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Molecular Cloning and Expression of Two Cytosolic Copper–Zinc Superoxide Dismutases Genes from *Nelumbo nucifera*

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Abstract Two cytosolic copper–zinc superoxide dismutase (cytCuZnSOD) complementary deoxyribonucleic acid were achieved in *Nelumbo nucifera* (Elian). The active sites and common characteristics of cytCuZnSOD family were showed by homology modeling. The two recombinant proteins expressed by PET-32a vector showed the similar SOD activity (89.94±0.54 U/mg) and could maintain more than 90% activity after incubation at 65°C. The subcellular location by green fluorescent protein revealed that these two isoforms were all located in cytosol and nucleus. The cyt*CuZnSODs* were expressed in various parts of *N. nucifera*, which were expressed highest in the leafstalks and young leaves and lowest in the roots. The cyt*CuZnSOD* messenger ribonucleic acids isolated from wounded leaves significantly increased at 1.5 h after treatment (HAT) with the highest expression at 3 HAT, after which the level decreased.

Keywords CuZnSODs · Recombinant proteins · Real-time PCR

Introduction

Superoxide dismutases (SODs) were the first line of defense against the toxic superoxide radical (O_2^-), which could react with membrane lipids, deoxyribonucleic acid (DNA) strands, and protein to cause irreversible damage to the cells [1]. Four styles of SODs were classified in eukaryotic cell according to their metal cofactor and subcellular localization: manganese SOD (MnSOD) located in mitochondrial matrix, intracellular copper–zinc SOD (icCuZnSOD) found in the cytosol and also in the chloroplast of plants, extracellular CuZnSOD (ecCuZnSOD) found in the extracellular matrix of tissues such as lymph and plasma [2], and iron SOD (FeSOD) located in mitochondria and chloroplast. Unlike most

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other organisms that usually had only one of each class of SODs in the various cellular compartments, plants had multiple forms of each class encoded by more than one gene [3]. Two styles of CuZnSODs were found in high plants: cytosolic CuZnSOD (cytCuZnSOD) and chloroplastic CuZnSOD (chlCuZnSOD) [4]. Numerous studies indicated that some factors could affect the expression of CuZnSOD such as irradiation, oxidative stress, metal stress, drought, and cold [1, 5].

Nelumbo nucifera is considered as molecular living fossil. Virtually as an important economic aquatic plant, all parts of the *N. nucifera* (such as seed, rhizome, leaf, stalk, and petal) are used as food or medicine in Asia. Seeds of the sacred lotus still hold the world record for longevity [6] and could maintain 100% viability even after 24 h treatment in an air oven at 100°C [7]. To the best of our knowledge, only open-reading frames of *CuZnSOD* gene were obtained in *N. nucifera* (GenBank accession nos. DQ22734 and DQ223864), and there were exactly few reports about this gene in aquatic plant.

This study was aimed to clone the complete sequences of *cytCuZnSOD* complementary CNAs (cDNAs) in *N. nucifera*, to analyze the activity of recombinant proteins and subcellular location, and to evaluate their expression in various parts and when *N. nucifera* responded to short-time mechanical wounding.

Materials and Methods

Plant Material

N. nucifera was kept in Lotus Research Centre of Wuhan University, China. All the materials were prepared and put into liquid nitrogen immediately.

Clone and Analysis of the Complete Sequence of *cytCuZnSOD* cDNAs

The ribonucleic acids (RNAs) were extracted from the terminal buds, and reverse transcription was performed according to the method reported [8]. Full-length *cytCuZnSOD* cDNAs of *N. nucifera* were obtained by the procedures of rapid amplification of cDNA ends (RACE) method. The degenerate primers pair (cytCuZnSOD DF and cytCuZnSOD DR) was designed to amplify 5′ partial cDNAs (Table 1). The settings for the thermal profile included an initial denaturing at 94°C for 5 min, followed by 35 cycles of (94°C for 30 s, 50°C for 30 s, and 72°C for 40 s), and finally an extension at 72°C for 10 min. The fragment was ligated into pGEM-T vector (Promega), the product of ligation was transformed into competent *Escherichia coli* DH5α, and the 5′ partial fragment was sequenced.

For the 3′-RACE, the reverse transcription was performed by PT1 primer. One successive polymerase chain reaction (PCR) was carried out with the primer GSP and PT2 (Table 1). The procedures were the same as 5′ partial cDNA amplification. The whole sequences were cloned and sequenced (GenBank accession nos. GQ149102 and GQ149103).

Similarity and Phylogenetic Analysis of CuZnSOD

The deduced amino acid sequences of *N. nucifera* cytCuZnSOD were compared with other species that was available in Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was created using CLUSTAL W. And the subsequent phylogenetic tree based on the amino acid sequences was performed by the parsimony method using the MEGA software version 4 [9]

Table 1 Degenerate and specific primers used in the experiment

Primer name	Sequence (5′-3′)
cytCuZnSOD DF	ATGGTGAARGCNGTYGYNGT
cytCuZnSOD DR	ACTCTNCCNCCNGCRTTNCC
PT1	GTTTTCCCAGTCACGAC(T)17
GSP	AAGGGAGGACATGAACTTAGC
PT2	GTTTTCCCAGTCACGAC
cytCuZnSOD PF1	CGGGATCCATGGTGAAGGCTGTTGCG
cytCuZnSOD PR1	CGAAGCTTTTAACCCTGCAAGCCA
cytCuZnSOD PF2	CGGGATCCATGGTGAAGGCGGTTGTC
cytCuZnSOD PR2	CGAAGCTTTTAACCCTGCCAGCCA
cytCuZnSOD F1	ATGGTGAAGGCTGTTGCG
cytCuZnSOD F2	ATGGTGAAGGCGGTTGTC
cytCuZnSOD R	TCCTGTTACTTTAGTTGAT
5 S rRNA F	GGATGCGATCATACCAGCAC
5 S rRNA R	GGGAATGCAACACGAGGACT

Homology Modeling of cytCuZnSOD

The three-dimensional (3D) structural models of cytCuZnSOD were constructed by homology modeling according to the published protocols [10], and the SOD from *Potentilla atrosanguinea* (PDB code. 2Q2L) was used as the template [11].

Expression and Effect of Temperatures on Recombinant cytCuZnSOD Proteins

For cloning *cytCuZnSOD* cDNAs in PET-32a (+) expression vector, two set of primers (cytCuZnSOD PF and cytCuZnSOD PR) were designed based on its coding sequence

Fig. 1 Comparison of CuZnSOD coding sequences by CLUSTAL W. Differences were indicated, while identical bases were represented by *asterisk* (*). Highly conserved amino acids critical for Cu (His 45, 47, 62, and 119) and Zn (His 70, His 79, His 62, and Asp 82) binding were shown. The amino acids formed the disulfide bonds (Cys 56 and Cys 145) were highlighted in *gray*. The ones formed the salt bridges (Arg 78 and Asp 82) were highlighted in *black*. The two conserved CuZnSOD signatures were shown in *red* letters

Table 2 Species, protein, and GenBank accession numbers of CuZnSOD sequences used for phylogenetic analysis

Species	Accession no.	Size	Identity, similarity (%) of cytCuZnSOD1/cytCuZnSOD2
Plant cyt			
Allium sativumi	ADB28989	152	81, 88/80, 87
Ananas comosus	Q9SQL5	152	86, 89/85, 88
Arabidopsis thaliana	CAA43270	152	81, 87/80, 86
Brassica cleracea var. *capitata*	P09678	152	78, 85/78, 84
Brassica juncea	Q42611	152	79, 86/79, 86
B. juncea	Q42612	152	78, 84/77, 84
Brassica rapa subsp. *pekinensis*	AAC25568	156	80, 85/80, 84
Cicer arietinum	CAA10132	152	78, 88/78, 87
Cucumis sativus	ACA49507	152	82, 86/82, 85
Dimocarpus longan	ACT54525	152	82, 90/82, 90
Glycine max	Q7M1R5	152	81, 89/80, 88
Gossypium hirsutum	ABA00453	152	84, 90/84, 90
Helianthus annuus	CAH06454	153	83, 87/83, 87
Ipomoea batatas	Q07796	152	84, 90/83, 89
Lotus japonicus	AAP81872	152	80, 97/79, 86
Melastoma malabathricum	BAJ07302	152	85, 89/86, 90
Oryza sativa Indica Group	A2XGP6	152	82, 86/83, 87
O. sativa Japonica Group	Q0DRV6	152	82, 86/83, 87
O. sativa Japonica Group	P28757	152	82, 88/81, 87
Pennisetum glaucum	ABP65325	152	83, 87/84, 88
Pisum sativum	AAA33659	152	78, 87/77, 86
Populus suaveolens	ABF48717	152	86, 91/85, 90
Populus tremuloides	AAD01605	152	86, 91/85, 90
Populus trichocarpa	ABN58428	152	86, 92/86, 91
Raphanus sativus	AAD05576	152	81, 87/80, 86
Solanum lycopersicum	Q43779	152	83, 88/82, 88
S. lycopersicum	CAA60826	152	83, 88/82, 88
Spinacia oleracea	P22233	152	79, 88/80, 89
S. oleracea	CAA37866	152	79, 88/80, 89
Zantedeschia aethiopica	O65174	152	79, 87/78, 86
Zea mays	P11428	152	82, 88/82, 87
Z. mays 4A	NP_001105423	152	84, 87/83, 86
Z. mays 4AP	P23346	152	85, 88/84, 88
Plant chl			
Arabidopsis thalianai	O78310	216	62, 72/62, 72
Bruguiera gymnorhiza	CAM98444	227	62, 71/61, 72
Chenopodium murale	ABO70347	218	61, 72/ 61, 72
Gossypium arboreum	ABL63518	215	62, 72/61, 71
Medicago sativa	O65198	202	62, 72/62, 72
Oryza sativa Japonica Group	BAD09607	203	64, 74/63, 73
O. sativa Japonica Group	BAD13222	203	64, 74/63, 73
Pisum sativum	P11964	202	62, 74/62, 73

Table 2 (continued)

Species	Accession no.	Size	Identity, similarity (%) of cytCuZnSOD1/cytCuZnSOD2
Solanum lycopersicum	P14831	217	61, 73/60, 72
Spinacia oleracea	P07505	222	65, 74/64, 73
Triticum aestivum	AAB67990	201	64, 72/62, 72
T. aestivum	AAB67991	201	64, 73/63, 72
Zantedeschia aethiopica	O65175	216	60, 70/59, 69
Zea mays	BAI50562	206	64, 74/63, 74
Z. mays	BAI50563	206	64, 74/63, 74
Animal cyt			
Bos taurus	P00442	152	58, 69/59, 70
Cristaria plicata	ACI28282	155	60, 70/61, 71
Drosophila melanogaster	P61851	153	60, 70/62, 71
Drosophila simulansi	P61852	153	60, 70/62, 71
Equus caballus	P00443	154	53, 66/54, 67
Oryctolagus cuniculus	P09212	153	54, 65/55, 65
Rattus norvegicus	P07632	154	54, 68/55, 68
Xenopus laevis	P15107	151	56, 67/58, 69
Animal ec			
Homo sapiens	P08294	240	36, 51/46, 58
Mus musculus	NP_035565	251	46, 57/46, 58
Oryctolagus cuniculus	CAA91785	244	44, 55/45, 56
Rattus norvegicus	NP_037012	244	48, 59/49, 60

(Table 1). The 5′ end of primers contained a restriction endonuclease site of *Bam*HI and *Hin*dIII, respectively. Prokaryotic expression and Western blotting were performed according to the published protocols [12] The unit of SOD activity was defined as the amount of enzyme required to result in a 50% of inhibition of nitro blue tetrazolium photoreduction to formazan at 560 nm [13]. The effect of temperature on cytCuZnSOD activity was assayed at pH 7.4 according to the method reported [14].

Subcellular Location of cytCuZnSOD

The modified plasmid PCAMBIA 1302 was obtained from a professor of Mengxiang Sun. The product of double digestion described above was used to clone the modified PCAMBIA 1302 vector for green fluorescent protein (GFP) fusions. The recombinant plasmid construction and subcellular location were performed according to the method reported [15].

cytCuZnSOD mRNas Differential Expression in Various Parts

Fresh materials, such as roots, young leaves, terminal buds, and leafstalks, were collected at the time when the young leaves had unfolded for 1 week. The special primers for *cytCuZnSOD1* (cytCuZnSOD F1 and cytCuZnSOD R) and *cytCuZnSOD2* (cytCuZnSOD F2 and cytCuZnSOD R) transcripts were designed by detecting the amino acid variants at position 6, according to the method reported [16]. The *5 S rRNA* gene (GenBank accession no. EF121364) was chosen as the control gene, and the primers (5 S rRNA F and 5 S rRNA R)

Fig. 2 Phylogenetic analysis was performed by the parsimony method using MEGA4 software. Numbers in the branches represented the bootstrap values (%) from 1,000 replicates

were designed (Table 1). Real-time PCR was carried out in a fluorometric thermal cycler (Rotor Gene 2000) using the DNA binding dye SYBR Green I (TOYOBO) for the detection of PCR products. The amplification program consisted of 1 cycle at 94°C for 60 s, followed 30 cycles of 94°C for 20 s and 60°C for 20 s. The relative gene expression data was obtained according to the method reported [17].

Expression of *cytCuZnSOD* mRNAs in Short-Term Mechanical Wounding

For wounding treatment, all the materials grown in the greenhouse were corrected when the leaves had unfolded for 1 week. The leaves of *N. nucifera* were treated for 1.5, 3, 4.5, and 6 h by exerting pressure with a needle puncher [18]. The intact leaves were used as the control. After treatment, the RNAs of materials were isolated, and the expression of *cytCuZnSOD* messenger RNAs (mRNAs) was demonstrated by real-time PCR as described above.

Statistical Analysis

All experiments were repeated three times. Statistical analysis was performed according to the report [19].

Results

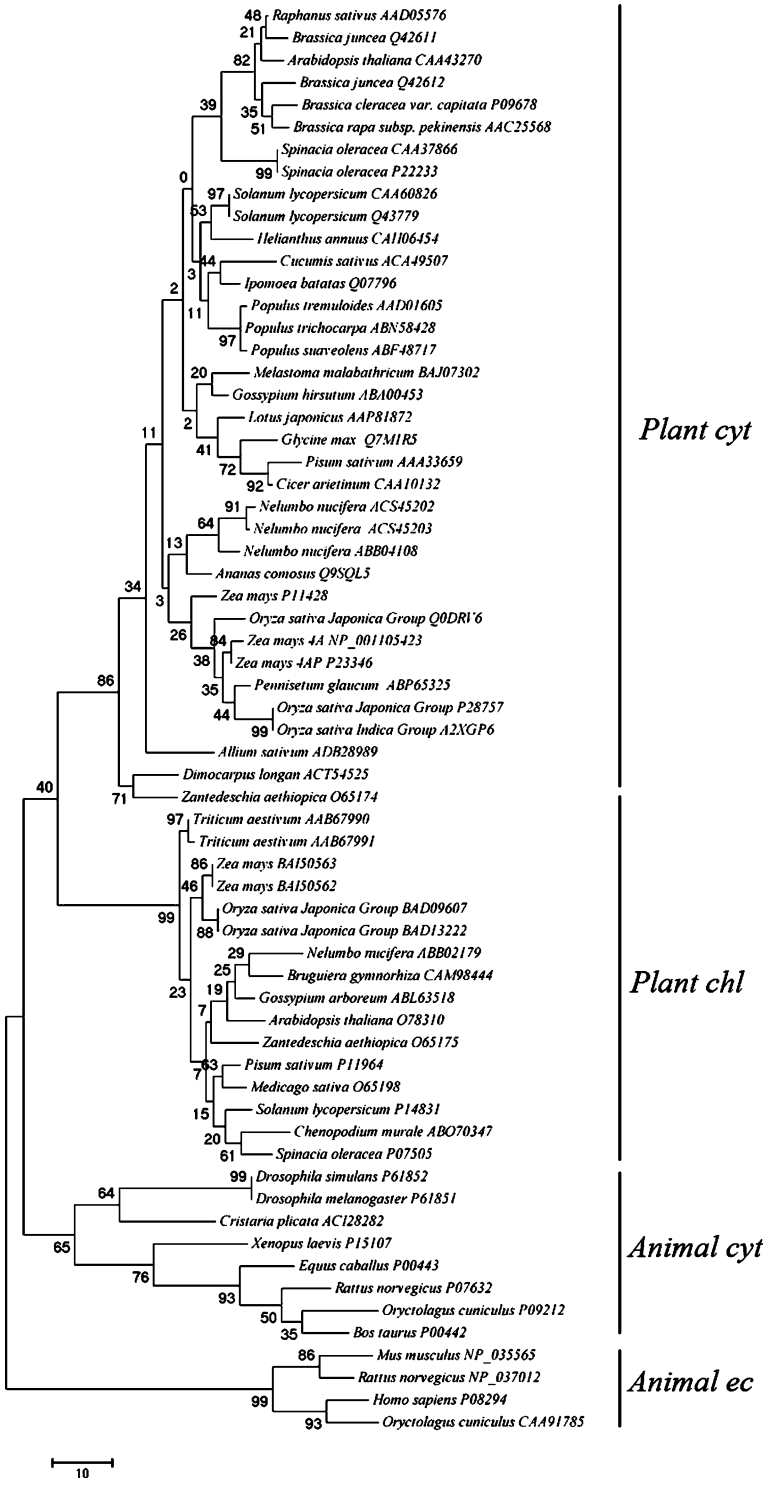
Cloning and Analysis of *cytCuZnSOD* cDNAs

Two functional forms of *cytCuZnSOD* cDNAs, named *cytCuZnSOD1* and *cytCuZnSOD2*, were cloned in *N. nucifera*. Both of them contained the start code ATG, stop code TAA, poly (A) tails, a putative polyadenylation signal (AATAAA), and 459 bp open-reading frame. The 3′ untranslated regions of *cytCuZnSOD1* and *cytCuZnSOD2* contained 264 and 254 bp, respectively.

The two proteins deduced had the same theoretical isoelectric point (5.68), with slight difference in molecular weights (cytCuZnSOD1 15492.26 Da and cytCuZnSOD2 15567.29 Da). Alignment of amino acid sequences revealed that *N. nucifera* cytCuZnSOD contained (1) the Cu and Zn signatures, (2) four copper binding sites, (3) four zinc binding sites, (4) a disulfide bond, and (5) one salt bridge (Fig. 1). Seven different nucleotide acids in the coding sequence were detected between these two transcripts: three of them showed slight changes while the other four more than changed the amino acids. These positions were Ala-6, Leu-110, and Leu-150 for cytCuZnSOD1 and Val-6, Ser-110, and Trp-150 for cytCuZnSOD2 (Fig. 1).

Similarity and Phylogenetic Analysis of CuZnSOD

The deduced amino acid sequences of *N. nucifera* cytCuZnSODs showed similarities with those of plants (84–92%) and animals (65–71%). The isoforms showed similarities with chlCuZnSOD from plants (69–74%), while showed similarities with ecCuZnSOD from animals (51–60%) (Table 2). The phylogenetic relationships indicated that CuZnSOD could be classified into three distinct clusters: ecCuZnSOD, cytCuZnSOD, and chlCuZnSOD. The two isoforms obtained in this article and the one previous reported (DQ227345) were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Arabidopsis thaliana CAA43270
Brassica juncea Q42612
Brassica cleracea var. capitata P09678
Brassica rapa subsp. pekinensis AAC25568
Spinacia oleracea CAA37866
Spinacia oleracea P22233
Solanum lycopersicum CAA60826
Solanum lycopersicum Q43779
Helianthus annuus CAI06454
Cucumis sativus ACA49507
Ipomoea batatas Q07796
Populus tremuloides AAD01605
Populus trichocarpa ABN58428
Populus suaveolens ABF48717
Melastoma malabathricum BAJ07302
Gossypium hirsutum ABA00453
Lotus japonicus AAP81872
Glycine max Q7M1R5
Pisum sativum AAA33659
Cicer arietinum CAA10132
Nelumbo nucifera ACS45202
Nelumbo nucifera ACS45203
Nelumbo nucifera ABB04108
Ananas comosus Q9SQL5
Zea mays P11428
Oryza sativa Japonica Group Q0DRV6
Zea mays 4A NP_001105423
Zea mays 4AP P23346
Pennisetum glaucum ABP65325
Oryza sativa Japonica Group P28757
Oryza sativa Indica Group A2XGP6
Allium sativum ADB28989
Dimocarpus longan ACT54525
Zantedeschia aethiopica O65174
Triticum aestivum AAB67990
Triticum aestivum AAB67991
Zea mays BA150563
Zea mays BA150562
Oryza sativa Japonica Group BAD09607
Oryza sativa Japonica Group BAD13222
Nelumbo nucifera ABB02179
Bruguiera gymnorhiza CAM98444
Gossypium arboreum ABL63518
Arabidopsis thaliana O78310
Zantedeschia aethiopica O65175
Pisum sativum P11964
Medicago sativa O65198
Solanum lycopersicum P14831
Chenopodium murale ABO70347
Spinacia oleracea P07505
Drosophila simulans P61852
Drosophila melanogaster P61851
Cristaria plicata ACI28282
Xenopus laevis P15107
Equus caballus P00443
Rattus norvegicus P07632
Oryctolagus cuniculus P09212
Bos taurus P00442
Mus musculus NP_035565
Rattus norvegicus NP_037012
Homo sapiens P08294
Oryctolagus cuniculus CAA91785
Plant cyt
Plant chl
Animal cyt
Animal ec
10

grouped together with cytCuZnSOD, while the other one (DQ223864) was homologous to chlCuZnSOD (Fig. 2).

The Homology Model

The target protein and template protein shared a high degree of homology (77.0%). It was found that *P. atrosanguinea* SOD was selected as the best structure template for the query sequences of cytCuZnSOD (Fig. 3a). The Ramachandran plot provided by PROCHECK documented that most of the model residues were in most favorable regions, and none of the residues generously allowed and disallowed regions.

The Activity and Thermal Stability of Recombinant Proteins

The recombinant cytCuZnSOD proteins were highly expressed in competent *E. coli* BL21 (Fig. 3b), and these two recombinant proteins showed the similar SOD activities (89.94± 0.54 U/mg). The purified enzymes were kept stable during the temperature range of 35–65°C. They maintained more than 90% activity even after treatment at 65°C. When the enzymes were incubated at 75°C for 30 min, the activity decreased sharply, retaining only around 50% activity of the maximum. They still held approximately 20% activity after incubation at 95°C for 30 min (Fig. 3c).

Subcellular Location

The onion epidermal cells were transiently transformed with the corresponding expression plasmid, and the GFP fluorescence visualized by fluorescence microscopy indicated that cytCuZnSOD-GFP accumulated in cytosol and nucleus (Fig. 4).

Datum Analysis of Real-Time PCR

All the R^2 values of *5 S rRNA* (0.998), *cytCuZnSOD1* (0.997), and *cytCuZnSOD2* (0.990) were close to 1. The real-time PCR efficiencies of *cytCuZnSOD*1 (1.03) and *cytCuZnSOD2* (1.04) were approximately equal to that of the *5 S rRNA* (1.03). So the $2^{-\Delta\Delta Ct}$ methods could be used to analyze the relative quantity. The *cytCuZnSODs* were expressed in various parts of *N. nucifera*. Both were expressed highest in the leafstalks and young leaves while lowest in the roots. The levels of these two transcripts were similar in the young leaves, roots, and terminal buds. The *cytCuZnSOD2* mRNA was correspondingly 1.4-fold as high as *cytCuZnSOD1* in leafstalks (Fig. 5a).

Real-time PCR analysis of RNAs isolated from wounded leaves showed that the *cytCuZnSOD* mRNAs strongly increased at 1.5 HAT with the highest expression at 3 HAT, after which the level decreased. The level of mRNA was the same as the control at 4.5 HAT. Then *cytCuZnSOD* mRNAs decreased at 6 HAT relative to control (Fig. 5b).

Discussion

Two *cytCuZnSOD* cDNAs were obtained in *N. nucifera*, which indicated that *N. nucifera* had multiple transcripts of *cytCuZnSOD*. Perhaps the occurrence of multiple transcripts of *cytCuZnSOD* in plants was reflective of the multiple and diverse roles played by this enzyme in plants [20]. The isoforms shared high level of sequence identities (77–86%) with

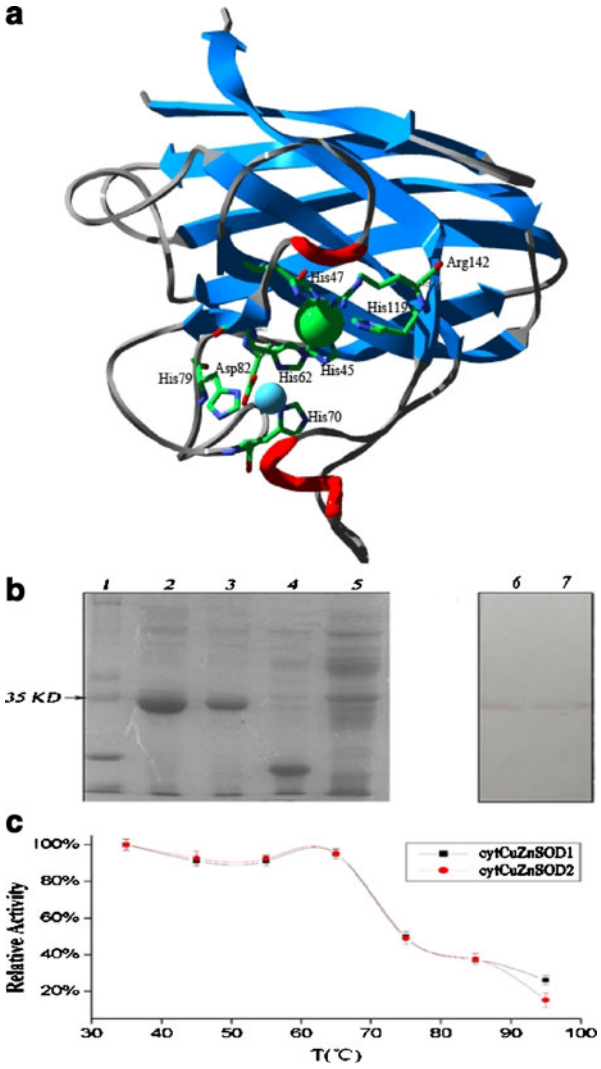

Fig. 3 **a** The predicted 3D structure of cytCuZnSOD in *N. nucifera*. The copper (His 45, 47, 62 and 119) and zinc (His 62, His 70, His 79, and Asp 82) binding sites were shown. **b** The expression of cytCuZnSOD during 15 h induction time. *Lane 1* is the molecular standard marker. *Lane 2* and *lane 3* represented the amount of PET-32a-cytCuZnSOD1 and PET-32a-cytCuZnSOD2, respectively. *Lane 4* represented the amount of control induced with PET-32a. *Lane 5* is the total cellular soluble fraction from *E. coli* BL21 without induce. *Lane 6* and *lane 7* represented the Western analysis the expression of cytCuZnSOD1 and cytCuZnSOD2. **c** The thermal stability of recombinant cytCuZnSOD protein. The enzymes were induced for 30 min at different temperatures. Then the activities were estimated under the standard assay condition (OD_{560}). The experiments were repeated three times

cytCuZnSOD of plant while low level of sequences identities (59–65%) with chlCuZnSOD. ecCuZnSOD, cytCuZnSOD, and chlCuZnSOD had similar sequences, while there were variations that unambiguously differentiated them. cytCuZnSOD and chlCuZnSOD of plant had a consensus head sequence (KAVAVL) in the N-terminus and a consensus tail sequence (GIIGL) in the C-terminus (data not shown), which were not found in ecCuZnSOD. Except

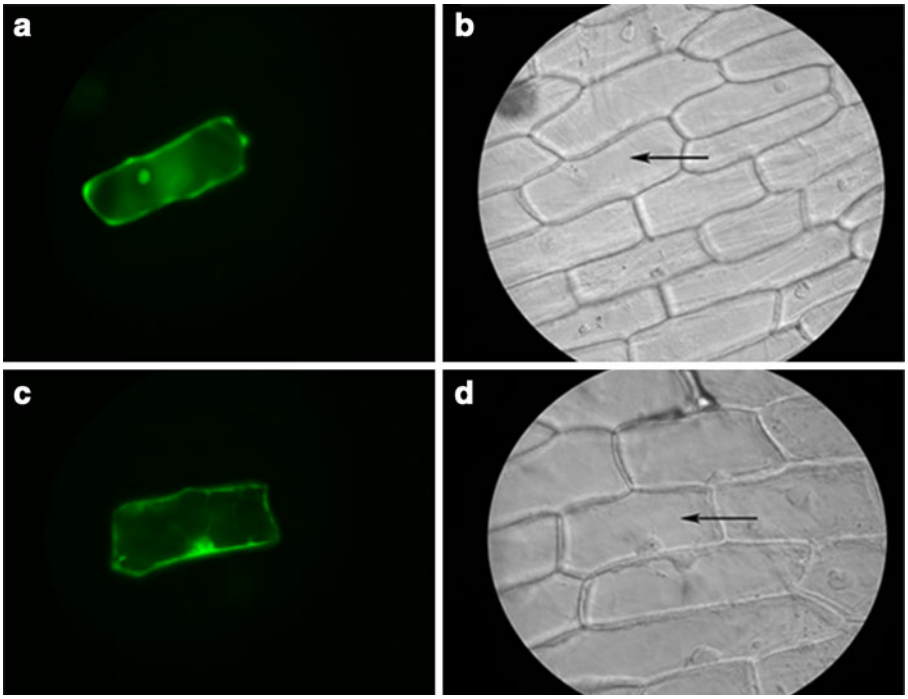

Fig. 4 Subcellular location of cytCuZnSOD proteins. The photographs were taken in the dark field for green fluorescence (**a** and **c**) and bright light for morphology of the cells (**b** and **d**). The subcellular location of cytCuZnSOD1 (**a** and **b**) and cytCuZnSOD2 (**c** and **d**) were shown

for the differences in the length of amino acid, terminal amino acid, and phylogenetic analysis [21], chlCuZnSOD also had a signal peptide which was not found in cytCuZnSOD. All the characteristics of *CuZnSODs* we cloned (such as 152 amino acids in size, Glycine in terminal amino acid, no signal peptide predicted by ExPASy tools, and grouping together with cytCuZnSODs in phylogenetic analysis) indicated that they might be *cytCuZnSODs*. The phylogenetic analysis showed that the cytCuZnSOD and chlCuZnSOD of plant originated from a common ancestor and later differentiated according to their cell compartment (Fig. 2) and consistent with the phylogenetic analysis in other report [22].

The model suggested the common characteristics of cytCuZnSODs family. Each subunit was primarily composed of eight anti-parallel β-strands, which were involved in dimmer formation. The single salt bridge at Arg 78–Asp 82 and disulfide bridge at Cys 56–Cys 145 stabilized the loop regions containing the metal ligands. The Cu^{2+} ligands (His 45, His 47, 62, and 119) and Zn^{2+} ligands (His 62, His 70, His 79, and Asp 82) were showed in Fig. 3a. Although the theoretical model must be confirmed by experimental structural determination, the three amino acids changes in the isoforms were in regions that did not appear to have a critical role for structure or catalysis of the proteins. So we speculated that the isoforms had no differences in activity, which was consistent with the assay of activity.

The thermal stability of the cytCuZnSOD had been well reported in animals and prokaryotes [23, 24], while few reports were available in plants. The SOD enzymes could

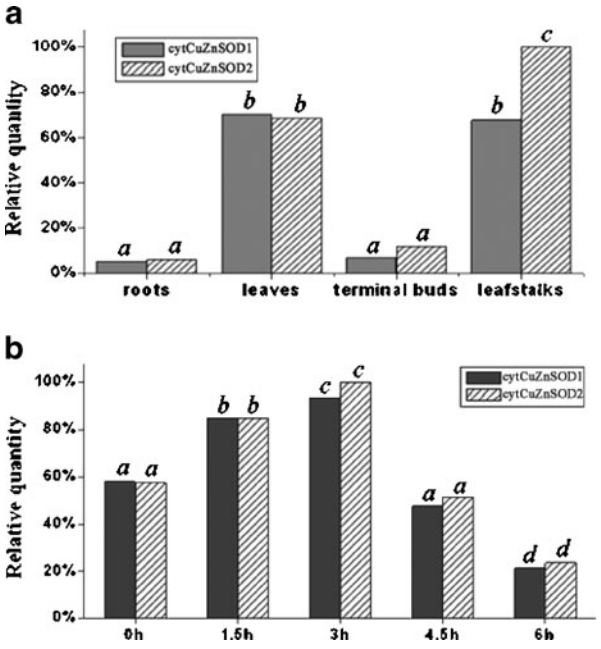

Fig. 5 **a** Relative amounts of *cytCuZnSOD* mRNAs which were isolated from four different parts (roots, leaves, terminal buds, and leafstalks) were determined by real-time PCR. **b** Relative amounts of *cytCuZnSOD* mRNAs in response to short-time mechanical wounding were determined by real-time PCR. The experiments were repeated three times. Different letters represented significant difference at $p<0.05$

only retain 50% of their activity after incubation at 50 and 60°C in *Lens esculanta* and brussel sprouts, respectively [25, 26]. However, the recombinant cytCuZnSOD proteins in *N. nucifera* retained more than 90% of their activity after incubation at 65°C, which was even more than the activity of SOD from the termophilic bacterium *Rhodothermus* sp. XMH10 [14]. The high stability of cytCuZnSOD observed under high temperature was possibly related to the strong stress resistance and longevity of *N. nucifera* seeds. Subcellular location documented that the isoforms were located in cytosol and nucleus (Fig. 4), consistent with the location of cytCuZnSOD in other species [27].

Real-time PCR showed that *cytCuZnSOD* mRNAs were expressed in roots, terminal buds, young leaves, and leafstalks. However, the amount of *cytCuZnSODs* mRNAs varied considerably between organ styles (Fig. 5a). The expression level of *cytCuZnSOD2* mRNA was relatively higher than *cytCuZnSOD1* in leafstalks, which might indicate that two transcripts played different roles in these parts. Compared with the *N. nucifera MnSOD* [8], we concluded that the expression of *cytCuZnSOD* and *MnSOD* had some similar characteristics in *N. nucifera*. First, the parts rich in chloroplast and mitochondria tended to express relatively higher level of SODs, because chloroplast and mitochondria were mainly resources of reactive oxygen species (ROS) through electron-transport chain of photosynthesis [28] and respiration [29, 30]. Second, the parts above the water expressed relative higher level of SODs, for the levels of SODs were enhanced under hyperoxia and that they were repressed under anoxia and hypoxia in *N. nucifera* [31]. Third, the differential expressions were detected during various physiological developments, and the

same results were found in wheat [32] and tobacco [33]. The young leaves and leafstalks contained several green tissues and lived above water, so their mRNA levels were relative higher than the terminal buds and roots.

Postharvest losses in *N. nucifera* resulting from environmental stresses, such as mechanical wounding, were enormous, which was also a potential infection site for pathogens. Recent studies showed that plants responded to these stresses by the production of ROS, which might function as a systemic signal in stress responses and systemic acquired resistance [34]. SODs catalyzed the breakdown of superoxide radicals and provided the first line of defense against oxygen toxicity, which were involved in stress resistance and longevity [35]. So SOD activity in plants was increased to remove these ROS caused by the various abiotic and biotic stresses [36, 37]. CuZnSOD was the most prominent SOD in high plants [38]. Present researches mainly focused on the expression of *CuZnSOD* mRNA in response to long-term wounding. The *CuZnSOD* mRNAs increased until 24 and 16 HAT, respectively, in *Manihot esculenta* [39] and *Pinus sylvestris* [20]. However, few responses to short-time wounding were reported. Our studies showed that the *N. nucifera cytCuZnSOD* mRNAs strongly increased within 3 HAT, after which the level decreased (Fig. 5b). It suggested that the cytCuZnSOD played a defensive role in the initial stages of oxidative stress and might be transcriptionally regulated. cytCuZnSOD might play a potential role as an antioxidant protein, which regulated the level of ROS by extracellular stimuli such as short-time mechanical wounding.

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