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Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid: Evidence of an improved detoxification mechanism against reactive oxygen species

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Ascorbate content and the activities of some key enzymes involved in the detoxification from reactive oxygen species were investigated in germinated embryos of two *Zea mays* L. inbred lines (B73 and Mo17) and of their heterotic F1 hybrid (B73 × Mo17). The F1 hybrid showed a higher ascorbate biosynthetic capability owing to a higher activity of L-galactono-γ-lactone dehydrogenase (EC 1.6.5.4), the last enzyme in ascorbate biosynthesis. Ascorbate peroxidase (EC 1.11.1.11), ascorbate free radical reductase (EC 1.6.5.4) and dehydroascorbate reductase (EC 1.8.5.1) activities were much higher in the F1 hybrid than in either inbred line, whereas catalase (EC 1.11.1.6) activity was similar in the three genotypes. Native polyacrylamide gel electrophoresis (PAGE) analysis showed three forms of cytosolic ascorbate peroxidase, both in parental lines and in the F1 hybrid. On the other hand, a complex pattern of proteins with dehydroascorbate reductase activity was observed, with the hybrid combining the different dehydroascorbate-reducing proteins expressed by the inbred lines. The possible involvement of the enzymes of the ascorbate system in the phenomenon of hybrid vigour is discussed.

Introduction

Organisms living in an oxygen-containing atmosphere have evolved a wide range of detoxification pathways to overcome the problems deriving from uncontrolled reactive oxygen species (ROS) production. These defence mechanisms are based on metabolites with antioxidant properties, such as ascorbate (ASC), glutathione, α-tocopherol and enzymes, including superoxide dismutase, which converts superoxide to hydrogen peroxide, catalase and ascorbate peroxidase, which co-operate in removing H_2O_2 (Asada 1994, Foyer et al. 1994).

In plant cells, ASC peroxidase (APX), which specifically uses ASC as a physiological reductant (Tommasi et al. 1987, Asada 1992) is considered a crucial component in the metabolic defence against oxidative stress. It acts by a series of coupled redox reactions, both in photosynthetic (Asada 1994) and non-photosynthetic tissues (Arrigoni 1994). The presence of APX has been documented in higher plants (Chen and Asada 1989, Paciolla et al. 1996) and different forms of the enzyme have been localized in peroxisomes and mitochondria, chloroplasts (both stromal and thylakoid-bound), cytosol and cell walls (De Gara and Tommasi 1999 and references herein). Furthermore, cDNA and genomic clones encoding cytosolic APX have been isolated and sequenced in higher plants (Jespersen et al. 1997), including maize (Van Breusegem et al. 1995).

In the ASC-dependent mechanism of ROS detoxification (the ascorbate-glutathione pathway), ASC utilization leads to the formation of the intermediate ascorbate free radical (AFR; also called monodehydroascorbate radical in the literature). This compound can either be reduced by AFR

Abbreviations – AFR, ascorbate free radical (monodehydroascorbate radical); APX, ascorbate peroxidase; ASC, ascorbate; DHA, dehydroascorbic acid; GSH, glutathione – reduced form; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

reductase, an enzyme present in several cell compartments, and by photosynthetic electron flow through ferredoxin. The AFR that is not reduced to ASC spontaneously dismutates yielding ASC and dehydroascorbic acid (DHA). The latter compound is then reduced back to ASC by DHA reductase using glutathione (GSH) as the electron donor. GSH is finally regenerated by NADPH-dependent glutathione reductase (Noctor and Foyer 1998, De Gara and Tommasi 1999).

Although the role of ASC redox enzymes in plant defence mechanisms still needs more clarification, it is generally assumed that the activities of the enzymes of the ascorbate-glutathione pathway (and specifically that of APX) confer general resistance to an array of environmental stresses (Foyer et al. 1994, Allen et al. 1997 and references therein; De Gara and Saracino 1997).

In the plant life cycle, a phase of dramatic and critical changes in ROS production is germination; particularly in those seeds that reach maturity in a dehydrated state (the so-called orthodox seeds). During germination of such seeds, cells switch from quiescent to very active metabolism in which the degradation of storage polymers and the biosynthetic processes co-ordinately occur. The recovery of respiratory activity, with an initial steep increase in O_2 consumption during imbibition (phase I of germination) and a second respiratory burst when the radicle protrudes from the surrounding tissues (phase III of germination), and the mobilization of storage fatty acid are the main pathways of ROS production (Bewley 1997). Since ROS scavenging capacities are almost lacking in dry seeds but they rapidly increase during germination, the correct temporal activation and the efficiency of ROS scavenging capabilities are of great importance for germination (Gidrol et al. 1994, De Gara et al. 1997).

On this basis, with the aim of investigating the involvement of ASC-dependent H_2O_2 scavenging in plant growth, we have compared the main features of some enzymes involved in ROS detoxification in embryos, at phase III of germination, of two maize inbred lines, i.e. B73 and Mo17, which represent two of the most widely used parental lines in commercial hybrid production and in their corresponding F1 single cross hybrid, which have a higher growth and productivity (hybrid vigour) than the parental lines (Stuber 1998).

The results reported in this study show that, from the first stages of plant development, ASC biosynthetic capability and the activity of the enzymes of the ASC system, but not that of catalase, are markedly increased in the F1 hybrid in comparison with those of the parental lines.

Materials and methods

Plant material

Two widely used inbred lines of *Zea mays* L. (B73 and Mo17) and their heterotic hybrid (B73 × Mo17) were used in this study. Self-pollination and B73 × Mo17 crosses were made in the summer of 1997. For each genotype, batches of 40 caryopses were surface sterilized in 1% sodium hypochlorite for 15 min, rinsed three times with distilled water and placed in glass Petri dishes (15 cm in diameter) with two sheets of moist filter paper. After 60 h of germination in the dark at 22°C, germinating embryos homogeneous for development stage (1.5–2 mm of radicle length and lacking a visible coleoptile) corresponding to early stage III germination (Bewley 1997) were withdrawn with the help of a stereomicroscope and used for analysis of enzymatic activities and ASC/DHA contents.

Vigour tests

The vigour of the three genotypes was tested by measuring the germination rate and the root length. Batches of 40 caryopses were placed in Petri dishes as previously described. At 1-day intervals the germinated caryopses were counted and root length of germinated embryos measured with the help of a stereomicroscope.

Enzyme assays

For each genotype, the embryos, withdrawn as previously described, were homogenized in an ice cold porcelain mortar with a grinding medium composed of 50 m*M* Tris-HCl (pH 7.8), 0.3 *M* mannitol, 1 m*M* EDTA, 10 m*M* $MgCl_2$, 0.05% (w/v) cysteine; in a 1:2 and 1:4 ratio (w/v) for electrophoretic and spectrophotometric assays, respectively. The homogenates were centrifuged for 15 min at 30000 *g*, and the supernatant was used for the enzymatic determinations. Because no ASC was added to the grinding medium only the cytosolic component of APX, which is not inactivated in a medium without ASC, was detected (Amako et al. 1994).

Spectrophotometric determination of ASC redox enzymes was assayed as reported in Arrigoni et al. (1992) with minor modifications, by using a Beckman (Fullerton, CA, USA) DU 7000 spectrophotometer.

DHA reductase (EC 1.8.5.1) activity was assayed following the increase in absorbance at 265 nm owing to the GSH-dependent production of ASC. The reaction mixture contained 0.1 *M* phosphate buffer, pH 6.2, 2 m*M* GSH and 50–100 μg protein. The reaction was started upon addition of 1 m*M* DHA. The rate of non-enzymatic DHA reduction was subtracted (extinction coefficient 14 mM^{-1} cm^{-1}).

AFR reductase (EC 1.6.5.4) was tested by measuring the oxidation rate of NADH at 340 nm in a reaction mixture composed of 0.2 m*M* NADH, 1 m*M* ASC, 50–100 μg protein and 50 m*M* Tris-HCl, pH 8.0. The reaction was started by adding 0.2 units of ASC oxidase (Boehringher Mannheim, Germany) to generate saturating concentration of AFR (extinction coefficient 6 mM^{-1} cm^{-1}).

APX (EC 1.11.1.11) activity was determined following the H_2O_2-dependent oxidation of ASC at 265 nm in a reaction mixture composed of 50 μ*M* ASC, 90 μ*M* H_2O_2, 50–100 μg protein and 0.1 *M* phosphate buffer, pH 6.4. The non-enzymatic H_2O_2-dependent oxidation of ASC was subtracted (extinction coefficient 14 mM^{-1} cm^{-1}).

Catalase (EC 1.11.1.6) activity assay was performed according to Beaumont et al. (1990) by following the H_2O_2 dismutation at 240 nm in a reaction mixture composed of

0.1 *M* phosphate buffer, pH 7.0, 50–100 μg protein and 18 m*M* H_2O_2 (extinction coefficient 23.5 mM^{-1} cm^{-1}).

L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3) activity was assayed in the mitochondrial fraction obtained as previously reported (De Gara et al. 1997). The enzyme activity was assayed as reported by Ôba et al. (1995), with minor modifications, by following the cytochrome *c* reduction at 550 nm in a mixture reaction composed of 0.1 *M* Tris-HCl, pH 8.0, 60 μ*M* cytochrome *c*, 2 m*M* L-galactono-γ-lactone (GL) and 10–30 μg mitochondrial protein (extinction coefficient 27 mM^{-1} cm^{-1}).

The ASC biosynthetic capability of the inbred lines and their heterotic F1 hybrid was also tested by measuring the in vivo capability of the embryos to convert GL into ASC as reported in De Gara et al. (1994). Briefly, batches of 60 h germinated embryos from each genotype were incubated with 5 m*M* GL, the last precursor of ASC biosynthesis, or water (control) or were immediately used to detect their ASC content (time 0). After 24 h of GL or water incubation the embryos' ASC content was again determined. The in vivo ASC biosynthetic capability was measured as the difference (expressed in fold) in the ASC content of the incubated embryos to that at time 0.

Protein measurement was performed according to Bradford (1976), using BSA as standard.

Native polyacrylamide gel electrophoresis (PAGE) of APX and DHA-reducing proteins were performed according to De Gara et al. (1997), using 14 × 24 cm slab gels. After completion of the electrophoretic run, gels were incubated under agitation for 10 min in a reaction medium containing 0.1 *M* phosphate buffer, pH 6.2, 4 m*M* ASC and 4 m*M* H_2O_2 for detecting proteins with APX activity. To verify the H_2O_2-dependence of APX bands, a gel was also incubated in a reaction medium without H_2O_2. For DHA reductase activity gels were incubated with 0.1 *M* phosphate buffer, pH 6.2, containing 4 m*M* GSH and 2 m*M* DHA. Gels were then washed with distilled water and stained by incubation for 10 min in a 0.125 *M* HCl solution containing 0.1% (w/v) ferrichloride and 0.1% (w/v) ferricyanide, their reaction with ASC yielding a coloured product.

After staining, band intensities were analysed utilizing the UTHSCA Image Tool software.

Determination of ASC and DHA contents

ASC and DHA were measured according to Kampfenkel et al. (1995) on a deproteinized supernatant obtained by homogenization of withdrawn embryos with ice cold 5% (w/v) metaphosphoric acid and centrifugation at 20000 *g* for 15 min.

Statistics

Statistical differences between mean values were determined with the Student's *t*-test. Number of samples and/or independent experiments are indicated in tables and figure legends.

Results

The germination capability and the rate of root elongation were tested in two parental lines, i.e. B73 and Mo17, and in their F1 hybrid B73 × Mo17 as parameters indicative of hybrid vigour. The caryopses of either inbred lines and heterotic F1 hybrid had almost 100% germinability; however, the rate of germination was significantly higher in the F1 hybrid than in the parental lines (Table 1). The higher germination rate of the hybrid was confirmed by measurements of root elongation, since root length was greater in the hybrid than in the parental lines (Table 1).

During the first stages of germination of caryopses, the recovery of oxidative metabolism causes an increase in ROS production, the damaging effects of which are limited by to what extent ROS-scavenging strategies are also activated (Puntarulo et al. 1988, Klapheck et al. 1990, De Gara et al. 1997). Indeed, activities of ROS-scavenging enzymes, in particular catalase (CAT) and APX, were tested in the three genotypes. For this and all the following analysis, the germinating embryos were chosen so that their development stage was homogeneous (1.5–2 mm radicle length and lacking a visible coleoptile – corresponding to early phase III of germination, see Materials and methods). Such a procedure was performed to avoid the differences in enzyme activities and in ASC/DHA contents being the result of the different development stage instead of an effective difference between the three genotypes.

CAT activity was similar in the parental lines and in the F1 hybrid; by contrast, the APX activity of the F1 hybrid was clearly higher than that of both parental lines (Fig. 1). The high APX activity detected with the spectrophotometric assay was confirmed and further substantiated by means of native PAGE. Three forms of cytosolic APX were observed in all three genotypes, with the F1 hybrid clearly showing higher band intensity resulting from increased activity (Fig. 2).

ASC biosynthesis was significantly higher in the hybrid than in the parental lines, both when it was assayed in cell-free extract by measuring the activity of L-galactono-γ-lactone dehydrogenase (Fig. 3A), the mitochondrial enzyme catalysing the conversion of GL to ASC (Ôba et al. 1995, Wheeler et al. 1998), and when it was tested as the embryos in vivo capability to convert GL to ASC (Fig. 3B).

Table 1. Germination rate and root growth of B73 and Mo17 inbred lines and their F1 hybrid B73 × Mo17. Caryopsis germinability and root length were tested on batches of 40 caryopses for each genotype. The results are the mean ± SD of three replicates.

Genotype	Germination capability (%)			Root length (mm)	
	48 h	72 h	120 h	48 h	72 h
B73	33 ± 3	43 ± 5	97 ± 2	1.2 ± 0.6	2.2 ± 1.1
Mo17	7 ± 2	15 ± 3	95 ± 4	0.7 ± 0.3	1.2 ± 0.6
B73 × Mo17	50 ± 7	90 ± 6	99 ± 1	2.2 ± 0.9	2.9 ± 1.3

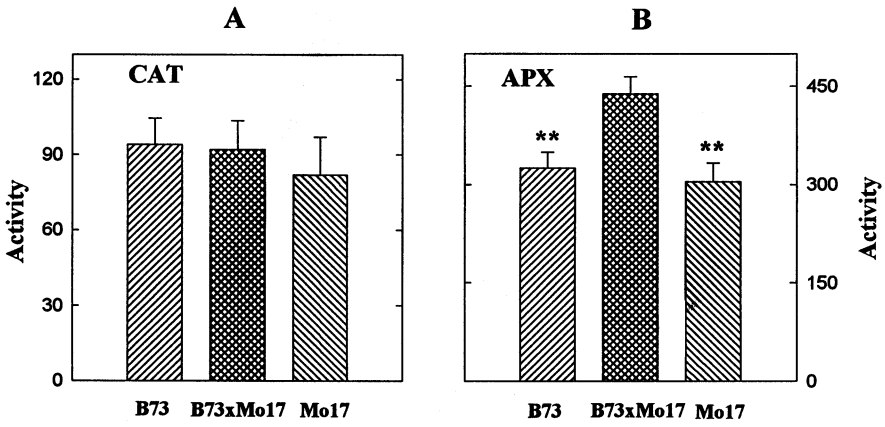

Fig. 1. Hydrogen peroxide scavenging enzymes in B73 and Mo17 inbred lines and in their F1 hybrid B73 × Mo17. The activities of (A) catalase (CAT, nmol H_2O_2 reduced [mg protein]$^{-1}$ min^{-1}) and (B) ascorbate peroxidase (APX, nmol ASC oxidized (mg protein)$^{-1}$ min^{-1}) were measured in embryos germinated for 60 h at 22°C in the dark. The values are the mean with SD of six experiments. ** Indicates values that differ significantly from the hybrid at $P < 0.01$.

ASC is always oxidized via the one-electron reaction producing AFR that is partly reconverted to ASC by AFR reductase and partly undergoes disproportionation producing ASC and DHA. Because the higher APX activity found in the F1 germinating embryos inevitably produces a higher amount of AFR and, probably, of DHA, the differences in AFR reductase and DHA reductase activities between F1 hybrid and parental lines were also analysed. Data reported in Fig. 4 show that the activities of the enzymes of ASC regeneration in the ascorbate-glutathione pathway were also higher in the F1 hybrid than in the parental lines, thus suggesting a possible involvement of the ASC system in the selective advantages of hybrid vigour. Moreover, the spectrophotometrically measured DHA reductase activity (Fig. 4B) was due to the presence of different proteins with DHA reductase activity, as can be observed by native PAGE (Fig. 5). The parental lines have some DHA-reducing proteins in common and some specific to themselves. DHA-reducing proteins that are apparently peculiar to the parental lines are summed in the F1 hybrid as a result of codominat expression. Similar results were observed in the electrophoretic patterns of the F1 crosses derived from other sets of parental lines, Lo951 × Lo924 and Lo1067 × Lo1096 (data not shown).

Fig. 6 reports the ASC and DHA levels in germinated embryos of the parental lines and their heterotic hybrid. The differences in ASC contents of the three genotypes are not statistically significant; by contrast, the DHA content of the germinating hybrid embryos is lower than that of the parental lines.

Discussion

Rapid generation of reactive oxygen intermediates is a characteristic event occurring during seed germination (Gidrol et al. 1994, De Gara et al. 1997).

Previous studies have also shown that during the first stages of germination, ROS scavenging constitutes a critical event because in dry seeds superoxide dismutase is present even if with a modest activity, but both APX and CAT are almost absent, whereas their activity progressively increases during germination (Cakmak et al. 1993, Gidrol et al. 1994, De Gara et al. 1997). Indeed, the efficiency of H_2O_2 removal in germinating embryos could be particularly important for seedling development. Accordingly, in order to clarify whether the efficiency of H_2O_2-scavenging capability during germination could contribute to hybrid vigour, the APX and CAT activity in the embryos, collected early in phase III of germination, of two inbred lines (Mo17 and B73) and their F1 hybrid (Mo17 × B73) was analysed, which greatly differed in hybrid vigour (Table 1; Stuber 1998).

The present data showed that APX activity is higher in the F1 hybrid than in its parental lines, whereas catalase has the same level of activity in the three genotypes here investigated (Figs. 1 and 2). This finding suggests a possible selective advantage deriving from increased ASC-dependent

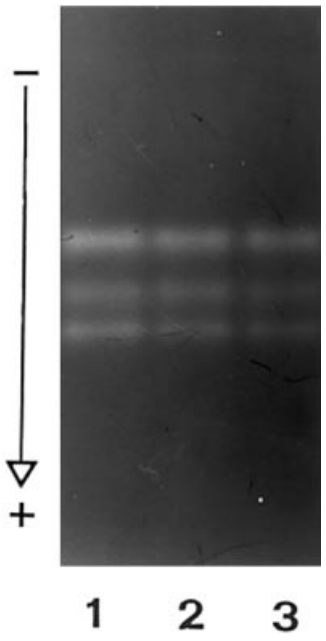

Fig. 2. Native-PAGE of ascorbate peroxidase. Extracts from 60 h germinated embryos of B73 × Mo17 (lane 1) B73 (lane 2) and Mo17 (lane 3) were subjected to native-PAGE as indicated in Materials and methods. Protein (300 μg) was loaded in each lane. When the intensities of the bands were compared using UTHSCSA Image Tool software, the B73 bands were 30%, 20% and 30%, for low, intermediate and high migration rate bands respectively, less intense than the corresponding hybrid bands, whereas the three Mo17 bands were about 50% less intense than the corresponding hybrid bands.

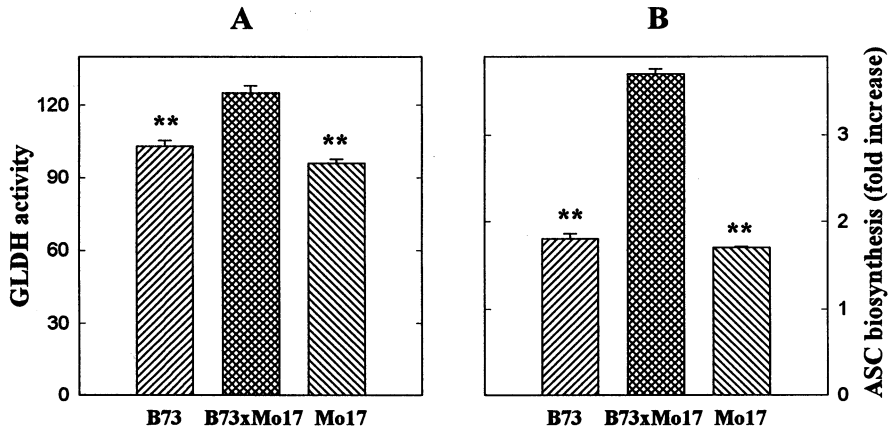

Fig. 3. ASC biosynthetic capability in B73 and Mo17 inbred lines and in their F1 hybrid B73 × Mo17. (A) L-galactono-γ-lactone dehydrogenase activity (GLDH, nmol Cyt *c* reduced [mg protein]$^{-1}$ min^{-1}) and (B) in vivo conversion of GL to ASC in B73, Mo17 and B73 × Mo17 germinating embryos. The values are the mean with SD of six experiments. ** Indicates values that differ significantly from the hybrid at $P < 0.01$.

H_2O_2 scavenging at the very beginning of plant development. It is worth noting that the hybrids maintain their improved H_2O_2-scavenging capability during the following development, since leaves from 10-day grown seedlings had a 30% higher APX activity in the hybrid than in the parental lines (De Gara L, Paciolla C, Arrigoni O. unpublished results). The fact that APX but not catalase increases in the F1 hybrid can be explained on the basis of the different K_m for H_2O_2 of the two enzymes. It is likely that the higher growth rate of the F1 hybrid requires a higher basal oxidative metabolism and, as a consequence, causes an overproduction of H_2O_2, even if at not so high a level as to induce oxidative stress. Such H_2O_2 overproduction could be sufficient to cause an increase in APX, an enzyme with a very high affinity for H_2O_2 but not of CAT, the affinity of which for the substrate is many times lower than that of APX.

The higher F1 hybrid APX activity was apparently associated with higher ASC biosynthetic capability. Accordingly, Conklin et al. (1997) reported that APX activity is remarkably lower in the *Arabidopsis* mutant *vtc1*, which is deficient in ASC biosynthesis.

The present study has also shown that the activities of the enzymes involved in the reduction of the ASC oxidized products, i.e. AFR reductase and DHA reductase, are significantly higher in the F1 hybrid (Fig. 4). This underlines that the regulation of all the components of the ASC system is closely associated in a complex balance. It has been recently reported that the activities of AFR reductase and DHA reductase are related to the necessity of avoiding DHA overproduction (Arrigoni 1994), and not simply to the replenishment of the ASC pool, as generally assumed. This view is also supported by the observation that even a very low DHA concentration can inhibit the activities of several enzymes (Fiorani et al. 1997, Morell et al. 1997). Therefore, in the F1 hybrid, higher AFR reductase activity may be a strategy to limit AFR disproportionation, thus lowering DHA formation. Moreover, an increased capability to reduce DHA by DHA reductase might efficiently contribute to avoiding the accumulation of the DHA produced in cell metabolism. As a confirmation of this, the hybrid had the lowest DHA content (Fig. 6).

The ASC/DHA contents of the three genotypes are consistent with the differences in the activities of the ASC redox enzymes. Despite the hybrid's higher ASC biosynthetic capability, the ASC contents of the three genotypes are similar, probably because the higher growth rate of the hybrids requires a higher ASC consumption, as is also substantiated by a higher APX activity. Furthermore, the lower DHA content of the hybrid perfectly agrees with a more efficient reduction of both AFR and DHA.

The presence in the F1 hybrid of a codominant expression of the proteins with DHA-reducing capability present in the parental lines also seems to contribute to increasing the DHA removal in the F1 hybrid (Fig. 5). The presence of several proteins with DHA-reducing capability in maize embryos raises the question as to whether all or some of

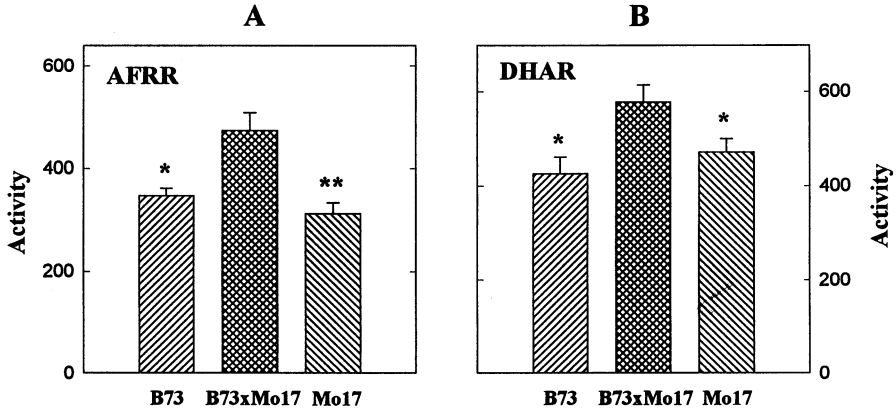

Fig. 4. ASC recycling enzymes in B73 and Mo17 inbred lines and in their F1 hybrid B73 × Mo17. (A) AFR reductase (AFRR, nmol NADH oxidized [mg protein]$^{-1}$ min^{-1}) and (B) DHA reductase activities (DHAR, nmol DHA reduced (mg protein)$^{-1}$ min^{-1}) of B73, Mo17 and B73 × Mo17 germinating embryos were spectrophotometrically measured as indicated in 'Materials and methods'. The values are the mean with SD of six experiments. *,** Indicate values that differ significantly from the hybrid at $P < 0.05$ and $P < 0.01$ respectively.

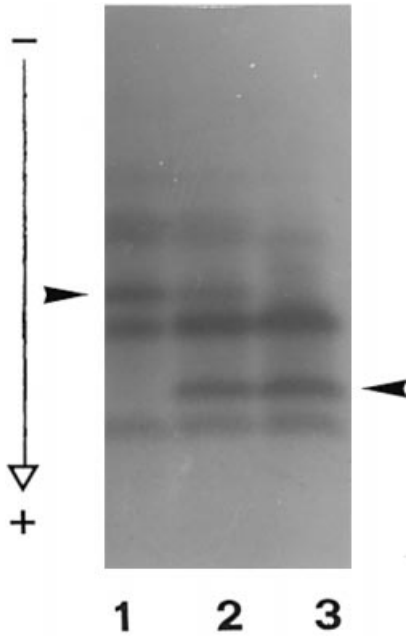

Fig. 5. Electrophoretic patterns of the DHA-reducing proteins active in B73 and Mo17 inbred lines and in their F1 hybrid B73 × Mo17. Proteins with DHA-reducing capability were identified by native PAGE with GSH as electron donor. The arrows indicate the proteins peculiar to B73 or Mo17 that are also present in the hybrids. Lane 1, B73; lane 2, B73 × Mo17; lane 3, Mo17; Protein (400 μg) was loaded in each lane.

these proteins are actually involved in DHA reduction in vivo. Recent findings have shown that distinct proteins sharing redox-active dicysteine sites (such as thioredoxins, thioredoxin reductase, thioltransferase, protein disulphide isomerase and some protease inhibitors) can all reduce DHA in vitro. According to data reported in the literature (Morell et al. 1997), it is likely that some of the DHA-reducing proteins detected by the native-PAGE analysis correspond to thioredoxins or other such redox proteins.

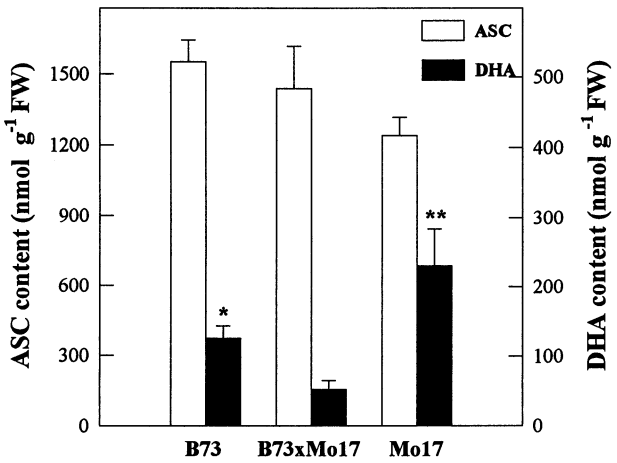

Fig. 6. ASC and DHA contents in B73, Mo17 inbred lines and in their F1 hybrid B73 × Mo17. The ASC and DHA contents were measured in 60 h germinated embryos of the three genotypes. The values are the mean with SD of three experiments. *,** Indicate values that differ significantly from the hybrid at $P < 0.05$ and $P < 0.01$ respectively.

Although the in vivo occurrence of such reduction has been questioned (Wells and Xu 1994, May et al. 1997, Morell et al. 1997), the fact that the hybrid, characterized with the highest number of DHA-reducing proteins, had the lowest DHA content, and other indications from literature (De Gara et al. 1997, De Tullio et al. 1998) support the hypothesis that the DHA-reducing capability of the proteins detected by native PAGE is not a mere artefact, due to in vitro conditions, but might have a physiological relevance. On this basis, further characterization of those DHA-reducing proteins that differentiate the hybrid from the parental inbred lines is worthwhile, with the aim of better understanding their role in hybrid vigour and their relevance in ASC metabolism.

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