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Salt- and osmotic stress-induced choline monooxygenase expression in *Kochia scoparia* is ABA-independent

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Abstract

Choline monooxygenase (CMO) is the first regulatory enzyme in the biosynthetic pathway for glycine betaine, an effective osmoprotectant in *Kochia scoparia*, a highly drought- and salt-tolerant species. In seedlings, CMO transcript levels are rapidly increased in response to both NaCl and osmotic stress treatments. The mRNA level in shoots was substantially higher than in roots. The rapid induction seen in whole plants was in contrast to the apparent down-regulation observed in suspension-cultured *K. scoparia* cells in response to the same salt stress. Treatment with exogenous abscisic acid (ABA) or fluridone shows that CMO induction proceeds *via* an ABA-independent signal transduction pathway. Examination of the CMO upstream regulatory region reveals a number of stress response-related elements, some of which may be involved in the stress tolerance shown by this species.

Additional key words: gene expression, glycine betaine, iso-osmotic treatments, Northern hybridization, reverse transcription quantitative PCR.

Introduction

Drought and salinity stress are among the major factors limiting plant growth and productivity. In response to these stresses, members of some plant families have developed the ability to accumulate compatible solutes (low molecular mass, highly soluble organic compounds). These substances contribute to cellular osmotic adjustment, stabilize and protect enzymes from denaturation, and ensure membrane integrity (Ashraf and Foolad 2007). A key compatible solute is the amino acid derivative glycine betaine (GB), which is synthesized in the chloroplast stroma from choline *via* two oxidation reactions, the first of which is catalyzed by choline monooxygenase (CMO; EC 1.14.15.7), a soluble ferredoxin- and oxygen-dependent enzyme (Rhodes and Hanson 1993). GB accumulates in members of the *Chenopodiaceae*, *Amaranthaceae*, *Gramineae*, *Compositae*, *Malvaceae*, and other plant families in response to stress (Ashraf and Foolad 2007). *Kochia scoparia* (L.) Schrader (= *Bassia scoparia* [L.] A.J. Scott) (*Chenopodiaceae*, *Caryophyllidae*) is an herbaceous annual plant with exceptional tolerance to drought, salt, and temperature stresses (Becker 1978).

Accumulation of GB is primarily due to induced CMO gene expression, as shown in several species in response to salinity (Rasinasabapathi *et al.* 1997, Russell *et al.* 1998, Meng *et al.* 2001, Shen *et al.* 2002, Kern and Dyer 2003, Wang and Showalter 2004) and drought (Russell *et al.* 1998). Similarly, CMO activity increased after salt, drought, and heat stresses in *Amaranthus tricolor* (Meng *et al.* 2001) and after salt stress in *K. scoparia* (Kern and Dyer 2003).

The role of abscisic acid (ABA) in the signal transduction pathways responsible for CMO gene induction remains unclear. CMO transcription was induced by ABA treatment in *Atriplex hortensis* (Shen *et al.* 2002) and *Amaranthus tricolor* (Bhuiyan *et al.* 2007). In contrast, other authors reported that CMO protein contents remained unchanged in *A. tricolor* in response to ABA treatment (Meng *et al.* 2001). ABA did not induce CMO expression in *Atriplex prostrata* (Wang and Showalter 2004) or *Atriplex nummularia* (Tabuchi *et al.* 2005).

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Abbreviations: ABA - abscisic acid; ABREs - ABA-responsive elements; ARF - auxin response factor; BADH - betaine aldehyde dehydrogenase; CMO - choline monooxydase; DRE - dehydration-responsive element; GB - glycine betaine; qRT-PCR - reverse transcription quantitative PCR.
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GB's known effectiveness in protecting against abiotic stresses (Chen and Murata 2011) has led to widespread interest in conferring stress tolerance *via* transgenic approaches. Transformation with cDNAs for GB-synthesizing enzymes like CMO and/or betaine aldehyde dehydrogenase (BADH; EC 1.2.1.8), the second enzyme of GB biosynthesis in plants, results in varying degrees of stress protection in transgenic plants (reviewed in Khan *et al*. 2009). These studies confirm that GB accumulation enhances tolerance to a number of abiotic stresses, including damage caused by reactive oxygen species.

Understanding the regulation of CMO expression *via* promoter analysis is thus of interest, as has been done for other stress-related regulatory networks (Shinozaki and Yamaguchi-Shinozaki 2007). CMO genes and upstream sequences have been reported from *Suaeda liaotungensis* (Li *et al.* 2007) and *A. tricolor* (Bhuiyan *et al.* 2007). In *S. liaotungensis*, *PLACE* analysis (Higo *et al.* 1999) of the 2 204 bp upstream region detected the presence of multiple *cis*-acting elements for salt, cold, ABA, drought, and wounding stress responses (Li *et al.* 2007). Similarly, numerous elements were found in the *A. tricolor* CMO upstream region, and the element(s) controlling CMO induction by NaCl were localized within 410 bp upstream of the transcriptional start site (Bhuiyan *et al.* 2007). ABA-responsive elements (ABREs; Nakashima *et al*. 2009) were found at -884 and -1 349 bp in the *A. tricolor* CMO upstream region.

We now report the characterization of CMO expression in *K. scoparia* cell suspension cultures and seedling shoot and root tissues. Expression levels are quantified by both Northern blots and qRT-PCR in response to NaCl and iso-osmotic drought stresses. CMO induction by salt is shown to be ABA-independent. DNA sequence analysis of the CMO promoter and upstream region reveals the presence of a number of stress-related *cis*-elements.

Materials and methods

Wild type *Kochia scoparia* (L.) Schrader biotype S1 (Kern and Dyer 2003) was used for all studies. Seeds were planted in sand in 17 cm-diameter pots and placed in the greenhouse under natural irradiance in combination with mercury vapor lamps (photon flux density, PFD 165 μmol m^{-2} s^{-1}) with a 16-h photoperiod and 23/18 ºC day/night temperatures. Seedlings were watered daily with half-strength Murashige and Skoog (1962; MS) salts for 5 d, followed by 12 d of water stress treatment. Stresses were imposed by the addition of iso-osmotic concentrations of 100 mM NaCl or 18 % PEG-8000 (*J.T Baker, Inc.*, Phillipsburg, NJ, USA) as described by Ahmad *et al.* (2007). Moisture content in pots was maintained gravimetrically by weighing pots every 3 d. Shoot tissue from 24 treated and control plants was harvested and pooled at the beginning of water stress treatments and after 3, 6, 24, 72, 168, and 288 h. The tissue was flash frozen in liquid nitrogen and stored at -80 ºC. Except for the 3- and 6-h time points, all harvests were done at the same time of day in order to account for possible circadian regulation of CMO expression, as shown in *A. hortensis* (Shen *et al.* 2002).

Cell suspension cultures were initiated as described by Encina *et al.* (2001) with some modifications. *K. scoparia* seeds were sterilized in a 10 % solution of sodium hypochlorite plus 0.1 % *Tween 20*, washed thoroughly in sterile distilled water and germinated overnight in the dark on wet sterile filter paper. Seedlings were transferred to Petri dishes containing half-strength MS medium supplemented with 30 g dm^{-3} sucrose and solidified with 2.5 g dm^{-3} *Phytagel* (*Sigma-Aldrich*, St. Louis, MO, USA). Dishes were maintained for 7 d in the growth chamber at 23 ± 1 ºC and a 16-h photoperiod under cool white florescent tubes (PFD of 42 μmol m^{-2} s^{-1}). Seedling leaves and stems were detached from roots, finely chopped, and 1 g aliquots of tissue were added to 25 cm^3 of full-strength liquid MS salts medium containing 2 mg dm^{-3} kinetin and 0.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) in 50-cm^3 Erlenmeyer flasks. Cultures were incubated on a rotary shaker at 100 rpm initially in the dark and then under a 16-h photoperiod after 2 weeks. Aliquots (5 cm^3) of cell cultures were transferred into 50 cm^3 of fresh medium every 2 weeks.

Preliminary experiments compared salt stresses imposed by 10, 100, and 200 mM NaCl (data not shown), from which the 200 mM treatment was chosen for further study. Sterile NaCl was added to a final concentration of 200 mM, or an equivalent volume of sterile water was added for control treatments. Cells were collected by vacuum filtration after 1, 3, 6, and 24 h, and 2, 3, 4, and 5 d of salt stress, flash frozen in liquid nitrogen, and stored at -80 ºC. The experiment was repeated three times with similar results.

For ABA and fluridone treatments, seeds were sterilized and germinated as described above and placed on 9 cm diameter Petri dishes containing solidified (15 g dm^{-3} *Bacto* agar) half-strength MS medium with 2 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 5.7). Dishes were oriented vertically and incubated in the growth chamber at 21 ºC and a 16-h photoperiod with PFD of 42 μmol m^{-2} s^{-1}. After 5 d, seedlings were transferred onto 53 μm nylon mesh on fresh agar medium containing 10 μM fluridone, 10 μM ABA (*Sigma-Aldrich*), or 200 mM NaCl in various combinations. Fluridone and ABA concentrations were chosen as described by Kikuchi *et al.* (2006), dissolved in dimethyl sulfoxide (DMSO) and 95 % ethanol, respectively, and further diluted in water to make 200 and 100 mM stocks, respectively, as described by Zhang and Gusta (2009). Dishes were placed in a plexiglass container to control evaporation and whole seedlings were harvested after 24 h (fluridone) or 24 and 48 h (ABA), flash frozen in liquid nitrogen, and stored at -80 ºC. The experiment was repeated once with similar results.

Total RNA was extracted using *TRIzol* reagent (*Invitrogen Life Technologies*, Carlsbad, CA, USA), followed by chloroform extraction and 2-propanol precipitation according to the manufacturer's protocol with minor modifications. Samples were incubated at -20 ºC overnight, slowly shaken on a rotary shaker at room temperature for 10 min, centrifuged at 12 000 *g* for 10 min at 4 ºC, washed with 75 % ethanol, briefly air dried, and resuspended in 50 mm^3 diethyl pyrocarbonate-treated water. RNA was further treated with *TURBO* DNase (*Ambion, Inc*/*Applied Biosystems*, Austin, TX, USA) at 37 ºC for 30 min, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 100 % ethanol and redissolved in water. Total RNA content was measured on a *NanoDrop* spectrophotometer (*NanoDrop Technologies*, Wilmington, DE, USA) and RNA integrity was checked by electrophoresis in a 1 % agarose-formaldehyde gel. First-strand cDNA synthesis was performed using *SuperScript* III reverse transcriptase (*Invitrogen Life Technologies*) using 1 µg of total RNA primed with random hexamers following the manufacturer's instructions.

Quantitative real-time reverse transcriptase PCR (qRT-PCR) was conducted using *SYBR GreenER SuperMix* universal qPCR cocktail (*Invitrogen*). CMO specific primers were designed using *PRIMER 3* software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html; Rozen and Skaletsky 2000) with a targeted GC content of 45 - 55 %. Forward (5'-AGTGCATGC ATTTCACAACG-3') and reverse (5'-TGAGTTTCA GCTTGGGTTGC-3') primers were used to amplify a 150-bp amplicon. Normalization of cDNA content in each reaction was accomplished by co-amplifying a 151-bp amplicon from 18S rRNA as described by Balaji *et al.* (2003), using 5'-GTGACGGGTGACGGAGAATT-3' and 5'-GACACTAATGCGCCCGGTAT-3' as reverse and forward primers, respectively. PCR was carried out in a *RotorGene 6000* thermal cycler (*Corbett Life Science*, Mortlake, NSW, Australia). Each 0.02 cm^3 reaction mixture contained *SYBR GreenER* qPCR master mix, 0.002 cm^3 of 10× diluted cDNA and 200 nM each primer. Amplification conditions were: 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s. A melt curve analysis was performed from 60 to 95 ºC, and CMO expression was normalized against 18S rRNA expression using the comparative quantitation function of the *RotorGene* software.

Northern blotting and hybridizations were carried out as described by Kern *et al.* (2005) with slight modifications. Briefly, total RNA (5 µg per lane) was separated on a 1 % agarose gel containing 2 % formaldehyde, blotted to positively charged *Osmonics Magna* nylon transfer membrane, and followed by UV-cross-linking. A CMO cDNA probe was generated by a *SpeI/EcoRI* digest of the *K. scoparia* CMO coding region to create a 370-bp fragment, which was agarose gel purified using a *QIAquick* gel extraction kit (*Qiagen*, Valencia, CA, USA). Radioactive labeling, probe hybridization and X-ray autoradiography were done as described by Kern and Dyer (2003). Ethidium bromide-stained RNA gels demonstrate equal loading of lanes for each blot.

A *K. scoparia* genomic library was constructed in *Lambda gt10* (*Promega*, Madison, WI, USA) and screened according to Sambrook *et al.* (1989). Briefly, DNA was isolated using the CTAB protocol (Doyle and Doyle 1990), partially digested with *EcoRI*, size-fractionated by pulsed field electrophoresis, and fragments between 1.5 and 7 kb were recovered by gel purification. DNA was ligated and packaged according to manufacturer's instructions (*Promega*), and the library was screened using a radiolabeled 245-bp partial CMO cDNA as described by Kern and Dyer (2003).

DNA was isolated from hybridizing *Lambda* plaques using the *Promega Wizard Lambda Prep* DNA purification system (*Promega*) and the *Lambda* mini kit (*Qiagen*) according to manufacturers' instructions. DNA sequencing was conducted by *Geneway Company* (Hayward, CA, USA) using lambda forward and reverse primers (*Promega*) and sequence-specific primers designed using *Primer3* software (Table 1). Canonical promoter sequences were identified using the *Softberry TSSP* package (Mount Kisco, NY; http://www.softberry.com; Shahmuradov *et al.* 2003).

Table 1. Primers used for PCR amplification and sequencing of the *Kochia scoparia* CMO promoter region.

Name	Sequence (5' - 3')
3pCMO	GCACTTGGCCCTTATGGCCGCACTAGCCATCAAATTAAAGAGGC
5pCMO-493	CCAGCTGGCCAAATCGGCCATGTTGATTATAAACTTAATATGGC
5pCMO-894	CCAGCTGGCCAAATCGGCCACCAATACTTCAATTCTCTCATCC
5pCMO-1247	CCAGCTGGCCAAATCGGCCTATGATGGTCACCATCCCATC

Results and discussion

K. scoparia cell suspensions were treated with 200 mM NaCl and steady-state CMO mRNA levels were observed by Northern blotting (Fig. 1). Unexpectedly, the initially low transcript levels declined rapidly upon exposure to

salt stress, reaching barely detectable levels by 3 and 6 h. Transcript levels stayed low and did not recover to pre-treatment levels until 2 d after treatment. Similar results were seen in three independent replications of the experiment (data not shown), and are in direct contrast to our previous work using whole plants of *K. scoparia* that demonstrated a rapid, massive induction of CMO mRNA in response to salt stress (Kern and Dyer 2003).

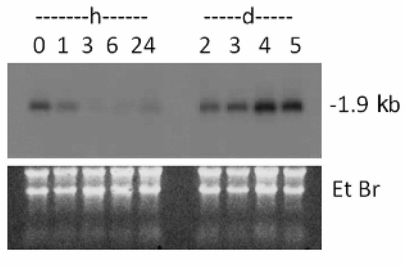

Fig. 1. CMO mRNA levels (top panel) and ethidium bromid-stained total RNA (bottom panel) isolated from suspension cultured *Kochia scoparia* cells at the indicated hours (h) and days (d) after treatment with 200 mM NaCl.

Physiological responses to environmental stresses may be different in cell suspension cultures than in whole plants, depending on the species. In highbush blueberry (*Vaccinium corymbosum* L. × *Vaccinium darrowi* Camp), steady-state levels of two dehydrin mRNAs, previously shown to be induced by cold stress of whole plants, were decreased in cell cultures after osmotic and cold stresses (Parmentier-Line *et al.* 2002). For *K. scoparia*, the lack of CMO induction in cultured cells after salt stress may indicate that the stress is perceived differently by cells than in whole plants. In *Atriplex semibaccata* and *Atriplex halimus*, species closely related to *K. scoparia*, both stem cell cultures and whole plants accumulated similar amounts (on a dry mass basis) of GB and choline in response to salt and osmotic stresses (Koheil *et al.* 1992). It is possible that our suspension cells, since they were initiated from shoot tissues, responded differently than would *K. scoparia* cultured root cells. Although we did not measure GB content in *K. scoparia* cell cultures, our previous work documented that GB accumulated up to 4 % of total plant dry mass after NaCl treatment (Kern and Dyer 2003).

To compare CMO expression in *K. scoparia* seedling shoot and root tissues in response to stress, qRT-PCR was used. In shoots, CMO mRNA was induced rapidly in response to NaCl treatment, reaching a peak about 10-fold higher than control levels by 3 h after treatment (Table 2). mRNA levels remained about 6- to 8-fold higher than control levels through 288 h (12 d) after treatment. Seedlings were separately treated with an iso-osmotic concentration of PEG-8000 (Ahmad *et al.* 2007) to separate NaCl toxicity from osmotic stress. CMO mRNA induction in shoots followed the same general pattern as seen for the NaCl treatment, albeit at slightly lower levels. These results indicate that CMO induction is not due solely to NaCl toxicity, but is a response to osmotic stress, as imposed by either NaCl or PEG. Similar results were seen for drought-stressed plants (data not shown).

In roots, CMO induction by both stress treatments was substantially slower and of a lesser extent than seen in shoots (Table 2). NaCl treatment resulted in only about a 2-fold increase in mRNA levels, and this was not achieved until 24 h after treatment. Transcript levels remained at this level through the next 12 d. PEG treatment resulted in a similar overall pattern of induction, with transcript levels only slightly higher than those observed after NaCl treatment. For both NaCl and PEG stresses, accumulation of CMO mRNA in root tissues was much less than that observed in shoot tissues.

Table 2. Relative expression (arbitrary units) of CMO mRNA in *Kochia scoparia* shoots and roots after treatment with 100 mM NaCl or 18 % PEG-8000. Means ± SE, $n = 9$.

Tissue	Time [h]	Untreated	NaCl	PEG 8000
Shoots	0	0.19±0.05	0.24±0.05	0.25±0.03
	3	0.56±0.09	2.32±0.28	1.30±0.38
	6	0.59±0.08	1.56±0.30	1.97±0.21
	24	0.65±0.07	1.57±0.16	1.24±0.20
	72	0.54±0.06	1.57±0.04	1.22±0.16
	168	0.39±0.09	1.32±0.05	1.20±0.05
	288	0.29±0.06	1.08±0.11	0.59±0.08
Roots	0	0.39±0.05	0.38±0.04	0.40±0.05
	3	0.27±0.06	0.20±0.04	0.21±0.05
	6	0.30±0.07	0.31±0.05	0.36±0.09
	24	0.43±0.06	0.59±0.04	0.71±0.04
	72	0.31±0.03	0.58±0.03	0.81±0.07
	168	0.33±0.07	0.61±0.06	1.40±0.09
	288	0.31±0.09	0.59±0.07	0.79±0.05

A similar, differential pattern of CMO induction was observed in *Atriplex prostrata* seedlings, where treatment with 2 % NaCl resulted in a 5-fold increase in transcript levels in young shoots, but only a 2-fold increase in roots by 3 d after treatment (Wang and Showalter 2004). Significant differences between shoots and roots in the expression of a number of salt stress-responsive genes were documented in *Arabidopsis* (Ma *et al.* 2006), and there are likely differences among other species as well.

To determine if ABA is involved in the signal transduction pathway leading to enhanced CMO expression, *K. scoparia* seedlings were treated with fluridone, an inhibitor of ABA biosynthesis (Bartels and Watson 1978). Fig. 2*A* shows that fluridone did not block CMO induction in response to NaCl. This result was verified by a separate experiment in which seedlings were treated with ABA and the basal levels of CMO expression were not affected by ABA treatment (Fig. 2*B*). Results from separate experiments on older (4 - 5 true leaves) plants were similar (data not shown). Together, the results confirm that ABA does not participate in salt-induced CMO expression in *K. scoparia*, suggesting that

CMO induction proceeds through an ABA-independent pathway.

A 1 246 bp fragment upstream of the CMO coding region was sequenced using a set of sequence-specific primers. The resulting overlapping fragments were compiled and subjected to DNA sequence analysis (Fig. 3). The translational start site was predicted by comparison to other published CMO genomic and cDNA sequences (data not shown). Sequence analysis predicted a transcription start site, a putative TATA box at -55 bp upstream, and numerous CAAT boxes. Potential regulatory elements known to be involved in stress-related responses were detected, including two ABREs at -1107 and -264 bp, two anaerobic1 motifs (induced by anaerobic conditions) at -729 and +19 bp, an anaerobic3 motif at -6 bp, and a hypo-osmolarity responsive element (ACTCAT; Satoh *et al.* 2002) at -780 bp. Although the presence of two ABREs might indicate that CMO induction is ABA-dependent, a CE3-like motif (CGTGTC; Hobo *et al.* 1999), thought to be required for full ABA response, was not present on either strand of this DNA fragment. The dehydration-responsive element (DRE; TACCGACAT), required for ABA-independent gene induction in response to dehydration, salt, or cold stress in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994) was similarly not present. As found once in the *A. tricolor* CMO upstream region (Bhuiyan *et al.* 2007), the NaCl-inducible, GT-1 *cis*-element GAAAAA (Park *et al.* 2004) was found at -98 and -65 bp in the *K. scoparia* CMO upstream region. Promoter analysis also detected four instances of MYB transcription factor consensus recognition sequences at -648, -608, -208, and -122 bp. W-box motifs, the recognition sequence for WKRY transcription factors (Eulgem *et al.* 2000), were found at -442 and +66 bp.

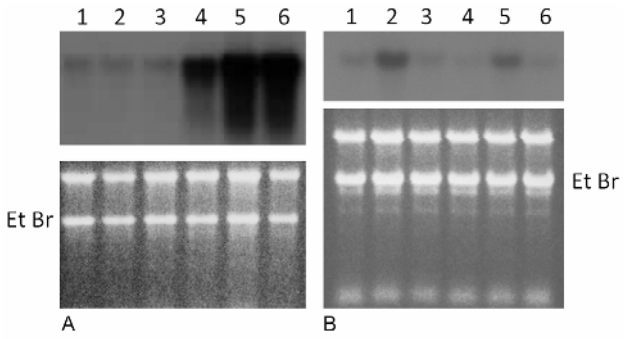

Fig. 2. *A* - CMO mRNA levels (*top panel*) and ethidium bromide-stained total RNA (*bottom panel*) from NaCl- and fluridone-treated *Kochia scoparia* seedlings. 5-d-old seedlings were treated with water (*lane* 1), 0.01 % DMSO (*lane* 2), 10 µM fluridone (*lane* 3), 100 mM NaCl (*lane* 4), 100 mM NaCl + 0.01 % DMSO (*lane* 5), or 100 mM NaCl + 10 µM fluridone (*lane* 6) and total RNA isolated after 24 h. *B* - CMO mRNA levels (*top panel*) and ethidium bromide-stained total RNA (*bottom panel*) from NaCl- and ABA-treated *Kochia scoparia* seedlings. 5-d-old seedlings were treated with 10 µM ABA (*lane 1*), 100 mM NaCl (*lane 2*), 10 µM ABA (*lane 3*), 0.25 % ethanol (*lane 4*), 100 mM NaCl + 10 µM ABA (*lane 5*), or water (*lane 6*) and total RNA isolated after 24 (*lanes 3 - 6*) or 48 h (*lanes 1 and 2*).

Because NaCl-induced CMO expression in *K. scoparia* was rapidly down-regulated by treatment with the auxinic herbicide *Dicamba* (Kern and Dyer 2003), we examined the CMO upstream region for auxin-related regulatory elements. Neither the auxin response factor (ARF) motif (TGTCTC; Guilfoyle and Hagen 2007) nor core sequences of the auxin-responsive NDE element in the SAUR 15A gene promoter (AAAACAA or CATATG; Xu *et al.* 1997) were found on either strand in either orientation in this DNA fragment. However, recent work suggests that ARF elements may not be involved in auxin-mediated down-regulation (Lee *et al.* 2009).

In summary, this work shows that NaCl-induced CMO expression in *K. scoparia* proceeds *via* an ABA-independent signal transduction pathway. Transcription levels in shoots are substantially higher than in roots, in response to both NaCl and osmotic stresses. The rapid induction seen in whole plants is not reflected in suspension-cultured *K. scoparia* cells in response to the same NaCl stress, where an apparent down-regulation of CMO expression is observed. Further research will be required to determine if the mechanism of salt tolerance in cultured cells is the same as that in whole plants. Examination of the CMO upstream regulatory region reveals several stress response-related elements, indicating potential avenues for further research on the drought and salt tolerance mechanisms in this species.

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