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Dormancy and germination in wheat embryos: ribonucleases and hormonal control

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Abstract

Acidic and neutral ribonucleases (RNases) were studied in embryos of *Triticum durum* cv. Cappelli and the effects of abscisic acid (ABA) and gibberellic acid (GA_3) were analysed. RNases activities increased during germination and were comparable in dormant and non-dormant embryos imbibed for 24 h. ABA generally inhibited ribonucleolytic activities, while GA_3 only affected dormant embryos. To assess whether changes in RNase activities during germination or following hormonal treatment required new transcriptional or translational action, cycloheximide or cordycepin were used. The action of inhibitors of acidic RNase activity was found only in non-dormant-embryos. Findings obtained in the present work concur with a change of the ribonucleolytic pattern in the shift from dormant to non dormant metabolism.

Additional key words: abscisic acid, cordycepin, cycloheximide, gibberellic acid.

Introduction

Freshly harvested grains of *Triticum durum* are dormant and are characterised by the presence of inhibitory substances both in embryos (Grilli *et al.* 1975) and in endosperms (Spanò *et al.* 1994). They slowly lose their dormancy during after-ripening and after some months of dry state storage the grains are completely released from dormancy (Meletti 1964).

Seed dormancy has been actively investigated with regard to many associated physiological, biochemical and molecular changes under various hormonal and environmental conditions (Li and Foley 1997). Although metabolic activities are generally non detectable in dry seed tissues (Lynch and Clegg 1986) and seed metabolism is very low (Bewley 1997), clearly some changes are occurring under these physiological conditions that eventually result in the loss of dormancy (Johnson and Dyer 2000, Leubner-Metzger 2005). The after-ripening dry storage of *T. durum* grains is accompanied by a reduction of content of proteins, RNA and poly(A)$^+$ RNA in embryos, and by a decline in the capacity of RNA to code for proteins (Grilli *et al.* 1986). A further characterisation of RNA metabolism may therefore be of value, in order to identify possible qualitative and quantitative changes in relation to the shift from a dormant to non-dormant metabolism. In fact dormancy is important in particular for cereal crops because it prevents germination of grains while still on the ear of the parent plant (preharvest sprouting).

Although the physiological role of ribonucleases remains obscure, RNases are ubiquitous in plants and show changes in activity during processes like wheat seed development (Grilli *et al.* 2002) and ageing (Spanò *et al.* 2007). RNases were classified as acidic (A), neutral salt-stimulated (B) and neutral salt-inhibited (C), according to Blank and McKeon (1991). Acidic RNases are ubiquitous and localised in the vacuole or endoplasmic reticulum or secreted from the cells (Gallie *et al.* 2002), while neutral RNases show changes during several developmental processes (Green 1994). The different forms of RNases were studied by enzymatic assay and SDS-PAGE with activity staining. A continuous presence of acidic RNases, with similar activities is characteristic of developing embryos while a progressive decrease in neutral RNases is typical of drying (Grilli *et al.* 2002). Since changes in seed water content seem to mark a point of transition characterised by qualitative and quantitative changes in enzyme activities, RNases were studied in both dry and imbibed dormant and non-dormant embryos. This study may be interesting as some changes induced during after-ripening have been evidenced after the

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Abbreviations: ABA - abscisic acid; A-RNases - acidic RNases; B-RNases - neutral salt-stimulated RNases; CHX - cycloheximide; Cor - cordycepin; C-RNases - neutral salt-inhibited RNases; D - dormant; GA_3 - gibberellic acid; GM - germination medium; ND - non dormant SDS-PAGE - sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

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start of imbibition (Johnson and Dyer 2000).

Seed dormancy and germination seem to be regulated by different hormones (Bhargava 1997) and substances. Nitric oxide plays a role in dormancy breaking or germination of several seeds as *Arabidopsis* (Batak *et al.* 2002, Bethke *et al.* 2004, 2006), barley (Bethke *et al.* 2004), lettuce (Beligni and Lamattina 2000), and *Paulonia tomentosa* (Giba *et al.* 1998). In apple embryos NO increases ethylene biosynthesis and its concentration in seeds. Ethylene lowers seed sensitivity to ABA, thus promoting dormancy breakage and stimulating seed germination (Gniazdowska *et al.* 2007). In fact not only the presence of ABA (Agrawal *et al.* 2001) but also the differential sensitivity to ABA may be important in maintaining mature seeds in a dormant state (Walker-Simmons 1987, Kawakami *et al.* 1997, Jacobsen *et al.* 2002). After the ABA controlled inhibition of germination has been overcome, GA_3 is important in the promotion and maintenance of germination (Bewley 1997, Finch-Savage and Leubner-Metzger 2006). As for ABA, sensitivity to GA_3 may be a key factor (Hilhorst *et al.* 1986, Karssen *et al.* 1989, Hilhorst and Karssen 1992). On this basis, this work evaluates the effect of ABA and gibberellic acid on RNases patterns in dormant and non dormant wheat embryos and the possible translational or transcriptional control in the role of these hormones.

Materials and methods

Plants and treatments: Uniformly-sized grains of *Triticum durum* Desf. cv. Cappelli were used immediately after harvesting or stored at -20 °C (D). One lot was stored at room temperature (19 ± 2 °C) for 10 months (ND). Dry after-ripening broke the dormancy in this cultivar. Grains (11 % moisture content) were surface sterilised for 3 min in NaOCl (1 %, v/v, available chlorine) and rinsed before use. Wheat grains were germinated in Petri dishes (four replicates each of 25 grains) on water-moistened *Whatman No. 2* filter paper at 23 ± 1 °C in the dark. The percentage germination was recorded after 24, 48, and 72 h, and the shoot and the root lengths of seedlings were measured at 72 h. Embryos isolated from dry (0 h) or imbibed (0.5, 24, 48 h) grains were frozen in liquid nitrogen until use for RNases extraction.

Embryos (axes + scutellum) were hand-isolated by mean of a gouge from sterilised grains and transferred to Petri dishes onto a layer of *Whatman No. 2* filter paper wetted with sterile germination medium (GM): 10 mM Tris HCl, pH 7, 20 mM KCl, 10 mg cm^{-3} sucrose and 50 μg cm^{-3} chloramphenicol (Caers *et al.* 1979). Germination was evaluated and embryos were considered germinated when the roots were at least 2 mm long. Five replications with 10 embryos were made for the control and for each hormonal solution.

Embryos utilised for RNase extraction were imbibed for 24 h in GM, in GM plus 20 μg cm^{-3} cycloheximide (GM + CHX) or GM plus 0.25 mM cordycepin (GM + Cor), and then frozen in liquid nitrogen until use. To study hormonal regulation of dormancy, GA_3 (GM + GA), or ABA (GM + ABA) were added at varying concentrations to the medium (0.1, 1.0, and 1000 μM for GA_3; 0.5, 5.0, and 50 μM for ABA). Cycloheximide or cordycepin were also used together with GA_3 (1000 μM) or ABA (0.5 μM).

RNases extraction and enzymatic assay: Embryos treated in the different ways, as described above, were ground in liquid nitrogen with a pestle and mortar to a fine powder. The extraction buffer, ice-cold 200 mM sodium acetate, pH 5.5 with 100 μM phenylmethylsulfonylfluoride (PMSF), was then added. Homogenates were centrifuged for 10 min at 14 000 *g* and the supernatant solutions were divided into small aliquots and stored in liquid nitrogen until used for electrophoresis and detection of protein content. All materials used were sterile.

Acidic, neutral salt-stimulated and neutral salt-inhibited RNases (Blank and McKeon 1991) were estimated by measuring acid-soluble nucleotides with *Torula* yeast RNA (10 μg cm^{-3}) as substrate according to Isola and Franzoni (1981) with minor modification (Grilli *et al.* 2002). The buffer in the reaction mixture was specific for each different class of RNase as described below. The enzyme activity of the extracts is expressed as U mg^{-1} protein. The enzyme unit (U) was determined according to Wilson (1975). The protein content was determined using a modified version of Lowry's method (Bensadoun and Weinstein 1976).

RNase activity: Electrophoresis was performed according to Blank *et al.* (1982) and Blank and McKeon (1991), with minor modifications (Spanò *et al.* 1999). RNase activity analyses were carried out using 12.5 % SDS-PAGE gels containing 2.4 mg cm^{-3} *Torula* yeast RNA. Equal amounts (25 μg) of proteins extracted from embryos were loaded onto gels. Following electrophoresis, the gels were washed, incubated and stained as previously described (Spanò *et al.* 2002). After the gels had been washed to remove SDS, they were incubated for 90 min at 37 °C in: 50 mM sodium acetate buffer, pH 5.7 for the A-RNases; 100 mM imidazole buffer, pH 8.0 for the C-RNases; 100 mM imidazole buffer, pH 8.0 containing 200 mM KCl for the B-RNases. Activity bands were visualised by staining with 0.2 % (m/v) toluidine blue and washing with buffer. Gels were scanned with a *GS-690* imaging densitometer (*Bio-Rad,* Hercules, CA, USA).

Results

Dormancy state of *Triticum durum* cv. Cappelli was evaluated using germination and growth parameters (Table 1). Dormant grains were characterised by a delay in germination and a low germinability (23 % at 72 h of imbibition). After a period of dry storage, dormancy was broken and grains were able to germinate. Only after-ripened grains showed root and shoot growth, while dormant grains showed only extrusion of roots without measurable growth of roots and shoots.

Embryos isolated and imbibed for 24 h in GM showed a higher percentage of germination than when enclosed in grains (Table 2). Nevertheless, germination was significantly lower in embryos from dormant grains (D-embryos) than in embryos from non-dormant grains (ND-embryos).

Table 1. Germination percentages after 24, 48 and 72 h and length of shoot and root at 72 h of dormant (D) and non-dormant (ND) caryopses of *Triticum durum*. Four replications (25 seeds of each) were used. Different letters show means that are significantly different at $P < 0.05$.

	Germination [%]			Length [mm]	
	24 h	48 h	72 h	shoot	root
D	0a	10a	23a	0a	0a
ND	24b	88b	100b	20b	47b

Table 2. Effects of ABA and GA on percentage germination of D and ND embryos of *T. durum* at 24 h. Five experiments for each treatment were performed. Different letters show means that are significantly different at $P < 0.05$.

	GM	ABA [μM]			GA_3 [μM]		
		0.5	5	50	0.1	10	1000
D	10a	2b	0b	0b	24c	45d	62e
ND	74a	32b	24b	8c	80a	88a	94a

The activity of acidic (A), neutral salt-stimulated (B) and neutral salt-inhibited (C) RNases was analysed both by enzymatic assay and by SDS-PAGE with activity staining in D- and ND-embryos during imbibition and the results obtained by the two methods concurred (Fig. 1). The activity of RNases always increased during imbibition, in particular in D-embryos (Fig. 1), where an early decrease in activity was recorded. Using SDS-PAGE (Fig. 1) A- and C-RNases were present as singular bands of activity with molecular masses 20 (p20) and 27 kDa (p27), respectively. Both p20 and p27 increased during germination, and the increase was particularly evident for C-RNases. Two forms of B-RNases with different molecular mass (24 kDa, p24 and 26 kDa, p26) were recorded. The main form, p26, was always present and its activity increased during imbibition, while p24, the secondary band with higher mobility, not detectable

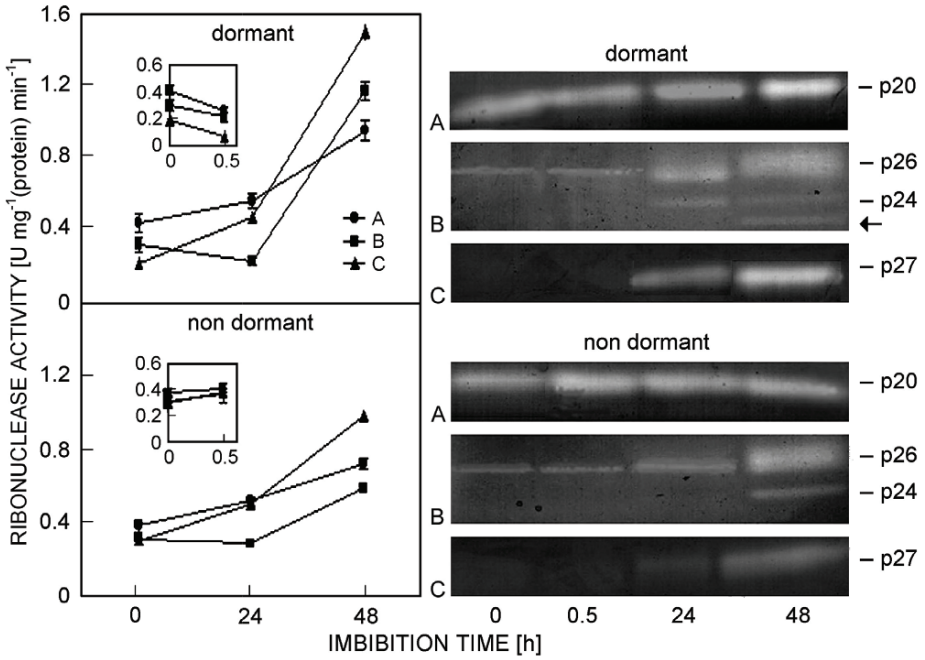

Fig. 1. Activity of acidic (*A*), neutral salt-stimulated (*B*), neutral salt-inhibited (*C*) RNases in dormant and non-dormant embryos at 0, 0.5, 24 and 48 h of imbibition. Results of enzymatic assays (*left*) and of electrophoresis (*right*) are shown. For enzymatic assays, RNases activity at 0 and 0.5 h are indicated in the box comprised in each graph. Results of enzymatic assays represent the mean of three replicates of three sets of independent experiments ± SE. The different forms of RNases are indicated; the degradation band is indicated by the *arrow*.

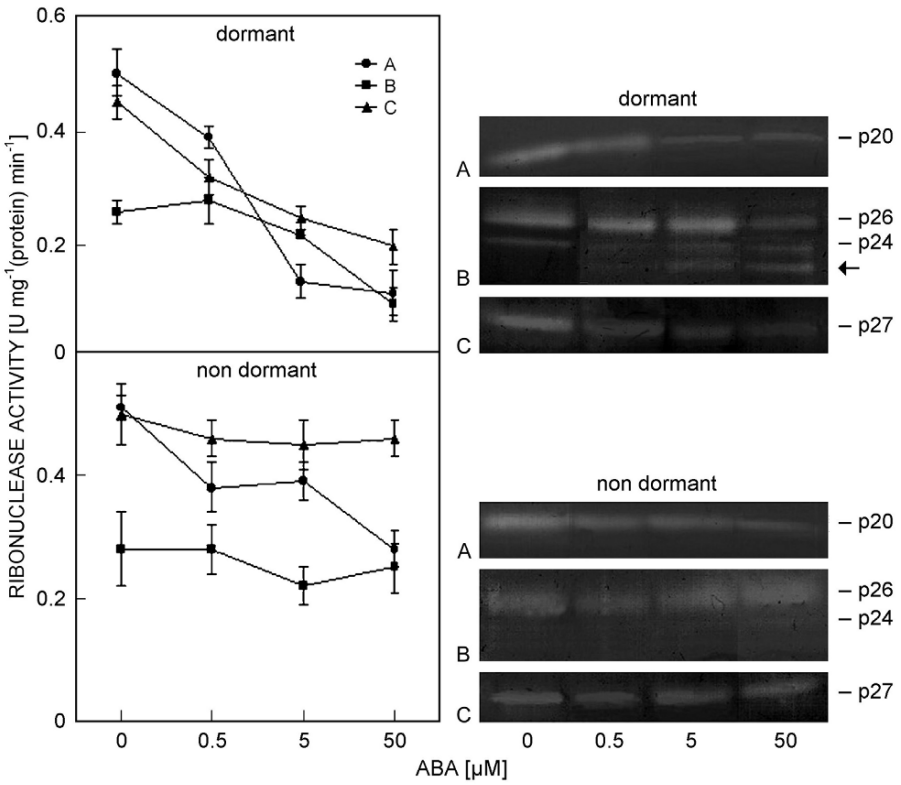

Fig. 2. Activity of acidic (*A*), neutral salt-stimulated (*B*), neutral salt-inhibited (*C*) RNases in dormant and non-dormant embryos at 24 h of imbibition in presence of different concentrations (0, 0.5, 5.0, 50 μM) of ABA. Results of enzymatic assays (*left*) and of electrophoresis (*right*) are shown. Results of enzymatic assays represent the mean of three replicates of three sets of independent experiments ± SE. The different forms of RNases are indicated; the degradation band is indicated by the *arrow*.

in dry embryos, appeared during imbibition after 24 h in D-embryos and after 48 h in ND-embryos. The appearance (48 h) in D-embryos of a third band of activity of B-RNases with higher mobility was recorded.

ABA inhibited germination, and no germination was recorded in D-embryos in the presence of higher ABA concentrations (Table 2). On the other hand, ND-embryos still germinated when treated with ABA, although germination was reduced with respect to the control. GA_3 always stimulated embryo germination to an extent that increased with increasing concentration of GA_3 in D-embryos and, in a lesser degree, in ND-embryos.

Different concentrations of ABA inhibited ribonuclease activities in D-embryos (Fig. 2) although the extent of inhibition varied for the different RNases. Only A-RNases were inhibited in ND-embryos, but the extent of inhibition was less than in D-embryos. ABA induced an earlier appearance (24 h) in D-embryos of the degradation band (Fig. 2), present in non treated D-embryos only after 48 h of imbibition (Fig. 1). In D-embryos GA_3 did not influence A-RNase activity while it slightly stimulated B-RNase and inhibited C-RNase. In ND-embryos GA_3 did not influence RNases activities significantly (Fig. 3). These findings are confirmed by electrophoresis (Fig. 3).

To assess whether changes in RNases activities recorded during imbibition in D- and ND-embryos required new transcriptional or translational action, CHX or Cord were added at the beginning of 24 h incubation (Table 3). In D-embryos, both the substances inhibited RNase activities, in particular C-RNase. On the other hand, ND-embryos were characterised by the strong activation of A-RNase by CHX and Cord, while B-RNase was insensitive and C-RNase was inhibited (Table 3).

On the basis of the influence of ABA and GA_3 on RNases, hormonal actions were analysed to establish whether they depended on new poly(A)$^+$ RNA and/or new protein synthesis. Embryos were therefore incubated with ABA (0.5 μM) or GA_3 (1000 μM) in the presence of CHX or Cord, and the activity of RNases was studied by enzymatic assay (Table 3) and SDS-PAGE with activity staining (data not shown as electrophoretic results were the same as the enzymatic ones). In D-embryos the strong inhibition by ABA of A-RNase (77 % of inhibition) was reduced by CHX and Cord. The contemporary presence of CHX or Cord with ABA resulted in an inhibitory action on B-RNase comparable with that recorded in the presence of the three substances used separately. The inhibitory action of ABA on C-RNase was increased when CHX and Cord were added. In ND-embryos, CHX and Cord did not relieve the inhibitory effect of ABA on A-RNase (Table 3).

When GA_3 had little or no effect on A-RNases for D- and ND-embryos and B- and C-RNases for

ND-embryos, the effect recorded when CHX and/or Cord were added to the GA_3 solution was due only to the presence of these two inhibitors. In D-embryos there was the nullification by CHX and Cord of the stimulatory effect of GA_3 on the activity of B-RNase. The inhibitory effect of CHX and Cord increased the inhibition by GA_3 on C-RNase in D-embryos.

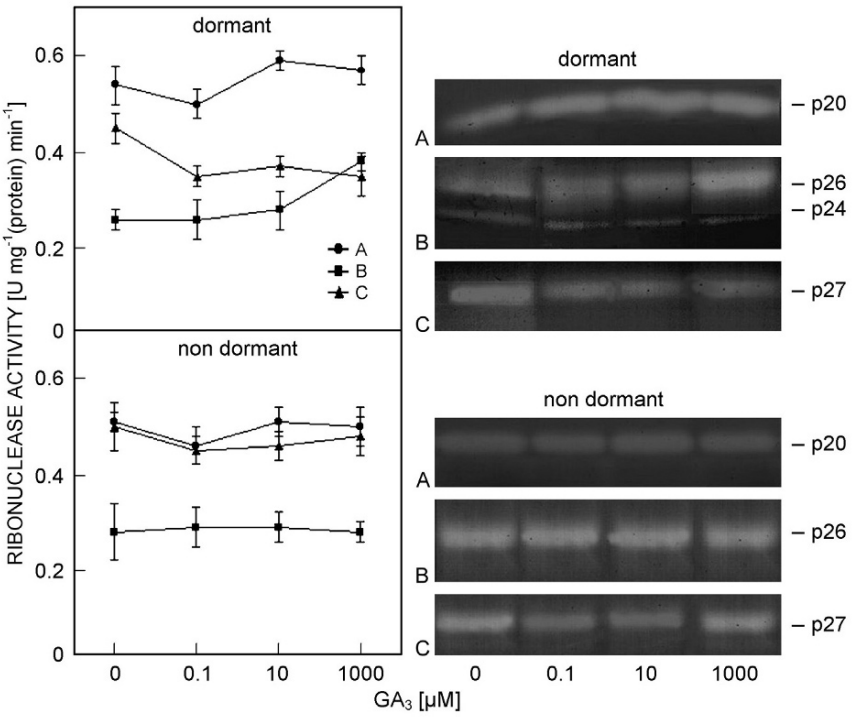

Fig. 3. Activity of acidic (*A*), neutral salt-stimulated (*B*), neutral salt-inhibited (*C*) RNases in dormant and non-dormant embryos at 24 h of imbibition in presence of different concentrations (0, 0.1, 10, 1000 μM) of GA_3. Results of enzymatic assays represent the mean of three replicates of three sets of independent experiments ± SE. The different forms of RNases are indicated.

Table 3. Changes in activity of acidic (A-RNase), neutral salt-stimulated (B-RNase), neutral salt-inhibited (C-RNase) in D- and ND-embryos after 24 h in GM, GM with CHX (20 μg cm^{-3}), Cord (0.25 mM), in the absence and presence of ABA (50 μM) or GA (1 mM). Data were subjected to one-way analysis of variance and differences among treatments were determined by Bonferroni post-test. Different letters denote significant differences at $P < 0.05$.

		GM	CHX	Cord	ABA	ABA+CHX	ABA+Cord	GA	GA+CHX	GA+Cord
D	A-RNase	0.54a	0.34b	0.31b	0.12c	0.29b	0.29b	0.57a	0.34b	0.28b
	B-RNase	0.20a	0.11b	0.13b	0.10b	0.10b	0.13b	0.38c	0.11b	0.11b
	C-RNase	0.45a	0.11b	0.12b	0.20c	0.13b	0.14b	0.35d	0.17c	0.12b
ND	A-RNase	0.51a	0.81b	0.90b	0.28c	0.58a	0.52a	0.50a	0.79b	0.87b
	B-RNase	0.28a	0.31a	0.34a	0.30a	0.31a	0.35a	0.28a	0.27a	0.32a
	C-RNase	0.50a	0.26b	0.31b	0.46a	0.34b	0.29b	0.48a	0.30b	0.32b

Discussion

The delay and the reduced capacity of germination shown by wheat grains immediately after harvesting are typical of relative dormancy (Meletti 1964, Bewley and Black 1994). Dry storage allows the breakage of dormancy and grains are able to germinate. The higher percentage of germination recorded in embryos isolated and imbibed for 24 h in GM than when enclosed in grains might be due to a more marked leaching effect and/or a failed endospermatic control, in accordance with the presence of inhibitory substances demonstrated in bran (aleurone layer and coats) of *Triticum durum* grains (Spanò *et al.* 1994). On the other hand, germination was significantly lower in embryos from dormant grains (D-embryos) than in embryos from non-dormant grains (ND-embryos),

showing the presence of true embryo dormancy. In fact, germination inhibitors have also been shown to be present in wheat embryos (Grilli *et al.* 1975). The state of dormancy in the intact grains could therefore be the result of an inhibitory effect of both endosperms and embryos.

In *T. durum* the inhibitory effect of ABA is concentration dependent as in *Hordeum* (Van Beckum *et al.* 1993) and in *T. aestivum* and the different response of D- and ND- embryos was probably due to a different sensitivity to applied ABA, as suggested by previous studies (Walker-Simmons 1987, Ried and Walker-Simmons 1990). GA_3 always stimulated embryo germination to an extent that increased with increasing concentrations of GA_3 in D-embryos and, less significantly, in ND-embryos.

RNase activities were similar in dry D- and ND-embryos, but differences were recorded upon imbibition. It is worth noting the initial decrease in activity of ribonucleases only in D-embryos. This decrease may be due to an early degradation or inactivation of stored RNases linked to the ripening process but not to the germination. The observed increase in RNases activities during later imbibition was probably associated with a RNA metabolism accompanying imbibition. Message-encoding proteins, that are important during seed maturation and drying, are likely to be degraded rapidly upon imbibition (Bewley 1997, Li *et al.* 2006), while other preformed mRNAs seem to have an important role during early imbibition and they might contribute to newly formed enzyme as in maize embryos (Enriquez-Arredondo *et al.* 2005).

The higher RNase activity in imbibing D- than in ND-embryos could be due to the need for a higher RNA turnover in D-embryos: dormant seeds have still not overcome the period of dry after-ripening when dormancy is gradually released. Using a cDNA-AFLP approach, dormancy-maintaining and dormancy-breaking genes were identified in *Nicotiana plumbaginifolia* seeds (Bove *et al.* 2005). After-ripening resulted, in the air-dried seed, in a new development program modulated at RNA level. The after-ripening in dry storage of *T. durum* grains is accompanied in embryos by a reduction of content of RNA, and poly(A)$^+$ RNA, and by a decline in the capacity of RNA to code for proteins (Grilli *et al.* 1986) and recent studies record a degradation of particular mRNAs in grains during dry after-ripening phase (Johnson and Dyer 2000). Dormant embryos of *T. durum*, differently from non dormant ones, might have the need to degrade these RNAs during imbibition before being able to germinate.

The appearance of p24 seems to be associated with embryo water content, in fact it progressively decreases during maturation drying (Grilli *et al.* 2002) and appeared with the progressive increase in water content during imbibition. On the other hand, results also suggest the importance of the physiological state: the band appeared more precociously in D- than in ND-embryos, although the imbibition rate was the same in the two physiological states (data not shown). Of particular interest was the appearance of the third band of activity of B-RNases after 48 h in D-embryos. The low molecular mass (18 kDa) leads to the suggestion that this band may be the result of a partial proteolysis of RNase with minor mobility as reported in literature (Chang and Gallie 1997). Noteworthy was the ABA-induced earlier appearance of this degradation band in D-embryos (24 h), and findings obtained suggest that ABA, endogenous or exogenous, could cause a partial degradation of B-RNase.

The use of CHX and Cord did not eliminate RNase activities and, in part or totally (B-RNase in ND-embryos), the enzymatic activity recorded after 24 h of imbibition did not depend on a new transcriptional or translational action and may therefore be due to a conserved RNase activity. Moreover, the strong stimulatory effect of the two substances on A-RNases activity in ND-embryos could be explained by the presence of inhibitory factors that required a new transcriptional and/or translational activity. Therefore, the use of the two inhibitors highlighted the different nature of the three RNases and the different responses in the two physiological states.

When the inhibition by ABA was relieved by CHX and Cord, as for A-RNase in D-embryos, the ABA effect could have been due to a factor requiring the new synthesis of mRNA and/or proteins. When the inhibitory action of ABA was not relieved by the two substances, as for A-RNase in ND-embryos, the hypothesis can be made that ABA could regulate the activity of the enzyme by a mechanism not requiring new synthesis, such as phosphorylation and de-phosphorylation, the regulative mechanism of RNase activities hypothesised in literature for wheat leaves (Chang and Gallie 1997).

GA_3 had little or no effect on activities of A-RNase for D- and ND-embryos, and B- and C-RNases for ND-embryos, and the effect recorded when CHX and/or Cord were added to the GA_3 solution was due only to the presence of these two inhibitors. In D-embryos the nullification by CHX and Cord of the stimulatory effect of GA_3 on the activity of B-RNases showed that the hormonal action required new synthesis. The inhibitory effect of CHX and Cord increased the inhibition caused by GA_3 on C-RNase in D-embryos.

Findings obtained in the present work concur with the change of the ribonucleolytic pattern in the shift from dormant to non-dormant metabolism evidenced after the start of imbibition. Although in general RNases activities are comparable in D- and ND- 24 h imbibed embryos, a higher fraction of new synthesised enzyme, or at least requiring new synthesis for regulation, is present in D- with respect to ND-embryos, as shown by the use of CHX and Cord. This is consistent with the hypothesis previously made of an initial (0.5 h) degradation of RNases in D-embryos and may be due to a higher turnover of RNases in D-embryos when RNA linked to maturation but not to germination might be eliminated. Another interesting finding is the possible action of inhibitors of A-RNase activity present only in ND-embryos, leading to the hypothesis of a different

control of enzymatic activity in D- and ND-embryos. In D-embryos the activity seems to be regulated by enzymatic turnover with a consistent contribution of new synthesised RNases to the overall activity. In ND-embryos, on the contrary, this ribonucleolytic activity is normally repressed by a new synthesised inhibitor. The use of ABA and GA_3 showed that the different forms of RNases have a different sensitivity to the hormonal action also in relation to the physiological condition of the grain. GA_3 did not influence RNases activities in a significant manner, while, generally, ABA had a greater inhibitory action in D- than in ND-embryos. The higher response generally recorded in D-embryos to ABA both in terms of germination and of nucleolytic enzymes might be partly due to a different sensitivity to applied ABA, in accordance with Walker-Simmons (1987), and partly to the synergistic action between exogenous ABA and embryo-endogenous inhibitors (Grilli *et al.* 1975).

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