Journal of
Experimental
Botany

RESEARCH PAPER

Redox regulation and storage processes during maturation in kernels of *Triticum durum*

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Abstract

Metabolic changes during the development and maturation of *Triticum durum* Desf. (L.) kernels were studied, with particular emphasis on changes in the redox state of ascorbate and glutathione, as well as in the activities of the enzymes responsible for the recycling of their oxidized forms (ascorbic free radical reductase, EC 1.6.5.4; dehydroascorbate reductase, EC 1.8.5.1; glutathione reductase, EC 1.6.4.2) and for detoxification or utilization of hydrogen peroxide (ascorbate peroxidase, EC 1.11.1.11; catalase, EC 1.11.1.6). In parallel with this analysis, the production and storage of reserve compounds was studied, in particular, soluble carbohydrates (mono- di-saccharides and fructans) and the transition from sulphydryl groups to disulphide bridges into proteins. The results indicate that both the activities of the ascorbate and glutathione redox enzymes and that of catalase are high before the start of drying maturation, after which they decrease. Moreover, analysis of the redox state of ascorbate and glutathione pairs and the sulphydryl to disulphide transition into proteins suggests that these three parameters are tightly related during kernel maturation, thus confirming the involvement of the two redox pairs in protein maturation as well as in protection against reactive oxygen species. The physiological implications of the changes in cellular redox state and in soluble carbohydrates for the acquisition of desiccation tolerance and reaching the resting phase in orthodox seeds are also discussed.

Introduction

Seed development and maturation is a highly orchestrated multi-step process during which embryos are formed and supplied with the carbohydrates, proteins and lipids needed for the subsequent germination. During seed development the increase in size is due to cell division and expansion, followed by a progressive accumulation of storage compounds. In many plants, the last step of seed maturation is dehydration, a process allowing seeds to delay germination until there are suitable environmental conditions (Steeves, 1983). Seeds that reach maturity in a highly dehydrated state, orthodox seeds, survive for long periods and can be stored for years under cold dry conditions without losing viability. By contrast, the so-called recalcitrant seeds do not undergo drying maturation, are very sensitive to desiccation, and lose viability in a very short time (Roberts, 1973). The desiccation tolerance acquired by orthodox seeds may be analogous in some aspects to the defence mechanisms carried out in vegetative tissues by tolerant species subjected to drying. In fact, the increase in abscisic acid (Mansfield and McAinsh, 1995; Rock and Quatrano, 1995) and the accumulation of specific polypeptides (Skriver and Mundy, 1990; Bradford and Chandler, 1992) are examples of processes that are common to both orthodox seed development and vegetative tissue responses to water depletion. Among orthodox seeds, cereal kernels have been intensively

investigated because of their economic importance. In particular, data are available on their capability of accumulating storage compounds and acquiring desiccation tolerance (Black *et al.*, 1999; Gebbing and Schnyder, 1999).

The aim of this paper was to analyse some parameters characterizing the development of *Triticum durum* Desf. (L.) kernels, including the storage of soluble and insoluble carbohydrates and the accumulation and maturation of proteins. Particular attention has been paid to ascorbate (ASC) and glutathione (GSH) metabolisms. These two redox pairs are involved in plant developmental processes as well as in the protection against reactive oxygen species (ROS), the production of which is well known to increase strongly in vegetative tissues subjected to water stress (Sgherri and Navarri Izzo, 1995; Noctor and Foyer, 1998; Asada, 1999; De Gara and Tommasi, 1999). ASC and GSH are responsive to ROS scavenging both directly and by means of enzymatic reactions. Among the latter, much attention has been given to the so-called ascorbate–glutathione cycle, a set of reactions in which ASC and GSH undergoing oxidation are continuously regenerated. The first enzyme of the cycle is ascorbate peroxidase (APX), that removes hydrogen peroxide by oxidizing ASC to the ascorbic free radical (AFR). The AFR formed is reduced to ASC by an NAD(P)H-dependent AFR-reductase (AFRR), or it disproportionates to ASC and dehydroascorbate (DHA). DHA, the final product of ASC oxidation, is then reduced to ASC by DHA-reductase (DHAR), an enzyme using GSH as reductant. Finally, GSH is regenerated from the glutathione disulphide (GSSG) produced in the latter reaction by GSSG-reductase (GR) by using the reducing power of NADPH. Changes in the ASC–GSH cycle enzymes occur in response to several kinds of stresses. Moreover, genetically modified plants over-expressing one of these enzymes are more resistant to abiotic stresses, whereas plants under-expressing one of them become more sensitive (Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). Changes in the ASC–GSH cycle also occur in developmental processes such as seed germination (Tommasi *et al.*, 2001) or leaf senescence (Borraccino *et al.*, 1994). In addition, a co-ordinated increase in their activities characterize the exponential growing phase in cultured tobacco cells, whereas all the enzymes have much lower activities in cells that have reached the stationary phase (de Pinto *et al.*, 2000). On these bases, changes in the ASC and GSH metabolisms could also be required for kernel development, during which, phases of intense metabolic activities are followed by programmed dehydration and achievement of a resting phase.

Materials and methods

Plant growth

Plants of *Triticum durum* Desf. (L.) (cv. Simeto) were grown in experimental fields in 1998–1999 in Rome on 10 m^2 plots with a sowing density of up to 450 seed m^{-2}. The plants were arranged in a randomized block design and collected on two segments of a 25 cm length on the same row. Irrigation, fertilization and plant protection were performed to ensure optimal plant growth. Ear flowering started on 4 May and plants were hand collected from 13 d after anthesis (DAA) (physiological stage of early milky phase) to 45 DAA (complete kernel development). The ears were separated from the stems and stored at –80 °C for analysis. Mid-ear kernels (grains) were used for the measurements of metabolite contents and enzyme activities.

Dry weight, water status and chlorophyll contents

Kernels (20–50) were dried at 100 °C until constant dry weight was achieved (24–36 h). Water content was expressed on a percentage fresh weight basis. Chlorophyll contents (chlorophyll *a* plus chlorophyll *b*) were assayed according to Zhang and Kirkham (1996) using 0.5–1 g of kernels for each experiment.

Carbohydrate and protein contents

The kernels previously dehydrated by lyophilization were ground in a laboratory mill. The carbohydrate-soluble analyses were carried out by a preliminary extraction with 96° ethanol for 1 h at 80 °C, followed by a water extraction for 2 h at 105 °C. The ethanol-soluble fraction mainly contained low molecular weight carbohydrates (e.g. mono – and di-saccharides), while, high molecular weight carbohydrates (e.g. fructans and oligosaccharides) were mainly present in the water-soluble fraction. Glucose and fructose contents were determined in the two fractions using enzymatic (glucose oxidase/ peroxidase kit; Sigma Diagnostic, St Louis, USA) or chemical (resorcine-HCl) procedures, respectively. Since fructans are exclusively constituted by fructose and glucose, their quantification allowed the measurement of the total content of fructan (Virgona and Barlow, 1991).

Starch determination was performed using the enzymatic assay procedure Total Starch (amyloglucosidase/α-amylase method) (American Association of Cereal Chemists, 2000) proposed by Megazyme (Warriewood, Australia)

Protein content was determined by the Dumas method (American Association of Cereal Chemists, 2000) with the automatic Leco FP-428 system (Leco Corporation, St Joseph, MI USA). Carbohydrate and protein contents were calculated on a per-kernel basis.

Analysis of ascorbate and glutathione pools

The kernels (0.5–1 g) were homogenized with eight volumes of cold 5% meta-phosphoric acid at 4 °C. The homogenate was centrifuged at 20 000 *g* for 15 min at 4 °C, and the supernatant was collected for analysis of ascorbate and glutathione contents and redox state as described in de Pinto *et al.* (1999). Ascorbate and glutathione contents were calculated on a per-kernel basis.

Enzyme assays

Kernels were ground in liquid N_2 with a mortar and pestle. Four volumes of a medium which consisted of 50 mM Tris-HCl (pH 7.8), 0.05% (w/v) cysteine, and 0.1% (w/v) BSA, were added just as the last trace of liquid N_2 disappeared. The thawed mixture was then ground and centrifuged at 20 000 *g* for 15 min. The supernatant was used for both spectrophotometric and native-PAGE analysis.

DHA reductase (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1), AFR reductase (NADH: ascorbate free radical oxidoreductase, EC 1.6.5.4) and ascorbate peroxidase (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) were assayed according to Di Cagno *et al.* (2001).

Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity assay was performed according to Beaumont *et al.* (1990) with minor modification, by following the H_2O_2

dismutation at 240 nm in a reaction mixture, which consisted of 0.1 M phosphate buffer, pH 7.0, 50–100 μg protein and 18 mM H_2O_2 (extinction coefficient 23.5 mM^{-1} cm^{-1}). Glutathione reductase (NADPH: glutathione disulphide oxidoreductase, EC 1.6.4.2) was measured as indicated in Osswald *et al.* (1992). Enzyme activities were calculated on a per-kernel basis.

Native polyacrylamide gel electrophoresis (PAGE) for APX and DHAR was performed as previously reported (De Gara *et al.*, 1997). Gels were photographed by using the gel documentation system GEL DOC 2000 by Biorad.

Analysis of proteic sulphydric groups

The identification of proteic –SH groups was performed by their labelling with mBBr according to Kobrehel *et al.* (1992). Kernels (0.5–2 g) were ground in liquid N_2 with a mortar and pestle. Four volumes of 2 mM mBBr (dissolved in acetonitrile) in 100 mM Tris-HCl, pH 7.5, buffer were added just as the last trace of liquid N_2 disappeared. The thawed mixture was then ground for 1 min, transferred to a microfuge tube and centrifuged at 30 000 *g* for 15 min at 4 °C. Ten μl of 10% SDS and 10 μl of 100 mM 2-mercaptoethanol were added to 80 μl of the mBBr-labelled extracts to stop the reaction and derivatize excess mBBr. Samples were then applied to gels for electrophoretic analysis.

SDS-PAGE analysis of mBBr-labelled extracts was performed at pH 8.5 in 12.5% gels of 1.5 mm thickness for approximately 14 h at a constant current of 8 mA. Electrophoresis was stopped when the solvent front, marked with bromophenol blue tracking die, migrated to approximately 1 cm from the bottom of the gel. After electrophoresis, gels were placed in 12% (w/v) trichloroacetic acid for 30 min to fix the proteins and were then transferred to a solution of 40% ethanol (v/v), 10% acetic acid (v/v) for 4–10 h to remove excess of mBBr. The fluorescence of proteins bound to mBBr was visualized by placing gels on a light box fitted with a UV light source (365 nm). Gels were photographed by using the gel documentation system GEL DOC 2000 by Biorad.

Results

Maturation parameters

Kernel development was studied from 13 day after anthesis (DAA) (early milky phase) to complete maturation (45 DAA). As expected, during the first 28 d of development, an increase in fresh weight and size of the kernels was observed. After this stage the water loss due to the dehydration process exceeded the dry weight increase due to reserve storage and, therefore, kernel dimensions decreased in terms of both fresh weight and size (Table 1).

Chlorophyll content was relatively stable until 17 DAA. At 21 DAA it decreased by about 20%, after which it progressively declined and, in the mature kernels, chlorophylls were not detectable (Table 1).

During the first period of maturation, kernels were particularly rich in low molecular weight carbohydrates including fructans (Table 2). As the development proceeded, fructan content per kernel decreased, whereas the mono- and di- saccharide contents first decreased from 17 to 28 DAA, then returned to a value comparable to the 13 DAA value in the mature kernels (Table 2). As expected, the starch content increased during maturation (Table 2). Under these growth conditions, this storage polysaccharide reached values of 35 mg per kernel that is about 60% of mature kernel dry weight. During kernel development, protein contents also increased (Table 2).

In order to analyse changes in the sulphydryl status of proteins during kernel maturation, the proteic –SH groups

Table 1. *Growth parameters, hydric state and chlorophyll content in wheat kernels collected from ears at different stage of maturation*

Days after anthesis	Fresh weight[a] (mg kernel^{-1})	Dry weight[a] (mg kernel^{-1})	Water content (% fresh weight)	Chlorophyll[a] (μg g^{-1} FW)	Kernel sizes[b] (mm kernel^{-1})
13	33±0.4	7.5±0.3	77	112±4	5.8±0.1
17	52±0.8	14.6±0.6	72	103±6	7.6±0.3
21	64±0.5	28.2±0.2	56	84±5	8.0±0.4
28	90±1.2	47.1±0.4	52	36±2	8.6±0.3
45	64±0.7	58.2±0.6	9	n.d.[c]	8.1±0.2

[a] The reported results are the mean values obtained from five different experiments ±standard error (SE).
[b] The reported results are the mean values obtained from the measurement of 50 kernels ±standard error (SE).
[c] n.d.=not detectable.

Table 2. *Changes in carbohydrates and proteins contents during wheat kernel maturation*

The values are the mean of five experiments ±SE.

Days after anthesis	Mono- and di-saccharides (mg kernel^{-1})	Fructans (mg kernel^{-1})	Starch (mg kernel^{-1})	Proteins (mg kernel^{-1})
13	0.92±0.02	1.66±0.01	1.3±0.30	1.1±0.01
17	0.95±0.01	1.87±0.01	5.8±0.30	2.2±0.01
21	0.75±0.03	1.82±0.01	12.9±0.20	7.1±0.01
28	0.58±0.02	1.02±0.01	24.4±0.40	7.1±0.03
45	0.92±0.06	0.80±0.04	34.4±0.32	9.5±0.03

were labelled with monobromobimane (mBBr), which reacts stoichiometrically with cysteine residues (Crawford *et al.*, 1989). The analysis showed a progressive increase in the number of proteic –SH groups during the first 21 DAA; this increase was particularly pronounced for proteins with a molecular mass ranging from 43–94 kDa (Fig. 1). At 28 DAA the proteic –SH groups available for interacting with mBBr started to decrease, particularly in the proteins with high molecular weight. Such a decrease is very probably due to the transition of sulphydryl groups to disulphide bridges. The changes in proteic redox state had the same behaviour when they were analysed on per kernel basis (Fig. 1) and when the same amount of proteins was loaded to the gel lanes (data not shown).

Ascorbate and glutathione redox state

The increase in weight and in the storage of reserve proteins occurring during kernel maturation renders the measurement of metabolites and enzymes on a per weight or per mg protein basis only slightly indicative of the physiological changes occurring during kernel maturation.
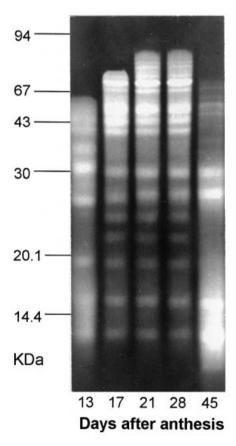

Fig. 1. Sulphydryl group–disulphide bridge transition in proteins of developing wheat kernels. SDS-PAGE of proteins extracted from kernels at different DAA. The presence of the –SH groups was labelled with mBBr. One-fifth of the total protein amount of a kernel was loaded in each lane.

For this reason, both ascorbate and glutathione contents and enzyme activities were calculated on a per kernel basis.

Remarkable variations in the ascorbate and glutathione pools were also observed during kernel development and maturation. The total ascorbate content (ASC+DHA) gradually increased from 13 to 21 d, remained constant for the following 7 d then decreased, reaching very low values in the mature kernels (Fig. 2A). In addition, the redox balance of the ascorbate pool significantly changed during the maturation period. At the beginning of kernel development the reduced form of vitamin C was predominant. In the following period of maturation, the ASC/DHA ratio decreased and DHA was the only form of vitamin C present in the mature kernels (Fig. 2A).

The glutathione pool also transiently increased during kernel maturation, reaching its maximum at 28 DAA (Fig. 2B). The redox balance of the glutathione pool was shifted more towards the oxidized form (GSSG) than that of ascorbate, since the GSH/GSSG ratio was around 1–1.5 during the whole maturation period, with the exception of the kernels collected at 28 DAA, the GSH/GSSG ratio of which decreased to about 0.4.

Enzymes of H_2O_2 removal and of the ascorbate and glutathione cycle

The redox enzymes of the ascorbate–glutathione cycle were also tested in order to follow their behaviour during kernel maturation. When determined on a per kernel basis, no significant differences were detected for APX during the first 28 DAA, but this H_2O_2 scavenging enzyme decreased until it was no longer detectable in the mature kernels (Fig. 3). Since three APX isoenzymes were active in wheat seedlings (Paciolla *et al.*, 1996; De Gara *et al.*, 1997), the presence of APX isoenzymes in wheat kernels and their pattern during maturation were analysed by native PAGE. Three APX bands, which had the same migration rate as seedling or germinating embryo isoenzymes (data not shown), were evident from 13–28 DAA of kernel development, but disappeared during the dehydration period (Fig. 4).

The activity of AFRR increased by about 50% from 13 to 17 DAA, remained constant for the following 10 d, after which it decreased to values lower than that of day 13 (Fig. 3). DHAR activity also transiently increased during kernel development, but unlike AFRR, this ASC recycling enzyme activity increased 5-fold over the period from 13 to 28 DAA and remained higher in mature kernels than at 13 d (Fig. 3).

Several proteins with DHA-reducing capability seemed to be involved in kernel maturation. At 13 DAA three proteins with DHA reducing capability were visible by native PAGE (Fig. 5). During maturation two new bands with lower migration rate appeared whereas the band with the highest migration rate disappeared (Fig. 5).
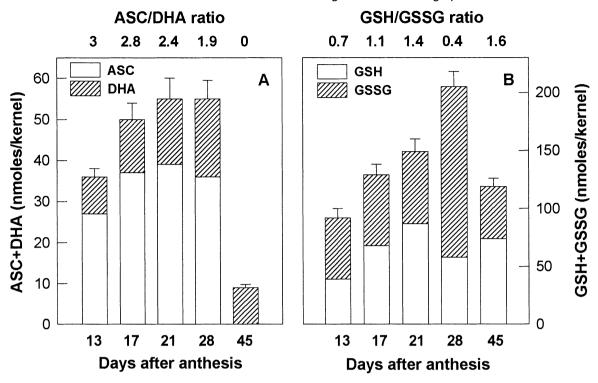
Fig. 2. Ascorbate (A) and glutathione (B) pools in wheat kernels at different stages of maturation. The reported values are the means of five experiments ±standard error (SE).

The activity of GR was constant from 13–21 DAA, after which it started to decrease, reaching values about 50% lower in the mature kernels than those reported during the first 21 d of kernel development (Fig. 3).

To obtain information on the global H_2O_2 removal capability of kernel cells during maturation, the activity of catalase (CAT) was also measured. As shown in Fig. 6, CAT almost doubled from 13 to 21–28 d, after which it rapidly decreased. Unlike APX, CAT was still present in the completely dehydrated kernels, even if with low activity.

Discussion

Data reported in this paper show that mono- and di-saccharides, fructans and starch change in different ways during kernel maturation. The behaviour of fructans mirrors that of ASC, indeed, the content of both these metabolites increases during the first 21 DAA. Since fructans are polymers of fructose, and sucrose is the fructosyl donor in their biosynthesis (Pollock and Cairns, 1991), the glucose release occurring in the tissues that actively synthesize fructans could be used as a precursor of ASC. However, at present, it is not known whether kernels are able autonomously to synthesize ASC or whether they import this metabolite from the mother tissues.

It has been reported that the rise in mono- and di-saccharides detected from 28–45 DAA is mainly due to an increase in sucrose (Abou-Guendia and D'Appolonia, 1972) and, according to the data present in the literature, this could be related to the acquisition of desiccation tolerance, a feature of particular relevance for orthodox seeds that reach maturity after a dehydration process. Indeed, the increase in sucrose, as well as in raffinose and dehydrins is concomitant and proportional to the induction of desiccation tolerance during seed maturation (Brenac *et al.*, 1997; Black *et al.*, 1999; Buitink *et al.*, 2000). The synthesis and degradation of fructans mark different stages of kernel maturation. Fructans, as well as being a form of carbohydrate reserve stored in vegetative organs by 15% of flowering plants, wheat included (Vijn and Smeekens, 1999), are also involved in drought tolerance (Wiemken *et al.*, 1995). Tobacco plants transformed with a bacterial gene for fructan synthesis have a higher resistance to drought stress than non-transformed plants (Pilon-Smits *et al.*, 1995). Under water stress, plants hydrolyse fructans as a strategy for decreasing osmotic potential into cells (Virgona and Barlow, 1991). The decrease in fructans observed in the kernel from 21 DAA could be triggered by the dehydration process. On the other hand, the changes in the soluble carbohydrate content occurring as a consequence of the decrease in fructans (that are soluble oligosaccharides) and the concomitant increase in the

synthesis of starch (insoluble polysaccharide) could contribute to up-regulate phloem unloading and be part of the

cross-talk between the whole plant and the developing seeds. Indeed, it has been proposed that a turgor-homeostat model might regulate phloem unloading and sugar uptake in embryos of several species (Weber *et al.*, 1997).
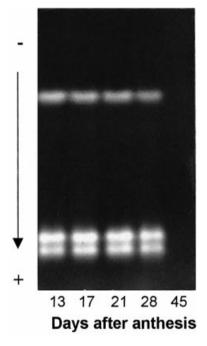

Fig. 3. Ascorbate–glutathione redox enzymes in wheat kernels at different stages of maturation. The reported values are the means of five experiments ± standard error (SE). 1 unit=1 nmol ASC oxidized min^{-1} kernel^{-1} (APX); 1 nmol NADH oxidized min^{-1} kernel^{-1} (AFRR), 1 nmol DHA reduced min^{-1} kernel^{-1} (DHAR); 1 nmol NADPH oxidized min^{-1} kernel^{-1} (GR).

Fig. 4. Native PAGE of ascorbate peroxidase isoenzymes in wheat kernels at different stages of maturation. Half of the protein content of a kernel was loaded in each lane.
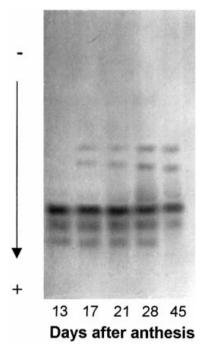

Fig. 5. Native PAGE of dehydroascorbate reducing proteins in wheat kernels at different stages of maturation. A quarter of the protein content of a kernel was loaded in each lane.
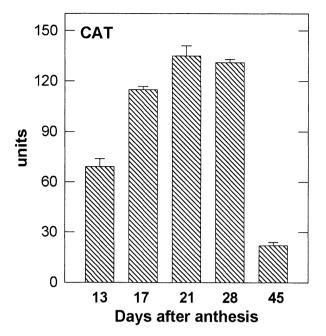
Fig. 6. Catalase activity in wheat kernels at different stages of maturation. The reported values are the means of five experiments ±standard error (SE). 1 unit=1 nmol H_2O_2 dismutated min^{-1} $kernel^{-1}$.

As far as the ROS–scavenger processes are concerned, the activities of APX and CAT suggest that the H_2O_2-detoxification requirement is almost constant during the first 28 d of kernel maturation; whereas, it decreases during dehydration, since the activities of the two enzymes responsible for H_2O_2-removal fall remarkably. The differences observed in their behaviours could be due to their different localization in cells and, as a consequence, to their involvement in metabolic pathways taking place at different times during kernel maturation. CAT is mainly present in the microbodies (Scandalios, 1994), organelles that, being involved in the fatty acid metabolism, are particularly active in lipid synthesizing cells. This could explain an increase in CAT activity during the first period of kernel development, when the synthesis of the storage lipids also increases (Morrison, 1988). On the other hand, APX, being more widely shared out in cell organelles (De Gara and Tommasi, 1999, and references reported here), could intervene in the removal of H_2O_2 produced by metabolic pathways with different timing during kernel maturation. Moreover, APX is always very active in dividing cells and tissues undergoing differentiation (De Gara *et al.*, 1996; de Pinto *et al.*, 2000), whereas it decreases in senescent tissues (Borraccino *et al.*, 1994). These reports perfectly agree with a high and quite stable APX activity from the beginning of kernel formation until the start of the dehydration phase, the period during which the cells undergo division and differentiation/storing processes. The loss in the H_2O_2-scavenging capability in the physiological context of orthodox seed dehydration is

not a critical event for seed viability, since the absence or very low activities of ROS-scavenging enzymes are typical features of all mature and viable orthodox seeds (Cakmak *et al.*, 1993; De Gara *et al.*, 1997; Tommasi *et al.*, 1999, 2001). A decrease in ROS generation also occurs during seed dehydration (Vertucci and Farrant, 1995). Indeed, the oxidative metabolism, that is the main source of ROS in non-green tissues, decreases together with the dehydration process, until reaching very low values in dehydrated seeds (Vertucci, 1989; Leprince *et al.*, 2000). Photosynthesis, another process responsible for ROS generation, is active during the first stages of kernel maturation, but at 28 DAA kernels contain a much lower amount of chlorophylls compared with the previous period and no chlorophylls are detectable after 45 DAA, thus confirming that a progressive decrease in this other physiological source of ROS also occurs during dehydration. However, the direct measurement of the changes in ROS production and ROS-scavenging enzymes in the same developing seed will be necessary in order to verify the existence of a causal relationship between a decrease in ROS production and APX and CAT decline in ripening seeds.

The presence in dry kernels of the enzymes of the ascorbate and glutathione recycle (i.e. AFRR, DHAR and GR) is of great importance for the first stage of germination. Since dry kernels are provided with DHA, these three recycling enzymes allow the ascorbate–glutathione cycle to be active as soon as they are reactivated by seed imbibition. ASC supplied by means of the reduction of its oxidized forms is necessary for the activation of the ASC-dependent metabolic pathway at the beginning of germination, since a lag of several hours is required for the start of ASC biosynthesis (De Gara *et al.*, 1997; Tommasi *et al.*, 2001).

The ASC–GSH recycling enzymes seem to have a different relevance during kernel maturation. AFRR activities resemble those of ASC utilization and, indeed, AFR production. Besides APX, several other enzymes, utilizing ASC as a physiological electron donor, show transient increase during kernel maturation. Among these are dioxygenases involved in the synthesis of gibberellin and abscissic acid (Prescott and John, 1996). The synthesis of these two phytohormones transiently increases during seed ripening (Frydman *et al.*, 1974; Rock and Quatrano, 1995); and, at least in the case of abscisic acid, this increase is due to the seed biosynthetic activity, not to hormone translocation from the parental tissues (Hole *et al.*, 1989). Another phytohormone synthesized by an ASC-depending enzyme is ethylene. It has been reported that, in wheat endosperm, production of ethylene reaches its highest level in the middle phase of kernel development (between 16–25 DAA), after which it decreases to undetectable values during the dehydration period (30–40 DAA) (Young and Gallie, 1999), when ASC is no longer available.

Among the enzymes whose activities increase during kernel development, DHAR has the highest increase in percentage terms. It is worth noting that protein disulphide isomerase has DHA reducing activity, because it utilizes DHA as an electron acceptor for maintaining two cysteine residues at its catalytic site in the oxidized form (Wells and Xu, 1994). In seed development, protein disulphide isomerase plays a key role in the maturation of storage proteins. The analysis of mBBR-labelled proteic –SH indicates that the transition from sulphydryl groups to disulphide bridges within proteins starts at 28 DAA, when the DHA reducing capability has the highest value. Thus, it is possible that some of the proteins, present during kernel maturation, that are detectable for their DHA-reducing activity by native PAGE, may be protein disulphide isomerases.

The GSH/GSSG pair is also involved in protein folding by sulphydryl groups–disulphide bridges transition (Kunert and Foyer, 1993). It has been reported that a low GSH/GSSG ratio facilitates the folding and assembly of newly synthesized secretory proteins in the endoplasmic reticulum (Hwang *et al.*, 1992). The GSH/GSSG ratio found in wheat kernels is similar to that detected in the endoplasmic reticulum (around 2) or even lower. It is also known that GSSG stimulates the rate of assembly of polymeric proteins by oxidizing specific cysteine residues (Jung *et al.*, 1997); moreover, it is responsible for the protection of specific thiol groups from the irreversible formation of intramolecular disulphide bonds (Kranner and Grill, 1996). In this reaction that occurs in seed dehydration and in vegetative tissues undergoing water stress conditions, a GSSG molecule reacts with free thiol groups of proteins producing a protein–glutathione complex and a molecule of GSH. Interestingly, the increase in the GSH/GSSG ratio that occurs after 28 d of kernel maturation (from a value of 0.4 at 28 d to a value of 1.6 at 45 d), in spite of GSSG-reductase activity decreasing during this period, can be explained by means of an involvement of GSSG reduction in the SH–S-S transition that mainly occurs 28 d after anthesis, as well as in protein-S-SG formation.

The data reported here also show that the redox state of the ascorbate and glutathione pools of kernels shifts towards the oxidized forms during maturation, unlike that which occurs in vegetative tissues or recalcitrant seeds, where the reduced forms represent 90–95% of the total pools (Hendry *et al.*, 1992; Tommasi *et al.*, 1999). Recently, it has been reported that the induction of an increase in DHA content in cultured cells is responsible for the decrease in their mitotic activity (de Pinto *et al.*, 1999; Potters *et al.*, 2000). The availability of ASC and GSH and changes in their redox state also affect gene expression (Noctor and Foyer, 1998; Catani *et al.*, 2001); thus, during kernel maturation, the levels and redox balance of the two redox pairs could also be involved in the activation/inactivation of specific metabolic pathways. On this basis, the peculiar redox state occurring during orthodox seed development and, in particular, the fact that at the end of their maturation processes only DHA is present in the seeds, raises the question as to whether such a shift toward the oxidized state is only a consequence of ASC utilization or whether it is a strategy carried out by orthodox seeds that contributes to reaching the resting stage.

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