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Analyses of Nine cDNAs for Salt-Inducible Gene in the Halophyte Sea Aster (*Aster tripolium* L.)

Migiwa TAKEDA[1]*, Yuichi UNO[2]*, Michio KANECHI[1] and Noboru INAGAKI[1]

[1]*Graduate School of Science and Technology, Kobe University, Kobe 657-8501, Japan*
[2]*Faculty of Agriculture, Kobe University, Kobe, Japan*
**Corresponding author* E-mail address: migiwat@hotmail.com and yuno@kobe-u.ac.jp

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Abstract

In order to understand responses of the halophyte sea aster (*Aster tripolium* L.) to salt stress at the molecular level, we used a differential screening strategy. Nine cDNAs of genes induced by salt stress were cloned by this method from sea aster. The clones were collectively named SASR (sea aster stress responsive). These cDNAs were mainly associated with osmoprotectant synthesis, protein metabolism, protein degradation or signal transduction. All the mRNAs corresponding to the SASR genes were accumulated under high salt conditions and the transcripts of 3 out of 9 genes were induced by osmotic stress.

Key words: Gene expression, Mannitol, NaCl stress, Sea aster (*Aster tripolium* L.).

Abbreviations

ATMPK3, *Arabidopsis thaliana* mitogen-activated protein kinase 3; SASR, sea aster stress responsive.

Salt stress has one of the most severe effect on the productivity of plants, by both toxicity of some ions and dehydration, similar to osmotic stress. The halophytes, actually growing in saline environment, have strong salt tolerance and compensations for such conditions at morphological and physiological levels (Orcutt and Nilsen, 1996). There is a possibility that halophyte has an advanced gene resources that are more useful for transgenic improvements than those of glycophyte. Some halophytes exclude Na^+ and Cl^- through glands and bladders, which are specialized structures. However, the halophyte sea aster (*Aster tolipolium* L.) does not have such morphological features (Flowers *et al.*, 1977) and still accumulates salt in saline habitant. The growth of sea aster was not inhibited by 150 mM but 300 mM NaCl (Uno *et al.*, 1996). Sea aster may utilize the Na^+ for the osmoregulation to decrease leaf osmotic potential under NaCl stress (Matsumura *et al.*, 1998). These suggest that sea aster seems to adapt to the saline environment at organization and cellular level. This adaptation mechanism is supposed to be mainly based on the control of gene expression. It has been reported that this plant expresses SAMIP (sea aster major intrinsic protein) genes encoding the aquaporin during the salt stress period (Uno *et al.*, 1998). However, there is no research that widely examined the gene expression in response to the salt stress in sea aster. Because salt tolerance is a complex trait involving: responses to cellular osmotic and ionic stresses, and their consequent secondary stresses (e.g. oxidative stress) and whole plant coordination (Zhu, 2000). It is necessary to search extensively the multiple genes that are concerned to salt stress with complexity. In this study, in order to understand responses of sea asters to salt stress at the molecular level, we used a differential screening strategy to isolate cDNA clones whose encoded proteins are differentially expressed in sea aster plants treated with 400 mM NaCl. We described the nature of the gene products and their expression under the NaCl and osmotic stresses in sea aster.

Seeds of sea aster were collected from plants at the mouth of the Sakata River in the Aioi Bay area, Hyogo Prefecture, Japan. Plants were grown from seeds on a vermiculite bed and irrigated with 0.1% Hyponex (N:P:K=1:2:1, Hyponex, Osaka, Japan) under illumination of approximately 200 μmol m^{-2} s^{-1} at 22℃ with a day/night cycle of 16/8 h in a chamber. Two-weeks-old plants were transferred to hydroponic conditions with 0.1% Hyponex. After 10 days from hydroculture,plants were treated with 400 or 0 mM NaCl. As an osmotic stress treatment,

plants were treated hydroponically with 600 mM mannitol to produce an osmotic stress equivalent to 400 mM NaCl. Poly(A)$^+$ RNA was purified from twenty grams of whole plants as described by Sambrook *et al.* (1989). A cDNA library was constructed with ZAP-cDNA Synthesis Kit (Stratagene, CA, U.S.A.). The salt stress cDNA library contained 6.4×10^6 pfu μg^{-1} cDNA. The double-stranded cDNAs were synthesized from poly(A)$^+$ RNA prepared from sea aster that had been harvested with or without exposure to 10 h of 400 mM NaCl, using the TimeSaver cDNA Synthesis Kit (Amersham Pharmacia Biotech, Aylesbury, UK). Three hundred thousand plaques were plated, transferred onto nylon filters, and differentially screened with [^{32}P]-labeled synthesized cDNAs. Hybridization was performed according to the method of Sambrook *et al.* (1989). The putative salt stress specific cDNA clones were sequenced using an ABI PRISM 310 Genetic Analyzer (ABI, San Jose, CA). The obtained sequences were compared with all known DNA sequences using the BLAST search programs on the network (NCBI; http://www.ncbi.nlm.nih.gov/blast/). Hybridization of RNA and DNA were performed as previously described (Uno *et al.*, 1998). Twenty μg of genomic DNA were digested to completion with *Eco*RI, *Hind* III or *Xho*I. The digestion products were fractionated in a 1% agarose gel, and blotted onto a nylon filter. Hybridization was carried out in the presence of ^{32}P labeled probes. For Northern hybridization as salt or osmotic stress treatments, plants were treated hydroponically with either 400 mM NaCl or 600 mM mannitol (equal to osmotic pressure for 400 mM NaCl) for 0, 1, 2, 5, 10, and 24 h. As controls, whole plants were subjected to nutrient water treatment. Forty μg of total RNA per lane were subjected to electrophoresis in a 1% agarose gel and blotted onto a nylon membrane, and the membrane was hybridized using ^{32}P labeled each SASR probe. The autoradiogram was scanned, and quantitative analysis was carried out with a public domain program NIH Image 1.62 (developed at the U.S. National Institutes of Health).

Although sea aster plants seemed to be wilted after the treatment with 400 mM NaCl for 10 h, they recovered their growth within 3 h (data not shown). The library was differentially screened using cDNAs prepared from normal (control cDNA) and treated plants (stress cDNA). The plaques produced a stronger hybridization signal with stress cDNA. The inserted DNA fragment from each phage clone was in vivo excised and used as a probe for Northern hybridization to confirm that it was ex-

Table 1 Characteristics of differentially expressed cDNA clones from sea aster plants treated with 400 mM NaCl.

Name	Accession number[1)]	Number of clones obtained	Variety of insert size[2)]	Insert size (bp)[3)]	Number of genes[4)]	Homology			References[6)]
						Annotation	Source	(%)[5)]	
SASR1	AB090878	2	2	819	4	Polyubiquitin	*Lycopersicon esculentum*	100	Christensen *et al.*, 1992
SASR20	AB090879	1	1	1596	2	Laccase	*Arabidopsis thaliana*	56	Wei *et al.*, 2000
SASR21	AB090880	2	2	910	1	Cysteine protease	*Ipomea batatas*	78	Koizumi *et al.*, 1993
SASR31	AB090881	1	1	1583	2	serine/threonine/tyrosine-specific protein kinase	*Arabidopsis thaliana*	78	Liu *et al.*, 2000
SASR45	AB090882	2	2	580	3	Metallothionein	*Mimulus guttatus*	69	Hsieh *et al.*, 1996 Oztur *et al.*, 2002
SASR50	AB090883	3	3	1870	2	Phosphoethanolamine N-methyltransferase	*Spinacia oleracea*	78	Nuccio *et al.*, 2000
SASR57	AB090884	9	6	955	1	C-4 sterol methyl oxidase	*Arabidopsis thaliana* (Accession No. AY087876)	60	
SASR69	AB090885	1	1	979	2	Dehydrin	*Helianthus annuus*	67	Masmoudi *et al.*, 2001
SASR81	AB090886	1	1	1372	1	Myo-inositol-1-phosphate synthase	*Nicotiana tabacum*	93	Ishitani *et al.*, 1996

[1)]The nucleotide sequences of cDNAs appear in the EMBL, GenBank, and DDBJ databases.

[2)]The number of insert that has different size among the obtained clones.

[3)]The longest size among the obtained clones.

[4)]Number of genes in the sea aster genome were estimated by genomic Southern blot analysis (Data not shown).

[5)]Homology percent was cited from the result of the BLAST search program and indicates a local homology between two proteins.

[6)]Previous reports of the cDNA homologue related to stress response, except for SASR57.

pressed differentially between the normal and stressed plants. Finally, 22 clones were selected and classified into 9 non-redundant SASR (sea aster stress responsive) groups of cDNAs after sequence and homology searches (**Table 1**). Some clones have a varied size of inserts. This result suggests that redundancy of the clones was not mainly caused by amplification but by abundance of cDNAs in the library. Gel blots of genomic DNA hybridized with radioactively-labeled SASR cDNA probes detected one to four major bands in DNA samples digested with *Eco*RI, *Hind*III or *Xho*I in each SASR (data not shown). It seemed that there must be at least one to four copies of each SASR sequence in the sea aster genome (**Table 1**). Identified cDNAs were mainly associated with osmoprotectant synthesis (SASR81), protein metabolism (SASR20, 50 and 57), protein degradation (SASR1 and 21) or signal transduction (SASR31). To our knowledge, it has been reported that eight of the SASR genes were stress-induced, especially SASR1, 20, 21, 31, 45, 50, 69 and 81 were transcriptionally upregulated during salt stress (references in **Table 1**).

We found that all the SASR genes were induced and accumulated under high-salt conditions, as time passed (**Fig. 1**). The level of mRNAs corresponding to the SASR45 began to accumulate after 2 h of salt stress, and those corresponding to the other eight SASR were gradually accumulated until 24 h after the onset of salt stress. The quantitative analysis was carried out with NIH Image, and the levels of mRNAs corresponding to each SASR in 24 h-NaCl stressed plants were increased 2-to 15-

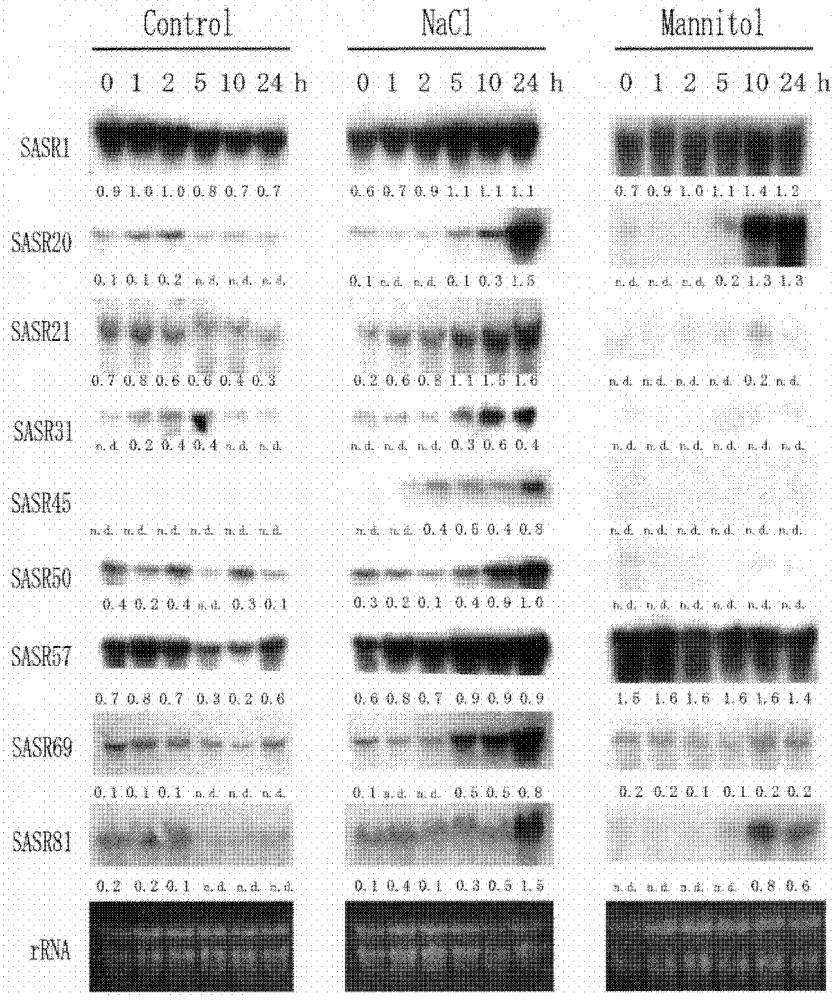

Fig. 1 Northern blot analysis of the SASR clones hybridized to total RNA from 2-weeks-old sea aster plants in response to isotonic treatment using 400 mM NaCl and 600 mM mannitol. Untreated samples were served as the control (Control). The plants were harvested at the indicated time intervals. A RNA blot containing 40 μg of RNA per lane was subjected to hybridization with the indicated probes. In each treatment, the hybridization and signal detection were performed under the same conditions. Ethidium bromide staining (rRNA) was used as a control for RNA loading. The autoradiogram was scanned, and the quantitative analysis was carried out with NIH Image. The relative expression ratios are indicated below the RNA gel blots. n.d., not determined.

fold compared to the levels in the pretreated (0 h-NaCl stressed) plants. In contrast, the level of mRNA from unstressed plants were low or began to decrease, as time passed. These observations indicate that these genes responded naturally to salt stress. While SASR gene under mannitol were classified into 3 groups. As shown in **Fig. 1**, the mRNAs corresponding to the SASR1, 57 and 69 were detectable all the time, and the level of these transcripts changed little over the 24 h period after mannitol treatment. Those corresponding to the SASR20, 21 and 81 were induced after 10 h of osmotic stress, especially the high levels from the SASR20 and 81 remained until 24 h. Those corresponding to the SASR31, 45 and 50 were undetectable.

The SASR1 gene has three tandem head-to-tail repeats of 228 bp encoding an ubiquitin monomer. Ubiquitin is thought to be primarily involved in the formation of conjugates with other proteins and signalling their selection under heat shock and other stresses (Finley *et al.*, 1987). The SASR1 gene was induced strongly by both salt and osmotic stresses (**Fig. 1**). As there are more than four homologs for SASR1 genes in sea aster (**Table 1**), this induction may indicates the simultaneous expression patterns of these genes. However, it is clear that at least one ubiquitin mRNA is accumulated during the stress period in sea aster.

The cDNA sequence of SASR20 has similarity to that of the gene encoding laccases (p-diphenol:O_2 oxidoreductase), which oxidise phenolic substrates using oxygen as the electron acceptor. In plants, the only function that has been proposed for laccases is their putative involvement in the oxidative polymerisation of phenolic compounds in lignin biosynthesis. This gene is also cloned by differential display-polymerase chain reaction method and identified as a salt-inducible gene in tomato (Wei *et al.*, 2000). SASR20 was induced by both salt and osmotic changes (**Fig. 1**) and may function as laccase concerning with lignin biosynthesis to strengthen the cell wall during the stress period.

The nucleotide sequence of the partial cDNA SASR21 contained a single open reading frame that encoded 232 amino acids. The protein encoded by SASR21 contains a putative conserved domain for cysteine protease at the C-terminus (from 110 to 232). The pea 15a genes that encode cysteine protease have been shown to respond to environmental stresses, such as cold or water deficiency (Guerrero *et al.*, 1990). It has been reported that osmotic changes caused by dehydration or high-salt conditions may trigger the induction of such genes (Koizumi *et al.*, 1993). SASR21 was also induced by osmotic change (**Fig. 1**). Cysteine protease could be involved in the degradation of vegetative storage proteins located in vacuoles, allowing the products of such degradation to be available for the synthesis of new proteins during stress or for osmotic adjustment, as suggested by Guerrero *et al.* (1990).

The SASR31 protein exhibited sequence homology to Arabidopsis protein kinase 1 (APK1), which has the activity to phosphorylate not only serine/threonine but also tyrosine (Hirayama and Oka, 1992). APK1 has the structural mosaic that the subdomains VI and VIII are close to serine/threonine kinases while the subdomain IX is to tyrosine kinase. SASR31 also has the same structure as that of APK1 (data not shown) and may have a similar kinase activity. It is unknown that an mRNA encoding APK1 accumulates during the stress period. It has been reported that the levels of transcripts for other serine/threonine kinases, which has weaker homology with SASR31 than APK1 does, increased by some stresses. The SOS2 (Salt Overly Sensitive 2) gene is induced by salt stress in the root (Liu *et al.*, 2000). The ATMPK3 (*Arabidopsis thaliana* mitogen-activated protein kinase 3) gene was increased markedly when plants were treated with touch stimuli, low temperature and salinity stress (Mizoguchi *et al.*, 1996). The transcription of SASR31 mRNAs was not induced under osmotic stress but salt stress. This indicates that the expression of SASR31 may not be triggered by the osmotic change but by the ionic change in sea aster.

At the amino acid level, SASR45 protein shared 69% identity with the metallothionein I homologue from *Mimulus guttatus* (de Miranda *et al.*, 1990). The metallothionein, a low molecular weight, cysteine-rich metal binding protein was isolated from animals, fungi, and plants, and be induced by hormones, heavy metals, and stress conditions (Zhou and Goldsbrough, 1994; Hsieh *et al.*, 1996; Foley *et al.*, 1997). The transcription of SASR45 mRNAs was induced under salinity conditions and the metallothionein encoding transcripts are also upregulated by salt and drought stress in barley (Oztur *et al.*, 2002). Its function with up-regulation under salt stress is still unknown.

The protein encoded by SASR50 exhibits homology to a phosphoethanolamine N-methyltransferase (PEAMT). The deduced amino acid sequence of the SASR50 contains two potential SAM-binding domains that are involved in preserving the methyl donor for each methylation step (data not shown). The N-methylation of phosphoethanolamine is the committing step in choline biogenesis in plants and PEAMT catalyzes the first N-methylation of phosphoethanolamine. The salinized spinach plants in-

creased PEAMT mRNA and the enzyme activity in leaves by about 10-fold, consistent with the high demand in stressed plants for choline to support glycinebetaine synthesis (Nuccio *et al.*, 2000). Sea aster PEAMT may also has some role in production of glycinebetaine as a compatible solute during the salt stress.

SASR57 was the most abundant clone among the 9 cDNAs we obtained. SASR57 protein has a conserved domain for sterol desaturase. This family includes C-5 sterol desaturase and C-4 sterol methyl oxidase that are involved in cholesterol biosynthesis and plant cuticular wax biosynthesis (Aarts *et al.*, 1995). The sterol methyl oxidase performs the first of three enzymic steps required to remove the two C-4 methyl groups leading to cholesterol (animal), ergosterol (fungal), and stigmasterol (plant) biosynthesis (Bard *et al.*, 1996). Northern hybridization analysis revealed that the transcription of SASR57 mRNAs was strongly induced by both salt and osmotic stress (**Fig. 1**). The observation that stress alters the level of the C-4 sterol methyl oxidase transcripts, as far as we know, has not been made before.

SASR69 protein was homologous to dehydrin protein (group 2 LEA protein). Group 2 LEA proteins usually contain a conserved sequence, DEYGN, at the N-terminnus, a cluster of serine residues, and a lysine-rich conserved sequence, KIKEKLPG. SASR69 protein contained all these motifs (data not shown). High levels of dehydrin transcripts have been observed in salt-stressed plants (Masmoudi *et al.*, 2001). They suggested that dehydrins may stabilize macromolecules through detergent and chaperone-like properties and may act synergistically with compatible solutes. Dehydrin would protect cytosolic structures from the deleterious effects of cellular dehydration (Cellier *et al.*, 1998). SASR69 might also function in protecting sea aster cells from osmotic stress with a tolerance mechanism leading to the maintenance of cellular turgor.

The cDNA sequence of SASR81 has a similarity to that of the gene encoding myo-inositol 1-phosphate (Ins-1P) synthase, which catalyzes the reaction from glucose 6-phosphate to Ins-1P in the pathway of inositol biosynthesis. In *Mesembryanthemum crystallinum*, Ins-1P synthase, which is induced by salt stress, was suggested to have a role in the production of osmoprotectants that are methylated derivatives of Ins-1P (Ishitani *et al.*, 1996). The transcription of SASR81 mRNAs was induced under both high-salt and high-mannitol conditions, suggesting it's relation to the production of osmoprotectants.

We expected that the sea aster might have the salt-responsive genes that are specific to the halophyte. However, most of SASR genes showed the same response as those of glycophytes. According to the study with the microarray, although the number of regulated genes of a glycophyte increases transiently within 10 h, a halophyte does gradually until 70 h after treatment with NaCl (Kawasaki *et al.*, 2001; Ozturk *et al.*, 2002; H.J. Bohnert and J.C. Cushman personal communication). The 10 h treatment used in this study might not be enough to induce a number of genes that associate with salt stress in sea aster. Otherwise the halophytes express the similar genes as those of the glycophytes and utilize the post-transcriptional modification system for their salt tolerance.

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