J Plant Growth Regul (2014) 33:272–284
DOI 10.1007/s00344-013-9369-x

Catalase Inhibition Affects Glyoxylate Cycle Enzyme Expression and Cellular Redox Control during the Functional Transition of Sunflower and Safflower Seedlings

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Received: 8 April 2013 / Accepted: 1 July 2013 / Published online: 25 August 2013

Abstract Oilseed crops are an important natural resource because they can be used for food and renewable energy production. However, oilseed seedling establishment and vigor depend upon the capacity to overcome functional transition, a developmental stage characterized by the consumption of the remaining oil reserves, through β-oxidation and glyoxylate cycle, and the onset of autotrophic metabolism. The increased growth and the acclimation to full photosynthetic activity lead to production of reactive oxygen species and a reorganization of the cell antioxidant systems to achieve a new redox homeostasis. In the present study, catalase (CAT) was inhibited by 3-amino-1,2,4-triazole application during functional transition in sunflower and safflower seedlings to understand the effect of this antioxidant enzyme impairment on the mRNA expression of the glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MLS), as well as the superoxide dismutase (SOD) activity and ascorbate peroxidase (APX) activity and expression. CAT inhibition led to significant seedling growth reduction and increases in H_2O_2 content, SOD activity, and mRNA expression of CAT and APX in both species. However, APX activity was induced only in safflower plants. Additionally, ICL and MLS mRNA expressions were upregulated after 6 h of treatment when compared to the control values. These results indicate that under CAT impairment conditions, redox homeostasis at the functional transition phase was partially supported by the SOD and APX antioxidant systems to maintain the seedling photosynthetic establishment.

Keywords Oil seeds · CAT · Ascorbate peroxidase · Isocitrate lyase · Malate synthase · 3-AT

Electronic supplementary material The online version of this article (doi:10.1007/s00344-013-9369-x) contains supplementary material, which is available to authorized users.

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Introduction

Plant oils are a high-value renewable natural resource, composed of predominantly triacylglycerols (TAGs), which can be used either as a biofuel or for human nutrition (Dyer and others 2008; Milazzo and others 2013). Although oilseed crops such as soybean, sunflower, and safflower have enriched fatty acid composition compatible with high-scale oil production (Gunstone and others 2010), storage conditions can severely reduce seed and oil quality, leading to decreased germination and seedling establishment rates (Devaiah and others 2007). Therefore, many efforts have been made to increase seed storage time and circumvent these usual problems (Schwember and Bradford 2010; Ventura and others 2012).

Seedling establishment depends upon the capacity to overcome a crucial developmental period known as the functional transition, which is characterized by a change from a metabolic heterotrophic condition to an autotrophic one. During this phase, the late stage of reserve mobilization occurs, as well as organelle differentiation and the activation of many signaling pathways that promote plant acclimation to the photosynthetic state (Gonzalez and Vodkin 2007). β-Oxidation and the glyoxylate cycle in glyoxysomes are two essential metabolic pathways that consume reserves and support oilseed seedling establishment (Graham 2008). The first promotes lipid catabolism and produces acetyl-CoA, which in turn feeds the glyoxylate cycle. This cycle is a variation of the tricarboxylic acid cycle (TCA) and has two reactions that bypass the TCA cycle decarboxylation steps catalyzed by malate synthase (MLS) and isocitrate lyase (ICL). Briefly, the glyoxylate cycle uses two acetyl-CoA molecules to produce one succinate or oxaloacetate molecule that can be later utilized in carbohydrate biosynthesis (Theodoulou and Eastmond 2012).

During the functional transition, the intense growth and the increased flow of fatty acids toward β-oxidation in seed glyoxysomes and NADH supplied to the electron transport chains in mitochondria and chloroplast can generate reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-). Though these species are known to be involved in signaling events during this physiological phase when produced in an uncontrolled manner, ROS can promote oxidative damage to lipids, proteins, and nucleic acids, while disrupting the cell redox homeostasis (Nyathi and Baker 2006; Gill and Tuteja 2010; Hu and others 2012). However, plants possess both nonenzymatic and enzymatic antioxidant systems to limit ROS production and promote their scavenging. The nonenzymatic antioxidant system is composed of metabolites such as reduced glutathione (GSH), reduced ascorbate (AsA), phenols, α-tocopherol, and β-carotene. On the other hand, the enzymatic system includes many enzymes throughout plant cell compartments that either directly act on ROS, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and phenol peroxidases (POXs), or maintain a reduced electron donor pool, such as the ascorbate–glutathione cycle enzymes (Cavalcanti and others 2007).

In the functional transition, the CAT antioxidant system is particularly important in scavenging the hydrogen peroxide produced by β-oxidation and photorespiration (Gonzalez and Vodkin 2007; Graham 2008). This antioxidant system is confined to the peroxisomal/glyoxysomal matrix, having a high H_2O_2 scavenging rate. However, peroxisomal CAT activity alone is often insufficient to cope with excess H_2O_2. Because this ROS can cross biological membranes (either directly or through porins) (Bienert and others 2006), two APX isoforms complement H_2O_2 scavenging: one is attached to the outer face of the glyxyosomal membrane (gmAPX), and other is the cytosolic isoform (cAPX). Both isoforms have high specificity to degrade H_2O_2 using ascorbate as an electron donor (Graham 2008). gmAPX is metabolically paired to monodehydroascorbate reductase (MDAR) attached to the inner face of the glyoxysomal membrane, and the disruption of this system can impair TAG hydrolysis (Eastmond 2007). Also, an imbalance in redox state caused by high levels of reduced coenzymes such as NADH can reduce the glyoxylate cycle flow (Alberty 2006). These events must be linked to the control of H_2O_2 levels, especially in the functional transition.

Additionally, during seedling establishment, superoxide generation is a natural by-product of the electron transport chains, and its production is increased when the plant cell is exposed to different stressful situations (Gill and Tuteja 2010; Roach and others 2010; Lidon and others 2011). Xi and others (2010) demonstrated that plants with overexpression of CAT, Mn-SOD, and HPT1 (which perform the first irreversible reaction in tocopherols biosynthesis) had better germination and establishment rates under stress conditions; thus, the enzymatic and nonenzymatic antioxidant systems have an important role during seedling establishment.

The late phase of reserve mobilization and the early photosynthetic state need complex redox regulation to promote functional transition and seedling establishment. However, there are several remaining gaps in the understanding of the enzymatic antioxidant system's role during plant establishment, particularly glyoxyosomal CAT, which is usually reported as essential for plant establishment (Gonzalez and Vodkin 2007; Graham 2008). In this context, we conducted an irreversible inhibition of CAT, using 3-AT, during the functional transition phase in sunflower and safflower seedlings. Our aim was to examine whether the drastic reduction of CAT activity affects the response patterns of the APX and SOD antioxidant systems, and also to evaluate its possible influence on the gene expression of glyoxylate cycle enzymes ICL and MLS.

Materials and Methods

Plant Material and Growth Conditions

Experiments were carried out with sunflower (*Helianthus annuus* L. cv. Catissol 01) and safflower (*Carthamus tinctorius* L. cv. Lasting Orange). All seeds were stored for less than 3 months at 24 °C and 70 % relative humidity. The seed surfaces were sterilized with NaOCl 1 % (w/v) for 1 min and washed four times with distilled water for 1 min per wash. Next, sunflower and safflower seeds were imbibed in distilled water for 10 min and 1 min, respectively. The seeds were then dispersed equidistantly on three sheets of paper towels (280 × 380 mm) moistened with distilled water in the ratio of 1.5 and 2.5 times the mass of dry paper for the safflower and sunflower seeds, respectively. The paper towels were rolled to form a cylinder and placed in two plastic bags fastened with a tie and considered as an experimental unit. The plants were kept in a growth chamber under controlled conditions: 26 ± 1 °C, photoperiod of 12 h of light and 12 h of dark, 70 % relative humidity, and photosynthetically active radiation of 150 µmoL m^{-2} s^{-1}. Two days after the sowing, the seedlings were transferred to new paper towels containing 0 mM (control) and 10 mM (treatment) of 3-amino-1,2,4-triazole (3-AT), corresponding to approximately 2 mg of 3-AT per single plant. 3-AT is a potent inhibitor of CAT enzymes, which increases cell H_2O_2 levels (Gechev and others 2002). The seedlings were kept in treatment for 48 h and the determinations were performed at 0, 6, 12, 24, and 48 h after transfer. All the determinations were carried out using four repetitions of the experimental unit.

Seedling Growth, Fresh and Dry Weights, and Determination of Chlorophyll Contents

Seedling growth and fresh weight were measured after each harvest. Then, seedlings were dried in an oven at 80 °C for 48 h, under artificial air circulation, and the dry weight was obtained. Quantification of chlorophyll was realized according to the method described by Arnon (1949). Briefly, 0.025 g of dry tissue was ground in a mortar in the presence of 5 mL of acetone at 80 % (v/v). Then the mixture was centrifuged at 800×*g* for 5 min at room temperature. The supernatant was collected and the absorbance at 645, 652, and 663 nm was measured by spectrophotometer to determine the content of chlorophyll *a*, *b*, and total.

Acid Extraction

Acid extraction was performed following the method described by Cavalcanti and others (2007), with minor modifications. Shoots samples (0.13 g) were ground in a mortar to a fine powder in the presence of liquid nitrogen, and then mixed for 5 min in 940 µL of ice-cold trichloroacetic acid solution at 1 % (w/v), containing activated charcoal at 0.5 % (w/v) to facilitate protein adsorption (Sekaran and others 1996; Kopper and others 2008). To this mixture 60 µL of 5 % Triton X-100 was added. The homogenate was then centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatant was collected immediately to determine the content of H_2O_2 and malondialdehyde.

Lipid Peroxidation and Hydrogen Peroxide Content

Lipid peroxidation was determined using the thiobarbituric acid reactive substances (TBARS) method (Heath and Packer 1968), with some modifications. Aliquots from acid extraction (described above) were added to a solution containing thiobarbituric acid (TBA) at 0.5 % (w/v) and trichloroacetic acid at 20 % (w/v). The tubes were sealed and the mixture was incubated in a water bath at 95 °C for 1 h and then cooled in ice to stop the reaction. The samples were centrifuged at 5,000×*g* for 10 min at room temperature (25 °C). The absorbances at 532 nm (specific reaction) and 660 nm (nonspecific reaction) were measured. The MDA–TBA content was calculated from the molar extinction coefficient of 155 mM^{-1} cm^{-1} and the results were expressed in nmol MDA–TBA g^{-1} FW.

The H_2O_2 content was measured by its specific reaction with potassium iodide (KI) (Jaleel and others 2008). Aliquots (500 µL) from the acid extraction were mixed with 500 µL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI; then the mixture was incubated for 30 min in the dark. The absorbance at 340 nm was measured and the H_2O_2 content was estimated based on a standard curve of H_2O_2 prepared in trichloroacetic acid at 1 % (w/v). The results were expressed as µmol H_2O_2 g^{-1} FW.

Protein Extraction and Quantification

Protein extraction was performed using the method described by Kagawa and others (1973). Briefly, shoot samples (0.1 g) were ground in a mortar to a fine powder in the presence of liquid nitrogen and then homogenized in 940 µL of ice-cold 165 mM Tris-HCl buffer (pH 7.5) containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM ascorbic acid. After the addition of 60 µL of 5 % Triton X-100, the samples were centrifuged at 13,000×*g* for 20 min at 4 °C. The supernatant was collected and stored at −20 °C for subsequent determinations of enzymatic activities and total soluble proteins (Bradford 1976).

Enzymatic Activities

CAT (EC 1.11.1.6) activity was determined by adding 50 µL of the protein extract to 2.95 mL of a solution containing 50 mM potassium phosphate buffer (pH 7.0) and H_2O_2 at 20 mM. The activity was measured by H_2O_2 oxidation at 240 nm at 30 °C (Havir and McHale 1987), over 5 min, with readings every 30 s. The enzyme activity was calculated using the H_2O_2 molar extinction coefficient ($\varepsilon_{240\ nm}$) of 40 mM^{-1} cm^{-1} (Velikova and others 2000) and expressed as consumption of H_2O_2 (µmol min^{-1} mg^{-1} protein).

APX (EC 1.11.1.11) activity was determined by monitoring ascorbic acid oxidation at 290 nm according to the method of Nakano and Asada (1981) and following the modifications suggested by Koshiba (1993). The reaction mixture contained the protein extract, L-ascorbic acid at 0.5 mM, and potassium phosphate buffer at 50 mM, pH 6.0. The activity was started after the addition of H_2O_2 at 30 mM, and the decreasing of absorbance was observed over 5 min, with readings every 30 s. Enzyme activity was calculated using an ascorbic acid molar extinction coefficient ($\varepsilon_{290\ nm}$) of 2.8 $mM^{-1}\ cm^{-1}$ and expressed as µmoL H_2O_2 min^{-1} mg^{-1} protein.

SOD (EC 1.15.1.1) activity was determined according to the method of Van Rossum and others (1997) as modified by Rosa and others (2010). In this method, the protein extract prevents NBT (p-nitroblue tetrazolium) photoreduction, thus avoiding formation of blue formazan. The reaction mixture (200 µL) contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, and 75 µM NBT. The reaction was started by adding 10 µM of riboflavin, followed by exposure to a 30-W fluorescent lamp for 30 s. The positive control contained no enzymatic extract and the blank reaction was kept in the dark. After the reaction, the absorbance was measured at 560 nm in a microplate spectrophotometer (Epoch BioTek®). One SOD activity unit (AU) was defined as the amount of enzyme required to inhibit 50 % of the NBT photoreduction and the activity was expressed as AU mg^{-1} protein (Beauchamp and Fridovich 1971).

RNA Extraction, Reverse Transcription, and qRT-PCR

Total RNA extraction was performed using the Concert Plant RNA® reagent (Invitrogen) according to the manufacturer's instructions, and the purified RNA was stored at −80 °C. The integrity of extracted RNA was checked by electrophoresis in agarose gel at 2 % in nondenaturing conditions, free from RNAses, in TBE buffer (Tris–boric acid at 0.045 M and EDTA at 0.001 M) and in the presence of ethidium bromide. The integrity of 26S and 18S ribosomal RNA was visualized in a UV transluminator. The cDNAs were obtained using the ImProm-II^{TM} (Promega) reverse transcriptase enzyme according to the manufacturer's instructions, and the resulting cDNAs were stored at −80 °C. RNA and cDNA quantities were estimated by a NanoDrop ND-2000 spectrophotometer (Thermo Scientific).

Specific primers were designed to exon–exon mRNA junctions of CAT, cytosolic ascorbate peroxidase, malate synthase, and isocitrate lyase. The BLAST tool (Altschul and others 1997) and sequences of the respective enzymes from *Arabidopsis* were used to search databases of EST (expressed sequence tags) and WGS (whole genome shotgun) of safflower, sunflower, and phylogenetically related species. All sequences were obtained from databases of the National Center for Biotechnology Information (NCBI).

The qRT-PCR reactions were conducted in an ABI 7500 Real-Time PCR System (Applied Biosystems) using Power SYBR® Green Master Mix (Applied Biosystems), according to the manufacturer's instructions. The RT-qPCR program was 95 °C for 10 min for denaturation; then 40 cycles of 95 °C for 15 s, 52–55 °C for 0.5–1 min for primer annealing, and 60 °C for 32 s for extension; and then a melting curve (95 °C for 15 s, 52–55 °C for 60 s, and 95 °C for 15 s) was performed to verify any nonspecific fragment amplification (data not shown). The expression was calculated relative to actin (Nicot and others 2005) and according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). A summary of the primer features and qRT-PCR reactions is given in Table 1 and Supplementary Table 1.

Statistical Analysis

The data are presented as mean ± sample standard deviation (SD). Significance analysis was performed by analysis of variance (ANOVA) followed by Student's *t*-test, and the

Table 1 Summary of RT-qPCR assays

Organism	Transcript name	Primer F (5'–3')	Primer R (5'–3')	Amplicon (bp)
Safflower	*CAT*	CACAACAATCACTATCATGG	GGAAAGTAATCGACCTCCTC	65
	cAPX	GATTTCCATCAGCTTGCTGG	CTTGGTAGCATCAGGAAGAC	126
	ICL	ACCCATGGCTGCATCTTTCT	GCCATATGATTGGCGGAGGT	178
	MLS	ACGAGCTGAGAATATGGCCG	CCATTTGGAACACTGCTGGC	86
	α-Actin	GCTTCCCGATGGTCAAGTCA	GGATTCCAGCTGCTTCCATTC	101
Sunflower	*CAT*	CACAACAATCACTATCATGG	GGAAAGTAATCGACCTCCTC	65
	cAPX	GATTTCCATCAGCTTGCTGG	CTTGGTAGCATCAGGAAGAC	126
	ICL	ACCCATGGCTGCATCTTTCT	GCCATATGATTGGCGGAGGT	178
	MLS	ACGAGCTGAGAATATGGCCG	CCATTTGGAACACTGCTGGC	86
	α-Actin	GCTTCCCGATGGTCAAGTCA	GGATTCCAGCTGCTTCCATTC	101

For supplemental information about thermal cycles and sequences used for primer design, see Supplementary Table 1 values were considered statistically different when $p < 0.05$. The programs PAST (Hammer and others 2001) and R (Ihaka and Gentleman 1996, www.r-project.org) were used.

Results

Catalase Inhibition Reduces Root Growth but not Seedling Establishment

In both sunflower and safflower, the 3-AT–treated seedlings had prominent root growth inhibition, which was clearly noticed 24 h after treatment (Fig. 1a, b and Table 2). However, this growth inhibition was not associated with any visible damage to the cotyledons. In addition, the seedling chlorophyll content displayed moderate changes at 12 and 24 h, returning to control levels after 48 h of treatment in both species (Fig. 1c, d). Interestingly, CAT-specific activity levels were greater in safflower than in sunflower. The deleterious effect of 3-AT on CAT activity was apparent within 6 h of treatment, with significant decreases in CAT levels in both sunflower and safflower (Fig. 2a, b). After 48 h, recovery of CAT activity in the 3-AT–treated seedlings was detected. Nevertheless, the levels were still significantly lower than those registered in control plants.

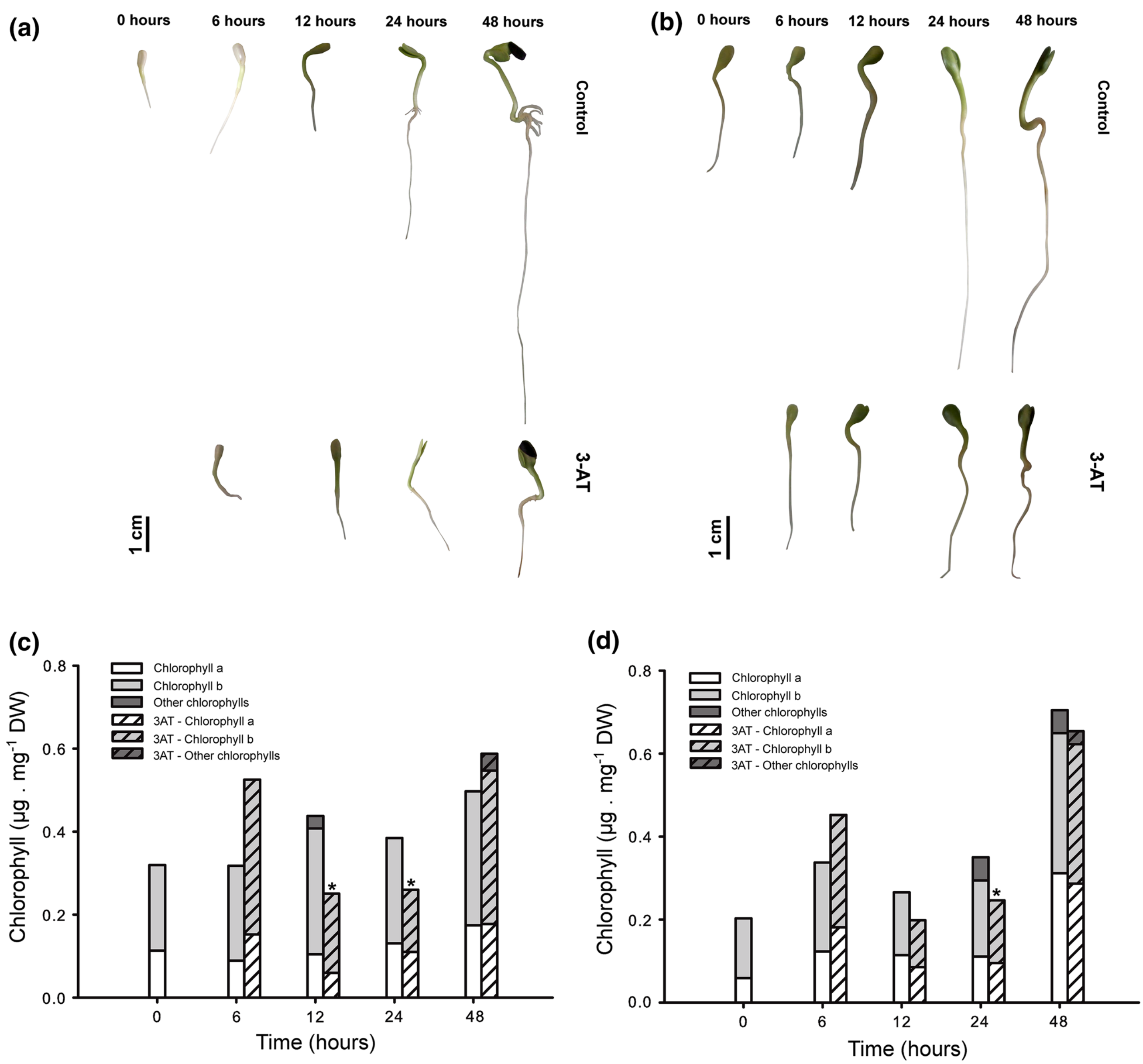

Fig. 1 Effect of 3-AT treatment on seedling growth and chlorophyll content of sunflower (**a, c**) and safflower (**b, d**) seedlings. *Values* represent the mean ± SD ($n = 4$). *Single asterisk* (*) at the top of the *errors bars* indicates statistically different means ($p < 0.05$)

Table 2 Changes in Growth and relative dry weight of sunflower and safflower seedlings submitted to 0 (Control) or 10 mM 3-AT treatment

Time (h)	Growth (cm)		Dry weight (%)	
	Control	3-AT	Control	3-AT
Sunflower				
0	2.21 ± 0.31		21.72 ± 1.51	
6	2.88 ± 0.52 a	2.94 ± 0.54 a	20.65 ± 2.43 a	14.04 ± 6.70 a
12	3.16 ± 0.44 a	3.30 ± 0.42 a	14.41 ± 1.30 b	16.24 ± 9.92 a
24	7.48 ± 1.10 bA	4.71 ± 1.01 bB	8.14 ± 2.30 c	6.95 ± 0.63 a
48	13.04 ± 2.94 cA	5.38 ± 0.75 bB	10.71 ± 1.08 cA	4.08 ± 0.11 bB
Safflower				
0	3.71 ± 0.51		33.89 ± 6.33	
6	4.72 ± 0.90 aA	3.47 ± 0.71 aB	31.79 ± 4.35 a	26.18 ± 3.23 a
12	5.49 ± 1.12 a	5.37 ± 0.79 b	19.21 ± 1.00 b	15.01 ± 2.28 b
24	7.04 ± 1.43 bA	4.68 ± 1.03 bB	8.17 ± 1.46 cA	15.41 ± 3.93 bB
48	11.84 ± 1.70 cA	7.34 ± 0.60 cB	9.90 ± 0.32 c	9.73 ± 0.57 c

Uppercase and lowercase letters indicate statistically different means ($p < 0.05$) between harvest times (rows) and between treatments (columns), respectively

Other Antioxidant Enzymes Play Crucial Roles in Constraining ROS Effects during Functional Transition

3-AT application induced distinct responses by the APX and SOD systems from both species (Fig. 3). Although the 3-AT–treated sunflower plants showed significant decreases in APX activity at the 6–12 h interval, a gradual recovery was observed between 24 and 48 h, sufficient enough to reach control plant levels (Fig. 3a). In contrast, the APX activity in safflower seedlings was significantly increased at 12 and 48 h (Fig. 3b).

At the end of the experimental period (48 h), SOD activity in 3-AT–treated plants was increased in both species (Fig. 3c, d). In sunflower, the treated seedlings showed similar activity levels until 24 h, but had higher values at 48 h when compared to control values at the same time (Fig. 3c). In safflower, SOD activity was induced 24 h after 3-AT application and was further increased at 48 h (Fig. 3d). In contrast to CAT and APX activities, the safflower plants had lower SOD activity levels than those observed in sunflower seedlings.

In both species there appeared to be a delay between H_2O_2 content and lipid peroxidation. In sunflower, the higher H_2O_2 accumulation occurred at 6 h and the higher value for lipid peroxidation was observed at 48 h (Fig. 4a, c), whereas in safflower seedlings the H_2O_2 accumulation occurred at 6 and 12 h and the lipid peroxidation maximum was recorded at 24 h (Fig. 4b, d).

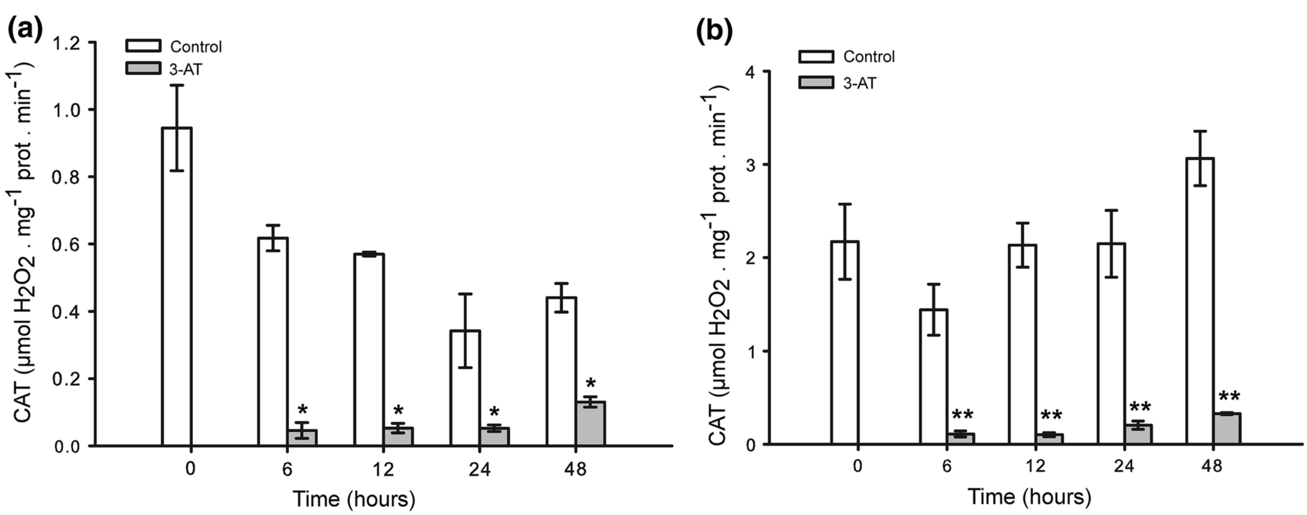

Fig. 2 Effect of 3-AT treatment on the CAT activity of sunflower (**a**) and safflower (**b**) seedlings. *Values* represent the mean ± SD ($n = 4$). *Double asterisks* (**) at the top of the *errors bars* indicate statistically different means ($p < 0.01$) and a *single asterisk* (*) indicates statistically different means ($p < 0.05$)

Effects of Catalase Inhibition on Gene Expression of Antioxidant Enzymes and Glyoxylate Cycle Enzymes

CAT and cAPX mRNA expression patterns were distinct in the two species (Fig. 5). In sunflower, photosynthetic establishment seems to promote a gradual decrease in CAT expression, regardless of the treatment, but it was faster in the 3-AT–exposed plants (Fig. 5a). In addition, this treatment induced a significant increase in sunflower CAT mRNA expression at 6 h. On the other hand, the cAPX expression gradually increased in the treated seedlings until a drastic decrease at 48 h, in both control and treated plants (Fig. 5c). Meanwhile, the safflower seedlings subjected to 3-AT showed a substantial increase in CAT expression at 24 and 48 h (Fig. 5b). Also, these plants showed a peak of cAPX mRNA expression at 6 h, before a drastic overall decrease at 12 and 24 h (Fig. 5d).

In both sunflower and safflower seedlings, the 3-AT treatment and the subsequent inhibition of CAT activity triggered an upregulation of ICL and MLS mRNAs at 6 h compared to that in the control plants (Fig. 6 and Table 3). In safflower, an increase in ICL mRNA expression was also observed at 12 h and no further significant variations were verified (Fig. 6b). However, in sunflower seedlings, the relative expression of both mRNAs declined during photosynthetic establishment, that is, after 12 h (Fig. 6a). From this point until the end of the experimental period, the ICL expression was reduced in the treated seedlings. MLS mRNA expression followed this same pattern, although it was less pronounced (Table 3). The downregulation of the MLS mRNA in safflower was very remark-

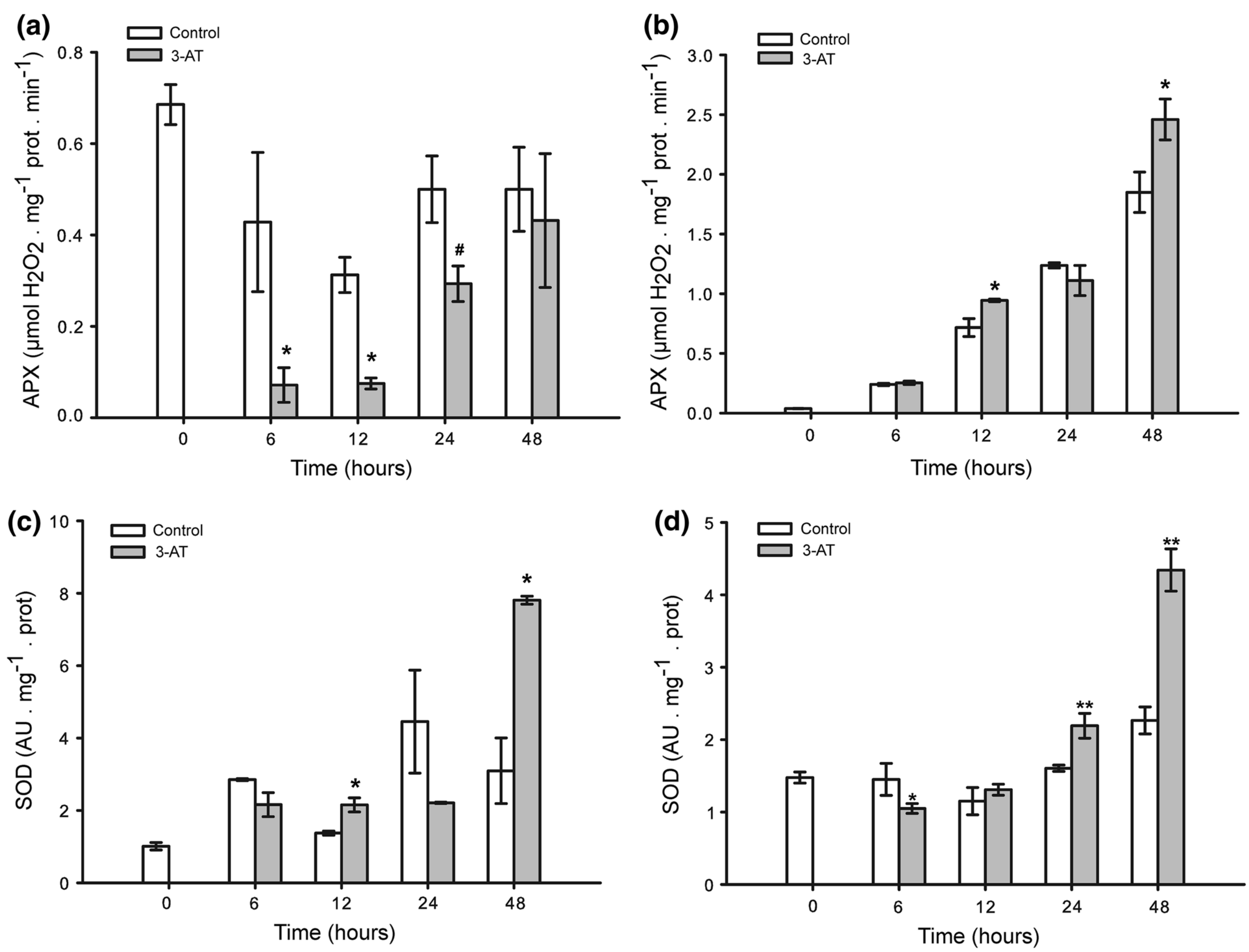

Fig. 3 Effect of 3-AT treatment on the APX and SOD activities of sunflower (**a, c**) and safflower (**b, d**) seedlings. *Values* represent the mean ± SD ($n = 4$). *Double asterisks* (**) at the top of the *errors bars* indicate statistically different means ($p < 0.01$), *single asterisk* (*) indicates statistically different means ($p < 0.05$), and *sharp character* (#) indicates statistically different means ($p < 0.1$)

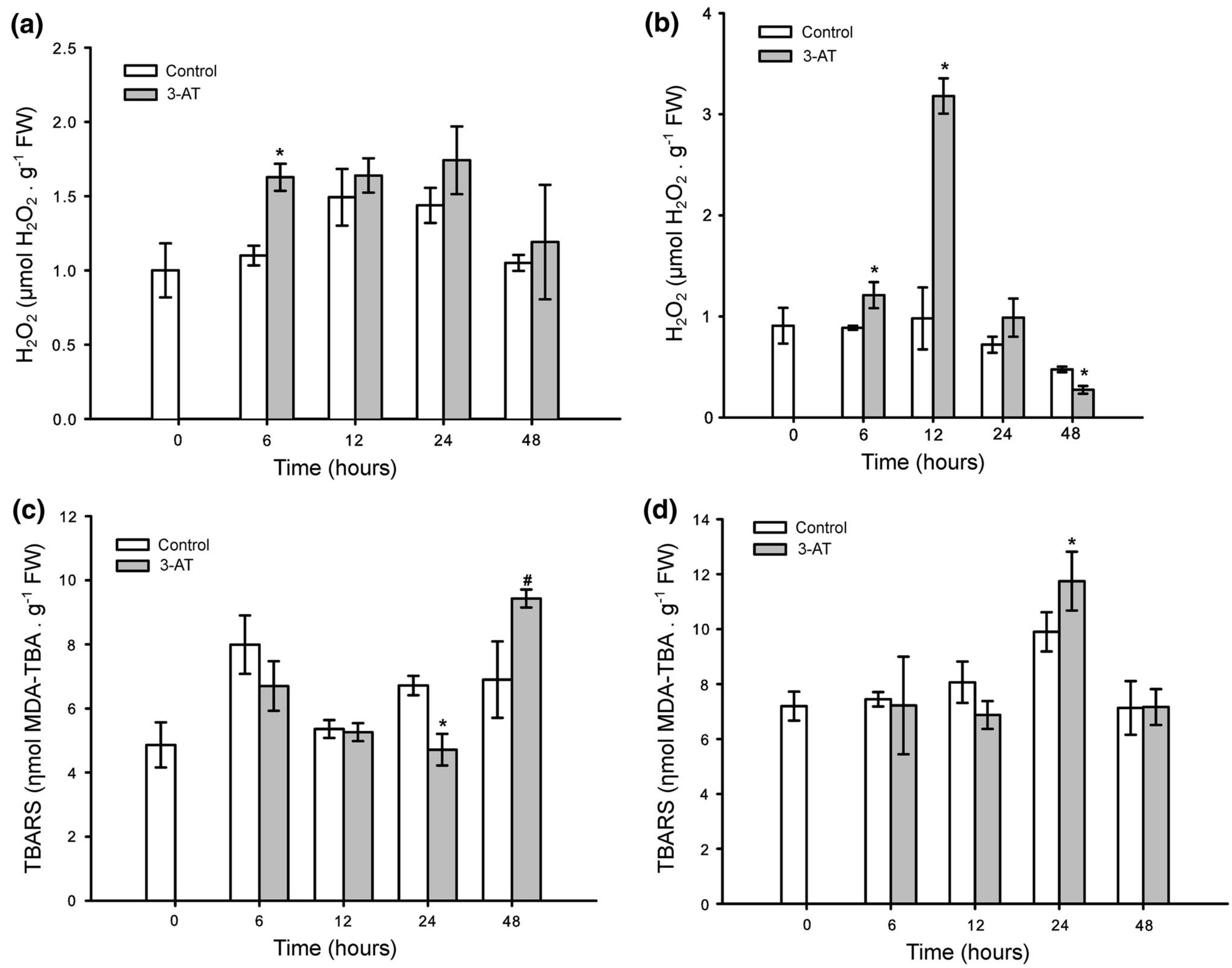

Fig. 4 Effect of 3-AT treatment on the H_2O_2 and TBARS contents of sunflower (**a, c**) and safflower (**b, d**). *Values* represent the mean ± SD ($n = 4$). *Single asterisk* (*) at the top of the *errors bars* indicates statistically different means ($p < 0.05$) and a *sharp character* (#) indicates statistically different means ($p < 0.1$)

able in that it was undetectable after 12 h in both control and treated plants (Table 3).

Discussion

3-AT treatment during functional transition in sunflower and safflower effectively inhibited seedling CAT activity and constitutes a successful approach to understanding the consequences of impairment of this important antioxidant system. Our results clearly demonstrate that the treated oilseed seedlings displayed residual CAT activities when compared to the control values (Fig. 2). The best-characterized visual effect of this deficiency was the marked root growth reduction (Fig. 1a, b and Table 2), which is often associated with peroxisome malfunction (Zolman and others 2005). During the 12–24-h interval, slight reductions in chlorophyll content were also observed (Fig. 1c, d), which is a previously described effect of 3-AT (Heim and Larrinua 1989). However, at 48 h, the chlorophyll contents of treated plants did not change significantly, suggesting that CAT inhibition does not prevent seedling photosynthetic establishment.

The deficiency in CAT activity leads to meaningful increases in H_2O_2 content, particularly early in the experimental period, but the increases were not associated with the estimated lipid peroxidation values at 6 and 12 h (Fig. 4). The germination and post-germination phases are characterized by intense growth, with constant cell membrane reorganization (Chapman 1998; Tavares and others 1998; Armstrong and others 2000 Simontacchi and others 2003; Gonzalez and Vodkin 2007). This might be a reason for the absence of significant differences in the MDA–TBA content at earlier times. On the other hand, regulation of

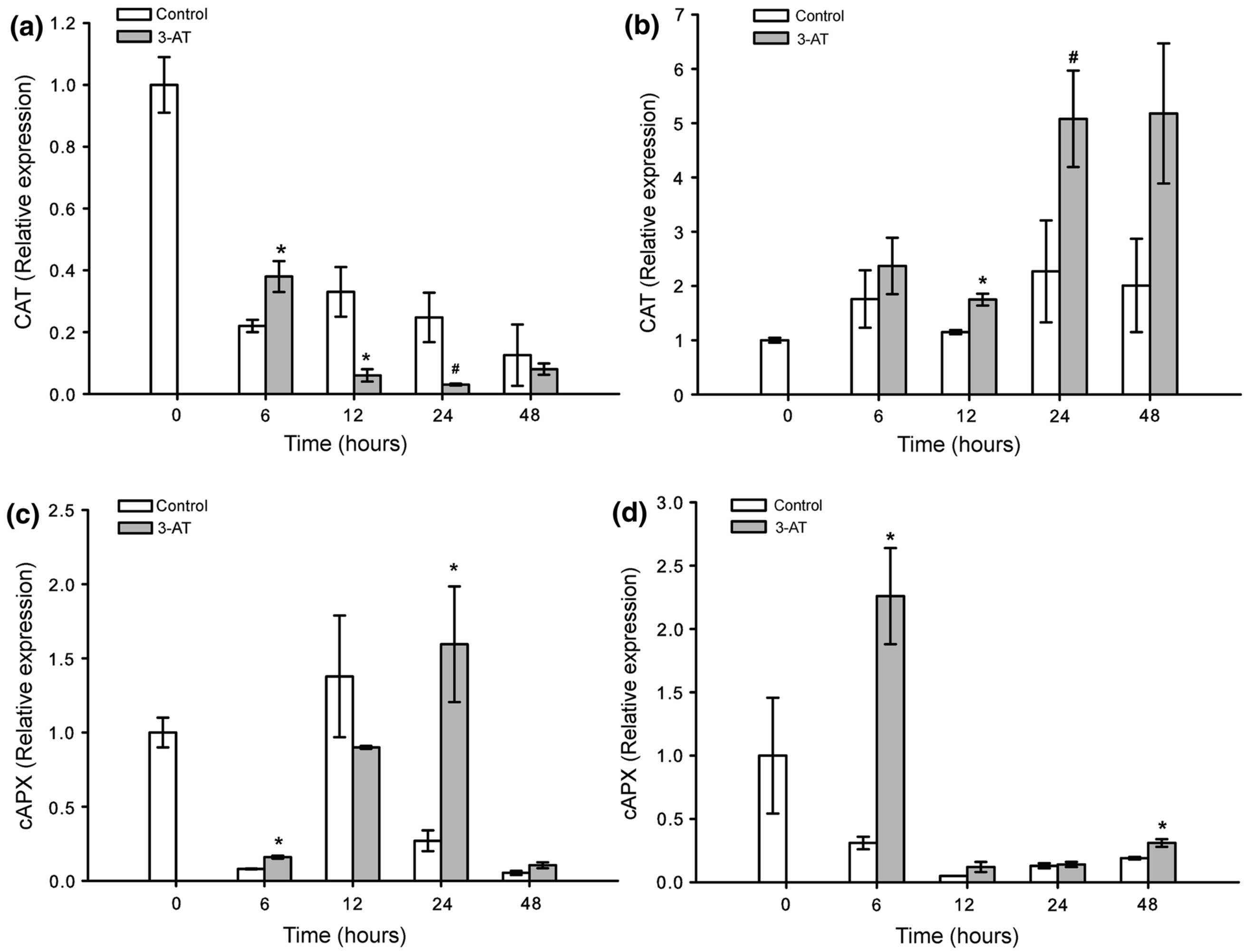

Fig. 5 Effect of 3-AT treatment on the CAT and cAPX mRNA expression patterns of sunflower (**a, c**) and safflower (**b, d**) seedlings. α-Actin mRNA was used as a housekeeping gene expression control. *Values* represent the mean ± SD ($n = 4$) and are relative to 0-h samples. *Single asterisk* (*) at the top of the *errors bars* indicates statistically different means ($p < 0.05$) and a *sharp character* (#) indicates statistically different means ($p < 0.1$)

H_2O_2 levels during the latter stages of photosynthetic establishment (24 and 48 h) could be due to the increase in APX activity and expression (Barba-Espín and others 2010), most noticeably in safflower, and possibly by other enzymes of the ascorbate-glutathione cycle, especially monodehydroascorbate reductase (MDAR), as demonstrated by Eastmond (2007).

Previous studies suggest that the SOD system has an essential role under these conditions. Superoxide overproduction causes severe alterations in *Salix nigra* germination (Roqueiro and others 2012), and MnSOD overexpression promotes a marked improvement in *Arabidopsis* post-germination establishment (Xi and others 2010). Therefore, the observed increases in SOD activity in 3-AT–treated plants at the later stages of photosynthetic establishment can indicate a demand for regulation of superoxide production, especially in a redox imbalance situation. During functional transition, the following processes are directly linked to O_2^- production rates: photophosphorylation (chloroplast), oxidative phosphorylation and TCA cycle (mitochondria), and β-oxidation and glyoxylate cycle (glyoxysome). The first process leads to increases in the $NADPH/NADP^+$ ratio and the latter three enhance the $NADH/NAD^+$ ratio, overreducing the electron transport chains and easing the electron escape to molecular oxygen, generating superoxide (Nyathi and Baker 2006; Gill and Tuteja 2010). Indeed, O_2^- levels are often amplified in plants and protoplasts exposed to high light intensities (Robert and others 2009; Lidon and others 2011). Therefore, the early photosynthetic stage during plant establishment can increase ROS production and generate signaling waves, where superoxide can have a fundamental role. Superoxide is also produced by respiratory burst oxidase homolog (RBOH) proteins, such as the NADPH

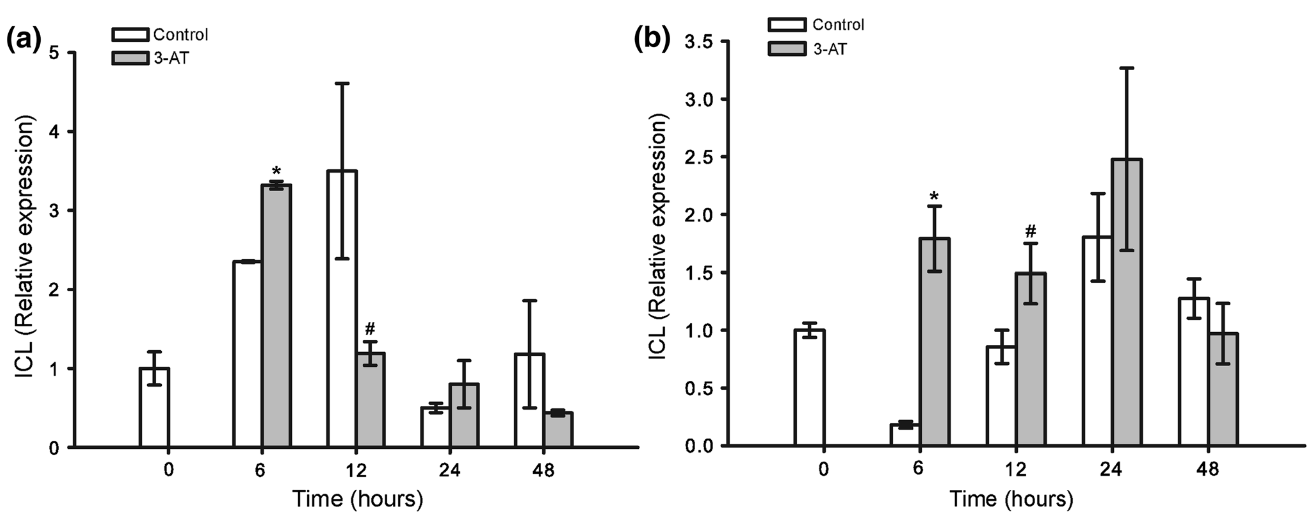

Fig. 6 Effect of 3-AT treatment on the ICL mRNA expression of sunflower (**a**) and safflower (**b**) seedlings. α-Actin mRNA was used as a housekeeping gene expression control. *Values* represent the mean ± SD ($n = 4$) and are relative to 0-h samples. *Single asterisk* (*) at the top of the *errors bars* indicates statistically different means ($p < 0.05$) and a *sharp character* (#) indicates statistically different means ($p < 0.1$)

Table 3 Relative expression of malate synthase transcript

Organism	Hours	Control	3-AT
Sunflower	0	1 ± 0.09	–
	6	0.31 ± 0.14	0.63 ± 0.08*
	12	0.33 ± 0.11	0.22 ± 0.05
	24	0.12 ± 0.02	0.11 ± 0.02
	48	0.06 ± 0.02	0.16 ± 0.04
Safflower	0	1 ± 0.03	–
	12	0.72 ± 0.072	1.72 ± 0.33[#]

α-Actin mRNA expression was used as a housekeeping gene expression control. Values represent the mean ± SD ($n = 4$) and are relative to 0-h samples. *Single asterisk* (*) indicates statistically different means ($p < 0.05$), *sharp character* (#) indicates statistically different means ($p < 0.1$)

oxidase enzymes, which are key signaling participants in a plethora of signal transduction pathways that coordinate plant essential processes such as plant cell growth and acclimation to abiotic stresses (Foreman and others 2003; Mittler and others 2011; Suzuki and others 2011). Taking these facts into consideration, we believe that the increase in SOD activity is necessary to prevent oxidative stress during seedling establishment, especially late in functional transition, with an intensification of photosystem activities along with cell expansion and growth (Nyathi and Baker 2006). The induction of SOD activity and the increase in the MDA–TBA level in treated sunflower seedlings at 48 h might be directly related to the aforementioned metabolic events (Fig. 4c). Nevertheless, the 3-AT–treated safflower seedlings did not show increases in lipid peroxidation at the same time (Fig. 4d). Based in our results, we propose that safflower seedlings under CAT impairment conditions either have a superior ability to regulate O_2^- production and the consequent H_2O_2 levels because these plants have higher APX activity rates during the establishment, or the diminished TBARS levels are associated with a growth restriction, which was indeed observed.

CAT has a key position in the detoxification of the H_2O_2 produced in β-oxidation during seedling establishment, but the APX antioxidant system is also important in the detoxification of H_2O_2 that could leak from the glyoxysome (Graham 2008). These enzymes present distinct substrate affinity, kinetics, and regulatory mechanisms (Gara and others 2010), which might reflect specialized functions in cell ROS control. CAT inhibition caused different responses in CAT and cAPX mRNA expression patterns in both species. In sunflower seedlings, CAT expression decays, whereas the APX antioxidant system seems to supplement H_2O_2 scavenging in the latter stages of seedling establishment. On the other hand, treated safflower seedlings showed a progressive increase in CAT activity and expression, suggesting that CAT is important for seedling establishment, despite the reduced activity. Also, there is an intense upregulation of cAPX transcription in the treated safflower seedlings within 6 h, whereas the increase in the activity is seen only at later times. In this case, we suggest that this increase was a consequence of an amplified signalization response to CAT drastic inhibition (Willekens and others 1997; Gechev and others 2008; Mhamdi and others 2010). It must be emphasized that cytosolic APX mRNA expression does not reflect the behavior of all APX transcripts, considering that they are differentially expressed in other species and physiological conditions (Rosa and others 2010; Bonifacio and others 2011; Lazzarotto and others 2011).

Our results indicate that both systems are indeed necessary in this stage, playing distinct but complementary roles in H_2O_2 scavenging and signaling events during seedling establishment (Barba-Espín and others 2011). The present study focused on the CAT and APX systems, and the contributions of other mechanisms that maintain redox homeostasis in the early stages of establishment were not evaluated. However, according to De Paula and others (1996) and Fisk and others (2006), these other mechanisms also perform an important role, especially in sunflower.

The expression pattern of the ICL and MLS transcripts in both species was similar in control seedlings, and a progressive decrease in the expression was detected. However, when CAT was inhibited, there was a rapid increase in the expression of ICL and MLS at 6 or 12 h, which may indicate greater synthesis to replace oxidized enzymes (Anand and others 2009) and, consequently, a greater flow of metabolites from the glyoxylate cycle. After 12 h, a tendency for a rapid decrease in expression was observed, which may indicate an adjustment to photosynthetic establishment. Moreover, the main functional transition metabolic pathways (glyoxylate cycle, β-oxidation, TCA cycle, and photosynthesis) can increase the $NADH/NAD^+$ and $NADPH/NADP^+$ ratios

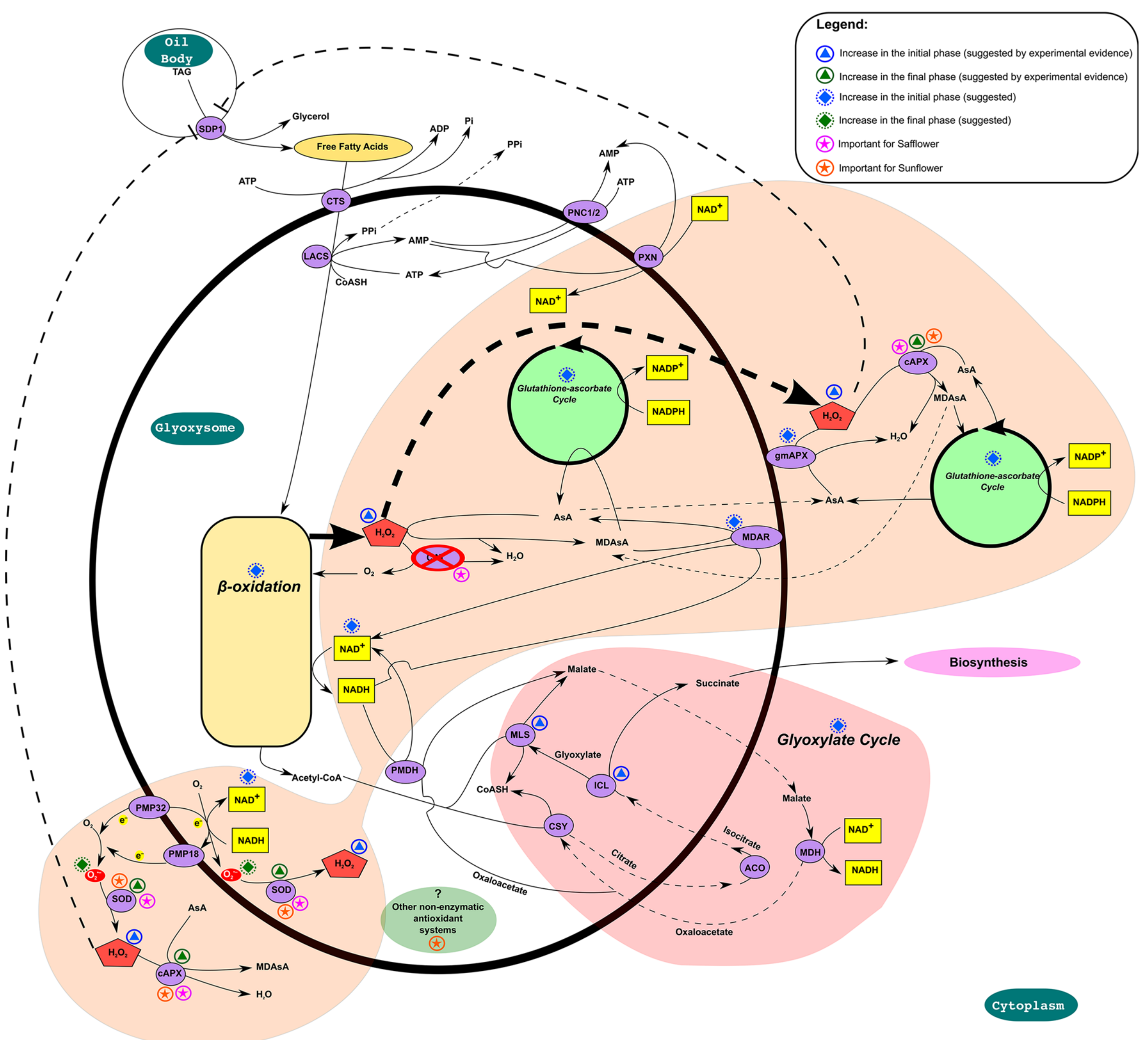

Fig. 7 Proposed model to explain the cell mechanisms that attenuate CAT inhibition by 3-AT during functional transition for both species. The model used experimental evidence obtained in the present study and from previous reported studies (Corpas and others 2001; Gonzalez and Vodkin 2007; Graham 2008; Bernhardt and others 2012). SDP1, triacylglycerol lipase; CTS, COMATOSE ATP-binding cassette transporter; *LACS* long-chain acyl-CoA synthetase, *CAT* catalase, *MLS* malate synthase, *ICL* isocitrate lyase, *CYS* citrate synthase, *MDH* malate dehydrogenase, *PMDH* glyoxysomal/peroxisomal malate dehydrogenase, *ACO* aconitase, *PNC1/2* peroxisomal nucleotide carriers 1 and 2; *PXN*, *NAD*$^+$ carrier, *cAPX* cytosolic ascorbate peroxidase, *gmAPX* glyoxysomal/peroxisomal membrane ascorbate peroxidase, *MDAR* monodehydroascorbate reductase, *SOD* superoxide dismutase, *PMP18* peroxisomal membrane protein 18, *PMP32* peroxisomal membrane protein 32, *AsA* ascorbate, *MDAsA* monodehydroascorbate

(Baker and others 2006; Bernhardt and others 2012). Eastmond (2007) and Graham (2008) suggested that MDAR has an essential role in mobilization of reserves. Thus, this enzyme can be associated with NADH reoxidation and ascorbate production to support the increase in APX activity observed in the present study and in that by Karyotou and Donaldson (2005).

Traditionally, it is thought that seedling establishment is favored through increasing the activity or transcription of CAT (Contento and Bassham 2010; Xi and others 2010), but there are still no reports of indirect influence of the CAT antioxidant system in the pattern of expression of the enzymes of the glyoxylate cycle (Fig. 7). The results of this study indicate that, when there is a drastic reduction in CAT activity, the transcription of marker enzymes of the glyoxylate cycle is increased to adjust to a new establishment condition, and SOD and APX antioxidant systems are induced to alleviate oxidative damage (Fig. 7). That said, we have characterized the response pattern of two species of the same taxonomic family and found that there are similar responses in the transcriptional level. However, these patterns are strongly influenced by other biochemical factors, characterizing a species-specific response.

Acknowledgments The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio à Pesquisa do Estado do Rio Grande do Norte (FAPERN), and Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES) for financial support. The authors are also very grateful to the editors and the two anonymous reviewers for the valuable comments and suggestions that helped to improve the manuscript.

Conflict of interest The authors have no conflict of interest or financial ties to disclose.

References

Alberty RA (2006) Thermodynamics and kinetics of the glyoxylate cycle. Biochemistry 45:15838–15843

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Anand P, Kwak Y, Simha R, Donaldson RP (2009) Hydrogen peroxide induced oxidation of peroxisomal malate synthase and catalase. Arch Biochem Biophys 491:25–31

Armstrong GA, Apel K, Rüdiger W (2000) Does a light-harvesting protochlorophyllide a/b-binding protein complex exist? Trends Plant Sci 5:40–44

Arnon DI (1949) Copper enzymes in isolated chloroplasts polyphenoloxidase in beta vulgaris. Plant Physiol 24:1–15

Baker A, Graham IA, Holdsworth M, Smith SM, Theodoulou FL (2006) Chewing the fat: β-oxidation in signalling and development. Trends Plant Sci 11:124–132

Barba-Espín G, Diaz-Vivancos P, Clemente Moreno MJ, Albacete A, Faize L, Faize M, Pérez Alfocea F, Hernández JA (2010) Interaction between hydrogen peroxide and plant hormones during germination and the early growth of pea seedlings. Plant Cell Environ 33:981–994

Barba-Espín G, Diaz-Vivancos P, Job D, Belghazi M, Job C, Hernández JA (2011) Understanding the role of H_2O_2 during pea seed germination: a combined proteomic and hormone profiling approach. Plant Cell Environ 34:1907–1919

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Bernhardt K, Wilkinson S, Weber APM, Linka N (2012) A peroxisomal carrier delivers NAD + and contributes to optimal fatty acid degradation during storage oil mobilization. Plant J 69:1–13

Bienert GP, Moller ALB, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP (2006) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem 282:1183–1192

Bonifacio A, Martins MO, Ribeiro CW, Fontenele AV, Carvalho FEL, Margis-Pinheiro M, Silveira JAG (2011) Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant Cell Environ 34:1705–1722

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cavalcanti FR, Santos Lima JPM, Ferreira-Silva SL, Viégas RA, Silveira JAG (2007) Roots and leaves display contrasting oxidative response during salt stress and recovery in cowpea. J Plant Physiol 164:591–600

Chapman KD (1998) Phospholipase activity during plant growth and development and in response to environmental stress. Trends Plant Sci 3:419–426

Contento AL, Bassham DC (2010) Increase in catalase-3 activity as a response to use of alternative catabolic substrates during sucrose starvation. Plant Physiol Biochem 48:232–238

Corpas FJ, Barroso JB, del Río LA (2001) Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. Trends Plant Sci 6:145–150

De Paula M, Perez-Otaola M, Darder M, Torres M, Frutos G, Martimez-Honduvilla CJ (1996) Function of the ascorbate-glutathione cycle in aged sunflower seeds. Physiol Plant 96:543–550

Devaiah SP, Pan X, Hong Y, Roth M, Welti R, Wang X (2007) Enhancing seed quality and viability by suppressing phospholipase D in Arabidopsis. Plant J 50:950–957

Dyer JM, Stymne S, Green AG, Carlsson AS (2008) High-value oils from plants. Plant J 54:640–655

Eastmond PJ (2007) Monodehyroascorbate reductase4 is required for seed storage oil hydrolysis and postgerminative growth in Arabidopsis. Plant Cell 19:1376–1387

Fisk ID, White DA, Carvalho A, Gray DA (2006) Tocopherol—An intrinsic component of sunflower seed oil bodies. J Am Oil Chem Soc 83:341–344

Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JDG, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422:442–446

Gara L, Locato V, Dipierro S, Pinto MCD (2010) Redox homeostasis in plants. The challenge of living with endogenous oxygen production. Respir Physiol Neurobiol 173:S13–S19

Gechev T, Gadjev I, Van Breusegem F, Inzé D, Dukiandjiev S, Toneva V, Minkov I (2002) Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell Mol Life Sci 59:708–714

Gechev TS, Ferwerda MA, Mehterov N, Laloi C, Qureshi MK, Hille J (2008) *Arabidopsis* AAL-toxin-resistant mutant atr1 shows enhanced tolerance to programmed cell death induced by reactive oxygen species. Biochem Biophys Res Commun 375:639–644

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

Gonzalez DO, Vodkin LO (2007) Specific elements of the glyoxylate pathway play a significant role in the functional transition of the soybean cotyledon during seedling development. BMC Genomics 8:468

Graham IA (2008) Seed storage oil mobilization. Annu Rev Plant Biol 59:115–142

Gunstone FD, Harwood JL, Dijkstra AJ (2010) The lipid handbook, 3rd edn. CRC Press, Boca Raton

Hammer Ø, Harper D, Ryan P (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9

Havir EA, McHale NA (1987) Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol 84:450–455

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Heim DR, Larrinua IM (1989) Primary site of action of amitrole in *Arabidopsis thaliana* involves inhibition of root elongation but not of histidine or pigment biosynthesis. Plant Physiol 91:1226–1231

Hu J, Baker A, Bartel B, Linka N, Mullen RT, Reumann S, Zolman BK (2012) Plant peroxisomes: biogenesis and function. Plant Cell 24:2279–2303

Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. J Comput Graph Stat 5:299–314

Jaleel CA, Sankar B, Murali PV, Gomathinayagam M, Lakshmanan GMA, Panneerselvam R (2008) Water deficit stress effects on reactive oxygen metabolism in *Catharanthus roseus*; impacts on ajmalicine accumulation. Coll Surf B Biointerfaces 62:105–111

Kagawa T, McGregor DI, Beevers H (1973) Development of enzymes in the cotyledons of watermelon seedlings. Plant Physiol 51:66–71

Karyotou K, Donaldson RP (2005) Ascorbate peroxidase, a scavenger of hydrogen peroxide in glyoxysomal membranes. Arch Biochem Biophys 434:248–257

Kopper RA, Kim A, Van T, Helm RM (2008) Adsorption of peanut (*Arachis hypogaea*, Leguminosae) proteins by activated charcoal. J Agric Food Chem 56:10619–10624

Koshiba T (1993) Cytosolic ascorbate peroxidase in seedlings and leaves of maize (*Zea mays*). Plant Cell Physiol 34:713–721

Lazzarotto F, Teixeira FK, Rosa SB, Dunand C, Fernandes CL, de Vasconcelos Fontenele A, Silveira JAG, Verli H, Margis R, Margis-Pinheiro M (2011) Ascorbate peroxidase-related (APx-R) is a new heme-containing protein functionally associated with ascorbate peroxidase but evolutionarily divergent. New Phytol 191:234–250

Lidon FJC, Teixeira M, Ramalho JC (2011) Decay of the chloroplast pool of ascorbate switches on the oxidative burst in UV-B-irradiated rice. J Agron Crop Sci 192:130–144

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Van Breusegem F, Noctor G (2010) Catalase function in plants: a focus on *Arabidopsis* mutants as stress-mimic models. J Exp Bot 61:4197–4220

Milazzo MF, Spina F, Vinci A, Espro C, Bart JCJ (2013) *Brassica* biodiesels: past, present and future. Renew Sustain Energy Rev 18:350–389

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, Van Breusegem F (2011) ROS signaling: the new wave? Trends Plant Sci 16:300–309

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Nicot N, Hausman J-F, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907–2914

Nyathi Y, Baker A (2006) Plant peroxisomes as a source of signalling molecules. Biochim Biophys Acta 1763:1478–1495

Roach T, Beckett RP, Minibayeva FV, Colville L, Whitaker C, Chen H, Bailly C, Kranner I (2010) Extracellular superoxide production, viability and redox poise in response to desiccation in recalcitrant *Castanea sativa* seeds. Plant Cell Environ 33:59–75

Robert G, Melchiorre M, Racca R, Trippi V, Lascano HR (2009) Apoplastic superoxide level in wheat protoplast under photooxidative stress is regulated by chloroplast redox signals: effects on the antioxidant system. Plant Sci 177:168–174

Roqueiro G, Maldonado S, del Ríos M C, Maroder H (2012) Fluctuation of oxidative stress indicators in *Salix nigra* seeds during priming. J Exp Bot 63:3631–3642

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira JAG, Ferreira-Silva SL, Abreu-Neto J, Margis R, Margis-Pinheiro M (2010) Cytosolic APx knockdown indicates an ambiguous redox response in rice. Phytochemistry 71:548–558

Schwember AR, Bradford KJ (2010) Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. J Exp Bot 61:4423–4436

Sekaran G, Mariappan M, Raghavan KV (1996) Adsorption of bovine serum albumin from salt solution onto activated carbon. Bioprocess Biosyst Eng 15:165–169

Simontacchi M, Sadovsky L, Puntarulo S (2003) Profile of antioxidant content upon developing of *Sorghum bicolor* seeds. Plant Sci 164:709–715

Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R (2011) Respiratory burst oxidases: the engines of ROS signaling. Curr Opin Plant Biol 14:691–699

Tavares RM, Morais F, Melo N, Pais MSS (1998) Thylakoid membrane reorganization during *Zantedeschia aethiopica* spathe regreening: Consequence of the absence of Δ^3-trans-hexadecenoic acid in photochemical activity. Phytochemistry 47:979–984

Theodoulou FL, Eastmond PJ (2012) Seed storage oil catabolism: a story of give and take. Curr Opin Plant Biol 15:322–328

Van Rossum MWP, Alberda M, van der Plas LH (1997) Role of oxidative damage in tulip bulb scale micropropagation. Plant Sci 130:207–216

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Sci 151:59–66

Ventura L, Donà M, Macovei A, Carbonera D, Buttafava A, Mondoni A, Rossi G, Balestrazzi A (2012) Understanding the molecular pathways associated with seed vigor. Plant Physiol Biochem 60:196–206

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inze D, Van Camp W (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. EMBO J 16:4806–4816

Xi DM, Liu WS, Yang GD, Wu CA, Zheng CC (2010) Seed-specific overexpression of antioxidant genes in *Arabidopsis* enhances oxidative stress tolerance during germination and early seedling growth. Plant Biotechnol J 8:796–806

Zolman BK, Monroe-Augustus M, Silva ID, Bartel B (2005) Identification and functional characterization of *Arabidopsis* PEROXIN4 and the interacting protein PEROXIN22. Plant Cell 17:3422–3435