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Two NCA1 isoforms interact with catalase in a mutually exclusive manner to redundantly regulate its activity in rice

Jianzhe Liu[†], Lili Cui[†], Zongwang Xie[†], Zhisheng Zhang, Ee Liu and Xinxiang Peng^{*}

Abstract

Background: NCA1 (NO CATALASE ACTIVITY 1) was recently identified in *Arabidopsis* as a chaperone protein to regulate catalase (CAT) activity through maintaining the folding of CAT. The gene exists mainly in higher plants; some plants, such as *Arabidopsis*, contain only one *NCA1* gene, whereas some others such as rice harbor two copies. It is not yet understood whether and how both isoforms have functioned to regulate CAT activity in those two-copy-containing plant species.

Results: In this study, we first noticed that the spatiotemporal expression patterns of *NCA1a* and *NCA1b* were very similar in rice plants. Subsequent BiFC and yeast three-hybrid experiments demonstrated that both *NCA1a* and *NCA1b* show mutually exclusive, rather than simultaneous, interaction with CAT. For a further functional analysis, *nca1a* and *nca1b* single mutants or double mutants of rice were generated by CRISPR/Cas9. Analysis on these mutants under both normal and salinity stress conditions found that, as compared with WT, either *nca1a* or *nca1b* single mutant showed no difference at phenotypes and CAT activities, whereas the double mutants constantly displayed very low CAT activity (about 5%) and serious lesion phenotypes.

Conclusions: These results suggest that *NCA1a* and *NCA1b* show mutually exclusive interaction with CAT to regulate CAT activity in a functionally-redundant manner in rice.

Keywords: Catalase, Function, Interaction, Isoforms, NCA1, Rice

Background

Catalase (CAT) is a heme-containing enzyme which typically catalyzes the dismutation of H₂O₂ to produce H₂O and O₂ in plants [1]. CAT usually consists of polypeptides of 50–70 kDa in mass that are organized into tetramers, with each monomer bearing a heme prosthetic group [2]. Known to date, all plants, including monocots and dicots such as tobacco, *Arabidopsis*, maize, pumpkin, and rice, contain three CAT genes [1]. The three genes of *Arabidopsis*, named as *CAT1*, *CAT2*, and *CAT3*, encode three corresponding proteins which consist of 492 amino acids, with high similarity between the amino acid sequences. The three genes, identified in the rice genome, were named as *CATA*, *CATB* and *CATC*,

respectively [3]. Comparisons of gene structures and functions found that *OsCATA* corresponds to *AtCAT3*, *OsCATB* to *AtCAT1*, and *OsCATC* to *AtCAT2* [4].

AtCAT1 is mainly expressed in pollen and seeds, while *AtCAT2* is predominantly expressed in photosynthetic active tissues and *AtCAT3* in vascular tissues and senescent leaves [4]. CAT is known to exist in the peroxisome although evidence emerged that CAT activity was detected in different organelles [4]. While *AtCAT3* knock-out only slightly reduced CAT activity; deletion of *AtCAT2* reduced the activity by 80%; on the contrary while deletion of *AtCAT1* had no effect on CAT activity [4, 5]. No obvious phenotypes were observed in the *CAT1* or *CAT3*-deficient *Arabidopsis*, whereas the *cat2* mutant displayed defects in many processes, including photorespiration and pathogenesis, salicylic acid-dependent hypersensitive response-like lesion formation [6], autophagy-dependent cell death [7], and altered gene expressions during both biotic and abiotic stresses, including

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cold, heat, and drought [5, 8]. In rice, the role of the three isoforms in catalase activity is poorly understood. Our recent results showed that CATC accounts for majority of CAT activities in rice leaves [9], and its knockout mutant exhibited cell-death phenotype, similar to the *cat2* mutant of *Arabidopsis* [10].

Plant CAT is a key antioxidant enzyme indispensable for plants to cope with adverse environmental stresses, thus, understanding how catalases are regulated is particularly important. Regulation at its transcriptional levels has been extensively investigated and many factors are shown to be involved in the regulation, such as circadian rhythm, reactive oxygen species (ROS), senescence and ABA signaling [11–14]. CAT can be also regulated at the protein level. In *Arabidopsis*, LSD1 (LESION SIMULATING DISEASE 1), a zinc finger protein, affected programmed cell death through interacting with CAT proteins in the cytosol and altering their activities [15, 16]. Hsp17.6CII, a peroxisomal small heat shock protein and chaperone, was shown to interact with CAT2 in peroxisome and to increase CAT activity [17]. In addition, CAT3 activity may be modulated through protein phosphorylation by CPK8 in response to drought stress [18]. Calmodulin [19], nucleoside diphosphate kinase 1 [20], salt overly sensitive 2 (SOS2) [21] and triple gene block protein 1 [22] have been also reported to be able to interact with CAT. But how these interaction proteins regulate CAT is still not well known. NCA1 (NO CATALASE ACTIVITY 1) was recently identified in *Arabidopsis*, which regulates CAT2 activity in response to salt, cold, and high pH. NCA1 acts as a chaperone protein that may fold catalase to a functional structure, the binding of a zinc ion in the N-terminal RING-finger domain of NCA1 is essential for the full function of CAT2 and that the tetratricopeptide repeat (TPR) domain in the NCA1 C terminus mediates interaction with CAT2 [5, 7]. More interestingly, NCA1 interacts with CAT in the cytosol, whereas the CAT function is carried out in the peroxisome [5]. Some plant species contain only one NCA1 gene, while some have two homologs in the genome. Whether the two isoforms in those two-copy-containing plant species function equally or differently in regulating CAT activity and how they realize the function remains to be investigated.

As a monocotyledonous model plant, rice harbors two NCA1 genes, so it is particularly interesting to address the above question in rice. In this study, the rice two NCA1 isoforms were comparatively investigated in terms of function and mechanism. The results demonstrated that NCA1a and NCA1b show mutually exclusive interaction with CAT to regulate its activity in a functionally-redundant manner in rice.

Results

NCA1 genes exist mainly in higher plants

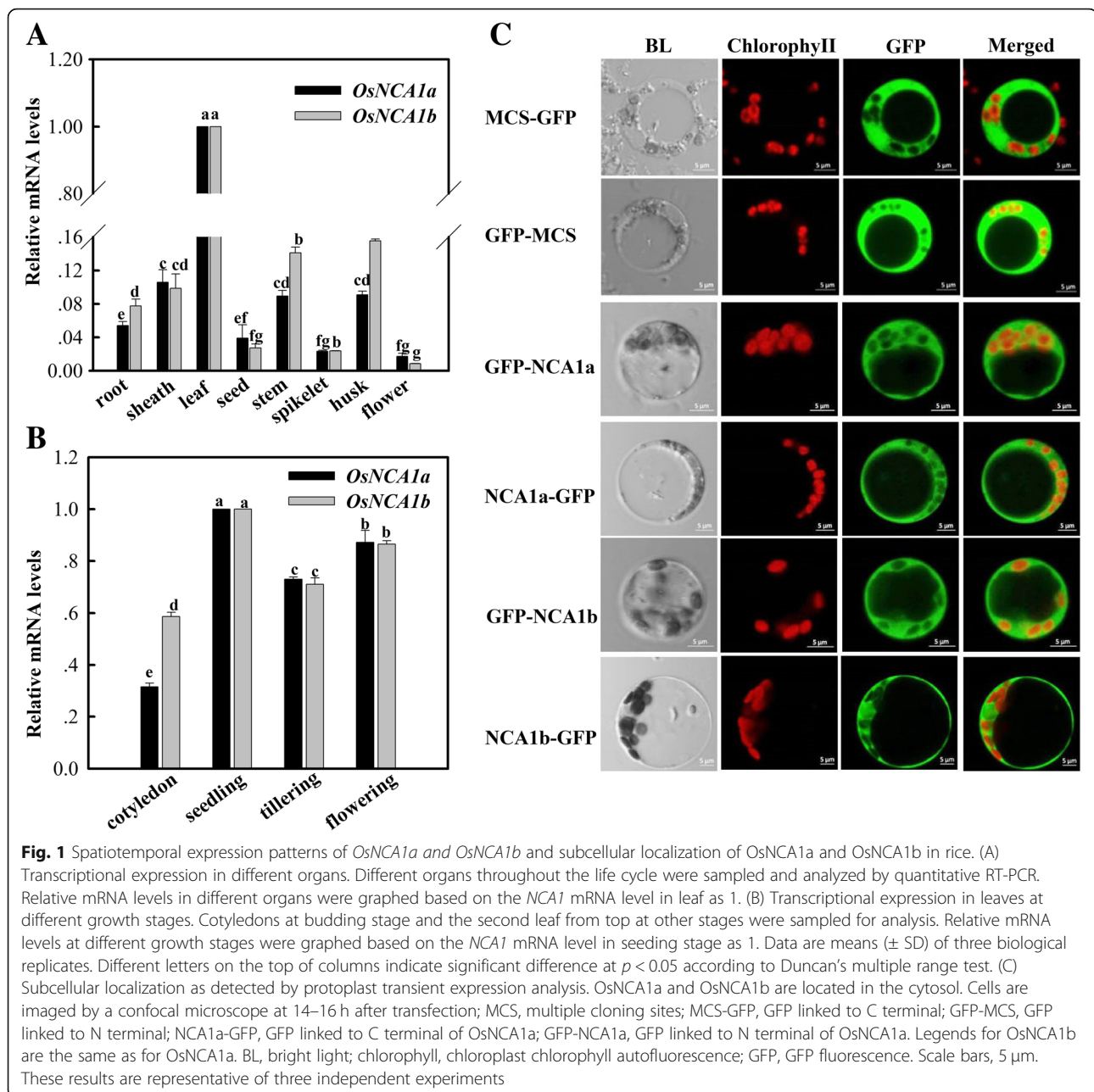
NCA1 was first identified in *Arabidopsis*, with only one gene in its genome [7], whereas two NCA1 genes are identified in the rice genome, hereby named as OsNCA1a (Os01g0104100) and OsNCA1b (Os02g0795300), both the mRNA sequences and polypeptides of these three NCA1 genes are highly similar (Additional file 1 Table S1). To examine the evolution of OsNCA1a and OsNCA1b, a phylogenetic tree was generated using the full-length protein sequences of OsNCA1a and OsNCA1b as input. It was found that some species have one NCA1 gene and some have two. Most of the organisms with the NCA1 gene are higher plants (Additional file 2 Figure S1). No NCA1 homologous gene was found in *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Zosteramarina*, *Spirodelapolyrhiza*, *Sphagnum*, *Ostreococcus lucimarinus*, *Oropetium*, *Micromonas pusilla*, *Marchantiapaleacea*, *Dunaliella Salina*, *Coccomyxa subellipsoidea*.

Spatiotemporal expression patterns and subcellular localization

OsNCA1a and OsNCA1b were distinctly expressed in different organs, with the highest abundance in leaves (Fig. 1a). Similar transcript levels were detected in rice leaves at different growth stages, except lower in the cotyledon (Fig. 1b). It was also noticed that the two genes displayed very similar expression patterns, pointing towards a possibility that OsNCA1a and OsNCA1b may have functioned redundantly. To examine the subcellular localization of the two proteins, both C-terminal and N-terminal GFP-tagged OsNCA1a and OsNCA1b were generated and transfected to rice protoplasts. Confocal microscopy observation revealed that both OsNCA1a and OsNCA1b are localized in the cytosol (Fig. 1c), consistent with the location of AtNCA1 in *Arabidopsis* [5].

OsNCA1a and OsNCA1b can activate CAT equally

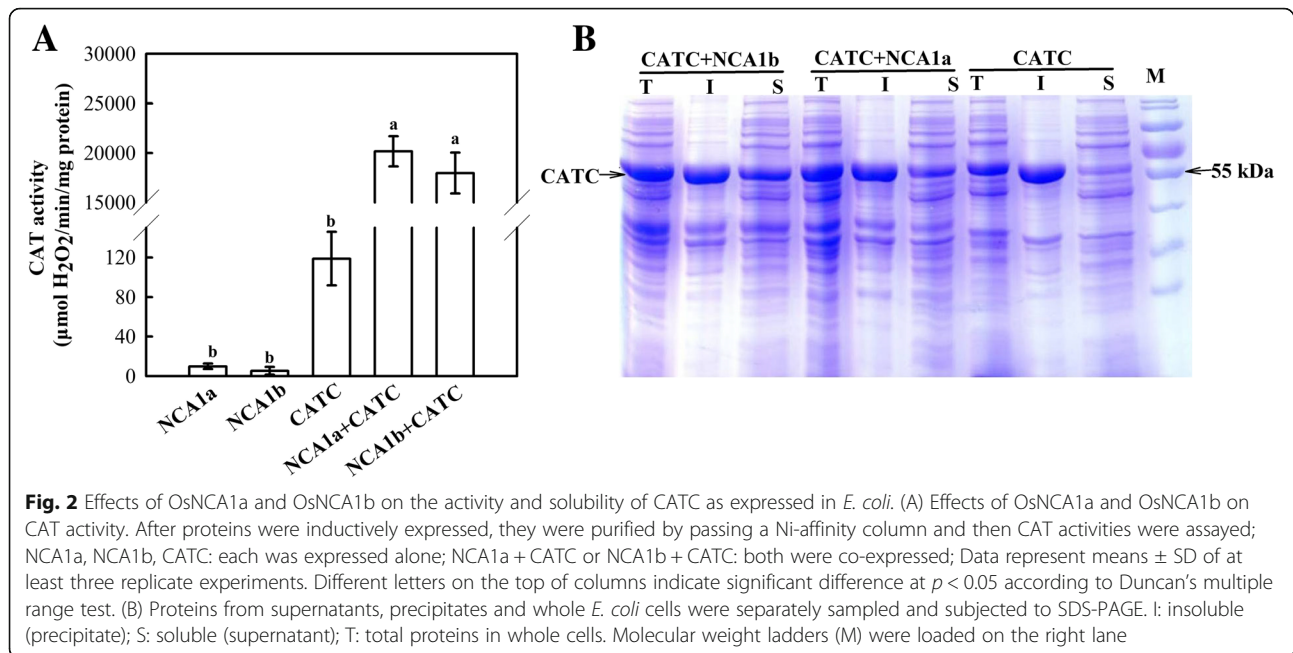
Only one NCA1 gene exists in *Arabidopsis*, which was shown to regulate CAT activity by maintaining the folding of CAT [5], whereas there are two homologs in rice (Additional file 2 Figure S1). Since transcriptional analysis (Fig. 1) has implicated that both NCA1 genes may have functioned similarly in rice, whether the two isoforms can activate CAT equally or differently remains a next interesting question. As shown in Fig. 2a, when OsCATC was expressed alone in *E. coli*, CAT activity was relatively low, with a specific activity of around 120 $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$; when either OsNCA1a or OsNCA1b was co-expressed with OsCATC, CAT activity was increased by more than 160 folds, up to 20,000 $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$. This result



clearly indicates that either *OsNCA1a* or *OsNCA1b* is able to equally activate CAT in rice. It was meanwhile noticed that, when *OsCATC* was expressed alone, most of CAT proteins were contained in the inclusion body as insoluble proteins (Fig. 2b); when either *OsNCA1a* or *OsNCA1b* was co-expressed with *OsCATC*, much more CAT proteins were distributed in the supernatant as soluble proteins (Fig. 2b). This result means that both *OsNCA1a* and *OsNCA1b* can help CAT to fulfill a correct folding, based on the fact that proteins with correct folding will become more soluble and appear in the supernatant in *E. coli*, vice versa [23].

OsNCA1a and *OsNCA1b* show mutually exclusive interaction with CAT

First, the BiFC experiment was conducted using rice protoplast transient expression system. As shown in Fig. 3a, both *NCA1* isoforms can interact with CAT, while interaction did not occur between *NCA1a* and *NCA1b*. The next question is whether both isoforms interact simultaneously, or either interacts alternately, with CAT to realize the function. To answer this question, yeast three-hybrid assay was carried out. The results showed that both *NCA1a* and *NCA1b* could not interact with either *NCA1a* or *NCA1b* (the yeast transformed with



pBridgeBD-NCA1a and pGADT7-NCA1a/pGADT7-NCA1b could not grow on SD-MWLHA media), suggesting that one single molecule of either NCA1a or NCA1b is the functional form to activate CAT (Fig. 3b). Furthermore, the results may also implicate that CAT has only one binding domain to interact with one molecule of NCA1 and is unable to act as a scaffold protein for more than one NCA1 molecule to drive the reporter gene expression in yeast three-hybrid system (the yeast transformed with pBridgeBD-CATC and pGADT7-NCA1a / pGADT7-NCA1b could grow on SD-MWLHA media, while the yeast transformed with pBridgeBD-NCA1a + MCSII-CATC and pGADT7-NCA1a / pGADT7-NCA1b could not grow on SD-MWLHA media; the expression of CATC from MCSII site was verified by CAT activity measurement) (Fig. 3b and c). Thus it can be further inferred that CAT interacts with either NCA1a or NCA1b in a molecular ratio of 1:1, in support of the previous result [5].

Phenotypes and CAT activity of the *nca1a/nca1b* single and double mutants

In order to further reveal the physiological function of *NCA1a* and *NCA1b* in rice, their single and double mutants were generated by CRISPR/Cas9. Sequence alignment of every sgRNA targeting region revealed that the three types of mutants were obtained, respectively, with *NCA1a* or *NCA1b* or both mutated (Fig. 4). Under normal conditions, either the *nca1a* or *nca1b* single mutant showed no any phenotypic difference throughout the growth period as compared with WT, whereas the

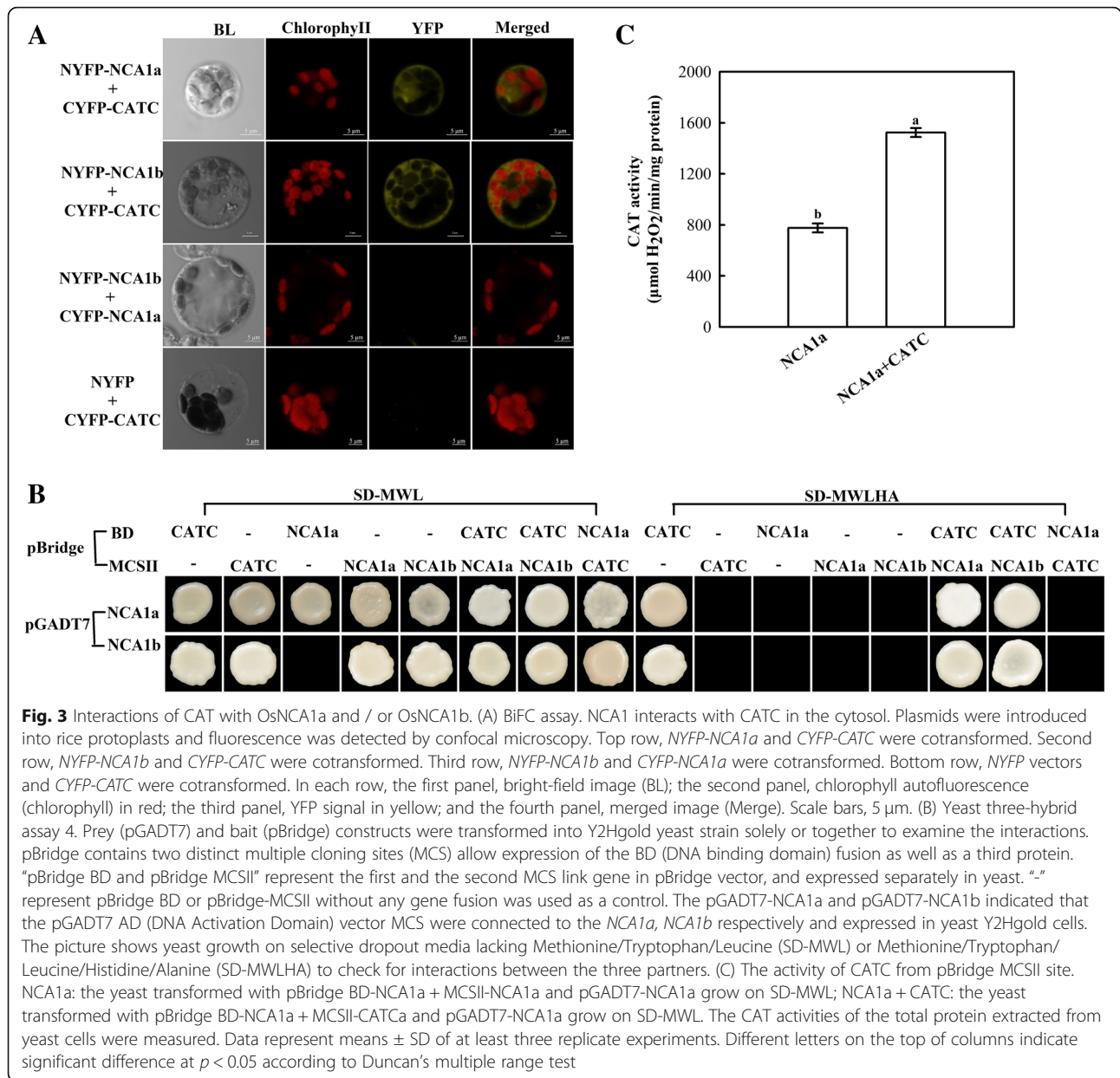
nca1a/nca1b double mutant displayed obvious lesion and even albino symptoms on leaves throughout the growth stages (Fig. 5a). When the double mutant was grown under high CO₂ (3500 ppm), which suppresses photorespiration and hence H₂O₂ production, the lesion phenotype was abolished (Fig. 5b), though CAT activity was still decreased by more than 95% in the double mutant, with no significant difference between air and high CO₂ conditions (Fig. 5c and d). Therefore, the lesion phenotype of the *nca1a/nca1b* double mutant may have resulted from the accumulated photorespiratory H₂O₂.

Effect of salinity stress on the *nca1a/nca1b* single and double mutants

Only one *NCA1* gene exists in *Arabidopsis* and its mutation has been shown to be hypersensitive to salinity [5]. Here we tested effect of salinity stress on the *nca1a/nca1b* single and double knockout mutants of rice. First, phenotypic observation showed that no obvious difference occurred between either of the single mutants and WT under salinity stress, although the double mutant was shown to be more seriously damaged as compared with WT (Fig. 6a). Activity assay showed that the double mutant still had only 5% CAT activity of WT plants, similar to the result under normal condition (data not shown), whereas activity in either of the single mutants was not significantly different from WT plants (Fig. 6b).

Discussion

Catalase (CAT) is an important antioxidant enzyme in various organisms. Usually there exist 3 CAT genes in



OsNCA1a:
WT 1 ATGAGCTC.....377 TCATT.....883 GTGG
nca1a 1 A - - GCTC.....377 TCATT
nca1a/nca1b 1 ATGAGCTC.....377 T - - T.....883 GTGTG

OsNCA1b:
WT 80 CATGTCCTGTC.....183 CTATGAC.....386 TCAT
nca1b 80 CATGTC- - - -.....183 CTATGA(delete 18bp) GCTTGG 212
nca1a/nca1b 80 CATGTCCTGTC.....386 T - T

Fig. 4 Molecular evaluation of the CRISPR-Cas9 generated *nca1a/nca1b* single and double mutants. The nucleotide underlined with "-" indicates mutations with the nucleotide newly inserted. "-" stands for deletion of nucleotides; Numbers with black character indicate the nucleotide position counting from ATG of the open reading frame

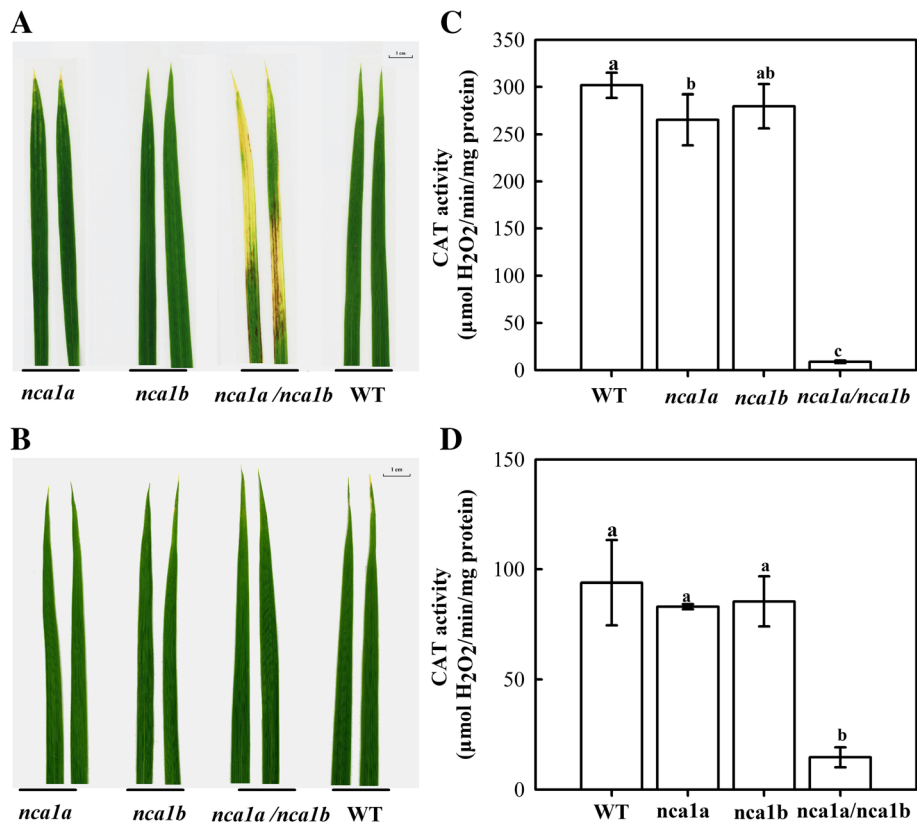


Fig. 5 Phenotypes and CAT activity of the *nca1a/nca1b* single and double mutants under normal air and high CO₂ conditions. The plants were hydroponically grown in an environment-controlled growth chamber for 15 d (temperature: 30 °C day/25 °C night; light intensity: 600 μmol m⁻² s⁻¹; humidity: 60%), under normal air condition 400 ppm CO₂ (A); air enriched with 3500 ppm CO₂ (B); CAT activity under normal air condition (C); CAT activity under 3500 ppm CO₂ (D). Data represent means ± SD of at least three replicate experiments. Different letters on the top of columns indicate significant difference at p < 0.05 according to Duncan's multiple range test

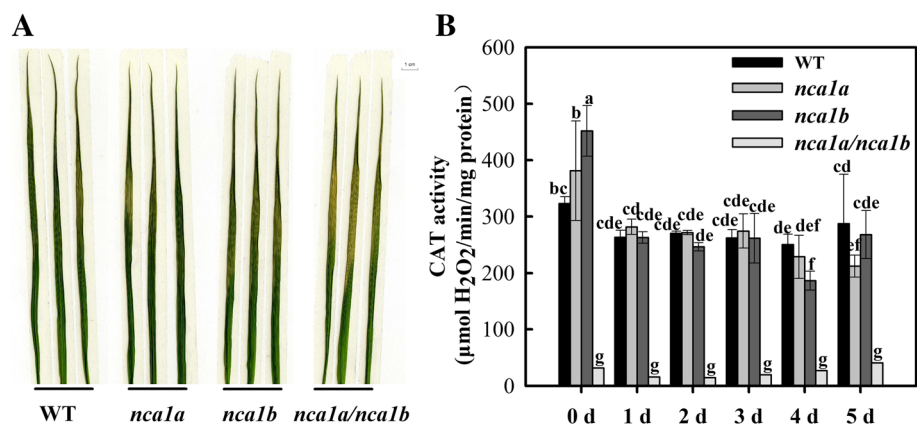


Fig. 6 Phenotype and CAT activity for the *nca1* mutants in response to salinity stress. The plants were hydroponically grown in an environment-controlled growth chamber until 3.5-leaf old (temperature: 30 °C day/25 °C night; light intensity: 600 μmol m⁻² s⁻¹; humidity: 60%), then treated with 150 mM NaCl. CAT activity was assayed at different times after the treatment (B) and the picture of the stressed leaves was taken at 5 days after the treatment (A). Data represent means ± SD of at least three replicate experiments. Different letters on the top of columns indicate significant difference at p < 0.05 according to Duncan's multiple range test

plant genomes, including *Arabidopsis*, rice, tobacco, maize, pumpkin etc. [1]. Besides scavenging H_2O_2 , CAT plays essential roles in plant growth, development, and response to stress [1]. In *Arabidopsis*, knockout of *CAT2* leads to 80% loss of catalase activity and it is hypersensitive to salinity, strong light, and low temperature, but tolerance to LiCl and Hydroxyurea (HU) [5, 24, 25]. Rice CATC accounts for the large majority of catalase activity, and it is distributed mostly in rice leaves [9]. Since CAT protein are abundant in plants, overexpression of CAT genes normally does not significantly increase plant catalase activity or affect plant growth and response to stress. However, there have been a few studies showing that introduction of bacterial catalases into plants may increase plant catalase activity and plant resistance to oxidative stress, NaCl, and high light [1]. Despite the importance of this enzyme, its regulatory mechanism, particularly at the protein level, is not well understood. NCA1 protein was recently identified, which was shown to serve as a chaperone to activate CAT by helping it to fold correctly [5, 7]. The NCA1 gene exists in higher plants, with species having one gene and some harboring two copies (Additional file 2 Figure S1). According to the phylogenetic tree, there appears no regular pattern for its classification and evolution. Whether the two isoforms in those two-copy-containing plant species function redundantly or differently in regulating CAT activity and how they realize the function are not yet known. As a monocotyledonous model plant, rice harbors two *NCA1* genes, so it is particularly interesting to address the above question in rice. First of all, our spatiotemporal expression pattern analysis, in combination with biochemical studies, demonstrates that OsNCA1a and OsNCA1b could function equally in activating CAT (Fig. 1; Fig. 2).

Subsequent BiFC and yeast three-hybrid experiments established that OsNCA1a and OsNCA1b showed mutually exclusive interaction with CAT in cytosol (Fig. 3; Fig. 4). CAT is a peroxisomal matrix protein and the nascent peroxisomal proteins are translated on the cytoplasmic free polysomes [9], so the OsNCA1a and OsNCA1b could regulate the CAT activity in the cytosol, this is consistent with the results of Li et al. (2015) [5]. Furthermore, they also detected that the nascent CAT in the cytosol interacts with NCA1 and form complexes in a 1:1 ratio. Our yeast three-hybrid result still indicated that in rice CAT interacts with either NCA1a or NCA1b in a molecular ratio of 1:1. It is currently not clear how CAT interacts with NCA1. Previous studies have shown that the TPR domain functions as a protein interaction scaffold for the formation of different protein complexes [26].

Li et al. (2015) also reported that *Arabidopsis* defective of NCA1 was highly sensitive to salinity stress [5]. We also have originally imagined that, in those plant species

containing two *NCA1* genes, the two *NCA1* genes may be simultaneously required under certain circumstances, likely under adverse conditions. However, by using *nca1a/nca1b* single and double knockout mutants, it was revealed that both genes function redundantly under both normal and salinity stress conditions (Fig. 5; Fig. 6). Such functionally-redundant genes have been frequently noticed in organisms. For instance, Blanvillain et al. (2008) identified two *XPO1* (*EXPORTIN1*) genes in *Arabidopsis*, i. e, *XPO1a* and *XPO1b*, which were shown to be functionally redundant to regulate the maternal-to-embryonic transition [31]. Two TRAF (Tumor necrosis factor receptor-associated factor) proteins MUSE 13 and MUSE14 were identified in *Arabidopsis*, which serve as redundant immune regulators [32, 33]. It is not yet understood why such redundant genes/proteins exist naturally, but we believe that the two *NCA1* genes/proteins may be simultaneously required under certain circumstances yet to be identified, based on the previous reports that CAT activity can be regulated by various stresses including drought, cold, H_2O_2 , methyl viologen (MV), high light or heat shock.

Conclusions

Our study found that in rice there exist two *NCA1* isoforms (*NCA1a* and *NCA1b*) that interact with CAT in a mutually exclusive manner to function-redundantly regulate CAT activity. It has been further demonstrated that CAT interacts with either *NCA1a* or *NCA1b* in a molecular ratio of 1:1. While redundant genes/proteins occur commonly in organisms, our study newly noticed that the two homologous proteins interact in a mutually exclusive manner to realize their function. We believe that the two *NCA1* genes/proteins may be simultaneously required under certain circumstances yet to be identified, based on the previous reports that CAT activity can be regulated by various stresses including drought, cold, H_2O_2 , MV, high light or heat shock [27–30].

Methods

Plant materials and growth conditions

Rice (*Oryza sativa* L) cv. Zhonghua 11 (japonica cultivar group) kept by our laboratory was used for generating the *NCA1a/NCA1b* single and double knockout mutants and for the functional analyses. Pre-germinated seeds were grown in Kimura B complete nutrient solution in a greenhouse condition [34]. Conditions of each treatment in growth chamber were specified in the legend of each figure.

Plasmid construction

The complete ORFs of *OsNCA1a* (Os01g0104100) and *OsNCA1b* (Os02g0795300) were cloned from rice leaves

by RT-PCR, the complete ORFs of *OsCATC* (Os03g0131200) were cloned from expression vectors described previously [9], and then the ORFs of *OsNCA1a*, *OsNCA1b*, and *OsCATC* were subcloned into the *pBridge* and *pGADT7* vectors for yeast three hybrid assay (Biosciences Clontech, Palo Alto, USA). For expression of the recombinant proteins in *E. coli*, the *OsNCA1a/b* sequences were inserted into the *pET28a* vector at the *BamH* I and *Xho* I sites and *OsCATC* sequence was inserted into *pETDuet-1* first MCS between the *EcoR* I and *Hind* III sites. For subcellular localization, the ORFs of *OsNCA1a*, *OsNCA1b* were introduced into *pYL322-DI*, under the control of the CAMV35S promoter. The *pYL322-DI* vector was kindly provided by Dr. Yao-Guang Liu, College of Life Sciences, South China Agricultural University. For constructing BiFC vectors, the ORFs of *OsNCA1a*, *OsNCA1b* and *OsCATC* were inserted into *pSAT6-cEYFP-C1* (CYFP) vector or *pSAT6-nEYFP-C1* (NYFP) vector [36]. The primers used for PCR were listed in Additional file 3 Table S2.

Subcellular localization and bimolecular fluorescence complementation (BiFC)

Rice protoplasts were isolated as previously described Zhang et al. (2011) [35]. For subcellular localization, 10 μ g of the *pYL322-DI-OsNCA1a/OsNCA1b-GFP* constructs was introduced into 100 μ L protoplasts by the PEG-mediated transfection, and then the protoplasts were incubated in dark at 25 °C for 16–24 h.

For BiFC, vector of *pSAT6-cEYFP-C1* (CYFP); vector of *pSAT6-nEYFP-C1* (NYFP); *CYFP-CATC* + *NYFP-NCA1a/NCA1b*: *CATC* fused with *eYFP* at the C-terminal and *NCA1a/NCA1b* with *eYFP* at the N-terminal were then co-transfected into rice protoplasts; *CYFP-NCA1a* + *NYFP-NCA1b*: *NCA1a* fused with *eYFP* at the C-terminal and *NCA1b* with *eYFP* at the N-terminal were then transfected into rice protoplasts; *CYFP-CATC* + *NYFP*: *CATC* fused with *eYFP* at the C-terminal and *NYFP* as a blank vector were transfected into rice protoplasts. 5 μ g of *NYFP*-tagged constructs and 5 μ g *CYFP*-tagged constructs were co-transfected into 100 μ L protoplasts by the PEG-mediated transfection. Then, the protoplasts were incubated in dark at 25 °C for 12 h [35, 36]. The confocal images were finally captured using the ZEISS LSCM 780 system.

RNA isolation and qRT-PCR

Total RNA was extracted from rice leaves using Trizol reagent (Life Technologies, USA), and treated with RNase free-DNaseI (Amersham, USA). The quality and quantity of the purified RNA was assessed with a NanoDrop-1000 (NanoDrop, USA). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Japan). Specific primer pairs were designed for the qRT-PCR of

each *NCA1* gene, and the specificity of these primers were evaluated using NCBI Primer-BLAST (Additional file 3 Table S2). The qRT-PCR was performed in 10 μ L of reaction mixture consisting of 5 μ L of 2 \times SYBR Green PCR Master Mix (Toyobo, Japan), 0.2 μ M of each primer, and 2 μ L of appropriate diluted cDNA. Transcript levels of each gene were measured by the DNA Engine Opticon2 Real-Time PCR detection system and opticon monitor software (Bio-Rad, USA) according to the manufacturer's instructions. The data were normalized to the amplification of the *OsActin1* gene (Os03g0718100). Method of presenting quantitative real-time PCR data is the comparative C_T method ($2^{-\Delta\Delta C_T}$ method).

Expression of the recombinant proteins in *E. coli* and their purification

The *pET28a-OsNCA1a*, *pET28a-OsNCA1b*, *pETDuet1-NhisCATC*, and *pETDuet1-NhisCATC-OsNCA1* plasmids were transformed into *E. coli* Rosseta (DE3) cells and screened on Luria-Bertani (LB) plates containing kanamycin or ampicillin, and then colony PCR was performed. Protein expression was induced at 16 °C with 1 mM of isopropyl-thio- β -D-1-thiogalactopyranoside (IPTG) overnight. The recombinant protein was purified with nickel column according to the manufacturer's protocol. The resulting proteins were separated using SDS-PAGE.

Enzyme assays and protein measurement

The CAT activity was measured using a UV-spectrophotometer 20 in a reaction mixture containing 50 mM PBS (pH 7.4), 25 mM H_2O_2 at 30 °C. The consumption of H_2O_2 was detected at 240 nm and the CAT activity was calculated using the extinction coefficient for H_2O_2 of 43.6 $M^{-1} cm^{-1}$ [37]. The protein contents were determined using Coomassie brilliant blue G250 [38].

Generation *nca1/nca2* single and double knockout mutants by CRISPR/Cas9

The *nca1/nca2* single and double mutant was generated using CRISPR/Cas9 method according to Ma et al. (2015) [39]. First, two sgRNAs targeting each *OsNCA1* at different locations were cloned into *pYLSgRNA-OsU6a* and *pYLSgRNA-OsU6b*, respectively, then the vector *pYL-CRISPR/Cas9 pubi-H* containing two U6-gRNA units for *OsNCA1a/OsNCA1b* was constructed and transformed into rice via *Agrobacterium tumefaciens* (strain EHA105). Eight independent lines were obtained, respectively, to examine the function of CRISPR/Cas9. The genomic DNA was extracted from transgenic plants and primer pairs flanking the designed target site were used for PCR amplification. The amplicons were sequenced and sequence alignment of every sgRNA targeting region

revealed that three independent mutants (*nca1a*, *nca1b*, and *nca1a/nca1b*) are reliable with loss of function of the single and double target genes (Fig. 4b).

Yeast three hybrid assay

Yeast cultures and three-hybrid procedures were carried out according to standard methods [40]. In the yeast three-hybrid assay cells were transformed with the bicistronic vectors *pBridge* and *pGADT7* (Biosciences Clontech, Palo Alto, USA). *pBridge* contains two distinct multiple cloning sites to allow expression of the BD (DNA binding domain) fusion as well as a third protein. When *pBridge* is used in conjunction with the *pGADT7 AD* (DNA activation domain) fusion vector a ‘three-hybrid’ system can be established that is dependent on the expression of a third protein. Co-transformation of appropriate *BD* and *AD* constructs (*AD-NCA1a*, *AD-NCA1b*, *BD-CATC*, *BD-NCA1a*, *BD-NCA1b*) into the yeast Y2Hgold cells (Biosciences Clontech, Palo Alto, USA). Yeast transformed with both bait (BD) and prey (AD) vectors were spotted on selective dropout media without Methionine/Tryptophan/Leucine (SD-MWL) or Methionine/Tryptophan/Leucine/ Histidine/Alanine (SD-MWLHA) to check for interaction between the three partners. In each case, a control with an empty vector was tested for self-activation of the constructs.

Statistical analysis

The data were subjected to statistical analysis using Duncan’s multiple range test at the 5% ($P < 0.05$) confidence levels. Data Processing System (DPS) software [41]. were used for data statistics analysis.

Additional files

Additional file 1: Table S1. Similarities of *Arabidopsis* and rice GLO genes at the level of mRNA and protein. (DOCX 58 kb)

Additional file 2: Figure S1. Phylogenetic relationships among NCA and orthologous proteins from other species. (DOCX 18 kb)

Additional file 3: Table S2. Primers used in this study. (DOCX 20 kb)

Abbreviations

BiFC: Bimolecular fluorescence complementation; CAT: Catalase; IPTG: Isopropyl-thio- β -D-1-thiogalactopyranoside; NCA1: NO CATALASE ACTIVITY 1; qRT-PCR: Real-time quantitative PCR

Acknowledgements

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Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors’ contributions

LJZ conducted experiments, original data organization. CLL prepared protein samples for analysis, grew rice plants. XZW performed stress treatment and the qRT-PCR analysis. ZZS and LEE wrote the manuscript. PXX wrote and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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