



Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph



Initial characterisation of low and high seed dormancy populations of *Lolium rigidum* produced by repeated selection

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ARTICLE INFO

Article history:

Received 22 February 2010

Received in revised form 22 April 2010

Accepted 22 April 2010

Keywords:

α -Amylase

Cytokinins

Inheritance

Lolium rigidum

Seed dormancy

ABSTRACT

The physiological and biochemical bases of seed dormancy in *Lolium rigidum* (annual ryegrass) are largely unknown, and study of this process is complicated by the outcrossing nature of the species and the strong influence of environment on seed dormancy. In order to identify heritable biochemical factors contributing to seed dormancy in *L. rigidum*, seeds from a field-collected population were used to select sub-populations with consistently low or high seed dormancy over four generations. Low-dormancy seeds showed constitutive α -amylase activity prior to imbibition, higher concentrations of polyphenols and cis-zeatin, and lower abscisic acid and cis-zeatin riboside concentrations than high-dormancy seeds. Selection for high dormancy was associated with a reduction in response to dark-stratification for 21 d at 20 °C (an effective means of releasing dormancy in the original, unselected population) over successive generations, but fluridone remained effective in breaking dormancy. Crossing of low- and high-dormancy populations indicated that dormancy level was not dependent upon the maternal genotype of the seed, and that the constitutive α -amylase activity and high seed anthocyanin concentrations characteristic of the low-dormancy populations were not correlated to high basal germination ability.

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1. Introduction

Primary seed dormancy is usually dependent upon both genetic and environmental factors. *Lolium rigidum* (annual ryegrass) is an outcrossing weed which is widespread in cropping systems in many regions of the world and, in this species, the effect of environment on seed dormancy has been the main focus of previous work. A warm, dry maternal environment during seed development and maturation resulted in the production of less-dormant seeds (Steadman et al., 2004); in contrast, long-term climatic conditions had little influence on the dormancy level of populations of *L. rigidum* seeds collected from across the Western Australian grain belt (Owen et al., unpublished). Compared to the information available on environmental effects, the genetic component of seed dormancy in *L. rigidum* is relatively uncharacterised. In the self-pollinating species *Bromus tectorum*, seed dormancy is mainly controlled by genetics (Meyer and Allen, 1999), whilst in oat (Foley and Fennimore, 1998) and wheat (Tan et al., 2006) there

is a strong genotype \times environment interaction. There is little information about the heritability of seed dormancy in cross-pollinated species, although different populations of *Knautia arvensis* produced seeds with different levels of dormancy when grown under uniform conditions, suggesting that there are genetic determinants of dormancy which are not strongly linked to the seed maturation environment in this species (Vange et al., 2004). In the dioecious *Ameranthus tuberculatus*, deep seed dormancy (defined as requiring release by cold- and dark-stratification) was demonstrated to be an inherited trait (Leon et al., 2006).

In the current study, genetically imposed factors affecting dormancy in *L. rigidum* seeds were investigated by repeatedly selecting sub-populations (from a single original field-collected population) that were grown under the same conditions and which possessed either low or high seed dormancy at maturation. The selected sub-populations were characterised physiologically and biochemically in an attempt to identify potential determinants of dormancy in *L. rigidum* seeds. Although abscisic acid (ABA) is not involved in maintenance of dormancy in mature, imbibed *L. rigidum* seeds (Goggin et al., 2009), it is currently unknown whether it plays a role in dormancy induction, as is the case in *Arabidopsis* (Finch-Savage and Leubner-Metzger, 2006). Therefore, endogenous ABA levels were measured in dry, mature seeds from the low- and high-dormancy populations. Cytokinins were measured at the same time to determine if these plant growth regulators, which are well-characterised

Abbreviations: ABA, abscisic acid; BAP, 6-benzylaminopurine; CK, cytokinins; F, fluridone; GA₄, gibberellin A₄; HD, high-dormancy; KIN, kinetin; LD, low-dormancy; TEA-HCl, triethanolamine hydrochloride.

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in studies on seed development (Emery and Atkins, 2006) but often neglected with respect to dormancy, showed different patterns of accumulation in the two sub-populations. As mobilisation of stored starch reserves is required for successful seed germination and subsequent early seedling growth (e.g. Perata et al., 1992), it was hypothesised that the low-dormancy seeds might be prepared for rapid germination by having higher, earlier induced activity of α -amylase, and higher activity of enzymes involved in soluble carbohydrate metabolism. Thus, α -amylase, triosephosphate isomerase and enolase were also measured in low- and high-dormancy seeds. Finally, crosses were made between plants from the low- and high-dormancy sub-populations in a preliminary assessment of the heritability of primary seed dormancy and its biochemical determinants in *L. rigidum*.

2. Materials and methods

2.1. Chemicals

All chemicals and enzymes were obtained from Sigma–Aldrich (Sydney, Australia).

2.2. Generation of sub-populations producing low- and high-dormancy seeds

Seeds were collected in November 2000 from a population of *Lolium rigidum* Gaud plants infesting a continuously cropped wheat field at Wongan Hills, Western Australia (30°53'S, 116°43'E). The basal level of germination after 42 d incubation at alternating 25/15 °C with a 12 h photoperiod (combined fluorescent and incandescent light at a fluence rate of 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was 17%. The response of this population to dark- and light-stratification at various temperatures is characterised in Steadman (2004).

From this original population, stored at -20°C following harvest, 'low-dormancy' (LD) and 'high-dormancy' (HD) seeds were selected. The criteria for each category were that LD seeds would start to germinate within 3 d upon imbibition under germination conditions, whilst HD seeds would require six weeks of dark-stratification at 20°C to enable subsequent germination. In order to prevent seeds with relatively low dormancy being selected in the HD group, the six-week dark-stratification period was interrupted at the three-week point by transferral of the seeds to germination conditions for one week. Any seeds that germinated during this time were removed, and the remainder of the seeds were returned to the dark for a further three weeks. The seedlings from selected LD and HD seeds were grown to maturity during 2003 in potting mix (50% pine bark, 25% peat moss, 25% washed river sand) in an outdoor plot at the University of Western Australia (the mean maximum and minimum temperatures during the growing season were approximately 20°C and 10°C , respectively). All plants commenced flowering within two weeks of each other, and these were segregated into LD and HD groups by pollen-proof enclosures. The resulting seeds (designated as generation 1, i.e. LD1 and HD1) were harvested at maturity (early December 2003), threshed by hand and cleaned in an air column to remove chaff and empty seeds, and then stored at -20°C in sealed foil bags.

Three further rounds of selection were performed (in 2006, 2007 and 2008) as described above, except that LD and HD plants were grown in separate (but adjacent) glasshouses, free from other ryegrass plants. In both glasshouses, the benches were in the same orientation and received the same light conditions; and average day and night temperatures were 25 and 15°C , respectively. The third generation of selected seeds (LD3 and HD3) was used to create individual crosses (made to discount the unlikely possibility that seed dormancy in *L. rigidum* is controlled by a single gene) as well

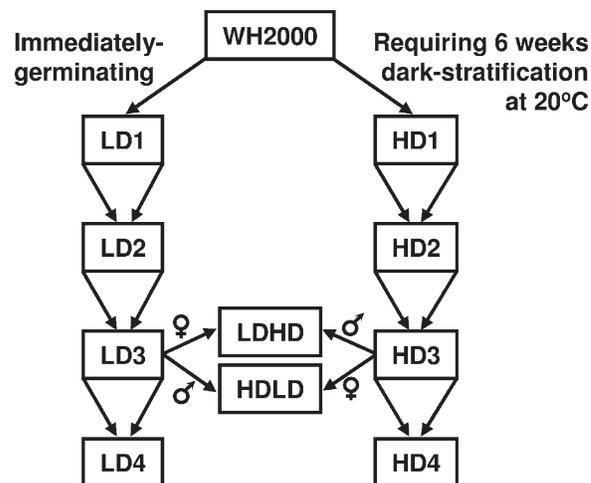


Fig. 1. Method of producing 'pure' LD and HD populations from the original field-collected (WH2000) population and of crossing individual LD3 and HD3 plants to obtain separate batches of seeds from both mother plants.

as the bulk LD4 and HD4 populations (Fig. 1). LD3 and HD3 plants were placed together in pollen-proof enclosures under glasshouse conditions, and the resulting seeds from each plant collected separately in December 2008, so that potential maternal effects could be identified.

All mature seeds were threshed and cleaned between 1 and 10 d after harvest and stored at -20°C in sealed foil bags immediately following cleaning. Seed moisture content at the time of freezing was 6–10% across all generations and populations, and their viability was unaffected by freezing (own observations and see Wen et al., 2009). However, storage at -20°C resulted in a very gradual loss of dormancy of all seeds, so that baseline germination after 2–3 years of storage was higher than at harvest (this did not decrease the magnitude of the difference between the LD and HD populations: see Fig. 2). Viability tests using the tetrazolium method (Steadman, 2004) demonstrated that seeds from generations 1–4 had 95–100% viability, whilst that of the LD3 \times HD3 crosses ranged from 70–100%.

2.3. Germination assays

Seed germination tests were set up on 1% (w/v) agar as described in Goggin et al. (2009). The dormancy status of each generation of seeds was checked immediately following harvest by measuring their response to imbibition under standard germination conditions (alternating 25/15 °C with a 12 h photoperiod, light intensity 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and to 21 d dark-stratification at 20°C followed by transferral to germination conditions. The effects of fluridone (F, 50 μM ; an inhibitor of carotenoid biosynthesis which inhibits seed ABA production in several species: Feurtado et al., 2007 and references therein), gibberellin A₄ (GA₄, 10 μM), KNO₃ (50 mM) and the cytokinins benzylaminopurine (BAP, 50 μM) and kinetin (KIN, 50 μM) on non-stratified seeds from generation 3 were later assessed by incubating seeds on agar containing these compounds, under standard germination conditions. Negative controls in these experiments were 0.1% (v/v) dimethylsulphoxide for the F and GA₄ treatments, 50 mM KCl for the KNO₃ treatment, and water for BAP and KIN. Germination was counted weekly until 42 d after the start of imbibition; four replicates of 50 seeds were used for each germination test and treatment. Ungerminated seeds that were dead (as determined by their collapse under gentle pinching of the embryo region, for which pilot studies gave the same results as a tetrazolium test) or empty were excluded from calculations.

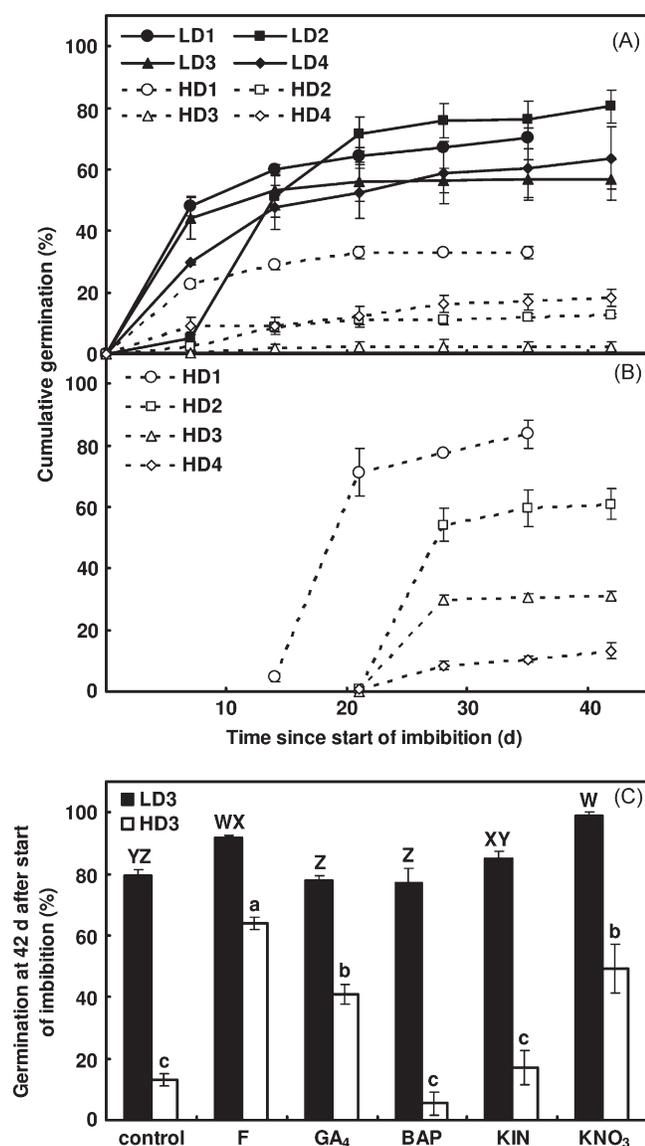


Fig. 2. Germination characteristics of *Lolium rigidum* seeds selected over four generations for low or high dormancy. (A) Germination of non-stratified seeds (i.e. imbibed under standard germination conditions of 25/15 °C with a 12 h photoperiod) immediately following harvest; (B) effect of dark-stratification at 20 °C on dormancy release in HD seeds immediately following harvest (HD1 was dark-stratified for 14 d, HD2–HD4 for 21 d, before transfer to germination conditions); (C) response of non-stratified generation 3 seeds (after storage at –20 °C for 2 years) to treatment with plant growth regulators over the entire 42 d at 25/15 °C with a 12 h photoperiod: F, 50 μM fluridone; GA₄, 10 μM gibberellin A₄; BAP, 50 μM benzylaminopurine; KIN, 50 μM kinetin; KNO₃, 50 mM potassium nitrate. Values are means of four replicates ± SE; in (C), letters above columns indicate significant differences between treatments (upper-case for LD3, LSD was 7.2; lower-case for HD3, LSD was 13.5).

2.4. Measurement of plant growth regulators and polyphenols

For measurement of plant growth regulators, dry LD3 and HD3 seeds were briefly (1–4 h) imbibed in water to facilitate excision of the embryos. Isolated embryos were freeze-dried and analysed for eight endogenous CKs and for endogenous ABA as described in Quesnelle and Emery (2007) and Ross et al. (2004), respectively. Three independent replicates of approximately 200 embryos were measured for each population.

Total polyphenols in dry seeds were extracted and measured as described by Randhir and Shetty (2005). Briefly, seeds were soaked in 10 vol 95% (v/v) ethanol at –20 °C for 72 h, then homogenised

in the same solution and clarified by centrifugation. Polyphenols were detected by the addition of Folin-Ciocalteu phenol reagent followed by Na₂CO₃, and the absorbance was recorded at 725 nm. Gallic acid (0–15 μg) was used as a standard. Anthocyanins were extracted by homogenising seeds in 50% (v/v) acetic acid according to the method of Konczak-Islam et al. (2003). Anthocyanin concentration was expressed as the unitless ‘colour value’, based on the absorbance of the extract at 530 nm and the dry weight of the seed sample.

2.5. Measurement of enzyme activities

Activity of seed α-amylase (EC 3.2.1.1) was measured using zymograms (each experiment was performed at least twice, with replicate gels run each time) as described in Goggin and Colmer (2007), but using 30 μg protein per lane. Triosephosphate isomerase (TPI; EC 5.3.1.1) and enolase (EC 4.2.1.11) measurements on dry and stratified seeds from generations 3 and 4 were carried out according to Esnouf et al. (1982) and Bergmeyer (1983), respectively, using kinetic spectrophotometric assays. Seeds were extracted in 4 vol 100 mM triethanolamine hydrochloride (TEA-HCl) (pH 7.4), 1 mM EDTA, 5% (v/v) glycerol, 0.002% (v/v) Triton X-100, 2 mM ascorbate, 5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and the cleared supernatant after centrifugation at 12 000 × g for 7 min (1 mL) was desalted into 2 mL 100 mM TEA-HCl (pH 7.4), 1 mM EDTA using Sephadex G-25 PD10 columns (Pharmacia).

Each enzyme assay was performed in a volume of 1 mL and contained 100 μL enzyme extract. Boiled extracts were used as negative controls. Enzyme activities were calculated from the change in absorbance in the linear range of the reaction, using an extinction coefficient for NADH of 6.2 mM⁻¹ cm⁻¹. Total soluble protein was measured using the method of Bradford (1976).

2.6. Statistical analysis

Each parameter was measured in three or four independent biological replicates, and data were analysed using single-factor ANOVA and the least significant difference test at the 5% level of significance. If experiments were carried out more than once and there was no significant difference between experiments, the data were pooled.

3. Results

3.1. Germination characteristics of LD and HD populations

Non-stratified LD seeds germinated to a final percentage of 57–81% at 42 d after the start of imbibition under optimal germination conditions, whilst HD seeds only reached 3–18% germination in the same time period (Fig. 2A). Dark-stratification for 21 d at 20 °C, a reliable means of releasing dormancy in the original field-collected *L. rigidum* population (Steadman, 2004; Goggin et al., 2009), became progressively less effective in each HD generation, to the point that germination of HD4 was unaffected by 21 d dark-stratification (Fig. 2B; note that HD1 was only dark-stratified for 14 d but still reached a higher final germination percentage than the other generations).

Fluridone, which was capable of replacing dark-stratification in the field-collected populations characterised in Goggin et al. (2009), was similarly able to break dormancy in HD3 seeds (Fig. 2C). GA₄ and KNO₃ were approximately as effective as dark-stratification in HD3 seeds, whilst BAP and KIN had no effect on their germination (Fig. 2C). Fluridone and nitrate also stimulated the (already-high) germination of the LD3 seeds, whereas BAP, KIN and GA₄ did not (Fig. 2C).

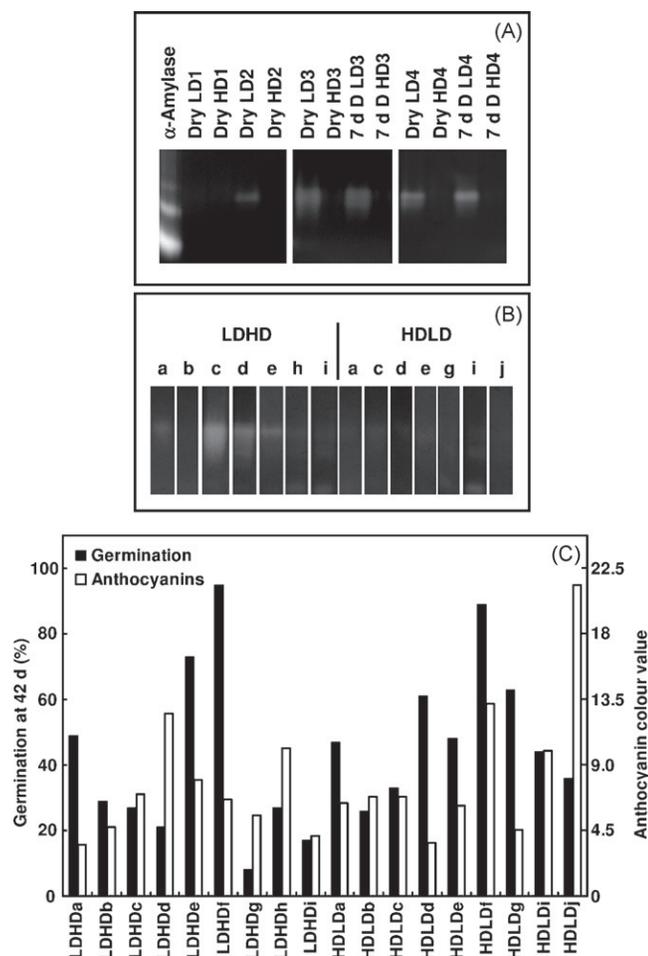


Fig. 3. α -Amylase activity in all populations and germination and anthocyanins in crossed populations. (A) Dry (generations 1–4) and 7 d dark-stratified (generations 3 and 4) seeds from the 'pure' LD and HD populations were assayed for α -amylase activity using zymograms to detect bands of activity. At least two independent experiments were carried out for each sample, with duplicate gels run each time. Representative gels are shown; α -amylase from *Aspergillus oryzae* was run as a positive control in the left lane. (B) Dry seeds of the crossed populations in which there was sufficient seed were assayed once for α -amylase activity. (C) Germination at 42 d after the start of imbibition and dry seed anthocyanin colour value in the crossed populations. One replicate of each crossed population was measured.

Ten single crosses between LD3 and HD3 plants were made, and the seeds collected from each parent were kept separate, so that 20 populations (10 with LD mother plants, designated LDHdA–j, and 10 with HD mother plants, designated HDLdA–j; Fig. 1) were obtained. Two populations (LDHdJ and HDLdH) produced non-viable seeds and were not included in subsequent analyses. Because of the low numbers of seeds in most populations, measurements were performed on one replicate per population only. Incubation at 25/15 °C with a 12 h photoperiod resulted in a wide range of germination percentages amongst populations, with no correlation between dormancy status and the genotype of the mother plant (Fig. 3C). Similarly, the averaged germination percentage at 42 d after the start of imbibition of the LDHD populations (38 ± 10) was not significantly different from that of the HDLD populations (50 ± 6). The average germination of the HDLD populations was higher than that of the HD2, HD3 and HD4 populations (Fig. 2A), but there was no clear pattern in a comparison of the LDHD populations with the LD or HD populations.

Table 1

Concentration of anthocyanins and total polyphenols in dry LD and HD seeds. Values are means \pm SE ($n=4$); means which are significantly different ($P<0.05$) between different populations are denoted by different letters. LDHD_{av} and HDLD_{av} refer to the mean value of anthocyanins from the individual populations ($n=9$) shown in Fig. 3. nd, not determined due to insufficient seed numbers.

Population	Anthocyanin colour value	Polyphenols ($\mu\text{mol g}^{-1}$ dwt)
LD1	57 ± 5^{ab}	540 ± 49^b
HD1	12 ± 2^{fg}	390 ± 25^c
LD2	62 ± 3^a	nd
HD2	46 ± 5^{bc}	nd
LD3	44 ± 1^c	884 ± 34^a
HD3	22 ± 3^e	543 ± 26^b
LD4	32 ± 3^d	616 ± 33^b
HD4	16 ± 3^{ef}	398 ± 38^c
LDHD _{av}	8 ± 1^g	nd
HDLD _{av}	10 ± 2^{fg}	nd

3.2. Endogenous seed hormone and polyphenol concentrations

An observed difference in colour between extractions of LD and HD seeds prompted an analysis of their anthocyanin concentrations. In each generation, LD seeds had a higher anthocyanin colour value than their HD counterparts (Table 1). The linear correlation between germination percentage (at 35 d after the start of imbibition: see Fig. 2A) and anthocyanin colour value of dry seeds was weakly positive ($r^2=0.4$). Total polyphenols, measured in generations 1, 3 and 4 (seed numbers in generation 2 were insufficient), showed the same pattern as anthocyanins (Table 1), although the correlation between germination and polyphenol concentration was weaker ($r^2=0.2$). The anthocyanin concentrations of seeds from the LD3 \times HD3 crosses were highly variable between crossing events, were not correlated to the dormancy status of the seeds, and did not reflect the anthocyanin concentration of the seeds from which the mother plants were grown (i.e. LD3 and HD3) (Fig. 3C).

The seeds from generation 3, which were the most abundant and showed the greatest difference in germination between LD and HD, were used to investigate embryo hormone concentrations. There were no significant differences between LD3 and HD3 embryos for most of the CK forms measured, with the average embryo concentrations being (in pmol g^{-1} dwt): trans-zeatin, 611 ± 96 ; trans-zeatin riboside, 11 ± 1 ; dihydrozeatin, 346 ± 87 ; dihydrozeatin riboside, 14 ± 3 ; isopentenyl adenine, 268 ± 59 ; isopentenyl adenosine, 24 ± 1 . However, embryos from LD3 seeds had a 2.5-fold higher concentration of cis-zeatin than those from HD3 seeds, whilst cis-zeatin riboside and also ABA concentrations were both 2-fold higher in HD3 embryos (Table 2).

3.3. Activity of carbohydrate-metabolising enzymes in LD and HD seeds

A possible explanation for the rapid germination of LD seeds is the constitutive expression of higher levels of enzymes involved in substrate mobilisation and carbohydrate metabolism, so that energy for embryo expansion is available immediately upon imbibition. Therefore, the activities of α -amylase, TPI and enolase were measured. α -Amylase is the first enzyme of starch mobilisation and hence is closely linked to the germination ability of cereal seeds. TPI and enolase activities were selected from the suite of glycolytic

Table 2

Concentration of abscisic acid (ABA), cis-zeatin (cZ) and cis-zeatin riboside (cZR) in dry LD3 and HD3 seeds. Values are means \pm SE ($n=3$); means which are significantly different ($P<0.05$) between different populations are denoted by different letters.

Population	ABA (nmol g^{-1} dwt)	cZ (pmol g^{-1} dwt)	cZR (pmol g^{-1} dwt)
LD3	2.4 ± 0.4^b	124 ± 4^a	63 ± 2^b
HD3	4.5 ± 0.2^a	50 ± 15^b	125 ± 7^a

enzymes because they were identified as being more highly (>5-fold) expressed in HD3 seeds (unpublished data).

α -Amylase activity was present in dry, mature LD seeds from generations 2, 3 and 4, whilst it was close to undetectable in the corresponding HD seeds (Fig. 3A). This difference between populations was maintained upon 7 d dark-stratification (due to limited seed numbers, only dark-stratified seeds from generations 3 and 4 were assayed) (Fig. 3A), demonstrating that constitutive α -amylase activity in the dry LD seeds was not an artefact of the seed maturation process.

TPI activity was the same in both populations, across generations 3 and 4, and in dry or 7 d dark-stratified seeds, with an overall average activity of $520 \pm 25 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$. Enolase activity was also consistent across the different sample types, with an average activity of $73 \pm 5 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$.

To further test whether constitutive α -amylase activity in dry seeds is a characteristic of genetically non-dormant *L. rigidum* seeds, the LD3 \times HD3 crosses with sufficient seed numbers were also assayed for this enzyme. Rather than correlating to dormancy status, detectable α -amylase activity in dry seeds was more likely to be found in the seeds having an LD maternal genotype (Fig. 3B), regardless of whether the LD3 \times HD3 population itself was dormant or non-dormant (Fig. 3C).

4. Discussion

4.1. Selection of low and high seed dormancy in *Lolium rigidum*

This study has confirmed that seed dormancy has a genetic component in *L. rigidum*, as the progeny of plants grown selectively from either low- or high-dormancy seeds, under the same glasshouse conditions, produced seeds with a similar dormancy status to their parents. Each generation of HD seeds progressively lost the ability to germinate following 21 d of dark-stratification, which was a sufficient dormancy-release treatment in the original population (Steadman, 2004). Leon et al. (2006) demonstrated by crossing wild dormant and non-dormant populations of *A. tuberculatus* that seed dormancy level and the ability to release dormancy via stratification (in this case, cold and dark conditions) are controlled by different genes in this species, thus providing a means for maintaining wide variability in germination times and ensuring survival of the species under different environmental conditions. Although this phenomenon of separate genes mediating dormancy level and dormancy release was not specifically studied in the current work, it is possible that a similar situation exists in *L. rigidum* seeds, as HD3 seeds displayed the highest initial level of dormancy, but were also more responsive to dark-stratification than the HD4 seeds (Fig. 2A, B).

In spite of the fact that 21 d dark-stratification was relatively ineffective as a dormancy-breaking treatment in HD3 seeds, fluridone remained an effective means of bypassing dormancy (also in HD4 seeds: data not shown). The mechanism of action of fluridone in dormancy release in annual ryegrass seeds is currently unclear, as ABA concentrations in stratified seeds remained the same in the presence or absence of fluridone (Goggin et al., 2009). Germination of HD3 seeds was also stimulated (but to a lesser extent) by GA₄, a known germination stimulant, and by nitrate, which is thought to act by altering ABA and/or GA signalling in the seed (Alboresi et al., 2005). The two CK tested, BAP and KIN, had no effect on germination of LD3 or HD3 seeds at 50 μM , but previous studies have shown that the effects of exogenous CK on seed germination are variable (Nikolić et al., 2006 and references therein), and it is likely that specific CK have different effects in different species; additionally, the concentration applied in the current study may have been too low (e.g. Zhang et al., 2006). Moreover, BAP and KIN are potent

in processes normally affected by trans-CK but they may not be relevant to processes affected by the cis-CK (discussed below).

4.2. Plant hormones and polyphenols in mature selected seeds

Most of the CK measured were present at similar levels in embryos of dry LD3 and HD3 seeds, but there were significant differences between populations in terms of their cis-zeatin (higher in LD3) and cis-zeatin riboside (higher in HD3) concentrations. It is thus possible that cis-zeatin is involved in establishing the dormancy level of mature *L. rigidum* seeds, with the free base acting to keep dormancy low. This fits with the idea that the nucleobase (unconjugated) CK are the primary, if not only, active forms (e.g. Yamada et al., 2001; Kurakawa et al., 2007), and therefore, riboside derivatives may only be active upon conversion to the nucleobases. In seed research the idea that CK nucleobases are the only active forms has been around for some time; for example, it was implicated to explain the role of an adenosine nucleosidase activity on CK-ribosides in wheat germ (Chen and Kristopeit, 1981). Although the role of CK in seed dormancy and germination is not as well-studied as its involvement in early seed development, Chiwocha et al. (2005) found that wild-type seeds of *Arabidopsis thaliana* contained higher concentrations of biologically active CK than higher-dormancy mutant seeds, which were richer in inactive, conjugated CK. The apparent bottleneck of conversion of cis-zeatin riboside to cis-zeatin seen in the *L. rigidum* HD3 population may, therefore, contribute to its strong dormancy.

The difference in riboside to free base conversion in the LD3 vs. HD3 seeds occurs only with cis-isomers of zeatin, and it happens against a backdrop of trans-isomers (traditionally considered to be much more biologically active) present at a similar magnitude of concentration. However, it is becoming clear that in some tissues, such as developing pea seeds, cis-CK can have similar activity to that of their trans-counterparts (Quesnelle and Emery, 2007). Overall, it is therefore possible that cis-zeatin may play a role in determining the dormancy level of *L. rigidum* seeds, most likely in an interaction with other plant growth regulators such as ABA and GA, whose roles in seed dormancy and germination are well-established.

Although ABA concentration was shown not to be involved in maintenance of dormancy following imbibition in dark- and light-stratified *L. rigidum* seeds (Goggin et al., 2009), the fact that HD3 seeds had a 2-fold higher embryo ABA concentration at maturity than LD3 seeds suggests that, as is the case in *Arabidopsis* and other species (reviewed in Finch-Savage and Leubner-Metzger, 2006), ABA is involved in induction of dormancy in annual ryegrass seeds, with a higher concentration corresponding to a higher initial level of dormancy. It is possible that the sensitivity of LD3 seeds to ABA may also be lower than in HD3 seeds, and that the ability to decrease sensitivity to ABA during dark-stratification (Goggin et al., 2009) is impaired over successive HD generations. However, this is difficult to test in these populations for which the level of germination in the absence of exogenous ABA is already so low.

Interestingly, the LD seeds had consistently higher total polyphenol and anthocyanin concentrations than their HD counterparts, suggesting that the concentration of these compounds might be linked to the dormancy level of mature seeds. Studies in several species including *Arabidopsis*, wheat, millet (Debeaujon et al., 2000 and references therein) and tomato (Downie et al., 2004) have shown that seeds which contain higher levels of condensed tannins (proanthocyanidins) or phenolic acids are often more dormant than those with low concentrations of these pigments, due to the fact that these compounds make penetration of the cell wall by the embryo more difficult and/or impede permeability to oxygen and water. Based on the results of the current study, it appears that this is not the case in *L. rigidum*, as the populations with higher concentrations of phenolic compounds had

lower dormancy. The correlation between germination percentage and anthocyanin concentration was more strongly positive than that between germination and total polyphenols. In *Arabidopsis* seedlings, it has been demonstrated that cytokinins, in an interaction with blue light, promote the synthesis of anthocyanins (Chen et al., 2006). Therefore, it is possible that the higher concentration of cis-zeatin in LD3 seeds contributes to their higher anthocyanin concentration compared to HD3 seeds. However, the reason for the higher anthocyanin levels in low-dormancy seeds is difficult to ascertain. These pigments can be involved in protection against light and other stresses, so it may be that they accumulate in *L. rigidum* seeds that have been selected for low dormancy in preparation for the rapid emergence of the young seedling. More work needs to be done on the differential accumulation of anthocyanins in LD and HD seeds in order to elucidate their potential role in seed dormancy.

4.3. Activity of carbohydrate-metabolising enzymes does not mediate dormancy status in selected seeds

In spite of the fact that TPI and enolase were differentially expressed in LD3 vs. HD3 seeds (unpublished data), a subsequent analysis of the activity of these enzymes in dry and imbibed seeds from generations 3 and 4 demonstrated that differential expression did not correlate to differential enzyme activities. Therefore, although the higher expression of enolase may be important in terms of its other role as a stress-induced transcription factor, its activity as a glycolytic enzyme, and that of TPI, is apparently not related to dormancy status. The initial concentration of water-soluble carbohydrates in LD and HD seeds, measured in generations 1–4, was also not different between populations (data not shown), confirming previous findings that dormant seeds are not impaired in soluble carbohydrate metabolism (reviewed in Bewley, 1997).

It was also hypothesised that LD seeds may be able to germinate immediately upon imbibition under favourable conditions because of rapid induction of enzymes related to germinative processes such as substrate mobilisation. A likely candidate was α -amylase, as studies on cereal and other seeds have shown that whilst seeds able to express α -amylase are capable of rapid germination, those for which expression of this enzyme is inhibited are also inhibited in germination (e.g. Kato-Noguchi and Macías, 2008; Perata et al., 1992). The unexpected constitutive presence of α -amylase activity in dry seeds of the LD2, LD3 and LD4 populations, and its maintenance upon imbibition under non-germination conditions, suggests that immediate preparedness for starch mobilisation may indeed be linked to the dormancy status of these seeds.

4.4. Crossing of LD and HD populations

Although the LD and HD populations were well-separated from each other in terms of germination behaviour and a range of biochemical parameters, there was no clear segregation of traits when these populations were crossed. Individual reciprocal crosses of LD3 and HD3 plants resulted in progeny with highly variable levels of seed dormancy, and there was no evidence of a correlation between high anthocyanin concentration, constitutive α -amylase activity and low dormancy as there was in the 'pure' LD populations. This suggests that primary dormancy is controlled by multiple genes in *L. rigidum*, as is the case in many grass species (reviewed in Foley and Fennimore, 1998). Interestingly, the seeds resulting from individual crosses only displayed constitutive α -amylase activity if they were produced by LD mother plants (but note that there were also some LDHD crosses without α -amylase activity), regardless of their own dormancy status. The reason for the selection of high levels of anthocyanins and α -amylase in 'pure' LD seeds relative to HD seeds is currently unknown, since they are not necessarily required

for low dormancy in the LDHD populations. Due to the low number of seeds in each crossed population, measurements requiring excised embryos (i.e. ABA and CK) could not be performed on these samples.

5. Conclusions

Seed dormancy in *L. rigidum* contains a genetic component, and high or low dormancy can be selected for over successive generations, with high-dormancy seeds gradually losing the ability to respond to dark-stratification. Although seeds from 'pure' low-dormancy and high-dormancy populations showed clear differences in constitutive expression of α -amylase activity and their level of phenolic compounds, these differences did not correlate to dormancy status in the progeny of low-dormancy \times high-dormancy crosses, suggesting that dormancy is controlled by several genes. Other differences between low- and high-dormancy seeds, such as ABA and cis-zeatin concentration, also appear to be related to their dormancy status, but will need to be measured in the crosses to confirm this. Further crossing and backcrossing events may also help to elucidate the pattern of dormancy inheritance, and the key factors contributing to primary dormancy, in *L. rigidum* seeds.

Acknowledgements

This work was funded by the Australian Research Council and the Natural Science and Engineering Research Council of Canada.

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