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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. 71.2–80.9%) 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.

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 Rio, 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

Abbreviations: 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.

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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, 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 (Zimmermann 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 °C/16 h day and 23 °C/8 h night cycle. Plantlets sprouted from the storage roots provided detached mature leaves for dark and ethephon treatments at 28 °C/16 h and 23 °C/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 reverse transcription-polymerase chain reaction (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 h 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