Annals of Botany **110**: 1641–1650, 2012
doi:10.1093/aob/mcs213, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Selection for low dormancy in annual ryegrass (*Lolium rigidum*) seeds results in high constitutive expression of a glucose-responsive α-amylase isoform

Danica E. Goggin* and Stephen B. Powles

Australian Herbicide Resistance Initiative, School of Plant Biology M086, The University of Western Australia, 35 Stirling Highway, Crawley 6009, Australia
** For correspondence. E-mail danica.goggin@uwa.edu.au*

Received: 8 July 2012 Returned for revision: 7 August 2012 Accepted: 16 August 2012 Published electronically: 21 September 2012

• *Background and Aims* α-Amylase in grass caryopses (seeds) is usually expressed upon commencement of germination and is rarely seen in dry, mature seeds. A heat-stable α-amylase activity was unexpectedly selected for expression in dry annual ryegrass (*Lolium rigidum*) seeds during targeted selection for low primary dormancy. The aim of this study was to characterize this constitutive activity biochemically and determine if its presence conferred insensitivity to the germination inhibitors abscisic acid and benzoxazolinone.
• *Methods* α-Amylase activity in developing, mature and germinating seeds from the selected (low-dormancy) and a field-collected (dormant) population was characterized by native activity PAGE. The response of seed germination and α-amylase activity to abscisic acid and benzoxazolinone was assessed. Using an alginate affinity matrix, α-amylase was purified from dry and germinating seeds for analysis of its enzymatic properties.
• *Key Results* The constitutive α-amylase activity appeared late during seed development and was mainly localized in the aleurone; in germinating seeds, this activity was responsive to both glucose and gibberellin. It migrated differently on native PAGE compared with the major activities in germinating seeds of the dormant population, but the enzymatic properties of α-amylase purified from the low-dormancy and dormant seeds were largely indistinguishable. Seed imbibition on benzoxazolinone had little effect on the low-dormancy seeds but greatly inhibited germination and α-amylase activity in the dormant population.
• *Conclusions* The constitutive α-amylase activity in annual ryegrass seeds selected for low dormancy is electrophoretically different from that in germinating seeds and its presence confers insensitivity to benzoxazolinone. The concurrent selection of low dormancy and constitutive α-amylase activity may help to enhance seedling establishment under competitive conditions.

Key words: α-amylase, annual ryegrass, benzoxazolinone, dormancy, germination, *Lolium rigidum*, seed, xylanase inhibitor protein.

INTRODUCTION

Starch hydrolysis in germinating cereal and grass caryopses (hereafter referred to as seeds) provides essential soluble sugars for the emerging seedling prior to the commencement of photosynthesis. Of the four enzymes required for the degradation of starch, α-amylase (1,4-α-D-glucan glucanohydrolase; EC 3·2·1·1) is considered to be the most important in seeds as it is capable of hydrolysing intact starch grains (Beck and Ziegler, 1989). A range of studies have shown that α-amylase activity and/or protein is absent in dry mature cereal seeds and that expression is induced upon commencement of germination (e.g. MacGregor *et al.*, 1984; Perata *et al.*, 1992; Bewley and Black, 1994; Guglielminetti *et al.*, 1995), with transcripts appearing first in the embryo and then in the aleurone a few days later (Loreti *et al.*, 2003; Chen *et al.*, 2006). Hormonal signals from the embryo regulate α-amylase expression in the aleurone, whilst expression in the embryo itself is largely regulated by sugars (Chen *et al.*, 2006). Although there can be uncertainty over whether α-amylase is required for germination itself or merely for post-germination seedling growth (summarized in Asatsuma *et al.*, 2005), recent studies on the expression of specific rice α-amylase genes or their regulatory proteins raise the strong possibility that whilst bulk mobilization of reserve carbohydrates in the endosperm is a post-germination process (Bewley and Black, 1994), seed germination is dependent upon the expression of certain α-amylase isoforms in the embryo (e.g. Asatsuma *et al.*, 2005; Lu *et al.*, 2007; Magneschi and Perata, 2009).

Most studies on the possible link between α-amylase and seed dormancy (as distinct from germination) focus on the genetic defects of late-maturity α-amylase and pre-harvest sprouting in crop cereals, where high levels of α-amylase synthesized late during grain development or retained in the pericarp cause premature starch degradation which is concomitant with the germination of seeds that are still attached to the ear (Kindred *et al.*, 2005; Lin *et al.*, 2008). Annual ryegrass (*Lolium rigidum*) seeds are a useful system for studying dormancy in isolation from germination because dormancy release requires a period of imbibition in the dark (referred to as dark stratification), whereas stimulation of germination requires alternating light and temperature, i.e. freshly harvested non-dormant or dark-stratified seeds will not germinate if imbibed under constant temperature, light or darkness (Steadman, 2004). Annual ryegrass seeds that were relieved of dormancy via dark stratification did not show induction of

α-amylase activity until soon after the seeds were transferred to germination-promoting conditions of alternating light and temperature (Goggin *et al.*, 2011*b*). However, seeds that were selected over several generations for the ability to germinate without prior dark stratification developed a constitutive, heat-stable α-amylase activity that was present even in the dry seeds (Goggin *et al.*, 2010). As these selected seeds still possessed a low level of dormancy (in that they would not germinate unless imbibed under alternating light and temperature), it is unlikely that the constitutive activity was analogous to the late-maturity or retained α-amylase activity found in cereals prone to pre-harvest sprouting. Therefore, this study aimed to investigate: at what point in seed development the constitutive α-amylase activity appears in seeds selected for low dormancy and whether it provides protection against inhibitors of α-amylase expression; whether the constitutive α-amylase activity is a isoform distinct from that present in germinating seeds; and whether there are electrophoretic or enzymatic differences between α-amylases in dry compared with germinating annual ryegrass seeds.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma-Aldrich (Sydney, Australia) unless otherwise stated.

Seed material

The dormant population of *Lolium rigidum* seeds was collected from plants infesting a wheat field at Wongan Hills (30°53′S, 116°43′E) in October 2007 and was characterized in Goggin *et al.* (2009). The low-dormancy population was the result of five generations of deliberate selection for low dormancy as described in Goggin *et al.* (2010), and was harvested in November 2010. The original parent population used for selection was collected in 2000 from the same field as the dormant seeds, and was of a similar dormancy level to the dormant 2007 population (Goggin *et al.*, 2009). Both populations were stored in sealed foil bags at −20 °C within 1 week of harvest and threshing in order to maintain their original dormancy level. Under optimum germination conditions (day/night temperatures of 25/15 °C and a 12 h photoperiod of cool white fluorescent light, fluence rate 45 μmol m^{-2} s^{-1} over 400–700 nm), germination at 7 d after the start of imbibition was >70 % in non-stratified low-dormancy seeds. In contrast, non-stratified seeds from the dormant population showed 0 % germination after 7 d, whilst dark-stratified (i.e. dormancy-released) seeds germinated to 40–60 % after being transferred to germination conditions for 7 d. To obtain germinating seeds for α-amylase analysis and purification, seeds were sown on 1 % (w/v) agar and incubated for 3 d under optimal germination conditions (dormant seeds were first stratified in the dark at 20 °C for 21 d before transfer to germination conditions). At this stage, those seeds that were visibly germinating (approx. 50 % in the low-dormancy population and approx. 25 % in the dark-stratified dormant population) had radicles of 1–2 mm and the coleoptiles were just starting to protrude from the seed coat. Therefore, it should be noted that the α-amylase activity present in these seed samples could be involved in germination itself, in post-germination seedling growth, or both.

Hereafter, the term 'low-dormancy' is used to refer to the fifth generation of selected seeds, and 'dormant' refers to the 2007 field-collected population, whether or not the actual dormancy status of the latter has been altered by dark stratification.

α-Amylase activity during seed development

Twenty plants germinated from the dormant seed population and 20 from the low-dormancy population were used to produce new generations of seeds, with plants being allowed to cross-pollinate only within their own population as described in Goggin *et al.* (2010). Three replicates of two flowering spikes were tagged at anthesis and collected at 15, 30, 45 and 58 d after anthesis. Seeds were immediately removed from the spikes by hand and stored at −20 °C until used for detection of α-amylase activity.

Extraction and affinity purification of α-amylase

A modification of the method of Sharma *et al.* (2000) was used to purify heat-stable α-amylase from annual ryegrass seeds using sodium alginate as an affinity matrix. Seeds were homogenized in 6 vols of cold grinding buffer [100 mM Tris–HCl pH 7·6, 10 mM $CaCl_2$, 0·00025 % (v/v) Triton X-100, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 % (v/v) glycerol] and the extract clarified by centrifugation (2000 *g* for 10 min at 4 °C), heated at 70 °C for 15 min to inactivate other starch-hydrolysing enzymes (Guglielminetti *et al.*, 1995), and clarified again (8000 *g* for 10 min at 4 °C). The 30–65 % ammonium sulfate cut (Koshiba and Minamikawa, 1981) of the heated, cleared extract was desalted into buffer 1 [50 mM sodium acetate, pH 5·2, 10 mM $CaCl_2$, 0·1 % (v/v) Triton X-100] on PD-10 Sephadex G-25 columns (Pharmacia) and incubated with sodium alginate, followed by washing with $CaCl_2$ and elution (once) with maltose as described in Sharma *et al.* (2000). The eluted protein was desalted into buffer 1, concentrated by acetone precipitation, and resuspended in a minimal volume of buffer 1. Three independent purifications were performed on dry and germinating seeds from each population for analysis of enzyme activity, and another two were performed for PAGE analysis. Total soluble protein was measured according to Bradford (1976) using BioRad Dye Reagent Concentrate (Hercules, CA, USA), with bovine serum albumin (0–25 μg) as a standard.

α-Amylase activity assays

Three methods of detecting α-amylase activity in seed extracts were used, depending upon the desired information. Semi-quantitative detection of different α-amylase isoforms in crude, heated seed extracts was performed using native PAGE zymograms (30 μg of protein per gel lane from an extract of 50–100 seeds) with β-limit dextrin as a substrate (Goggin *et al.*, 2010). Regions of α-amylase activity showed up as clear yellow bands on a purple-black background following staining with iodine (Perata *et al.*, 1992).

The progress of enzyme purification and the pH and temperature optima of the purified α-amylases were assessed using a spectrophotometric starch–iodine method based on that of Hunjan (2006). Standard reactions were set up in a total volume of 500 μL, containing 50 mM sodium acetate (pH 5·2), 10 mM $CaCl_2$, 250 μg of boiled soluble potato starch and 0·5–5 μL of sample, and incubated at 37 °C for 30 min (pilot studies showed that the reaction rate was linear over this time). Reactions were stopped with the addition of 5 μL of concentrated HCl, and the remaining starch was detected by the addition of 500 μL of KI/I_2 (2·3 mM/0·23 mM) solution, followed by measurement of the absorbance at 580 nm. Boiled soluble starch (0–125 μg) was used as a standard. To determine the pH optima of the purified enzymes, reactions were performed in buffer adjusted to pH 4–8 in 0·25 unit increments. Temperature optima were assessed over the range of 15–73 °C.

Kinetic measurements of purified α-amylase activity were performed by detecting the reducing sugars formed from starch hydrolysis according to the method of Guglielminetti *et al.* (1995). Reactions, in a total volume of 200 μL, contained 50 mM sodium acetate (pH 5·2), 10 mM $CaCl_2$, 0·5–2·5 μg of boiled soluble starch and 1–5 μL of purified enzyme, and were incubated at 37 °C for 25 min. Reactions were stopped with the addition of 150 μL of DNS solution (40 mM dinitrosalicylate, 400 mM NaOH, 1 M sodium potassium tartrate) and boiled for 5 min. Glucose (0–1·5 μmol) was used as a standard. Samples and standards were diluted 14-fold with water and the absorbance at 530 nm recorded. Hanes–Woolf plots were used to calculate K_m and V_{max} (Cornish-Bowden, 2004).

Xylanase inhibitor protein (XIP) activity assay

As XIP was found to co-purify with ryegrass seed α-amylase (see the Results), the method of Gebruers *et al.* (2001) was used to measure XIP activity in seed extracts and purified α-amylase preparations. Xylanase (0·03 U per reaction) from *Thermomyces lanuginosus* and seed protein (either crude seed extract or purified α-amylase) were combined in a total volume of 50 μL and incubated at room temperature for 25 min, followed by 5 min at 37 °C. To initiate the xylanase reaction, 50 μL of 1 % (w/v) 4-*O*-methyl-D-glucurono-D-xylan dyed with Remazol brilliant blue R (RBB–xylan) was added to the mixture and this was incubated at 37 °C for 30 min before the reaction was stopped with ethanol. Precipitated RBB–xylan was removed by centrifugation and the absorbance of the supernatant at 590 nm recorded. Activity was expressed as the amount of protein required to inhibit by 50 % the xylanase-mediated release of the xylan-conjugated dye.

Denaturing gel electrophoresis

SDS–PAGE was performed as in Laemmli (1970) and two-dimensional (2-D) PAGE as in Goggin *et al.* (2011*a*). Gels were stained with colloidal Coomassie Brilliant Blue G-250, using the method of Candiano *et al.* (2004).

Peptide sequencing by mass spectrometry

Peptide sequencing of protein spots excised from 2-D gels was done at the Lotterywest State Biomedical Facility, Proteomics Node, Western Australian Institute for Medical Research, using electrospray ionization-tandem mass spectrometry. Trypsin-digested samples were separated by a Dionex UltiMate 3000 Nano LC (Thermo Scientific, CA, USA) coupled to a 4000 QTRAP mass spectrometer (AB SCIEX, MA, USA). Proteins were identified by comparing spectra with the Ludwig NR Database (taxonomy: Viridiplanteae) using Mascot software (Matrix Science, London, UK), provided by the Australian Proteomics Computational Facility.

Statistical analysis

Untransformed numerical data were analysed by one-factor analysis of variance (ANOVA) at a significance level of 5 %. Differences between pairs of means were compared using the least significant difference (l.s.d.) test.

RESULTS

α-Amylase activity during seed development

α-Amylase activity was detectable at 15 and 30 d after anthesis in both low-dormancy and dormant seeds, but disappeared after 30 d after anthesis in the latter (Fig. 1A, B; arrows indicate different isoforms numbered according to their speed of migration through the gel). Activity in the low-dormancy progeny seeds was maintained until maturity at 58 d after anthesis, but the major band present at 45 and 58 d after anthesis (isoform 1) migrated more slowly than those in the younger seeds (isoforms 2 and 3) (Fig. 1A). Upon germination, the major α-amylase bands in low-dormancy progeny seeds were isoform 1 and the slower migrating isoform 4 (Fig. 1A). In germinating dark-stratified dormant progeny seeds, isoforms 2, 3 and 4 were visible, with isoform 2 being the most intense; if isoform 1 was present in germinating dormant seeds, it was more diffuse than the equivalent band in the low-dormancy seeds (Fig. 1B).

Localization of seed α-amylase activity

The localization of α-amylase activity in seeds was investigated by excising the embryo-containing third of the seeds and separately measuring activity in the embryo-containing and embryo-less seed parts. In dry low-dormancy seeds, a more intense signal, mainly corresponding to isoform 1, was obtained from the embryo-less portion (Fig. 1C). After 3 d imbibition of low-dormancy seed parts under germination conditions, isoforms 2 and 3 were the major isoforms in the embryo-containing portion, whilst isoforms 1 and 4 were present in the embryo-less seed parts (Fig. 1C). Inclusion of 10 μM gibberellin A_4 (GA_4) in the germination agar stimulated the activity of isoforms 1 and 4 in the embryo-less portions and induced the appearance of faster migrating isoforms 5 and 6, whilst inclusion of 100 mM glucose caused the disappearance of isoform 2 in the embryo-containing seed parts and almost completely inhibited activity in the embryo-less seed parts (Fig. 1C). Dry embryo-containing and embryo-less seed

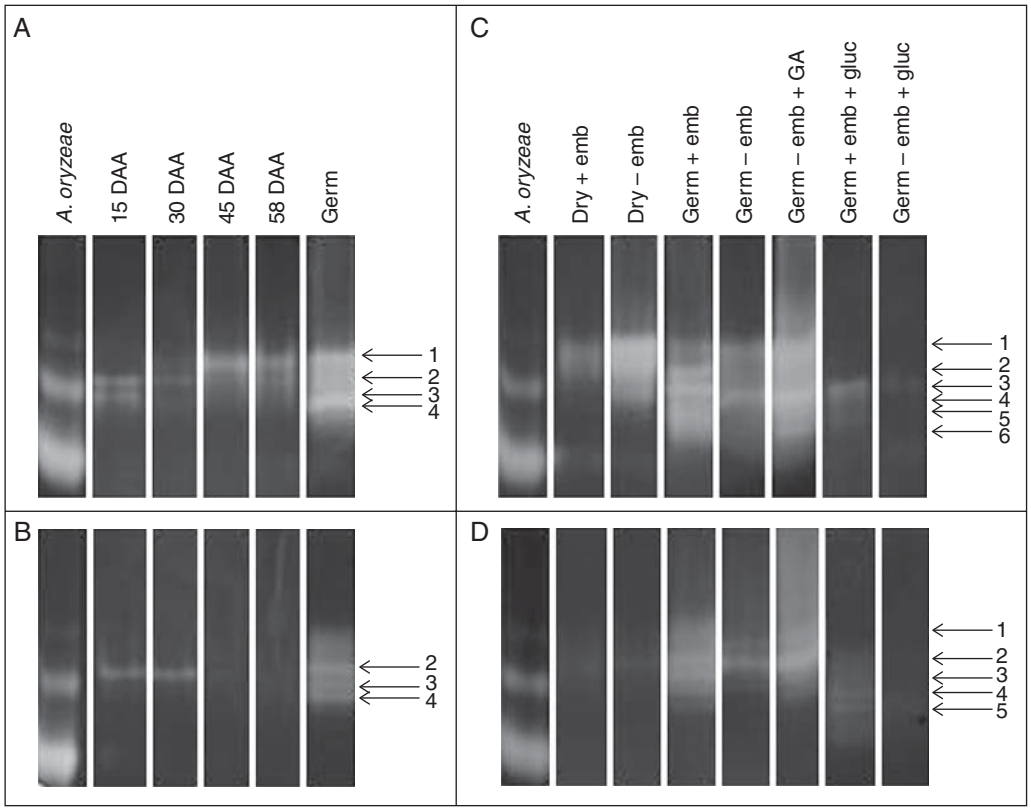

FIG. 1. α-Amylase activity in developing and mature annual ryegrass seeds. α-Amylase activity in heated extracts of low-dormancy (A, C) and dormant (B, D) seeds was detected by native PAGE, using β-limit dextrin as a substrate. Developing seeds (A, B) were assayed at 15, 30, 45 and 58 days after anthesis (DAA) and after 3 d germination (Germ) of mature seeds. Mature seeds were also dissected (C, D) into embryo-enriched (+emb) and embryo-less (−emb) parts before being germinated for 3 d in the presence of 10 μM gibberellin A_4 (+GA) or 100 mM glucose (+gluc). Untreated dry and germinating seed parts were also included. Prior to germination in (B) and (D), dormant seeds were dark-stratified for 21 d to release dormancy. A commercial preparation of α-amylase from *Aspergillus oryzeae* was included as a positive control. Representative gels are shown from three (A) or two (B) independent seed collections/treatments, using approx. 50 seeds or seed parts per sample. Arrows indicate the position of the various α-amylase isoforms, numbered according to their migration speed.

parts from the dormant population had no detectable α-amylase activity (Fig. 1D). Dormant seeds that had been dark stratified to relieve dormancy, then dissected and placed under germination conditions for 3 d, showed a stimulation of activity of isoforms 2 and 3 (and, to a lesser extent, isoforms 4 and 5) in the embryo-containing seed parts, whilst the embryo-less portions had low activity of isoform 2 which was stimulated in the presence of exogenous GA_4, along with fainter signals from isoforms 1, 3 and 4 (Fig. 1D). Germination in the presence of 100 mM glucose prevented the stimulation of isoforms 2 and 3 in the embryo-containing seed parts, but the low levels of isoforms 4 and 5 remained (Fig. 1D). The low activity in the embryo-less, dark-stratified dormant seed parts was decreased further by 100 mM glucose (Fig. 1D).

Effect of plant growth regulators on germination and α-amylase activity

Abscisic acid (ABA) and benzoxazolinone, both of which inhibit germination and the synthesis of α-amylase (Beck and Ziegler, 1989; Kato-Noguchi *et al.*, 2010), were used to determine if the constitutive α-amylase activity in low-dormancy seeds would permit germination under conditions inhibiting *de novo* α-amylase synthesis. Dark-stratified dormant seeds did not germinate at all in the presence of 50 μM ABA, and 1 mM benzoxazolinone inhibited germination to <25 % of control values (Fig. 2A). In contrast, germination of the low-dormancy seeds was only slightly adversely affected by 1 mM benzoxazolinone, whereas 50 μM ABA did not inhibit germination completely (Fig. 2A). The relative sensitivity of low-dormancy and dark-stratified dormant seeds to ABA was further investigated by treating the seeds with 0, 0·5, 1, 5, 10 or 50 μM ABA. Inhibition of seed germination to <5 % of the ABA-free control, at 7 d after the start of treatment, was observed at 10 μM ABA for low-dormancy seeds but at only 1 μM ABA for dark-stratified dormant seeds (data not shown).

Incubation of seeds on 50 μM ABA or 1 mM benzoxazolinone under germination conditions for 3 d resulted in a decrease of α-amylase activity to undetectable levels in dark-stratified dormant seeds, whilst activity in low-dormancy seeds was decreased by only 50 % in the presence of benzoxazolinone and was still detectable (at 14 % of the untreated control) in seeds treated with ABA (Fig. 2B).

Enzymatic properties of α-amylase purified from dry and germinating seeds

As a positive control for the alginate affinity purification method, α-amylase from wheat (*Triticum aestivum* cv. Westonia) seeds was purified in parallel with that from

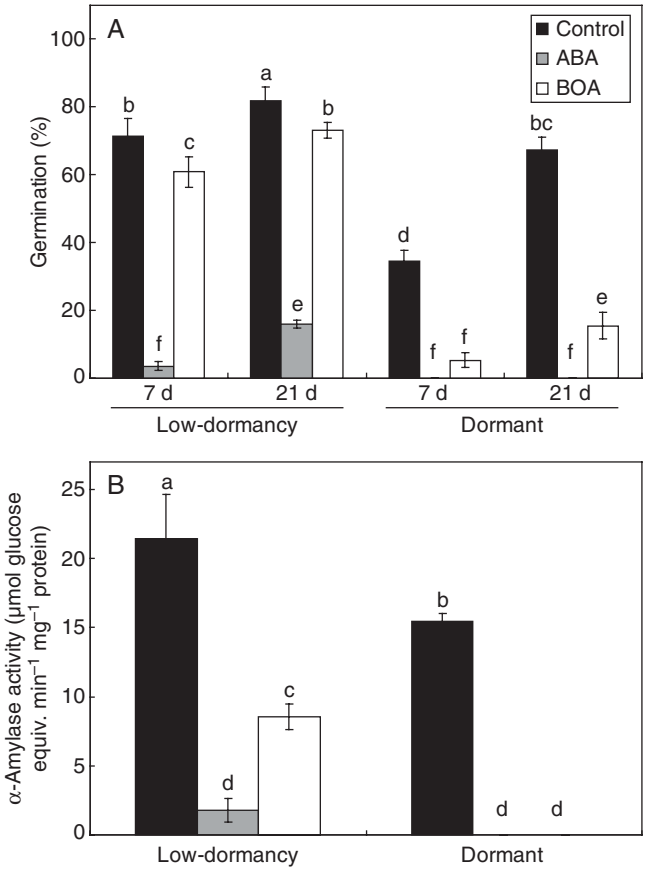

FIG. 2. Effect of plant growth regulators on seed germination and α-amylase activity. Seeds were incubated in the presence or absence of 50 μM abscisic acid (ABA) or 1 mM benzoxazolinone (BOA) under optimum germination conditions (25/15 °C, 12 h photoperiod). Germination was measured after 7 and 21 d exposure to the plant growth regulators (A), and α-amylase activity in heated seed extracts was measured after 3 d exposure (B). Dormant seeds were first dark-stratified in the absence of plant growth regulators for 21 d to release dormancy. In (B), α-amylase activity was quantified using the dinitrosalicylate-based detection of reducing sugars (Guglielminetti *et al.*, 1995) released from the β-limit dextrin substrate. Values represent means ± s.e. ($n = 4$); different letters above columns denote significant differences between treatments ($P < 0.05$).

low-dormancy and dormant ryegrass seeds. Heat-stable α-amylase activity was purified 5- to 6-fold with a yield of 2 % from low-dormancy (dry and germinating) and dark-stratified dormant (germinating only) seeds, and 64-fold with a yield of 3 % from germinating wheat seeds (a representative purification is shown in Table 1). There was negligible to no α-amylase activity isolated from dry dormant or wheat seeds (Table 1). The pH and temperature optima of the purified ryegrass α-amylases showed negligible differences between the various seed sources, with a broad pH optimum of 4–5 for the activity from dry and germinating low-dormancy seeds, and 4–6 for that from germinating dark-stratified dormant seeds (Fig. 3A), and a temperature optimum of 53 °C in all populations (Fig. 3B). The wheat enzyme showed little change in activity over pH 4–6·5, and an optimum temperature profile identical to that of ryegrass (data not shown). When assayed at 53 °C in the absence of added calcium chloride, the activity of all ryegrass α-amylases was more than halved (data not shown).

Although hydrolysis of starch does not necessarily follow Michaelis–Menten kinetics (Dona *et al.*, 2010), the apparent K_m and V_{max} of the purified α-amylase activities were calculated from Hanes–Woolf plots as a means of comparing samples. There were no significant differences ($P = 0.07$) between the K_m values of the three ryegrass enzyme preparations (Table 2), although that of the germinating dark-stratified dormant seeds appeared lower than those of the low-dormancy seeds. By comparison, the K_m of purified wheat α-amylase appeared to be around 8-fold lower than the ryegrass activities. The V_{max} of α-amylase activity from germinating low-dormancy seeds was an order of magnitude higher than that of the other two ryegrass enzymes and similar to that of wheat (Table 2).

Denaturing gel analysis and peptide sequencing of purified α-amylases

SDS- and 2-D-PAGE analysis of the α-amylase activity eluted from the alginate affinity matrix showed intensely staining, approx. 29 kDa proteins of pI 8–9 in each of the ryegrass seed preparations, along with multiple, less abundant proteins of 30–43 kDa and pI 5–6 (Figs 3C and 4A–D). Peptide sequencing of the 30–43 kDa SDS–PAGE bands did not identify any as α-amylase, and all were designated as uncharacterized proteins from various cereal species (data not shown). The approx. 29 kDa proteins (spots 1, 5, 8, 9 and 12 in Fig. 4) were all identified by peptide sequencing as XIP (Table 3). The SDS–PAGE banding patterns of the eluted wheat proteins were more complex than those of the ryegrass proteins, but there was no highly abundant XIP band (Fig. 3C: note that 30 μg of protein per lane was loaded for the ryegrass samples rather than 2 μg as for the wheat samples, because only the XIP band was visible when using 2 μg of ryegrass protein). Only spot 6 (43 kDa, pI 5·6: Fig. 4B) from germinating low-dormancy seeds and spot 18 (42 kDa, pI 6·3: Fig. 4F) from germinating wheat seeds were identified as α-amylase; the other alginate-binding proteins of similar size or high abundance that were sequenced were either not identified or were matched to serpins (serine protease inhibitors) or peroxidase (Table 3).

Xylanase inhibitor protein activity from dry and germinating ryegrass seeds, and from germinating wheat seeds, was tracked through a new round of the alginate-binding procedure. XIP activity was detected in all samples, with a purification factor of 4–13 and a yield of 1–4 % (Table 1).

DISCUSSION

The occurrence of α-amylase activity in dry grass seeds is rare, although de Lespinay *et al.* (2010) recently found activity in dry *Poa pratensis* seeds. Therefore, the constitutively high α-amylase activity that appeared unexpectedly in dry annual ryegrass seeds upon selection for low dormancy (Goggin *et al.*, 2010) was analysed biochemically to determine if its properties were different from those of α-amylases in germinating seeds, and if its presence conferred insensitivity to inhibitors of α-amylase synthesis.

TABLE 1. *Alginate affinity purification of α-amylase (AA) and xylanase inhibitor protein (XIP) activity from low-dormancy (LD) and dormant (D) ryegrass seeds and from wheat seeds*

	Volume (mL)		Activity (U mL^{-1})		Total activity (U)		Total protein (mg)		Specific activity (U mg^{-1})		Yield (%)		Fold purification	
Sample	AA	XIP	AA	XIP	AA	XIP	AA	XIP	AA	XIP	AA	XIP	AA	XIP
Dry LD														
Crude extract	18·5	37·5	626	269	11 589	10 074	20	95	593	106	100	100	1	1
Heated extract	18·5	37	429	336	7929	12 440	9·0	45	879	274	68	123	1·5	2·6
30–65 % cut	3	9	1435	2023	4305	18 210	5·3	14	812	1275	37	181	1·4	12
Elution	0·1	0·1	2550	2887	255	289	0·10	0·26	2659	1101	2·2	2·9	4·5	10
Germ LD														
Crude extract	10	54	1781	364	17 806	19 657	17	175	1053	112	100	100	1	1
Heated extract	9·5	52·5	1481	420	14 065	22 033	6·7	62	2103	358	79	112	2	3·2
30–65 % cut	2	9	2866	886	5731	7975	2·4	15	2431	537	32	41	2·3	4·8
Elution	0·075	0·15	5153	1011	386	152	0·06	0·19	6761	812	2·2	0·8	6·4	7·2
Dry D														
Crude extract	8·8	32·5	304	291	2676	9454	18	86	147	110	100	100	1	1
Heated exteact	8·5	31·5	18	332	150	10 449	11	42	14	249	5·6	111	0·1	2·3
30–65 % cut	3	9	0	865	0	7784	4·1	16	0	493	0	82	0	4·5
Elution	0·06	0·1	44	3797	2·6	380	0·10	0·27	25	1387	1·6	4·0	0·2	13
Germ D														
Crude extract	23	47·5	1099	541	25 278	25 679	26	90	963	285	100	100	1	1
Heated extract	21·5	45	623	324	13 402	14 567	11	52	1229	278	53	57	1·3	1
30–65 % cut	3	9	2288	546	6863	4914	3·7	8·5	1858	576	27	19	1·9	2
Elution	0·1	0·1	4770	1642	477	164	0·10	0·12	4974	1420	1·9	0·6	5·2	5
Dry wheat														
Crude extract	24	ND	1801	ND	43 229	ND	79	ND	548	ND	100	ND	1	ND
Heated extract	23	ND	601	ND	13 828	ND	36	ND	384	ND	32	ND	0·7	ND
30–65 % cut	3	ND	0	ND	0	ND	3·9	ND	0	ND	0	ND	0	ND
Elution	0·075	ND	0	ND	0	ND	0·50	ND	0	ND	0	ND	0	ND
Germ wheat														
Crude extract	13	27	1876	290	24 393	7833	42	90	577	87	100	100	1	1
Heated extract	12·5	26	1798	86	22 470	2226	11	42	2038	54	92	28	3·5	0·6
30–65 % cut	2	3	3083	104	6167	313	1·5	6·4	4117	49	25	4·0	7·1	0·6
Elution	0·07	0·1	9352	492	655	49	0·02	0·16	36 963	306	2·7	0·6	64	3·5

α-Amylase enzyme units are defined as μg soluble starch consumed min^{-1}, XIP units as the amount of enzyme required to inhibit *Thermomyces lanuginosus* xylanase activity by 50 %.

ND, not determined.

α-Amylase activities in dry and germinating ryegrass seeds have different native PAGE migration and hormone response patterns

The major difference between seeds from the low-dormancy and dormant populations was that α-amylase isoform 1 (mainly aleurone localized, with the faint signals in the embryo-containing seed parts probably due to the small amounts of aleurone tissue in the top third of the seed) appeared between 30 and 45 d after anthesis in developing low-dormancy seeds and was maintained until maturity and into germination (Fig. 1). The activity of isoform 1 was inhibited by germination of seeds on glucose, but was stimulated by germination on gibberellin, reminiscent of rice Amy3E (also known as Amy8) (Chen *et al.*, 2006). Early studies showed that rice Amy3E is predominantly localized to the aleurone, as is ryegrass isoform 1, but is expressed during seedling elongation rather than at the earliest stages of germination (Terashima *et al.*, 1995). Later work demonstrated that Amy3E is also present in the rice embryo, and that whilst glucose represses *Amy3E* expression in this tissue, the gene responds only to gibberellin in the aleurone (Chen *et al.*, 2006). Therefore, if ryegrass isoform 1 (inhibited by glucose in the aleurone) is indeed analogous to Amy3E, it is possible that the intricate interplay between its glucose-responsive and gibberellin-responsive promoter elements, as described by Chen *et al.* (2006), is different in rice and ryegrass.

Isoform 2 (present during early seed development and reappearing upon germination: Fig. 1A, B) and isoform 4 were also responsive to both glucose and gibberellin, but their expression patterns were different in the low-dormancy and dormant populations. Isoform 2 was prominent in the embryo of both populations and also in the aleurone of the dark-stratified dormant seeds, whilst high levels of expression of isoform 4 were confined to the aleurone of the low-dormancy seeds. The matching localization and relative intensities of isoforms 1 and 4 suggest that they could be the same gene product with differential post-translational modifications that cause them to migrate differently on native PAGE (Bewley and Black, 1994). Isoform 3 (also present in low-dormancy but not dormant seeds during the earliest stages of seed development) was localized mainly to the embryos of germinating seeds and was insensitive to glucose in low-dormancy seeds, but was inhibited by glucose in dark-stratified dormant seeds. Therefore, it is possible that in the two seed populations, different gene products with similar migration speeds on native PAGE (or the same gene with different promoter elements) are expressed.

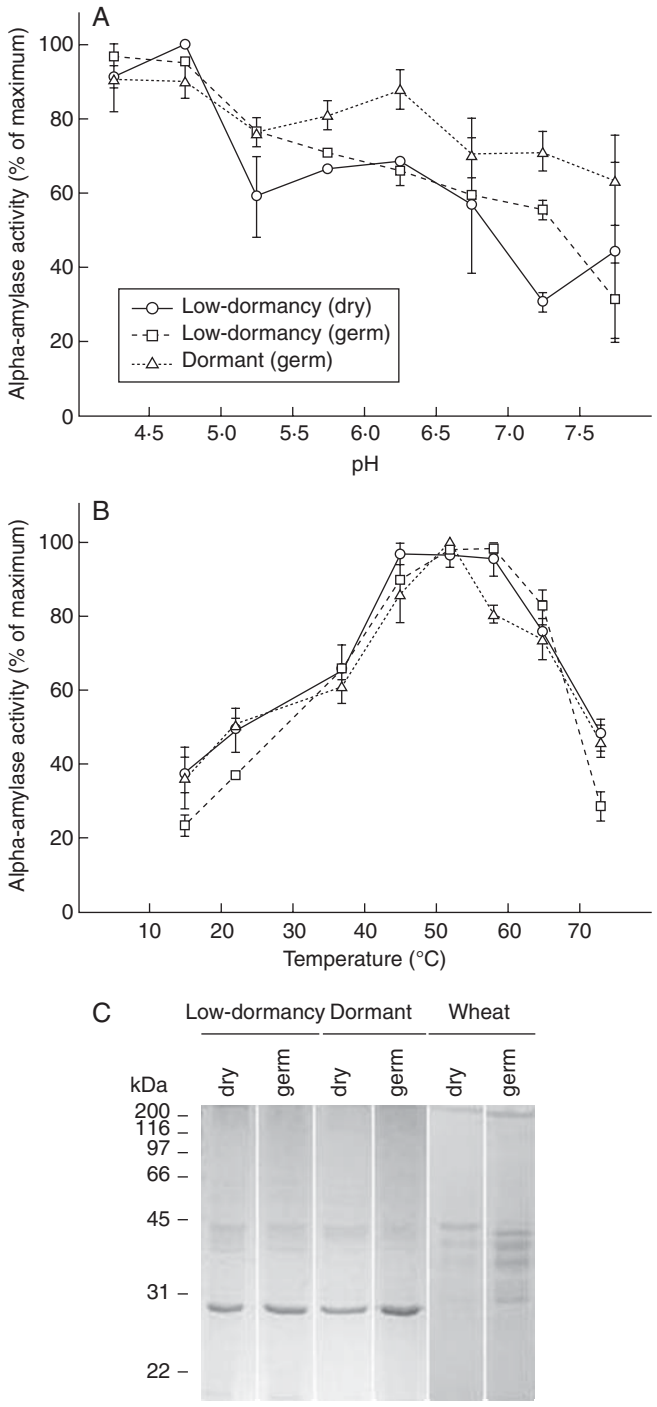

FIG. 3. Purification of α-amylase from dry and germinating seeds. (A) The pH and (B) temperature optima of the ryegrass α-amylase activity eluted from an alginate affinity matrix were determined using the starch–iodine quantitative assay method. Values are means ± s.e. of three independent purifications. Proteins eluted from the alginate matrix following incubation with extracts from dry or germinating (germ) ryegrass and wheat seeds were analysed by SDS–PAGE (C), using 30 μg of protein per lane for ryegrass and 2 μg per lane for wheat. A representative gel is shown, using ryegrass proteins pooled from three independent purifications. Molecular mass standards are shown to the left of the gel.

TABLE 2. *Kinetic properties of α-amylase activity purified from low-dormancy (LD) and dormant (D) ryegrass seeds and from wheat seeds*

Enzyme source	Starch K_m (mg mL^{-1})	V_{max} (μmol min^{-1} mg^{-1} prot)
Dry LD	34·2 ± 2·3[a]	37·5 ± 0·4[b]
Germinating LD	30·4 μ 5·5[a]	218 ± 9·5[a]
Germinating D	19·8 ± 2·3[a]	50·1 ± 1·0[b]
Germinating wheat	3·4	180

Activity is defined as μmol glucose equivalents formed min^{-1} mg^{-1} protein.

Values for the ryegrass enzymes are means ± s.e. of three independent enzyme purifications, and different superscript letters within columns denote a significant difference between means ($P < 0·05$).

The wheat data are from a single purification and were not included in the statistical analysis.

Overall, it appears that selection for low dormancy in undomesticated ryegrass seeds has resulted in an alteration of the relative expression levels, and possibly also post-translational modifications, of seed α-amylases. The high constitutive expression of an aleurone-localized α-amylase that is responsive to both glucose and gibberellin could reflect the need for greater flexibility in response to environmental variables in a population which will germinate immediately upon imbibition (providing there is sufficient light), thus leaving the young seedlings to cope with whatever conditions are encountered after germination. If the seedlings can establish quickly, assisted by the rapid mobilization of endosperm starch reserves, they will have a greater chance of survival.

Constitutive α-amylase activity renders low-dormancy seeds less sensitive to benzoxazolinone

It was hypothesized that the presence of constitutive α-amylase activity in the low-dormancy seeds would lower their sensitivity to ABA and the allelopathic compound benzoxazolinone, both of which inhibit synthesis of α-amylase. This hypothesis was supported by the fact that incubation of (dark-stratified) dormant and low-dormancy seeds on benzoxazolinone greatly inhibited germination and α-amylase activity in the former, but had only a minor effect on the latter (Fig. 2). Similarly, seed germination and α-amylase activity were more sensitive to ABA in the dark-stratified dormant than in the low-dormancy population. In an ecological context, seed dormancy is a strategy which prevents seeds from germinating under sub-optimal conditions or at an inappropriate time of year, and allows a population to stagger its germination so that at least part of the population encounters favourable temperatures and moisture levels and a lack of competition from other plants (Finkelstein *et al.*, 2008). The low-dormancy ryegrass population, in contrast, was produced by imposing a selection pressure to germinate rapidly and synchronously. Many plants, particularly those in the Poaceae, secrete high levels of benzoxazinoids to discourage pathogen and insect attack and to inhibit seed germination and seedling growth of their neighbours (Kato-Noguchi *et al.*,

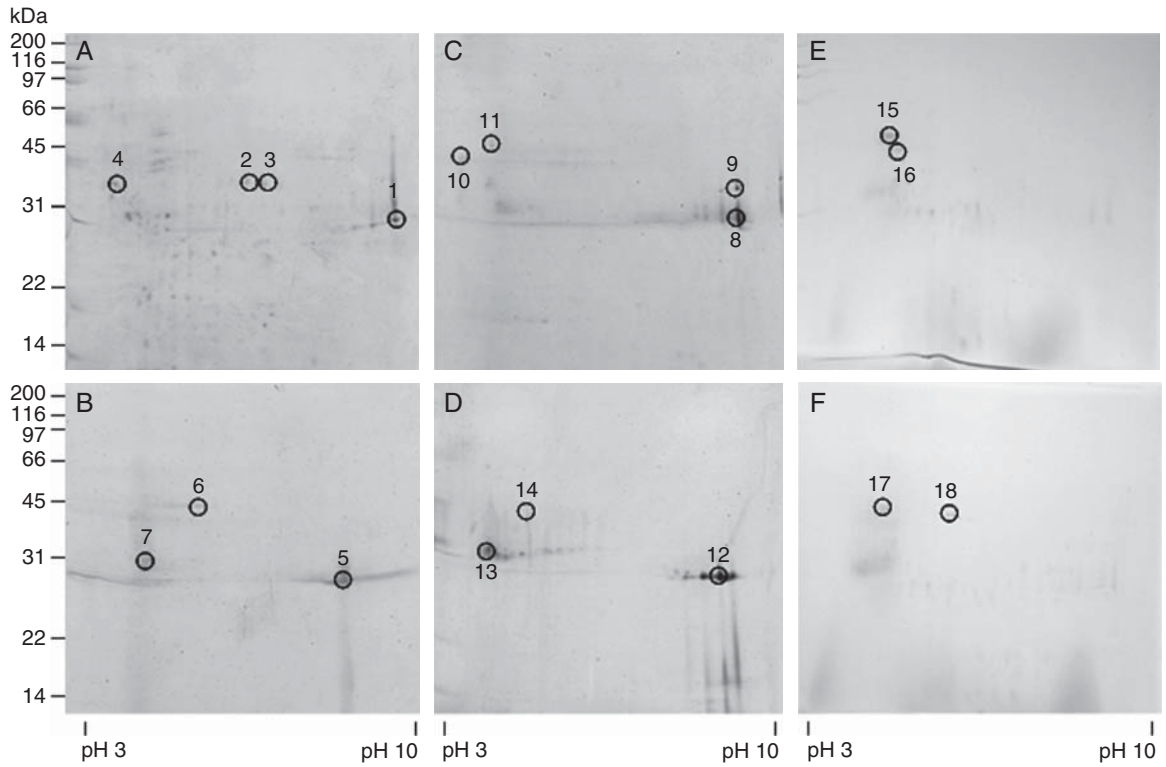

FIG. 4. Two-dimensional PAGE analysis of α-amylase preparations. Protein eluted from the alginate affinity matrix (60 μg of protein per gel, pooled from at least three independent purifications for the ryegrass samples) from (A) dry low-dormancy, (B) germinating low-dormancy, (C) dry dormant, (D) germinating dormant, (E) dry wheat and (F) germinating wheat seeds was analysed by 2-D-PAGE stained with colloidal Coomassie Brilliant Blue G-250. Spots that were used for peptide sequencing, based on their abundance and/or similarity to the expected size of α-amylase (approx. 45 kDa), are circled. Molecular mass standards are shown to the left of the gel, with pH at the bottom.

TABLE 3. *Identification of purified proteins by tandem mass spectrometry*

Spot no.	Observed M_r (kDa)	Observed pI	Protein ID (UniProt accession no.)	MOWSE protein score (no. of peptides matched)	Deduced M_r (kDa)	Deduced pI
1	29·3	9·0	XIP from *Triticum aestivum* (A7BJ77)	122 (2)	32·9	8·5
2	45·7	6·0	No hits	N/A	N/A	N/A
3	45·7	6·2	Unknown protein from *Pseudotsuga menziesii* (P85925)	51 (2)	1·4	5·8
4	44·2	4·7	No hits	N/A	N/A	N/A
5	30·2	8·0	XIP from *Triticum aestivum* (A7BJ77)	125 (2)	32·9	8·5
6	43·3	5·6	α-Amylase from *Dactylis glomerata* (D0VEB9)	294 (5)	43·2	4·9
7	32·6	5·2	Predicted protein from *Hordeum vulgare* var. *distichum* (F2DIK1)	122 (2)	33·8	4·9
8	29·2	8·5	XIP from *Triticum aestivum* (A7BJ77)	109 (2)	32·9	8·5
9	34·3	8·5	XIP from *Tritucum aestivum* (A7BJ77)	130 (2)	32·9	8·5
10	41·0	4·7	Serpin-Z2A from *Triticum aestivum* (Q9ST57)	72 (2)	43·3	5·5
11	45·4	5·0	No hits	N/A	N/A	N/A
12	28·7	8·5	XIP from *Triticum aestivum* (A7BJ77)	97 (2)	32·9	8·5
13	31·5	5·0	Serpin 2 from *Triticum aestivum* (C0LF31)	96 (2)	43·4	5·1
14	39·6	5·6	Serpin-Z2A from *Triticum aestivum* (Q9ST57)	50 (2)	43·3	5·5
15	47·0	4·9	No hits	N/A	N/A	N/A
16	42·3	4·9	Peroxidase from *Triticum aestivum* (Q8LK23)	755 (11)	38·8	8·1
17	45·7	4·8	Serpin 2 from *Triticum aestivum* (C0LF30)	470 (6)	43·4	5·1
18	41·8	6·3	α-Amylase from *Hordeum vulgare* var. *distichum* (C3W8N0)	154 (4)	47·3	5·8

Based on the search parameters used, MOWSE protein scores of >50, with two or more matched peptides, were considered to represent a significant hit.
N/A, not applicable.

2010, and references therein). The evolution of a constitutive α-amylase in the low-dormancy ryegrass population could therefore confer a combination of rapid seedling establishment and resistance to benzoxazolinone, permitting the seedlings to survive if the seeds are imbibed under highly competitive conditions. It would be interesting to determine if the low-dormancy ryegrass population also produces higher levels of endogenous benzoxazinoids to improve its competitive ability further.

Properties of purified ryegrass α-amylase activities from dry and germinating ryegrass seeds

The enzymatic properties of α-amylase isoform 1 from dry seeds and isoforms 1, 2, 3 and 4 (collectively) from germinating seeds, purified using an alginate affinity matrix, were very similar. The pH and temperature optima, heat stability and requirement for Ca^{2+} were consistent with the wheat seed α-amylase purified using alginate affinity in the current study, and with other plant α-amylases isolated in previous studies (extensively summarized in Kumari *et al.*, 2010). However, the ryegrass seed α-amylases had an apparently higher K_m (20–30 vs. 1–3 mg starch mL^{-1} for most α-amylases), suggesting a lower affinity for soluble starch.

In spite of the high α-amylase activity in the alginate-binding protein fraction of heated seed extracts, the α-amylase protein was not readily visualized on denaturing PAGE: only two protein spots, from germinating low-dormancy and wheat seeds, were identified by peptide sequencing as α-amylase. If the highly abundant XIP that co-purified with ryegrass α-amylase activity could have been removed from the enzyme preparation, α-amylase may have been more readily visualized. The molecular mass (43 kDa) of the α-amylase identified by peptide sequencing in germinating low-dormancy seeds is consistent with that of previously characterized plant α-amylases, and the pI (5·6) corresponds to the finding of Cornford and Hill (1994) that grasses from outside the Triticodae supertribe, such as ryegrass, possess only the low pI (4·9–6·0) family of α-amylases. Interestingly, the wheat low pI α-amylases have a lower affinity for starch than the high pI family (Terashima *et al.*, 1995), so if the low pI ryegrass α-amylase is similar, this could also explain its high apparent K_m against soluble starch. As no α-amylase protein could be identified in the preparations from dry low-dormancy seeds or in dark-stratified, germinating seeds from the dormant population, it is uncertain as to which isoform was identified in the germinating seeds. It is also possible that only a sub-set of the seed α-amylase activities bound to the affinity matrix (e.g. Cornford and Hill, 1994).

The unexpected co-purification of ryegrass α-amylase and xylanase inhibitor protein

Xylanase inhibitor protein is expressed during the mid-to-late stages of seed development and during maturation and germination (Croes *et al.*, 2009). It is thought to protect the seed from pathogen attack by inhibiting microbial xylanases, thus preventing degradation of the cell wall (Dornez *et al.*, 2010). XIP may also act as an inhibitor of seed α-amylase, as demonstrated by the starch-dependent inhibition of barley seed α-amylase by purified wheat XIP (Sancho *et al.*, 2003). However, given that the interaction between XIP and α-amylase is not observed in the presence of calcium ions (Sancho *et al.*, 2003), and that high concentrations of calcium chloride were used during all stages of α-amylase purification and analysis in the current study, the high apparent K_m of purified ryegrass α-amylase is probably not due to inhibition by XIP. The high level of XIP protein in the ryegrass α-amylase preparation does, however, provide an explanation for the lower specific activity and purification factor of ryegrass α-amylase compared with the alginate-purified wheat α-amylase (Table 1). The fact that wheat XIP was not also highly enriched during the α-amylase purification process suggests that ryegrass XIP is more abundant or has a higher affinity for alginate than the wheat enzyme. It is possible that ryegrass seeds have higher constitutive XIP levels than wheat seeds because they need protection from microbial attack during the time that they are lying dormant or quiescent in the soil seed bank, but this requires further study.

In summary, the constitutive α-amylase activity selected during selection for low dormancy in annual ryegrass seeds appears during the late stages of seed development and is maintained throughout seed maturation and germination. As it appears to be localized in the aleurone, it is unlikely to be involved in the stimulation of germination, but rather would contribute to hydrolysing bulk starch reserves for early seedling growth and establishment (Asatsuma *et al.*, 2005; Magneschi and Perata, 2009). It is likely that the competitive ability of the low-dormancy seeds would be improved by (1) the potential for immediate (perhaps concomitant with germination initiation) mobilization of endosperm starch reserves and (2) the insensitivity to the allelochemical benzoxazolinone, both conferred by the constitutive presence of a sugar- and hormone-responsive α-amylase activity. Improved competitive ability would be of great importance if the seeds, due to their lack of dormancy, germinated under conditions sub-optimal for ryegrass seedling establishment.

ACKNOWLEDGEMENTS

We thank Proteomics International for the ESI-MS/MS protein analysis, and Drs R. Long and Q. Yu, and two anonymous reviewers, for helpful comments on the manuscript. This work was supported by the Australian Research Council (grant number LP0990923).

LITERATURE CITED

Asatsuma S, Sawada C, Itoh K, Okito M, Kitajima A, Mitsui T. 2005. Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. *Plant and Cell Physiology* **46**: 858–869.

Beck E, Ziegler P. 1989. Biosynthesis and degradation of starch in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 95–117.

Bewley JD, Black M. 1994. *Seeds: physiology of development and germination*, 2nd edn. New York: Plenum Press.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72**: 248–254.

Candiano G, Bruschi M, Musante L, *et al*. 2004. Blue-silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* **25**: 1327–1333.

Chen P-W, Chiang C-M, Tseng T-H, Yu S-M. 2006. Interaction between rice MYBGA and the gibberellin response element controls tissue-

specific sugar sensitivity of α-amylase genes. *The Plant Cell* **18**: 2326–2340.

Cornford CA, Hill RD. 1994. α-Amylase isozymes and BASI-like proteins in seeds of different grass species. *Seed Science Research* **4**: 285–291.

Cornish-Bowden A. 2004. *Fundamentals of enzyme kinetics*, 3rd edn. London: Portland Press.

Croes E, Gebruers K, Luyten N, Delcour JA, Courtin CM. 2009. The three classes of wheat xylanase-inhibiting proteins accumulate in an analogous way during wheat ear development and germination. *Journal of Plant Physiology* **166**: 1253–1262.

Dona AC, Pages G, Gilbert RG, Kuchel PW. 2010. Digestion of starch: *in vivo* and *in vitro* kinetic models used to characterise oligosaccharide or glucose release. *Carbohydrate Polymers* **80**: 599–617.

Dornez E, Croes E, Gebruers K, *et al*. 2010. Accumulated evidence substantiates a role for three classes of wheat xylanase inhibitors in plant defense. *Critical Reviews in Plant Sciences* **29**: 244–264.

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

Gebruers K, Debyser W, Goesaert H, Proost P, Van Damme J, Delcour JA. 2001. *Triticum aestivum* L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities. *Biochemical Journal* **353**: 239–244.

Goggin DE, Steadman KJ, Emery RJN, Farrow SC, Benech-Arnold RL, Powles SB. 2009. ABA inhibits germination but not dormancy release in mature imbibed seeds of *Lolium rigidum* Gaud. *Journal of Experimental Botany* **60**: 3387–3396.

Goggin DE, Emery RJN, Powles SB, Steadman KJ. 2010. Initial characterisation of low and high seed dormancy populations of *Lolium rigidum* produced by repeated selection. *Journal of Plant Physiology* **167**: 1282–1288.

Goggin DE, Powles SB, Steadman KJ. 2011*a*. Selection for low or high primary dormancy in *Lolium rigidum* Gaud seeds results in constitutive differences in stress protein expression and peroxidase activity. *Journal of Experimental Botany* **62**: 1037–1047.

Goggin DE, Powles SB, Toorop PE, Steadman KJ. 2011*b*. Dark-mediated dormancy release in stratified *Lolium rigidum* seeds is associated with higher activities of cell wall-modifying enzymes and an apparent increase in gibberellin sensitivity. *Journal of Plant Physiology* **168**: 527–533.

Guglielminetti L, Yamaguchi J, Perata P, Alpi A. 1995. Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. *Plant Physiology* **109**: 1069–1076.

Hunjan GK. 2006. Presence of an alpha-amylase isozyme with high temperature optima in the wheat variety tolerant to high temperature at juvenile plant stage. *Acta Physiologiae Plantarum* **28**: 205–215.

Kato-Noguchi H, Macías FA, Molinillo JMG. 2010. Structure–activity relationship of benzoxazinones and related compounds with respect to the growth inhibition and α-amylase activity in cress seedlings. *Journal of Plant Physiology* **167**: 1221–1225.

Kindred DR, Gooding MJ, Ellis RH. 2005. Nitrogen fertilizer and seed rate effects on Hagberg falling number of hybrid wheats and their parents are associated with α-amylase activity, grain cavity size and dormancy. *Journal of the Science of Food and Agriculture* **85**: 727–742.

Koshiba T, Minamikawa T. 1981. Purification by affinity chromatography of α-amylase – a main amylase in cotyledons of germinating *Vigna mungo* seeds. *Plant and Cell Physiology* **22**: 979–987.

Kumari A, Singh VK, Fitter J, Polen T, Kayastha AM. 2010. α-Amylase from germinating soybean (*Glycine max*) seeds – purification, characterization and sequential similarity of conserved and catalytic amino acid residues. *Phytochemistry* **71**: 1657–1666.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

de Lespinay A, Lequeux H, Lambillotte B, Lutts S. 2010. Protein synthesis is differentially required for germination in *Poa pratensis* and *Trifolium repens* in the absence or in the presence of cadmium. *Plant Growth Regulation* **61**: 205–214.

Lin R, Horsley RD, Schwarz PB. 2008. Associations between caryopsis dormancy, α-amylase activity, and pre-harvest sprouting in barley. *Journal of Cereal Science* **48**: 446–456.

Loreti E, Alpi A, Perata P. 2003. α-Amylase expression under anoxia in rice seedlings: an update. *Russian Journal of Plant Physiology* **50**: 737–742.

Lu C-A, Lin C-C, Lee K-W, *et al*. 2007. The SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling growth in rice. *The Plant Cell* **19**: 2484–2499.

MacGregor AW, MacDougall FH, Mayer C, Daussant J. 1984. Changes in levels of α-amylase components in barley tissues during germination and early seedling growth. *Plant Physiology* **75**: 203–206.

Magneschi L, Perata P. 2009. Rice germination and seedling growth in the absence of oxygen. *Annals of Botany* **103**: 181–196.

Perata P, Pozueta-Romero J, Akazawa T, Yamaguchi J. 1992. Effect of anoxia on starch breakdown in rice and wheat seeds. *Planta* **188**: 611–618.

Sancho AI, Faulds CB, Svensson B, Bartolomé B, Williamson G, Juge N. 2003. Cross-inhibitory activity of cereal protein inhibitors against α-amylases and xylanases. *Biochimica et Biophysica Acta* **1650**: 136–144.

Sharma A, Sharma S, Gupta MN. 2000. Purification of wheat germ amylase by precipitation. *Protein Expression and Purification* **18**: 111–114.

Steadman KJ. 2004. Dormancy release during hydrated storage in *Lolium rigidum* seeds is dependent on temperature, light quality, and hydration status. *Journal of Experimental Botany* **55**: 929–937.

Terashima M, Katoh S, Thomas BR, Rodriguez RL. 1995. Characterization of rice α-amylase isozymes expressed by *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **43**: 1050–1055.