Catalase activity and expression in developing sunflower seeds as related to drying

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Abstract
Changes in catalase (CAT) activity and in CAT isoform pattern and expression were investigated in developing sunflower (Helianthus annuus L.) seeds during desiccation on the mother plant and after artificial drying on the flowerheads. Seeds regularly desiccated during their development on the mother plant and reached mass maturity at c. 42 d after flowering (DAF). Freshly harvested seeds did not germinate at any stage of development because they were dormant, but their dormancy was broken after 5–6 months of dry storage. Immature seeds were desiccation-tolerant at 24 DAF since they were able to germinate fully after artificial drying on the flowerheads followed by dry storage. CAT activity increased in non-dehydrated seeds during their development, reaching a maximum a little after seed mass maturity and after artificial drying in immature seeds. This stimulation of CAT activity by natural and artificial drying was related to changes in CAT isoform pattern. Of the four constitutive CAT subunits, that of 59 kDa was always present, but dehydration induced the synthesis of a 55 kDa subunit. This synthesis of the CAT 55 kDa subunit resulted from an activation of the CATA1 gene, suggesting that the regulation of catalase activity and synthesis by drying occurred at the transcriptional level. The increase in CAT activity induced by seed drying was associated with a decrease in hydrogen peroxide level and in lipid peroxidation. These results suggest that CAT plays a role during seed desiccation by preventing dehydration-related oxidative damage and that H₂O₂ may play a role in the regulation of CAT gene expression and the transduction pathway of the dehydration signal.

Key words: Catalase, Helianthus annuus, hydrogen peroxide, seed development, seed drying.

Introduction
Reactive oxygen species (ROS) are of increasing interest in seed physiology. Most contributions to date in this field have been concerned with the role of ROS in the loss of vigour and viability during the prolonged storage of desiccation-tolerant orthodox seeds. Indeed, lipid peroxidation induced by these compounds has been widely cited as being a major cause of seed ageing (Priestley, 1986; McDonald, 1999). Dehydration of recalcitrant (desiccation-intolerant) seeds has often been associated with the impairment of antioxidative mechanisms leading to oxidative damage and to numerous lethal lesions (Hendry et al., 1992; Finch-Savage et al., 1993; Vertucci and Farrant, 1995; Li and Sun, 1999; Pammenter and Berjak, 1999). Conversely, tolerance to drought of vegetative tissues or resurrection plants is thought to be associated with an up-regulation of antioxidant genes (Ingram and Bartels, 1996; Sherwin and Farrant, 1998). However, the involvement of such mechanisms in the maturation of orthodox seeds is so far poorly documented with, as far as the authors are aware, studies performed only on Vicia faba (Arrigoni et al., 1992) and bean (Bailly et al., 2001) seeds.

In sunflower, by manipulating seed germinability with accelerated ageing and controlled imbibition (formerly known as priming) treatments, it has been shown that germination rate is closely related to catalase (CAT) activity (Bailly et al., 1998, 2000, 2002). Other antioxidant enzymes, such as superoxide dismutase and glutathione reductase, are also present in sunflower seeds, but they do

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not appear to play a major role in germination (Bailly et al., 2000, 2002).

Catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) is a tetrameric enzyme which dismutates H$_2$O$_2$ into water and oxygen. In mature seeds, it is located mainly in glyoxysomes (Willekens et al., 1995; Scandalios et al., 1997). In sunflower, eight isoforms, CAT1 to CAT8, have been identified (Eising et al., 1990). The first group of isoenzymes, from CAT1 to CAT5, results from the association of four 55 and 59 kDa subunits in various proportions. The second group includes the isoforms CAT6 to CAT8 which are formed only by four 55 kDa subunits differing in their charge. They are the product of at least four different genes (CATA1 to CATA4): CATA1 (GenBank accession number L28740, Kleff et al., 1994) and CATA2 (GenBank accession number AF243517) code for matrix catalases whereas CATA3 and CATA4 (GenBank accession numbers AF243518 and AF243519, respectively) code for core catalases. CAT activity is essential for the removal of the potentially toxic hydrogen peroxide produced under various stress conditions and then for the avoidance of oxidative-stress-related damage (Willekens et al., 1995). In oily seeds, CAT is particularly important in the early events of seedling growth because it removes H$_2$O$_2$ produced during β-oxidation of the fatty acids (Bewley and Black, 1994). CAT is also important in fine-tuning the cellular H$_2$O$_2$ and then in modulating the related signalling pathways (Mittler, 2002; Neill et al., 2002). Indeed, numerous recent studies have shown that H$_2$O$_2$ is a central component in the transduction of various signals such as responses to pathogen and abiotic stresses (Wu et al., 1995; Foyer et al., 1997; Lamb and Dixon, 1997; Desikan et al., 2001; Mittler, 2002) or programmed cell death (Solomon et al., 1999; Bethke and Jones, 2001).

Considering the increasing evidence on the role of H$_2$O$_2$ and the contribution of CAT to various physiological processes, it was of a particular interest to investigate if this enzyme plays a role in sunflower seed development. The present work therefore focuses on changes in CAT activity and expression during seed development on the mother plant and after artificial drying of seeds at three stages of development. The aims of this study were to determine whether CAT plays a role in preventing H$_2$O$_2$ accumulation and related damage during seed development, and to investigate the possible effects of dehydration on CAT expression.

Materials and methods

Plant material and drying conditions

Sunflower (Helianthus annuus L., cv. Fructidor) plants were grown in the fields of Limagrain located near Montélimar (Drôme, France). Five hundred plants were tagged at the beginning of flowering on 19 July 1999. Flowerheads were then periodically hand-collected from 24–58 d after flowering (DAF). For each developmental stage, seeds were collected from the middle rings of the flowerheads. Control seeds were immediately frozen in liquid nitrogen, without drying, and stored at −30 °C until required. Additional fresh seeds were used for moisture content determinations. Other seed batches were air-dried by placing the flowerheads at room temperature for at least 7 d until the seed moisture content reached approximately 4.5% fresh weight (FW). They were then stored dry at 20 °C and 70% relative humidity for 5–6 months to break their dormancy (Corbineau et al., 1990) before germination tests.

Germination tests and seed moisture content determination

Germination assays were performed by placing seeds (6 replicates of 50 seeds each) in boxes containing filter paper moistened with deionized water at 20 °C and a photoperiod of 12 h. The results presented correspond to the mean percentages of normal seedlings obtained after 10 d, according to ISTA (International Seed Testing Association) (1993).

Seed moisture content and dry matter mass were determined by oven-drying achenes (about 60 g fresh weight) at 105 °C for 17 h. Moisture content was calculated on a per seed or fresh weight basis.

Enzyme extraction and assays

All enzyme assays were performed with naked seeds, i.e. seeds from which the pericarp was removed. All extraction procedures were carried out at 4 °C. Naked seeds (about 1 g FW) were ground in 20 ml of potassium phosphate buffer (0.1 M, pH 7.8) containing 2 mM α-dithiothreitol, 0.1 mM EDTA, 1.25 mM polyethylene glycol 4000, and 20% (p/v) polyvinylpolypyrolidone, and mixed for 15 min. The homogenate was centrifuged at 16 000 × g for 15 min, the supernatant filtered through Miracloth, desalted on a PD 10 column (Pharmacia), and used for assays.

Catalase (CAT, EC 1.11.1.6) activity was determined as previously described by Bailly et al. (1996) by spectrophotometrically following H$_2$O$_2$ consumption at 240 nm. The results were expressed as specific activity, i.e. as nmol H$_2$O$_2$ decomposed min$^{-1}$ mg$^{-1}$ protein and correspond to the means ±SD of the values obtained with nine measurements carried out on three different extracts (three measurements per extract). Catalase activity was expressed per mg of extractable protein (specific activity) rather than on a per seed basis since the amount of extractable protein did not vary among the various seed samples (it was always close to 1.3 mg seed$^{-1}$) whereas dry and fresh seed weights markedly changed during seed development. Indeed, sunflower seeds contain c. 15–20% albumins, which are extractable with the buffer used in this study, and c. 80% reserve protein (globulins, prolamins, and glutelins) (Lusas, 1985), which accumulate during seed-filling, but were not extracted by the unsalted potassium phosphate buffer (Madhusudhan et al., 1986). The protein content of the extracts was determined using the BioRad assay kit with bovine serum albumin as standard.

Western blots

Naked seeds (0.3 g FW) were ground in liquid nitrogen with a mortar and pestle and homogenized in 1.5 ml of 30 mM TES (pH 7.5) containing 20 mM NaCl and 1 mM PMSF. The homogenate was then centrifuged at 14 000 × g for 15 min at 4 °C. Ice-cold acetone (4 vols) containing 10 mM β-mercaptoethanol was added to the supernatant (1 vol.) to precipitate the proteins. After 16 h at −20 °C, the mixture was centrifuged at 14 000 × g for 30 min at 4 °C, and the resulting pellet was air-dried and dissolved in 1 ml SDS-PAGE sample buffer.

For all protein extracts from either fresh or artificially dried seeds 15 μg protein per lane were loaded onto an 11% acrylamide running gel and a 4% acrylamide stacking gel, and were separated by SDS-PAGE. After separation, the proteins were transferred electrophoretically (20 V, 35 min) onto nitrocellulose using a Trans-blot semi-dry system (BioRad). Membranes were fixed with Blotto (5% (w/v)
non-fat dry milk in TBS) for 1 h at 25 °C, and incubated at 25 °C for 1.5 h in the presence of catalase antibodies diluted in Blotto. After washing three times for 10 min with Blotto, they were incubated for 1 h at 25 °C with alkaline-phosphatase conjugated goat anti-rabbit IgG (Sigma) also diluted in Blotto. A secondary antibody was localized using 4-nitroblue-tetrazolium chloride (NBT) and 5bromo-4-chloro-3-indolyl-phosphate (BCIP). Three polyclonal antibodies against catalase (anti-CAT5, anti-CAT6, and anti-CAT59, provided by Professor R Eising, Institut für Botanik, Münster, Germany) were used in this study. Anti-CAT5 detected 55 kDa and 59 kDa catalases, anti-CAT6 detected mainly 55 kDa catalase, and anti-CAT59 detected 59 kDa subunits of catalase (Kleff et al., 1997; Tenberge et al., 1997). To avoid misinterpretation, extracts from fresh and dried seeds were run on the same gels and blotted simultaneously.

**Northern blots**

Total RNA was extracted according to Verwoerd et al. (1989) using 10 naked seeds per sample and separated (10 µg per lane for all extracts) in 1% agarose-formaldehyde gel (Sambrook and Russell, 2001). RNA loading was checked using ethidium bromide staining. The RNAs were transferred to nylon filter (Biodyne B, Pall) by capillary action with 100 ml of 1% agarose-formaldehyde gel (Sambrook and Russel, 2001) and fixed by UV crosslinking (Stratalinker, Stratagene). DNA probes were labelled with a [32P]-dCTP (Amersham Pharmacia Biotech) using the 'Ready-To-Go DNA labelling beads' kit (Amersham Pharmacia Biotech) and purified with the ‘ProbeQuant G-50 Micro Columns’ (Amersham Pharmacia Biotech) as described by the manufacturer. A part of the CATA1 (bp 1522–1710) and CATA3 (bp 1501–1800) cDNAs encoding for sunflower catalase 55 kDa and 59 kDa subunits (GenBank accession numbers L28740 and AF243518, Kleff et al., 1994; kindly provided by M Heinze, Institut für Botanik, Münster, Germany), respectively, were used as probes for northern blot analyses. CATA1 and CATA3 probes were designed in coding regions showing no homology in order to avoid any cross-hybridization. Filters were hybridized at 55 °C in 0.5 M sodium phosphate buffer, pH 7.2, 5% SDS, and 10 mM EDTA. A first wash was performed at 55 °C in 1× SSC, 0.1% SDS and a second wash at 55 °C in 0.1× SSC, 0.1% SDS. Filters were exposed overnight on phosphor screens and radioactivity was quantified using a Phosphorimagwer (Storm 840) and ImageQuant software (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Radioactivity quantification was expressed as arbitrary units corresponding to the intensity of the signal after subtraction of background value. The data were then normalized using samples common to all filters as the reference. The results presented correspond to the means ± SD of values of quantifications obtained with four replicates.

**Malondialdehyde measurements**

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content from 0.5 g FW of naked seeds, according to Heath and Parker (1968). Results are expressed as µmol g⁻¹ dry matter of seeds and correspond to the means of measurements carried out with five extracts ± SD.

**Evaluation of hydrogen peroxide contents**

H₂O₂ contents of naked seeds were determined according to O’Kane et al. (1996). Seeds (0.5 g FW) were ground in a mortar and homogenized with 5 ml of perchloric acid (0.2 N). After 15 min of centrifugation at 13 000 g at 4 °C, the resulting supernatant was neutralized to pH 7.5 with 4 N KOH and then centrifuged at 1 000 g for 3 min at the same temperature. The supernatant was immediately used for spectrophotometric determination of H₂O₂ at 590 nm using a peroxidase-based assay. The reaction mixture contained 12 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 1.3 mM 3-methyl-2-benzothiazolidone hydrazone, 20 µl (0.25 units) horseradish peroxidase (Sigma), and 50 µl of the collected supernatant for a total volume of 1.5 ml. The reaction was started with the addition of peroxidase. The increase in absorbance at 590 nm was monitored after 5 min at 25 °C and compared with the absorbance obtained with known amounts of H₂O₂. Results were expressed as nmol H₂O₂ g⁻¹ DW and correspond to the means obtained with five different extracts ± SD.

**Results**

**Seed development**

Figure 1A shows the changes in dry weight and in water content, expressed on a per seed basis or as % FW, of seeds during their development on the mother plant. Mean seed dry weight increased approximately linearly from 24 to 42 DAF. At the end of seed reserve accumulation, i.e. at 42 DAF, it reached 53.6 mg. From 24–55 DAF, mean seed moisture content decreased almost linearly from 63.2% to 13.5% FW. At mass maturity, its value was 38.4% FW. However, when expressed on a per seed basis, water content decreased mainly after 38 DAF.

Freshly harvested seeds did not germinate at all (data not shown) because they were dormant (Corbineau et al., 1990). After drying in ambient conditions and storage for 5–6 months at 20 °C and 70% relative humidity, seeds were no longer dormant; 100% germinated and 85–100% produced normal seedlings (Fig. 1B). A slight decrease in normal seedlings, related to the appearance of abnormal seedlings in the ISTA germination tests, at 58 DAF resulted from contamination by Botrytis under the pericarp due to excessive rainfall at the end of seed development.

**Catalase activity**

CAT specific activity was low in fresh seeds collected at 29 DAF (Fig. 2A), but it increased strongly during seed development until 50 DAF then remained almost constant. A negative sublinear relationship was evident between CAT specific activity measured in freshly harvested seeds and their moisture content expressed as % FW or as g H₂O seed⁻¹ (Fig. 2B).

Artificial drying of seeds on the flowerheads up to 42 DAF resulted in a marked increase in CAT specific activity (Fig. 2A). The more immature the seeds, i.e. the higher their moisture content, the stronger the stimulatory effect of drying on enzyme activity. After 42 DAF, when the moisture content of fresh seeds dropped below 30% FW, CAT specific activity was almost similar in fresh and artificially dried seeds (Fig. 2A).

**CAT isoform pattern and gene expression**

The characterization of CAT isoforms and the analysis of CAT gene expression were carried out with freshly harvested and artificially dried seeds collected at 29
(immature seeds), 42 (end of reserve accumulation), and 58 (fully mature seeds) DAF.

The subunit composition of catalase was analysed by immunoblot after SDS-PAGE using antibodies against the 55 kDa and 59 kDa peptides (Fig. 3). In fresh seeds collected at 29 DAF, blots from SDS-gels showed a single weak band at 59 kDa with antibodies against either CAT5 (Fig. 3A) or the 59 kDa subunit (Fig. 3B), and a weakly visible band at 55 kDa with antibodies against CAT6 (Fig. 3C). Overloading gels with increasing amounts of proteins (up to 25 μg) from fresh seeds at 29 DAF did not result in a more visible 55 kDa band with either antibodies against CAT5 or CAT6 (data not shown). Seed development was characterized by an increase of the band at 59 kDa and, more markedly, of the band at 55 kDa in fresh seeds collected at 42 DAF and mainly at 58 DAF, revealing the synthesis of both subunits. In fully mature fresh seeds the 59 kDa and 55 kDa subunits were equally represented.

In seeds artificially dried on the flowerheads, both 55 kDa and 59 kDa subunits were present at all the developmental stages (Fig. 3). The intensity of the band corresponding to the 55 and 59 kDa subunits appeared to be stronger in seeds dried at 29 DAF than in those dried at 42 and 58 DAF.

Northern blot analyses revealed a very weak expression of the CATA1 gene in fresh seeds collected at 29 DAF. This was hardly visible on northern blots (Fig. 4A) but was clearly revealed by image analysis (Fig. 4B). Seed development on the mother plant was marked by a progressive induction of the transcript which increased from 29 DAF to 42 DAF and, more markedly, from 42 DAF to 58 DAF (Fig. 4B).

Drying of seeds collected at 29 and 42 DAF resulted in a marked increase in CATA1 expression (Fig. 4A). Expression of CATA1 was similarly high in all dried seeds, whatever their developmental stage, but was lower than that measured in mature fresh seeds (Fig. 4).

The CATA3 gene was expressed in sunflower seeds, but the CATA3 probe used in this study did not allow a precise quantification of the transcripts. It was low in immature seeds collected at 29 DAF and increased during seed development or after artificial drying (data not shown).
Hydrogen peroxide content and lipid peroxidation

H$_2$O$_2$ content was high (around 800 nmol g$^{-1}$ DW) in fresh seeds collected at 29 DAF, but it decreased during seed development to reach 300 nmol g$^{-1}$ DW at full maturity (58 DAF) (Fig. 5A). Artificial drying of seeds collected at 29 and 42 DAF resulted in a decrease in H$_2$O$_2$ level to about 500 nmol g$^{-1}$ DW. By contrast, H$_2$O$_2$ content slightly increased after artificial drying of mature seeds collected at 58 DAF.

MDA content was around 100 μmol g$^{-1}$ DW in fresh seeds collected at 29 DAF and was almost 2-fold lower (56–58 μmol g$^{-1}$ DW) in those collected at 42 DAF and 58 DAF (Fig. 5B). Artificial drying of immature (29 DAF) seeds induced a decrease in MDA content to a value close to that measured in fresh mature seeds, but did not affect the MDA content of fully developed (42 DAF) or mature (58 DAF) seeds (Fig. 5B).

Discussion and conclusion

Sunflower seed development displayed the classical developmental pattern of orthodox seeds (Bewley and Black, 1994). Seed filling, which in sunflower mainly consists in the accumulation of triacylglycerols in the cotyledons (Miquel and Browse, 1995), appeared to be concomitant with seed dehydration (Fig. 1A). However, true desiccation, in the strict sense, probably began at c. 38 DAF since the amount of water per seed started to decrease sharply at this time (Fig. 1A). This dehydration pattern, therefore, appears to be similar to that of other developing seeds, such as those of pea and bean (Sanhewe and Ellis, 1996; Corbineau et al., 2000; Bailly et al., 2001) for which desiccation occurs mainly at the end of seed filling, i.e. during the maturation drying phase. Dry mass matter accumulation ceased at 42 DAF, which corresponded to a seed moisture content of 38% FW and a mean seed dry weight of 6 mg.

Freshly harvested sunflower seeds (i.e. seeds which were not dried) were unable to germinate at 20 °C whatever their stage of development (data not shown). This inability to germinate results mainly from an embryo dormancy which is easily broken during dry storage (Corbineau et al., 1990). Seeds which were stored dry before sowing fully germinated whatever their date of harvest (Fig. 1B). Data presented in Fig. 1B also show that sunflower seeds were desiccation-tolerant very early in their developmental programme, at least when they were slowly dried on the flowerheads.

CAT specific activity in developing sunflower seeds was closely related to their water content. The drier the seeds were, the higher it was (Fig. 2). In the case of immature seeds, artificial drying increased this activity (Fig. 2A). During natural dehydration on the mother plant there was even a linear relationship between seed moisture content and CAT specific activity (Fig. 2B). It seems, therefore, that the latter might be an indicator of the seed developmental stage. In fresh sunflower seeds collected at 29 and 42 DAF, the 59 kDa subunit was much more represented than the 55 kDa one (Fig. 3), suggesting that isoforms CAT1 or CAT2 were predominant since the 59 kDa
subunits are known to dominate in these two isoforms (Eising et al., 1990). In fresh mature seeds, i.e. those collected at 58 DAF, the 55 kDa subunit was at least as well represented as the 59 kDa subunit. This result indicates the appearance of new isoforms that might be CAT3, CAT4, or CAT5, since they are hybrids of 55 kDa and 59 kDa subunits, or belong to the group CAT6 to CAT8. Artificial drying of the immature seeds also induced a marked synthesis of the 55 kDa subunit and, to a lesser extent, of the 59 kDa subunit. It must be remembered that western blot analysis gives indications about the synthesis of catalase subunits, but does not necessarily reflect the enzyme activity which depends on the assembly of the four subunits constitutive of CAT. This might explain why the slight apparent decrease in 55 and 59 kDa subunits in artificially dried seeds from 29–58 DAF (Fig. 3) was not correlated with the small increase of CAT activity in the same seeds (Fig. 2). The precise identification of CAT isoforms and the determination of their respective activity would help clarify this point, but this was not the purpose of the present study since these isoforms, as well as their subcellular localization, have already been described in sunflower cotyledons (Eising et al., 1990; Kleff et al., 1997; Tenberge et al., 1997). These results, however, demonstrate that the CAT isoform pattern changes during seed development, and that this change is probably triggered by seed desiccation. Heterogeneity of catalase has already been demonstrated in mature seeds of cotton (Kunce and Trelease, 1986), sunflower (Eising et al., 1990), and maize (Scandalios et al., 1997). Eising et al. (1990) have suggested that CAT4 and CAT5 are the predominant isoforms in dry mature sunflower seeds. The nomenclature of CAT genes, CAT4 to CAT8 (Kleff et al., 1994), differs from the nomenclature of the isoforms, CAT1 to CAT8. CAT4 and CAT2 are assumed to code for the 55 kDa subunits localized in the matrix of peroxisomes, whereas CAT3 and CAT4 genes are supposed to code for the 59 kDa subunits localized in the core of the peroxisomes. Owing to the high homology between CAT4 and CAT2 and their similar size, the CAT4 probe used here did not discriminate between these two genes. However, this probe was designed in the C-terminal domain of CAT4 and therefore did not show homology with CAT3 and CAT4 genes, thus avoiding cross hybridization with these two transcripts. Analysis of CAT4 transcript expression confirmed the appearance of the 55 kDa isoform in artificially dried seeds and in mature fresh seeds, suggesting that induction of this isoform occurred at the transcriptional level (Fig. 4). Alternatively, the increase in catalase transcripts induced by drying might also be related to a possible effect of water loss on mRNA half-life, since dry seeds are rich in long-lived mRNAs compared with immature ones (Bewley, 1979). CAT3 and CAT4 genes show high homology with CAT1 and CAT2 but differ from them by an additional C-terminal domain. The CAT3 probe designed in this small zone (bp 1501-1800) did not allow a precise identification of their expression pattern, however, it permitted a progressive increase in CAT3 or CAT4 expression during seed development to be shown (data not shown).

These results suggest that the increase in catalase specific activity during seed development and artificial drying (Fig. 2) corresponds mainly to an increase in the transcription of gene(s) coding for the 55 kDa subunits of matrix catalase, as shown by western blot analysis. Several authors (for reviews see Leprince et al., 1993; Pammenter and Berjak, 1999) have suggested that ROS scavenging through antioxidant enzyme activities might be involved in the acquisition of desiccation tolerance by orthodox seeds; this is the case, for example, in bean seeds (Bailly et al., 2001). In other respects, Ingram and Bartels (1996) and Ramanjulu and Bartels (2002) have mentioned several genes that are up-regulated by dehydration in plants. Among these genes, the LEA-related ones are the most widely cited but others are also concerned, including ROS scavenging enzyme genes such as those of ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Ingram and Bartels, 1996; Sherwin and Farrant, 2001).
Catalase and sunflower seed development

The results obtained concerning the changes in hydrogen peroxide and MDA contents during sunflower seed development provide insights on the functional role of CAT. The amount of H$_2$O$_2$ was high in fresh immature seeds, but it decreased during seed desiccation on the plant (Fig. 5A). In artificially dried seeds, the level of H$_2$O$_2$ was almost the same, whatever the stage of development, and it was close to that found in mature seeds desiccated on the plant. Hydrogen peroxide is known to form the highly reactive hydroxyl radical (OH) through the Fenton reaction, leading to lipid peroxidation (Scandalios, 1997). The results of this study show that a high amount of H$_2$O$_2$ corresponded to a high level of MDA (Fig. 5). However, they do not show any increase in lipid peroxidation during natural seed desiccation (Fig. 5A). Therefore, the increase in CAT activity during seed development or after artificial drying might also be involved in the decrease of H$_2$O$_2$ content. Hydrogen peroxide is known to form the highly reactive hydroxyl radical (OH) through the Fenton reaction, leading to lipid peroxidation (Scandalios, 1997). The results of this study show that a high amount of H$_2$O$_2$ corresponded to a high level of MDA (Fig. 5). However, they do not show any increase in lipid peroxidation during natural seed desiccation (Fig. 5A). Taken all together these data address the question of the functional role of CAT with respect to dehydration-related damage, and they suggest that the ability of cells to withstand loss of water might be closely related to H$_2$O$_2$ scavenging. It is indeed admitted that water stress induces ROS generation, which can, in turn, lead to the various cellular and metabolic damage associated with oxidative stress (Smirnoff, 1993).

The regulation pathway of CAT expression in response to dehydration, nevertheless, remains to be elucidated since several modes of action involving the antioxidant-responsive element (ARE) and/or the ABA-responsive element (ABRE) in the gene promoter region, have so far been described (Polidoros and Scandalios, 1999; Guan et al., 2000). In sunflower, ABA content peaks quite precociously during seed development, but then decreases during further seed desiccation (Le Page-Degivry et al., 1990). ABA synthesis is, therefore, probably not directly related to the dehydration transduction pathway leading to CATAI activation. Regulation of CAT expression in developing sunflower seeds might, therefore, be related to H$_2$O$_2$, probably via a mechanism close to that described by Polidoros and Scandalios (1999) who have shown that ROS induces catalase expression through ARE-like motifs. It is, therefore, proposed that H$_2$O$_2$ might play a key regulatory role in controlling CAT gene expression during seed desiccation. The characterization of the promoter region of sunflower CAT genes would help to clarify the effects of H$_2$O$_2$ on their expression.

In addition, the question of the role of the balance between CAT activity and H$_2$O$_2$ concentration in the control of the developmental pattern of sunflower seeds must also be addressed, since H$_2$O$_2$ scavenging through CAT activity modulates the signalling capacity of H$_2$O$_2$. Seed desiccation is associated with a shift from a developmental to a germinative mode (Kermode, 1995). Beside the deleterious oxidative effects of hydrogen peroxide, the involvement of this compound in the reorientation of genome functioning cannot be excluded, because of the numerous genes it regulates. Desikan et al. (2001), for example, have identified many genes that are up- or down-regulated by hydrogen peroxide. Lastly, CAT is also known to play a role in detoxifying H$_2$O$_2$ generated by reserve lipid mobilization during germination (Bewley and Black, 1994). Its synthesis might therefore accompany glyoxysome biogenesis during sunflower seed development (Trelease, 1984). A better understanding of the role of CAT and H$_2$O$_2$ in sunflower seed development would require their subcellular as well as their tissue and organ location to be investigated. CAT is known to be located mainly in peroxisomes and glyoxysomes (Willekens et al., 1995) whereas H$_2$O$_2$, which may diffuse from its production sites, may originate from the mitochondrial respiratory chain (Moller, 2001), xanthine oxidase activity in the peroxisomal matrix (Corpas et al., 2001), or NADPH oxidases of the plasma membrane (Grant and Loake, 2000).

In conclusion, this set of data demonstrates that sunflower seed development is associated with marked changes in CAT expression and activity. The tight regulation of CAT by artificial or natural drying suggests that hydrogen peroxide removal may be crucial for avoiding desiccation-related oxidative stress.

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