Seed Science Research (2014) **24**, 79–89
doi:10.1017/S0960258514000051

Fluctuating temperatures terminate dormancy in *Cynara cardunculus* seeds by turning off ABA synthesis and reducing ABA signalling, but not stimulating GA synthesis or signalling

H. Roberto Huarte[1]*, Virginia Luna[2], Eduardo A. Pagano[3], Jorge A. Zavala[4] and Roberto L. Benech-Arnold[5]

[1]Facultad de Ciencias Agrarias, Universidad Católica Argentina, 183 Ramón Freire, 1426, CABA, Argentina; [2]Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, 5800, Rio Cuarto, Argentina; [3]Cátedra de Bioquímica; Facultad de Agronomía, Universidad de Buenos Aires, 4453 San Martín Avenue, 1417DSE, CABA, Argentina; [4]INBA/Cátedra de Bioquímica; Facultad de Agronomía, Universidad de Buenos Aires, 4453 San Martín Avenue, 1417DSE, CABA, Argentina; [5]IFEVA/Cátedra de Cultivos Industriales/CONICET/Facultad de Agronomía, Universidad de Buenos Aires, 4453 San Martín Avenue, 1417DSE, CABA, Argentina

(Received 18 October 2013; accepted after revision 25 February 2014)

Abstract

Fluctuating temperatures terminate seed dormancy in many species, including *Cynara cardunculus* (L.) var. *sylvestris*. Termination of physiological dormancy requires low ratios of abscisic acid (ABA)/gibberellins (GA). In a previous paper we have shown that physiological responses to fluctuating temperatures comprise a reduction of abscisic acid (ABA) content and sensitivity. However, a possible stimulation of GA synthesis was also suggested as part of the mechanism. That possible stimulation, as well as the identification of potential regulatory sites for ABA and GA metabolism and signalling involved in the termination of dormancy by fluctuating temperatures, are yet to be determined. In this study, we measured GA content and sensitivity in seeds incubated under constant and fluctuating temperatures. We also assessed the expression of several genes involved in ABA and GA metabolism and signalling. Our results show that fluctuating temperatures reduce ABA/GA ratios through a reduction in ABA accumulation during incubation but without altering GA synthesis as compared to that observed under constant temperatures. On the other hand, fluctuating temperatures did not increase sensitivity to GA. Fluctuating temperatures reduced the expression of *CycaNCED* and *CycaABI5* (ABA synthesis and signalling genes) with a temporal pattern that coincides with the interruption of ABA accumulation that precedes germination of seeds incubated under fluctuating temperatures. However, fluctuating temperatures did not modify the expression of *CycaCYP707A2* (ABA inactivation) as compared to that observed under constant temperatures. Consistent with our determinations of GA content and sensitivity, fluctuating temperatures did not modify the expression of GA synthesis (*CycaGA3ox*) and signalling genes (*CycaRGL2* and *CycaGAI*) in relation to that observed at constant temperatures. These results show that fluctuating temperatures terminate dormancy in *Cynara cardunculus* seeds through an interruption in ABA accumulation and a reduction in ABA signalling exerted at the level of *CycaNCED* and *CycaABI5* expression.

Keywords: abscisic acid, dormancy, fluctuating temperatures, gene expression, gibberellins

Introduction

Dormancy could be defined as the failure of an intact viable seed to complete germination in a specified period of time under any combination of normal physical environmental factors that are otherwise favourable for its germination (Bewley, 1997; Baskin and Baskin, 2007). Dormancy is a common trait in non-domesticated plants which increases the ability of a species to avoid competition between individuals of the same species and prevents germination out of place or season (Finkelstein *et al.*, 2008). Seeds are dormant at the time of dispersal from the mother plant (Hilhorst, 1995) and dormancy is progressively lost as a consequence of seed interaction with environmental signals such as

*Correspondence
Email: robertohuarte@uca.edu.ar

soil temperature and moisture (Benech-Arnold *et al.*, 2000). However, in many species, dormancy is terminated only when seeds are exposed to a second set of signals, such as light, nitrate and fluctuating temperatures (Footitt *et al.*, 2011). This second set of signals indicates if existing conditions are suitable to terminate dormancy and induce the completion of germination (Finch-Savage and Footitt, 2012).

In species with physiological dormancy, abscisic acid (ABA) and gibberellins (GA) have an antagonistic role (Yamaguchi, 2008; Nambara *et al.*, 2010; Linkies and Leubner-Metzger, 2012). The action of ABA and GA is supported by numerous genetic studies using biosynthesis and signalling mutants. In particular, the ratio between the contents of these two hormones and their respective signalling pathways are important in regulating induction, maintenance and termination of dormancy (Finkelstein *et al.*, 2008). The termination of dormancy requires low ABA/GA ratios. In contrast, the maintenance of dormancy is associated with high ABA/GA ratios (Finch-Savage and Leubner-Metzger, 2006). In addition to hormone content, dormancy termination is characterized by a decrease in ABA sensitivity and an increase in GA sensitivity (Linkies and Leubner-Metzger, 2012).

Hormone content is the result of two processes: synthesis and catabolism (Umezawa *et al.*, 2006). A reduction in ABA content by the effect of a dormancy-terminating factor is due to a slow, or even turned off, synthesis, an increased inactivation or to the co-ordinated action of both processes. ABA synthesis is mainly modulated by NCED (9-*cis*-epoxycarotenoide dioxygenase) and the activity of this enzyme has been proposed as a key regulatory step in ABA synthesis (Holdsworth *et al.*, 2008). ABA inactivation comprises two processes: ABA hydroxylation or conjugation (Yamaguchi *et al.*, 2007). ABA hydroxylation is accomplished by the activity of ABA 8′-hydroxylase (Millar *et al.*, 2006) and ABA conjugation is achieved by the ABA-glycosyltransferase (Nambara and Marion-Poll, 2005). Likewise, an increment of GA content could be related to a higher GA synthesis, a reduction in GA deactivation or the parallel action of both processes (Yamauchi *et al.*, 2007).

The way in which light terminates seed dormancy altering ABA/GA ratios has been well studied (e.g. Seo *et al.*, 2009). Light modulates the transcription of many genes involved in ABA and GA synthesis and catabolism (Yamaguchi, 2008; Nambara *et al.*, 2010) allowing, as a result, a reduction in the ABA/GA ratio. Toyomasu *et al.* (1993, 1998) observed that light promotes GA synthesis through an up-regulation of a gene that encodes a GA 3-β-hydroxylase (i.e. the enzyme that catalyses the last step in bioactive GA synthesis). In addition to this, Yamauchi *et al.* (2007) determined that light down-regulates a GA 2-oxidase gene, which codes for an enzyme that inactivates GA. On the other hand, light reduces seed ABA content; in this regard, Seo *et al.* (2006) observed a negative effect on the expression of *AtNCED*6, while Toyomasu *et al.* (1994) determined an up-regulation of the expression of *CYP707A2*, a gene encoding an ABA 8′-hydroxylase. Light treatments are also known to modify hormone signalling networks (Arana *et al.*, 2006; Piskurewicz *et al.*, 2008; Graeber *et al.*, 2012).

In contrast, the way in which fluctuating temperatures alter ABA/GA ratios to terminate dormancy is poorly understood (Hu *et al.*, 2012). *Cynara cardunculus* (L.) var. *sylvestris* seeds display an almost absolute requirement for fluctuating temperatures for dormancy termination. A water relations analysis of seed germination at fluctuating and constant temperatures using Gummerson's hydrotime model (Gummerson, 1986) was carried out using *C. cardunculus* seeds and revealed that incubation under fluctuating temperatures displaces the mean base water potential (ψ_b (50)) towards more negative values (Huarte and Benech-Arnold, 2005). This implies that fluctuating temperatures terminate dormancy in this species through an enhancement of embryo potential to overcome a physical restraint for germination. This displacement of ψ_b (50) towards more negative values has also been observed in ABA mutants or in seeds incubated in the presence of GA (Ni and Bradford, 1992, 1993; Alvarado and Bradford, 2005) suggesting a hormonal control behind the above-mentioned displacement as a result of incubation under fluctuating temperatures. Indeed, studies in *C. cardunculus* (L.) have shown that fluctuating temperatures terminate dormancy by reducing ABA content and sensitivity (Huarte and Benech-Arnold, 2010). Furthermore, germination behaviour at constant and fluctuating temperatures and changing ABA/GA ratios, using exogenously applied ABA, GA and their synthesis inhibitors (i.e. fluridone and paclobutrazol, respectively), revealed that fluctuating temperatures could terminate dormancy by eliciting a reduction in ABA/GA ratio (Huarte and Benech-Arnold, 2010). Indeed, GA_3-treated and fluridone-treated seeds incubated under constant temperatures germinate as if they had been incubated under fluctuating temperatures; in contrast, the presence of paclobutrazol in the incubation medium when the seeds were incubated under fluctuating temperatures, inhibited germination as if they had been incubated under constant temperatures (Huarte and Benech-Arnold, 2010). Taken together, these results suggest that fluctuating temperatures terminate dormancy by means of a change in ABA/GA ratio through a reduction in ABA content and sensitivity, but also through an increase in GA content and/or sensitivity. Alternatively, fluctuating temperatures could modify ABA/GA ratio just by reducing ABA content and sensitivity without modifying GA synthesis and sensitivity. In this context, the reduction

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

of ABA content at fluctuating temperatures might be expected to be triggered at the level of gene expression through a negative regulation in the expression of an ABA synthesis gene (*NCED*) or a positive regulation of an ABA inactivation gene (*CYP707A2*) as has been observed for other signals that terminate dormancy, such as light or nitrate (Nambara *et al.*, 2010). Likewise, the reduction in ABA sensitivity at fluctuating temperatures could be exerted by a reduction in the expression of ABA signalling genes (e.g. *ABI5*) (Raghavendra *et al.*, 2010). ABI5 is a positive regulator of ABA signalling through the induction of transcription of genes involved in processes promoted by ABA (Chen *et al.*, 2008). Similarly, if fluctuating temperatures stimulate GA synthesis or sensitivity and this stimulation takes place at the level of gene expression, this should be reflected in a positive regulation of a GA synthesis gene (e.g. GA 3-oxidase) (Yamaguchi, 2008) or a negative regulation of GA signalling genes such as RGL2 and GAI (Hartweck, 2008).

The objectives of this paper were to determine if: (1) the reduction of ABA content that results from incubation under fluctuating temperatures is exerted through changes in the transcription of ABA synthesis (*NCED*) and/or inactivation genes (*CYP707A2*); (2) dormancy termination by fluctuating temperatures includes the promotion of GA synthesis and/or sensitivity; and (3) if an eventual enhancement of GA content or sensitivity by fluctuating temperatures is exerted at the level of transcription of GA synthesis genes (i.e. *GA3ox*) and/or GA signalling genes (i.e. *RGL2* and *GAI*).

Materials and methods

Plant material

C. cardunculus (L.) mature achenes (hereafter termed 'seeds') were hand collected from a plot at the School of Agricultural Sciences, Argentine Catholic University, Buenos Aires, Argentina (34°34′W, 58°26′S) during January 2010 and 2011, and from an infested roadside in Azul, Buenos Aires Province, Argentina (36°47′W, 59°42′S) at the time of their natural dispersal. After cleaning, seeds were kept in paper bags at −18°C to maintain their initial level of dormancy.

Role of GA in seed responses to fluctuating temperatures

Gibberellin extraction and quantitation by LC–ESI–MS–MS

Analysis of gibberellins was carried out on seeds collected in 2010. Seeds were incubated in distilled water for 0, 1, 3, 4 and 5 d. After each incubation period, three samples (biological triplicates) of embryos were excised from the achenes and frozen in liquid N_2. Samples were freeze-dried, ground to powder with a mortar and pestle, and weighed (100–200 mg per sample). Extraction of gibberellins was performed with 5 ml of methanol:water (80:20, pH 2.8) at 4°C. After centrifugation (1 min, maximum speed), buffer was collected and the pellet was then re-extracted with 2 ml of fresh buffer (pH 2.8) for an additional 4 h. A 50 ng aliquot of each of deuterated GA_1, GA_3, GA_4, GA_8 and GA_{34} (Lew Mander, Australian National University, Canberra, Australia) was added as internal standards. Extracts were transferred to 50-ml tubes and mixed with ethyl acetate and partitioned. Then, the organic phase was extracted and evaporated at 37°C in a Speed-Vac. After methanol evaporation, the volume of the remaining aqueous fraction was adjusted with water to 15 ml, and the pH was lowered to 2.5 with diluted HCl. The 15 ml aqueous extract was then partitioned three times against 5 ml of ethyl acetate (water saturated). After solvent evaporation, samples were taken to the University of Río Cuarto (Córdoba, Argentina) for hormone analysis.

Liquid chromatography (LC). Analyses were performed using an Alliance 2695 (Separation Module, Waters, Milford, Massachusetts, USA) quaternary pump equipped with auto-sampler. A Restek C18 (Restek, Bellefonte, Pennsylvania, USA) column (2.1 × 100 mm, 5 μm) was used at 28°C, with injected volume 10 μl. The binary solvent system used for elution gradient consisted of 0.2% acetic acid in H_2O (solvent B), and methanol (MeOH; solvent A), at a constant flow-rate of 200 μl min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied [*t* (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration.

MS/MS experiments. MS/MS experiments were performed on a Micromass Quatro UltimaTM^{PT} double quadrupole mass spectrometer (Micromass, Manchester, UK). All analyses were performed using turbo ion spray source (ESI) in negative ion mode with the following settings for gibberellins: capillary voltage −3250 V, energy cone 35 V, RF Lens1 (20), RF Lens 2 (0.3), source temperature 100°C, desolvation temperature 350°C, gas cone 100 l h^{-1}, gas desolvation 701 l h^{-1}, collision cell potential of 15 V and multiplier (650). MS/MS parameters were optimized in infusion experiments using individual standard solutions of each hormone. MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the double quadrupole mass spectrometer, and mass was analysed using the second analyser of the instrument. In negative mode, the spectrum for

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

each hormone gave deprotonated molecules [M−H]. Quantitation was performed by injection of samples in multiple reaction monitoring (MRM) modes, since many compounds could present the same nominal molecular mass. The combination of parent mass and unique fragment ions was used to selectively monitor hormones. MRM acquisition was performed by monitoring the 348/242 and 350/244 transitions for GA_1 and ($^2H_2$)-GA_1; 345/221 and 347/223 for GA_3 and ($^2H_2$)-GA_3; 332/244 and 334/246 for GA_4 and ($^2H_2$)-GA_4; 364/276 and 366/278 for GA_8 and ($^2H_2$)-GA_8, and 347/ 242 and 349/244 for GA_{34} and ($^2H_2$)-GA_{34} respectively, with dwell 2000 ms for each transition. Data were acquired and analysed using MassLynxTM 4.1 and QuanLynx^{TM} 4.1 (Micromass) software. For quantitation, values were obtained from a calibration curve previously constructed using known amounts of each hormone and their pure standard (Sigma, St. Louis, Missouri, USA)/deuterated internal standard ratio. Data were subjected to analysis of variance (Statistix 8.0, Analytical Software, Tallahassee, Florida, USA). Tukey's test at 5% level of probability was used for comparison between means.

Sensitivity to GA_3

Sensitivity to GA_3 as affected by the incubation regime (constant or fluctuating) was determined in the 2011 seed lot by means of two different approaches.

The first approach (test 1) involved incubation in 7 ml of a mixed solution of GA_3 (Phytotechnology Laboratories, Shawnee Mission, Kansas, USA) (0, 10, 50, 250, 1000 and 1500 μM) plus 750 μM of Trinexapac-ethyl (TE). The pH of each solution was adjusted to 6.7. To determine the dose of TE that inhibits germination (750 μM), seeds were dark incubated for 14 d at fluctuating (20°C, 12 h/10°C, 12 h) and constant temperatures (15°C) in a range of TE concentrations (0, 125, 250, 500, 750 and 1000 μM). Germination in the presence of 750 and 1000 μM of TE was similarly reduced in both thermal treatments to 15 and 10% (750 and 1000 μM, respectively).

The second approach (test 2) involved incubation in 7 ml of a mixed solution of GA_3 (10, 50, 250, 1000 and 1500 μM) plus 250 μM ABA (Phytotechnology Laboratories). To determine the ABA dose that inhibits germination (250 μM) seeds were dark incubated in the presence of ABA (0, 50, 100 and 250 μM) at fluctuating (20°C, 12 h/10°C, 12 h) and constant temperatures (15°C).

In both tests, treatments were factorial combinations of five GA_3 doses and two thermal conditions (15°C versus 20/10°C). The germination data were subjected to analysis of variance (Statistix 8.0, Analytical Software). Tukey's test at 5% level of probability was used for comparison between means.

RNA extraction and cDNA synthesis

Three replicates of 20 seeds were incubated for 0, 1, 3 and 5 days at fluctuating (20/10°C) and constant (15°C) temperatures. Between 15 and 17 embryos (≥350 mg) per sample were isolated from pericarp and frozen in liquid N_2 and stored at −20°C until used for RNA extraction. RNA was extracted from embryos using the mRNA Isolation Kit (Roche Molecular Biochemicals, GmbH, Mannheim, Germany) and cDNA was made with at least 10 ng of RNA sample by means of Revert Aid M-Mul V Reverse transcriptase system (Fermentas International Inc., Burlington, Ontario, Canada). RNA concentration was estimated by absorbance at 260 nm with a fluorometer Qubit® 2.0 (Invitrogen, Carlsbad, California, USA). The integrity and purity of RNA was checked with a 260/280 nm absorbance ratio.

RNA was converted to cDNA as follows: (1) the RNA concentration of each sample was determined; (2) according to the RNA concentration, a solution containing variable volumes of miliQ water and RNA plus 1 μl oligo-p (dT) (Biodynamics, Buenos Aires, Argentina) to reach a final volume of 11 μl was made. Each sample was placed in a thermocycler for 5 min at 70°C and 3 min at 4°C. Afterwards, 8 μl of a solution containing 4 μl of reverse transcriptase buffer (RT), 2 μl of dinucleotides (dNTPs) 10 mM, 0.5 μl of RNAse inhibitor (Fermentas) and 1.5 μl of MiliQ water was added. The reaction was continued for 5 min at 37°C when an additional 1 μl of RT (Fermentas) was added, with the following conditions: for 60 min at 42°C, 10 min at 70°C and finally reducing the temperature to 4°C. The cDNA was stored at −20°C until used.

Search for Cynara *sequences encoding putative orthologues for ABA and GA metabolism and signalling proteins and RT-PCR*

Transcript accumulation for *NCED, CYP707A2, ABI5, GA3ox, GAI* and *RGL2* genes was estimated by semi-quantitative reverse transcriptase–polymerase chain reactions (RT-PCR). The cDNA sequences were obtained from seed RNA through RT-PCR. Degenerate primer pairs were designed to amplify fragments between 192 and 620 bp (Table 1) within highly conserved regions of candidate genes from other species (Table 1). Aligned sequences used for *NCED* primer design were: DQ173543.1 (GenBank accession number) (*Rumex palustris* NCED9), XM002862948.1 (*Arabidopsis lyrata* NCED9), NM106486.2 (*Arabidopsis thaliana*, NCED9), AB120110.1 (*Lactuca sativa*, NCED4). For *CYP707A2*, sequences used were: AB235920.1 (*Lactuca sativa* LsABA8ox4), NM128466.2 (*A. thaliana* CYP707A2), GU559990.1 (*Prunus avium* CYP707A3), DQ145932.1 (*Hordeum vulgare* subsp. *vulgare* ABA 8′-hydroxylase 1). For *ABI5*, sequences used were:

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

Table 1. Primers used for semi-quantitative RT-PCR

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified fragment (bp)
Actin	CCGGTATTGTGCTGGATTCT	GTCAAGAGCGACGTATGCAA	230
NCED	ACRATGATCCAYGAYTTCGC	CAATCKGGTACKTCGATCCA	192
CYP707A2	GGWTACAACTCCATGCCTYT	ACCTCGAATCTWGAAGGRTC	590
ABI5	GAGATGACNCTBGAGGAGTT	TTCTTGATCATCCTCCTCTG	620
GA3ox	ATGTGGTCYGAAGGNTTCAC	GGACARGCYGGGTAKGAATT	260
GAI	CTCAAGTTCGCYCACTTCAC	GTGMAGCTCGAAMACMGAGT	384
RGL2	GGGCTTAAWCARGGGATGCA	CGGTGRAGCTCRAAWACCGA	290

R = A + G; Y = C + T; N = A + C + G + T; M = A + C; W = A + T.

EU964768.1 (*Zea mays*), NM129185.3 (*A. thaliana*), AB193553.1 (*Triticum aestivum*), XM002970700.1 (*Selaginella moellendorffii*), AY150676.1 (*H. vulgare* subsp. *vulgare*). For *GA 3-oxidase*, the following sequences were used: AB012205.1 (*L. sativa*), AB303422.1 (*Allium fistulosum*), AB010991.1 (*Solanum lycopersicum*), AB613270.1 (*Torenia fournieri*), AB032198.1 (*Nicotiana tabacum*), DQ641497.1 (*R. palustris*), AJ006453.1 (*Cucurbita maxima*). For *GAI*, sequences used were: AY781175.1 (*Oryza sativa*), DQ062091.1 (*Sacharum officinarum*), NM101361.2 (*A. thaliana*), EU112606.1 (*Helianthus annus*). For *RGL2*, sequences used were: NM111216.2 (*A. thaliana*), XM002519168.1 (*Ricinus communis*) and DQ007884.1 (*Malus* × *domestica*).

PCR conditions for *NCED* were as follows: 4 min at 94°C (first cycle); 50 s at 94°C, 1 min at 54°C, 1 min at 72°C (40 cycles) and 7 min at 72°C (last cycle). PCR conditions for *ABI5* were as follows: 4 min at 94°C; 50 s at 94°C, 45 s at 55°C, 1 min at 72°C (38 cycles) and 7 min at 72°C. PCR conditions for *GA3ox* were as follows: 4 min at 94°C, 50 s at 94°C, 50 s at 55°C, 1 min at 72°C (37 cycles) and 7 min at 72°C. PCR conditions for *GAI, RGL2* and *CYP707A2* were as follows: 4 min at 94°C, 50 s at 94°C, 1 min at 50°C, 1 min at 72°C (38 cycles) and 7 min at 72°C.

Actin was selected as a housekeeping gene and used to normalize the amount of starting template. For *Actin* a specific primer was designed by means of OligoPerfect™ Designer (Invitrogen) from a sequence of *Cynara scolymus* (GenBank accession no. AM744951.1). PCR conditions were as follows: 4 min at 94°C (first cycle), 50 s at 94°C, 50 s at 55°C, 1 min at 72°C (34 cycles) and 7 min at 72°C (last cycle).

PCR products were separated on 1.5% agarose gels, stained with SYBR Green (Invitrogen) and visualized by the UVP Doc-It LS Image Acquisition Software (UVP, Upland, California, USA). A 100-bp DNA ladder (Invitrogen) was used as a standard molecular marker. Amplified fragments were cloned into the pGEM®-T Easy vector system (Promega, Madison, Wisconsin, USA). Sequence analysis was performed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, California, USA). The final sequence was analysed and homology searches were carried out using the National Center for Biotechnology Information (NCBI) BLASTx algorithm (www.ncbi.nlm.nih.gov/BLAST). They contained cDNA sequences of genes with very high similarity to *NCED, CYP707A2, ABI5, GA3ox, GAI* and *RGL2* genes of other plant species (GenBank databases). Sequences are shown in Supplementary Figure S1 (available online). They were named and those with a length up to 200 bp were registered in GenBank: *CycaNCED, CycaCYP707A* (accession no. KF769950), *CycaABI5* (KF769951), *CycaGA3ox* (KF769952), *CycaGAI* (KF769953), *CycaRGL2* (KF769954) and *Cycaactin*. A ratio of intensities between bands obtained for each gene and those obtained for *Cycaactin* (in both cases analysed with the National Institutes of Health (NIH) image program) for each thermal treatment and time of incubation was calculated.

Results

Determination of fluctuating temperature requirements to terminate seed dormancy in C. cardunculus *seeds*

To corroborate the requirement of fluctuating temperatures to terminate *C. cardunculus* seed dormancy, seeds were incubated at fluctuating (20°C, 12 h/10°C 12 h) or constant (15°C, 24 h) temperatures. The exposure of seeds to fluctuating temperatures increased total germination ($P = 0.0019$) (Fig. 1). The effect of fluctuating temperatures on germination was observed from day 5 on (mean ± SE: 20 ± 10.4% and 0% for 20/10°C and 15°C, respectively). Maximum germination increased to 81.6 ± 10.1% and 6.6 ± 1.6% for 20/10°C and 15°C, respectively, by day 9 and no further germination was scored until the end of the experiment (day 14).

Role of GA in seed responses to fluctuating temperatures

GA content during seed incubation

The embryonic content of the active GAs (GA_1 and GA_4) [ng (g DW)$^{-1}$] was similar in both thermal

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

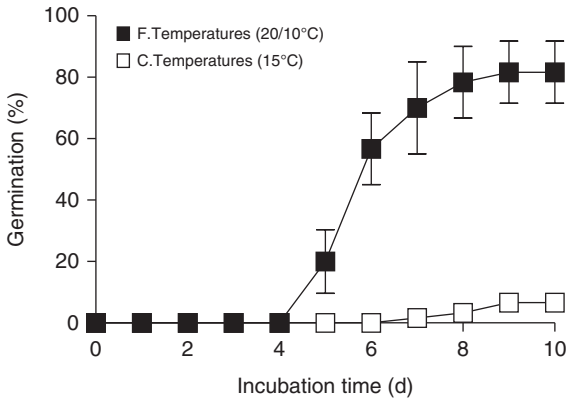

Figure 1. Cumulative germination time courses of *Cynara cardunculus* seeds incubated at fluctuating (20°C/10°C, 12 h thermoperiod) (closed symbols) or constant temperatures (15°C) (open symbols). Data are means of triplicates ± SEs.

treatments during a 5 d incubation period (just prior to the onset of germination in seeds incubated at 10/20°C) (Fig. 2). Similarly, GA_8 content (GA_1 catabolite) did not differ between thermal regimes (Fig. 2C). GA_{34} (GA_4 catabolite) was not detected. To confirm these results, on dry seeds and seeds incubated for 5 d, a second GA quantitation was carried out. In this assay, GA_3 and GA_{34} contents were also studied. The GA content was similar among dry seeds and seeds incubated for 5 d at constant or fluctuating temperatures ($P = 0.3$, $P = 0.65$, $P = 0.15$, $P = 0.44$ and $P = 0.15$ for GA_1, GA_3, GA_4, GA_8 and GA_{34}, respectively) (Fig. 3). These results do not support the possibility of a stimulation of GA synthesis by fluctuating temperatures.

Effect of fluctuating temperatures on seed sensitivity to GAs

Sensitivity of seeds to GA_3 at fluctuating or constant temperature was determined in two independent tests through incubation in GA_3 solutions at different concentrations. Sensitivity to GA_3 was determined as its effectiveness to overcome the inhibitory effect imposed on germination by a solution of Trinexapac-ethyl (TE, 750 μM) (test 1) or ABA (250 μM) (test 2), both at fluctuating and constant temperatures. In both tests, the two main effects, thermal treatment (20/10°C and 15°C) and seed responses to increasing doses of GA_3, differed, as well as their interaction ($P < 0.05$) (Fig. 4A and B). In test 1, germination did not differ between 20/10°C and 15°C when seeds were incubated in the presence of GA_3 (10, 50, 250, 500 and 1500 μM). Germination was similar between water at 20/10°C and 1500 μM of GA_3 at 20/10°C or 15°C. Germination in TE at fluctuating or constant temperatures or water at constant temperatures was low. Likewise, in test 2, germination was also similar between 20/10°C and 15°C when seeds were treated with GA_3 (10, 50, 250, 1000 and 1500 μM) (Fig. 4B). Germination in all ABA + GA solutions was less than that scored in water at 20/10°C or in GA_3 at constant or fluctuating temperatures. Total germination in water at 15°C or ABA (250 μM) at 20/10°C and 15°C was scarce. These results indicate that the effect of fluctuating temperatures is not through an increase in sensitivity to GA.

Identification of Cynara *genes encoding putative ABA and GA metabolism and signalling enzymes*

In order to study the effect of both thermal treatments on the expression of genes that are involved in

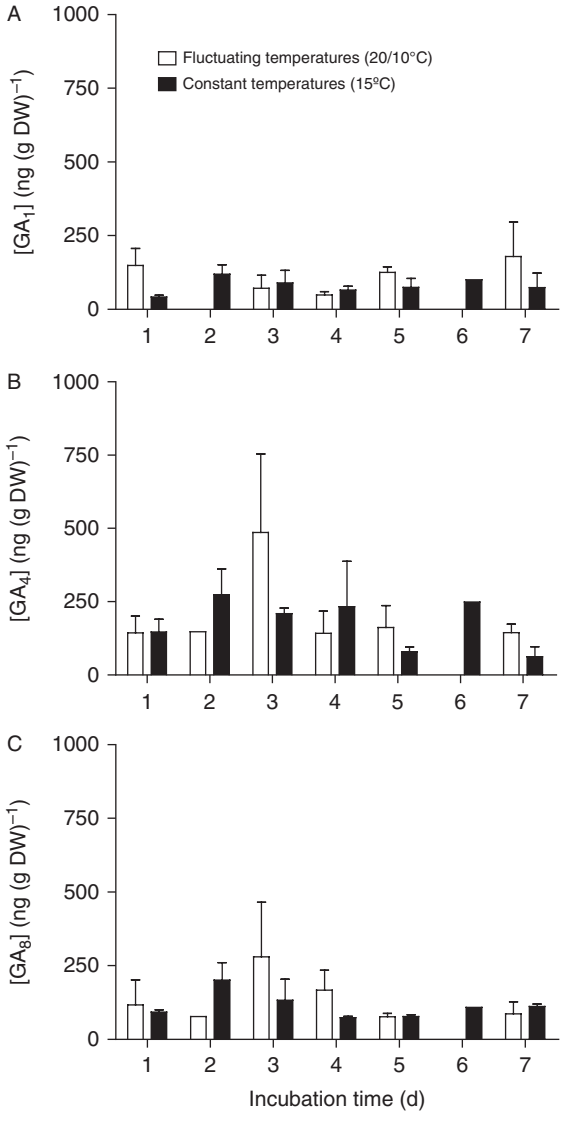

Figure 2. Embryonic content of GA_1 (A), GA_4 (B) and GA_8 (C) as a function of incubation time for *Cynara cardunculus* seeds exposed to fluctuating (20°C, 12 h/10°C, 12 h) (solid bars) or constant (15°C, 24 h) (open bars) temperatures. Values represent the mean of three biological replicates ± SEs.

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

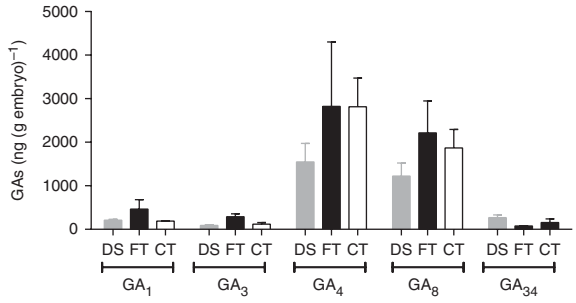

Figure 3. Embryonic content of GA_1, GA_3, GA_4, GA_8 and GA_{34} in dry seeds (DS, grey bars) or after 5 d of incubation at fluctuating (FT, closed bars) and constant temperatures (CT, open bars). Data are means of three biological replicates ± SEs.

ABA and GA synthesis, inactivation and signalling, degenerate primers matching highly conserved nucleotide sequences were designed for *NCED, CYP707A2, ABI5, GA3ox, GAI* and *RGL2*. A total of six cDNA fragments belonging to putative candidate genes (*CycaNCED, CycaCYP707A2, CycaABI5, CycaGA3ox, CycaGAI* and *CycaRGL2*) were isolated. The PCR products (size ranged between 189 and 620 bp) were purified, cloned and sequenced to confirm their putative identity. The obtained sequences are shown in Supplementary Figure S1 (available online). Sequences were compared to all known proteins in the GenBank database using BLASTx. Results of bioinformatics analysis are shown in Supplementary Table S1 (available online). Each of the six sequences identified in *C. cardunculus* appeared as most closely related to the proposed candidate proteins, supporting their expected identities.

Expression of ABA and GA metabolism and signalling genes during incubation at fluctuating or constant temperatures

In order to evaluate if fluctuating temperatures alter ABA and GA metabolism and signalling in relation to constant temperatures acting at the level of gene transcription, the expression pattern of *CycaNCED*, *CycaCYP707A2* and *CycaABI5* (ABA synthesis, inactivation and signalling, respectively), and *CycaGA3ox*, *CycaRGL2* and *CycaGAI* (GA synthesis the former, and GA signalling the remainder) was analysed during incubation under fluctuating and constant temperatures. Incubation under fluctuating temperatures reduced expression of genes involved in ABA synthesis and signalling (Fig. 5A and C) in relation to that observed under constant temperatures. Indeed, *CycaNCED* expression could not be detected beyond 1 d of incubation when it was performed at fluctuating temperatures. In contrast, at constant temperatures, the expression of *CycaNCED* was detected throughout 5 d of incubation (Fig. 5A). Fluctuating temperatures reduced the expression of *CycaABI5* in relation to that observed at constant temperatures (Fig. 5C). Differences were just found on day 5 of incubation ($P = 0.017$). The expression of *CycaCYP707A2* was not affected by the thermal treatment ($P = 0.26$, 0.92 and 0.11 for days 1, 3 and 5, respectively) (Fig. 5B). These results are in full agreement with our previous results reporting ABA content and ABA sensitivity reductions as a result of incubation under fluctuating temperatures that terminate dormancy (Huarte and Benech-Arnold, 2010). In contrast, fluctuating temperatures did not modify the expression of GA synthesis (*CycaGA3ox*) and signalling (*CycaRGL2* and *CycaGAI*) genes in relation to that observed at constant temperature (Fig. 5D–F). Transcript expression for *CycaGA3ox* was detected by 3 d after incubation and did not differ between 20/10°C and 15°C ($P = 0.20$ and 0.77, for days 3 and 5 respectively). Likewise, transcript expression for *CycaRGL2* and *CycaGAI* was similar among treatments ($P = 0.26$, 0.07 and 0. 34 for days 1, 3 and 5, respectively, and $P = 0.15$ and 0.97 for days 1 and 5, respectively). These results are also in

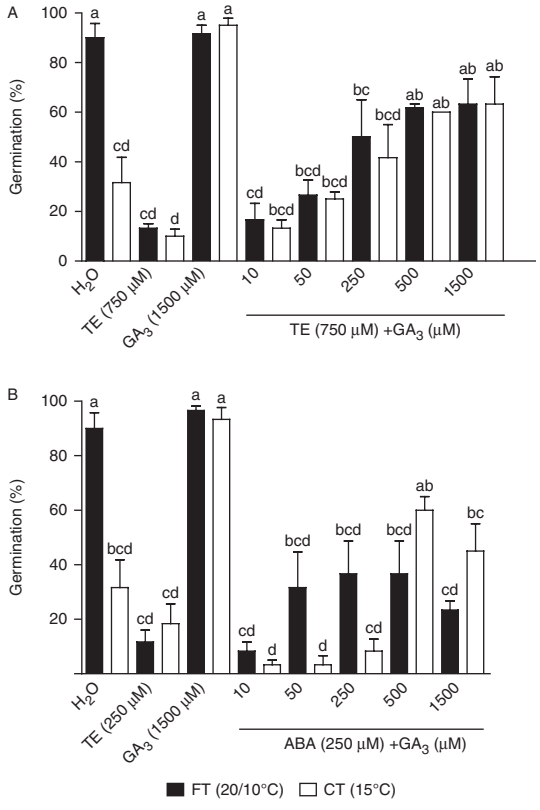

Figure 4. Final germination percentages of *Cynara cardunculus* seeds incubated at fluctuating temperatures (20°C, 12 h/10°C, 12 h) (closed bars) or constant (15°C, 24 h) (open bars) temperatures incubated in the indicated solutions. Vertical bars indicate the SEs. Similar letters at the top of each bar indicate no differences according Tukey's test ($P < 0.05$).

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

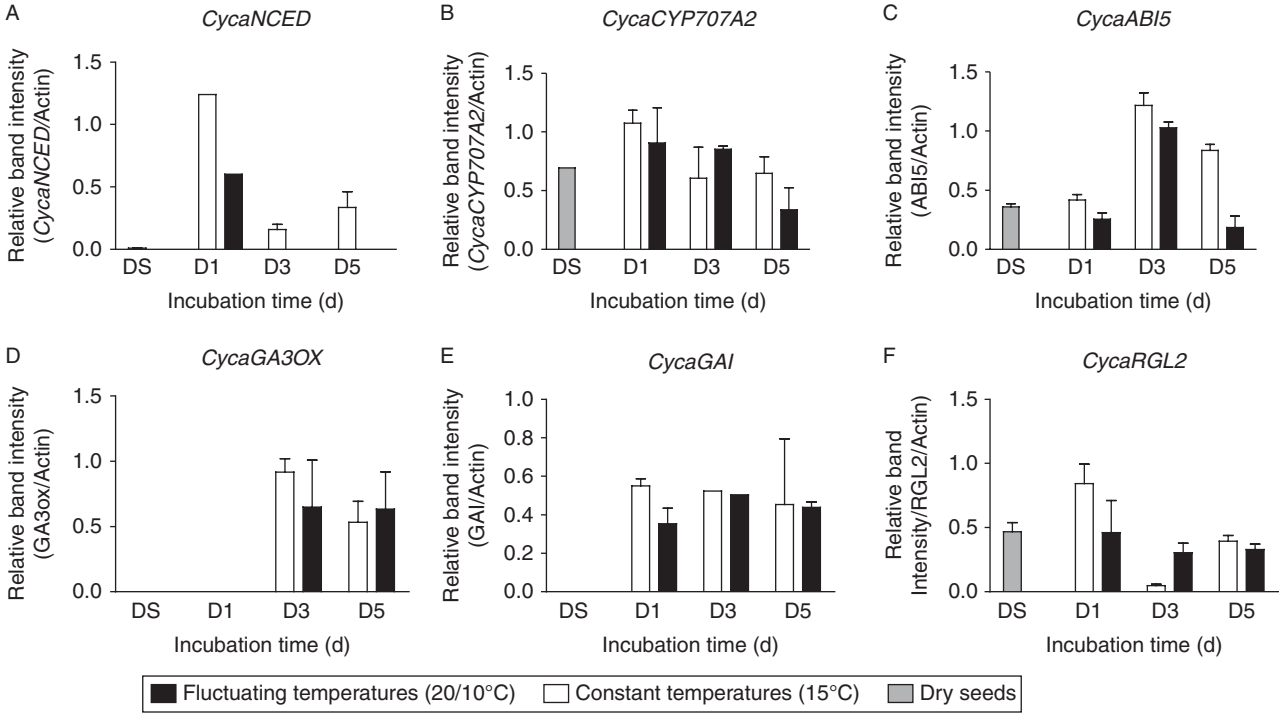

Figure 5. Expression analysis relative to actin mRNA of *CycaNCED* (A), *CycaCYP707A2* (B), *CycaABI5* (C), *CycaGA3ox* (D), *CycaGAI* (E) and *CycaRGL2* (E) in embryos isolated from achenes on 0, 1, 3 and 5 d of incubation. RT-PCR was performed as described in the Materials and methods section. Mean values from three biological replicates are shown. Bars indicate SEs.

agreement with results reported in this paper showing that stimulation of germination by fluctuating temperatures is neither through an enhancement of GA synthesis, nor through an increase in GA sensitivity (Figs 2–4).

Discussion

Fluctuating temperatures were proposed as an environmental signal involved in the termination of seed dormancy, together with light and nitrates (Bewley and Black, 1994; Benech-Arnold *et al.*, 2000; Finch-Savage and Footitt, 2012). Furthermore, for seeds buried in the soil seed bank, the perception of fluctuating temperatures is a most relevant signal since it provides information about the spatial position of seeds (Thompson and Grime, 1983; Ghersa *et al.*, 1992). Despite the fact that seed responses to fluctuating temperatures are widespread, knowledge about physiological mechanisms underlying such responses is still insufficient (Hu *et al.*, 2012). The termination of seed dormancy is associated with changes in hormone contents (i.e. a low ABA/GA ratio), as well as in hormone sensitivity (i.e. high GA sensitivity and/or low ABA sensitivity) (Kucera *et al.*, 2005; Finkelstein *et al.*, 2008; Graeber *et al.*, 2012). Several studies reported changes in ABA/GA ratio and sensitivity in response to light or nitrate (e.g. Toyomasu *et al.*, 1993, 1998; Sawada *et al.*, 2008a, b, c; Matakiadis *et al.*, 2009). In contrast, changes in ABA/GA ratio and hormone sensitivity as a result of exposure to fluctuating temperatures that terminate dormancy have been partially assessed. In a previous paper (Huarte and Benech-Arnold, 2010), we have reported that fluctuating temperatures terminate *C. carduncul*us dormancy through a reduction in ABA content and sensitivity. To account for a lower ABA content and sensitivity in seeds exposed to fluctuating temperatures, the expression of genes involved in ABA synthesis, catabolism and signalling should be different between both thermal treatments, so long as the effect of the stimulatory thermal regime is exerted at the level of gene transcription. Fluctuating temperatures reduced mRNA abundance of *CycaNCED* from day 1 on and *CycaABI5* at day 5 (Fig. 5A and C). In contrast, mRNA abundance of *CycaCYP707A2* did not differ between treatments (Fig. 5B). These results suggest that fluctuating temperatures terminate seed dormancy by means of a negative regulation of the transcription of ABA synthesis and signalling genes (i.e. putative *CycaNCED* and *CycaABI5*). This is consistent with the results of Argyris *et al.* (2008) and Toh *et al.* (2008) who found a negative regulation of ABA synthesis genes at temperatures permissive for germination. Also, and similar to our results, both studies did not show changes in the expression of ABA catabolism genes (e.g. *LsABA8ox4*). On the other hand,

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

the inhibition of germination by constant (Fig. 1) or supraoptimal temperatures reported by Argyris *et al.* (2008), Toh *et al.* (2008) and Huo *et al.* (2013), seems to be exerted through a positive regulation of the expression of ABA synthesis genes. Fluctuating temperatures down-regulated the expression of the ABA-signalling gene *CycaABI5* from day 3 onwards. ABI5 is a positive regulator of ABA signalling (Lopez-Molina *et al.*, 2002). The low expression of *CycaABI5* under fluctuating temperatures, might determine reduced protein abundance, thus resulting in low sensitivity to ABA inhibitory action. This result agrees with the results of our physiological approach where fluctuating temperatures reduced ABA sensitivity of germination (Huarte and Benech-Arnold, 2010). Therefore, it appears that the stimulation of germination by fluctuating temperatures operates, at least in part, through the modulation of the expression of ABA synthesis and signalling genes. Seed sensitivity to ABA is related to ABI5 content, and a close relationship exists between ABA content and ABI5 protein (Lopez-Molina *et al.*, 2001). Therefore, environmental signals that lead to a reduction of ABA content (i.e. light, nitrates and fluctuating temperatures in this case) would also reduce the expression of *ABI5.* Nevertheless, ABI5 content and stability are not modulated by ABA content only. Lopez-Molina *et al.* (2003) and Stone *et al.* (2006), reported that AFP (ABI5 binding protein) and KEG (Keep On Going) target ABI5 for subsequent degradation by the ubiquitine–proteosome system. Although the reduction in ABA content triggered by incubation under fluctuating temperatures also occurs with other signals that terminate dormancy, such as light and nitrates, the regulatory site for ABA content is not always a reduced rate of ABA synthesis. For instance, *Lactuca sativa* light-treated seeds exhibit two regulatory sites at the level of gene transcription to control ABA content (Sawada *et al.*, 2008a, b): a negative regulation of two ABA synthesis genes *LsNCED2* and *LsNCED4* and a positive regulation of the ABA catabolism gene *LsABA8ox4*. In turn, for nitrate-treated seeds, the regulatory site for ABA content is only an up-regulation of the ABA catabolism gene *CYP707A2* (Matakiadis *et al.*, 2009). Recently, Foley *et al.* (2012) analysed the trancriptome of *Euphorbia esula* seeds exposed to fluctuating and constant temperatures and found that fluctuating temperatures did not modify the transcription of *ABI3* and *ABI5* after 3 d of seed exposure to an alternating thermal regime, a time at which *E. esula* seeds are still far from the onset of germination (Foley and Chao, 2008). Transcriptional changes at times closer to germination onset were not provided, thus precluding a more direct comparison with our results.

In a previous paper we have shown that the manipulation of the seed hormone balance through the use of exogenously applied ABA, GA and their respective synthesis inhibitors, under fluctuating and constant temperatures, allows for the modification of seed germination behaviour (Huarte and Benech-Arnold, 2010). From these results, it was observed that those experimental conditions that imposed a low ABA/GA ratio (i.e. fluridone and GA_3 application) were as effective as fluctuating temperatures to terminate dormancy. One explanation for these results was that fluctuating temperatures stimulate germination through an enhancement of GA synthesis and/or sensitivity in addition to the observed reduction in ABA content. The contents of GA_1 and GA_4 did not differ throughout incubation under fluctuating or constant temperatures (Figs 2 and 3). These results show that GA synthesis was equally active at both thermal regimes. It appears, then, that at constant temperatures, a continuous ABA synthesis is the mechanism responsible for dormancy maintenance (i.e. high ABA/GA ratio). Therefore, using fluridone in the incubation medium impaired ABA synthesis and, accordingly, reduced ABA/GA ratio, since GA synthesis is active regardless of the thermal treatment. For this reason, seeds treated with fluridone and incubated under a constant temperature behaved as if they had been exposed to fluctuating temperatures. Conversely, fluctuating temperatures reduced ABA content without affecting GA synthesis, resulting in a low ABA/GA ratio that allowed for the termination of dormancy. The presence of paclobutrazol in the incubation medium when seeds were incubated at fluctuating temperatures reduced GA content and reset the ABA/GA ratio to that imposed by incubation under constant temperatures. Hence, paclobutrazol-treated seeds incubated under fluctuating temperatures behaved as if they had been incubated under constant temperatures. In each one of these scenarios GA *de novo* synthesis appeared as a requisite for germination of seeds from this species to occur. The determination of active GA content was consistent with the expression level of *CycaGA3ox* (Fig. 5D). Selection of expression of *CycaGA3ox* among other genes involved in GA synthesis was due to the fact that *GA 3-oxidase* gene, coding the enzyme committed to the final inter-conversion into the bioactive GA_4 or GA_1, is the gene up-regulated by Pfr, the active form of phytochrome (Yamaguchi, 2008). Fluctuating temperatures did not enhance seed sensitivity to exogenous GA_3 either (Fig. 4A, B), thus suggesting that an increase in GA sensitivity is not part of the physiological mechanism through which fluctuating temperatures terminate seed dormancy. This is further supported by the fact that fluctuating temperatures did not down-regulate the expression of GA signalling genes (*GAI* and *RGL2*) (Fig. 5E and F).

These results, together with those reported by Huarte and Benech-Arnold (2010), allow us to give an explanation of the mechanisms underlying the

https://doi.org/10.1017/S0960258514000051 Published online by Cambridge University Press

termination of dormancy by fluctuating temperatures in *C. cardunculus* and to denote the hormonal nature of the process. Fluctuating temperatures terminate seed dormancy by a reduction of ABA synthesis and sensitivity with no changes in GA synthesis, catabolism and sensitivity.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0960258514000051

Acknowledgements

We are grateful to Natalia Ilina and Jessica Barneto for their guidance of H.R.H. in molecular experiments, Eduardo Fernandez for his help with the statistical analyses and Oscar Masciarelli for measurements of gibberellins.

Financial support

This work was supported by Universidad Católica Argentina grant 2010-2012 to H.R.H.

Conflicts of interest

None.

References

Alvarado, V. and Bradford, K.J. (2005) Hydrothermal time analysis of seed dormancy in true (botanical) potato seeds. *Seed Science Research* **15**, 77–88.

Arana, M.V., De Miguel, L.C. and Sánchez, R.A. (2006) A phytochrome-dependent embryonic factor modulates gibberellin responses in the embryo and micropylar endosperm of *Datura ferox* seeds. *Planta* **223**, 847–857.

Argyris, J., Dahal, P., Hayashi, E., Still, D.W. and Bradford, K.J. (2008) Genetic variation for lettuce seed thermoinhibition is associated with temperature-sensitive expression of abscisic acid, gibberellin, and ethylene biosynthesis, metabolism, and response genes. *Plant Physiology* **148**, 926–947.

Baskin, J.M. and Baskin, C.C. (2007) A classification system for seed dormancy. *Seed Science Research* **14**, 1–16.

Benech-Arnold, R.L., Sánchez, R.A., Forcella, F., Kruk, B.C. and Ghersa, C.M. (2000) Environmental control of dormancy in weed seed banks in soil. *Field Crops Research* **67**, 105–122.

Bewley, J.D. (1997) Seed germination and dormancy. *The Plant Cell* **9**, 1055–1066.

Bewley, J.D. and Black, M. (1994) *Seeds: Physiology of development and germination*. New York, Plenum Press.

Chen, H., Zhang, J., Neff, M.M., Hong, S.W., Zhang, H., Deng, X.W. and Xiong, L. (2008) Integration of light and abscisic acid signaling during seed germination and early seedling development. *Proceedings of the National Academy of Sciences of the USA* **105**, 4495–4500.

Finch-Savage, W.E. and Footitt, S. (2012) To germinate or not to germinate: a question of dormancy relief not germination stimulation. *Seed Science Research* **22**, 243–248.

Finch-Savage, W.E. and Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytologist* **171**, 501–523.

Finkelstein, R., Reeves, W., Ariizumi, T. and Steber, C. (2008) Molecular aspects of seed dormancy. *Annual Review of Plant Biology* **59**, 387–415.

Foley, M.E. and Chao, W.S. (2008) Growth regulators and chemicals stimulate germination of Leafy Spurge (*Euphorbia esula*) seeds. *Weed Science* **56**, 516–522.

Foley, M.E., Chao, W.S., Dogramaci, M., Horvath, D.P. and Anderson, J.V. (2012) Changes in the transcriptome of dry leafy spurge (*Euphorbia esula*) seeds imbibed at a constant and alternating temperature. *Weed Science* **60**, 48–56.

Footitt, S., Douterelo-Soler, I., Clay, H. and Finch-Savage, W.E. (2011) Dormancy cycling in *Arabidopsis* seeds is controlled by seasonally distinct hormone-signaling pathways. *Proceedings of the National Academy of Sciences of the USA* **108**, 20236–20241.

Ghersa, C.M., Benech-Arnold, R.L. and Martinez-Ghersa, M.A. (1992) The role of fluctuating temperatures in germination and establishment of *S. halepense* (L.) Pers. II. Regulation of germination at increasing depths. *Functional Ecology* **6**, 460–468.

Graeber, K., Nakabayashi, K., Miatton, E., Leubner-Metzger, G. and Soppe, W.J.J. (2012) Molecular mechanisms of seed dormancy. *Plant, Cell and Environment* **35**, 1769–1786.

Gummerson, R.J. (1986) The effect of constant temperatures and osmotic potentials on the germination of sugar beet. *Journal of Experimental Botany* **37**, 729–741.

Hartweck, L.M. (2008) Gibberellin signaling. *Planta* **229**, 1–13.

Hilhorst, H.M. (1995) A critical update on seed dormancy I. Primary dormancy. *Seed Science Research* **5**, 61–73.

Holdsworth, M.J., Bentsink, L. and Soppe, W.J.J. (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179**, 33–54.

Hu, X.W., Huang, X.H. and Wang, Y.R. (2012) Hormonal and temperature regulation of seed dormancy and germination in *Leymus chinensis*. *Plant Growth Regulation* **67**, 199–207.

Huarte, H.R. and Benech-Arnold, R.L. (2010) Hormonal nature of seed responses to fluctuating temperatures in *Cynara cardunculus* (L.). *Seed Science Research* **20**, 39–45.

Huarte, R. and Benech-Arnold, R.L. (2005) Incubation under fluctuating temperatures reduces mean base water potential for seed germination in several non-cultivated species. *Seed Science Research* **15**, 89–97.

Huo, H., Dahal, P., Kunusoth, K., McCallum, C.M. and Bradford, K.J. (2013) Expression of 9-cis-epoxycarotenoid dioxygenase 4 is essential for thermoinhibition of lettuce seed germination but not for seed development or stress tolerance. *The Plant Cell* **25**, 884–900.

Kucera, B., Cohn, M.A. and Leubner-Metzger, G. (2005) Plant hormone interactions during seed dormancy

release and germination. *Seed Science Research* **15**, 281–307.

Linkies, A. and Leubner-Metzger, G. (2012) Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination. *Plant Cell Reports* **31**, 253–270.

Lopez-Molina, L., Mongrand, S. and Chua, N.H. (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the USA* **98**, 4782–4787.

Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T. and Chua, N.H. (2002) ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *Plant Journal* **32**, 317–328.

Lopez-Molina, L., Mongrand, S., Kinoshita, N. and Chua, N.H. (2003) AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes & Development* **17**, 410–418.

Matakiadis, T., Alboresi, A., Jikumaru, Y., Tatematsu, K., Pichon, O., Renou, J.P., Kamiya, Y., Nambara, E. and Truong, H.N. (2009) The *Arabidopsis* abscisic acid catabolic gene CYP707A2 plays a key role in nitrate control of seed dormancy. *Plant Physiology* **149**, 949–960.

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

Nambara, E. and Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56**, 165–185.

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

Ni, B.R. and Bradford, K.J. (1992) Quantitative models characterizing seed germination responses to abscisic acid and osmoticum. *Plant Physiology* **98**, 1057–1068.

Ni, B.R. and Bradford, K.J. (1993) Germination and dormancy of abscisic acid- and gibberellin-deficient mutant tomato (*Lycopersicon esculentum*) seeds: sensitivity of germination to abscisic acid, gibberellin, and water potential. *Plant Physiology* **101**, 607–617.

Piskurewicz, U., Jikumaru, Y., Kinoshita, N., Nambara, E., Kamiya, Y., Lopez-Molina, L. and Gene, D. (2008) The gibberellic acid signaling repressor RGL2 inhibits *Arabidopsis* seed germination by stimulating abscisic acid synthesis and ABI5 activity. *The Plant Cell* **20**, 2729–2745.

Raghavendra, A.S., Gonugunta, V.K., Christmann, A. and Grill, E. (2010) ABA perception and signalling. *Trends in Plant Science* **15**, 395–401.

Sawada, Y., Aoki, M., Nakaminami, K., Mitsuhashi, W. and Tatematsu, K. (2008a) Phytochrome and gibberellin-mediated regulation of abscisic acid metabolism during germination of lettuce seeds. *Plant Physiology* **146**, 1386–1396.

Sawada, Y., Aoki, M., Nakaminami, K., Mitsuhashi, W., Tatematsu, K., Kushiro, T., Koshiba, T., Kamiya, Y., Inoue, Y., Nambara, E. and Toyomasu, T. (2008b) Phytochrome and gibberellin-mediated regulation of abscisic acid metabolism during germination of photoblastic lettuce seeds. *Plant Physiology* **146**, 1386–1396.

Sawada, Y., Katsumata, T., Kitamura, J., Kawaide, H., Nakajima, M. and Asami, T.B. (2008c) Germination of photoblastic lettuce seeds is regulated via the control of endogenous physiologically active gibberellin content, rather than of gibberellin responsiveness. *Journal of Experimental Botany* **59**, 3383–3393.

Seo, M., Hanada, A., Kuwahara, A., Endo, A., Okamoto, M., Yamauchi, Y., North, H., Marion-Poll, A., Sun, T.P., Koshiba, T., Kamiya, Y., Yamaguchi, S. and Nambara, E. (2006) Regulation of hormone metabolism in *Arabidopsis* seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. *Plant Journal* **48**, 354–366.

Seo, M., Nambara, E., Choi, G. and Yamaguchi, S. (2009) Interaction of light and hormone signals in germinating seeds. *Plant Molecular Biology* **69**, 463–472.

Stone, S.L., Williams, L.A., Farmer, L.M., Vierstra, R.D. and Callis, J. (2006) KEEP ON GOING, a RING E3 ligase essential for *Arabidopsis* growth and development, is involved in abscisic acid signaling. *The Plant Cell* **18**, 3415–3428.

Thompson, K. and Grime, J.P. (1983) A comparative study of responses to diurnally fluctuating temperatures. *Journal of Applied Ecology* **20**, 141–156.

Toh, S., Imamura, A., Watanabe, A., Nakabayashi, K., Okamoto, M., Jikumaru, Y., Hanada, A., Aso, Y., Ishiyama, K., Tamura, N., Iuchi, S., Kobayashi, M., Yamaguchi, S., Kamiya, Y., Nambara, E. and Kawakami, N. (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in *Arabidopsis* seeds. *Plant Physiology* **146**, 1368–1385.

Toyomasu, T., Tsuji, H., Yamane, H., Nakayama, M., Yamaguchi, I., Murofushi, N., Takahashi, N. and Inoue, Y. (1993) Light effects on endogenous levels of gibberellins in photoblastic lettuce seeds. *Journal of Plant Growth Regulation* **12**, 85–90.

Toyomasu, T., Yamane, H., Murofushi, N. and Nick, P. (1994) Phytochrome inhibits the effectiveness of gibberellins to induce cell elongation in rice. *Planta* **194**, 256–263.

Toyomasu, T., Kawaide, H., Mitsuhashi, W., Inoue, Y. and Kamiya, Y. (1998) Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seeds. *Plant Physiology* **118**, 1517–1523.

Umezawa, T., Okamoto, M., Kushiro, T., Nambara, E., Oono, Y. and Seki, M. (2006) CYP707A3 a major ABA 8′-hydroxylase involved in dehydration and rehydration response in *Arabidopsis thaliana*. *Plant Journal* **46**, 171–182.

Yamaguchi, S. (2008) Gibberellin metabolism and its regulation. *Annual Review of Plant Biology* **59**, 225–251.

Yamaguchi, S., Kamiya, Y. and Nambara, E. (2007) Regulation of ABA and GA levels during seed development and germination in *Arabidopsis*. pp. 224–247 *in* Bradford, K.J.; Nonogaki, H. (Eds) *Seed development, dormancy and germination*. Oxford, Blackwell Publishing.

Yamauchi, Y., Takeda-Kamiya, N., Hanada, A., Ogawa, M., Kuwahara, A., Seo, M., Kamiya, Y. and Yamaguchi, S. (2007) Contribution of gibberellin deactivation by AtGA2ox2 to the suppression of germination of dark-imbibed *Arabidopsis thaliana* seeds. *Plant and Cell Physiology* **48**, 555–561.