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Catalase activity is modulated by calcium and calmodulin in detached mature leaves of sweet potato

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ABSTRACT

Catalase (CAT) functions as one of the key enzymes in the scavenging of reactive oxygen species and affects the H_2O_2 homeostasis in plants. In sweet potato, a major catalase isoform was detected, and total catalase activity showed the highest level in mature leaves (L3) compared to immature (L1) and completely yellow, senescent leaves (L5). The major catalase isoform as well as total enzymatic activity were strongly suppressed by ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). This inhibition could be specifically and significantly mitigated in mature L3 leaves by exogenous $CaCl_2$, but not $MgCl_2$ or $CoCl_2$. EGTA also inhibited the activity of the catalase isoform *in vitro*. Furthermore, chlorpromazine (CPZ), a calmodulin (CAM) inhibitor, drastically suppressed the major catalase isoform as well as total enzymatic activity, and this suppression was alleviated by exogenous sweet potato calmodulin (SPCAM) fusion protein in L3 leaves. CPZ also inhibited the activity of the catalase isoform *in vitro*. Protein blot hybridization showed that both anti-catalase SPCAT1 and anti-calmodulin SPCAM antibodies detect a band at the same position, which corresponds to the activity of the major catalase isoform from unboiled, but not boiled crude protein extract of L3 leaves. An inverse correlation between the major catalase isoform/total enzymatic activity and the H_2O_2 level was also observed. These data suggest that sweet potato CAT activity is modulated by $CaCl_2$ and SPCAM, and plays an important role in H_2O_2 homeostasis in mature leaves. Association of SPCAM with the major CAT isoform is required and regulates the in-gel CAT activity band.

Introduction

Catalase (CAT) has long been considered as one of the major antioxidant enzymes and functions mainly in the removal of excessive H_2O_2 by conversion to water and oxygen in all aerobic organisms. CAT is generally composed of a multigene family and has been reported in many plant species, including *Arabidopsis* (Frugoli et al., 1996), barley (Skadsen et al., 1995), cotton (Ni et al., 1990), maize (Guan and Scandalios, 1995), sunflower (Eising et al., 1990), and tobacco (Havir et al., 1996). Different isoforms respond differentially to developmental cues and environmental stimuli (Guan and Scandalios, 1995; Zimmermann et al., 2006; Du et al., 2008). In *Arabidopsis*, three CAT isoforms (CAT1, CAT2 and CAT3) were detected in activity gels (Zimmermann et al., 2006; Du et al., 2008). CAT2 was found to be the major and predominant isoform in leaves of vegetative mature plants, and to became gradually less in leaves of reproductive and senescent plants. Isoforms CAT3 and CAT1 were much less enhanced during leaf senescence (Zimmermann et al., 2006). In tobacco, there were also three CAT isoforms (CAT-1, CAT-2 and CAT-3) identified, and CAT-1 was found to be the predominant one in mature leaves (Havir and McHale, 1987; Havir et al., 1996). The various isoforms were differentially detected in veinal and interveinal tissues of leaves (Niewiadomska et al., 2009). In sweet potato, a major CAT activity band was also found in mature leaves, and temporarily enhanced by ethephon, an ethylene-releasing compound (Chen et al., 2011). An ethephon-inducible CAT *SPCAT1*, which exhibited characteristics similar to the major CAT has been recently cloned (Chen et al., 2012b).

CATs affect various physiological functions *via* the modulation of H_2O_2 homeostasis at different developmental stages or under environmental stresses (Mhamdi et al., 2010). Therefore, a light-dependent source of H_2O_2 *via* photorespiration in the peroxisomes acts as a signal molecule and its concentration is regulated by CAT. In the *Arabidopsis* CAT2 mutant (*cat2*), severe reduction of growth

Abbreviations: CAM, calmodulin; CAT, catalase; CCI, chlorophyll content index; CPZ, chlorpromazine; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPCAM, sweet potato calmodulin.

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and biomass, induction of defense gene expression, increase of cell death and lesion development were observed and correlated with the oxidative stress generated from photorespiration under different day length condition (Queval et al., 2007). Transgenic tobacco plants expressing an antisense construct of CAT1 showed severely reduced CAT activity, a higher level of pathogenesis-related protein 1 and enhanced resistance to tobacco mosaic virus (Takahashi et al., 1997). In sweet potato, ethephon-mediated promotion of H_2O_2 generation and leaf senescence could be attenuated by exogenous SPCAT1 fusion protein (Chen et al., 2012b). CATs also function as a cellular sink for H_2O_2, and are essential for stress tolerance (Willekens et al., 1997). Therefore, increased susceptibility to abiotic stress, including paraquat, salt, and ozone, was observed in the tobacco CAT1 mutant (*cat1*), which could be complemented by exogenous CAT (Willekens et al., 1997). In cotton, transgenic plants ectopically expressing Cu/Zn superoxide dismutase (*GhSOD1*) and CAT (*GhCAT1*) resulted in synergistic effects on enhanced tolerance to methyl viologen and salt stress (Luo et al., 2013).

Modulation of plant CAT activity has been previously reported and is likely associated with calcium ions and calmodulin (CAM) (Yang and Poovaiah, 2002,2003). Treatment of excised maize stems with different concentrations of $CaCl_2$ for 8 h resulted in the increase of total CAT activity in a dose-dependent manner (Jiang and Zhang, 2003). In wheat, hardening of plantlets with $CaCl_2$ increased total CAT activity (Agarwal et al., 2005; Kolupaev et al., 2008), and the survival rate under heat stress (Kolupaev et al., 2008). Pretreatment of apple fruits with $CaCl_2$ also increased total CAT activity, reduced ethylene biosynthesis, prolonged the shelf-life and improved fruit quality during post-harvest storage (Shirzadeh et al., 2011). In mustard, $CaCl_2$ enhanced total CAT activity and improved tolerance to salt stress in excised leaves (Khan et al., 2012). Total CAT activity and the reduction of the glutathione content were much lower in Ca-deficient rice leaves, which exhibited enhanced toxicity to Cd as compared to control leaves (Cho et al., 2012). In *Amaranthus lividus*, $CaCl_2$ elevated total CAT activity in shoots and roots under both heat and chilling stresses (Bhattacharjee, 2009). Maize leaves treated with abscisic acid (ABA) or H_2O_2 showed significantly enhanced total CAT activity, possibly *via* a CAM-associated mechanism (Xu, 2010). In tobacco, relative CAT activity was drastically reduced *in vitro* in the absence of exogenous $CaCl_2$ or potato CAM PCM6 fusion protein (Yang and Poovaiah, 2002). These reports suggest the possible association of $CaCl_2$ and CAM with the modulation of plant CAT activity.

Sweet potato CAT has been reported in storage roots and leaves. For storage roots, CAT has been partially purified with ammonia sulfate and detected with anti-CAT antibody (Esaka and Asahi, 1982; Sakajo and Asahi, 1986). A full-length cDNA encoding the corresponding CAT has also been cloned (Sakajo et al., 1987a) and shown to be inducible in wounded root tissue (Sakajo et al., 1987b). In different developmental stages of leaves, a major CAT isoform was identified by in-gel activity assay. Its enzymatic activity was the highest in mature leaves and could be temporarily enhanced by ethephon and darkness (Chen et al., 2011). A full-length cDNA of ethephon-inducible *SPCAT1* has been cloned from leaves, which exhibited similar expression pattern with the major CAT isoform and could be repressed by ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Chen et al., 2012b). Its open reading frame also contained a conserved CAM binding domain found in CATs of different plant species, including *Arabidopsis* and tobacco (Chen et al., 2012b). In this manuscript, we report for the first time that the major CAT isoform and total enzymatic activity are modulated by $CaCl_2$ and sweet potato calmodulin (SPCAM), and are inversely correlated with the H_2O_2 level in sweet potato mature leaves. Association of sweet SPCAM with the major CAT isoform is required and regulates the in-gel CAT activity band.

Materials and methods

Plant materials

The storage roots of sweet potato [*Ipomoea batatas* (L.) Lam.] were grown in a growth chamber at 28 °C/16 h day and 23 °C/8 h night cycle. Plantlets sprouted from the storage roots provided (a) different developmental stages of leaves for temporal expression experiments, and (b) detached mature leaves for different effector treatments. Leaves were arbitrarily divided into L1 to L5 according to their size and developmental stage: L1 has folded, unopened immature leaves; L2 unfolding but not fully expanded leaves; L3 fully expanded mature leaves; L4 and L5 partially or completely yellow senescent leaves, respectively (Chen et al., 2011). Samples collected from L1, L3 and L5 stages were used for catalase (CAT) activity assays and protein blot hybridization. L3 leaves were mainly collected between the 3rd and the 7th positions counted downward from the shoot apex. Detached L3 leaves were also used for treatments with different effectors, including ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), $CaCl_2$/$MgCl_2$/$CoCl_2$, chlorpromazine (CPZ) and sweet potato calmodulin (SPCAM) fusion protein for inhibition and reversion of CAT activity. Leaf morphology, chlorophyll content, Fv/Fm and H_2O_2 amount were analyzed simultaneously as described below.

EGTA and $CaCl_2$ treatments

EGTA, a Ca^{2+} ion chelator, was used to study the effect of Ca on the major CAT isoform and total enzymatic activity of sweet potato leaves in both *in vivo* and *in vitro* conditions. For *in vivo* EGTA inhibition of the CAT activity assay, detached L3 leaves were placed on wet paper towels containing 3 mM 2-(N-morpholino)-ethane sulphonic acid (MES) buffer pH 5.8 plus different concentrations of EGTA (0, 0.1, 0.2 and 0.5 mM), and kept at 28 °C in the dark for 4 d (Khan et al., 2010). Leaves were individually collected for analysis of leaf morphology, chlorophyll content, Fv/Fm, qualitative and quantitative CAT activity, and H_2O_2. For *in vitro* EGTA repression of the CAT activity assay, different concentrations of EGTA (0, 0.1, 0.2 and 0.5 mM) were added directly into the crude protein extract (2 μg) from L3 leaves and incubated at 4 °C for 2 d before qualitative in-gel CAT activity assay with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For the reversion of EGTA-mediated effects, detached leaves were placed on wet paper towels containing 3 mM MES buffer pH 5.8, 0.5 mM EGTA and different concentrations of $CaCl_2$ (0, 1 and 2 mM) for 4 d. For the specificity of reversion on EGTA-mediated effects, the same method was used except that $CaCl_2$ was replaced by 2 mM $MgCl_2$, or $CoCl_2$. Leaves were individually collected and analyzed for leaf morphology, chlorophyll content, Fv/Fm, in-gel CAT activity, and H_2O_2.

CPZ and SPCAM fusion protein treatments

CPZ, a calmodulin (CAM) inhibitor, and SPCAM fusion protein were used to study their effects on the major CAT isoform and total enzymatic activity. For *in vivo* CPZ inhibition of the CAT activity assay, L3 leaves were detached and placed on a wet paper towel containing 3 mM MES buffer pH 5.8 supplemented with different concentrations of CPZ (0, 1, 3 and 10 μM), then kept in the dark for 3 h (Khan et al., 2010). Leaves were individually collected for analysis of leaf morphology, chlorophyll content, Fv/Fm, qualitative and quantitative CAT activity and H_2O_2. For *in vitro* CPZ inhibition