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Title: Expression of a cloned sweet potato catalase SPCAT1 alleviates ethephon-mediated leaf senescence and H2O2 elevation

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Abstract: In this report a full-length cDNA, SPCAT1, was isolated from ethephon-treated mature L3 leaves of sweet potato. SPCAT1 contained 1479 nucleotides (492 amino acids) in its open reading frame, and exhibited high amino acid sequence identities (ca. 71.2% to 80.9%) with several plant catalases, including Arabidopsis, eggplant, grey mangrove, pea, potato, tobacco and tomato. Gene structural analysis showed that SPCAT1 encoded a catalase and contained a putative conserved internal peroxisomal targeting signal PTS1 motif and calmodulin binding domain around its C-terminus. RT-PCR showed that SPCAT1 gene expression was enhanced significantly in mature L3 and early senescent L4 leaves and was much reduced in immature L1, L2 and completely yellowing senescent L5 leaves. In dark- and ethephon-treated L3 leaves, SPCAT1 expression was significantly enhanced temporarily from 0 to 24 h, then decreased gradually until 72 h after treatment. SPCAT1 gene expression levels also exhibited approximately inverse correlation with the qualitative and quantitative H2O2 amounts. Effector treatment showed that ethephon-enhanced SPCAT1 expression was repressed by antioxidant reduced glutathione, NADPH oxidase inhibitor diphenylene iodonium (DPI), calcium ion chelator EGTA and de novo protein synthesis inhibitor cycloheximide. These data suggest that elevated reactive oxygen species H2O2, NADPH oxidase, external calcium influx and de novo synthesized proteins are required and associated with ethephon-mediated enhancement of sweet potato catalase SPCAT1 expression. Exogenous application of expressed catalase SPCAT1 fusion protein delayed or alleviated ethephon-mediated leaf senescence and H2O2 elevation. Based on these data we conclude that sweet potato SPCAT1 is an ethephon-inducible peroxisomal catalase, and its expression is regulated by reduced glutathione, DPI, EGTA and cycloheximide. Sweet potato catalase SPCAT1 may play a physiological role or function in cope with H2O2 homeostasis in leaves caused by developmental cues and environmental stimuli.

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August 4, 2011

Dear Editor-in-Chief:

We would like to submit a revised manuscript (Ms. Ref. No.: JPLPH-D-11-00273) originally entitled "**Cloning and expression of a sweet potato catalase SPCAT1 alleviated ethephon-mediated leaf senescence and H_2O_2 elevation**" co-authored by **Hsien-Jung Chen, Sin-Dai Wu, Guan-Jhong Huang, Che-Yu Shen, Mufidah Afiyanti, Wei-Jhen Li, and Yaw-Huei Lin** to ***Journal of Plant Physiology*** for publication. The original title has slightly modified as "**Expression of a cloned sweet potato catalase SPCAT1 alleviates ethephon-mediated leaf senescence and H_2O_2 elevation**" as suggested by the Editorial office comments. All the materials are original, no part has been submitted for publication elsewhere, and all authors have agreed for submission.

Thank you very much for your time and truly help. Anything else that I can help to speed the process, please feel free to inform me.

Sincerely yours,

Yaw-Huei Lin, Ph. D.
Retired Professor and Research Fellow

Response to the reviewers' comments

For comments of Reviewer #1

1. Fig.4D and page13 line51: "H_2O_2 amount was the highest in L1 leaves", how do you explain this result? In Arabidopsis, Brassica raps and tobacco plant all showed low amount of H_2O_2 in young leaves.

Ans: We do not know why the young L1 leaves contained the highest amount of H_2O_2. However, our data showed that the catalase was also lower at the L1 leaf stage. Therefore, these data may partly explain why the H_2O_2 amount was the highest in L1 leaves. In addition, small antioxidant molecules (such as reduced glutathione and ascorbic acid) and scavenging enzymes (such as glutathione peroxidase, glutathione reductase, monoascorbate reductase and dehydroascorbate reductase) may also affect H_2O_2 amount via glutathione-ascorbate cycle. The information about the change of these components during leaf development mostly remains unclear in sweet potato and should be addressed in the future in order to understand the changes of H_2O_2 amount in developing leaves.

In Arabidopsis, Brassica rape and tobacco plants all showed low amount of H_2O_2 in young leaves according to the reviewer's comments. In sweet potato, the L1 leaf is the stage with folding, unopened very young leaves. It just comes out from the apical shoot. In Figure 4A, it was forced to open without folding in order to take a picture. In Figure 4D, it remained folding, unopened. The L2 leaf is the stage with open, immature leaves. The H_2O_2 amount also dropped from L1 to L2 and continued to L3 mature young leaves. Therefore, we do not know why the H_2O_2 amount was lower in young leaves of Arabidopsis, Brassica rape and tobacco plants compared to that of sweet potato young leaves. One possibility may be due to the difference that the definition of so-called young leaves for experiments in different plant systems was not the same. For example: Are the young leaves of Arabidopsis, Brassica rape and tobacco plants used for experiments equal to the stage of L1 (folding, unopened very young leaves with the highest H_2O_2 amount), L2 (open, immature young leaves with higher H_2O_2 amount) or L3 (young mature leaves with lower H_2O_2 amount) leaves of sweet potato?

2. Fig.6 the results of semi-qRT-PCR are not so clearly showed the changing of transcript level. The quantitative RT-PCR is suggested.

Ans: We agree with the reviewer's comment. Use of quantitative RT-PCR is a better way to solve the question. In addition, the mature L3 leaf was the leaf stage used for ethephon induction and inhibitor experiments. At the stage, the catalase gene expression was higher compared to the other L1, L2 and L5 stages (Figure 4C). The induction of catalase by ethephon and the repression by inhibitors may not be so clear as raised by the reviewer's comment likely due to the higher catalase gene expression level (basal line) for mature L3 leaves. Therefore, use of another leaf stage such as L2, which did not express significant catalase amount, may reduce the effect of high catalase level (basal line) of mature L3 leaves.

3. page 4 lane49, 58, page7 lane42, page16 lane55 "gene structure" should be "protein structure"

Ans: We agree with the reviewer's comments and correct "gene structure" as "protein structure" (lines 31 and 37, page 4; line 27, page 7; line 35, page 16 of revised manuscript).

For comments of Reviewer #2

Reviewer #2: This manuscript is acceptable for publication with minor grammatical revision as deemed necessary by the Editor. Otherwise the information presented is of high quality and presents new information to the scientific community.

REQUESTS BY THE EDITORIAL OFFICE

Please have the English edited to conform to accepted standards of English style and usage.

Q: FOR EXAMPLE, the title:
Cloning and expression have to be separated, since the cloning did not contribute to the alleviation, only the expression did. You could say "Cloning and expression analysis.... revealed....." or similar. Write alleviates (instead alleviated), and much more.

Ans: We agree with the editorial comments and revise the title as below:

"Expression of a cloned sweet potato catalase *SPCAT1* alleviates ethephon-mediated leaf senescence and H_2O_2 elevation"

Q: Check manuscript for compliance with "Instructions for Authors". EXAMPLES (NOT a complete list) of formatting problems is given in the following. Especially the reference section is poorly formatted or formatted for a different journal.

Ans: We rechecked and revised the manuscript format again according to the "Instruction for Authors" especially the "Reference section" (From line 1, page 20 to line 32, page 23 of revised manuscript).

Q: give authority (L.) Lam. in M&M only (not Summary etc.)

Ans: The "*Ipomoea batatas* (L) Lam." (original Line 5, page3; line 56, page 5) was deleted from any section of the text except the "Material and Methods".

Q: add list of abbreviations

Ans: A list of abbreviations was added and showed as below:

Abbreviation: DAB, diaminobenzidine; DPI, diphenylene iodonium; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; MES, 2-(N-morpholino) ethanesulphonic acid; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction (lines 32 to 35, page 3 of revised manuscript)

Q: write 2009; 2010a (no "and") also 1987a; b (no "and")

Ans: The text was corrected as suggested and showed below:

(Chen et al., 2004; 2006; 2008a; 2009; 2010a) (line 3, page 6 of revised manuscript)
(Sakajo et al., 1987a; b) (line 5, page 6 of revised manuscript)

Q: Reference Section (not formatted for this journal)
no periods after initials and journal abbreviations
year in incorrect position
issue number not bold

page numbers in short form (669-76)

Ans: We rechecked and corrected the format of the “Reference section” again according to the “Instruction for Authors” (From line 1, page 20 to line 32, page 23 of revised manuscript).

Q: Chen YC 2008b, where is "a"?

Ans: There are two “Chen et al., 2008” cited in the “Introduction” section and “Discussion” section, respectively. However, the two “Chen et al., 2008” are not the same author. One is for “Chen HJ” and the other is for “Chen YC”. In order to differentiate, we mark them as “Chen et al., 2008a” in the Introduction section for “Chen HJ” and “Chen et al., 2008b” in the Discussion section for “Chen YC”.

For reference “Chen et al., 2008a” (original line 3, page 6 of Introduction section):
“Chen HJ, Wen IC, Huang GJ, Hou WC, Lin YH. Expression of sweet potato asparaginyl endopeptidase caused altered phenotypic characteristics in transgenic *Arabidopsis*. Bot Stud 2008a; 49: 109-17.”

For reference “Chen et al., 2008b” (original line 20, page 19 of Discussion section):
“Chen YC, Lin HH, Jeng ST. Calcium influxes and mitogen-activated protein kinase kinase activation mediate ethylene inducing ipomoelin gene expression in sweet potato. Plant Cell Environ 2008b; 31: 62-72.”

Q: list alphabetically Gonzales - Guan - Gould ?

Ans: These references were re-scheduled as suggested and shown below (lines 11 to 18, page 21 of revised manuscript):

González E. The C-terminal domain of plant catalase: implications for a glyoxysomal targeting sequence. Eur J Biochem 1991; 199: 211-5.

Gould S, Keller G, Subramani S. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. J Cell Biol 1987; 105: 2923−31.

Gould SJ, Keller GA, Hosken N, Wilkinson J, Subramani S. A conserved tripeptide sorts proteins to peroxisomes. J Cell Biol 1989; 108:1657-664.

Guan L, Scandalios JG. Developmentally related responses of maize catalase genes to salicylic acid. Proc Natl Acad Sci USA 1995; 92: 5930-4.

Q: Niewiadomska all names incorrectly copied (with index letters)

Ans: The reference was corrected and list below (lines 15 to 18, page 22 of revised manuscript):

Niewiadomska E, Polzien L, Desel C, Rozpadek P, Miszalski Z, Krupinska K. Spatial patterns of senescence and development-dependent distribution of reactive oxygen species in tobacco (*Nicotiana tabacum*) leaves. J Plant Physiol 2009; 166: 1057-68.

Q: book titles with upper-case word-beginnings (A Laboratory Manual); journal titles NOT (see Wang et al.)

Ans: The reference was corrected as suggested and list below (lines 37 to 38, page 22 of revised manuscript):

Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning $^{\text{A laboratory manual}}$, 2^{nd} ed. Cold Spring Harbor Laboratory Press; 1989.

Q: Figures
4A leaf but 4D leaves (be consistent)

Ans: We revised and replaced the X-axis label (leaves) of Figure 4D with leaf (Figure 4 of revised manuscript).

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Running title: Sweet potato catalase

Expression of a cloned sweet potato catalase *SPCAT1* alleviates ethephon-mediated leaf senescence and H_2O_2 elevation

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ABSTRACT

In this report a full-length cDNA, *SPCAT1*, was isolated from ethephon-treated mature L3 leaves of sweet potato. *SPCAT1* contained 1479 nucleotides (492 amino acids) in its open reading frame, and exhibited high amino acid sequence identities (ca. 71.2% to 80.9%) with several plant catalases, including *Arabidopsis*, eggplant, grey mangrove, pea, potato, tobacco and tomato**.** Gene structural analysis showed that *SPCAT1* encoded a catalase and contained a putative conserved internal peroxisomal targeting signal PTS1 motif and calmodulin binding domain around its C-terminus. RT-PCR showed that *SPCAT1* gene expression was enhanced significantly in mature L3 and early senescent L4 leaves and was much reduced in immature L1, L2 and completely yellowing senescent L5 leaves. In dark- and ethephon-treated L3 leaves, *SPCAT1* expression was significantly enhanced temporarily from 0 to 24 h, then decreased gradually until 72 h after treatment. *SPCAT1* gene expression levels also exhibited approximately inverse correlation with the qualitative and quantitative H_2O_2 amounts. Effector treatment showed that ethephon-enhanced *SPCAT1* expression was repressed by antioxidant reduced glutathione, NADPH oxidase inhibitor diphenylene iodonium (DPI), calcium ion chelator EGTA and *de novo* protein synthesis inhibitor cycloheximide. These data suggest that elevated reactive oxygen species H_2O_2, NADPH oxidase, external calcium influx and *de novo* synthesized proteins are required and associated with ethephon-mediated enhancement of sweet potato catalase *SPCAT1* expression. Exogenous application of expressed catalase SPCAT1 fusion protein delayed or alleviated ethephon-mediated leaf senescence and H_2O_2 elevation. Based on these data we conclude that sweet potato *SPCAT1* is an ethephon-inducible peroxisomal catalase, and its expression is regulated by reduced glutathione, DPI, EGTA and cycloheximide. Sweet potato catalase *SPCAT1* may play a physiological role or function in cope with H_2O_2 homeostasis in leaves caused by developmental cues and environmental stimuli.

*Keywords***:** Catalase, Ethephon, Glutathione, Leaf, Sweet potato

Abbreviation: DAB, diaminobenzidine; DPI, diphenylene iodonium; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; MES, 2-(N-morpholino) ethanesulphonic acid; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction

Introduction

Leaf is the main place of photosynthesis and serves as a source of carbohydrate for sink nutrients in plants. Leaf senescence affects the efficiency of photosynthesis and is influenced by endogenous and exogenous factors, including plant growth regulators, starvation, wound, dark, ozone, UV, and other environmental stresses (Yoshida 2003; Lim et al., 2007). Elevated oxidative stresses caused by environmental stimuli, including ozone, UV-B, and wounding have been reported and enhanced ethylene production via ACC synthase and ACC oxidase (Wang et al., 2002). In ozone treatment, ethylene also enhances reactive oxygen species (ROS) generation, which in turn leads to senescence and cell death (Wang et al., 2002). Examples concerning the role of elevated oxidative stress have been reported in natural senescence of pea leaves (Pastori and del Río, 1997), induced senescence by ethylene in sweet potato leaves (Chen et al., 2010b), JA (Hung and Kao, 2004a) and ABA (Hung and Kao, 2004b) in rice leaves, and wounding in tomato leaves (Orozco-Cardenas and Ryan, 1999).

Catalase is one of the major H_2O_2-scavenging enzymes and functions mainly in the removal of excessive H_2O_2 generated during developmental processes or by environmental stimuli into water and oxygen in all aerobic organisms (Mhamdi et al., 2010). Plant catalases are composed of a multigene family and have been reported in different species. There are 1 identified in sweet potato storage root (Sakajo and Asahi, 1986), castor bean (González, 1991) and tomato (Drory and Woodson, 1992), 2 in cottonseed (Ni et al., 1990) and *Hordeum vulgare* (Skadsen et al., 1995), 3 in tobacco (Willekens et al., 1994), maize (Guan and Scandalios, 1995), *Arabidopsis* (Frugoli et al., 1996) and pumpkin (Esaka et al., 1997).

Plant catalase is a tetrameric heme-containing enzyme, and is mainly localized in peroxisomes that are bound by a single membrane and contain hydrogen peroxide-generating oxidases. Thus, catalase enzyme plays an important role in scavenging hydrogen peroxide accumulated in peroxisomes (Gillham and Dodge, 1986). Plant peroxisomal proteins including catalases require particular peroxisomal targeting signal (PTS) for import into peroxisomes. Protein structure analysis showed that at least two types of PTSs have been identified. The peroxisomal targeting signal type 1 (PTS1) is based on consensus tripeptides at the C-terminus. Gould et al. (1987 and 1989) first identified firefly luciferase C-terminal SKL as a PTS. Mullen et al. (1997) analyzed the importance of cottonseed catalase into peroxisomes in tobacco BY-2 cells, and demonstrated the C-terminal tripeptides as the PTS1 of cottonseed catalase. In addition, protein structure and transgenic analysis also demonstrated that an internal consensus tripeptide PTS1-like motif (QKL) around or at the C-terminus of pumpkin

Cat1 catalase was identified and directed pumpkin Cat1 catalase import into peroxisome (Kamigaki et al., 2003). For type 2 peroxisomal targeting signal (PTS2), the consensus amino acid sequence (RL/IX5H/QL) located within the N-terminal pre-sequence of a small subset of peroxisomal proteins was identified, proteolytically processed and directed their import into peroxisomes (Gietl, 1996; De Hoop and Ab, 1992; Subramani, 1993).

Gene expression of various plant catalases is regulated temporally and spatially and differentially responds to developmental and environmental stimuli (Guan and Scandalios, 1995; Zimmermann et al., 2006; Du et al., 2008). In *Arabidopsis*, three major catalase CAT1, CAT2 and CAT3, were identified and isolated (Frugoli et al., 1996). CAT2 is the predominant catalase in *Arabidopsis*, and its expression increased and reached maximum at mature leaves. For CAT3 and CAT1, their expression levels were much less than CAT2, and was enhanced in senescent leaves (Zimmermann et al., 2006). In tobacco, there were three catalase genes isolated and named as CAT1, CAT2, and CAT3. Gene expression patterns demonstrated that CAT1 and CAT2 were detected in non-senescent leaves, however, the amount of CAT2, but not CAT1, was significantly reduced in senescent leaves compared to non-senescent leaves. For CAT3, it was detected in both non-senescent and senescing leaves (Niewiadomska et al., 2009).

The catalase activity levels were inversely correlated with the cellular H_2O_2 amounts of plants (Zimermann et al., 2006). Therefore, a light-dependent source of H_2O_2 via photorespiration in the peroxisomes is regulated by catalase (Queval et al., 2007). Transgenic tobacco plants expressing antisense construct of peroxisomal CAT-1 displayed severely reduced catalase activity and developed chlorosis and necrosis on some of the lower leaves due to the elevated H_2O_2 levels (Takahashi et al., 1997). In *Arabidopsis*, the growth of a peroxisomal catalase 2 knock-out mutant (cat2), which also contained higher H_2O_2 amounts, was severely decreased in rosette biomass under ambient air (Queval et al., 2007). Calmodulin, a ubiquitous calcium-binding protein, has been reported to bind and activate some plant catalases in the presence of calcium, but calcium CaM does not have any effect on bacterial, fungal, bovine, or human catalases. In *Arabidopsis*, the putative calmodulin binding domain of CAT3 catalase was identified and confirmed (Yang and Poovaiah, 2002; 2003). These results demonstrate that plant peroxisomal catalases contain calmodulin binding domain that mediates the activation of catalase catalytic activity and down-regulation of H_2O_2 levels.

Sweet potato is an important food crop in the tropics and subtropics including Taiwan. In our laboratory, ethephon, an ethylene-releasing compound, can promote senescence in detached sweet potato leaves. Several ethephon-inducible senescence-

associated genes have previously been cloned and characterized, including isocitrate lyase (Chen et al., 2000), metallothionein (Chen et al., 2003), and cysteine proteases (Chen et al., 2004; 2006; 2008a; 2009; 2010a). Research about sweet potato catalase, however, was limited. A full-length cDNA encoding putative catalase had been cloned previously from sweet potato storage root (Sakajo et al., 1987a; b), however, its physiological role and function remained unclear. In our laboratory, a major leaf-type catalase was identified and characterized with in-gel activity assay in sweet potato. Its enzymatic activity was enhanced in mature leaves, and was induced by dark and ethephon. The leaf-type catalase expression level exhibited negative correlation with cellular H_2O_2 level (Chen et al., 2011). In this report, an ethephon-inducible catalase cDNA *SPCAT1* was also cloned and characterized for the first time from sweet potato leaves. A possible role of sweet potato catalase *SPCAT1* in cope with H_2O_2 homeostasis in natural and induced senescent leaves was also addressed.

Materials and methods

Plant materials

The storage roots of sweet potato (*Ipomoea batatas* (L.) Lam.) were grown in the growth chamber at 28^0C/16 h day and 23^0C/8 h night cycle. Plantlets sprouted from the storage roots provided detached mature leaves for dark and ethephon treatments at 28^0C/16 h and 23^0C/8 h cycle in the dark, and different developmental stages of leaves for temporal and spatial expression experiments. Leaves were arbitrary divided into L1 to L5 according to their size and different developmental stages. L1 was the stage with folding, unopened immature leaves. L2 was the stage with unfolding but not fully-expanded immature leaves. L3 was the stage with fully-expanded mature leaves. L4 and L5 were the stages with partial and completely yellowing senescent leaves, respectively. Samples collected were used for leaf morphology, photochemical Fv/Fm, chlorophyll content, diaminobenzidine (DAB) staining, H_2O_2 determination, and gene expression with RT-PCR as described below. Detached L3 mature leaves treated with 1 mM ethephon and dark control were used for PCR-selective subtractive hybridization and rapid amplification of cDNA ends (RACE) experiments as described below.

PCR-selective subtractive hybridization and RACE PCR

Sweet potato L3 mature leaves were detached and treated with 1 mM ethephon for 6 and 24 hours according to the report of Chen et al. (2003), then the two samples were combined together for PCR-selective subtractive hybridization and RACE PCR for full-length catalase cDNA cloning. The dark-treated leaves were used as control. Total RNAs were isolated separately from the samples of dark control and 1 mM ethephon-treated L3 mature leaves as described above according to the method of Sambrook et al. (1989). The mRNAs were purified with a purification kit (Promega) and used for the differentially-expressed first strand cDNA synthesis with a PCR-selective subtractive hybridization kit (Clontech) following the protocols supplied by the manufacturer. The differentially expressed cDNAs of 1 mM ethephon-treated leaves after subtraction by that of dark control mature leaves were ligated to pGEM-T easy vector for *E. coli* DH5α competent cell transformation. Recombinant plasmids were isolated for DNA sequencing using an ABI PRIZM 337 DNA Sequencer. Nucleotide sequence data were analyzed using the NCBI Blast program. The RACE PCR method with the Marathon cDNA amplification kit (Clontech) was used to isolate the 5' and 3' ends of the target catalase cDNAs according to the protocols provided by the manufacturer. Finally, a primer pair (5’ primer: CATTATTCTCTCT GTCCCCTCATCTCCATG; 3’ primer: TGCCTTTAATTCCACCTCTCTTACATCG TC) was used to amplify the full-length cDNA encoding the putative sweet potato catalase. The amplified cDNA products were purified from agarose gel after electrophoresis with QIAquick Gel Extraction kit (QIAGEN), then cloned directly into pGEM-T easy vector (Promega). Recombinant plasmids were isolated and the insert DNAs were used for DNA sequencing with an ABI PRIZM 337 DNA Sequencer. The catalase cDNA was renamed as *SPCAT1* and its nucleotide and amino acid sequence was submitted to NCBI GenBank for registration.

Protein structure analysis and phylogenetic tree construction

After NCBI/blast comparison, the amino acid sequence of sweet potato catalase *SPCAT1* (accession no. GU230147) was aligned with published plant catalases, including *Arabidopsis* CAT1 (accession no. Q96528), *Arabidopsis* CAT2 (accession no. P25819), *Arabidopsis* CAT3 (accession no. Q42547), eggplant CAT (accession no. X71653), grey mangrove CAT1 (accession no. Q53ZT2), pea CAT (accession no. P25890), potato CAT1 (accession no. U27082), tobacco CAT1 (accession no. Z36975), tobacco CAT2 (accession no. Z36976), tobacco CAT3 (accession no. Z36977), and tomato CAT1 (accession no. M93719), for (a) phylogenetic tree construction, (b) identification of conserved internal peroxisomal targeting signal (PTS1) around the C-terminus, and (c) putative consensus calmodulin binding domain.

For phylogenetic tree construction, the distances among entries were calculated with neighbor-joining method (Thompson et al. 1994), and the BLOSUM series matrix (80, 62, 45, 30) of software ClustalW2 was used as parameters in the alignment with default setting for gap penalty. The phylogenetic tree was drawn using NJ plot.

Temporal and spatial expression of sweet potato catalase SPCAT1

In order to analyze the temporal and spatial expression of sweet potato catalase *SPCAT1*, samples were collected from stem, root, skin and flesh of storage root, and different leaf developmental stages (L1 to L5) as described above. These samples were analyzed for catalase *SPCAT1* expression with RT-PCR as described below for temporal and spatial expression patterns. For different developmental stages of leaf samples (L1 to L5), additional assays were also performed, including leaf morphology, chlorophyll content, photochemical Fv/Fm, DAB staining and determination of H_2O_2 amount as described below.

Ethephon and dark treatments

For dark treatment, detached L3 mature leaves were placed on a wet paper towel containing 3 mM 2-(N-morpholino)ethanesulphonic acid (MES) buffer pH 5.8, and kept at 28^0C/16 h and 23^0C/8 h cycle in the dark. Samples were collected individually at 0, 6, 24, 48 and 72 h after treatment. For ethephon treatment, detached mature leaves (L3) were also placed on a wet paper towel containing 3 mM MES buffer pH 5.8 plus 1 mM ethephon, and kept at 28^0C/16 h and 23^0C/8 h cycle in the dark. Samples were also collected individually at 0, 6, 24, 48 and 72 h after treatment. Both samples from dark and ethephon treatments were analyzed for leaf morphology, chlorophyll content, photochemical Fv/Fm, catalase *SPCAT1* expression with RT-PCR, DAB staining and determination of H_2O_2 amount as describes below.

Effector treatment

Influence of effectors such as antioxidant reduced glutathione, NADPH oxidase inhibitor DPI, calcium ion chelator EGTA, and *de novo* protein synthesis inhibitor cycloheximide on ethephon-enhanced sweet potato catalase *SPCAT1* gene expression were studied. Detached L3 mature leaves were pretreated with (+) or without (-) 1 mM reduced glutathione, 100 μM DPI, 5 mM EGTA, or 20 μg/mL cycloheximide for ca. 15 to 30 min prior to 1 mM ethephon treatment on a wet paper towel containing 3 mM 2-(N-morpholino)ethanesulphonic acid (MES) buffer pH 5.8. Leaves were kept at

28^0C/16 h and 23^0C/8 h cycle in the dark for 24 h and 72 h, respectively, then were individually collected and analyzed using RT-PCR with the primer pair (5' primer: CATTATTCTCTCTGTCCCCTCATCTCCATG; 3' primer: TGCCTTTAATTCCAC CTCTCTTACATCGTC) to amplify the full-length cDNA for sweet potato catalase *SPCAT1* expression.

Construction, overexpression, and purification of SPCAT1 fusion protein from E. coli

The full-length catalase *SPCAT1* cDNA in the recombinant pGEM-T easy vector was used as templates to amplify the PCR products encoding the putative SPCAT1 protein (from the 1st to 492nd amino acid residues) with the 5' (Catalase A: ATGCCCATGGATCCTTCAAAGTATCGTCCA) and 3' (Catalase B: ATGCCCATGGTTACATCGTCGGTCTTATGT) primers. The ATG and TTA underlined indicated the initiation codon and stop codon, respectively. A *Nco*I cutting site (CCATGG printed in black on gray) was also introduced into the 5' and 3' primer pair. The amplified PCR products were purified first from agarose gel after electrophoresis with QIAguick Gel Extraction kit (QIAGEN), then ligated with *Nco*I digested PET30a vector (Novagen) for *E. coli* DH5α competent cell transformation according to the protocols provided by the supplier. The recombinant PET-30a vector containing the correct catalase *SPCAT1* construction was isolated and transferred into BL21 (DE3) competent cell again. The transformants were isolated and used for catalase SPCAT1 fusion protein induction, detection, and purification according to the protocols provided by the supplier.

For SDS-PAGE of catalase SPCAT1 fusion protein, the cells were induced by 1 mM IPTG and harvested individually at the time intervals (0, 1, 2, 3, 4 and 5 h, respectively) after treatment. The cell pellet from 1.5 ml culture after centrifugation at 10,000 xg for 10 min was re-suspended in phosphate buffer saline, then sonicated, and finally mixed with 5x SDS sample buffer (60 mM Tris-HCl pH 6.8, 50% glycerol, 2% SDS, 28.8 mM β-mercaptoethanol, 0.1% bromophenol blue) and boiled at 95^0C for 5 min. Then, equal volume of samples was loaded into 12.5% SDS-PAGE for analysis.

For catalase SPCAT1 fusion protein purification, cells after induction with 1 mM IPTG for 5 h were collected and centrifuged at 10,000 xg for 10 min and re-suspended in phosphate buffer saline. The expressed fusion proteins were extracted from cell pellet with 8 M urea in binding buffer B (8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl, pH 8.0), and then applied to His-tag affinity column for purification according to the protocols from Novagen. The column was washed with denaturing wash buffer (Buffer C: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 6.3), and then eluted the

catalase SPCAT1 fusion protein with denaturing elution buffer (buffer D: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 5.9) for the first time and denaturing elution buffer (buffer E: 8 M urea; 0.1 M NaH2PO4; 0.01 M Tris-Cl pH 4.5) for the second and third time. The different portions collected during purification procedure were used for SDS-PAGE as described above. The eluted proteins in buffer D and buffer E were combined together and used for exogenous application of purified catalase SPCAT1 fusion protein to ethephon-treated sweet potato leaves.

For exogenous application of catalase SPCAT1 fusion protein, the purified fusion protein (0.2 and 2.0 mg, respectively) was mixed together with 1 mM ethephon in 3 mM MES buffer pH 5.8 (final volume of 50 mL), and used for exogenous application to detached L3 mature leaves in order to study its influence on ethephon-mediated effects of leaf senescence, including leaf morphology, chlorophyll content, photochemical Fv/Fm, and quantitative determination of H_2O_2 amount as described below.

Treatment of catalase SPCAT1 fusion protein

For influence of exogenous catalase SPCAT1 fusion protein on ethephon-mediated effects, detached L3 mature leaves were placed on a wet paper towel containing 3 mM MES buffer pH 5.8, 1 mM ethephon and different amount of purified catalase SPCAT1 fusion protein (0, 0.2 and 2.0 mg, respectively), then kept at 28^0C/16 h and 23^0C/8 h cycle in the dark. Samples were collected individually at 0 and 72 h after treatment, and analyzed for leaf morphology, chlorophyll content, photochemical Fv/Fm, DAB staining and determination of H_2O_2 amount as describes below.

Leaf morphology

Leaves from treatments mentioned above were scanned with a scanner for morphological record and comparison. Each experiment was repeated at least three times and a representative one was shown (Chen et al., 2010b).

Measurement of chlorophyll content

Leaves from treatments mentioned above were measured and recorded directly with non-invasive CCM-200 Chlorophyll Content Meter. Each leaf sample was measured at least five different leaf areas, and each treatment was repeated at least three times. The data were expressed as mean ± S.E. (Chen et al., 2010b).

Measurement of photochemical Fv/Fm

Leaves from treatments mentioned above were measured and recorded with non-invasive Chlorophyll Fluorometer (WALZ JUNIOR-PAN). The photochemical Fv/Fm is used to determine the maximal quantum efficiency of photosystem II primary photochemistry. In healthy leaves, this value is close to 0.8 and independently of the plant species. Therefore, the photochemical Fv/Fm was measured, recorded and compared among control and treated samples. Each leaf sample was measured at least five different leaf areas, and each treatment was repeated at least three times. The data were expressed as mean ± S.E. (Chen et al., 2010b).

DAB staining

DAB staining method was used to qualitatively detect the H_2O_2 generation in leaves after treatments, and was basically according to the method described by Hu et al. (2005) with minor modification. Leaves from treatments mentioned above were collected separately and stained with 1 mg/ml DAB solution pH 3.8 at 37^0C for 2 hours. After DAB staining, leaves were boiled in ethanol for 10 minutes, then cooled down to room temperature and photographed (Chen et al., 2010b).

Measurement of H_2O_2 amount

For quantitative measurement of H_2O_2 amount, leaves from treatments mentioned above were analyzed basically according to the method reported by Kuzniak et al. (1999). There were about ten leaf discs (1 cm in diameter) incubated in 2 mL reagent mixture (50 mM phosphate buffer pH 7.0, 0.05% guaiacol and horseradish peroxidase (2.5 U mL^{-1})) for 2 h at room temperature in the dark. Four moles of H_2O_2 are required in order to form 1 M of tetraguaiacol, which has an extinction coefficient of ε = 26.6 cm^{-1} mM^{-1} at 470 nm. The absorbance in the reaction mixture was measured immediately at 470 nm and expressed as μmole H_2O_2 g^{-1} leaf fresh weight. Each treatment was repeated at least three times. The data were expressed as mean ± S.E.

RT-PCR analysis

Total RNA was isolated from (a) different developmental stages of leaves (L1 to L5), (b) different organs including roots, stems, L3 mature leaves, skin and flesh of storage roots, (c) time course of dark-treated or 1 mM ethephon-treated L3 mature

leaves, and (d) dark-treated, 1 mM ethephon-treated, and effector plus 1 mM ethephon-treated L3 mature leaves for 24 h and 72 h, respectively, as described above. The *SPCAT1* 5' primer (CATTATTCTCTCTGTCCCCTCATCTCCATG) and 3' primer (TGCCTTTAATTCCACCTCTCTTACATCGTC) were used to amplify the full-length catalase PCR products for analysis with agarose gel electrophoresis. In addition, a primer pairs (5' primer: ATGTCGGACAAGTGCGGAAACTGCG; 3' primer: TTAGTGGCCACAGGTGCGGTCGGTA) for G14, which encoded a constitutively-expressed metallothionein-like protein, was used as an endogenous control (Chen et al., 2003). The RT-PCR condition was 94^0C 3 min for 1 cycle; 94^0C 1 min, 55^0C 45 sec, 72^0C 90 sec for 25 cycle; then, 72^0C 7 min for 1 cycle.

Results

Nucleotide and amino acid sequences of catalase SPCAT1

In order to clone ethephon-inducible genes, sweet potato L3 mature leaves were treated with 1 mM ethephon for 6 and 24 h and used for differentially-expressed gene isolation. With PCR-selective subtractive hybridization and RACE PCR techniques, a full-length cDNA (accession no. GU230147) was cloned and named as *SPCAT1*. There were 1479 nucleotides (492 amino acids) in its open reading frame (Fig. 1). NCBI/Blast comparison showed that *SPCAT1* exhibited high amino acid sequence identities (ca. 71.2% to 80.9%) with several plant catalases, including *Arabidopsis*, eggplant, grey mangrove, pea, potato, tobacco and tomato. The 65^{th} His (H), 104^{th} Ser (S) and 138^{th} Asn (N) printed in white on black represent the conserved catalytic amino acid residues. The C-terminal 480^{th} Gln (Q), 481^{st} Lys (K) and 482^{nd} Val (V) amino acid residues printed in white on black represent the putative consensus internal peroxisomal targeting signal PTS1 (Figs. 1 and 2A). Amino acid residues from the 415^{th} Gly (G) to 451^{st} Val (V) printed in black on gray represent the putative calmodulin binding domain (Figs. 1 and 2B). These data suggest that sweet potato *SPCAT1* encodes a putative peroxisomal catalase, which is likely regulated and activated by calmodulin and calcium.

Amino acid sequence alignment and phylogenetic relationship of *SPCAT1* with several plant catalases, including *Arabidopsis* CAT1 (accession no. Q96528), *Arabidopsis* CAT2 (accession no. P25819), *Arabidopsis* CAT3 (accession no. Q42547), eggplant CAT (accession no. X71653), grey mangrove CAT1 (accession no. Q53ZT2), pea CAT (accession no. P25890), potato CAT1 (accession no. U27082), sweet potato SPCAT1 (accession no. GU230147), tobacco CAT1 (accession no.

Z36975), tobacco CAT2 (accession no. Z36976), tobacco CAT3 (accession no. Z36977) and tomato CAT1 (accession no. M93719) were shown in Figure 3. Sweet potato SPCAT1 exhibited close association with a group of plant catalase, including grey mangrove CAT1, tobacco CAT1 and CAT3, pea CAT, and *Arabidopsis* CAT1 and CAT2. However, SPCAT1 displayed a more distantly-related association with another group of plant catalases, including *Arabidopsis* CAT3, eggplant CAT, potato CAT1, tobacco CAT2, and tomato CAT1. These data suggest that sweet potato catalase SPCAT1 may have physiological role or function more related to grey mangrove CAT1, tobacco CAT1, and *Arabidopsis* CAT2.

Temporal expression of catalase SPCAT1 in leaves

There were 5 leaf stages (L1 to L5) divided according to leaf size and different developmental stages as described in "Materials and Methods" section (Fig. 4A). The chlorophyll content increased gradually from L1 (ca. 13.24%) and L2 (ca. 74.80%) leaves, and reached maximum at L3 leaves (100%), then decreased gradually from early L4 senescent leaves (ca. 38.86%) until completely yellowing L5 senescent leaves (ca. 6.93%) (Fig. 4A). The photochemical Fv/Fm was not much different among L1, L2, L3 and L4 leaves, and was significantly decreased in completely yellowing L5 senescent leaves (ca. 59%) (Fig. 4B). These data demonstrate that L3 leaves are the fully-expanded mature leaves, therefore, contain the highest chlorophyll content and photochemical Fv/Fm.

The temporal expression patterns of *SPCAT1* were studied with RT-PCT according to Chen et al. (2003). *SPCAT1* gene expression was remarkably enhanced at L3 mature and early L4 senescent leaves compared to immature L1 and L2 leaves and completely yellowing L5 senescent leaves (Fig. 4C). However, a metallothionein-like protein gene, *G14*, which was isolated previously from sweet potato leaves, exhibited constitutive expression pattern in all tissues assayed and was used as an endogenous control (Chen et al., 2003). No significant variation of *G14* expression level was found among different leaf developmental stages analyzed (Fig. 4C). Qualitative DAB staining and quantitative determination of H_2O_2 amounts in leaves of different developmental stages were also measured. The results showed that H_2O_2 amount was the highest in L1 leaves, and decreased gradually from L2 leaves to the lowest in L3 and L4 leaves, then increased again in completely yellowing L5 senescent leaves (Fig. 4D). An inverse correlation between catalase *SPCAT1* expression level and H_2O_2 amount in different developmental leaf stages of sweet potato was observed. These data suggest a possible role of sweet potato catalase *SPCAT1* in association with the H_2O_2 homeostasis during leaf development.

Dark and ethephon enhance catalase SPCAT1 expression

Effects of dark and ethephon on leaf senescence, chlorophyll content, photochemical Fv/Fm, H_2O_2 amount, and catalase *SPCAT1* expression were studied. Dark did not promote significant leaf senescence (Fig. 5A), decrease of chlorophyll content (Fig. 5B), and reduction of photochemical Fv/Fm (Fig. 5C) within 72 h treatment. These data suggest that dark may not be a key regulator in promotion of leaf senescence. However, dark did affect the catalase *SPCAT1* expression and H_2O_2 amount in treated detached leaves. Catalase *SPCAT1* expression was remarkably enhanced from 0 to 24 h, and then decreased gradually until 72 h after dark treatment in detached L3 mature leaves. However, the metallothionein-like protein gene, *G14*, exhibited constitutive expression pattern and no significant variation was found in all tissues assayed (Fig. 5D). Qualitative DAB staining and quantitative determination of H_2O_2 amounts in dark-treated detached leaves showed that the H_2O_2 amount was the lowest from 0 to 24 h, and then increased gradually and reached a maximum at 72 h after dark treatment in detached L3 mature leaves (Figs. 5E and 5F). An inverse correlation between catalase *SPCAT1* expression level and H_2O_2 amount in dark-treated detached L3 mature leaves was observed. These data suggest a possible role of sweet potato catalase *SPCAT1* in association with the H_2O_2 homeostasis in dark-stressed leaves.

Effects of ethephon on leaf senescence, chlorophyll content, photochemical Fv/Fm, H_2O_2 amount, and catalase activity were also studied. Ethephon did cause significant promotion on leaf senescence (Fig. 5A), decrease of chlorophyll content (Fig. 5B), and reduction of photochemical Fv/Fm (Fig. 5C) at 72 h after treatment. These data also consistent with the key role of ethylene in promotion of leaf senescence. Ethephon also affected catalase *SPCAT1* expression and the H_2O_2 amount in treated detached L3 mature leaves. Catalase *SPCAT1* expression was significantly enhanced from 0 to 24 h, and then decreased gradually until 72 h after ethephon treatment in detached L3 mature leaves. The enhancement of *SPCAT1* expression by ethephon was greater than that by dark from 0 to 24 h after treatment (Fig. 5D). However, the metallothionein-like protein gene, *G14*, exhibited constitutive expression pattern and no significant variation was found in all tissues assayed (Fig. 5D). Qualitative DAB staining and quantitative determination of H_2O_2 amounts in ethephon-treated detached leaves showed that the H_2O_2 amount was also the lowest from 0 to 24 h, and then increased gradually and reached maximum at 72 h after ethephon treatment in detached L3 mature leaves (Figs. 5E and 5F). The increase of H_2O_2 amount by ethephon was also greater than that by dark from 0 to 72 h after treatment (Figs. 5E

and 5F). An inverse correlation between catalase *SPCAT1* expression level and H_2O_2 amount in ethephon-treated detached L3 mature leaves was also observed. These data suggest a possible role of sweet potato catalase *SPCAT1* in association with the H_2O_2 homeostasis in ethephon-treated leaves.

Ethephon-enhanced catalase SPCAT1 expression was repressed by reduced glutathione, DPI, EGTA and cycloheximide

Factors involved in the enhancement of catalase *SPCAT1* expression by ethephon in L3 mature leaves was also studied with different effectors at 24 h and 72 h after treatment. Ethephon-mediated enhancement of catalase *SPCAT1* expression was repressed by pretreatment with antioxidant reduced glutathione (Fig. 6A), NADPH oxidase inhibitor DPI (Fig. 6B), calcium ion chelator EGTA (Fig. 6C), and *de novo* protein synthesis inhibitor cycloheximide (Fig. 6D) to a level similar to that of dark control (Fig. 6). These data suggest the association of elevated H_2O_2 level, NADPH oxidase, external calcium influx, and *de novo* synthesized proteins with ethephon-mediated enhancement of catalase *SPCAT1* gene expression.

Ethephon-mediated leaf senescence and elevated H_2O_2 level were delayed or attenuated by exogenous catalase SPCAT1 fusion protein

Sweet potato catalase *SPCAT1* full-length cDNA was constructed with PET30a expression vector and induced to express the corresponding SPCAT1 fusion protein by 1 mM IPTG within 5 hours after treatment (Fig. 7A). The expressed catalase SPCAT1 fusion protein was purified (Fig. 7B) and used for exogenous application with ethephon together. Influence of exogenous catalase SPCAT1 fusion protein on ethephon-mediated effects on leaf senescence, chlorophyll content, photochemical Fv/Fm, and H_2O_2 level was shown. Ethephon significantly promoted leaf senescence (Fig. 7C), decrease of chlorophyll content (Fig. 7D), reduction of photochemical Fv/Fm (Fig. 7E), and elevation of H_2O_2 levels (Fig. 7F) in leaves at 72 h after treatment. These ethephon-mediated effects were all significantly delayed or alleviated by exogenous catalase SPCAT1 fusion proteins (Fig. 7). Ethephon-mediated leaf senescence were significantly delayed or alleviated by exogenously applied purified catalase SPCAT1 fusion protein at both doses, 0.2 mg and 2.0 mg, respectively (Fig. 7C). For chlorophyll content, it was assigned as 100% for D3 control sample. The chlorophyll content decreased significantly to about 14.4% for ethephon-treated E3 sample, and the reduction was remarkably alleviated by exogenous application of purified catalase SPCAT1 fusion protein at doses of 0.2 mg (ca. 24.4%) and 2.0 mg

(32.3%), respectively (Fig. 7D). For photochemical Fv/Fm, the value of D3 control sample was assigned as 100%. The photochemical Fv/Fm value decreased significantly to about 54.4% for ethephon-treated E3 sample, and the reduction was remarkably delayed by exogenous application of purified catalase SPCAT1 fusion protein at doses of 0.2 mg (ca. 81.3%) and 2.0 mg (74.5%), respectively (Fig. 7E). For H_2O_2 level, the amount of D3 control sample was assigned as 100%. The H_2O_2 amount increased significantly to about 216.9% for ethephon-treated E3 sample, and the increase was remarkably attenuated by exogenous application of purified catalase SPCAT1 fusion protein at doses of 0.2 mg (ca. 126.3%) and 2.0 mg (ca. 136.2%), respectively (Fig. 7F). These data clearly demonstrate that sweet potato catalase SPCAT1 can alleviate ethephon-mediated leaf senescence and H_2O_2 elevation *in vitro*. A role of sweet potato catalase SPCAT1 in association with the regulation of H_2O_2 homeostasis and attenuation of leaf senescence caused by ethephon in sweet potato leaves is also suggested.

Discussion

Plant catalases have been intensively studied and function mainly in the removal of excessive H_2O_2 generated during developmental processes or by environmental stimuli into water and oxygen in all aerobic organisms (Mhamdi et al., 2010). Catalases are generally composed of a multigene family and various plant catalase isoforms are temporally and spatially regulated and may respond differentially to developmental and environmental stimuli (Zimmermann et al., 2006). Sweet potato catalase *SPCAT1* exhibited high amino acid sequence homologies (71.2% to 80.9%) with several plant catalases, including *Arabidopsis*, eggplant, grey mangrove, pea, potato, tobacco and tomato (Fig. 1). From RT-PCR, *SPCAT1*-encoded products could be detected at mRNA levels (Figs. 4 and 5). The open reading frame of *SPCAT1* was also constructed in recombinant PET30a vector and expressed in *E. coli* BL21 (DE3) cells. A fusion protein with molecular mass near 55 kDa was detected (Fig. 7A). Exogenous application of expressed catalase SPCAT1 fusion protein delayed or alleviated ethephon-mediated H_2O_2 elevation and leaf senescence (Fig. 7). These data, therefore, provide evidence to support sweet potato catalase *SPCAT1* as a functional gene.

Protein structural analysis showed that sweet potato catalase SPCAT1 contained the putative consensus internal PTS1 motif (QKV) around the C-terminus (Figs. 1 and 2A). In pumpkin, the CAT1 catalase C-terminal portion was fused with green fluorescence protein (GFP) and expressed in tobacco BY-2 cells in order to identify

the possible peroxisomal targeting signal. The results showed that removal of the tripeptides at the C-terminus did not affect pumpkin CAT1 targeting to peroxisome. However, the amino acid region (QKL) from 13 to 11 around or at the C-terminal portion functions as an internal PTS1 sequence. Deletion of the tripeptides affects CAT1 import into peroxisome. Analysis of the binding of CAT1 to PTS1 receptor (Pex5p) by the yeast two-hybrid system revealed that CAT1 can bind with the PTS1 receptor (Pex5p) (Kamigaki et al., 2003). These data indicates that pumpkin CAT1 is imported into peroxisomes by the PTS1 system. Comparison of several plant catalases showed that the internal PTS1 sequence around the C-terminal region from the positions 13 to 11 were also found in catalases of castor bean, cotton, maize, sweet potato, *Arabidopsis*, sunflower, barley, pea, tobacco and tomato as mentioned in Figure 2A. The amino acid sequences of catalases from *Arabidopsis* (QKL), barley (QKL), pea (QKL), sunflower (QKI), tobacco (QKL) and tomato (QKV) showed that QKL/I/V was conserved in the positions 13–11 (González, 1991; Kamigaki et al., 2003). Our data agree with these reports and suggest the consensus QKV tripeptide may function as an internal PTS1 for sweet potato catalase SPCAT1 import into peroxisome similar to PTS1 (QKL) for pumpkin CAT1. Further experiments are required in order to prove the role of QKV in peroxisomal targeting in sweet potato.

In *Arabidopsis*, two C-terminal deletion mutants of CAT3 was used to identify the possible cambodulin binding domain with ^{35}S-CaM-binding assays. In the presence of calcium, the calmodulin binding region was restricted to the 415th to 451st amino acid region of CAT3. Synthetic peptide according to the consensus sequence also binds to the expressed calmodulin, and the binding was repressed in the presence of EGTA (Yang and Poovaiah, 2002). Sweet potato SPCAT1 also contained the putative consensus calmodulin binding domain within the C-terminal portion similar to *Arabidopsis* CAT3 (Figs. 1 and 2B). These data suggest that the enzymatic activity of sweet potato SPCAT1 may also be regulated and activated by calmodulin similar to the report of *Arabidopsis* CAT3.

Mhamdi et al. (2010) described that plant catalases were probably divided into three classes in *Arabidopsis*, maize, pumpkin, rice and tobacco based on the report of Willekens et al. (1995). Class I contains *Arabidopsis* CAT2, maize CAT2, pumpkin CAT2, rice CATC and tobacco CAT1. Class II contains *Arabidopsis* CAT3, maize CAT3, pumpkin CAT3, rice CATA and tobacco CAT2. Class III contains *Arabidopsis* CAT1, maize CAT1, pumpkin CAT1, rice CATB and tobacco CAT3. Phylogenetic tree analysis showed that SPCAT1 exhibited more closely-related association with class I plant catalases including *Arabidopsis* CAT2 and tobacco CAT1 than class III plant catalases such as *Arabidopsis* CAT1 and tobacco CAT3. Sweet potato SPCAT1 exhibited more distantly-related association with class II plant

catalases including *Arabidopsis* CAT3 and tobacco CAT2 (Fig. 2). In sweet potato, *SPCAT1* expression level was higher in L3 mature and early L4 senescent leaves, however, was lower in immature L1, L2 and completely yellowing L5 leaves (Fig. 4). In tobacco, the CAT1 was the predominant one and present earlier 8 days post-germination and continuously increased and reached maximum 21 days post-germination, then decreased gradually (Havir and McHale, 1987). In *Arabidopsis* leaves, the CAT2 is the major catalase isoform. Its activity increased and reached maximum at mature leaves, then, decreased gradually after transition from vegetative to reproductive phases and senescence of plant (Zimmermann et al., 2006). Our data agree with these reports and suggest that sweet potato *SPCAT1* likely belongs to class I catalase and exhibits expression pattern similar to *Arabidopsis* CAT2 and tobacco CAT1 during leaf development.

In sweet potato, *SPCAT1* gene expression level was enhanced temporarily by dark and ethephon treatments (Fig. 5). These data agree with our previous enzymatic catalase activity assay (Chen et al., 2011). Similar results were observed for grey mangrove CAT1 gene expression, which was also temporarily enhanced by salt treatment (Jithesh et al., 2006). Sweet potato *SPCAT1* gene expression level also exhibited negative correlation with H_2O_2 amount in dark-treated, ethephon-treated and different developmental stages of leaves (Figs. 4 and 5). Exogenous application of purified catalase SPCAT1 fusion protein (0.2 mg and 2.0 mg, respectively) delayed or alleviated ethephon-mediated leaf senescence and elevation of H_2O_2 amount in sweet potato leaves (Fig. 7). In *Arabidopsis*, the growth of a peroxisomal catalase 2 knock-out mutant (cat2) was severely decreased in rosette biomass under ambient air, and significant increase of intracellular H_2O_2 level was also observed (Queval et al., 2007; Mhamdi et al., 2010). Transgenic tobacco plants expressing antisense construct of peroxisomal CAT1 displayed severely reduced catalase activity and developed chlorosis and necrosis on some of the lower leaves due to the remarkable elevation of H_2O_2 amount (Takahashi et al., 1997). In transgenic tobacco plant Cat1AS expressing tobacco CAT1 antisense construct, the catalase activity was significantly reduced to ca. 10% that of wild type control. The CAT1 deficiency plant also exhibited reduced H_2O_2-removing capacity and, consequently, led to higher steady-state levels of H_2O_2 inside leaves, chlorophyll bleaching and glossary necrotic spots on leaves under stresses such as high light, paraquat, H_2O_2 and ozone treatments. Exogenous application of commercial bovine catalase complemented the bleaching effects caused by high light exposure in CAT1 deficiency plant leaves (Willekens et al., 1997). Our results agree with these reports and support a role of sweet potato catalase *SPCAT1* similar to *Arabidopsis* CAT2 and tobacco CAT1 in the regulation of plant cellular

homeostasis of reactive oxygen species H_2O_2, which in turn affects plant physiological and developmental processes including leaf senescence.

Ethephon-enhanced sweet potato *SPCAT1* gene expression level was repressed by reduced glutathione, DPI, EGTA and cycloheximide (Fig. 6). A close relationship between intracellular H_2O_2 and cytosolic calcium in response to biotic and abiotic stresses was also observed and reported. Zhao et al. (2007) demonstrated that ethephon activated a plasma membrane Ca^{2+}-permeable channel in tobacco suspension cells with patch-clamp technique and confocal microscopy. In tobacco cell suspension culture, an elicitor, quercinin, induced ethylene biosynthesis and H_2O_2 formation. Ethylene at low concentrations proved to be necessary for induction and maintenance of H_2O_2 production in tobacco cells treated with quercinin (Koehl et al., 2007). In grey mangrove, the CAT1 gene expression was also induced by exogenous H_2O_2 application (Jithesh et al., 2006). Chen et al. (2008b) reported that sweet potato wound-inducible ipomoelin (*IPO*) gene expression was induced by ethephon. *IPO* gene expression was completely repressed by diphenylene iodonium (DPI), an inhibitor of NADPH oxidase which caused the elevation of intracellular oxidative stress H_2O_2 (Jih et al., 2003). In sweet potato, ethephon-induced leaf senescence, reactive oxygen species H_2O_2 elevation, senescence-associated cysteine protease SPCP1 gene expression was also repressed by EGTA, reduced glutathione, and cycloheximide in detached leaves (Chen et al., 2010b). Our data agree with these reports and suggest that ethephon-mediated enhancement of sweet potato catalase *SPCAT1* gene expression likely requires NADPH oxidase, elevated H_2O_2 level, external calcium influx and *de novo* synthesized proteins. Based on these data, we conclude for the first time that an ethephon-inducible catalase *SPCAT1* was cloned and characterized from sweet potato leaves. Sweet potato catalase *SPCAT1* may function in the assistance of reactive oxygen species H_2O_2 homeostasis in leaves in order to cope with developmental cues and/or environmental stimuli.

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Legends of figures

Fig. 1. The nucleotide and deduced amino acid sequences of full-length cDNA of sweet potato catalase *SPCAT1*. The left was the number of nucleotide sequence and the right was the number of deduced amino acid sequence. ATG (underlined) and TAA (underlined) represent the start codon and the stop codon, respectively. The 65-His (H), 104-Ser (S) and 138-Asn (N) printed in white on black represent the conserved catalytic amino acid residues. The amino acid residues from 415-Gly (G) to 451-Val (V) printed in black on gray represent the putative calmodulin binding domain of catalase. The C-terminal 480-Gln (Q), 481-Lys (K) and 482-Val (V) amino acid residues printed in white on black represent the putative conserved internal peroxisomal targeting signal PTS1.

Fig. 2. Amino acid sequence alignment of putative conserved internal peroxisomal targeting signal PTS1 motif and calmodulin binding domain of plant catalases. (A) peroxisomal targeting signal PST1 motif. (B) Calmodulin binding domain. Plant catalases used for analysis include *Arabidopsis* CAT1 (accession no. Q96528), *Arabidopsis* CAT2 (accession no. P25819), *Arabidopsis* CAT3 (accession no. Q42547), eggplant CAT (accession no. X71653), grey mangrove CAT1 (accession no. Q53ZT2), pea CAT (accession no. P25890), potato CAT1 (accession no. U27082), pumpkin CAT1 (accession no. D55645), pumpkin CAT2 (accession no. D55646), pumpkin CAT3 (accession no. D55647), sweet potato catalase (X05549), sweet potato SPCAT1 (accession no. GU230147), tobacco CAT1 (accession no. Z36975), tobacco CAT2 (accession no. Z36976), tobacco CAT3 (accession no. Z36977), and tomato CAT1 (accession no. M93719).

Fig. 3. Phylogenetic tree analysis of sweet potato catalase SPCAT1 with other plant catalases, including *Arabidopsis* CAT1 (accession no. Q96528), *Arabidopsis* CAT2 (accession no. P25819), *Arabidopsis* CAT3 (accession no. Q42547), eggplant CAT (accession no. X71653), grey mangrove CAT1 (accession no. Q53ZT2), pea CAT (accession no. P25890), potato CAT1 (accession no. U27082), sweet potato SPCAT1 (accession no. GU230147), tobacco CAT1 (accession no. Z36975), tobacco CAT2 (accession no. Z36976), tobacco CAT3 (accession no. Z36977), and tomato CAT1 (accession no. M93719).

Fig. 4. Temporal and spatial gene expression of sweet potato catalase *SPCAT1*. (A) Leaf morphology and chlorophyll content. (B) Photochemical Fv/Fm. (C) Temporal expression of sweet potato catalase *SPCAT1* in different leaf developmental stages. (D)

Qualitative DAB staining and quantitative determination of H_2O_2 amount. The leaf morphology, chlorophyll content, photochemical Fv/Fm, H_2O_2 determination, and gene expression of sweet potato catalase *SPCAT1* with RT-PCR were analyzed in different leaf developmental stages (L1, L2, L3, L4 and L5) of sweet potato. Stages of L1 to L5 leaves were described in "Materials and methods". The data were shown as mean ± S.E.. The experiments were performed three times and a representative one was shown.

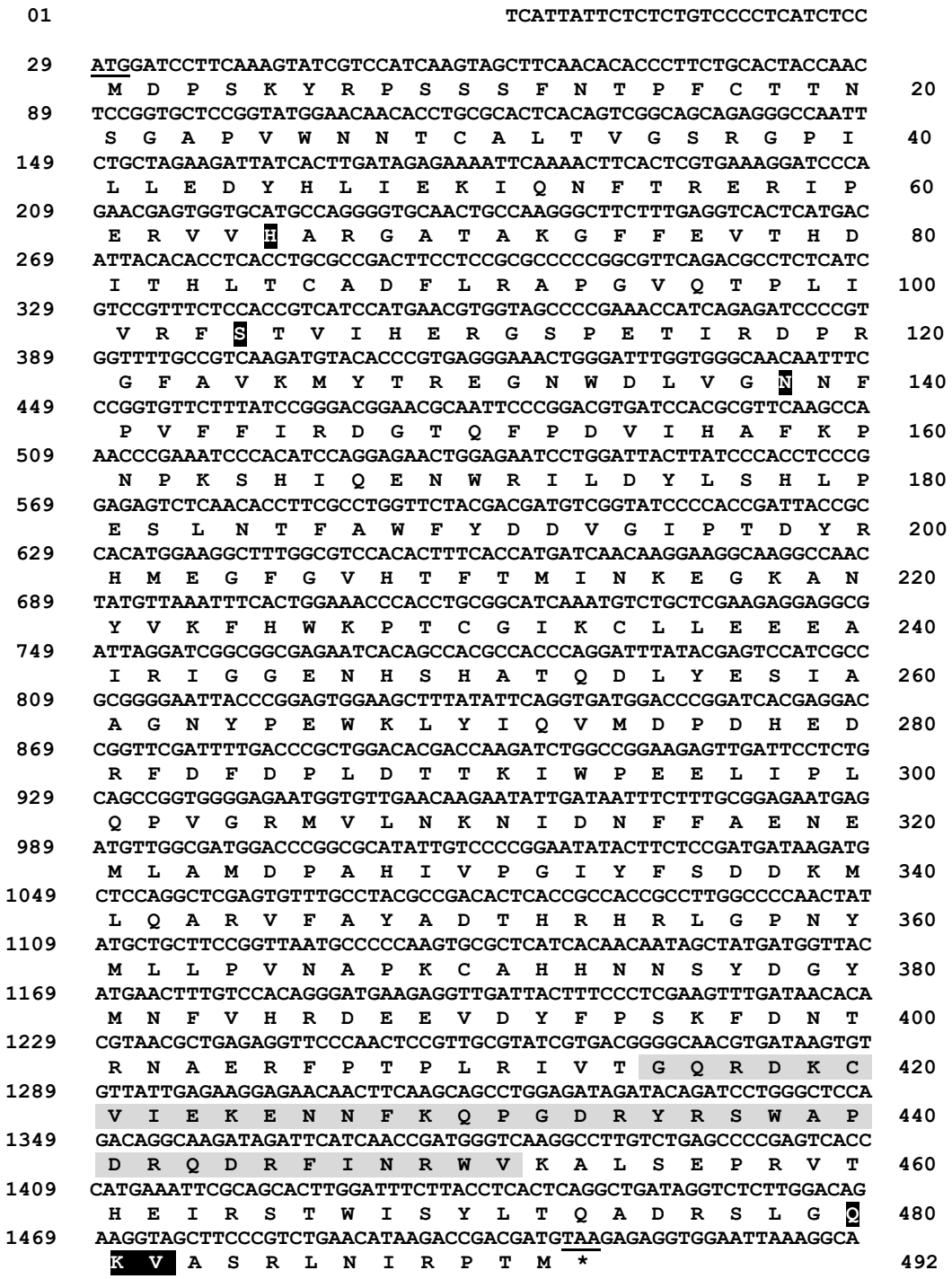
Fig. 5. Effects of dark and ethephon on sweet potato catalase *SPCAT1* gene expression. (A) Leaf morphology. (B) Chlorophyll content. (C) Photochemical Fv/Fm. (D) RT-PCR products of sweet potato catalase *SPCAT1*. (E) DAB staining and (F) H_2O_2 amount. The leaf morphology, chlorophyll content, photochemical Fv/Fm, H_2O_2 determination, and gene expression of sweet potato catalase *SPCAT1* with RT-PCR were analyzed in detached L3 mature leaves treated with dark and 1 mM ethephon for 0, 6 h, 24 h, 48 h and 72 h, respectively. D and E denote dark and ethephon treatments, respectively. The data were shown as mean ± S.E.. The experiments were performed three times and a representative one was shown.

Fig. 6. Effects of antioxidant reduced glutathione, NADPH oxidase inhibitor DPI, calcium ion chelator EGTA, and *de novo* protein synthesis inhibitor cycloheximide on ethephon-induced sweet potato catalase *SPCAT1* gene expression. (A) Reduced glutathione. (B) DPI. (C) EGTA. (D) Cycloheximide. Detached L3 mature leaves were pretreated with (+) or without (-) 1 mM reduced glutathione, 100 μM DPI, 5 mM EGTA, or 20 μg/mL cycloheximide for ca. 15 to 30 min prior to 1 mM ethephon treatment. D and E denote dark and ethephon treatment, respectively, for 24 h and 72 h. The experiments were performed three times and a representative one was shown.

Fig. 7. Induction, expression and purification of sweet potato catalase SPCAT1 fusion protein and its influence on ethephon-mediated leaf senescence and H_2O_2 level. (A) Induction and expression of sweet potato catalase SPCAT1 fusion protein in *E. coli* BL21 (DE3) cells harboring recombinant PET30a vector by 1 mM IPTG inducer for 5 hours. The recombinant PET30a vector contains a full-length catalase *SPCAT1* cDNA insert from initiation codon to stop codon. (B) Purification of catalase SPCAT1 fusion protein. The lanes of 1, 2, 3, 4 and 5 represent crude extract of catalase SPCAT1 fusion protein from *E. coli* BL21 (DE3) cells after 1 mM IPTG induction for 5 hours, the unbound fraction after the crude extract applied to the affinity column, eluent after wash with denaturing wash buffer C, eluent after wash with denaturing elution buffer D, eluent after wash for the first time with denaturing elution buffer E, and eluent after

wash for the second time with denaturing elution buffer E, respectively. The arrowhead indicates the expressed catalase SPCAT1 fusion proteins. Effects of exogenous application of purified catalase SPCAT1 fusion protein on ethephon-mediated leaf senescence and H_2O_2 elevation were also shown. (C) Leaf morphology. (D) Chlorophyll content. (E) Photochemical Fv/Fm. (F) H_2O_2 amount. The detached L3 mature leaves treated with dark and 1 mM ethephon plus purified catalase SPCAT1 fusion proteins (0, 0.2 and 2.0 mg, respectively) for 3 days. D and E denote dark and ethephon, respectively. These data were shown as mean ± S.E.. The experiments were performed three times and a representative one was shown.

Figure 1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L L E D Y H L I E K I Q N F T R E R I P 60
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E R V V H A R G A T A K G F F E V T H D 80
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I T H L T C A D F L R A P G V Q T P L I 100
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G F A V K M Y T R E G N W D L V G N N F 140
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P V F F I R D G T Q F P D V I H A F K P 160
509 AACCCGAAATCCCACATCCAGGAGAACTGGAGAATCCTGGATTACTTATCCCACCTCCCG
N P K S H I Q E N W R I L D Y L S H L P 180
569 GAGAGTCTCAACACCTTCGCCTGGTTCTACGACGATGTCGGTATCCCCACCGATTACCGC
E S L N T F A W F Y D D V G I P T D Y R 200
629 CACATGGAAGGCTTTGGCGTCCACACTTTCACCATGATCAACAAGGAAGGCAAGGCCAAC
H M E G F G V H T F T M I N K E G K A N 220
689 TATGTTAAATTTCACTGGAAACCCACCTGCGGCATCAAATGTCTGCTCGAAGAGGAGGCG
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I R I G G E N H S H A T Q D L Y E S I A 260
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V I E K E N N F K Q P G D R Y R S W A P 440
1349 GACAGGCAAGATAGATTCATCAACCGATGGGTCAAGGCCTTGTCTGAGCCCCGAGTCACC
D R Q D R F I N R W V K A L S E P R V T 460
1409 CATGAAATTCGCAGCACTTGGATTTCTTACCTCACTCAGGCTGATAGGTCTCTTGGACAG
H E I R S T W I S Y L T Q A D R S L G Q 480
1469 AAGGTAGCTTCCCGTCTGAACATAAGACCGACGATGTAAGAGAGGTGGAATTAAAGGCA
K V A S R L N I R P T M * 492

Figure 2

A. Peroxisomal targeting signal (PTS1)

```
D55646     EALSDSKVTHEVRNIWISYWTQADRSLGQKIASRMNARPNM
D55647     EALSDPRVTDEVRNIWISYWSQADRSLGQKIASRLNVRPNI
Z36977     DALSDPRVTHEIRSIWFSYWSQADKTLGQKIASRLNVRPTM
D55645     DALSDTRVTHEIRSIWISYWSQADRSLGQKLASHLNVRPSI
P25819     DALSDPRITHEIRSIWISYWSQADKSLGQKLASRLNVRPSI
P25890     LSDTDPRITHEIRSIWVSYWSQADRSLGQKLASHLNMRPSI
Q42547     DILSEARLTHEIRGIWTSYWLKADRSLGQKLASRLNVRPSM
Q53ZT2     DALSDPRLTLEIRSIWVSYWSQADKSFGQKLASRLNVRPTM
Q96528     EALWEPRVTHEIRSIWISYWSQADKSLGQKLATRLNVRPNF
Z36975     EALSDPRITYEIRTIWISYWSQADKSLGQKLASRLNVRPSI
GU230147   KALSEPRVTHEIRSTWISYLTQADRSLGQKVASRLNIRPTM
M93719     ESLSDPRVTHEIRSIWISYLSQADKSCGQKVASRLTVKPTM
U27082     ESLSDPRVTHEIRSIWISYLSQADKSCGQKVASRLTVKPTM
X71653     ESLSDPRVTHEIRSIWISYLSQADKSCGQKVASRLLVKPTM
X05549     KALSEPRVTHEIRSTWISYLTQADRSLGQKVASRLNIRPTM
Z36976     EHLSDPRVTYEIRSIWISYLSQADKSCGQKVASRLTLKPTM
```

B. Calmodulin binding domain

```
D55646     LSGKRERCVIPKENHNFKQAGDRYRSWAPDRQERFVNRFVEA
D55647     LTGKRERCVIPKEN NFKQAGDRYRSWAPDRQDRFVKRFVEA
Z36977     LTGKRDKCIIEKENN-FKQPGERYRSWAPDRQERFICRWVDA
D55645     CSGKRERCIIEKENN-FKEPGERYRSWTPDRQERFVRRWVDA
P25819     CSGKRERCIIEKENN-FKEPGERYRTFTPERQERFIQRWIDA
P25890     LSARREKCNIPKQNH-FKQAGERYRTWAPDRQERFLRRWVEA
Q42547     YTGIRTKCVIKKENN-FKQAGDRYRSWAPDRQDRFVKRWVEI
Q53ZT2     ITGRRDRRVIEKEDN-FKQAGDRYRSWAPDRQERFLRRWVDA
Q96528     CSGNREKCFIGKENN-FKQPGERYRSWDSDRQERFVKRFVEA
Z36975     CTGKREKCVIQKENN-FKQPGERYRSFTPDRQERFIRRWVET
GU230147   VTGQRDKCVIEKENN-FKQPGDRYRSWAPDRQDRFINRWVKA
M93719     LNGRRTNCVIPKENN-FKQAGERYRSWEPDRQDRYINKWVES
U27082     LNGRRTNCVIPKENN-SKQAGERYRSWESDRQDRYINKWVES
X71653     LTGRREKCVIPKENN-FKQAGERYRTWEPDRQDRYINKWVES
X05549     VTGQRDKCVIEKENN-FKQPGDRYRSWAPDRQDRFINRWVKA
Z36976     LNGRREMCVIEKENN-FKQAGERYRSWEPDRQDRYVSKWVEH
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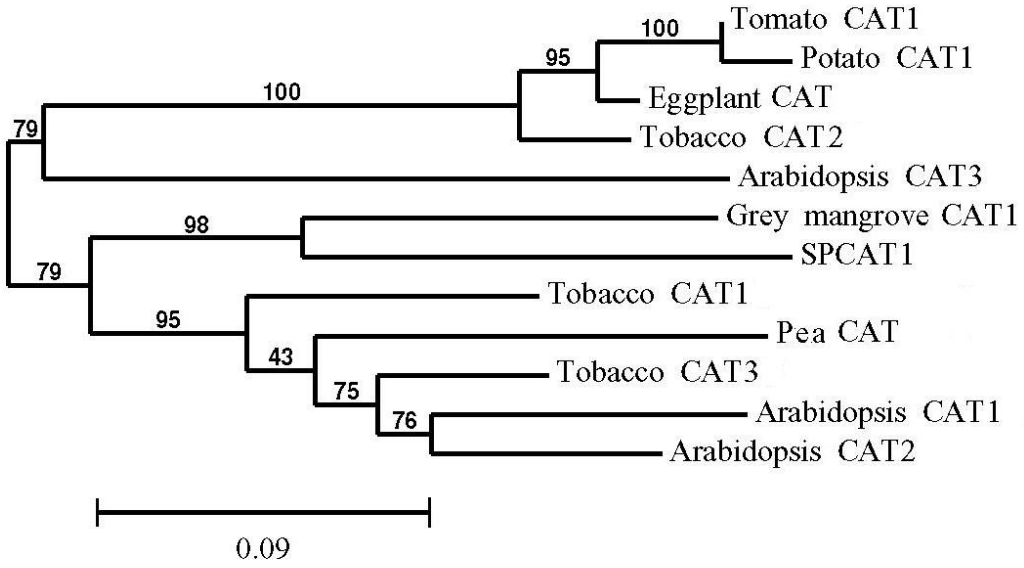
Figure 3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Arabidopsis CAT1
Arabidopsis CAT2
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95
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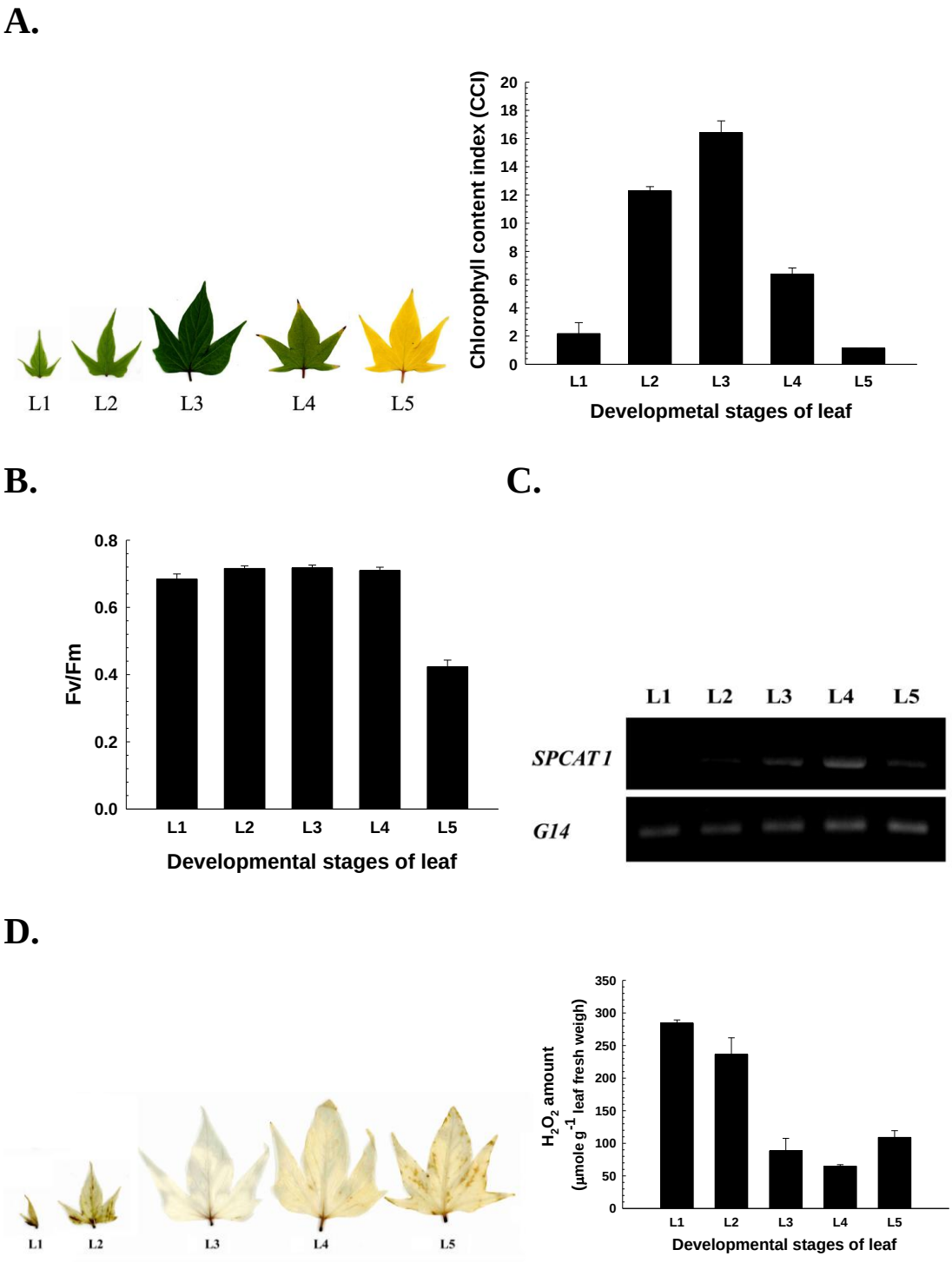
98
79
95
43
75
76
0.09

Figure 4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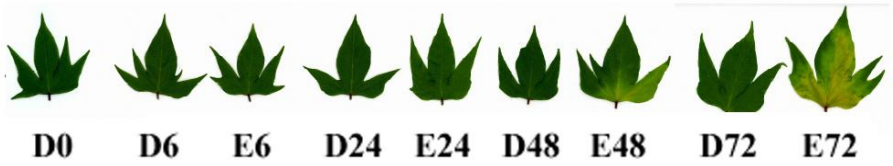
L1
L2
L3
L4
L5
Chlorophyll content index (CCI)
0
2
4
6
8
10
12
14
16
18
20
L1
L2
L3
L4
L5
Developmetal stages of leaf
B.
Fv/Fm
0.0
0.2
0.4
0.6
0.8
L1
L2
L3
L4
L5
Developmental stages of leaf
C.
L1
L2
L3
L4
L5
SPCAT1
G14
D.
L1
L2
L3
L4
L5
H2O2 amount
(μmole g-1 leaf fresh weigh)
0
50
100
150
200
250
300
350
L1
L2
L3
L4
L5
Developmental stages of leaf

Figure 5

A.



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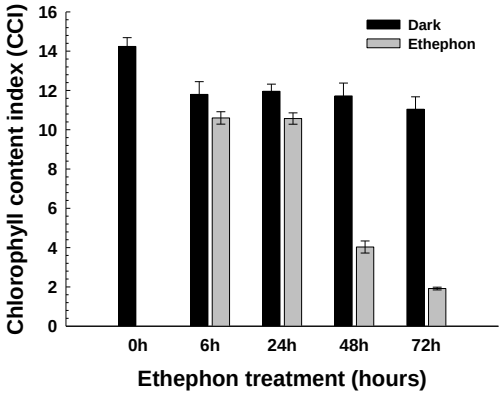

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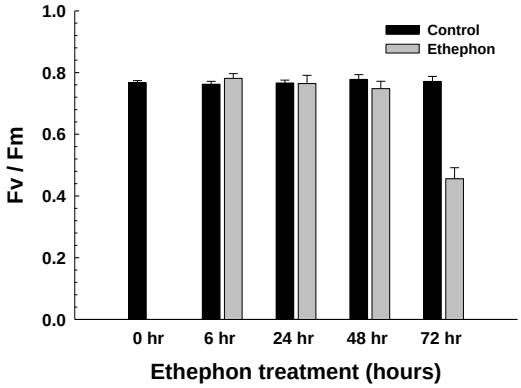

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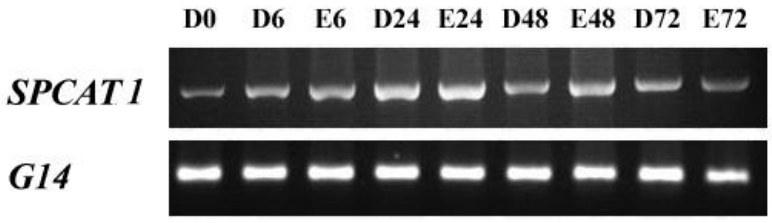


E.

F.

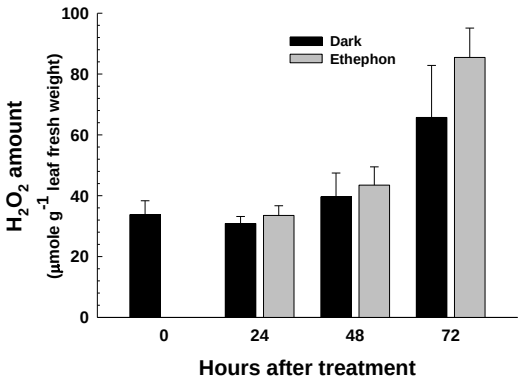
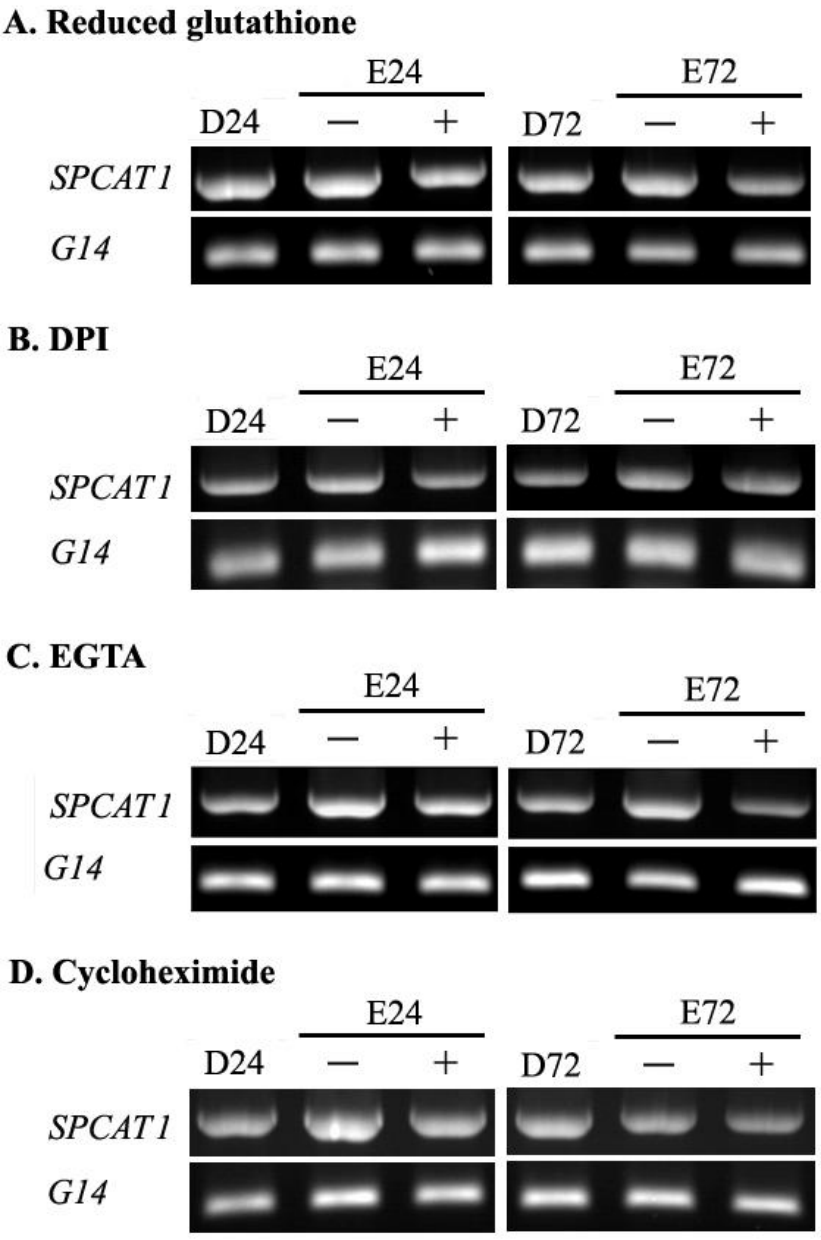


Figure 6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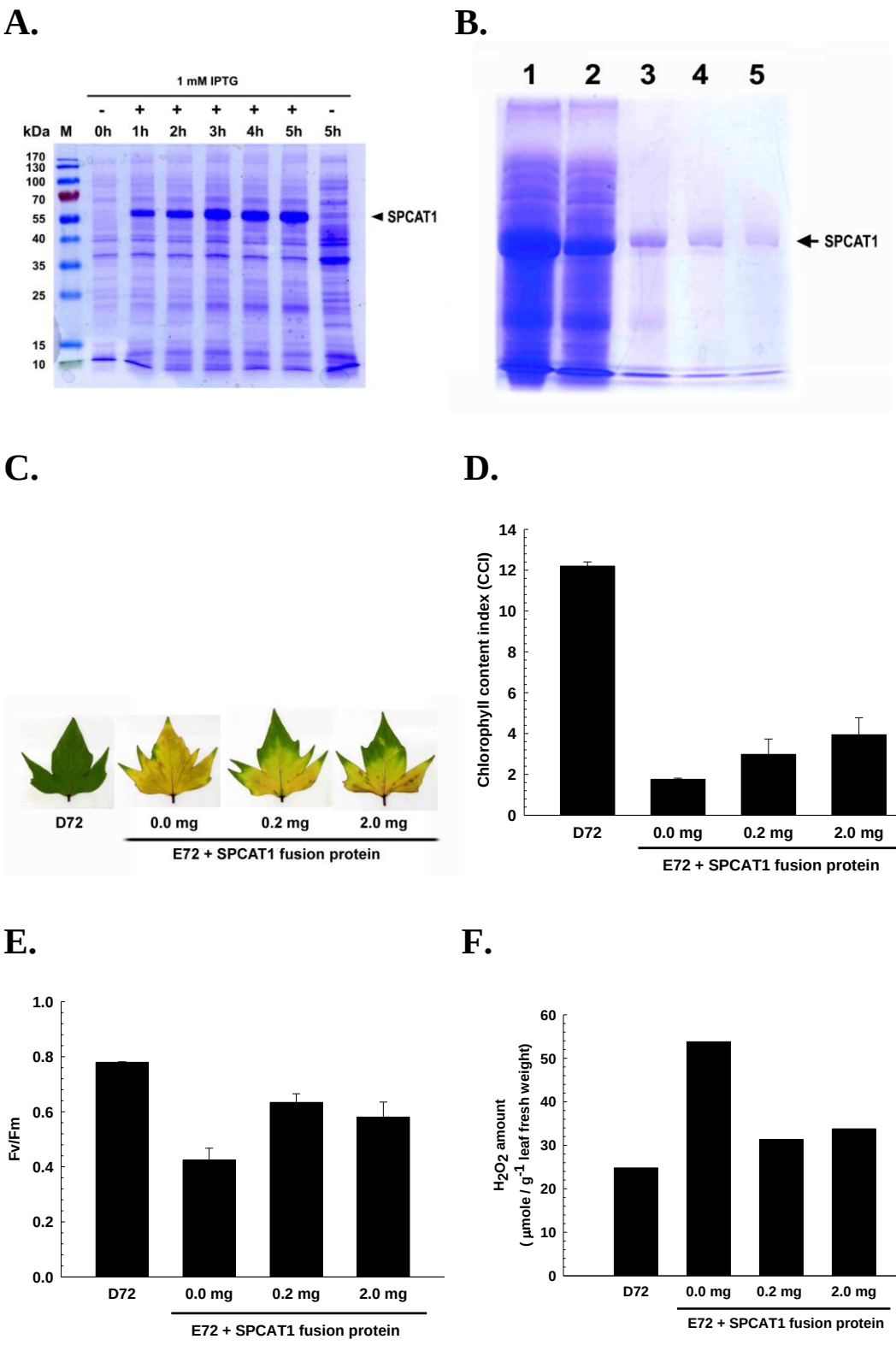
E72
D24
—
+
D72
—
+
SPCAT1
G14
B. DPI
E24
E72
D24
—
+
D72
—
+
SPCAT1
G14
C. EGTA
E24
E72
D24
—
+
D72
—
+
SPCAT1
G14
D. Cycloheximide
E24
E72
D24
—
+
D72
—
+
SPCAT1
G14

Figure 7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1 mM IPTG
- + + + + + -
kDa M 0h 1h 2h 3h 4h 5h 5h
170
130
100
70
55
40
35
25
15
10
SPCAT1
B.
1 2 3 4 5
SPCAT1
C.
D72
0.0 mg
0.2 mg
2.0 mg
E72 + SPCAT1 fusion protein
D.
Chlorophyll content index (CCI)
14
12
10
8
6
4
2
0
D72
0.0 mg
0.2 mg
2.0 mg
E72 + SPCAT1 fusion protein
E.
Fv/Fm
1.0
0.8
0.6
0.4
0.2
0.0
D72
0.0 mg
0.2 mg
2.0 mg
E72 + SPCAT1 fusion protein
F.
H2O2 amount
(μmole / g-1 leaf fresh weight)
60
50
40
30
20
10
0
D72
0.0 mg
0.2 mg
2.0 mg
E72 + SPCAT1 fusion protein