Similar Regulation Patterns of Choline Monooxygenase, Phosphoethanolamine N-Methyltransferase and S-Adenosyl-L-Methionine Synthetase in Leaves of the Halophyte Atriplex nummularia L.

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Glycinebetaine (betaine) highly accumulates as a compatible solute in certain plants and has been considered to play a role in the protection from salt stress. The betaine biosynthesis pathway of betaine-accumulating plants involves choline monoxygenase (CMO) as the key enzyme and phosphoethanolamine N-methyltransferase (PEAMT), which require S-adenosyl-L-methionine (SAM) as a methyl donor. SAM is synthesized by SAM synthetase (SAMS), and is needed not only for betaine synthesis but also for the synthesis of other compounds, especially lignin. We cloned CMO, PEAMT and SAMS isogenes from a halophyte Atriplex nummularia L. (Chenopodiaceae). The transcript and protein levels of CMO were much higher in leaves and stems than in roots, suggesting that betaine is synthesized mainly in the shoot. The regulation patterns of transcripts for SAMS and PEAMT highly resembled that of CMO in the leaves during and after relief from salt stress, and on a diurnal rhythm. In the leaves, the betaine content was increased but the lignin content was not changed by salt stress. These results suggest that the transcript levels of SAMS are co-regulated with those of PEAMT and CMO to supply SAM for betaine synthesis in the leaves.

Keywords: Atriplex nummularia — Choline monoxygenase — Glycinebetaine — Phosphoethanolamine N-methyltransferase — S-adenosyl-L-methionine synthetase — Salt stress.

Abbreviations: AnCMO, Atriplex nummularia CMO; AnPEAMT, A. nummularia PEAMT; AnSAMS, A. nummularia SAMS; betaine, glycinebetaine; CMO, choline monoxygenase; COMT, caffeic acid 3-O-methyltransferase; ORF, open reading frame; PEAMT, phosphoethanolamine N-methyltransferase; SAM, S-adenosyl-L-methionine; SAMS, SAM synthetase.

The nucleotide sequences reported in this paper has been submitted to DDBJ under accession numbers AB183561 (AnSAMS1), AB183562 (AnSAMS2), AB183563 (AnSAMS3), AB183564 (AnSAMS4), AB183565 (AnSAMS5), AB112481 (AnCMO), AB196771 (AnPEAMT) and AB112477 (AnLFP).

Introduction

Halophytes can accumulate high levels of ions and organic solutes in the cells to reduce the water potential, so that they can grow in soil with high concentrations of NaCl (Flowers et al. 1977). Since enzymes in salt-adapted halophytes are Na+ sensitive, they accumulate compatible solutes, which hardly inhibit the enzymic activities, in the cytoplasmic compartment and Na+ in vacuoles in order to regulate turgor (Jacoby 1999). Chenopods, which include many halophyte species, accumulate glycinebetaine (N,N,N-trimethylglycine; hereafter betaine) in the cytoplasmic compartment and NaCl in vacuoles to reduce the water potential especially under salt stress (Flowers et al. 1977, Storey and Wyn Jones 1977, Coughlan and Wyn Jones 1980, Leigh et al. 1981, Matoh et al. 1987).

Betaine is synthesized by two-step oxidation of choline via betaine aldehyde by choline monoxygenase (CMO) and betaine aldehyde dehydrogenase in betaine-accumulating plants (Hanson and Hitz 1982, Sakamoto and Murata 2000), and both their mRNAs and proteins accumulate under salt stress in chenopods (Weretilnyk and Hanson 1989, McCue and Hanson 1992, Russell et al. 1998, Shen et al. 2002). The choline precursor, phosphocholine, is synthesized by phosphoethanolamine N-methyltransferase (PEAMT), which catalyzes successive S-adenosyl-L-methionine (SAM)-dependent N-methylations of phosphoethanolamine (Smith et al. 2000). The activity of PEAMT is also up-regulated by salt stress in spinach and sugar beet (Summers and Weretilnyk 1993, Weretilnyk et al. 2001). S-Adenosylhomocysteine derived from SAM after transmethylation is used for SAM resynthesis via homocysteine and methionine, and the resynthesis pathway involves S-adenosylhomocysteine hydrolase and adenosine kinase. The two enzyme activities in spinach and sugar beet increased under salt stress, but not in non-accumulators of betaine, tobacco and canola (Weretilnyk et al. 2001). Thus, these betaine accumulators may increase the consumption of SAM for betaine synthesis under salt stress.

SAM is synthesized from methionine and ATP by SAM synthetase (SAMS; EC 2.5.1.6). The SAMS gene is considered to be a housekeeping gene because SAM is an essential substance for living cells as a methyl group donor and as a precursor of ethylene, polyamines and nicotianamine (Tabor and Tabor 1984, Moffatt and Weretilnyk 2001). Many housekeeping genes are expressed constantly, but mRNA levels of SAMS genes in Arabidopsis thaliana are about 20 times higher in

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stems and roots than in leaves (Peleman et al. 1989a, Peleman et al. 1989b). The expression analysis of β-glucuronidase genes under the control of SAMS gene promoters indicated that SAMS genes in Arabidopsis seem to be expressed in lignifying tissues (Peleman et al. 1989a, Peleman et al. 1989b). SAMS in elicitor-treated cells of alfalfa is co-induced with caffeic acid 3-O-methyltransferase (COMT), which is a key enzyme of lignin biosynthesis, and the expression pattern of SAMS is similar to that of COMT at various developmental stages in different organs of alfalfa (Gowri et al. 1991). These studies suggest that lignification consumes a relatively large amount of SAM as compared with other biological reactions in certain tissues of some plants. The SAMS mRNA levels in tomato and Catharanthus roseus are increased by salt stress (Espartero et al. 1994, Schröder et al. 1997), and Espartero et al. (1994) discussed the effect of SAM in relation to cell wall synthesis and cell modification such as lignification.

Enzymes synthesizing betaine from choline have been introduced into non-betaine-accumulating plants by molecular genetic manipulation, and some of the transgenic plants had higher tolerance against various environmental stresses than the wild type (Sakamoto and Murata 2000, Shen et al. 2002). However, the amount of betaine they accumulated was much less (up to about 5 μmol (g FW)^{-1}; Sakamoto and Murata 2000) than that accumulated in many salt-adapted chenopods (>30 μmol (g FW)^{-1}; Storey and Wyn Jones 1977, Coughlan and Wyn Jones 1980, McCue and Hanson 1992). Since choline synthesis is a limiting factor of betaine synthesis in transgenic plants such as Arabidopsis, Brassica napus and tobacco (Nuccio et al. 1998, Nuccio et al. 2000a, Huang et al. 2000), chenopods seem to have an efficient mechanism for the synthesis of choline and probably its precursors. By examining the mechanism, we may promote the transgenic approaches to enhance the stress tolerance in plants.

We searched for salt-induced genes by differential screening to examine the tolerance of the halophyte Atriplex nummularia L. (Chenopodiaceae) against salt stress, and found SAMS and CMO genes. Other SAMS isogenes and PEAMT gene were obtained by screening a cDNA library. In this study, we show that the regulation patterns of mRNAs for SAMSs and PEAMT highly resembled that for CMO in the leaves and discuss the roles and the regulation system of SAMSs and PEAMT in A. nummularia.

Results

Cloning of CMO, SAMS and PEAMT genes

A thousand clones were picked up at random from the full-length cDNA library constructed from the salt-treated shoot of A. nummularia, and arrayed onto membranes. The clones were differentially screened with probes made from salt-stressed and control leaves of A. nummularia. Some clones were obtained by the differential screening.

The deduced amino acid sequence of one clone showed high identity to SAMSs of various plants such as tomato SAM1 (92.9%; Espartero et al. 1994), rice SAMS (92.4%; Van Breusegem et al. 1994) and Arabidopsis sam-1 (90.1%; Peleman et al. 1989a). Therefore, the cDNA seemed to encode SAMS, and so this gene was named AnSAMS1 (Atriplex nummularia SAMS 1). The soluble protein extracted from His-tagged AnSAMS1 expressing M15[pREP4] cells had 84-fold the SAMS activity of that from the non-transformed M15[pREP4] cells (15.4 and 0.184 nmol min^{-1} mg^{-1}, respectively), and SAMS activity of purified His-tagged AnSAMS1 (109 nmol min^{-1} mg^{-1}) was much higher than that of the extracted total proteins. These results indicate that this protein has SAMS activity.

The deduced amino acid sequence of another clone had high sequence identity to CMOs of some plants such as Atriplex hortensis (97.7%; Shen et al. 2002), Atriplex prostrata (95.9%; Wang and Showalter 2004), spinach (82.9%; Rathinasabapathi et al. 1997) and sugar beet (73.9%; Russell et al. 1998). This deduced amino acid sequence contained the sequence of chloroplastic-targeting peptides, the conserved sequence of the Rieske-type [2Fe–2S] cluster and the conserved motif for the mononuclear Fe-binding domain like other CMOs (Rathinasabapathi et al. 1997, Russell et al. 1998, Shen et al. 2002). Thus, the gene was considered to code for CMO and was named AnCMO (Atriplex nummularia CMO).

We screened the cDNA library using the PEAMT gene of spinach as a probe, and obtained a clone, whose deduced amino acid sequence had high sequence identity to spinach PEAMT (92.0%; Nuccio et al. 2000b). The gene was considered to code for PEAMT and was named AnPEAMT (Atriplex nummularia PEAMT).

SAMS, CMO and PEAMT gene families in A. nummularia

DNA gel blots were performed on genomic DNAs from A. nummularia, Atriplex lentiformis and spinach, using the AnSAMS1 open reading frame (ORF) fragment as a probe (Fig. 1). There is not an EcoRI or XbaI site in the ORF of AnSAMS1 cDNA. Four or five genomic DNA fragments from A. nummularia, but only one or two fragments from A. lentiformis or spinach were detected under a high stringency condition (Fig. 1). Under a low stringency condition, the other three or four fragments from A. nummularia and the other one or two fragments from A. lentiformis or spinach were detected (Fig. 1). These results indicate that these chenopods would have some SAMS isogenes.

To estimate the expression rates of these SAMS isogenes in A. nummularia, we screened the above cDNA library with the ORF fragment of AnSAMS1 as a probe with the same low stringency condition. Twenty-one positive cDNA clones were obtained by screening about 2,000 plaques of the above cDNA library. These clones encoded AnSAMS1 (three clones) or four other genes named AnSAMS2 (11 clones), AnSAMS3 (three clones), AnSAMS4 (three clones) and AnSAMS5 (one clone).
The molecular organization of CMO genes in these chenopods was also determined by DNA gel blotting, using the AnCMO ORF fragment as a probe (Fig. 1). Under a low stringency condition, the probe hybridized to several DNA fragments from spinach when genomic DNA was digested with EcoRI, and to one DNA fragment when digested with XbaI (Fig. 1). Since there are three EcoRI and no XbaI sites in the ORF of spinach CMO (accession number: U85780), the...
hybridization pattern suggests the presence of one gene encoding CMO in the spinach genome. Under a low stringency condition, a large number of genomic DNA fragments from *A. nummularia* were found, whereas only two or three fragments from *A. lentiformis* were detected (Fig. 1). These hybridization patterns of *A. nummularia* and *A. lentiformis* genomic DNAs were almost the same as those under a high stringency condition (Fig. 1). These results suggest that the AnCMO gene is highly duplicated in the *A. nummularia* genome.

The genomic organization of the PEAMT gene in *A. nummularia* was also determined, using the AnPEAMT ORF fragment as a probe (Fig. 1). Under low and high stringency conditions, the probe hybridized to four DNA fragments from *A. nummularia* when genomic DNA was digested with *Xba*I (Fig. 1). Since there is no *Xba*I site in the ORF of AnPEAMT cDNA, AnPEAMT may have some copies in the *A. nummularia* genome, but the other possibility exists.

### Tissue-specific expression levels of SAMS isogenes, AnCMO and AnPEAMT

We analyzed the tissue-specific expression levels of SAMS isogenes, AnCMO and AnPEAMT in *A. nummularia* grown hydroponically. The ORF region of AnSAMS1 and antiserum against AnSAMS1 were used as the hybridization probe for RNA gel blotting and as primary antibody for immunoblotting, respectively, because the detection of the total level of the SAMS genes is important simply to indicate co-regulation of SAMS, CMO and PEAMT transcripts. Since nucleotide sequences of the putative ORF region and putative protein sequences of AnSAMS1 are highly similar to those of AnSAMS2, AnSAMS3, AnSAMS4 and AnSAMS5 (>97 and 99%, respectively), the probe and the antiserum will detect total levels of their products. The total levels of mRNA and protein for these SAMS isogenes in leaves and stems were increased by salt stress, but those in roots were not (Fig. 2). SAMS activity was also increased by salt stress in leaves, but not in roots (Fig. 3). The mRNA and protein levels of AnCMO were increased by salt stress in leaves, stems and roots, but the absolute levels in roots were much lower than those in leaves or stems (Fig. 2). The transcript level of AnPEAMT was increased by salt stress in leaves, but not in stems or roots (Fig. 2A).

### Betaine and lignin contents

We examined the contents of betaine and lignin in the tissues of *A. nummularia*. Betaine contents in leaves, stems and roots were increased by salt stress (Fig. 4). Lignin contents were higher in stems and roots than in leaves, and only the content in stems was increased by salt stress (Fig. 4). Betaine contents were similar to or higher than the lignin contents in leaves, but lower than the lignin contents in either stems or roots in both stressed and control plants on a weight basis (Fig. 4).

### Transcript regulations of the SAMS isogenes, AnCMO and AnPEAMT in leaves

We examined the transcript expression of these SAMS isogenes, AnCMO and AnPEAMT in leaves during and after relief from salt stress (Fig. 5A). The total level of mRNA for SAMS isogenes was nearly constant for 4 d under the control condition (data not shown), but began to increase at 3 h of salt stress, reaching a peak at 1 d of salt stress, and then maintained high levels under salt stress. This level reached the control level at 6 h after relief from salt stress, but did not decrease at 1 d after...
additional treatment with 500 mM NaCl. Transcript regulation patterns of AnCMO and AnPEAMT during and after relief from salt stress highly resembled that of the total level of mRNAs for SAMS isogenes.

Many salt-induced genes are regulated with ABA (Hartung et al. 1999). We also examined the responses of SAMS isogenes, AnCMO and AnPEAMT to ABA treatment (Fig. 5B). The total level of transcripts for the SAMS isogenes, and transcript levels for AnCMO and AnPEAMT in the leaves were not induced by the treatment. As a positive control for ABA-induced gene expression, we show the ABA response of a putative lipid transfer protein, which was cloned by the above differential screening.

Shen et al. (2002) reported that the CMO transcript in A. hortensis is regulated by a possible circadian rhythm. The AnCMO transcript in leaves was also regulated on a daily rhythm, peaked from 9 a.m. to 12 a.m. and declined to low levels in the evening and night under natural daylight conditions (Fig. 6). The total level of mRNA for SAMS isogenes and the mRNA level for AnCMO and AnPEAMT were almost coordinately regulated with the AnCMO transcript.

Discussion

Expression of SAMS isogenes

The nucleotide sequences of AnSAMS2, AnSAMS3, AnSAMS4 and AnSAMS5 have high identity to that of AnSAMS1. The detected DNA fragments from A. nummularia under a high stringency condition by DNA gel blotting were more abundant than those from spinach or A. lentiformis (Fig. 1). These results suggest that an ancestral gene of AnSAMS1 duplicated these SAMS isogenes relatively recently in a progenitor of A. nummularia. Therefore, the five gene products would have the same function.

Since some other DNA fragments from the chenopods were detected by DNA gel blotting under a low stringency condition (Fig. 1), these chenopods should have some isozymes of SAMS as in other plants (Peleman et al. 1989a, Peleman et al. 1989b, Espartero et al. 1994, Schröder et al. 1997). The total expression levels of the five SAMS isogenes in the salt-stressed shoot would be higher than that of the other isogenes only detected under a low stringency condition in the DNA gel blotting, because 21 clones encoding any of the five SAMS isogenes, no clone encoding other SAMS isogenes, were screened from the cDNA library using the low stringency condition. The expression pattern of SAMS activity is highly similar to that of the total transcript level of the five SAMS isogenes on the RNA gel blot (Fig. 2, 3). These results suggest that SAMS activity in the tissues would be controlled mainly by transcript regulations of the five SAMS isogenes.

Regulation of AnCMO expression

The levels of mRNA and protein for AnCMO were much higher in leaves and stems than in roots (Fig. 2). The tissue-specific expression pattern of AnCMO suggests that betaine is synthesized mainly in the shoot of A. nummularia. However, the betaine content in roots was not much lower than that in either leaves or stems (Fig. 4). Since betaine can move through the phloem (Hanson and Hitz 1982), most betaine in roots would be imported from the shoots.

The level of mRNA for AnCMO was downregulated by relief from salt stress (Fig. 5A). The levels of mRNA and protein for and enzymic activity of CMO in sugar beet also decreased to control levels at 3 d after relief from salt stress (Russell et al. 1998). Betaine was reported to have strong
resistance to metabolic degradation (Hanson and Wyse 1982), and production of betaine is energy consuming (Raven 1985). Therefore, in order not to synthesize superfluous betaine, *A. nummularia* should have a rapid AnCMO down-regulating mechanism to respond to the relief from salt stress.

*AnCMO* transcript was not induced by ABA treatment (Fig. 5B). Transcripts of CMOs in other *Atriplex* species are also hardly produced by ABA treatment; at least the level of induction is much lower than that induced by salt treatment (Shen et al. 2002, Wang and Showalter 2004). In *Atriplex* species, therefore, CMO induction under salt stress would be independent of the endogenous ABA level as Wang and Showalter (2004) discussed.

**Gene expression of SAMS and AnPEAMT for betaine synthesis**

SAMS is not only a methyl donor for betaine or lignin synthesis but also a substrate for the synthesis of ethylene and polyamines. The key regulatory enzyme in ethylene synthesis in higher plants is considered to be L-aminoacyclopropane-1-carboxylic acid synthase (Yang and Hoffman 1984), and a transcript induction of SAMS is not required for an increase of ethylene production even in a senescing flower (Woodson et al. 1992), which synthesizes substantial amount of ethylene. Therefore, it is seemed that biosynthesis of ethylene consumes a relatively lower amount of SAM than biosynthesis of the other products. Normally, spermidine and spermine are not accumulated at such high levels as betaine in betaine-accumulating plants. For example, the contents of spermidine and spermine in *Atriplex halimus* grown on saline soil are only 5.8 and 4.5 nmol (mg protein)$^{-1}$, respectively (Friedman et al. 1989). Since betaine content was 24 nmol (mg FW)$^{-1}$ in the leaves of *A. nummularia* under the control condition in this report, the polyamine contents are expected to be much lower than the betaine content in *A. nummularia*. Thus, it is anticipated that the amounts of SAM required for syntheses of ethylene and polyamines are much lower than that for synthesis of betaine.

Since not only lignin but also betaine is a stable final product in plant metabolism (Hanson and Wyse 1982), the contents of these products would be almost equivalent to the synthesized amounts in the tissue, although there is a possibility of betaine transportation through the phloem. Therefore, in some cases, whether betaine synthesis is more SAM consuming than lignin synthesis or not would be determined by comparing betaine content with lignin content. Lignin is synthesized by dehydrogenative polymerization of $p$-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, and its composition varies with the species and site (Christensen et al. 2000, Hatfield and Vermerris 2001). SAM is not consumed for synthesis of $p$-coumaryl alcohol, but, for synthesis of coniferyl alcohol and sinapyl alcohol, one and two molecules of SAM, respectively, are consumed (Christensen et al. 2000). Because the synthesis of one molecule of betaine (mol. wt 117 Da) demands three molecules of SAM, even if lignin was polymerized only from sinapyl alcohol (mol. wt 210 Da), betaine synthesis would consume about 2.5-fold the number of SAM molecules consumed by lignin synthesis on a weight basis. In the leaves under the control condition, the betaine content was almost the same as the lignin content (Fig. 4). Therefore, the synthesized SAM may be consumed for betaine synthesis more than for lignin synthesis in leaves under the control condition. The betaine content was increased but the lignin content was not changed by salt stress in the leaves (Fig. 4). Therefore, in order to supply methyl groups mainly for betaine synthesis, the protein levels of SAMSs would be increased in the leaves by salt stress. In stems, the betaine content was about a half of the lignin content, and betaine and lignin were increased by salt stress (Fig. 4). Thus, the induction of SAMSs in stems by salt stress seemed to reflect the increase of lignin content as well as that of betaine content. Since betaine is probably synthesized mainly in the shoot, the SAMS genes in roots may be expressed to supply SAM predominantly for the synthesis of some products other than betaine such as lignin, polyamines and ethylene.

When [$^{14}$C]choline is supplied to excised spinach leaves, the major labeled product is betaine (Coughlan and Wyn Jones 1982). Since *A. nummularia* accumulates betaine in the leaves more than spinach does, the majority of synthesized choline would be consumed for betaine synthesis in the leaves of *A. nummularia*. Therefore, the transcript level of *AnPEAMT* would be increased by salt stress to supply choline mainly for betaine synthesis in the leaves.

**Co-regulation of SAMS, PEAMT and AnCMO genes in leaves**

The regulation patterns of the total level of mRNAs for SAMS isogenes and the mRNA level for *AnPEAMT* in the leaves highly resembled that of the mRNA for *AnCMO* during and after relief from salt stress (Fig. 5A), and on a diurnal rhythm (Fig. 6). Because SAM is the substrate for various methyltransferases and a precursor of ethylene, polyamines and nicotianamine (Tabor and Tabor 1984, Moffatt and Weretilnyk 2001), both an excess and a deficiency of SAMS activity may have detrimental effects on numerous biological reactions. In fact, both overexpression and suppression of SAMS cause abnormal phenotypes in transgenic tobacco (Boerjan et al. 1994). Choline is needed to synthesize phosphatidylcholine, which is a major membrane lipid in non-plastid plant membranes (Moore 1990), and silencing of PEAMT leads to phenotypes of abnormal morphology and stress sensitivity in *Arabidopsis* (Mou et al. 2002). Inadequate PEAMT activity is also expected to cause a negative effect in the plant cell. Therefore, plant cells may have systems for regulating SAMS and PEAMT expression in order to maintain specific pool sizes of SAM and choline, respectively. Because considerable amounts of SAM and choline seemed to be consumed for betaine synthesis in the leaves, *A. nummularia* should control the expression of SAMS and PEAMT along with that of CMO during and after relief from salt stress.
Materials and Methods

Plant growth conditions

*Artemisia nummularia* and *A. lentiformis* seeds were purchased from B & T World Seeds sarl (France). For cDNA library construction and differential screening, cuttings were obtained from a 3-year-old shrub of *A. nummularia*, which was directly grown in soil in a greenhouse at temperatures of approximately 35°C/15°C (day/night) in natural daylight. The cuttings were grown on vermiculite with nutrient solution (Tabuchi et al. 2003) at 25°C under continuous light (150 µmol m⁻² s⁻¹) from fluorescent tubes (FFH32EX-N-HG, NEC, Tokyo, Japan) in a growth chamber. The nutrient solution contained 1 mM NaCl, because the growth is poor when the Na⁺ concentration is too low (Bownell 1968). When shoot height reached 10 cm, salt stress was given by the stepwise addition of NaCl (100 mM for 2 d, 200 mM for 2 d, 300 mM for 2 d, 400 mM for 2 d, and then 500 mM for 12 d) to the nutrient solution.

For the experiments, *A. nummularia* seeds were germinated on vermiculite, and the seedlings were grown hydropotentially in the nutrient solution at 25°C under continuous light (150 µmol m⁻² s⁻¹) from fluorescent tubes in a growth chamber. For salt stress, when the average shoot length reached 4.5 cm, the nutrient solution was supplemented with 250 mM NaCl for 2 d, and then 500 mM NaCl for 2 d. For relief from the salt stress, the salt-stressed seedlings were transferred to the control nutrient solution. For ABA treatment, the nutrient solution was supplemented with 20 µM (±)-ABA (Wako Pure Chemicals, Osaka, Japan) for 1 d. Fully expanded true leaves at the third to sixth nodal position counted from the base were used as the leaf samples. For a diurnal rhythm experiment, mature leaves were obtained from the 3-year-old shrub, when the sun rose at about 4.50 a.m. and set at about 7.10 p.m.

Construction of a cDNA library of salt-stressed plant

Total RNA was extracted from the salt-stressed shoot by the AGPC method (Chomczynski and Sacchi 1987), and purified further with a Quickprep Micro RNA Purification Kit (Amersham Biosciences, Uppsala, Sweden). A 3'& 3'-TriplEx2 cDNA library was constructed from poly(A) RNA with a SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, U.S.A.). The primary library contained 5.5 x 10⁶ plaque-forming units. After library amplification, a part of the phage library was converted to a plasmid library by incubation of the mixtures at 25°C for 2 min, followed by probe synthesis and labeling were performed with Taq polymerase from the pTriplEx2 plasmids containing the full-length cDNA insert of *AnSAMS1* or *AnCMO* from the plasmid library. The primers of the 5' ends of *AnSAMS1* and *AnCMO* were 5'-GGATCCGCACATGGCAGCAAGTGCAACA, respectively. The 3'-λTriplEx2 Sequencing Primer (Clontech) was used as the primer of the 3' ends of *AnSAMS1* and *AnCMO*. The PCR fragments of *AnSAMS1* and *AnCMO* were digested with *HindIII* and *SalI*, respectively, after digestion of BamHI, and then each fragment was ligated to the BamHI and SalI sites of pQE-42 (Qiagen, Valencia, CA, U.S.A.). The constructs were transformed into Escherichia coli strain M15[pREP4] (Qiagen).

For preparation of antibodies, His-tagged full-length polypeptide of *AnSAMS1* and His-tagged polypeptide fragment of *AnCMO* were expressed in the *E. coli* cells and purified with Ni-NTA agarose (Qiagen) under denaturing conditions according to the manufacturer’s manual.

For detection of *SAMS* activity of His-tagged *AnSAMS1*, the cultures containing the His-tagged *AnSAMS1* expression plasmid were grown in LB medium supplemented with 100 mg 1⁻¹ ampicillin and 50 mg 1⁻¹ kanamycin at 37°C to a density of 0.5–0.6 A₆₀₀ nm⁻¹, and then induced for His-tagged *AnSAMS1* with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. His-tagged *AnSAMS1* was purified with Ni-NTA agarose under native conditions according to the manufacturer’s manual.

Expression in Escherichia coli and purification of *AnSAMS1* and *AnCMO*

The *AnSAMS1* and *AnCMO* cDNA fragments were amplified with Taq polymerase from the pTriplEx2 plasmids containing the full-length cDNA insert of *AnSAMS1* or *AnCMO* from the plasmid library. The primers of the 5' ends of *AnSAMS1* and *AnCMO* were 5'-GGATCCGCACATGGCAGCAAGTGCAACA, respectively. The 3'-λTriplEx2 Sequencing Primer (Clontech) was used as the primer of the 3' ends of *AnSAMS1* and *AnCMO*. The PCR fragments of *AnSAMS1* and *AnCMO* were digested with *HindIII* and *SalI*, respectively, after digestion of *BamHI*, and then each fragment was ligated to the *BamHI* and *SalI* sites of *pQE*-42 (Qiagen, Valencia, CA, U.S.A.). The constructs were transformed into *Escherichia coli* strain *M15* (pREP4) (Qiagen).

DNA and RNA gel blot analysis

Genomic DNAs were purified from the 3-year-old shrub of *A. nummularia*, *A. lentiformis*, and spinach purchased from a market, as described by Rogers and Bendich (1994). After digesting with restriction enzymes, the fragments were separated on 0.5% agarose gels. Total RNA was isolated from various plant tissues as described by Nagy et al. (1988). Total RNA, denatured by formamide and formaldehyde, was separated on 1.5% agarose gels containing formaldehyde. Nucleic acids were transferred onto Hybond-N⁺ by capillary blotting. The ORF fragments of cDNA clones corresponding to *AnSAMS1*, *AnCMO*, AnPEAMT and AnLTP and the fragments of *Arabidopsis* 18S rDNA (Long et al. 2002) were α-32P-dCTP labeled using the BcaBEST Labeling kit (Takara Bio Inc., Tokyo, Japan). The membranes were hybridized in hybridization buffer (Church and Gilbert 1984) at 30 min for 18S rRNA or at 65°C overnight for the other genes, washed at 65°C in 1 x SSC, 1% SDS for low stringency washing, and then in 0.1 x SSC, 0.1% SDS for high stringency washing for RNA gel blotting. The membranes were analyzed with a Typhoon 9210 (Amershams Biociences).
molecular masses of 43.1–43.2 and 42.6 kDa, respectively, and proteins with apparent molecular masses of 44 and 42 kDa were recognized with the AnSAMS1 and AnCMO antisera, respectively (Fig. 2). These results indicate that these antisera can be used for detection of these SAMSs and AnCMO, respectively, by immunoblot.

**Immunoblotting**

Tissues were ground into a fine powder in liquid N\(_2\). The tissue powder (0.1 g) was suspended in a 0.1 ml of a mixture of 2% SDS, 2% \(\beta\)-mercaptoethanol, 100 mM Tris–HCl, pH 6.8 and 20% glycerol, immediately boiled for 5 min, and centrifuged at 10,000 \(\times g\) for 10 min. The proteins of the supernatants were quantified by the Bradford assay (Bradford 1976) with bovine serum albumin (BSA) as a standard. The proteins separated by 10% SDS–PAGE were electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked in a 5% solution of reconstituted skimmed milk powder prepared in phosphate-buffered saline containing 0.1% Tween-20 (PBST), and then probed with the anti-AnSAMS1 or anti-AnCMO antiserum in PBST for 1 h. After washing with PBST, the proteins recognized by the primary antibody were revealed with a goat anti-rat IgG coupled to horse-radish peroxidase (Chemicon, Temecula, CA, U.S.A.). The complexes were visualized using the substrates hydrogen peroxide and 3,3′-diaminobenzidine.

**SAMs assay**

The SAMs assays were done essentially as described by Boerjan et al. (1994). Plant tissues were ground into a fine powder in liquid N\(_2\). The tissue powder (0.1 g) was suspended with sonication in 0.2 ml of extraction buffer (100 mM Tris–HCl, pH 7.5, 2 mM EDTA, 20% glycerol, 20 mM \(\beta\)-mercaptoethanol, 1 mM dithiothreitol), and centrifuged at 10,000 \(\times g\) for 10 min. The supernatants were desalted by passage through a Sephadex G-50 (Amersham Biosciences) column that had been with the extraction buffer. The desalted proteins were quantified by the Bradford assay (Bradford 1976) with BSA as a standard. The desalted protein was incubated in 50 \(\mu\)l of reaction mixture (30 mM MgSO\(_4\), 10 mM KCl, 20 mM ATP, 5 mM unlabeled methionine, 0.75 \(\mu\)Ci of \([\text{\textsuperscript{35}}\text{S}]\)methionine (Amersham Biosciences), 100 mM Tris–HCl, pH 8.0). Control reactions contained all reagents except for ATP. After incubation for 1 h at 25°C, 20 \(\mu\)l of the reaction mixture was spotted on a phosphocellulose filter disc (P81; Whatman, Maidstone, U.K.) in duplicate. The filters were air dried, washed three times with ice-cold water for 5 min, and transferred to scintillation vials containing 1 ml of 1.5 M ammonium hydroxide. After 5 min, Hionic-Flour (Packard, Meriden, CT, U.S.A.) was added, and the sample was counted by scintillation spectrometry.

**Betaine assay**

The tissue was ground in 1 ml of ethanol with a mortar and a pestle, and the mortar was washed with 1 ml of ethanol three times. The combined extract was evaporated in vacuo, resuspended in water, and centrifuged at 10,000 \(\times g\) for 5 min. Betaine contents of the supernatant were estimated as described by Bessieres et al. (1999), except that the betaine amount was determined by high-performance liquid chromatography (HPLC) on a Cosmosil 5C18-AR column (4.6×250 mm; Nacalai Tesque, Kyoto, Japan).

**Lignin assay**

The tissues were ground into a fine powder in liquid N\(_2\). Lignin contents of the fine powder were estimated using the acetyl bromide procedure (Iiyama and Wallis 1990).

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**References**


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