Plant Omics Journal POJ

POJ 7(6):438-444 (2014) ISSN:1836-3644

Stress-inducible expression of a *Cleistogenes songorica ALDH* gene enhanced drought tolerance in transgenic *Arabidopsis thaliana*

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Abstract

Aldehyde dehydrogenases (ALDHs) have been considered as general detoxifying enzymes which eliminate abiotic stress in a variety of organisms. The ALDH12A participates in preventing proline toxicity. To targeted mining drought responsive genes from *Cleistogenes songorica*, a xerophytic grass distributed in the arid-desert grasslands of Inner Mongolia China, cDNA libraries from leaves and roots of drought-stressed seedlings were constructed. Here, we cloned an *ALDH12A* homologue, *CsALDH12A1* (GenBank No. FJ972824). The *CsALDH12A1* cDNA is 2,016 bp and encodes a deduced polypeptide of 551 amino acids, approximately 93% identical to the *Sorghum bicolor* homologue. Quantitative RT-PCR was conducted to examine the expression pattern. The results showed that *CsALDH12A1* transcripts accumulated a six-fold abundance in response to drought stress. Furthermore, transgenic *Arabidopsis* plants expressing *CsALDH12A1* under the abiotic stress inducible *rd29A* promoter showed enhanced tolerance to drought stresses. The Malondialdehyde (MDA) content of the transgenic plants with *rd29A::CsALDH12A1* were significantly lower (P<0.01) than that in non-transgenic plants, which confirm the crucial role of ALDH12A1 in the detoxification of reactive aldehydes produced from lipid per-oxidation. The data presented here suggest that *CsALDH12A1* plays a crucial role in abiotic stress tolerance during plant development.

Keywords:*Cleistogenes songorica*; Drought stress; Gene expression; MDA; Putative aldehyde dehydrogenase; Transgenic *Arabidopsis*
Abbreviations: ALDH_Aldehyde dehydrogenase; MDA_Malondialdehyde; WT_Wild type

Introduction

Drought is the most devastating abiotic stress affecting crop productivity, which is caused by insufficient rainfall and/or altered precipitation patterns (Toker et al., 2007). Oxidative stress is one of the major causes of cellular damage and cell death (Mittler 2002; Ramanjulu and Bartels 2002). During oxidative stress, lipid peroxidation chain reaction results in producing chemically reactive cleavage products which include alkanes, aldehydes, ketones, and hydroxy acids (Esterbauer et al., 1991). Aldehyde dehydrogenases (ALDHs) have been considered as general detoxifying enzymes which eliminate biogenic and xenobiotic aldehydes in a $NAD(P)^+$-dependent manner (Yoshida et al., 1998). In plants, the ALDH super family contains 13 distinct members: ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH21, ALDH22, ALDH23 and ALDH24 (Brocker et al., 2013). Six of them (ALDH10, ALDH12, ALDH21, ALDH22, ALDH23 and ALDH24) are unique to plants. Many *ALDH* genes in plants are stress-responsive, and change its expression following exposure to a wide variety of stress factors including dehydration, water logging, heavy metals, high salinity, heat, cold, oxidation, ultra violet light and many others (Chugh et al., 2011; Yang et al., 2012). The mitochondrial Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) in *Arabidopsis* (ALDH12A1) probably participates in preventing proline toxicity (Deuschle et al., 2001). Expression-profiling arrays to understand *ALDH12A* gene response to drought stress have been used on "model" species such as *Arabidopsis* (Seki et al., 2002) and more recently on crop species such as rice (*Oryza sativa*) (Zhang et al., 2011), maize (*Zea mays*) (Jimenez-Lopez et al., 2010), and grapevine (*Vitis vinifera*) (Zhang et al., 2012). Subcellular localization of *ALDH12A* genes showed Mitochondrion localized in *Arabidopsis* (Seki et al., 2002) and rice (Kotchoni et al., 2010). In *Arabidopsis*, over expression of the *Ath-ALDH3* gene improves stress tolerance by scavenging toxic aldehydes and reducing lipid peroxidation (Sunkar et al., 2003). However, the direct function of the *ALDH12A* gene response to drought stress is unknown. Drought tolerance of transgenic plants over expressing or inducing the expression of *ALDH12A* has not been demonstrated yet. It is therefore intriguing to investigate whether transgenic plants inducing *ALDH12A* expression are able to tolerate drought stress. *Cleisotogenes songorica* is a xerophytic C4 grass native to the arid-desert grasslands of Inner Mongolia, China. It is an desiccation tolerant grass adapted to environments with mean annual precipitation as low as 100mm. *C. songorica* has attracted particular attention as a species valuable in the study of drought adaptation in grasses (Zhang et al., 2011) and also as potential germplasm for the development of new varieties to improve unproductive degraded grasslands in China (Wei et al., 2009). This paper reports on the sequences and expression profile of the *CsALDH12A1* gene in order to understand desiccation tolerance mechanisms. The direct function of the *ALDH12A* gene response to drought stress was investigated through the effect of induced expression of

CsALDH12A1 in transgenic *Arabidopsis* plants exposed to drought stress.

Results

Cloning and bioinformatics analysis of the CsALDH12A1 gene

A 2,016 bp fragment of *C. songorica CsALDH12A1* was amplified and deposited in the GenBank as accession No. FJ972824. Sequence analysis indicated that *CsALDH12A1* cDNA contained an open reading frame of 1,653 bp, a 85 bp 5' UTR, 244 bp in the 3' UTR, and had a poly(A) tail (Supplementary Fig 1). The ORF encodes a protein of 551 amino acids. Bioinformatics of homology comparison in the bioinformatics database (http://bioinformatics.psb.ugent.be/plaza/) indicated that *CsALDH12A1* homologous genes are widely present in eukaryotic organisms, while 31 homologous genes belonged to 24 species of eukaryotes were found in *ALDH12A1*-owned subfamily. *CsALDH12A1* showed high identity with the orthologs from *Sorghum bicolor* (93%), *Zea mays* (92%), *Oryza sativa* Indica Group (91%), *Hordeum vulgare* (90%), *Brachypodium distachyon* (90%) and *Triticum aestivum* (90%), respectively (Fig 1). The conserved amino acids characteristics for ALDHs were present including the possible NAD^+ binding domain FTGSSV, the catalytic domain VKLEDAG and the Cys active bridging domain (Kirch et al., 2004). Results from the 3D protein structure prediction, with SWISS model software, were presented in Supplementary Fig 2. To compare the plant ALDH12A superfamily, as well as to conduct a comprehensive analysis of the evolution of the gene family, a phylogenetic analysis of the ALDH12A protein was carried out in a number of plant species-including *A. thaliana* (thale crest), *Chlamydomonas reinhardtii* (unicellular algae), *C. songorica* (xerophytic grass), *O. sativa* (rice), *Physcomitrella patens* (moss), *Populus trichocarpa* (poplar tree), *Selaginella moellindorffii* (gemmiferous spikemoss), *S. bicolor* (sorghum) and *Volvox carteri* (colonial algae), *Vitis vinifera* (grapevine) and *Z. mays* (maize). *ALDH12A* genes and associated sequence information used in the analyses are listed in Table 1. All plant ALDH12 orthologues identified share >60 % sequence identity and therefore fall into the same subfamily, namely ALDH12A (Supplementary Fig 3). ALDH12A subfamilies in *C. songorica*, *O. sativa*, *S. bicolor*, and *Z. mays* seem to cluster together, and directly diverged from the single-celled algae *V. carteri* and *C. reinhardtii*. Finally, the predicted ALDH12A forms in moss plants of *P. patens* and *S. moellindorffii* are quite isolated from the single-celled algae plants and other angiosperms.

Expression analysis of CsALDH12A1 genes

To examine the expression pattern of the *CsALDH12A1* gene in different tissues, semi-quantitative PCR and realtime PCR were carried out. Semi-quantitative RT-PCR demonstrated that *CsALDH12A1* transcripts accumulated in both leaves and roots during drought stress over the period of 10 days (Fig 2a). Relative quantitative RT-PCR showed a 6 fold abundance in roots of 10 day stressed plants compared with unstressed plants, and very low expression in drought-stressed leaves (Fig 2b).

Phenotypic analysis of transgenic plants

To address the function of the *CsALDH12A1* gene, the constructs of pPZP200 *rd29A*::*CsALDH12A1* transgenic *Arabidopsis* lines were grown in parallel with WT plants under well-watered conditions and under drought stress. Under well-watered conditions, no obvious difference was detected between transgenic and WT plants. However, the transgenic plants showed enhanced tolerance to drought stress conditions. When water was withheld for 14 days, both WT and transgenic plant leaves were wilted, with a wilt rate of 100% and 96%, respectively (data not shown). Upon re-watering, most of the transgenic plants recovered rapidly, whereas only a few wild-type plants had regrowth (Fig 3). To determine lipid peroxidation accumulation under environmental stresses, MDA content was measured in WT and two transgenic *Arabidopsis* lines as shown in Fig 4. There was no significant difference ($p>0.05$) in MDA content between the WT and two transgenic lines in the non-moisture stress treatment (CK). However, the MDA content dramatically increased up to 95%, 32% and 33% in WT, transgenic rd29A:ALDH-2 and rd29A:ALDH-6 lines after 14d of dehydration. The MDA content of the transgenic plants with *rd29A::CsALDH12A1* were significantly lower ($P<0.01$) than that in non-transgenic plants. The MDA content between two rehydrated transgenic lines showed no significant difference ($p>0.05$). These results indicated that expression of *rd29A*::*CsALDH12A1* helped to maintain the membrane permeability and as a result enhanced the tolerance of plants to drought stress.

Discussion

Drought is perceived as the most significant environmental stress in agriculture worldwide, and improving yield under drought is therefore a major goal for plant breeding (Cattivelli et al. 2008). Plant responses to drought stress are complex. They involve multiple genes and confounding genotype-by-environment interactions. These complexities can limit the magnitude and rate of genetic gain in conventional breeding strategies targeting the improvement of drought tolerance. Modern genomics and genetic approaches coupled with advances in precise phenotyping are expected to be more effectively unravel the genes and metabolic pathways that confer drought tolerance in crops (Mir et al., 2012). The significant advances made in the model plant systems of major crop species provide an opportunity to identify candidate genes associated with drought tolerance. During the last few decades efforts have been made to develop transgenic lines in different crops, showing improved tolerance to drought stress (Ashraf 2010). In *C. songorica*, 3579 ESTs were generated earlier from normalized cDNA libraries of drought stressed seedlings. Transcripts of 13 of these 22 unigenes were shown to be at least three fold more, or less abundant in drought-stressed leaves or roots, with 8 increased and 5 decreased in relative transcript abundance (Zhang et al., 2011). Drought stress can induce the rapid and excessive accumulation of reactive oxygen species (ROS) in plant cells (Sunkar et al., 2003) and gradually accentuate injury to leaf cell membranes through lipid peroxidation with plant growth. The ALDH activity increase is considered as an efficient defense strategy to eliminate the toxic aldehydes caused by ROS (Rodrigues et al., 2006). The *Arabidopsis* genome contains 14 genes belonging to the *ALDH* gene superfamily, encoding members of nine distinctive protein families (Kirch et al., 2004). In addition, ALDH expression is variable and widespread throughout plant tissues and also developmentally regulated (Missihoun et al., 2011). Plant ALDH proteins are found in numerous subcellular compartments-including cytosol, mitochondria, plastids (chloroplasts, chromoplasts and leucoplasts), peroxisomes and microsomes (Kotchoni et al., 2010; Missihoun et al., 2011; Mitsuya et al., 2009). The single-celled algae *V. carteri* and the angiosperm *Arabidopsis* express putative *ALDH12A* proteins that share 61 % sequence identity and 74 % sequence similarity, which shows a high degree of conservation between evolutionarily

Table 1. ALDH12 family members: unified nomenclature and gene information

Species	Gene name	NCBI gene ID	Other names/ aliases	NCBI protein ID	Phytozome ID	Chrm	Scaffold	Exon #	AA #	References
Arabidopsis thaliana	*ALDH12A1*	836373	K19B1.14; K19B1_14; P5CDH	NP_568955.1	AT5G62530	5	-	16	556	(Kirch et al., 2004)
Chlamydomonas reinhardtii	*ALDH12A1*	159477663	Cr_Aldh12A	XP_001696928.1	Cre12.g520350	12	-	12	548	(Wood and Duff 2009)
Cleistogenes songorica	*ALDH12A1*	972824	-	-	-	-	-	-	551	(Zhang et al., 2011)
Oryza sativa	*ALDH12A1*	4339448	Os05g45960; OsALDH12	EEE64501.1	No entry	Un	-	Un	716	(Gao and Han, 2009)
Physcomitrella patens	*ALDH12A1*	5923366	Pp_Aldh12A	XP_001760169	Pp1s41_177V6	-	41	17	571	(Wood and Duff, 2009)
Populus trichocarpa	*ALDH12A1*	7491541	Pt-FIS1.3; POPTRDRAFT_581353	XP_002330119.1	POPTR_0015s07550	15	-	16	566	-
Selaginella moellindorffii	*ALDH12A1*	9650766	-	XP_002968656.1	90262	-	11	15	526	-
Sorghum bicolor	*ALDH12A1*	8068986	-	XP_002441445.1	Sb09g026810	9	-	15	549	-
Vitis vinifera	*ALDH12A1*	100251938	VpALDH12A1	XP_002273569.1	GSVIVT01008047001	17	-	16	555	(Zhang et al., 2012)
Volvox carteri	*ALDH12A1*	9623193	-	XP_002958122.1	69010	-	37	10	550	-
Zea mays	*ALDH12A1*	No entry	ZmALDH12A1	AAL70108.1	GRMZM2G090087	6	-	15	549	(Jimenez-Lopez et al., 2010)

Exon and amino acid figures obtained from NCBI entries or Phytozome. Un undetermined

Table 2. Primers used to clone and analyze *CsALDH12A1*

Primer name	(5'-3') Nucleotide sequence	Purpose
CsALDH_F	TCCGATGAGCCGCCTCCTCT	cDNA Cloning of *CsALDH12A1*
CsALDH_R	TTAAGTCGCGGAAGGAAGCGCC	
CsALDH12A1_F_{rt}	TGCTTATGCTTGCAGTGGTC	RT-PCR of *CsALDH12A1*
CsALDH12A1_R_{rt}	TCATCCTTTCACAGGCTTCC	
CsGAPDH_F_{rt}	CTCTGCCCCTAGCAAAGATG	RT-PCR of *CsGAPDH*
CsGAPDH_R_{rt}	GAGCTTGCCCTCAAAAACAG	
CsALDH12A1_F_{Qrt}	GCATCCATCATCGTCTCTGA	Q-RT-PCR of *CsALDH12A1*
CsALDH12A1_R_{Qrt}	TCAAGGCGCTTCTCATATCC	
CsGAPDH_F_{Qrt}	GTCAGCCAAGGACTGGAGAG	Q-RT-PCR of *CsGAPDH*
CsGAPDH_R_{Qrt}	ACACATCGACTGTTGGGACA	
rd29A_F	AGAATCTCAAACACGGAG	Cloning of *rd29A*
rd29A_R	ACTAAGTTTATAGAGAGACTG	
CsALDHattB1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGAGCCGCCTCCTCTCGCGGCGGC[1]	Gateway cloning *CsALDH12A1* into vector
CsALDHattB2-R	GGGGACCACTTTGTACAAGAAAGCTGGGT ACAAATTAAGTCGCGGAAGGAAGCG[2]	

[1,2] Underlined sequences, attB1 and attB1 adaptor of Gateway cloning vector

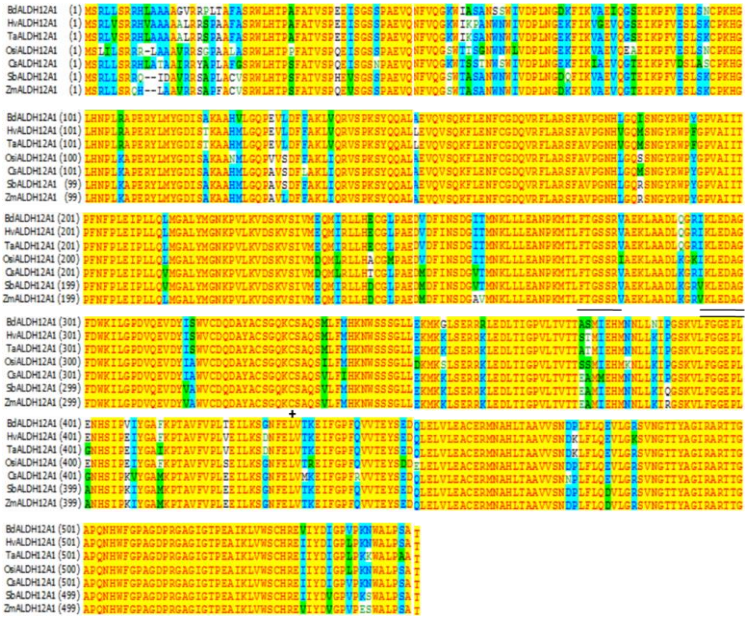

Fig 1. Multiple amino acid sequences alignment of the ALDH-like proteins derived from *Sorghum bicolor* (GenBank accession no. XP_002441445, 93%), *Zea mays* (GenBank accession no. AF467541, 92%), *Oryza sativa* Indica Group (GenBank accession no. EEC79594, 91%), *Hordeum vulgare* (GenBank accession no. AF467539, 90%), *Brachypodium distachyon* (GenBank accession no. XP_003568012, 90%) and *Triticum aestivum* (GenBank accession no. AF467542, 90%). The conserved amino acids characteristics for ALDHs are present as the possible NAD+ binding site FTGSSV (single underlined), the catalytic site VKLEDAG (double underlined) and the Cys as the active site (+).

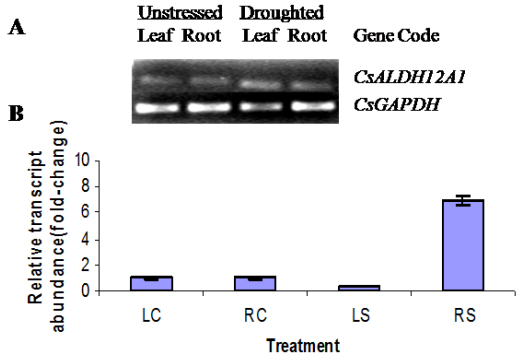

Fig 2. Expression patterns of *CsALDH12A1* mRNA under dehydration stress. A, B The semi-quantitative RT-PCR and the quantitative RT-PCR results of *CsALDH12A1* gene expression during hydrate to dehydration. LC and RC, leaf and root from WT without stress; LS and RS, leaf and root from transgenic *CsALDH12A1* line with 10d dehydration.

distant species and strong selective pressure to maintain gene function (Brocker et al., 2013). The *ALDH12A* protein of *C. songorica* shares 93% sequence identity to *S. bicolor*, 76% to *A. thaliana*, and 63% to *V. carter* (data not shown). The first genome-wide expression study of rice *ALDH* genes under osmotic stress revealed organ specific adaptation to stress, and five genes (*OsALDH2-4*, *OsALDH3-4*, *OsALDH7*, *OsALDH18-2* and *OsALDH12*) were induced more than 2-fold in drought-stressed young leaf (Gao and Han, 2009). *ALDH12A1* in *Arabidopsis* encodes P5CDH localized in the mitochondrial matrix and is highly induced by exogenous proline application and salinity (Deuschle et al., 2001). Expression of *ALDH12A*1 is regulated by a series of nat-siRNA processing steps under salt stress (Borsani et al., 2005). Moreover, the ALDH7B4 gene promoters were compared between Brassicaceae. The *Cis*-acting elements including two conserved ACGT-containing motifs near to the translation start codon were found to be essential for the responsiveness to osmotic stress in Arabidopsis leaves and in seeds (Missihoun et al., 2014). Here, we presented the expression of *CsALDH12A1* under drought stress at the seedling stage. *CsALDH12A1* was up-regulated more than 6-fold specifically in drought-stressed roots (Fig 2B). Genes encoding different types of antioxidants have been engineered in different plants for achieving enhanced drought tolerance. Over-expression of some stress-induced *ALDH* superfamily genes from different model plants could enhance the stress tolerance of transgenic plants. However, little has been done to evaluate the effects of the *ALDH12A* gene family on drought stress tolerance. Former research in various plant species indicates that the expression of stress inducible genes driven by constitutive promoters like CaMV *35S*, rice *actin 1* and maize *ubiquitin*, result in improved stress tolerance, but with a penalty on plant growth and productivity (Behnam et al., 2006; Kasuga et al., 2004). Utility of stress-inducible promoters for overexpression of transgenes in heterologous systems have been used earlier to minimize the undesirable effects on plant growth (Kasuga et al., 2004; Pellegrineschi et al., 2004). The *rd29A*-regulated transgene expression confers enhanced tolerance against drought, salt and cold stress (Behnam et al., 2006; 2007; Checker et al., 2012). Therefore, the stress-inducible *rd29A* promoter from *Arabidopsis* was used to drive the expression of the *CsALDH12A1* gene, with the aim of minimizing the undesirable effects on plant growth. We compared stress tolerance between transgenic and WT plants by subjecting the plants to simulate drought stress conditions. Morphological evaluations of *rd29A::CsALDH12A1* plants exhibited better growth and improved stress tolerance than WT plants under controlled conditions, as revealed by delayed leaf wilting and high recovery from dehydration stress of *rd29A::CsALDH12A1* plants (Fig 3). It has been reported that MDA levels in WT *Arabidopsis* plants increased with salt stress (NaCl or KCl), but the comparative increase in MDA levels of plants over-expressing either ALDH3I1 or ALDH7B4 was less than in WT plants (Kotchoni et al., 2006). We also found that under drought stress, MDA content of the transgenic plants was significantly (P<0.01) lower than that in non-transgenic plants (Fig 4), which confirms the crucial role of ALDH12A in detoxification of reactive aldehydes produced from lipid per-oxidation. These results further indicate that the stress-inducible *rd29A* promoter is effective to induce the expression of multifunctional genes for improving drought stress tolerance.

Materials and Methods

Plant materials, growth conditions and stress treatment

C. songorica seeds were collected from native grasslands in the Alashan region, Inner Mongolia. Seeds were initially germinated in a sand bed and 3-week-old seedlings were transplanted individually into 8 cm pots containing a sand vermiculite mixture (1:1 by volume), with an 18/6 h photoperiod, day/night temperature of 28/20℃, and a photosynthetic photon flux density of 240 μmol m^{-2} s^{-1}. Around

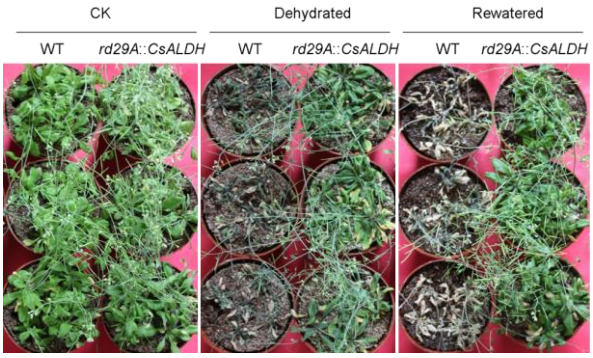

Fig 3. Phenotype of wild type and transgenic *Arabidopsis* plants under watering, dehydrated and rewatered.

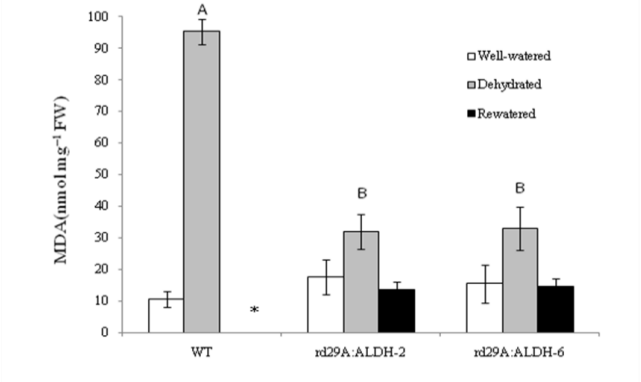

Fig 4. MDA measurement during the hydration to desiccation treatment in *Arabidopsis ALDH* lines
Lipid peroxidation as measured by MDA levels in transgenic and wild type Arabidopsis plants in well-watered plants (CK), 14 days after dehydration and 5 days after recovery. Capital letters in the same column significantly differ ($P < 0.01$). * WT did not recover.

100 fifty five days old plants were subjected to moisture stress by withholding watering for up to 10 days. Leaf and root samples were collected at 0 and 10 days of drought stress, flash frozen, and stored at −80℃. Wild type *Arabidopsis* (genotype Col-0) seeds were used for genetic transformation by vacuum infiltration.

Gene cloning, sequencing and bioinformatics analysis

Four cDNA libraries were constructed from leaves and roots sampled from drought-stressed *C. songorica* seedlings (Zhang et al., 2011), with a EST that showed similarity to putative aldehyde dehydrogenase MIS1 of *Zea mays* (AF467541). Based on the nucleotide sequence of putative aldehyde dehydrogenase MIS1 of *C. songorica*, gene-specific primers of CsALDH_F and CsALDH_R were designed to amplify the ORF of *CsALDH12A1*. The amplified PCR products were examined on 1 % agarose gels, cloned into a pGEMT-Easy vector (Promega Corp., Madison, WI) and sequenced at Shanghai Shenggong Biotechnological Ltd. (Shanghai, China). Sequence similarities were examined with the GenBank database using the BLAST program (http:/ /blast.ncbi.Nlm.nih.gov/Blast.cgi). For nucleic acid and amino acid sequence alignment DNAMAN 5.2.2 software (Lynnon Biosoft) was used. The phylogenetic relationship was analyzed by multiple alignments of plant *ALDH12A1* cDNAs via Vector NTI advance 10 Suit (Invitrogen, Carlsbad, CA). The 3D (dimension) structure of CsALDH12A1 protein was also predicted by SWISS-MODEL software.

Semi-quantitative PCR and real-time PCR

Total RNA was extracted from the leaf and root tissues sampled from the moisture stressed plants and also the non-stressed control, using a RNeasy plant mini kit (Qiagen, Germany) and treated with RNase-free DNase I. About 1 μg of total RNA was used for first strand cDNA synthesis. The semi-quantitative PCR was carried out in a total volume of 30 μL including 0.005 μmol L^{-1} dNTP, 3 μL 10×Dynazyme buffer, 1 U *Taq* polymerase, 0.5 μmol L^{-1} of each primer, and 0.5 μg cDNA. A putative GAPDH encoding sequence (*CsGAPDH*, FJ972819) from *C. songorica* (Zhang et al., 2009) was used as a constitutively expressed reference gene. Specific primers (Table 2) were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The real-time PCR was assayed in a 10 μL qRT-PCR reaction containing 5 μL 2×SYBR Green mix (Applied Biosystems) and 1μL cDNA (1:10 dilution). Cycling conditions were 95 ℃ for 10 min; then 40 cycles of 95 ℃ for 30 s, and 60 ℃ for 30 s. The *CsGAPDH* was used as the internal reference gene to normalize the gene expression levels of different samples. The gene specific primers designed using Primer Express software are presented in Table 2. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative changes in gene expression from quantitative real-time PCR experiments (Livak and Schmittgen, 2001; Zhang et al., 2009). -ΔΔCt was calculated from the equation: $-\Delta\Delta Ct = -(Ct_{target} - Ct_{GAPDH})_{drought\text{-}stressed\ group} - (Ct_{target} - Ct_{GAPDH})_{control\ group}$. The data are presented as the fold change in transcript level normalized to the *CsGAPDH* gene, relative to that in non-moisture stress plants. All experiments were conducted with three biological, and two technical replicates.

Construction of stress-inducible expression vector and generation of transgenic A. thaliana

Plasmid pPZP200-*hph*-*rd29A*-*CsALDH12A1*-*35St* was developed using Gateway™ cloning techniques following the instructions provided by the manufacturer (Invitrogen, USA). The *rd29A*, *CsALDH12A1* and *35St* was first introduced into entry vectors using the BP recombination reaction, to create pDONR P4-P1- *rd29A*, pDONR 221-*CsALDH12A1* and pDONR P2R-P3-*35St*. The three above entry vectors and the destination vector pPZP200-*hph*-R4R3 were used to perform the MultiSite Gateway LR recombination reaction and generate the expression vector. Each step was verified by sequencing the clones. Electro competent *Agrobacterium tumefaciens AGL-1* cells were electroporated with the pPZP200-*hph*-*rd29A*-*CsALDH12A1*-*35St* construct, and *hygromycin phosphotransferase* (*hph*) gene as selectable marker genes was used. Isolated colonies were cultured to transform WT *Arabidopsis* (Col) plants by a Vacuum infiltration method (Bechtold and Pelletier 1998). The T1 plants were selected on Hygromycin (15 μg ml^{-1}) and confirmed by PCR. T2 generation lines which showed 3:1 segregation for Hygromycin resistance were carried forward to T3 generation. Presence of the transgene in transgenic *Arabidopsis* was confirmed by PCR using promoter specific forward primers and the *CsALDH12A1* specific reverse primer. PCR confirmed homozygous T3 generation lines, transgenic rd29A:ALDH-2 and rd29A:ALDH-6 lines, were used for the drought stress tolerance assay.

Drought tolerance analysis of transgenic Arabidopsis

For assaying drought stress tolerance, one week old seedlings of WT and transgenic *Arabidopsis* plants expressing

CsALDH12A1 in MS media were transplanted into soil in a plant growth chamber maintained at 22/20 ℃ day/night, 16/8 h day/night and 80 μmol·m^{-2}·s^{-1}. Three week old plants were stress treated by withholding irrigation for 14 days and then re-watered for recovery.

Malondialdehyde measurement of transgenics

Malondialdehyde was estimated in 100 mg of fresh T3 transgenic *Arabidopsis* samples following the procedure of Draper and Hadley (Draper and Hadley 1990). The plant material was ground using a mortar and pestle and then transferred into 1.5 ml eppendorf tubes with 1.5 ml of chilled 0.1% (w/v) TCA solution. The mixture was vortexed, incubated for 5 min and then centrifuged for 10 min at 10,000 rpm at 4ºC. The supernatant was transferred into new tubes; one aliquot of 0.6 ml the supernatant in 0.6 ml of 20% (w/v) TCA with 0.5% (w/v) TBA solution and another with a 20% (w/v) TCA solution (without TBA) and incubated for 30 min at 100ºC in a water bath. The mixtures were centrifuged for 10 min at 10,000 rpm. The absorbance of supernatant was measured at 532 nm and 600 nm. The amount of MDA was calculated using the following formula (where FW= fresh weight):

MDA equivalents (nmol/mL) = $[(OD_{532TCA+TBA}-OD_{600TCA+TBA}) - (OD_{532TCA}-OD_{600TCA})/157000] \times 10^6$

MDA equivalents (nmol/g FW) = 2 × MDA equivalents (nmol/mL) × Total volume of the extracts (mL) / g FW

Statistical analysis

For water deficit experiment, all treatment has three replicants and each replicant has at least 5 plants. All these plants were arranged as Random Completed Block Design and rotated twice a week to ensure consistency. One-way ANOVA was performed on the data Using SPSS 12.0 statistical analysis software, and LSD approach was used for multiple comparisons to check the significant differences.

Conclusion

In conclusion, this study isolated *CsALDH12A1* cDNA and evaluated the transcript expression patterns in response to drought stress. The results of quantitative RT-PCR and induced expression in transgenic *Arabidopsis* showed improved the tolerance when exposed to drought stress. These data suggest that *CsALDH12A1* gene are potential candidates for improving tolerance to drought stress in *Cleistogenes songorica*. Further studies are also required to examine the exact biochemical roles of *CsALDH12A1* in developmental process and stress tolerance in the future.

Acknowledgments

We thank Dilini Gunawardana for her technical assistance. This work was supported by grants from Ministry of S&T, China (31101759, 2014CB138704), the Department of Agriculture and Animal Husbandry, Gansu province (GNSW-2011-16), Program for Changjiang Scholars and Innovative Research Team in University (IRT13019) and Fundamental Research Funds for the Central Universities (lzujbky-2013-87).

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