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Research article

Cloning and functional analysis of four *O*-Acetylserine (thiol) lyase family genes from foxtail millet

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ABSTRACT

Cysteine is the first organic molecule generated during the assimilation of sulfate. As such, cysteine and its derivatives are always essential signal molecules and thus have important roles in the regulation of many plant processes. *O*-acetylserine (thiol) lyase (OASTL) catalyzes the last step of the biosynthesis of cysteine. At present, detailed and comprehensive work about these enzymes has only been reported from the plant *Arabidopsis thaliana*, though sporadic studies on OASTL have been conducted on other dicots, such as spinach and soybean. However, few reports on the functions of OASTLs in monocots have been found in the literature. Here in this study, we obtained four *SiOASTL* genes (*SiOASTL7*, *SiOASTL8*, *SiOASTL9* and *SiOASTL10*) from foxtail millet and analyzed their potential functions. Phylogenetically, the four *SiOASTL* genes did not belong to any published subfamily of the *OASTL* genes; instead they constituted a new subfamily specific to the *OASTL* genes from monocots. In sequencing, we found that with the exception of the pseudogene *SiOASTL8,* proteins encoded by the other three genes exhibited high similarity with OASTL proteins from Arabidopsis, though the critical PLP-binding sites of both *SiOASTL7* and *SiOASTL10* were missing. The enzymatic activity assays demonstrated that *SiOASTL9* has the ability to catalyze the biosynthesis of both cysteine and S-sulfocysteine, while *SiOASTL7* and *SiOASTL10* did not possess any previously reported catalyzing abilities. In addition, the gene expression pattern analysis showed that all four genes were widely expressed in various tissues of foxtail millet, and all had a preference in the leaves. Under abiotic stresses, the expression of these genes could be induced by salt and drought stress. Our finding that cadmium could only up-regulate the transcription of *SlOASTL8* and *SlOASTL9*, further indicates the diversified responses of *SiOASTLs* to abiotic stresses.

1. Introduction

Sulfur (S) is an essential macronutrient for plant growth and accounts for 0.1%–1% of the dry weight of plants (Hawkesford and De Kok, 2006; Takahashi et al., 2011). Sulfur-containing compounds are also involved in many processes of plant growth and their environmental adaptation (Zhao et al., 2008). For example, during plant growth and development, moderate use of sulfur fertilizer can enhance crop yield and quality (Zhao et al., 2008). Whether present in nature or added as a fertilizer, sulfur is absorbed by plant roots in the form of sulfate, and then metabolized into the sulfur-containing compound cysteine (Hawkesford and De Kok, 2006).

Cysteine plays important roles in the structure, stability and catalytic function of many proteins (Droux et al., 1998; Romero et al., 2014). Moreover, cysteine is also a precursor molecule for the synthesis of many sulfur-containing metabolites, such as the cofactors, vitamins, and Fe–S clusters (Romero et al., 2014) necessary for the growth and development of many plant species. The biosynthesis of cysteine is catalyzed by serine acetyltransferase (SAT) and *O*-acetylserine(thiol) lyase (OASTL) sequentially (Jez and Dey, 2013). SAT catalyzes the generation of *O*-acetylserine (OAS) from serine and acetyl-CoA, while OASTL catalyzes the last step of cysteine biogenesis by combining OAS and sulfide with 5′-pyridoxal-5′-phosphate (PLP) as a cofactor (Jez and Dey, 2013; Romero et al., 2014). These two enzymes form the cysteine

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synthase complex (CSC) to regulate the synthesis of cysteine (Feldman-Salit et al., 2009). The interactions between these two proteins strongly modify their kinetic properties. CSC actives SAT, but the activity of OASTL is greatly reduced when it is in the complex. The formation of the complex is regarded as a sensor of the sulfurous state of the plant, and is dependent on the available amount of OAS and sulfide in the cell (Romero et al., 2014).

As the enzyme that catalyzes the last step of cysteine biosynthesis, OASTL proteins are widely distributed in plant and microbial cells (Hirai and Saito, 2008). Meanwhile, OASTL is also a family of proteins with multiple functions, including its role in cysteine biogenesis. For example, L-cysteine desulfhydrase 1 (DES1) can use L-cysteine as a substrate to generate the important gasotransmitter hydrogen sulfide (H_2S) (Alvarez et al., 2010). The Arabidopsis CYS-C1 can convert Cys and cyanide into H_2S and β-cyanoalanine in mitochondria to alleviate the toxicity of cyanide (Yamaguchi et al., 2000), and as such, functions as a β-cyanoalanine synthase (CAS). Moreover, the Arabidopsis CS26 can catalyze the generation of S-sulfocysteine (S-Cys) from OAS and thiosulfate as an S-sulfocysteine synthase (SSCS) (Bermudez et al., 2010). Recently, it has also been shown that CysK (bacterial OASTL) and its paralogs influence transcription in Gram-positive bacteria in the presence of a binding partner like CysE (bacterial SAT), indicating its potential to be a transcription factor (Campanini et al., 2015).

In plants, *OASTL* genes are involved in environmental stress responses (Fediuc et al., 2005; Ning et al., 2010; Xie et al., 2013). For example, under heavy metal and high salt treatment, the expression levels of *OASTL* genes in *Phragmites and Typha* are up-regulated (Fediuc et al., 2005), and overexpression of *GmOASTL4* in tobacco enhance the tolerance of plants to cadmium stress (Ning et al., 2010). The expression levels of *OASTL* genes are also affected when the plants are exposed to nutrient starvation or oxidative stress (Nakamura et al., 1999; Dominguez-Solis et al., 2001), and the over-expression of some *OASTL* genes can improve the adaptation of plant to oxidative stress in several species (Sirko et al., 2004; Fediuc et al., 2005).

To date, Arabidopsis is the only higher plant species in which the functions of OASTLs have been extensively and deeply investigated (Jost et al., 2000; Heeg et al., 2008), though studies are also sporadically reported using other dicots, such as spinach, soybean, tobacco, rape, etc. (Takahashi and Saito, 1996; Zhang et al., 2008; Ning et al., 2010; Xie et al., 2013). Comparatively, studies of OASTLs in monocots are still lacking. Rice, wheat, Phragmites, and Typha are the only four monocots in which the functions of OASTLs have ever been reported, though the research is still very limited (Youssefian et al., 1993; Nakamura et al., 1999; Fediuc et al., 2005).

Foxtail millet (*Setaria italica* (L.) P. Beauvois) is a kind of crop with great commercial value in the agricultural industry (Tian et al., 2016). In China, foxtail millet is mainly grown in the northwest, as it is drought-tolerant, adaptable, and gives high and stable yields. Nowadays, with the completion of its genomic sequencing, foxtail millet has become a new model plant for the study of monocots (Bennetzen et al., 2012; Zhang et al., 2012). In this study, four *SiOASTL* genes were cloned from foxtail millet and the potential functions of these proteins were analyzed. We demonstrated that the four millet *OASTL* genes belong to a new subclade specific to monocots phylogenetically, and only the protein encoded by *SiOASTL9* still possessed the ability to catalyze with PLP as a cofactor. All four genes were widely expressed in different plant tissues, and their responses to abiotic stresses were distinct from each other.

2. Materials and methods

2.1. Plant material and growth condition

Foxtail millet cv. Jingu-21 was used as the experimental material. The seeds were surface-sterilized with 75% (v/v) ethanol for 30 s and 6% (v/v) NaClO for 10 min, and then rinsed with distilled water and placed in 9 cm Petri dishes with four layers of sterile gauze. The seeds were germinated in the dark at 23 °C, and thereafter grown in a condition of 23 °C/16 h day and 18 °C/8 h night at a relative humidity of 60% (Tian et al., 2016).

2.2. Cloning and sequence analysis of the four SiOASTL genes

Total RNA was extracted from the millet seedlings using the Trizol Total RNA Extraction Kit (TaKaRa, Japan). cDNA was synthesized using total RNA as the template with the First-Strand cDNA Synthesis Kit (ABM, Canada), according to the manufacturer's instructions. Using the *Arabidopsis* OASTL protein sequences as the query sequences, millet cDNA sequences with high similarity to the *Arabidopsis* OASTL were obtained from the foxtail millet gene database through a tBLASTn search. Primers were designed using the Primer Premier5.0 software (Singh et al., 1998). The primers used are listed in Table S1. The PCR programs were as follows: a pre-denaturation of 10 min at 94 °C, followed by 35 cycles of 30 s each at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, then a final elongation step of 7 min at 72 °C. The PCR products were electrophoresed on a 1% agarose gel and the target bands were purified as previously described (Meng et al., 2007). The DNA fragments were then cloned into the pMD-18T vector (TaKaRa, Japan) for sequencing.

The physical and chemical characterization of the SiOASTL proteins were predicted by the Expert Protein Analysis System (ExPASy) (http://cn.expasy.org/) (Krogh et al., 2001). Multiple alignment analysis of the sequences was performed with ClustalX and GenDoc software (Larkin et al., 2007). The phylogenetic tree was constructed by MEGA7.0 software using the neighbor-joining (NJ) method (Kumar et al., 2016).

2.3. Prokaryotic expression and purification of the three SiOASTL proteins

In order to exogenously express the SiOASTL proteins in *E. coli*, the coding domain sequences of *SiOASTL7*, *SiOASTL9* and *SiOASTL10* were first cloned into the expression vector pCold to produce pCold-*SiOASTL7*, pCold-*SiOASTL9* and pCold-*SiOASTL10* constructs respectively. The recombinant constructs were then validated by PCR and double enzymatic digestion, and transferred into the *E. coli* expression strain BL21 (DE3). Expression of the recombinant proteins was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) at 16 °C for 24 h, and the proteins were purified on a nickel-charged nitrilotriacetic acid (Ni-NTA) resin affinity chromatography column (CWBIO, http://www.cwbiotech.com/). The purified proteins were then detected using SDS-PAGE and stored at −20 °C before subsequent biochemical analysis.

2.4. Enzymatic activity assays

The abilities of the three SiOASTL proteins to generate H_2S using cysteine (L-cysteine or D-cysteine) as the substrate were measured according to the method described previously (Jin et al., 2011). OASTL activity was measured in a reaction mixture with a total volume of 1 ml: the reaction mixture contained 500 mM Tris-HCl (pH 7.5), 5 mM OAS, 10 mM Na_2S, 50 mM DTT, 50 μM pyridoxal-5-phosphate (PLP) and 100 μl enzyme extract. The reaction mixture was first incubated at 30 °C for 10 min, then 200 μl acidic ninhydrin reagent were added, and the tube was placed in boiling water for 10 min. To terminate the reaction, 500 μl ethanol was added to the reaction mixture and cysteine was determined by measuring the absorbance of the reaction mixture at 560 nm. The concentration of cysteine was calculated according to the method described previously (Bonner et al., 2005). S-sulfocysteine synthase activity was measured in an assay similar to that of OASTL, using OAS and $Na_2S_2O_3$ as the substrates. The sodium salt of S-sulfocysteine (Abcam, USA) was used to make the standard curve.

For SiOASTL9, kinetic analysis was also performed. The Michaelis constants (Km) of SiOASTL9 with either sulfide or thiosulfate as the substrate were obtained by varying the concentrations of these two substrates from 2.5 mM to 10 mM, respectively. Data from at least 3 replicate experiments for each substrate were collected and analyzed from non-linear regression using Michaelis-Menten equation as described before (Wirtz et al., 2004).

2.5. Extraction of RNA and quantitative real-time PCR analysis

Roots, stems, leaves and spikes were obtained from 6-month-old plants for tissue-specific expression pattern analysis. For expression pattern analysis under abiotic stresses, 5-day-old seedlings were treated with NaCl (250 mmol L^{-1}), $CdCl_2$ (5 mmolL^{-1}) or dried and assayed for gene expression at 0, 3, 6, 9, 12 and 24 h after treatment. Here, the drought treatment was conducted as previously described in the literature (Jin et al., 2011). All tissue samples were used immediately or stored at −80 °C until use, and the unstressed plants were used as controls.

Next, the total RNAs of roots, stems, leaves, spikes, as well as seedlings from different stress treatments, were extracted and reverse transcribed as described above. The quantitative real-time PCR primers were designed as described above, and *SiACTIN* was used as the internal control (Table S1). The expression levels of the target genes were detected with a Bio-Rad CFX96 Fluorescence Quantitative PCR Detection System (Bio-Rad CFX96, USA), and the relative expression levels of these genes were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. Statistical analyses

For all experiments, three independent biological replicates were carried out for each sample, with each replicate value being the mean of three technical replicates (triplicates). The data were presented as the mean ± standard error (SE). SPSS 19.0 software was used for all data analysis (IBM SPSS, Chicago, IL, USA), with *($p < 0.05$) and **($p < 0.01$) being used to indicate statistically significant effects.

3. Results

3.1. Cloning and sequence analysis of the four SiOASTL genes

In order to obtain the coding domain sequences (CDS) of *OASTL* genes in foxtail millet, the Arabidopsis OASTL protein was used as a query. Ten CDSs with high similarity to the query sequence were then found through tBLASTn searching. Phylogenetically, proteins encoded by the 10 millet *SiOASTL* genes were divided into two parts. Five proteins (SiOASTL1-5) were clustered with dicot OASTL proteins (from Arabidopsis and tomato) in different subclades as reported before, while the other five (SiOASTL6-10) constituted a new subfamily specific to the OASTL proteins from monocots, with proteins from *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Brachypodium distachyon*, *Dendrobium Catenatum* and *Musa acuminata* (Fig. 1). Interestingly, four out of these monocot-specific SiOASTLs were located in chromosome 4: SiOASTL6 (Millet_GLEAN _10027562), SiOASTL7 (Millet_GLEAN_10027560), SiOASTL9 (Millet_GLEAN_10027558) and SiOASTL10 (Millet_GLEAN_10027561) – and their locations on the genome were closely related like tandem repeats. Meanwhile, the proteins encoded by these four genes also shared high similarities (ranging from 70% to 89%).

To explore the function and diversification of these monocot-specific genes, we next tried to clone the CDSs of these genes from whole foxtail millet tissue cDNA. The amplification of *SiOASTL7*, *SiOASTL8*, *SiOASTL9* and *SiOASTL10* was successfully obtained, though we found that *SiOASTL8* could not encode a functional OASTL-like protein owing to the frameshift mutation caused by alternative splicing (Fig. 2a). The CDS of *SiOASTL6* could not be cloned at all, and our following studies were therefore focused on *SiOASTL7*, SiOASTL8, *SiOASTL9* and *SiOASTL10*. The structures of the four *SiOASTL* genes were also analyzed: both *SiOASTL7* and *SiOASTL9* were composed of five exons and four introns, *SiOASTL8* consisted of 7 exons and 6 introns, while *SiOASTL10* consisted of six exons and five introns (Fig. 2b).

Proteins encoded by *SiOASTL7*, *SiOASTL9* and *SiOASTL10* were 268, 285 and 284 amino acid residues, with theoretical molecular masses of 30 kDa, 30.5 kDa and 30.5 kDa, respectively. To further characterize the SiOASTL proteins, a multiple protein sequence alignment was carried out among SiOASTLs and OASTL proteins from other plant species. For most of the OASTL family members, PLP was considered to be a cofactor to facilitate catalysis, and in general, these proteins are highly conserved at the PLP binding site (PXXSVKDR) (Yamaguchi et al., 2000). In our results, the PLP binding sites of SiOASTL9 were conserved. However, the PLP binding sites of both SiOASTL7 and SiOASTL10 were missing, indicating that these two proteins might not use PLP as a cofactor, and as such, lose their catalyzation abilities (Fig. 3). It has been reported that the reaction mechanisms between CAS and OASTL are very similar, but these two enzymes prefer distinct substrates (Yi et al., 2012). By examining the three critical amino acids that determine the catalyzing specificity of SiOASTL9, we found that two out of three of these amino acids are similar to those found in an OASTL, indicating that SiOASTL9 might not a be CAS (Fig. 3).

3.2. Purification of SiOASTL recombinant proteins and enzyme activity assays

In order to analyze the catalyzing abilities of SiOASTL7, SiOASTL9 and SiOASTL10 proteins, all the three proteins were exogenously expressed in *E. coli* as His-tagged fusion proteins and purified through Ni-NTA affinity chromatography. The purified proteins were detected on an SDS-PAGE gel, where only one band was found for each purified protein (Fig. 4a). Their abilities to produce H_2S, cysteine and S-sulfocysteine were then analyzed using the purified recombinant proteins, with the protein produced by the empty pCold vector being used as a negative control. H_2S production by each of the three proteins was lower than that of the control group, indicating that none of the proteins had the ability to produce H_2S (Fig. 4b). The ability of each of the three proteins to synthesize cysteine or S-sulfocysteine was also assessed: our experimental results showed that only SiOASTL9 exhibited the ability to synthesize cysteine and S-sulfocysteine, while SiOASTL7 and SiOASTL10 could not act as a catalyst for the biosynthesis of cysteine or S-sulfocysteine (Fig. 4c and d). To decipher the preferred substrate of SiOASTL9, we performed kinetic analysis on SiOASTL9 with sulfide and thiosulfate as the substrate, respectively. And our results showed that the K_m^{sulfide} of SiOASTL9 is 6.4 ± 0.5 mM, while the $K_m^{\text{thiosulfate}}$ of SiOASTL9 is 26.2 ± 1.8 mM, indicating that the preferred activity of SiOASTL9 might be an OASTL.

3.3. Tissue-specific expression pattern analysis of the four SiOASTL genes

In order to further investigate the potential functions of *SiOASTL* genes in different tissues and organs of foxtail millet, the expression patterns of *SiOASTL* genes were determined by real-time PCR. The four genes were widely expressed in various tissues and organs of foxtail millet. Similar expression patterns were found for *SiOASTL7* and *SiOASTL9*, with maximum expression level in the leaves and minimum expression in the spike. *SiOASTL8* also exhibited the highest expression levels in the leaves, but its expression level in spike was also higher. Meanwhile, *SiOASTL10* also showed a vegetative tissue-specific expression pattern, as it was highly expressed in the stem and leaves (Fig. 5).

3.4. Responses of the SiOASTL genes to abiotic stresses

Previously, it has been shown that OASTL genes are involved in the responses of plants to abiotic stresses, with high salt and heavy metal being the two stresses most frequently studied (Fediuc et al., 2005; Xie

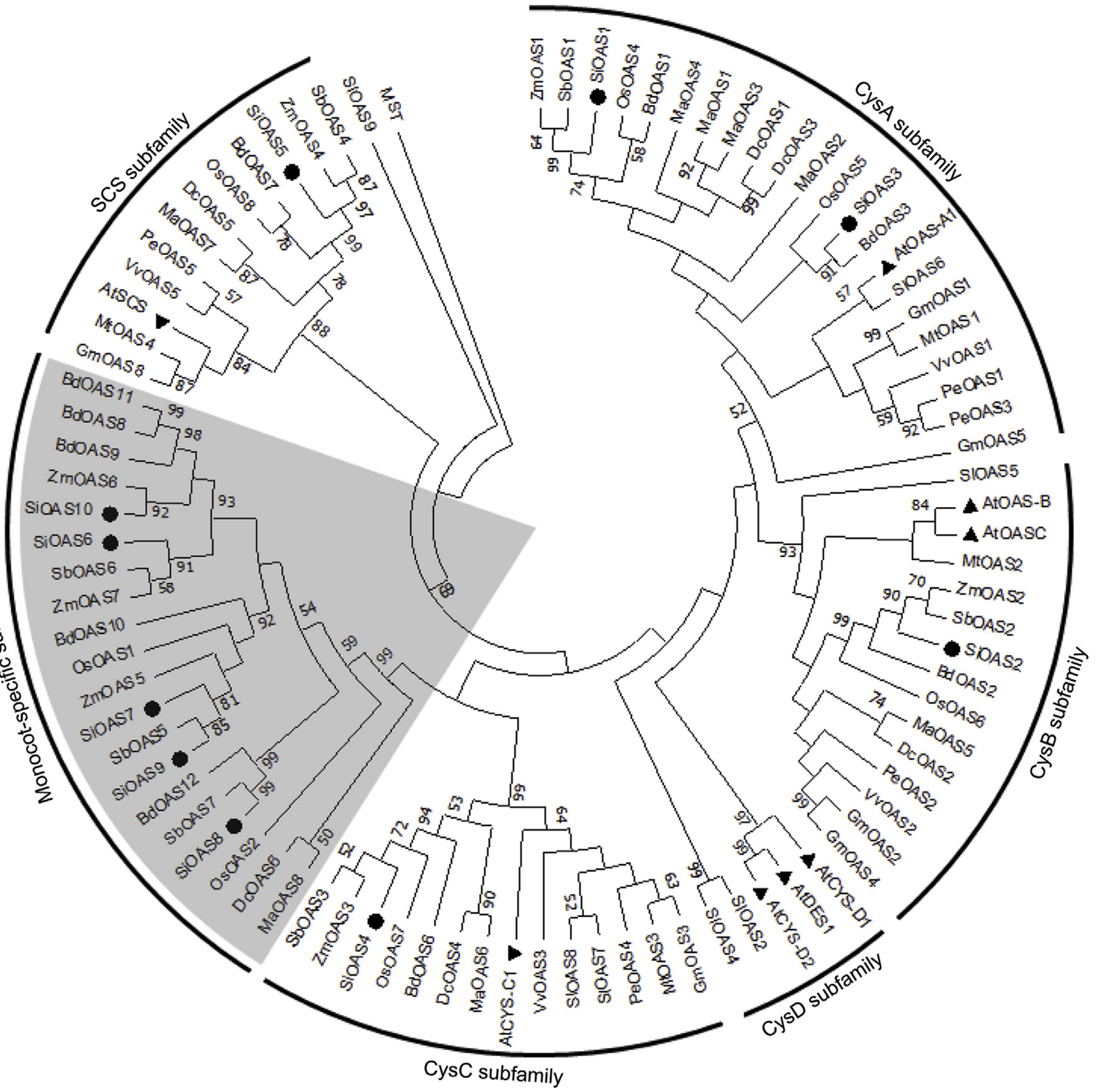

Fig. 1. Phylogenetic analysis of OASTL proteins from millet and other higher plants. The gray shading region represents the OASTL subclade specific to monocots. The animal MST protein was used as an outgroup. Proteins prefixed by the term 'At', 'Bd', 'Dc', 'Si', 'Gm', 'Mt', 'Ma', 'Os', 'Pe', 'Sl', 'Sb', 'Vv' and 'Zm' indicate OASTLs from Arabidopsis thaliana, Brachypodium distachyon, Dendrobium catenatum, Foxtail millet, Glycine max, Medicago tubercuata, Musa acuminata, Oryza sativa, Populus euphratica, Solanum lycopersicum, Sorghum bicolor, Vitis vinifera and Zea mays respectively. Numbers on the branches are bootstrap values > 50%.

et al., 2013). Moreover, drought stress causes differential acclimation responses of sulfur metabolism (Ahmad et al., 2016). Since OASTL proteins are important players during sulfur metabolism, the response of *SiOASTL* genes to drought stress were also analyzed in our study.

Here, changes in transcript abundance of the four *SiOASTL* genes were determined following exposure to three different stresses (salt, cadmium and drought), with unstressed plants used as controls for comparison (Fig. 6). The results showed that the four *SiOASTL* genes behaved distinctly under the three abiotic stresses. Under salt stress treatment, *SiOASTL7*, *SiOASTL9*, *SiOASTL10* exhibited similar responses: the expression levels of all the three genes reached a maximum at 9 h, after which the expression levels decreased gradually (Fig. 6a). However, compared to the striking expression changes of *SiOASTL7* and *SiOASTL10*, the transcription changes of *SiOASTL9* seemed to be more moderate. The expression of *SiOASTL8* could also have been stimulated by salt stress, but its response was much later, as the expression level of

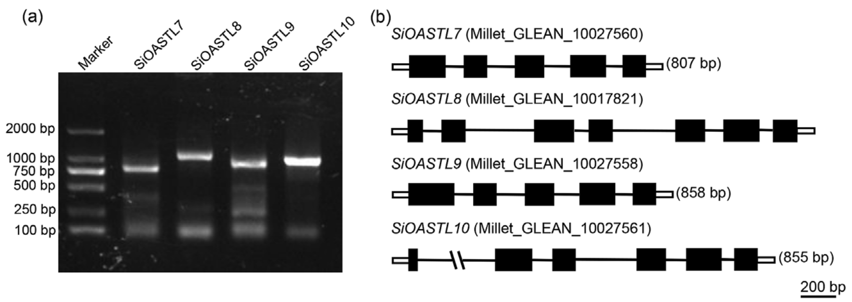

Fig. 2. Cloning and gene structure analysis of the four *SiOASTL* genes. (a) Amplification of the cDNA sequences of the four *SiOASTL* genes. (b) Gene structures of the four *SiOASTL* genes. Black bars represent exons, and lines represent introns. Blanks indicate 5′ and 3′ non-coding areas. Splicing sites were predicted based on the alignment results between cDNA and the genomic sequences. Numbers in the brackets indicated the length of cds for each gene, and the scale indicates 200 bp.

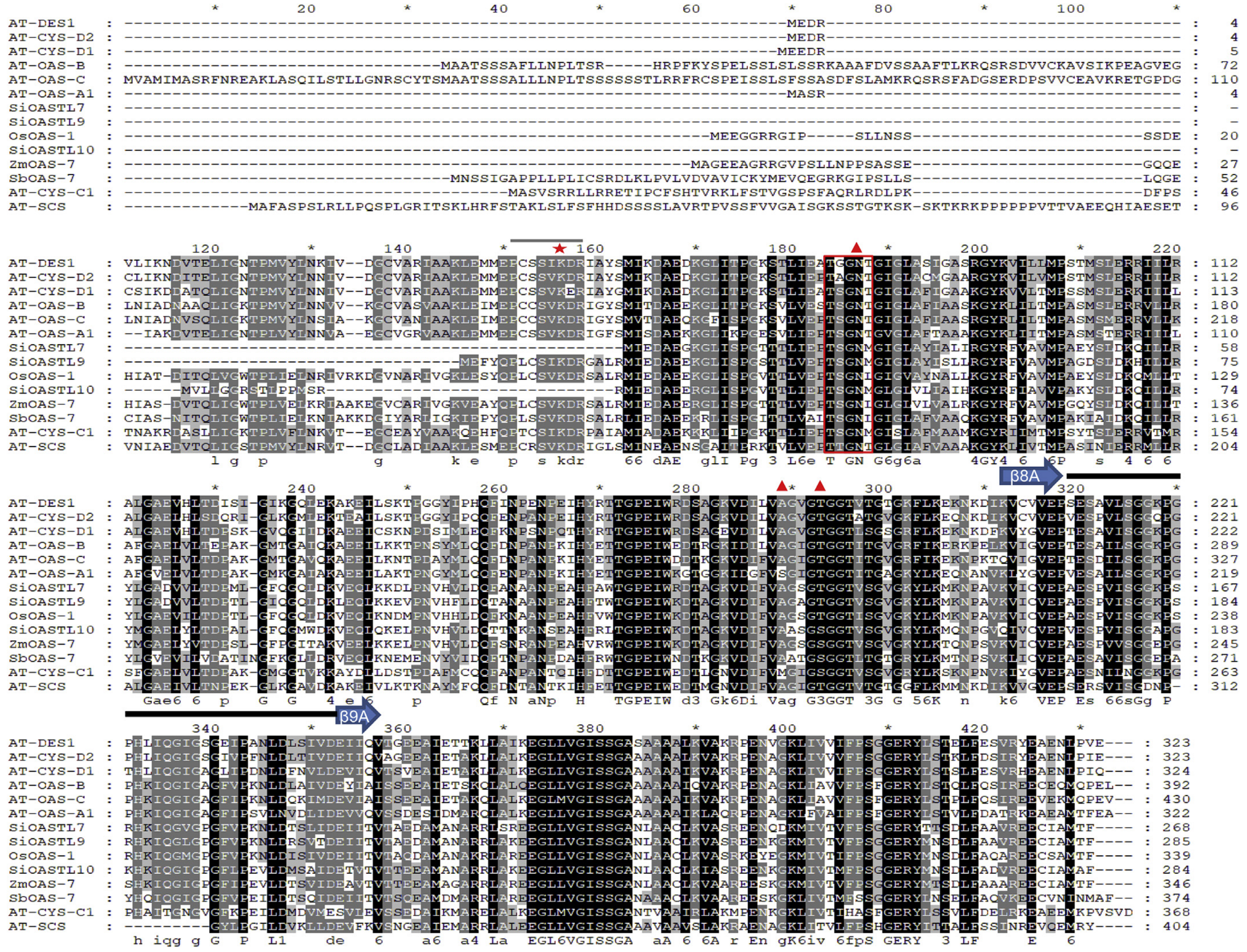

Fig. 3. Multiple sequence alignment of SiOASTLs and OASTL proteins from other species. The PLP-binding site is represented by a gray line, and the pentagram on the line highlighted the critical 'K'; the SAT protein-interaction site (β8A–β9A loop) is shown by a black line; amino acids critical for the substrate distinguishing between CAS and OASTL were indicated by triangles.

SiOASTL8 peaked at 24 h after high-salt treatment. Under drought stress treatment, the responses of *SiOASTL7* and *SiOASTL10* were similar to that of salt stress, but the response of *SiOASTL9* seemed to be earlier, as the expression level of *SiOASTL9* reached a maximum value at 6 h (Fig. 6b). The response of *SiOASTL8* to drought stress was again late: it reached its highest expression level at 12 h after treatment, and kept the high expression level until 24 h. Interestingly, under Cd treatment, only the transcription of *SiOASTL8* and *SiOASTL9* was induced. The expression levels of these two genes was greatly up-regulated from 6 h, with maximum induction at 9 h following exposure. However, the exposure did not cause a significant change in the expression levels of *SiOASTL7* and *SiOASTL10* (Fig. 6c). These results suggested that these genes might have different functions in plant response to abiotic stresses.

4. Discussion

OASTL proteins were first identified as being catalyzing enzymes in the last step of cysteine synthesis and serves as the origin of their name. Nowadays, with more and more research carried out in plants, especially Arabidopsis, OASTL-like proteins have been found to be multifunctional (Yamaguchi et al., 2000; Alvarez et al., 2010; Bermudez et al., 2010). Notably, most of the metabolites processed by OASTL are important signal molecules (such as H_2S), making OASTL proteins critical regulators during plant development and environmental adaption (Romero et al., 2014).

Most OASTL family members with catalytic activity use PLP as their cofactor. "PXXSVKDR" is a highly conserved domain for PLP binding in OASTL proteins, among which lysine (K) plays a critical role (Yamaguchi et al., 2000). However, in our study, both SiOASTL7 and SiOASTL10 missed this binding site, indicating the loss of catalytic ability of these two proteins. The enzyme activity assays we conducted confirmed this hypothesis: among the three proteins, only SiOASTL9 possessed the ability to catalyze the biosynthesis of cysteine and S-sulfocysteine, while SiOASTL7 and SiOASTL10 exhibited no activity with respect to any of the catalyzing functions reported before. As a result, we speculated that these two proteins might have other non-catalytic functions (Campanini et al., 2015). On the other hand, the protein sequence properties of SiOASTL7/9/10 have also indicated that they did not possess the ability to catalyze the detoxification of cyanide. As we reported above, both SiOASTL7 and SiOASTL10 could not bind PLP, so these two proteins could not function as a CAS, where a functional CAS needs a PLP as its cofactor. Meanwhile, it has been reported that the reaction mechanisms between CAS and OASTL are very similar, but these two enzymes prefer distinct substrates. It has been shown that there are three amino acids near the PLP-Cys ligand that affect the

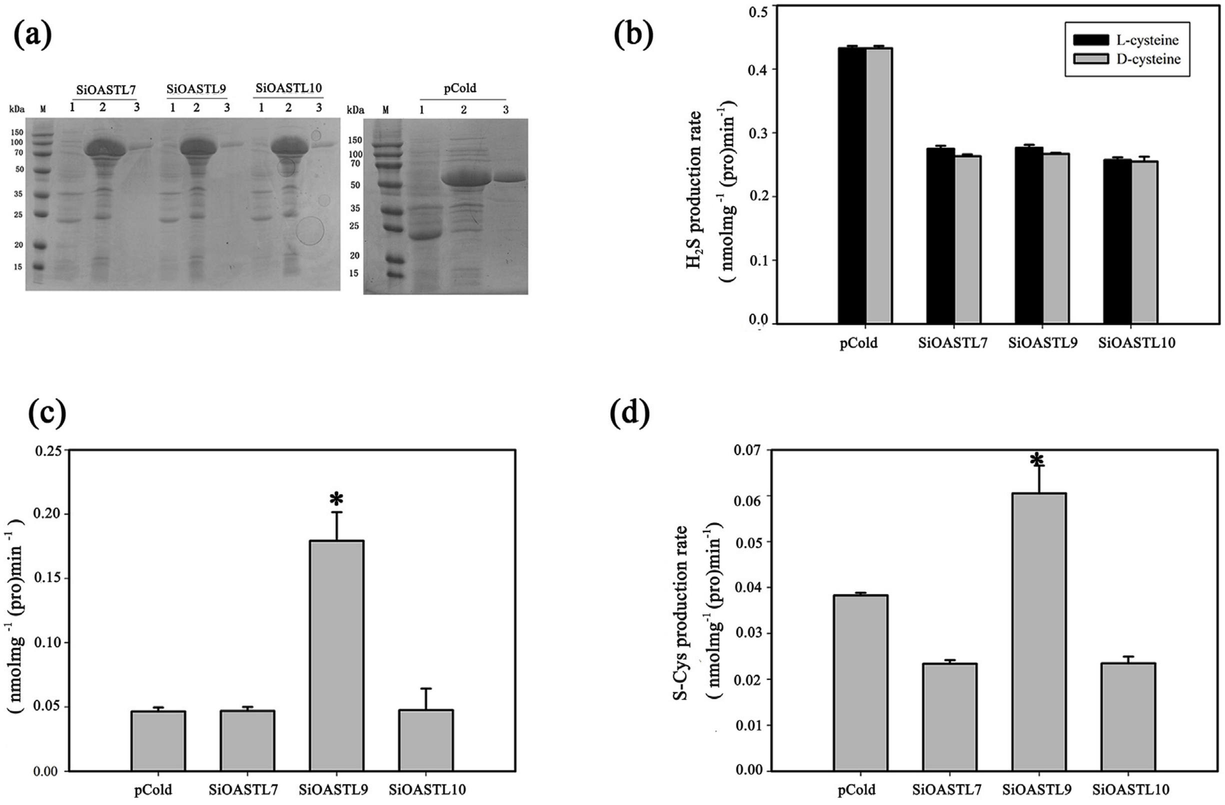

Fig. 4. Enzymatic activity assays of SiOASTL7, SiOASTL9, SiOASTL10. (a) SDS-PAGE analysis of the induced and purified fusion proteins of SiOASTLs, as well as the proteins produced by the empty pCold factor. Lane M: protein molecule marker; Lane1: protein extracts of SiOASTL7, SiOASTL9, SiOASTL10 and pCold before induction; Lane2: proteins of SiOASTL7, SiOASTL9, SiOASTL10 and pCold after induction; Lane3: proteins of SiOASTL7, SiOASTL9, SiOASTL10 and pCold purified by Ni-NTA affinity chromatography.(b) The production of H_2S using L-cysteine (black) and D-cysteine (gray) as substrates, respectively. (c) The OASTL activity of purified proteins; OAS and Na_2S were used as substrates.(d) The SSCS ability of these purified proteins with OAS and $Na_2S_2O_3$ as substrates. Each sample was performed with three replicates. The data was presented as mean ± SE (n = 3 independent replicates), * represents a significant difference ($P < 0.05$) from the control (pCold).

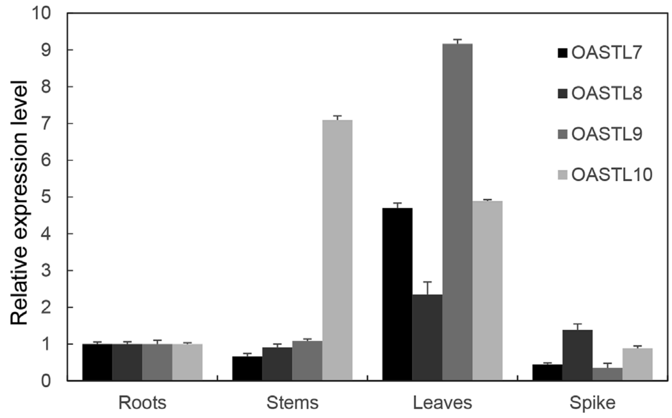

Fig. 5. The expression patterns of the four *SiOASTL* genes among different tissues of the foxtail millet plants. The *SiACTIN* gene was used as the internal gene. The expression levels are fold-change values compared with the root. The data was presented as mean ± SE (n = 3 independent replicates).

specificities of these two enzymes, and they are covaried between the two. Evidence has also shown that when all the three related amino acids of GmOASS are mutated to what are frequently found in a CAS, the GmOASS changes into a CAS (Yi et al., 2012). Here in our study, SiOASTL9 had the ability to be an OASTL, and among the three amino acids that determine the specificity of an enzyme, there were two amino acids that were identical to what is found in an OASTL – thus, SiOASTL9 could not function as a CAS. Phylogenetically, the millet SiOASTL4 was clustered in the same subclade with Arabidopsis Cys-C1, and it is possible that the ability to detoxify cyanide was obtained by SiOASTL4. We conclude this observation needs further investigation.

Further, because SiOASTL9 had the ability to catalyze the biosynthesis of both cysteine and S-sulfocysteine, we were interested about its preferred activity. We performed kinetic analysis on SiOASTL9 with sulfide and thiosulfate as the substrates, respectively. We found that the K_m value of SiOASTL9 was much lower when the sulfide was used as the substrate, indicating that the preferred activity of SiOASTL9 was to be an OASTL.

It has also been reported previously that OASTL plant proteins can be divided into five subgroups (Yamaguchi et al., 2000). Here, among the 10 OASTL proteins found in millet, five were clustered with eudicot proteins in different clusters, indicating that the functions of these proteins might be similar among eudicot and monocot. However, the other SiOASTLs were clustered in a separate subgroup. In this subgroup, not a single eudicot protein was included, and as such, we consider this group as a subclade specific to monocots (Fig. 1). Members of this subclade might have novel functions different from what have been found in eudicots. Functionally, as shown in Fig. 6, we found that the expression levels of all the four *OASTL* genes in millet were significantly up-regulated under drought and high salt stress, and it will be interesting to further explore the function of these genes to elucidate the mechanisms by which they help plants function under these stresses.

In the enzymatic activity assay, we found that SiOASTL9 was the only protein among the three that possessed the ability to catalyze the biosynthesis of cysteine (Fig. 4c), while under Cd stress, only the expression of *SiOASTL8* and *SiOASTL9* could be up-regulated. Some reports have shown that cysteine plays an important role under heavy

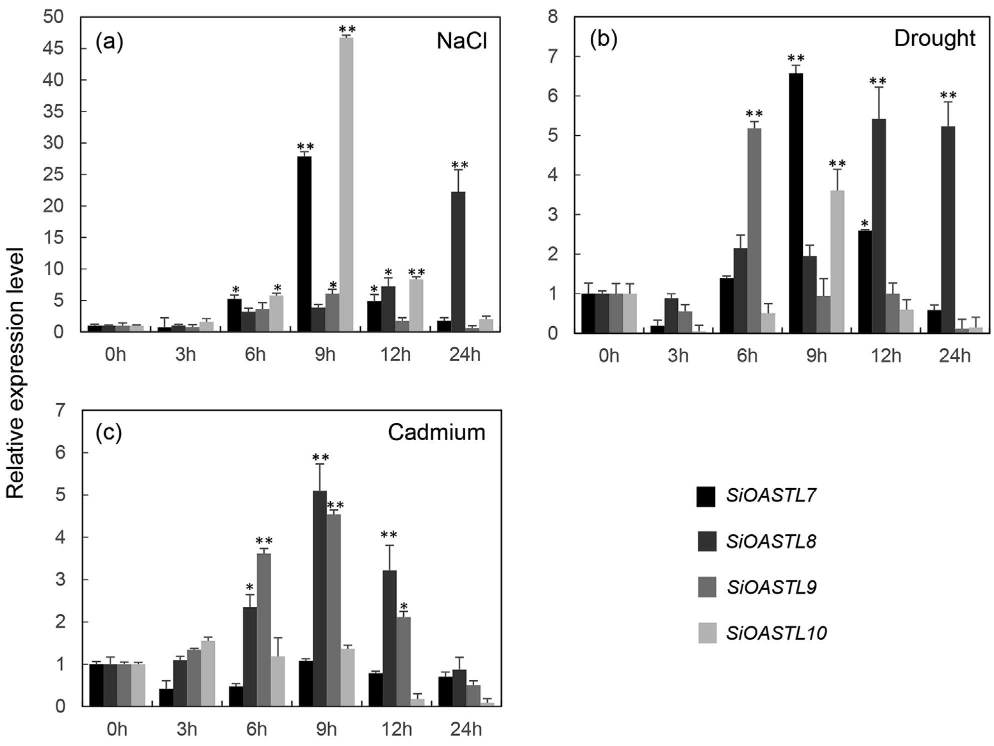

Fig. 6. Expression level changes of the four *SiOASTL* genes under abiotic stresses. (a) Expression level changes of *SiOASTL* genes under NaCl treatment, (b) Expression level changes of *SiOASTL* genes under drought treatment, (c) Expression level changes of *SiOASTL* genes under Cd treatment. Five-day-old seedlings were treated with different stresses [NaCl (250 mmol L^{-1}), CdCl$_2$ (5 mmolL^{-1}) and drought] for 0, 3, 6, 9, 12 and 24 h. *SiACTIN* was used as an internal control. The data was presented as mean ± SE (n = 3 independent replicates), *($p < 0.05$) and **($p < 0.01$) indicate significant differences.

metal stress. Cysteine treatment promoted the accumulation of the heavy metal (HM) chelators phytochelatins (PCs) and metallothioneins (MTs) to assist *A. thaliana* in tolerating Cr^{6+} stress (Fang et al., 2016). In addition, other studies have also shown that glutathione (GSH) can bind to Cd directly, synthesize phytochelatins and cysteine-rich proteins to alleviate plant damage during plant response to Cd stress, and eliminate excess ROS to regulate redox homeostasis (Li et al., 2018). Moreover, Glutathione is a tripeptide formed by the dehydration of glutamic acid, cysteine and glycine. It is an important antioxidant and free radical scavenger *in vivo* in that it acts as a detoxifier by combining free radicals and heavy metals (Romero et al., 2014). In our study, the finding that SiOASTL9 possessed the ability to catalyze the biosynthesis of cysteine might explain why this gene is involved in heavy metal response in foxtail millet. However, the specific regulatory mechanism still needs to be evaluated. Regarding *SiOASTL8*, it is interesting to see that the transcription of this gene could also be induced by various abiotic stresses, though it could not encode a functional OASTL-like protein. This phenomenon has actually been discovered in *OASTL* genes from many other species. *OAS-A2* from Arabidopsis (Jost et al., 2000) and *SlOAS3* from tomato (Liu et al., 2018) are two pseudogenes which can also not encode intact OASTL-like proteins owing to the frameshift mutation caused by alternative splicing. However, these genes might have other functions: it has been reported that in the *oas-a2* mutant, the production rate of H_2S was up-regulated under drought stress, indicating the involvement of *OAS-A2* in the H_2S signaling pathway during the plant resistance to drought stress (Jin et al., 2017). However, the exact functional mechanism of these genes still needs further investigation.

Previous studies have shown that *OASTL* genes have multiple functions during plant development. It has been proven that at least one major OASTL is required for the successful fertilization of Arabidopsis (Birke et al., 2013). In rape, the OASTL protein BnDES1 has been shown to promote the formation of roots (Xie et al., 2013), while the Arabidopsis CS26 is essential for its photosynthetic performance under long-day conditions (Bermudez et al., 2010). In our study, results of the expression pattern analyses indicated that all four *SiOASTL* genes exhibited higher expression levels in vegetative organs such as stems or leaves, indicating that these genes might play critical roles in the vegetative organs of foxtail millet. *SiOASTL8* was the only one among the four genes that showed a relatively high expression level in spike, representing the functional diversification of these monocot-specific OASTL genes during the millet plant development.

In this study, four monocot-specific *SiOASTL* genes were cloned from foxtail millet and characterized to explore their potential functions. Overall, our results suggest that though these genes shared high similarities in their sequence, their functions are diversified. There might be unidentified functions for the monocot OASTLs like SiOASTL7 and SiOASTL10 that have lost their catalyzing abilities. More research endeavors should be made to fully decipher the evolution and diversification of OASTL proteins in plants.

Author contributions

Danmei Liu, Yanxi Pei and Juan Li designed the experiments and wrote the manuscript. Juan Li, Danmei Liu and Juanjuan Lu performed the experiments. Danmei Liu and Juan Li analyzed the data. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.032.

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