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Glycine Betaine Biosynthesis Is Induced by Salt Stress but Repressed by Auxinic Herbicides in *Kochia scoparia*

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Abstract

Kochia scoparia biotypes that are susceptible or resistant to the auxinic herbicide dicamba were used to characterize expression levels of choline monooxygenase (CMO) and glycine betaine accumulation in response to salt stress and herbicide treatment. A 1180-bp cDNA was isolated using differential display and 3′ RACE with a deduced amino acid sequence that was more than 90% similar to the carboxy terminal 290 residues of CMOs from four related plant species. Salt stress led to a substantial increase in CMO mRNA and enzyme levels in *K. scoparia* biotypes, and the accumulation of up to 80 μmol g^{-1} fresh weight glycine betaine. In contrast, dicamba treatment was followed by the rapid attenuation of CMO message and protein levels, with a recovery of expression in the resistant but not the susceptible biotype. CMO mRNA and enzyme levels similarly declined, and recovered in the resistant biotype, after dicamba treatment of plants that were previously salt stressed for 4 days. The opposing effects of these two stresses may represent a regulatory scheme in which competition for the substrate choline leads to a repression of glycine betaine biosynthesis to make sufficient choline available for auxin-mediated growth processes.

Key words: Auxinic herbicide; Choline monooxygenase; Dicamba; Osmotic adjustment

Introduction

Glycine betaine (GB) is synthesized by several plant families in response to salt or osmotic stress, and serves as a compatible solute to protect proteins and membranes from the damaging effects of salts as well as functioning in cytoplasmic osmoregulation (Yancey 1994; Papageorgiou and Mutata 1995). Choline monooxygenase (CMO) catalyzes the primary, regulatory step of GB biosynthesis by oxidizing choline to betaine aldehyde, which is subsequently oxidized to GB by betaine aldehyde dehydrogenase (BADH). CMO is apparently unique to members of the Chenopodiaceae and Amaranthaceae (Russell and others 1998) and appears to be a soluble ferredoxin- and oxygen-dependent enzyme localized primarily in the chloroplast stroma. The enzyme is an unusual oxygenase, being insensitive to carbon monoxide and having char-

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acteristics of iron-sulfur Rieske-type proteins not related to cytochrome P450-type monooxygenases (Burnet and others 1995).

GB is constitutively synthesized at low levels in some species of the Chenopodiaceae (Grieve and Maas 1984) including *Kochia scoparia* (Selvaraj and others 1995) and production is significantly increased in response to osmotic stress (Guy and others 1984; Russell and others 1998). GB accumulation occurs as a result of CMO gene induction, as documented in *Spinacia* (Rasinasabapathi and others 1997), *Beta* (Russell and others 1998), and *Amaranthus* (Wang and others 1999). Expression of BADH, the second enzyme of the GB pathway, is also induced by osmotic stress in *Amaranthus hypochondriacus* (Legaria and others 1998) and *Hordeum vulgare* (Ishitani and others 1995). Although plant responses to osmotic and other stresses are often mediated by abscic acid (ABA) (Franks and Farquhar 2001), the effects of phytohormones on CMO expression have not been investigated.

Kochia scoparia (L.) Schrader (= *Bassia scoparia* (L.) A.J. Scott) (cyclolobeae, Chenopodiaceae, Caryophyllidae) is an annual herbaceous dicot native to Russia (Hitchcock and others 1964) that has established extensive populations in cultivated fields, waste areas and disturbed ruderal habitats across much of the United States and southern Canada (Durham and Durham 1979; Eberlein and Fore 1984). The species is highly invasive and competitive in agronomic cropping systems, due to high fecundity, extensive seed dispersal via its tumbleweed growth habit, and exceptional tolerance to environmental stresses such as drought, salinity, temperature extremes, and disturbance (Becker 1978).

Widespread use of the sulfonylurea herbicide chlorsulfuron in the late 1980s led to the selection of herbicide-resistant *K. scoparia* populations (Dyer and others 1993; Primiani and others 1990), and more recently, reliance on auxinic herbicides has selected for dicamba-resistant biotypes (Miller and others 1997). The mechanism of dicamba resistance is currently unknown, but is not due to altered herbicide uptake, translocation, or metabolism (Cranston and others 2001). As part of an ongoing effort to understand mechanisms of herbicide resistance in weedy plant species, we used mRNA differential display to isolate genes whose expression levels were rapidly altered in response to dicamba treatment (Kern 2002). One gene thus identified appears to encode CMO, and significantly, its mRNA levels rapidly declined after dicamba treatment of whole plants. This report extends the characterization of this response, in addition to monitoring expression levels as affected by a combination of osmotic and herbicide stresses imposed on dicamba-resistant and susceptible plants.

Methods and Materials

3′ RACE

The 245 bp KSR2 partial CMO cDNA obtained by mRNA differential display (Kern 2002) was used to obtain a more complete cDNA using 3′ RACE (3′ RACE System for Rapid Amplification of cDNA Ends®; Gibco Life Technologies) according to manufacturer's instructions. Poly(A)$^+$ RNA (50 ng) was incubated at 70°C in the presence of 0.5 μM anchored primer 5′-GGCCACGCGTCGACTAGTA $CT_{(17)}$-3′ in 12 μl nuclease-free water for 10 min, followed by 5 min on ice. Reverse transcription was performed in a 20 μl reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 100 μM each dNTP, and 200 units Superscript II® Reverse Transcriptase (Gibco Life Technologies) at 42°C for 50 min. Reactions were terminated by heating to 70°C for 10 min followed by RNase H digestion. cDNA (5 ng) was then amplified in a 50 μl PCR reaction containing 1 unit AmpliTaq Gold® DNA polymerase (Perkin Elmer), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 nM specific primer (5′-AGTGCATGCA TTTCACAACG-3′), 200 nM universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′), and 200 μM each dNTP. Cycling conditions included an initial 3-min denaturation step at 94°C, followed by 35 cycles of 94°C for 30 sec, 55°C for 60 sec, 72°C for 30 sec, and a final extension step of 72°C for 10 min in a Perkin Elmer 9600 thermocycler. Twenty μl of the PCR reaction was analyzed using agarose gel electrophoresis and ethidium bromide staining to verify the expected amplification product (1.2 Kb), and the band was excised and ligated into the pCR 2.1/TOPO TA cloning vector (Invitrogen Corp.). DNA sequencing of both strands using the M13 Forward (−40) and Reverse primers was performed at the Washington State University DNA Sequencing Facility (Pullman, WA) using the BigDye™ dideoxy termination method. DNA sequences were compared to sequence databases using the BLASTN and BLASTX algorithms (Altschul and others 1997). Deduced amino acid sequences were aligned with known sequences using the Genetics Computer Group GCG (v. 10.0) PILEUP program.

Plant Material and Stress Treatments

The dicamba-resistant HRd biotype was derived from a resistant *K. scoparia* field population and

subjected to six generations of recurrent herbicide selection in the greenhouse as described (Cranston and others 2001). In addition to dicamba resistance, HRd is resistant to the auxinic herbicides MCPA and picloram, as well as unrelated sulfonylurea herbicides. The S1 biotype was derived from a field population of dicamba-susceptible *K. scoparia* plants and grown under pollen isolation conditions for three generations as described (Cranston and others 2001). Both HRd and S1 biotypes were maintained as families of about 30 individuals rather than using single-seed descent to avoid symptoms of inbreeding depression, as previously observed for this highly outcrossing species (Mulugeta 1991).

Seeds were planted 0.5 cm deep in 54 by 26 by 6 cm flats containing soil mix (1:1:1 Bozeman silt loam:washed sand:peat moss), grown under natural light supplemented with mercury vapor lamps (14 hour daylength; 24/18°C day/night; 165 μmol m^{-2} s^{-1} PPF), and fertilized and watered as needed. After 6 weeks, HRd and S1 plants were subjected to salt stress by adding solutions of NaCl dissolved in tap water. Plants were treated with increasing NaCl concentration by sequentially adding two soil volumes (4 L) of 150 mM, 300 mM, 500 mM, and 750 mM NaCl every 2 days. Plant material (10 g fw) from at least six plants was pooled into liquid nitrogen 0, 2, 4, 6, and 8 days after NaCl treatments began. For dicamba treatment, 3- to 5-cm tall plants were sprayed with 70 g ai ha^{-1} dicamba (formulated as the dimethylamine salt; Banvel® herbicide, BASF Corporation) using a greenhouse belt sprayer delivering a carrier volume of 94 L water ha^{-1}. For combination treatments, plants were treated with dicamba 8 days after NaCl treatments began and harvested after 0, 0.5, 1, 1.5, 2, 6, 12, 24, and 48 hours by pooling shoot tissue (10 g fw) from at least six plants as above.

Northern Blot Analysis

Frozen tissue (0.5 g fw) was pulverized to a powder under liquid nitrogen using a prechilled mortar and pestle and transferred to a 1.5-ml centrifuge tube. Total RNA was extracted using a phenol/chloroform extraction procedure modified from Ausubel and others (1994). One-half ml TIE extraction buffer (0.2 M Tris [pH 8.2], 0.1 M lithium chloride [LiCl], 5 mM EDTA), 0.15 ml TLE-equilibrated phenol, and 0.15 ml chloroform were mixed with the frozen tissue, and the tube was incubated at 50°C for 30 min with occasional vortexing. After centrifugation for 1 min at 14,000*g* at room temperature, the aqueous phase was transferred to a new tube containing 0.3 ml phenol:chloroform (1:1), vortexed, and centrifuged as before. The aqueous layer was twice more extracted with phenol:chloroform, and total RNA was precipitated by adding 0.33 volumes of 8 M LiCl and incubating for 16 hours at 4°C. After centrifugation for 30 min at 14,000*g* and 4°C, the RNA pellet was washed with 0.5 ml 2 M LiCl, resuspended in 100 μl sterile water, and ethanol precipitated. The RNA was again recovered by centrifugation as above, resuspended in 100 μl sterile water, and stored at −80°C. RNA obtained by this method was judged to be free of degradation by denaturing agarose gel electrophoresis and ethidium bromide staining.

Total RNA (12 μg per lane) was denatured for 5 min at 65°C in 50% formamide/6% formaldehyde, electrophoresed at 8 volts/cm for 90 min in a 1% agarose gel containing 2% formaldehyde, stained with ethidium bromide to verify equal loading, transferred overnight to a positively charged nylon membrane (Osmonics Magna nylon transfer membrane, 0.45 μm pore size), and bound to the membrane by UV-crosslinking. To make cDNA probes, 80 ng of purified KSR2 insert was labeled with 50 μCi ^{32} P-dCTP (specific activity >3000 Ci/mmol; DuPont/NEN) using a random hexamer priming kit (Ready-To-Go labeling beads, Amersham Pharmacia) to a specific activity of more than 10^9 dpm/μg DNA and unincorporated nucleotides were removed using G50 Sephadex columns (ProbeQuant G50 micro columns, Amersham Pharmacia). The probe was denatured and added to RNA blots preincubated for 4 hours at 65°C in 4 ml of aqueous hybridization buffer (Sambrook and others 1989). Blots were hybridized overnight at 65°C, washed once for 15 min in 100 ml 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na_3 citrate·$2H_2O$, pH 7.0) at room temperature, followed by sequential 20 min washes at 65°C in 100 ml 2× SSC, 1× SSC, and 0.5× SSC. Blots were exposed to Kodak X-OMAT X-ray film for 10 min to 1 week, depending on signal intensity as detected by a Geiger-Mueller counter. Relative changes in hybridization intensities for each mRNA were determined using a Bio-Rad PhosphorImager, and approximations of relative mRNA abundance were made by comparing signal intensities and times required to achieve detectable hybridization signals among blots.

Protein Extraction

Frozen shoot tissue (1 g fw) was pulverized to a powder under liquid nitrogen using a prechilled mortar and pestle, transferred into a 50 ml Falcon tube, and extracted with 20 ml ice-cold buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA,

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                    1                                                  50
Kochia scoparia     CRAGEGKVHA FHNVCTHRAS ILACGSGKKS CFVCPYHGWV FGMNGDLTKA
                    148
Spinacia oleraceae  SRDGEGKVHA FHNVCTHRAS ILACGSGKKS CFVCPYHGWV FGMNGDLTKA
                    149
Atriplex nummularia CRDGEGKVHA FHNVCTHRAS ILACGSGKKS CFVCPYHGWV YGMNGSLTKA
                    158
Beta vulgaris       SRDGQGELHA FHNVCTHRAS ILACGSGKKS CFVCPYHGWV YGLDGSLAKA
                    151
Amaranthus tricolor CRDGQGKVHA FHNVCTHRAS ILACGTGKKS CFVCPYHGWV FGLDGSLMKA
                                  ^ ^                 ^ ^

                    51                                                 100
Kochia              TQA.ETQTFD AKELGLVALK VAVWGPFVLI SLDKTLPE.T .DVGTEWLGK
Spinacia            SKAKPEQNLD PKELGLVPLK VAVWGPFVLI SLDRSLEE.G GDVGTEWLGT
Atriplex            SKATPEQSLN PDELGLVPLK VAVWGPFILI SLDRSSRE.V GDVGSEWLGS
Beta                SKATETQNLD PKELGLAPLK VAEWGPFILI SLDRSLDA.N ADVGTEWIGK
Amaranthus          TK.TENQVFD PKELGLVTLK VAIWGPFVLI SLDRSGSEGT EDVGKEWIGS

                    101                                                150
Kochia              SAEDVKAHAF DPSLQFIHRS EFPMECNWKV FCDNYLDSSY HVPYAHKYYA
Spinacia            SAEDVKAHAF DPSLQFIHRS EFPMESNWKI FSDNYLDSSY HVPYAHKYYA
Atriplex            CAEDVKAHAF DPNLQFINRS EFPIESNWKI FSDNYLDSSY HVPYAHKYYA
Beta                SAEDVKAHAF DPNLKFTHRS EFPMECNWKV FCDNYLDSSY HVPYAHKYYA
Amaranthus          CAEEVKKHAF DPSLQFINRS EFPMESNWKV FCDNYLDSAY HVPYAHKYYA
                                                    ♦  ♦       ♦  ♦

                    151                                                200
Kochia              TELDFDTYDT QMIENVVIQR VGGNKNKTDG IDRLGNQAFY AFAYPNFAIE
Spinacia            TELNFDTYDT QMIENVTIQR VEGSSNKPDG FDRVGIQAFY AFAYPNFAVE
Atriplex            TELDFDTYQT DMVGNVTIQR VAGTSN..NG FNRLGTQAFY AFAYPNFAVE
Beta                AELDFDTYNT EMIEKCVIQR VGSSSNKPDG FDRLGTEAFY AFIYPNFAVE
Amaranthus          AELDFDTYKT DLLEKVVIQR VASSSNKPNG FDRLGSEAFY AFIYPNFAVE

                    201                                                250
Kochia              RYGPWMTTMH VQPLGLRKCK LVVDYYIEDS KLEDKDYIEK GIAINDNVQS
Spinacia            RYGPWMTTMH IHPLGPRKCK LVVDYYIENS MLDDKDYIEK GIAINDNVQR
Atriplex            RYGPWMTTMH IVPLGPRKCK LVVDYYIEKS KLDDKDYIEK GIAINDNVQK
Beta                RYGTWMTTMH VVPMGQRKCK LVVDYYLEKA MLDDKAYIDK GIAINDNVQK
Amaranthus          RYGPWMTTMH IGPLGPRKCK LVVDYYLENA MMNDKPYIEK SIMINDNVQK

                    251                                                293
Kochia              EDKVLCESVQ RGLETPSYST GRYVMPIEKG IHHFHCWLHQ VLQ*
Spinacia            EDVVLCESVQ RGLETPAYRS GRYVMPIEKG IHHFHCWLQQ TLK*
Atriplex            EDVVLCESVQ KGLETPAYRS GRYVMPIEKG IHHFHCWLHQ VLK*
Beta                EDKVLCESVQ RGLETPAYRS GRYVMPIEKG IHHFHCWLHE TLQ*
Amaranthus          EDVVLCESVQ RGLETPAYRS GRYVMPIEKG IHHFHCWLHQ TLN*
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Figure 1. Alignment of deduced amino acid sequence from *K. scoparia* WCMO cDNA with CMO sequences from *Spinacia oleraceae* (accession U85780), *Atriplex nummularia* (AB112481), *Beta vulgaris* (AF023132), and *Amaranthus tricolor* (AF290974). Amino acid sequence identities among the cDNAs are in bold and stop codons are indicated by asterisks. Conserved cysteine/histidine pairs involved in the putative Rieske-type cluster are underscored by a ^. Conserved asparagine/histidine residues involved in mononuclear iron binding are underscored by a ♦.

20 mM sodium borate, 50 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride, 10 mM DTT, 15 µl Antifoam A (Sigma Chemical Co.), and 0.2 g polyvinylpolypyrrolidone by homogenization with a Polytron® tissue homogenizer (Brinkmann Co.). Samples were filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem-Novabiochem Corp.) and centrifuged at 10,000*g* and 4°C for 10 min. The protein was precipitated by adding 8 volumes of −20°C acetone, recovered by centrifugation at 10,000*g* and 4°C for 15 min, and the pellet resuspended in 0.5 ml sam-

ple buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 mM sodium borate, 50 mM ascorbic acid, and 10 mM DTT. Total protein was determined according to Bradford (1976) using BSA as a standard. Prior to storage at −20°C, samples were diluted 1:1 with 2× SDS sample buffer (50 mM Tris-HCl, [pH 6.8], 2% [w/v] SDS, 10 mM EDTA, 20% [v/v] glycerol, 20 mM DTT, and 100 μg/ml bromophenol blue), incubated at 85°C for 5 min, centrifuged at 14,000*g* for 5 min at room temperature, and the supernatant transferred to a new 1.5 ml centrifuge tube.

Western Blot Analysis

Protein samples (15 μg per lane) were separated by SDS-PAGE on 12 % acrylamide gels at 50 volts for 16 hours and transferred to polyvinylidene fluoride-coated nylon membranes (BioTrace PVDF; Pall Gelman Sciences) by electrophoretic transfer at 50 volts constant current for 4 hours in transfer buffer containing 10 mM Tris (pH 8.3), 200 mM glycine, and 15% (v/v) methanol. Immunodetection was performed by adding 4 μl mouse polyclonal antibody raised against the *Spinacia oleraceae* CMO protein (graciously provided by Dr. Andrew Hanson, University of Florida) to protein blots preincubated for 3 hours in 4 ml T-TBS (100 mM Tris [pH 7.5], 0.9% [w/v] NaCl, 0.1% [v/v] Tween 20) supplemented with 5% (w/v) nonfat dried milk. Blots were incubated with gentle agitation at room temperature for 16 hr with the CMO antibody and washed three times for 5 min each in 5 ml T-TBS. Next, 2.5 μl goat anti-mouse IgG/alkaline phosphatase (Sigma Chemical Co.) in 5 ml T-TBS plus 5% nonfat dried milk was added and the blot was incubated with gentle agitation at room temperature for 2 hr. The blots were washed three times for 5 min each in 5 ml T-TBS followed by two washes for 5 min each in alkaline phosphatase buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$), and developed using a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium salt substrate system (Sigma Chemical Co.). Intensities of 42 and 43 kD immunoreactive protein bands were quantified by scanning densitometry of blots using 1D Molecular Imaging® software (v. 3.0.1, Kodak Digital Sciences). Results were compared to standard curves generated from 0, 5, 10, 15, and 20 μg of protein extracts.

Glycine Betaine Extraction and ^{1}H NMR

GB was extracted from *K. scoparia* plants using a method modified from Fan and others (1993). Frozen shoot tissue (1 g fw) was pulverized to a powder under liquid nitrogen using a prechilled mortar and pestle and extracted with 3 ml 5% (v/v) perchloric acid in water with occasional vortexing at 0°C for 60 min. Twenty μl of Antifoam A was added and the extracts adjusted to pH 7.0 with 5 M K_2CO_3.The extracts were centrifuged at 10,000*g* and 4°C for 20 min and 1 ml of the supernatant was lyophilized and redissolved in 0.5 ml deuterium dioxide. An internal standard of alanine (10 mg) dissolved in deuterium dioxide was added to each sample prior to analysis.

Nuclear magnetic resonance (NMR) spectra were collected on a Bruker DRX500 NMR spectrometer operating at a proton frequency of 500.13 MHz. Time domain data were collected at a spectral width of 6000 Hz using 32 k complex points. A total of 16 scans were averaged for each spectrum. The proton pulse length was 8 μsec and a 7.7 sec recycle was used. The spectra were zero filled and Fourier transformed to give a final frequency domain size of 16 k real points. The spectra's chemical shift scale was referenced to the residual water peak at 4.8 ppm, and GB concentrations in samples were determined by comparing sample peak areas to a standard curve generated from known amounts of GB dissolved in deuterium dioxide.

Results and Discussion

Analysis of the CMO 3′RACE Product

RT-PCR 3′ RACE of the KSR2 partial-length cDNA yielded a 1180-bp PCR product that was designated as WCMO (GenBank accession AY392096). DNA sequencing revealed an 870-bp open reading frame potentially encoding 290 amino acids with an additional 310 bp of 3′ untranslated region. PILEUP sequence analysis indicated that the deduced amino acid sequence was more than 90% similar to the carboxy terminal 290 residues of choline monooxygenases from *Spinacia oleraceae*, *Atriplex nummularia*, *Beta vulgaris*, and *Amaranthus tricolor* (Figure 1). The deduced amino acid sequence contains several motifs present in CMOs from other plants and related prokaryotic genes encoding oxygenase-like proteins. First, the cysteine/histidine consensus sequence for a Rieske-type [2Fe-2S] cluster (Cys-X-His-X_{15-17} -Cys-X_2 -His) is present in *K. scoparia* CMO, as it is in all CMOs isolated to date. Second, CMO and other monooxygenase cDNAs from numerous bacterial species contain a proposed consensus sequence for coordination sites of mononuclear, nonheme iron (Glu/Asp-X_{3-4}-Asp-X_2-His-

X_{4-5}-His; Jiang and others 1996). The same motif is present in plant CMOs except that the central asparagine and histidine residues are three residues apart instead of two. N-terminal regions of CMOs from *Spinacia* and *Beta* contain putative stromal targeting peptides of 65 and 60 residues, respectively, and an additional approximately 85 residues of conserved (>90% identical) sequence (Russell and other 1998) that was not present in the truncated *K. scoparia* WCMO cDNA.

CMO Expression in Response to Salt Stress

The radiolabeled KSR2 probe recognized a 1.9-Kb mRNA that was present at moderately low levels in resistant (R) and susceptible (S) *K. scoparia* plants in the absence of stress (Figure 2B). However, NaCl treatment was followed by significant accumulations of CMO mRNA. Message levels increased more than 20-fold after S plants had been treated with 500 mM NaCl for 2 days (Figure 2C). In R plants, CMO mRNA levels accumulated more slowly during salt stress but reached levels similar to those in S plants after 8 days of NaCl treatment.

Mouse polyclonal antibodies raised against *Spinacia oleraceae* CMO (Rathinasabapathi and others 1997) recognized two protein bands of 42 and 43 Kd (hereafter referred to as Kochia immunoreactive protein or KIP) in protein extracts from *K. scoparia* (Figure 3A). KIP accumulations generally followed the pattern seen for CMO mRNA, and by 4 days after treatment, S and R plants had about 8- and 9-fold more protein than untreated plants, respectively (Figure 3B). After 8 days, KIP levels in R and S plants were about 15 times their original levels. CMO enzyme levels increased in salt-stressed *Amaranthus tricolor* (Wang and others 1999), *Beta vulgaris*, and *Amaranthus caudatus* (Russell and others 1998) plants by 4 days after treatment with 300 mM NaCl. However, it is evident that *K. scoparia* has the ability to continue accumulating KIP under conditions of more severe osmotic stress, as imposed by 750 mM NaCl.

Other reports using western blotting have detected a single CMO protein of about 45 Kd in *Beta vulgaris*, *Amaranthus caudatus* (Russell and others 1998), and *Amaranthus tricolor* (Wang and others 1999). Although it is possible that the presence of two KIP bands in *K. scoparia* is a degradation artifact, it is also reasonable to speculate that the two bands represent products of different genes. Consistent with this idea, Southern analysis indicated that there are two to four CMO genes in the *K. scoparia* diploid genome (data not shown).

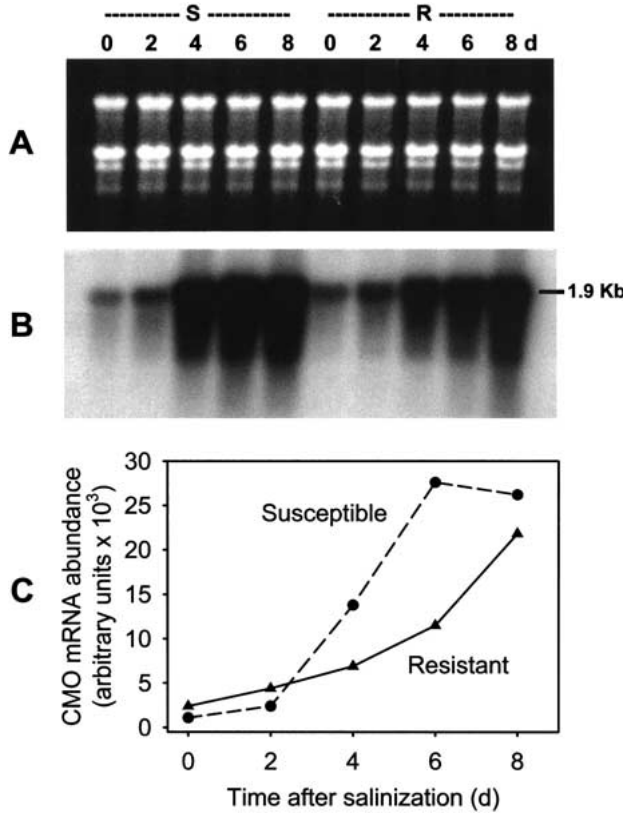

Figure 2. Steady state CMO mRNA levels in susceptible (S) and dicamba-resistant (R) *K. scoparia* plants in response to salt stress. (**A**) Ethidium bromide-stained agarose gel containing total RNA isolated 0, 2, 4, 6, and 8 days after salinization treatment. (**B**) RNA blot from panel A was probed with KSR2. (**C**) PhosphorImager analysis of mRNA abundance from blot in panel B.

Prior to salinization, GB was present at about 10 μmol g^{-1} fw (Figure 3C), which is consistent with the low constitutive levels of GB seen in other members of the Chenopodiaceae (Weretilnyk and Hanson 1990). However, GB levels increased more than 6-fold to 67 and 80 μmol g^{-1} fw in S1 and HRd plants, respectively, after 14 days of NaCl treatment.

The increases in CMO mRNA and KIP levels, as well as GB content, in response to salt stress are consistent with the natural salt and drought tolerance of *K. scoparia*, a species that is highly adapted to xeric environments. In intermountain sagebrush steppe habitats, *K. scoparia* is one of the very few species found growing in localized areas of extremely saline soil (saline seeps). During the course of NaCl stress imposed in these experiments, plant growth essentially ceased, perhaps due to the metabolic costs of GB production, as has been implicated for other osmoregulating compounds (Maggio and others 2002). At the end of the experiments, GB

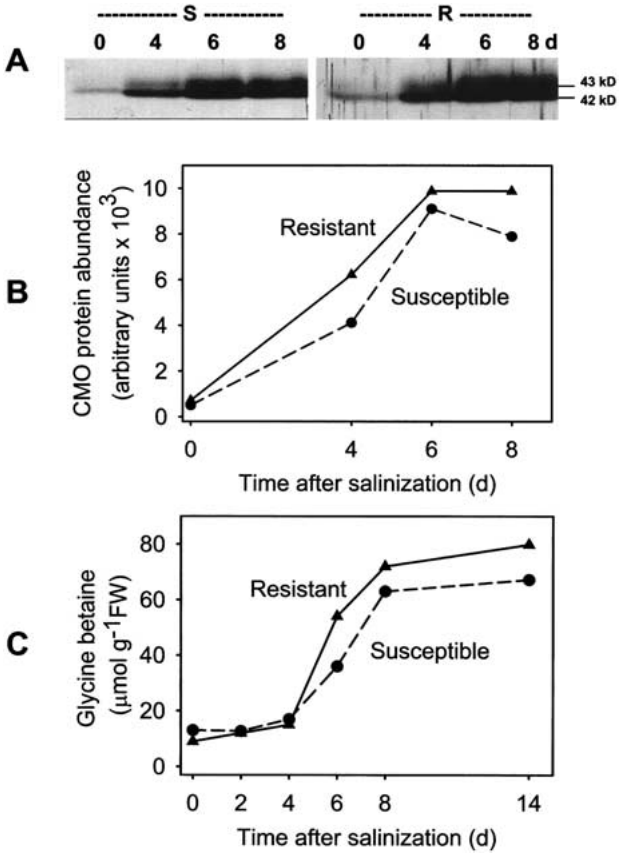

Figure 3. Accumulation of CMO protein and GB in susceptible (S) and dicamba-resistant (R) *K. scoparia* plants in response to salt stress. (**A**) Protein extracted 0, 2, 4, 6, and 8 days after salinization treatment began was probed with mouse anti-CMO antibodies. (**B**) Densitometry analysis of 42 and 43 kD protein bands in panel A. (**C**) GB concentrations in tissue samples from panel A.

had accumulated up to 4% of the total plant dry weight (data not shown), and thus likely represents a significant carbon and nitrogen sink.

Salinity stress is known to cause a number of changes in gene expression, and some of the initial responses seem to be common to other abiotic stresses like drought and cold (Kreps and others 2002). Recent efforts at the genome (Bohnert and others 2001), transcriptome (Kreps and others 2002) and proteome (Salekdeh and others 2002) levels have identified several key responses to alterations in hydration and ion homeostasis, although functions for a significant number of the genes thus implicated remain unknown. Nonetheless, such knowledge has led to real progress in conferring transgenic salt tolerance to crop plant species using CMO (Sakamoto and Murata 2000) and other approaches (reviewed in Jain and others 2003).

CMO Expression in Response to Dicamba Treatment in R and S *K. scoparia*

Steady-state CMO mRNA levels were moderately low in untreated plants (Figure 4A). However, the CMO message was rapidly attenuated in HRd and S1 plants after treatment with 70 g ai ha^{-1} of dicamba, with the apparent down-regulation occurring somewhat more rapidly in R than in S. By 90 min after treatment, both biotypes exhibited a greater than 10-fold reduction in CMO mRNA, and levels remained low in S plants but returned to near steady-state levels in R by 24 hr after treatment (Figure 4B). The difference in CMO mRNA levels between the biotypes after 24 hr may be associated with their different physiological responses to dicamba treatment. By this time, S plants had begun to show injury symptoms such as leaf epinasty, whereas R plants did not. The failure of CMO mRNA levels to recover in S plants may thus be due to the eventual lethal effects of dicamba. Similar reductions in CMO mRNA levels were seen in R and S plants after treatment with 2,4-D, another auxinic herbicide (data not shown).

Western analysis indicated that KIP levels closely followed the kinetics of CMO mRNA abundance (Figure 5A). Basal KIP levels were moderately low and became nearly undetectable by 6 and more hours after treatment. KIP levels remained low in S plants, whereas they recovered slightly in R plants by 48 hours after treatment (Figure 5B). The rapid decline in CMO mRNA and KIP levels in response to dicamba treatment appears to be due to gene repression, although an alteration in mRNA stability could also explain these results. Elevated CMO enzyme levels similarly declined in *Beta vulgaris* after removal of drought stress (Russell and others 1998), although the molecular mechanism was not examined. It is currently unknown if the reductions in GB biosynthetic activity seen after dicamba treatment and upon relief from drought stress are controlled by the same mechanism.

In contrast to the rapid decline in CMO mRNA and KIP levels seen after dicamba treatment, GB concentrations transiently increased in both R and S *K. scoparia* plants (Figure 5C). In S plants, GB levels increased for the first hour after dicamba treatment but then steadily declined to about one-third their initial levels after 24 hr. The GB increase in R plants continued for 2 hr after dicamba treatment, before falling to a similar level as in S plants after 24 hr. The continued accumulation of GB for 1 (S plants) or 2 hr (R plants) after dicamba treatment may reflect residual enzymatic activity of CMO (and BADH) before down-regulation. The reduction of

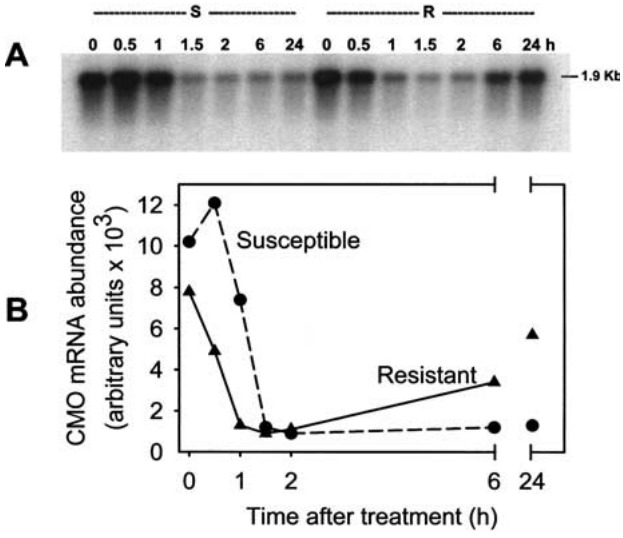

Figure 4. Steady state CMO mRNA levels in susceptible (S) and dicamba-resistant (R) *K. scoparia* plants in response to treatment with 70 g ai ha^{-1} dicamba. (**A**) RNA isolated 0, 0.5, 1, 1.5, 2, 6, and 24 h after dicamba treatment was probed with KSR2. (**B**) PhosphorImager analysis of mRNA abundance from blot shown in panel A.

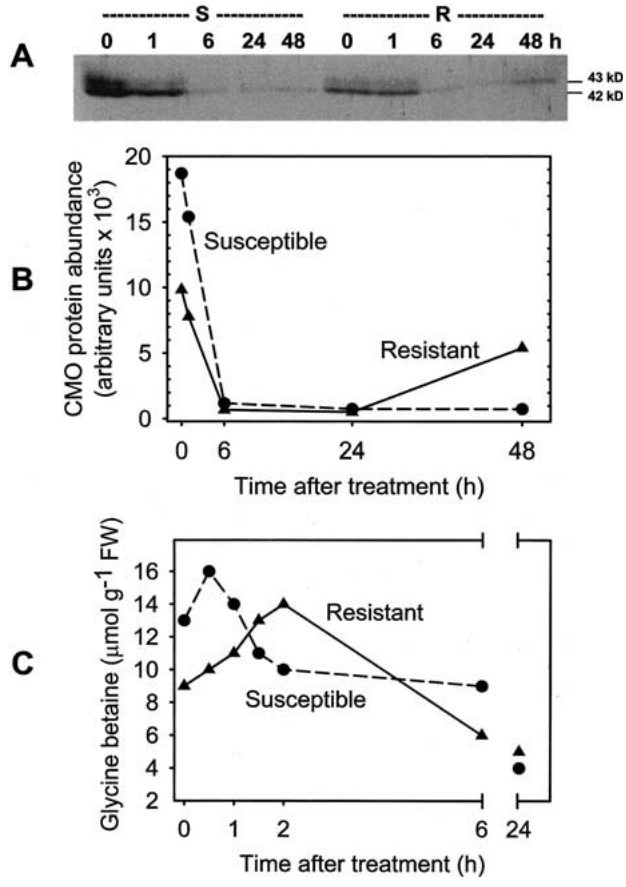

Figure 5. Reduction of CMO protein and GB levels in susceptible (S) and dicamba-resistant (R) *K. scoparia* plants in response to dicamba treatment. (**A**) Protein extracted 0, 1, 6, 24, and 48 h after dicamba treatment was probed with mouse anti-CMO antibodies. (**B**) Densitometry analysis of 42 and 43 kD protein bands in panel A. (**C**) GB concentrations in tissues harvested 0, 0.5, 1, 1.5, 2, 6, and 24 h after dicamba treatment.

GB to levels significantly below those found in untreated tissue supports the idea that GB is metabolized at a low constitutive rate, as has been shown in *Beta vulgaris* (Russell and others 1998) and *Sorghum bicolor* (Wood and others 1996).

CMO Expression in Response to Dicamba Treatment in Salt-stressed *K. scoparia*

Because the auxinic herbicides dicamba and 2,4-D caused a rapid decline in CMO mRNA, KIP, and GB levels in unstressed plants, experiments were conducted to determine whether these herbicides had similar effects on plants that had previously been exposed to salt stress. In plants acclimated for 4 days to 750 mM NaCl, CMO mRNA levels declined about 2-fold (S plants) and 11-fold (R plants) by 1 hr after treatment with dicamba (Figure 6). mRNA levels remained at reduced levels in S plants throughout the course of the experiment, as was seen in Figure 2 for non salt-stressed S plants treated with dicamba. However, in this case, CMO mRNA abundance declined to a new level lower than before herbicide treatment, but much higher than basal levels in non salt-stressed plants (marked with an asterisk in Figure 6). KIP and GB levels also declined after dicamba treatment, although more slowly than in non salt-stressed plants (data not shown). Thus, CMO expression (and GB biosynthesis) remained responsive to treatment with an auxin mimic, whether the pathway was operating at low, constitutive levels or was previously induced to much higher levels by salt stress.

In contrast to the results seen in S plants, CMO mRNA levels in dicamba-treated R plants fell about 11-fold, before recovering to levels slightly higher than before herbicide treatment (Figure 6). The transient nature of this attenuation is markedly similar to the decline and subsequent recovery of CMO mRNA seen in Figure 2 for non-salt stressed, dicamba-treated R plants. It is tempting to attribute the recovery of CMO expression to a time-dependent metabolism of dicamba *in vivo* but previous studies showed that dicamba remains essentially unmetabolized by 24 hr after treatment in both R and S *K. scoparia* (Cranston and others 2001). The continued responsiveness to dicamba seen in salt-stressed plants of both biotypes indicates that the

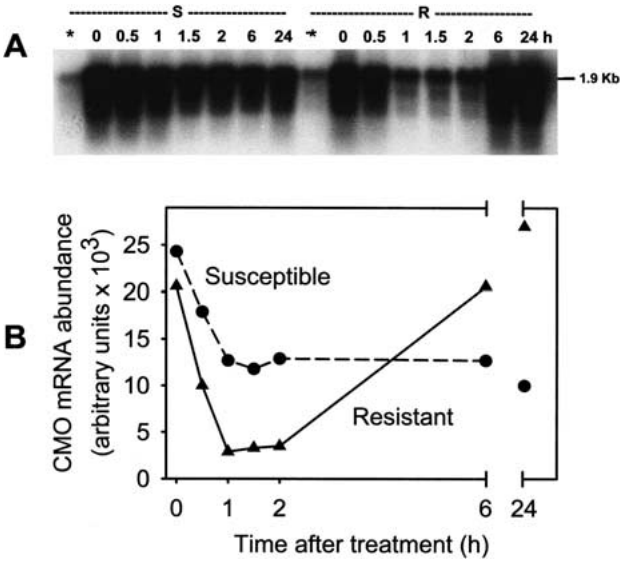

Figure 6. Steady state CMO mRNA levels in salt-stressed susceptible (S) and dicamba-resistant (R) *K. scoparia* plants after treatment with 70 g ai ha^{-1} dicamba. (**A**) RNA isolated 0, 0.5, 1, 1.5, 2, 6, and 24 h after dicamba treatment was probed with KSR2. mRNA levels in non salt-stressed, unsprayed plants are indicated by asterisks. (**B**) PhosphorImager analysis of mRNA abundance from blot shown in panel A.

herbicide was being absorbed and translocated, even though herbicide efficacy typically declines under stressful environmental conditions (Ashton and Crafts. 1981). The fact that CMO expression levels were restored in R plants suggests that the mechanism of dicamba resistance is operable even under conditions of severe stress, and that herbicide resistance is probably unrelated to the salt stress response.

This is the first report documenting a rapid decline in CMO expression (and the resulting rate of GB biosynthesis) in response to treatment with auxin or an auxin mimic. A number of other auxin down-regulated genes have been isolated, including the soybean *ADR* gene family (Datta and others 1993) and genes encoding soybean vegetative storage proteins (Berger and others 1995), a xyloglucan endotransglycosylase (Catala and others 2001), Class I β-1,3-glucanases (Vögelie-Lange and others 1994), and other enzymes involved in plant defense (Ozeki and others 1990; Pasquali and others 1992). Although the biological significance of such down-regulation is unclear in some of these cases, we speculate that the attenuation of GB biosynthesis by auxin may represent regulatory competition for the substrate choline as it is needed for lipid synthesis associated with auxin-mediated growth. Free choline is reversibly derived from phosphatidylcholine or phosphocholine in the cytosol, and exists in a small, metabolically active pool and a relatively larger storage (probably vacuolar) pool (Hanson and Rhodes 1983). In plant species that do not accumulate GB, the metabolic flux of choline is tightly regulated, and only very low free concentrations (1–2 μmol g^{-1} fw) are maintained as needed for phosphatidylcholine production (Rhodes and Hanson 1993). In support of this idea, the availability of choline was shown to be the primary limiting factor preventing enhanced GB production in transgenic tobacco plants expressing a spinach CMO gene (Nuccio and others 1998). Although detailed information about choline flux is not available for GB accumulators like *K. scoparia*, it seems obvious that its regulation must be much more plastic to supply the massive amounts needed for GB production in the chloroplast. In this regard, the curtailing of GB production may be necessary to make available sufficient choline in the same organelle for *de novo* lipid synthesis required for auxin-mediated growth.

Although the studies reported here did not directly test the effect of ABA treatment on CMO gene expression, a well-known effect of osmotic stresses similar to those employed here is an overall increase in ABA levels, leading to pysiological responses consistent with reducing evaporative water loss (Jensen and others 1996). In this regard, an *Atriplex centralasiatica* gene encoding BADH, the second enzymatic step of GB biosynthesis, was recently shown to be induced by ABA (Yin and others 2002). Thus, regulation of the GB pathway, as controlled by CMO expression levels, may represent a pathway that is repressed and induced by auxin and ABA, respectively. It may be of interest to pretreat *K. scoparia* plants with an auxinic herbicide before salinization, to compare CMO regulation with the ABA-inducible GAD1 and GAD2 genes (Jacobsen and Olszewski 1996) and the desiccation-related LEA protein EDP31 gene (Kiyosue and others 1992), that did not respond to ABA after auxin pretreatment. In summary, CMO expression appears to be regulated by a possibly complex interaction of hormones and osmotic stress. A better understanding of this regulation will enhance our knowledge of plant response to environmental stresses, and may facilitate transgenic efforts to improve crop tolerance to salt and drought.

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