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Identification of *Zantedeschia aethiopica Cat1* and *Cat2* catalase genes and their expression analysis during spathe senescence and regreening

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Abstract

Plants encode catalase (CAT; EC 1.11.1.6) as multigene families, which may reflect the multiple and diverse roles played by this enzyme. Catalases from higher plants can be subdivided into three distinct types, according to their phylogenetic relationship. However, there is not a specific correlation of phylogeny and function within these groups, as catalases from the same type can play different specific roles. We report on the characterization of two monocot catalase genes from *Zantedeschia aethiopica* (a C_3-type Araceae) and its expression analysis during spathe senescence and regreening. *Z. aethiopica* CAT2 appears to play an exclusive role in scavenging photorespiratory H_2O_2, but is more phylogenetically related to the unspecific *Zea mays* CAT3 and *Oryza sativa* CATA than to the photorespiratory-related *Zea mays* CAT2 and

Oryza sativa CATC. *Z. aethiopica* CAT1 is more phylogenetically related to *Zea mays* CAT1 and *Oryza sativa* CATB and appears to have a dual role in scavenging glyoxysomal and peroxisomal H_2O_2.

Keywords: *Zantedeschia aethiopica*; senescence; regreening; peroxisomal metabolism; catalase; peroxisomal targeting signal

1. Introduction

Catalase (CAT; EC 1.11.1.6) is a tetrameric, heme-containing enzyme found in all aerobic organisms. Because of its wide distribution and capacity to rapidly dismutate hydrogen peroxide (H_2O_2) into water and oxygen, it has been considered as one of the primary enzymatic mechanisms used by aerobic organisms to degrade H_2O_2, a toxic intermediate of oxygen metabolism. Catalase is unique among H_2O_2-scavenging enzymes in that it degrades H_2O_2 without consuming cellular reducing equivalents, providing the cell with a very efficient mechanism to remove hydrogen peroxide.

Catalase has been found in all plants examined so far and has been intensively studied biochemically, genetically, and molecularly mainly in the agronomic important species *Zea mays* L. [1]. This enzyme is particularly abundant in peroxisomes of green leaves, where it destroys H_2O_2 arising from the oxidation of photorespiratory glycolate, and in the glyoxysomes of germinating seeds, where it destroys H_2O_2 released during the ß-oxidation of fatty acids [2]. The enzyme is also an important component of the system involved in detoxification of reactive oxygen species that increase following biotic and abiotic stresses [3, 4]. In accordance to these functions, many environmental factors such as O_2 and CO_2 concentrations [5], light [6], and temperature [7], have been shown to influence catalase activity in mature plants. There are also evidences suggesting that catalase isozymes undergo specific changes during development, such as in cottonseed maturation and germination [8], sunflower cotyledons after germination [9], tobacco seedling

development and leaf maturation [10], maize [11] and pumpkin [12] development.

Many plants encode catalase as multigene families, which may reflect the multiple and diverse roles of this enzyme. In *Arabidopsis thaliana*, three catalase genes encode individual subunits that associate to form at least six isozymes [13]. Also, three catalase genes have been identified in *Z. mays* [14-16], *Nicotiana plumbaginifolia* [17] and *Oryza sativa* [18, 19]. Several reports on differential developmental and spatial expression pattern of catalase genes have been contributing for assigning specific cellular function to each catalase gene [12, 20-25]. In *N. plumbaginifolia* the temporal and spatial patterns of catalase gene expression, as well as a differential regulation of each gene by light, suggest the association of each specific gene product to a particular H_2O_2-producing process and to a specific type of peroxisome [22]. *N. plumbaginifolia Cat1*, highly expressed in photosynthesizing tissues, is under control of a circadian clock and is positively regulated by light, suggesting that CAT1 is involved in scavenging H_2O_2 in leaf peroxisomes. The expression of *N. plumbaginifolia Cat3* in seeds as well as in mature and senescing petals accounts for a possible correlation with glyoxysomal metabolism. *N. plumbaginifolia Cat2* is uniformly expressed in different organs with a cellular predominance in vascular tissues, suggesting no correlation with a known type of peroxisomes. However, the rapid increase of *Cat2* transcript levels after different stress treatments suggests that CAT2 may play an important role in scavenging of H_2O_2 produced during stress conditions [22].

In monocotyledonous plants, the functional organization of catalases into distinct classes is still ambiguous. It was suggested that *Z. mays* CAT2 and *Oryza sativa* CATC might be involved in scavenging the H_2O_2 generated during photorespiration [20, 25]. Although each of the maize catalases exhibits a unique and complex pattern of developmental expression throughout the life cycle, the involvement of *Z. mays* CAT2 isozyme exclusively on scavenging photorespiratory H_2O_2 is still not clear [1]. CAT1 is the only maize catalase isozyme expressed in mature pollen, milky endosperm, aleurone cells, and scutellum during kernel development, which could indicate its involvement in glyoxysomal activity. However, upon germination *Cat2* is rapidly induced and CAT2 soon becomes the predominant isozyme in scutellum [26]. This profile parallels that of the glyoxysomes, suggesting an additional involvement of CAT2 on scavenging glyoxysomal H_2O_2 [1]. The spatial expression of *Z. mays Cat3* gene is similar to *N. plumbaginifolia Cat2*, showing highest transcript levels in stems and vascular tissues of leaves, which suggests they play a similar role within the plant. However, CAT2 from *N. plumbaginifolia*, by having a consensus peroxisomal targeting signal, is expected to be localized in peroxisomes [17] whereas CAT3 from *Z. mays* seems to be mitochondrial [27].

We report on the identification of two catalase genes (*Cat1* and *Cat2*) from the C_3 monocot calla lily (*Zantedeschia aethiopica*). In this species, the inflorescence (spadix) consists of small closely packed flowers, being the feminine located in the lower part. Enveloping the spadix there is a large, leaf-like bract (spathe) that exhibits natural senescence and regreening during the time course of development [28]. Soon after their formation, the photosynthetic

spathes undergo senescence. Fruiting inhibits the ongoing spathe senescence and induces its regreening which is accompanied by the recover of photosynthetic ability [29-31]. The present study addresses the metabolic role of *Z. aethiopica* catalases by studying *Cat1* and *Cat2* expression during spathe senescence and regreening, as well as during leaf development.

2. Materials and Methods

2.1. Plant material

Plants of *Zantedeschia aethiopica* (L.) Spreng. grown under natural conditions were used for all experiments. Spathes in different stages of senescence - floral bud spathe (FS), senescence intermediates (S1 and S2) and white spathe (WS), as well as in different stages of regreening – regreening intermediates (R1 and R2) and regreened spathe (RS) were harvested. Spathe portions between 2 and 4 cm from the peduncle were used. Young (still curled) and full-developed leaves were used after mid-rib removal.

2.2. Electron microscopy and catalase cytochemistry

Sections (1mm^3) of *Z. aethiopica* regreened spathes were fixed with 2% (w/v) formaldehyde (derived from paraformaldeyde), 2.5% (v/v) glutaraldehyde (grade I, Sigma, St. Louis) in 0.1 M cacodylate buffer (pH 7.2), for 2 h at 4°C. After three washes in the same buffer the samples were postfixed with 1% (w/v) OsO_4 (Sigma, St. Louis) in the same buffer, for 2 h, at room temperature. The material was then dehydrated in an acetone series and embedded in Epon-Araldite [32]. Ultrathin sections were stained with uranyl

acetate/lead citrate and observed in a Jeol 200 LX electron microscope at 80 kV.

For cytochemical localization of catalase activity, the material was fixed overnight with 4% (v/v) glutaraldehyde (grade I, Sigma, St. Louis) in 0.1 M cacodylate buffer (pH 7.2), at 4°C. After 3 washes in the same buffer, samples were incubated in 50 mM glycine-NaOH buffer (pH 10) containing 0.2% (w/v) 3,3'-diaminobenzidine and 0.06% (v/v) H_2O_2, for 1 h, at 37°C, in the dark [33]. Control was performed by adding 20 mM 3-amino-1,2,4-triazole to the incubation medium. Postfixation, dehydration and embedding were performed as described above. Ultrathin sections were visualized unstained.

2.3. Cell fractionation

Z. aethiopica regreened spathes were homogenized in a Waring blender homogenizer (3 pulses of 3 seconds each, at maximum speed) using a 1:4 (w/v) ratio of material/homogenization buffer [50 mM Hepes-KOH (pH 7.5) containing 0.4 M sucrose, 10 mM NaCl, 1 mM EDTA, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, 0.1% (w/v) BSA and 2% (w/v) polyvinylpolypyrrolidone]. All subsequent operations were performed at 4°C. The homogenate was filtered through 8 layers of cheesecloth and one layer of nylon cloth (20 μm pore size; TETKO, Elmsford, NY) and centrifuged at 2 000 *g*, for 10 min. The supernatant was recovered and centrifuged at 12 000 *g*, for 15 min. The pellet, corresponding to 150 g of plant material, was resuspended in 2-4 ml of 50 mM Hepes-KOH (pH 7.5) containing 0.4 M sucrose and 1 mM EDTA, and aliquots of 0.8 ml were loaded on the top of a linear sucrose density gradient [30% (w/w) to 60% (w/w) sucrose gradient,

prepared in 50 mM Hepes-KOH (pH 7.5) containing 1 mM EDTA, overlaid on a cushion of 3 ml 65% (w/w) sucrose prepared in the same buffer] [34]. After centrifugation at 68 000 *g*, in a Beckman SW 28 rotor for 4.5 h, the gradient was fractionated from the bottom into 1 ml fractions.

2.4. Preparation of crude extracts

Z. aethiopica spathes and leaves were harvested, immediately frozen in liquid nitrogen and ground to a fine powder in a cold mortar. Approximately 1 g of powder was homogenized, at 4°C, in 4 ml of 50 mM phosphate buffer (pH 7.0), 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine and 4% (w/v) polyvinylpolypyrrolidone. After centrifugation at 15 000 *g*, for 15 min, at 4°C, the supernatant was recovered.

2.5. Enzymatic assays

Catalase (EC 1.11.1.6) activity was determined by following the decomposition of H_2O_2 at 240 nm [35]. Fumarase (EC 4.2.1.2) activity was determined following formation of fumarate at 240 nm [36]. One unit (U) of catalase or fumarase activity is defined as the amount of enzyme causing the decomposition of 1 μmol of H_2O_2, or the production of 1 μmol of fumarate, respectively, per minute, at 25°C. Hydroxypyruvate reductase (EC 1.1.1.29) was determined by following the oxidation of NADH at 340 nm, according to Liang et al. [37]. Isocitrate lyase (EC 4.1.3.1) was evaluated by measuring the formation of glyoxylate in the presence of $K_3Fe(CN)_6$ at 535nm, according to Zelitch [38].

2.6. Protein and chlorophyll determination

Protein was quantified by the Coomassie Blue blue dye method, using BSA as a standard [39]. Chlorophyll was quantified according to Arnon [40].

2.7. mRNA purification and cDNA library construction

Total RNA from leaves and spathes were extracted using the phenol-SDS method [41]. For cDNA library preparation, poli(A)$^+$ RNA from 550 μg of young leaves total RNA was purified using Oligo (dT) beads (Dynabeads Oligo $(dT)_{25}$, Dynal). *Z. aethiopica* cDNA library was constructed using 5.2 μg poly(A)+ RNA with ZAP Express™ cDNA synthesis/Gigapack III Gold cloning kits (Stratagene) according to the manufacturer's instructions.

2.8. cDNA library screening and sequence analysis of Cat cDNAs

Screening of *Z. aethiopica* cDNA library for *Cat* cDNA clones was performed using the *Cat1* cDNA from *N. plumbaginifolia* as probe [17, Accession No. **Z36975**]. Duplicate plaque filters (Hybond-N+; Amersham) were hybridized at 42°C for 16 h with the referred ^{32}P-labeled probe and successive washings were performed until the final concentrations of 1 x SSC and 0.1% SDS, at 60°C, for 30 min. After a second round of screening, cDNA inserts were excised *in vitro* from positive phage clones as pBK-CMV plasmids.

The inserts of *Cat* cDNA clones were sequenced using universal T3 and T7 and new designed primers on PE/Applied Biosystems 377 and 3700 sequencers (Applied Biosystems Inc., Darmstadt, Germany) using BigDye

terminator chemistry. Nucleotide and amino acid sequences analysis were performed using DNASTAR package software version 1.58 (Lasergene).

2.9. RNA blot hybridization

Aliquots (20 μg) of total RNA isolated from spathes and leaves were separated by electrophoresis on 1.2% formaldehyde agarose gel and blotted onto a Hybond-N membrane (Amersham) [41]. Hybridizations with ^{32}P-labeled *Z. aethiopica Cat1* and *Cat2* coding regions were performed under the same conditions as described above. The final washing step was performed in 0.1x SSC and 0.1% SDS, at 68°C, for 30 min.

3. Results

3.1. Analysis of Z. aethiopica CAT1 and CAT2

Screening of about 3x10^5 independent clones from *Z. aethiopica* cDNA library with *Cat1* fragment from *Nicotiana plumbaginifolia* [17] resulted on the identification of a positive clone (pCAT11) containing a 1917 bp insert. This cDNA sequence (*Cat1*; Accession No. **AF207906**) includes a 68 bp 5'-untranslated sequence, a 1479 bp open reading frame and a 370 bp 3'-untranslated sequence (including a poly(A)+ tail of 20 bp). The deduced protein sequence consists of 492 amino acid residues with an expected molecular weight of 57 kDa. A previously identified *Z. aethiopica* CAT2 (Accession No. **AF332973**) has the same number of amino acid residues, which determines a similar molecular weight. The amino acids reported to be involved in catalytic activity (His^{65}, Ser^{104} and Asn^{138}) and in direct association

with the heme group (Val^{64}, Arg^{102}, Thr^{105}, Pro^{141}, Pro^{326}, Arg^{344}, and Tyr^{348}) [42, 43] are conserved in *Z. aethiopica* CAT1 and CAT2.

Amino acid sequence comparison clearly distinguished *Z. aethiopica* CAT1 from *Z. mays* CAT3 and *O. sativa* CATA (Table 1), both members of an unspecific group of monocot catalases [19, 44]. The nucleotide identity of *Z. aethiopica Cat1* coding sequence was higher with maize *Cat1* and rice *CatB* than with maize *Cat2* and *Cat3* or rice *CatA* and *CatC* (Table 1). Also the coding sequence of *Z. aethiopica Cat1* has a GC content of 47.6%, very close to GC content of maize *Cat1* (51.2%) and rice *CatB* (49.0%). *Z. aethiopica Cat2* shares a higher nucleotide identity with *Z. mays Cat1* than with maize *Cat2* and *Cat3*, and it shares higher identity with rice *CatA* coding sequence than with rice *CatB* and *CatC* (Table 1). However, *Z. aethiopica Cat2* has a 54.9% GC content that is still closer to the GC content of maize *Cat1* and rice *CatB* than to the other maize and rice *Cat* coding sequences (63.5 to 65.8%).

The alignment of amino acid sequences of edited monocotyledonous catalases (including both *Z. aethiopica* catalases) with those of *N. plumbaginifolia* and *A. thaliana* catalases generated a tentative phylogenetic relationship among these sequences and a corresponding tree of shortest length was designed (Fig. 1). As expected from the nucleotide sequence analysis, *Z. aethiopica* CAT1 appears to be more phylogenetically related to *Z. mays* CAT1 and *O. sativa* CATB. *Z. aethiopica* CAT2 appears to be more phylogenetically related to *Z. mays* CAT3 and *O. sativa* CATA. According to the classification based on phylogenetic relationships [45], *Z. aethiopica* CAT1 is a Type I and *Z. aethiopica* CAT2 is a Type III catalase.

3.2. Peroxisomal targeting signals in Z. aethiopica catalases

The amino acid sequences relevant for the import of plant catalases into peroxisomes seems to reside on C-terminal end (PTS1-like motifs) [46-48]. The comparison of *Z. aethiopica* CAT1 and CAT2 C-terminal regions with other plant catalases is depicted in Fig. 2. The carboxyl terminus of *Z. aethiopica* CAT1 possesses the SerArgLeu sequence (at positions 484-486) located 7 amino acid residues from the C-terminal end. This sequence seems to correspond to the C-terminal internal motif Ser/Ala/Cys-Lys/Arg/His-Leu/Met/Phe, originally proposed as catalase PTS1 [46]. In contrast, *Z. aethiopica* CAT2 exhibits the AsnArgLeu tripeptide in the same position, which agrees with the suggestion that the plant catalase PTS1 is not the internal SerArgLeu motif but the C-terminal degenerate tripeptide Pro-Ser/Thr/Asn-Met/Ile [48]. Accordingly, the C-terminal tripeptide of *Z. aethiopica* CAT2 is ProSerMet. Contrarily to *Z. aethiopica* CAT2, CAT1 C-terminal tripeptide does not match the PTS1 sequence proposed by Mullen et al. [48]. As *Z. aethiopica* CAT1 is the only plant catalase sequence that has ProThrLeu as the C-terminal tripeptide, we have investigated if the subcellular localization of catalase could be other than in peroxisomes, as it seems to be the case of mitochondrial *Z. mays* CAT3 [27].

3.3. Subcellular localization of catalase activity

The subcellular localization of catalase activity was investigated by cell fractionation studies and also by cytochemical localization of enzyme activity. A regreened spathe homogenate was fractionated after a sucrose-density

centrifugation and the corresponding fractions were assayed for catalase activity and for chlorophyll and fumarase activity, as markers for chloroplasts and mitochondria, respectively (Fig. 3). Catalase activity was observed as a single peak, distinct from those corresponding to chlorophyll and fumarase activity. These results suggest that in *Z. aethiopica* catalase is only located in peroxisomes. To further investigate subcellular localization of catalase, cytochemical assay for enzyme activity was performed in *Z. aethiopica* regreened spathe (Fig. 4). The results showed that catalase activity is observed as electron-dense deposits only in peroxisomes, being absent in other cell compartments such as mitochondria, chloroplast and cytoplasm.

3.4. Catalase activity during Z. aethiopica spathe and leaf development

Cell-free extracts from *Z. aethiopica* spathe and leaf developmental stages were assayed for catalase activity and results were expressed on protein basis (Fig. 5A). During spathe senescence and at the beginning of regreening, corresponding to the transition of floral bud spathe (FS) to the second stage of regreening (R2), a 4-fold increase on catalase activity was observed. During transition from the second stage of regreening (R2) to regreened spathe (RS), a decline of about two-fold on catalase activity was observed. An evident increase on catalase activity (up to five-fold) was observed during transition from young leaf (YL) to full-developed leaf (L).

3.5. Expression analysis of Cat1 and Cat2 during Z. aethiopica spathe and leaf development

The expression of *Z. aethiopica Cat1* and *Cat2* was analyzed by Northern blotting, using the corresponding coding regions as probes (Fig. 5B). The expression of *Cat1* and *Cat2* genes had a significant increase during the transition of young leaf (YL) to full-developed leaf (L). As far as spathe development is concerned, a different expression pattern was observed for each catalase gene. *Cat1* expression increased during spathe senescence and at the beginning of regreening, from floral bud spathe (FS) until the second stage of regreening (R2), reaching a total of 9-fold increase. The levels of *Cat2* transcripts decreased during spathe senescence, from floral bud spathe (FS) until white spathe (WS), attaining not detectable transcript levels in this stage. During spathe regreening, *Cat2* expression was strongly induced until the second stage of regreening (R2). In the final stages of spathe regreening (R2 to RS transition), a decrease in *Cat1* and *Cat2* expression levels was observed.

3.6. Isocitrate lyase and hydroxypyruvate reductase activity during Z. aethiopica spathe development

To investigate whether the differential expression of *Z. aethiopica Cat1* and *Cat2* could be related to the glyoxysomal or peroxisomal metabolism, the activity of isocitrate lyase (ICL), a key enzyme of glyoxylate cycle, and hydroxypyruvate reductase (HPR), a key enzyme of glycolate pathway, were determined during the time course of spathe development (Fig. 6). The results showed that during *Z. aethiopica* spathe development, ICL activity increases

until the first stage of regreening (R1), decreasing afterwards. On contrary, the activity of HPR is reduced during spathe whitening and increases during regreening, attaining maximum value in regreened spathe stage (RS).

4. Discussion

In *Z. aethiopica* two different catalase cDNAs (*Cat1* and *Cat2*) were identified. The topology of the phylogenetic tree constructed based on the sequences of mono- and dicotyledonous catalases is consistent with previous studies on plant catalases [22, 44, 45, 49]. According to the classification based on phylogenetic relationships [45], *Z. aethiopica* CAT1 is a Type I catalase and CAT2 is a Type III. Comparison of grass catalase sequences suggested the existence of three subclades, each including one of the three known *Zea mays* catalases [49]. Phylogenetic analysis of *Z. aethiopica* catalases revealed that CAT1 is phylogenetically related to *Z. mays* CAT1 and *O. sativa* CATB, which are reported as playing a role on glyoxysomal metabolism [1]. *Z. aethiopica* CAT2 is included in the subclade comprising *Z. mays* CAT3 and *O. sativa* CATA, which constitute a group of monocot isozymes with unspecific function and are the most divergent from all other known catalases [25, 44]. Neither *Z. aethiopica* CAT1 nor CAT2 seems to be phylogenetically related to monocot catalases involved in scavenging photorespiratory H_2O_2 (*Z. mays* CAT2 and *O. sativa* CATC). It cannot be excluded the possibility of the existence of another catalase gene in *Z. aethiopica*, as is the case in almost all other plant species. After re-screening the cDNA library but using

Z. aethiopica Cat1 and *Cat2* coding regions as probes under low stringency conditions, no different catalase cDNA could be found.

Catalase has been referred as a marker enzyme for peroxisomes [50], although a mitocondrial localization of *Z. mays* CAT3 has been reported [27]. In *Z. aethiopica,* catalase seems to be exclusively located in peroxisomes, since its activity was only detected in peroxisomal fractions obtained from regreened spathes. This result was corroborated by cytochemical localization of catalase activity.

In contrast to what is described for other peroxisomal proteins, the targeting signal for catalases has not been fully identified. Although catalases exhibit PTS1-like motifs within their C-termini, it remains unclear which specific sequences are necessary for import into peroxisomes. In what concerns *Z. aethiopica*, CAT1 carboxyl terminus exhibits the first proposed catalase PTS1 (Ser/Ala/Cys-Lys/Arg/His-Leu/Met/Phe) [46], while CAT2 carboxyl terminus does not. On the contrary, *Z. aethiopica* CAT2 protein have the C-carboxyl tripeptide ProSerMet, matching the degenerate Pro-Ser/Thr/Asn-Met/Ile sequence proposed as the effective PTS1 of plant catalases by Mullen et al. [48]. *Z. aethiopica* CAT1 is unique among plant catalases in having ProThrLeu as the C-carboxyl tripeptide, which is different from the degenerate PTS1 sequence proposed by Mullen et al. [48]. This result indicates that the substitution of the last amino acid residue (Met or Ile) by another nonpolar amino acid residue (such as Leu) could also result in an effective PTS1. However, the presence of the polar amino acid tyrosine at the carboxyl

terminus of *Helianthus annuus* catalases seems also to result in a signal for protein import into peroxisomes [2].

The peroxisomal location of *Z. aethiopica* catalases together with C-terminal sequence differences found in CAT1 and CAT2 suggest that neither PTS1 reported by González et al. [46] nor the PTS1 tripeptide proposed by Mullen et al. [48] are exclusively necessary for targeting catalase to peroxisome. More recently, Kamigaki et al. [47] reported that the C-terminal 10-amino acid sequence, comprising both previously suggested PTS1, was not required for the import into peroxisomes. According to these authors, the effective PTS1 tripeptide is the internal degenerate motif Gln-Lys-Leu/Ile/Val, located 11-13 amino acid residues from the C-terminal end. In agreement, this motif is fully conserved in both *Z. aethiopica* catalases. Other sequences could also play an accessory role in targeting efficiency [47, 51] as seems to be the case of SerArgLeuAsnValArg located 4 amino acid residues from the C-terminal end [51]. Although *Z. aethiopica* CAT1 and CAT2 do not exhibit this exact sequence, the amino acid differences resulted from substitutions by amino acids from the same chemical category.

The differential expression of catalase genes has been correlated to a specific cellular function. A photorespiratory role for *N. plumbaginifolia Cat1* and *O. sativa CatC* was proposed by detection of highest expression levels in cells with high photosynthetic activity and not in non-photosynthetic cells [22, 25]. During *Z. aethiopica* development, the spathe undergoes senescence, which is characterised by the loss of photosynthetic activity, as chloroplasts change into amyloplasts [31]. When fruiting occurs, spathe senescence is

inhibited and the white spathe changes into a green leaf-like organ (regreened spathe), as the differentiation of amyloplasts into functional chloroplasts takes place [28]. The expression levels of *Z. aethiopica Cat2* closely follows the variation in hydroxypyruvate reductase activity, as well as the changes on photosynthetic and photorespiratory rates observed during the time course of spathe whitening and regreening [30, 31]. Also, during *Z. aethiopica* leaf development, a strong induction of *Cat2* expression was observed. These results suggest that *Z. aethiopica* CAT2 is specifically associated to H_2O_2 decomposition that occurs in leaf-type peroxisomes.

The increase on catalase activity during *Z. aethiopica* spathe regreening could be associated to the increased photosynthetic and photorespiratory rates that occur during this developmental phase; however, a role for catalase other than scavenging photorespiratory H_2O_2 should be suggested to explain the increase in catalase activity during spathe senescence, since spathe photosynthetic ability declines during whitening [30, 31]. During leaf senescence, leaf-type peroxisomes are converted into glyoxysomes and the glyoxylate cycle is induced [52-55]. As a senescence process, *Z. aethiopica* spathe whitening might also involve transition of leaf-type peroxisomes into glyoxysomes. Our results suggest that the increase on isocitrate lyase activity during spathe whitening could reflect the induction of glyoxysomal metabolism for the conversion of lipids into sugars. As a result, the increase in catalase activity observed during the transition from floral bud spathe (an incipient photosynthetic organ) to the late stage of spathe whitening could be explained

by the expression of a catalase form suitable for destroying H_2O_2 produced during ß-oxidation of fatty acids.

The high expression level of specific catalase genes in tissues where glyoxysomal metabolism is induced (*e.g.* germinating fat-storing seeds, senescing tissues), allowed the correlation of specific catalase genes to the scavenging of glyoxysomal H_2O_2 [1, 22]. As the transcript levels of *Z. aethiopica Cat1* increase during spathe whitening, probably CAT1 is associated to scavenging of glyoxysomal H_2O_2; however, as *Cat1* expression is also strongly induced during *Z. aethiopica* spathe regreening, an additional role of CAT1 in scavenging photorespiratory H_2O_2 can be suggested. This assumption is also supported by the strong induction of *Cat1* expression during the transition from young to full-developed leaf.

Based on the expression profiles of genes encoding catalase isozymes, plant catalases have been classified into three different classes [reviewed by 2, 56]. Class I is characterized by high levels of expression in photosynthetic tissues, suggesting that they are involved in the degradation of H_2O_2 produced during photorespiration. Class II is expressed in vascular tissues and has an unspecified cellular function. Class III is highly abundant in seeds and young seedlings and is believed to be involved in degrading H_2O_2 produced during fatty acid ß-oxidation in glyoxysomes. According to this classification, *Z. aethiopica* CAT2 can be included in Class I, since it shows highest expression levels in photosynthetic tissues, closely following the increase in hydroxypyruvate reductase activity. In *Z. aethiopica* CAT2 there is no correlation between the classification based on putative cellular function and

that based on phylogenetic analysis. *Z. aethiopica* CAT2 [Class I according to Willekens et al. [56], and Type III according to Klotz et al. [45]] is more phylogenetically related to Class II *Zea mays* CAT3 and *Oryza sativa* CATA, which do not have a established function [19, 44], than with other Class I catalases.

Z. aethiopica CAT1 can be included in Class III, since its expression profile followed that of isocitrate lyase activity. In addition, *Z. aethiopica* CAT1 clustered together with *Z. mays* CAT1, which is expressed in dark-grown leaf tissue, in young roots and in the early stages of seed germination [1] and with *O. sativa* CATB whose gene is highly expressed in seedlings roots [25]. Our results suggest that besides being associated to scavenging of glyoxysomal H_2O_2, *Z. aethiopica* CAT1 could also be implicated on photorespiratory metabolism, since it is highly expressed in mature leaf. Therefore, from the expression analysis of *Z. aethiopica* catalase genes, CAT2 seems to be specifically associated to photorespiratory metabolism and CAT1 seems to have a dual function in scavenging leaf-type peroxisomal and glyoxysomal H_2O_2. A similar situation was reported on *Z. mays*, where *Cat2* expression pattern also points out for a function on scavenging photorespiratory and glyoxysomal H_2O_2 [1, 26]. Additionally, although *Z. mays* CAT1 has been associated to glyoxysomal metabolism, it is able to compensate the absence of CAT2 in null mutants [7, 57]

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Figure legends

Fig. 1 – Phylogenetic tree representing the relationship between *Z. aethiopica* CAT1 and CAT2 and other higher plant catalases. Amino acid sequences were aligned with the MegAlign program (DNASTAR) using Clustal Method with PAM250 residue weight table. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree indicates the distance between sequences. Catalase types based on phylogenetic relationships [45] are indicated. Catalase classes grouped by their putative function [56] are shadowed as follows: class I in light gray, class II in dark gray and class III in black. See Fig. 2 for accession numbers.

Fig. 2 – Comparison of C-terminal sequence of plant catalases. The tripeptide sequences GlnLysLeu [47], SerArgLeu [46] and ProSerIle [48] proposed as peroxisome targeting signal are boxed in gray. The sequences are given as standard one-letter symbols and points indicate the residues that match the consensus. The numbers refer to the amino acid sequence positions. Accession numbers of sequences are depicted in brackets.

Fig. 3 – Distribution of protein and chlorophyll contents and enzyme (catalase and fumarase) activities following sucrose density centrifugation (30 % (w/w) to 60% (w/w) sucrose) of a peroxisome-enriched fraction from *Z. aethiopica* regreened spathes. Fractions (1 ml) were numbered from the bottom of the gradient.

Fig. 4 – Ultrastructural aspects of *Z. aethiopica* regreened spathe cells. Regreened spathe mesophyll cells exhibit full-organized chloroplasts with well-structured grana and stromal regions, and a large number of peroxisomes and mitochondria (1). After incubation in diaminobenzidine reaction medium for cytochemical localization of catalase activity, electron-dense deposits are only observed in peroxisomes, being absent in mitochondria and chloroplasts (2 and 3). In the presence of catalase activity inhibitor aminotriazole, no electron-dense deposits are observed in peroxisomes (4). Bars in figures indicate 1 μm.

Fig. 5 – Evaluation of catalase activity and expression analysis of *Cat1* and *Cat2* genes during *Z. aethiopica* spathe and leaf development. (A) Catalase activity was determined on extracts obtained from three independent pools of leaves or spathes in different developmental stages. Bars represent SE of three to five independent experiments. The letters above bars indicate significant differences at $P<0.05$. (B) Northern analysis of *Cat1* and *Cat2* genes. Total RNA samples (20 μg per lane) were separated on formaldehyde agarose, blotted and hybridized with ^{32}P-labeled *Cat1* and *Cat2*. Ethidium bromide staining of RNA gel was used to insure equal loading among lanes. (FS - floral bud spathe; S1 and S2 - senescence intermediates; WS – white spathe; R1 and R2 - regreening intermediates; RS - regreened spathe; YL - young leaf; L – mature leaf).

Fig. 6 – Evaluation of isocitrate lyase (ICL) and hydroxypyruvate reductase (HPR) activity during the time course of *Z. aethiopica* spathe development. The activity of the glyoxysomal key enzyme ICL and the leaf-type peroxisomal key enzyme HPR were determined in extracts obtained from three independent pools of spathes in different developmental stages. Bars represent SE of three independent experiments. (FS - floral bud spathe; S1 and S2 - senescence intermediates; WS – white spathe; R1 and R2 - regreening intermediates; RS - regreened spathe).