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ORIGINAL ARTICLE

Molecular cloning and characterization of an *ASR* gene from *Cucumis sativus*

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Abstract Abscisic acid-, stress- and ripening-induced (ASR) proteins are widely present in the plant kingdom and play important roles in different biological processes. However, no reports of ASR proteins are available in cucumber. In this study, an *ASR* gene (*CsASR1*) was identified and characterized from *Cucumis sativus*. CsASR1 exhibited a high content of disorder-promoting amino acids, indicating that it is an intrinsically disordered protein (IDP). CsASR1 protein was highly homologous to ASR proteins from other plant species. Expression of *CsASR1* was induced by diverse abiotic stresses such as heat, PEG and NaCl, as well as by signaling molecules such as ABA and H_2O_2, suggesting a close relationship between CsASR1 and abiotic stress. Overexpression of *CsASR1* could increase the tolerance against salinity and osmotic stress in *E. coli*. Transgenic *Arabidopsis* plants overexpressing *CsASR1* exhibited higher germination rate than WT plants on MS medium containing various concentrations of NaCl. In addition, overexpression of *CsASR1* in *Arabidopsis* resulted in significantly improved salt tolerance due to the increased activity of SOD and elevated transcripts of *SOS3* and *LEA4-5*. Finally, CsASR1 could protect the activity of lactate dehydrogenase (LDH) from heat-induced inactivation. Taken together, our results demonstrate that CsASR1 plays an important role in abiotic stress tolerance, and it may function as an IDP to confer abiotic stress tolerance by protecting some stress-related proteins from inactivation under stress conditions.

Keywords *Cucumis sativus* · ASR · Abiotic stress · Salt tolerance · Intrinsically disordered protein (IDP)

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Yong Zhou and Lifang Hu have contributed equally to this work.

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Introduction

Under natural conditions, plants are frequently exposed to various stresses which adversely affect their growth, development and productivity (Hu et al. 2014; Pěnčík et al. 2015). Plants adapt to these adverse conditions by developing complex networks through the transcriptional regulation of stress responsive genes (Ingram and Bartels 1996). Many of these genes involved in the response networks of various stresses have been identified, but the mechanisms underlying their functions have not been completely clarified.

ASRs are small and hydrophilic proteins specifically observed in plants. The first ASR protein was isolated from tomato (*Lycopersicon esculentum*) and was shown to be coded by *Asr1* (Iusem et al. 1993). More *ASR* genes have since been identified from various species of monocot, dicot and gymnosperm plants, but so far there has been

no report of such genes in the Brassicaceae family such as *Arabidopsis* and *Thellungiella* (Carrari et al. 2004; Philippe et al. 2010; Wong et al. 2006; Yang et al. 2005).

The *ASR* genes belong to a small gene family and the copy number varies from one copy in grape to nine copies in maize (Gonzalez and Iusem 2014; Philippe et al. 2010; Virlouvet et al. 2011). The structures of ASR proteins are simple with two highly conserved regions: a short N-terminal consensus sequence containing 6–7 His residues that might constitute a zinc-binding site, and a large DNA-binding domain located at the C-terminal region consisting of about 70 amino acids with a highly conserved ABA/WDS domain (abscisic acid/water deficit stress domain; PF02496 in PFAM) (Çakir et al. 2003; Henry et al. 2011; Jia et al. 2016; Rom et al. 2006). Previous studies have revealed that a putative nuclear targeting signal is present at the C-terminal region of ASR proteins, making most ASR proteins localized in the nucleus, where they possibly function as transcription factors or as chaperones (Frankel et al. 2006; Konrad and Bar-Zvi 2008; Wang et al. 2005). However, ASR proteins can also be localized in the cytosol, where they have been shown to have chaperone-like activities to stabilize a number of proteins against denaturation caused by heat and freeze-thaw cycles (Konrad and Bar-Zvi 2008; Philippe et al. 2010; Wang et al. 2005).

There have been many reports about the expression of *ASR* genes in various organs and growth stages such as senescence, pollen maturation, fruit development and ripening among different species, and their expression has been demonstrated to be responsive to ABA and different environmental stress conditions, including drought, cold, salt and other stresses (Chen et al. 2011; Frankel et al. 2006; Gonzalez and Iusem 2014; Jia et al. 2016; Joo et al. 2013; Li et al. 2016; Maskin et al. 2001; Philippe et al. 2010; Yang et al. 2005). These results suggest that *ASR* genes are involved in various abiotic and biotic stresses. For example, overexpression of tomato *Asr1* gene in tobacco resulted in a decreased water loss rate and improved salt tolerance (Kalifa et al. 2004). Transgenic tobacco plants overexpressing *SbASR-1* from *Salicornia brachiate* and *BdASR1* from *Brachypodium distachyon* also exhibited higher salt tolerance (Jha et al. 2012; Wang et al. 2016). In addition, overexpression of *LLA23* from lily (*Lilium longiflorum*), *MpAsr* from plantain (*Musa paradisiaca*) and *MaASR* from banana (*Musa acuminata* L. AAA group cv. Brazilian) in *Arabidopsis* also resulted in increased tolerance to drought and salinity (Dai et al. 2011; Yang et al. 2005; Zhang et al. 2015). Moreover, overexpression of either *OsASR1* or *OsASR3* in transgenic rice plants contributed to strong tolerance to drought and cold stress (Joo et al. 2013; Kim et al. 2009). Very recently, overexpression of *OsASR5* was shown to enhance the osmotic and drought tolerance in *E. coli*, rice and *Arabidopsis* (Li et al. 2016). Many studies have demonstrated that the increased expression of *ASR* genes can improve abiotic stress tolerance in tobacco, rice and *Arabidopsis*, suggesting that they function as positive regulators of stress signaling. However, the exact molecular mechanisms underlying their functions remain unclear.

Cucumber (*Cucumis sativus*), a major vegetable crop consumed worldwide, has a high transpiration rate and sensitivity to drought due to its high demand for water. ASRs have been studied previously in many other plants, but not in cucumber. In this study, we identified an *ASR* gene named *CsASR1* from cucumber, and studied its transcript profiles in response to various abiotic stresses such as heat, PEG and NaCl as well as signaling molecules such as ABA and H_2O_2. Overexpression of *CsASR1* conferred tolerance against abiotic stresses including NaCl and sorbitol in *E.coli*. In addition, its overexpression in *Arabidopsis* also resulted in elevated tolerance to salt and increased germination rate. In the present study, we performed a detailed functional analysis of *CsASR1* to explore its roles in abiotic stress tolerance. A better understanding of the mechanisms through which ASRs regulate stress signaling is significant for promoting abiotic stress tolerance and production of crops.

Materials and methods

Plant materials and growth conditions

Cucumber (*C. sativus* L. cv. Chinese long No. 9930) and *Arabidopsis thaliana* (Col-0 ecotype) were used in this study. The cucumber plants were planted in the field of Jiangxi Agricultural University (Nanchang, China). Wild-type and *CsASR1* transgenic *Arabidopsis* plants were grown on soil at 22–24 °C in a plant growth chamber with a 14-h light/10-h dark cycle.

For the salt, abscisic acid (ABA), H_2O_2 and drought treatments, cucumber seedlings were grown in liquid Murashige and Skoog (MS) medium containing 200 mM NaCl, 100 μM ABA, 10 mM H_2O_2, and 300 mM PEG-6000, respectively. For the heat treatment, cucumber seedlings in the growth chamber (light cycle: 14 h light/10 h dark) were transferred to 50 °C under light conditions.

Cloning and bioinformatic analysis of *CsASR1*

To identify novel *ASR* genes in cucumber, the conserved ABA/WDS domain (abscisic acid/water deficit stress domain; PF02496 in PFAM) was used as a query probe to blast the public cucumber databases. Two predicted *ASR-like* genes were identified and visualized using the genome browser of the cucumber database (http://cucumber.genomics.org.cn). Next, one of the predicted *ASR-like* genes was

amplified from cDNA templates synthesized from RNA mixtures which were extracted from cucumber leaves with primers in Table S1. The PCR product was sub-cloned into pGEM-T (Promega, USA) and sequenced.

The gene structure of *CsASR1* was analyzed with the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). The disordered regions of CsASR1 were analyzed by Protein Disorder prediction system (PrDOS) (http://prdos.hgc.jp/cgi-bin/top.cgi) (Ishida and Kinoshita 2007). The folding prediction was carried out by the FoldIndex program at http://bioportal.weizmann.ac.il/fldbin/findex (Prilusky et al. 2005). The theoretical pI, MW and the grand average of hydropathicity (GRAVY) of the putative protein were determined by the ProtParam program (http://web.expasy.org/protparam/). Analysis of signal peptide and subcellular localization were performed using SignalP (http://genome.cbs.dtu.dk/services/SignalP) and iPSORT (http://ipsort.hgc.jp/). The multiple sequence alignments of CsASR1 and other ASR proteins were carried out with ClustalW software (Larkin et al. 2007). Phylogenetic trees were constructed using the MEGA4 software by the neighbor-joining method (Tamura et al. 2007).

RNA extraction, RT-PCR and quantitative RT-PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). The first-strand cDNA was synthesized from 3 μg RNA treated with DNase I with the oligo$(dT)_{15}$ primer and the Superscript™ III RNase H-Reverse Transcriptase kit (Invitrogen, USA) in a 25-μL reaction mixture. The RT-PCR program was performed as follows: 1 cycle at 94 °C for 5 min, followed by 28–30 cycles of 40 s at 94 °C, 40 s at 58 °C, and 40 s at 72 °C, and then a final extension at 72 °C for 7 min. *CsActin* and *AtTubulin4* listed in Table S1 were used as the internal controls in cucumber and *Arabidopsis*, respectively. Quantitative real-time PCR (qRT-PCR) was performed with SYBR premix Extaq (TaKaRa, Japan) and the LightCycler 480 System (Bio-Rad, USA). The reaction procedure was as follows: 94 °C for 5 min, and then 40 cycles of 94 °C for 5 s, 55 °C for 10 s and 72 °C for 15 s. The relative expression level was normalized to *CsActin* by the $2^{-\Delta\Delta CT}$ method for quantification (Livak and Schmittgen 2001). The primers for qRT-PCR analyses are listed in Table S1. Three biological replications were performed in all reactions.

Production and solubility of CsASR1 in *E. coli*

To generate the recombinant construct of *CsASR1*, the open reading frame (ORF) of *CsASR1* was amplified from the cDNA of cucumber leaves and cloned into pET32a, resulting in a pET32a-*CsASR1* construct. The construct was confirmed by sequencing and transformed into *E. coli* BL21 (DE3) strain. The bacterial cultures were grown in LB medium at 37 °C until the OD_{600} reached 0.6, and then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. The cells were re-suspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) after centrifugation at 5000×*g* for 5 min. The cell pellets were sonicated and centrifuged, and the cell pellets and soluble cell extracts were detected by SDS–PAGE analysis.

Abiotic stress tolerance assay of *E. coli* transformants

The abiotic stress tolerance assay of *E. coli* transformants was performed using the method of a previous study with some modifications (He et al. 2012). The IPTG induction was conducted as described above, and then the cultures of pET32a and pET32a-CsASR1 (named as BL/pET32a and BL/CsASR1, respectively) in liquid LB were adjusted to an OD_{600} of 1.0. Subsequently, the IPTG-induced transformant strains were diluted (1:25), and 100 μL of the diluted BL/pET32a and BL/CsASR1 were spread on IPTG LB agar plates supplemented with 0.1, 0.2, 0.3, 0.4 mM concentration gradient of NaCl, as well as 300, 600, 900 mM concentration gradient of sorbitol, respectively. After incubation overnight at 37 °C, the colony number on each plate was measured to determine the cell viability as described previously (Liu and Zheng 2005).

LDH activity assay

Lactate dehydrogenase (LDH) from rabbit muscle (Sigma, USA) was diluted to 1 mg/mL with PBS buffer. The lactate dehydrogenase (LDH) activity in *E. coli* was measured in vivo using an enzyme assay kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). For the thermal inactivation assay, CsASR1 proteins were added to equal volumes of LDH at the mass ratio of 1:5. The enzyme mixture was incubated at 65 °C for 15 and 30 min, and water and BSA were used as negative and positive controls, respectively. All samples were assayed in triplicate.

Plasmid construction and plant transformation

To create the overexpression construct, the coding sequence of *CsASR1* was amplified and inserted into PHB using the following primers: 5′-aaaaAAGCTTATGTCGGAGGAAAACCGCCAC-3′ and 5′-aaaaCTGCAGTTAGAATATATGGTGATGCTTC-3′, resulting in a 35S::*CsASR1* construct. The construct was introduced into *A. thaliana* by *Agrobacterium tumefaciens*-mediated transformation according to a previous study (Clough and Bent 1998). Transgenic

seedlings were selected by plating on 1/2 MS medium containing hygromycin (50 mg/L) and confirmed by PCR with the following primers: 5′-TGGTGGAATGGAAGAAGA GG-3′ and 5′-CTAGCTTGTGCCTGTGTCCA-3′. The antibiotic-resistant T_2 homozygous transgenic lines were selected for further analysis.

Analysis of transgenic plants under salt stress

All *Arabidopsis* seeds were harvested and stored under identical conditions. For the salt tolerance test during seed germination, seeds of *CsASR1*-overexpressing transgenic line (OE1) and WT plants were placed at 4 °C for 3 days in the dark to break dormancy, and then surface sterilized, germinated on MS medium with 0, 50 or 100 mM NaCl. The germination rates were recorded every day. For salt tolerance test on plants, 3-week-old soil-grown seedlings of OE1 and WT were irrigated with the 200 mM NaCl solution every other day for up to 14 days. The rosette leaves were harvested at 14 days after treatment, and the activity of superoxide dismutase (SOD) was measured following a previously described method (Tianpei et al. 2015).

Statistical analyses

Statistical significance was calculated with SPSS 16.0 software. Statistical significance ($P<0.05$ or $P<0.01$) were determined based on Student's *t*-test in a two-tailed analysis. Data are mean ± SD calculated from three biological replicates.

Results

Isolation and sequence analysis of *CsASR1* from cucumber

A total of two *ASR* genes (Gene IDs: Csa005659 and Csa005660) were identified from cucumber. Compared with Csa005660, Csa005659 encodes a protein with a lot more disorder-promoting amino acids, such as Glu (18.7%), Gly (18.3%), Lys (9.7%), Tyr (8.0%), Ala (7.6%), His (7.6%) and Ser (6.6%), implying that Csa005659 may encode an intrinsically disordered protein (IDP). This gene (designated as *CsASR1*) was chosen for further functional studies and its characteristics are listed in Table S2. Analysis of the disordered regions in CsASR1 by PrDOS suggested that over 93.4% of the amino acid residues of CsASR1 were disordered (Fig. 1a). Moreover, the folding prediction of CsASR1 with the FoldIndex program indicated that it might be a disordered protein (Fig. 1b). Additionally, the theoretical isoelectric point (pI) was 5.01, and the grand average of hydropathicity (GRAVY) was −1.458, implying that the CsASR1 protein is strongly hydrophilic (Kyte and Doolittle 1982). These results suggested that CsASR1 is an IDP.

Amino acid sequence alignment of CsASR1 and other ASR proteins from monotcot and dicot species is displayed in Fig. 2. It was shown that the amino acid sequence of CsASR1 was 45, 52, 44, 45 and 53% identical to that of ASRs from soybean (*Glycine max*), *Litchi chinensis* (LcASR), *S.brachiata* (SbASR-1), apple (MdASR1), strawberry (FaASR) and lychess (LcAsr), respectively (Fig. 2). Phylogenetic analyses indicated that CsASR1 was closely related to the dicot ASR group, especially CmASR, FaASR, MdASR1 and GmASR (Fig. 3). All these results suggested that the *CsASR1* gene encodes a putative ASR protein in cucumber.

Transcript profiles of *CsASR1*

The spatial expression pattern of *CsASR1* was examined by RT-PCR, and the results showed that *CsASR1* was ubiquitously expressed in selected tissues (Fig. 4a).

Most of the *ASR* genes were up-regulated under various stress treatments, including drought, cold, salinity and exogenous ABA (Frankel et al. 2006; Shen et al. 2005). We also assessed whether *CsASR1* is regulated by abiotic stresses like other *ASRs* by qRT-PCR. As shown in Fig. 4b–d, the expression level of *CsASR1* was responsive to different treatments to various degrees. Under PEG treatment, the expression of *CsASR1* increased by 33-fold after 3 h treatment, decreased at 6 h, subsequently reached the highest level (115-fold) after 12 h, and ultimately decreased (14-fold) at 24 h (Fig. 4b). Compared with PEG treatment, similar expression level of *CsASR1* gene was observed under NaCl treatment, but the highest expression level was observed at 24 h (Fig. 4b). Under heat treatment, the expression of *CsASR1* was induced gradually, and peaked at 24 h (Fig. 4c). It is well known that stress treatment can induce the accumulation of various signal molecules. Thus, we also examined the effects of H_2O_2 and ABA on *CsASR1* transcription. Under H_2O_2 treatment, the transcription of *CsASR1* was induced, and was enhanced strongly at 6 and 12 h followed by a decrease at 24 h (Fig. 4c). Under ABA treatment, the transcript level of *CsASR1* peaked at 6 h in leaves and then decreased (Fig. 4d). These results indicated that *CsASR1* can be differentially regulated by multiple abiotic stresses and signal molecules.

Heterologous expression of the CsASR1 protein in *E. coli*

To investigate the biological functions of CsASR1, the transformant cells of both pET32a-*CsASR1* and pET32a were induced by IPTG at 37 °C for 4 h, and examined by

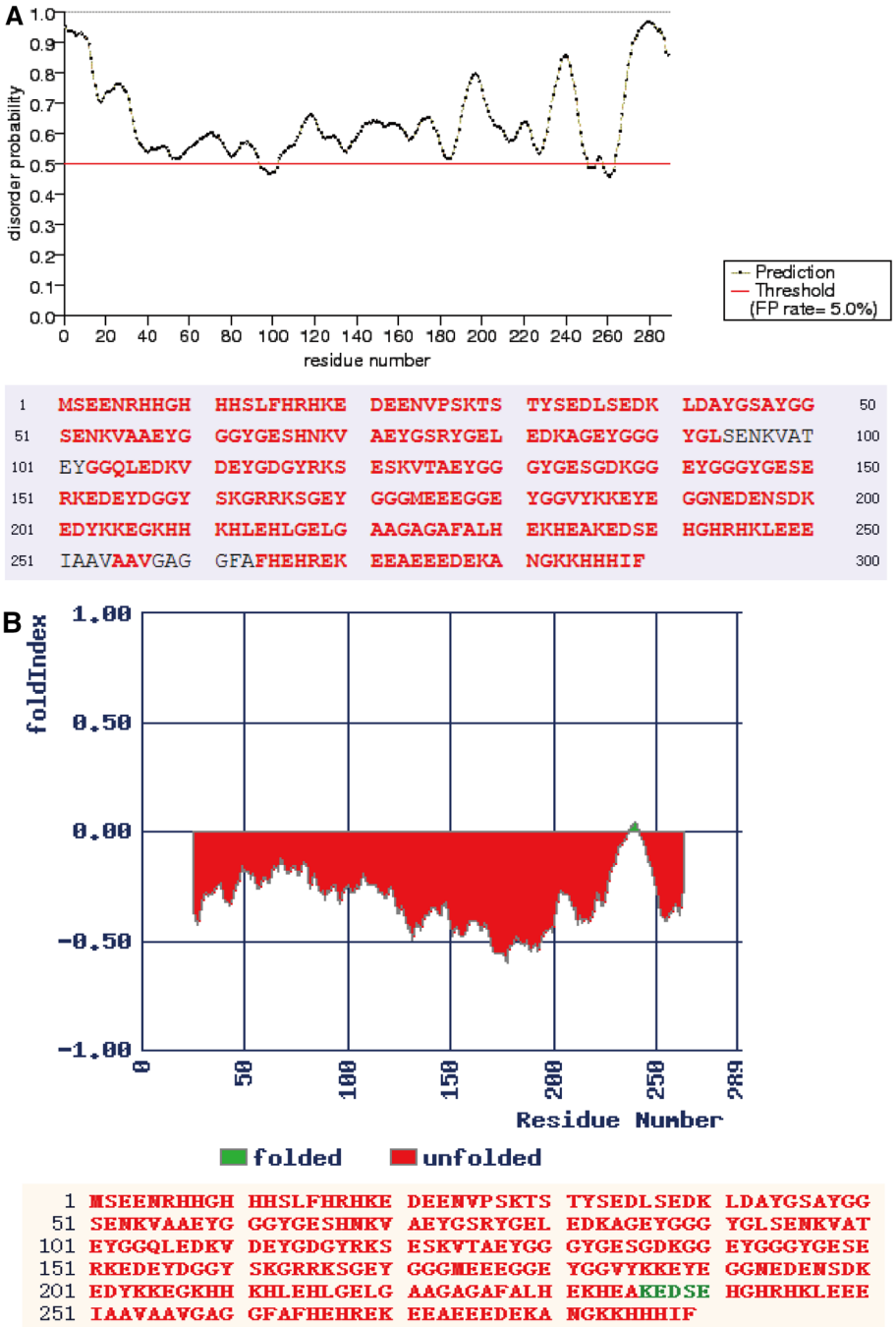

Fig. 1 CsASR1 as a disordered protein. **a** Analysis of the disordered regions in CsASR1 *via* PrDOS program. The folding characteristics of the CsASR1 protein. Disorder probability above 0.5 represents the disordered amino acid residues. *Red* Disordered residues. *Black* Ordered residues. **b** CsASR1 folding prediction using the FoldIndex program with the values predicted in the window =10 and step =1. *Positive and negative numbers* represent ordered and non-ordered regions, respectively

SDS–PAGE. The predicted molecular mass of CsASR1 protein was 31.7 kDa. As shown in Fig. 5a, a specific band at about 60 kDa was detected in the BL/CsASR1 cell pellets (Fig. 5a), which was higher than the expected theoretical size (31.7 kDa of CsASR1 plus 21.1 kDa of TrxA intein fusion protein). This abnormal phenomenon of protein size may be related to the net charge of protein molecules, which was also reported in SbASR-1 (Goldgur et al. 2007; Tiwari et al. 2015). It is worth noting that the specific band was observed in both cell pellets and soluble cell extracts from BL/CsASR1 cells (Fig. 5b), indicating that the CsASR1 fusion protein was soluble.

Overexpression of *CsASR1* in *E. coli* enhanced growth under salinity and osmotic stress

To better understand the biological functions of CsASR1, the growth of *E. coli* transformants was analyzed under salinity and osmotic stress conditions. As shown in Fig. 6a, BL/pET32a and BL/CsASR1 cells had similar growth on

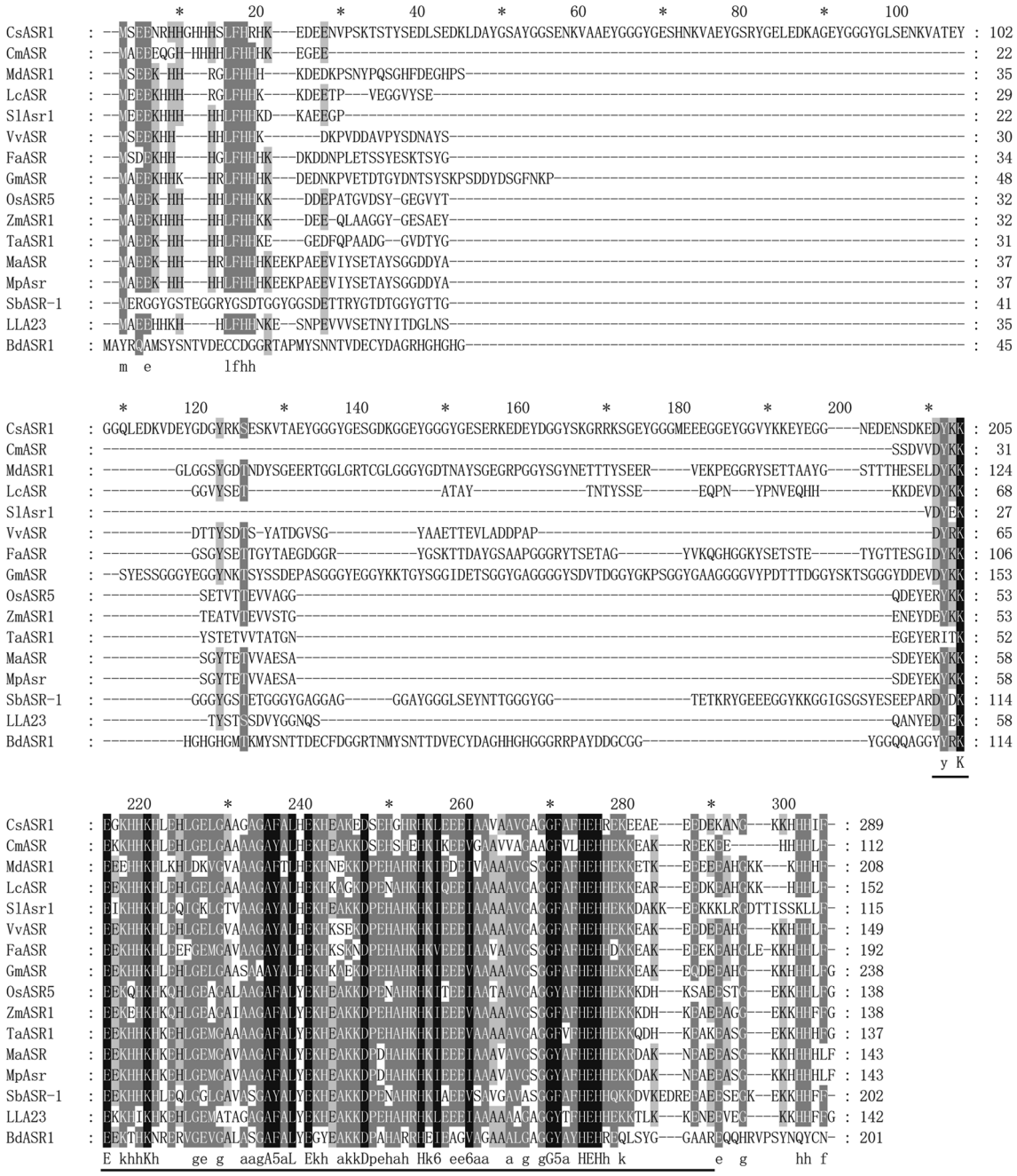

Fig. 2 Multiple amino acid sequence alignment of CsASR1 with homologues from different plant species. Amino acid sequences were aligned using the ClustalW software. Similar amino acids were indicated with *black foreground* and *light gray*. The highly-conserved abscisic acid/water deficit stress (ABA/WDS) domain annotated by query against InterPro is underlined. The amino acid sequences are listed in Table S3

LB medium in overnight grown culture. Although both of them lost viability under NaCl treatment, the BL/CsASR1 strain cells showed obviously higher viability compared with the control cells under the conditions of 100 and 200 mM NaCl.

We also tested the cell viability of transformants under osmotic condition. The viability of BL/CsASR1 cells was greater than that of BL/pET32a cells under sorbitol treatment at 600 mM (Fig. 6b). BL/CsASR1 exhibited almost no difference from BL/pET32a under sorbitol treatment at 300 mM, and all of the cells died under sorbitol treatment at 900 mM (Fig. 6c), implying that the expression of the CsASR1 protein contributes to BL21 cell tolerance to water-deficit stress caused by sorbitol. All these results

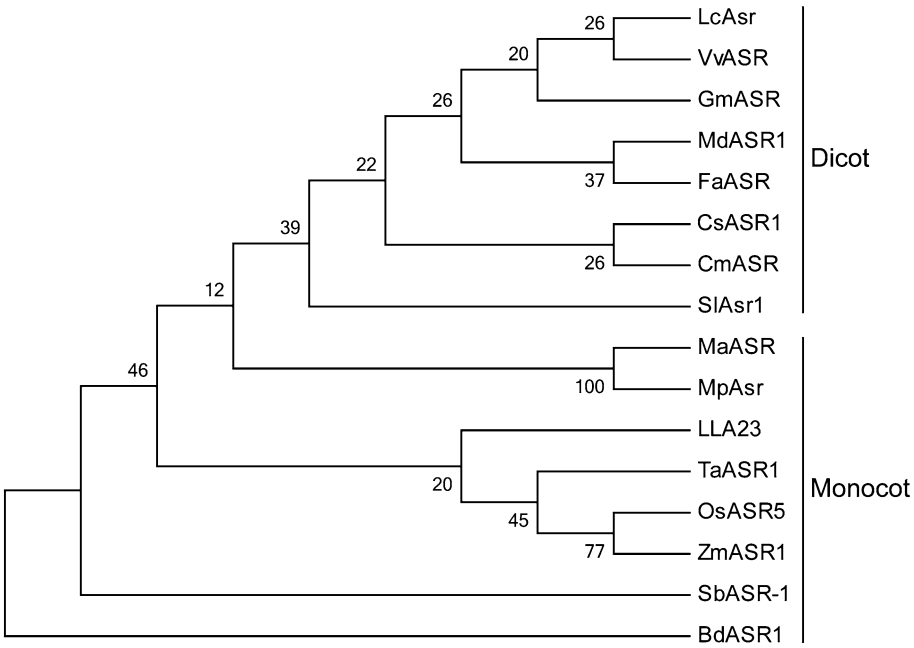

Fig. 3 Phylogenetic trees of ASR proteins from different plants species. Amino acid sequences were aligned using the ClustalW software and a phylogenic tree was constructed using the MEGA4 software with 1000 bootstrap. The amino acid sequences used to build up the phylogenic tree are listed in Table S3

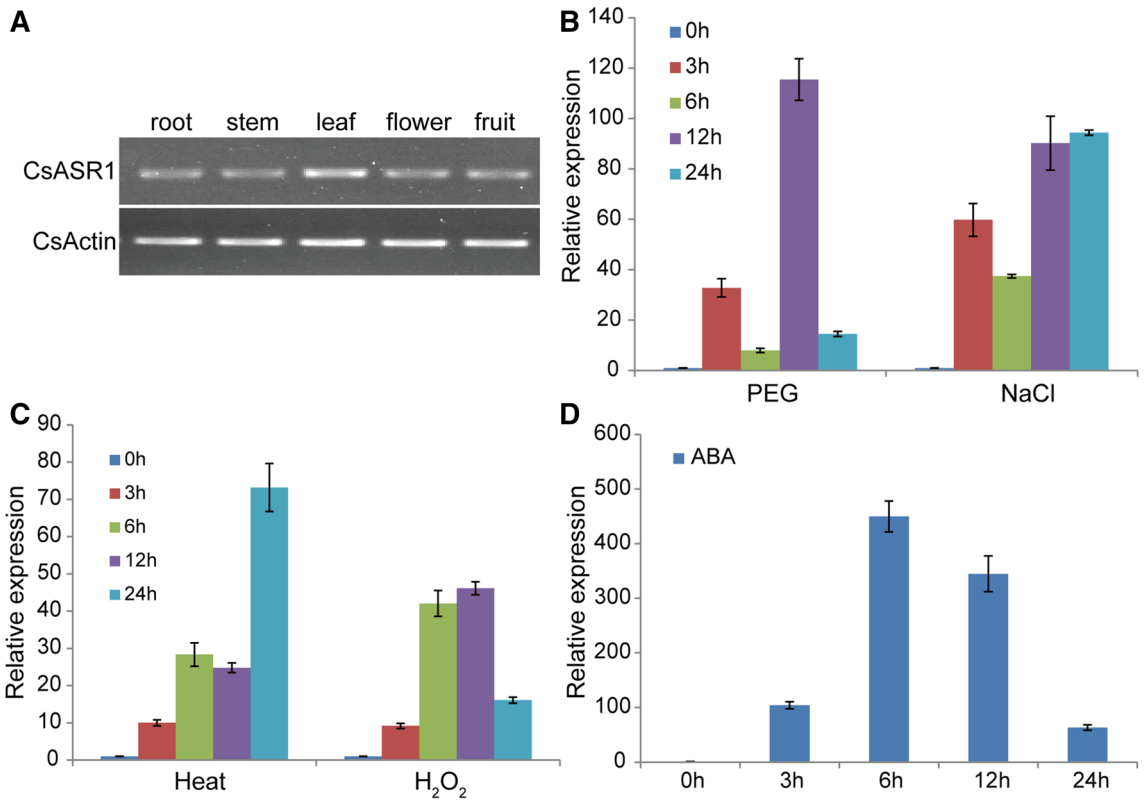

Fig. 4 Transcript profiles of *CsASR1* determined by RT-PCR and qRT-PCR. **a** RT-PCR analysis of the transcript profile of *CsASR1* in root, stem, leaf, flower, and fruit. **b–d** Expression patterns of *CsASR1* in leaves in response to PEG and NaCl (**b**), heat and H_2O_2 (**c**), and ABA (**d**). Samples were collected from leaves at 0, 3, 6, 12, and 24 h during each stress treatment. The values are means (±SD) of three replicates

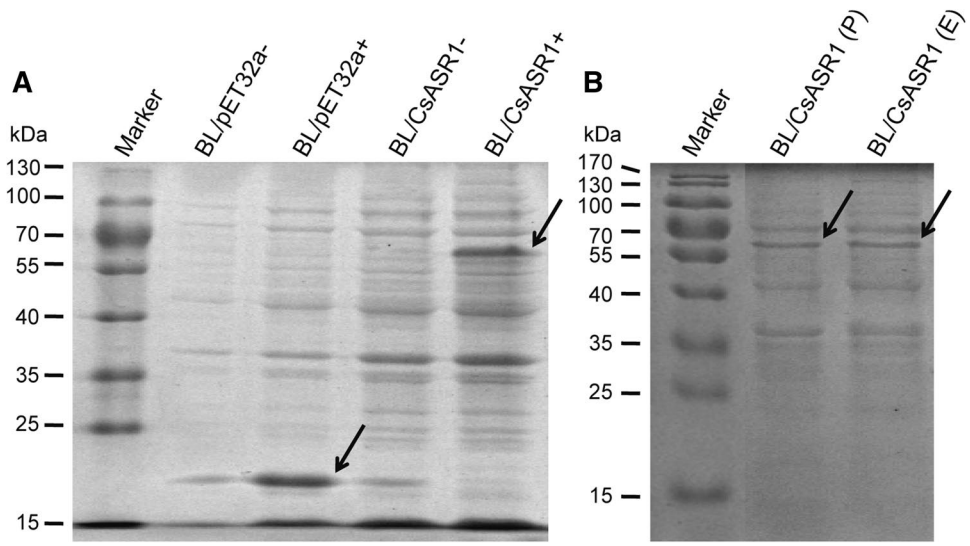

Fig. 5 SDS–PAGE analysis of recombinant pET32a-*CsASR1* fusion protein in *E.coli*. **a** Total protein extracted from IPTG-induced cultures (*Lane 3* and *5*) and non-induced controls (*Lane 2* and *4*). *Lane 1*, molecular weight marker. *Lanes 2* and *3*, cells were transformed with pET32a. *Lanes 4* and *5*, cells were transformed with pET32a-*CsASR1*. **b** Solubility analysis of pET32a-*CsASR1* fusion protein by SDS–PAGE. P: cell pellets from BL/CsASR1 cells. E: soluble cell extracts from BL/CsASR1 cells. *Arrows* indicate the expressed proteins

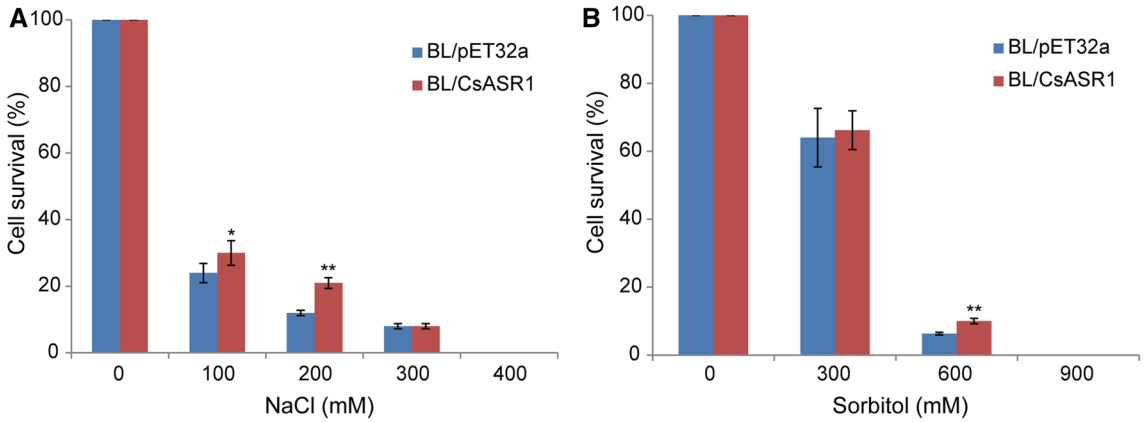

Fig. 6 Overexpression of *CsASR1* in *E. coli* enhanced growth during abiotic stresses. **a** and **b** Survival of IPTG-induced *E. coli* harboring pET32a and pET32a-*CsASR1* in LB medium under different abiotic stress conditions including NaCl (**a**) and sorbitol (**b**), respectively. At the end of the treatment, the cell viability of *E. coli* determined by counting the colony-forming units were measured by plating an aliquot of *E. coli* cells on LB medium and allowing them to form colonies overnight at 37 °C. Data sets marked with *asterisks* indicate significant differences (*$P < 0.05$, or **$P < 0.01$, Student's *t*-test)

indicated that the *CsASR1* gene could enhance the tolerance to salinity and osmotic stress.

CsASR1 protein prevented LDH from inactivation under heat stress

To examine the protective effect of CsASR1 protein on enzyme activity under heat stress, the ability to prevent the LDH activity loss was tested after 65 °C treatment. The LDH activity was measured as described previously, and BSA and PBS buffer were used as the positive and negative controls, respectively (Yang et al. 2015). As shown in Fig. 7, without protectant, the activity of LDH was sharply decreased after treatment at 65 °C and had only 16.06% and 13.64% of its initial activity upon incubation for 15 min and 30 min, respectively. Addition of CsASR1 or BSA could significantly minimize the loss of LDH activity. LDH activity was 2.0-, and 2.0-fold higher with the presence of CsASR1 than with PBS buffer after treatment at 65 °C for 15 and 30 min, respectively. Although the ability of CsASR1 to minimize the loss of the LDH activity was lower than positive control BSA, these results suggested that the CsASR1 protein could confer stabilization of the LDH under heat treatment in vitro.

CsASR1 overexpression conferred salinity tolerance in *Arabidopsis*

Considering that *CsASR1* was induced by multiple abiotic stresses, the *CsASR1* coding sequence driven by the 35S

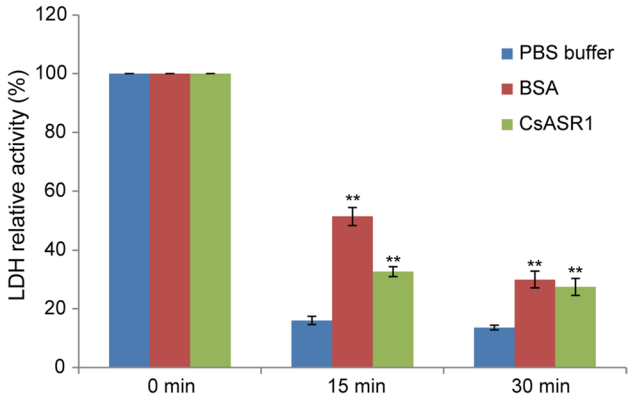

Fig. 7 CsASR1 protein protected LDH activity from inactivation under heat stress. The relative activity of LDH during heating at 65 °C for the indicated times. The data represent the mean ± SD of three independent experiments. Data sets marked with asterisks indicate significant differences (**$P < 0.01$, Student's *t* test)

promoter was introduced into the wild-type (WT) Col-0 to overexpress *CsASR1*, and two lines (OE1 and OE2) with constitutive expression were selected for further analysis (Fig. 8a).

To assess whether *CsASR1* overexpression could enhance the tolerance to salt stress, we measured the germination rates of the transgenic line (OE1) and WT seeds on MS agar plates supplemented with 0, 50 or 100 mM NaCl. Under normal growth conditions, the transgenic and WT plants showed similar germination rates of approximately 99% at 5 days after sowing (Fig. 8b). In contrast, the germination rate was dramatically decreased in OE1 and WT plants in the presence of 50 and 100 mM NaCl, and the inhibitory effect became more significant with increasing NaCl concentration (Fig. 8c, d). It was noteworthy that the germination rate of transgenic seeds was less inhibited by NaCl compared with the WT seeds (Fig. 8c, d). Take 5 days for example, in the presence of 50 mM NaCl, 98.7% of OE1 seeds were germinated, which was much higher than that of WT seeds (82.9%) (Fig. 8c). The addition of 100 mM NaCl decreased the percentage of germination in the wild type to 57.7%, whereas the germination rate of OE1 seeds remained at 98.7% under the same condition (Fig. 8d). These results indicated that *CsASR1* overexpression confers salinity tolerance to *Arabidopsis* during seed germination.

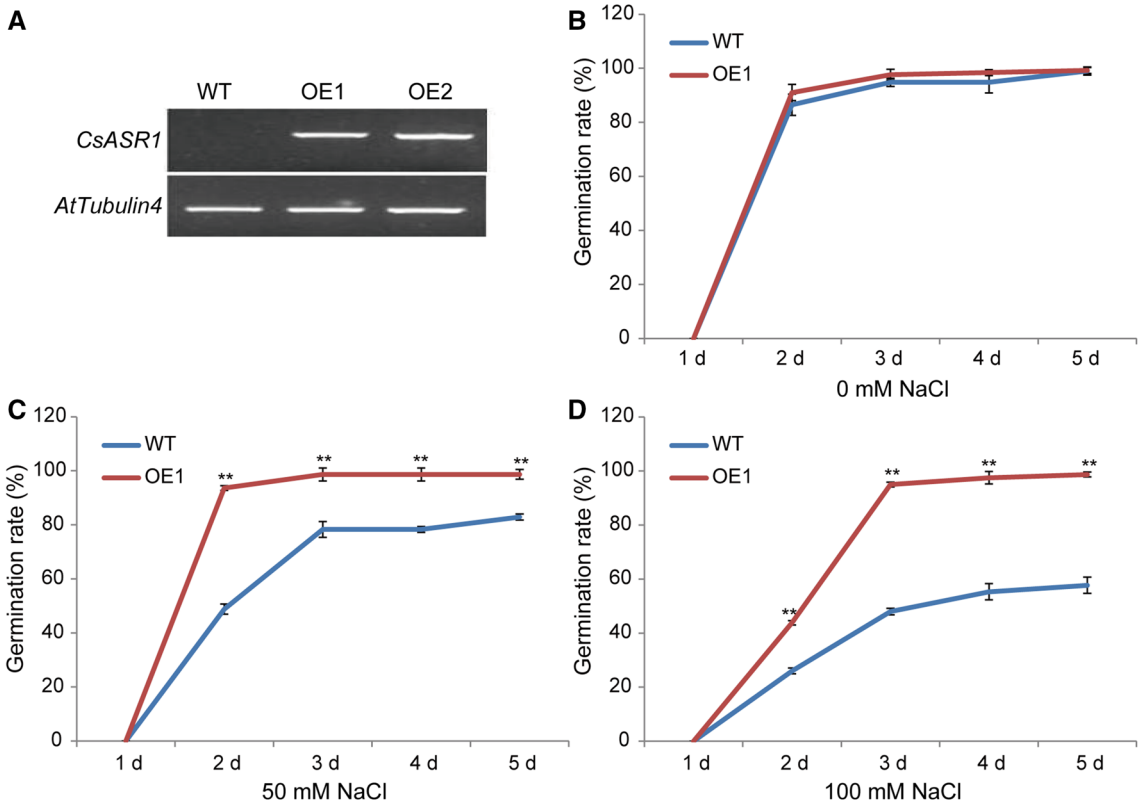

Fig. 8 Overexpression of *CsASR1* and germination of transgenic and WT plants under salt stress. **a** RT-PCR analysis of *CsASR1* expression in two transgenic lines (OE1 and OE2) and WT plants. The *AtTubulin4* gene was amplified as a control. **b–d** The seed germination rates of transgenic line (OE1) and WT plants were measured on MS agar plates supplemented with 0 (**b**), 75 (**c**), and 100 mM (**d**) NaCl at 1–5 days after sowing. Error bars indicate SD (n = 3). **Represent significant differences from the WT at values of $P < 0.01$, as determined by Student's *t*-test

To further verify the roles of *CsASR1* in salt tolerance, the performance of *CsASR1*-overexpressing transgenic plants under salt stress in soil was also evaluated. After 14 days of 200 mM NaCl treatment, obvious yellowing of leaves and retardation of growth were observed in the WT plants but not in the transgenic plants, indicating that the overexpression of *CsASR1* could improve the tolerance to high salinity in *Arabidopsis* (Fig. 9a). To investigate the causes for the phenotypic changes, we measured the activity of SOD, an important enzyme in protecting cells against various stresses. Under normal growth conditions, the SOD activity in OE1 line was 54.7 unit (100 mg protein)$^{-1}$, whereas that of the WT plants was 48.3 unit (100 mg protein)$^{-1}$. After 14 days of salt stress treatment, the SOD activity was increased by approximately 1.3-fold in WT plants compared with in those without the treatment, while transgenic lines showed a 1.5-fold increase, and the final SOD activity in the leaves of transgenic plants was approximately 1.3-fold higher than that in the leaves of WT plants (Fig. 9b).

The stress responsive genes including *AtCBL4*/*SOS3* (salt overlay sensitive) and *LEA4-5* that encodes late embryogenesis abundant protein are ideal molecular markers for salt stress in *Arabidopsis* (Bies-Etheve et al. 2008; Liu and Zhu 1998). Thus, we further examined the expression levels of *SOS3* and *LEA4-5* by RT-PCR. As shown in Fig. 9c, the transcripts of *SOS3* and *LEA4-5* in the transgenic lines were remarkably higher than those in the WT plants, which might be the reason for the improvement of salt stress tolerance in the transgenic lines. These findings indicated that overexpression of *CsASR1* in *Arabidopsis* could confer higher tolerance to salt stress.

Discussion

Over the past few decades, many *ASR* genes have been identified and characterized in the plant kingdom. However, there has been no report about the *ASR* genes in cucumber (*C. sativus* L.), an economically and nutritionally important vegetable crop cultivated and consumed worldwide. In the present study, an *ASR* gene named *CsASR1* was isolated and characterized for the first time from *C. sativus*. CsASR1 contains an N-terminal His-rich region and a C-terminal conserved ABA/WDS domain, and shows a significant homology to other homologous ASRs from various plant species, especially dicotyledons (Figs. 2, 3). Some previous studies have demonstrated that *ASR* genes are upregulated by different developmental and environmental signals, including drought, cold, salt, and ABA (Hsu et al. 2011; Joo et al. 2013; Li et al. 2016; Maskin et al.

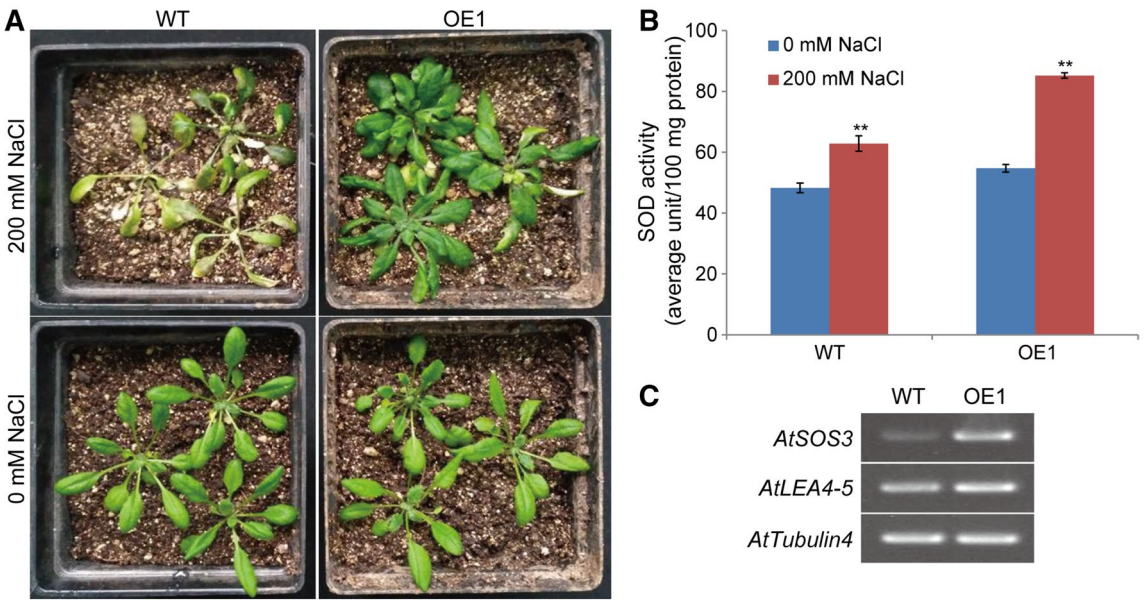

Fig. 9 Phenotypes of transgenic plants under normal and salt stress conditions. **a** Photographs of transgenic line (OE1) and WT plants before and after salt stress. Three-week-old soil grown seedlings of transgenic lines and WT were irrigated with the 200 mM NaCl solutions every other day for up to 14 days. **b** Determination of SOD activity in OE1 and WT plants with and without salt stress. Samples were collected from 3-week-old seedlings before and after 14 days of salt stress. Error bars indicate SD (n = 10). **Represent significant differences from the WT at values of $P < 0.01$, as determined by Student's *t* test. **c** Transcript levels of stress-related genes (*SOS3* and *LEA4-5*) in transgenic and WT plants assayed by RT-PCR. RNA samples were extracted from leaves from 3-week-old seedlings after 14 days of salt stress

2001; Philippe et al. 2010; Wang et al. 2016). In this study, remarkable increase in the transcript level of *CsASR1* was observed under various abiotic stresses (Fig. 4b–d). These results suggest that *CsASR1* is a member of *ASR* gene family, and is likely involved in stress adaptation of cucumber.

It is generally known that the genes induced by abiotic stresses may play positive roles in abiotic stress tolerance (Li et al. 2016). In addition, CsASR1 is a hydrophilic protein rich of Glu, Gly, Lys, Tyr, Ala, His and Ser. The high content of charged amino acids contributes to the increase of hydrophilicity of ASR proteins, which can increase the accessibility to water molecules during stress conditions (Padaria et al. 2016), implying that CsASR1 may play a role under stress conditions. In the present study, overexpression of the *CsASR1* gene in *E. coli* resulted in significantly increased tolerance to salinity and osmotic stress (Fig. 6), suggesting that CsASR1 may play a role in response to abiotic stresses.

It has been known that some protective mechanisms to mediate responses to various stresses might be common in prokaryote and eukaryote (Garay-Arroyo et al. 2000). To further understand the biological functions of CsASR1 under abiotic stress conditions, *CsASR1* was overexpressed in *Arabidopsis* plants, which lacks an *ASR* homolog and thus can be an excellent plant to study the biological functions of *ASR* genes (Yang et al. 2005). We found that the overexpression of *CsASR1* in *Arabidopsis* could confer tolerance to salt stress with increased SOD activity and higher expression levels of *SOS3* and *LEA4-5* (Figs. 8, 9). AtCBL4/SOS3 has been shown to play a role in salt resistance of *Arabidopsis* (Liu and Zhu 1998). *AtLEA4-5* is induced by various abiotic stresses, and transgenic *Arabidopsis* plants overexpressing *AtLEA4-5* showed higher tolerance to salt stress compared with WT plants (Olvera-Carrillo et al. 2010). Thus, the phenotypes of *CsASR1*-overexpressing plants may be partly due to the changes in the expression of some stress-related genes.

The subcellular localization of ASR proteins was previously reported to be in cytosol (Goldgur et al. 2007), nucleus (Çakir et al. 2003; Feng et al. 2016; Hu et al. 2013, 2014; Saumonneau et al. 2008; Tiwari et al. 2015), or both (Chen et al. 2011; Kalifa et al. 2004; Takasaki et al. 2008; Wang et al. 2005), even in multiple cellular compartments such as nucleus, cytoplasm and chloroplasts (Arenhart et al. 2014; Li et al. 2016). In many plant species, the nucleus-localized ASR proteins act as transcription factors to regulate specific promoters such as hexose transporters and ABA responsive genes (Padaria et al. 2016). The ASR1 protein of tomato is unstructured in cytoplasm and presents chaperone-like activity due to its high hydrophilicity that stabilizes other proteins against denaturation caused by heat and freeze–thaw cycles (Goldgur et al. 2007; Konrad and Bar-Zvi 2008). According to the SignalP and iPSORT prediction, CsASR1 protein lacks any recognizable signals, including mitochondrial targeting signal, or chloroplast transit peptides, implying that CsASR1 is not a nucleus-localized protein.

The intrinsically disordered proteins (IDPs), due to their enrichment in disorder-promoting charged amino acid residues (Glu, Lys, Arg, Gly, Gln, Ser, Pro, and Ala), do not have any fixed conformation (Charfeddine et al. 2017; Tiwari et al. 2015). Both the disordered regions and the folding prediction of CsASR1 suggested that CsASR1 is an IDP, which is similar to SlASR1 in *Solanum lycopersicum* (Goldgur et al. 2007), MpASR in *M. paradisiaca* (Dai et al. 2011), SlASR in *Suaeda liaotungensis* (Hu et al. 2014), and SbASR-1 in *S. brachiata* (Jha et al. 2012; Tiwari et al. 2015). Because of the structural flexibility and hydrophilicity, IDPs can allow easier protein–protein interactions for the protection of other cellular proteins under stress conditions and help them in transcriptional regulation of other genes for modulation of biological functions (Jha et al. 2012; Uversky and Dunker 2010). For example, SlASR1 can fold into an ordered structure and form homodimer upon binding with zinc ions (Goldgur et al. 2007), and cytosolic SlASR1 could confer stability to a lot of proteins in response to heat and freeze–thaw cycles under in vitro experiments (Konrad and Bar-Zvi 2008). TaASR1 may act as a transcriptional regulator and bind to DNA in a Zn^{2+}-dependent manner during the transition from a disordered to an ordered state, and plays a role in regulating the expression of stress defense genes (Hu et al. 2013). The MpASR protein also showed a partially ordered structure and became less sensitive to proteolysis after binding with Zn^{2+} (Dai et al. 2011). Since CsASR1 is a typical IDP instead of a nucleus-located protein, it can help to protect the activity of LDH from damage under heat stress. Similar results were observed for MpASR from plantain (Dai et al. 2011). Hence, like MpASR and SlASR1, CsASR1 possibly functions as a chaperone-like protein to help plants adapt to abiotic stresses by protecting some stress-related proteins from inactivation under stress conditions.

In conclusion, we have cloned and functionally characterized an *ASR* gene named *CsASR1* from *C. sativus*. Our results show that CsASR1 protein might function as an IDP, and thus could protect *E. coli* under heat stress and increase abiotic stress resistance in *Arabidopsis* under salt stress by protecting some stress-related proteins from inactivation. Further studies are required to generate transgenic cucumber plants by silencing and overexpressing *CsASR1* in cucumber to further clarify its biological role and reveal its importance in tolerance against different stresses. Besides, the *CsASR1* gene has the potential to be used to improve the tolerance to abiotic stresses in plants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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