

Ectopic expression of *CisAF7*, an Alfin1-like gene from *Citrus sinensis*, confers tolerance to several abiotic stresses in *Escherichia coli*

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Abstract

In order to identify the function of a sweet orange (*Citrus sinensis* [L.] Osbeck cv. Valencia) Alfin1-like (AL) gene, *CisAL7*, *in vitro* functional analyses were performed using a prokaryotic heterologous expression system (*Escherichia coli*). *CisAL7* was cloned and expressed in a pET28a(+) system. *E. coli* cells containing the recombinant plasmid or empty vector as a control were treated by multiple stresses. In LB (Luria-Bertani) solid media, *Escherichia coli* harboring *CisAL7* gene was remarkably more tolerant to stresses, including high salinity (0.6 M NaCl or 0.6 M KCl), high temperature (50°C), and low temperature (4°C), than control cells. These results indicate that *CisAL7* protein may play a positive role in responsive to abiotic stresses above. This provides the first experimental evidence that AL7 enhances abiotic tolerance of *E. coli* cells.

Keywords: *CisAL7*; Abiotic stress; *Citrus sinensis*; Alfin1-like protein; *E. coli*.

Abbreviations: AL_Alfin1-like; *C. sinensis* Annotation Project_CAP; ORF_open reading frame; NLS_nuclear localization signal; TF_transcription factor.

Introduction

Fruit trees are constantly subjected to variable abiotic stresses. Among them, high salinity and extreme temperatures are the major environmental factors to constrain plant growth and development (Krasensky and Jonak, 2012; Mahajan and Tuteja, 2005). To successfully survive these stresses, plants regulate the expression of genes responsible for synthesis of effector proteins and metabolites which partake in stress responsive and tolerance (Shekhawat and Ganapathi, 2013; Shinozaki and Yamaguchi-Shinozaki, 2000). Transcription factors (TFs) are important regulators of gene expression under stress conditions, and act to control the expression of a broad range of target genes by binding to specific *cis*-acting element in their promoters (Nakashima et al., 2009). Characterization of genes involved in synthesis of TFs is important because they influence a variety of environmental stress-related genes. The AL protein (*MsAlfin-1*) was first discovered to be a TF in alfalfa (*Medicago sativa*) (Winicov, 1993). After that a number of AL proteins have been identified throughout eukaryotic and prokaryotic kingdom. The members of this family conserved in N-termini DUF3594 domain and C-termini PHD-finger motif with approximately 130 and 50 amino acid residues, respectively (Song et al., 2013). The alfalfa root-specific *MsAlfin-1*, a PHD finger protein, was identified as salt-induced TFs and enhanced the stress tolerance by ectopic expression in transgenic plants (Winicov, 2000). *MsAlfin-1* could act as a regulatory factor *in vitro*, both calli and transgenic plants overexpressing *MsAlfin-1* grew better in the presence of increased levels of NaCl than parent wild-type or

vector-transformed controls whereas calli expressing *MsAlfin-1* in the antisense orientation were more sensitive to salt, demonstrating that *MsAlfin-1* functions in salt tolerance in alfalfa (Winicov, 2000; Winicov and Bastola, 1999). Although the exact role of these AL genes in plants is still being elucidated, recently, the AL genes have been found in many other land plants, such as *Arabidopsis* (Lee et al., 2009), rice (Xiong et al., 2005), *Solanum tuberosum* (Liu and Wang, 2007), *Populus euphratica* (Wang et al., 2005) and *Glycine max* (Wei et al., 2009). Additionally, functional analysis using both T-DNA insertion mutants and overexpression lines revealed that *AtAL7* functions as a negative role in salt stress tolerance of *Arabidopsis*, suggesting the presence of adaptive evolution among AL gene family members (Song et al., 2013). Citrus, which ranks as the world's most widely cultivated fruit crop (Wang et al., 2014), is listed among the world's healthiest food commodities (<http://www.whfoods.com/genpage.php?tname=foodspice&dbid=37>). In comparison with rice and *Arabidopsis*, the research on citrus stress response pathways, apart from a few recent studies, has historically not been commensurate with its inclusion among the most important fruit products of the world (Wang et al., 2014). Extremes of temperature and high salinity are the major abiotic environmental factors affecting citrus plant growth and productivity. Hence investigations into its stress response pathways in citrus plant are urgently warranted. Sweet orange (*C. sinensis*) was selected as a model fruit tree for genome sequencing recently (Xu et al., 2013). This provides a great deal of information to carry on researches

on gene families from whole genome view. We identified several DUF3594 domain and PHD-finger motif containing putative AL sequences in citrus database available with *C. sinensis* Annotation Project (CAP) (<http://citrus.hzau.edu.cn/orange>) (data not shown). One such AL gene *Cs3g01400* was found to be similar to *AtAL7* and *MsAlfin-1*. Owing to its similarity to *AtAL7*, we hereafter named it *CisAL7*. As described above, *AtAL7* and *MsAlfin-1* plays a negative and positive role in salt stress tolerance, respectively. Thus, it is worthy of investigations into the possible roles (i.e., negative or positive) played by *CisAL7* in the abiotic stresses response pathways in citrus plant. Until now, to our knowledge, experimental evidence regarding *in vitro* functional studies of AL proteins in *Escherichia coli* (*E. coli*) has not been reported.

To answer this question, in this study, a novel abiotic stress response AL gene was cloned from sweet orange (*C. sinensis* [L.] Osbeck cv. Valencia) and then transformed into *E. coli*. We found that overexpression of *CisAL7* gene enhances heat, cold and salt tolerance of *E. coli* cells. The *in vitro* functional validation in *E. coli* indicates that this gene is a potential candidate for resistance to salinity and extreme temperature in plants. To our knowledge, this is the first report of AL7 from fruit trees involved in abiotic stress response in *E. coli*. Studies on AL7 provide significant knowledge to reveal the function of ALs in plants.

Results

Clone and sequence analysis of *CisAL7* ORF

Firstly, we successfully amplified the complete open reading frame (ORF) by reverse transcription polymerase chain reaction (RT-PCR) from sweet orange (*C. sinensis* [L.] Osbeck cv. Valencia) total RNA using specific primers. The complete ORF was 756 bp in length, which encoded a residue polypeptide with a calculated molecular weight of *c.* 28.1 kDa and isoelectric point (pI) of 5.12. Additionally, the *CisAL7* (*Cs3g01400*) C-terminus contained a basic acid region (KRAR) which played a role as a nuclear localization signal (NLS) (Chelsky et al., 1989) (Fig. 1).

Identification of functional *cis*-acting elements in a promoter is a crucial step toward understanding the gene function (Ibraheem et al., 2010). We made use of the Plant-CARE software online and predicted the putative *cis*-acting regulatory elements of *CisAL7* promoter with 1500 bp upstream of translation start site ATG. The results showed that promoter sequence contains various putative stress response-related *cis*-acting elements such as Box-W1, GC-motif, TC-rich repeats, MBS, TC-rich repeats, W box, etc. (Table 1). For example, two Box-W1 motifs were identified and have been shown to be fungal elicitor responsive elements. Three CGTCA-motifs and three TGACG-motifs were found, which are involved in the MeJA-responsiveness. GC-motif is an enhancer-like element involved in anoxic specific inducibility. Two GT1-motifs were identified as light responsive elements. One MBS and three TC-rich repeats were previously identified as MYB binding site involved in drought-inducibility and *cis*-acting element involved in defense and stress responsiveness, respectively. One TCA-element and two W boxes were found, which are involved in salicylic acid responsiveness and direct fungal elicitor stimulated transcription of defense genes and activation of genes involved in response to wounding, respectively. Such an enriched presence of stress response-related *cis*-acting elements may suggest a critical role of *CisAL7* in stress response.

Amino acid sequence analysis and alignment

By query GenBank/EMBL data libraries, we found 17 *CisAL7* homologous proteins. Fig. 2 shows the alignment result of the deduced amino acid sequences of ALs from *Arabidopsis thaliana*, *Oryza sativa* and *Medicago sativa*. The sequences of ALs are highly conserved among all the species tested. As shown in Fig. 2A, structural analysis of the *CisAL7* protein demonstrated that it possesses most of the characteristics of AL protein family. These include the conserved DUF3594 domain and PHD-finger (Song et al., 2013). Further analysis of the phylogenetic relationship between *CisAL7* and some AL family members from different species revealed that 3 AL proteins and *CisAL7* were significantly clustered together on a single branch of the tree; in particular, *CisAL7* had a high sequence identity to the reported *AtAL7* (Song et al., 2013) (86%) and *MsAlfin-1* (Winicov, 1993; Winicov, 2000) (83%) (Fig. 2B). These features suggest that the *CisAL7* protein belongs to AL protein family, which may have similar functions in different species.

Expression of *CisAL7* fusion protein in recombinant *E. coli* and Western blot analysis

CisAL7 was expressed in *E. coli* and confirmed by SDS-PAGE. Expression cassette of pET28a-*CisAL7* was transformed into *E. coli* strain BL21 (DE3). We extracted the total protein of transformants after induction and then carried out SDS-PAGE. As shown in Fig. 3, the specific band of *c.* 31.6 kDa was detected in the total protein of pET28a-*CisAL7* transformed *E. coli* on Coomassie blue-stained gels (Fig. 3A). No signal was either detected in the empty plasmid (pET-28a) transformed *E. coli* BL21 (DE3) strains and pET28a-*CisAL7* transformants without isopropylthio- β -D-galactoside (IPTG) been induced. These results showed the efficient expression of the *CisAL7* fusion protein in *E. coli*. Western blot using mouse anti-His-tag monoclonal antibody also proved the reactivity of the fusion protein as shown in Fig. 3B.

Overexpressed *CisAL7* in *E. coli* enhanced resistance to abiotic stresses

To ascertain the stress-resistant function of *CisAL7*, survival on solid medium was tested under a variety of stress conditions. Cultures of *CisAL7* or empty vector transformed *E. coli* were spotted on LB plates for heat shock (50°C), chilling (4°C), 0.6 M NaCl and 0.6 M KCl treatments. The quantity of the *E. coli* cell carrying *CisAL7* gene was approximately the same as that of the control empty vector under non-stress conditions as shown in Fig. 4. In contrast, in the presence of heat shock, chilling, NaCl and KCl at 1 to 10⁻³ dilutions, the quantity of the cell carrying *CisAL7* gene was significantly more than that of empty vector (Fig. 4). The results above revealed that *CisAL7* confers strong stress-tolerance to *E. coli* cell against abiotic stresses. Meanwhile, it is proved that the *E. coli* expression system is a simple and convenient method to identify the functions of some stress-tolerant genes from plants.

Discussion

Plants have a series of fine mechanisms for responding to environmental changes, these mechanisms are involved in many aspects of physiology, biochemistry, genetics, development, and molecular biology, in which the adaptive machinery related to molecular biology is the most important (Shao et al., 2007). AL proteins were identified as a salt-induc-

Table 1. Distribution and sequences of stress-related *cis*-acting regulatory elements in the 1.5 kb upstream promoter region of *CisAL7*.

Site name	Position	Strand	Core sequence (5'→3')
Box-W1	-1154 to -1159	+	TTGACC
	-1197 to -1202	-	TTGACC
CGTCA-motif	-107 to -111	+	CGTCA
	-549 to -553	+	CGTCA
	-1236 to -1240	+	CGTCA
GC-motif	-536 to -541	+	CCCCCG
GT1-motif	-1162 to -1170	+	GTGTGTGAA
	-1373 to -1378	+	GGTTAA
MBS	-122 to -127	+	TAACTG
TC-rich repeats	-334 to -343	+	ATTCTCTAAC
	-367 to -376	+	ATTTTCTCCA
	-1058 to -1067	+	ATTTTCTTCA
TCA-element	-92 to -101	-	CAGAAAAGGA
TGACG-motif	-107 to -111	-	TGACG
	-549 to -553	-	TGACG
	-1236 to -1240	-	TGACG
W box	-1154 to -1159	+	TTGACC
	-1197 to -1202	-	TTGACC

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1   ATGGAAGCGATACCGCACCCCTATACCTAGAAGCTGTAGAGGAGGTTTTCAGCGATTTTAAG
   M E A I P H P I P R T V E E V F S D F K
61  GGCAGACGGGCTGGCTTGATTAAGGCTTTAACAGCTGATGTTGAGAAGTTCTACCAGCAG
   G R R A G L I K A L T A D V E K F Y Q Q
121 TGCGATCTGAAAAAGAGAAGCTGTGCCTATACGGGCTTCTAATGAGACATGGGAAGTT
   C D P E K E N L C L Y G L P N E T W E V
181 AATCTGCTGTGAGGAGGTGCCTCCGAGCTCCTGAGCCAGCATTAGGTATAAACTTT
   N L P V E E V P P E L P E P A L G I N F
241 GCTAGAGATGGAATGCAGGAGAAGGACTGGTTATCACTGGTTGCAGTGACAGTGATTC
   A R D G M Q E K D W L S L V A V H S D S
301 TGTTGCTTGCTGTAGCTTTTATTTTGGTGCACGCTTGGATTGGCAAAAATGAAAGG
   W L L A V A F Y F G A R F G F G K N E R
361 AAGAACTTTTCCAGATGATAAATGATCTCCAACCATCTTTGAAGTTGAACTGGAAT
   K K L F Q M I N D L P T I F E V V T G N
421 GCTAAGCAACCAAGGACCTATCTGCTAATCACAATAGCAGCAAAAAGCAAATCGAGTGGA
   A K Q P K D L S A N H N S S K S K S S G
481 AAGTCTCGTCCAGCTGAATCACAACCAAGCAGTGAAGATGTCTCCACCACCGAAGGAC
   K S R P A E S Q T K A V K M S P P P K D
541 GATGATGATAGCGGTGATGAAGAAGGGAAGATGATGAACAGGGTGCCACCTGTGGAGCA
   D D D S G D E E E E D D E Q G A T C G A
601 TGCGGGATAACTATGGCACTGACGAATTCGGATCTGCTGCGATATCTGTGAGAAATGG
   C G D N Y G T D E F W I C C D I C E K W
661 TTCAACGGCAAATGTGTAAGATTACACCTGCGAAGGCCGACACATCAAGCAGTACAAG
   F H G K C V K I T P A K A E H I K Q Y K
721 TGCCCCAGCTGCAGTAGCAAGAGGCTAGAGTTTAA
   C P S C S S K R A R V *

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Fig. 1. Nucleotide and deduced amino acid sequence of the *CisAL7* ORF. The shaded area is forecast a nuclear localization signal (NLS). The stop codon (TAA) is indicated with an asterisk.

ced TF and enhanced the stress tolerance by ectopic expression protein from sweet orange (*C. sinensis*), subsequently, we analyzed its promoter region and many stress-related *cis*-acting elements were found. Therefore, we speculated that the gene might be activated by a variety of stresses. The resistance experiments show that the over-expression the *CisAL7* protein enhanced the tolerance of *E. coli* recombinants to diverse stresses: high-temperature, low-temperature, and high-salinity stress. Previous studies have also suggested that part of AL proteins are stress-protective (Wei et al., 2009; Winicov, 2000; Winicov and Bastola, 1999). The results of the present study, obtained using the *E. coli* expression system, also demonstrate a positive effect of *CisAL7* protein on growth in solid media

in transgenic plants. Here, we isolated a putative AL containing high salt and extreme temperature stresses. On the contrast, previous functional studies based on both T-DNA insertion mutants and overexpression lines indicated that salt-stress-induced *AtAL7* functions as a negative role in salt tolerance of *A. thaliana* (Song et al., 2013). These discrepancies between studies may be due to adaptive evolution occurred in the members of *AL* gene family or for other reasons that require further investigation. The salt tolerance recombinant bacteria show that the *CisAL7* gene may be involved in the function of the protective protein to avoid damage in the host cells. This may be due to having the common protective mechanisms in prokaryotes and eukaryotes

A

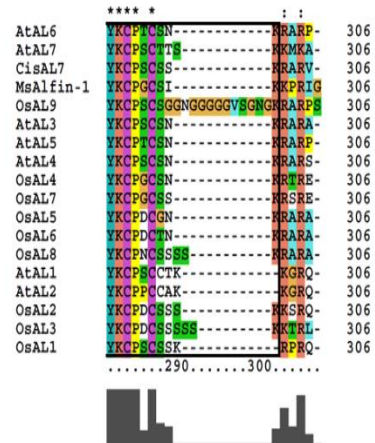
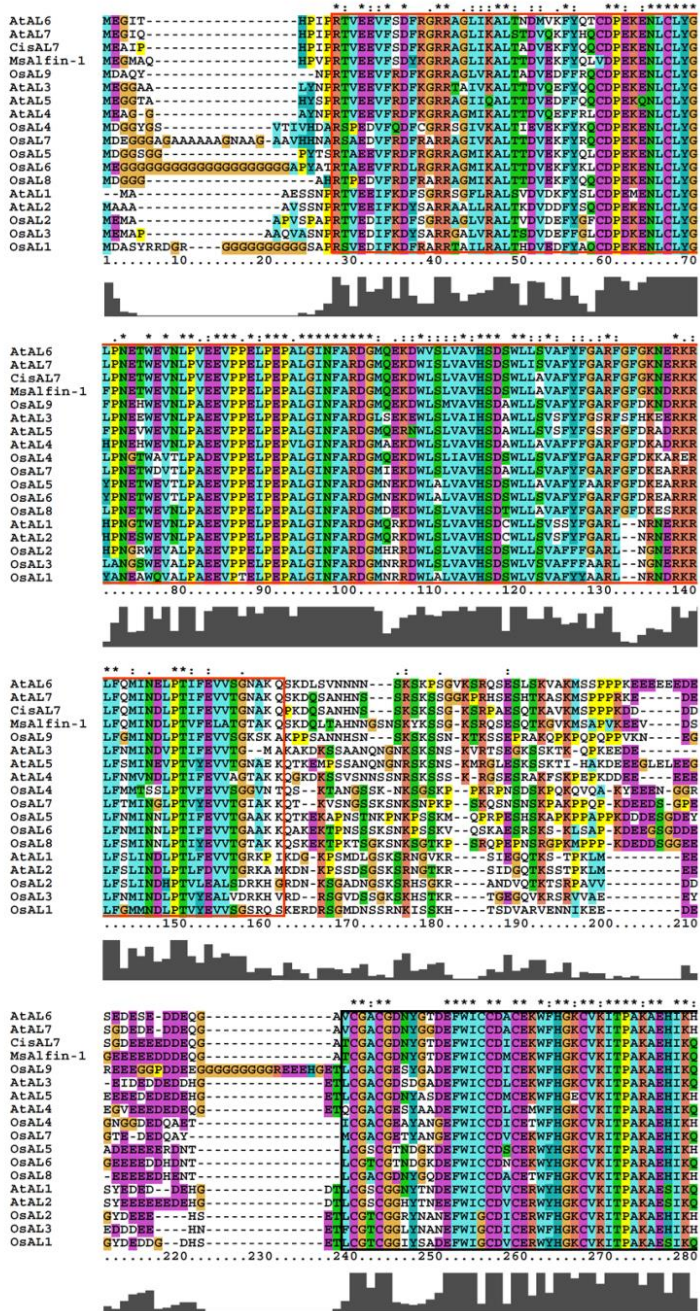


Fig 2. Multiple sequence alignment and phylogenetic tree analysis of amino acid sequences of CisAL7 with the other 17 AL proteins. (A) Multiple sequence alignment of amino acid sequences of CisAL7 with the other AL proteins. The DUF3594 domain and PHD-finger are indicated by red and black boxed letters. (B) The phylogenetic tree of plant AL proteins. An un-rooted phylogenetic tree was generated by Neighbor-Joining (NJ) method with MEGA 6.06 based on the predicted amino acid sequences of the putative CisAL7 and their homologs in other species. The branch lengths are proportional to divergence, with the scale of '0.05' representing 5% change. Sequence data can be found in the GenBank/EMBL data libraries under the following accession numbers: MsAlfin-1, AAA20093; AtAL1, At5g05610; AtAL2, At3g11200; AtAL3, At3g42790; AtAL4, At5g26210; AtAL5, At5g20510; AtAL6, At2g02470; AtAL7, At1g14510; OsAL1, LOC_Os05g07040; OsAL2, LOC_Os07g12910; OsAL3, LOC_Os03g60390; OsAL4, LOC_Os04g36730; OsAL5, LOC_Os05g34640; OsAL6, LOC_Os01g66420; OsAL7, LOC_Os02g35600; OsAL8, LOC_Os11g14010; OsAL9, LOC_Os07g41740.

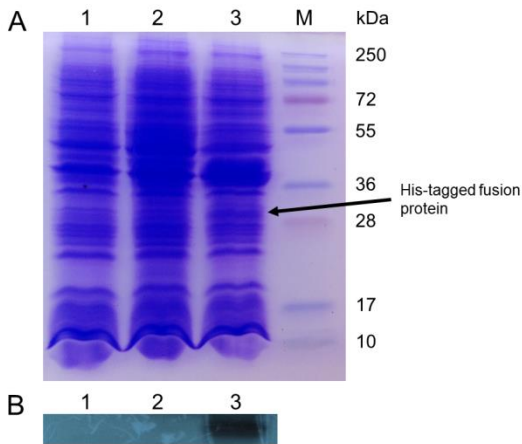


Fig 3. Overexpression of the *CisAL7* gene in *E. coli* BL21 (DE3) and detection of His-tagged fusion protein by Western blotting. (A) SDS-PAGE analysis of expressed product for pET28a-CisAL7 in *E. coli* BL21 (DE3). M: molecular-mass markers; Lane 1: expression of pET-28a induced after 2 hours; Lanes 2-3: expression of pET28a-CisAL7 induced after 0 and 2 hours, respectively. The arrow indicates expressed product of pET28a-CisAL7, the expected molecular weight of the fusion protein is about 31.6 KDa. (B) Western blot analysis of CisAL7 protein using anti-His-tag monoclonal antibody. Lanes 1-3 are same as lines in (A) described above.

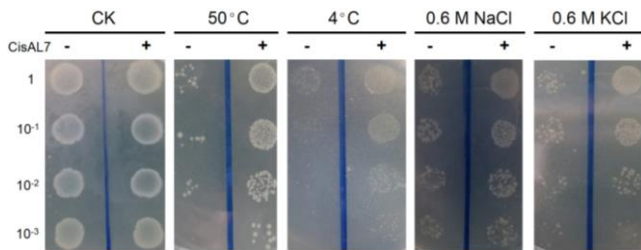


Fig 4. Survival test of *E. coli* cell carrying CisAL7 (+) or empty vector (-) on various stress conditions. 10 µL cultures from 1 to 10⁻³ dilutions were spotted on LB basal plates (CK) and plates treated with 50°C, 4°C, 0.6 M NaCl or 0.6 M KCl

contribute to the increase of abiotic stress tolerance of the bacteria host cells (Du et al., 2014; Lan et al., 2005; Peng et al., 2013; Yadav et al., 2014; Yamada et al., 2002; Yang et al., 2005; Yun and Zheng, 2005; Zhou et al., 2014). In our experiment test, we found that the recombinant CisAL7 protein expressed in *E. coli* could effectively improve the salinity and extreme temperature tolerance of the engineered strain when compared with the control samples. This enhancement of salinity and extreme temperature tolerance further indicates that the expression of CisAL7 in host cells is able to confer their protective function against protein damage, cellular apoptosis, and membrane disruption; its exact function in plants needs to be further examined.

Materials and Methods

Plant material and bacterial strains

Citrus plants, ‘Valencia’ sweet oranges (*C. sinensis* [L.] Osbeck), were grown on the green house bench under natural light conditions at Wuhan Bioengineering Institute in China. *E. coli* strain DH5α (Invitrogen, USA), BL21 (DE3) (Novagen, USA) and plasmid pET28a(+) (Novagen, USA) were kept in the Center of Applied Biotechnology in Wuhan Bioengineering

Institute, China.

Total RNA isolation and cDNA synthesis

Total RNA were extracted from young leaves of ‘Valencia’ sweet orange grown in normal soil with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Subsequently, the RNA was treated with DNaseI (TaKaRa, Dalian, China) to remove the residual genomic DNA. Preferably with 2 µg of RNA synthesized of first strand cDNA using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).

Cloning and sequencing of CisAL7

In order to identify *AL* genes present in the sweet orange genome, BLASTP analysis at CAP using alfalfa *MsAlfin-1*, *Arabidopsis* and rice *ALs* as query sequences was performed. *CisAL7* (*Cs3g01400*) was found to be more phylogenetic closer to *AtAL7* and *MsAlfin-1* than other citrus *AL* genes. We have selected it for functional studies. The forward primers with *Nde* I restriction enzyme site 5'-GGAATTCATATGGAAGCGA-TACCGCACC-3' and reverse primers *Bam*H I restriction enzyme site 5'-CGCGGATCCTTAAACTCTAGCCCTCT-TG-3' were designed for isolation of *CisAL7* complete ORF from ‘Valencia’ sweet orange, restriction enzyme recognition sites are underlined. The PCR was performed in the reaction mixture with total volumes of 25 µL, including 80 ng cDNA as template, 4 µL of 2.5 mM dNTP mixture (2.5mM each), 2.5 µL of 10× *LA Taq* Buffer II (Mg²⁺ plus), 1.25 U of *LA Taq* (TaKaRa, Dalian, China), 0.5 µL of forward primer (100 pM), 0.5 µL of reverse primer (100 pM). The PCR program was set as follows: pre-denaturation at 94°C for 3 min; 30 cycles of denaturation at 98°C for 10 s, and annealing-elongation at 68°C for 55 s; and 10 min at 72°C for the final elongation. RT-PCR products were purified and ligated with T-Vector pMD19 (Simple) (TaKaRa, Dalian, China), and finally transformed into competent *E. coli* DH5α cells according to a standard transformation method (Sambrook and Russell, 2001). Three to five positive clones were sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Multiple sequence alignment and phylogenetic analysis

Alignment of selected sequences was performed using program ClustalX 2.1 (Larkin et al., 2007). The corresponding phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis version 6.06 (MEGA 6.06) software (Tamura et al., 2013) by employing the Neighbor-Joining method and the bootstrap test was replicated 1000 times. All the parameters were taken to the default settings.

Cis-acting regulatory elements analysis

Using database associated search tools, 1.5 kb upstream from the translational start site of *CisAL7* was scanned for the presence of putative *cis*-acting regulatory elements identical with or similar to the motifs registered in Plant CARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al., 2002) and New PLACE (<https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action=page&page=newplace>) (Higo et al., 1999).

Construction of expression vector pET28a-CisAL7

In order to construct the expression vector pET28a-CisAL7, the PCR amplification product of *CisAL7* complete ORF was

ligated into the pET28a(+) expression vector at *Nde* I-*Bam*H I site to express *CisAL7* protein fused with His-tag and T7-tag at the N terminus. The recombinant plasmid pET28a-*CisAL7* was transformed into *E. coli* BL21 (DE3). The BL21 (DE3) cell with pET28a-*CisAL7* plasmid was named as BL21(pET28a-*CisAL7*). Containing the pET28a plasmid of BL21 (DE3) cells as blank control was named BL21(pET28a).

Expression of *CisAL7* gene in *E. coli*

Both BL21(pET28a-*CisAL7*) and BL21(pET28a) were cultured overnight at 37°C in Luria-Bertani (LB) agar medium supplemented with 100 mg/L ampicillin. Then, a pick single colony was inoculated into fresh LB with 100 mg/L ampicillin with gentle shaking at 37°C. When the optical density at 600 nm (OD₆₀₀) reached 0.5, IPTG was added into cultures to a final concentration of 0.5 mM and continue to grow 0-2 hours to induce the target protein in the recombinant cells. The cell cultures were centrifuged at 13,000×g for 2 min, and discarded the supernatant. Precipitate was added PBS and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (5×), then placed in a boiling water bath and boiled for 10 min. The samples were separated on 15% SDS-PAGE. Finally, gels were stained with Coomassie brilliant blue G-250 before destaining in decolorization solution.

Immunoblotting of the recombinant protein

After separation of proteins by SDS-PAGE, Western blotting was performed for target protein identification. For this purpose, all protein bands were transferred to a nitrocellulose membrane, using transfer buffer and electrophoresis. Afterwards, the nitrocellulose membrane was blocked in Tris buffered saline (TBS) with Tween-20 containing 50 g/L skimmed milk for 2 h. The membrane was then washed three times with PBS containing 1% BSA and 0.05% Tween-20 (PBST) and incubated with mouse anti-His-tag monoclonal antibody for 2 h at room temperature. The strips were washed three times with TBS (5 min each time) and then incubated with horseradish peroxidase-conjugated second antibody for 2 h, washed again with TBS as described previously, and the final reaction was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate.

Assay for abiotic stress tolerance of *E. coli* transformants

The BL21(pET28a-*CisAL7*) and BL21(pET28a) were used for stress tolerance assay. IPTG induction and cell culture described the same as above. A final concentration of all induced cultures, OD₆₀₀ value reached 1.0. To test the temperature stress tolerance of *CisAL7* transformants, the cell cultures of BL21(pET28a-*CisAL7*) and BL21(pET28a) were exposed at 4°C for 12 h or 50°C for 1 h, respectively. Then the cultures were diluted 10-fold, 100-fold, 1000-fold and 10 µL of diluted sample was spotted onto the LB agar plates. These plates were incubated overnight at 37°C. For salt tolerance assay, the sample were diluted 10-fold, 100-fold, 1000-fold and then 10 µL of diluted sample was spotted onto the LB agar plates in the presence or absence of 0.6 M NaCl or KCl. Then the plates were incubated at 37°C overnight. All data were duplicated in at least three independent experiments with consistent results.

Conclusion

In this study, *CisAL7* was isolated and molecularly

characterized. The function analyses of the *CisAL7* protein were performed in *E. coli*. The effective prokaryotic recombinant *CisAL7* expression system was established. The *CisAL7* protein was expressed in *E. coli* efficiently. The over-expression of *CisAL7* can improve the tolerance of recombinant *E. coli* under abiotic stresses including high salinity, high temperature and low temperature, suggesting that *CisAL7* may play an important role in plant to adapt to adverse environments. Furthermore, our results indicated that *CisAL7* is a promising gene for improving novel citrus cultivar(s) with high abiotic stress tolerance, which further decelerate the citrus yield loss.

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