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Full Length Research Paper

Molecular cloning, expression and computational analysis of a water stress inducible copper-containing amine oxidase gene (*CuAO*) from tea plant [*Camellia sinensis* (L.) O. Kuntze]

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Copper-containing amine oxidase (CuAO) is the enzyme known to play diversity of function in plant responses to environmental stresses through its reaction products. Here, for the first time we report full length cDNA encoding CuAO protein from a drought tolerant tea cultivar. It was found to be 785 bp long with a 70 bp 5′-UTR, 193 bp 3′-UTR, 522 bp mORF and a polyA adenylational signal. It codes for a poly-peptide of 173 amino acids having predicted molecular weight and isoelectric point of 19 KDa and 7.75 respectively. Heterologous expression and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the protein in *Escherichia coli* revealed similar size as predicted by *in silico* analysis. Blastp analysis and template based homology modeling in Phyre2 has identified a copper amine oxidase domain with ligand binding site for copper at residue 123 (Histidine) which suggests its probable role in plant responses to environmental stresses. Interestingly, no signal peptide sequence was detected in the predicted protein which is in contrast to the CuAO so far reported in plants. Although, *in slico* analysis of the protein have indicated its probable structure and functions, further functional characterization is needed to better understand its role during drought and other environmental stresses in tea.

Key words: *Camellia sinensis*, copper amine oxidase, homology modeling, molecular cloning.

INTRODUCTION

Amine oxidases are enzymes reported widely in plants, animals and microorganisms (Mclntitre and Hartmann, 1993). It catalyzes the oxidative deamination of polyamines (PAs) that are known to function in responses to environmental stimuli such as osmotic stress, mineral deficiency, salinity (Bouchereau et al., 1999) and pathogen infections (Walters, 2003) in plants and therefore it is likely that these enzymes performs a diversity of functions. The copper-containing amine oxidase (CuAO) is one of the diamine oxidases that catalyze the oxidation of diamines Putrescine (Put) and Cadaverine at the primary amino groups (Cohen, 1998) to produce hydrogen peroxide (H_2O_2), Δ^1-pyrroline (P5C) and ammonia. The H_2O_2 thus produced is a potent reactive oxygen species (ROS) and involved in abscisic acid (ABA) induced stomatal closure (An et al., 2008) during dehydration stress in plants. The Δ^1-pyrroline is further reduced by P5CR (pyrroline-5-carboxylate reductase) to produce proline (Adams and Frank, 1980; Delauney et al., 1993),

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a well known osmoregulator (Delauney and Verma, 1993) and osmoprotectants (Venekamp et al., 1989) involved in the protection of plant cells from damage due to water stress (Shinozaki and Yamaguchi-Shinozaki, 1997). Thus, CuAO are involved not only in polyamine homeostasis but also involves in a numbers of important physiological processes in plants through its reaction products. Although, the protein has been reported to be present at high levels in dicot (Federico and Angelini, 1991), only few of the genes encoding it have been isolated and characterized from some leguminous species; *Arabidopsis thaliana* and *Euphorbia characias* (Koyanagi et al., 2000; Tipping and McPherson, 1995; Rea et al., 1998; Moller and McPherson, 1998; Padiglia et al., 2002). Till date, there is no report of cloning and characterization of the gene from tea.

Tea being perennial is subjected to recurring drought stress resulting in crop lost every year (Barua, 1989; Jain, 1999). Therefore, isolation and functional characterization of genes induced in drought and other environmental stresses could be an essential step for improvement of abiotic stress tolerance in tea. In the present study, we reported the full length isolation and cloning of *Camellia sinensis CuAO* gene (*CsCuAO*) from a drought tolerant (Annual Report, TRA, 2006) tea cultivar TV23 (Tocklai vegetative clone 23) followed by its computational analysis and heterologous expression in *Escherichia coli* and purification. qRT-PCR was performed to study the effect of drought stress on expression of the gene.

MATERIALS AND METHODS

Drought treatment and sample collection

To study the expression of the *CsCuAO* gene under water stress, an induced water stress experiment was conducted under green house conditions (day temperature of 26 ± 2°C, night temperature of 20 ± 2°C, 60% humidity, and natural photoperiod) in earthen pots (height, 36 cm; bottom diameter, 22 cm; top diameter, 35 cm and containing sandy-loam soil having pH 4.8 to 5.1, bulk density of 1.3 to 1.4 g/mL, and single super phosphate 500 g/cubic meter of soil). Drought stress was induced in TV23 plant by withholding water (after the plants acclimatized for 45 days in pot condition), while the control plants were regularly watered to field capacity. The extent of drought induction during the entire experiment was monitored by measuring different parameters like Leaf water potential (Ψ_L) (Scholander et al., 1965), relative water content of leaf (RWC) (Barrs, 1968), water use efficiency (WUE) (Wibbe and Blanke, 1995) and soil water content (SWC). Based on morphological observations, we identified three stages of drought induction: moderate stress (MS), severe stress (SS) and after severe stress (ASS). Leaf samples (bud+2 leaves) were collected at all three stages.

Ribonucleic acid (RNA) isolation and rapid amplification of cDNA ends (RACE)

Total RNA was isolated from both experimental and control samples (MS stage) using RNAqueous kit (Ambion, Cat. No. AM1912) following the manufacturers protocol and used for full length gene isolation. An EST (submitted GeneBank accession no HS396211) from an in-house SSH library of TV23 showing high homology with *CuAO* gene in the public database was used in the present study for full length isolation and cloning of *CsCuAO.* The in-house library was constructed using PCR-Select cDNA Subtraction Kit (Clontech, USA, Cat. No. 637401) between experimental (tester) and control (driver) plants of TV23 of MS stage to identify the differentially expressed drought responsive transcripts.

Based on EST sequence, 5´ gene specific primer (5´-TACAGTCGGCATGATTGGGAAGT-3´) was designed and RACE-PCR was performed (94°C for 5 min, 35 cycles of 94°C for 5 s, 65°C for 20 s, 68°C for 1 min and final extension at 68ºC for 5 min) in a thermal cycler (MasterCycler Gradient, Eppendorf) to amplify the unknown 5´ end (as 3´end of the EST was having PolyA tail) of *CsCuAO* using Platinum*Taq* High Fidelity DNA Polymerase (Invitrogen, Catalogue no. 11304-011) following the protocol mentioned in the GeneRacer Kit (Invitrogen, Cat. Nos. L1500-01; L1500-02; L1502-01; L1502-02). The amplified product was cloned into pGEM-T Easy vector (Promega Corporation, USA, Cat. No. A1360) and sequenced (using a 3130 XL Genetic Analyzer, Applied Biosystems) and compared with the EST sequence to get the full length cDNA of *CsCuAO.*

In silico analysis of the full length cDNA and predicted protein

Homology search of the isolated cDNA was done using Blastx programme of NCBI (National Centre for Biotechnology Information). The mORF and corresponding amino acid sequences were predicted using NCBI's ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) while UTRs (Untranslated region) were identified using UTRScan (http://itbtools.ba.itb.cnr.it/utrscan) and UTRSite (http://utrsite.ba.itb.cnr.it/).

The deduced amino acid sequences of the mORF was blasted using blastp programme of NCBI and the first six CuAO proteins showing high homology with the query protein were retrieved and aligned using Clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and sequence manipulation suite 2 (http://www.bioinformatics.org/sms2/). The presence of domains, patterns and motifs were searched using SMART (Simple Modular Architecture Research Tool) on line tool (http://smart.embl-heidelberg.de/). Theoretical molecular weight and isoelectric point (pI) was computed using Compute pI/Mw tool (http://web.expasy.org/compute_pi/).

3D structure prediction/Homology modeling

Topology of the predicted protein was computed using Phyre2 (Protein homology/analogy recognition engine) server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).
Further, the ligand binding site and transmembrane helix were predicted using 3DLigandSite (http://www.sbg.bio.ic.ac.uk /3dligandsite/) and PSIPRED Protein Structure Prediction Server (http:// bioinf.cs.ucl.ac.uk/psipred/) respectively (Wass et al., 2010; Nugent and Jones, 2009). Presence of signal peptide was predicted in SignalIP server (http://www.cbs.dtu.dk/services/SignalP/).

Cloning, heterologous expression and protein purification

The predicted mORF was amplified (94°C for 5 min, followed by 35 cycles of 94 °C for 30 s, 67°C for 20 s, 68ºC for 1 min and final extension at 68°C for 5 min) using forward (**5´**-GACGACGACAAGATGAAAGCTGTGAGGAATGTGGCCAG-3´) and reverse (5´-GAGGAGAAGCCCGGTCAGGCAGAAGCAGAAGCC-3´) primers designed according to the instructions given in the user manual of pET-43.1 Ek/LIC Vector Kit (Novagen, Cat. No. 71072-3) so that the amplified product incorporates all the tags (Nus-tag, His-tag and S-Tag) (tag size 546 amino acids) at the N-terminal end. The amplified cDNA was cloned into pET-43.1 Ek/LIC Vector, trans-

formed and sequenced followed by retransformation into *E. coli* host strains BL21 (DE3) pLysS (Novagen) for protein expression at different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG). The protein was purified using Dynabeads His-Tag isolation and pull down kit (Invotrogen, Cat. No.101.04D) and run in sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-(PAGE) for size verification.

Expression of *CsCuAO* in response to water stress

Total RNA isolated from three stages (MS, SS, ASS) of experimental and control plants were reverse transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche, Germany, Cat. No. 04379012001) following the manufacturers protocol. Quantitative real time PCR (qRT-PCR) was carried out in triplicates (LightCycler 480 II, Roche, Germany) using forward (5´-GCTTCGATCTAAAACCGGTCA-3´) and reverse (5´-AGCTGCAAACAACACCCAAG-3´) primers following the instructions given in LightCycler 480 SYBR green I Master kit (Cat. No. 04707516001, Roche, Germany). Constitutively expressed 18S housekeeping gene (GeneBank Accession no. AY563528) was used as internal control (Gohain et al. 2011) using forward (5´-GGCCGGCTCCGTTACTTTG-3´) and reverse (5´-GTTTCAGCCTTGCGACCATACTC-3´) primers. The following amplification programme was used: pre incubation at 95°C for 5 min, 45 cycles of 95°C for 10 s, 65°C for 10 s and 72°C for 30 s followed by melting curve analysis (95°C for 5 s, 65°C for 1 min and 97°C for continuous acquisition). The data acquisition was done employing the 2nd derivative maximum method (Tichopad et al., 2003, 2004), as computed by the software of LightCycler (Roche Diagnostics) Carousel-based system.

RESULTS

Drought treatment

The induction of progressive drought during the experiment was clearly evident from the morphological observations (Figure 1A to C). MS stage (20th day) was characterized by start of partial drooping of young leaves and complete drooping of mature leaves. SS stage (25th day) was characterized by complete drooping of all the young leaves and senescence of mature leaves while ASS stage (27th day) was characterized by start of drying of young leaves and senescence of mature leaves. Statistical analysis of the data (Figure 1D) collected at three stages also clearly showed induction of progressive drought with time.

Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR)

5´–RACE-PCR resulted into amplification of a single band of approximate size 500 bp (Figure 2A). The cloning and subsequent sequence analysis of this fragment has shown overlapping sequences of its 3´ end with the 5´ end of the EST under consideration. This clearly indicates that the amplified fragment represent the 5´ end of the transcript from which EST was derived. Since the EST had a PolyA tail, there was no need to perform the 3´-RACE-PCR. The full length cDNA was then reconstituted by combining these two sequences. It was found to be 785 bp in length (Figure 3).

In silico analysis of full length cDNA of *CsCuAO*

The blastx analysis of the full length cDNA showed high homology with the *CuAO* of other plant species from the public database. The cDNA was found to have a 70 bp 5′-UTR, 193 bp 3′-UTR, 522 bp mORF, a polyA tail and a polyA adenylational signal (Figure 3). The presence of these features clearly indicates that the isolated cDNA is a true full length cDNA and has been submitted to NCBI (GeneBank accession number JN561075). The mORF is predicted to code for a polypeptide of 173 amino acids (Figure 3) which upon blastp analysis have shown a putative copper amine oxidase domain. The top six amine oxidase proteins showing maximum homology with the deduced amino acid sequence of *CsCuAO* upon blastp analysis are *Ricinus communis* (86%), *Solanum lycopersicum* (83%), *Canavalia lineate* (82%), *Vitis vinefera* (83%), *Arabidopsis thaliana* (77%) and *M. tranculata* (80%) (Figure 4).

In silico analysis and homology modeling of predicted protein

The deduced protein is predicted to have a molecular weight and pI of 19 KDa (assuming 110 KDa per amino acid) and 7.75 respectively. Signal peptide and transmembrane domain prediction could not detect any signal peptide sequence and domain in the predicted protein. SMART tool analysis revealed the presence of a copper amine oxidase domain (Pfam accession no. PF01179) from residues 1 to 161 with high e-value of 4.2e-59 which was also revealed by NCBI's blastp analysis.

Template based homology modeling in Phyre2 successfully modeled the predicted protein (Figure 5A) based on protein data bank (PDB) template c1ksiA which is a homodimer of chain A and B; each having three domains (Kumar et al., 1996). The largest domain of chain A (227 to 647) representing copper amine oxidase domain was used as template for modeling the predicted protein. Seven β- strand and four α-helices were identified in the predicted model with significant confidence level. The alignment of amino acid sequences of the copper amine oxidase domain of the predicted protein (residues from 1-166) and template (residues from 482-647) has shown highly conserved residues between them (not shown). A copper binding site at residue 123 (histidine) was also identified in the predicted model (Figure 5B). However, there are binding sites detected for other metallic heterogens like Nickel and Zinc ions in the predicted binding site at histidine residue. The average distance between the binding site residue and ligand was found to be zero indicating a very close association of ligand and histidine residue.

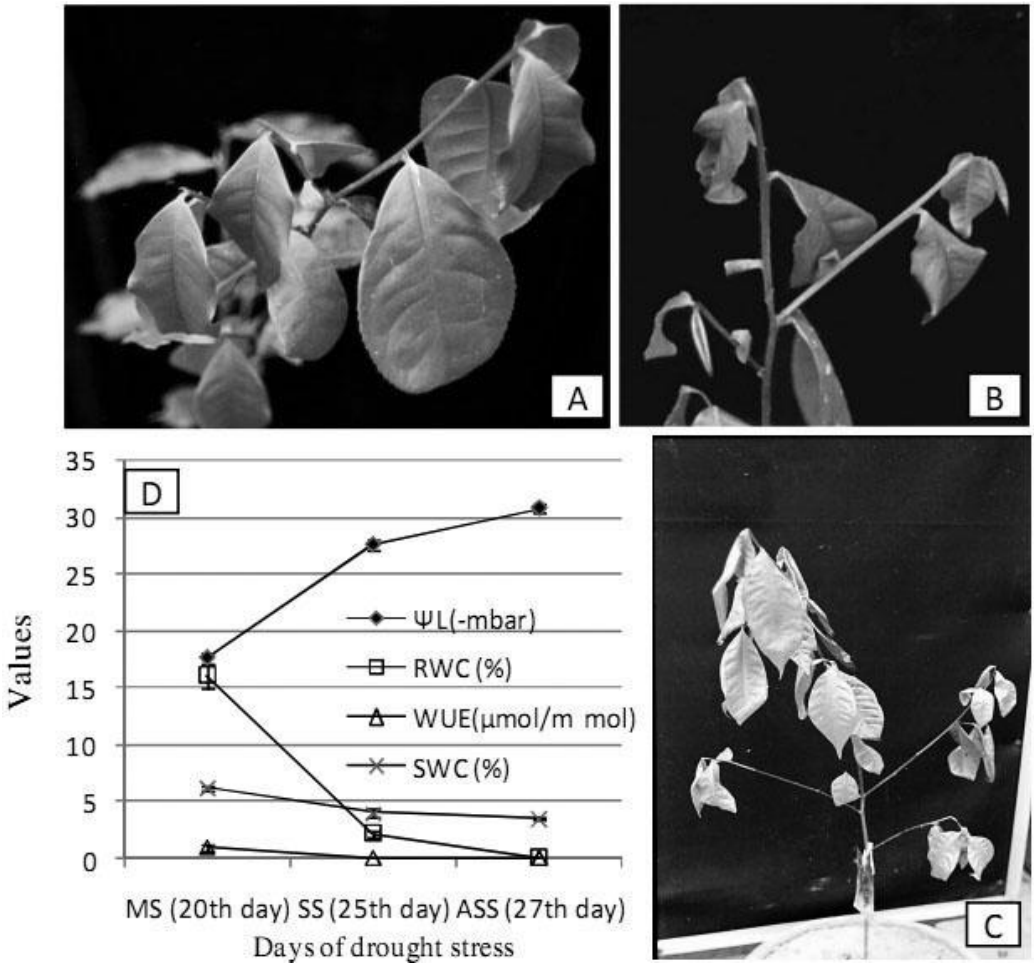

Figure 1. The induced water stress experiment showing three stages of drought in TV23 plant. A , MS stage on 20th day. B, SS stage on 25th day. C, ASS stage on 27th day of drought induction. Leaf samples from these three stages were used in the expression studies of *CsCuAO*. D, Physiological parameters recorded (mean ± SE of 7 replicates, significant at 5% level) at three stages. On 25th day the SWC was 4% which is just sufficient to survive the plant. At this stage plant was just able to maintain some photosynthetic activities and leaf water status as indicated by low value of WUE and RWC, indicating severe stress condition. On 27th day the values were further reduced resulting more severe stress.

Heterologous expression and real-time-polymerase chain reaction (qRT-PCR)

The amplification of the mORF resulted into amplification of a 548 bp fragment (Figure 2B) which was cloned into pET vector for expression. The incubation of the positive clone at 37°C for 8 to 10 h at 1.5 mM IPTG concentration was found to be optimum for the maximum induction of the predicted protein (Figure 5C). The induction was found to be suboptimum at IPTG concentrations more or less than 1.5 mM. The SDS-PAGE analysis of the purified protein revealed a molecular weight of approximately 79 KDa which includes a tag of 60 KDa. Therefore, the molecular weight of the predicted protein was 19 KDa (6879K Da minus 60 KDa) which is equal to the size predicted in Compute pI/Mw tool.

To know the effects of drought on expression of the gene, we monitored its expression in leaf tissues at three stages of induced drought. The result shows that the gene is strongly induced by drought and its expression increases several fold with the progression of drought in three stages in the experimental plant (Figure 6A), while very low level of expression was detected in the control plant (Figure 6B).

DISCUSSION

The water stress during the induced drought experiment was very much evident from morphological changes in the experimental plants (Figure 1A to 1C) as well as from decreasing values of physiological parameters compared

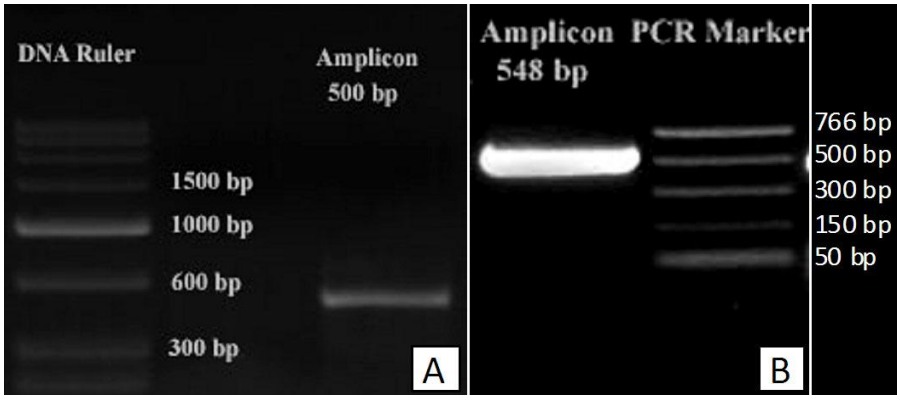

Figure 2. Amplification of full length cDNA of *CsCuAO.* A, 500 bp amplified product of 5´-RACE-PCR. B, 548 bp amplified product of the mORF which was eluted and ligated to pET vector for cloning and expression in *E. coli.*

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001 caattccttcgtgaatgtcaatctcaaaagggaacagacctcccc

046 ggcggagacccctcgtaggagctac

071 atgaaagctgtgaggaatgtggccaggactgagaaggacgcccag
    M  K  A  V  R  N  V  A  R  T  E  K  D  A  Q
116 gttaagttcaagctctatgacccatctgaattccacgtgatcaat
    V  K  F  K  L  Y  D  P  S  E  F  H  V  I  N
161 ccgtctaagaagacacgggttgggaatccggttgggtacaagctg
    P  S  K  K  T  R  V  G  N  P  V  G  Y  K  L
206 gttcctgctggcacggccgctagcttgctagatcctgaggatcct
    V  P  A  G  T  A  A  S  L  L  D  P  E  D  P
251 cctcagaagagaggcgcattcacaaataatcaaatttgggtcact
    P  Q  K  R  G  A  F  T  N  N  Q  I  W  V  T
296 ccgtacaaccagaccgagcaatgggctggcggcttgtttgcctac
    P  Y  N  Q  T  E  Q  W  A  G  G  L  F  A  Y
341 caaagccaaggtggggacactcttgcaacatggtctgaaaggtat
    Q  S  Q  G  G  D  T  L  A  T  W  S  E  R  Y
386 cggccaattgagaacaaggacatcgtgctgtggtacactttaggc
    R  P  I  E  N  K  D  I  V  L  W  Y  T  L  G
431 tttcatcgcgtgccgtgtcaagaggacttcccaatcatgccgact
    F  H  R  V  P  C  Q  E  D  F  P  I  M  P  T
476 gtatcttcgagcttcgatctaaaaccggtcaacttctttgagaac
    V  S  S  S  F  D  L  K  P  V  N  F  F  E  N
521 aatccgattctgaggatccctcctaatgttgaaaaagacctaccc
    N  P  I  L  R  I  P  P  N  V  E  K  D  L  P
566 aactgcaaggcttctgcttctgcctga 592
    N  C  K  A  S  A  S  A  *

593 gaaactatgccgatccatagagccttttgttgtaagatgatgctg

638 tgaacacatcaagtatgtacaagactactgtacttgaaacttggg

683 tgttgtttgcagctaagctaatttaataaaaaattcgtatctaaa

728 gggtgtgtgtaagaacagtacttgaatgttggatgtttctttgtg

773 agtcaaatttggtaaaaaaaaaaaaaaaaaaaaaaaaa
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Figure 3. Full length sequence of *CsCuAO*. Predicted ORF (middle part), 5´ and 3´-UTRs (upper and lower part) are separated by horizontal lines. The PolyA adenylation signal sequence is underlined.

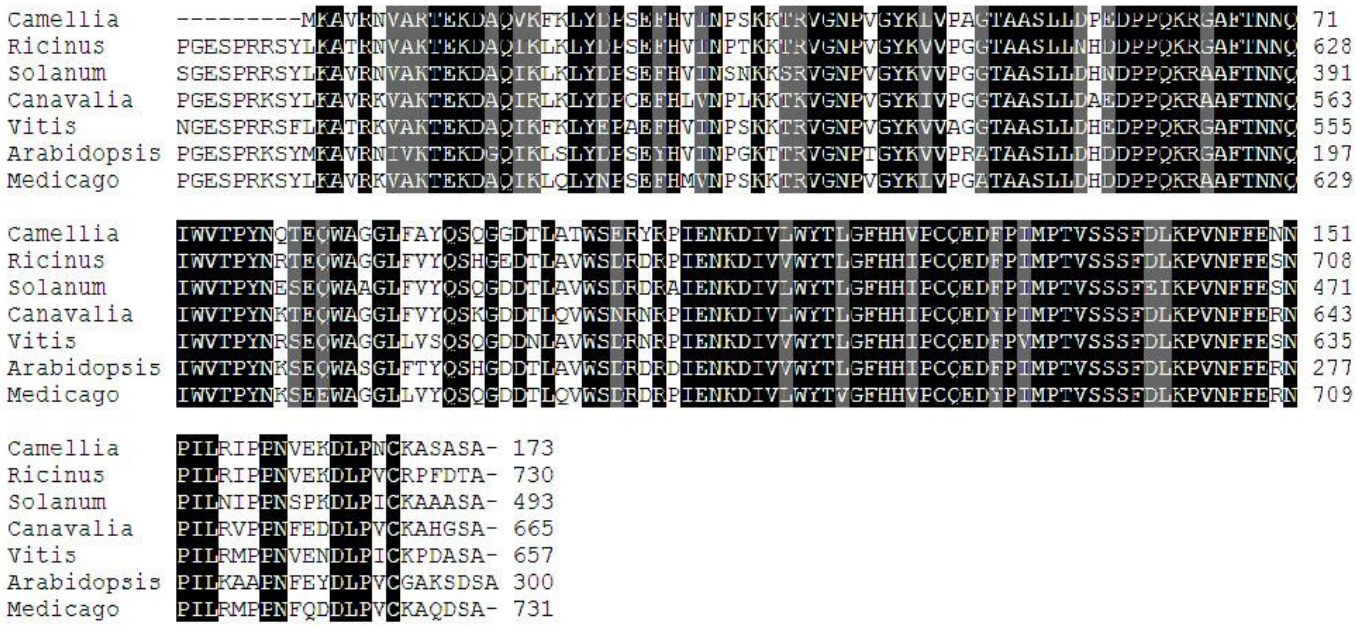

Figure 4. Multiple sequence alignment of deduced amino acid sequence of *CsCuAO* with six amine oxidase sequence from the public database having maximum homology (80-86%). Residues that are identical among the sequences are given a black background, and those that are similar among the sequences are given a gray background. The accession numbers of these sequences are JN561075 (*C. sinensis*), XP_002509596.1 (*R. communis*), CAI39243.1 (*S. lycopersicum*), AAD49420.1 (*C. lineate*), XP_002275872.1 (*V. vinifera*), NP_192965.1 (*A. thaliana*) and XP_003601419.1 (*Medicago tranculata*).

to the control plants (Figure 1D). On 25th day of drought induction, three of the parameters (RWC, WUE, and SWC) approached zero which indicated that the plant was under severe water stress condition. With the progresssion of drought, the Ψ_L became very high indicating that the plants were under severe water stress condition. An attempt for revival of the plant by rewatering at ASS was found unsuccessful, while at SS it could easily revive upon rewatering. Therefore, the genes that are expressed at MS and SS are very much important for survival of the plant under water stress condition. These groups of genes might function during water stress condition and help the plant to improve its drought tolerance.

In the present study, we have reported for the first time the full length cDNA of *CuAO* of tea. High homology of the isolated cDNA with other plant *CuAO* gene indicate that it represent full length *CsCuAO*. However, it was found to be smaller (785 bp) than all the full length *CuAO* reported in plants (Rossi et al., 1992; Tipping and McPherson, 1995; Moller and McPherson, 1998; Koyanagi et al., 2000). Identification of a copper amine oxidase domain and a copper ion binding site at histidine residue indicates its involvement in cell differentiation and growth, wound healing, detoxification and cell signaling as suggested by Kumar et al. (1996). The *CuAO* isolated from pea seedling (Koyanagi et al., 2000) and Arabidopsis (Moller and McPherson, 1998) have been reported to have a signal sequence at its N-terminal end. This signal sequence was not found in the present study indicating that the CuAO in tea is neither a secretory nor a membrane protein. Therefore, further biochemical characterization is necessary to know its localization and function.

Expression analysis has shown a very strong induction of the *CsCuAO* gene at three stages during the induced drought experiment. This high expression of the gene must have some role to play that probably helped the plant to survive up to 25 days water stress condition during the drought experiment. This enzyme has been reported to maintain PA homeostasis in plants, in addition to participate in a number of physiological processes during environmental stresses including drought, through its reaction products like H_2O_2 and proline (Cona et al., 2006). The H_2O_2 is a ROS and have been reported to be involved in ABA induced stomatal closure (An et al., 2008) that aid in dehydration stress tolerance in plants by reducing transpirational water loss. ABA is a general stress hormone that accumulates under drought and known to activate a number of stress responsive genes that functions in drought adaptation or tolerance in plants (Shinozaki and Yamaguchi-Shinozaki, 1997). The proline is an osmoprotactant whose accumulation is known to enhance drought tolerance in plants. Therefore, it is likely that the higher expression level of the gene observed in the present study might contribute for drought tolerance of TV23, possibly through its reaction products as shown in Figure 7. However, drought tolerance of plant is a complex multigenic trait often manifested by its physiological and biochemical reactions involving interplay of a vast array of genes. Therefore, further investigations are required to understand the significance of high level

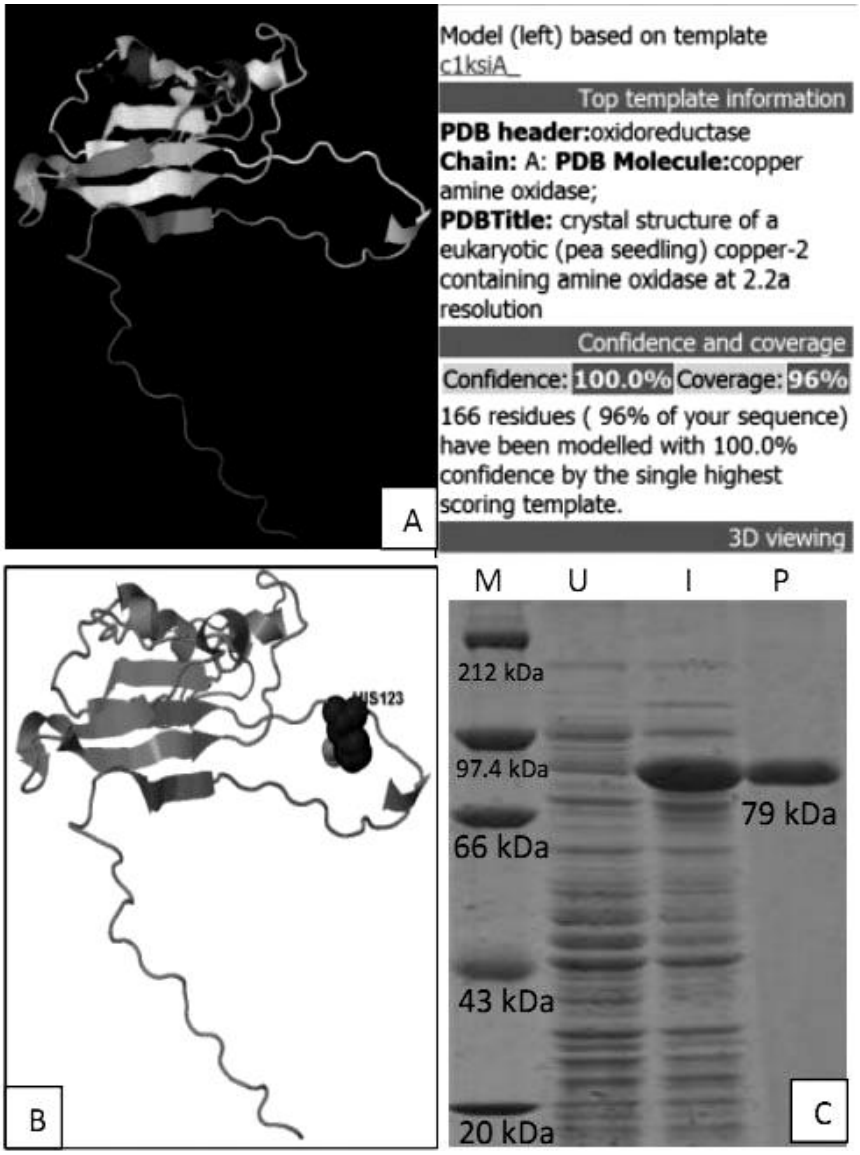

Figure 5. Template based homology modeling and heterologous expression of the predicted protein. A, Model of *CsCuAO* protein based on template c1ksiA [crystal structure of eukaryotic (pea seedling) copper-2 containing *amine oxidase*] predicted with 100% confidence, 53% identity and 96% query coverage (166 residue). The high confidence level and identity indicates that there is a very high probability that the used template for modeling is a true homologue of the query protein. B, Predicted Ligand binding sites (His123) is shown in black balls and heterogens in grey balls. C, SDS-PAGE showing His-tag purified protein (M, Protein marker; U, uninduced culture; I, induced culture; P, purified protein).

expression of this gene, along with others, under water stress environment in tea.

Conclusion

The cDNA encoding CuAO from *Camellia sinensis* was cloned, expressed and purified from *E. coli*. The size was determined by *in silico* analysis as well as by SDS-PAGE. Identification of copper amine oxidase domain has indicated its possible involvement in environmental stresses. Although, expression analysis has revealed a very strong induction of the gene under drought, its role in tea need further study using different techniques like nuclear magnetic resonance (NMR), X-ray crystallography to get further insights into its function and structure.

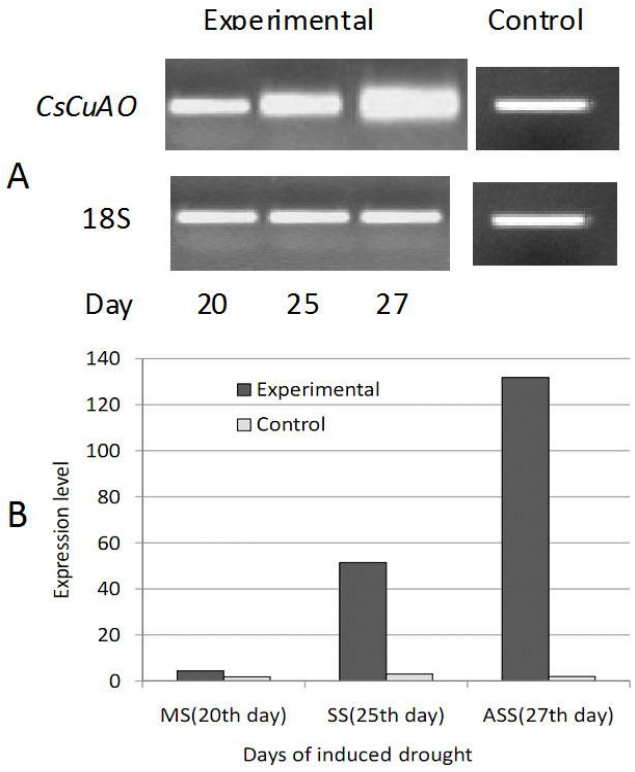

Figure 6. Relative expression of the *CsCuAO* at three stages of drought induction. A, Showing relative transcript abundance of *CsCuAO* in control and experimental plants at three stages. B, Showing fold increase of expression level of *CsCuAO* with the increase of drought stress in three stages.

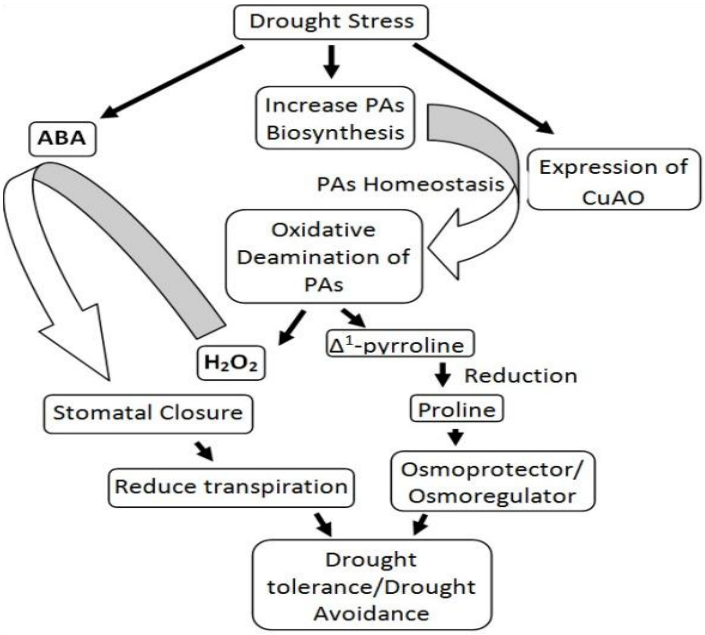

Figure 7. The schematic representation of events that takes place during drought stress in plants mediated by oxidative deamination products of polyamines (PAs) by CuAO.

Further, functional characterization of this protein in model plants can be expected to through light towards its role during water stress and may provide a candidate gene for future improvement of drought tolerance in tea and others crops.

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