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ORIGINAL ARTICLE

Molecular cloning and characterization of drought stress responsive abscisic acid-stress-ripening (*Asr1*) gene from wild jujube, *Ziziphus nummularia* (Burm.f.) Wight & Arn

Jasdeep Chatrath Padaria[1] · Radha Yadav[1] · Avijit Tarafdar[1,3] · Showkat Ahmad Lone[1] · Kanika Kumar[1] · Palaiyur Nanjappan Sivalingam[2]

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Abstract Drought is a calamitous abiotic stress hampering agricultural productivity all over the world and its severity is likely to increase further. Abscisic acid-stress-ripening proteins (ASR), are a group of small hydrophilic proteins which are induced by abscisic acid, stress and ripening in many plants. In the present study, *ZnAsr1* gene was fully characterized for the first time from *Ziziphus nummularia*, which is one of the most low water forbearing plant. Full length *ZnAsr1* gene was characterised and *in silico* analysis of ZnASR1 protein was done for predicting its phylogeny and physiochemical properties. To validate transcriptional pattern of *ZnAsr1* in response to drought stress, expression profiling in polyethylene glycol (PEG) induced *Z. nummularia* seedlings was studied by RT-qPCR analysis and heterologous expression of the recombinant *ZnAsr1* in *Escherichia coli*. The nucleotide sequence analysis revealed that the complete open reading frame of *ZnAsr1* is 819 bp long encoding a protein of 273 amino acid residues, consisting of a histidine rich N terminus with an abscisic acid/water deficit stress domain and a nuclear targeting signal at the C terminus. In expression studies, *ZnAsr1* gene was found to be highly upregulated under drought stress and recombinant clones of *E. coli* cells expressing ZnASR1 protein showed better survival in PEG containing media. *ZnAsr1* was proven to enhance drought stress tolerance in the recombinant *E.coli* cells expressing ZnASR1. The cloned *ZnAsr1* after proper validation in a plant system, can be used to develop drought tolerant transgenic crops.

Keywords *Ziziphus nummularia* · RT-qPCR · *ZnAsr*1 gene · Phylogenetic analysis · SDS-PAGE

Introduction

Crop plants in their natural environments are frequently exposed to different abiotic stresses like high salinity, extremes of both high and low temperatures and water deficiency or drought. Whether plants are susceptible or tolerant to these adverse condition depends on expression of multiple stress responsive genes and their coordinated action, which also cross-talk with other modules of signal transduction involved in stress related pathways. Over long evolutionary scales, plants in response to different environmental stresses have attained mechanisms by which they can assess these stresses and modulate their physiological mechanisms accordingly. In recent years, several stress responsive genes have been investigated particularly so in model plant systems *Arabidopsis*. But physiological and molecular functions of only a few such genes have been predicted from their translated amino acid sequences. A large group of these stress responsive genes are thought to be under the control of stress induced abscisic acid (ABA) [1].

Drought stress is a serious limiting factor that significantly influences agricultural production by preventing maximum crop yields. Onset of water deficit in plants leads to induction of various drought responsive genes including *Asr*. ASR, a group of plant-specific proteins is potentially

✉ Jasdeep Chatrath Padaria
jasdeep_kaur64@yahoo.co.in

[1] National Research Centre on Plant Biotechnology, PUSA Campus, New Delhi, India

[2] Central Institute for Subtropical Horticulture, Bikaner, Rajasthan, India

[3] Present Address: International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Telangana, India

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involved in drought tolerance mechanisms through an ABA mediated pathway [2, 3]. *Asr1* gene is reported to participate in regulation of ROS homeostasis through activation of both, the antioxidant system and the genes associated with drought stress [4]. Moreover, *Asr* has also been suggested to act as a transcription factor for certain drought stress responsive genes [5]. Although, the exact function of ASR proteins remain to be established, they have been identified to play an important role in improving plant's adaptability to different abiotic stresses including the water deficit stress [6–8].

Ziziphus spp. (ber) belonging to the family Rhamnaceae, possess intrinsic and dual capability of drought avoidance and drought tolerance by means of various physiological mechanisms. These mechanisms include, stimulation of antioxidative metabolic pathways, osmotic adjustment and sensitive stomatal closure to minimize dehydration during adverse conditions [9–11]. *Z. nummularia* (Burm.f.) Wight & Arn., a perennial herb commonly known as jhar ber is native to western India, southeastern Pakistan and southern Iran. *Z. nummularia* along with seventeen other species are wildly distributed in dry and hot climates of the central India, northwestern plains and dry areas in the peninsular region [12, 13]. Both, the domestic and wild species of *Ziziphus* are found in the Indian deserts, but species *Z. mauritiana* and *Z. rotundifolia* are found only in the northwestern Indian desert along with the traditional species, *Z. nummularia*.

Ziziphus nummularia responds to drought, salinity and temperature stress [14–17] through a series of variations at physiological and developmental levels by inducing expression of a number of stress responsive genes. Amongst other signaling molecules produced, ABA production plays a key role in induction of cold and drought stress responsive genes [18–21], and has been proven to result in the accumulation of various stress responsive molecules during drought stress [21].

In the present work, we have characterized the complete open reading frame of *Asr1* gene in *Z. nummularia* (*ZnAsr1*) and the gene was also sequenced after amplification in genomic DNA. The transcript patterns of *ZnAsr1* in response to PEG induced drought stress, and the expression profiling of the recombinant ZnASR protein was also studied. This is the first report describing the characterization of *Asr* gene from *Z. nummularia*.

Materials and methods

Plant material and treatment

Seeds of *Z. nummularia* were procured from the national repository of Central Institute for Arid Horticulture Bikaner, India. The seeds (after removing the hard coat) were planted in soilless medium in a growth chamber under phytotron conditions.

In order to study the expression pattern of *Asr1* gene in *Z. nummularia*, twenty seedlings were grown under controlled conditions in a plant growth chamber for one month. Seedlings were subjected to 30 % polyethylene glycol M.W. 6000 (PEG 6000) treatment and samples were harvested after 6, 12, 24, 48 and 72 h of PEG treatment. The osmotic potential was calculated as

$$\psi\pi = -C\ R\ T_{(K)}$$

where "$\psi\pi$" refers to the osmotic potential, "C" the molar concentration of solutes (0.05 M), "R" the universal gas constant (i.e., 8.314472 $JK^{-1}\ mol^{-1}$), and "$T_{(K)}$" the experimental temperature in Kelvin (K). Whole seedlings were harvested, flash frozen in liquid nitrogen, and stored at −80 °C for further downstream processing.

Extraction of total RNA and genomic DNA

Total RNA from control and treated seedlings was isolated using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to manufacturer's instructions. Genomic DNA was extracted from seedlings of *Z. nummularia* by a modified CTAB method [22]. The quality and quantity of extracted RNA and genomic DNA were determined by agarose gel electrophoresis and UV spectrophotometry [23] (NanoDrop 2000, Thermo Scientific, USA).

Generation of full length cDNA by 5′ RACE and 3′ RACE

5′ RACE: For the amplification of 5′ and 3′ cDNA ends of *ZnAsr1* gene, 1 µg of total RNA was used to produce cDNA by means of SMARTer™ RACE cDNA amplification kit (Clontech, USA). First PCR was carried out using gene specific Asr1 primer (0.2 µM), 5 µl of 10× Universal Primer A Mix (UPM), 5 µl 10× advantage 2 PCR buffer, 0.2 mM dNTP Mix, 1 µl 50× Advantage 2 polymerase mix and 2.5 µl of 5′ RACE ready cDNA. The total reaction was carried out in 50 µl volume and the amplification conditions were as: 5 cycles each at 94 °C for 30 s, 72 °C for 3 min and 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min, followed by 25 cycles of amplification at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min. The PCR product thus generated was used as template in a primary nested PCR, which was performed with gene specific primer Asr2 and Nested Universal Primer A (NUP). The amplification conditions were kept the same as used for the first PCR. Using the product of primary nested PCR as template, the secondary nested PCR was performed with primers Asr3 and NUP at the same amplification conditions. The final PCR product was cloned into the pJET1.2/

blunt cloning vector (Thermo Scientific, USA) and sequenced bi-directionally.

3′ RACE: First PCR for the amplification of 3′ cDNA ends was performed with Asr4 primer (0.2 µM), 5 µl 10× UPM, 5 µl 10× advantage 2 PCR buffer, 0.2 mM dNTP Mix, 1 µl of 50× Advantage 2 polymerase mix and 2.5 µl of 3′ RACE ready cDNA. First and second nested PCR cycles were performed as described in 5′ RACE with primers Asr5 and Asr6, respectively. The purified 3′ RACE PCR products were cloned into pJET1.2/blunt and sequenced bi-directionally. The full length cDNA sequence of *ZnAsr1* was obtained by comparing and aligning the sequences of 5′ and 3′ RACE products using BioEdit version 7. 1. 11 [24].

Phylogenetic and *in silico* analysis

In order to study the phylogenetic relationship of *ZnAsr1* with that of the known *Asr*s, the sequences of the later were retrieved from the GenBank database (Table 1). Multiple sequence alignment of *ZnAsr1* was performed using Clustal X (version 1.81) [25] and pair-wise sequence identities of the nucleotide and amino acid were compared using GeneDoc (version 2.6.002) (Table 1). Maximum likelihood phylogenetic trees based on the nucleotide and deduced amino acid sequences were constructed using program MEGA6 [26]. The physiochemical properties of ZnASR protein like aliphatic index, amino acid composition, chemical formula, grand average of hydropathicity (GRAVY), instability index, molecular weight, theoretical pI and total number of positive and negative residues were calculated by the ProtParam tool.

Full length amplification of the *ZnAsr1* genomic sequence

The 3′ and 5′ RACE sequences were aligned to yield the complete coding sequence of *ZnAsr1* by CLUSTAL W program. To obtain the complete genomic sequence of *ZnAsr1* from *Z. nummularia*, four gene specific overlapping primers, Asr7 and Asr8 to amplify the 5′ half of the *ZnAsr1* genomic region, and Asr9 and Asr10 to amplify the 3′ half, including intronic region were designed.

RT-qPCR analysis

The time dependent expression patterns of *ZnAsr1* in response to PEG induced drought stress was studied by RT-qPCR analysis. The first strand cDNA was synthesized from the total RNA of the PEG treated *Ziziphus* seedlings by the SuperScript®III first strand synthesis system (Invitrogen, USA) using oligo (dT) primer. *Ziziphus elongation factor*1 (*Zjef1*) gene (accession no. EU916202) was used as an internal control [27]. RT-qPCR analysis was performed using specific primers for both *Asr* as well as *Zjef1* (Table 2) to amplify 154 and 111 bp products, respectively. RT-qPCR amplification mixtures (20 µl) contained 100 ng template cDNA, 0.5 µM of each primer and 2× KAPA SYBR FAST qPCR master mix buffer (10 µl) (KAPA Biosystems, USA). Reactions were run on the LightCycler® 480 II (Roche, Switzerland). The cycling conditions consisted of 4 min polymerase activation at 94 °C and 40 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Experiments were performed on three biological replicates with three technical replicates for

Table 1 List of the accession nos. of *Asr* genes retrieved from the NCBI database for analyzing the nucleotide and amino acid sequences

Plants	Family	Accession no	Length of sequences		% Sequence similarity	
			CDS (bp)	Deduced amino acids	Nucleotide	Amino acid
Calystegia soldanella	Convolvulaceae	AB047594	747	248	41	41
Citrus maxima	Rutaceae	U18972	297	98	25	27
Ginkgo biloba	Ginkgoaceae	AAR23420	546	181	34	41
Solanum lycopersicum Asr1	Solanaceae	AAB64185	348	115	24	30
Solanum lycopersicum Asr2	Solanaceae	CAA52873	345	114	26	32
Oryza sativa	Poaceae	AF039573	416	138	27	33
Prunus armeniaca	Rosaceae	U93164	603	200	44	48
Prunus persica	Rosaceae	AF317062	582	193	43	47
Glycine max	Fabaceae	AK244579	717	238	45	48
Prunus mume	Rosaceae	AB434496	618	205	45	49
Ricinus communis	Euphorbiaceae	XM_002524250	426	141	31	37
Hevea brasiliensis	Euphorbiaceae	AY221984	327	108	27	32
Vitis pseudoreticulata	Vitaceae	DQ336286	450	149	31	38
Ziziphus nummularia	Rhamnaceae	KC816463	822	273	–	–

Table 2 Description and list of primer sequences used in this study

Application	Primer designation	Primer sequences (5′ → 3′)	Description
RACE amplification	Asr1_R	GGTGCTTCTTTCCATGAGACTCC	5′ RACE: outer primer
	Asr2_R	CAAAACCTCCAGATCCAACTGCAG	1st internal nested primer
	Asr3_R	GCCTGTGACTATGCTCTGTGTCTTTC	2nd internal nested primer
	Asr4_F	CAAGCATCTTGAGCACCTGGGTGAGC	3′ RACE: Outer primer
	Asr5_F	CACGAGGCAAAGAAAGACACAGAGC	1st internal nested primer
	Asr6_F	CAGTGGCTGCAGTTGGATCTGG	2nd internal nested primer
Amplification of complete genomic DNA	Asr7_F	ACACAACCTCCTTTAGACCTCC	Primers designed for amplification of 5′ half of the genomic DNA including 5′ UTR
	Asr8_R	ATACAATAATATATTATTGATCATATTGC	
	Asr9_F	CTCACAAGTGTACGGTAGTGGTGG	Overlapping primers designed for amplification of 3′ half of the genomic DNA including 3′ UTR
	Asr10_R	GTGCTTCTTTCCATGAGACTCC	
RT-qPCR analysis	Asr11_F	ACACAGAGCATAGTCACAGGCACA	Gene specific primers designed from Exon 2
	Asr12_R	AGGTGATGGTGGTGCTTCTTTCCA	
	Zjef 1_F	GCTGACTGTGCTGTTCTCATC	Primers designed from *Ziziphus jujuba* elongation factor gene used as a reference gene
	Zjef 2_R	GACACCAAGAGTGAAAGCGAG	
Cloning in expression vector	Asr13_F	CGGGATCCATGGCTGAAGAGAAGCACCATCACC	Gene specific forward primer with *Bam*HI site. Underline indicate the restriction site of restriction enzyme
	Asr14_R	CGAGCTCTTAGAAAAGGTGATGGTGGTGCTTCTTTCCATG	Gene specific reverse primer with *Sac*I site. Underline indicate the restriction site of restriction enzyme

samples from each treatment. Melting curves were analyzed to confirm the specificity of the reactions. Ct (threshold cycles) value were calculated by the $2^{-\Delta\Delta CT}$ method [28]. Ct values from three replicates were averaged and normalized with the Ct values of the internal control *Zjef1*, and standard deviations and errors were calculated.

Expression of recombinant *pET28a-ZnAsr1* in *E. coli*

A pair of primers, Asr13 and Asr14 with *Bam*HI and *Sac*I sites, respectively, were designed to amplify the complete ORF of *ZnAsr1* (Table 2). Template cDNA was prepared as described above and the amplification conditions were set as: 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplified product (822 bp) was digested with *Bam*HI and *Sac*I, purified and ligated to *Bam*HI and *Sac*I pre-digested expression vector *pET28a* (Novagen, USA). The recombinant clones containing *pET28a-ZnAsr1* plasmid were identified by restriction digestion followed by sequencing both the strands of plasmid to confirm the correct orientation of the ORF. The recombinant *pET28a-ZnAsr1* plasmid was consequently transformed into the expression host, *E. coli* strain BL21 (DE3) (Novagen, USA).

A single transformed colony of *E. coli* strain BL21 harboring *pET28a-ZnAsr1* was inoculated into LB broth amended with kanamycin at 50 μg mL^{-1} and incubated at 37 °C with shaking at 200 rpm for 16 h. An aliquot from the primary culture was transferred to fresh LB and allowed to grow until the OD_{600} reached 0.6. The expression of recombinant *pET28a-ZnAsr1* was induced by the addition of IPTG (isopropyl-β-D-thiogalactoside) at 1 mM concentration. Aliquots of 2 mL each were collected, before adding IPTG and at 3, 6 and 24 h post induction. Total protein was extracted and the over-expression of the recombinant protein was analysed by resolving on 12 % SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250 to ascertain the molecular weight of the expressed protein.

Study of *E. coli* cells with *ZnAsr1* gene under osmotic stress by liquid culture assay

To compare the response of recombinant *E. coli* BL21 (DE3) cells containing *pET28a-ZnAsr1* and those containing *pET28a* only under osmotic stress, a liquid assay was performed. Both the types of recombinant *E. coli* BL21 (DE3) cells were cultured in LB as mentioned above. After 12 h of IPTG induction, equal number of cells from each culture

were transferred to 20 mL of fresh LB media containing kanamycin at 50 μg mL^{-1} followed by the addition of 10 % PEG 6000 to induce drought stress. The suspensions were allowed to grow at 37 °C with continuous shaking at 200 rpm. Aliquots of the bacterial suspension were collected at different time intervals (2, 4, 8 and 12 h after addition of PEG 6000) and the optical density (OD) was calculated spectrophotometrically. Cell numbers were calculated using the *E. coli* OD_{600} calculator (http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp).

Results

Molecular characterization and *in silico* sequence analysis of *ZnAsr1*

The complete cDNA sequence of the *ZnAsr1* gene obtained by 5′ RACE and 3′ RACE was 1203 bp long including 122 bp upstream and 259 bp downstream untranslated regions, and a poly (A) tail. Two polyadenylation signals (AATAA) were identified in *ZnAsr1* transcripts positioned 87 and 210 bp downstream of the stop codon (Fig. 1a, b). The complete sequence of the *ZnAsr1* ORF is available at GenBank (Accession no. KC816463).

The open reading frame of *ZnAsr1* is 819 bp long encoding a protein of 273 amino acids. The ProtParam calculated molecular weight of this protein was 27.88 kDa with a theoretical pI of 6.13. Glycine was the most abundant amino acid in the ZnASR1 protein sequence (encompassing 30 % of the total number of amino acids), followed by glutamic acid (9.5 %) and histidine (8.4 %). Furthermore, the ZnASR1 sequence carried 38 negatively charged (aspartic acid and glutamic acid) and 28 positively charged (arginine and lysine) residues. The predicted number of atoms of ZnASR1 was 3696 with chemical formula of $C_{1167}H_{1724}N_{386}O_{418}S_1$. The instability index (II) of ZnASR was checked by ProtParam, which classified the protein as stable with a II score of 19.72 (<40). The negative value (−1.322) of the GRAVY indicated that the ZnASR1 protein was hydrophilic.

The N terminus of ASR proteins is rich in histidine residues in all aligned ASR sequences from different species except in *Citrus maxima*. Using motif search server, we found an ABA_WDS (from amino acids 185–262) functional domain. The occurrence of a putative nuclear signal RKEAKEQDEESYGKKHHHH at the C terminus of ZnASR1 indicated that it is a nuclear protein.

Phylogenetic analysis

The sequence identity matrix of ZnASR1 based on nucleotide and deduced amino acid sequences were studied (Fig. 2). *ZnAsr1* shared maximum identity 45 and 41 % with the *Asr* gene of *Glycine max* (Accession no. AK244579) and *Prunus mume* (Accession no. AB434496), based on nucleotide sequences and amino acid sequences, respectively; the shared minimum identity was 24 % with the *Asr1* gene of *Solanum lycopersicum Asr1* (Accession no. AAB64185), based on nucleotide sequence (Table 1). However, ZnASR1 showed only 21 % homology with the amino acid sequence of *C. maxima* (Accession no. U18972). An unrooted phylogenetic tree was constructed to study the evolutionary relationship of ZnASR1 with the known ASRs of other plant species. In the phylogenetic tree constructed based on the nucleotide sequences, *ZnAsr1* grouped with the *Asr*'s of *G. max* and *Prunus* species (Fig. 3a); while as, in the phylogenetic tree constructed from the deduced amino acid sequences, ZnASR1 clustered with the ASRs of *C. maxima* and *Prunus* species (Fig. 3b).

Full length amplification of *ZnAsr1* from genomic DNA

A pair of primers (Asr9 and Asr10) was designed to amplify the full length genomic DNA of the *Asr1* gene in *Z. nummularia*. The primers were designed respectively, from the 5′ and 3′ untranslated regions (UTRs) of the *ZnAsr1* cDNA sequence. PCR was performed using genomic DNA as template and a 1367 bp long fragment was obtained. The genomic analysis revealed that the fragment encompassing 822 bp of cDNA sequence composed of two exons (E1 and E2 of 636 bp and 186 bp, respectively) interrupted with one intron (300 bp) (Fig. 1a). A High AT contents (A 34 % and T 48.3 %) and splicing sites, 5′ GT and AG 3′, indicate that the *ZnAsr1* intron has the typical structural characteristics of a plant intron.

Expression patterns of *ZnAsr1* under drought stress

The time dependent expression patterns of *ZnAsr1* in response to PEG treatment were analyzed using RT-qPCR. Seedlings after treatment with PEG responded strongly, showing 7.8-fold increase in *ZnAsr1* transcripts at 6 h. The expression declined (3.57-fold) abruptly at 12 h followed by a gradual decrease (2.13-fold) for up to 72 h post-treatment (Fig. 4). The RT-qPCR results indicated that *ZnAsr1* showed differential pattern of expression with time to the PEG induced drought stress.

Bacterial expression of *pET28a-ZnAsr1* in *E. coli*

ZnASR1 protein was successfully induced at 3 h of IPTG treatment and expressed with an N-terminal histidine-tag in recombinant *E. coli* BL-21. Based on the SDS-PAGE

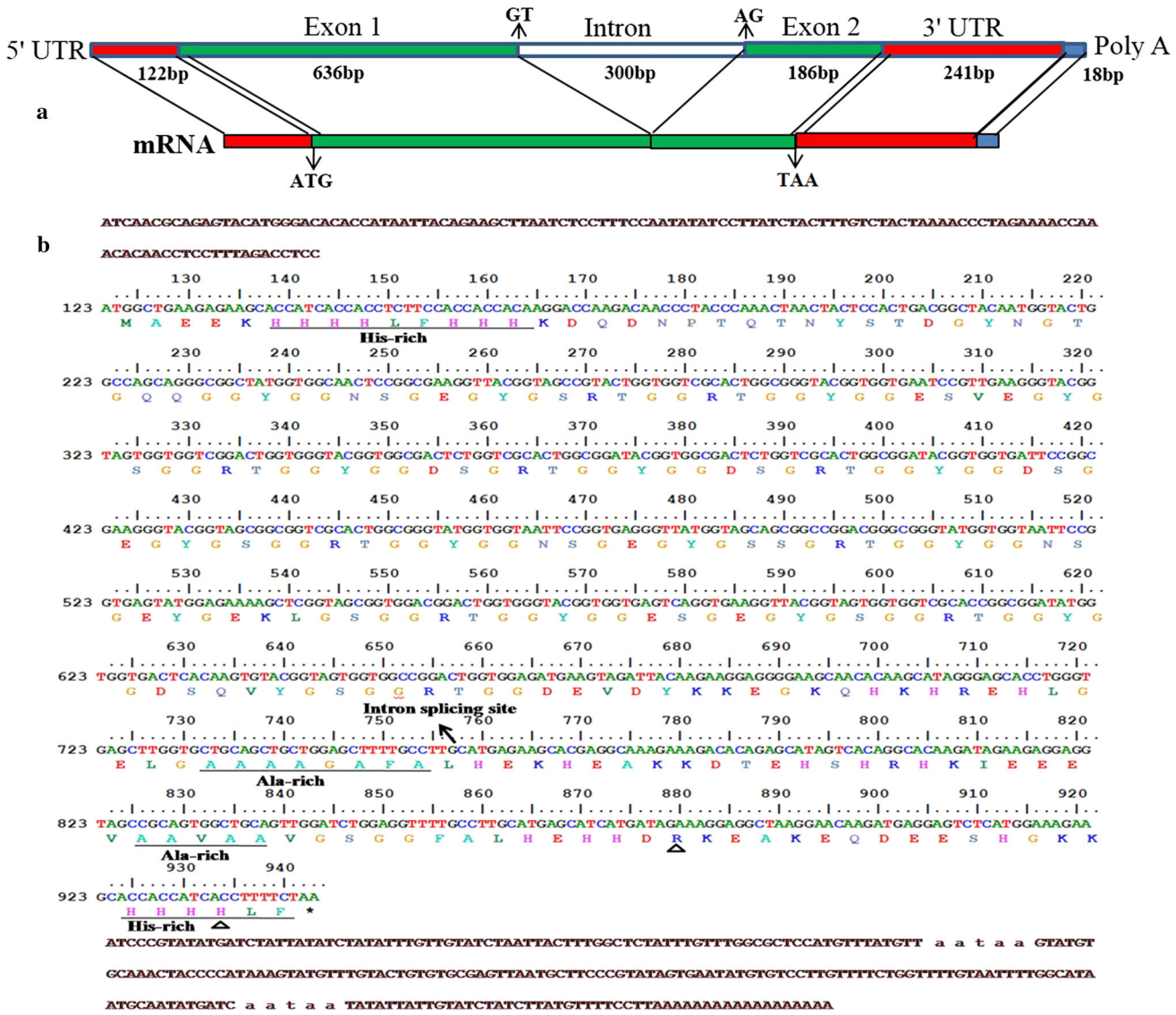

Fig. 1 **a** Schematic representation of genomic DNA and mRNA structure of *ZnAsr1*. Genomic DNA contains two exons that are separated at a 5′ GT site and 3′ AG site by one small intron (300 bp). **b** The full length cDNA sequence (including 5′ UTR, 3′ UTR and poly A tail) and deduced amino acid sequences of *ZnAsr1*. The possible nuclear localization signal is marked by 'Δ···Δ'. C terminus and N terminus histidine (His) rich and two alanine (Ala) rich regions are marked by an underline. Two putative polyadenylation signals AATAA are marked by lower case letters. The arrow indicates the intron splicing site in mRNA sequences

analysis, the molecular mass of ZnASR1 fusion protein was observed to be 32 kDa; comprising of 27.8 kDa of ZnASR1 protein fused with the 4.5 kDa N terminal His tag sequence of *pET28a* vector. It was observed that protein of interest continued to accumulate for up to 24 h post IPTG induction (Fig. 5a).

Overexpression of *ZnAsr1* in *E. coli* enhances growth during drought stress

The ability of the recombinant and the control cells of *E.coli* to multiply in 10 % PEG6000 amended LB medium was studied by growing them under IPTG induction conditions for 12 h. There was least if any difference in the cell densities of the recombinant and the control *E.coli* cells in LB only medium; whereas, the recombinants cells showed significant increase in cell density compared to the control cells in LB supplemented with 10 % PEG6000 (Fig. 5b). Up to 4 h of inoculation, both the recombinant and the control cell growth was exponential; however, cell density of the recombinant cells compared to that of the control cells improved significantly after 6 h and continued to increase further till 8 h of inoculation. The distinct difference in the cell

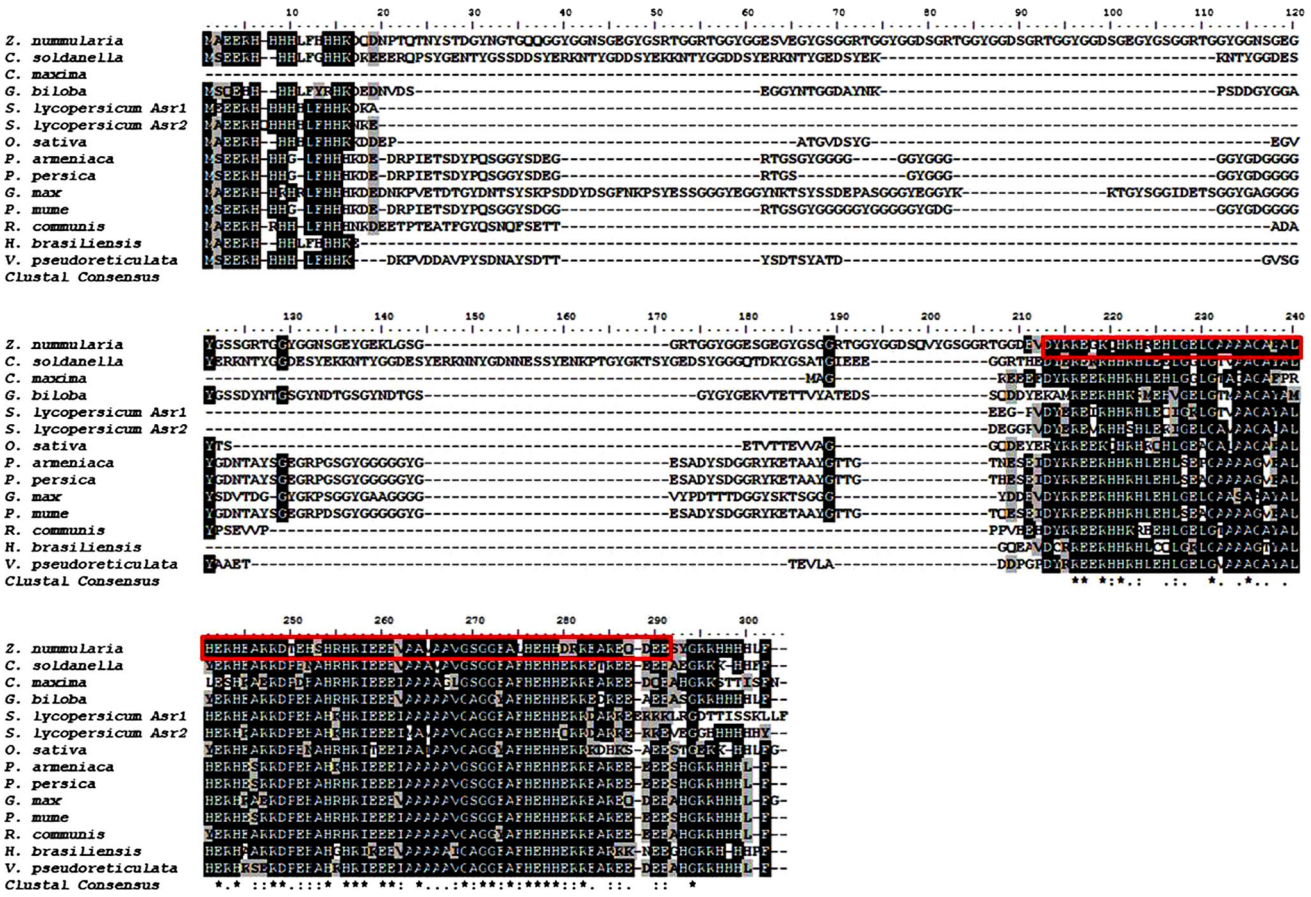

Fig. 2 Multiple alignment of the deduced amino acid sequence of ZnASR1 with ASRs from other plant species using CLUSTAL W. Similar and identical amino acid residues were shaded in *gray* and *black*, respectively. The conserve residues are indicated by *hash*. The ABA/WDS motif is marked by a *rectangular box*. *Lower case letters* in *italics* indicate the amino acid residues in unshared motifs of ZnASR1

densities of the recombinant and the control cells suggests that *ZnAsr1* imparts tolerance to drought stress in the recombinant *E. coli*.

Discussion

After the first report of the *Asr* gene in commercial tomatoes [29], more than 30 ASR orthologs have been characterized in different plants across the kingdom plantae [30], including Strawberry (*Fragaria* × *ananassa*) [31], *Ginkgo biloba* [32], tomato [29], grape [3], lily [33], rice [34], banana [35], potato [36], maize [6], *C. maxima* [37] and *Calystegia soldanella* [38]. However, *Arabidopsis*, a model plant for studying biological function in plants, surprisingly lacks a homolog to *Asr* [39]. To date, no ASR proteins have been reported in *Ziziphus* spp. In the present study, full length cDNA encoding the ASR protein was characterized for the first time at molecular level and functionally validated in *Z. nummularia*.

The complete CDS of *ZnAsr1* gene was found to be 819 bp long consisting of two exons (E1 and E2) of 636 and 183 bp respectively. The length of both exon 1 and exon 2 is generally conserved across *Asr* gene family (approximately 200 bp), but in case of *ZnAsr1* we obtained exon 1 of 636 bp in length, the longest reported so far (Fig. 1). Longer exons (603, 609 and 594 bp) in case of *Asr3* from *Sorghum bicolor*, *Hordeum vulgare* and *Zea mays* respectively have also been reported earlier [8]. Moreover, there have also been reports of both exon 1 and exon 2 being fused like in case of *Z. mays*, *Asr7-1*, *Asr7-2*, *Asr7-3* and in case of *Sorghum bicolor Asr6* and *Asr7*. Based on the *in silico* analysis, ZnASR1 was predicted to be a highly hydrophilic in nature with its hydrophilicity being ascribed to the high glycine content, the later has a high hydrophilicity index [40]. The sequence of amino acids of ZnASR1 showed a high percentage (29 %) of glycine residues, which is higher than that found in other ASR proteins. ASR has been reported to get accumulated under stress [7], the accumulation may be due to the

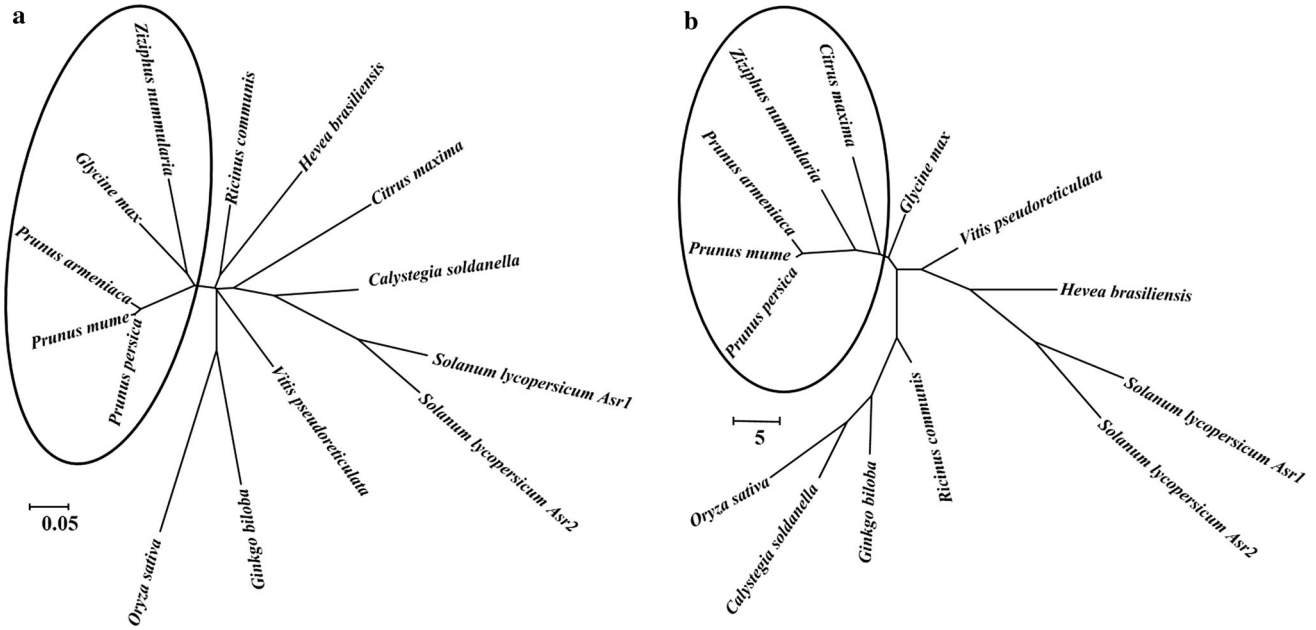

Fig. 3 Maximum likelihood tree showing the phylogenetic relationship of *ZnAsr1* with *Asr* genes from different plant species retrieved from GenBank under the accession no. listed in Table 1, **a** tree based on the nucleotide sequences of the complete CDS . **b** tree based on the translated deduced amino acids by employing program MEGA 6 (bootstrap of 1000). *Scale bar* represents the genetic distance that is proportional to the number of amino acid/nucleotide differences between branch nodes. *Round circle* indicates the cluster where *ZnAsr1* is placed

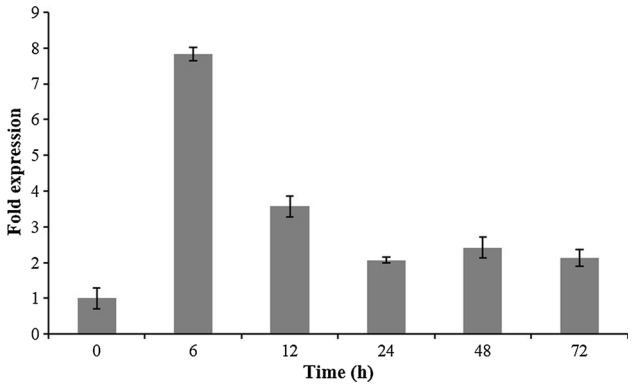

Fig. 4 RT-qPCR analysis of *Asr1* gene expression in *Z. nummularia*. Transcript levels of the *Asr1* gene was determined by RT-qPCR in *Z. nummularia* treated with 30 % of PEG 6000 for 0, 6, 12, 24, 48 and 72 h. Fold change in gene expression and time (in hours) are shown on X and Y axis, respectively. The data were generated from three biologically independent experiments. Standard deviation was calculated and represented as *error bars*

presence of charged amino acids which in turn contribute to the increased hydrophilicity. It can be concluded that the high content of glycine increases the hydrophilic nature of ZnASR1 and increases the accessibility to water molecules during stress conditions. Moreover, the average molecular weight of ASR, owing to their difference in the total number of residues varies from one plant species to another.

RT-qPCR analysis showed upregulation of *ZnAsr1* in response to PEG induced drought stress in the tested seedlings. The maximum induction was observed after 6 h of treatment (Fig. 4), and similar findings were reported in *G. biloba* where transcript levels of *GbAsr* were increased by approximately five-folds compared to the control after 48 h of dehydration stress induced by ABA treatment [32]. In lily, the role of ASR in mediating stress responsive ABA signaling has been reported [40].

It was also reported that the transgenic line *35S::MpAsr* (*Asr* gene transformed from *Musa paradisica*) of *Arabidopsis* in comparison to the wild type exhibited improved tolerance to drought stress [30]. However, the increased tolerance in *Arabidopsis* may be a result of competition between the DNA binding protein (ASR) on the promoter binding site and some endogenous transcription factor like ABI4 [41]. Proteomic experiments on ZnASR1 can lead to knowledge about the actual biological function of ASR under adverse stress conditions and an understanding of the regulatory mechanism in *Z. nummularia* will help in formulating rational strategies to develop crop plants tolerant to adverse environmental conditions by using the *Asr* gene in breeding or genetic engineering.

The nuclear targeting signal identified in mRNA transcript of *ZnAsr1* at the 3′ end indicates that the protein is supposed to be localized in the nucleus. It is also known that the zinc binding site in ZnASR1 consists of a stretch of seven His residues present at the N terminus of the protein.

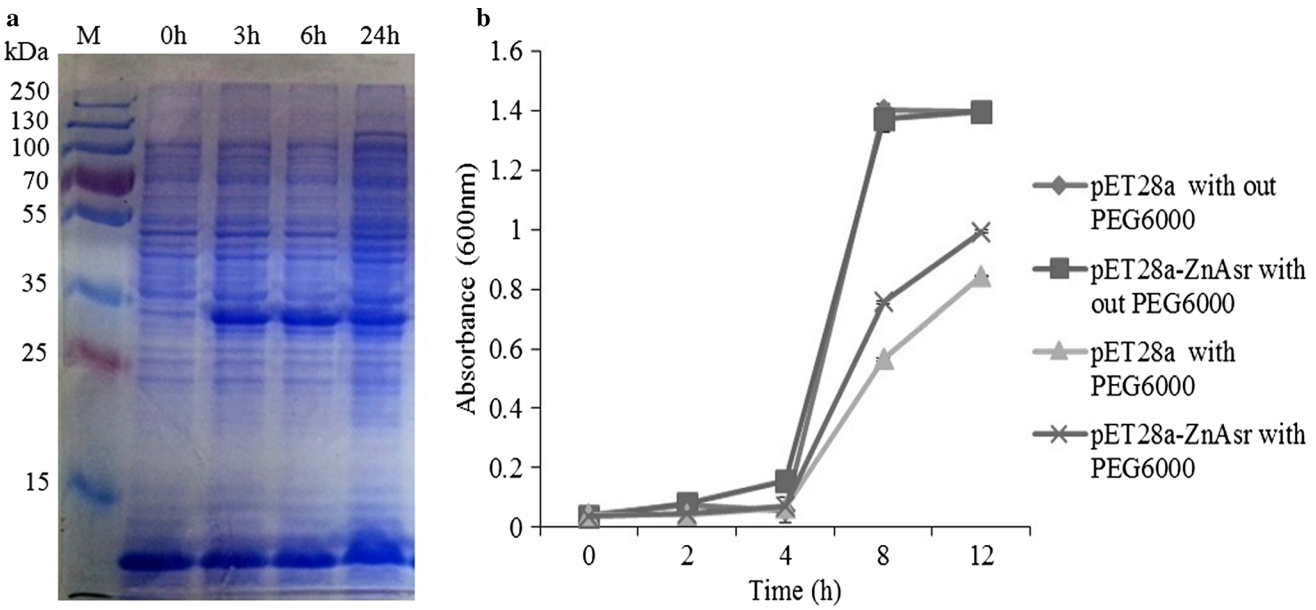

Fig. 5 Expression and accumulation of recombinant ZnASR1 protein in a bacterial system (*E. coli* strain BL21). **a** Coomassie brilliant blue R250 stained SDS/PAGE showed banding patterns of total cellular protein under induced condition at different time intervals after induction along with PageRuler Plus Prestained protein ladder, **b** growth patterns of pET28a and recombinant *pET28a-ZnAsr1* clones in LB medium with and without PEG6000 (10 %)

It is well reported that *Asr1* gene from tomato and rice is natively unordered protein [42] and has chaperone-like activity [30, 43, 44]. *Asr1* gene has Zn^{2+}-dependent DNA-binding activity, becomes structured and dimerized when it binds to Zn^{2+} and is translocated to the nucleus [42, 45, 46], where it binds to the specific promoter sequences and regulates gene expression [3, 39, 47, 48]. The presence of specific ABA/WDS motifs is reported in ASR proteins [38] and in water deficit stress–induced proteins (WDS) [49]. The recombinant ZnASR1 protein imparted tolerance to *E. coli* for better growth under PEG induced drought stress, confirming its role in drought response (Fig. 4). Generally tobacco, *Arabidopsis* and yeast mutants are used as model to carry out the functional validation and characterization of genes from plants, but there are reports of *E. coli* being used as host for functional validation of stress responsive genes from plant origin [50–52]. *E. coli* cells transformed with a dehydration-responsive element binding (DREB) transcription factor isolated from *Salicornia brachiata* (*SbDREB2A*) showed improved growth under drought and excess salt stresses [51]. The group 3 *LEA* protein PM2 from soybean imparted recombinant *E. coli* with improved tolerance to salt stress [53]. NAC4 protein from horse gram (*Macrotyloma uniflorum*) showed tolerance against salt (6 % NaCl), heavy metal (100 mM $CuSO_4$) and water stresses (6 % PEG) in *E. coli* cells [54]. The phenomenon of imparting stress tolerance in both prokaryotic and eukaryotic system may be due to a common protective mechanism acting in both prokaryotes and eukaryotes [40, 55, 56].

In many plant species, ASR proteins are localized in the nucleus where they act as transcription factors and regulate specific promoters [3, 39, 57] of hexose transporters and ABA responsive genes. Abscisic acid is a plant hormone which often interacts with various transcription factor commonly involved in signaling of drought stress and its levels in a cell are often linked to the plant tissues experiencing drought stress [30]. Drought tolerance mechanism in plants may [20] or may not be [30] controlled by an ABA responsive pathway. Furthermore, ABA regulates many *Asr* and other genes involved in sugar transport, plant development, other environmental stresses and seed maturation [58]. It also moderates the effect of various abiotic stresses by stimulating a range of stress-responsive genes, the latter are involved in the production of various osmolytes and LEA-like proteins, which ultimately lead to improvement in the plant's tolerance to stress [1, 59, 60].

Conclusion

In the present study, full-length cDNA of *Asr1* gene from *Z. nummularia* was isolated for the first time and its expression was studied. The up regulated transcript profile of *ZnAsr1* in *Z. nummularia* seedlings at 30 % PEG 6000 induced stress and over expression of recombinant ZnASR

protein in *E. coli* cells grown in LB medium containing 10% PEG6000, confirmed the role of *Asr1* gene under drought conditions. As *Z. nummularia* is naturally tolerant to drought stress and hence a putative reservoir of drought responsive genes, isolation and characterization of a full length CDS of *Asr1* in *Z. nummularia* is very useful for further studies on abiotic stress tolerance in the plant system. Moreover, advanced studies are needed to identify the molecular interaction mechanism of *Asr1* in drought tolerance in *Z. nummularia*.

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