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Isolation Of A Novel Sulfite Oxidase Gene From *Codonopsis lanceolata* And Analysis Of The Response Related To Abiotic Stresses

Pulla Rama Krishna[1,2], Myung Kyum Kim[1], Senthil Kalai Selvi[2], Yu-Jin Kim[1], Deok Chun Yang[1]*
[1]Dept. of Oriental Medicinal Material and Processing, College of Life Science, Kyung Hee University, (SOUTH KOREA)
[2]Dept. of Biochemistry and Biotechnology, Avinashilingam University, Coimbatore, (INDIA)
Tel : +82-31-201-2688 ; Fax : +82-31-202-2687
E-mail : deokchunyang@yahoo.co.kr
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ABSTRACT

A cDNA clone containing a sulfite oxidase(SO) gene, designated ClSO, was isolated from a medicinal plant *Codonopsis lanceolata*. ClSO is predicted to encode a precursor protein of 396 amino acid residues, and its sequence shares high degrees of homology with a number of other SOs. The expression of ClSO in different *C.lanceolata* organs was analyzed using reverse transcriptase (RT)-PCR. The results showed that ClSO expressed high in stems of intact plant, while expressed at low level in leaves and roots. In addition, the expression of ClSO under different abiotic stresses was analyzed at different time points. Among the all the various stresses, hydrogen peroxide, salt, sulfur and light triggered a significant induction of ClSO within 2-8h post-treatment. The positive responses of ClSO to the above abiotic stimuli suggested that ClSO may help to protect the plants against environmental stresses.

KEYWORDS

Abiotic stress;
Codonopsis lanceolata;
Sulfite oxidase(SO).

INTRODUCTION

Sulfite can be oxidized to sulfate by the molybdenum containing enzyme, sulfite oxidase(SO; E.C 1.8.3.1). SO enzymes are essential mononuclear molybdenum (Mo) proteins involved in sulfur metabolism of animals, plants and bacteria. There are three such enzymes presently known: (1) SO in animals, (2) SO in plants and (3) sulfite dehydrogenase(SDH) in bacteria. X-ray crystal structures of the enzymes from all three sources (chicken SO, *Arabidopsis thaliana* SO, and *Starkeya novella* SDH) show nearly identical square pyramidal coordination around the Mo atom, even though the overall structures of the proteins and the presence of additional cofactors vary[5]. All sulfite oxidizing enzymes, except plant SO, possess two redox centers located in the Mo and heme domains. Intraprotein electron transfer(IET) processes between these two centers are critical in enzymatic turnover[5]. The vertebrate SO is a mitochondrial enzyme containing a heme domain such as cytochrome c serving as the physiological electron acceptor[2], where as plant SO is the smallest Mo containing enzyme and lacks contiguous redox-active centers such as FAD, heme or Fe-S[4,2].

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Human sulfite oxidase deficiency leads to severe neurological abnormalities that often result in death in infancy[7,15]. Brain damage may be due to the accumulation of a toxic metabolite, possibly SO_3^{2-}, which is a strong nucleophile that can react with a wide variety of cell components. It has been reported that sulfite reacts with protein disulfides to form sulfonated cysteine derivatives, and since the integrity of disulfide bonds is crucial to the tertiary structure and thus protein function, the disruption of protein structure by sulfitolysis may result in altered cellular activities leading to biochemical lesions[1,15]. Alternatively, a deficiency in the reaction product(sulfate, SO_4^{2-})may disturb normal fetal and neonatal development of the brain[20]. In addition, the nature of the lesion in human sulfite oxidase deficiency(with the central nervous system(CNS) being disproportionately affected) suggests that the principal problem is likely to be lipid peroxidation rather than amino acid metabolism[13]. Specifically, the cell membranes of the CNS myelin sheath are unique in possessing high concentrations of sulfatides and related lipids, which is likely the root cause of the sensitivity of the CNS to SO deficiency.

It has been speculated that SO is required for removing excess sulfite that accumulates upon decomposition of sulfur-containing amino acids or sulfated metabolites[10-12]. In contrast to this pathway, in sulfur assimilation, plants reduce the ubiquitous sulfate ion through a series of steps that includes activation by ATP sulfurylase and subsequent reduction to the sulfite by APS reductase[14,19]. The sulfite is then reduced by sulfite reductase(SiR; EC 1.8.7.1) through a process that transfers six electrons from ferredoxin to produce the fully reduced sulfide for incorporation into amino acids[8,18,21]. The enzyme catalyzes a two-electron transfer reaction in which the electrons from sulfite reduce the molybdenum that is a cofactor of sulfite oxidase. Thus, sulfite can be processed in plants by multiple pathways, and the exact physiological role of SO activity has yet to be established. Recent attempts to mimic the physiological electron acceptor cytochrome c by a modified electrode are gaining more interest in order to develop novel sulfite biosensors[3,6].

Codonopsis lanceolata is one of the most important medicinal plants of the family *Campanulaceae*, which consists of many species including many important medicinal plants. The related medicinal plants belonging to the family include *Platycodon grandiflorum*, *Codonopsis pilosula* and *Adenophora* species[9]. Despite their medicinal importance, little study has been carried out regarding genomic study of that plant. In this study, we report the isolation of a novel sulfite oxidase gene from *C.lanceolata* and analysis of the expression of the gene in response to various abiotic stresses.

EXPERIMENTAL

Materials

Codonopsis lanceolata plantlets were planted in glass bottles that contained a 70ml Murashige and Skoog medium with 3%(w/v) sucrose and 0.7% plant agar. The plants were grown in growth room at 25/18^{0}C and a 16-h photoperiod. General electrical lamps were used delivering irradiance of 8Wm^{-2}. For abiotic stress study, the plants were treated with abiotic stimuli in about 1 month after planting. For analysis of gene expression in different organs, samples were collected from leaves, roots and stems of *C.lanceolata* plants at 1 month after planting.

Sequence analyses

The full-length ClSO gene was analyzed using softwares BioEdit, Clustal X, Mega 3 and the other databases listed below: NCBI (http://www.ncbi.nlm.nih), SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsaautomat.pl/page=npsasopma..html).

Stress assays

To investigate the response of the ClSO gene to various stresses, the third leaves(from the top) with petioles from *C.lanceolata* were used. For treatment with hydrogen peroxide(H_2O_2-10 mM), salt (NaCl-100mM), and sulfur (Na_2SO_3-10mM), leaves samples were incubated separately in media containing each chemical at 25^{0}C for 48h. Chilling stress was applied by exposing the leaves to a temperature of 4^{0}C. To investigate relationship between ClSO gene expression in light and dark, leaves were kept for 2 days in dark room in order to inactivate photosynthesis and leaves were incubated under electrical lamp(OSRAM FL40SSEX-D/36) and in the dark room for 48h, respectively. In all cases, stress treatments were carried out on the MS media containing sterile water(chilling,

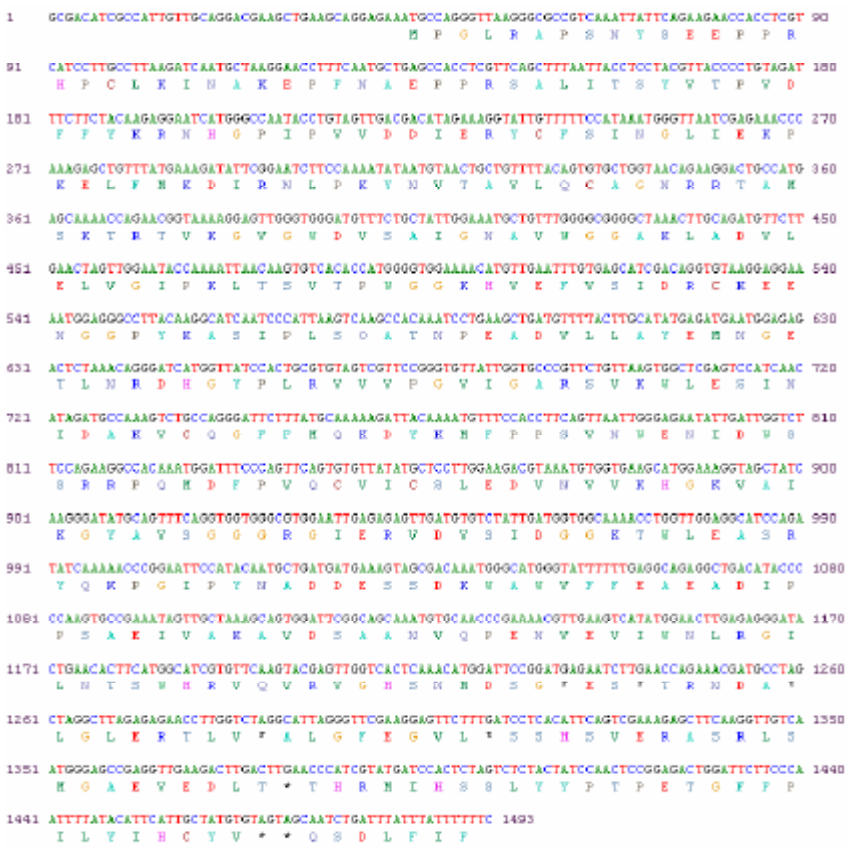

Figure 1 : Nucleotide sequence and deduced amino acid sequence of a ClSO cDNA isolated from *C.lanceolata*. Numbers on the right represent nucleotide positions. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The asterisk denotes the translation stop signal.

light and dark) and with treatment solution(H_2O_2, NaCl and Na_2SO_3) under continuous light. All treated plant materials were immediately frozen in liquid nitrogen and stored at -70^{0}C until required.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from various whole plant tissues (leaves, stems, roots) of *C.lanceolata* using RNeasy mini kit(Qiagen, Valencia, CA, USA). For RT-PCR, 800ng of total RNA was used as a template for reverse transcription using oligo$(dT)_{15}$ primer(0.2mM) (INTRON biotechnology, Inc., South Korea) for 5min at 75^{0}C. Then reaction mixture was incubated with AMV reverse transcriptase(10U/μl) (INTRON Biotechnology, Inc., South Korea) for 60min at 42^{0}C. The reaction was inactivated by heating the mixture at 94^{0}C for 5 min. PCR was then performed using a 1μl aliquot of the first strand cDNA in a final volume of 25μl containing 5pmol of specific primers for coding region of ClSO gene (forward, 5'- TCC TGA AGC TGA TGT TTT AC-3'; reverse, 5'-GCC ATG AAG TGT TCA GTA TC-3'). As a control, the primers specific to *C.lanceolata* actin gene were used (forward, 5'-CGA GAA GAG CTA CGA GCT ACC CGA TGG-3'; reverse, 5'-CTC GGT GCT AGG GCA GTG ATC TCT TTG CT-3'). PCR was carried out using 1μl of Taq DNA polymerase(Solgent Co., South Korea) in a thermal cycler programmed as follows: an initial denaturation for 5min at 95^{0}C, 30 amplification cycles[30sec.at 95^{0}C (denaturation), 30sec at 56^{0}C(annealing), and 90sec at 72^{0}C(polymerization)], followed by a final elongation for 10min at 72^{0}C. Actin gene was PCR-amplified in the same PCR conditions as ClSO gene with the same amplification cycles(30 cycles) and was used as an internal control to normalize each sample for variations in the amounts of RNA used.

RESULTS AND DISCUSSIONS

Isolation and characterization of the full-length

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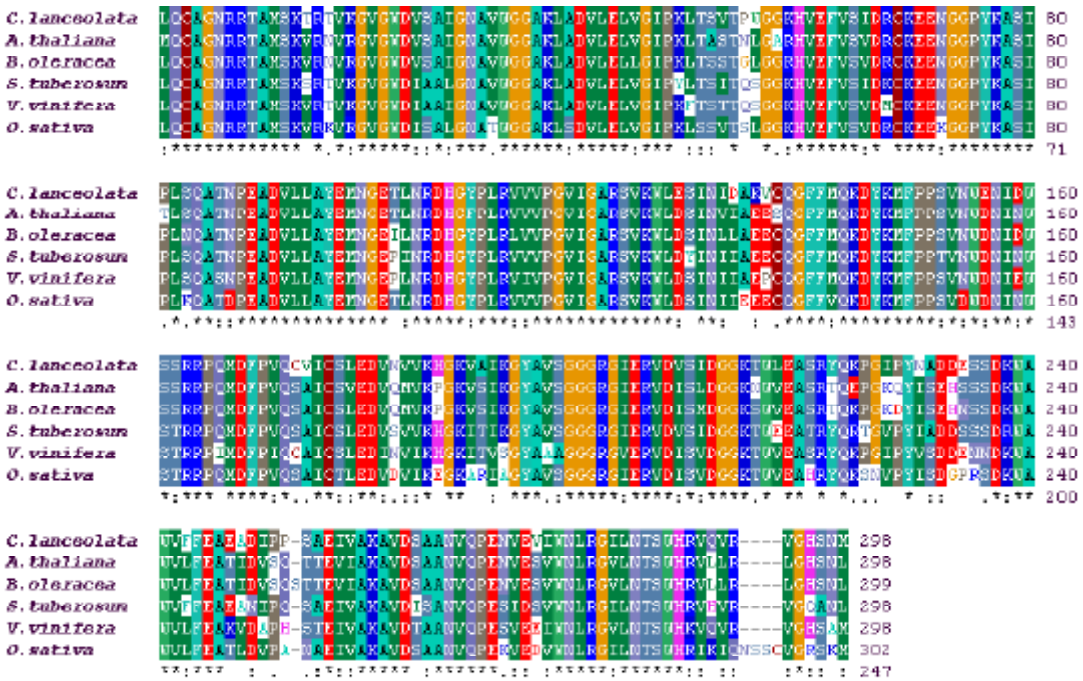

Figure 2 : Alignment of ClSO with most closely related SOs form 1. *Codonopsis lanceolata* (BAE48793); 2. *Solanum tuberosum* (ABB86275); 3. *Vitis vinifera* (CAN70050); 4. *Brassica oleracea* (ABD65019); 5. *Arabidopsis thaliana* (NP_001030620); 6. *Oryza sativa* (NP_001062326). Amino acid residues that are identical in all six sequences are indicated by asterisks, while well conserved residues are indicated by colons (identity at least four in five amino acids). Gaps introduced for optimal alignment are marked with dashes.

cDNA of the ClSO gene

As part of a genomic project to identify genes in the medicinal plant *Codonopsis lanceolata*, a cDNA library consisting about 1,000 cDNAs were previously constructed. A cDNA encoding a sulfite oxidase(SO), designated ClSO was isolated and sequenced. The sequence data of ClSO have been deposited in gen bank under accession number AB243086. As shown in figure 1, ClSO is 1493bp in length, and it has an open reading frame(ORF) of 1188bp nucleotides with a 42-nucleotide upstream sequence and a 263-nucleotide downstream sequence. The ORF of ClSO starts at nucleotide position 43 and ends at position 1230. ClSO encodes a precursor protein of 396 amino acids residues with no predicted signal peptide at the n-terminal. The calculated molecular mass of the matured protein is approximately 43.9 kDa. Total number of negatively charged residues(Asp+Glu) was 47 while the total number of positively charged residues(Arg+Lys) was 46.

Homology analysis

A GenBank Blastp search revealed that ClSO has the highest sequence homology to the potato(*Solanum tuberosum*) SO(ABB86275) with 85.3% identity and 92% similarity. Figure 2 shows a sequence alignment of ClSO and other closely related SOs. ClSO also shares a high degree homology with grapes(*Vitis vinifera*) SO(CAN70050)(83% identity and 91% similarity), Wild cabbage*(Brassica oleracea)* SO(ABD65019) (81% identity and 86% similarity), *Arabidopsis thaliana* SO(NP_001030620)(79% identity and 91% similarity). In contrast, ClSO shares lower degrees of homology with other than plants. For example, the homology scores are 46% identity and 59% similarity for a man(*Homo sapiens*) SO(NP_000447), and 41% identity and 60% similarity for a fruit flies (*Drosophila melanogaster*) SO(NP_573331)(TABLE 1). Figure 2 shows a sequence alignment result of ClSO and other closely related SOs and TABLE 1 shows sequence similarities between them. Phylogenetic analysis of twenty two SOs has been carried out using the Clustal X program (Figure 3).

Secondary structure analysis of ClSO protein

Secondary structure analysis and molecular modeling for ClSO were performed by SOMPA. The

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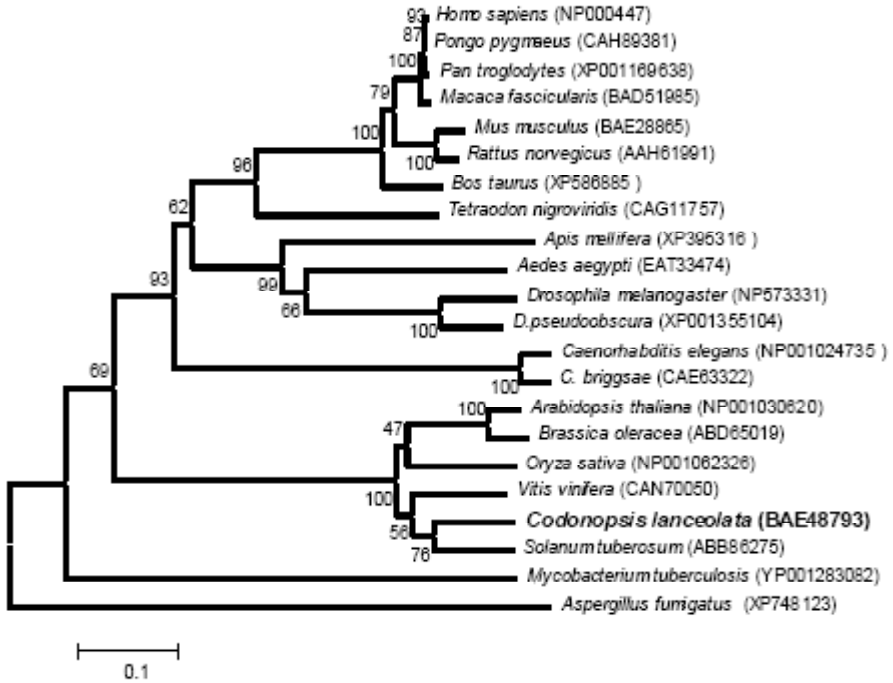

Figure 3 : Phylogenetic tree based on SO amino acid sequences, showing the phylogenetic relationships between ClSO and other SOs. Neighbor-joining method was used and a barrepresents 0.05 substitutions per amino acid position

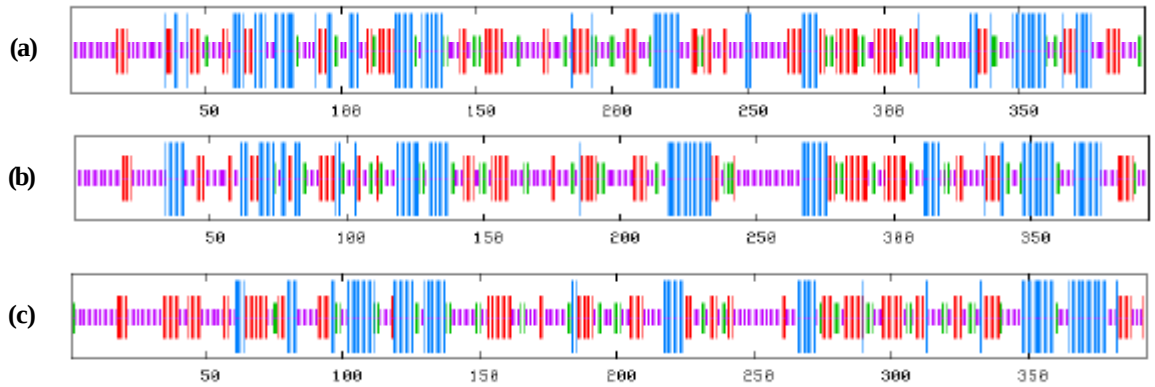

Figure 4 : Comparison of the secondary structures of SOs(a) ClSO, (b) potato(*Solanum tuberosum*)(c) grape (*Vitis vinifera*)

secondary structure analysis revealed that ClSO consists of 90α-helices, 44 β-turns jointed by 99 extended strands, and 163 random coils. This is highly similar to the secondary structure of potato SO(*Solanum tuberosum*), which contains 102 α-helices, 44 β-turns jointed by 86 extended strands, and 161 random coils and to the grapes(*Vitis vinifera*) SO, which contains 83 α-helices, 43 β-turns jointed by 104 extended strands.

The differential expression of ClSO in different *C.lanceolata* organs

The expression patterns of ClSO in different *C.lanceolata* organs were examined using reverse transcriptase (RT)-PCR analysis. From the results shown in figure 5, it is clear that ClSO is constitutively expressed in leaves, stems and roots. Among these organs, relatively higher levels of ClSO mRNA were observed in stems. A moderate level of ClSO was observed in roots and leaves.

Expression of ClSO in response to chilling

In figure 6A, the expression level of ClSO gradually increased after chilling. It is the first report that expression of sulfite oxidase is increased by chilling.

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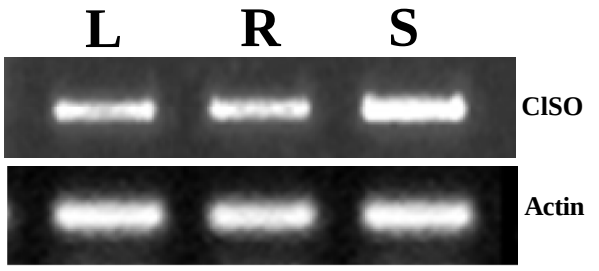

Figure 5 : Expressions of ClSO gene in different organs of *C.lanceolata*. Total RNAs were extracted from leaves (L), root (R) and stems (S). Actin was used as a control

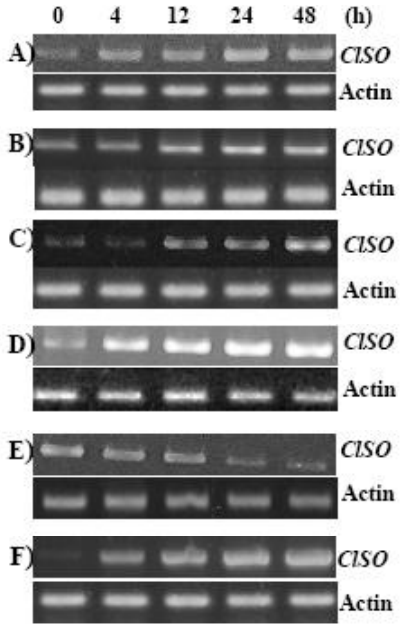

Figure 6 : RT-PCR analyses of the expressions of ClSO gene of *C.lanceolata* at various time points (h) post-treatment with various stresses

(A) chilling (4^{0}C) ; (B) 10mM H_2O_2; (C) 100mM NaCl; (D) Light; (E) Dark and (F) 10mM Na_2SO_3 treatment. Actin was used as an internal control

Expression of ClSO in response to oxidative stress

Figure 6B shows the accumulation of ClSO mRNA in response to hydrogen peroxide(10mM H_2O_2). Exogenous application of hydrogen peroxide appeared to cause an induction of ClSO gene at 4h post-treatment. The expression level increased gradually. It is the first report that expression of sulfite oxidase is increased by H_2O_2 oxidative stress.

Expression of ClSO in response to hyper-osmotic stress

Figure 6C, shows the accumulation of ClSO mRNA in response to salt stress(100mM NaCl), ClSO expression gradually increased.

Expression of ClSO in response to light and darkness

Figure 6D and 6E, shows that ClSO gene expression was gradually increased under light stress and gradually decreased under dark stress. Robert et al[10,11] found that SO expression was highest in *A.thaliana* after six to nine hours light exposure, but night time protein amount was slightly decreased. This point have been justified our result in light and dark stress.

Expression of ClSO in response to sulfur stress

Finally, figure 6F shows that ClSO gene expression induced at 4h post treatment of sulfur (Na_2SO_3) and increased the expression level gradually. The reason for increase the level of transcription of sulfite oxidase gene is due to excess sulfur content in the medium. It has been speculated that SO is required for removing excess sulfite that accumulates upon decomposition of sulfur-containing amino acids or sulfated metabolites[11,12].

CONCLUSION

In conclusion, we have cloned a new sulfite oxidase gene from *Codonopsis lanceolata* and named ClSO. As discussed above, its sequence characterization and expression pattern strongly suggest that ClSO plays a positive role in stress tolerance in *Codonopsis lanceolata*.

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