Cloning and salt-induced, ABA-independent expression of choline mono-oxygenase in *Atriplex prostrata*

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Certain plants accumulate glycinebetaine, a type of osmoprotectant, in response to salinity. Glycinebetaine is synthesized in these plants via the two-step oxidation of choline, and the first step is catalysed by choline mono-oxygenase (CMO; EC 1.14.15.7). Cloned by RT-PCR and 5′-RACE, the cDNA of *Atriplex prostrata* CMO (*ApCMO*) is 1669bp in length and encodes a full-length protein of 438 amino acids. The deduced amino acid sequence of *ApCMO* revealed a Rieske-type [2Fe-2S] cluster motif and a mononuclear non-heme Fe binding motif, and shares 82.9% identity and 87.2% similarity with the deduced amino acid sequence of spinach CMO. Accumulation of CMO transcript and glycinebetaine both increased in response to NaCl treatment. Without salt treatment, CMO mRNA was detected in stems and 5-day-old seedlings, but not in leaves, roots and older seedlings. With salt treatment, CMO mRNA accumulated dramatically in stems, leaves and roots, with the most abundant accumulation occurring in young stems. Although abscisic acid may initiate global physiological reactions in response to osmotic stress, it did not induce the expression of CMO in *A. prostrata*. In summary, salt-induction of CMO mRNA in *A. prostrata* is more substantial than that reported in spinach and sugar beet, and the plant may serve as a useful model to study regulation of glycinebetaine synthesis by environmental stress.

**Introduction**

In order to survive plants have developed different mechanisms in response to salt stress. Among the known mechanisms associated with salt tolerance, accumulation of osmoprotectants such as glycinebetaine and proline is of particular interest because of the potential to genetically engineer this pathway into non-salt-tolerant crops. Glycinebetaine, one of the quaternary ammonium compounds, is a well-known osmoprotectant that effectively stabilizes enzymes critical to physiological functions (Papageorgiou and Murata 1995). Under salt stress, the level of glycinebetaine in salt-tolerant plants typically rises (Khan et al. 1998). In higher plants, glycinebetaine is synthesized by the two-step oxidation of choline with betaine aldehyde serving as an intermediate. The first step is catalysed by choline mono-oxygenase (CMO; EC 1.14.15.7) in the presence of ferredoxin, whereas the second step is catalysed by betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8) in a NAD\(^+\)-dependent reaction. Both enzymes are located in the stroma of chloroplasts and increase in activity in response to salinity treatments (Weigel et al. 1986, Brouquisse et al. 1989).

After being identified as a unique plant oxygenase containing a Rieske-Type [2Fe-2S] centre, CMO was first cloned from spinach by RT-PCR using primers corresponding to amino acid sequences in this iron–sulphur centre (Rathinasabapathi et al. 1997). To date, this enzyme, which catalyses the committed step of glycinebetaine synthesis, has been cloned from spinach (Rathinasabapathi et al. 1997), sugar beet (Russell et al. 1998), *Atriplex hortensis*, and amaranth (Meng et al. 2001). Little is known about the expression of CMO
Materials and methods

Plant material and growing conditions

Seeds of *Atriplex prostrata* were collected at an abandoned salt mine owned by the Morton Salt Co. at Rittman, OH, USA, and germinated in flats filled with sand in an environmental growth chamber (Conviron, Pembina, ND, USA) under a 25°C:5°C, 12 h:12 h day:night regime (400–700 nm, 400 μmol m⁻² s⁻¹). Ten days after germination, seedlings were transferred to 4-inch pots filled with sand at a density of four seedlings per pot. Pots were watered weekly. Solutions supplemented with or without 10 mM NaCl and maintained in an environmental growth chamber (Minigro II, Interpump Ltd., USA) with the following cycle profile: initial denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extending at 72°C for 1 min for each cycle, and a final extension (i.e. polishing) at 72°C for 7 min. The sequence of the primers (synthesized by Integrated DNA Technologies, Inc, Coralville, IA) used in RT-PCR are: CMO3- TGGTGGATTCTTCTCAT-CTTTCTCAAT-AGG; C-MO5S- AATCATGGCAGCAAGTGCAA; CMO5AS- GGTCAAAAGCATGGGCCTTA.

For 3′-RACE, 2 μg of mRNA isolated from total RNAs using a mRNA isolation kit (Qiagen Inc., Chatsworth, CA, USA) was used to carry out first and second strand cDNA synthesis. The resulting double-stranded cDNAs were then ligated to Marathon⁺ cDNA adaptors (Clontech, Palo Alto, CA, USA) prior to 3′-RACE. CMO6 (5′-CACGATTCAAGGGTGGC- TGGGACTTC-3′), a GC-rich primer, was designed based on the sequence of previous RT-PCR products (Fig.1A). CMO6 and the adapter primer amplified the 3′ end product of ApCMO with the touchdown PCR program according to the instruction of Marathon⁺ RACE (Clontech).

Cloning of ApCMO by RT-PCR and 3′ RACE

Total RNA from leaves treated with 2% (w/v) NaCl for 3 days was reverse-transcribed into cDNAs in the presence of MMLV-reverse transcriptase (EC 2.7.7.49; Promega, Madison, WI, USA). PCR reactions were then carried out using TaqPlus® Precision DNA polymerase (EC 2.7.7.6; Stratagene, La Jolla, CA, USA) with the following cycle profile: initial denaturation of cDNAs at 95°C for 2 min, 30 cycles of amplification by denaturing at 95°C for 1 min, annealing at 50°C for 1 min and extending at 72°C for 1 min for each cycle, and a final extension at 72°C for 7 min. The sequence of the primers (synthesized and by Integrated DNA Technologies, Inc, Coralville, IA) used in RT-PCR are: CMO3- TGGTGGATTCTTCTCAT-CTTTCTCAAT-AGG; C-MO5S- AATCATGGCAGCAAGTGCAA; CMO5AS- GGTCAAAAGCATGGGCCTTA.

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Subcloning and sequence analysis of PCR products

All RT-PCR and RACE products were subcloned using the TOPO cloning kit by Invitrogen (Carlsbad, CA, USA) with the vector, pCR®II-TOPO (3.9 kb). Subcloned cDNAs were sequenced using the BigDye Terminator Cycle Sequencing Reactions kit (Perkin-Elmer, Foster City, CA, USA) and an automated DNA sequencer (ABI 310, Perkin-Elmer). Sequence comparison with other databases was performed through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) via BLAST. Sequence alignments and analysis were performed using AlignX® of Vector NTI suite 6 (InforMax, Inc., Bethesda, MD, USA).

RNA isolation and northern blotting

Total RNAs were isolated from tissues of *A. prostrata* from various treatments as described using RNeasy® Plant Total RNA Kit (Qiagen Inc.). Total RNAs were subjected to electrophoresis and gels were then transferred to Zeta-probe GT blotting membranes (Bio-Rad, Hercules, CA, USA) in 10× SSC. Membranes were fixed by a Stratalinker® UV crosslinker (Model 1800; Stratagene) at an energy of 120000 J for approximately 2 min and then air-dried. A partial length CMO cDNA, ApCMO-0.7, was labelled with [α-32P]dCTP using the Prime-a-Gene® Labeling System (Promega) and used as a probe. Prehybridization and hybridization reactions were carried out in 7% (w/v) SDS, 250 mM sodium phosphate buffer (pH 7.2) at 65°C. Washing was performed in one-quarter strength prehybridization solution at room temperature for 5 min and twice at 65°C for 10 min.

Autoradiographs of northern blots and ethidium bromide-stained gels which were photographed were scanned using a digital imaging system (Fluar-S® Multi-Imager MAX; Bio-Rad) and analysed with Quantity One® quantification software (Version 4.2.2; Bio-Rad) to quantify levels of rRNA and CMO transcript. Levels of CMO transcript were normalized with respect to the amount of rRNA in each lane, determined by ethidium-bromide staining of the original gel. Northern blots showed here were typical examples obtained from two
Fig. 1. Cloning of *A. prostrata* CMO. A, a schematic map of the 1669 bp composite cDNA sequence encoding CMO in *A. prostrata*. , Primers used for RT-PCR (see Materials and methods for primer sequences); , cleavage site of restriction enzymes; , positions of start (ATG) and stop (TGA) codons. B, Nucleotide and deduced amino acid sequence of *ApCMO*. The binding site of Rieske type Fe-S cluster is underlined with the key Cys and His marked with asterisks. The dotted underline represents the non-heme Fe binding motif, in which the conserved residues in this reported motif are marked with daggers (Jiang et al. 1996). The putative transit peptide is underlined twice. The start and stop codons are boxed. The accession number of *ApCMO* is: AY082068.
sets of biological replicates with multiple northern blots performed on each replicate.

Measurement of glycinobetaine

Glycinobetaine was extracted with boiling water and measured with a HPLC (HP 1050 modular 3D; Hewlett Packard, Boise, ID, USA) as described in Khan et al. (1998). Betaine (Sigma) standards were run at 1, 10 and 100 mM.

Results

Cloning of CMO by RT-PCR and RACE

An alternative approach to conventional cDNA library screening for cloning CMO is to clone by RT-PCR and RACE given that there were known cDNA sequences encoding CMOs. For cloning CMO from *A. prostrata*, sequences of spinach and sugar beet CMOs were aligned to identify consensus regions. Two primers, CMO3 and CMO4, were designed based on these consensus regions to amplify a partial length CMO cDNA from *A. prostrata*. CMO3, which encodes one of the two Rieske-type Fe-S cluster binding sites, and CMO4, which is located in the consensus region near the end of the 3'-coding sequence, amplified a 749-bp fragment (*ApCMO-0.7*) (Fig. 1A). This fragment was homologous to both spinach and sugar beet CMOs based on a BLAST search and subsequent sequence alignments. CMO6, which is rich in GC content, was designed based on the sequence of *ApCMO-0.7* and used to perform 3'-RACE (Fig. 1A). The resulting poly-A-tail-containing 726-bp fragment (*ApCMO-3*) contains a 323-bp perfect match with the 3'-end of *ApCMO-0.7*. CMO5AS and CMO5SS, which were designed based on the sequence of spinach CMO with the later corresponding to the start codon, amplified a 779-bp fragment (*ApCMO-5*) (Fig. 1A). *ApCMO-5* overlapped with *ApCMO-0.7* by 241 bp and it was homologous to the 5'-end of spinach and sugar beet CMO cDNAs (Rathinasabapathi et al. 1997, Russell et al. 1998).

Based on these three overlapping clones (and other clones not shown), a composite cDNA encoding *A. prostrata* CMO (*ApCMO*) was assembled. *ApCMO* is 1669 bp in length and encodes 438 amino acid polypeptide, as shown in Fig. 1B. This cDNA has a 3'-untranslated region of 348 bp. According to the prediction of ChloroP (Version 1.1, http://www.cbs.dtu.dk), *ApCMO* contains an N-terminal chloroplast transit peptide (score: 0.567). Based upon the comparisons of the CMO polypeptide (Rathinasabapathi et al. 1997), Ala59 should be the cleavage site for the transit peptide. In order to further confirm the localization of this cloned CMO according to its sequence, the deduced amino acid sequence was submitted to the PSORT (prediction of protein localization sites, http://psort.nibb.ac.jp) web site. PSORT predicted that *ApCMO*, as well as the other known CMO clones, is most likely localized in the chloroplast stroma (certainty = 0.625) based on the following: (1) an apolar signal for intrachloroplastic sorting (aa 59–65) was found immediately after the transit peptide; and (2) *ApCMO* scores very high for the consensus sequence for intrachloroplastic sorting within the transit peptide (aa 1–58) (Fig. 2A).

According to Motif Finder (http://motif.genome.ad.jp), *ApCMO* contains both subunits of the Fe-S cluster binding sites that are the characteristic of Rieske iron-sulphur proteins. In fact, the registered Rieske-type [2Fe-2S] cluster motif in PROSITE is Cys-X-His-X5–17–Cys-X-X-His (where X represents any amino acid) and *ApCMO* contains this registered motif (underlined region in Fig. 1B), which is highly conserved in all reported CMOs (Fig. 2B). In addition, *ApCMO* also contains another consensus sequence, a mononuclear non-heme Fe binding motif: Glu/Asp-X3–4-Asp-X2-His-

Fig. 2. Amino acid sequence alignments of CMOs from spinach, sugar beet, and *A. prostrata*. A, N-terminal region. The N-terminal regions of *A. hortensis*, spinach, amaranth, and sugar beet CMOs (Fig. 2A), and the experimentally determined N-terminal residue of the purified spinach CMO polypeptide (Rathinasabapathi et al. 1997), Ala59 should be the cleavage site for the transit peptide. In order to further confirm the localization of this cloned CMO according to its sequence, the deduced amino acid sequence was submitted to the PSORT (prediction of protein localization sites, http://psort.nibb.ac.jp) web site. PSORT predicted that *ApCMO*, as well as the other known CMO clones, is most likely localized in the chloroplast stroma (certainty = 0.625) based on the following: (1) an apolar signal for intrachloroplastic sorting (aa 59–65) was found immediately after the transit peptide; and (2) *ApCMO* scores very high for the consensus sequence for intrachloroplastic sorting within the transit peptide (aa 1–58) (Fig. 2A).

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binding motifs, the amino acid sequences are completely identical in spinach, *Atriplex* and sugar beet.

When the nucleotide sequence of *Ap*CMO was aligned to that of spinach and sugar beet, it shared 73.4% and 72.8% identity, respectively. At the amino acid level, the identity between *A.prostrata* and spinach is 82.9 and 95.8% at the nucleotide level and 96.3% at the amino acid level when *A.hortensis* CMO was compared with *A.prostrata* CMO.

**CMO expression in *A.prostrata* is salt-inducible**

In order to examine how salinity affects CMO expression, *A.prostrata* was treated with 0% (control), 1 and 2% (w/v) NaCl for 3 d. Total RNA was then isolated from young stems (i.e. the first internode from the top of the plant) and young leaves (i.e. fully expanded leaves from the top of the plant) to perform northern blotting. When the membrane was probed with *Ap*CMO-0.7, a 1.9-kb transcript was detected (Fig. 3A). In young stems, this transcript increased two-fold when plants were treated with 1% (w/v) NaCl (=170 mM) and increased by approximately five-fold when plants were treated with 2% (w/v) NaCl (=340 mM). In young leaves, there was almost no expression of CMO when the plants were not treated with salt. When treated with 1% NaCl, CMO expression was induced to 1.6-fold of the expression in 0% YS, whereas with 2% NaCl, the induction was further enhanced to 2.5-fold of the expression with 1% NaCl (i.e. four-fold the expression in 0% YS) (Fig. 3A).

The accumulation of CMO transcript paralleled accumulation of glycinebetaine. In stems of *A.prostrata* treated with NaCl, there is a two- to three-fold increase in glycinebetaine content. In 1% NaCl-treated leaves, a two-fold increase in glycinebetaine content was observed, while a three-fold increase was detected in 2% NaCl-treated leaves (Fig. 3B).

**CMO expression in *A.prostrata* is regulated developmentally and in an organ-specific manner**

Since CMO mRNA levels in young leaves and stems were different (Fig. 3), it was of interest to investigate whether various organs differentially respond to NaCl treatment with respect to CMO expression. Total RNAs were isolated from roots as well as old and young stems and leaves after entire plants were treated with 2% NaCl for 3 days. Without salt treatment, CMO transcripts were detected only in the stems (Fig. 4). When plants were treated with 2% NaCl, CMO transcripts were present in roots, stems and leaves. In order to obtain a more accurate estimate of changes in *Ap*CMO transcript levels, a densitometric analysis of Northern blots was performed (Fig. 4). Compared to 0% YS, an approximately five-fold increase after salt treatment was detected in young stems whereas in young leaves the increase was ~ four-fold. In old stems, there was still a three-fold increase relative to the level of CMO transcript in 0% YS. In old leaves, the salt-induction of CMO expression was minimal. In roots, with both old and young roots included, the increase was moderate (about two-fold more than that detected in control young stems).

Not only was CMO differentially expressed in old and young tissues, it was also differentially expressed in seedlings of different ages. CMO transcripts were first detected in 3-day-old seedlings (3 days after germination), and accumulation of the transcript reached a maximum in 5-day-old seedlings (Fig. 5). The CMO transcript, however, was barely detectable in 10- and 15-day-old seedlings.

**Effects of exogenous ABA on CMO expression**

In addition to participating in various developmental and physiological processes including stomatal closure...
and root growth, abscisic acid (ABA) may initiate global physiological reactions in response to osmotic stress (reviewed by Zhu et al. 1997). Therefore, it was of interest to investigate whether ABA can transduce signals related to salt stress in *A. prostrata* and induce CMO expression. As shown in Fig.6, ABA alone did not induce CMO expression. The only induction here was observed when plants were treated with 2% (w/v) NaCl.

**Discussion**

Using RT-PCR and RACE, a composite cDNA (*ApCMO*) encoding CMO from *A. prostrata* was cloned and characterized. This composite *ApCMO* is derived from *ApCMO-5′*, *ApCMO-0.7*, and *ApCMO-3′* which substantially overlap with one another (Fig.1A). These overlapping regions, not including the primer sites, match almost perfectly with less than five mismatches which were fully resolved with other RT-PCR clones (data not shown). *ApCMO* encodes a 438 amino acid protein that includes a transit peptide typical for chloroplast stromal targeting (Cline and Henry 1996). The size of the transit peptide was predicted to be 67 residues, according to ChloroP. However, based on the N-terminal sequence of the purified polypeptide of spinach CMO determined by Rathinasabapathi et al. (1997), as well as the alignment between the N-terminal sequence of the purified polypeptide of spinach CMO determined by Rathinasabapathi et al. (1997), as well as the alignment between the N-terminal regions to *ApCMO* and available CMOs (Fig.2A), the most probable cleavage site for the *ApCMO* transit peptide is at Ala59. Interestingly, Ala59 was excluded by ChloroP as the cleavage site, although it scored higher than Ala68, because it is one amino acid outside the algorithm-predicted window of possible cleavage sites, indicating such computer predictions may be misleading and require more cautious consideration. *ApCMO* is 72.8–96.3% identical and 80.8–97.3% similar to CMOs of other members in the Chenopodiaceae (i.e. *A. hortensis*, spinach, and sugar beet). Among CMOs of these four species, *A. prostrata* is most related to *A. hortensis* and sugar beet is least related to others. As a betaine-accumulator from another family, *Amaranth* CMO is more closely related to CMOs of...
the chenopods than to Arabidopsis. These observations are in agreement with known phylogenetic relationships. The deduced amino acid sequence of ApCMO contains two motifs, the Rieske-type [2Fe-2S] cluster and the mononuclear non-heme Fe binding site (Fig.1B), which are also conserved in all reported CMOs (Fig.2B, C; Rathinasabapathi et al. 1997, Russell et al. 1998, Meng et al. 2001). Although the Rieske cluster is also present in various dioxygenases and chlorophyll oxygenase, the presence of these two motifs together make CMO unique.

Although CMO was cloned from spinach, sugar beet, Atriplex hortensis, and amaranth earlier, the expression of CMO at mRNA level was not studied in much detail except to observe that it is induced by salt. In A. prostrata, CMO expression is up-regulated by NaCl (Fig.3), which is consistent with other research (Rathinasabapathi et al. 1997, Russell et al. 1998, Meng et al. 2001). Indeed, with higher salt concentrations, accumulation of CMO transcript was observed to increase in A. prostrata. In parallel, glycinebetaine accumulates as a function of different salt concentration. Taken together, these data are consistent with the finding that CMO catalyses the rate-limiting step of glycinebetaine production (McNeill et al. 2000). In addition, CMO expression in response to salt treatment differs in an organ-specific manner (Fig.4). CMO transcript is most abundantly expressed in stems, and the induction by 340 mM NaCl is also the most pronounced in stems. In young leaves and roots, NaCl-induced CMO expression patterns were dramatic and infinitely large as CMO expression in corresponding unsalinized tissues were undetectable.

Up-regulation of CMO expression by NaCl is less pronounced in older leaves and stems (Fig.4), indicating that glycinebetaine biosynthesis may be more active in younger tissues. One explanation is that young tissues are generally more sensitive to salt stress than older tissues. Glycinebetaine is not degraded once it accumulates in the cytosol (Nuccio et al. 1998), therefore older tissues, may not need to activate the pathway as much as younger tissues. Another piece of evidence demonstrating that CMO is differentially expressed at different developmental stages is that the CMO transcripts accumulate in 3- and 5-day-old seedlings without salt induction (Fig.5). However, CMO is not expressed later in 10- or 15-day-old seedlings. This may be because 3- and 5-day-old seedlings require glycinebetaine to deal with the particular physiological conditions unique to these developmental times.

Many observations indicate that osmotic stress due to salt, drought and water stress, leads to an increase in endogenous levels of ABA, which in turn induces expression of osmotic stress responsive genes (Skriver and Mundy 1990, Ishitani et al. 1997). Moreover, such genes can be induced by the exogenous application of ABA. However, accumulation of ApCMO mRNA was not dependent upon exogenous ABA (Fig.6). Using the same blots hybridized with a partial cDNA of ApBADH, ABA-induced expression of BADH in A. prostrata was clearly observed (L.W. Wang, 2002, Dissertation, Ohio Univ., OH). This ABA-dependent expression was also reported in barley (Ishitani et al. 1995). This indicates that the CMO gene at least in A. prostrata is not one of the ABA-responsive genes whose transcript levels increase many-fold within hours of ABA application. Such ABA-responsive genes share sequence similarity within their respective 5’ upstream regions (Guillotin et al. 1990). In particular, specific nucleotide sequences or ABA-responsive elements (ABREs) in the promoter region are necessary for the induction of these ABA-responsive genes (Marcotte et al. 1989, Shen and Ho 1997). Without the sequence of the CMO gene from Atriplex or any other species with the possible exception of Arabidopsis (see below), it is impossible to search for an ABRE in CMO genes. Recently, however, a CMO-like protein in Arabidopsis was documented (protein ID: CAB43664), and no ABRE (i.e. ACGTGGC in either orientation) can be found upstream of its start codon. Therefore, it appears unlikely that an ABRE is present and ABA is unlikely to induce CMO expression.

There are other examples of osmotic stress-responsive genes whose expression is independent of ABA (Binzel and Dunlap 1995, Gosti et al. 1995). In Mesembryanthemum crystallinum (ice plants), the transition from the C 3 photosynthetic pathway to Crassulacean acid metabolism (CAM) is one mechanism for water conservation and/or salt tolerance. A CAM-specific isozyme, phosphoenolpyruvate carboxylase, is strongly induced by salt, but ABA is not necessary for the expression of phosphoenolpyruvate carboxylase (Yen et al. 1995). Therefore, different signal transduction pathways (i.e. ABA-dependent and ABA-independent) exist to regulate the expression of osmotic stress-induced genes in plants. Based on data presented here, the expression of A. prostrata CMO gene is ABA independent.

It will be useful to obtain genomic clones and elucidate their regulatory elements from members of the Chenopodiaceae in order to investigate how signals are transduced and activate CMO expression. Since CMO expression in response to salt stress in A. prostrata is more substantial than that reported for other members of the Chenopodiaceae, such as spinach and sugar beet (Rathinasabapathi et al. 1997, Russell et al. 1998), Atriplex may serve as a particularly useful model plant for studies of regulatory mechanisms related to the activation of the glycinebetaine biosynthetic pathway in response to environmental stress.

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