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Barley molybdenum cofactor sulfurase (MCSU): sequencing, modeling, and its comparison to other higher plants

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Abstract: Molybdenum cofactor sulfurases (MCSUs) are important enzymes for plant development and response to environmental queues, including processes such as nitrogen metabolism and regulation of the abscisic acid levels in plant tissues. We cloned and sequenced *MCSU* gene from barley and performed in silico comparison with rice, tomato, and *Arabidopsis*. Physico-chemical properties and subcellular predictions were found to be similar in different plant species. All MCSUs had three critical domains: aminotransferase class-V (Pfam: PF00266), MOSC N-terminal beta barrel (Pfam: PF03476), and MOSC (Pfam: PF03473). Secondary structure analysis revealed that random coils were the most abundant, followed by α-helices and extended strands. Predicted binding sites of MCSUs were different in barley and *Arabidopsis*, whereas rice and tomato showed the same pattern. A conserved triple-cysteine motif was detected in all MCSUs with cys438-cys440-cys445, cys431-cys433-cys438, cys428-cys430-cys435, and cys425-cys427-cys432 in barley, rice, *Arabidopsis*, and tomato, respectively. Furthermore, a 3D structure analysis indicated that structural divergences were present in all MCSUs, even in the core domain structure. Phylogenetic analysis of MCSUs revealed that monocot–dicot divergence was clearly observed with high bootstrap values. The results of this study will contribute to the understanding of *MCSU* genes and proteins in plants. The data of this study will also constitute a scientific basis for wet-lab and in silico studies of MCSUs.

Key words: Barley, in silico analysis, molybdenum cofactor sulfurase, sequencing, 3D structure, plant protein

1. Introduction

The plant hormone abscisic acid (ABA) appears to influence several physiological and developmental events. ABA plays a major role in the adaptation to abiotic environmental stresses, seed development, and germination (Zeevaart and Creelman, 1988). Thus, an understanding of the ABA biosynthetic pathway and its regulation is of crucial importance in elucidating the factors that mediate both stress responses and plant development.

Molybdenum (Mo) is an essential micronutrient for plant growth, development, and yield (Hewitt, 1983). Biologically active Mo is complexed by a cofactor, molybdopterin. Molybdenum cofactor (MoCo) combines with apoproteins to interact with other components of the enzyme for electron transport (Mendel, 1997, 2013). Sulfurylated MoCo is a cofactor of ABA-aldehyde oxidase (AO) that functions in the last step of ABA biosynthesis (Xiong et al., 2001; Chen et al., 2003). The Mo-enzymes in plants can be subdivided into two groups: nitrate reductase (NR) and sulfite oxidase (SO), which possess a dioxo-Mo center and are activated by insertion of MoCo, xanthine dehydrogenase (XDH), and AO; however, they require a separate final step of maturation after insertion of MoCo. XDH and AO have a mono-oxo MO center and need the addition of a terminal inorganic sulfur to the Mo-center in order to gain enzymatic activity. The mitochondrial amidoxime-reducing component (mARC) has been identified in mitochondria of mammals, and catalyzes the reduction of *N*-hydroxylated substances (Havemeyer et al., 2011). Like mammals, plant genomes encode two mARC isoforms, which have not yet been investigated in detail (Chamizo-Ampudia et al., 2011; Krompholz et al., 2012; Bittner, 2014). mARC proteins represent the simplest eukaryotic molybdenum enzymes in that they

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consist of only a single distinct MCSU C-terminal domain and bind only one prosthetic group, the molybdenum cofactor. Moreover, with an average molecular weight of 35 kDa, mARCs are even smaller than that of plant sulfite oxidase. In contrast to all other known eukaryotic molybdenum enzymes, which are dimers, mARC proteins are monomeric. These proteins share a significant degree of homology with the molybdenum cofactor-binding domain of eukaryotic molybdenum cofactor sulfurase proteins, the latter catalyzing the post-translational activation of aldehyde oxidase and xanthine oxidoreductase (Wahl et al., 2010).

Biochemical analysis of mutants that are deficient in ABA has facilitated the elucidation of the biosynthetic pathway of ABA in higher plants. These mutants include *viviparous10* (*vp10*) and *viviparous15* (*vp15*) in maize; *flacca*, *notabilis*, and *sitiens* in tomato; *dr* in potato; *aba1* in *Nicotiana plumbaginifolia*; *nar2a* in barley; and *sir* and *aba3* in *Arabidopsis thaliana* (Walker-Simmons et al., 1989; Taylor, 1991; Schwartz et al., 1997; Zhao et al., 2003; Dai et al., 2005; Porch et al., 2006). Genetic mapping and functional complementation studies have demonstrated that the pleiotropic *flacca* phenotype in tomato is a result of the absence of MoCo sulfuration (Sagi et al., 2002). Although cDNA encoding molybdenum cofactor sulfurases have been isolated from several organisms (Watanabe et al., 2000; Bittner et al., 2001; Ichida et al., 2001; Sagi et al., 2002; Komoto et al., 2003), detailed biochemical information is available only for the molybdenum cofactor sulfurase ABA3 from *A. thaliana*. ABA3 is a homodimeric protein consisting of two monomers of ~92 kDa each, individually subdivided into two domains (Seo et al., 2000). The NH2-terminal domain possesses a pyridoxal-5-phosphate cofactor, which is crucial for the activity of the protein in which sulfur is abstracted from L-cysteine (Sagi et al., 2002; Heidenreich et al., 2005). Based on sequence similarity, mARC proteins are related to the molybdenum cofactor sulfurases C-terminal domains (MOSC) of ABA3 and related sulfurase systems. Their main structural features are a β-barrel-like structure N-terminal to the MOSC domain itself and a strictly conserved cysteine residue at the extreme C-terminal end (Anantharaman and Aravind, 2002). The β-barrel-like structure is predicted to have specific roles in the interaction with the substrates of MOSC domain proteins, while the conserved cysteine residue accepts sulfur from the sulfur-generating systems, such as cysteine desulfurases, prior to transfer on other proteins for yet unknown scopes. The MCSU proteins encoded by the *Arabidopsis ABA3*/*LOS5* gene and tomato *FLACCA* gene are both composed of two functional domains: a NifS-like sulfurase domain at the N-terminus and a MCSU conserved domain (MOSC) at the C-terminus (Bittner et al., 2001; Anantharaman and Aravind, 2002). The NifS domain functions as cysteine sulfurase using pyridoxal phosphate (PLP) as a cofactor. It transfers the sulfur from L-cysteine to target molecules and releases L-alanine (Heidenreich et al., 2005; Lehrke et al., 2012). The conserved C-terminal is considered to function in the recognition of molybdenum enzymes (Bittner et al., 2001). Previous studies of the *MCSU* mutants showed that mutation at either the N-terminal or C-terminal portion of the protein disrupted MCSU function and reduced AO activity and ABA content in plant tissues (Wollers et al., 2008). In humans, lesions in MoCo sulfurase give rise to Xanthinuria type II disease (Ichida et al., 2001). Genes for *ma-1* (GenBank AF162681; (Amrani et al., 2000)) and for similar loci in *Aspergillus nidulans* (hxB, GenBank AF128114; (Amrani et al., 2000)), cattle (*MCSU*, GenBank AB036422; (Watanabe et al., 2000)), humans (hmcs, AA689476; (Amrani et al., 2000); GenBank XP 008726; (Ichida et al., 2001)), and *Arabidopsis* (*aba3*, GenBank AF325457 (Seo et al., 2000) and AY034895 (Xiong et al., 2001)) were identified. Recently, Huang et al. (2009) identified the rice MCSU cDNA (OsMCSU), which is the first *MCSU* gene cloned in monocot species. According to the functional domain analysis of the predicted amino acid sequence, the OsMCSU protein contains a Nifs domain at its N-terminus and a MOSC domain at the C-terminus. Here, we report for the first time on the cloning, sequencing, and molecular modeling of MoCo sulfurase in barley. We perform a comparative analysis of MoCo sulfurase protein in higher plants, including barley (*Hordeum vulgare* subsp. *vulgare*), *Oryza sativa*, *Arabidopsis thaliana*, and *Solanum lycopersicum*, by using bioinformatics tools.

2. Materials and methods

2.1. Plant materials and growth conditions

Three-week-old leaf material from soil-grown plants (*Hordeum vulgare* L.) cv. Morex was grown in pots in a greenhouse at 22 ± 2 °C with a relative humidity of 70% and was used for DNA extraction using a standard protocol (Song et al., 1995).

2.2. Cloning and sequencing MoCo sulfurase gene in barley

The genomic DNA of barley (*Hordeum vulgare* L.) cv. Morex was used as the DNA template in initial PCR reactions. C-terminal portion of MoCo sulfurase homolog from cultivated barley (*Hordeum vulgare*) EST (AV834055) was chosen for primer designing. Amplifications were performed on a GeneAmp PCR System 9700 thermal cycler (Perkin–Elmer Applied Biosystems, USA). The resulting 945 bp DNA fragment was purified by using EZ-Gel Extraction Kit (Biological Industries, Israel). The PCR products were cloned into pGEM-T Easy Vector (Promega, USA) and sequenced. The barley cv. Morex BAC library has been described previously (Yu

et al., 2000). Seventeen high density colony filters of barley (*H. vulgare*) BAC clones (6.64x genome coverage) were obtained from Clemson University, SC, USA and hybridized with the *MoCo sulfurase* gene probe labeled by ^{32}P-dCTP. Positive clones were screened by *MoCo sulfurase* gene specific primers. DNA samples were isolated from the positive BAC clones using PSI Clone BAC DNA Kit (Princeton Separations, USA) from 5 mL of 24-h *E. coli* cultures containing 20 µg/mL of chloramphenicol. *Hind*III digestion of fragments were separated on a 1% agarose gel in 0.5xX TBE using Chef Mapper XA Pulse Field Electrophoresis System (Bio-Rad), then blotted onto a nylon membrane and probed with [^{32}P]-labeled MoCo sulfurase probe. Prehybridization and hybridization were carried out at 65 °C in 2.5xX SSPE (sodium phosphate monobasic), 1% SDS, 0.01% sodium pyrophosphate, and 0.1% PAES (polyanetolensulfonic acid). Washes were performed at 65 °C with 0.5xX SSC and 0.1% SDS in the final wash. Primary and secondary genome walking amplifications in the 5 and 3 prime direction on BAC DNA and genomic DNA were carried out with the combination of DNA Walking ACP (DW-ACP) primers provided by the kit, together with *MoCo sulfurase* gene specific primers. The resulting secondary PCR products were gel separated, isolated, and cloned into pGEM-T Easy Vector (Promega, USA), according to standard manufacturer protocol, and sequenced. Gene specific primers were used for performing 3'-rapid amplification of cDNA ends to obtain the full length of *MoCo sulfurase* gene. The FirstChoice RLM-RACE Kit (Ambion, USA) was applied according to manufacturer instructions using the mRNA poly-(A)$^+$. RACE products were generated using the gene-specific outer and inner primers. Single amplification product from RACE experiments was cloned into pGEM-T Easy Vector and sequenced. 5' end sequence of MoCo sulfurase gene was obtained by transposon insertion into MoCo sulfurase BAC clone. A total of 250 ng of MoCo sulfurase BAC DNA was used in a standard EZ::TN <oriV/KAN-2> insertion reaction (EZ::TN Insertion Kit Epicentre, USA); the reaction was stopped and the DNA stored at –20 °C. Transformation of TransforMax EPI300 *E. coli* with 1 µL of transposon-inserted BAC DNA was performed according to manufacturer's instructions. Positive clones were selected on LB plates containing kanamycin (50 µg/mL) and chloramphenicol (12.5 µg/mL). For the induction of BAC clones retrofitted with EZ::TN <oriV/KAN-2> transposon, standard manufacturer protocol was followed. DNA samples were purified and sequenced with gene specific primer together with TN FP-1 forward primer supplied with the kit.

2.3. Sequence data and analyses

The MCSU protein sequences of higher plant species (*Oryza sativa*, *Arabidopsis thaliana*, and *Solanum lycopersicum*) were retrieved from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/) (Table 1). Physico-chemical analyses (number of amino acids, molecular weight, and theoretical pI) were performed with the ProtParam tool (http://web.expasy.org/protparam/) (Gasteiger et al., 2005) (Table 1). Secondary structure prediction, subcellular localizations, conserved protein motifs, and predicted domain analyses were evaluated by SOPMA server (Geourjon and Deleage, 1995), CELLO v.2.5 server (Yu et al., 2006), Wolf PSORT (Horton et al., 2007), MEME (Multiple Em for Motif Elicitation) server, and Pfam (http://www.sanger.ac.uk/software/pfam/search.html) server (Punta et al., 2012), respectively. Interacting partners of barley MoCo sulfurase and its co-expressed genes were predicted using String software (http://string-db.org/) (Franceschini et al., 2013). These analyses were performed by using MCSU protein sequences submitted to the online bioinformatics server mentioned above.

2.4. 3D structure modeling and validation

The 3D structures were predicted by using Protein Homology/analogY Recognition Engine V 2.0 (Phyre^2) server (Kelley and Sternberg, 2009) and Swiss-Pdb Viewer 4.1.0 (Guex and Peitsch, 1997), respectively. Furthermore, predicted binding sites and heterogen presence in these

Table 1. Physico-chemical properties, predicted domains, and subcellular predictions in higher plant species.

Species	Access no.	Length (aa)	MW (kDa)	*pI*	Predicted Pfam domain	Subcellular prediction by CELLO	Subcellular prediction by Wolf PSORT
**H. vulgare*	EU673449	831	92.21	6.84	AT, MOSC N, MOSC	Nuclear	Nuclear
O. sativa	ABH88164	824	91.75	7.16	AT, MOSC N, MOSC	Nuclear	Nuclear
A. thaliana	NP_564001	819	91.80	6.56	AT, MOSC N, MOSC	Cytoplasmic or nuclear	Nuclear
S. lycopersicum	NP_001234144	816	91.16	6.80	AT, MOSC N, MOSC	Plasma membrane	Nuclear

AT: Aminotransferase class-V (Pfam: PF00266), MOSC N: MOSC N-terminal beta barrel domain (Pfam: PF03476), MOSC: MOSC domain (Pfam: PF03473). * indicates the new sequence obtained from this study.

sites were analyzed with 3D Ligand Site server (http://www.sbg.bio.ic.ac.uk/~3dligandsite/) (Wass et al., 2010). Model evaluation was conducted with Rampage Ramachandran plot analysis (Lovell et al., 2003).

2.5. Phylogenetic analysis

A total of 16 MoCo sulfurase protein sequences were used for phylogenetic analysis, and multiple alignment of MoCo protein sequences was performed by using Clustal W (Higgins et al., 1996). Phylogenetic analysis was carried out with MEGA 5.1 (Tamura et al., 2011) by bootstrap analyses with 1000 replications, including Poisson model and complete deletion parameters.

3. Results and discussion

3.1. Primary sequence analyses

In this study, *MoCo sulfurase* gene was cloned and sequenced (accession number: EU673449.1) in barley. The annotation of nucleotide sequence predicted the presence of 23 exons (2496 bp), 22 introns (3514 bp), 1347 bp of sequence 5'-UTR (untranslated region) to the initiation codon, and 700 bp of sequence 3'-UTR to the stop codon that encoded a preprotein of 831 amino acids. We also produced the modeling of MoCo sulfurase in barley (hereafter HvMCSU) and compared it with the rice, tomato, and *Arabidopsis* orthologs. Protein BLAST analysis revealed that HvMCSU shared 80%, 59%, and 57% identity with rice, tomato, and *Arabidopsis* orthologs, respectively. Physico-chemical analysis showed that the predicted length, molecular weight, and pI values of HvMCSU are 831 amino acids, 92.21 kDa, and 6.84, respectively (Table 1). These findings were similar to those obtained from *Arabidopsis*, tomato, and rice orthologs (Xiong et al., 2001; Sagi et al., 2002; Huang et al., 2009). MOSC domain could be an essential component in the formation of diverse metal–sulfur clusters containing conserved cysteine residues (Anantharaman and Aravind, 2002). Based on domain analysis, all of the analyzed MCSUs had an amino transferase class-V domain (Pfam: PF00266), MOSC N-terminal beta barrel domain (Pfam: PF03476), and a MOSC domain (Pfam: PF03473) (Figure 1). Previously, this conserved domain architecture was detected in rice (Huang et al., 2009) and tomato (Sagi et al., 2002). The subcellular localization of MCSUs, predicted by both CELLO and PSORT severs, indicated that most of these proteins are associated with nuclear localizations (Table 1). Nevertheless, the tomato MCSU was predicted to be localized in the plasma membrane by the CELLO server. Based on conserved motif analysis, it was observed that five common motifs were detected in all MCSUs. Furthermore, tomato and *Arabidopsis* contained additional motif 2 and motif 1, respectively (Figure 2; Table 2). According to Pfam analysis, motifs 3, 4, and 5 were related to amino transferase class-V domain, MOSC N-terminal beta barrel domain, and MOSC domain, respectively. The motif structure of barley MCSU was highly similar to that of rice, suggesting that motifs of MCSU proteins were well conserved in different monocots.

3.2. Secondary structure analyses

The secondary structure predictions of MCSUs for barley, rice, *Arabidopsis*, and tomato showed that all species had a higher percentage of random coils and alpha helices (Table 3). Alpha helices, extended strand, and random coils ranged from 30.39% to 35.26%, 17.09% to 19.30%, and 46.09% to 51.35%, respectively. In general, similar secondary structures were observed in MCSUs. Molybdenum enzymes (Mo-enzymes) are crucial for many metabolic processes, including nitrate assimilation and phytohormone synthesis in plants (Schwarz and Mendel, 2006). These conserved secondary structures could be related to the stability of protein folding, which plays an essential role in metabolic pathways. Random coils are an important component in protein flexibility and conformational changes for enzymatic turnover (Miller, 2004). Flexibility also contributes to changes in protein structure with interaction of binding sites (Teilum et al., 2009). The highest value of random coils in MCSUs may be relevant to protein functions.

3.3. Predicted binding sites of MCSUs

Predicted binding sites of MCSUs differed in barley and *Arabidopsis* (Table 4). Tomato and rice showed the same residue patterns in predicted binding sites with various positions, including Gly-Ala-Thr-Asn-Asn-Asn-Asp-Ala-Lys-Ser-Tyr-Leu. However, barley and *Arabidopsis*

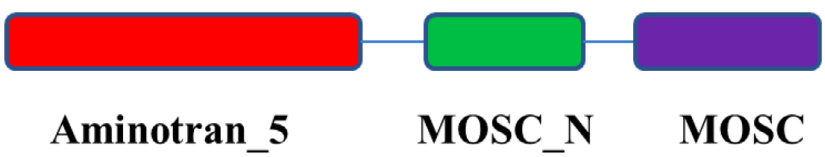

Figure 1. Domain structure of barley MCSU based on Pfam database. Aminotransferase class-V domain (Pfam: PF00266), MOSC N-terminal beta barrel domain (Pfam: PF03476), and MOSC domain (Pfam: PF03473) were positioned between 236–497, 536–656, and 673–828 residues, respectively.

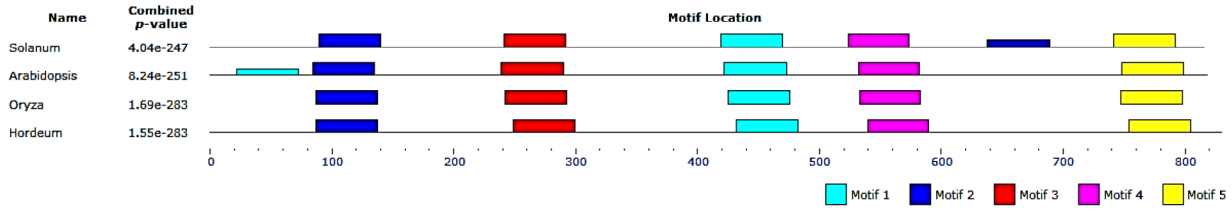

Figure 2. The diagrams of the conserved protein motifs in MCSUs by using MEME server. Each motif was represented in boxes with different colors: motif 1: cyan; motif 2: blue; motif 3: red; motif 4: pink; and motif 5: yellow.

Table 2. The most conserved protein motifs in MCSU protein sequences in higher plants.

Motif number	Width sequence	Protein sequences	Pfam domain
1	50	HLRTGCFCNPGACAKYLGLSHSDLVSNFEAGHVCWDDNDIINGKPTGAVR	Not found
2	50	RHQVLKYFNASPRDYKCIFTSGATAALKLVGECFPWSRESCYMYTMENHN	Not found
3	50	WMVLIDAAKGCATEPPNLSVYPADFVVCSFYKIFGYPTGLGALIVKNDAA	Aminotransferase class-V
4	50	SITIYPIKSCQGFSVQQWPLTTTGLLHDREWMLQGSTGEILTQKKVPEM	MOSC N
5	50	FTSMGGCNRCQMINIHQEAGQVQKSKEPLATLASYRRVKGKILFGILLRY	MOSC

Table 3. Secondary structure of MCSUs by using SOPMA server.

Secondary structure	*H. vulgare*	*O. sativa*	*A. thaliana*	*S. lycopersicum*
Alpha helix	35.26%	34.22%	34.43%	30.39%
Extended strand	18.65%	19.30%	17.09%	18.26%
Random coil	46.09%	46.48%	48.47%	51.35%

indicated different binding site patterns (in bold), including Gly-Ala-Thr-**Glu**-**Asp**-**Lys**-**Gly**-Asn-Asp-Lys-Ser-Tyr for barley and Gly-Ala-Thr-**Asn**-**His**-Asn-Asp-**Ala**-Lys-Ser-Tyr for *Arabidopsis*. Proteins with the same function could contain similar residues in active sites and observe various positions in binding sites; thus it can be suggested that mutations may affect the binding sites' conformation (Bowie et al., 1990; Magliery and Regan, 2005). In this study, although rice and barley (belong to Poaceae family) showed 80% sequence identity, they did not share a similar binding site pattern. Notably, tomato and rice contained the same putative binding site, despite belonging to a different plant family. These differences in the predicted binding sites may be affected by mutations in MCSU gene structure during the course of evolution. The majority of cysteine desulfurase proteins contain significantly more cysteines. The N-terminal domain of the MoCo sulfurase ABA3 includes a conserved triple-cysteine motif with cys428-cys430-cys435, and this motif was observed in all MoCo sulfurases. Thus, these cysteine residues contribute to active site formation (Lehrke et al., 2012). The cys438-cys440-cys445, cys431-cys433-cys438, cys428-cys430-cys435, and cys425-cys427-cys432 were detected in amino transferase class-V domain of barley, rice, *Arabidopsis*, and tomato, respectively, indicating that these residues may contribute to the same function as active site formation. The interacting partners of barley MCSU were predicted (Figure 3) (Franceschini et al., 2013), and some common proteins were detected in barley, including Fe-S metabolism-associated domain containing protein, aldehyde oxidase, AP2 domain containing protein, nitrate reductase, ABC transporter, and bZIP transcription factor. Fe-S proteins were discovered in nitrogen fixation (NIF) machinery in bacteria (Rees and Howard, 2000). In eukaryotes, Fe-S proteins are observed in mitochondria, cytosol, and nucleus. Iron–sulfur clusters are ancient cofactors for proteins that play a significant role in electron transport, enzyme catalysis, and regulation

Table 4. Predicted binding sites of MoCo sulfurase proteins in higher plants.

Species	Residue	Amino acid	Contact	Av. distance
H. vulgare	109	Gly	7	0.57
	110	Ala	16	0.65
	111	Thr	25	0.00
	160	Glu	25	0.00
	161	Asp	7	0.67
	162	Lys	25	0.00
	163	Gly	8	0.53
	218	Asn	24	0.04
	255	Asp	25	0.00
	258	Lys	25	0.00
	278	Ser	15	0.16
	280	Tyr	24	0.09
O. sativa	109	Gly	9	0.61
	110	Ala	16	0.63
	111	Thr	25	0.00
	135	Asn	25	0.14
	137	Asn	25	0.00
	216	Asn	25	0.07
	248	Asp	25	0.00
	250	Ala	9	0.37
	251	Lys	25	0.00
	271	Ser	19	0.15
	273	Tyr	25	0.04
	282	Leu	23	0.23
A. thaltana	106	Gly	13	0.58
	107	Ala	14	0.62
	108	Thr	25	0.00
	132	Asn	18	0.40
	133	His	25	0.00
	207	Asn	25	0.00
	245	Asp	25	0.00
	247	Ala	10	0.46
	248	Lys	25	0.00
	268	Ser	21	0.25
	270	Tyr	25	0.01
S. lycopersicum	111	Gly	10	0.59
	112	Ala	14	0.60
	113	Thr	24	0.00
	137	Asn	24	0.14
	139	Asn	24	0.01
	210	Asn	23	0.06
	247	Asp	24	0.00
	249	Ala	8	0.45
	250	Lys	24	0.00
	270	Ser	18	0.22
	272	Tyr	24	0.03
	281	Leu	22	0.02

of gene expression (Lill and Mühlenhoff, 2005). Forty different Mo enzymes have been found in living organisms so far, and only four are detected in plants, including nitrate reductase, sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase (Schwarz and Mendel, 2006). Nitrate reductases (NR; EC 1.6.6.1-3) are molybdoenzymes that reduce nitrate to nitrite. Eukaryotic nitrate reductases are member of the sulfite oxidase family of molybdoenzymes that serve as transferring electrons from NADH or NADPH to nitrate (Campbell, 1999; Tischner, 2000). NRs have three forms: NADH-specific, NADPH-specific, and NAD (P) H-bi-specific forms (Campbell and Kinghorn, 1990). Aldehyde oxidase (AO, EC 1.2.3.1) is a cytoplasmic enzyme with FAD, iron, and MoCo as prosthetic groups. AO and xanthine dehydrogenase (XDH, EC 1.1.1.204) show similar amino acid sequences, suggesting that they evolved from a common ancestor (Hille, 2002; Schwarz and Mendel, 2006). Plant AOs are encoded by multigene families and play a crucial role in the biosynthesis of phytohormones ABA and processes auxins (Milborrow, 2001). ABC transporters play an important role in various processes, including pathogen response, surface lipid deposition, phytate accumulation in seeds, and transport of the phytohormones auxin and abscisic acid. Hence, ABC transporters are involved in organ growth, plant nutrition, plant development, response to abiotic stress, and interaction of the plant with its environment (Kang et al., 2011). *Arabidopsis thaliana bZIP* (At*bZIP*) factors can affect diverse biological processes such as pathogen defense, light and stress signaling, seed maturation, and flower development (Jakoby et al., 2002).

3.4. The 3D structure analysis

According to primary sequence identity, HvMCSU indicated 80%, 59% and 57% identity with rice, tomato, and *Arabidopsis* orthologs, respectively. However, some structural divergences were observed with various positions and numbers of the α-helix and β strands using Phyre[2] server in predicted 3D models of MCSUs (Figure 4). The 3D structure analysis detected 16, 16, 14, and 16 α-helices and 18, 15, 15, and 17 β sheets in *Hordeum*, *Arabidopsis*, *Oryza*, and *Solanum*, respectively. Rich β strand structures were observed in MCSUs (Figures 4A–4D). It can be proposed that the α-helices and the β sheets cover dominantly in 2D and the 3D structure of MCSUs and may contribute to domain conformation (Ananthraman and Aravind, 2002). Protein domains contain a stable 3D structure and are more conserved than sequences. Some changes are observed in protein folding during their evolution, even in the generation of new folding patterns (Lehrke et al., 2012). Structural divergences in MCSUs can be explained by some evolutionary genomic forces such as duplication, recombination, loss and transfer of a domain between species, and changes within the genomic

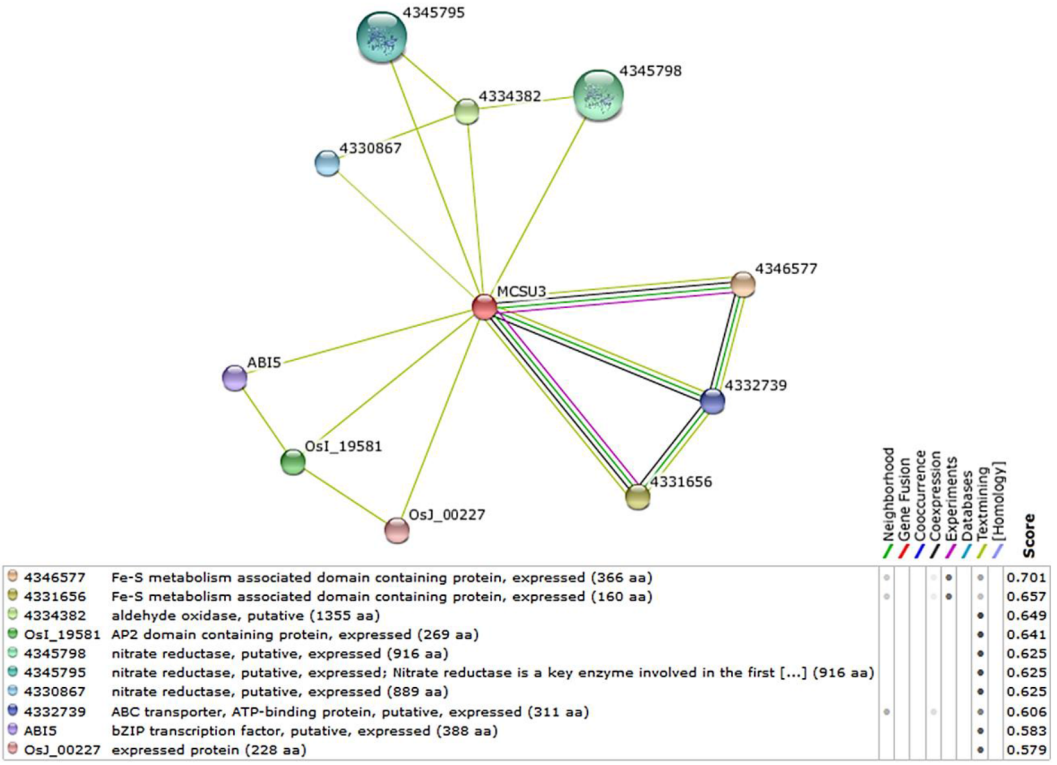

Figure 3. In silico prediction of interacting partners for *MoCo sulfurase* gene of barley by using STRING. The box shows a list of putative interacting partners of the barley *MoCo sulfurase* gene. STRING automatically highlighted the corresponding nodes in the network, and the interactions contain direct (physical) and indirect (functional) associations (Franceschini et al., 2013).

content of domains (Rees and Howard, 2000). Insertions and deletions (indels) are one of the most important effects that lead to structural divergence, although major conformational characteristics of proteins cannot be altered considerably (Lill and Mühlenhoff, 2005). Another possible explanation of structural divergence in MCSUs is indel events that affect the MCSU gene structures in evolutionary history. The MOSC domain is a superfamily including beta-strand-rich domains detected in the MoCo sulfurase (Ananthraman and Aravind, 2002). The function of the MOSC-N domain is unknown, but it may be related to the adoption of a beta barrel fold (Zhang et al., 2011). These rich β strand structures were detected in Figure 4 and indicated in blue. The MOS-C β-barrel domain in the MOS-like family contains universally conserved cysteine in N-terminal (Ananthraman and Aravind, 2002). In this study, conserved cysteine residues were identified in MOSC-N and MOSC domains of MCSUs, including Cys550, Cys657, Cys680, Cys761, and Cys764 for barley; Cys542, Cys650, Cys673, Cys755, and Cys758 for *Arabidopsis*; Cys543, Cys650, Cys673, Cys754, and Cys757 for rice; and Cys534, Cys642, Cys665, Cys748, and Cys751 for tomato. The Ramachandran plot analysis was used for model validation and revealed that 91.1%, 91.4%, 92%, and 93.6% were in the favored region; 5.7%, 6.4%, 5.4%, and 5.7% in the allowed region; and 3.1%, 2.2%, 2.6%, and 0.7% in the outlier region, indicating that the 3D models were of fairly good quality.

3.5. Phylogenetic analysis of MCSUs

In order to analyze the phylogenetic relationship between MCSUs in higher plants, a phylogenetic tree was generated using MEGA 5.1, and bootstrapped with 1000 replicates (Figure 5). A total of 16 plant species were used and two major groups were observed with dicots (subgroups A1, A2, and A3) and monocots (group B). Dicots and monocots diverged clearly with a high bootstrap value: 85% for dicots and 100% for monocots. Specifically, *S. lycopersicum* (tomato) was separated from the other dicots, which may reflect species-specific genome dynamics in MCSU gene evolution. Thus, these groups may indicate a divergent evolution of MCSU genes in monocots and dicots. Sagi et al. (2002) reported that C-terminal portion of *MoCo sulfurase* genes in tomato was separated from other plant sequences in phylogenetic tree, and these data corroborated our findings. In subgroup A2, the Fabaceae taxa with the highest bootstrap value (100%)

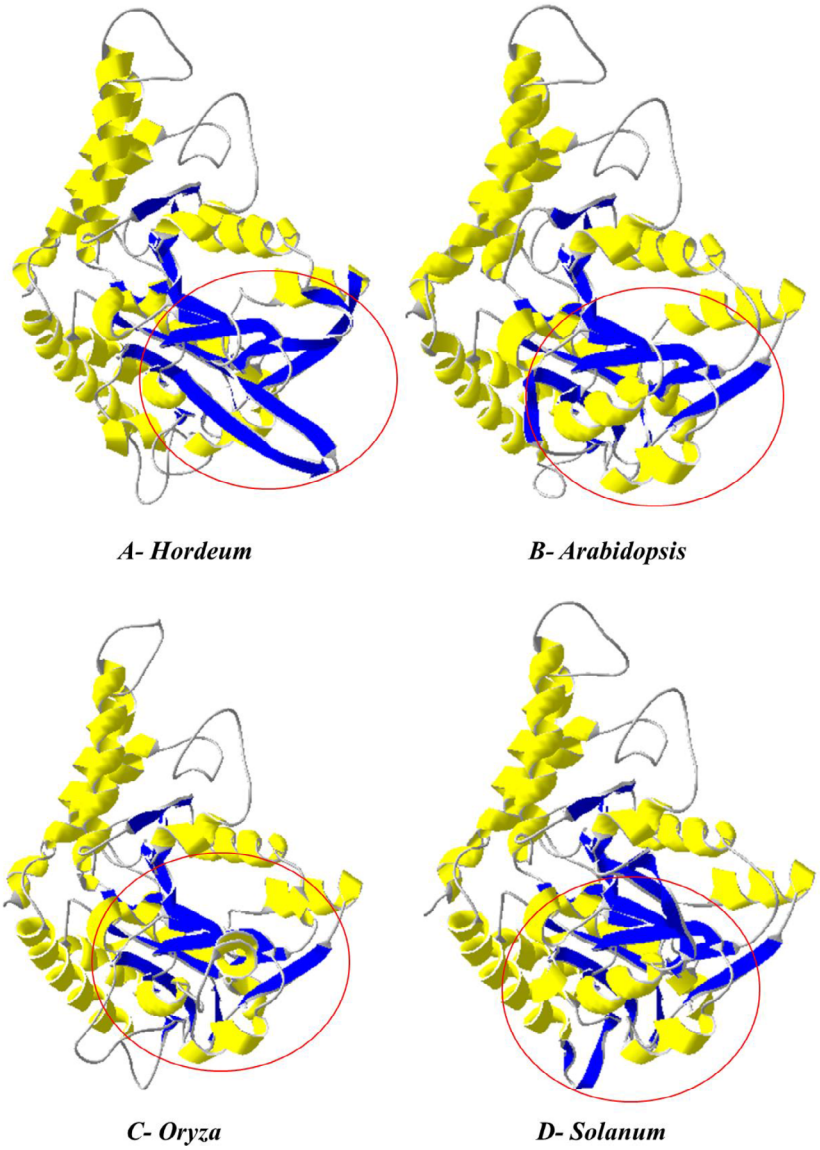

Figure 4. 3D structure of MCSUs in higher plants, including *H. vulgare* (4A), *A. thaliana* (4B), *O. sativa* (4C), and *S. lycopersicum* (4D). The structural divergences and rich beta strands were indicated as an empty red circle. Also, α-helices and β sheets were presented as yellow and blue, respectively.

were clustered together, including *Medicago truncatula*, *Glycine max*, and *Phaseolus vulgaris*. Plants in the same genus with highest bootstrap value (100%) also grouped together, such as *Citrus* and *Arabidopsis* taxa in subgroup A1. Particularly, barley clustered with *Aegilops tauschii*, showing the highest bootstrap value (100%) in group B, and this clade represents the subfamily Pooideae. Thus, our results validate the systematic division of monocot and dicot species (Daly et al., 2001).

In conclusion, MoCo enzymes are important in global carbon, nitrogen, and sulfur cycles. The protein sequences of MCSUs were analyzed using bioinformatics tools, including barley (obtained in the current study), rice, tomato, and *Arabidopsis*. The observed structural divergences in the 3D modeling of proteins and domains could be the result of the mutations in MCSU gene accumulated during the evolutionary history of these plant species. The dicot and monocot divergences were obtained clearly with high bootstrap value in phylogenetic tree, supporting an independent evolutionary origin. The results obtained in the current study could provide a better scientific basis for understanding.

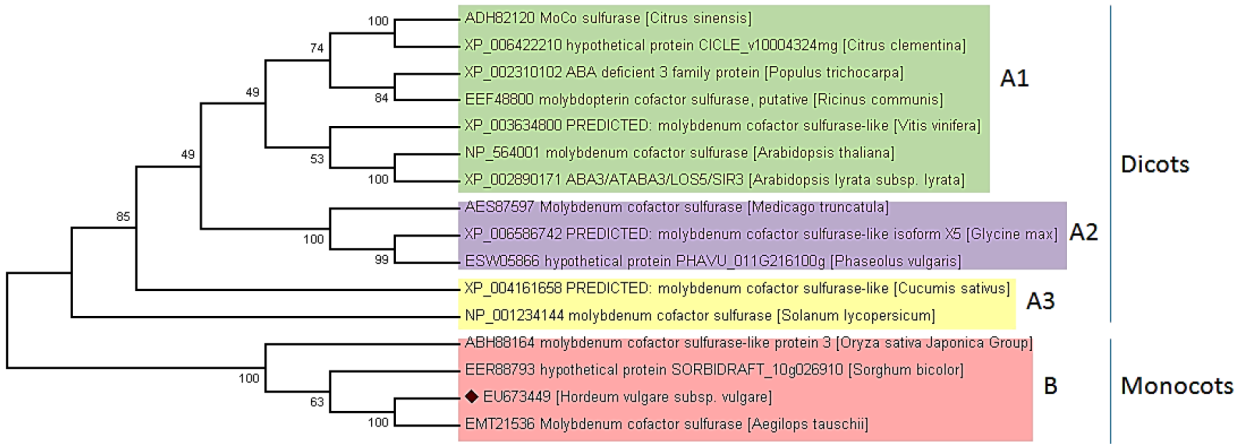

Figure 5. Phylogenetic analysis of MCSU proteins from higher plant species by using the neighbor-joining method with MEGA 5.1. Accession numbers and protein names are shown in a phylogenetic tree. Bootstrap values are indicated against each branch with 1000 replications.

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