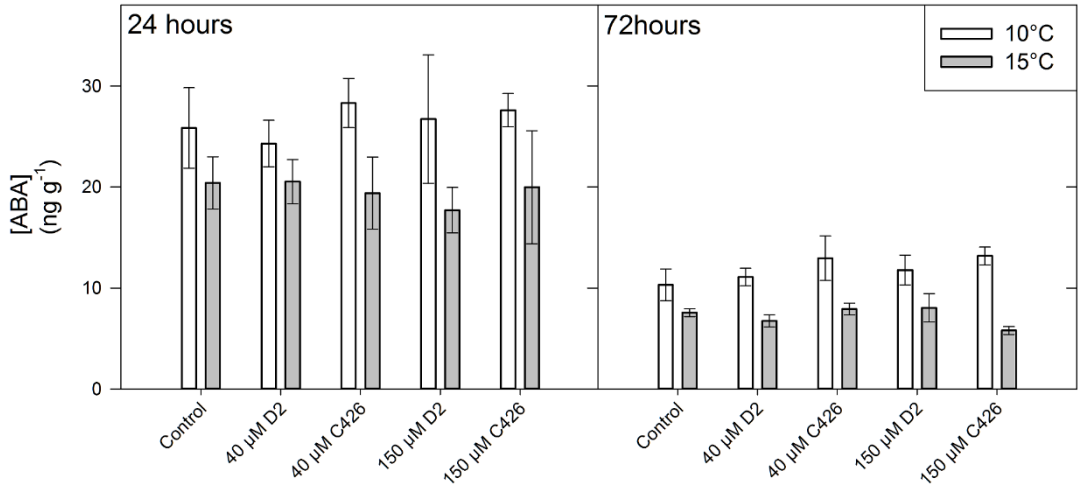

Figure 6.8. The effect of imbibition in NCED inhibitor solutions on ABA accumulation. Seed of NK Falkone were imbibed on vermiculite containing aqueous solutions of 40 µM or 150 µM D2; 40 µM or 150 µM C426, or without inhibitor (control). Seed were imbibed at 10°C or 15°C. Samples were taken at 24 hours and 72 hours after sowing and ABA was quantified. Means of 5 replicates (7 embryos each) per treatment are indicated. Error bars indicate standard error.

6.6 Discussion

6.6.1 ZmNCEDs are induced and ABA levels maintained under cold test conditions

The vermiculite cold test reduced germination of the low-vigour seed lot 4B (Chapter 3), and the effect of the cold was reversible, potentially indicating the induction of a dormant state. Dormancy induction and maintenance often depends on the biosynthesis of ABA (Finch-Savage and Leubner-Metzger, 2006). NCED, which catalyses the last committed and rate-limiting step in ABA biosynthesis, is differentially expressed temporally and spatially allowing control of ABA related processes (Frey, *et al.*, 2012; Lefebvre, *et al.*, 2006; Schwartz, *et al.*, 1997; Tan, *et al.*, 2003; Tan, *et al.*, 1997; Taylor, *et al.*, 2005). Therefore it was hypothesised that *ZmNCED* expression may increase during imbibition of maize under cold conditions,

resulting in increased ABA accumulation, which could explain the reversible non-germinating state observed.

Samples were collected from lot 4B during imbibition under cold test and control (warm test) conditions for quantification of *ZmNCED* expression and ABA accumulation. Cold test conditions clearly induced *ZmNCED* expression, particularly of *ZmNCED1* and *ZmNCED9,* and reduced the decline in *ZmNCED3b* expression. However, compared to seed directly imbibed at 25°C, seed transferred after seven days at 5°C to 25°C showed a rapid reduction in *ZmNCED* expression. Overall, cold test treatment resulted in accumulation of *ZmNCED* transcripts. It may then be expected that ABA would also accumulate during the cold treatment, however this was not the case. During imbibition at 5°C, the ABA concentrations actually were reduced at 72 hours, indicating that ABA was catabolised (or leached) more rapidly than it was synthesised. This may indicate a lag between the *ZmNCED* induction and ABA biosynthesis.

Whilst ABA does not accumulate at 5°C, between 72 hours and 168 hours in the cold, the ABA concentration does not decrease further. This could be an indicator that the induction of *ZmNCED* balanced the rate of ABA loss through increased ABA biosynthesis. 2 hours after transfer from 5°C to 25°C there is a rapid increase (although only significant compared to the 72 hour time point) in ABA concentration, even though the total *ZmNCED* expression does not increase. This is perhaps as a result of the increase in temperature accelerating the rate at which *ZmNCED* expression can lead to ABA biosynthesis (i.e. due to increased rate of catalysis). The rapid decrease in *ZmNCED* expression 6 hours after transfer is simultaneous to the rapid reduction in ABA accumulation, and levels are maintained up to 24 hours after transfer. Thus the overall speed of decrease in ABA concentration in seed transferred from 5°C to 25°C is more rapid than in seed directly imbibed at 25°C. However the cold test seed had been imbibed for seven days which may accelerate the process compared to dry seed that are imbibed at 25°C. Thus it is possible that *ZmNCED* transcripts accumulate during the cold, this may allow the rapid ‘spike’ in ABA concentration when seed are transferred to 25°C, which could induce dormancy. The ABA stays at a lower level, but perhaps higher than seeds not cold treated to maintain dormancy.

The trend in ABA concentration bares some similarities and differences to high-temperature induced secondary dormancy in barley (Leymarie, *et al.*, 2008). Similar to what was seen in barley, the ABA concentration decreased compared to dry seed and then stabilised over time under non-permissive conditions. However, in maize, *NCED* expression increased overall, compared to dry seed whereas it was reduced in barley. Additionally, there is an increased level of *NCED* expression and ABA concentration in secondary dormant barley seeds after transfer to permissive conditions (Leymarie, *et al.*, 2008). However, in maize both *NCED* expression and ABA levels are reduced after seed are transferred from germination non-permissive temperatures (5°C) to germination permissive temperatures (25°C). It would be interesting to measure the expression of genes related to ABA catabolism and their products under these conditions to see if ABA catabolism is the reason for the reduction in ABA concentration during imbibition (rather than leaching, for example).

6.6.2 ZmNCED expression under cold test conditions varies between seed lots

10 maize seed lots, provided by Syngenta, varied in vigour according to vigour indicators. One such indicator of vigour is the percentage of normal seedling establishment at 25°C following prior imbibition of seeds at 10°C on paper (discussed in Chapter 3), which was performed by Syngenta. In Chapter 3, a modified protocol in which germination was measured after seven days at 5°C and three days at 25°C in vermiculite differentiated between the vigour of four of these seed lots. High and low vigour seed lots of variety 1 were identified (1B and 1A, respectively); and high and low vigour seed lots of variety 4 were identified (4A and 4B, respectively). This vermiculite cold led to an induction of *ZmNCED* in low vigour seed lot 4B. The highest levels of *ZmNCED* expression were observed between 168 hours of imbibition at 5°C and 2 hours after transfer from 5°C to 25°C.

It was hypothesised that high vigour seed lots may express *ZmNCED* at lower levels after 168 hours at 5°C which may then result in decreased ABA accumulation and this may partly explain their increased ability to germinate after transfer to 25°C in the cold test. In lettuce, thermoinhibition of germination between varieties can vary due to differences in expression of *LsNCED4* and ABA accumulation (Argyris, *et al.*, 2008; Huo, *et al.*, 2013). Here it is observed that the different maize seed lots and

varieties also exhibit varied levels of *ZmNCED* expression, although the relationship to seed vigour is unclear.

The expression of *ZmNCED*s in 10 seed lots after 168 hours at 5°C was examined. Only four of these seed lots were characterised under the 5°C vermiculite cold test, but vigour was approximated in the 10°C cold test (Figure 3.3). Thus *ZmNCED* expression was compared to vigour for each seed lot, by plotting the sum number of *ZmNCED* transcripts against the germination following a 10°C paper cold test (Figure 6.5 A). In varieties 1,2, 4 and 5 the low vigour seed lot of the variety, determined by the 10°C cold test, tended to have a higher number of total *ZmNCED* transcripts. The same trend is evident in two varieties tested in the 5°C vermiculite cold test (Figure 6.5 B). The difference in *ZmNCED* expression in Variety 1 was very small compared to the differences in germination. However, the low vigour seed lot of Variety 1 (1A) exhibits lower germination even under optimal conditions (discussed in Chapter 3). So it may be necessary to compare the *ZmNCED* expression data to the difference between cold test and control germination in order to see if *ZmNCED* expression is affecting vigour. Testing all these seed lots in the 5°C vermiculite cold test and control conditions will allow this comparison to be made.

ZmNCED expression was seed lot, and variety, dependent. In particular, the strong induction of *ZmNCED9* at 5°C was only apparent in seed lots of variety 4, and was significantly elevated in the low-vigour seed lot (4B). Interestingly, lot 1A and lot 1B which both do not show a large decrease in germination after vermiculite cold test conditions (compared to control conditions) exhibit the lowest levels of *ZmNCED9* expression and significantly lower total expression of *ZmNCED* compared to lot 4B. However the total expression of *ZmNCED*s in the high vigour seed lot 4A is not significantly different to that seen in the low-vigour seed lot 4B.

Further characterisation of all seed lots under the vermiculite cold test conditions may allow for further correlations between expression of particular *ZmNCED*s and vigour, due to the diversity in *ZmNCED* expression observed. It is also possible that the *ZmNCED* expression at different time points (e.g. 2 hours after transfer, or the high *ZmNCED3b* expression in dry seed), or ABA accumulation, are more important in determining germination under vermiculite cold test conditions.

Because there appears to be both an effect of variety and seed lot on *NCED* expression under the cold test, the *NCED* expression may vary as a result of both genetic factors (e.g. transcription factors involved in controlling *NCED* expression) and the environmental conditions of seed production (i.e. including epigenetic factors) and storage. However more lots from each variety would need to be tested in order to confirm genetic effects.

6.6.3 ZmNCED expression and ABA concentrations are elevated during imbibition at low temperatures

Application of exogenous ABA to maize seed imbibed at an optimal temperature appeared to delay maize germination in a similar way to lower temperatures (Chapter 3). Dormancy is not always expressed as the complete ability or inability of a seed to germinate given a set of imbibition conditions. In tobacco, primary dormancy is reduced by after-ripening. Some dormant seed, rather than display an inability to germinate, exhibit an ABA dependent delay in germination (Grappin, *et al.*, 2000). It is possible that part of the delay in germination of maize under low-temperatures, is too, attributable to ABA biosynthesis. Compared to 20°C, low temperatures did indeed elevate overall levels of *ZmNCED* expression, attributable to the slower decline of *ZmNCED3b* expression and induction of *ZmNCED1* expression at 10°C, similar to that seen at 5°C in the cold test. A similar trend was seen at 15°C, but without the induction of *ZmNCED1*. ABA levels were also elevated at low temperatures, although they declined over time. It is possible that this delayed reduction in ABA concentration leads to the delay in germination at low temperatures. It would be interesting to see if ABA and *ZmNCED* expression levels are maintained, or rise, at 10°C beyond 72 hours, since germination does not begin until one week after sowing. There may too be a lag between the induction of *ZmNCED1* between 48 hours and 72 hours and the resulting ABA biosynthesis (i.e. as a result of a reduced rate of metabolism at lower temperatures). Thus sampling embryos from beyond 72 hours (e.g. up to germination, at ca. 9 days) might provide more insight in to the regulation of ABA accumulation in NK Falkone imbibed at low temperatures.

6.6.4 NCED inhibitors did not reduce ABA concentration during imbibition at low temperatures

Treatment of seed imbibing at 10° and 15°C did not reduce the seed ABA concentration 24 hour or 72 hours after imbibition. There are multiple possible reasons for this to occur. Firstly it is possible that the inhibitors are not strong enough inhibitors of NCED, or that there are issues with uptake. The former reason is supported by the observation of relatively weak inhibition observed *in vitro* (Chapter 4; Sergeant et al., 2009; Awan *et al*., in preparation). However, hydroxamic acids have been shown to promote germination in situations where ABA concentrations are elevated, and reduce ABA accumulation (Awan *et al*.,in preparation; Chapter 3). Unfortunately, lower concentrations (150 µM) of inhibitors had to be used than previously used to see effects in the cold test (1 mM) due to limitations in the available compound provided by Syngenta (i.e. it is expensive to synthesise). Thus it is still possible that inhibition of ABA biosynthesis would be observed with increased compound concentrations.

It is also possible that the ABA concentration is not dependent on ABA biosynthesis. Indeed the dry seed appear to contain the highest concentration of ABA, so it may be that the differences in ABA concentration at different temperatures are dependent on the loss of ABA through leaching or catabolism. A similar trend is seen in barley, where ABA is at the highest concentration in dry seed and declines over time under suboptimal conditions. However, this maintenance of ABA concentration is likely dependent on ABA biosynthesis since fluridone reduces the ABA concentration (Leymarie, *et al.*, 2008). Therefore it appears to be the balance between ABA biosynthesis and catabolism which is controlling the ABA levels. Additionally the elevation of *ZmNCED* expression seen in maize imbibed at low temperatures is highly suggestive of increased ABA biosynthesis.

Therefore it seems likely that the compound treatments were ineffective at reducing ABA concentration and this may explain the lack of effect of the compounds on stimulating germination of maize imbibed at low temperatures. Investigation of the effects of more concentrated or more potent ABA biosynthesis inhibitors may yield more insight into the role of ABA in germination at lower temperatures.

6.6.5 Conclusions

ZmNCED expression is clearly affected by imbibition under suboptimal temperatures, at which some *ZmNCED*s (particularly *ZmNCED1*, *ZmNCED3b* and *ZmNCED9*) appear to be particularly abundant. This increase in *ZmNCED* expression does not lead to large increases in ABA accumulation under cold test conditions (perhaps because of catabolism) or under low temperature imbibition, although there is a trend of maintained ABA levels under sub-optimally low temperature conditions. These results are generally supportive of the role of ABA in retarding germination under cold test and low-temperature conditions. The conditions tested would be suitable for further testing of new *NCED* inhibitors and novel germination stimulating compounds (Chapter 7).

Further examination is required, for example, in the investigation of other mechanisms involved in seed dormancy and germination (e.g. ABA catabolism and sensitivity, and gibberellin related mechanisms). Additionally the mechanism through which the effect of seven days at 5°C is reversed by a drying treatment could be investigated though measurement of gene expression and hormone quantification. Correlations between *ZmNCED* expression, ABA accumulation and cold test performance of different seeds lots, should also be investigated further.

7 Screening Chemical Libraries for Germination Stimulating Compounds

7.1 Introduction

7.1.1 The need for novel germination stimulating compounds

As discussed in the main introduction, there is a general need for novel germination stimulating compounds. In Chapter 3, there was evidence for small effects of hydroxamic acid CCD inhibitors in improving maize vigour under cold test conditions. However, hydroxamic acids failed to affect maize germination or ABA accumulation following imbibition under suboptimal conditions in other experiments. The current lead compound, hydroxamic acid D4, most likely improves germination in tomato and lettuce through inhibition of NCED and the resulting decreased ABA biosynthesis (Awan *et al*., in preparation). However, D4 appears to exhibit relatively weak inhibition of NCED: *in vitro,* 100 µM D4 results in ca. 30% inhibition of *Le*NCED1 (and *Zm*NCEDs in Chapter 4), but almost complete inhibition of *Le*CCD1 and *At*CCD8 (Harrison, *et al.*, 2015; Sergeant, *et al.*, 2009). This weak inhibition appears to be reflected in the concentrations required for germination stimulation effects *in vivo*. In (Awan *et al*., in preparation), 1 mM D4 (0.312 g l^{-1}) was required for maximum germination stimulating activity, however 0.5 mg l^{-1} norflurazon was more effective than 1 mM D4. Application of norflurazon is, however, phytotoxic to germinated seedlings, due to inhibition of phytoene desaturate and consequent depletion of carotenoids. Indeed, D4 failed to affect maize germination or ABA levels in multiple experiments in Chapters 3 and 6. Thus there is likely scope for the development of a more potent NCED inhibitor which stimulates germination by screening compounds structurally related to D4.

Alternatively, it is possible that inhibition of another step in ABA biosynthesis or signalling, or perturbation of an alternative signalling pathway, could result in germination stimulation. For this reason, compounds which exhibit germination stimulating activity, but are structurally dissimilar, are also of interest as they may represent compounds that have different modes of action. It is also possible that structurally dissimilar compounds could be new lead NCED inhibitors.

However, the experimental methods used in Chapter 3 (i.e. vigour tests of maize seed) do not lend themselves to the screening of novel compound libraries due to the high volumes of solution that would be required. Thus alternative means for identifying more potent NCED inhibitors or novel treatments are required.

7.1.2 High-throughput screening

High-throughput screening (HTS), in this case, is a method of quickly testing a large number of compounds in an assay to identify those that yield desirable results. HTS has been developed and extensively used for discovering novel lead compounds, particularly for drug discovery in the pharmaceutical industry (Mayr and Bojanic, 2009). HTS has become increasingly more high-throughput with advances in robotics and miniaturisation driving potential compound screening rates from 10 k compounds per day up to 200 k compounds per day (Mayr and Bojanic, 2009; Persidis, 1998). HTS has also been adopted in the agrochemical industry, particularly for identifying novel pesticides and fungicide using *in vivo* and *in vitro* methods (Drewes, *et al.*, 2012).

Concerns in high-throughput screening

High-throughput screens typically depend on a reliable assay from which a positive 'hit' compound will always elicit the desired result, and be identified, and that inactive compounds will never elicit the desired result. That is to say that the assay would have a low false positive and low false negative rate. This is of particular importance as replication in high-throughput assays is typically very low (compounds are perhaps only screened once), to both maximise the number of compounds that can be tested, and minimise the amount of compound that is used.

The suitability of an assay for high-throughput can be summarised by a number of parameters, including the 'Z-factor' (Zhang, *et al.*, 1999). This factor takes into account the variation of the compound library sample and the control, and the separation between the means of the sample and control (Equation 7.1). Thus in a good assay, the difference between mean signal produced by the positive control and the sample (Z) or the negative control (Z′) should be separated by more than 3 control standard deviations plus 3 sample (Z) or negative control (Z′) standard deviations (Z or $Z' = 1$). However this method does not necessarily describe the ability of the assay to detect weakly active compounds, since it compares the

negative and positive (perhaps the most strongly active) control (Mayr and Bojanic, 2009). In reality, there are also many more considerations, such as determining the day-to-day variations, between plate, and within plate position effects, which can essentially mean that screen quality parameters, such as the Z-factor, vary within experiments (Gribbon, *et al.*, 2005). An assay which has high quality (e.g. Z-factor) and low variability between experiments is desirable to enable low, or no, replication screens for large compound libraries.

$$Z = 1 - \frac{(3\sigma_s + 3\sigma_c)}{|\mu_s - \mu_c|}$$

Equation 7.1. Calculation of the Z-factor. σ_s, standard deviation of the sample signal sample of compounds (i.e. 'background signal'); μ_s, mean of the signal from a sample of compounds; σ_c, standard deviation of the positive control signal; μ_c, mean of the positive control signal; where the signal is the output of the assay. For Z′, σ_s and μ_s are replaced with the signal from a sample of negative controls.

7.1.3 Chemical genetics and high throughput screening

As discussed in the Chapter 1 (Section 1.9), chemical genetics is the investigation of biological systems through the use of small molecules (O'Connor, *et al.*, 2011). In Chapter 3 and 6, hydroxamic acid CCD inhibitors were used to probe the roles of *ZmNCED*s in maize germination and ABA accumulation, an example of reverse chemical genetics. This is analogous to reverse genetics, where the equivalent procedure would be to knock-out the *ZmNCED* genes to uncover their roles.

In the screen described in this chapter, the result could be a combination of reverse and forward chemical genetics. The reverse chemical genetics element would be identifying new compounds which inhibit NCED – which could then be used to study the role of NCED in other plants. Alternatively, compounds will be found that act by another mode-of-action, and therefore further study will be required to identify these mechanisms – forward chemical genetics.

7.1.4 The basis of the assay

Tetracycline inducible expression of *LeNCED1* in tobacco was developed in Thompson et al., 2000. Application of tetracycline leads to the derepression of the promoter sequence controlling the expression of the *LeNCED1* transgene (Gatz, *et al.*, 1992). This results in over-production of abscisic acid, at least in the case of

vegetative tissue (Thompson, *et al.*, 2000). In the present work, preliminary results also indicated that seed were responsive to the tetracycline treatment, as germination was inhibited, presumably due to over-production of ABA. This was also observed with constitutive over-expression of *NCED* in tobacco and tomato and also with dexamethasone inducible *PvNCED* expression on tobacco (Qin and Zeevaart, 2002; Thompson, *et al.*, 2000). The dormancy-inducing effects of constitutive over-expression are reversible by norflurazon, which inhibits ABA biosynthesis through inhibition of phytoene desaturase (Bartels and Watson, 1978; Thompson, *et al.*, 2000). Thus it is possible that the dormant seed could be used to screen compounds for germination stimulating activity, particularly inhibitors of ABA biosynthesis or signalling (Figure 7.1).

Tobacco seed harbouring the tetracycline inducible *LeNCED1* represent a useful material for the development of a high-throughput screen: Firstly the inducible nature of the *NCED* expression may allow for tailoring of the level of dormancy exhibited by the seeds, which may enable the assay strength to be altered (i.e. a less deep dormancy may identify relatively 'weak' germination stimulating compounds). Secondly, the tobacco seed are small enough to be used in the wells of a microtiter plate (unlike maize, the eventual target), reducing the amount of screen compound has to be used. This is useful since the screen compounds are often not in abundance.

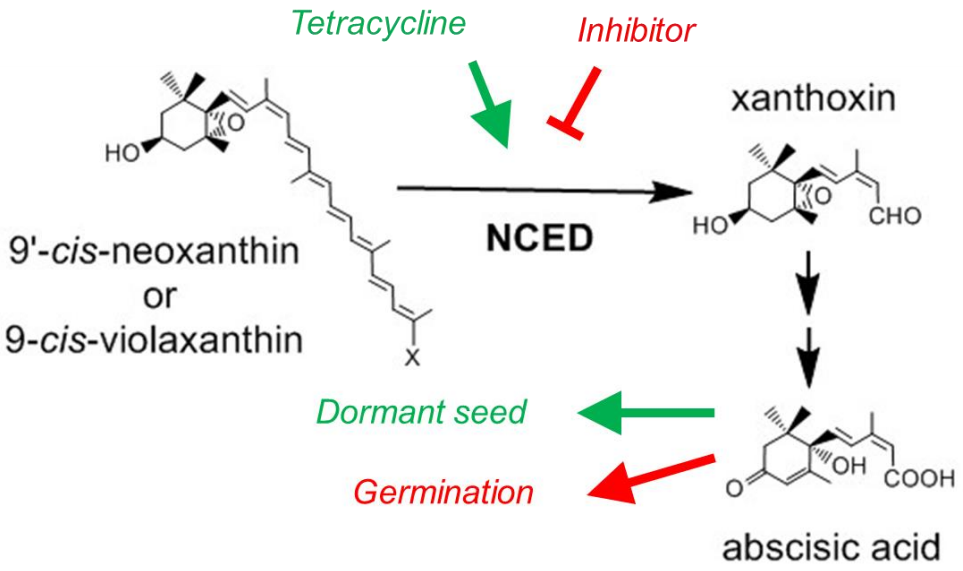

Figure 7.1. Screening for germination stimulating compounds using tobacco seed harbouring tetracycline-inducible *LeNCED1*. Tetracycline is added to induce *LeNCED* expression, resulting in dormant seed. Compounds that inhibit NCED will be identified by their activity to restore germination.

7.1.5 Libraries to screen for compounds with germination stimulating activity

Two compound libraries were provided by Syngenta. The first library contained 5074 unique compounds, diverse in structure ('5K compound library'). A separate second library contained 88 unique compounds which were chosen by collaborators at Syngenta ('88 compound library'). Compounds in the second library were selected as potential leads as germination stimulating compounds and they were designed based on the mode of action of the hydroxamic acids. Also included in the second library were abamineSG, a compound with reported NCED-inhibition activity (Kitahata*, et al.,* 2006), and hydroxamic acid D15 which also inhibits CCDs (Harrison*, et al.,* 2015; Van Norman*, et al.,* 2014). The structure of the other library compounds was not disclosed by Syngenta. Thus it was expected that the 88 compound library would contain a higher proportion of germination stimulating compounds than the 5K compound library.

7.1.6 Aims and objectives

The overall aim of this chapter was to identify new lead compounds that stimulate germination for use in chemical genetics studies and in the development of novel seed treatment technologies. This would potentially lead to new NCED inhibitors, allowing the role of NCEDs to be probed in other systems (particularly seeds), or compounds with new modes of action. These findings may ultimately yield compounds with agrochemical purposes, in particular stimulating crop germination under suboptimal field conditions.

To identify *new types* of lead compounds that stimulate germination, the following objectives were pursued:

- Create and employ a high-throughput screen that can be used to quickly identify germination stimulating compounds from libraries containing large numbers of compounds (i.e. the 5K library).
- Determine if any potential hits identified in the screen of the library were reproducibly active and assess differences in efficacy of any new lead compounds by using the screening assay with replication.

To aid design and development of the *current* lead compounds (i.e. hydroxamic acids) the following objectives were pursued:

- Determine the potency of the 88 compounds in stimulating germination, compared to each other and to D4, by utilising the screening assay with replication and at different compound concentrations.

7.2 The Effect of Tetracycline-Induced *LeNCED1* Expression on the Germination of Tobacco Seed

The effects of tetracycline-induced *LeNCED1* expression were investigated to determine the potential use of the seed in an assay to identify germination stimulating compounds. Tobacco seed harbouring tetracycline-inducible *LeNCED1* (pBHT-NCED – as described in Thompson et al., 2000) were imbibed on filter paper wetted with solutions of varying tetracycline concentration with or without norflurazon. Tobacco seed harbouring only the tetracycline repressor (*tetR* – as described in Thompson et al., 2000) were used as a control (Figure 7.2). Different stages of germination were observed: testa rupture, endosperm rupture and the emergence of green cotyledons ('green leaf emergence'). Both the average time to these events and the frequency of the events (percentage) were calculated, although mean time to cotyledon emergence was not used since cotyledon emergence did not occur in some of the treatments.

The percentage of testa rupture was not affected by tetracycline or norflurazon, and did not differ between the two genotypes. Testa rupture occurred significantly earlier in seeds harbouring tetracycline inducible *LeNCED1*, although testa rupture was not significantly altered by tetracycline concentration in either genotype. Differences exceeding the 5% LSD are observed – particularly between 0.16 mg l^{-1} and 0.63 mg l^{-1}, for example, although there does not appear to be an overall trend related to tetracycline concentration. Norflurazon may have had an effect in reducing mean testa rupture time in BHT seeds at 0.625 mg l^{-1} tetracycline (difference exceeds 5% LSD), but no other significant effects of norflurazon were apparent in terms of testa rupture.

The percentage of endosperm rupture was significantly reduced in seed harbouring tetracycline-inducible *LeNCED1* (BHT) at 10 mg l^{-1} tetracycline compared to lower concentrations. At 10 mg l^{-1} tetracycline, the percentage endosperm rupture of the BHT seed was also significantly lower than seed harbouring only *TetR* (TetR).

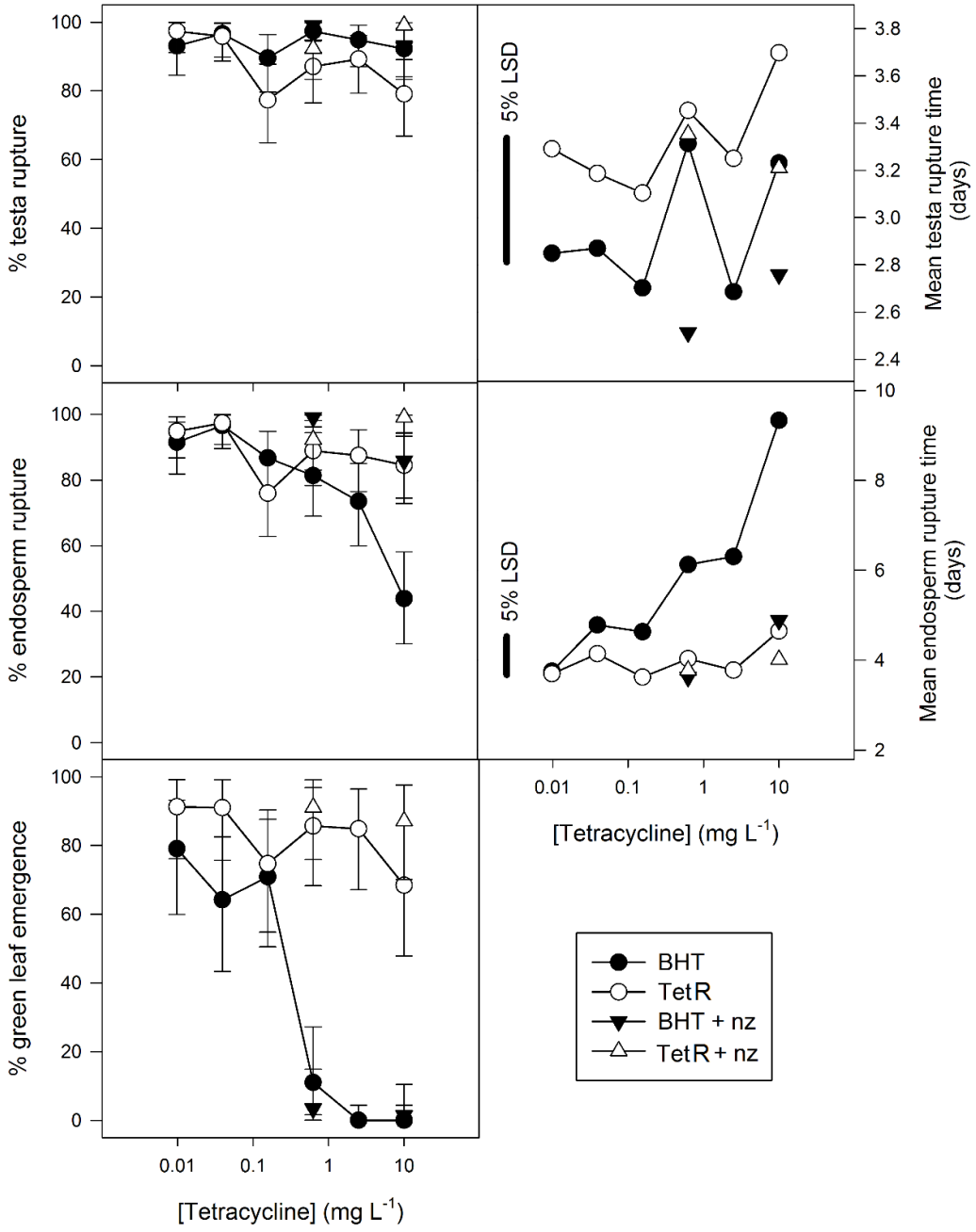

Figure 7.2. The effect of tetracycline on germination of tobacco seeds harbouring tetracycline-inducible *LeNCED1*. Tobacco seeds harbouring the tetracycline inducible *LeNCED* (BHT), or control seeds containing only the tetracycline repressor (TetR) were imbibed on filter paper soaked with solutions containing different concentrations of tetracycline, with (+nz) or without (-nz) norflurazon (0.5 mg l^{-1}). Germination (testa rupture, endosperm rupture, green cotyledon emergence) was scored daily. Mean time to germination and final percentage by the different measurements are indicated. Percentage data were angular transformed (arcsin(√x)) before analysis by Genstat ANOVA. ±½ LSD error bars are indicated for back-transformed data and a 5% LSD reference bar is provided for untransformed data.

The TetR seed were not affected by tetracycline concentration. Norflurazon had a significant effect in increasing the percentage endosperm rupture at 10 mg l^{-1} tetracycline in BHT seed, and had no significant effect at other tetracycline

concentrations in either genotype. At the lowest concentrations of tetracycline (0 – 0.16 mg l^{-1}) the mean time to endosperm rupture was similar in both genotypes. At concentrations above 0.16 mg l^{-1} tetracycline, the mean endosperm rupture time in BHT seed was larger than in TetR seed, in the absence of norflurazon. A general trend of increase in endosperm rupture time with increasing tetracycline concentrations was seen in BHT seed. Endosperm rupture took more than twice as long at 10 mg l^{-1} tetracycline than at 0.01 mg l^{-1} tetracycline. Addition of norflurazon to media reversed the effect of tetracycline in increasing endosperm rupture time in BHT seeds, and the addition of norflurazon had no influence on the TetR seeds in terms of endosperm rupture time.

Tetracycline concentrations of 0.01 to 0.16 mg l^{-1} had no effect on BHT or TetR seeds in terms of the percentage of cotyledon emergence. Between 0.16 mg l^{-1} and 0.63 mg l^{-1} tetracycline, the percentage of cotyledon emergence in BHT seeds dropped significantly from 71% to 11% and no green leaf emergence was observed in BHT seeds at the two highest tetracycline concentrations. No significant effect of tetracycline on cotyledon emergence in the TetR seeds was observed. Norflurazon had no additional effect in addition to the tetracycline treatment in either of the genotypes. At 2.5 mg l^{-1} tetracycline, TetR seeds showed 85% cotyledon emergence, but BHT seeds 0% cotyledon emergence.

In summary, neither seed genotypes were very sensitive to any of the tetracycline nor norflurazon treatments in terms of testa rupture time, although the testa of TetR seeds ruptured later than BHT seeds. TetR seeds were not sensitive to the tetracycline or norflurazon treatments in any of the parameters measured. However BHT seeds, harbouring the tetracycline inducible *LeNCED1* showed reduced endosperm rupture and cotyledon emergence percentages as well as a large effect in endosperm rupture time increase at higher tetracycline concentrations. Norflurazon only reversed the effect of the tetracycline on BHT seeds in terms of endosperm rupture, but not cotyledon emergence. A green leaf emergence-based assay to identify germination promoters (e.g. by ABA biosynthesis inhibitors) was planned based on these results.

7.3 Development of a green leaf area based assay in 25-Well Plates

Preliminary results indicated that the hydroxamic acid inhibitors also restored germination in seed over-expressing *LeNCED1* in response to addition of tetracycline (Appendix 7.1). However, the seedlings were etiolated, leading to seedlings crossing into adjacent wells of a 96-well plate, and exhibited very little green leaf area. Incubation of plates directly under lights led to germination of seed imbibed on tetracycline solutions, and was presumed to be due to degradation of tetracycline by light.

To improve green leaf area for photographic measurement, seed were imbibed on a nutrient supplemented agar medium under different lighting regimes, to allow the tetracycline to act on seeds before transfer to light. This would allow seeds that had germinated, despite the tetracycline treatment, to grow under light conditions. It was hypothesised that adjusting the lighting regime may therefore allow better discrimination between germinated and ungerminated seeds by measurement of green leaf area. BHT seeds were imbibed on an agar medium under different lighting regimes in the presence of varied tetracycline concentrations with and without 1 mM D4. Plates were photographed daily between 6 and 10 days after imbibition. The measurement of green leaf area 10 days after sowing is shown in Figure 7.3.

In plates that were transferred to light conditions 3 days after sowing there was no effect of tetracycline in the absence of D4 on leaf area after 10 days. Under 1 mM D4, 0.20 mg l^{-1} tetracycline resulted in a decrease in leaf area compared to 0 mg l^{-1} tetracycline (Figure 7.3 A). In plates transferred to light conditions 4 days after sowing, tetracycline concentrations of 0.10 and above significantly reduced day 10 leaf area, with leaf area tending to decrease further at higher tetracycline concentrations. The addition of 1 mM D4 partially reversed this effect, and under 1 mM D4 only 0.20 mg l^{-1} tetracycline significantly reduced leaf area compared to 0 mg l^{-1} tetracycline (Figure 7.3 B).

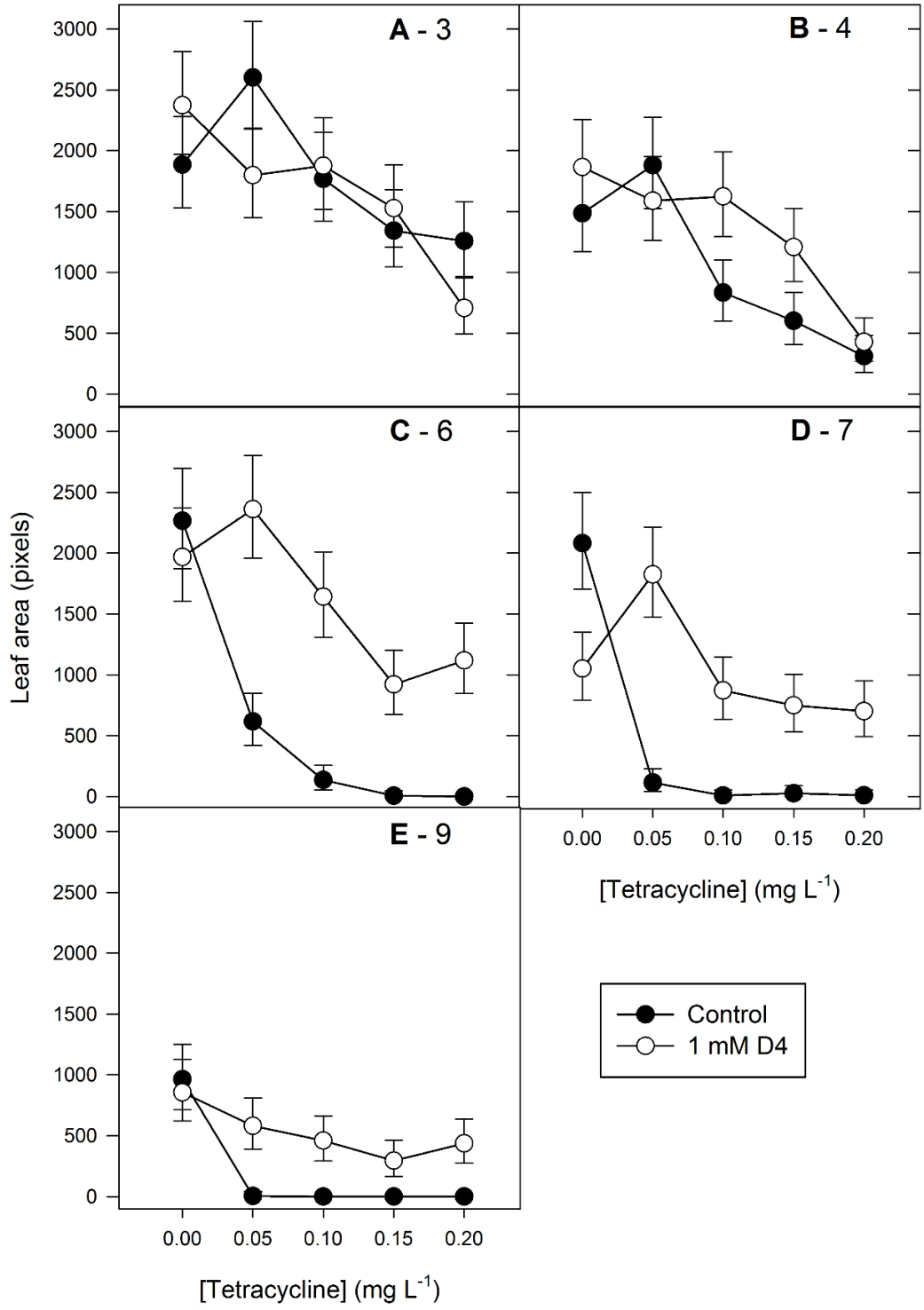

Figure 7.3. The effect of the lighting regime, tetracycline concentration and D4 on cotyledon and leaf emergence in tobacco harbouring tetracycline-inducible *LeNCED1*. Tobacco seed harbouring tetracycline-inducible *LeNCED1* were transferred in a 0.2% agar seed suspension to 0.6% agar containing ½ MS salts and imbibed in the presence of 0.00 to 0.25 mg l^{-1} tetracycline with 1 mM D4 or 0.2% DMSO (control). Plates were placed in the dark at 25°C and transferred to 16 h light per day at different times: A, transferred after 3 days; B, after 4 days; C, after 6 days; D, after 7 days; E, after 9 days. Images of plates on day 10 were analysed using ImageJ to quantify average green cotyledon area of seedlings in each well. Data were transformed (√x) before analysis by Genstat ANOVA. ±½ maximum 5% LSD error bars are indicated for back-transformed data (n = 4 or 6).

In plates transferred to light conditions 6 days after sowing, day 10 leaf area was significantly reduced between each rise of 0.05 mg l^{-1} tetracycline up to 0.15 mg l^{-1} tetracycline at which point leaf area was approximately zero. The addition of 1mM D4 reversed this effect, with no significant reduction in day 10 leaf area seen between 0 mg l^{-1} and 0.10 mg l^{-1} tetracycline. However, leaf area was still reduced by 0.15 mg l^{-1} and 0.20 mg l^{-1} tetracycline in the presence of 1 mM D4. At these tetracycline concentrations, leaf area was still only reduced to half by 0.20 mg l^{-1} tetracycline compared to 0 mg l^{-1} tetracycline in the presence of 1 mM D4, indicating D4 was still partially reversing the effect of tetracycline (Figure 7.3 C). The difference in leaf area between 0 mM D4 and 1 mM D4 at 0.10 mg l^{-1} tetracycline is evident in the photograph shown in Figure 7.4, and is most clear in plates transferred to the light on Day 6 (Figure 7.4 B).

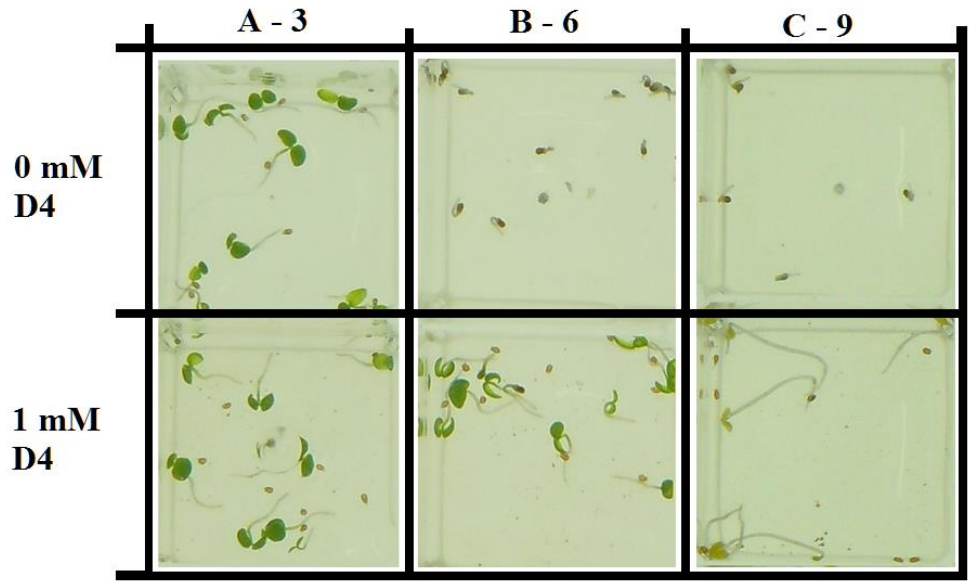

Figure 7.4. The effect of the lighting regime, tetracycline concentration and D4 on seedling growth of tobacco harbouring tetracycline-inducible *LeNCED1*. Tobacco seed harbouring tetracycline-inducible *LeNCED1* were transferred in a 0.2% agar seed suspension to 0.6% agar containing ½ MS salts and imbibed in the presence of 0.10 mg l^{-1} tetracycline with 1 mM D4 or 0.2% DMSO (control). Plates were placed in the dark at 25°C and transferred to 16 h light per day at different times: A, transferred after 3 days; B, after 6 days; C, after 9 days. Images were taken on day 10 (images from the same experiment described in Figure 7.3).

Trends were similar in plates transferred to light conditions 7 days after sowing, although the 1 mM D4 appeared to inhibit green leaf area at 0 mg l^{-1} tetracycline. At 0.05 mg l^{-1} tetracycline and above, 1 mM D4 restored the almost complete inhibition

of green leaf emergence seen in absence of D4. Leaf area restoration by 1 mM D4 was only complete at 0.05 mg l^{-1} tetracycline (Figure 7.3 D). Seedlings on these plates did however appear to exhibit some etiolation compared to seedlings transferred 6 days after transfer.

In plates transferred to light conditions 9 days after sowing, the day 10 green leaf area never reached the maximum leaf areas seen in the previous light treatments. Tetracycline concentrations of 0.05 mg l^{-1} and above appeared to completely prevent green leaf area in the absence of 1 mM D4. In the presence of 1 mM D4 the effects of the tetracycline were completely reversed except at 0.15 mg l^{-1} tetracycline where D4 only partially restored leaf area compared to 0 mg l^{-1} tetracycline (Figure 7.3 E). These seedlings were very etiolated compared to seedlings in previous light treatments (Figure 7.4 C)

In summary, the addition of light to the assay prevents etiolation and allows quantification of green leaf area. However, transferring the seeds / seedlings to light too early (5 days or less) prevents the effects of tetracycline on leaf area at day 10, whereas transferring the seedlings too late (7 days or more) allows etiolation of seedlings to occur. 1 mM D4 can reverse the effects of 0.05 mg l^{-1} and 0.10 mg l^{-1} tetracycline on day 10 leaf area in plates transferred to light on day 6. This reinforced the idea that an assay based on this treatment and measurement of green leaf area would be able to identify other NCED inhibitors or germination promoters.

7.4 Screening of the 5K Compound Library

To identify new lead compounds from a diverse chemical library, the 5K compound library was screened for germination stimulating activity using the final screening method (Section 2.4). Due to limitations of lighting space, the assay was separated in to four batches of 16 assay plates.

7.4.1 Performance of the negative and positive controls in the screen

An analysis of control wells on all plates was carried out in order to confirm the assay was performing as expected (i.e. to see if negative and positive controls could be distinguished by leaf area measurements). Additionally plate and batch effects were assessed to determine if the hit threshold needed to be scored on a within-plate or within-batch basis.

Negative controls consisted of 0.8 mg l^{-1} tetracycline, which should result in a low green leaf area due to inhibition of germination and establishment of tobacco seed. Positive controls consisted of 0.8 mg l^{-1} tetracycline plus the current lead compound, D4 (1 mM), which should reverse the effects of 0.8 mg l^{-1} tetracycline and lead to higher green leaf area than in negative controls. In general, the controls behaved as expected, with the positive control mean leaf area increasing between days 9 and 13 whilst the negative control mean did not appear to increase (Figure 7.5). However Z′-factors (Section 7.1.2) were below zero (Table 7.1), which is suboptimal for a single-replicate assay: the lower separation of positives and negative controls; and higher spread increases the likelihood of detecting false positives or false negatives. Preliminary assays using only negative and positive control wells had indicated that more optimal *Z′*-factors of above 0.20 were possible. Since negative control values were consistently very low, with 81% being zero values on day 13, the low Z′-factor is likely due to the spread and low leaf area values being recorded for the D4 positive control. Nonetheless, the Z′-factors indicated that the best separation of positive and negative controls occurred on day 13 (Table 7.1). Despite the relatively poor Z′-factor scores, the maintenance of low leaf areas in negative controls meant that leaf areas that were higher than the majority of negative controls were likely to be 'real' hits in the assay. However, false negatives were perhaps more likely for compounds with similar efficacy to 1 mM D4.

It was apparent that the increase in positive control leaf area differed between batches (for example, the mean positive control √leaf area at day 13 in batch 1 is much lower than in batch 2). ANOVA confirmed that there were both significant effects of batch ($p < 0.001$) and plate, nested within batch ($p = 0.002$) (Appendix 7.2). Within-plate column position effects did not appear to affect positive control leaf area. The effect of column was not significant using ANOVA ($p = 0.262$) or when using the GenStat unbalanced ANOVA procedure to account for plate effects ($p = 0.645$) (Appendix 7.3). It was therefore decided to classify hits on a per-plate basis.

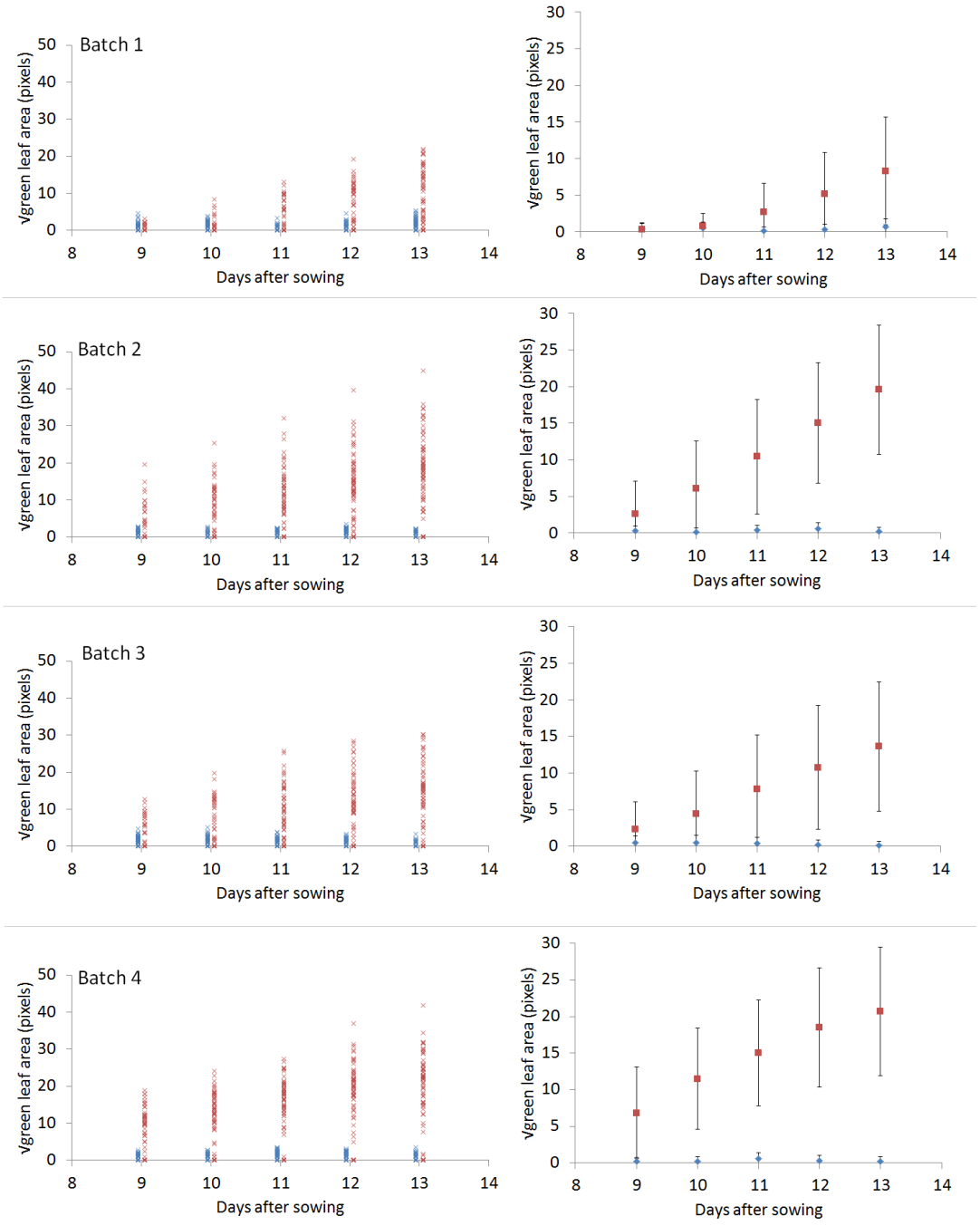

Figure 7.5. Performance of control treatments in the 5K screen. Four batches of 16 plates were required for the 5K screen. Each plate contained 12 negative control wells, 4 positive control and 80 assay wells. Photographs of plates from the 5K screen were taken daily 9 days to 13 days after sowing. Individual data points are indicated on the left for each batch and means ± standard deviation error bars are indicated on the right graph for each batch. Data were square-root transformed prior to analysis. Negative control data is indicated in blue and positive control data is indicated in red.

Table 7.1. Z′-factor calculated for control treatments in the 5K library screen. Four batches of 16 plates were required for the 5K screen. Each plate contained 12 negative control wells, 4 positive control and 80 assay wells. Photographs of plates from the 5K screen were taken daily 9 days to 13 days after sowing. The Z′-factor was calculated for each batch on square-root transformed data for each day. The best Z′-factor for each batch is highlighted in **bold**.

Day	Batch 1	Batch 2	Batch 3	Batch 4
9	-65.69	-5.78	-6.83	-2.11
10	-25.88	-2.52	-4.27	-1.00
11	-4.33	-1.54	-2.30	-0.68
12	-2.98	-0.89	-1.59	-0.46
13	**-2.33**	**-0.46**	**-1.08**	**-0.37**

Table 7.2. Frequency distribution of leaf area values in the 5K compound library assay for compound wells and negative control wells. 5074 compounds and 768 negative controls were assayed in the initial screen of the 5K library. Leaf areas were calculated from photographs of plates 13 days after sowing. The data were square-root transformed and the distribution of values from compound assay wells and negative control wells is indicated below. The frequency is also expressed as a percentage of the total number of wells assayed for each group.

	Compound wells		Negative control wells	
√leaf area (pixels)	**Number**	**(%)**	**Number**	**(%)**
$x = 0$	3454	68.07	625	81.38
$0 < x \leq 5$	1058	20.85	137	17.84
$5 < x \leq 10$	164	3.23	3	0.39
$10 < x \leq 15$	124	2.44	1	0.13
$15 < x \leq 20$	96	1.89	0	0.00
$20 < x \leq 25$	76	1.50	1	0.13
$25 < x \leq 30$	51	1.01	0	0.00
$30 < x \leq 35$	23	0.45	0	0.00
$35 < x \leq 40$	18	0.35	1	0.13
$40 < x \leq 45$	5	0.10	0	0.00
$45 < x \leq 50$	3	0.06	0	0.00
$50 < x \leq 55$	0	0.00	0	0.00
$55 < x \leq 60$	2	0.04	0	0.00
Total	5074	100.00	768	100.00

7.4.2 Identification of potential hit compounds

Similar to the negative controls the majority of wells treated with assay compounds exhibited zero leaf area (Table 7.2). However there was a higher proportion of non-zero leaf areas in the compound wells, indicating that some of the compounds may stimulate germination and seedling growth. Potential hit compounds were defined as having a leaf area larger than all negative controls on their respective plate. Based on this criterion 907 potential hits were identified. The removal of four possible outlier

negative controls (out of the total of 768 negative control wells), due to their extremely high leaf area values resulted increased the number of potential hits to 965 (Appendix 7.4). This represented 19% of all assay wells. However the majority (566) of the potential hit compound wells exhibited leaf areas of 100 pixels or less. Follow-up assays were carried out to confirm the germination stimulating activity of compounds identified in wells with relatively high leaf areas.

7.5 Confirmation of potential hit compounds from the 5K Library

Due to a shortage of time, 236 potential hit compounds were initially selected by eye for re-testing. Replication was used in the secondary screens to confirm the activity of compounds in restoring germination and seedling growth; and to gauge the efficacy of the compounds compared to the current lead compound, D4. 122 potential hit compounds from batches 1 and 2 of the 5K library screen were assayed in 'follow-up 1' and 114 potential hit compounds from batches 3 and 4 of the 5K library screen were assayed in 'follow-up 2.' There were three assay replicates for each potential hit compound, and 76 assay replicates for the positive (1 mM D4) and negative controls.

7.5.1 Follow-up 1: Confirmation of hits from screen batches 1 & 2

Separation of positive and negative controls by leaf area appeared to be weaker in the follow-up assay ('follow-up 1') of potential hit compounds from batches 1 and 2 of the 5K library screen (Figure 7.6). This appeared to be mainly due to the absence of an increase in leaf area in some of the positive controls. The lack of separation of controls resulted in a less optimal *Z′*-factor than the initial 5K screen, but the separation was still highest on day 13 (Table 7.3). 86% of the negative controls had a leaf area of zero. Only three negative controls (out of 76) had √leaf area values of higher than 2.8 √pixels on day 13. To see if compounds differed significantly in their ability to increase leaf area in the assay, the square-root transformed leaf areas (Figure 7.7) were analysed by ANOVA (Appendix 7.5). Compound treatment had a significant effect on day 13 √leaf area ($p < 0.001$). Post-hoc comparisons were made using *t*-tests, and false discovery rate (FDR) was controlled at 5% (for Chapter 7.5 and 7.6) using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995)resulting in only *p*-values of below 0.033 being declared as significant.

Table 7.3. Z′-factor calculated for control treatments in hit compound follow-up tests. Hit compounds from the 5K screen were assayed in two batches, each with 3 replicates. 76 positive and 76 negative controls were included. Photographs of plates were taken daily 9 days to 13 days after sowing. The Z′-factor was calculated for each batch on square-root transformed data for each day. The best Z′-factor is highlighted in **bold**. In follow-up 2, negative controls exhibited larger leaf area values than the positive controls, rendering the Z′-factor inapplicable.

Day	Follow-up 1	Follow-up 2
9	-80.55	
10	-19.18	
11	-7.89	N/A
12	-5.78	
13	**-5.33**	

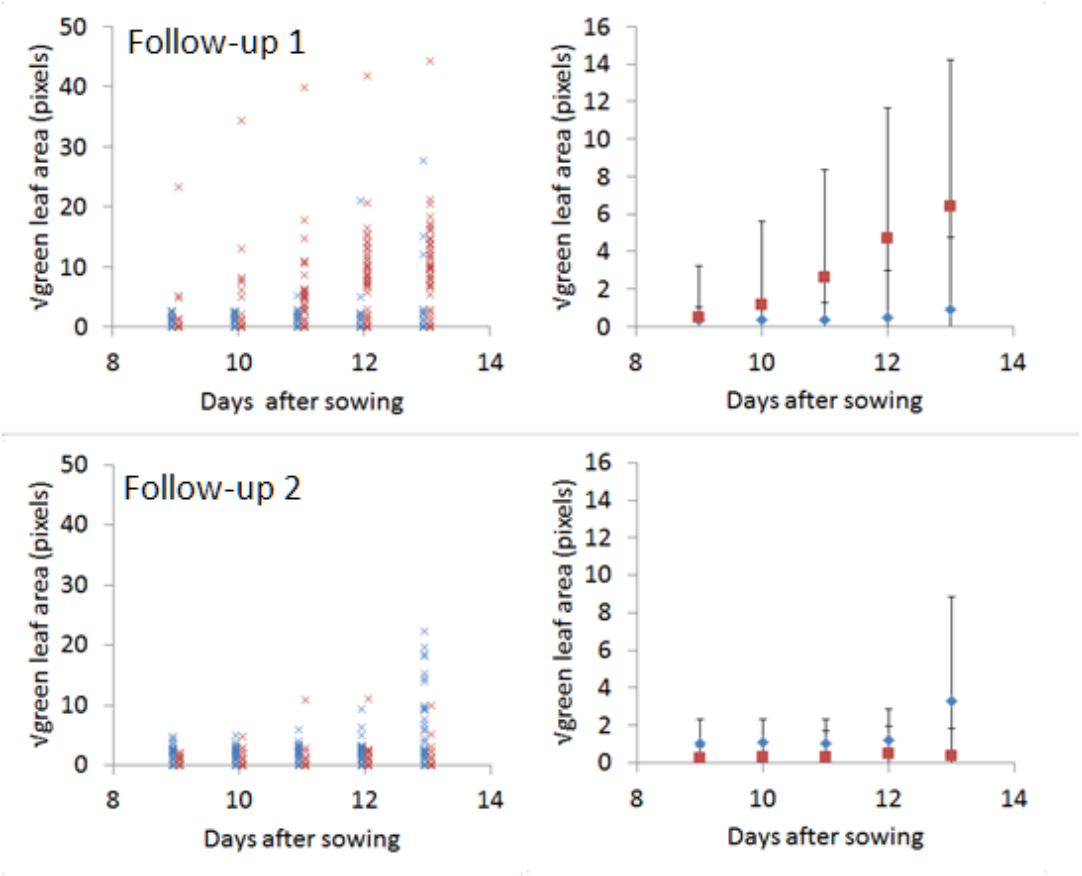

Figure 7.6. Performance of control treatments in the follow-up assays of potential hits from the 5K library. Potential hit compounds from the 5K screen were assayed in two batches, each with 3 replicates across multiple plates. At least 12 positive and 12 negative controls were included on each plate. Photographs of plates were taken daily 9 days to 13 day after sowing. Individual data points are indicated on the left for each batch and means ± standard deviation error bars are indicated on the right graph for each batch. Negative control data is indicated in blue and positive control data is indicated in red.

Comparisons were limited to comparisons of compounds vs. negative control and compounds vs. positive control, as well as positive controls vs. negative controls. This was done to confirm the activity of potential hit compounds, and to identify compounds which may be more active than 1 mM D4 in the assay.

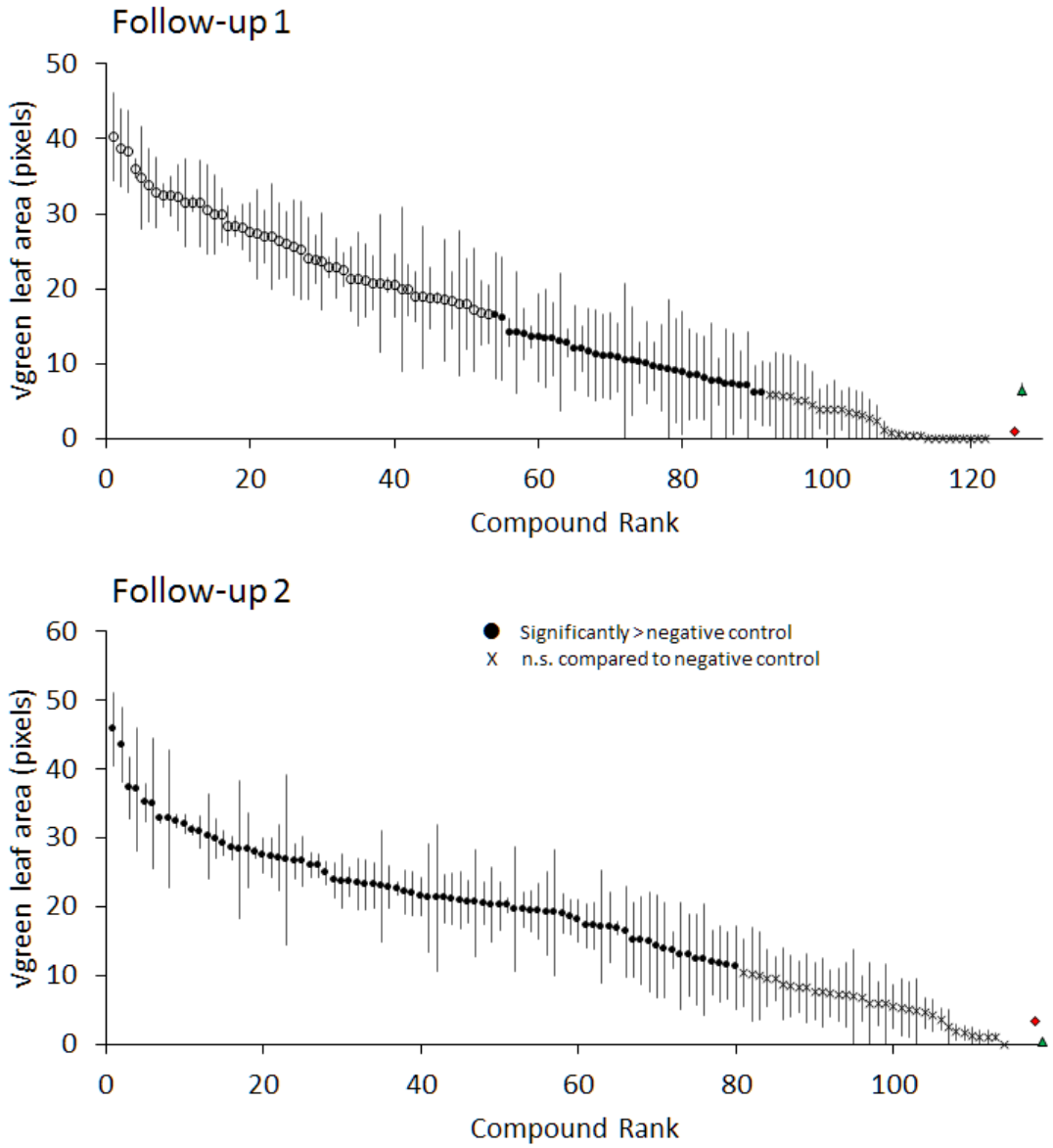

Figure 7.7. Confirmation of germination stimulating activity in selected hits from the 5K compound library. Hit compounds from the 5K screen were retested at 10 ppm in two batches (follow-up 1 and follow-up 2), each with 3 replicates. 76 negative (no compound) and 76 positive controls (1 mM D4) were included in each follow-up. Day 13 leaf area data was square-root transformed prior to analysis and the effect of compound analysed by ANOVA (Appendix 7.5). Compound means are sorted by rank from highest to lowest (left to right). The negative control is indicated by the red diamond, and positive control by the green triangle at the left of each panel. Student's *t*-tests were used to compare each compound to the controls, and significance is indicated in the legend. A minimum significance level of $p < 0.033$ was determined to control the FDR to 5% using the Benjamini-Hochberg procedure. Significance compared to positive control is not indicated in follow-up 2 as positive control leaf area was significantly lower than negative control. Error bars indicate standard error.

As expected the positive control, 1 mM D4, significantly increased day √leaf area compared to the negative control, by 5 √pixels. 91 of the potential hit compounds (75%) elicited significantly larger leaf areas than the negative control, confirming their potential germination and seedling growth stimulatory activity. Furthermore, 53 of these potential hit compounds (43% of the total) stimulated significantly larger leaf areas (at 10 ppm) than 1 mM D4 (312 ppm). There was no evidence that the remaining 31 compounds (25%) affected leaf area in the assay compared to the negative control; and 9 of these compounds had zero leaf area values for all three replicates, indicating that some of the potential hits identified in the initial 5K screen were false positives.

7.5.2 Follow-up 2: Refinement of hits from screen batches 3 & 4

To see if compounds differed significantly in their ability to increase leaf area in the assay, the square-root transformed leaf areas were analysed by ANOVA (Appendix 7.5). Compound treatment had a significant effect on day 13 square-root leaf area ($p < 0.001$). Again, post-hoc comparisons were made using *t*-tests and FDR was controlled at 5% using the Benjamini-Hochberg procedure. Positive and negative controls were compared to each other; and each compound was compared with both the positive and negative control illustrated in (Figure 7.7). Unexpectedly, the positive control assay wells exhibited a significantly lower leaf area than that negative control wells. Despite the suboptimal performance of the positive control, the negative controls still exhibited low leaf areas and thus provided a sufficient baseline for comparison of the other compounds.

65 of the potential hit compounds (70%) elicited significantly larger leaf areas than the negative control, confirming their potential germination and seedling growth stimulatory activity. There was no evidence that the remaining 34 compounds (30%) affected leaf area in the assay compared to the negative control.

7.5.3 Summary

The screen identified 965 potential hit compounds. Out of a selection of 236, 171 compounds were confirmed as hits in follow up screens. This indicated that the initial 5K screen did refine the number of 'hit' compounds (from 19% to 72%), but also indicated that false positives (28%) did occur in the initial 5K screen. The identified hit compounds included 65 compounds that, at 10 ppm, appeared to be more potent than 1 mM D4 in stimulating leaf area on day 13 of the assay.

7.6 Screening and Refinement of 88 Potential Lead Compounds

To quantify the efficacy of the 88 potential lead compounds in germination stimulation, the compounds were screened using the same assay used for the 5K follow-up assays. Many of the compounds proved to be phytotoxic (i.e. causing bleaching of leaves, or delayed seedling growth) in preliminary screens, as shown in Figure 7.8.

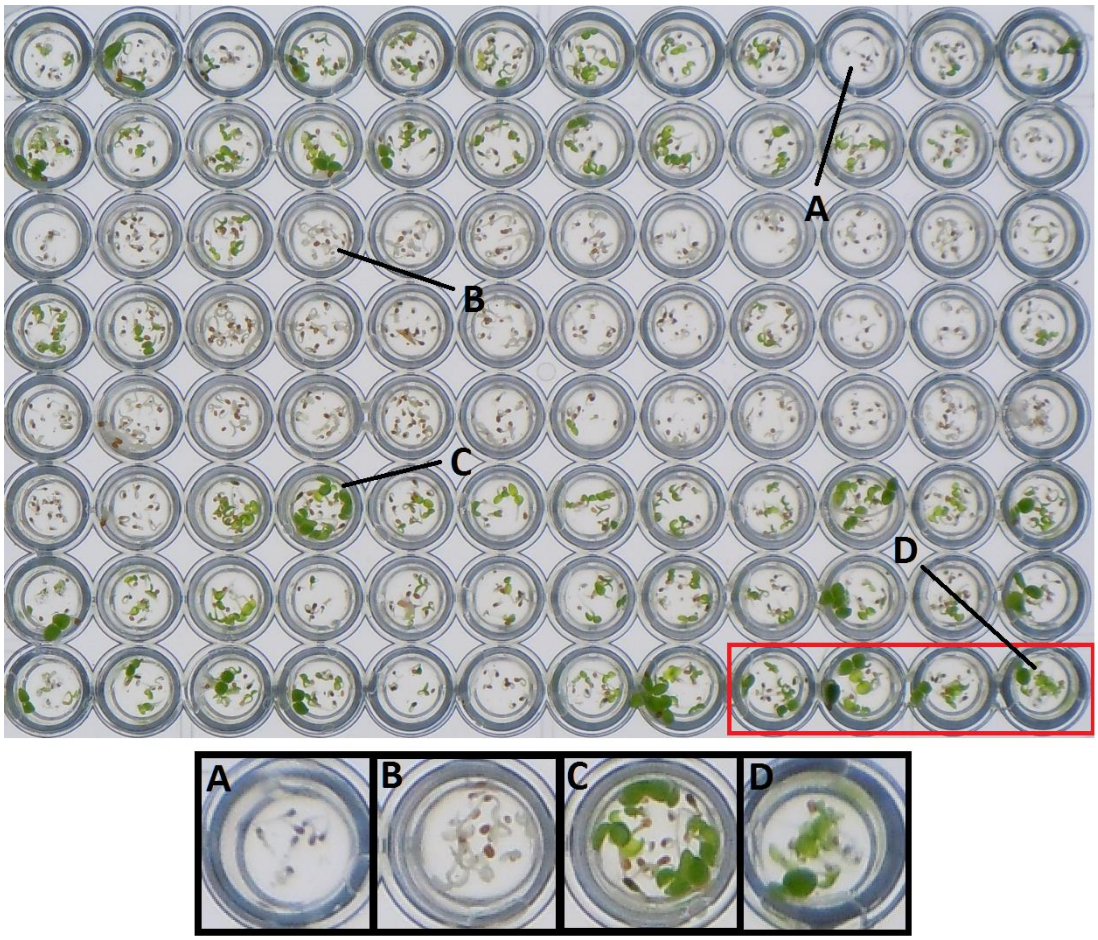

Figure 7.8. Phytotoxicity of compounds in the 88 compound library. This is a photograph of an assay microtiter plate from a preliminary screen (early in assay development) in which compounds were added at 1 ppm to assay wells containing 0.2 mg l^{-1} tetracycline and 1% DMSO. The plate was incubated for 6 days in the dark then under 16 h light per day for 4 days. The 88 compounds are distributed in wells 1 to 92 (from left to right, from the top). It is evident that some, but not all of the compounds cause bleaching of leaves. A indicates a well exhibiting poor seedling growth; B indicates a well with bleached (white) seedlings; C indicates normal seedlings. The last four wells (outlined in red box) do not contain library compound (e.g. D). Magnified images of four highlighted wells (A-D) are shown.

All 88 compounds were screened on each replicate plate with space for 6 negative controls and 2 positive controls (1 mM D4) per plate. Compounds were screened at 10 ppm, 1 ppm, 0.1 ppm and 0.01 ppm. This was done for two reasons. Firstly, the compound efficacy may be gauged by the ability of the compound to promote

germination even at lower concentrations. Secondly compounds which stimulate germination at low concentrations may be phytotoxic at higher concentrations. Since there were only 88 compounds to screen (rather than 5074) screening at four concentrations with four replicates per compound was possible. As expected, the positive control showed significantly increased √leaf area compared to the negative control between days 10 and 13. The Z′-factor was highest on day 13 at -0.48 (Figure 7.9), thus the day 13 √leaf area for all compounds was analysed.

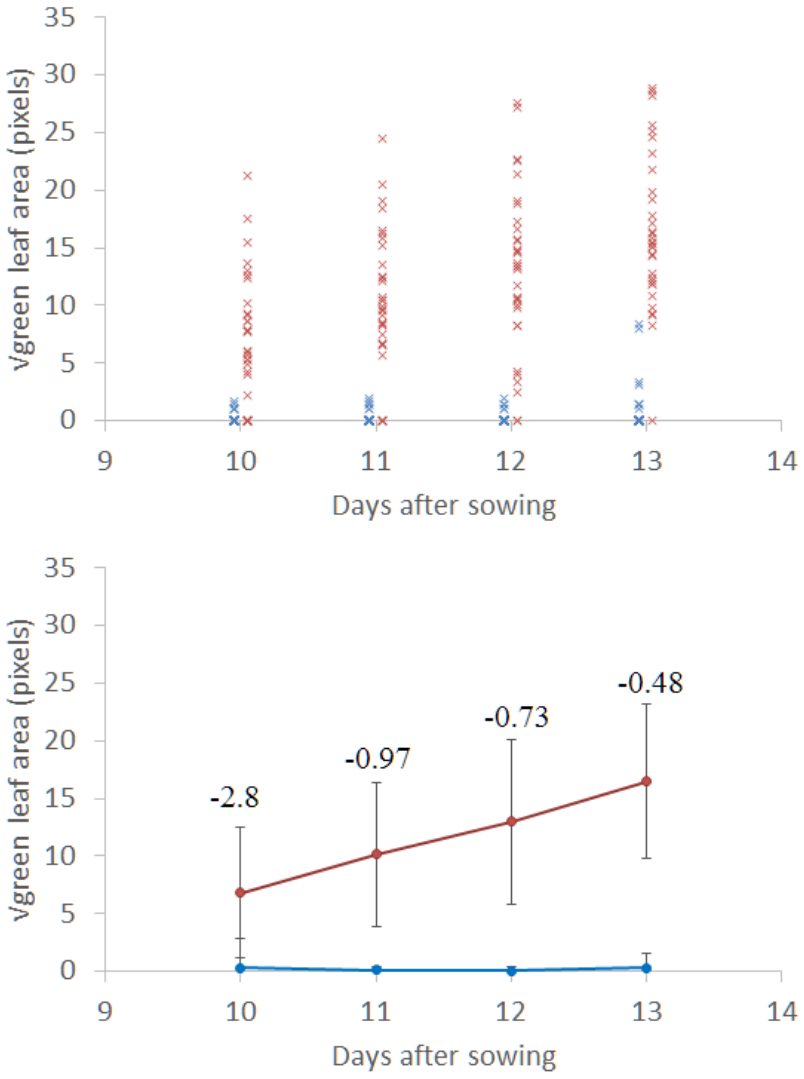

Figure 7.9. Performance of controls in the 88 compound library screen. 88 compounds from the 88 compound library were tested in the screening assay at concentrations of 10 ppm, 1 ppm, 0.1 ppm and 0.01 ppm. 4 replicates were performed per compound per concentration. Compounds and controls were blocked by plate (1 assay compound replicate, 2 positive controls and 6 negative controls per plate), and different concentrations run on different plates. Plates were photographed between days 10 and 13. Green leaf area data was square-root transformed. Negative controls are indicated in blue, and positive controls in red. The top graph shows individual data points, and the bottom graph indicates means and standard deviation. The Z′-factor value for each day is indicated above the data points on the bottom graph.

To distinguish between the efficacies of the compounds at different concentrations, and to compare to the positive control, 1 mM D4, day 13 √leaf area on was analysed (Figure 7.10). ANOVA indicated significant effects of the compound library at all concentrations (p <0.001 for each concentration) (Appendix 7.6).

To determine which individual compounds induced significantly different day 13 leaf areas than the negative and positive controls, compound leaf areas were compared to the controls using *t*-tests. Due to the large number of comparisons being made, the FDR was controlled at 5% (for Chapter 7) using the Benjamini-Hochberg procedure.

At each concentration there were compounds that had significantly higher leaf area values than the positive control 1 mM D4: 2 at 10 ppm (C21 and C69); 2 at 1 ppm (C70 and C72), 5 at 0.1 ppm (C19, C27, C36, C72 and C93) and 1 at 0.01 ppm (C36). Thus 8 compounds, at least at one concentration, resulted in a significantly larger day 13 √leaf area than 1 mM D4. Many compounds resulted in significantly larger √leaf area than control that was not significantly different to the effect of 1 mM D4. This indicated many compounds had equivalent germination stimulating or seedling growth promoting activity to 1 mM D4, even at relatively lower concentrations.

All compounds resulted in a larger day 13 √leaf area, in at least one concentration, than the negative control. This included abamineSG (C95) and hydroxamic acid D15 (C48). AbamineSG was a hit at 1 ppm, 0.1 ppm and 0.01 ppm, and D15 was a hit at 0.1 ppm and 0.01 ppm. Neither of these two compounds were a hit at 10 ppm indicating they may exert phytotoxicity at concentrations higher than their optimum for stimulating day 13 √leaf area in the assay. Many of the compound appeared to exert phytotoxicity at 10 ppm as they did not allow leaf areas significantly larger than the negative control at 10 ppm; but allowed leaf areas significantly larger than the negative control at concentrations of 0.01, 0.1 and 1 ppm (e.g. C1, C68 and C85).

A scatter plot was generated to exhibit the optimum concentration for each compound (crudely predicted from the √leaf area values from the four concentrations tested, Appendix 7.7), and the maximum average leaf area for each compound, out of the four concentrations tested (Figure 7.11).

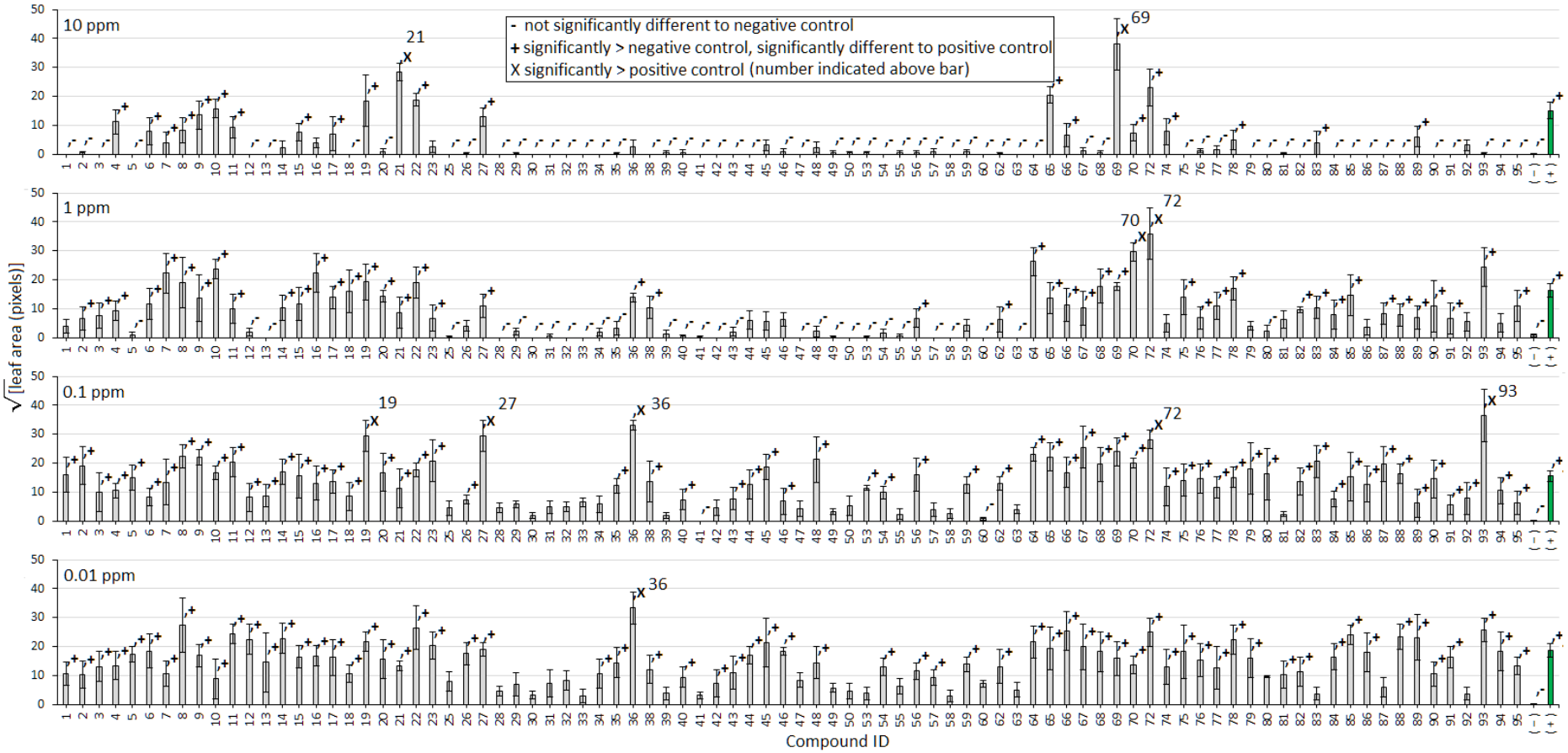

Figure 7.10. Assay of the germination stimulating activity of the 88 compound library at four different concentrations. The 88 compound library was assayed at 0.01, 0.1, 1 and 10 ppm (n = 4 per compound per concentration). Day 13 √leaf area is presented for compound, negative control (-) and positive control (+) (1 mM D4). Student's *t*-tests were used to compare each compound to the controls, and significance is indicated in the legend. FDR was limited to 5% (Benjamini-Hochberg procedure). Significance of comparisons to controls are indicated in the legend. Error bars indicate standard error.

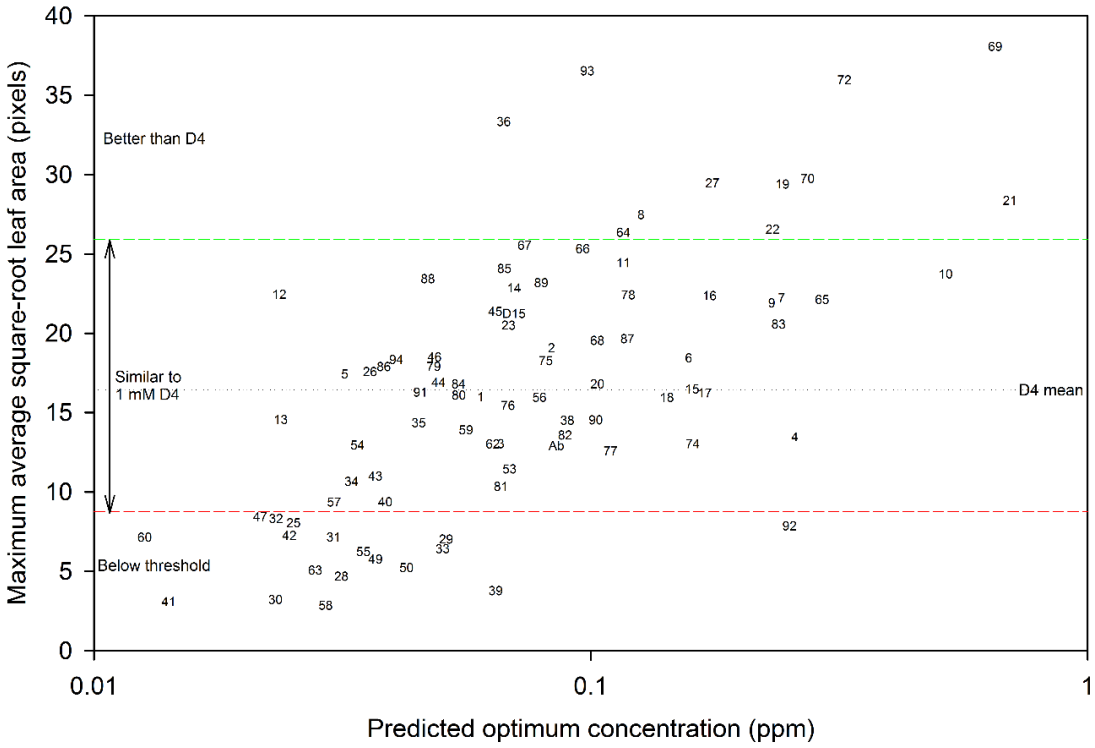

Figure 7.11. Best performance of compounds from the 88 compound library and predicted optimum concentration. Compounds were screened at 0.01 ppm, 0.1 ppm, 1 ppm and 10 ppm with four replicates per compound per concentration. The best average day 13 √leaf area (pixels) from the four concentrations is plotted for each compound against the predicted optimum concentration (Appendix 7.7). The red line indicates the maximum leaf area of a negative control well (the threshold for a hit compound). The D4 mean is indicated by a dotted grey line, and the green line indicates the D4 mean plus 5% LSD (calculated by ANOVA, Appendix 7.6). Compounds with mean √leaf areas larger than the green line are significantly larger than the mean √leaf area for D4. Negative control values were very close to zero (mean = 0.3 ± 0.1 standard error), and are not displayed.

Some compounds allow the most leaf area for that compound at high concentrations (e.g. compounds 69, 21, 10 and 92). This indicates that they are most stimulatory to germination and establishment when applied at higher concentrations. Compound 69 allows a maximum leaf area larger than D4, and its optimal concentration for increased leaf area is close to 1 ppm. Compounds like compound 69 are likely to: a) be most stimulatory to germination at high concentrations; b) not phytotoxic at these high concentrations.

On the other hand, whilst the optimal concentration of increasing leaf area in compound 92 is also predicted to be between 0.1 and 1 ppm, it is not able to significantly stimulate leaf area at any concentration. Compounds like compound 92 are likely to: a) be not, or very weakly stimulatory to germination at any concentration; b) not be phytotoxic at high concentrations.

Compounds which have a lower optimal concentrations tend to have lower maximum average leaf areas. This is likely due to the phytotoxic activity of some of these compounds at higher concentrations, which means that they allow the most leaf area growth when applied at lower concentrations. Some of these compounds do not promote leaf area even at the low concentrations, as the maximum leaf area produced is low (e.g. compounds 60, 41, and 47). Compounds like compound 60 are likely to: a) not be stimulatory to germination at any concentration; b) be most phytotoxic at high concentrations.

However, some compounds which have low optimal concentrations e.g. compound 12, can stimulate germination (to a similar level as D4) at a low concentration. This indicates that compound 12 may be phototoxic at high concentrations, but stimulates germination at low concentration. Compounds like compound 12 are therefore likely to: a) be stimulatory to germination at low concentrations; b) be phytotoxic at high concentrations.

Some compounds have optimal concentrations at intermediate concentrations (such as compound 93 and 36). This may be indicative of a bi-phasic dose-response, where an intermediate concentration is required for promotion of green leaf area, but the lower leaf areas are observed at higher concentrations as the compound becomes phytotoxic.

Compounds that result in significantly more leaf area than 1 mM D4 have optimums at a range of concentrations (Figure 7.11) and exhibit varied dose response characteristics (Figure 7.12). Compound 69, which resulted in the highest ranked leaf area at 10 ppm, also stimulates leaf area at 1 ppm, 0.1 ppm and 0.01 ppm, indicating that like D4 it requires relatively higher concentrations to be most effective (D4 is most effective above 300 ppm). Compound 36 is most effective at inducing day 13 leaf area at 0.1 ppm (being similarly effective at 0.01 ppm), but results in leaf areas below the threshold at 10 ppm. This may indicate the compound stimulates day 13 leaf area at relatively lower concentrations, but is phytotoxic at higher concentrations. Some compounds (e.g. compound 93) exhibit a biphasic dose-response, where assay leaf area increased up to the optimal dose, but beyond the optimal dose, the stimulatory effect was lessened, potentially indicating that some compound possess both stimulatory and phytotoxic effects.

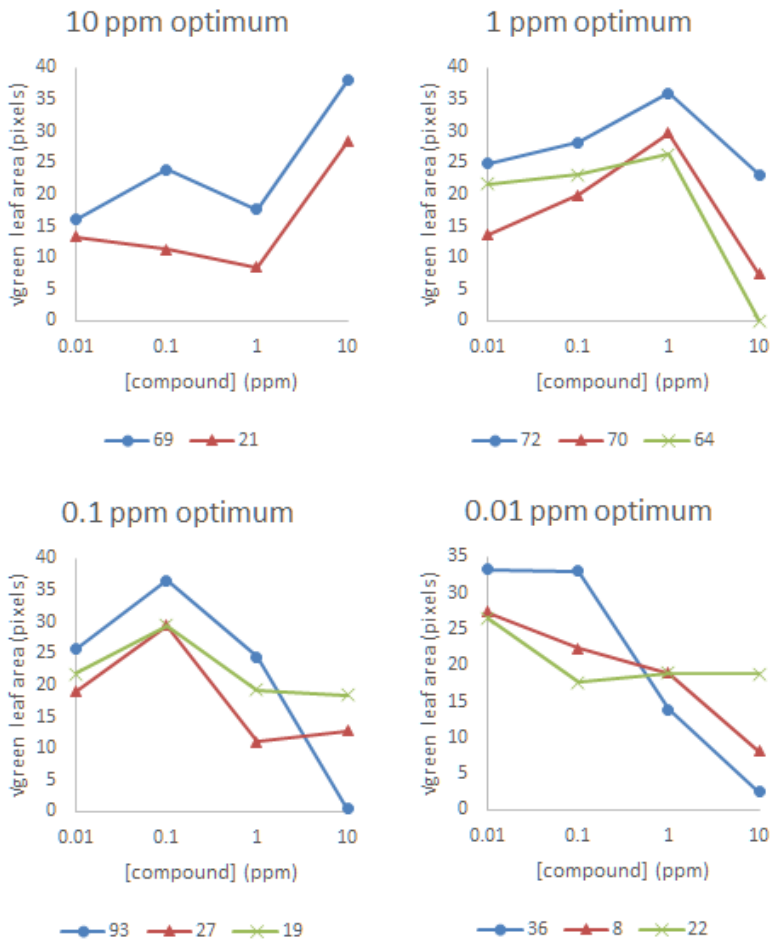

Figure 7.12. Dose response of top-ranked compounds from the 88 compound library screen. Compounds were screened at 0.01, 0.1, 1 and 10 ppm with four replicates per compound per concentration. The 11 compounds whose top-ranked leaf area was significantly larger than 1 mM D4 are presented here (C8, C19, C21, C22, C27, C36, C64, C69, C70, C72 and C93) and are separated by the concentration at which their peak leaf area was measured.

7.7 Discussion

7.7.1 Tetracycline induced LeNCED1 expression inhibits germination in tobacco seed

Tetracycline induced *LeNCED1* expression resulted in inhibition of germination. In particular, endosperm rupture was inhibited and took longer than in absence of tetracycline induction of *LeNCED1* (at 0 mg l^{-1} tetracycline, or in comparison with TetR seeds lacking tetracycline-inducible *LeNCED1*). Similarly, cotyledon emergence was very inhibited at tetracycline concentrations above 0.6 mg l^{-1} tetracycline. Germination of tobacco is sensitive to exogenously applied ABA, in a similar manner (Manz, *et al.*, 2005). The effect of tetracycline-induced *LeNCED1* on endosperm rupture was reversed by norflurazon, presumably by preventing the over-

accumulation of ABA due to *LeNCED1* overexpression through prevention of carotenoid biosynthesis (Bartels and Watson, 1978). Similar effects of dexamethasone-inducible expression of *PvNCED* were observed in tobacco (Qin and Zeevaart, 2002), and in methoxyfenozide-inducible *AtNCED6* expression in Arabidopsis (Martinez-Andujar, *et al.*, 2011). In the latter system, the effects of induced *AtNCED6* expression are also reversible by norflurazon. In these systems, the induction of a dormant state only appears to be temporary, with endosperm rupture and radicle emergence occurring eventually, similar to what was seen here.

That induced *LeNCED1* expression inhibited endosperm rupture and not testa rupture is consistent with experiments in which ABA is applied exogenously. Testa rupture is associated with the uptake of water at the end of phase II of imbibition, when the water content is approximately 50% of fresh weight. Endosperm rupture allows further uptake of water (phase III of imbibition), and is inhibited by ABA, which acts in part by inhibiting the breakdown of the endosperm tissue by ß-1,3-glucanase (Leubner-Metzger and Meins, 2000; Manz, *et al.*, 2005). ABA can inhibit germination and subsequent growth by reducing embryo growth potential, which may explain why the seeds that do exhibit endosperm rupture when imbibed at tetracycline concentrations above 0.63 mg l^{-1} do not also exhibit cotyledon emergence (da Silva, *et al.*, 2004). The lack of green leaf emergence in BHT seed imbibed at tetracycline concentrations above 0.63 mg l^{-1} in the presence of norflurazon does not result from a bleaching effect due to carotenoid biosynthesis inhibition. TetR seedlings produce green cotyledons in the presence of norflurazon when imbibed in the dark. It is possible that the inhibition of ABA biosynthesis is therefore incomplete, or that norflurazon also acts to inhibit germination through another mechanism in the presence of induced *LeNCED1* expression.

7.7.2 Green leaf area as an indicator of germination – discovery of a potential assay

The idea of using green leaf area to assess the effectiveness of a germination stimulator came to fruition when leaf area was measured in the 25-well plate format, although clearly the assay was very sensitive to factors such as light and tetracycline concentration, but usefully green leaf area was also sensitive to the concentration of D4 in the presence of tetracycline.

Observation of the cotyledon emergence following imbibition in the dark indicated that some light would be necessary to prevent seedling etiolation (which also leads to seedlings crossing across wells), and to increase the green leaf area for photographic measurement. However simply incubating the assay in the light resulted in a loss of the effect of tetracycline in inhibiting germination. I hypothesised that this was due the photodegradation of tetracycline, as tetracycline is sensitive to light, pH and temperature, amongst other factors (Chen, *et al.*, 2008). To allow the tetracycline to affect seeds, before the assay was transferred to light to allow greening, the assay was incubated for some time in the dark before being transferred to light conditions. Allowing 4 days or more in the dark before transfer to light restored effects of tetracycline on inhibiting germination, however transferring 7 days or later led to the etiolation and reduced leaf area previously seen. A dark period of 6 days was deemed to be optimal. A tetracycline concentration of 0.05 mg l^{-1} under this regime allowed complete restoration of leaf area by 1 mM D4, and also indicated a sensitivity to varied concentrations of D4. This could mean that similar germination stimulators or ABA signalling inhibiting compounds may be discernable under such conditions.

However, the 25-well plate format was not particularly suited for high-throughput purposes. The assay required 1 ml (too large for use of novel compounds) and was not suited for multichannel pipetting. A 96-well plate format was pursued for further optimisation of the assay for high-throughput purposes, and was used to screen the 5K compound and 88 compound libraries for germination stimulating activity.

7.7.3 Discovery of many new germination stimulating compounds

The compound screening assay developed in Chapter 7 enabled the identification of germination stimulating compounds from compound libraries. This identification is based on the ability of a compound to accelerate germination and establishment in transgenic tobacco seeds which normally would not germinate owing to *NCED* overexpression.

The screen of the 5K library identified 965 potentially germination stimulating compounds from a structurally diverse compound library (Appendix 7.4), and 171 of these were confirmed in follow-up screens. The top 5 ranked compounds from each follow-up, and the four top hits that have not yet been reassessed, are shown in Table

7.4. The top ranked compounds are of particular interest because they are likely to be more potent stimulators of germination than hydroxamic acid D4. Firstly, because the compounds stimulated more green leaf area than D4. Secondly because the compounds were screened at 10 ppm in comparison to the positive control D4, which was added at 312 ppm. This confirms that the screen was adequate to identify germination stimulators that are more potent than D4. Furthermore, even the hit compounds that did not stimulate the highest leaf areas may be a useful resource. Structure-activity relationships may be derived from analysis of the many hit compounds (i.e. at Syngenta), potentially leading to the design of new lead compounds (which then could be tested again in the assay). The compounds are also likely to have been used in other screens, and some targets of the identified compounds may already be known.

A second library containing 88 candidate compounds that were selected or designed to stimulate germination based on known modes-of-action indicated that all of these compounds might be germination stimulators at least at one concentration.

Table 7.4. Selected germination stimulating compounds identified in 5K library. The table shows the top 5 compounds from each follow-up (Figure 7.7). Their identity (ID) is based on their plate position in the 5K compound library: compound plate number (1-53), row (A-H); column (1-12). Leaf areas measured for the original screen and replicate wells in the follow-up (FU1 to FU3), and the mean of the four values is indicated. The mean of the positive control (1 mM D4) is included for comparison. Additionally, four compounds which scored high leaf areas in the screen, but were not re-tested are included. Top 200 indicates if compounds were in the top 200 leaf areas of the screen.

Follow-up	Rank	ID	Top 200	√[green leaf area (pixels)]				
				Screen	FU1	FU2	FU3	Mean
1	1	**Pl20A11**	No	23	43	49	29	36
	2	**Pl25H8**	Yes	45	39	30	48	40
	3	**Pl23F6**	Yes	32	28	40	46	37
	4	**Pl20E12**	Yes	39	36	34	38	37
	5	**Pl20B5**	Yes	35	48	31	25	35
		D4						6
2	1	**Pl38H12**	Yes	33	38	44	56	43
	2	**Pl46H9**	Yes	45	37	40	54	44
	3	**Pl43G2**	Yes	41	45	30	36	38
	4	**Pl52B6**	Yes	36	32	24	54	37
	5	**Pl43A11**	Yes	41	40	35	31	37
		D4						0
Not yet re-screened		**Pl46B5**	Yes	35	N/A			
		Pl25A3	Yes	36				
		Pl12A5	Yes	44				
		Pl46A9	Yes	55				

There was evidence that some of these compounds were more potent stimulators of germination and establishment than 1 mM (312 ppm) D4. This indicated that such compounds are likely to be much more potent than the current lead compound, hydroxamic acid D4, as the compounds were screened at 10 ppm and lower. Some of these compounds were more potent than 312 ppm D4 at concentrations of 0.01 to 1 ppm. Thus some very potent germination or seedling growth stimulators are present in this library.

Additionally, because the screen is based on the emergence of green cotyledons, compounds which cause bleaching (e.g. compounds which inhibit carotenoid biosynthesis like norflurazon) are not likely to be detected. Thus these hit compounds are able to stimulate germination in the absence of leaf bleaching.

7.7.4 Germination stimulators may vary in optimal dose

In the 88 compound library, some potent compounds had peak germination stimulatory effects at concentrations of 0.01 to 1 ppm, but did not stimulate germination at higher concentrations, such as 10 ppm, resulting in a biphasic dose-response. This may be due to different dose-dependencies of the stimulatory activity and toxic activity of the compounds, which results in a hormetic dose response (Mattson, 2008). Compound toxicity can only be inferred from the presence of, and lack of, stimulatory activity at low and high concentrations, respectively, as inert compounds (neither stimulatory nor toxic at any concentration) would result in zero green leaf area. It is therefore possible that some of the compounds that result in low green leaf area at 0.01 ppm to 10 ppm may still be stimulatory to at concentrations below 0.01 ppm.

For logistical reasons, the 5K screen was performed only with compounds at 10 ppm. It is possible, that like in the 88 compound screen, there could also be potent germination stimulators which are effective at low concentrations but inactive or inhibitory at higher concentrations. It is also possible that some compound from either library may only be effective, like D4, at concentrations of above 10 ppm. However, such compounds are less desirable leads than the more potent compounds. Thus future screens of the 5K and 88 compound library at a lower concentrations (e.g. 1 ppb) may be useful to yield further leads.

It is possible that the phytotoxity of the compounds at higher concentrations is indicative of different mechanisms of action than NCED inhibition alone. A number of the compounds in the 88 compound library clearly induce leaf bleaching at 1 ppm (Figure 7.8). This is similar to the phenotype observed in carotenoid deficient mutants, or plants treated with norflurazon or fluridone (Bartels and Watson, 1978; Maluf, *et al.*, 1997). Perhaps it is possible that these compounds may stimulate induction of green leaf area at very low concentrations by partially depleting the carotenoid abscisic acid precursors, enough to stimulate germination, but not enough to cause severe leaf bleaching. Alternatively these compounds may also inhibit two different processes, for example by having a high affinity for NCED, but a low affinity for carotenoid biosynthesis-related enzymes. Hydroxamic acid inhibitors of CCD1, F1 and F2, have also been found to inhibit *p*-hydroxy-phenylpyruvate dioxygenase and result in chlorophyll bleaching (Sergeant, *et al.*, 2013). Thus it is possible that some compounds in the 88 compound library may also affect this process.

7.7.5 Use of compounds in chemical genetic studies

As discussed in the introduction of this chapter, it is possible that some compounds stimulate germination through different modes of action than NCED inhibition. There are a plethora of potential mechanisms which could be targeted which result in improved seed germination (Villedieu-Percheron, *et al.*, 2014).

Confirmed hits from the 5K compound library will be screened for inhibitory effects on LeCCD1a expressed *in vivo* in *E. coli* (using the methods described in Sergeant et al., 2009). This technique is based on the measuring pigment produced by carotenoid synthesising *E. coli* colonies and should establish if any of the identified compounds are likely to be CCD inhibitors. Assays of inhibition of NCED *in vitro* (which is more laborious), e.g. as done with *Zm*NCED in Chapter 4, would be required to confirm or rule out NCED inhibition as potential mode of action of these compounds. It is possible that some of the compounds could be potent and NCED-specific inhibitors. Additionally, compounds which do not inhibit NCED may be very interesting for further study because they may yield novel, or yet unknown, targets for stimulation of germination and emergence. A number of approaches for investigating the mode-of-action of the compounds are possible. For example: transcriptomic analyses could reveal which signalling pathways are affected by the

modulator; proteomics analyses such as affinity chromatography or yeast-three-hybrid assays could be used to identify the protein targets; or forward genetic screening could be used to identify mutants with insensitivity to the identified compounds (McCourt and Desveaux, 2010; Toth and van der Hoorn, 2010).

A very interesting prospect is to test the activity of the identified germination stimulators in breaking the cold-induced dormancy in maize seed (Chapter 3).

7.7.6 In relation to other chemical genetics studies in plants

The screening of the 5K compound library in this chapter is the first step in a forward chemical genetics approach to identify new targets for germination stimulation. Similar chemical genetics studies have previously identified both agonists (Park, *et al.*, 2009; Zhao, *et al.*, 2007) and antagonists (Kim, *et al.*, 2011) of ABA signalling by screening compound libraries. Pyrabactin was identified in a microtitre plate assay not too dissimilar to the one outlined in this Chapter, except it was identified as a germination inhibitor, rather than a stimulator (Park, *et al.*, 2009). Pyrabactin, a selective ABA receptor agonist, facilitated the forward genetics screen in which the ABA receptor family was elucidated (Park, *et al.*, 2009; Zhao, *et al.*, 2007). DFPM, was identified as an ABA signalling antagonist based on its ability to down regulate ABA-responsive genes in a ß-glucuronidase reporter gene system (Kim, *et al.*, 2011). This led to identification of components of a plant immune response pathway which regulates ABA signalling (Kim, *et al.*, 2011). Thus compounds identified in chemical genetics screens can result in the elucidation of novel signalling pathways. These previous screens (Kim, *et al.*, 2011; Zhao, *et al.*, 2007) used Arabidopsis because of its small seed size which lends to it use in microtitre plate format. In this Chapter, tobacco seed were used which also have the benefit of being small enough for microtitre plate format.

A smaller library of compounds which were selected as potential NCED inhibitors were also screened here. Previous studies have tested the efficacy of designed compounds *in vitro* against target NCEDs (Boyd, *et al.*, 2009; Sergeant, *et al.*, 2009). However, this is not a particularly high-throughput method for identifying NCED inhibitors because of the requirement to use a HPLC based assay (Harrison, 2014). Additionally, compounds which exhibit activity *in vitro* may not yield desirable results *in vivo*. This was the case for a few hydroxamic acid inhibitors which exhibit

promising *in vitro* NCED inhibition; but actually have deleterious effects on germination (Awan *et al.*, in preparation) (e.g. D8; Table 1.3). Thus a direct screen, *in vivo* for the phenotype of interest, seed germination in seed highly expressing *NCED*, may be more efficient.

7.7.7 Summary and conclusion

A very useful screen has been developed for identifying novel germination stimulating compounds, and has already yielded some success in this chapter. Many compounds have been identified for use in further chemical genetics studies, and this may eventually provide insight in to novel modes of action for germination stimulation. Furthermore, compounds which a promising candidates for potent NCED inhibitors have been identified. This study may therefore yield compounds that can be applied in the development of novel seed treatment technology.

8 General Discussion and Conclusion

8.1 Aims and objectives of the project

8.1.1 Project rationale

Physiological dormancy can prevent seed germination under conditions otherwise suitable for germination, and is often dependent on the phytohormone ABA. NCED, a member of the CCD enzyme family, catalyses the last committed and rate-limiting step in ABA biosynthesis. Novel hydroxamic acid inhibitors of CCDs were found to stimulate germination in seeds imbibed under germination-inhibiting conditions, or in seeds over-expressing *NCED* (Awan *et al*., in preparation). Hydroxamic acids inhibited NCEDs *in vitro*, and reduced ABA accumulation *in vivo*. Therefore it was thought that hydroxamic acids stimulate germination by reducing ABA accumulation (Awan *et al*., in preparation). However, there were no NCED-specific inhibitors within the collection of hydroxamic acid CCD inhibitors (Table 1.3).

Low vigour maize seed lots germinate poorly (e.g. non-germination, poor seedling growth) following or during imbibition at suboptimal temperatures. It was hypothesised that low vigour in maize could, in part, be due to an induction of physiological dormancy. Thus ABA may have a role, and this could be probed through the use of NCED inhibitors. If this is the case, then germination under suboptimal conditions could be improved through the use of small biologically active molecules, such as NCED inhibitors, if applied to commercial seed lots as a seed treatment.

There were therefore two main aims of the project: 1 - To determine which, if any, maize *NCED* genes control germination, vigour and ABA biosynthesis at suboptimal temperatures; and 2 - To identify novel germination stimulating compounds and NCED-specific inhibitors. The objectives that were set in order to meet these aims, and the progress made towards meeting these objectives, are outlined in Table 8.1.

Table 8.1. Completion of objectives defined in the main introduction.

#	Objective	Done?	Comments
1a	Identify conditions which inhibit maize germination (is it dormancy?)	Yes	Identified conditions, probed idea of dormancy
1b	Probe the role of ABA biosynthesis under the identified conditions using hydroxamic acid NCED inhibitors.	Partially	A small, but significant, effect of hydroxamic acids on maize germination under cold was found. However inhibitors had little or no effect in most other cases.
1c	Identify or confirm putative maize *NCED*s using a bioinformatics approach.	Yes	Confirmed five maize *NCED*s; *in vitro* activity confirmed for 4; identified potential *NCED*-like pseudogenes
1d	Measure expression of *ZmNCED* and ABA accumulation during imbibition under suboptimal conditions.	Yes	Some maize NCEDs were confirmed to respond to imbibition at suboptimal temperatures
2a	Clone *ZmNCED*s for expression in *E. coli* to aid *in vitro* identification of *Zm*NCED-specific inhibitors.	Yes	*NCED*s cloned and expressed in *E. coli.* *Zm*NCEDs available for identification of *Zm*NCED specific inhibitors.
2b	Develop a high-throughput assay to screen chemical libraries for germination stimulating compounds.	Yes	Yes
2c	Screen a diverse chemical library for germination stimulating compounds	Yes	More than 5000 chemicals were screened, ca. 100 compounds identified as having potential value as germination stimulants.
2d	Test designed ABA biosynthesis/ NCED inhibitors for germination stimulation.	Yes	Potent germination stimulators were identified as a basis for further compound design.

8.1.2 Completion of objectives and summary of outcome

Overall significant progress was made towards achieving the aims and most of the objectives were completed (Table 9.1). It was found that cold could induce a reversible state of non-germination in maize seeds (Chapter 3), suggesting the induction of dormancy. Hydroxamic acid inhibitors had a small, but significant, effect in reversing the non-germinating state (Chapter 3), particular *NCED*s were up-regulated and ABA concentration was modulated during cold treatments (Chapter 6). This supported the hypothesis that cold induces physiological dormancy in maize seed.

A screen was developed (Chapter 7) to identify novel germination-stimulating compounds, and it succeeded in identifying ca. 100 efficacious compounds from a library of 5000 diverse compounds, and a number of these compounds were more potent than the original lead compounds D2 and D4 (Chapter 8).

*ZmNCED*s were cloned and expressed in *E.coli*. Hydroxamic acid D2 and D4 were shown to inhibit ZmNCEDs *in vitro* (Chapter 4). Purified NCEDs may be used to measure the *in vitro* NCED inhibition activity of the newly identified germination stimulating compounds. The specificity of the newly selected compounds, which includes ones that are more potent than D2 and D4, has not yet been tested: these could contain the ideal NCED-specific compounds, and they sould be tested with the in vitro assays with both NCED and other CCDs, and against maize germination in the cold.

8.2 The Role of NCEDs in Maize Germination at Suboptimal Temperatures

8.2.1 Does imbibition of maize at suboptimal temperatures induce dormancy?

8.2.1.1 Imbibition at low temperatures leads to a reversible state of non-germination

The induction of dormancy, which restricts the range of environmental conditions (e.g. temperature) that allow seed germination, is a reversible process: whilst seed may not germinate under particular conditions, they remain viable and able to germinate provided dormancy can be broken (Finch-Savage and Leubner-Metzger, 2006). Although maize primary dormancy is well-documented because of its role in preventing vivipary (Tan, *et al.*, 1997; White, *et al.*, 2000; White and Rivin, 2000), mature, dry maize seed are generally considered as non-dormant and typically exhibit the ability to germinate over a wide range of temperatures (Blacklow, 1972). However some of the wild relatives of maize do exhibit various types and depths of dormancy (Avendano Lopez, *et al.*, 2011). Some of the subspecies enter dormancy during summer high temperature and wet conditions, whereas in others dormancy is associated with low temperatures during autumn and winter (Avendano Lopez, *et al.*, 2011).

In Chapter 3, it was observed that low vigour seed (lot 4B) germinated when imbibed at permissive temperatures (25°C). However, three to seven days of imbibition of seed at 5°C prevented approximately one third of these seed from germinating when subsequently transferred to otherwise permissive conditions (25°C). Two main hypotheses can be suggested to explain this: 1. cold imbibition results in damage and a loss of viability in the seeds such as can occur in imbibitional chilling injury (Bedi and Basra, 1993); or 2. Cold imbibition induces a state of dormancy. Imbibition of seeds from some species that exhibit nondeep physiological dormancy at temperatures unfavourable for germination can result in induction of secondary dormancy [e.g. Arabidopsis (Cadman, *et al.*, 2006); many others (Baskin and Baskin, 2001)]. Secondary dormancy and deepened primary dormancy also occur in domesticated crop species, such as barley [at supraoptimal temperatures, and in hypoxia (Hoang, *et al.*, 2013; Leymarie, *et al.*, 2008)]; but an extensive literature search has not revealed any such report of maize dormancy being induced by cold temperatures.

By definition, if the non-germinating state occurred as a result of dormancy, it would be reversible (and not reversible if due to cold induced loss of viability). Desiccation treatments have previously been observed to release dormancy in other species [*Chenopodium albium* (Bouwmeester and Karssen, 1993b); *Orobanche* spp. (Kebreab and Murdoch, 1999); *Sisymbrium officinale* (Bouwmeester and Karssen, 1993a); *Spergula arvensis* (Karssen, *et al.*, 1988)]. In these cases, seed desiccation has been used as one method of determining the difference between non-viable and dormant seeds. In Chapter 3, there was an indication that drying the maize seed following imbibition at 5°C may restore the ability of the seed to germinate at 25°C. The experiment will need replicating to confirm this. If this can be replicated, it will show that seed remained viable during cold imbibition. This observation would stimulate further research to define conditions in which maize germination occurs, and the mechanisms responsible; it is very important for seed producers to understand why only a proportion of seeds in a seed batch are susceptible to cold induced dormancy because their aim is to produce seeds that will germinate uniformly in a wide range of field conditions.

ABA signalling is a widely conserved mechanism in the induction and maintenance of nondeep physiological dormancy (Graeber, *et al.*, 2012). Hydroxamic acid

inhibitor D4, which inhibits *Le*NCED1 *in vitro* (ca. 35%) stimulated germination, and reduced ABA accumulation, in transgenic tomato seed overexpressing *LeNCED1* and in wild-type tomato seed imbibed in osmoticum (Awan *et al.*, in preparation). Hydroxamic acid CCD inhibitors, D4 and D2, also moderately inhibited *Zm*NCEDs *in vitro* (ca. 35%) (Chapter 4). Low vigour maize seed imbibed at 5°C with hydroxamic acid inhibitors D2 or D4, exhibited a small but significant decrease in non-germination when transferred to 25°C than seed imbibed without the inhibitors. However the effect in reversing cold-induced dormancy was not as strong as the desiccation treatment (Chapter 3). If the only mechanism of germination inhibition is ABA accumulation; some of the newly selected compounds from the 88 compound and 5K compound libraries (Chapter 8) could potentially be as effective as the desiccation treatment.

ABA biosynthesis inhibition has been shown to be inefficient at reducing ABA accumulation, and breaking dormancy, in secondary dormant barley seeds (Leymarie, *et al.*, 2008), and does not fully recover germination in secondary dormant seeds of Arabidopsis unless combined with GA (Cadman, *et al.*, 2006). Since other mechanisms than ABA signalling are likely to be involved in secondary dormancy [e.g. GA signalling and *DOG1* expression (Footitt, *et al.*, 2011)] and D2 and D4 are unlikely to cause 100% inhibition of ABA accumulation *in vivo*, this data is consistent with the hypothesis that there is a role for ABA in secondary dormancy induction in maize (seed lot 4B) imbibed at suboptimal temperatures.

Like many other species [e.g. Arabidopsis (Okamoto, *et al.*, 2006); barley (Leymarie, *et al.*, 2008); and tobacco (Manz, *et al.*, 2005)], maize germination was delayed by exogenous ABA. However, ABA did not result in a persistent non-germinating state, perhaps indicating changes in ABA sensitivity, catabolism and ABA-independent mechanisms are also involved in the non-germinating state induced by cold temperatures. It is possible that application of higher ABA concentrations is required to observe a persistent effect of ABA. However, approximately 40 µg of ABA was added per seed, which far exceeds the amount of endogenous ABA found per seed based on mean embryo ABA content (< 30 ng per seed). Similarly, exogenous ABA application or ABA produced via overexpression of ectopic *NCED*s does not always induce lasting dormancy in other species [e.g. tobacco (Manz, *et al.*, 2005; Qin and Zeevaart, 2002)].

8.2.1.2 Imbibition at low temperatures led to ZmNCED1 and ZmNCED9 induction, maintenance of ZmNCED3b transcripts and altered ABA accumulation

NCED, which catalyses the last committed and rate-limiting step in ABA biosynthesis is commonly found to be transcriptionally up-regulated in conditions that stimulate ABA accumulation such as water deficit (Iuchi, *et al.*, 2001; Qin and Zeevaart, 1999) and dormancy (Cadman, *et al.*, 2006; Hai Ha, *et al.*, 2014). Thus, if imbibition at 5°C induced dormancy, it may be associated with an increase in *NCED* expression and an increase in ABA accumulation.

The role of the five maize NCEDs (identified in Chapter 4) during germination at suboptimal conditions was investigated using RT-qPCR (Chapter 6). Expression of *ZmNCED1* and *ZmNCED9* in embryos was very strongly induced, and *ZmNCED3b* expression persisted at a higher level during imbibition at 5°C compared to 25°C, resulting in an accumulation of total *NCED* gene family transcripts at 5°C. However, the induction of *NCED* at 5°C did not correlate with an increase in embryo ABA concentration.

Compared to dry seed, ABA concentration was reduced significantly by imbibition for 24 hours at 25°C, whereas it was not reduced significantly after 168 hours of imbibition at 5°C. Furthermore a short pulse of ABA biosynthesis was observed two hours after transfer of seeds from 5°C to 25°C (170 hours from beginning of cold treatment): This increase in ABA accumulation was not significant compared to the 168 hour time point, but was significant compared to the 72 hour time point, so it may have partially occurred during imbibition at 5°C. Nonetheless the ABA concentration reduced significantly between 6 and 24 hours after transfer of cold test treated seeds to 25°C. A similar trend is seen in barley seed under induction of secondary dormancy by imbibition at supraoptimal temperatures (Leymarie, *et al.*, 2008): in this case ABA concentration was high in dry seed, and when seeds were imbibed in permissive conditions there was a rapid decline in ABA concentration. Under conditions which induce secondary dormancy, ABA concentration was maintained at a higher level and NCED expression increased. As observed here in maize, the ABA levels peaked again when the barley seeds were transferred from supraoptimal to optimal temperatures (Leymarie, *et al.*, 2008).

The main difference in maize seed lot 4B, observed here, is that the ABA concentration 'peak' upon transfer from suboptimal to optimal temperatures appears to be very transient. Here the ABA concentration peaks at 2 hours after transfer, and declines to significantly lower than dry seed ABA concentrations after 6 hours, whereas in barley, the ABA concentration peak is observed 24 hours after transfer to optimal conditions (Leymarie, *et al.*, 2008). The study on barley does not measure ABA beyond the 24 hour time point (Leymarie, *et al.*, 2008), so it is not known if the increase in ABA concentration in barley seeds is also transient, but it appears to persist longer in barley (Leymarie, *et al.*, 2008) than in maize. Imbibition of barley seed under continuous blue light also induces secondary dormancy, as it reduces their ability to germinate in darkness at 10°C (Hai Ha, *et al.*, 2014). In some cases, the ABA concentration also reduces after transfer from dormancy inducing (blue light) to optimal (darkness) conditions. However the ABA concentration is still maintained at levels higher than seen in dry seed of barley (Hai Ha, *et al.*, 2014). This suggests the hypothesis that it is the dynamic change in ABA concentration rather than the absolute concentration that is important in transition between dormant and non-dormant states.

Thus the patterns of *NCED* expression and ABA accumulation are at least partially consistent with an induction of secondary dormancy in maize by suboptimal temperatures. However, it is likely that other mechanisms (e.g. GA signalling) are also involved in the induction of dormancy in maize seed at low temperatures. Future experiments to elucidate these mechanisms are proposed in Section 8.4.1.1.

8.2.1.3 Dormancy in seed lots of varying vigour

The ability to germinate at 25°C following imbibition at 5°C varied between seed lots. High-vigour seed lots (4A and 1B) typically exhibited over 95% germination at 25°C after being imbibed for 7 days at 5°C, compared to the low vigour seed lot 4B which typically exhibited less than 75%. This is interesting as seed lots 4A and 4B belong to the same variety, indicating that the difference in vigour is due to environmental rather than genotype effects (but not age, as seed lots were harvested at similar times). It is known that seed vigour of maize can vary even within the same harvest, and vigour can correlate to kernel shape, size and position on the ear, as well as the size of the ear itself (Graven and Carter, 1990; Msuya and Stefano, 2010; Muchena and Grogan, 1977), although the vigour-shape/size relationship

depends on conditions. For example, small round seed had similar cold test results to other seed types, but generally poorer field emergence (Graven and Carter, 1990). However, smaller seed appear to have better germination at low water potentials (Muchena and Grogan, 1977).

The low vigour seed lot 4B typically contained smaller (lower seed weight) and rounder seeds than the high vigour seed lot of the same variety (4A) (visual observation). It is known that the two seed lots, 4A and 4B originate from the same harvest year, are from the same production facility, and that they were sampled from two contiguous batches (numbers *xx*28 and *xx*29) (Personal communication, Barbara Westland, 4 November 2013) and it can be speculated that they are from the same production, but have been size graded. Thus, potential for dormancy induction at 5°C may be dependent on seed size or shape, and maturation position (which relates to seed size) on the ear. Indeed, in other species, dormancy levels vary between seed that mature on the same parent plant, and this is sometimes also dependent on maturation position of the seed [e.g. celery (Thomas, *et al.*, 1979); parsley (Thomas, 1996)].

It would also be interesting to study the effect of maturation conditions on the potential for cold induced dormancy in maize. Environmental conditions preceding and during seed maturation in other species can determine dormancy depth. For example in Arabidopsis, maternal temperature history (Chen, *et al.*, 2014), seed maturation temperature (Chiang, *et al.*, 2011) and nitrate availability during seed maturation (Alboresi, *et al.*, 2005; Matakiadis, *et al.*, 2009) can all affect dormancy levels in seed.

8.2.1.4 NCED control of dormancy in seed lots of varying vigour

A strong induction of *ZmNCED1* and *ZmNCED9* was observed in the low vigour seed lot 4B during imbibition at 5°C, and *ZmNCED3b* expression was maintained compared to a decline observed in seed imbibed at 25°C. It is possible that this increased *ZmNCED* expression contributes to the induction of a dormant state, preventing germination when transferred from 5°C to 25°C. Thus it could be expected that higher *ZmNCED* expression would be found after 7 days at 5°C in low vigour seed lots, compared to high vigour seed lots.

The low vigour seed lot of Variety 4 (4B) had higher (but not significantly) sum expression of all *ZmNCED*s than the high vigour seed lot 4A. Notably, Variety 4 showed an induction of *ZmNCED9* by cold treatment, and that induction was significantly higher in the lower vigour seed lot. It is possible therefore that *ZmNCED9* expression could correlate to reductions in cold test vigour in this variety. The high and low vigour seed lots of Variety 1 differed in viability, and only relatively small effects of 5°C imbibition on germination at 25°C were observed, indicating the two seed lots did not differ as much in vigour as Variety 4. The two Variety 1 seed lots also expressed very similar levels of *ZmNCED* after 7 days at 5°C, and expressed *ZmNCED9* at much lower levels than Variety 4. Thus it is possible that high expression of *ZmNCED9* is responsible for an induction of dormancy in lot 4B under cold conditions.

ZmNCED expression after 7 days at 5°C was also measured in six other seed lots, and was found to vary significantly within and between varieties (Figure 6.4). Results from a 10°C paper germination test indicate that these seed lots may also vary in both viability and vigour. Thus these seed lots should be characterised in the 5°C cold test (and control) conditions to investigate correlations between *ZmNCED* expression and vigour.

8.2.1.5 ZmNCED3b is expressed at relatively high levels in dry seed

Here, it was shown that ABA concentration and *ZmNCED3b* expression were relatively high in the dry seed of lot 4B. High ABA concentration in dry seed is sometimes associated with primary dormancy (Ali-Rachedi, *et al.*, 2004; Grappin, *et al.*, 2000; Groot and Karssen, 1992), but not always (Millar, *et al.*, 2006). It is possible that storage of *ZmNCED3b* transcripts in dry seed allows for accelerated ABA biosynthesis upon imbibition, a mechanism which is required for dormancy maintenance (Ali-Rachedi, *et al.*, 2004; Alvarado and Bradford, 2005; Lepagedegivry and Garello, 1992; Leymarie, *et al.*, 2009). It was shown here that *ZmNCED3b* transcripts were quickly reduced under optimal germination conditions, but not under cold conditions. Measurement of the *NCED* (particularly *ZmNCED3b*) transcript abundance in dry seed of seed lots of different vigour might indicate if *ZmNCED3b* transcript abundance affects the ability of the maize seed to enter a dormant state under cold conditions.

If not indicative of the ability to synthesise ABA *de novo* upon imbibition, the *ZmNCED3b* transcript abundance in dry seed may be reflective of the rate of ABA biosynthesis during seed maturation. Both the dry seed transcriptome and ABA concentration can be altered by changes in maturation conditions which affect dormancy status [e.g. nitrate (Matakiadis, *et al.*, 2009)]. However, transcriptomes of dormant (Arabidopsis ecotype Cvi) and non-dormant (Arabidopsis ecotype Columbia) dry seed have been found to be very similar, indicating that stored mRNAs largely reflect transcripts required for developmental purposes, rather than dormancy in this species (Kimura and Nambara, 2010). Regulation at the translational level is also important in the determination of seed germination and vigour (Gallandt and Rajjou, 2015). Thus *ZmNCED* expression in dry seed is a potential indicator of vigour in the 5°C vermiculite cold test, but tests on a more diverse collection of seed lots are required to demonstrate this.

8.2.1.6 Conclusion – does cold induce dormancy in maize?

Imbibition of maize seed lot 4B at suboptimal temperatures results in a partially reversible (by hydroxamic acids, and maybe seed drying) state of non-germination that is associated with an increase in *ZmNCED* expression and maintenance of ABA concentration. This is consistent with the hypothesis that the non-germination of some seed results from the induction of secondary dormancy that involves ABA.

However, there are some inconsistencies. For example, *ZmNCED* expression and ABA accumulation drop rapidly between 2 hours and 6 hours after seed of lot 4B are transferred from 5°C to 25°C, to similar levels seen in seed imbibed directly at 25°C; yet the non-germinating state of some seeds is still maintained. It is possible that ABA accumulation is maintained only in dormant seed and that differences were not observed because of random sampling from a population of seed in which only a proportion are dormant. Thus this could be resolved by measuring ABA accumulation in seeds which persist in a state of non-germination beyond 24 hours. It is also possible that ABA at 5°C and the ABA pulse after transfer to 25°C induces another mechanism which is responsible maintaining dormancy at 25°C. Other dormancy mechanisms such as ABA sensitivity or GA signalling may be involved, so these too need to be studied. Suggestions for future work are outlined in 8.4.1.1.

Nonetheless, the evidence is strongly supportive of a role of *ZmNCED*s in germination at 25°C following imbibition at 5°C, and the reversibility of the non-germinating state is strongly supportive of the induction of dormancy.

8.2.2 Do low temperatures reduce rate of germination through ABA signalling?

8.2.2.1 Hydroxamic acids did not affect ABA accumulation or germination rate at low temperatures

Typically, percentage maize germination is high over a wide range of temperatures. However, suboptimal temperatures that allow high germination percentages, still reduce germination rate (i.e. increase MGT) (Blacklow, 1972). The rate of germination (= germination / time) is usually linearly related to the difference in temperature, above a base temperature threshold (T_b), and the rate increases up to the optimal temperature for germination (T_o). Above T_o, germination rate decreases linearly with temperature up to the ceiling, or maximum, temperature (T_c) that permits germination (Bradford, 2002). Thus suboptimal temperatures will always act to slow germination compared to optimal temperatures. Seeds within a lot vary in the amount of thermal time [time x (temperature - T_b)] required for germination, and this causes a spread in the time at which the population of seeds germinate (Bradford, 2002). Hydrothermal time, which also incorporates a basal water potential (Ψ_b) required for germination is often used to predict seed germination (Alvarado and Bradford, 2005; Bradford, 2002). If the conditions do not exceed the basal requirements (e.g. Ψ_b) for some seed fractions, then these seed fractions will not germinate, leading to a reduced germination percentage.

Dormancy may increase Ψ_b, amount of thermal time (or hydrothermal time) required for germination and modify other parameters such as T_c and T_b (all of which could differ between seeds within a seed lot), usually resulting in a reduced final germination percentage or reduced germination rate for a given set of conditions (Alvarado and Bradford, 2005). Under conditions which permit germination, therefore, "residual dormancy" can still be observed in the mean and distribution of germination rates (Bradford, 2002). Dormancy breaking treatments, such as after-ripening or fluridone can therefore decrease the amount of hydrothermal time required for germination, increasing the rate of germination under a given set of conditions (Alvarado and Bradford, 2005).

Treatment of high (4A) and low vigour (4B) seeds with ABA resulted in an increase in MGT when seed were imbibed at 15°C or 25°C. Additionally, collaborators at Syngenta reported that addition of hydroxamic acids, such as D2, decreased MGT in seed of maize cultivar NK Falkone imbibed at suboptimal temperatures (David Brocklehurst, Syngenta, personal communication, September 22, 2014). Thus it is hypothesised here that germination rates of maize may be reduced at suboptimal temperatures due to the induction of ABA biosynthesis which acts to increase the amount of thermal time required for germination and increases residual dormancy. Seeds from one lot of maize variety NK Falkone had a mean T_b of 4.5°C (data not shown): temperatures above 10°C allowed germination of nearly all seeds and mean germination rate (1 / MGT) increased linearly with temperature at 15°C and 20°C. NK Falkone was germinated at 10°C and 15°C in the presence of hydroxamic acid CCD inhibitor D2, previously confirmed to inhibit NCEDs *in vitro* (Chapter 4; Sergeant, et al., 2009), and C426, a new compound developed by Syngenta. These inhibitors had no significant effect on germination times (e.g. MGT, T_{10}, T_{50}, T_{90}), or germination uniformity (T_{90-10}) at the two temperatures (Chapter 3). However, it was later found that the inhibitors did not significantly affect seed ABA concentrations either, and so a role of ABA in low temperature germination rate could not be ruled out. Low temperatures are associated with elevated NCED transcription and ABA concentration

A role of ABA could not be probed through the use of current hydroxamic acid CCD inhibitors, due to their lack of effect on ABA accumulation in NK Falkone. Therefore the expression of the five *ZmNCED*s and ABA accumulation during low temperature imbibition was measured (Chapter 6). *ZmNCED3b* was again found to be accumulated at high levels in dry NK Falkone seed, and low temperatures decrease the rate of decline in *ZmNCED3b* transcripts. *ZmNCED1* was induced at 5°C and 10°C, but not at 15°C, perhaps indicating a specific low-temperature germination role for *ZmNCED1*. Overall, *NCED* transcripts were elevated for longer at lower temperatures, and the trend in ABA concentration reflected this too. Thus it remains possible that ABA could contribute to residual dormancy and decreased germination rates at suboptimal temperatures. More potent ABA biosynthesis inhibitors and a responsive seed lot may enable this to be shown experimentally.

8.2.3 Effect of suboptimal temperatures on post-germination growth

Suboptimal temperatures affect maize seedling growth in a similar manner to the way in which they affect germination (i.e. seedling growth rate can also be described with a thermal-time model) (Blacklow, 1972). Seed vigour can be defined as the "***the sum of properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments***" (ISTA, 2015b). Thus seed vigour can be determined by both germination rates and percentages, but also by seedling growth rates and emergence characteristics. The effect of 5°C cold test conditions, here, was mainly as a result of the induction of a non-germinating state in low-vigour seed (Chapter 3); but it is possible seedling growth rate is also increased in high-vigour seed. For example, emerged seedlings of seed lot 4A are larger than those of 4B following a cold test (Figure 3.6 C). This could be as a result of delayed germination or a reduced rate of post-germination seedling growth, or both.

Seedling growth rate can be affected by genetic and pre-germination environmental factors, much in the same way that germination can be. The initial heterotrophic seedling growth rate may depend on the rate and efficiency of mobilisation of seed reserves (Eagles, 1982; Rajjou, *et al.*, 2012); it may also depend on seed integrity in a similar manner to germination. For example, artificially aged soybean seeds mobilised a significantly smaller fraction of seed reserves (Mohammadi, *et al.*, 2011). The potential for heterotrophic growth in maize is dependent on the amount of seed reserves, and smaller seed produce smaller seedlings than larger seed, which have more reserves (Msuya and Stefano, 2010).

However, hormonal regulation of seedling growth may also be an important factor. In rice, ABA production in seedlings growing under cold conditions can be inhibitory to seedling growth (Mega, *et al.*, 2015). Moderate inhibition of ABA accumulation, which was achieved by overexpression of *ABA 8′-hydroxylase* responsible for ABA catabolism, promoted rice seedling growth under cold conditions (Mega, *et al.*, 2015). However strong inhibition of ABA accumulation by overexpression of *ABA 8′-hydroxylase* inhibited seedling growth; indicating that low levels of ABA are necessary for growth (Mega, *et al.*, 2015). An effect of the hydroxamic acid CCD inhibitors on post-germination growth under suboptimal temperatures was not studied, but would be a priority for future study (alongside measurement of *ZmNCED* expression and ABA measurements). Additionally, it

could be hypothesised that ABA produced in the seed during cold imbibition may affect subsequent seedling growth. Indeed, in Arabidopsis, seedlings remain highly sensitive to growth inhibition by ABA immediately after germination, which can initiate a drought avoiding quiescent state (Lopez-Molina, *et al.*, 2001). Again, hydroxamic acid CCD inhibitors did not significantly stimulate growth of seedlings subsequent to cold test conditions (Chapter 3), although hydroxamic acid treated seedlings tended to be larger than those of the acetone control treatment. Experiments in Chapter 6 indicate that the inhibitors may have very little effect on seed ABA concentrations, therefore this hypothesis cannot be rejected and warrants further study, e.g. with more potent inhibitors or through genetic approaches.

8.2.4 Importance of findings

New potential roles for maize *NCED*s and ABA biosynthesis in germination at suboptimal temperatures have been identified (Table 8.2). In particular, *ZmNCED3b* which currently appears to have specific expression in mature seeds, and *ZmNCED1* and *ZmNCED9* which are induced by cold temperatures.

Table 8.2. Roles for members of the maize *NCED* gene family.

Gene	Expression / Potential Roles	References [a]
ZmNCED1	Primary dormancy induction [b]	1
	Water deficit response [b]	1
	ABA accumulation in developing seed [b]	1, 2, 3
	Most abundant NCED in a variety of tissues	2,3,4
	Expressed in leaves and roots	2,3,4
	Low-temperature germination induced	**Here**
ZmNCED2	Drought stressed ovaries	4, 5
	Otherwise generally minor expression	2,3,4
ZmNCED3a	Expressed in leaves and roots	2,4
	Otherwise generally minor expression	2,4
ZmNCED3b	High transcript levels in mature, dry seed	**Here**
	Degradation decelerated in low temperature germination	**Here**
	Otherwise generally minor expression	2,4
ZmNCED9	Expressed in developing seeds, particularly endosperm	2,4
	Expressed in leaves and roots	2,4
	Low-temperature germination induced (variety-specific)	**Here**

[a] References: 1 (Tan, *et al.*, 1997); 2 (Vallabhaneni, *et al.*, 2010); 3 (Capelle, *et al.*, 2010); 4 Queries at http://qteller.com and http://bar.utoronto.ca/maizeefp/ discussed in Chapter 4; 5 (Kakumanu, *et al.*, 2012). [b] Role confirmed in loss-of-function mutant *vp14*; otherwise based on transcriptomic data.

The primary *NCED*s which control germination in maize may not be seed-specific, unlike Arabidopsis, for example, which has two seed-specific *NCED*s (Lefebvre, *et al.*, 2006). Such differences are not surprising since NCED genes appear to have duplicated and diverged following the monocot/dicot split (Vallabhaneni, *et al.*, 2010) (also indicated in Figure 4.1). Importantly, there is evidence that low temperatures can induce a reversible state of dormancy in maize. This may represent a target for further development of novel seed treatment strategies which can improve vigour of more dormant seed lots. The implications of this are discussed further in Section 8.5.2.

8.3 Chemical Genetics of Seed Germination

8.3.1 A high-throughput assay for identification of germination stimulating compounds

A high-throughput assay was developed for screening compound libraries for germination stimulating compounds. The assay was based on the detection of green cotyledon emergence quantified by image analysis of photographs (Chapter 7) of tobacco seeds whose germination and establishment is normally inhibited due to tetracycline-induced ectopic expression of *LeNCED1*.

Some high-throughput assays for seed germination already exist. For example, the 'Germinator' package allows the high-throughput scoring of seed germination (Joosen, *et al.*, 2010). This particular package enables the high-throughput scoring of endosperm rupture of seeds imbibed on filter paper and can describe multiple germination parameters based on a curve-fitting approach (Joosen, *et al.*, 2010). However, such an approach may not lend itself to the screening of large compound libraries as the method requires placement of seed on filter paper and enough compound solution (in the described methods, around 7 ml per sample) (Joosen, *et al.*, 2010). The method described in Chapter 7 required only 200 µl solution per sample and allows seed placement by pipette although it does not provide as detailed or direct output parameters (germination is based on leaf area). However, because the screen is based on measurement of green leaf area, it identifies compounds that stimulate germination and post-germination growth, and will not select agronomically less interesting compounds that inhibit post-germination growth or cause bleaching.

A comparable assay was developed for identifying germination stimulating compounds in root parasitic plants (Pouvreau, *et al.*, 2013). Like the tobacco seed assay utilised in Chapter 7, this was also performed in microtitre plate format but used colour change in tetrazolium salt (MTT) as an indicator of radicle emergence (Pouvreau, *et al.*, 2013). A similar MTT staining method could work in combination with the transgenic tobacco seed (Chapter 7) to identify compounds which specifically stimulate radicle emergence.

Whilst the tobacco seed assay was high-throughput and useful for identifying germination stimulating compounds from large chemical libraries, it was perhaps not as successful in determining relative compound efficacy at any given concentration because of the variation in leaf areas. For this purpose, identified compounds may need to be re-tested using a different method, perhaps such as using the Germinator package (Joosen, *et al.*, 2010), an MTT based assay (Pouvreau, *et al.*, 2013), or more simply by counting radicle emergence on paper. It is also possible that the current tobacco assay could be photographed more frequently to allow a 'real-time' based assay (e.g. time until *x* number of green pixels), which may allow better discrimination between compound germination stimulating efficacies. Thus there may be a trade-off between having a high-throughput in a screening assay (i.e. using one replicate per compound and one measurement) and having additional compound detail, which is less high-throughput (i.e. repeated measurements or replication).

Additionally, compounds could be screened for effectiveness in restoring germination in different seed or dormancy-inducing conditions. For example, seeds of Arabidopsis ecotype Cvi could be placed in deep primary or secondary dormancy states (outlined in Cadman, *et al.*, 2006) to identify chemical modulators, which may interact with specific types of dormancy. The small size of Arabidopsis seed would fit with microtiter plate formats. Because deeply dormant seeds do not germinate in the light (Cadman, *et al.*, 2006) the assay could be run under illumination to maintain the target dormancy mechanism, while at the same time allowing quantifiable growth and greening of germinated seed if dormancy was broken by a compound.

8.3.2 Identified germination stimulating compounds

The screen of the diverse 5k library identified many germination stimulating compounds, a number of which were confirmed in follow-up assays and shown to be

more potent than the current lead hydroxamic acid germination stimulating compound D4 (Chapter 7). Similarly, some compounds identified in the targetted 92-compound library also appear to be more potent germination stimulators than D4, even at much lower concentrations.

The mode of action of these compounds is unknown; however it seems likely that the screen may be biased towards identifying compounds which regulate ABA biosynthesis and signalling, although there are a multitude of mechanisms that may result in germination stimulation (Chapter 1). However, a preliminary assay of carotenoid accumulation in *E. coli* expressing CCDs indicates that some of the compounds may exhibit CCD inhibition activity (data not shown). Further work is required to determine the effectiveness of these compounds in stimulating germination in other systems and determining their mode-of-action, and this may yield new targets for germination stimulation. All of the efficacious compounds should be screened using the aforementioned *E. coli* assay (described in Sergeant, *et al.*, 2009) and assayed for *in vitro* inhibition of NCEDs and CCDs (NCED-specific inhibitors could be identified).

8.3.3 A NCED-specific inhibitor is still required

Whilst the current lead hydroxamic acid inhibitors of NCED, D2 and D4, were found to inhibit *Zm*NCEDs *in vitro* (Chapter 4) they appear to inhibit other CCDs (CCD1 and CCD8) more potently than NCED *in vitro* (Harrison, *et al.*, 2015; Sergeant, *et al.*, 2009) and it is possible that they may affect other metalloenzyme families (i.e. in the same way NDGA inhibits NCEDs and lipoxygenases). However, D2 and D4 do not inhibit the 15/15′ cleavage activity of *Mus musculus* ß-carotene-15-15′-oxygenase (cleaves ß-carotene to produce retinal) suggesting they specifically inhibit particular CCDs. Thus that inhibition of non-CCD metalloenzymes is less likely.

Whilst *in vitro* inhibition of NCED (Harrison, 2014; Sergeant, *et al.*, 2009), *in vivo* ABA accumulation reduction, and a stimulatory effect on NCED-inhibited seed germination (Awan *et al.*, in preparation) point to NCED inhibition being the mode-of-action of D2 and D4 in germination stimulation, it is not completely proven. Doubt is raised because a number of other compounds, such as abamine and D-series hydroxamic acids (Table 1.3) which inhibit NCEDs *in vitro* do not stimulate germination in the same situations despite the observation that abamine and

abamineSG can elicit *in vivo* NCED inhibition in vegetative tissues (Han, *et al.*, 2004; Kitahata, *et al.*, 2006). However, this may be explained by problems with seed-uptake or non-specific negative effects which mask the stimulatory effect of *in vivo* NCED inhibition.

Identification and use of an NCED-specific inhibitor could be used to indicate that the effects of D2 and D4 are not mediated through inhibition of other CCDs, and will aid future reverse chemical genetics studies of NCED. However, existing evidence suggests that it is unlikely that the mode-of-action of hydroxamic acids in germination stimulation is through inhibition of CCDs other than NCED. This is because CCD7 and CCD8 catalyse the biosynthesis of the germination promoting hormone strigolactone (Alder, *et al.*, 2012; Toh, *et al.*, 2012) and loss-of-function *ccd1* and *ccd4* mutants have not been identified in screens for mutations affecting germination; nor are there reports of altered germination phenotypes in *ccd1* and *ccd4* mutants whereas ABA insensitive and ABA deficient mutants, including *NCED* mutants, often display reduced dormancy phenotypes.

Despite their apparent low potency, the current lead compounds D2 and D4 still represent an improvement on fluridone and norflurazon, which inhibit PDS and thus inhibit formation of all carotenoids (Bartels and Watson, 1978) and apocarotenoids and cause photobleaching. Encouragingly, the most recent work in which inhibitors were pre-incubated for an increased amount of time with ZmNCEDs before addition of the substrate, indicates that D2 and D4 may be more effective inhibitors (> 95%) of NCED (Harrison, 2014) than previous *in vitro* assays have shown (Sergeant et al 2009), so further chemical modifications to improve uptake and *in vivo* stability may be a way to improve efficacy in seeds.

8.4 Recommended Future Work

8.4.1 Dormancy in maize

*8.4.1.1 Further study of dormancy mechanisms and the role of **ZmNCED**s*

Further investigation of cold induced dormancy in maize is required. In particular, the following mechanisms could be studied: ABA catabolism, which can be measured by *CYP707A* expression or measurement of ABA catabolites; GA biosynthesis and metabolism, to see if ABA:GA ratio is modulated as is common in

physiological dormancy (Finch-Savage and Leubner-Metzger, 2006; Graeber, *et al.*, 2012); and other dormancy modulators such as *DOG1* (Ashikawa, *et al.*, 2014; Bentsink, *et al.*, 2006; Footitt, *et al.*, 2011). '-Omics' (e.g. transcriptomics, proteomics, metabolomics) approaches to study non-dormant, including cold imbibed and desiccated seed, and dormant seed based on conditions identified here may lead to discovery of new mechanisms. Such approaches have been successful in identifying physiological mechanisms of dormancy in other species; for example the transcriptomic investigation of dormant Arabidopsis seeds (Cadman, *et al.*, 2006; Finch-Savage, *et al.*, 2007). Coverage of the whole transcriptome could be achieved using an RNA-seq approach (Kakumanu, *et al.*, 2012; Marioni, *et al.*, 2008).

Additionally, further chemical genetics approaches or dormancy breaking treatments could be used. Desiccation and hydroxamic acid CCD inhibitor treatments were studied in detail, but addition of other phytohormones (such as GA) and nitrates, for example, may also yield further insight into the reversibility of the non-germinating state. In terms of the future study of the roles of ABA, a more potent, and specific, inhibitor of ABA signalling is required (e.g. a specific NCED inhibitor). It is possible that one of the more efficacious compounds identified in screen of the 5K library is one of these. It is also reasonable to assume that some of the very potent germination stimulators identified in the 88 compound library are potent NCED inhibitors, as the design of these compounds was based on hydroxamic acids.

Moreover, a reverse genetics approach based on the *ZmNCED* roles identified could be pursued to see if dormancy depends on a particular *ZmNCED* function. Conventional techniques such as transposon insertional mutagenesis, or chemical mutagenesis and selection by TILLING, could be used to generate knock-out lines in maize but this would be fairly resource intensive (i.e. screening mutants; backcrossing) (McCarty and Meeley, 2009; Weil and Monde, 2009). More recently developed methods can allow directed mutagenesis which may be less intensive, such as the CRISPR/Cas9 system (Xing, *et al.*, 2014). However, knock-out lines could be problematic due to pleiotropic effects. A *ZmNCED1* knockout mutant, *vp14*, is already available. However seed development and primary dormancy induction may be disrupted in this mutant, so it would not be known if changes in the phenotype observed (e.g. 5°C cold test vigour) are as a result of differences in seed development, or differences in the imbibed matured seed.

Perhaps, instead, a chemically inducible RNA intereference (RNAi) approach could be used to study the effects of knocking down single or multiple *NCED*s during cold imbibition (McGinnis, *et al.*, 2007; Padidam, 2003). Whilst there are technical difficulties with this approach such as reduced transgene transmission, transgene silencing, incomplete target silencing (McGinnis, *et al.*, 2007), and the requirement of a suitable chemical inducer for seed germination studies, such a system could be very useful for studying the role of *ZmNCED*s during germination. Additionally, all of these studies would depend on the ability to reliably and reproducibly produce low vigour seed lots (i.e. sensitive to dormancy induction at 5°C) to study the effects of the mutations or transgenes.

However, identification of a potent NCED inhibitor (which might have already been found here in Chapter 8) combined with expression studies to understand the most likely NCED target protein, could be a relatively rapid chemical genetic approach to studying *ZmNCED* gene functions during germination; and it can simply be used on existing seed lots already known to exhibit low vigour without the need to produce new seed lots from genetically altered lines. Conversely, inducible over-expression could be used to show NCED inhibition of germination through ABA biosynthesis under permissive conditions (similar to shown in tobacco here).

8.4.1.2 A wider range of relevant suboptimal conditions

As indicated in Figure 3.1 maize is cultivated in diverse geographical locations. Thus the environmental extremes which might limit successful germination and establishment in different environments can be very contrasting.

This thesis focussed on the effect of suboptimal temperatures on maize germination, which can occur during early spring sowing at high latitudes and it was found that these conditions may induce dormancy in some seed lots. Cold temperatures are not the only stress associated with this environment; soil saturation, i.e. excess water, can also occur and this can intensify the effect of cold (Saab, 2014; Stoll and Saab, 2015). It was also found here that a soaking treatment induced the non-germinating state earlier, and that this too was partially reversible by a desiccation treatment. The soil may also be compacted due to the use of heavy machinery, or can be capped or form surface crusts due to heavy rainfall, solar drying and/or microbial biofilms; this can lead to increased resistance of the soil to seedling penetration in combination

with wet, hypoxic conditions (Latif, *et al.*, 2008; Nasr and Selles, 1995) which can hinder seedling emergence and crop establishment. To study the impact of these conditions there is a need for experimental systems that can reliably simulate field conditions so that genetic and physiological impacts on vigour can be evaluated. It is possible that the effects of cold temperatures on seed vigour may affect the ability of seedling to emerge in penetration resistant soils. For example, ABA produced by rice seedlings limited growth in rice under the cold (Mega, *et al.*, 2015). It could be hypothesised then that ABA production could also limit the ability of seedlings to penetrate resistant soil.

8.4.2 Novel germination stimulating compounds

A high-throughput screen was developed, and was successful in identifying germination stimulating compounds from a library of over 5000 compounds. The screen could be used to screen further large libraries of compounds to identify yet more compounds. Further study is required to confirm the modes-of-action of the germination stimulators identified here. Compounds should firstly be screened for NCED and CCD inhibition activity. More complicated studies will be required to elucidate the modes-of-action in compounds which do not inhibit NCED.

The most efficacious compounds identified could be tested for germination stimulating activity in alternative situations to tobacco overexpressing *LeNCED1*. The effects of these compounds on cold test germination of the low vigour seed lot 4B would be of particular interest.

8.5 Project Conclusion

8.5.1 Summary of findings

The role of NCEDs and ABA in maize germination were studied using a variety of approaches. The effects of cold on maize germination were investigated, and it was found that imbibition at cold temperatures may induce physiological dormancy in low vigour seed lots. This dormancy induction was largely reversible through a desiccation treatment. Hydroxamic acid CCD inhibitors were used to probe NCED functions in maize seed imbibed in these suboptimal temperatures and a significant, but small, dormancy breaking effect was observed under cold test conditions.

A bioinformatics approach allowed the identification for subsequent cloning of five *ZmNCED*s. Four out of five of these were expressed in *E. coli* and the proteins were able to be purified for *in vitro* inhibition studies. NCED activities were confirmed for these four and were equally and moderately inhibited (ca. 35%) by hydroxamic acid CCD inhibitors D2 and D4. The hypothesis that cold conditions induce dormancy in maize was reinforced through the observation of up-regulation of *ZmNCED1*, *ZmNCED3b* and *ZmNCED9* under cold conditions and observed changes in ABA accumulation.

A high-throughput assay was developed to identify germination stimulating compounds. The assay was successful in identifying a number of efficacious germination stimulants from a library of more than 5000 compounds. The assay also confirmed strong germination stimulating activity in a small library of compounds designed or predicted to inhibit NCED. Some of the compounds identified were shown to be much more potent germination stimulators than the original lead compound D4.

8.5.2 Implications of findings

At high latitudes, early spring sowing of maize can lead to increased yields. Sub-optimal temperatures during this period can delay or prevent germination in maize, leading to crop stands of suboptimal density and uniformity, and ultimately a loss in yield. Here it was shown that suboptimal temperatures can induce dormancy in low vigour maize seed lots. This means that there is potential for the effects of cold to be reversed through dormancy breaking treatments in these seed. This would allow for improvement of germination even under cold conditions, and may allow seed to be sown earlier in the season than might otherwise be possible. Such a treatment may also allow for the sale and use of low vigour seed lots which would have otherwise failed quality control tests, increasing the profitability of seed production.

Furthermore, it was shown here that suboptimal temperatures lead to induction of particular *ZmNCED* genes, and modulated ABA accumulation, which may be responsible for induction of dormancy. These *ZmNCED*s may be targets for conventional or biotech breeding strategies to produce maize lines which are less likely to enter dormancy at low temperatures. Additionally chemical seed treatment strategies could be focused on the inhibition of ABA biosynthesis (e.g. NCED

inhibitors). Here, the means to identify chemical inhibitors of maize NCEDs has also been established, and candidate compounds can be screened *in vitro* for their inhibition of the target *Zm*NCEDs.

Moreover, a number of efficacious candidate germination stimulants have been identified, and more a likely to be identified through further screening. Some of the compounds found here could be potent NCED or ABA biosynthesis inhibitors, and some may have novel modes of action. It has already been shown that some of these compounds are more potent than the current lead compound, D4. These compounds therefore not only represent potential active ingredients in future seed treatments, but a potential resource for chemical genetics studies of dormancy. Because dormancy is an issue in many crop species, these chemicals may also have value in a number of seed treatment strategies.

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Appendices

Appendix 3.1. SPSS ANOVA output for data shown in Figure 3.3. Transformed percentage of seedlings (arcsin(√x)) was analysed using the model Variety/Lot.

Category	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Normal germinated	Corrected Model	9	.706[a]	0.078	9.131	<0.001
	Intercept	1	57.811	57.811	6730.392	<0.001
	Variety	4	0.184	0.046	5.364	0.002
	Variety * Lot	5	0.522	0.104	12.145	<0.001
	Error	30	0.258	0.009		
	Total	40	58.774			
	Corrected Total	39	0.964			
Abnormal	Corrected Model	9	.497[b]	0.055	7.547	<0.001
	Intercept	1	2.485	2.485	339.864	<0.001
	Variety	4	0.175	0.044	6	0.001
	Variety * Lot	5	0.321	0.064	8.784	<0.001
	Error	30	0.219	0.007		
	Total	40	3.2			
	Corrected Total	39	0.716			
Non-germinated	Corrected Model	9	.357[c]	0.04	4.096	0.002
	Intercept	1	70.668	70.668	7302.404	<0.001
	Variety	4	0.126	0.031	3.246	0.025
	Variety * Lot	5	0.231	0.046	4.775	0.003
	Error	30	0.29	0.01		
	Total	40	71.316			
	Corrected Total	39	0.647			

[a] R Squared = .733 (Adjusted R Squared = .652);[b] R Squared = .694 (Adjusted R Squared = .602);[c] R Squared = .551 (Adjusted R Squared = .417).

Appendix 3.2. SPSS ANOVA output for data shown in Figure 3.4 Transformed percentage of normal seedlings (arcsin(√x)) was analysed using the model Regime*Orientation*Variety/Lot.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Corrected Model	23	1.651[a]	0.072	4.313	<0.001
Intercept	1	62.286	62.286	3743.224	<0.001
Cold duration	2	0.155	0.078	4.659	0.020
Variety	1	0.047	0.047	2.81	0.107
Orientation	1	0.068	0.068	4.063	0.055
Cold duration.Variety	2	0.038	0.019	1.15	0.333
Cold duration.Orientation	2	0.001	0	0.026	0.974
Variety.Lot	2	1.051	0.526	31.584	<0.001
Variety.Orientation	1	0.084	0.084	5.069	0.034
Cold duration.Variety.Lot	4	0.16	0.04	2.404	0.078
Cold duration.Variety.Orientation	2	0.036	0.018	1.089	0.353
Variety.Lot.Orientation	2	0.003	0.002	0.092	0.912
Cold duration.Variety .Lot.Orientation	4	0.007	0.002	0.109	0.978
Error	24	0.399	0.017		
Total	48	64.336			

[a] R Squared = .805 (Adjusted R Squared = .619).

Appendix 3.3. SPSS ANOVA output for data shown in Figure 3.6. Transformed percentage of normal seedlings, abnormal seedlings or non-germinated seedlings (arcsin(√x)) was analysed using the model Medium*Variety/Lot.

Category	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Useable (normal + small)	Corrected Model	7	1.953[a]	0.279	33.568	<0.001
	Intercept	1	31.855	31.855	3833.298	<0.001
	Variety	1	0.033	0.033	3.929	0.059
	Medium	1	1.06E-06	1.06E-06	0	0.991
	Variety.Lot	2	1.91	0.955	114.912	<0.001
	Variety.Medium	1	0.009	0.009	1.053	0.315
	Variety.Lot.Medium	2	0.001	0.001	0.085	0.919
	Error	24	0.199	0.008		
	Total	32	34.007			
	Corrected Total	31	2.152			
Abnormal	Corrected Model	7	.396[b]	0.057	10.245	<0.001
	Intercept	1	2.169	2.169	392.786	<0.001
	Variety	1	0.001	0.001	0.106	0.747
	Medium	1	0.002	2.00E-03	0.335	0.568
	Variety.Lot	2	0.37	0.185	33.478	<0.001
	Variety.Medium	1	0.016	0.016	2.979	0.097
	Variety.Lot.Medium	2	0.007	0.004	0.67	0.521
	Error	24	0.133	0.006		
	Total	32	2.697			
	Corrected Total	31	0.528			
Non-germinated	Corrected Model	7	1.492[c]	0.213	17.599	<0.001
	Intercept	1	7.16	7.16	591.053	<0.001
	Variety	1	0.022	0.022	1.785	0.194
	Medium	1	0	0.00E+00	0.018	0.894
	Variety.Lot	2	1.462	0.731	60.364	<0.001
	Variety.Medium	1	0.004	0.004	0.304	0.586
	Variety.Lot.Medium	2	0.004	0.002	0.177	0.839
	Error	24	0.291	0.012		
	Total	32	8.943			
	Corrected Total	31	1.783			

[a] R Squared = .907 (Adjusted R Squared = .880); [b] R Squared = .749 (Adjusted R Squared = .676); [c] R Squared = .837 (Adjusted R Squared = .789).

Appendix 3.4. SPSS ANOVA output for data shown in Figure 3.7. Transformed percentage of germinated seedlings, abnormal seedlings or non-germinated seedlings (arcsin(√x)) was analysed using the model Conditions*Variety/Lot.

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Normal Germinated	Corrected Model	7	.923a	0.132	17.988	<0.001
	Intercept	1	42.81	42.81	5838.003	<0.001
	Condition	1	0.133	0.133	18.077	0.001
	Variety	1	0.018	0.018	2.437	0.138
	Condition.Variety	1	0.02	0.02	2.773	0.115
	Variety.Lot	2	0.609	0.305	41.541	<0.001
	Condition.Variety.Lot	2	0.143	0.072	9.772	0.002
	Error	16	0.117	0.007		
	Total	24	43.851			
	Corrected Total	23	1.041			
Abnormal	Corrected Model	7	.129a	0.018	7.089	0.001
	Intercept	1	0.052	0.052	20.132	<0.001
	Condition	1	0.029	0.029	11.236	0.004
	Variety	1	0.029	0.029	11.236	0.004
	Condition.Variety	1	0.013	0.013	4.915	0.041
	Variety.Lot	2	0.042	0.021	8.075	0.004
	Condition.Variety.Lot	2	0.016	0.008	3.043	0.076
	Error	16	0.042	0.003		
	Total	24	0.223			
	Corrected Total	23	0.171			
Non-germinated	Corrected Model	7	.819a	0.117	16.72	<0.001
	Intercept	1	1.184	1.184	169.319	<0.001
	Condition	1	0.097	0.097	13.866	0.002
	Variety	1	0.006	0.006	0.915	0.353
	Condition.Variety	1	0.034	0.034	4.885	0.042
	Variety.Lot	2	0.55	0.275	39.309	<0.001
	Condition.Variety.Lot	2	0.131	0.066	9.376	0.002
	Error	16	0.112	0.007		
	Total	24	2.115			
	Corrected Total	23	0.931			

Appendix 3.5. SPSS ANOVA output for experiment highlighted in Figure 3.12. Seed of *LeNCED1* overexpressing tomato seed sp12 were treated with 1 mM D4, 15 mM D4, 0.5 mg l^{-1} norflurazon or respective controls (solvent only, control for D4 treatments and 0.0025% ethanol, control for norflurazon treatment). Water, acetone and dichloromethane were used as solvents for treatments. Seed were imbibed on filter paper at 25°C in the dark and germination scored daily. ANOVA model used terms solvent*treatment/compound.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Corrected Model	14	4.820[a]	0.344	5.675	<0.001
Intercept	1	1653.269	1653.269	27252.92	<0.001
Solvent	2	0.105	0.052	0.863	0.432
Treatment	3	3.328	1.109	18.287	<0.001
Solvent.Treatment	6	0.383	0.064	1.051	0.413
Treatment.Concentration	1	0.664	0.664	10.943	0.002
Solvent.Treatment.Concentration	2	0.278	0.139	2.293	0.118
Error	30	1.82	0.061		
Total	45	1691.069			
Corrected Total	44	6.64			
Corrected Model	14	4.820a	0.344	5.675	<0.001
Intercept	1	1653.269	1653.269	27252.92	<0.001

[a] R Squared = .726 (Adjusted R Squared = .598)

Appendix 3.6. SPSS ANOVA output for experiment highlighted in Figure 3.13 Lot 4A or Lot 4B seed were pierced, pierced and treated with 15 µl acetone, or pierced and treated with 15 µl 10 mM (±)-abscisic acid in acetone and imbibed in vermiculite wet to field capacity at 15°C or 25°C. Radicle emergence was scored daily. Final percentage germination was transformed (arcsin(√x)), MGT calculated and effects of treatments analysed by ANOVA model seed lot*treatment*temperature.

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
MGT	Corrected Model	11	24597.743[a]	2236.158	65.78	<.001
	Intercept	1	290796.746	290796.7	8554.2	<.001
	Temperature	1	11044.765	11044.77	324.89	<.001
	Lot	1	98.711	98.711	2.904	0.101
	Treatment	2	13065.152	6532.576	192.16	<.001
	Temperature.Lot	1	0.001	0.001	0	0.996
	Temperature.Treatment	2	193.112	96.556	2.84	0.078
	Lot.Treatment	2	173.231	86.616	2.548	0.099
	Temperature.Lot.Treatment	2	22.772	11.386	0.335	0.719
	Error	24	815.866	33.994		
	Total	36	316210.355			
	Corrected Total	35	25413.609			
Final percent germination	Corrected Model	11	.334[b]	0.03	3.454	0.005
	Intercept	1	77.581	77.581	8834.2	<.001
	Temperature	1	0.012	0.012	1.398	0.249
	Lot	1	0.019	0.019	2.126	0.158
	Treatment	2	0.211	0.105	12.002	<.001
	Temperature.Lot	1	0.01	0.01	1.168	0.291
	Temperature.Treatment	2	0.003	0.002	0.199	0.821
	Lot.Treatment	2	0.01	0.005	0.543	0.588
	Temperature.Lot.Treatment	2	0.069	0.034	3.905	0.034
	Error	24	0.211	0.009		
	Total	36	78.126			
	Corrected Total	35	0.544			

[a] R Squared = .968 (Adjusted R Squared = .953); [b] R Squared = .613 (Adjusted R Squared = .0435)

Appendix 3.7. Genstat ANOVA output for experiment highlighted in Figure 3.14(A). Seed of 12CN011 (Variety 4, lot B) were soaked with acetone solutions containing 1 mM D2 or 1 mM D4, or acetone alone, or without pre-treatment (control). Seed were imbibed in vermiculite wet to field capacity at 5°C for 7 days then transferred to 25°C (Cold test) or imbibed directly at 25°C (Direct). Germination was scored after three days at 25°C. Two-way ANOVA (regime*compound) was performed, with replicates as the block structure, on transformed data (arcsin(√x)).

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Normal	Rep stratum	2	0.00632	0.00316	0.42	
	Regime	1	3.07733	3.07733	405.83	<.001
	Compound	3	0.04358	0.01453	1.92	0.173
	Regime.Compound	3	0.04259	0.0142	1.87	0.181
	Residual	14	0.10616	0.00758		
	Total	23	3.27599			
Abnormal	Rep stratum	2	0.00063	0.00032	1.43	
	Regime	1	0.00427	0.00427	19.26	<.001
	Compound	3	0.00087	0.00029	1.3	0.312
	Regime.Compound	3	0.00067	0.00022	1	0.420
	Residual	14	0.0031	0.00022		
	Total	23	0.00954			
Non-germinated	Rep stratum	2	0.01144	0.00572	2.28	
	Regime	1	0.73148	0.73148	291.53	<.001
	Compound	3	0.00857	0.00286	1.14	0.367
	Regime.Compound	3	0.00291	0.00097	0.39	0.765
	Residual	14	0.03513	0.00251		
	Total	23	0.78952			

Appendix 3.8. Genstat ANOVA output for experiment highlighted in Figure 3.14 (B and C). Seed of 12CN011 (Variety 4, lot B) were soaked with acetone solutions containing 1 mM D2 or 1 mM D4, or acetone alone, or without pre-treatment (control). Seed were imbibed in vermiculite wet to field capacity at 5°C for 7 days then transferred to 25°C (Cold test) or imbibed directly at 25°C (Direct). Root and shoots were measured after 4 days of growth subsequent to transfer. One-way ANOVA (compound) was performed, with replicates as the block structure, on transformed data (arcsin(√x)) for control and cold test seedlings (n=3 per treatment).

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Cold test root mass	Rep stratum	2	137.06	68.53	0.93	
	Treatment	3	586.64	195.55	2.65	0.143
	Residual	6	442.88	73.81		
	Total	11	1166.58			
Cold test shoot mass	Rep stratum	2	1052.2	526.1	2.29	
	Treatment	3	3123.4	1041.1	4.52	0.055
	Residual	6	1381.1	230.2		
	Total	11	5556.7			
Cold test Root : Shoot	Rep stratum	2	0.03293	0.01646	1.16	
	Treatment	3	0.09045	0.03015	2.12	0.200
	Residual	6	0.0855	0.01425		
	Total	11	0.20888			
Control test root mass	Rep stratum	2	79.71	39.85	0.54	
	Treatment	3	654.93	218.31	2.93	0.122
	Residual	6	446.78	74.46		
	Total	11	1181.43			
Control test shoot mass	Rep stratum	2	418.9	209.5	1.63	
	Treatment	3	520.6	173.5	1.35	0.343
	Residual	6	769.5	128.3		
	Total	11	1709.1			
Control Root : Shoot	Rep stratum	2	0.17902	0.08951	1.89	
	Treatment	3	0.03883	0.01294	0.27	0.842
	Residual	6	0.28343	0.04724		
	Total	11	0.50128			

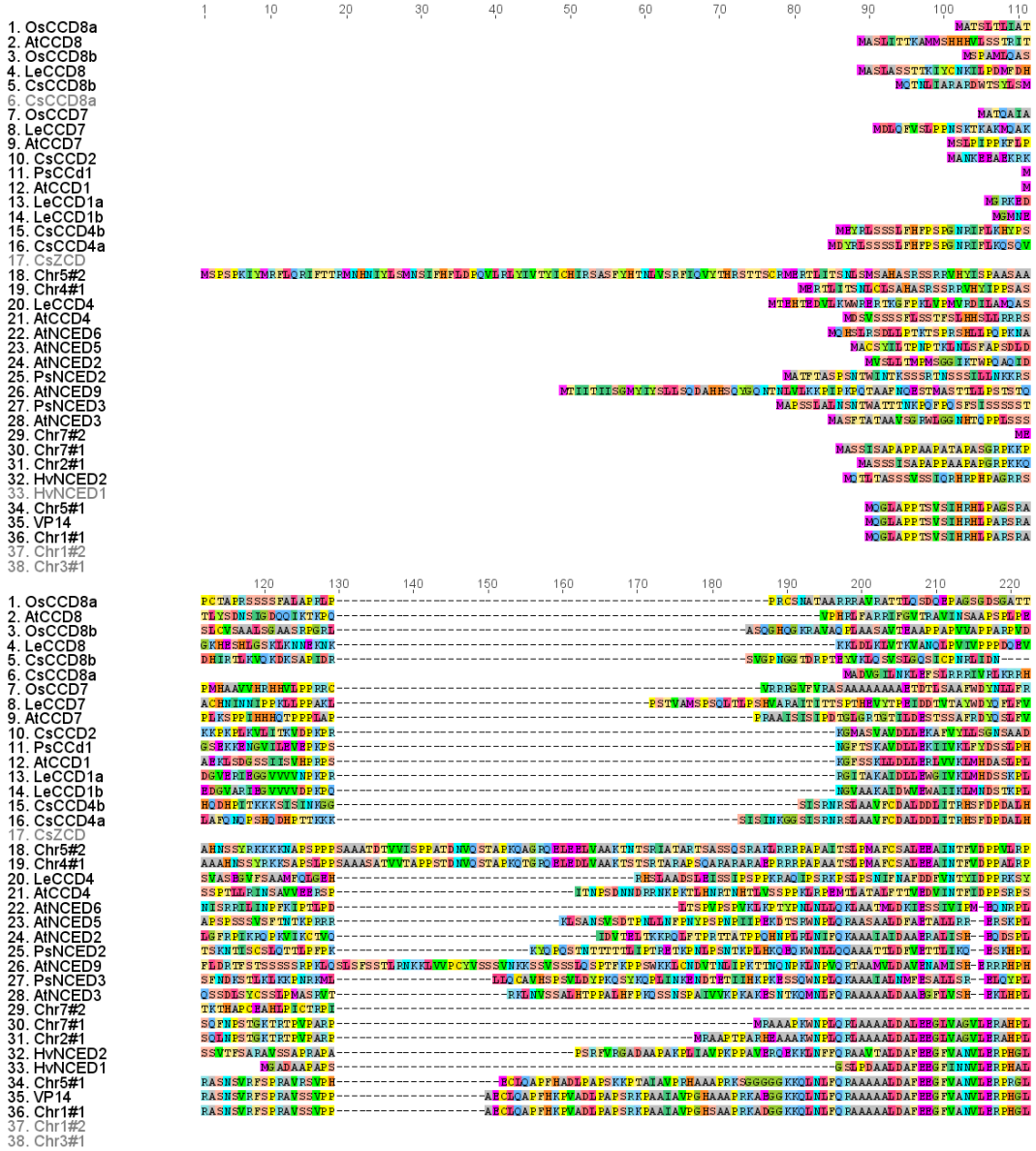
Appendix 3.9. ANOVA output for experiment highlighted in Figure 3.15. Seed of 12CN011 (Variety 4, lot B) were imbibed in 1 mM D2 or 1 mM D4 or Control (0.1% DMSO) solutions for 7 days at 5°C before being transferred to 25°C. Germination was scored and root and shoot mass measured after 3 days at 25°C. Germination data was transformed data (arcsin(√x)). Hydroxamic acid treatments D2 and D4 were pooled for some analyses.

D2 and D4 data separate

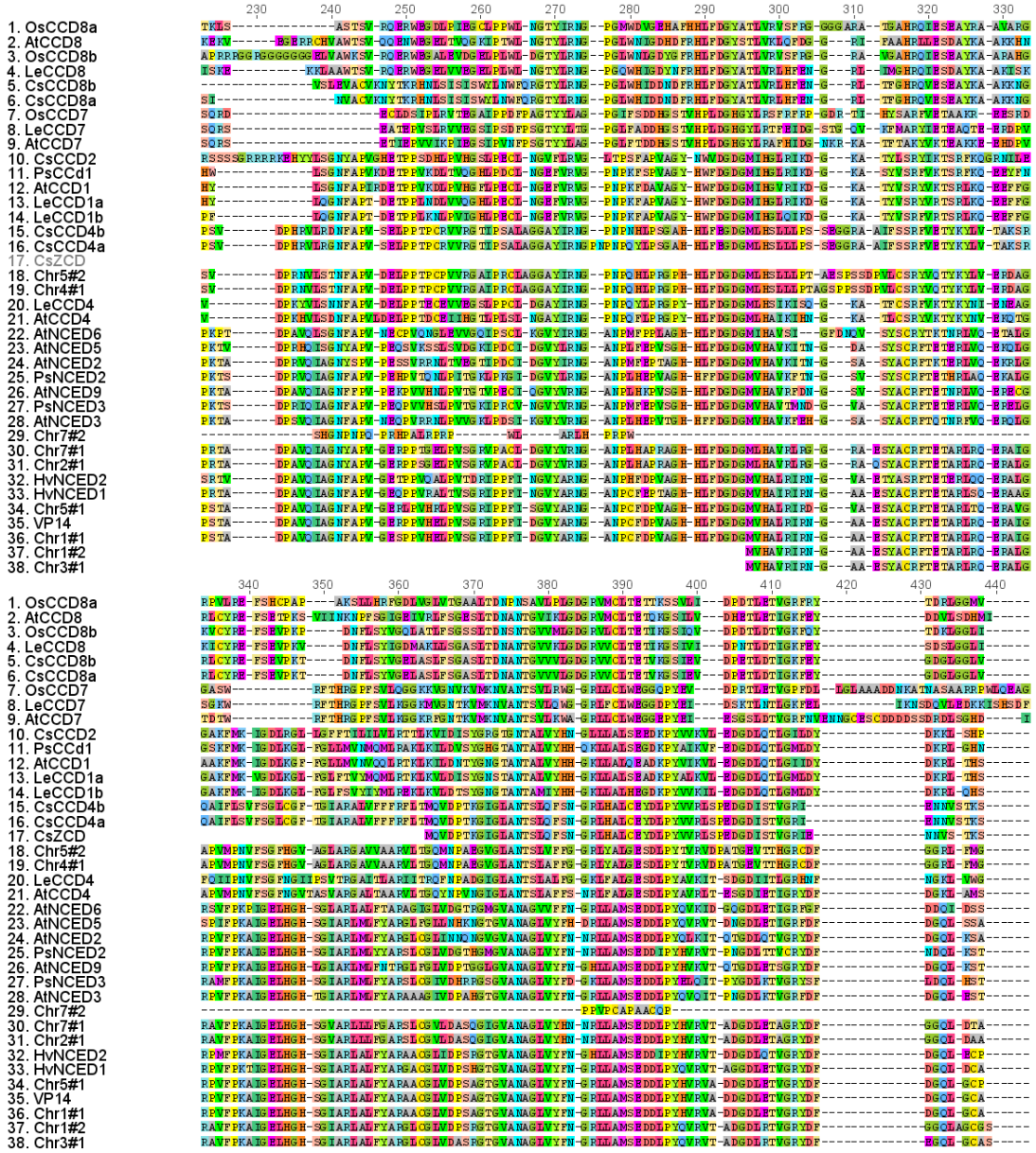
Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Normal	Between Groups	2	0.136	0.068	3.379	0.061
	Within Groups	15	0.302	0.02		
	Total	17	0.439			
Abnormal	Between Groups	2	0.047	0.024	2.002	0.17
	Within Groups	15	0.177	0.012		
	Total	17	0.224			
Non-germinated	Between Groups	2	0.088	0.044	2.397	0.125
	Within Groups	15	0.276	0.018		
	Total	17	0.364			
Germinated	Between Groups	2	0.088	0.044	2.397	0.125
	Within Groups	15	0.276	0.018		
	Total	17	0.364			
Root	Between Groups	2	73.028	36.514	0.72	0.503
	Within Groups	15	760.468	50.698		
	Total	17	833.496			
Shoot	Between Groups	2	93.137	46.569	0.442	0.651
	Within Groups	15	1578.877	105.258		
	Total	17	1672.015			
Root : shoot	Between Groups	2	0.086	0.043	1.024	0.383
	Within Groups	15	0.631	0.042		
	Total	17	0.717			

Pooled hydroxamic acids (D2 + D4 data)

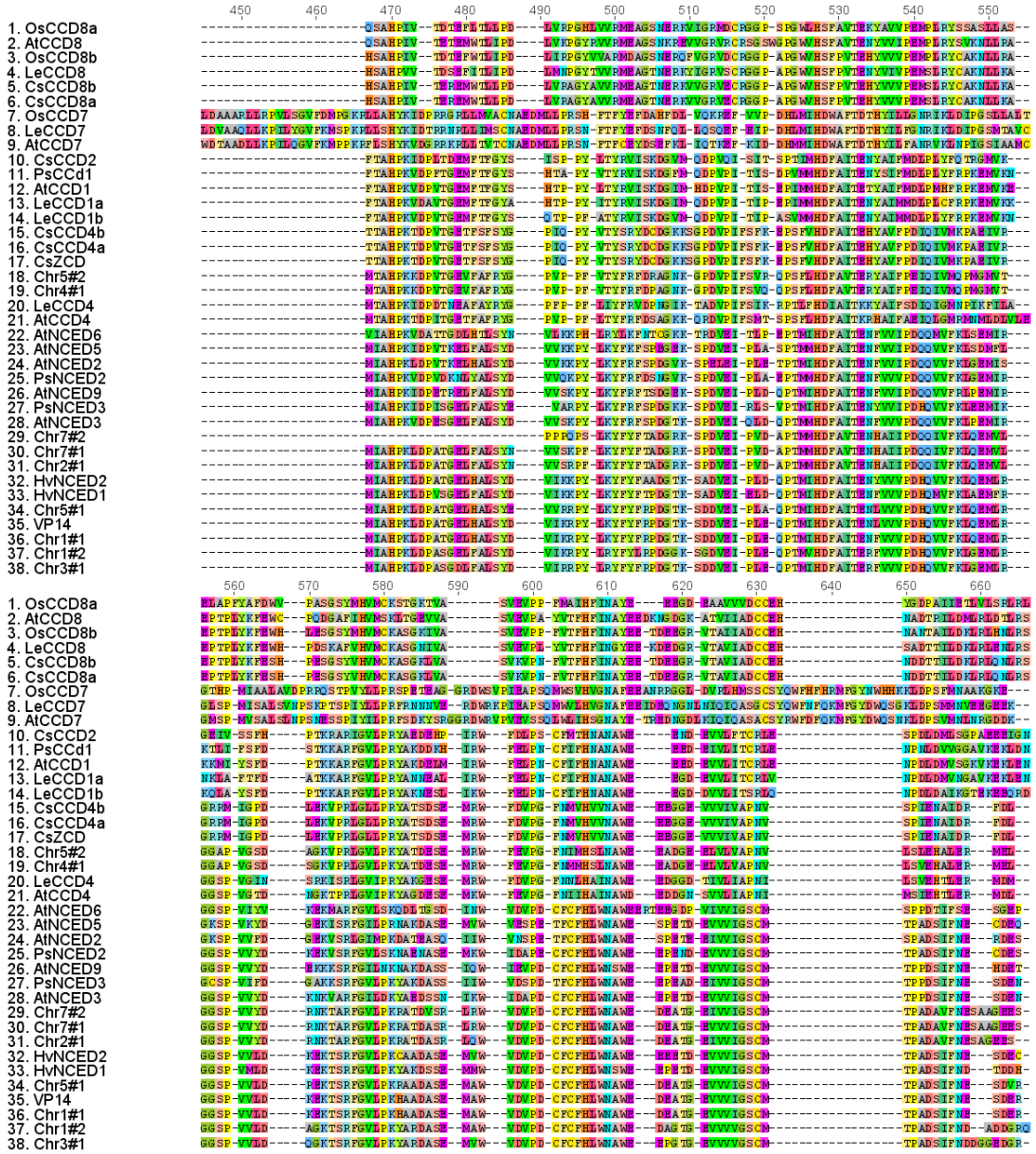
Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Normal	Between Groups	1	0.112	0.112	5.51	0.032
	Within Groups	16	0.326	0.02		
	Total	17	0.439			
Abnormal	Between Groups	1	0.033	0.033	2.765	0.116
	Within Groups	16	0.191	0.012		
	Total	17	0.224			
Non-germinated	Between Groups	1	0.072	0.072	3.976	0.063
	Within Groups	16	0.292	0.018		
	Total	17	0.364			
Germinated	Between Groups	1	0.072	0.072	3.976	0.063
	Within Groups	16	0.292	0.018		
	Total	17	0.364			
Root	Between Groups	1	8.933	8.933	0.173	0.683
	Within Groups	16	824.562	51.535		
	Total	17	833.496			
Shoot	Between Groups	1	89.093	89.093	0.901	0.357
	Within Groups	16	1582.922	98.933		
	Total	17	1672.015			
Root : shoot	Between Groups	1	0.078	0.078	1.955	0.181
	Within Groups	16	0.639	0.04		
	Total	17	0.717			



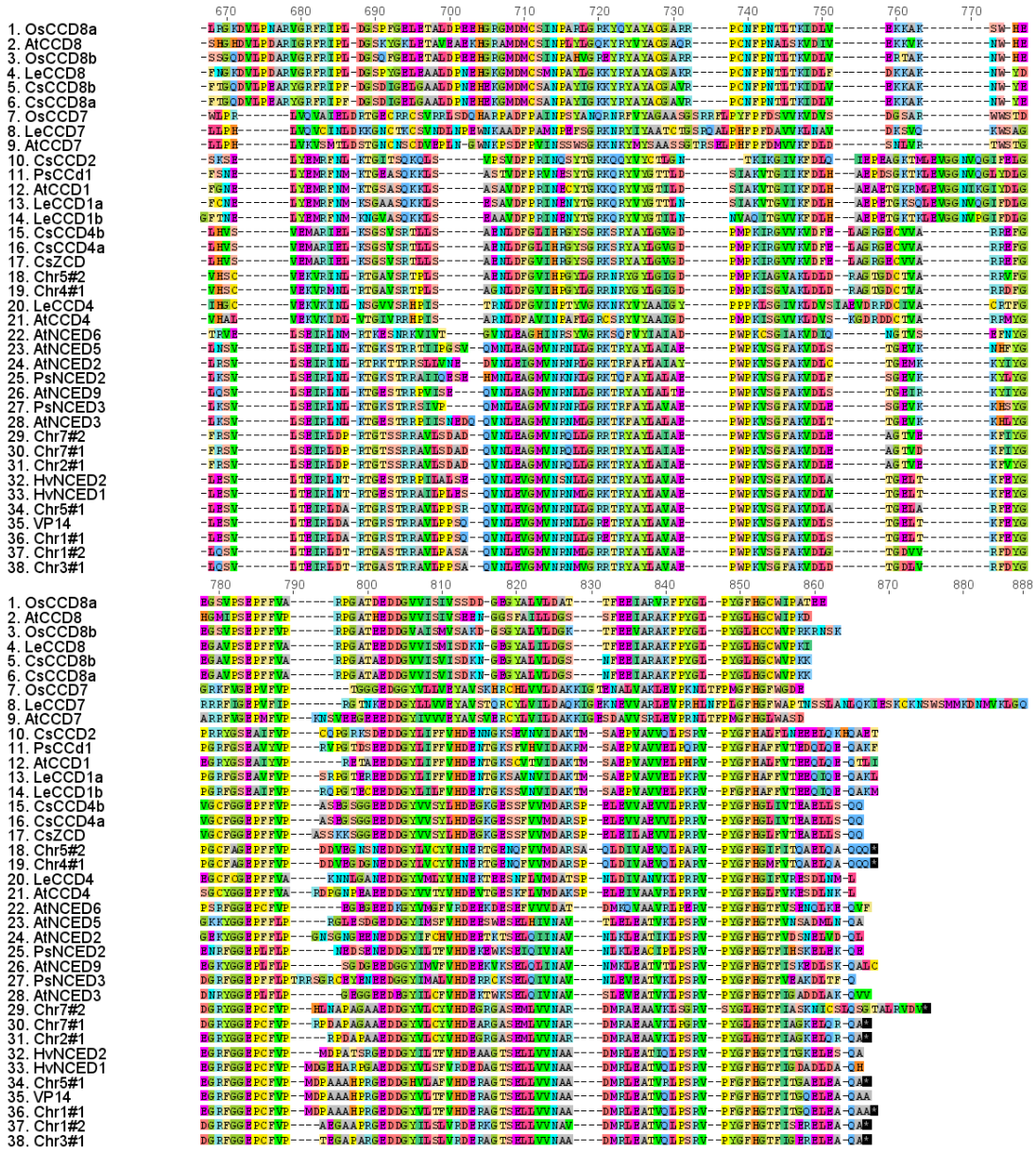
Appendix 4.1. Geneious MUSCLE alignment of nine identified ORFs and CCDs. (Page 1 of 4)



Appendix 4.1. Geneious MUSCLE alignment of nine identified ORFs and CCDs. (Page 2 of 4)



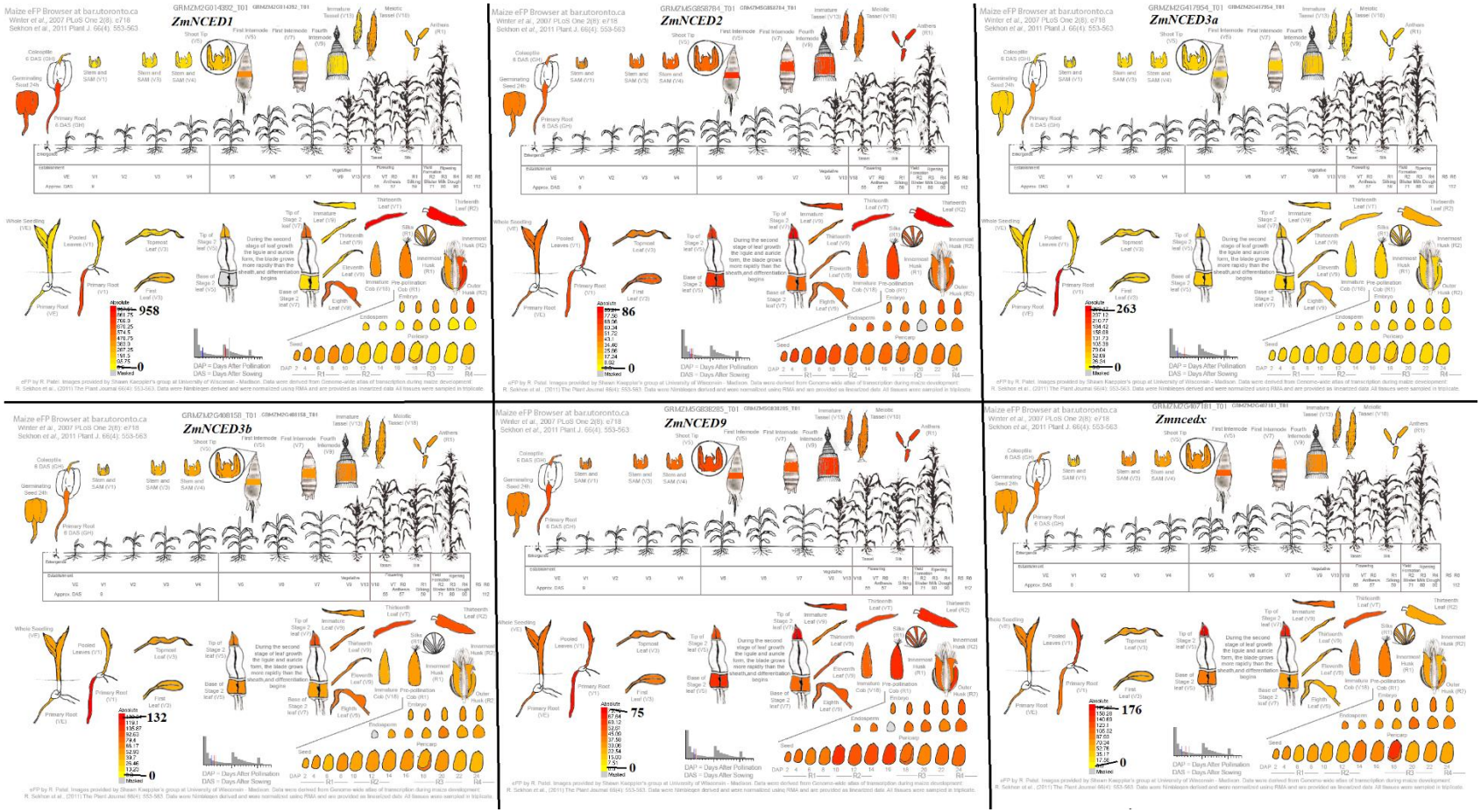
Appendix 4.1. Geneious MUSCLE alignment of nine identified ORFs and CCDs. (Page 3 of 4)



Appendix 4.1. Geneious MUSCLE alignment of nine identified ORFs and CCDs. (Page 4 of 4)

Appendix 4.2. Table of homologous full length cDNAs and ESTs for putative *NCEDs*. Sequences of interest were identified by using genomic DNA sequences from the B73 reference as a query at NCBI BLAST.

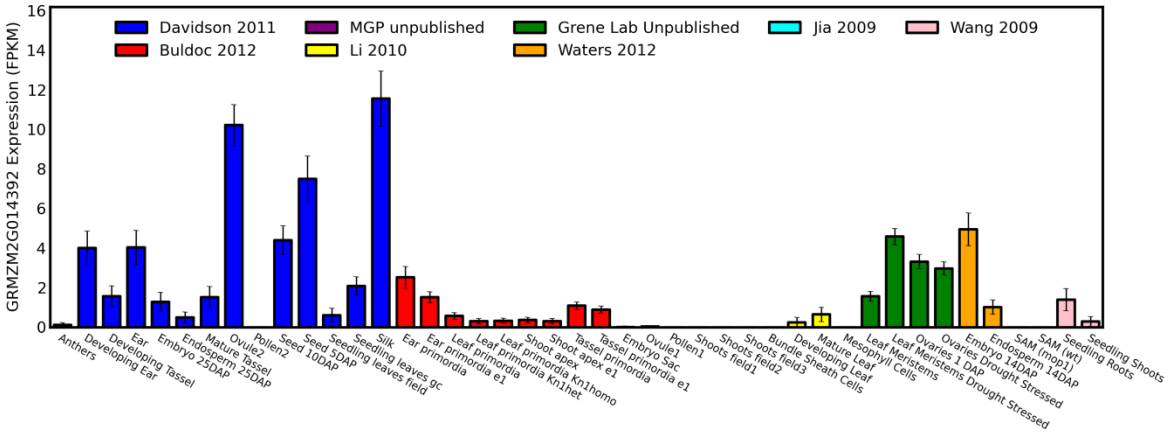
ZmNCED	**Full length cDNA accessions**	**EST accessions**
ZmNCED1	NM_001112432.2	FL009215.1, EE036360.2 EE036359.2, (7 more with >98% identity).
ZmNCED2	NM_001154055.1	DV032362.1, DV520145.1, EC895196.1, DY618542.1 (27 more with >98% identity)
ZmNCED3a	BT062179.1	DY541318.1, DR798039.1, DV533388.1, DR966527.1 (14 more with >98% identity)
ZmNCED3b	Not available	DV533388.1 (only 97%)
ZmNCED9	Not available	EE036359.2 (only 95%)
Zmncedx	NM_001196156.1	EB816223.1, EB816224.1, EB816223.1, EE285552.2, DV514610.1
Zmncedy	Not available	EE044100.2, CF634282.1, DR960651.1 (21 more with >98%)



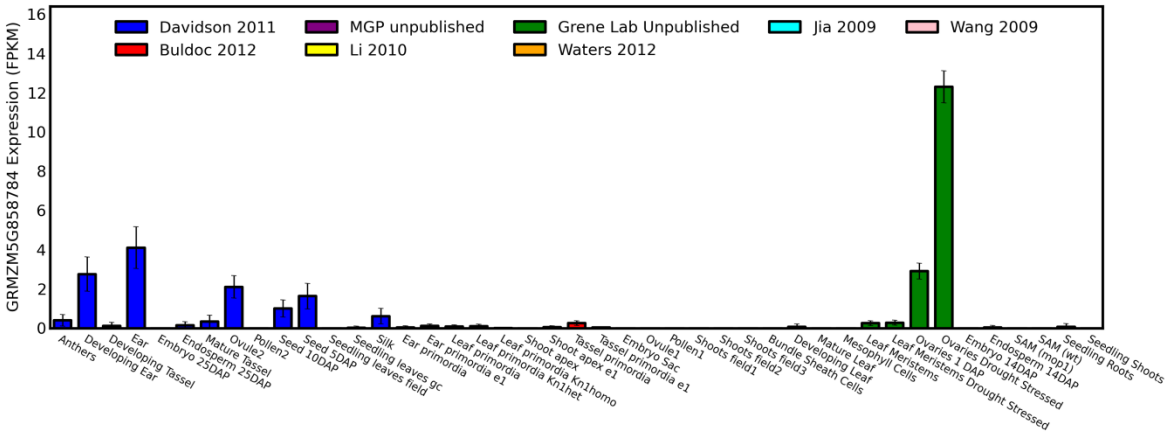
Appendix 4.3. eFP browser results (image output) – expression of *NCED* genes in different maize tissues based on microarray data. The gene models associated with the identified *NCED* loci (Table 4.2) were used to query maize eFP browser (bar.utoronto.ca/efp_maize/cgi-bin/efpWeb.cgi). The output images are shown, with the scale bar annotated with maximum and minimum average values**.**

Appendix 4.4. qTeller results (image output) – expression of *NCED* genes in different maize tissues based on RNA-seq data. The gene models associated with the identified *NCED* loci (Table 4.2) were used to query qTeller (http://qteller.com/qteller3/generate_figures.php).

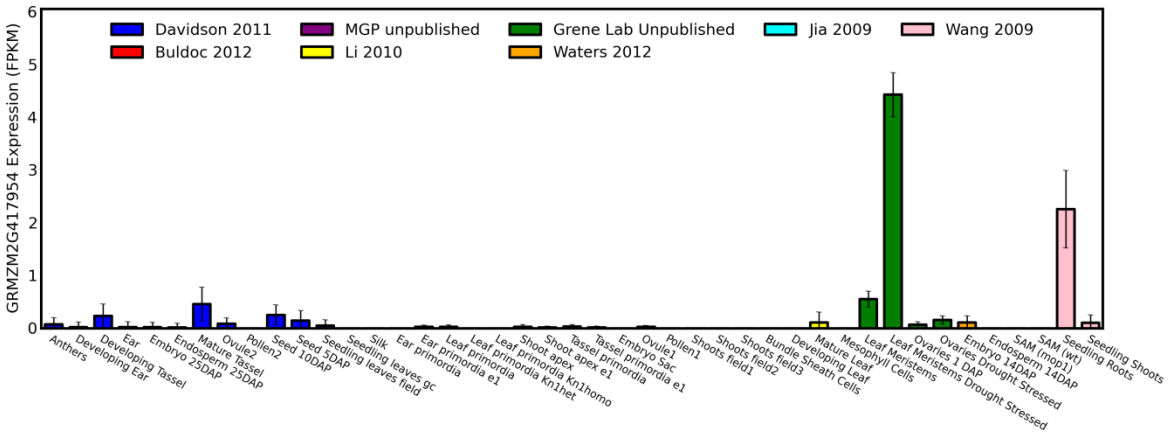
ZmNCED1



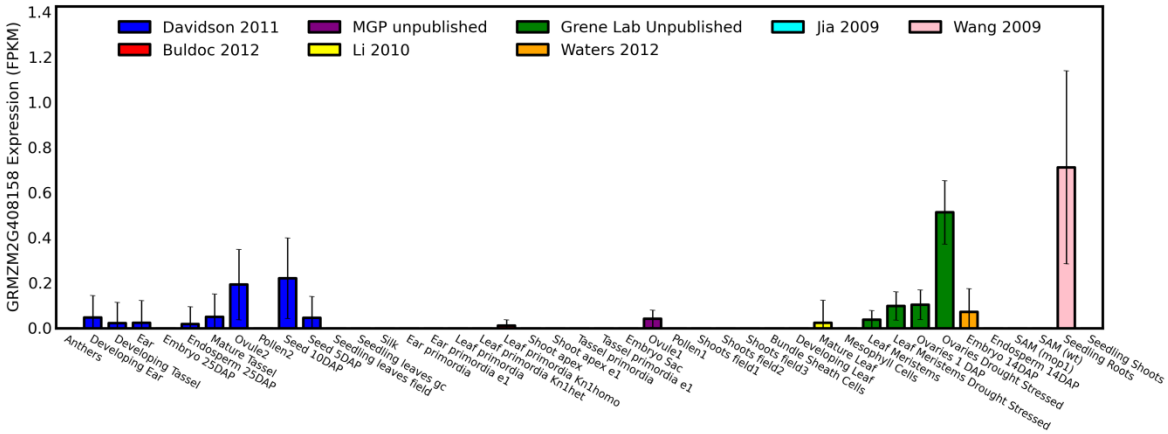
ZmNCED2



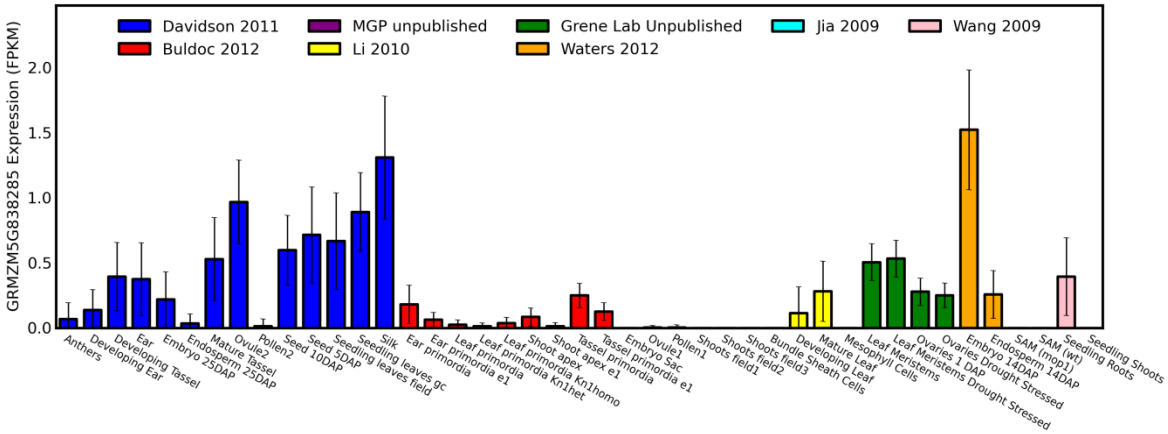
ZmNCED3a



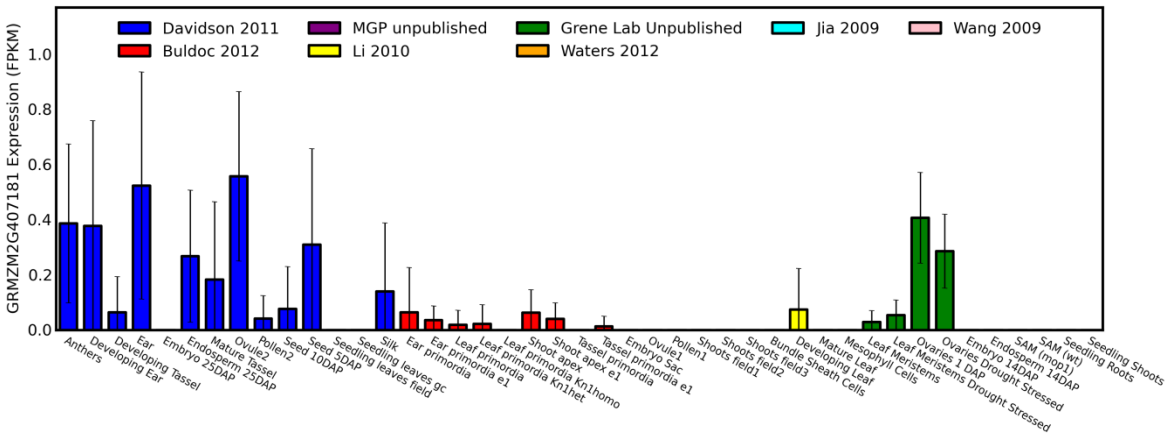
ZmNCED3b



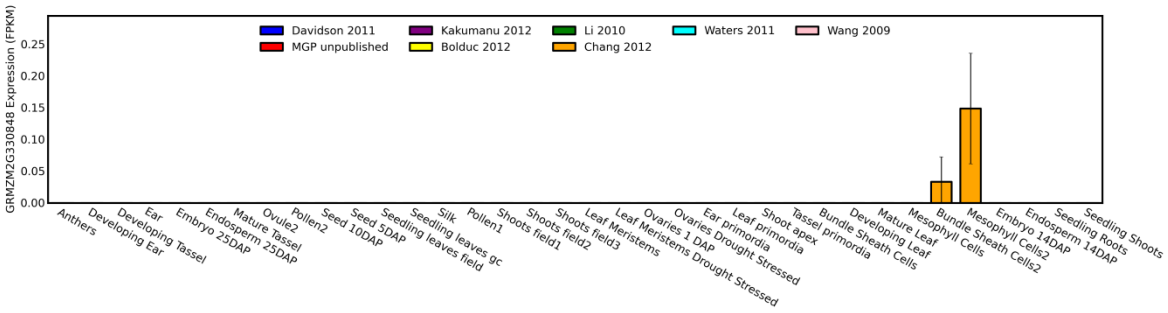
ZmNCED9



Zmncedx



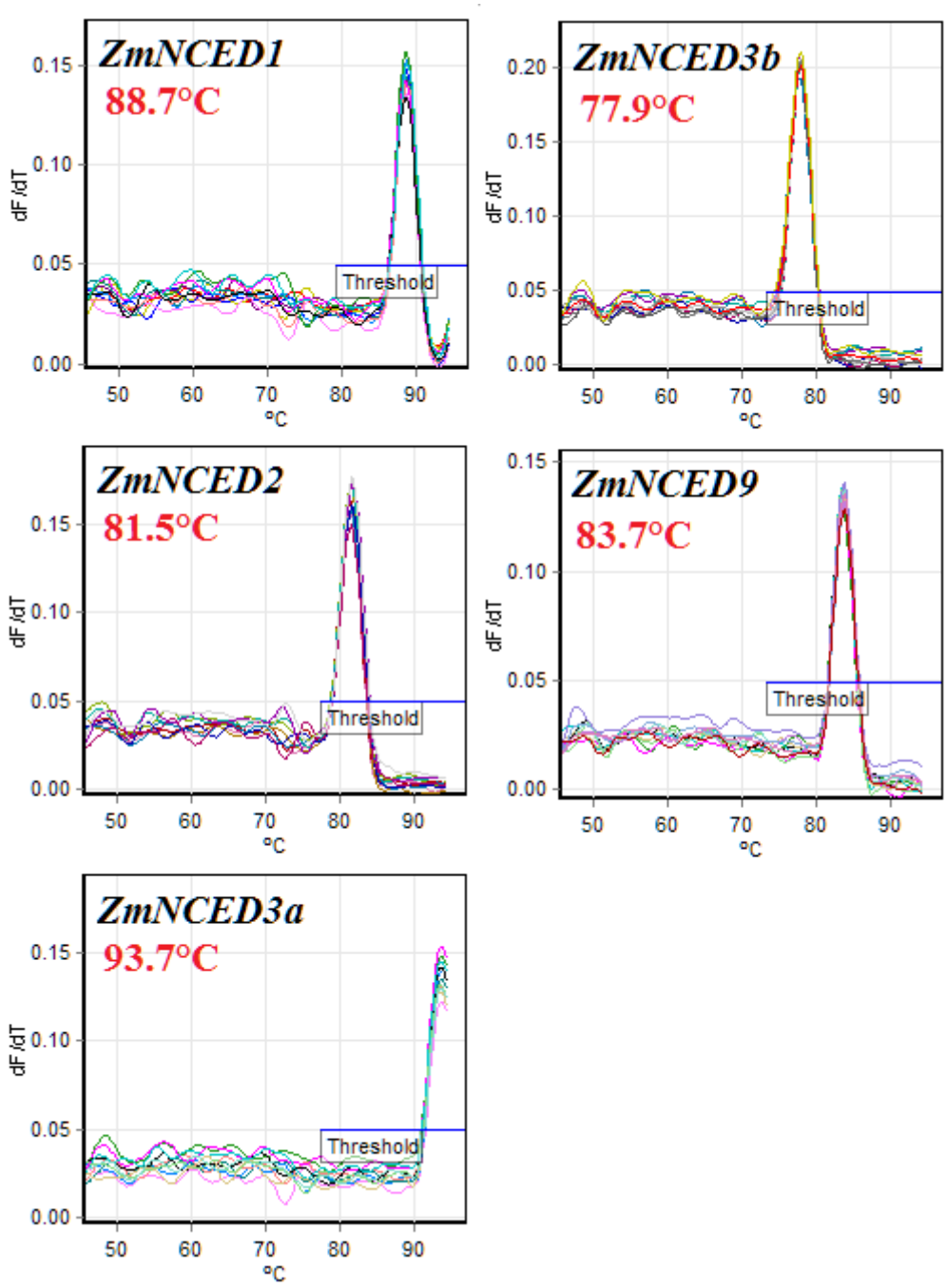
Zncedy



Appendix 4.5. SPSS ANOVA for experiment outlined in Figure 4.10. Percentage inhibition of activity (compared to control without inhibitor present) of *Zm*NCEDs *in vitro* by hydroxamic acid CCD inhibitors D2 and D4 was measured.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Corrected Model	7	5290.144a	755.735	1.533	0.216
Intercept	1	31981.42	31981.42	64.861	<0.001
NCED	3	3015.865	1005.288	2.039	0.143
Inhibitor	1	4.083	4.083	0.008	0.928
NCED * Inhibitor	3	2274.741	758.247	1.538	0.237
Error	19	9368.471	493.077		
Total	27	47604.18			
Corrected Total	26	14658.62			

[a] R Squared = .361 (Adjusted R Squared = .125)



Appendix 5.1. Melt curves for chosen *ZmNCED* primer pairs. Curve plot and attributes calculated in Rotor-Gene Q series software. Peak temperatures are indicated in red.

Appendix 6.1. *ZmNCED* expression and ABA accumulation in Variety 4 lot B under vermiculite cold test conditions ANOVA. Samples were collected as outlined in Figure 6.1. RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5. Seeds were subject to cold test conditions or control conditions. One-way ANOVA was calculated on log-transformed data for each treatment time point in Genstat (n = 3). Means represented are in Figure 6.2 and Figure 6.3.

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ZmNCED1	Treatment	11	18.27944	1.661767	179.36	<0.001
	Residual	24	0.222365	0.009265		
	Total	35	18.5018			
ZmNCED2	Treatment	11	2.51813	0.22892	5.28	<0.001
	Residual	24	1.04048	0.04335		
	Total	35	3.55861			
ZmNCED3a	Treatment	11	7.11801	0.64709	7.69	<0.001
	Residual	24	2.01867	0.08411		
	Total	35	9.13668			
ZmNCED3b	Treatment	11	11.91678	1.083344	176.54	<0.001
	Residual	24	0.147275	0.006136		
	Total	35	12.06406			
ZmNCED9	Treatment	11	18.86793	1.71527	152.38	<0.001
	Residual	24	0.27015	0.01126		
	Total	35	19.13808			
Total *NCED*	Treatment	11	4.55131	0.413755	123.98	<0.001
	Residual	24	0.080093	0.003337		
	Total	35	4.631403			
ABA	Treatment	11	1222.6	111.15	6.64	<0.001
	Residual	24	401.44	16.73		
	Total	35	1624.03			

Appendix 6.2. Expression of *ZmNCED*s in different seed lots under vermiculite cold test conditions ANOVA. Maize seeds from different seed lots (from Variety 1 lot A to Variety 5 lot B, left to right) were imbibed at 5°C for seven days in vermiculite (168 hour time point indicated in Figure 6.1). Embryos were isolated, RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5. A one-way ANOVA was performed on log-transformed data, blocked by replicate in Genstat (n = 3). Means indicated in Figure 6.4.

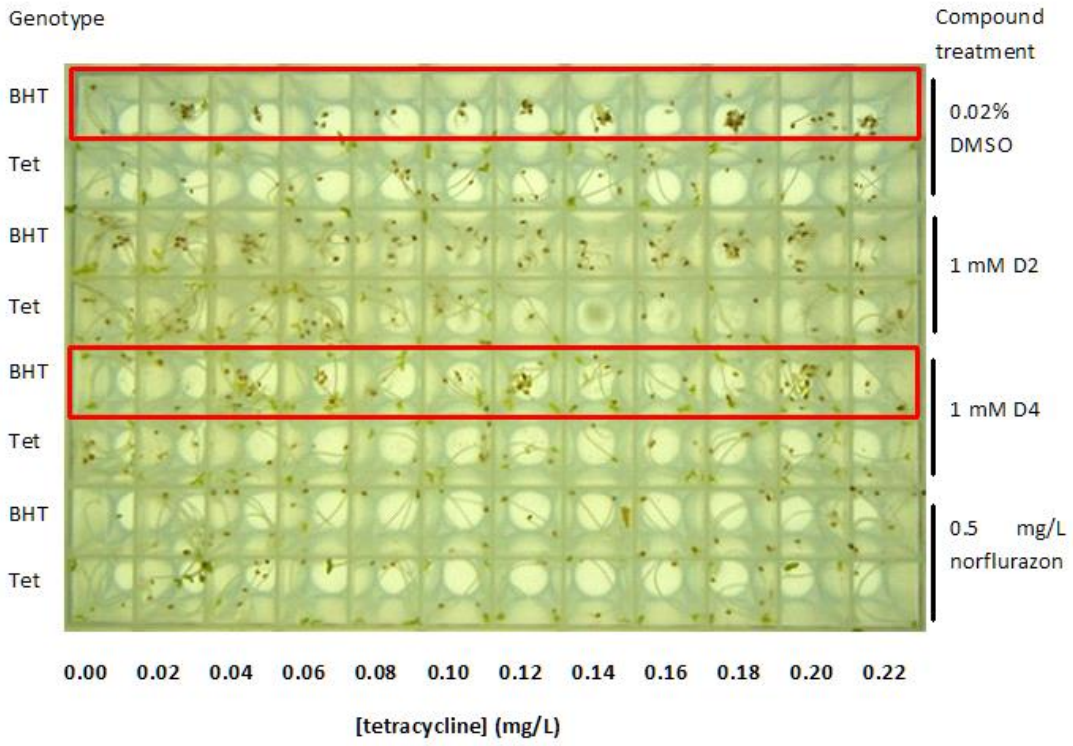
Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ZmNCED1	Replicate stratum	2	0.001397	0.000699	0.11	
	Sample	9	0.330816	0.036757	5.62	<0.001
	Residual	18	0.117677	0.006538		
	Total	29	0.449891			
ZmNCED2	Replicate stratum	2	0.03838	0.01919	1.69	
	Sample	9	0.18441	0.02049	1.81	0.136
	Residual	18	0.20395	0.01133		
	Total	29	0.42675			
ZmNCED3a	Replicate stratum	2	0.01013	0.00506	0.08	
	Sample	9	2.24533	0.24948	3.82	0.007
	Residual	18	1.17595	0.06533		
	Total	29	3.4314			
ZmNCED3b	Replicate stratum	2	0.0182	0.0091	0.43	
	Sample	9	2.63145	0.29238	13.85	<0.001
	Residual	18	0.38009	0.02112		
	Total	29	3.02974			
ZmNCED9	Replicate stratum	2	0.03188	0.01594	0.87	
	Sample	9	27.13164	3.01463	163.65	<0.001
	Residual	18	0.33158	0.01842		
	Total	29	27.4951			
Total *NCED*	Replicate stratum	2	0.00409	0.002045	0.3	
	Sample	9	0.59893	0.066548	9.88	<0.001
	Residual	18	0.121207	0.006734		
	Total	29	0.724226			

Appendix 6.3 *ZmNCED* expression and ABA accumulation during maize imbibition at different temperatures ANOVA. Seed of NK Falkone were imbibed at 10°C, 15°C or 20°C. Samples (3 replicates of 20 embryos) were taken at 6 hours, 24 hours, 48 hours and 72 hours after sowing. Dry seed were also sampled. RNA was purified from samples and *ZmNCED* expression quantified using the RT-qPCR assay and RNA standards developed in Chapter 5, and pulverised sample was sent for ABA quantification. Two way ANOVA (temperature*time, blocked by replicate) on on log-transformed data for *ZmNCED* and untransformed data for ABA was performed in in Genstat (n=3). Means indicated in Figure 6.6and Figure 6.7.

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ZmNCED1	Replicate stratum	2	0.09441	0.04721	1.59	
	Time	4	5.38217	1.34554	45.32	<0.001
	Temperature	2	5.36036	2.68018	90.26	<0.001
	Time.Temperature	8	4.15215	0.51902	17.48	<0.001
	Residual	28	0.8314	0.02969		
	Total	44	15.82049			
ZmNCED2	Replicate stratum	2	0.02175	0.01087	0.62	
	Time	4	0.42814	0.10703	6.15	0.001
	Temperature	2	0.21213	0.10607	6.1	0.006
	Time.Temperature	8	1.33965	0.16746	9.62	<0.001
	Residual	28	0.48725	0.0174		
	Total	44	2.48892			
ZmNCED3a	Replicate stratum	2	0.00773	0.00387	0.05	
	Time	4	5.35315	1.33829	16.06	<0.001
	Temperature	2	0.3655	0.18275	2.19	0.13
	Time.Temperature	8	2.03094	0.25387	3.05	0.014
	Residual	28	2.33384	0.08335		
	Total	44	10.09117			
ZmNCED3b	Replicate stratum	2	0.00189	0.00095	0.06	
	Time	4	18.03365	4.50841	294.03	<0.001
	Temperature	2	4.11974	2.05987	134.34	<0.001
	Time.Temperature	8	3.19174	0.39897	26.02	<0.001
	Residual	28	0.42934	0.01533		
	Total	44	25.77636			
ZmNCED9	Replicate stratum	2	1.0298	0.5149	1.41	
	Time	4	3.0117	0.7529	2.06	0.112
	Temperature	2	9.0248	4.5124	12.38	<0.001
	Time.Temperature	8	6.1999	0.775	2.13	0.067
	Residual	28	10.2097	0.3646		
	Total	44	29.4759			
Total *NCED*	Replicate stratum	2	0.00121	0.0006	0.04	
	Time	4	6.29112	1.57278	92.67	<0.001
	Temperature	2	2.87953	1.43977	84.83	<0.001
	Time.Temperature	8	2.76438	0.34555	20.36	<0.001
	Residual	28	0.47521	0.01697		
	Total	44	12.41145			
ABA	Replicate stratum	2	61.5	30.75	2.23	
	Time	4	3679.79	919.95	66.68	<0.001
	Temperature	2	195.88	97.94	7.1	0.003
	Time.Temperature	8	777.26	97.16	7.04	<0.001
	Residual	28	386.32	13.8		
	Total	44	5100.76			

Appendix 6.4. The effect of imbibition on NCED inhibitor solutions on ABA accumulation ANOVA. Seed of NK Falkone were imbibed on vermiculite containing aqueous solutions of 40 µM or 150 µM D2; 40 µM or 150 µM C426, or without inhibitor (control). Seed were imbibed at 10°C or 15°C. Samples were taken at 24 hours and 72 hours after sowing and ABA was quantified. Genstat two-way ANOVA (treatment*temperature, blocked by replicate) was calculated separately for 24 hours and 72 hours (n = 5 per treatment). Means indicated in Figure 6.8.

Variate	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ABA 24 h	Replicate stratum	4	1014.36	253.59	5.72	
	Treatment	4	22.93	5.73	0.13	0.971
	Temp	1	604.17	604.17	13.62	<.001
	Treatment.Temp	4	52.68	13.17	0.3	0.878
	Residual	36	1596.74	44.35		
	Total	49	3290.88			
ABA 72 h	Replicate stratum	4	7.873	1.968	0.26	
	Treatment	4	17.145	4.286	0.57	0.687
	Temp	1	269.227	269.227	35.69	<.001
	Treatment.Temp	4	30.104	7.526	1	0.421
	Residual	36	271.583	7.544		
	Total	49	595.932			



Appendix 7.1. Hydroxamic acids D2 and D4 restore germination in tobacco seed over-expressing *LeNCED1*. BHT and Tet seeds were sown onto 0.6% agar treated with a range of tetracycline concentrations and compound treatments (as indicated). Plates were placed in the dark at 25°C. Key points to see are the difference between row A and row E, highlighted with red rectangles.

Appendix 7.2. Batch and plate effectts on positive controls in the 5K screen (Day 13) ANOVA. Four batches of 16 plates were required for the 5K screen. Each plate contained 12 negative control wells, 4 positive control and 80 assay wells. Photographs of plates from the 5K screen were taken daily 9 days to 13 days after sowing. Data were square-root transformed prior to analysis. Slice = plate. ANOVA was performed in GenStat using the model Batch/Slice. Means shown in

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Batch	3	6403.27	2134.42	34.84	<0.001
Batch.Slice	60	6413.82	106.9	1.74	0.002
Residual	192	11762.69	61.26		
Total	255	24579.78			

Appendix 7.3. Control column position effect on positive controls in the 5K screen (Day 13) ANOVA. Four batches of 16 plates were required for the 5K screen. Each plate contained 12 negative control wells, 4 positive control and 80 assay wells. Photographs of plates from the 5K screen were taken daily 9 days to 13 days after sowing. Data were square-root transformed prior to analysis. Slice = plate. A. ANOVA of column effect. B. Unbalanced ANOVA of column effect with plate ('slice') included as a nuisance term.

	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
A: ANOVA	Column	11	1300.4	118.22	1.24	0.262
	Residual	244	23279.37	95.41		
	Total	255	24579.78			
	Change	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
B: Unbalanced ANOVA	+ Slice	63	12817.09	203.45	3.28	<0.001
	+ Column	11	542.2	49.29	0.8	0.645
	Residual	181	11220.49	61.99		
	Total	255	24579.78	96.39		

Appendix 7.4. ID of hit compounds from the 5K library. Compound identification (ID) based on library plate positions: Plate number (1-53); Row (A-H); Column (1-12). Ordered by assay √leaf area.

ID	√LA	ID	√LA	ID	√LA	ID	√LA	ID	√LA	ID	√LA
28H8	59	4A11	29	46E9	24	20D7	20	27F3	17	51C7	14
46A9	55	22C2	29	27F5	23	20F2	20	10E3	17	16C9	13
42A5	47	9A5	29	26C6	23	44B7	20	28A4	17	34C11	13
25H8	45	15F5	29	53H8	23	41G8	20	12F8	16	40C9	13
46H9	45	17B12	28	36D1	23	29D12	20	21D6	16	2A9	13
12A5	44	13F7	28	31F4	23	46C4	19	44F10	16	29B3	13
43A11	41	51B10	28	17E10	23	42B6	19	10C6	16	1D1	13
19A5	41	27A4	28	22E3	23	2H1	19	21F9	16	11E1	13
43G2	41	48H2	28	3A12	23	25F1	19	53D7	16	47F1	13
8H1	40	51H5	28	49C4	23	14E10	19	10D7	16	4A6	13
20E12	39	23E2	28	9F2	23	45H2	19	7D11	16	24B11	13
18G5	39	14B9	28	42F4	23	7D8	19	14A9	16	20A1	13
16A3	39	3D4	27	11H8	23	17E6	19	12A6	16	20B3	13
46E12	39	25F10	27	12G12	23	45F10	19	19A7	16	22G7	13
30H2	39	52G11	27	16A5	23	10A12	19	9A12	16	9B6	13
36G1	38	13G1	27	20A11	23	29B10	19	12E7	16	30G12	13
29D8	38	42C6	27	23B10	22	46B4	19	32D6	16	3A4	13
19D6	38	16B10	27	43B8	22	10G12	19	24E9	16	36A9	13
19G6	38	19G12	27	4B1	22	21G4	19	46C2	16	49D10	13
13E6	37	53E1	27	44F4	22	2B5	19	1H12	16	16D2	13
16E7	37	16B3	27	15A3	22	7E1	19	9G8	16	50B4	13
13H1	37	2F5	26	37H10	22	53H6	18	22A8	16	36H11	13
32C1	37	17B4	26	8D5	22	53E7	18	24B3	15	4D3	13
25A3	36	16D11	26	7B12	22	6C10	18	50C11	15	11D2	13
52B6	36	24G1	26	45D6	22	34B7	18	52C1	15	53E8	13
5B7	36	36A1	26	52B3	22	2D10	18	19C3	15	7G9	13
20B5	35	11F8	26	2B7	22	38C12	18	41D3	15	26A6	12
46B5	35	47E2	26	20C8	22	45G7	18	45F11	15	21E12	12
32G7	35	53G5	26	39G11	22	6A9	18	16E2	15	29C9	12
39B2	34	22H10	26	23H3	22	39H9	18	50E1	15	43D1	12
9E12	34	17D12	26	51E1	22	26E10	18	33B6	15	38A3	12
39B6	34	41G6	26	33B9	22	49E3	18	40E1	15	40D2	12
18G7	33	5B5	26	6F5	22	53C5	18	44E12	15	26G1	12
22D5	33	47F6	26	12E9	21	20G9	18	51E4	15	18D6	12
29G9	33	42B8	25	24B4	21	24H9	18	18B9	15	0A0	12
38H12	33	24C6	25	7H2	21	20E3	18	49B3	15	48E9	12
6F7	33	24G9	25	27B11	21	38D6	18	30H4	15	44H11	12
29F5	32	13H9	25	49B7	21	30B7	18	21H12	15	4C1	12
41A1	32	32F8	25	9C4	21	20G12	18	26B4	15	8A12	12
19B7	32	9F1	25	15G1	21	2B8	18	9C8	15	45A7	12
23F6	32	33D5	25	23C10	21	10A5	18	8G7	14	20E1	12
5H2	32	42G4	25	43C12	21	40F3	18	28A8	14	25B1	12
3H3	31	23C11	25	36G10	21	17G10	17	29C5	14	31A1	12
18A4	31	19C5	25	17B11	21	25A5	17	6H11	14	51B12	12
27A8	31	2C6	25	52C9	21	51C4	17	19H12	14	9G11	12
2A7	31	31A2	25	12C7	21	27H3	17	24C11	14	9H11	12
16F2	31	49C6	25	9B8	21	6A1	17	2B3	14	15B10	12
7D5	30	20G5	25	23G11	21	19D9	17	6D6	14	7F9	12
36A10	30	5A10	25	48A10	20	21E7	17	51G2	14	19F10	12
21B9	30	6F2	25	23C3	20	20D2	17	40H11	14	42C8	12
46C3	30	17B8	25	53C3	20	52F11	17	41G3	14	14D5	11
24H12	30	17C12	24	4H5	20	5A4	17	51G11	14	31D12	11
52C3	30	31F7	24	14F4	20	11A12	17	26A12	14	50H8	11
20F5	30	49C5	24	26C10	20	16F8	17	5A6	14	23B9	11
46E1	30	46B6	24	15F2	20	45G2	17	14D9	14	32E9	11
18E6	29	32G5	24	10B12	20	8D2	17	19E12	14	40E3	11
27C2	29	24A3	24	11B5	20	8A11	17	27D8	14	41E4	11
49F1	29	31B10	24	27C5	20	33H7	17	37H9	14	25A6	11
18D9	29	18G8	24	16G11	20	53A9	17	12E12	14	8F7	11
30A11	29	12G5	24	45H7	20	22A11	17	24F8	14	27H6	11

Appendix 7.4 continued

ID	√LA	ID	√LA	ID	√LA	ID	√LA	ID	√LA	ID	√LA
21C2	11	52H12	9	7D4	7	4A12	5	2F10	4	25E8	3
26D3	11	19D11	9	33D3	7	14F12	5	10E4	4	26A10	3
12F12	11	43D7	9	49A9	7	23B8	5	20A2	4	28B7	3
17G8	11	16H11	9	22B7	7	31D2	5	50A5	4	34E4	3
33C6	11	26H6	9	3B7	7	40D5	5	5B4	4	46F10	3
44B6	11	9B11	9	52E12	7	43C10	5	17H3	4	15C12	3
14D10	11	42C5	9	12H8	7	49E4	5	22D8	4	17C11	3
30H6	11	46A1	9	15F10	7	15B11	5	28C11	4	18F4	3
41F12	11	21G10	9	22A9	7	22B9	5	31G6	4	18H11	3
42B11	11	24B8	9	41F1	7	25E10	5	35B9	4	21D10	3
6G11	11	24G6	9	44C6	7	31B8	5	7A3	4	24E8	3
16G5	11	25G10	9	2G7	7	37G11	5	7C6	4	26B9	3
39G12	11	29H9	9	4A2	7	52A1	5	20H12	4	26C4	3
2H5	11	49H9	9	19C1	7	21H8	5	42G3	4	35A8	3
8C12	11	53C11	9	43D11	7	25C3	5	45F5	4	38D9	3
11G10	11	25F11	9	9H5	7	43C5	5	49G8	4	39B10	3
17C9	11	29G3	9	18F1	7	2H8	5	14F10	4	45H12	3
26G5	11	12A8	9	37A11	7	5F4	5	17H8	4	49F8	3
51E2	11	18D8	9	41A11	7	19H11	5	39D5	4	5D2	3
17A4	10	16A1	9	6A12	7	32A6	5	41D12	4	7C7	3
7D10	10	11C6	9	19E6	7	17D8	5	45G1	4	7F7	3
18C3	10	26E4	9	28A10	7	26E11	5	48A5	4	11H11	3
41F7	10	26C12	9	44G5	7	42D5	5	0H0	4	14C3	3
4B3	10	42E6	9	16F7	6	10A7	5	2F11	4	15F12	3
44B2	10	42E3	8	22E8	6	15B9	5	2H11	4	17H2	3
4D8	10	48E6	8	25E6	6	21E6	5	7E8	4	17C10	3
5E1	10	21F1	8	42G12	6	27G7	5	13C7	4	21E11	3
40A12	10	34E11	8	44H5	6	29A7	5	16A8	4	23A8	3
43A5	10	36C11	8	26C1	6	1B5	5	38G5	4	30D2	3
17D6	10	5G11	8	53H9	6	2A2	5	43F11	4	31A8	3
14A8	10	14F7	8	11G2	6	10B6	5	46A11	4	31E2	3
40A9	10	25C8	8	18D2	6	18D3	5	53B10	4	36E5	3
43A12	10	46D8	8	22E9	6	20A10	5	6H1	4	38D10	3
4G10	10	3G3	8	24F7	6	31G5	5	25F3	4	48B10	3
3C3	10	17A7	8	49F11	6	46A4	5	31E5	4	49D4	3
26D4	10	39B1	8	7B9	6	7F1	5	41G5	4	53B12	3
6E1	10	51D12	8	8C7	6	38B8	5	49E9	4	2G12	3
19D12	10	24E3	8	9F11	6	20F4	5	53G6	4	4E11	3
44D4	10	41A10	8	10E12	6	32C12	5	2E5	3	5G10	3
52F8	10	12D9	8	10H8	6	39H2	5	15H2	3	6D11	3
8H5	10	30G6	8	26B6	6	48F1	5	22F12	3	11H6	3
10A6	10	5H8	8	11C3	6	7A5	5	24G5	3	12H1	3
13E10	10	22A10	8	26H4	6	11G4	5	44H3	3	14H11	3
15B12	10	25C12	8	23D9	6	20D11	5	45A12	3	16G4	3
49H8	10	39H6	8	37B6	6	22E7	5	49F10	3	20A12	3
6D5	10	53F5	8	7E12	6	52A6	5	52H11	3	21A1	3
7E10	10	41H1	8	23F7	6	11F7	4	2E8	3	21D3	3
18D1	10	46B7	8	31G11	6	19F4	4	4H4	3	23F10	3
45B11	10	1B10	8	41H9	6	27E2	4	7A7	3	24H2	3
6A7	10	20G6	8	42B7	6	35D7	4	18H6	3	25C2	3
7C1	9	51A8	8	3D10	6	41C2	4	25F2	3	25D4	3
10B11	9	19F11	8	19G5	6	42H6	4	31C2	3	27B8	3
12A12	9	28A11	8	22E1	6	44A9	4	40A2	3	29A12	3
22C7	9	43C2	8	42F2	6	45B1	4	41C1	3	30G2	3
42D9	9	18A6	7	10H6	6	7E7	4	44D1	3	33G6	3
40B8	9	17G2	7	19E11	6	11B12	4	48D11	3	33D8	3
43A7	9	22D9	7	43D8	6	16E1	4	1E11	3	36A2	3
14G8	9	24C10	7	52G6	6	18G2	4	1F11	3	41B9	3
20B12	9	49G11	7	53H1	6	25G2	4	14A2	3	41H10	3
27H2	9	35C4	7	53C8	6	50H3	4	20G2	3	45C12	3

Appendix 7.4 continued

ID	√LA	ID	√LA	ID	√LA	ID	√LA	ID	√LA
48C4	3	38A5	2	24F4	2	37E6	1	21A5	1
49H3	3	44B3	2	27F7	2	41B4	1	53D12	1
49F9	3	45E11	2	33A3	2	41D7	1	53F8	1
3H4	2	45F7	2	32F9	2	41G2	1	53G7	1
5G2	2	49B6	2	34E3	2	42E2	1	53H11	1
5E9	2	49E5	2	36C5	2	42H8	1		
11A11	2	50H11	2	36H10	2	43B5	1		
12A3	2	51A2	2	38D8	2	43F4	1		
15A8	2	51B9	2	39A7	2	42G11	1		
16H10	2	52B8	2	40A1	2	45B12	1		
19A10	2	3F5	2	40B5	2	45D12	1		
22B8	2	2G9	2	41B8	2	45E12	1		
22G9	2	5C1	2	41F5	2	45G6	1		
24A9	2	7E6	2	41A12	2	45H11	1		
25B2	2	7H7	2	42B3	2	51B3	1		
25H1	2	12F7	2	42D11	2	51C10	1		
27H9	2	12G6	2	43F6	2	51E8	1		
31A10	2	16B5	2	45A4	2	52A2	1		
38H6	2	16C5	2	45A9	2	52C4	1		
39G3	2	16D1	2	45E7	2	52D3	1		
41D8	2	21A2	2	49A7	2	52H7	1		
46A10	2	22A7	2	49B11	2	53A7	1		
48H5	2	27H8	2	49D7	2	0C0	1		
49C10	2	33B3	2	49G6	2	53G11	1		
50B12	2	36F4	2	50D12	2	21H5	1		
51A7	2	37A3	2	50F12	2	32B5	1		
51D10	2	36G11	2	51H8	2	32B8	1		
51F8	2	38D3	2	52B7	2	32H5	1		
3B5	2	39D1	2	53E10	2	35C1	1		
3C2	2	42H7	2	53H12	2	35D2	1		
4A5	2	43B6	2	4A10	1	34E9	1		
4B10	2	50F2	2	4G11	1	35G2	1		
5B12	2	49H6	2	5H1	1	36A8	1		
5C12	2	51G5	2	6A11	1	36B4	1		
5E12	2	51G10	2	7A6	1	36B5	1		
5H10	2	51H10	2	7B8	1	36B6	1		
5H12	2	3C4	2	7H3	1	36B7	1		
6C11	2	3E2	2	19A4	1	36B10	1		
7F8	2	4C7	2	18B11	1	36H2	1		
7G5	2	5F1	2	18B12	1	36H4	1		
7H8	2	4H11	2	19C4	1	36H7	1		
11B10	2	6D12	2	19E3	1	37E3	1		
12A7	2	7F6	2	21A5	1	37G7	1		
12D7	2	7H4	2	21B5	1	37H7	1		
14E12	2	12A4	2	21F4	1	43A2	1		
15F1	2	11E11	2	21C12	1	43C1	1		
16C1	2	12F5	2	22G5	1	43E1	1		
16D9	2	11H12	2	32A1	1	42F10	1		
16G9	2	12H5	2	32E7	1	42H12	1		
16G12	2	16B2	2	32H1	1	45A6	1		
19H4	2	19B3	2	33A5	1	45A8	1		
20C12	2	19E1	2	33H3	1	45B6	1		
21B10	2	20A8	2	35A2	1	45D8	1		
22F8	2	20E8	2	35H2	1	45E8	1		
22E10	2	20F8	2	35B11	1	52A3	1		
24B2	2	20G4	2	35E9	1	52A8	1		
26H9	2	21G8	2	36A3	1	51C11	1		
33H6	2	24A4	2	36A6	1	52C8	1		
35G12	2	23D12	2	36C9	1	52G7	1		
37H8	2	24F1	2	36E4	1	52H8	1		

Appendix 7.5. Effect of selected hit compounds in the rescreens on day 13 leaf area ANOVA. Hit compounds from the 5K screen were rescreened in two batches (rescreen 1 and rescreen 2), each with 3 replicates across multiple plates. At least 12 positive and 12 negative controls were included on each plate. Photographs of plates were taken daily 9 days to 13 days after sowing. Leaf area data was square-root transformed and the effect of compound analysed by the GenStat unbalanced ANOVA procedure including plate ('slice') as a nuisance term. Negative controls were not included in the analysis due to strongly non-normal distribution (most values = 0).

Rescreen	Change	d.f.	s.s.	m.s.	v.r.	F pr.
1	+ Slice	5	3099.22	619.84	9.18	<0.001
	+ Compound	122	46690.98	382.71	5.67	<0.001
	Residual	314	21207.41	67.54		
	Total	441	70997.61	160.99		
2	+ Slice	4	560.04	140.01	2.45	0.046
	+ Compound	114	51545.4	452.15	7.91	<0.001
	Residual	291	16632.95	57.16		
	Total	409	68738.39	168.06		

Appendix 7.6. Effect of compounds in the 88 compound library on day 13 leaf area in the assay ANOVA. 88 compounds from the 88 compound library were tested in the screening assay at concentrations of 10 ppm, 1 ppm, 0.1 ppm and 0.01 ppm. 4 replicates were performed per compound per concentration. Compounds and controls were blocked by plate (1 assay compound replicate, 2 positive controls and 6 negative controls per plate), and different concentrations run on different plates. Plates were photographed between days 10 and 13. Green leaf area data was square-root transformed and analysed by GenStat ANOVA. Negative controls were excluded from the analysis due to the largely non-normal distribution as 92% of negative controls had a zero value.

[compound] (ppm)	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
10	Slice stratum	3	287.92	95.97	4.01	
	Compound_ID	88	17576.19	199.73	8.35	<0.001
	Residual	268	6407.7	23.91		
	Total	359	24271.81			
1	Slice stratum	3	277.88	92.63	1.46	
	Compound_ID	88	21486.07	244.16	3.84	<0.001
	Residual	268	17021.37	63.51		
	Total	359	38785.33			
0.1	Slice stratum	3	527.7	175.9	2.08	
	Compound_ID	88	20914.01	237.66	2.81	<0.001
	Residual	268	22677.44	84.62		
	Total	359	44119.16			
0.01	Slice stratum	3	1690.89	563.63	6.38	
	Compound_ID	88	16370.28	186.03	2.11	<0.001
	Residual	268	23678.05	88.35		
	Total	359	41739.22			

Appendix 7.7. Crude prediction of optimum concentration for compound activity in the screen.

The following formula was used to calculate the optimal concentration (c_{opt}) for maximum leaf area: $c_{opt} = \frac{\sum c_i.A_i}{\sum A}$

where A_i is the average √leaf area at concentration c_i and $\sum A$ is the sum of √leaf areas.