Note

Plant Biotechnology, 19 (4), 277-281 (2002)

Characterization and Expression of a Water Stress Responsive Gene from a Seashore Plant *Calystegia soldanella*

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Received 7 February 2002; accepted 25 June 2002

Abstract

We have isolated a cDNA clone encoding the 28.3 kDa polypeptide homologous to tomato ASR (abscisic acid, stress and ripening) proteins from *Calystegia soldanella*. The protein, designated CSASR (*C. soldanella* ASR), had not only homologous regions to ASR, but also a unique insert consisting of 139 amino acids containig eight repeating motifs of 11- to 12-mer amino acids near the N-terminus. The strongest expression of the CSASR gene was observed in anther, but no expression was found in the other parts of the flower in *C. soldanella*. The gene was also expressed in the leaf, but little in the stem or the root. Drought, NaCl, abscisic acid, and cold induced the gene expression in the shoot, but only drought and cold induced expression in the root. The findings suggested that CSASR might be involved in the reproduction and survival of *C. soldanella* in the seashore.

Accession number: AB047594

Key words: abscisic acid, ASR gene, *Calystegia soldanella*, seashore plant, water stress

Water stress is a major abiotic stress that decreases the viability of plants. Many plants respond to water stress at the developmental and physiological levels. A large number of genes are expressed by water stress, such as for a scavenging system of reactive oxygen species, biosynthetic enzymes of osmolytes, water/ion-channels, proteinases, signal transductions, LEA (late embryogenesis abundant) proteins (Shinozaki and Yamaguchi-Shinozaki, 1997), and ASR (abscisic acid, stress and ripening) proteins (Iusem *et al.*, 1993). The ASR gene named ASR1 was first identified in *Lycopersicon esculentum* (Iusem *et al.*, 1993). The accumulation of ASR1 transcripts and protein were detected predominantly in fruits and induced by drought, salt stress, and treatment with ABA (abscisic acid). Recently, ASR families have been isolated from *Solanum tuberosum* (van Berkel *et al.*, 1994), *Citrus maxima* (Canel *et al.*, 1995), *Pinus taeda* (Padmanabhan *et al.*, 1997), *Oryza sativa* (Vaidyanathan *et al.*, 1999), and *Lilium longiflorum* (Huang *et al.*, 2000). Most of these genes were also inducible by water deficit, salinity and exogenous ABA. ASRs contain bipartite nuclear localization signals (NLS) at the C-terminus. The *L. esculentum* ASR1 is known to be localized in the nuclei and bound to DNA in the Zn^{2+} dependent manner *in vitro* (Gilad *et al.*, 1997). It is likely that ASR may protect DNA in the nuclei from the water deficit.

Some plants choose their habitats in the sandy soil of seashores, where the complicated environmental factors assemble, such as desiccation, salinity, extreme temperature, intensive daylight and gale. Therefore, such plants can possess adaptive mechanisms to survive in the seashore. Despite this point of view, studies on their responses to environmental cues at the molecular levels have been demonstrated scarcely. Firstly, to elucidate the molecular mechanisms of stress tolerance in seashore plants, we tried to isolate water stress inducible genes from *Calystegia soldanella*, that is a common seashore plant distributed in temperate and subtropical regions of Asia including Japan, Europe, Australia, and America (Iwatsuki *et al.*, 1993). In this paper we report the isolation of the ASR gene from *C. soldanella* and expression patterns under the water stress. This is the first report to describe genetic analysis of *C. soldanella*.

C. soldanella growing at the sandy area of Ohya seashore in Shizuoka, Japan was used for the following experiments. Since *C. soldanella* makes several sizes of colonies in the sandy soil, we harvested the plants from one colony chosen (ca.

100 m^2) to obtain the samples growing under the same environmental conditions. The cDNA library was constructed from a whole plant harvested in June, 1999. In order to check tissue specific expression of the CSASR (*C. soldanella* ASR) gene, leaf, stem, root, flower, bud, and seed were obtained in June, 2000 and the separation of flower to organs was done in June, 2001. The indoor experiments of water stress were demonstrated by using whole plants obtained from September to October, 2000. Water stresses were treated as follows. For drought stress, plants were directly put on filter paper and incubated at 25 ℃ . During the incubation the water contents in the plants were measured and the degree of dehydration was monitored. Water content in shoot and root decreased linearly from 90 to 30% in 4 days and from 80 to 10% in 2 days, respectively. This showed that the treatment provided enough desiccation to plants. To chill plants, samples were transplanted to vermiculite, equilibrated with the deionized water, and placed in the chamber at 4 ℃ . Plants were treated with NaCl or ABA by hydroponics containing 200 mM NaCl or 50 μM ABA at 25 ℃ . Control plants were grown in wet vermiculite with deionized water. After sampling, plants were divided into shoot and root and kept at -80 ℃ until use for the RNA extraction.

Total RNA was extracted from whole plants of *C. soldanella* by the phenol/SDS method (Hussey and Hunsperger, 1996) and precipitated by LiCl. Poly(A)$^+$ RNA was isolated from the total RNA by a polyA Spin mRNA Isolation Kit (New England BioLabs, Tokyo, Japan). A cDNA library was constructed by using the cDNA Synthesis, ZAP-cDNA Synthesis and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, CA, USA) according to the manufacturer's instructions. Approximately 1.1 × 10^5 recombinant plaques were transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech, Tokyo, Japan) and screened by hybridization with the cDNA clone of citrus dehydrin (Hara *et al.*, 1999, 2001). The probe was labelled and detected by a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany). For the screening, hybridization was performed at 58 ℃ in the hybridization buffer; 5 × SSC (1 × SSC: 15 mM sodium citrate buffer containing 150 mM NaCl), 0.1% (w/v) N-lauroylsarcosine, 1% (w/v) blocking reagent, and 0.02% (w/v) SDS. The filters were washed twice in 2 × SSC-0.1% (w/v) SDS for 5 min at room temperature, then twice in 0.1%SSC-0.1% (w/v) SDS for 15 min at 58 ℃ . Positive plaques were isolated and converted into the pBluescript (SK) vector by an *in vivo* excision according to the Stratagene's instructions. Nucleotide sequencing of full length clone was performed using a SequiTherm EXCEL II Long-Read Premix DNA Sequencing Kit-LC (Epicentre Technologies, WI, USA) on a DNA sequencer (Model 4000L, Aloka, Tokyo, Japan), after the generation of a series of deleted plasmids. A computer search of DDBJ databases was done with the BLAST programs (Altschul *et al.*, 1990). Total RNA was fractionated by formaldehyde-agarose gel electrophoresis and blotted onto a Hybond-N^+ membrane. The membranes were hybridized with a DIG-labelled CSASR cDNA as a probe at 68 ℃ in the hybridization buffer. After hybridization, the blots were washed with 2 × SSC (1 × SSC: 15 mM sodium citrate buffer containing 150 mM NaCl) - 0.1% (w/v) SDS for 5 min at room temperature and 0.1 × SSC-0.1% (w/v) SDS for 15 min at 68 ℃ . Positive signals were visualized according to the instruction manual of the DIG Labeling and Detection Kit.

CSASR cDNA contains 1060 nucleotides and the 5' and 3' noncoding regions were 72 and 241 nucleic acids in length, respectively. The longest open reading frame encodes a predicted polypeptide of 248 amino acids, and the calculated molecular weight and isoelectric point were 28329 and 5.1, respectively. CSASR protein was rich in Glu (17%), Gly (13%) and Lys (12%) but lack in Cys and Trp. The hydrophilicity/hydrophobicity plot in the GENETYX-MAC software showed that the protein was predominantly hydrophilic (data not shown). These features of CSASR resembled those of other ASRs (Silhavy *et al.*, 1995, Padmanabhan *et al.*, 1997, Vaidyanathan *et al.*, 1999).

Fig. 1 showed the deduced amino acid alignment of the genes hit in the BLAST search due to the similarity to CSASR. The homologous regions of these proteins were localized in the two parts, *ca.* twenty amino acid residues in the N-terminus and *ca.* ninety residues in C-terminus. On the other hand, spacer sequences inserted between the two homologous areas were varied with plant species. There were eight repeating motifs, DDSYERKNTYG and its derivatives, in the spacer region of CSASR. The tandem repeats were also found in the DS2 from *Solanum chacoense* (Silhavy *et al.*, 1995). A bipartite NLS (Varagona *et al.*, 1992), KKETKEEEEEAEGKKKH, was present at the C-terminus of CSASR protein. This sequence is supposed to be an evidence that *L. esculentum* ASR1 was a nuclear protein (Gilad *et al.*, 1997).

The tissue specific expressions of the CSASR gene in *C. soldanella* were analysed in **Fig. 2**. Since *C. soldanella* plant contains much mucus which interferes with RNA extraction and Northern hy-

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CSASR     1:MSEEKHHH--LFGHHKDKEEERQPSYGENTYGSSDDSYERKNTYGDDSYEKKNTYGGDDS 58
DS2       1:----KHHFGGLFHHHKNEEEDTPVEKTTYEETTYGESEKTSTYGEKTSYGDDTYGEKTTT 56
ASR1      1:MEEEKHHHHHLFHHKDKAEEGPV----------------------------------- 23
CI21A     1:MEEEK-HHHHLFHHKDKEEEGPV----------------------------------- 22
LLA23     1:MAEE-HHKHHLFHHNKESNPEVVVSETNYITDGLNSTYSTSSDVYGGNQSQANYE----- 54
LP3       1:MSEEK-HHHHLLHHKKEDESENVPSEVVCAETTTAYGDEVIQSADVYAAGEVNDDKFAEY 59
OSASR1    1:MAEEK-HHHHLFHHKKDDEPATGVDSYGEGVYTSETVTTEVVAGGQDEYE---------- 49
                  *   *  *

CSASR    59:YERKNTYGEDSYEKKNTYGGDESYERKNTYGGDESYEKKNTYGGDESYERKNNYGDNNES 118
DS2      57:FGDDNKYGEKTSYGDDTYGEKPTSYGGDNTYGEKTSYGKGDDNKYGEKTSYGEGDDNKYG 116
ASR1     24:------------------------------------------------------------
CI21A    23:------------------------------------------------------------
LLA23    55:------------------------------------------------------------
LP3      60:E----------------------------------------------------------- 60
OSASR1   50:------------------------------------------------------------

CSASR   119:SYENKPTGYGKTSYGED-SYGGGQTDKYGSATGIE-EEGGRTHE---------------- 160
DS2     117:EKTSYGDSG----YGEKPSYGGGDDNKYGEKTSYGNEEGGYGGGVGETTNYEENESETKT 172
ASR1     24:------------------------------------------------------------
CI21A    23:------------------------------------------------------------
LLA23    55:------------------------------------------------------------
LP3      61:------------------------------------------------------------
OSASR1   50:------------------------------------------------------------

CSASR   161:--DYEKEKKHHKHLEELGGLGTVAAGAYALYEKHEAKKDPENAHRHKIEEEVAAAVAVGS 218
DS2     173:SEDYKEEKKHHKHLEEIGGLGAVAAGAFALHEKHKAEKDPENAHKHKIEEGIAAAAAIGA 232
ASR1     24:--DYEKEIKHHKHLEQIGKLGTVAAGAYALHEKHEAKKDPEHAHKHKIEEEIAAAAAVGA 81
CI21A    23:--DYEKETKHHKHLEQIGKLGTVAAGAYALHEKHEAKKDPEHAHKHKIEEEIAAAAAVGA 80
LLA23    55:--DYEKEKKHIKHKEHLGEMATAGAGAFALYEKHEAKKDPEHAHRHKLEEEIAAAAAAGA 112
LP3      61:--KARKEEKHHKHLEELGGLGTVAAGAFALHEKHASKKDPENAHRHKIEEEIAAAAAVGA 118
OSASR1   50:--RYKKEEKQHKHKQHLGEAGALAAGAFALYEKHEAKKDPENAHRHKITEEIAATAAVGA 107
                * *  **    *     *** ** ***   **** ** **  *  **  * *

CSASR   219:GGFAFHEHHEKKETKEEEEEAEGKKKHHFF                               248
DS2     233:GGFAFHEHHEKKEAKEEEEEAEGKKKHHFFN                              263
ASR1     82:GGFAFHEHHEKKDAKKEEKKKLRGDTTISSKLLF                           115
CI21A    81:GGFALHEHHEKKDAKKEQKKAEGGHHHLF                                109
LLA23   113:GGYTFHEHHEKKTLKKENEEVEGKKHHFFG                               142
LP3     119:GGYVFHEHHEKKESKEEEKEAEGKKHHHLFYVRCH                          153
OSASR1  108:GGYAFHEHHEKKKDHKSAEESTGEKKHHLFG                              138
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Fig. 1 Comparison of deduced amino acid sequences of the ASR proteins. CSASR: *Calystegia soldanella* (present study), DS2: *Solanum chacoense*, ASR1: *Lycopersicon esculentum*, CI21A: *Solanum tuberosum*, LLA23: *Lilium longiflorum*, LP3: *Pinus taeda*, and OSASR1: *Oryza sativa*. Asterisks shows the identical amino acids among the seven polypeptides. Eight arrows on the sequence of CSASR indicates tandem repeating motifs. A bar shows the NLS sequence.

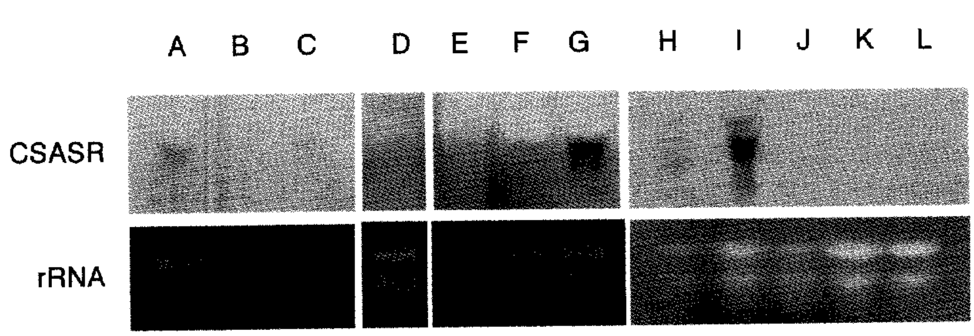

Fig. 2 Expression of CSASR gene in the different tissues of *C. soldanella*. A: leaf, B: stem, C: root, D: mature seed, E: immature seed, F: flower bud, G: open flower, H: pistil, I: anther, J: filament, K: ovary, and L: petal. Each lane contains 15 μg total RNA. The RNA blots were hybridized with DIG-labelled CSASR cDNA.

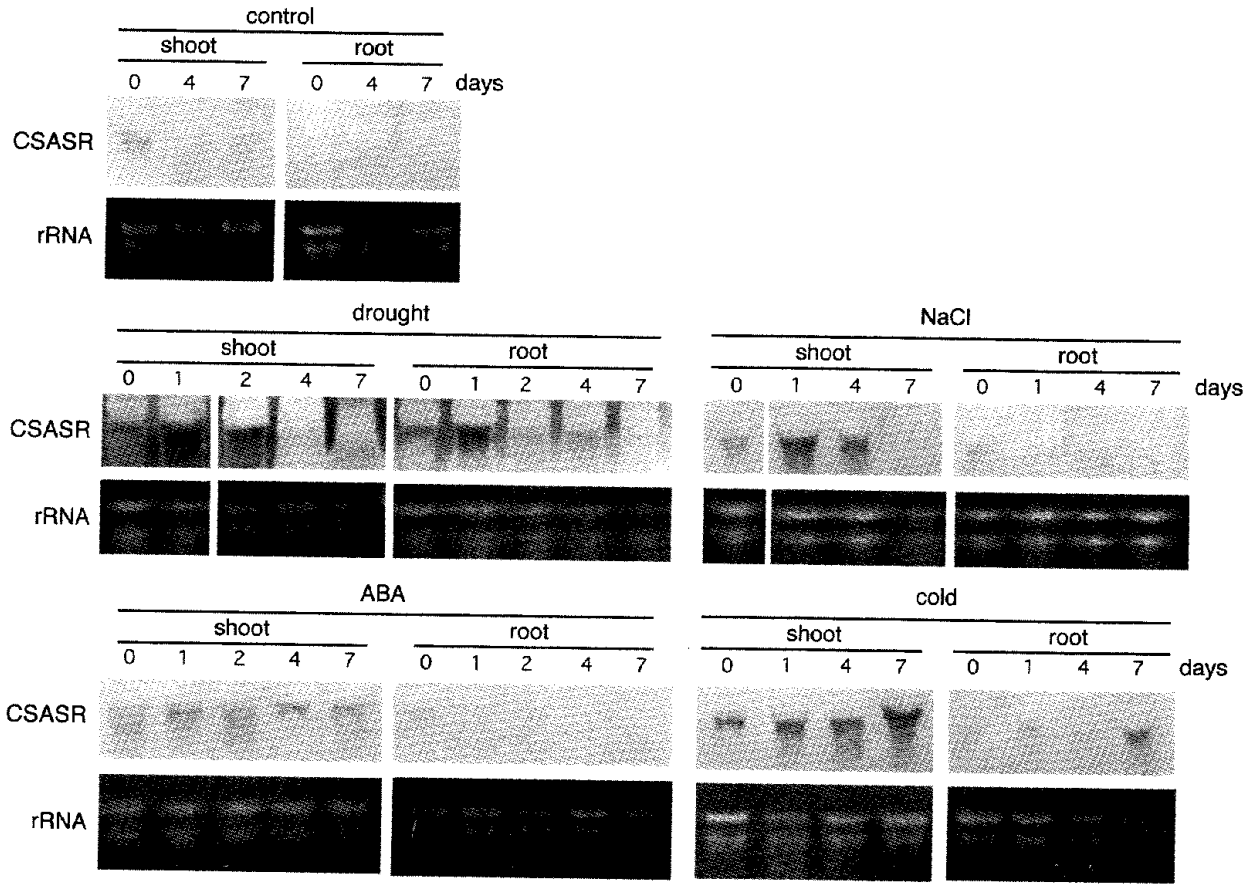

Fig. 3 Effects of stimuli (drought, NaCl, ABA, and cold) on the accumulation of CSASR transcripts in shoot or root of *C. soldanella*. Total RNA was extracted from shoot or root after the number of days of incubation shown in the figure. Each lane contains 15 μg total RNA. The RNA blots were hybridized with DIG-labelled CSASR cDNA. In each treatment, the hybridization and the signal detection were performed under the same conditions.

bridization, several lanes smeared in **Fig. 2** and **3**. The main organ expressing the CSASR gene was the flower. We found that the strong expression was indicated only in an open flower, but no expression was indicated in a flower bud or a seed. When the open flower was separated into parts, the transcripts were present only in the anther. Similar results were reported in LLA23, an ASR gene from *L. longiflorum*, which was expressed only in the later stage of pollen maturation (Huang *et al.*, 2000). It is likely that the expression in pollen may be a common nature in ASR families.

In the vegetative organs, the CSASR gene was somewhat expressed in a leaf, but not in a stem or a root. The expression in the shoot containig leaf and stem, however, was remarkably induced by drought, NaCl, exogenous ABA and cold (**Fig. 3**). The expression in root responded only by drought and cold, and the degree of expression was relatively low.

The time courses of CSASR gene expressions were classified into two types, the transient expressions found in the drought stress in shoot and root, and the NaCl stress in shoot, and also the continuous expressions in the ABA treatment in shoot and the cold stress in shoot and root. The data showed that there were at least two independent pathways in the signal transductions for the gene expression of CSASR in *C. soldanella*.

CSASR was shown to have two features as the anther protein and the water stress inducible protein. Thus, CSASR is supposed to be an important protein not only to accomplish pollination but also to protect desiccated cells when *C. soldanella* lives at the seashore. Further research such as transgenic analyses controlling CSASR gene expression will elucidate functions of the gene in plants.

Acknowledgement

This study was supported in part by a Grant-in-Aid (No. 12460078) for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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