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Seed dormancy is a dynamic state: variable responses to pre- and post-shedding environmental signals in seeds of contrasting *Arabidopsis* ecotypes

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Abstract

Seeds have evolved to be highly efficient environmental sensors that respond not only to their prevailing environment, but also their environmental history, to regulate dormancy and the initiation of germination. In the present work we investigate the combined impact of a number of environmental signals (temperature, nitrate, light) during seed development on the mother plant, during post-shedding imbibition and during prolonged post-shedding exposure in both dry and imbibed states, simulating time in the soil seed bank. The differing response to these environments was observed in contrasting winter (Cvi, Ler) and summer (Bur) annual *Arabidopsis* ecotypes. Results presented show that environmental signals both pre- and post-shedding determine the depth of physiological dormancy and therefore the germination response to the ambient environment. The ecotype differences in seed response to ambient germination conditions are greatly enhanced by seed maturation in different environments. Further variation in response develops following shedding when seeds do not receive the full complement of environmental signals required for germination and enter the soil seed bank in either dry or imbibed states. Species seed dormancy characteristics cannot therefore be easily defined, as seed dormancy is a dynamic state subject to within-species adaptation to local environments.

Keywords: afterripening, *Arabidopsis*, nitrate, seed dormancy, seed germination, soil seed bank, temperature

Introduction

Seeds have evolved to be highly efficient sensors and interpreters of not only the prevailing environment, but also their environmental history, in order to regulate physiological dormancy and the initiation of germination. This takes the form of sensing the maternal environment (light, nitrate and temperature) (Sawhney *et al.*, 1985; Alboresi *et al.*, 2005; Kendall *et al.*, 2011; Kendall and Penfield, 2012; Penfield and Springthorpe, 2012; He *et al.*, 2014; Huang *et al.*, 2014) and the environment of the soil seed bank (light, nitrate and temperature) (Footitt *et al.*, 2011, 2013, 2014; Finch-Savage and Footitt, 2012; Penfield and Springthorpe, 2012). This enables seeds to use environmental signals to determine the time and place of seed germination and subsequent seedling emergence (Baskin and Baskin, 1998; Finch-Savage and Leubner-Metzger, 2006; Footitt *et al.*, 2011, 2013, 2014). Within a species, the initial depth of primary dormancy is determined during seed development by ecotype genetics and the prevailing environment (Baskin and Baskin, 1998; Kendall *et al.*, 2011; Donohue, 2014; He *et al.*, 2014; Huang *et al.*, 2014). If seeds are not exposed to suitable environmental signals for dormancy removal upon shedding, they enter the soil seed bank. Here, in either the dry or imbibed state, they continually adjust their dormancy status, in either direction, by sensing and integrating a range of environmental signals (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage and Footitt, 2012). The signals related to slow seasonal change are used for temporal sensing, to determine the time of year. In response to these signals, seeds alter their depth of

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dormancy and their sensitivity to other signals that inform of the spatial environment. These spatial signals indicate in a more immediate way that conditions are suitable for germination, and so trigger the termination of dormancy and therefore induce germination completion. Seeds are sensitive to a variety of spatial signals such as light and nitrate (discussed below). The response to each temporal, and subsequently spatial, signal appears to remove successive blocks to germination. However, this process usually needs to be carried out in a set order for it to lead to germination completion, i.e. spatial signals are only effective if temporal sensing has enhanced sensitivity to them. Thus a dormancy continuum is thought to exist, driven in both directions by environmental signals, and when all dormancy layers are removed germination occurs (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage and Footitt, 2012). In the annual dormancy cycle, if the correct spatial window does not occur, sensitivity is lost and the temporal window will close for another year. This dynamic environmental response allows multiple species to compete successfully within species-rich natural communities (Baskin and Baskin, 1998, 2006; Walck *et al.*, 2011). There are limited data available regarding differences in impact of environmental signals on dormancy in winter- and summer-annual species. Therefore in the present work we consider the combined impact of a number of environmental signals (described below) upon the depth of seed dormancy in *Arabidopsis* ecotypes with contrasting life cycles, to improve understanding of environmental adaptation.

Genetics × maternal environment determines depth of primary dormancy

Natural genetic variation results in *Arabidopsis* ecotypes with very different primary dormancy when produced in the same environment (Koornneef *et al.*, 2004; Alonso-Blanco *et al.*, 2009). This variation is altered by differences in the environment during seed maturation, to determine the depth of primary dormancy (Donohue, 2009; Kendall and Penfield, 2012; Penfield and Springthorpe, 2012; He *et al.*, 2014; Huang *et al.*, 2014). Temperature is the principal environmental factor driving differences in depth of dormancy, with lower maturation temperatures tending to enhance dormancy.

Changes in the dry state (afterripening)

Following shedding, dormancy is constantly changing (declining) in the dry state due to afterripening (AR) (Bewley, 1997). In general, seeds are considered as 'dry' when they have less than 0.2 g water (g dry weight)$^{-1}$ (Bewley *et al.*, 2013). AR is a process regulated by time and environment (temperature and moisture), which increases the sensitivity of seeds to spatial environmental signals that allow completion of germination (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage *et al.*, 2007; Carrera *et al.*, 2008).

Changes following imbibition (temperature as a temporal signal)

In a temperate seasonal environment it is most likely that seeds will imbibe water soon after shedding and then exist at varying levels of moisture content. Under these conditions, it is widely accepted that temperature is an important temporal signal that influences both the induction and loss of seed dormancy, and therefore sensitivity to spatial signals (Probert, 2000; Finch-Savage and Leubner-Metzger, 2006). In general, low temperature (cold stratification) releases seed dormancy of many summer-annual plants, reflecting the loss of dormancy over winter so that germination occurs the following spring (Baskin and Baskin, 1998). Conversely, the germination of many winter annuals often requires exposure to warm temperatures (warm stratification) over summer to remove dormancy, resulting in germination in the autumn. Both summer- and winter-annual *Arabidopsis* ecotypes exist (e.g. Bur and Cvi or Ler, respectively, used in the present work; Baskin and Baskin, 1998; Footitt *et al.*, 2013; Huang *et al.*, 2014). During cold stratification, two separate processes may occur: a rapid loss of primary dormancy and also a slower induction of secondary dormancy (Totterdell and Roberts, 1979). In *Arabidopsis*, extended low-temperature exposure of multiple ecotypes leads to secondary dormancy (Finch-Savage *et al.*, 2007; Penfield and Springthorpe, 2012).

Light as a spatial signal

Light is another critical environmental signal influencing seed germination, especially in small-seeded species such as *Arabidopsis* (Yamauchi *et al.*, 2004). Light can inform the seed that disturbance of the soil has occurred, or that the seed is on, or very near, the surface. The seed can be sensitive to a range of wavelengths that inform it about the spatial environment and its suitability for germination and seedling survival. For example, the ratio of red/far red wavelengths alters as light passes through a leaf canopy, informing about the presence of competing plants (as reviewed in Pons, 2000). In *Arabidopsis*, dormancy release and the completion of germination of both summer- and winter-annual ecotypes can have an absolute light dependency (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007; Footitt *et al.*, 2013).

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Nitrate as a spatial signal

Nitrate has long been known as one of the key variables in the loss of seed dormancy and promotion of germination (Baskin and Baskin, 1998; Alboresi *et al.*, 2005; Finch-Savage *et al.*, 2007; Matakiadis *et al.*, 2009). Seeds use nitrate sensing as a gap detection mechanism, as nitrate levels inform about the presence of competing plants that deplete soil nitrate (Pons, 1989). In the deeply dormant *Arabidopsis* Cvi ecotype, nitrate can substitute for the long period of dry storage (7–12 months afterripening) or several days of cold stratification required for dormancy release (Finch-Savage *et al.*, 2007). Sensitivity to nitrate differs between ecotypes and is high in the Bur ecotype (Chardon *et al.*, 2010). Results of exogenous nitrate applications to wild-type and nitrate reductase (NR) deficient *Arabidopsis* seeds supported the role of nitrate as a signal during germination, and revealed that this involves interaction with the abscisic acid (ABA) or gibberellic acid (GA) signalling pathways, as nitrate induced expression of the ABA catabolism gene *CYP707A2* (Ali-Rachedi *et al.*, 2004; Alboresi *et al.*, 2005; Matakiadis *et al.*, 2009). In addition, nitrate feeding experiments in *Arabidopsis* showed that supplying higher nitrate levels to the mother plant (maternal nitrate supply) increased seed nitrate levels, and these were negatively correlated with dormancy levels in the mature seed (Alboresi *et al.*, 2005; Matakiadis *et al.*, 2009).

Here we demonstrate how seeds of different ecotypes act as environmental sensors to sense and interpret the environmental signals during seed development and in the post-shedding environment. We show how these histories combine to influence the ecotypic differences in physiological dormancy and germination phenology. To do this we produced seeds from both winter- and summer-annual *Arabidopsis* ecotypes under different conditions of nitrate and temperature and then investigated different imbibition environments on their germination characteristics. We report on this series of experiments to develop a more detailed understanding of the combined impact of pre- and post-shedding environments on the flexibility of the dormancy/germination response within a single species.

Materials and methods

Seed production

Seeds from three *Arabidopsis* ecotypes (Burren (Bur), Cape Verdi Islands (Cvi), Landsberg erecta (Ler)) were produced under controlled conditions on three occasions. In 2009 and 2010 seeds were produced in a temperature-controlled glasshouse (23/17°C, 16/8 h, light/dark) and harvested in July and March, respectively. The glasshouse was vented and heated to control temperature and had supplementary lighting to maintain light levels and photoperiod. In 2012 seeds were produced in temperature-controlled incubators (15/15°C, 12/12 h, light/dark; 20/20°C, 12/12 h, light/dark).

Compost (Levingtons FI compost:sand:vermiculite) was mixed to provide two levels of nitrate [ratios 6:1:1 and 4:2:2 providing 422 and 154 mg (kg dry weight)$^{-1}$ nitrate, respectively] and added to P24 cellular trays (36 × 24 cm containing 24 cells). Each tray was then placed in a second tray lined with capillary matting to ensure all the plants had a uniform water supply when watered from below. The trays were covered with transparent propagator lids for at least 4 d following sowing, to establish seedlings, which were thinned to one per cell after 7 d. Watering stopped when seeds had reached maturity (all siliques yellow and dry). Seeds were then harvested and dried to equilibrium (ERh; 6 d) with relative humidity of 55% above a saturated calcium nitrate solution [measured seed moisture content 9.9% dry weight (DW) basis]. Seed yield and 1000-seed weight were measured before sealing in aluminium-foil bags and storage at −80°C for germination experiments. A proportion of the freshly harvested Bur and Cvi seeds were placed in separate sealed bags to afterripen (AR) at 20°C and 55% ERh for 8 months, matching the requirement for completion of AR in previous studies of Cvi (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007). In further experiments, seeds were placed in Eppendorf tubes (1.5 ml) within 50-ml screw-cap tubes sealed with Nescofilm at 20°C for periods up to 210 d before germination was recorded at a range of conditions, as described below. In all experiments there were three replicates of 40 seeds for each ecotype/treatment combination. A seed was considered to have germinated when the radical had emerged through the testa and endosperm.

Seed germination

Seeds were surface-sterilized in a 0.125% sodium hypochlorite solution (household bleach: 5% sodium hypochlorite, diluted to 2.5% v/v) for 5 min and then washed three times with distilled water. Germination experiments were either conducted on a thermogradient table [model GRD1 LH, Grant Instruments Ltd, Cambridge, UK (Murdoch *et al.*, 1989)] or in temperature-controlled incubators.

The thermogradient table was used to provide a linear range of constant temperatures from 5 to 30°C using a 10 × 10 matrix of cells, each containing a single germination box (7 × 7 cm). Temperature was incremented within each column of the matrix and was

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constant in each row. Seeds were sown in transparent polystyrene boxes (7 × 7 m; Stewart Plastics Ltd, Croydon, UK) containing 0.7% agarose (30 ml per box) to provide a reservoir of water below one sheet of 3MM chromatography paper (Camlab, Cambridge, UK). Boxes were placed in constant light (white fluorescent) or darkness. Boxes used for the latter were wrapped in black sticky-back plastic (Fablon, H-A Interiors Ltd, Cramlington, Northumberland, UK) and placed under a wooden cover to exclude light.

In all other experiments, seeds were incubated on two layers of 3MM chromatography paper in clear plastic boxes (8 × 12 cm) (Stewart Plastics Ltd) containing 8 ml of distilled water, or 1 mM or 10 mM KNO_3. Germination was recorded in the light and in the dark for 28 d. Seeds with dark treatments were sown and germination was recorded in the dark under a green safe light (Kodak 7B safelight filter/Green, Kodak Limited, London, UK); at all other times germination boxes were wrapped in a double layer of aluminium foil.

Prolonged dark incubation

Fresh seeds were incubated on water in the dark at a range of temperatures (5–30°C) and for different periods of time up to 200 d. Seeds were incubated on two layers of 3MM chromatography paper with 4 ml of distilled water in transparent polystyrene boxes (7 × 7 m; Stewart Plastics Ltd). All procedures were carried out in a dark room under a green safe light and boxes were wrapped with two layers of aluminium foil and sealed inside a polyethylene freezer bag. Water (2 ml) was applied monthly in complete darkness (dark room without safe light) to those seeds in prolonged dark incubation (≥35 d) to offset any loss of water. At the end of the assigned dark incubation period boxes were transferred to 20°C in constant light for another 28 d to record germination.

Measurement of seed nitrate content

Triplicate 150 mg samples of dry seeds were ground using a pestle and mortar, and transferred to a 20-ml scintillation vial that was weighed before and after drying at 80°C for 16 h. Deionized water (10 ml) was added and the samples were shaken for 1 h and then centrifuged for 5 min at 5000 rpm. The supernatant was filtered using nitrogen-free filter paper, and analysed for nitrate-nitrogen (NO_3-N) by a steam distillation method using a FOSS FIAstar 5000 Flow Injection Analyser (Gerber Instruments, Effretikon, Switzerland) for end-point determination (Bremner and Keneney, 1965).

Data analysis

Analysis of variance was used to detect the differences between variates. Statistical analysis was carried out using the software package GenStat (VSN International, Hemel Hempstead, UK, 2012). All percentage germination data were first angular transformed.

Results

To increase understanding of the flexibility of germination responses within a single species, we compared the impact of environmental signals pre- and post-shedding on dormancy and the germination of seeds from contrasting winter- (Cvi or Ler) and summer-annual (Bur) ecotypes of *Arabidopsis*. In a series of experiments the seeds were produced under different controlled environments (nitrate × temperature) and, to indicate behaviour in the soil seed bank, seeds were exposed to a range of dry (afterripening: 20°C, 55% ERh) and moist (prolonged imbibition) post-shedding environments.

Effect of seed production regime (nitrate and temperature) on subsequent dormancy and germination

Seedlings of Bur, Ler and Cvi ecotypes were grown in media with high and low levels of nitrate [422 and 154 mg (kg DW)$^{-1}$, respectively]. Rate of growth and plant size was reduced in low nitrate; this effect was extreme in Cvi seedlings, which eventually died without producing seeds. Thus in limiting-nitrate conditions we compared the summer-annual ecotype Bur to the winter-annual ecotype Ler. Seeds were produced in a glasshouse maintained at 23/17°C (16 h light/8 h dark) to determine the effect of the nitrate regimes on subsequent seed germination characteristics. In a second experiment, the interaction with seed production temperature was investigated by producing seeds in incubators under two temperature regimes (15/15°C, 12/12 h, light/dark; 20/20°C, 12/12h, light/dark).

Effect of nitrate regime supplied to the mother plant

Seed nitrate content was significantly ($P < 0.001$) affected by both ecotype and maternal nitrate supply. Ler plants grown on high-N compost produced seeds with significantly ($P < 0.001$) higher nitrate content [743.2 ± 3.9 mg (kg DW)$^{-1}$] than those on low-N compost [145.2 ± 1.4 mg (kg DW)$^{-1}$]. In contrast, Bur seed nitrate content was not significantly different between seeds of the two N regimes [202.9 ± 11.5 and 145.3 ± 3.5 mg (kg DW)$^{-1}$, respectively]. The depth of dormancy of these seeds was characterized by changes

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in germination sensitivity to exogenous nitrate (KNO_3) and light (Fig. 1). Under both nitrate regimes final germination at 25°C in the light was significantly ($P < 0.001$) lower in the more dormant Ler seeds, but at 10°C in the light final germination was greater than 95% in both ecotypes. Exogenous nitrate significantly ($P < 0.001$) enhanced germination of Ler seeds in the light from the high-nitrate regime, but high exogenous nitrate significantly ($P < 0.01$) delayed germination of Bur. In the dark there was little effect of maternal nitrate regime on Bur percentage germination, except for a reduction at 25°C in seeds from the low-N regime. In Ler, percentage germination was lower at 25°C and germination was delayed at 10°C in seeds from the low-N regime compared to the high-N regime.

Effect of nitrate and temperature regimes on seed yield, size and nitrate content

In Bur, seed yield per plant was significantly ($P < 0.001$) higher at 15°C than at 20°C, and plants in the high-N regime produced significantly ($P < 0.001$) higher yields at both temperatures (Table 1). In contrast, Ler seed yield was not reduced at the higher temperature under high N, and under low N seed yield increased at 20°C compared to 15°C. The effect on seed size also differed between ecotypes (Table 1). Bur plants produced significantly ($P < 0.05$) larger seeds than Ler in both growth regimes. Bur plants produced significantly ($P < 0.05$) larger seeds under low N at both 15 and 20°C, whereas low N led to significantly larger ($P < 0.05$) Ler seeds at 20°C.

Both temperature and N regime had significant ($P < 0.01$) effects on seed nitrate content (Table 1). In Ler, seed nitrate content increased dramatically in plants grown at 20°C compared to 15°C, especially in the high-N regime. However, Bur seeds had higher nitrate content when produced with high N at 15°C, but lower content when produced at 20°C. The reason for this is not obvious, suggesting that other factors are involved in determining seed nitrate content. In general, the maternal nitrate regime had a more significant ($P < 0.01$) effect on the nitrate content in Ler than in Bur seeds.

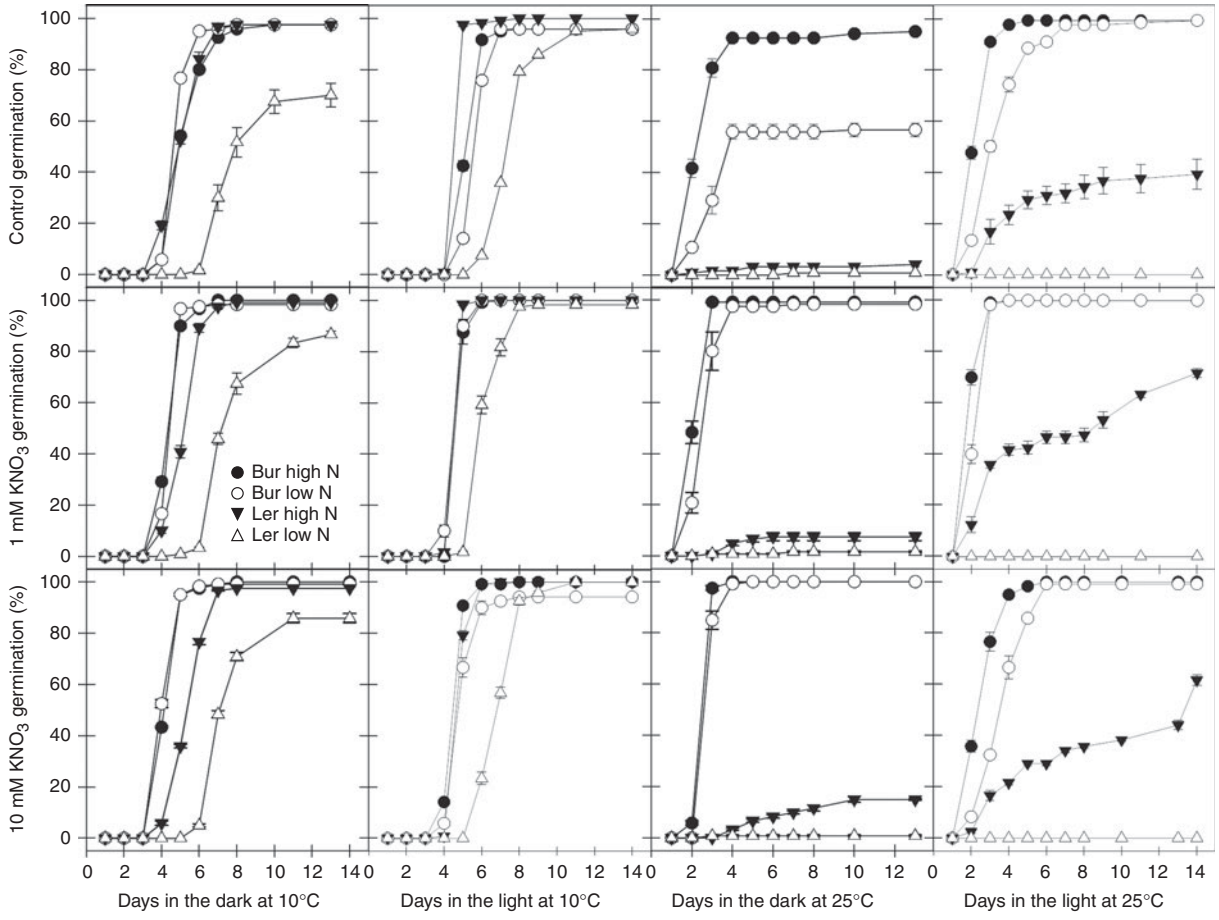

Figure 1. Germination responses in the dark and light to temperature (10°C and 25°C) and exogenous nitrate (1 and 10 mM) of Bur and Ler seeds produced under low- and high-nitrate regimes supplied to the mother plant at 23/17°C (16 h light/8 h dark). Data represent the mean ± standard error. Absent error bars indicate that the symbol is larger than the error.

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Table 1. The effect of seed production regime (temperature and nitrate) on seed yield, size and nitrate content

Ecotype	Nitrate regime	Growth temperature	Seed yield per plant (mg)	1000 seed weight (mg)	Nitrate content per 1000 seeds (mg)
Bur	High N	15°C	105.8 ± 15.1	29.1 ± 0.14	3.28 ± 0.47
Bur	Low N	15°C	53.3 ± 0.002	30.0 ± 0.06	2.26 ± 0.39
Bur	High N	20°C	24.9 ± 0.005	37.5 ± 0.08	2.19 ± 0.6
Bur	Low N	20°C	12.4 ± 0.002	38.5 ± 0.13	2.95 ± 0.4
Ler	High N	15°C	100.9 ± 0.008	19.5 ± 0.07	0.49 ± 0.05
Ler	Low N	15°C	42.4 ± 0.002	19.3 ± 0.03	0.55 ± 0.005
Ler	High N	20°C	92.9 ± 0.006	17.7 ± 0.12	5.24 ± 0.33
Ler	Low N	20°C	63.8 ± 0.007	18.7 ± 0.07	2.88 ± 0.14

Effect of growth regimes (nitrate and temperature) on seed sensitivity to temperature and nitrate following shedding

Final germination was significantly ($P < 0.001$) affected by ecotype, seed maturation temperature and imbibition temperature. For both ecotypes, lower maturation temperature enhanced seed dormancy and final germination was significantly higher in Bur than Ler (Fig. 2). Ler seed produced at 15°C exhibited increased dormancy with increasing imbibition temperatures, whereas seeds produced at 20°C were less dormant (higher final germination) when imbibed at 15°C or lower. Bur seeds germinated to high levels at all temperatures when seeds were produced at 20°C, but when produced at 15°C Bur seeds exhibited thermodormancy similar to that of Ler seeds.

The nitrate regime did not have a significant effect on subsequent germination of either ecotype, with the exception of Bur seeds germinated at 5°C, where germination of seeds from the low maternal N regime was significantly ($P < 0.05$) higher than that of seeds from the high-N regime. The depth of dormancy was further characterized by changes in germination sensitivity to exogenous nitrate (1 mM KNO_3). Ler seeds matured at 15°C responded to exogenous KNO_3 with higher germination at all but the highest temperature (Fig. 2). In contrast, Ler seeds matured at 20°C were less dormant and the temperature range for germination was broadened by exogenous nitrate. Bur seeds produced at 15°C, in particular, were more sensitive to KNO_3 than Ler seeds, consistent with a more shallow dormancy.

Effect of post-shedding environment on dormancy/germination: dry storage

Seeds of Cvi and Bur were produced at 23/17°C (16 h light/8 h dark) and their thermal responses in both light and dark were investigated before and after afterripening (AR; 8 months). There were significant ($P < 0.001$) effects of temperature, light and afterripening on germination of Bur and Cvi seeds. AR relieved dormancy in both Bur and Cvi, widening the temperature range at which maximal germination occurred (Fig. 3). In general, following AR Bur seeds germinated to high percentages at higher temperatures (>18–30°C), whereas Cvi seeds germinated to high percentages at lower temperatures (5–25°C).

Fresh Cvi seeds did not germinate at any temperature in either the presence or absence of light (Fig. 3). However, in the light, the AR seeds of Cvi had high germination percentage at temperatures ranging from 7 to 25°C, but germination was reduced above these temperatures. Fewer seeds germinated in the dark at temperatures up to 20°C, with no germination

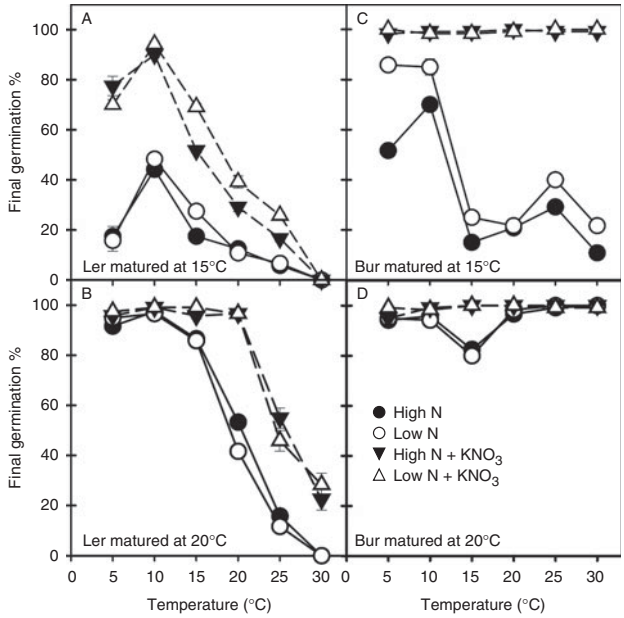

Figure 2. Germination responses in the light of Bur and Ler seeds, produced at 15 and 20°C on high- and low-N compost, to temperatures (5–30°C) and exogenous nitrate (1 mM KNO_3). (A) Ler matured at 15°C; (B) Ler matured at 20°C; (C) Bur matured at 15°C; (D) Bur matured at 20°C; (15°C: 15/15°C, 12/12 h, light/dark; 20°C: 20/20°C, 12/12 h, light/dark). Data represent the mean ± standard error. Absent error bars indicate that the symbol is larger than the error.

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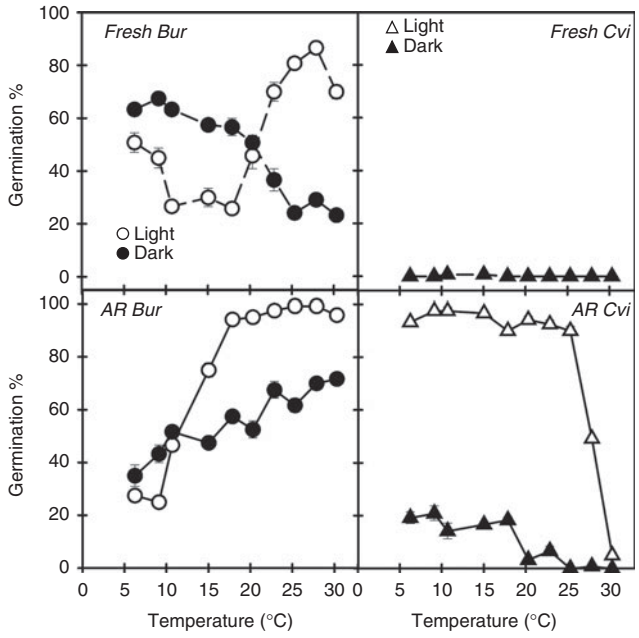

Figure 3. Final germination percentages of fresh and afterripened Bur and Cvi seeds on a temperature gradient in the light and in the dark. Data are the mean ± standard error. Absent error bars indicate that the symbol is larger than the error.

above this temperature. In contrast, in Bur seeds there was a significant interaction ($P < 0.001$) between the effects of light and temperature on the final percentage germination. In fresh Bur seeds, light had a positive effect on germination at high temperature, whereas in the dark increasing temperature had a negative effect on germination. Furthermore, germination in the dark was negatively correlated with temperature in fresh Bur seeds but positively correlated in AR seeds. In the light, AR extended the temperature range, with high percentage germination to lower temperatures. The increase in germination observed in the light at temperatures below 7°C is possibly the result of cold stratification.

The duration of AR and application of exogenous KNO_3 also significantly ($P < 0.001$) affected the final germination percentage (Fig. 4). Bur seeds were less dormant than those of Cvi at the start of the experiment, having 83.3% germination compared to 0% in Cvi. In Bur, germination was initially greater at 10°C than at 20°C on water, but was maximal at both temperatures by 56 d of AR. Nitrate addition resulted in high germination at both temperatures without AR. Germination of Cvi at 10°C increased significantly for up to 56 d of AR, but thereafter there was no significant increase (Fig. 4A). At 10°C there was no significant effect of adding nitrate. At 20°C in the presence of nitrate the response was similar to that at 10°C. However, unlike the response at 10°C, the increase in germination with increasing AR was significantly delayed in the absence of nitrate. In the latter case, final germination increased up to 210 d, but this increase was no longer significant after 105 d (Fig. 4B). At both germination temperatures germination on nitrate followed a similar time course, indicating that nitrate was able to overcome residual high-temperature thermodormancy at 20°C as AR time increased.

Depth of dormancy can be measured in terms of the AR time to reach 50% germination (AR_{50}) (Alonso-Blanco *et al.*, 2003). AR_{50} values of Cvi seeds increased when the germination temperature increased from 10 to 20°C (38.5 and 69.7 d, respectively). However, if seeds were incubated with 10 mM KNO_3 the AR_{50} value decreased at both incubation temperatures, becoming significantly ($P < 0.001$) smaller at the higher temperature (34.6 and 21.4 for 10 and 20°C, respectively). As dormancy was shallow in Bur, the AR_{90} values were calculated instead of AR_{50}. Less AR was required to achieve 90% germination at 20°C (27.8 d) than at 10°C (42.3 d). However, Bur seeds treated with KNO_3 germinated to 90% without AR at both 10 and 20°C.

Effect of post-shedding environment on dormancy/germination: moist storage

In these experiments dark incubation of imbibed seeds represents time spent in the soil seed bank following shedding. Fresh seeds of both Bur and Cvi were produced at 23/17°C (16 h light/8 h dark), imbibed and placed at a range of temperatures in the dark. The effect of dark incubation on depth of seed dormancy was determined by observing subsequent germination in the light.

Moist storage of Bur seed

Bur seeds were incubated in the dark at a range of temperatures from 5 to 30°C for periods of up to 200 d

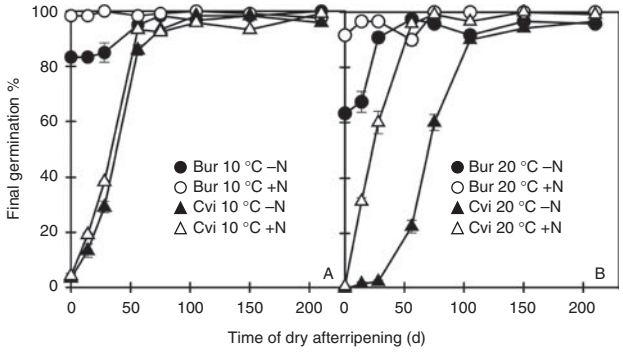

Figure 4. Final germination percentages of seeds in the light following dry afterripening for increasing periods. (A) 10°C ± KNO_3; (B) 20°C ± KNO_3; the concentrations of KNO_3 are: 1 mM KNO_3 for Bur seeds and 10 mM KNO_3 for Cvi seeds. Data represent the mean ± standard error. Absent error bars indicate that the symbol is larger than the error.

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before recording germination in the light at 20°C (Fig. 5). Seeds were significantly ($P < 0.001$) affected by the duration and temperature of dark incubation. The impact of dark incubation temperature was significant at all durations greater than 3 d. In general, dark incubation initially increased final germination percentage and then final percentage germination began to decline. The data suggested that there is a proportion of seeds that can germinate in the dark; dark incubation then enhances sensitivity to light; but then further dark induces secondary dormancy and thus decreasing light sensitivity. Consequently, final germination (germination in the dark plus germination following transfer to light) increases and then decreases with increasing dark incubation time. The rate of change in dormancy differs at each incubation temperature. The initial increase is fastest at 30°C and slower at lower temperatures. Interestingly, seeds incubated at the apparently neutral temperatures of 10 and 15°C changed least with increasing time in the dark (Fig. 5).

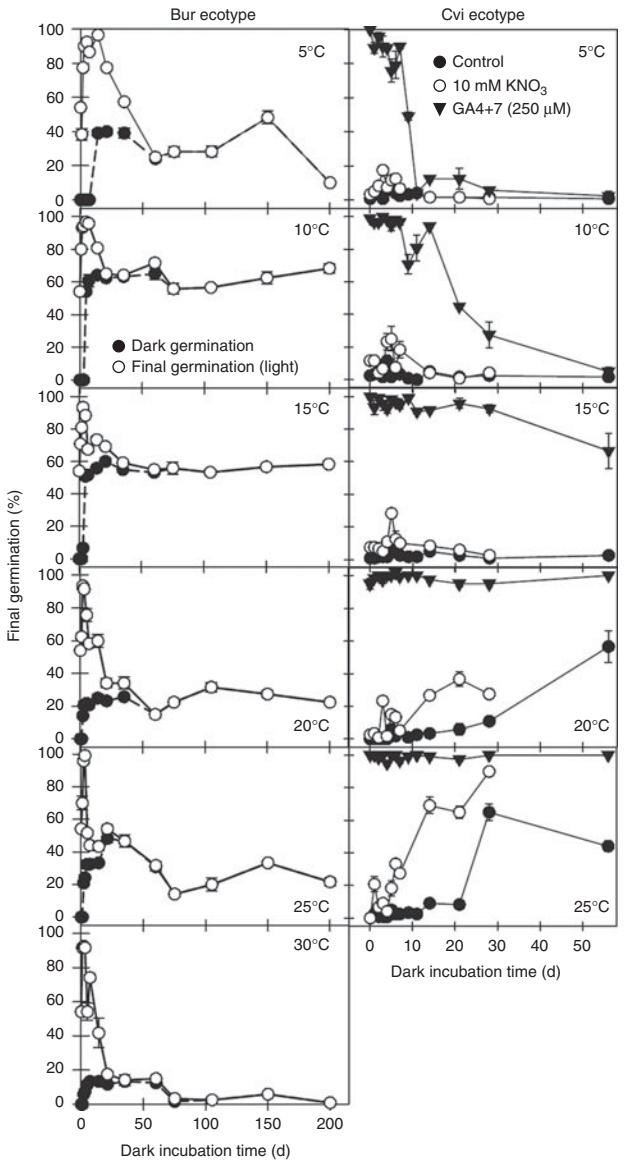

Figure 5. Germination responses of Bur and Cvi seeds after periods imbibed in the dark at different temperatures. For the Bur ecotype (left-hand panels) percentage germination in the dark (5–30°C; filled circles) and the final germination percentage after subsequent transfer to light at 20°C (dark plus light; open circles) are shown. For the Cvi ecotype (right-hand panels) there was no germination in the dark; therefore the final germination percentage after subsequent transfer to light at 20°C with either nitrate (10 mM KNO_3) or 250 μM GA (dark plus light; open circle, closed triangle, respectively) are shown. A buffer control (closed circle) only is shown for comparison, as buffer and water controls were not significantly different. Data are the mean ± standard error. Absent error bars indicate that the symbol is larger than the error.

Moist storage of Cvi

The impact of dark incubation on the deeply dormant winter annual Cvi (Fig. 5) differed greatly from that described above for the less dormant Bur. Cvi seeds did not germinate on water during incubation at temperatures below 15°C. Therefore to observe changes in the depth of dormancy in dark-imbibed seeds they were subsequently placed on either nitrate or 250 μM GA. Interestingly, the percentage of seeds germinating on GA declined rapidly after 6 d of incubation at 5°C, indicating that depth of dormancy increased. This increase in dormancy was slower at 10°C and slower still at 15°C. In contrast, percentage germination at 20 and 25°C on GA remained high, and percentage germination on nitrate, water and buffer all increased slowly with dark incubation time, indicating (opposite to that observed in Bur) that dormancy was being relieved. This relief was more rapid at 25 than at 20°C.

Discussion

It is clear from the data presented here that environmental signals during both seed maturation and post-shedding determine the depth of physiological dormancy and therefore the germination response to the ambient environment in *Arabidopsis* seeds. However, there is also a genetic component to depth of dormancy, and this becomes evident when seeds from widely different ecotypes representing winter (Cvi, Ler) and summer (Bur) annual phenotypes are produced in the same constant environments (Figs 1 and 2; He *et al.*, 2014, Huang *et al.*, 2014). These differences in seed response to ambient germination conditions are then greatly enhanced by seed maturation in different environments (e.g. Figs 1 and 2). Further variation in response develops following shedding when seeds do not receive the

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full complement of environmental signals required for germination and enter the soil seed bank in both dry and imbibed states (e.g. Figs 3, 4 and 5). Species seed dormancy characteristics cannot therefore be easily defined, as seed dormancy is a dynamic state subject to within-species adaptation to local environments.

Bur and Cvi ecotypes have similar responses to afterripening but distinct germination responses to temperature and light

Afterripening

Seed dormancy in both Bur and Cvi seeds was released by dry AR, resulting in a widening of the range of permissible germination temperatures. Thus, during AR, germination requirements became less specific, and the capacity of seeds to complete germination was enhanced. Seeds of Cvi are deeply dormant and so the effect of AR was particularly large in this ecotype. In contrast 80% of Bur seeds were able to germinate at shedding, in the absence of AR.

Temperature

The temperature during seed maturation played an important role in determining seed dormancy and germination potential of all three ecotypes. Seed maturation at lower temperature (15 rather than 20°C) enhanced dormancy; this temperature effect can override the influence of other environmental factors (light, nitrate). Similarly, seeds of the Col ecotype matured at 15°C were also reported to have decreased germination in response to cold stratification compared to those matured at higher temperatures (e.g. 20°C) (Donohue *et al.*, 2008; Kendall *et al.*, 2011).

Irrespective of maturation environment, fresh Bur seeds were less dormant than fresh Cvi seeds, and they had an opposite thermal response following AR. Cvi seeds tend to germinate more readily at lower temperatures, consistent with a winter-annual phenotype, while Bur seeds show the reverse response and germinate more readily at higher temperatures consistent with a summer-annual phenotype. In addition, short periods of low temperature (5°C) appeared to be an efficient way to release dormancy of Bur but not Cvi, which first requires a period of AR to become sensitive to low temperature (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007). Germination of Ler seeds produced at 20°C had high germination at low temperatures coupled to high-temperature thermodormancy. In contrast, Bur seeds produced in 20°C showed higher germination at high temperature. Interestingly, low maturation temperature (15°C) led to high-temperature thermodormancy in Bur seeds, but they still remained highly sensitive to exogenous nitrate. Picó (2012) provides evidence that the two phenotypes, i.e. winter- and summer-annual life cycles, can occur in the same population, with their proportions able to change systematically along an altitude gradient. The associated changes in maturation temperature along the gradient may alter depth of dormancy and therefore their behaviour from summer to winter annuals.

Following shedding, extended moist incubation at low temperature in the absence of light was shown to induce secondary dormancy in both Bur and Cvi (Fig. 5). The minimum duration of exposure to low temperature that induces secondary dormancy differs between the two ecotypes (i.e. 4 d in Cvi and 14 d in Bur). The time required to induce secondary dormancy in Bur is determined by temperature: the rate of induction is greater with higher temperatures. This response to warm, moist treatment is likely an adaptation to the cold/wet Burren climate, to avoid germination before winter in a warm spell following shedding in autumn. Cvi also responds to warm, moist treatment that releases dormancy, but incubation at low temperature enhanced dormancy. Footitt *et al.* (2011) showed that deep dormancy in Cvi in the field was lost in the warm, moist soils of the UK summer in less than 30 d, whereas a similar level of dormancy relief would require 200 d of dry afterripening. In its natural environment, rapid loss of dormancy in warm, moist conditions may enable rapid completion of dormancy relief by AR to allow germination when rain comes after the dry summer of the Cape Verdi Islands.

Light

The germination of the strongly dormant Cvi seeds had an absolute requirement for light, whatever conditions they were exposed to, but seed dormancy was not released by light without those seeds first being exposed to a period of AR. However, in fresh Bur seeds, light was not absolutely required for germination of all seeds, but exposure to light led to higher germination in Bur, especially at high temperatures. In view of this response, it is perhaps surprising that no seed germination was recorded in samples recovered from the soil over a 1-year cycle (Footitt *et al.*, 2013). This may be because seeds in the surface layers of soil are exposed to limiting water availability much of the time, and this mild stress enhances the tendency of seeds to enter seconary dormancy, introducing yet another source of variation in response. This is consistent with the observation that secondary dormancy is rapidly induced in the dark at low water potential (Pons, 1991). In Bur, seeds exhibit high sensitivity to light following short periods of dark incubation, but this sensitivity decreases as the temperature of dark incubation increases.

Nitrate

Nitrate played a positive role in the promotion of seed germination following shedding in all ecotypes, and

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could also reduce the time of AR required for the completion of germination. Seeds of Cvi have relatively deep dormancy and had a greater need for AR to increase sensitivity to other environmental signals, such as light, nitrate and temperature. Nitrate was found to play a positive role in the promotion of Cvi seed germination by Alboresi *et al.* (2005). The freshly harvested, but less dormant, Ler seeds exhibit thermodormancy at 25°C like Cvi seeds; however, nitrate provided exogenously during imbibition released dormancy in a dose-dependent manner. In contrast to Ler and Cvi, the shallowly dormant Bur seeds were always highly sensitive to nitrate. Interestingly, higher concentration of nitrate can delay the germination of Bur seeds by 2 d, which is consistent with previous work showing that Bur plants are poorly tolerant of high-nitrate nutrition (Chardon *et al.*, 2010).

The post-shedding response to nitrate is related to endogenous nitrate in the seed before shedding (Alboresi *et al.*, 2005). We therefore looked at the impact of nitrate level in the growing media of the mother plant. In our hands, Cvi was less tolerant of reduced nitrogen content and did not survive on compost with low N. Therefore Ler, another winter-annual ecotype, was substituted for Cvi in these experiments. High-nitrate feeding of the mother plants resulted in less dormant Ler seeds. In contrast, nitrate supplied to Bur mother plants had little impact on seed dormancy; seeds produced from both nitrate regimes showed similarly high sensitivity to exogenously supplied nitrate during subsequent imbibition. However, in this study, only Ler seeds produced under high N at 20°C had significantly higher nitrate content. The absence of a response in Bur may result from its reported high nitrogen use efficiency (Chardon *et al.*, 2010). In general, increasing maturation temperature increased the nitrate content of Ler seeds and enhanced the difference in seed nitrate content between seeds produced under different nitrate regimes. This indicates that temperature plays an important role in determining the seed nitrate content in Ler. In general, seed maturation temperature showed a predominant effect on dormancy in both Bur and Ler seeds (Fig. 2).

We have demonstrated the complexity in seed response to environmental signals during seed development and subsequent imbibition that magnifies the genetic variation in dormancy levels between ecotypes. These responses to environmental signals can be additive, synergistic or inhibitory, depending on the combination, providing an almost infinite spectrum of dormancy levels. Seeds are sometimes considered as a comparatively simple experimental system for the study of individual molecular mechanisms in single fixed environments. While this can advance our understanding of genes and their function, and so is very worthwhile, this approach masks the truly complex and dynamic ability of seeds to sense and interpret environmental signals to determine germination timing, a major life-cycle transition, through the regulation of dormancy.

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Conflicts of interest

None.

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