



Molecular studies of dicamba-resistant *Kochia scoparia* L.
by Anthony John Kern

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy in Plant Sciences
Montana State University
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Abstract:

Extensive use of the auxinic herbicide dicamba (3,6-dichloro-2-methoxybenzoic acid) in Montana grain production systems has selected for biotypes of *Kochia scoparia* that are insensitive to the herbicide. Dicamba is thought to induce the same physiological responses as the natural phytohormone auxin (indole-3-acetic acid), including changes in gene expression that may be involved in growth and developmental responses. Since the mechanism of dicamba resistance in *K. scoparia* is currently unknown, differential mRNA display techniques were conducted to compare patterns of dicamba-induced gene expression in resistant (R) and susceptible (S) biotypes. Examination of >80,000 mRNA fragments showed that changes in mRNA abundance occurred within minutes after dicamba treatment, and most changes were similar in R and S plants. From 106 cDNAs isolated, sequenced, and used as probes on northern blots, 14 represented mRNAs whose abundance changed after dicamba treatment. Of these 14 cDNAs, four were repressed similarly in R and S, two were similarly induced, and eight responded differentially to dicamba treatment in R and S. Eight cDNAs were assigned putative functions based on DNA or deduced amino acid sequence similarities to known genes, and included enzymes involved in basic carbon metabolism, cellular chloride uptake, photosynthesis, initiation of protein synthesis, synthesis and degradation of cell wall material, and a protein with a chaperone function. A partial cDNA encoding choline monooxygenase (CMO), an enzyme involved in the biosynthesis of the osmoprotectant glycine betaine (GB), was chosen for more detailed study. Characterization of expression patterns indicated that levels of CMO mRNA were increased by osmotic stress but rapidly declined after dicamba treatment. Levels of the CMO enzyme and GB were similarly reduced in R and S plants following dicamba treatment. However, R and S plants prestressed with NaCl showed differential CMO response after dicamba treatment, likely indicating a fundamental difference in dicamba translocation, perception, or signal transduction between the two biotypes. This research demonstrates that differential display is a useful technique for discovering changes in gene expression that may be initially involved in basic plant responses.

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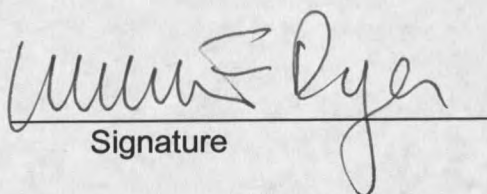
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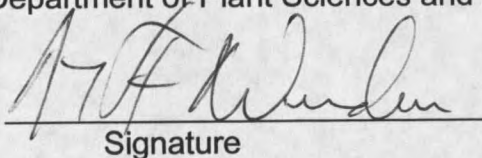
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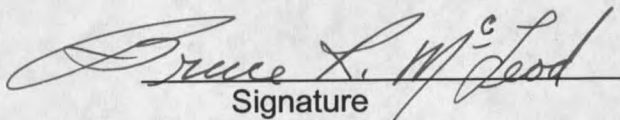
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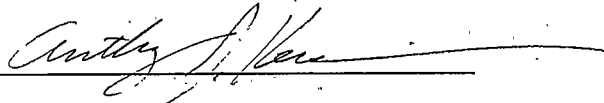

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ABSTRACT

Extensive use of the auxinic herbicide dicamba (3,6-dichloro-2-methoxybenzoic acid) in Montana grain production systems has selected for biotypes of *Kochia scoparia* that are insensitive to the herbicide. Dicamba is thought to induce the same physiological responses as the natural phytohormone auxin (indole-3-acetic acid), including changes in gene expression that may be involved in growth and developmental responses. Since the mechanism of dicamba resistance in *K. scoparia* is currently unknown, differential mRNA display techniques were conducted to compare patterns of dicamba-induced gene expression in resistant (R) and susceptible (S) biotypes. Examination of >80,000 mRNA fragments showed that changes in mRNA abundance occurred within minutes after dicamba treatment, and most changes were similar in R and S plants. From 106 cDNAs isolated, sequenced, and used as probes on northern blots, 14 represented mRNAs whose abundance changed after dicamba treatment. Of these 14 cDNAs, four were repressed similarly in R and S, two were similarly induced, and eight responded differentially to dicamba treatment in R and S. Eight cDNAs were assigned putative functions based on DNA or deduced amino acid sequence similarities to known genes, and included enzymes involved in basic carbon metabolism, cellular chloride uptake, photosynthesis, initiation of protein synthesis, synthesis and degradation of cell wall material, and a protein with a chaperone function. A partial cDNA encoding choline monooxygenase (CMO), an enzyme involved in the biosynthesis of the osmoprotectant glycine betaine (GB), was chosen for more detailed study. Characterization of expression patterns indicated that levels of CMO mRNA were increased by osmotic stress but rapidly declined after dicamba treatment. Levels of the CMO enzyme and GB were similarly reduced in R and S plants following dicamba treatment. However, R and S plants prestressed with NaCl showed differential CMO response after dicamba treatment, likely indicating a fundamental difference in dicamba translocation, perception, or signal transduction between the two biotypes. This research demonstrates that differential display is a useful technique for discovering changes in gene expression that may be initially involved in basic plant responses.

CHAPTER 1

INTRODUCTION

Since the introduction of 2,4-D (2,4-dichlorophenoxyacetic acid) in 1952, selective herbicides have been an integral part of modern agriculture, and are used in essentially all non-organic cropping systems to help ameliorate the impacts of weedy plants. While these herbicides have been generally very effective at reducing competition from weeds, the evolution of weed populations resistant to specific herbicides poses a serious threat to global agricultural production. In 1970, Ryan reported the first instance of herbicide resistance in *Senecio vulgaris* (Ryan, 1970), and by 2001, the *International Survey of Herbicide Resistant Weeds* documented over 250 populations of weeds resistant to different herbicides, with the numbers expected to continue to increase as herbicide use and selection continues (Heap 2001). Despite the problems herbicide-resistant weeds pose to agricultural production, these mutant phenotypes can provide useful tools to dissect important genetic and metabolic pathways. Weedy species are problematic in agricultural systems largely because of vigorous growth and reproduction, phenotypic plasticity, and the ability to grow under conditions of diverse stress. Some of these characteristics could be useful in an agronomic system if introduced into crop species (Snow et al., 1998), and a great deal of basic and applied research has focused on one weedy plant in particular (*Arabidopsis thaliana*; hereafter referred to as

Arabidopsis). Mutants in this model plant have been the focus of an unusual amount of research, especially related to efforts to elucidate genetic control over plant hormone physiology and response to environmental stress. It is likely that other weedy species may provide useful tools to address questions related to both basic plant biology and potential agronomic applications. This report addresses changes in expression patterns mediated by a mimic of the natural phytohormone auxin in a mutant of the weedy plant *K. scoparia*, which has evolved resistance to the synthetic auxin dicamba (3,6-dichloro-2-methoxybenzoic acid). This mutant may provide a unique tool to gain insight into the genetics of auxin physiology. Additionally, this report characterizes a gene from *K. scoparia* that has received considerable attention as a potential target for transgenic applications that aim to improve drought tolerance in crops.

Mechanisms of Herbicide Resistance

Mechanisms of herbicide resistance vary greatly across plant species, but all stem from changes in the physiological fate of the herbicide: how it is absorbed, transported within the plant, metabolized, or exerts its toxic effects. In most cases, herbicides exhibit their phytotoxic activities by binding to and inhibiting the normal biochemical activity of a single enzyme. Mutations in the genes encoding such enzymes that lower or abolish herbicide binding result in plants that are no longer sensitive to the herbicide. This so-called target site resistance is most thoroughly documented for the triazine and phenylurea herbicides, which normally inhibit photosynthesis by blocking electron transport

from plastoquinone Q_A (bound to the D2 protein in photosystem II) to plastoquinone Q_B (bound to the neighboring D_1 protein); this blockage is probably mediated by displacement of Q_B with the herbicide (Vermass et al., 1983). In resistant plants, one or more mutations in the amino acid sequence of D_1 severely reduce the binding affinity of the enzyme for the herbicide, thereby conferring resistance. Plants that are herbicide resistant because of target-site enzyme mutations (such as the D_1 protein, acetolactate synthase, and acetyl-coenzyme A carboxylase) account for well over half of the documented cases to date (Heap, 2001).

Altered herbicide movement has also been shown to confer the resistance trait in several plant species. Slow absorption of herbicides through the cuticle is thought to confer resistance to triasulfuron (2-(2-chloroethoxy)-n-[[[4-methoxy-6-methyl-1,3,5-triazin-2-yl) amino]carbonyl] benzene sulfonamide) in *Triticum aestivum* (Meyer and Muller, 1989), and several species of *Equisetum* exhibit natural tolerance to the herbicide glyphosate (*N*-phosphonomethyl glycine) due to similar mechanisms (Marshall et al., 1987). In *Erigeron philadelphicus* and *E. canadensis* (Tanaka et al., 1986), *Hordeum glaucum* (Bishop et al., 1987), *Conyza bonariensis* (Fuerst et al., 1985), and *Lolium perenne* (Faulkner, 1976), resistance to the herbicides paraquat (1:1-dimethyl-4,4'-bipyridinium dichloride) and diquat (6,7-dihydroipyridol[1,2-a:2'1'-c]pyrazidinium dibromide) is due to reduced translocation of the herbicide within the plant, even though rates of absorption were similar to susceptible biotypes. Sequestration of herbicides into

vacuoles has been documented for 2,4-D resistance in soybean (Schmitt and Sandermann, 1982) and is also thought to confer resistance to diclofop-methyl (methyl-2-[4-(2,4-dichlorophenoxy) phenoxy] propanoate) in *Avena fatua* populations (Bourgeois et al., 1997). Herbicide sequestration into extracellular cell wall matrices is associated with resistance to difenzoquat (1,2-dimethyl-3,5-diphenyl-1*H*-pyrazolium) in *Avena fatua* (Kern and Dyer, 1998a), and has been hypothesized as a mechanism of resistance to paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) in other species as well (Norman and Fuerst, 1997).

Herbicide resistance can be caused by alterations in herbicide metabolic pathways. In most cases, these metabolism-based cases of resistance stem from increased rates of herbicide catabolism or conjugation of herbicides with endogenous cellular components. Cytochrome P450-mediated hydroxylation, sulfoxidation, and deesterification reactions are responsible for metabolic detoxification of herbicides in several species (Brown, 1991). Conjugation of herbicides with reduced glutathione (Dean et al., 1991), sugars (Gronneau et al., 1988), or other compounds (Gronwald, 1994) can quickly mitigate herbicidal activity. The natural levels of tolerance exhibited to atrazine in *Zea mays* and certain biotypes of *Abutilon theophrasti* (Timmerman, 1989, Gronwald et al., 1989) are thought to be mediated by high levels of glutathione-S-transferase activity. While most metabolism-based mechanisms of herbicide resistance are due to increased rates of herbicide catabolism, other work has shown that decreased metabolism of certain herbicides like triallate (S-(2,3,3-trichloro-2-propenyl) bis(1-methylethyl)carbamothioate) that require an *in vivo* activation

reaction also confer resistance (Kern et al., 1998b).

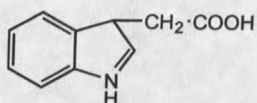
Under conditions of extensive herbicide selection pressure, the evolution and increase of herbicide-resistant plants is highly likely (Maxwell and Mortimer, 1994). In most cases, inheritance of resistance can be explained by the action of a single dominant gene, although recessive (loss of function) alleles have been documented in *Setaria viridis* and *Avena fatua* for resistance to diclofop and triallate, respectively (Jasieniuk et al., 1994; Kern et al., 2002). Herbicide-resistant weed populations typically become resistant to other members of the herbicide class and mode of action to which they were repeatedly exposed; this characteristic is termed cross-resistance. Populations of *Kochia scoparia* that developed resistance to chlorsulfuron (2-chloro-N-[[[(4-methoxy-6-methyl-1,3,5 triazin-2-yl)amino]carbonyl]benzenesulfonamide) were also resistant to several other sulfonylurea herbicides (Sivakumaran, 1992), and trifluralin (1,6-dinitro-N,N-dipropyl-4-(trifluoromethyl) benzeneamine)-resistant populations of *Eleusine indica* and *Setaria viridis* were also shown to be cross-resistant to numerous other dinitroaniline herbicides (Mudge et al., 1984). In some cases, however, the evolution of resistance to one chemical class of herbicides may also confer resistance to herbicides with different mechanisms of action. *Avena fatua* populations resistant to the thiocarbamate herbicides triallate and diallate (S-(2,3-dichloro-2-propenyl) bis(1-methylethyl) carbamothioate) were shown to be cross-resistant to the unrelated bipyridilium herbicide difenzoquat (Kern et al., 1996, Blackshaw et al., 1996). *Lolium rigidum* populations from Australia were shown to be resistant to over 20 different herbicides from nine different chemical

classes (Hall et al., 1994). However, multiple resistance mechanisms, including target-site mutations and increased metabolism-based herbicide detoxification, were present in resistant populations.

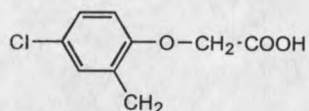
The auxinic herbicides

Auxinic herbicides have long played a key role in controlling dicot weed species in grass crops. Although the precise mode of action of the individual auxinic herbicides is unknown, it is widely thought that they act as synthetic versions of the phytohormone auxin (indole-3-acetic acid, IAA; Sterling and Hall, 1997). In low doses, the auxinic herbicides 2,4-D and dicamba are commonly used to replace auxin in plant cell tissue culture, supporting the idea that these herbicides act as synthetic auxin mimics. In higher doses, auxinic herbicides cause cell and plant death through uncontrolled growth, vascular tissue proliferation, and cell membrane destruction (Devine, 1993).

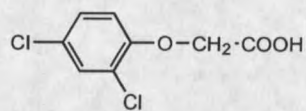
Despite this evidence, individual auxinic herbicides have chemical structures very different from auxin, although all have a carboxylic acid moiety (Figure 1). The phenoxy-carboxylic acids include MCPA (4-chloro-2-methylphenoxy acetic acid), 2,4-D, 2,4-DB (4-(2,4-dichlorophenoxy) butanoic acid), and mecoprop (2-(4-chloro-2-methylphenoxy) propanoic acid); dicamba and chloramben (3-amino-2,5-dichloro benzoic acid) are derivatives of benzoic acid; picloram (4-amino-3,5,6-trichloro 2-pyridinecarboxylic acid) and triclopyr ([[(3,5,6-trichloro-2-pyridinyl] oxy)acetic acid) are pyridine carboxylic acids, and quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) and quinmerac (7-chloro-3-



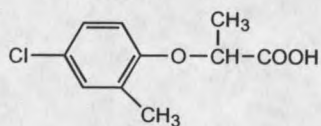
indole-3-acetic acid

Phenoxyacetic acids

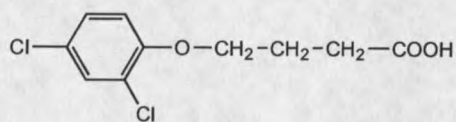
MCPA



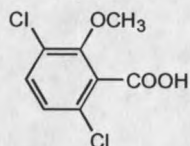
2,4-D



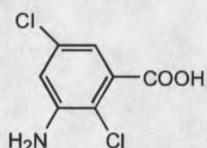
mecoprop



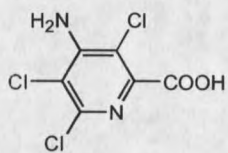
2,4-DB

Benzoic acid derivatives

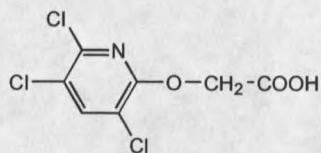
dicamba



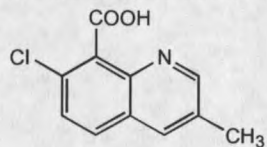
chloramben

Pyridine carboxylic acids

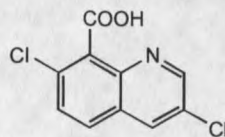
picloram



triclopyr

Quinoline carboxylic acids

quinmerac



quinclorac

Figure 1. Structures of auxin (indole-3-acetic acid) and representative auxinic herbicides. Revised from Sterling and Hall, 1997.

methyl 8-quinolinecarboxylic acid) belong to the quinoline carboxylic acid class of compounds.

Recent reviews (Grossmann, 2000; Sterling and Hall, 1997; Hansen and Grossmann, 2000) suggest that multiple modes of action, coupled with different perturbations in plant hormone homeostasis, exist for different auxinic herbicides. Soon after application of all auxinic herbicides, the rate of ethylene biosynthesis increases dramatically (Sterling and Hall, 1997). This is primarily thought to be the effect of rapid induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), the enzyme that catalyzes the rate-limiting step in ethylene biosynthesis (conversion of S-adenosyl methionine to ACC). In some plants, induction of ethylene biosynthesis is thought to be the primary mechanism by which the auxinic herbicides cause leaf epinasty, stem curvature, and leaf abscission (Wei et al., 2000). Additionally, Arabidopsis mutants defective in ethylene perception and tomato plants containing an overexpressed antisense version of the ACS gene were not sensitive to auxin (Sitrit and Bennett, 1998), whereas ethylene applications caused symptoms normally associated with auxinic herbicide application. Other evidence suggests that ethylene overproduction is not the causative agent of phytotoxicity after treatment with auxin: one byproduct of ethylene biosynthesis is cyanide, which is thought to play a role in plant death after treatment with quinclorac (Grossman, 1998). As with IAA, the auxinic herbicides have been shown to increase proton pumping into the tonoplast within minutes of application, subsequently activating cell wall degrading enzymes in support of the acid growth hypothesis (Salisbury

and Ross, 1992). Activation of these enzymes may cause irreversible degradation of plant cell wall materials, leading to necrosis and cell death (Sterling and Hall, 1997).

Resistance to the auxinic herbicides

Despite extensive use for over 50 years, there are relatively few examples of plant resistance to the auxinic herbicides. The few biotypes that have developed resistance generally have lower levels of fitness and survivability, making them less likely to become a widespread threat to agronomic systems utilizing these herbicides. In 1957, *Commelina diffusa* plants were isolated in Hawaii that were not controlled by field rates of 2,4-D (Hilton, 1957), but have not been documented since. Populations of wild carrot (*Daucus carota*) that were resistant to 2,4-D were a similarly minor problem (Whitehead and Switzer, 1963). Other examples include 2,4-D-resistant *Carduus* species (Bonner et al., 1998), *Centaurea solstitialis* (Sabba et al., 1998) resistant to picloram, MCPA-resistant populations of *Cirsium arvense* (Fogelfors, 1979; Solymosi et al., 1987), populations of *K. scoparia* resistant to dicamba, MCPA, butyrac, bicloram, and 2,4-D (Miller et al., 1997), as well as a few other cases (for review, see <http://www.weedscience.org>). With the exception of 2,4-D-resistant *Carduus* spp. and *K. scoparia*, most instances of auxinic-herbicide resistant weeds have resulted in relatively few agricultural problems.

Little is known about the mechanism(s) of resistance to the auxinic herbicides, although there typically are severe morphological effects associated

with the resistance trait. R biotypes of *Sinapis arvensis* tend to be shorter, more branched, darker green, have poorly-branched roots, and had aberrations in cytokinin and other hormone levels (Hall and Romano, 1995). R *S. arvensis* biotypes showed decreased binding of ^3H -IAA to unidentified microsomal proteins; however, it is unclear if such binding assays represent physiologically relevant binding activities (Webb and Hall, 1995). Studies on mecoprop resistance in *Stellaria media* (Coupland et al., 1991), picloram resistance in *Sinapis arvensis* (Peniuk et al., 1993), picloram resistance in *Centaurea solstitialis* (Fuerst et al., 1996) and dicamba resistance in *K. scoparia* (Cranston et al., 2001) indicated that resistance to the auxinic herbicides was not due to reduced herbicide uptake or translocation, or increased metabolism.

Arabidopsis auxin-related mutants

Insights into the mechanism of resistance to the auxinic herbicides may be gained by understanding the biochemical and genetic differences between wild-type plants and plants with altered auxin responsiveness. Numerous mutant phenotypes have been isolated from *Arabidopsis* and provide the opportunity to study auxin physiology at many different levels, and in some cases these studies have identified specific genes that result in altered auxin biosynthesis, metabolism, transport, or cellular perception/signal transduction.

Auxin is produced in plant meristems by transamination or decarboxylation of the amino acid tryptophan (Salisbury and Ross, 1992), and is often subsequently conjugated to free amino acids, short polypeptides, and

sugars. Alterations in auxin biosynthesis and metabolism have been shown to cause several auxin mutant phenotypes, including the Arabidopsis *trp1* mutant, which is deficient in tryptophan production and exhibits symptoms similar to auxin deficiency (Last et al., 1991). In contrast, other auxin-production mutants are overproducers: the *sur1* (*superroot1*) mutant exhibits morphology suggestive of severe auxin overproduction, including elongated hypocotyls, increased adventitious root formation, and epinasty (Boerjan et al., 1995). The *superroot2* mutant *sur2*, which also overproduces auxin and has a similar morphology to *sur1*, was generated using transposon mutagenesis. Tagging studies indicated that *SUR2* encodes a cytochrome P450 gene, and the gene is upregulated in both *sur1* and *sur2* populations (Delarue et al., 1998). These mutants, along with the *trp2* and *trp3* populations (Normanly et al., 1993), are thought to overproduce auxin via upregulation of cytochrome P450-mediated oxidation of indole-3-acetonitrile (IAN), suggesting the presence of a tryptophan-independent pathway for auxin biosynthesis. Indeed, two P450 genes have been isolated from Arabidopsis that may mediate this pathway (Hull et al., 2000), and other auxin-insensitive Arabidopsis mutants are defective in the *NIT1* gene, which is also thought to catalyze the formation of auxin from IAN (Normanly et al., 1997). The *sur2* mutants reverted to wild-type morphology when treated with exogenously-applied auxin or when grown on an acidic growth medium; concomitant decreases in both indole-3-acetonitrile and auxin content were noted (Barlier et al., 2000).

Aside from auxin production, other auxin mutants appear to have altered

auxin transport characteristics. It is thought that auxin transport is mediated by two important cellular proteins: auxin efflux carriers, which are located at the basal ends of cells and are responsible for basipetal flow of auxin in stems (Palme and Galweiler, 1999), and auxin influx proteins, which are thought to mediate cellular auxin uptake by a proton symport system (Lomax et al., 1995). The latter system suggests a plasmalemma-bound auxin receptor (an auxin binding protein) responsible for initiation of cellular secondary messenger cascades. The auxin binding protein ABP1 has been purified from maize (Löbner and Klämbt, 1985) and shows features characteristic of a protein located in the lumen of the endoplasmic reticulum (ER), as it contains a carboxy-terminal KDEL motif (Tillman et al., 1989). However, an auxin receptor has been shown to be located on the surface of the plasma membrane (Napier and Venus, 1995), and it is thought that the ABP protein is stored in the ER and secreted to the cell surface at levels which depend on the physiological state of the cell (Shimomura et al., 1999).

Perhaps the most-studied of these influx or efflux mutants is the *aux1* mutant from *Arabidopsis*. Initially created by mutagenesis (Maher and Martindale 1980), the *aux1* phenotype is characterized by root agravatropism and reduced lateral root formation (Pickett et al., 1990). *Aux1* is resistant to auxin and 2,4-D treatment (Yamamoto and Yamamoto 1998); but not to 1-naphthaleneacetic acid (NAA), which crosses membranes by simple diffusion, suggesting a defect in cellular auxin uptake. Bennett et al. (1996) recapitulated the *aux1* mutant from wild-type plants by transposon mutagenesis and showed

AUX1 has high sequence homology to amino acid permeases, which are also proton symporters. Promoter-GUS fusions showed that *AUX1* is expressed in root tips and in emerging lateral roots, but not in shoots, further supporting the hypothesis that the *AUX1* protein is involved in root auxin uptake (Marchant et al., 1999).

Auxin efflux carriers are thought to be the driving force of auxin transport, and physiological studies have shown that one of these proteins, *pin1*, exists on the basal ends of vascular parenchyma cells (Galweiler et al., 1998). Mutation in the *PIN1* gene results in altered inflorescence and vascular development consistent with perturbations in auxin transport. Additionally, treatment of wild-type plants with auxin transport inhibitors yields phenotypes similar to *pin1* mutants. These inhibitors (2,3,5-triiodobenzoic acid and *N*-1-naphthylphthalamic acid; NPA) act as direct inhibitors of auxin efflux proteins, as in the *tir3* auxin transport mutant, which has fewer sites that bind NPA in microsomal preparations than do wild-type plants (Ruegger et al., 1997). Other research suggests that reduced NPA binding is associated with reduced auxin polar transport activity. The *interfascicular fiberless/revoluta* (*ifl1/rev*) mutant is characterized by a pin-like inflorescence and lack of normal interfascicular fiber differentiation (Zhong et al., 1997), altered xylem differentiation (Zhong and Ye, 1999), and dark green leaves with delayed senescence. The *ifl1/rev* mutant shows a 40% reduction in NPA binding to plasma membranes compared to the wild-type, and expression of *PIN3* and other putative auxin efflux carrier genes is reduced in *ifl1/rev* mutants (Zhong and Ye, 2001). The *IFL1* gene encodes a

homeodomain leucine-zipper, and it is thought that mutations in this gene are responsible for decreased expression of the *PIN* auxin efflux carrier(s) and resultant phenotypes (Ratcliffe et al., 2000). A second putative auxin efflux carrier termed *AGR* (Utsuno et al., 1998) is allelic to *EIR1* (Luschnig et al., 1998), has been shown to be involved in root gravitropism, and encodes a protein with likely membrane-spanning domains that also has sequence homology to toxin efflux systems in *E. coli* (Luschnig et al., 1998). Other mutations in auxin efflux carrier-associated proteins appear to have similar effects as mutations in the efflux proteins themselves. The *rcn1* mutant is deficient in protein phosphatase 2A activity and exhibits defects in root curling and other phenotypes requiring differential cell elongation (Rashotte et al., 2001).

While auxin influx and efflux mutants have been fairly well characterized, less is known about mutants that have altered auxin perception or signal transduction, likely because these pathways have yet to be elucidated. Mutants of these types carry a variety of genetic lesions. The *axr1* mutant is one of only a few that show reduced auxin sensitivity in all tissues, reduced induction of auxin-responsive genes such as the SAUR genes (see below), and do not have altered endogenous auxin concentrations (Lincoln et al., 1990). Although the *AXR1* gene has been cloned and has sequence similarity with the ubiquitin-activating enzyme E1 (Leyser et al., 1993), it is not clear what role this gene plays in reduced auxin sensitivity (Leyser, 1997). The *arg1* mutant was generated through transposon mutagenesis and has been proposed to be deficient in signal transduction pathways after auxin treatment (Sedbrook et al.,

1999). The *ARG1* gene was positionally cloned using bulked segregant analysis (interestingly, *ARG1* was not transposon tagged, despite being generated using transposon mutagenesis; this may be due to a transposon excision event, leaving a footprint responsible for the mutant phenotype) and shown to encode a DnaJ-like protein, suggesting it may modify protein kinase or calmodulin activity by acting as a chaperone protein (Sedbrook et al., 1999).

Certainly, auxin-response mutants in *Arabidopsis* have given insight into auxin physiology and have provided strong evidence for an active transport system which includes auxin efflux and influx proteins. Additionally, studies investigating auxin autotrophs and auxotrophs show alternative metabolic pathways for auxin production, and clearly indicate severe morphological effects when *in vivo* auxin concentrations are perturbed. Despite this, these mutants provide limited information on the ultimate mechanism of auxin action (changes in cell physiology that may be mediated by alterations in gene expression). It is likely that genetic studies investigating changes in gene expression as affected by auxin will provide considerable insight into auxin action. Some of the experiments reported here investigate such changes in an auxin-mimic mutant of *K. scoparia*.

Genes responsive to auxin and auxinic herbicides

While it is not entirely clear if the auxinic herbicides affect plants in ways that are identical to IAA, numerous bioassays indicate both have similar effects on various aspects of plant growth such as cell elongation, tissue differentiation,

turgor pressure, and cell polarity (Abel and Theologis, 1996; Sitbon and Perrot-Rechenmann, 1997). Although molecular mechanisms (incontrovertible verification of an auxin receptor, secondary messenger cascades, and changes in gene expression that are ultimately responsible for alterations to plant growth) by which auxin exerts its effects have not been identified, considerable research has concentrated on the roles that auxin and auxin mimics have on changes in gene expression. It is important to note that while auxin and the auxinic herbicides (in particular, 2,4-D) are often used in these studies interchangeably, in some cases the two have different effects on gene expression (Mito and Bennett, 1995).

Most systems to date have used subtractive hybridization methodologies in auxin-starved cell suspension cultures or auxin-treated elongating tissues to isolate differentially-expressed genes. While dozens of these auxin-responsive genes have been isolated, considerable effort has focused on the expression events that occur within minutes after auxin application (Abel and Theologis, 1996); these are termed primary-response genes. It is thought that changes in the expression patterns of these primary-response genes occur independently of *de novo* protein synthesis, indicating that the cellular components necessary to change expression patterns are preexisting in the cell. It follows that these genes may be some of the most important in initiating the cascade of transcriptional events ultimately leading to the various physiological responses to auxin, and may offer the most insight into auxin action.

Many of the genes isolated to date are members of large gene families,

and homology-based PCR cloning has enabled researchers to isolate and identify multiple members of these families. The majority of auxin-responsive genes isolated to date are induced, or up-regulated, by auxin treatment and loosely fall into one of eight categories (Table 1).

Although auxin has been only loosely implicated in the initiation of cell division (Kitamiya et al., 2000), several cell cycle-associated cDNAs have been isolated that are responsive to auxin treatment. Homologs of the protein kinase p34 (*cdc2*) have been isolated from *Arabidopsis* (Martinez et al., 1990), *Medicago* (Hirt et al., 1993), *Nicotiana* (John et al., 1993), *Pisum* (Hemerly et al., 1993), and *Glycine* protoplasts (Miao et al., 1993). However, since cytokinin is also required for their induction, and induction requires several hours, they are probably not primary-response genes. In contrast, Xie et al. (2000) suggests that the rapidly activated *TIR1* (Gray et al., 1999) and *AXR1* (Leyser et al., 1993) genes, both of which encode proteins involved in the pathways of protein ubiquitination, allow pericycle cells (normally arrested in the G2 phase of the cell cycle) to re-enter the cell cycle and initiate lateral root formation.

Most genes induced by auxin treatment appear to be directly involved in biochemical processes mediated by IAA. Because of the broad array of gene transcription events associated with auxin treatment, it is not surprising that transcription of several putative DNA-binding proteins is induced by auxin treatment. The histone genes *DBP* and *H3C-II* were induced within 4 hours after auxin treatment in *Arabidopsis* and *Medicago* (Alliotte et al., 1989; Kapros et al., 1992), suggesting that expression of histones may be involved in auxin-mediated

Table 1. Representative auxin induced genes. Modified and updated from Sitbon and Perrot-Rechenmann (1997)

Gene	Function	Experimental system	Effective auxins	Response time	Reference
<u>Genes encoding DNA binding proteins</u>					
<i>PS/IAA4/5</i>	transcription factor	<i>Pisum</i> hypocotyls	20 μ M IAA, NAA, 2,4-D	10 min	Theologis et al., 1985
<i>DBP</i>	histone	<i>Arabidopsis</i> peduncles	1 μ M NAA	<4 hr	Alliotte et al., 1989
<i>H3C-11</i>	Histone	<i>Medicago</i> callus	100 μ M 2,4-D	<1 hr	Kapros et al., 1992
<i>AT-IAA1</i>	transcription factor	<i>Arabidopsis</i> seedlings	20 μ M IAA, NAA, 2,4-D	4 min	Abel et al., 1995
<i>SGBF-2</i>	G-box factor	<i>Glycine</i> hypocotyls	23 μ M 2,4-D	<2 hr	Hong et al., 1995
<i>ATHB-8</i>	homeobox protein	<i>Arabidopsis</i> leaves	10 μ M IAA, NAA, 2,4-D	<1 hr	Baima et al., 1996
<u>Genes encoding calcium-modulated proteins</u>					
<i>PCM-1</i>	calmodulin	<i>Fragaria</i> fruit	1 mM NAA	12 hr	Jena et al., 1989
<i>MBCAM-1</i>	calmodulin	<i>Vicia</i> leaves	500 μ M IAA	<3 hr	Botella and Ateca, 1994
<i>TCH3</i>	calcium-binding protein	<i>Arabidopsis</i> leaves	1 μ M IAA	<30 min	Antosiewicz et al., 1995
<i>ARCAM</i>	calmodulin	<i>Vicia</i> hypocotyls	1 μ M IAA	<1 hr	Okamoto et al., 1995
<i>VR-CDPK1</i>	Ca-dependent kinase	<i>Vicia</i> seedlings	500 μ M IAA	3 hr	Botella et al., 1996
<u>Genes encoding cell cycle-associated proteins</u>					
<i>CDC2</i>	CDC2 protein kinase	<i>Arabidopsis</i> roots	0.1 μ M IAA	<2 d	Martinez et al., 1990
<i>CDC2MS</i>	CDC2 protein kinase	<i>Medicago</i> cell culture	100 μ M 2,4-D (pulse)	>8 hr	Hirt et al., 1993
<i>P34CDC2</i>	CDC2 protein kinase	<i>Pisum</i> roots	50 μ M IAA	<1 hr	John et al., 1993
<i>CDC2-S5, CDC2-S6</i>	CDC2 protein kinase	<i>Glycine</i> roots	0.5 mM NAA	<24 hr	Miao et al., 1993
<u>Genes encoding cell wall-associated proteins and hydrolytic enzymes</u>					
<i>EII</i>	β -glucanase	<i>Hordeum</i> alurone	5 μ M IAA	<20 hr	Stakeski and Fincher, 1992
<i>DCPRP1</i>	proline-rich protein	<i>Daucus</i> roots	10 μ M IAA, NAA, 2,4-D	3-24 hr	Ebener et al., 1993
<i>SBPRP2</i>	proline-rich protein	<i>Glycine</i> roots	10 μ M IAA, NAA, 2,4-D	<20 hr	Suzuki et al., 1993
<i>HGRPNT3</i>	hydroxyproline-rich GP	<i>Nicotiana</i> roots	10 μ M IAA	1-2 hr	Vera et al., 1994
<i>TCH4</i>	xyloglucan transglycosylase	<i>Arabidopsis</i> seedlings	1 μ M IAA	<10 min	Xu et al., 1995
<i>EXT</i>	xyloglucan transglycosylase	<i>Arabidopsis</i> seedlings	1 μ M IAA	10-30 min	Xu et al., 1996
<i>EGL1</i>	β -1,4 glucanase	<i>Pisum</i> hypocotyls	5 μ M 2,4-D	<5 hr	Wu et al., 1996
<i>DD21.4-1</i>	α -expansin	<i>Pinus</i> hypocotyls	10 μ M IBA	<24 hr	Hutchison et al., 1999

Table 1 (continued). Representative auxin induced genes. Modified and updated from Sitbon and Perrot-Rechenmann (1997)

Gene	Function	Experimental system	Effective auxins	Response time	Reference
<u>Genes encoding hydrolitic enzymes</u>					
<i>ATSEH</i>	epoxide hydrolase	<i>Arabidopsis</i> plants	100 μ M 2,4-D, IAA	<1 hr	Kiyosue et al., 1994
<i>VR-ACS6</i>	ACC synthase	<i>Vigna</i> hypocotyls	500 μ M IAA	<4 hr	Yoon et al., 1997
<i>AT-ACS4</i>	ACC synthase	<i>Arabidopsis</i> seedlings	20 μ M IAA, NAA, 2,4-D	20 min	Abel et al., 1995
<i>ADC</i>	arginine decarboxylase	<i>Pisum</i> ovaries	45 μ M 2,4-D	<12 hr	Perez-Armador et al., 1995
<u>Genes encoding oxidative enzymes</u>					
<i>AOP1</i>	ascorbate oxidase	<i>Cucurbita</i> fruit	4.5 μ M 2,4-D	24 hr	Esaka et al., 1992
<i>AAO</i>	ascorbate oxidase	<i>Zea</i> roots	5 μ M 2,4-D	<48 hr	Kerk and Feldman, 1995
<i>PS-ACO</i>	ACC oxidase	<i>Pisum</i> seedlings	100 μ M IAA	<4 hr	Peck and Kende, 1995
<i>GERMIN</i>	oxalate oxidase	<i>Hordeum</i> seedlings	10 μ M IAA	<4 hr	Hurkman and Tanaka, 1996
<u>Genes encoding GSTs</u>					
<i>GH2/4</i>	GST	<i>Glycine</i> hypocotyls	2.2 μ M 2,4-D, NAA, IAA	15 min	van der Zaal et al., 1991
<i>CNT103</i>	GST	<i>Nicotiana</i> cell culture	2.2 μ M 2,4-D, NAA, IAA	15 min	van der Zaal et al., 1987
<i>PARA</i>	GST	<i>Nicotiana</i> protoplasts	4.5 μ M 2,4-D, NAA, IAA	10 min	Takahashi et al., 1991
<i>PARB</i>	GST	<i>Nicotiana</i> protoplasts	4.5 μ M 2,4-D	<20 min	Takahashi and Nagata, 1992
<i>HMGST-1</i>	GST	<i>Hyoscyamus</i> culture	10 μ M 2,4-D, 2,4,5-T	<24 hr	Bilang and Sturm, 1995
<u>Genes encoding proteins of unknown function</u>					
<i>SAUR</i>		<i>Glycine</i> hypocotyls	100 μ M 2,4-D, IAA	3 min	McClure and Guilfoyle, 1987
<i>GH3</i>		<i>Glycine</i> hypocotyls	100 μ M 2,4-D, IAA	<15 min	Hagen et al., 1985
<i>ARCA</i>		<i>Nicotiana</i> cell culture	0.9 μ M 2,4-D, NAA	2 hr	Ishida et al., 1993, 1996
<i>SAR1, SAR2</i>		<i>Fragaria</i> fruit	1 mM NAA	2 hr	Reddy et al., 1990
<i>MII-3</i>		<i>Vicia</i> leaves	500 μ M IAA	<2 hr	Chen et al., 1996
<i>GO15-13</i>		<i>Nicotiana</i> seedlings	1 μ M NAA	<2 hr	Roux et al., 1998

DNA replication. Other DNA-binding proteins with homologies to transcription factors have been isolated, and at least one (*Ps-iaa4/5*) has DNA binding domains characteristic of repressor proteins (Abel et al., 1994).

Not surprisingly, genes encoding proteins thought to be involved in secondary-messenger cascades have been shown to be up-regulated after exposure to auxin. Several studies have shown that treatment with auxin increases calcium levels within the cytosol (Irving et al., 1992; Gehring et al., 1990), suggesting that calcium is involved in these signal transduction pathways. In support of this idea, the calmodulin-homologs *PCM-1* from *Fragaria* (Jena et al., 1989), *MBCAM-1* and *ARCAM* from *Vicia faba* (Botella and Arteca, 1994), and *TCH3* from *Arabidopsis* (Okamoto et al., 1995) are induced by auxin treatment, although the time required for induction ranges from one to 24 hours (Antosiewicz et al., 1995). Similarly, the calcium-dependent protein kinase gene *VR-CDPK1* from *Vicia* was induced after auxin treatment, mechanical stimuli, or NaCl treatment (Botella et al., 1996). The role of these putative secondary messenger proteins in auxin perception or signal transduction has yet to be elucidated.

Glutathione-S-transferases (GSTs) are enzymes involved in the detoxification of numerous herbicides and various other electrophilic substances (Dean et al., 1991; Gronwald et al., 1989), and have been shown to be induced by auxin, ethylene, and many stress treatments. *PARA* and *PARB* from *Nicotiana* (Takahashi and Nagata, 1992; Takahashi et al., 1991) are induced within 20 minutes of auxin treatment, as are other GST-like proteins in *Nicotiana*

and *Glycine* (Table 1). The role of GSTs in the auxin response is unclear, but they may represent a general response to cell stress rather than a primary-response gene (Abel and Theologis, 1996).

Cell wall-associated proteins such as hydroxyproline-rich glycoproteins, glycoproteins, and glycine- and proline-rich glycoproteins are known to play a role in pathogenesis and wounding by increasing the barrier to pathogen infection and spread (Somssich and Hahlbrock, 1998). After auxin treatment, expression levels of *DCPRP1* in *Daucus* (Ebener et al., 1993), *SBPRP2* in *Glycine* (Suzuki et al., 1993), and *HGRPNT2* from *Nicotiana* (Vera et al., 1994) were increased, suggesting that auxin may be involved in stress responses. Activities of cell wall hydrolytic enzymes have independently been shown to be elevated by auxin treatment, possibly associated with concomitant acidification of the apoplast in preparation for cell elongation (Rayle and Cleland 1992). In *Arabidopsis*, *TCH4* and *EXT* (Xu et al., 1995, 1996), both encoding xyloglucan transglycosylases, were induced within 30 minutes after auxin treatment, although *tch4* is well known to be responsive to mechanical stimuli as well. A β -1,4-endoglucanase (*EGL1*) from *Pisum* increased almost 10-fold after treatment with 2,4-D (Wu et al., 1996). Similarly, the β -glucanase *EL1* from *Hordeum vulgare* is up-regulated after treatment with IAA (Slakeski and Fincher, 1992).

Increases in ethylene production have been documented within minutes of auxin treatment (Devine et al., 1993). Peck and Kende (1995) indicated that the *Pisum sativum* *PS-ACO* gene, encoding ACC oxidase (the rate-limiting step in ethylene biosynthesis), was induced within 4 hours after treatment with 100 μ M

IAA. Also, the Arabidopsis *AT-ACS4* gene encoding ACC synthase was induced within 20 minutes after auxin treatment.

While the majority of auxin-responsive genes investigated thus far are induced by auxin treatment, there are several examples of auxin down-regulated genes (Table 2). The most dramatically attenuated genes are the *ADR* family from *Glycine* (Datta et al., 1993), with transcript levels dropping over 100-fold within 24 hours after whole-plant treatment with 2,4-D. While this family of auxin down-regulated genes has no known function, genes encoding enzymes of common metabolism like tryptophan decarboxylase (Goddijn et al., 1992), chalcone synthase and phenylalanine ammonia lyase (Ozeki et al., 1990), and strictodine synthase (Pasquali et al., 1992) were down-regulated within 6 hours of auxin or 2,4-D treatment. While it is unknown why these aspects of plant metabolism are inhibited by auxin and auxin mimics, the attenuation of other genes involved in plant defense may provide more insight. An endo- β -1,3-glucanase from *Nicotiana* (*GL43*) is down-regulated by trace amounts of NAA (Mohnen et al., 1985), and both the mRNA and enzymatic activity of a class of chitinases (exemplified by *CHN50*) are reduced by NAA (Shinshi et al., 1987). Transgenic expression of the proteinase *PIN2* promoter indicated that this promoter region is responsive to auxin down-regulation within 18 hours after treatment (Kernan and Thornburg, 1989), and similar work suggests that another endo- β -1,3-glucanase from tobacco (*GLB1*) is down-regulated by auxin treatment (Vögelié-Lange et al., 1994). It is not clear what role the attenuation of these genes may have in response to auxin treatment, but these results are

Table 2. Representative auxin repressed genes. Modified and updated from Sitbon and Perrot-Rechenmann (1997)

Gene	Function	Experimental system	Effective auxins	Response time	Reference
<u>Genes encoding pathogenesis-related proteins</u>					
GL43	β -1,3-glucanase	<i>Nicotiana</i> pith culture	11 μ M NAA	<7 d	Mohnen et al., 1985
CHN50	chitinase	<i>Nicotiana</i> pith culture	11 μ M NAA	<7 d	Shinshi et al., 1987
CA125	unknown PR protein	<i>Capsicum</i> leaves	IAA, <i>Xanthomonas</i>	<24 hr	Jung and Hwang, 2000
<u>Genes encoding enzymes in secondary metabolism</u>					
TDC	tryptophan decarboxylase	<i>Catharanthus</i> culture	0.1 μ M NAA, 2,4-D, IAA	<2 hr	Goddijn et al., 1992
SSS	strictodine synthase	<i>Catharanthus</i> culture	10 μ M NAA	<6 hr	Pasquali et al., 1992
CHS	chalcone synthase	<i>Daucus</i> culture	0.5 μ M 2,4-D	<3 hr	Ozeki et al., 1990
PAL	phenylalanine ammonia lyase	<i>Daucus</i> culture	0.5 μ M 2,4-D	<3 hr	Ozeki et al., 1990
CGS	cystathione- γ -synthase	<i>Fragaria</i> fruit	1 μ M NAA (spray)	<24 hr	Marty et al., 2000
<u>Genes encoding miscellaneous proteins</u>					
SAR5	unknown function	<i>Fragaria</i> fruit	1 mM NAA (spray)	<2 hr	Reddy and Poovaiah, 1990
SAM46	superoxide dismutase	<i>Glycine</i> culture	10 μ M NAA	<4 hr	Crowell and Amasino, 1991
GB8	ribosomal protein	<i>Pisum</i> shoots	10 μ M NAA	<24 hr	Stafstrom and Sussex, 1992
ADR1-12	unknown function	<i>Glycine</i> seedlings	2.5 mM 2,4-D (spray)	<24 hr	Datta et al., 1993
VSP	vegetative storage protein	<i>Glycine</i> leaf petioles	1 μ M NAA	<6 hr	DeWald et al., 1994
NI-POX	peroxidase/GUS fusion	<i>Nicotiana</i> protoplasts	30 μ M IAA, NAA	<30 min	Klotz and Lagrimini, 1996

consistent with the finding that wounding leads to reduced auxin levels (Thornburt and Li, 1990; others), and causes increased expression of pathogenesis-related genes. These findings, coupled with the observation that the activities of other stress-related enzymes (superoxide dismutase and peroxidase) are also reduced upon treatment with 2,4-D (Crowell and Amasino, 1991; Klotz and Lagrimini, 1996), suggest that auxin perception may act as an "all-clear" signal, indicating that limited stresses are being placed upon the plant.

Other evidence that auxin may counteract stress-related signals in plants can be found in studies of the physiological and genetic changes mediated by the plant hormone abscisic acid (ABA), which can be reversed by auxin treatment. Heat, drought, salt, and cold stress on plants are all thought to cause a decrease in cellular turgor pressure (Tamminen et al., 2001), and this decrease in cellular water content causes significant responses in many plants. A well-known rapid effect of drought stress is an increase in ABA levels which in turn lead to stomatal closure (for review, see Jensen et al., 1996). Long-term growth under drought conditions or after treatment with ABA results in smaller stomata in *Tradescantia virginiana* (Franks and Farquhar, 2001), while treatment with auxin rapidly overcomes these effects, possibly mediated by increases in ethylene production (Merritt et al., 2001). Several ABA-induced stress genes are down-regulated by auxin treatment. For example, induction of *GAD1* and *GAD2* mRNAs, encoding wound-inducible proteinase inhibitors, is inhibited by auxin pretreatment (Jacobsen and Olszewski, 1996). The dessication-related LEA (late embryogenesis-abundant) protein EDP31 mRNA is also induced by ABA,

while transfer of developing embryos to auxin-free growth media also induces expression, suggesting a repressor role for auxin in the expression of this gene (Kiyosue et al., 1992). As a corollary, the auxin-induced expression of various genes encoding ACC synthases (Yoon et al., 1999; Botella et al., 1992; Kim et al., 1992) are suppressed by ABA treatment.

Despite these examples of the reciprocal control of physiological and genetic responses between auxin and ABA, there are numerous reports that confound our understanding of plant hormone interactions. As mentioned above, many auxin effects are thought to be mediated by induction of ethylene production, and auxin treatment has been shown to increase in *in vivo* ABA levels (Grossman, 1998). Certainly, additional research is needed to help elucidate the roles of both hormones in controlling plant response to stress.

Water deficit in plants

Water deficit is one of the most common environmental limitations of plant productivity (Boyer, 1982), and can be caused by drought, cold temperatures, or excessively saline soils. In all cases, plants must respond to decreased water potential in the soil, which limits water uptake into tissues. Of special interest are the stresses imposed on a plant as a result of saline soils, which not only decrease soil water potential, but also contain potentially toxic concentrations of sodium, carbonate, and chloride ions. At high concentrations, these ions (especially sodium) exert their toxic effects primarily by the denaturation of proteins and cell membranes (Cheesman, 1988), and plants have evolved

specialized strategies to overcome these effects. Some halophytes (plants that grow naturally in saline environments) have developed succulence to dilute sodium concentrations; as sodium content in the plant increases, the plant maintains turgor pressure and osmotic potential by absorbing more water (Salisbury and Ross, 1992). Other halophytes (such as *Atriplex* and members of the Chloridoideae) exude excess salt on the surface of leaves, and have developed specialized salt glands to actively pump sodium out of the cytoplasm (Troughton and Donaldson, 1972; Marcum, 1999). Since charged molecules do not typically move through a lipid bilayer by diffusion (Schachtman and Liu, 1999), plants must use active transporter systems to absorb micro- and macronutrients. Salt tolerance in some species can be attributed to the species' ability to exclude sodium while still absorbing essential nutrients, in particular potassium (Mumms, 1993). It has been shown that sodium ions compete with the active uptake of potassium, and potassium is typically present in the soil at much lower concentrations than sodium. Some halophytes such as mangrove exclude nearly 100% of the sodium in the soil (or brackish water), limiting cellular exposure to sodium (Ball, 1988). Genetic variation in salt tolerance in *Triticum* has been attributed to differing affinity of cation uptake channels, with tolerant cultivars excluding more sodium than susceptible cultivars while still maintaining potassium uptake (Davenport and Tester, 2000), and other cereals have been shown to accumulate sodium in old leaves and actively transport potassium to young leaves (Wolf et al., 1991, Colmer et al., 1995). Certain plants also actively pump sodium out of the cytoplasm and into the vacuole, probably powered by

the pH gradient across the tonoplast, utilizing Na^+/H^+ antiporters (Darley et al., 2000). The increased salt tolerance observed in wild *Lycopersicon* over that of cultivated varieties has been attributed to variability in these antiporters (Taha et al., 2000). Additional molecular evidence for the importance of vacuolar sequestration of sodium was found in transgenic maize suspension cells, where overexpression of a Na^+/H^+ antiporter significantly increased NaCl tolerance (Gruwel et al., 2001). Similar results have been noted in transgenic *Arabidopsis* overexpressing the AVP1-1 and AVP1-2 vacuolar proton pumps (Gaxiola et al., 2001).

A more common way for plants (especially nonhalophytes) to avoid sodium toxicity and associated dessication is by the production and accumulation of certain organic solutes, which maintain a more negative osmotic potential in tissues than in the soil and thus increase the ability of cells to retain water. These compounds, termed compatible solutes, can exist in cells at high concentrations without denaturing enzymes and cell membranes (Yancey et al., 1982). The sugar-alcohols glycerol, pinitol, mannitol, and arabitol have been identified as compatible solutes in yeast (Blomberg, 2000) and in the algae *Dunaliella* (Gimmler, 1989), the plant *Mesembryanthemum crystallinum* (Vera-Estrella et al., 1999), and other prokaryotic and fungal species. The amino acid proline has long been known to act as a compatible solute in plants, and dozens of plant species accumulate proline to high levels in response to drought or salt stress (for review, see Yoshida et al., 1995; Csonka and Hanson, 1991). Not surprisingly, genes encoding proline biosynthesis in *Arabidopsis* are induced

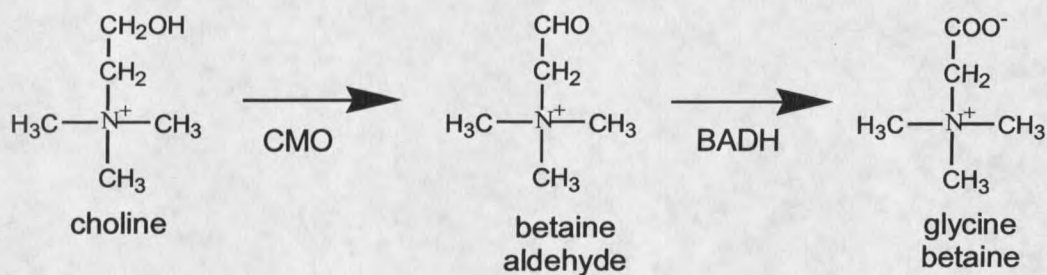
in response to drought and water stress, and this response appears to be dependent on ABA perception (Strizhov et al., 1997). There is considerable interest in genetic modification of crop plants to increase proline production in response to drought or salinity stress; however, the unavailability of appropriate genes is currently a major constraint in this effort (Jiban-Mitra and Mitra, 2001). To date, genetic modification to overproduce proline has met with limited success, except in the case of overexpression of the defense- and drought-related polypeptide osmotin (Barthakur et al., 2001).

Another compatible solute common in a wide variety of plant species is the quaternary ammonium compound glycine betaine (GB; for review, see Rhodes and Hanson, 1993). GB is zwitterionic but electrically neutral at physiological pH and appears to be a particularly effective compatible solute (LeRudulier et al., 1984). In addition to acting as an osmoregulant, GB acts as an osmoprotectant in stabilizing the structure of cell membranes *in vitro* as well as the tertiary and quaternary structures of enzymes like ribulose biphosphate carboxylase and components of photosystem II (Papageorgiou and Mutata, 1995) against salt stress (Gorham, 1995). GB may protect the cell against damage induced by free radicals (Jolivet et al., 1982), and Holmstrom et al. (2000) noted that GB protects photosynthetic machinery against light-induced damage in tobacco. Sakamoto and Murata (2001) suggested that enhanced tolerance to temperature stress in transgenic GB-producing *Arabidopsis* is due to a similar protection of membrane integrity. Bourot et al. (2000) showed that GB behaves like a chaperonin, and suggested that GB may stabilize the transcription

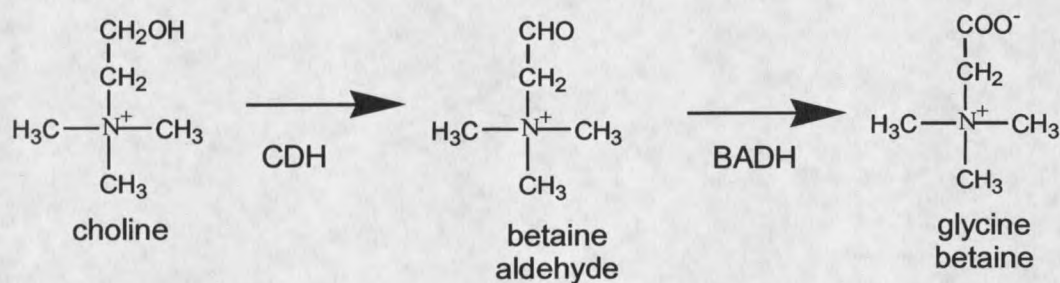
and translation machinery under conditions of stress. In this light, Allard et al. (1998) showed that exogenous GB activates different cold-inducible genes. Additionally, GB lowers the melting temperature of double-stranded DNA, which may facilitate transcription and DNA replication in high-salt environments (Rajendrakumar et al., 1997).

In all cases studied to date, GB is synthesized from choline utilizing one or two enzymes to carry out the oxidation of choline (Figure 2), and three distinct pathways are known. In *Arthrobacter globiformis* and *Arthrobacter pascens*, the oxidation reaction and synthesis of GB are carried out by the flavoenzyme choline oxidase (COD), releasing hydrogen peroxide (Ikuta et al., 1977). In mammalian cells and in *E. coli*, choline undergoes a dehydrogenation reaction mediated by the oxygen-dependent enzyme choline dehydrogenase (CDH), producing the toxic intermediate betaine aldehyde, which is then converted to GB by the NAD⁺-dependent enzyme betaine aldehyde dehydrogenase (BADH; Landfald and Strom, 1986). In plants, choline is oxidized by the enzyme choline monooxygenase (CMO), a ferredoxin-dependent soluble protein which is localized primarily in the chloroplast stroma (Rathinasabapathi et al., 1997), to produce betaine aldehyde. As in mammalian cells, the toxic betaine aldehyde intermediate is then converted to GB via the activity of BADH, which is also localized to the chloroplast stroma in members of the Chenopodiaceae. GB accumulates almost exclusively in the chloroplast of the chenopods, and likely serves to protect the photosynthetic machinery (McNeil et al., 1999). In contrast, BADH appears to be localized to the peroxisomes in members of the

1. Plants



2. *Escherichia coli*



3. *Arthrobacter globiformis*

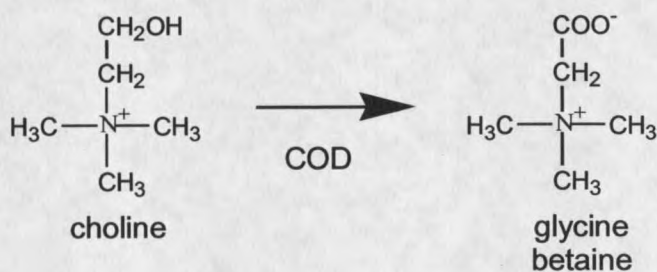


Figure 2. Biosynthetic pathways leading to GB production. Choline is oxidized by two enzymes in plants and *E. coli* and by a single enzyme in *Arthrobacter globiformis*. Abbreviations: CMO, choline monooxygenase; BADH, betaine aldehyde dehydrogenase; CDH, choline dehydrogenase; COD, choline oxidase. Revised from Sakamoto and Murata, 2000.

Graminaceae (Nakamura et al., 1997).

GB is only produced by certain members of the Chenopodiaceae, Gramineae, and Amaranthaceae (McCue and Hanson, 1992) and GB levels increase on exposure to drought or salt stress. Levels of GB increase approximately 10-fold in salt-stressed leaves of *Hordeum vulgare* (Arakawa et al., 1992), and is preferentially produced in young leaves (Nakamura et al., 1996). A similar induction was noted in sorghum, where GB levels increased up to as high as $70 \mu\text{mol GB g}^{-1}$ dry weight (Grieve and Maas, 1984). Other grasses that accumulate GB in response to drought or salt stress include *Triticum aestivum*, *Secale cereale*, and *Zea mays*, although there is considerable variation between cultivars. In corn, this variation is due to a nonsense mutation in the CMO gene in some maize genotypes, and the mutation is correlated with a decreased resistance to dry conditions (Lerma et al., 1990). Certain members of the Chenopodiaceae and Amaranthaceae appear to be the most vigorous producers of GB. For example, salt-stressed sugar beet and amaranth accumulate GB to $>30 \mu\text{mol GB g}^{-1}$ fresh weight (Russell et al., 1998).

Although numerous studies have documented increases in cellular GB concentration in response to water deficit (for review, see Rhodes and Hanson, 1993), only a few studies have investigated concomitant CMO and BADH expression in natural systems. The abundance of BADH mRNA was shown to increase under conditions of water stress in mangrove (Hibino et al., 2001), sugar beet (McCue and Hanson, 1992), rice (Kishitani et al., 2000), amaranth (Legaria et al., 1998), sorghum (Wod et al., 1996), spinach (Weretilnyk and

Hanson, 1990), and barley (Arakawa et al., 1992). Most of these studies reported a 2- to 10-fold increase in BADH mRNA under water stress conditions as compared to control plants. Immunological and *in vitro* assays for detecting BADH levels and activity have yielded similar results, although have only been performed in barley (Arakawa et al., 1992) and *Amaranthus tricolor* (Wang et al., 1999). Treatment with ABA also increased BADH mRNA levels in barley (Ishitani et al., 1995). However, no other studies have looked at the effects of ABA or other plant hormones on altering expression of BADH or other enzymes involved in GB biosynthesis. To date, CMO has only been isolated and cloned from members of the Chenopodiaceae (*Spinacia oleraceae*, *Beta vulgaris* (Russell et al., 1998), and *Atriplex hortensis* (Shen and Chen, 2001)) and Amaranthaceae (*Amaranthus tricolor* (Wang et al., 1999), and its expression patterns seem to follow that of BADH. CMO mRNA levels increased 7-fold in salinized leaves of spinach, with CMO enzyme activity increasing approximately 4-fold (Rathinasabapathi et al., 1997). Similar results were seen in *Beta vulgaris* and *Amaranthus caudatus* (Russell et al., 1998), as well as in *Amaranthus tricolor* (Wang et al., 1999).

As mentioned above, the chemical characteristics of GB make it an attractive target for use in the genetic modification of crops to increase their drought and salt tolerance (Sakamoto and Murata, 2000; Sakamoto and Murata, 2001), and attempts have been made using both bacterial COD genes and plant BADH and CMO genes. A spinach BADH controlled by the CaMV 35S promoter was introduced into tobacco and targeted to chloroplasts (Rathinasabapathi et al.,

1994). BADH activity effectively conferred resistance to betaine aldehyde (the toxic intermediate in GB production), evidently by its conversion to GB. However, transgenic plants failed to accumulate GB in the absence of exogenously supplied betaine aldehyde, evidently because tobacco has no *in vivo* CMO activity and could not produce betaine aldehyde. Tobacco transformed with a barley BADH cDNA showed increased levels of BADH mRNA in response to salt and ABA (Ishitani et al. 1995). In a similar study, Kishitani et al. (2000) introduced a barley BADH cDNA into rice and noted that transgenic lines were able to convert exogenous betaine aldehyde to GB, and showed an increased tolerance to salt, cold, and heat stress over untransformed lines.

To date, only one study has attempted to introduce CMO into a GB non-accumulator. Nuccio et al. (1998) used a spinach CMO cDNA under the control of the CaMV 35S promoter to transform tobacco. Although high CMO expression levels were noted (nearly as high as CMO mRNA in salinized spinach leaves), the tobacco plants were only able to synthesize GB to ~50 nmol g⁻¹ FW, which is far below the levels seen in natural accumulators. Although the authors did not speculate what role the absence of a functional BADH enzyme played in GB production, they did note that CMO enzyme levels (as indicated by Western blot) increased 3- to 5-fold upon salinization of the transformants, leading to the conclusion that post-transcriptional events modify CMO expression. Indeed, the authors verified an unusually fast disappearance of the CMO protein upon release from salt stress, a finding noted by other researchers (Russell et al., 1998).

The Nuccio study (1998) also provides interesting evidence that the endogenous supply of choline (the precursor to GB) strongly influences GB production. In this study, supplying exogenous choline increased GB production by about 50-fold in the transformed plants, suggesting that the endogenous choline supply limits GB production. Subsequent studies (McNeil et al., 2000; Huang et al., 2000) verified that choline production in tobacco must be increased before appreciable GB can be synthesized in this normally non-accumulating species. Indeed, *in vivo* choline production is increased in spinach plants undergoing salt stress (Summers and Weretilnyk, 1993; Weretilnyk et al., 1995), and the drought-induced enzymes involved in choline production have been recently characterized (Weretilnyk et al., 2001). Despite this potential limit, Sakamoto and Murata (2000) point out that tobacco contains only a small amount of choline, whereas rice and *Arabidopsis* have 10- to 30-fold higher concentrations of endogenous choline (Nuccio et al., 1998; Sakamoto et al., 1998; Hayashi et al., 1997), suggesting that some species are better targets for genetic modification to increase GB production.

In addition to using plant-derived enzymes for the production of GB, other research has utilized the bacterial COD gene to transform plants. The major advantage of this approach is that only a single transformation with the relevant gene (*codA*) is needed to introduce the pathway to convert endogenous choline to GB. Hayashi et al. (1997) produced transgenic *Arabidopsis* that overexpressed the *codA* gene from *Arthrobacter globiformis*, and noted that GB accumulation was as high as 5 $\mu\text{mol g}^{-1}$ fresh weight in salt-stressed plants.

Despite this relatively low production, the authors noted significantly enhanced tolerance to salt and cold stress, likely because the majority of GB was localized to chloroplasts, leading to physiologically significant GB concentrations in that organelle (Sakamoto et al., 2000). Subsequent work has shown that transformed plants had increased seed germination under high salt conditions (Hayashi et al., 1998), were less susceptible to photosynthetic inhibition under salt and cold stress (Sakamoto et al., 1998), were more tolerant of high temperatures (Alia et al., 1998a), had higher germination rates and better growth under cold stress (Alia et al., 1998b), and showed decreased susceptibility to freezing temperatures (Sakamoto et al., 2000) than wild-type plants.

Endogenous choline levels did not decrease in the transformed plants, although *in vivo* hydrogen peroxide levels doubled (Alia et al., 1999). Other studies utilizing the *CODA* gene have noted similar broad protection from stress in *Brassica juncea* (Prasad et al., 2000a, Prasad et al., 2000b) *Diospyros khaki* (Gao et al., 2000), and *Nicotiana* (Holstrom et al., 2000), even though GB production was nearly 1000-fold lower in these plants than in natural GB-accumulating plants. Clearly, cellular localization plays an important role in GB-mediated stress protection; targeting the *codA* protein to the chloroplasts had a more significant protective effect against salt-induced photosynthesis deactivation in rice plants than targeting it to the cytosol (Sakamoto et al., 1998).

While the one-enzyme *codA* system has been explored for transgenic applications to increase crop tolerance to drought stress, other researchers suggest that plant-derived systems (CMO and BADH) are a better choice for

manipulation for two reasons. First, CMO requires reduced ferredoxin, which links GB production to the light reactions of photosynthesis. This coupling would increase GB production during the day, when demand for osmotic adjustment is highest. Secondly, the CMO and BADH genes presumably have stress-responsive *cis* regulatory elements, which are needed to reproduce the natural pattern of drought-induced GB accumulation (Rathinasabapathi et al., 1997) and enable genetically modified plants to produce GB only when needed. The authors concluded that the isolation and characterization of CMO genes from new plant sources is an important step in bioengineering GB synthesis in crops.

CHAPTER 2

ISOLATION OF DIFFERENTIALLY EXPRESSED GENES IN RESPONSE TO
DICAMBA TREATMENT IN DICAMBA-RESISTANT AND
SUSCEPTIBLE *K. SCOPARIA*Introduction

Auxin and other phytohormones mediate a diverse array of plant growth and developmental responses. Although numerous physiological studies have documented these effects, the molecular mechanisms of hormone action remain largely unknown. One strategy to investigate hormone-mediated signal transduction pathways is through the study of genes that respond rapidly to hormone treatments. Until recently, isolation of hormone-responsive genes relied on interpreting physiological responses and subsequently cloning genes likely involved in these responses. For instance, it has long been known that auxin treatment dramatically increases ethylene production (Bonner et al., 1980). Molecular evidence for this induction came from assaying and purifying the ACC synthase enzyme, determining its amino acid sequence, and designing degenerate PCR primers to isolate the corresponding cDNA (Nakagawa et al., 1991). Induction at the mRNA level was verified by northern blotting. Subtractive hybridization screening strategies of cell lines treated with auxin have also led to the discovery of several auxin-responsive genes, such as the Aux/IAA gene family (Ainley et al., 1988, Conner et al., 1990, Yamamoto et al., 1992), the

SAUR gene family (McClure et al., 1989), and several genes encoding GSTs (Hagen et al., 1988, Takahashi and Nagata, 1992, Van der Zaal et al., 1987; see Table 1, pp. 18-19). Although PCR-based cloning approaches have led to isolation of homologs of these genes from different species (Van der Straeten et al., 1992, Kim et al., 1992, Trebitsh et al., 1997, Abel et al., 1995; Chen and Singh, 1999; de Billy et al., 2001), few other hormone-responsive genes in plants had been isolated until fairly recently.

In 1992, a new molecular technique was developed to isolate genes that are differentially expressed in response to specific treatments, or in different tissues in response to developmental cues (Liang and Pardee, 1992).

Differential Display (DD) is a reverse transcription/PCR-based approach to amplify mRNAs and radiolabel the resulting partial cDNAs, which are then run in adjacent lanes on a denaturing polyacrylamide sequencing gel, followed by autoradiography. Comparisons of individual band intensities between treatments potentially identifies mRNA species whose abundance has changed as a result of the treatment in question, and their cDNAs can be isolated from the gel, subcloned, and used as probes on northern blots. Because it utilizes PCR, DD is thought to be an improvement over traditional subtractive hybridization methodologies because of a lack of discrimination against rare messages and its technical ease of use (Yoshida et al., 1994).

DD relies on amplification of a subset of mRNAs primed at 1) the site of the polyadenylated 3' tail (the anchored primer), and 2) a random site on the first-strand cDNA upstream from the 3' tail (the arbitrary primer). Briefly, total

RNA samples are prepared from separate tissues undergoing the treatment of interest, and a subset of the mRNA is primed for reverse transcription of first-strand cDNA with an oligo-dT primer such as 5'-T₁₁CA-3'. In theory, this would only prime mRNAs ending with the sequence 5'-TGAAAAAAAAAAAA-3'. Each anchored primer would thus recognize one-twelfth of the total mRNA population (4x3 possibilities for the two 5' nucleotides, excluding thymidine from the penultimate position), permitting reverse transcription of only this subpopulation. After reverse transcription, partial second-strand synthesis is performed by annealing an arbitrary 10-mer, strand extension, and PCR amplification in the presence of ³³P-dATP.

Like most developing technologies, DD has seen many improvements since its introduction in 1992. A common problem with early DD screens was the generation of false positives (messages that appear to be differentially expressed by PCR amplification, but prove to be otherwise when examined by northern blotting). In 1997, Zegzouti et al. refined the technique by suggesting 1) the use of an anchored primer with only one selective base, such as T₁₁C, and 2) that PCR reactions be split prior to amplification and subsequently run in adjacent lanes on the DD gel. These changes were thought to reduce the percentage of false positives from as high as 70% down to perhaps 25% by screening for, and avoiding, amplification patterns that are not reproducible.

Since 1998, DD has seen significant utilization for the study of gene expression in response to plant hormone treatment, and this technique has more than doubled the number of hormone-responsive genes previously

characterized. More than 20 ethylene-responsive genes (Zegzouti et al., 1999; Hajouj et al., 2000), 14 ABA-responsive genes (Yoshida et al., 2001, Deleu et al., 1999, Karkthik et al., 2000), a gene induced by cytokinin treatment (Iwahara et al., 1998), and ten auxin-responsive genes have been isolated from various plant species. Interestingly, the recent DD-based studies show that significant numbers of such genes are down-regulated after hormone treatment, in contrast with earlier methodologies that isolated primarily hormone-induced genes. Of the auxin-responsive genes isolated using DD, six have homology to the *Aux/IAA* gene family (Dargeviciute et al., 1998), one is an expansin involved in acid-induced loosening of cellulose and xyloglucan networks (Hutchison et al., 1999), one has sequence homology with the SAUR genes (Watillon et al., 1998), and two others show sequence homology to the auxin-induced aldo-keto reductases (Mazeyrat et al., 1998) and lignin degrading enzymes (Watillon et al., 1998). All ten of these genes isolated by DD are induced by treatment with auxin or auxin analogs.

The research reported here used DD to screen two inbred biotypes of *K. scoparia* that were either susceptible (S) or resistant (R) to the auxinic herbicide dicamba. Previous experiments in our lab showed that dicamba resistance was not due to altered herbicide uptake, translocation, or metabolism patterns (Cranston et al., 2001). Since auxin is known to have rapid effects on the expression of specific genes and subsequent plant developmental responses (Guilfoyle et al., 1998), we hypothesized that dicamba (an auxin-mimic herbicide) would have similar effects on gene expression patterns in *K. scoparia* plants and

that these changes may be characteristic of auxin treatment. Using DD as a screening tool, these studies investigated 1) if dicamba treatment has a rapid effect on gene expression patterns in *K. scoparia*; 2) which genes are induced or repressed shortly after treatment; and 3) whether there is a difference in gene expression patterns between S and R *K. scoparia* as a result of dicamba treatment that may provide insight into the mechanism of dicamba resistance.

Methods and Materials

Plant material

The R biotype was derived from a population collected in a cultivated field near Fort Benton, MT, in 1992. The initial R population, designated as line R99, was derived from cuttings taken from 25 plants that survived field dicamba treatment. About 12% of the progeny from these cuttings showed no injury symptoms when treated with 70 g ai ha⁻¹ of dicamba in the greenhouse. Eighteen of the uninjured plants were individually transplanted into 15-cm diameter 3.8 L pots containing greenhouse soil mix (1:1:1 Bozeman silt loam:washed sand:peat moss), fertilized weekly, and watered as needed. At maturity, seeds from these plants were bulked and planted 0.5 cm deep in 54 by 26 by 6 cm flats and grown for 4 weeks in the Montana State University Plant Growth Center greenhouses 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 above. After 4 weeks, 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. Plants were returned to the greenhouse and evaluated 21 days after treatment. Bulk seeds from 20 plants that showed no injury symptoms were designated as line R99 and used as the R phenotype in all experiments reported here. Even after two generations of selection, the R99 line contained about 10% S individuals. The S254 line was generated from *K. scoparia* plants that were susceptible to dicamba treatment and seeds were obtained by growing untreated plants under isolation conditions for two generations as described above. Both R and S lines were maintained as families rather than developed using single-seed descent in order to avoid symptoms of inbreeding depression, as previously observed in this highly outcrossing species (Mulugeta, 1991). Previous reports (Cranston et al., 2001) showed that R99 is about 5-fold more tolerant to dicamba treatment than S254. Interestingly, the R phenotype of *K. scoparia* does not exhibit significant morphological changes, as do dicamba-resistant *Sinapis arvensis* plants (Hall and Romano, 1995), which are shorter, more branched, darker green, and more tolerant to cold temperatures than S plants. Similarly, auxin- and 2,4-D-resistant *Arabidopsis* mutants are typically stunted, show reduced apical dominance, have reduced lateral root formation, or are agravitropic (Leyser, 1997).

RNA extraction

Four-week-old R99 and S254 plants were sprayed with 70 g ai ha⁻¹ dicamba as above and shoot tissue was harvested at 0 (untreated), 30, 60, 90, or 120 minutes after treatment and frozen in liquid nitrogen. Frozen tissue (0.5 g fresh weight) was pulverized 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 et al. (1994). TLE (0.5 ml) extraction buffer (0.2 M Tris, pH 8.2, 0.1 M lithium chloride, 5 mM EDTA), 0.15 ml TLE-equilibrated phenol and 0.15 ml chloroform were added to the frozen powder, and the tube was vortexed and incubated at 50°C for 30 minutes. After centrifugation at 14,000g at room temperature to remove cell debris, 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 again removed and extracted in 1 volume of phenol:chloroform (1:1) two more times as above, 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 14,000g at 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 and resuspended in 100 µl sterile water and stored at -80°C. RNA obtained by this method was judged to be intact by denaturing agarose gel electrophoresis and ethidium bromide staining.

Differential display RT-PCR

Two approaches were utilized in the DD screens reported here. Initial efforts in 1997 and 1998 used the two-base selective anchored primer as described by Liang and Pardee (1992). After reports of reduced false positives by the Zegzouti group (1997) using the protocol changes mentioned above, a single-base discriminatory anchored primer coupled with parallel PCR reactions became the method of choice in 1998 to 2000. All other procedures were identical between the two screens. Total RNA extracted as above was treated with RNase-free DNase I (10 units/25 µg RNA; Promega Corp.) in 100 mM Tris-HCl (pH 8.0), 100 mM KCl, and 15 mM MgCl₂ for 60 min at 37°C. Samples were then extracted with an equal volume of water-saturated phenol:chloroform (1:1) and once with chloroform. RNA was ethanol precipitated, centrifuged 30 min at 14,000g at 4°C, washed with 70% ethanol, and resuspended in diethyl pyrocarbonate-treated water. Quantification of RNA samples was determined spectrophotometrically and by using denaturing agarose gel electrophoresis.

DNA-free RNA (2 µg) from the timepoints described above was incubated at 80°C in the presence of the anchored primer (2 µM; Table 3) for 5 min, cooled to 37°C over 20 min, and reverse-transcribed in a 20 µl reaction containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl₂, 10 mM DTT, 100 µM each dNTP, and 200 units MMLV Superscript® reverse transcriptase (GIBCO-BRL) for 120 min at 37°C. Reactions were stopped by heating to 75°C for 10 min. cDNA (10 ng) from each treatment was amplified in a 10 µl PCR reaction containing

Arbitrary primers used with dinucleotide anchored primers		Arbitrary primers used with mononucleotide anchored primers	
<u>Name</u>	<u>Sequence</u>	<u>Name</u>	<u>Sequence</u>
OPA01	CAGGCCCTTC	H-AP1	AAGCTTGATTGCC
OPA02	TGCCGAGCTG	H-AP2	AAGCTTCGACTGT
OPA03	AGTCAGCCAC	H-AP3	AAGCTTTGGTCAG
OPA04	AATCGGGCTG	H-AP4	AAGCTTCTCAACG
OPA05	AGGGGTCTTG	H-AP5	AAGCTTAGTAGGC
OPA06	GGTCCCTGAC	H-AP6	AAGCTTGCACCAT
OPA07	GAAACGGGTG	H-AP7	AAGCTTAACGAGG
OPA08	GTGACGTAGG	H-AP8	AAGCTTTTACCGC
OPA09	GGGTAACGCC	H-AP9	AAGCTTCATTCCG
OPA10	GTGATCGCAG	H-AP10	AAGCTTCCACGTA
OPA11	CAATCGCCGT	H-AP11	AAGCTTCGGGTAA
OPA12	TCGGCGATAG	H-AP12	AAGCTTGAGTGCT
OPA13	CAGCACCCAC	H-AP13	AAGCTTCGGCATA
OPA14	TCTGTGCTGG	H-AP14	AAGCTTGGAGCTT
OPA15	TTCCGAACCC	H-AP15	AAGCTTTAGAGCG
OPA16	AGCCAGCGAA	H-AP16	AAGCTTACGCAAC
OPA17	GACCGCTTGT		
OPA18	AGGTGACCGT		
OPA19	CAAACGTCGG		
OPA20	GTTGCGATCC		

Table 3. Primer sequences used for differential display. (Left) Arbitrary 10-mer primers used in combination with dinucleotide-specific primers T₁₁GC, T₁₁CA, or T₁₁GA for a total of 360 PCR reactions. (Right) Arbitrary 13-mer primers used in combination with mononucleotide-specific primers T₁₁G, T₁₁C, or T₁₁A for a total of 576 PCR reactions.

0.5 unit AmpliTaq Gold® DNA polymerase (Perkin Elmer), 60 mM Tris-HCl (pH 8.9), 18 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 3 μM each dNTP, 2 μM anchored primer, 2 μM arbitrary primer, and 2 μCi ^{33}P -dATP (DuPont-NEN). Cycling conditions included an initial 5-min denaturation step at 94°C , followed by 35 cycles of 94°C for 30 sec, 42°C for 60 sec, 72°C for 30 sec, and a final extension step at 72°C for 10 min in a Perkin Elmer 9600 thermocycler. When two parallel PCR reactions were used, the 20 μl reaction volume was split into two 10 μl reactions prior to thermocycling.

After thermocycling, 3 μl from each reaction was heated for 5 min at 95°C with loading dye (95% formamide, 20 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol FF) and separated by electrophoresis on a denaturing 6% polyacrylamide/7 M urea gel in Tris-borate-EDTA buffer at 50°C until optimum separation of bands in the 200- to 500-bp range was achieved (about 5 hr at 1900 volts constant voltage). The gel was blotted to Whatman 3MM paper, fixed with 10% acetic acid/5% methanol for 10 min, dried at 60°C on a slab gel dryer (Hoefer Scientific), and exposed to Kodak X-OMAT AR film for 6 to 16 hours at room temperature.

Differentially displayed cDNA bands were excised with a scalpel from the dried gel by aligning the exposed film over the gel (premarked with 0.001% ^{35}S -dATP [DuPont-NEN] in India ink) and eluting the cDNA in 100 μl water at 37°C for 60 min followed by 15 min at 94°C . cDNAs were recovered by ethanol precipitation, redissolved in 20 μl nuclease-free water, and a 1 μl aliquot was

used for reamplification in a 50 μ l PCR reaction using the above conditions, except that ^{33} P-dATP was omitted and the dNTP concentration was increased to 200 μ M.

Ten μ l of the PCR reaction was analyzed on a 1% agarose gel with ethidium bromide staining to verify the expected fragment size and specificity of amplification. One μ l of successful PCR reactions was then subcloned into the pCR 2.1/TOPO TA® cloning vector (Invitrogen Corp.) and used to transform competent cells (TOP10 *E. coli*; F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80/*lacZ* Δ M15 Δ *lacX74 recA1 deoR araD139 Δ (*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*). After culture and selection on Luria-Bertani medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per L, pH 7.0) supplemented with 100 μ g/ml ampicillin, master plates were made from 10 white colonies from each transformation, and the resulting insert sizes were determined by PCR as above, except that the M13 Universal Forward and Reverse primers (1 μ l of 0.1 ng primer/ μ l) were used in a 20 μ l PCR reaction. Anticipated insert sizes were verified by agarose gel electrophoresis/ethidium bromide staining as above, and corresponding single colonies were used to inoculate 3 ml cultures in Terrific Broth (0.017 M KH₂PO₄, 0.072 M K₂HPO₄, 12 g tryptone, 24 g yeast extract, and 4 ml glycerol per L) supplemented with 100 μ g/ml ampicillin. Plasmid DNA purifications were performed using the SET/alkaline lysis buffer method (Sambrook et al., 1989), and plasmids were digested with *EcoRI* (New England Biolabs) under standard conditions. DNA inserts were isolated by electrophoresing the digestion mix on a 1.5% agarose gel, excising the insert*

with a scalpel, and purifying the DNA from the gel matrix using the QIAQuick® Gel Extraction Kit (Qiagen Company). DNA inserts were resuspended in 50 µl sterile water and stored at -20°C.

Northern blot analysis

Analysis of differentially exposed bands was carried out by using the appropriate cDNA as a probe on northern blots. RNA (12 µg per lane) was denatured for 5 min at 65°C in 50% formamide/6% formaldehyde, electrophoresed at 50 volts constant voltage 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, 40 ng of purified insert was labeled with 50 µCi ³²P-dCTP (specific activity 3000 Ci/mmol; DuPont/NEN) using a random hexamer priming kit (Ready-To-Go® labeling beads, Amersham Pharmacia) and unincorporated nucleotides were removed using G50 Sephadex columns (ProbeQuant® G50 micro columns, Amersham Pharmacia). After verification of labeling efficiency (> 50% incorporation of label using a Geiger-Mueller counter), the probe was denatured by boiling for 5 min and added to RNA blots preincubated for 4 hours at 57°C in 4 ml of aqueous hybridization buffer (Sambrook et al., 1989). Blots were hybridized overnight at 57°C, washed once for 15 min in 100 ml 2X SSC (0.3 M NaCl, 0.03 M Na₃citrate (dihydrate), pH 7.0) at room temperature, followed by three 20 min washes at

57°C in 100 ml 2X SSC, 1X SSC, and 0.5X SSC, respectively. Blots were sealed in plastic bags while wet and exposed to film as above for 10 min to 1 week, depending on signal intensity as detected by a Geiger-Mueller counter. For rare messages, film was exposed at -80°C in the presence of an intensifying screen (DuPont Lightning Plus®). Equal loading of lanes was determined by visual inspection of ethidium bromide-stained RNA on northern blots (Figure 3).

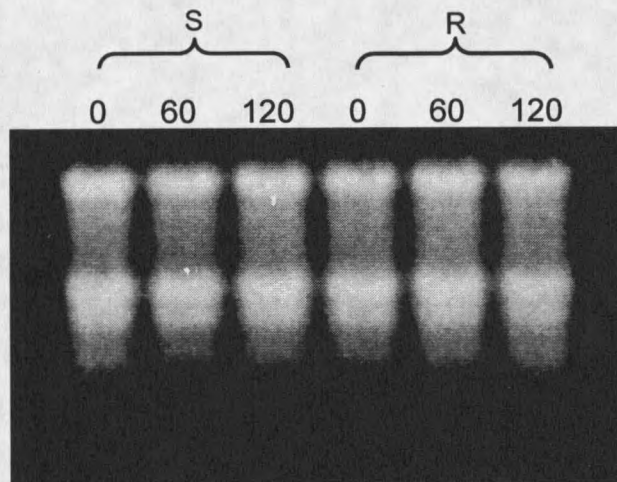


Figure 3. A representative northern blot containing RNA isolated 0, 60, or 120 minutes after dicamba treatment of susceptible (S) or dicamba-resistant (R) *K. scoparia*, showing intact RNA and uniform RNA loading of each lane.

Sequencing

Sequencing of isolated cDNA fragments in the pCR 2.1/TOPO TA plasmid was performed at the Washington State University DNA Sequencing Facility (Pullman, WA) using the BigDye dideoxy termination method. In all cases, primers used for sequencing were the M13 Forward (-40) and Reverse primers.

Sequence alignments

DNA sequences of selected cDNA fragments were compared to sequence databases using the National Center for Biotechnology Information's BLASTN and BLASTX algorithms (Altschul et al., 1997) using default settings. For further analyses, sequences were compared to published sequences using the Genetics Computer Group (Madison, WI) GCG® (v 10.0) PILEUP program.

DNA extraction

Genomic DNA was isolated from S254 *K. scoparia* using a method modified from Sambrook et al. (1989). Tissue was harvested, frozen, and pulverized as above. Extraction buffer (0.5 ml; 100 mM Tris, pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% (w/v) sodium dodecyl sulfate, 10 mM β -mercaptoethanol) was added and the sample was placed at 65°C for 30 min with occasional gentle mixing. Polysaccharides and protein were precipitated by adding 0.33 volume 5 M potassium acetate. The sample was incubated on ice for 60 minutes, centrifuged 20 min at 14,000g at room temperature, and the

supernatant removed and added to a new tube. DNA was precipitated by adding 1 volume ice-cold isopropanol, centrifuged as above, and washed with 0.5 ml ice-cold 70% ethanol. The DNA pellet was redissolved in 100 µl sterile water and stored at 4°C. Quality of genomic DNA obtained by this method was determined by agarose gel electrophoresis and ethidium bromide staining.

Cloning a gene fragment of 1-aminocyclopropane-1-carboxylate synthase

Auxins are known to stimulate ethylene production in a variety of plant tissues (Abeles, 1973), and numerous systems have shown that the mRNA for 1-aminocyclopropane-1-carboxylate synthase (ACC synthase), the enzyme mediating the rate-limiting step in ethylene production, is induced after auxin (Kim et al., 1992, Yoon et al., 1997, Arteca and Arteca, 1999) or 2,4-D (Jones and Woodson, 1999) treatment. Although dicamba is known to induce ethylene production in dicamba-susceptible *Sinapis* but not in R biotypes (Hall et al., 1993; Peniuk et al., 1993), the molecular basis for the dicamba-mediated induction in ethylene production has not been examined. To address this question, efforts were undertaken to clone the *K. scoparia* ACC synthase gene and determine whether dicamba treatment causes accumulation of the ACC synthase mRNA. Such studies would address 1) what role ACC synthase mRNA accumulation plays in ethylene biosynthesis in R and S *K. scoparia*, and 2) whether dicamba is perceived similarly to auxin by *K. scoparia* (as measured by an induction of ACC synthase transcript accumulation).

ACC synthase has been cloned from dozens of plant species and has

seven highly conserved regions, including the active site domain (Henskens et al., 1994). Because two of these conserved domains (amino acid sequences TNPSNPLGTT and MSSFGLV) do not span introns, PCR amplification using DNA as a template was successfully used to clone 290- and 328-bp fragments of ACC synthase genes from *Malus* and *Vigna*, respectively (Kim et al., 1992). Under my direct guidance, Mr. Thomas Emborg attempted a parallel study in *K. scoparia* while enrolled in BCHM 489 (Undergraduate Research Methods) at Montana State University.

ACC synthase DNA and inferred amino acid sequences were aligned by hand, spanning the conserved domains listed above from *Malus* (Kim et al., 1992), three *Vigna* cDNAs (Kim et al., 1992, Botella et al., 1992, Yoon et al., 1997), *Lycopersicon* (Rottman et al., 1991; Olson et al., 1995), *Dianthus* (Henskens et al., 1994), *Cucumis* (Trebitsh et al., 1997), two *Pelargonium* cDNAs (Wang and Arteca, 1995), and *Arabidopsis* (Van Der Straeten et al., 1992). Two degenerate primers corresponding to the highly conserved amino acid sequences TNPSNP (5' AC(CGA)AA(TC)CC(ATG)TC(AGC)AA(TC)CC 3') and KMSSFG (5' TCC(AG)AA(GA)CTNGACAT(TC)TT 3') were designed to limit degeneracy but account for all wobble seen in each codon in the above species. Primers were synthesized by Integrated DNA Technologies (Coralville, IA) and used in PCR amplifications of *K. scoparia* DNA. A PCR master mix (240 μ l) containing 100 ng genomic DNA, 1 μ M each primer, 50 mM Tris (pH 8.6), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, and 6 units AmpliTaq Gold® DNA polymerase (Perkin Elmer) was split into 12 aliquots and amplification was done

on a thermal gradient block in a RoboCycler® (Stratagene Company) thermocycler. Cycling conditions included an initial 5-min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 sec, 40°C to 62°C (gradient) for 30 sec, 72°C for 30 sec, and a final extension step at 72°C for 10 min. Amplification products were analyzed on a 1% agarose gel, and the 305 bp clone TACC1-6 from the 58°C annealing temperature was gel-purified, subcloned, sequenced, and used as a probe on northern blots as above.

Results and Discussion

Differential Display using di- and mono-nucleotide-specific anchored primers

Use of dinucleotide-specific anchored primers, coupled with individual 10-mer arbitrary primers, typically resulted in 20 to 70 radiolabeled amplification products per reaction in the 200-bp to 600-bp size range (Figure 4). A total of about 20,000 message fragments were evaluated using this experimental approach. A wide variety of message abundance and expression patterns was noted. Most messages (>90%) were not affected by dicamba treatment in R or S and therefore were not differentially expressed between the two biotypes (constitutive messages). About 1,000 (5%) of the messages were amplified in only R or S, and about 600 (3%) of the messages were induced or repressed equally in R and S up to 120 minutes after dicamba treatment. The relatively few messages that were differentially expressed between the two biotypes as a result of dicamba treatment were given priority for subcloning and verification of expression patterns by northern blotting. Of 44 cDNAs reamplified and used as probes on northern blots, 17 proved to be constitutively expressed (in contrast to the expression patterns noted on the DD film), 15 did not recognize a detectable message after 1 week of exposure, nine gave unusable northern blot results due to nonspecific hybridization (typically to ribosomal RNA), and three exhibited expression patterns consistent with the DD gel. Not including the cDNAs with an undetectable signal, these numbers indicate the occurrence of false positives to be nearly 90%.

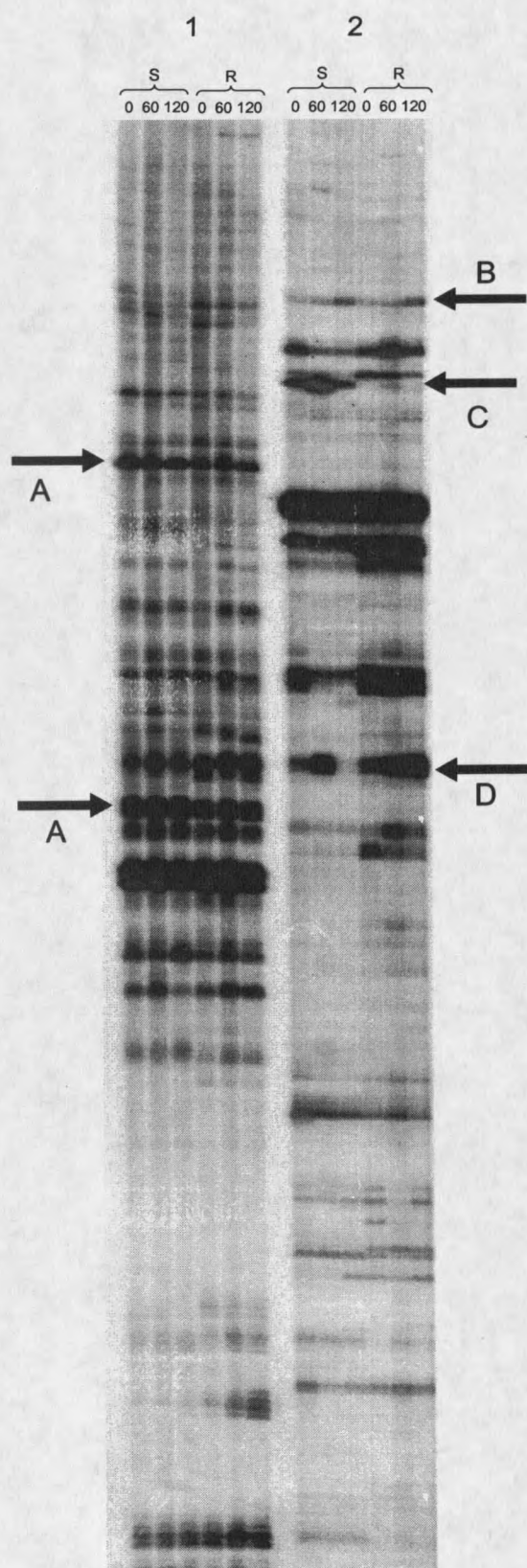


Figure 4. mRNA amplification and differential display using dinucleotide-specific anchored primers.

DD was performed using the anchored primer $T_{11}GC$ and arbitrary primer OPA07 (group 1) and OPA08 (group 2). The six lanes in each group correspond to RNA from S (left three lanes in each group) and R (right three lanes in each group) *K. scoparia* isolated 0, 60, or 120 minutes after treatment with dicamba.

In most cases, expression remained essentially unchanged as a result of dicamba treatment, as in "A". In other cases, expression patterns changed as a result of treatment but were not different between R and S, as in the example shown by "B". "C" and "D" denote cDNAs whose expression patterns differ between R and S and were changed as a result of treatment. In "C", the message is more abundant in untreated S than in R, and is transiently induced in S and only slightly in R. In "D", the message accumulates in S but only until 60 minutes after treatment, whereas it continues to increase in abundance in R up to 120 minutes after treatment.

DD using single-nucleotide discriminatory anchored primers and arbitrary 13-mers yielded similar patterns of expression, but this method proved to be more useful. As expected, the numbers of amplification products rose significantly, yielding about 80 to 200 amplification products per PCR reaction. Occurrence of differentially expressed messages remained low, and performing parallel PCR reactions effectively removed about 50% of the bands from further consideration, as they failed to give repeatable amplification patterns.

Approximately 60,000 message fragments were screened using this DD technique, and 62 were selected and successfully reamplified for use as probes in northern blots. Of these, 21 proved to be constitutively expressed, 24 did not recognize a detectable message after 1 week of exposure, six gave unusable northern blot results, and 11 exhibited differential expression patterns consistent with the DD gel. Not considering undetectable messages, the rate of false positives with this approach was about 70%.

mRNAs that were detectable and not constitutively expressed after dicamba treatment could be assigned to one of three categories: 1) mRNAs that were similarly up-regulated in R and S, 2) mRNAs that were similarly down-regulated in R and S, or 3) mRNAs that were differentially expressed between R and S after dicamba treatment. The cDNAs for two constitutively expressed mRNAs were also sequenced because of unusually high expression levels and this writer's interest. In all cases reported here, changes in gene expression were inferred from changes in steady-state mRNA levels as determined by northern

blotting. While other mechanisms may affect mRNA abundance, such as a change in the rate of mRNA degradation, the expression level of most genes is determined primarily by the rate of transcription (Alberts et al., 1989). I assumed that steady-state ("basal" levels of expression) mRNA abundance for each gene was exemplified in untreated tissue (i.e., 0 minutes after dicamba treatment).

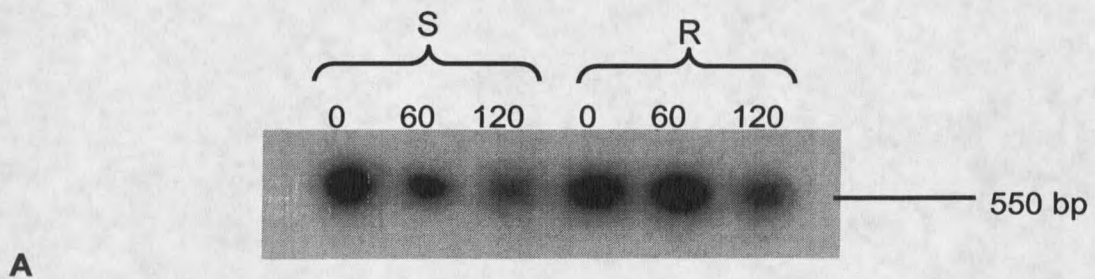
mRNAs down-regulated by dicamba treatment

C01R120

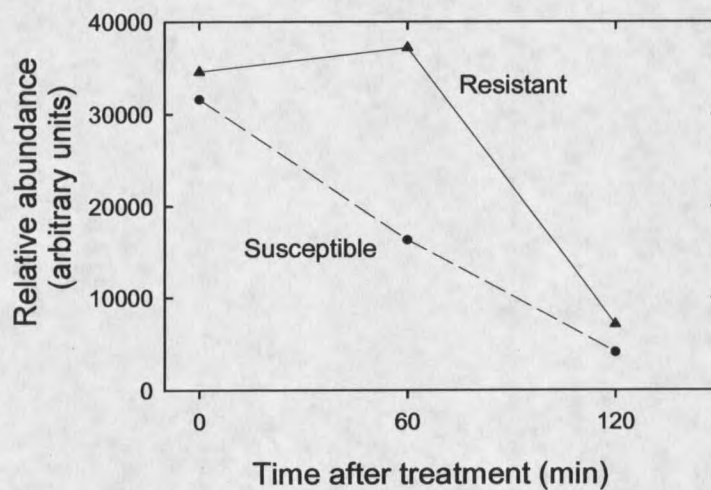
The 287-bp cDNA C01R120 represents a 550-b message expressed at moderate levels (16-hour film exposure) that is down-regulated in R and S after dicamba treatment (Figure 5). It is attenuated about 8-fold in S and 5-fold in R, and this down-regulation occurs somewhat more quickly in S than in R. No significant sequence identities were detected at the nucleotide or translated amino acid levels.

D416S0

cDNA D416S0 is a 245-bp fragment recognizing a 1.9-Kb message that is relatively abundant in untreated tissue (16-hour film exposure; Figure 6). By 120 minutes after dicamba treatment, the message is decreased about 7-fold in S and 10-fold in R. High sequence similarity was noted at the nucleotide (>80%) and translated amino acid levels (>77% identical; 88% similar) to the chloroplastic enzyme choline monooxygenase from *Spinacia oleracea*, *Beta*



A

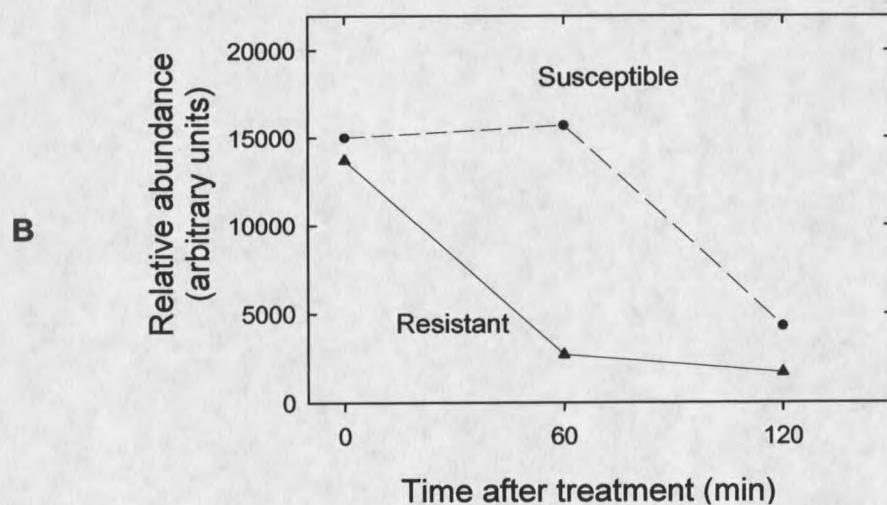
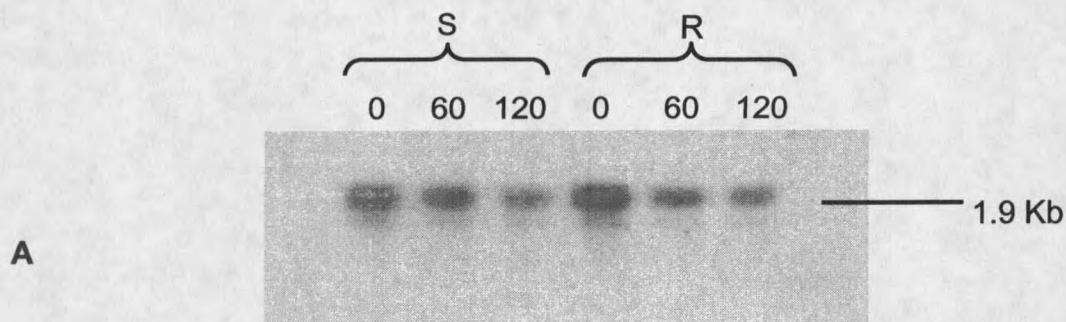


B

C

agcttgattgccnaacatatgcatgatcaactttgatggacctattcaaaggtttttttt
 ataaatattcgtgttgagagagtgttctattttatatttatgntgtttcgttttcgtttc
 aatgtataaantatggtttcgcatgtgtttgtggttattatgaattcaaagttttatttg
 ttttgccttcgtggngtggntgtattcattttattatatatatatatatcaataaat
 ggagtcttgtaataaccnttttatttgtttgtcgaaaaaaaaa

Figure 5. Expression of C01R120 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) cDNA sequence of clone C01R120.



C

ttgccgagctggcgaagggaaagtgcatttcacaacgttgactcatcgtgcttcg
 attctcgcttggtggaagtggcaaaaagtcctgctttgtgtgcccttaccatggatgggtt
 ttggcatgaatggagatctcacaaaagcaacccaagctgaaactcaaacttttgatgct
 aaggaacttgggctagtggccctaaaggttgagatgagggaccattcgttctgatcagc
 tcggc

Figure 6. Expression of D416S0 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of cDNA D416S0.

vulgaris, *Amaranthus tricolor*, *Atriplex hortensis*, and a predicted protein from *Arabidopsis*. This enzyme is involved in the biosynthesis of the osmoprotectant glycine betaine in certain members of the Chenopodiaceae and Amaranthaceae (Russell et al., 1998) and is the subject of Chapter 3.

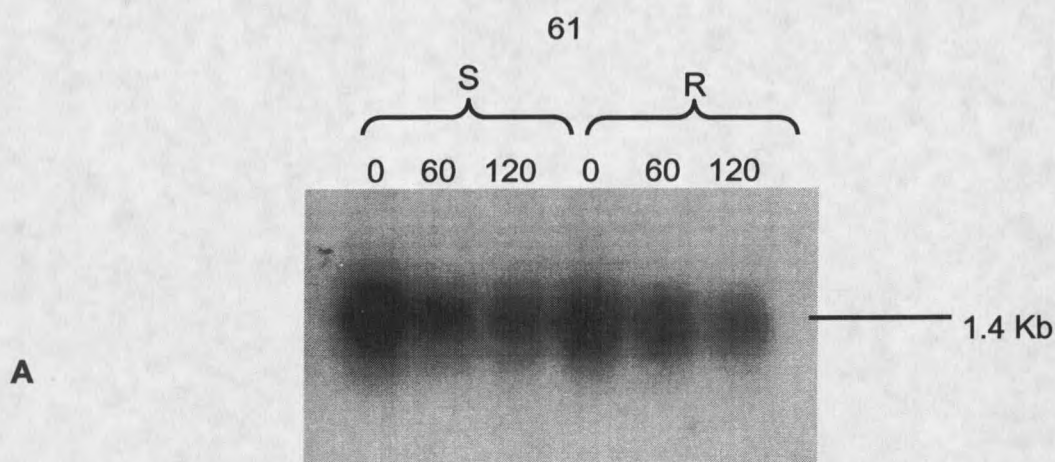
A05S0

The 382-bp A05S0 cDNA recognized a 1.4-Kb message in both R and S that was expressed at high levels in untreated tissues (16-hour exposure; Figure 7). The corresponding mRNA decreased in abundance by 30 minutes after treatment, and was attenuated about 10-fold in both R and S by 120 minutes after treatment. Database searches revealed no significant sequence identities at the nucleotide or translated amino acid levels.

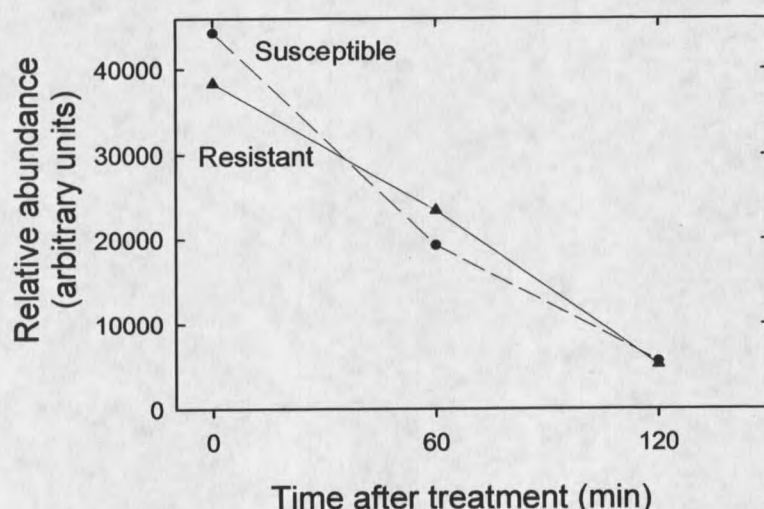
C03S0

Clone C03S0 is a 328-bp fragment that recognizes an abundant 2.1 Kb mRNA (Figure 8). After dicamba treatment, this message is down-regulated within minutes to levels nearly 20-fold lower than basal levels in both R and S.

Sequence identity searches revealed no sequence homologies at the DNA level, probably because the clone is mostly 3' untranslated sequence (Figure 9). However, a portion of the deduced amino acid sequence indicated strong homology to a short domain at the carboxyl terminus of several genes in the methylenetetrahydrofolate reductase (MTHFR) family of enzymes. MTHFRs have been found in bacteria and *Saccharomyces* (Hanson et al., 2000),



A



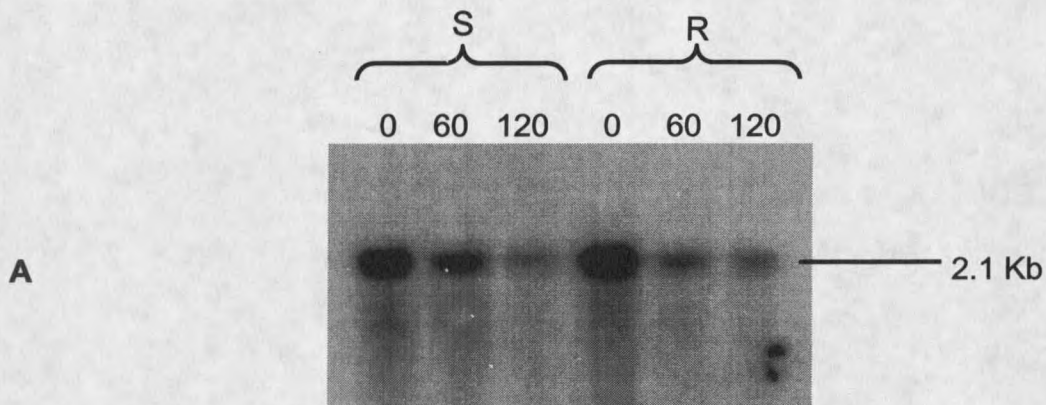
B

C

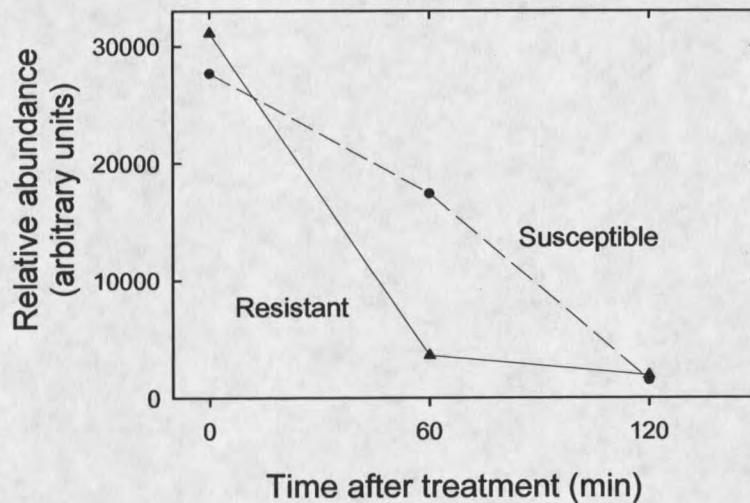
```

cttcgactgtgggatctcgacttcaaaatttcaccagtcgagtatgagaatgagatgaa
caacatatatatgcatcatatcatactgtgatcatctttttcggttatttttttactc
ttctgtttaatttcctgttcttcgagtactctgtataatatgcatgaaaaatgctgc
ttatgtacaataaggagaccatcatccttccttgttttcttttcttatgttcgttcttgc
aagctaacggatacgattaatcgcccttttgatgattagtcatgccgtaagcctgtaaga
catatgtaatctcattattatatcaatccatatactgtatggttttaattaatgtactct
gtaattattcacaaaaaaaaaaaa
  
```

Figure 7. Expression of A05S0 after dicamba treatment. (A) RNA isolated from S and R *K. scaparia* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of cDNA A05S0.



B



C

aagcttttggtcagcgtggttgacaatgattacatcaatggtaatttggttttcgggttttcg
 cttaaatttgagatgagacttcgatacgtcattgttcctacattcatatcttcttcctac
 attgtatcccattgggcttttactttccattgtttcaagtgttgattggttagatgtagt
 agagtttgtaatggcataaatcttggtgggattttgcaaattctcccaagcctcccaatgca
 aattgcatggcgagttgatgtaatgcgccttcagtctatttggttcgataaaaatagat
 attgcttctgggttttggaaaaaaaaaa

Figure 8. Expression of C03S0 after dicamba treatment. (A) RNA isolated from S and R *K. scaparia* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the C03S0 cDNA.

A

```

3  gctttggtcagcgtgggttgacaatgattacatcaatggtaatttgtttcggttttcgct
   A L V S V V D N D Y I N G N L F S V F A
63  taaatttgagatgagacttcgatacgtcattgttcctacattcatatcttcttcctacat
   *
123 tgtatcccattgggcttttactttccattgtttcaagtgttgattgtagatgtagtag
183 agtttgtaatggcataaatcttggtgggattttgcaaactctccaagcctccaatgcaaa
243 ttgcatggcgagttgatgtaatgcgcccttcagtctatttgtttcgataaaaaatagatat
303 ttgcttctggttttggaaaaaaaaa 328

```

B

<i>Kochia</i>	ALVSVVDNDY	INGNLF SVFA*
<i>Arabidopsis</i> AL138647	YLVSLVDNNY	INGDIF SVFADL*
<i>Arabidopsis</i> AF370515	YLVSLVENDY	INGDIF AVFADL*
<i>Zea</i>	YLVSLVDNDY	VQGDLF AAFKI*

Figure 9. C03S0 is mostly 3' untranslated region. (A) DNA (lower case) and in-frame translated amino acid sequence (upper case) of the C03S0 partial-length cDNA. The stop codon is indicated by an asterisk. (B) PILEUP analysis of C03S0 and similar amino acid sequences as recovered by BLAST. Amino acid sequences similar between the cDNAs are in bold; the stop codon for each is indicated by an asterisk.

Arabidopsis and *Zea* (Roje et al., 1999), and now *Kochia*, but the vast majority of the research on these enzymes has been in humans. MTHFRs irreversibly catalyze the reduction of 5,10-methylenetetrahydrofolate (produced from glycine, serine, or 5,10-methenyltetrahydrofolate) to 5-methyltetrahydrofolate, which constitutes the major form of folate in tissues and is the primary donor of methyl groups in one-carbon (C_1) metabolic pathways. Many C_1 transfers are mediated by the coenzyme tetrahydrofolate, which is produced by the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine (mediated by methionine synthase), creating methionine. Methionine is then used in protein synthesis or converted to S-adenosylmethionine, which acts as a universal donor for other methylation reactions. Reduced MTHFR activity results in an inability to produce 5-methyltetrahydrofolate, and therefore decreased homocysteine catabolism. Although there are several known MTHFR mutations in humans, the most common and severe mutation (Ala222Val) causes increased blood homocysteine levels and corresponding drops in folate and vitamin B₁₂ levels. MTHFR mutations cause severe hyperhomocysteinemia, premature atherosclerotic vascular disease, have been implicated in colorectal cancer (Choi, 2001), and can cause severe neurological disorders, especially in developing fetuses (Verhoeff et al., 1998). Because of variability in the activities of human MTHFRs, folate supplements are commonly suggested for pregnant humans. MTHFRs and other enzymes involved in plant C_1 metabolism have recently been suggested as targets for plant metabolic engineering, although biochemical data on these enzymes in plants are scarce (Roje et al., 1999).

In plants, methyl units are needed to synthesize amino acids, pantothenate, nucleic acids, lignin, alkaloids, and numerous other compounds. Auxin has been shown to induce DNA synthesis (Nagata, and Takahashi, 1992), and presumably a larger pool of nucleotide precursors would be needed under such conditions. Five,10-methylenetetrahydrofolate is also used as a methyl donor for thymidine monophosphate (a precursor required for DNA synthesis) by thymidylate synthase, which transfers a methyl group from 5,10-methylenetetrahydrofolate to deoxyuridine monophosphate (Cox et al., 1999). Since MTHFRs may compete with thymidylate synthase for the available 5,10-methylenetetrahydrofolate pool, it is possible that MTHFRs are down-regulated in response to auxin in order to increase substrate availability for subsequent DNA and RNA synthesis.

mRNAs up-regulated by dicamba treatment

C07S120

The 325-bp C07S120 clone was initially selected as a differentially expressed message (based on DD results) but proved to be regulated similarly in R and S (Figure 10). It recognizes a 2.7-Kb mRNA that is initially relatively scarce (48-hour film exposure) but increases in abundance about 5-fold in both R and S in response to dicamba treatment. Deduced amino acid sequence comparisons indicated that it has 75% identity with a 20-amino acid region near the carboxyl terminus of chloride channel proteins in *Nicotiana* and *Arabidopsis* (Figure 11).

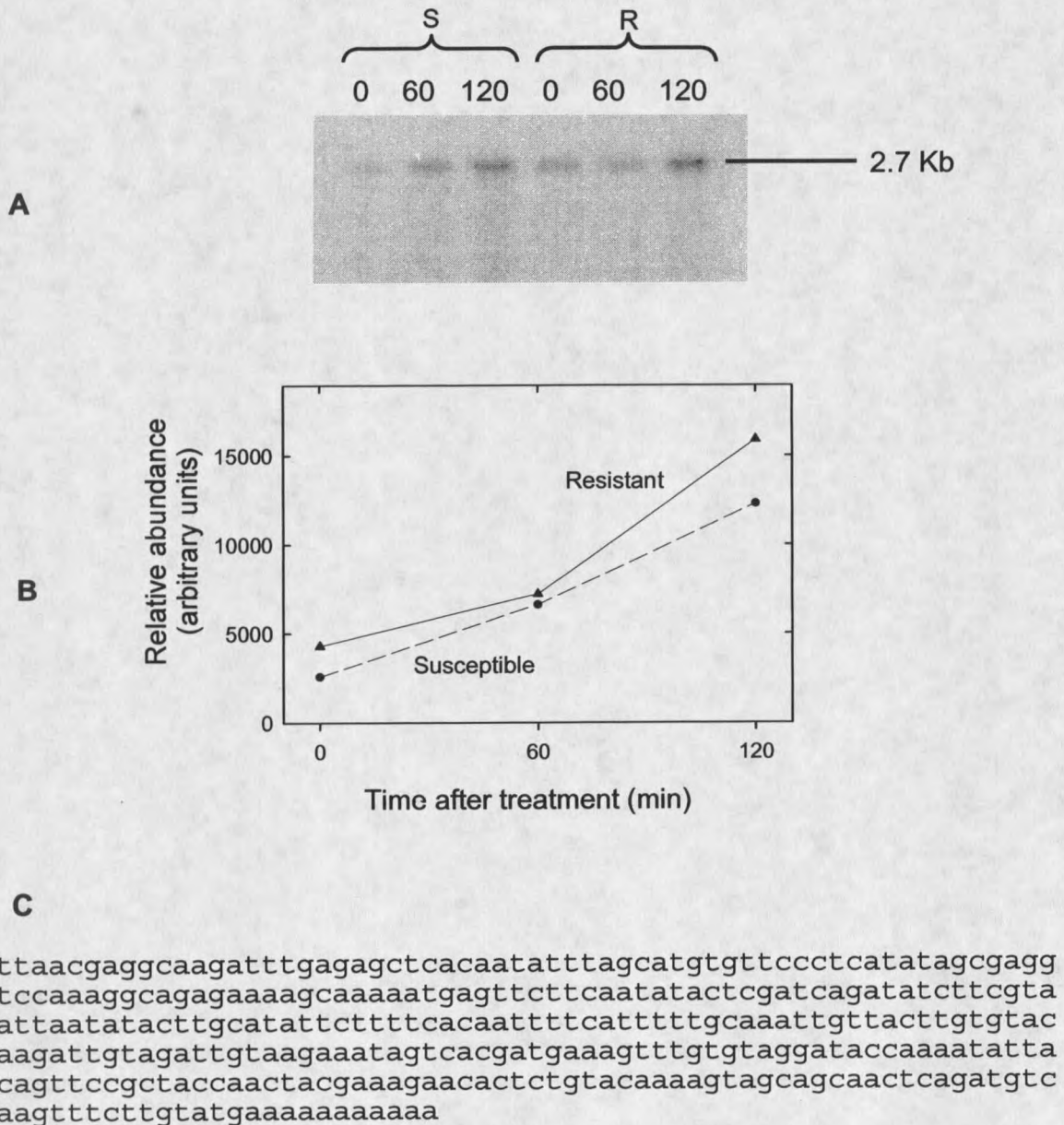


Figure 10. Expression of C07S120 after dicamba treatment. (A) RNA isolated from S and R *K. scolaria* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the C07S120 cDNA.

A

```

1 ttaacgaggcaagatttgagagctcacaatatttagcatgtgttcctcatatagcgagg
  L T R Q D L R A H N I * H V F P H I A R
61 tccaaaggcagagaaaagcaaaatgagttcttcaatatactcgatcagatatcttcgta
  S K G R E K Q K *
121 attaatatacttgcataattcttttcacaatttttcatttttgcaaattgttacttgtgtac
181 aagattgtagattgtaagaaatagtcacgatgaaagtttgtgtaggataccaaaatatta
241 cagttccgctaccaactacgaaagaacactctgtacaaaagtagcagcaactcagatgtc
301 aagtttcttgtatgaaaaaaaaa 325

```

B

<i>Kochia</i>	LTRQDLRAHN	I*HVFPHIAR	SKGREKQK*
<i>Nicotiana</i>	LTRQDLRAHN	ILSVFPHLEK	SKSGKKGN*
<i>Arabidopsis</i> AP001312	LTRQDLRAYN	ILQAFPLLEK	SKGGKTH*
<i>Arabidopsis</i> Z71445	LTRQDLRAYN	ILQAFPHLDK	HKSGKAR*

Figure 11. C07S120 is mostly 3' untranslated region. (A) DNA (lower case) and translated amino acid sequence (upper case) of the C07S120 partial-length cDNA. Potential stop codons are indicated by asterisks. (B) PILEUP comparisons of C07S120 and similar amino acid sequences as recovered by BLAST. Amino acid sequence identities between the cDNAs are in bold; the stop codon for each is indicated by an asterisk. Note the possible single base pair substitution in (A) that introduces a premature stop codon.

Other similarities are to gated anion channel proteins from *Arabidopsis*. No similarities at the DNA level were noted.

Auxin is known to cause proton extrusion into the apoplast, leading to loosening of non-covalent bonds in the cell wall. Harling et al. (1997) suggest that this electrochemical gradient also drives solute uptake into the cytosol,

which is likely a prerequisite for osmotically-driven cell expansion and subsequent elongation and tropic responses. Other findings suggest that chlorine is involved in stomatal movement, turgor, and possibly signal transduction (Tyerman, 1992, Schroeder, 1995). There is evidence that auxin directly stimulates chloride uptake in protoplasts by activation of plasma membrane chloride channels (Babourina et al., 1998). Similarly, it has long been known that auxin stimulates chloride uptake into *Avena* coleoptiles (Rubinstein and Light, 1973) and this uptake is an active process (Assman, 1993). Additional evidence for auxin-induced chloride influx into plant cells comes from studies showing that anion channel blockers prevent growth inhibition mediated by high auxin concentrations (Thomine et al., 1997), possibly by preventing excess chloride uptake. Additionally, Keller and VanVolkenburgh (1996) provide evidence for auxin induced chloride channels, and suggest that these channels are central in controlling the auxin-induced H^+ -ATPase-mediated hyperpolarization of the cell membrane.

Given these reports, it is possible that dicamba induces expression of these chloride channel proteins, possibly as a response to mediate chloride uptake across the plasma membrane. However, the hyperpolarization response to auxin is extremely fast in protoplasts (Keller and Van Volkenburgh, 1996), occurring within seconds. The much slower induction of this putative chloride transporter by dicamba treatment may represent part of a long-term growth response.

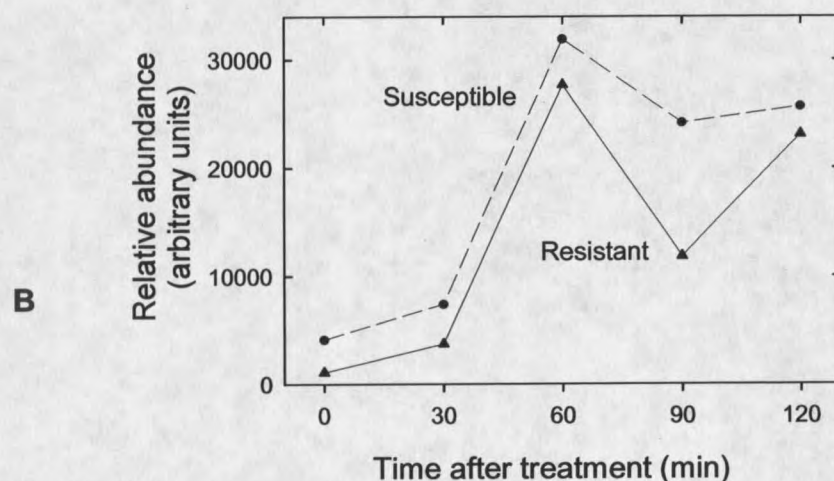
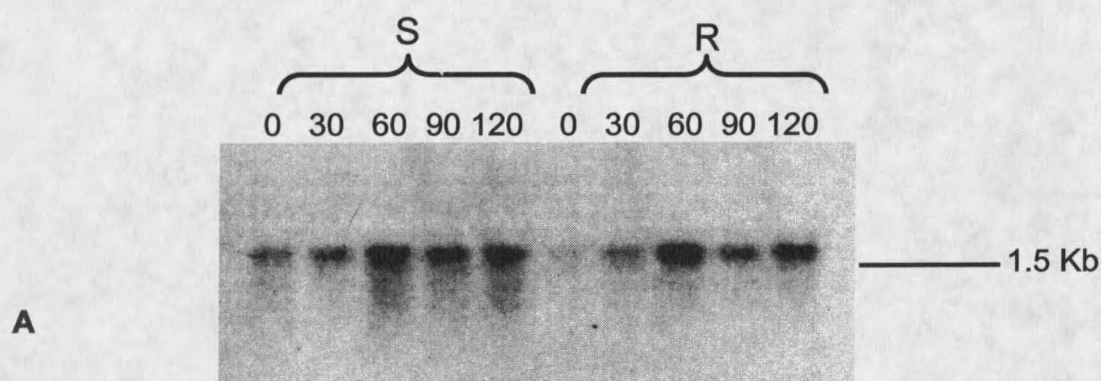
G14S120

The 280-bp G14S120 clone hybridized with a 1.5-Kb mRNA that was quite rare (1-week exposure) and induced in both R and S (Figure 12). Induction in S was about 8-fold while induction in R plants was about 25-fold. Basal expression levels were higher in S than in R. In both cases, peak induction came at 60 minutes after treatment, and mRNA abundance declined thereafter. Sequence identity searches revealed no significant similarities at either the DNA or amino acid level.

mRNAs differentially expressed between R and S
K. scoparia after dicamba treatment

A02S120

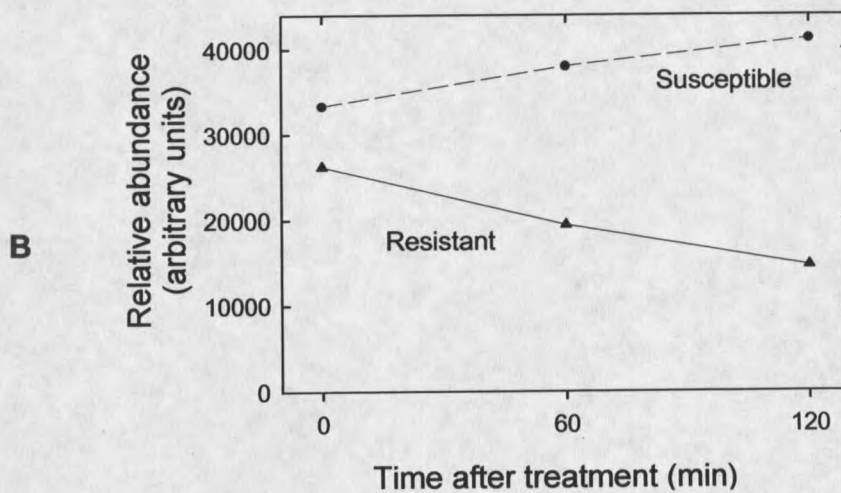
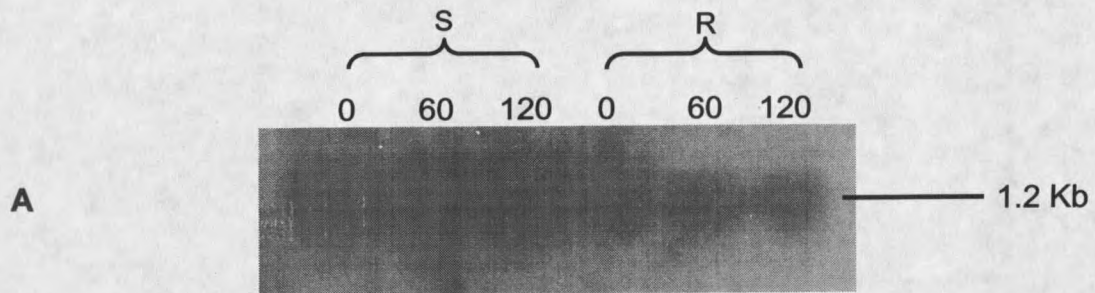
Basal levels of cDNA A02S120 were higher in S than in R, and increased in S but declined in R after dicamba treatment (Figure 13). S plants had about 3-fold higher levels of this 1.2-Kb mRNA by two hours after treatment than R plants. Sequence analysis of the 213-bp clone found no significant similarities at the nucleotide level; however, deduced amino acid sequence showed 84% identity to the nuclear-encoded chloroplast thylakoid lumen 5 kDa protein (Kapazoglou et al., 1995). Additionally, the same fragment showed >70% amino acid identity to the *Spinacia* PSII-T protein and to the carboxyl terminus of the *Gossypium* psbT gene (Figure 14), as well as to the large polycistronic chloroplastic transcript encoding the psbB-psbT-psbH-petB-petD suite



C

ataattaaaatgtgggtaagcataatgtgtgtctgctttgcttaattgtgtacttgagtg
 taagggtgctaataatgactattataaataaacaccatggcacccttcccctaaatagt
 tttcaaataattttggggggttaagcgaagaatagatcgcttatgaatgttttatctagtc
 atgtatggttttcattagtttaagtacatgaggaaaacattattgtgtgttgaatacagca
 tattaataacaacatattatcagttgtagtataaaaaaaaaa

Figure 12. Expression of G14S120 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 30, 60, 90, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the G14S120 cDNA.



C

aagcaaacgagggactgcagaagctaagaagaagtatgcgcccatttgcgtcactatgcc
 aactgccaggatctgtttacaagtgaggcatctcttgaaattccgatatgaaaatcagtag
 caatatctaataatgttgatattatgtttgtaatgttctgtaaaacttgactactttaa
 tgcattatgcgtcatttttgcgaaaaaaaaaaaa

Figure 13. Expression of A02S120 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the A02S120 cDNA.

A

```

2 agcaaacgagggactgcagaagctaagaagaagtatgcgcccatttgcgtcactatgcca
  S K R G T A E A K K K Y A P I C V T M P
62 actgccaggatctgttacaagtgaggcatctcttgaaattccgatatgaaaatcagtagc
  T A R I C Y K *
122 aatatctaataatgttgatattatgtttgtaatgttctgtaaaacttgactactttaat
182 gcattatgcgtcatttttgcgaaaaaaaaaaaaa 213

```

B

<i>Kochia</i>	SKRGTA EAKK	KYAPIC VTMP	TARICYK*
<i>Arabidopsis</i> (AY050320)	PKRGTE AAKK	KYAPVC VTMP	TAKICRN*
<i>Arabidopsis</i> (AF339682)	PKRGTE AGKK	KYAQVC VTMR	TAKICRY*
<i>Gossypium</i>	PKRGSA EAKK	AYAPVC VTMP	TARICRN*
<i>Spinacia</i>	PKRGTP EAKK	KYAPVX VTMP	SARIXYK*

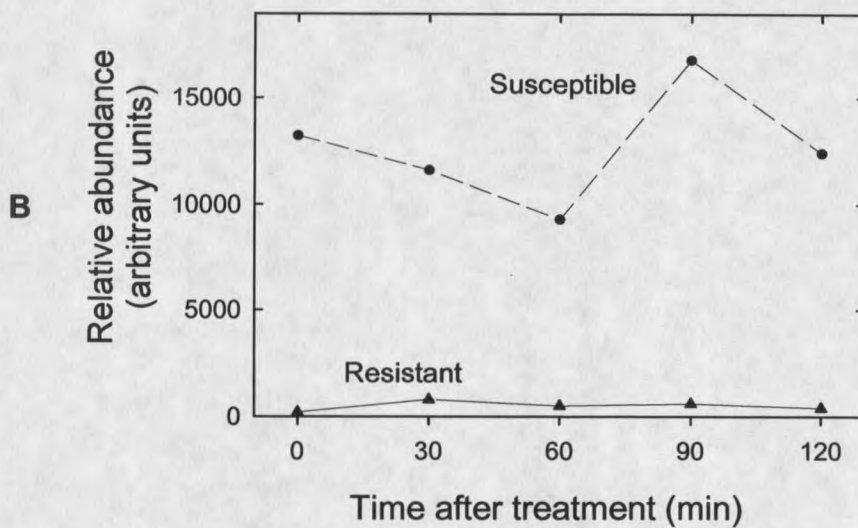
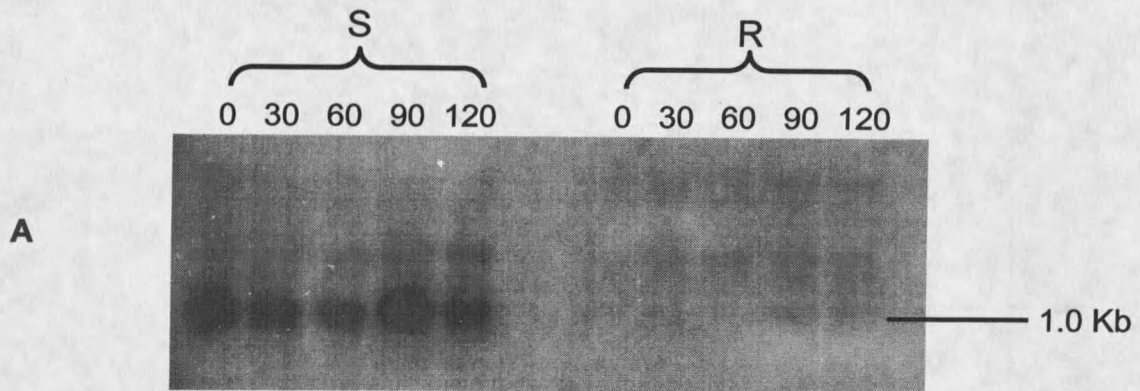
Figure 14. A02S120 is mostly 3' untranslated region. (A) DNA (lower case) and inferred amino acid sequence (upper case) of the A02S120 partial-length cDNA. The stop codon is indicated by an asterisk. (B) PILEUP comparisons of A02S120 and similar amino acid sequences as recovered by BLAST. Amino acid sequence identities between the cDNAs are in bold; the stop codon for each is indicated by an asterisk.

of chloroplastic proteins from *Arabidopsis* (Meurer et al., 1996). Based on amino acid similarities, it thus appears that A02S120 encodes a fragment of a light-regulated photosystem II component. The significance of the observed differential expression of this gene in R and S *K. scoparia* after dicamba treatment is unknown.

C07S60

The 1.0 Kb mRNA recognized by the 434-bp C07S60 clone was present in S but not detectable in R after a 48-hour film exposure (Figure 15). C07S60 also hybridized at moderate levels to rRNA in both R and S samples. DNA sequence analysis indicated this clone has >80% sequence identity to the translation initiation factor eIF-1 from *Sporobolus stapfianus* (Neale et al., 1999), and to homologs of the translation initiation factor SUI1 from *Arabidopsis*, *Salix*, *Eucalyptus*, and *Pimpinella*, and to the *Zea* translation initiation factor GOS2 (de Pater et al., 1992; Figure 16). At the deduced amino acid level, the sequence was >80% identical and 90% similar to translation initiation factors from a variety of organisms including *Arabidopsis*, *Oryza*, yeast, human, *Mus*, *Schizosaccharomyces*, and others.

While most translation initiation factors are expressed at very low levels (Neale et al., 1999), the mRNA for the C07S60 appears to be relatively abundant and may be slightly induced by 90 minutes after treatment. The eIF-1 translation initiation factor is induced upon drought stress in *Sporobolus*, but it is not known whether dicamba treatment presents a similar challenge to S *K. scoparia*. Of the 106 clones generated by DD and used as probes on northern blots in the studies reported here, C07S60 is one of only two that hybridized to one *K. scoparia* biotype and not the other. One possible explanation is that the clone was originally generated from a cDNA from the S biotype, and allelic differences between R and S were significant enough to prevent hybridization to R RNA.



C

aagcttcgactgttcaaggtctgaagaaggatttcagctacgagaagataactcaaagacc
tcaaaaaggagtttctgctgcaacggcaatgttgtagaggacaaggagctaggggaaggta
tccaactgcaaggcgatcagcgtaagaagggttgcaatttcctgacccaagccggtcttg
tgaagaaggatcggtatcaaaattcatgggtttttaagctttgattcgtgctgttgagatac
aagagcgattgatttgttcttgatagtttctgttttcgttttagggtttggtcgtttgat
gttggttggtttcagttgctttcgtttctgttgagtgttaatgcatcccatggatggcac
tggtagtctgttatgtattgagctttcgaactcgttgctattaaataaaaatcggtatgctt
acgtaaaaaaaaaa

Figure 15. Expression of C07S60 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 30, 60, 90, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the C07S60 cDNA.

A

```

3 gcttcgactgttcaagggtctgaagaaggatttcagctacgagaagataactcaaagacctc
  A S T V Q G L K K D F S Y E K I L K D L
63 aaaaaggagttctgctgcaacggcaatgttgtagcaggacaaggagctaggaagggttattc
  K K E F C C N G N V V Q D K E L G K V I
123 caactgcaaggcgatcagcgtaagaagggttgcaatttcctgacccaagccgggtcttggtg
  Q L Q G D Q R K K V A N F L T Q A G L V
183 aagaaggatcgatcaaaattcatggtttttaagctttgattcgtgctgttgagatacaa
  K K D R I K I H G F *
243 gagcgattgatttgttcttgatagtttctgttttcgttttaggggtttggcggttgatgt
303 tgttggtttcagttgcttttcgtttctgttgagtgttaatgcatcccatggatggcactg
363 gtagtctgttatgtattgagctttcgaactcgttgctattaaataaaatcggatgcttac
423 gtaaaaaaaaaa 434.

```

B

	1		50
<i>Kochia</i>	TVQGLKKDFS	YEKILKDLKK	EFCCNGNVVQ DKELGKVIQL QGDQRKKVAN
<i>Zea</i>	TVQGLKKEFS	YSKILKDLKK	EFCCNGTVVQ DPELGQVIQL QGDQRKNVSN
<i>Phleum</i>	TVQGLKKEFS	YSKILKDLKK	EFCCNGTVVQ DPELGQVIQL QGDQRKNVSN
<i>Sporobolus</i>	TVQGLKKEFS	YNKILKDLKK	EFCCNGTVVQ DPELGQVIQL QGDQRKNVSN
<i>Arabidopsis</i>	TVQGLKKEYS	YERILKDLKK	DFCCNGNVVQ DKELGKIIQL QGDQRKKVSQ
<i>Homo</i>	TVQGIADDYD	KKKLVPFKK	KFACNGTVIE HPEYGEVIQL QGDQRKNIQ

	51		68
<i>Kochia</i>	FLTQAGLVKK	DRIKIHGF*	
<i>Zea</i>	FLVQAGIVKK	EHIKIHGF*	
<i>Phleum</i>	FLVQAGIVKK	EHIKIHGF*	
<i>Sporobolus</i>	FLVQAGIVKK	EHIKIHGF*	
<i>Arabidopsis</i>	FLVQTGIARK	DQIKIHGF*	
<i>Homo</i>	FLVEIGLAKD	DQLKVHGF*	

Figure 16. C07S60 has high sequence similarity to SU11 translation initiation factors. (A) DNA (lower case) and translated amino acid sequence (upper case) of the C07S60 partial-length cDNA. The stop codon is indicated by an asterisk. (B) PILEUP comparisons of C07S60 and similar amino acid sequences as recovered by BLAST. Amino acid sequence identities between the cDNAs are in bold; the stop codon for each is indicated by an asterisk.

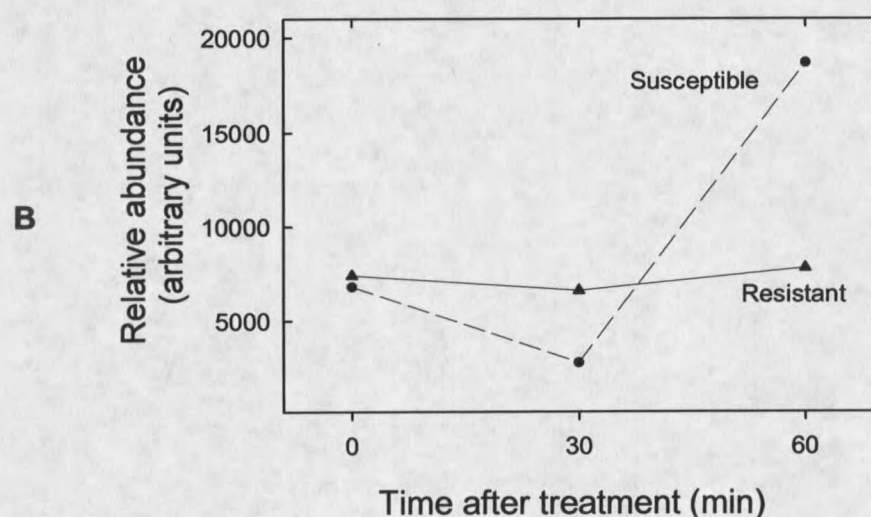
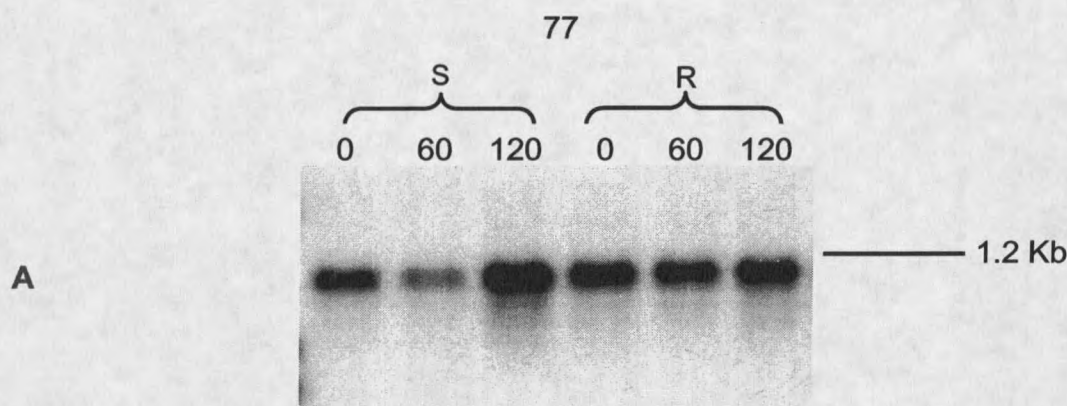
Alternatively, this may be a member of a multiple gene family that is expressed in S but not R. Both these explanations seem somewhat unlikely, however, given the high similarity across diverse species seen in Figure 16B.

G07R0

Figure 17 shows the expression patterns of the 1.2-Kb mRNA that hybridized to the 657-bp clone G07R0. The message appears to be differentially expressed in S but levels do not change in R. In S, the message is reduced by about half at 30 minutes after treatment, but increased to about 3 times basal levels by 60 minutes after treatment.

DNA sequence analysis of the clone indicated 82-89% similarity to xyloglucan endotransglycosylases (XETs) from *Arabidopsis*, *Carica*, and *Fagus*. The predicted amino acid sequence (Figure 18) was about 65% identical or 74% similar to dozens of xyloglucan endotransglycosylases (also known as endoxyloglucan transferases) and xyloglucan endo-1,4- β -D-glucanases from *Arabidopsis* and other dicots. Clone G07R0 has several characteristics that further suggest it is a xyloglucan endotransglycosylase, including the HDE(I/V)D(F/I/M)EFLG motif (underlined in Figure 19) and the four invariant cysteine residues thought to be involved in disulfide bridge formation (Xu et. al., 1995a).

Xyloglucan endo-1,4- β -D-glucanases have a cellulase activity and catalyze the internal hydrolysis of long xyloglycan chains, whereas XETs transfer a xyloglucan molecule onto another polysaccharide after the same internal cleavage (Arrowsmith and de Silva, 1995). Such enzymes appear to be critical in the creation, modification, and degradation of plant cell walls, which are composed of cellulose microfibrils embedded in a matrix of hemicellulose, proteins, and pectin (Xu et al., 1995b). In dicots, the primary hemicellulose is



C

aacgagggttacactgctggagttataacagcattctatctttcgaacaaccaagttcatcccgggcaccacg
 atgaagtagacatggaattccttgggacaacatttggaaccatggtttgcaacaaatgtgtacataa
 ggggtagtggagatggcacaattattggaagagagatgaaatttcatctatggtttgatccaactaagggat
 ttcaccattatgccattttttggagtcctcaagaaatcatatttttggtagatgatattccaataagaagat
 atcctagaaagagtgcagccacatatcctctaaggccaatgtgggtgatgggtcaatatgggatgcatcat
 cttgggcaaccgaagatggaaaatacaaagcaaattataattaccaaccatttgttgggtcaatacactaatt
 ttaaagctagtgggttgctccgcctacgccccacgctcatgtcgcccggtgtccgtctcgccctaccggtccg
 ggggccttaccctaaagcaaacctacgtgatgaagtgggtacaaagacattacatgatttataactattgca
 aggaccgtaaaanggaccattcacataccccggaatgttggttacgtcgccggttaataatttaaagttatata
 atggtgg

Figure 17. Expression of G07R0 after dicamba treatment. (A) RNA isolated from S and R *K. scolaria* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the G07R0 cDNA.

```

3  cgagggttacactgctggagttataacagcattctatctttcgaacaaccaagttcatccc
   R G Y T A G V I T A F Y L S N N Q V H P
63  gggcaccacgatgaagtagacatggaattccttgggacaacatttgaaaaccatatgtt
   G H H D E V D M E F L G T T F G K P Y V
123 ttgcaaacaaatgtgtacataaggggtagtggagatggcacaattattggaagagagatg
   L Q T N V Y I R G S G D G T I I G R E M
183 aaatttcattctatggtttgatccaactaagggtttcaccattatgccattttttggagt
   K F H L W F D P T K G F H H Y A I F W S
243 cccaaagaaatcatatttttggtagatgatattccaataagaagatattcctagaaagagt
   P K E I I F L V D D I P I R R Y P R K S
303 gcagccacatatcctctaaggccaatgtgggtgtatggctcaatatgggatgcatcatct
   A A T Y P L R P M W V Y G S I W D A S S
363 tgggcaaccgaagatggaaaatacaaagcaaattataattaccaaccattttgttggtcaa
   W A T E D G K Y K A N Y N Y Q P F V G Q
423 tacactaattttaagctagtgttgctccgcctacgccccacgctcatgtcgcccggtg
   Y T N F K A S G C S A Y A P R S C R P V
483 tccgtctcgccctaccggtccgggggccttaccctaaaagcaaacctacgtgatgaagtgg
   S V S P Y R S G G L T Q K Q T Y V M K W
543 gtacaaagacattacatgatttataactattgcaaggaccgtaaaanggaccattcacat
   V Q R H Y M I Y N Y C K D R K X D H S H
603 accccggaatgttggttacgtcgccgttaataattaagttatataatggtgg 655
   T P E C W L R R R * *

```

Figure 18. G07R0 open reading frame. DNA (lower case) and deduced amino acid sequence (upper case) of the G07R0 partial-length cDNA. Consecutive stop codons are indicated by asterisks.

xyloglucan, which is structurally similar to cellulose but has numerous xylosyl side chains. Since xyloglucans form tight hydrogen bonds with cellulose, it has been proposed that modification of xyloglucans and subsequent turgor-mediated cell expansion is necessary for cell growth (Fry et al., 1992, McCann et al., 1992, others). Xyloglucan degradation and turnover is associated with auxin-mediated cell elongation in *Pisum* (Labavitch and Ray, 1974), *Vigna* (Nishitani and Masuda, 1982), and *Oryza* (Revilla and Zarra, 1987).

	1					50
Kochia	RGYTAGVITA	FYLSNNQVHP	GHHDEVDMEF	LGT	TFGKPYV	LQTNVYIRGS
Arabidopsis	PGYTAGVITS	LYLSNNEAHP	GHHDEVDIEF	LGT	TFGKPYT	LQTNVYIRGS
Carica	PGYTAGVITS	FYLSNNEAHP	GHHDEVDIEF	LGT	TPGEPYT	LQTNVYIRGS
Tropaeolum	SGYTAGVITS	FYLSNNQDYP	GHHDEVDIEF	LGT	IPGKPYT	LQTNVYIRGS
Oryza	PGYTAGVNTA	FYLSNTEQYP	GHHDEVDMEF	LGT	VPGEPTT	LQTNVYIRGS
Nicotiana	GGDSAGVVTA	FYLSNN...	AEHDEVDIEF	LGN	RTGQPYI	LQTNVFTGGK
	51					100
Kochia	GDGTIIIGREM	KFHLWFDPTK	GFHHYAIKWS	PKEIIFLVDD	IPIRRYPRKS	
Arabidopsis	GDGKIIGREM	KFRLWFDPTK	DFHHYAILWS	PREIIFLVDD	IPIRRYPRKS	
Carica	GDRNIVGREV	KFHLWFDPTQ	DFHHYAILWT	PSEIVFFVDD	VPIRRYPRKS	
Tropaeolum	GDYNIIGREM	RIHLWFDPTQ	DYHNYAIYWT	PSEIIFVDD	VPIRRYPRKS	
Oryza	GDGNIVGREM	RFHLWFDPTA	GFHHYAILWN	PDQILFLVDD	VPIRRYPRKS	
Nicotiana	GD.....REQ	RIYLWFDPTK	GYHSYSVLWN	TFQIVIFVDD	VPIRAFKNKS	
	101					150
Kochia	A..ATYPL.R	PMWVYGSWD	ASSWATEDGK	YKANYNYQPF	VGQYTNFKAS	
Arabidopsis	A..STFPL.R	PMWLYGSWD	ASSWATEDGK	YKADYKYQPF	TAKYTNFKAL	
Carica	D..ATFPL.R	TMWVYGSWD	ASSWATDDGK	YKADYQYQPF	VGRYKNFKIA	
Tropaeolum	D..ATFPL.R	PLWVYGSVWD	ASSWATENGK	YKADYRYQPF	VGKYEDFKLG	
Oryza	E..GTFPE.R	EMWAYGSWD	ASDWATDGGK	YRADYRYQPF	VSRFADLKVG	
Nicotiana	DLGVKFPFNQ	PMKIYSSLWD	ADDWATRGGL	EKTDWSNAPF	TASYTSFHVD	
	151					200
Kochia	GCSAYAPRS.	..CRPVSVS.	.P.YRSGGLT	QKQTYVMKWV	QRHYMIYNYC	
Arabidopsis	GCTAYSSAR.	..CYPLSAS.	.P.YRSGGLT	RQHQAMRWV	QTHSMVYNYC	
Carica	ACRADGQAS.	..CRPPSVS.	.P.SGFGVLS	PQESAMEWA	QRNSLVYNYC	
Tropaeolum	SCTVEAASS.	..CNPASVS.	.P...YGQLS	QQQVAAMEWV	QKNYMVYNYC	
Oryza	GCATAAPPA.	..CSPVPAS.	.SGGSAALS	PQEAAMAWA	QRNAMVYNYC	
Nicotiana	GCEAATPQEV	QVCNTKGMRW	WDQKAFQDL	ALQYRRLRWV	RQKYTIYNYC	
	201					219
Kochia	KDRKXDHSHT	PECWLRRR*				
Arabidopsis	KDYKRDHSLT	PECWR*				
Carica	LDPKRDHTPT	PEC*				
Tropaeolum	DDPTRDHTLT	PEC*				
Oryza	QDYSRDHTFY	PEC*				
Nicotiana	TDRKRYPTLP	PECTKDRD*				

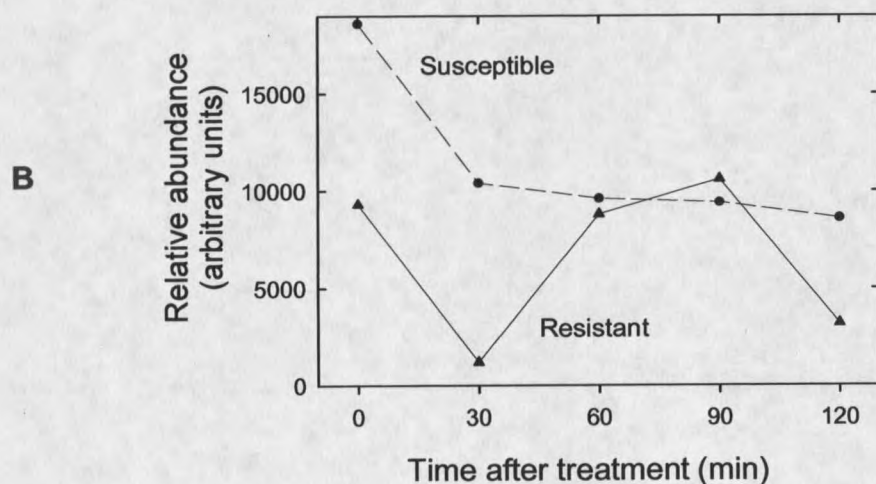
Figure 19. PILEUP comparisons of G07R0 and similar amino acid sequences as recovered by BLAST. Amino acid sequence identities among the cDNAs are in bold and stop codons are indicated by asterisks. Residues involved in the putative active site are underlined (Xu et al., 1995). Invariant cysteine residues are shown with a ^.

The mRNAs for the XETs TCH4 and EXT were induced within 10 minutes in *Arabidopsis* seedlings treated with low concentrations of auxin (Xu et al., 1995, Xu et al., 1996). Similarly, RNAs for two endo-1,4- β -D-glucanases from *Hordeum* (Slakeski and Fincher, 1992) and *Pisum* (Matsumoto et al., 1997) were induced after treatment with auxin and 2,4-D, respectively. In contrast, XET in *S. K. scoparia* appears to be initially down-regulated by 30 minutes after treatment, followed by the induction noted by Xu et al. The difference in expression patterns between R and S *K. scoparia* is consistent with the resistance trait in that S plants become epinastic within a few hours after treatment. In some species, epinasty is thought to be the result of uncontrolled cell elongation (Sterling and Hall, 1997), and the induction of this XET in S but not R plants may partly account for this symptom.

XETs and endo-1,4- β -D-glucanases are large families of enzymes in *Arabidopsis* and other dicots, and different members appear to respond to various stimuli (including auxin treatment) differently. Expression of the CEL1, CEL5, and BAC1 genes, which encode endo-1,4- β -D-glucanases, is thought to be involved in the abscission process and these enzymes are induced by ethylene treatment. As this induction is strongly inhibited by auxin (Tucker et al., 1988, Wu et al., 1996), there appears to be complex regulation of these classes of enzymes.

C08R60

The 522-bp clone C08R60 recognized a moderately-abundant (24 hour exposure time) 1.4-Kb message in both R and S (Figure 20). Basal mRNA levels were somewhat higher in S than in R, and levels fell slowly in S. However, mRNA levels declined rapidly in R by 30 minutes after treatment before returning to basal levels by 60 and 90 minutes after treatment. Sequence analysis of C08R60 indicated no nucleotide sequence similarities to any known genes, but the translated amino acid sequence was 71% identical and 83% similar to a hypothetical protein in Arabidopsis (Figure 21). Somewhat weaker similarities were noted between this clone and the human translation initiation factor HSPC182 (52% identical; 76% similar) as well as the human (50% identical; 68% similar) and yeast (40% identical; 59% similar) transcription initiation factor SSU72 (Pappas and Hampsey, 2000). Sequence similarities to these diverse genes prevents a putative identification of C08R60, but a more detailed analysis of the full-length clone could provide more information on the presence of functional domains common to translation or transcription initiation factors. If the apparently differential response between R and S can be confirmed on independent blots, this cDNA should be studied further.



C

```

gcttctcaacgctggcaagacaacgcacttgatggtgcttttgatggttgcttacatttgaagaaaaggct
tttgatatggttggtgaagatctataccaccgtgagcatgtacaaatgaagcctggtgtcatcatcaacctt
gaggtaaaagacaatcacgaggaagcagctattggcggctcgcttacactagattttatgccaagagattga
agagactgctgattcatgggaagacaagatcgatgagatcattgctgcttttgagagacaccataggcgcaa
gattgtctacactatttccttttattgaacataagatattgttttgtaggcaatgaccaacaatttgtaaca
gttctgttttacatgagcacagattcccgcatttcacatattcatgcggtcaciaaacgtgacatattgtat
agttctagcctatgttacttttgactcgtatacgagatgtccgactcggctaatttatgaaaattacctagat
tttagccaaaaaa
  
```

Figure 20. Expression of C08R60 after dicamba treatment. (A) RNA isolated from S and R *K. scopia* 0, 30, 60, 90, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the C08R60 cDNA.

A

```

1 gcttctcaacgctggcaagacaacgcacttgatgggtgcttttgatgttgcttacattt
  A S Q R W Q D N A L D G A F D V V L T F
61 gaagaaaaggctctttgatatgggtgtgaagatctataccaccgtgagcatgtaaaatg
  E E K V F D M V V E D L Y H R E H V Q M
121 aagcctgttgatcatcatcaaccttgaggtaaaagacaatcacgaggaagcagctattggc
  K P V V I I N L E V K D N H E E A A I G
181 ggtcgccttactactagattttatgccaagagattgaagagactgctgattcatgggaaga
  G R L T L D F M P R D *
241 caagatcgatgagatcattgctgcttttgagagacaccataggcgcaagattgtctacac
301 tatttccttttattgaacataagatatgttttgtaggcaatgaccaacaatttgtaaca
361 gttctgttttacatgagcacagattcccgcatctcatattcatgcggtcacaacgt
421 gacatattgtatagttctagcctatgttactttgactcgtatacgagatgtccgactcgg
481 ctaatttatgaaaattacctagatttttagccaaaaaaaaaaaa 522

```

B

	1		50
<i>Kochia</i>	ASQRWQDNAL	DGAFDVVLTF	EEKVFDMMVVE DLYHREHVQM KPVVIINLEV
<i>Arabidopsis</i>	APQRWQDNAG	DGVFDVVMTF	EEKVFDLVLE DLNNREQSLT KTIIVMNLEV
<i>Homo</i>	KPRPERFQNC	KDLFDLILTC	EERVYDQVVE DLNSREQETC QPVHVNVNDI
<i>Saccharomyces</i>	APEKWQEST-	-KVDFVFTC	EERCFDAVCE DLMNRGGKLN KIVHVINVNDI

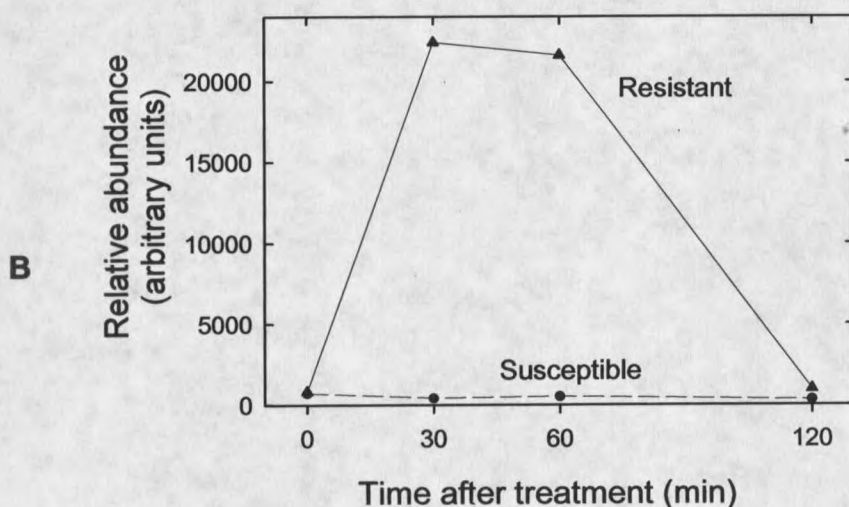
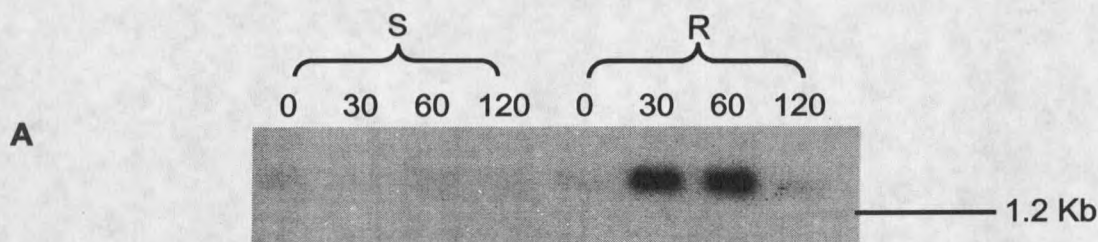
	51		79
<i>Kochia</i>	KDNHEEAAIG	GRLTLDPMR	D*
<i>Arabidopsis</i>	KDNHEEAAIG	GRLALELCQE	I*
<i>Homo</i>	QDNHEEATLG	AFLICELCQC	IQHTEDMEN
<i>Saccharomyces</i>	KDDDENAKIG	SKAILELADM	LNDKIEQCE

Figure 21. Translated amino acid sequence of the C08R60 cDNA. (A) DNA (lower case) and translated amino acid sequence (upper case) of the entire C08R60 partial-length cDNA. The stop codon is indicated by an asterisk. (B) PILEUP comparisons of C08R60 and similar amino acid sequences as recovered by BLAST. Amino acid sequence identities between the cDNAs are in bold; the stop codon for each is indicated by an asterisk.

C08R60-2

Clone C08R60-2 recognized a moderately abundant (16 hour exposure) 1.2-Kb message in both R and S that was induced over 20-fold by 30 minutes after treatment in R (Figure 22). Message levels remained high at 60 minutes after treatment, but dropped to nearly basal levels by 120 minutes after treatment. In S, the message remained at very low levels throughout the time course.

Sequence analysis showed that this 479-bp clone has 80% DNA sequence identity and 92% amino acid identity to an unknown, hypothetical protein in *Arabidopsis* (NCBI accession AF375454; Figure 23). Other amino acid similarities are to unknown, hypothetical proteins from *Volvox* (63% identical) and *Synechocystis* (64% identical). Hypothetical proteins are typically derived from large-scale genome sequencing efforts, and are identified by the presence of open reading frames recognized by sequence analysis software. These proteins have not been characterized biologically, so no putative function can be assigned to them. The dramatic increase in mRNA levels specifically in R plants after dicamba treatment suggests that this clone deserves further attention and may provide important insights into the physiological responses occurring in R plants.



C

```
ccgacccggggcgcagcccatgatgaagaatgttaatgaagggaaagggtatatttgctcctgtggtggttggtg
actaggaacatcattggcaaaaagaggttcaatcaacttagaggcaaagccattgcccttcactcacaggtt
ataacagagttttgcaagtcaataggagcagatgcaaagcaaagacaaggcttgattcgattggcaaagaag
aatggagagaaaattagggttccttgcataattaaattgaacatttgtttgaatttgc aaatgaaattgtt
gagacttgtgtaaaaccttatgtatacatttcttgtgtagaagtaatctgtagaaatttctgttgcaattgc
aattgcaaaattcatctaaatttggggattaaacttcaaattgatggtattattatttttaggtttagtaaaa
tcaaattagcaattttgtagttgttattgttattataaaaaaaaaa
```

Figure 22. Expression of C08R60-2 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 30, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the C08R60-2 cDNA.

A

```

1  ccgacccgggcgcagcccatgatgaagaatgttaatgaagggaaagggtatatttgctcct
   P T R A Q P M M K N V N E G K G I F A P
61  gtggtggttgtagactaggaacatcattggcaaaaagaggttcaatcaacttagaggcaaa
   V V V V T R N I I G K K R F N Q L R G K
121 gccattgcccttcactcacagggtataacagagttttgcaagtcaataggagcagatgca
   A I A L H S Q V I T E F C K S I G A D A
181 aagcaaagacaaggcttgattcgattggcaaagaagaatggagagaaattaggggttcctt
   K Q R Q G L I R L A K K N G E K L G F L
241 gcataattaaatttgaacatttgtttgaatttgtcaaatgaaattgttgagacttgtgta
   A *
301 aaaccttatgtatacatcttctgtgtagaagtaatctgtagaaatttctgttgcaattgc
361 aattgcaaaattcatctaaatttggggattaaacttcaaattgatggtattattatttta
421 ggtttagtaaaatcaaattagcaattttagtattgttattattataaaaaaaaaa 479

```

B

	1		50
<i>Kochia</i>	PTRAQPMKN	VNEGKGIFAP	VVVVTRNIIG KKRFNQLRGK AIALHSQVIT
<i>Arabidopsis</i>	AIRAVPMKN	VNEGKGLFAP	LVVVTRNLVG KKRFNQLRGK AIALHSQVIT
<i>Synechocystis</i>	~~~~~	~~~~~MFAP	IVILVRQQLG KAKFNQIRGK AIALHCQTIT
<i>Volvox</i>	PRRKPVTMMG	NKATTGPFAP	LVVVVRGAIG EKEFNQFRGK AISLHSQVIK
	51		81
<i>Kochia</i>	EFCKSIGADA	KQRQGLIRLA	KKNGEKLGF A*
<i>Arabidopsis</i>	EFCKSIGADA	KQRQGLIRLA	KKNGERLGF A*
<i>Synechocystis</i>	NFCNRVGIDA	KQRQNLIRLA	KSNGKTLGL A*
<i>Volvox</i>	DFCKLLGVDN	KQVQGVIRLA	KKNGEKLGF A*

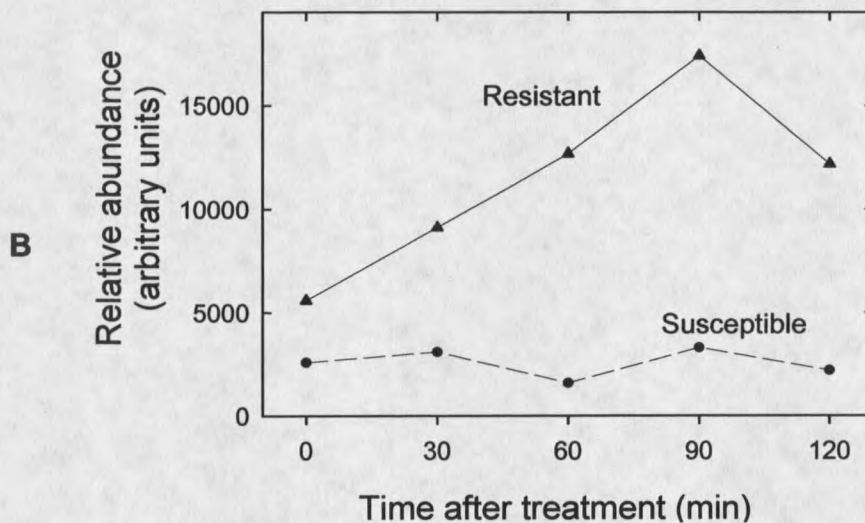
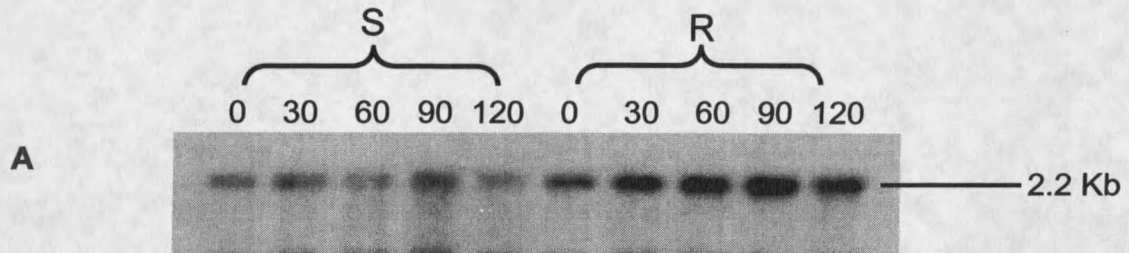
Figure 23. C08R60-2 is similar to hypothetical proteins in several photosynthetic organisms. (A) DNA (lower case) and translated amino acid sequence (upper case) of the C08R60-2 partial-length cDNA. The stop codon is indicated by an asterisk. (B) PILEUP comparisons of C08R60-2 and similar amino acid sequences as recovered by BLAST. Amino acid sequence similarities are in bold; the stop codon for each gene is indicated by an asterisk.

B16R120

Clone B16R120 recognized a moderately abundant (16 hour film exposure) 2.2-Kb message in both R and S Kochia (Figure 24). By 90 minutes after treatment there was about 8-fold more of the mRNA in R than in S K. scoparia. Expression was essentially constitutive in S plants after herbicide treatment, but increased about 3-fold in R. The 263-bp fragment showed no sequence similarity to known sequences at either the DNA or deduced amino acid level.

A13S60

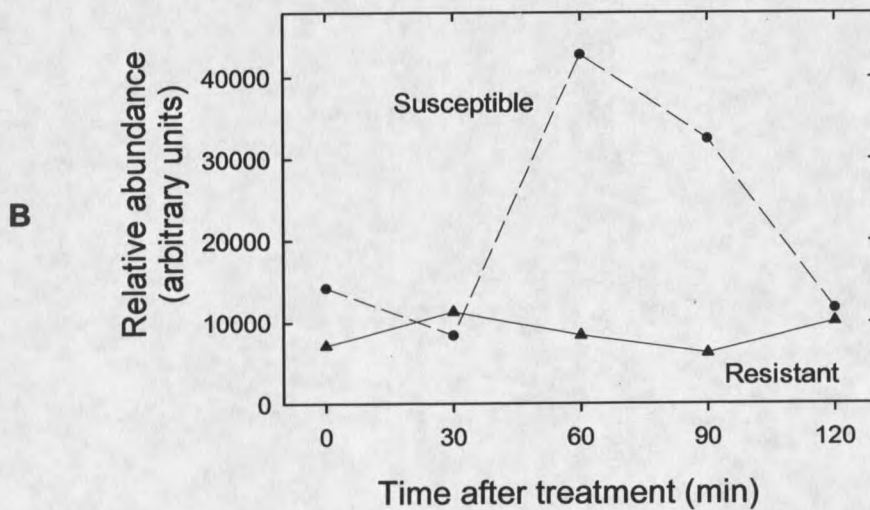
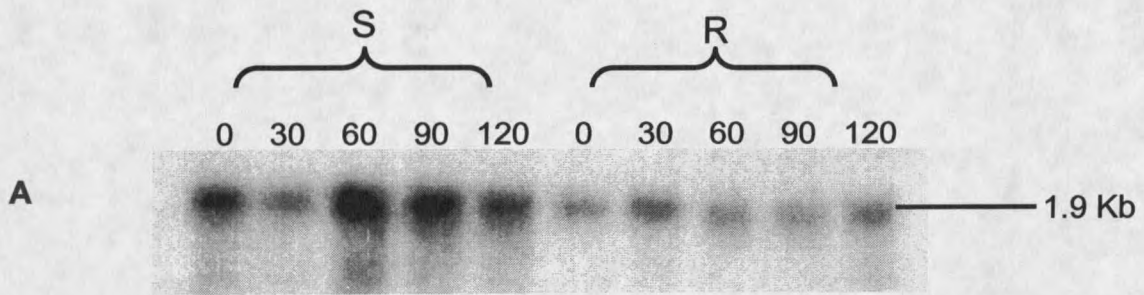
Expression levels of the moderately abundant (16 hour film exposure) A13S60 message increased in S and remained constitutive in R (Figure 25). The 231-bp fragment recognized a 1.9-Kb mRNA, which was induced about 5-fold in S by 60 minutes after treatment. Sequence analysis found no significant similarities to known sequences at the DNA or inferred amino acid level.



C

cgaaacaaacacgtcttctgattgtacttggaggatatcatatagattaatggtctttgttatgttttaga
 aatttaaggatctattgggtttgtagtggccactgtgacattaattcataacagattcagaaagttgtatca
 attatccaagaagtctggttagcaggataaccttttgccttttaggaattaggatgtttactatagttaagt
 ataatgataataataataaaaaaacatcttttattagaaaaaaaaa

Figure 24. Expression of B16R120 after dicamba treatment. (A) RNA isolated from S and R *K. scopia* 0, 30, 60, 90, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the B16R120 cDNA.



C

agtaggccatgagtgttggtcggttggttaacaagagatcattatgcatgtaatgtaataattaaggtgtga
 ttaaattaaaatgtacttttaagaagctaagcattgttgatttgattggaaaagggtgtttaaacttaggg
 ttgtgtgttatatgcgagggttaaatttatgtataatgcaaagcaaatgtattttgtgtt
 gcggaaaaaaaaa

Figure 25. Expression of A13S60 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 30, 60, 90, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the A13S60 cDNA.

C04R60

Clone C04R60 recognized a 1.5-Kb message in R tissues but was not detectable in S (Figure 26). Levels of the mRNA approximately doubled in R by 120 minutes after treatment. This is an exceedingly rare message, requiring a one-week exposure to detect. Sequence analysis of the 231-bp fragment showed 37% identity (52% similarity) over 52 amino acid residues to a *Plasmodium* DnaJ-like gene (NCBI accession H71602). DnaJ genes have been identified in dozens of species including plants, bacteria, yeast, and humans (Luke et al., 1991, Ahu et al., 1993, Caplan and Douglas, 1994). The gene products interact with the chaperone Hsp70 family of proteins, with specific interactions among individuals to perform certain cell functions related to molecular chaperones. Several DnaJ proteins respond to heat shock (Blumberg and Silver, 1991, Cyr et al., 1994), and one was shown to be induced by the synthetic auxins 2,4-D and naphthalene acetic acid (Frugis et al., 1999).

Despite the relatively low sequence homology between this clone and other DnaJ-like proteins, C04R60 may indeed represent a member of the diverse DnaJ family of heat shock proteins. Most DnaJs isolated to date are cytosolic, but two mitochondrial and chloroplastic forms have been isolated in *Arabidopsis* (Kroczyńska et al., 1996). Kroczyńska et al. (1996) and Schlicher and Soll (1997) report that these plastidic forms have very low overall sequence homology to cytoplasmic versions.

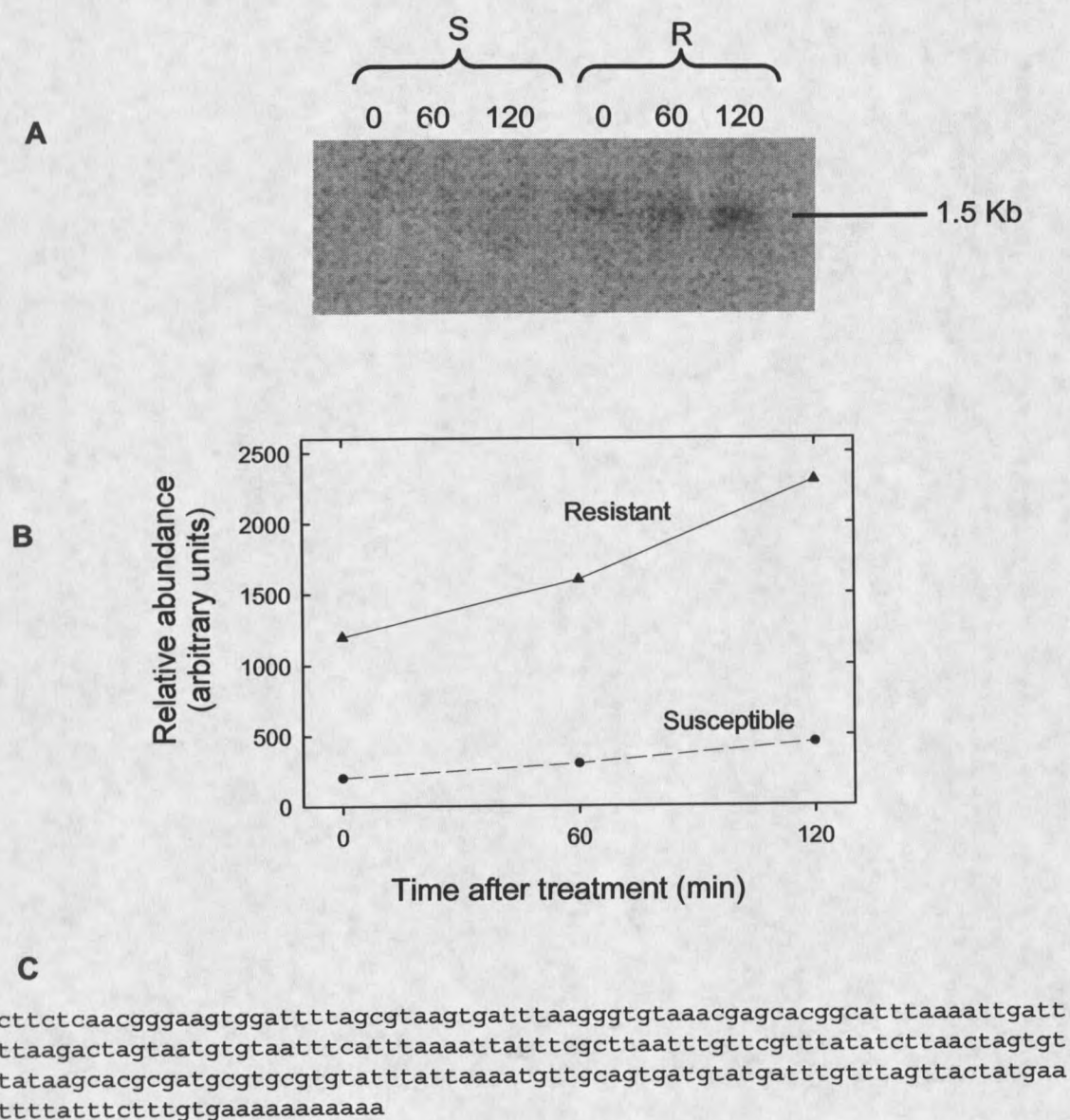


Figure 26. Expression of C04R60 after dicamba treatment. (A) RNA isolated from S and R *K. scoparia* 0, 60, or 120 minutes after treatment and probed with the radiolabeled cDNA. (B) Phosphorimager analysis of message abundance from blot (A). (C) Nucleotide sequence of the C04R60 cDNA.

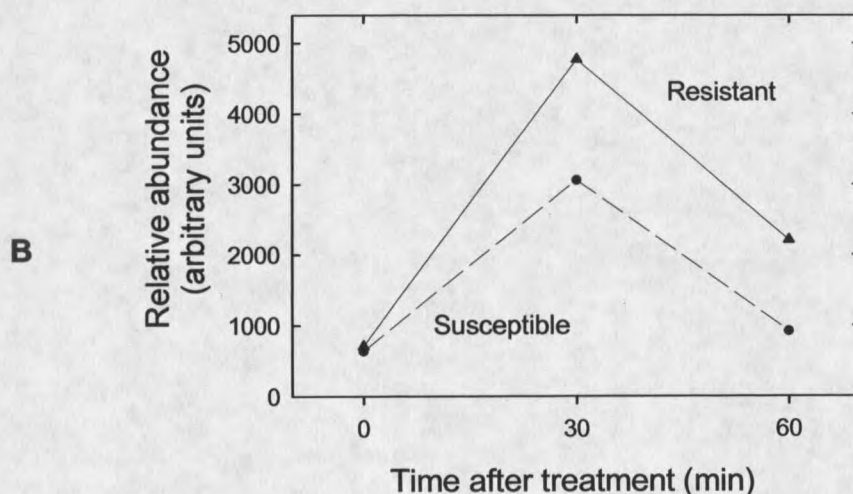
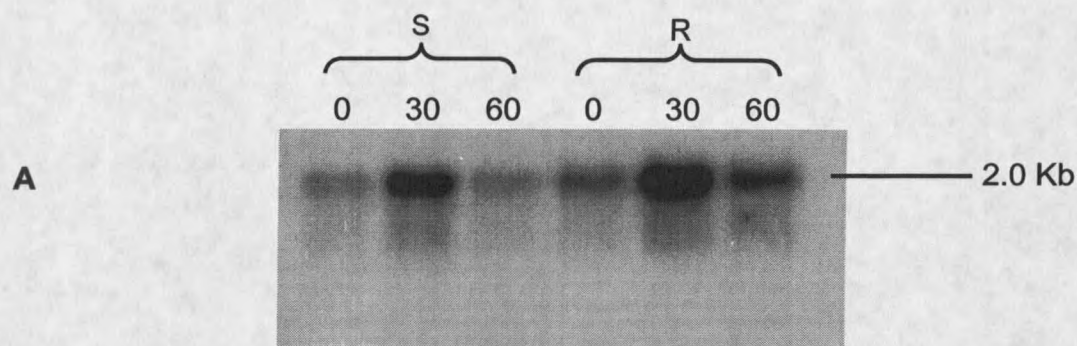
Cloning a fragment of the ACC synthase gene from *K. scoparia*

Translation of the 305-bp TACC1-6 clone revealed a 303-bp open reading frame potentially encoding a 101-amino acid polypeptide. DNA sequence analysis of the fragment showed strong similarity to ACC synthase genes from *Dianthus* (82% identical), *Citrus* (83% identical), *Solanum* (93% identical), *Carica* (83% identical), and other species. Amino acid sequence inferred from the DNA sequence also showed strong similarities to ACC synthase protein sequences from *Dianthus* (80% identical, 90% similar), *Petunia* (71% identical, 86% similar), *Pelargonium* (74% identical, 86% similar), and at least a dozen other species including *Arabidopsis*, *Cucumis*, *Vigna*, *Populus*, *Lycopersicon*, and *Passiflora* (Figure 27). The deduced amino acid sequence includes a highly conserved active site present in all ACC synthase genes isolated to date. Based on nucleotide and amino acid similarity, it is likely that TACC1-6 encodes a fragment of a *K. scoparia* ACC synthase gene. In particular, the active site has an inferred dodecapeptide sequence common to all ACC synthase genes isolated to date (SLSKD[M/L]G[L/F]PGFR), including the invariant lysine residue which binds pyridoxal 5' phosphate, a cofactor for this enzyme (Kende, 1989). ACC synthases occur in large gene families, and a few members are not induced by auxin treatment (Kim et al., 1992, Arteca and Arteca, 1999). To address whether the gene corresponding to TACC1-6 is induced by a synthetic auxin, the fragment was hybridized to a northern blot on RNA isolated from dicamba-treated S and R *K. scoparia*. As shown in Figure 28, the mRNA corresponding

	1		50
<i>Kochia</i>	TNPSNPLGTV	LDRETLSTIL	SFSTEKNIHL VCDEIYGATV FGYPNFVSIS
<i>Dianthus</i>	TNPSNPLGTV	LDKETLINIL	EFTNEKNIHL ICDEIYGATV FGEQKFISIS
<i>Lycopersicon</i>	TNPSNPLGTT	LDKDTLKSVL	SFTNQHNIHL VCDEIYAATV FDTPOFVSIA
<i>Petunia</i>	TNPSNPLGTT	LDRDTLKSLI	NFTNERNIHL VCDEIYAATV FNTPOFVSIA
<i>Citrus</i>	TNPSNPLGTT	LDRDTLKSLV	SFINDKNIHL VCDEIYAATV FSSPKFTSVA
<i>Passiflora</i>	TNPSNPLGTI	LDRDTLRSIV	HFVNEKNIHL VCDEIYAATV FRQPDFVSIS
	51		100
<i>Kochia</i>	EIMLDQDY..	.NPELIHIVY	<u>SLSKDLGFPG</u> FR VGIVYSYN DRVVSCARKM
<i>Dianthus</i>	EVIIDQPH..	.NPDLVHIVY	<u>SLSKDLGFPG</u> FR VGIVYSYN DNVVNCARKM
<i>Lycopersicon</i>	EILDEQEMTY	CNKDLVHIVY	<u>SLSKDMGLPG</u> FR VGIIYSFN DDVVNCARKM
<i>Petunia</i>	EILNDEKSNF	.NKDLVHIVY	<u>SLSKDMGLPG</u> FR IGIVYSYN DAVVNCARKM
<i>Citrus</i>	EVIQEMD...	CNRDLIHIIY	<u>SLSKDMGLPG</u> FR VGIVYSFN DTVVNCGRKM
<i>Passiflora</i>	EILQEDDIE.	CNLDLIHIVY	<u>SLSKDMGFPG</u> FR VGIIYSYN DAVVSCARKM
	101		
<i>Kochia</i>	SSFG		
<i>Dianthus</i>	SSFG		
<i>Lycopersicon</i>	SSFG		
<i>Petunia</i>	SSFG		
<i>Citrus</i>	SSFG		
<i>Passiflora</i>	SSFG		

Figure 27. PILEUP analysis of the deduced TACC1-6 amino acid sequence and ACC synthase sequences from other species. Highly conserved residues are in bold; the active site of the enzyme is underlined.

to TACC1-6 is induced about 5-fold in S and 7-fold in R by 30 minutes after treatment. However, expression levels decline quickly, reaching basal levels in S and near-basal levels in R by 60 minutes after treatment. Auxin induction of ACC synthase is rapid in many species, occurring within 2 hours after auxin treatment in isolated *Vigna* hypocotyls (Botella et al., 1992, Yoon et al., 1997) and *Pisum* shoots (Peck and Kende, 1995), and within 6 hours of 2,4-D treatment of *Pelargonium* leaves (Wang and Arteca, 1995). The studies reported here indicate that dicamba has similar effects, although it appears that



C

acaaaccggtcgaaccattagggacgggtcctagacagggagaccttgtcaactatactgtctttcagcact
 gagaagaacatccacttgggtgtgcatgagatatacggagccactgttttcgggtatcctaacttcggttagc
 atatccgagatcatgttgaccaagactataaccccgaactcattcacattgtgtacagcctttctaaagac
 ttgggattccccgggttttaggggtggggattgtgtactcgtacaacgatagagtctgtgagttgtgcacgtaa
 atgtcgagtttcgga

Figure 28. ACC synthase gene expression is induced by dicamba treatment. (A) Northern blot of RNA isolated from S and R *K. scoparia* 0, 30, and 60 minutes after dicamba treatment and probed with the radiolabeled TACC1-6 fragment. (B) Phosphorimager analysis of message abundance from the blot shown in (A). (C) Nucleotide sequence of the TACC1-6 cDNA.

the induction is more rapid than in the above systems. Additionally, the rapid attenuation seen in Figure 28 has not been reported for other species or auxin analogs, suggesting that ethylene production may be a transient effect of dicamba treatment in *K. scoparia*.

Although several studies have compared the kinetics of ethylene production after herbicide treatment of susceptible and auxinic herbicide-resistant plants (Hall et al., 1993; Peniuk et al., 1993), none have used molecular tools to note changes in expression of genes encoding ethylene biosynthetic enzymes. Ethylene production did not increase in biotypes of *Sinapis* resistant to 2,4-D, dicamba, and mecoprop in response to 2,4-D application, whereas ethylene production increased about 10-fold in S plants (Peniuk et al., 1993). However, this increase was not significant until 24 hours after treatment. In contrast, Coupland and Jackson (1991), using the same *Sinapis* biotypes, reported that ethylene production in R plants was greater within 6 hours of treatment with mecoprop, but S plants produced significantly more ethylene after 24 hours. Although these results are consistent with the greater induction of ACC synthase mRNA seen in R *K. scoparia* than in S (Figure 28), different auxinic herbicides were used in the two studies, and the results may not be comparable. The *K. scoparia* results indicate that ACC synthase is similarly induced in R and S biotypes, and thus it appears unlikely that reduced or altered ethylene production plays a role in dicamba resistance. Further, the induction of ACC synthase in both biotypes indicates that dicamba is perceived similarly to auxin in *K. scoparia*.

CHAPTER 3

EXPRESSION OF *K. SCOPARIA* CHOLINE MONOOXYGENASE IN
RESPONSE TO SALT STRESS AND DICAMBA TREATMENTIntroduction

In Chapter 2, clone D416S0 was shown to be rapidly down-regulated in response to dicamba treatment in R and S *K. scoparia*, and sequence analysis revealed strong similarity to genes encoding choline monooxygenase (CMO) from *Spinacia oleracea*, *Beta vulgaris*, *Amaranthus tricolor*, and *Atriplex hortensis*. Choline monooxygenase oxidizes choline to betaine aldehyde, which is subsequently oxidized by betaine aldehyde dehydrogenase (BADH) to produce glycine betaine (GB). GB 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). CMO is unique to plants (Rathinasabapathi et al., 1997) and appears to be a soluble ferredoxin- and oxygen-dependent protein localized primarily in the chloroplast stroma. In other organisms, flavoprotein dehydrogenases or oxidases (Sakamoto and Murata, 2001) catalyze the choline oxidation reaction. CMO is an unusual oxygenase, being insensitive to carbon monoxide and having characteristics of an iron-sulfur Rieske-type protein that is not related to cytochrome P450-type monooxygenases (Burnet et al., 1995).

It is established that salinization or drought treatments increase the levels

of CMO mRNA (Russell et al., 1998), protein (Rathinasabapathi et al., 1997), and the endproduct GB (Nakamura et al., 1996). Despite the fact that plant responses to abiotic stresses are often mediated by plant hormones like ABA (Franks and Farquhar, 2001), the effects of phytohormones on GB production are poorly understood. Studies to date have only investigated the effects of phytohormones on the expression of BADH. Legaria et al. (1998) showed that BADH mRNA and protein were induced by both osmotic stress and abscisic acid in *Amaranthus hypochondriacus*. Similarly, Ishitani et al. (1995) found that BADH mRNA was induced in *Hordeum vulgare* leaves by salt stress and treatment with ABA. The effects of phytohormone treatment on CMO expression have not been examined. Therefore, the goals of these studies were to: 1) characterize CMO expression in response to osmotic stress; 2) more fully characterize the dicamba-mediated attenuation of CMO expression in the absence of osmotic stress; 3) determine whether dicamba treatment can override osmotic stress-induced CMO expression; and 4) compare the responses of R and S *K. scoparia* to the above treatments.

Methods and Materials

3' RACE

Sequence analysis of clone D416S0 indicated that it had sequence similarity to internal sections of CMO cDNAs from other species and was not amplified from the 3' end of the message, as would be expected from clones generated using differential display. In general, the regions of similarity corresponded to bases 500 to 745 of 1.9-Kb full-length cDNAs from *Spinacia oleracea*, *Beta vulgaris*, *Amaranthus tricolor*, and *Atriplex hortensis*. In order to verify the identity of D416S0, 3' RACE (Rapid Amplification of cDNA Ends) was performed with the assistance of Mr. Harwood Cranston to isolate a longer cDNA for use in further studies.

Poly(A)⁺ RNA was purified from untreated S254 *K. scoparia* total RNA isolated as described above using the Straight A's® mRNA isolation system (Novagen, Inc.). Fifty µg total RNA was mixed with 10 mg oligo(dT)-coated Magnetite® magnetic beads in a 1.5-ml centrifuge tube using manufacturer-supplied extraction buffer (pH 7.0) at room temperature. Magnetic beads were captured using a magnet, the supernatant removed, and beads were washed 3 times using the manufacturer's wash buffer. Poly(A)⁺ RNA was recovered by incubating the beads in nuclease-free water at 60°C for 10 min, recapturing magnetic particles, and precipitating the supernatant with isopropanol. mRNA was pelleted by centrifugation at 14,000g for 10 min at 4°C and the pellet washed with 0.5 ml 70% ethanol. The final pellet was redissolved in 100 µl

nuclease-free water and subjected to another round of magnetic purification as above. Typical yields were 1 to 2 μg poly(A)⁺ RNA per mg of total RNA. Purity of poly(A)⁺ RNA generated using this method was verified by agarose gel electrophoresis, ethidium bromide staining, and northern blotting using radiolabeled D416S0 as a probe.

3' RACE was performed using the 3' RACE System for Rapid Amplification of cDNA Ends® cDNA amplification kit (Gibco Life Technologies). Poly(A)⁺ RNA (50 ng) was incubated at 70°C in the presence of 0.5 μM anchored primer 5'-GGCCACGCGTCGACTAGTACT₍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 MgCl₂, 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 MgCl₂, 200 nM HJC3 primer (5'- AGTGCATGCATTTTCAACG-3'; designed from the D416S0 sequence), 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, ligated into the pCRII/TOPO cloning vector, and sequenced as described previously.

NaCl treatment

Six-week-old R99 and S254 plants grown under the greenhouse conditions described previously were subjected to osmotic 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) from at least six plants was bulked and harvested into liquid nitrogen 0, 2, 4, 6, and 8 days after NaCl treatments began.

Dicamba treatment of NaCl-treated plants

Eight days after NaCl treatments began, R99 and S254 plants were sprayed with 70 g ai ha⁻¹ formulated dicamba as previously described and harvested after 0, 0.5, 1, 1.5, 2, 6, 12, 24, and 48 hours by bulking shoot tissue (10 g) from at least six plants as above.

Northern blot analysis

Analysis of CMO mRNA levels was performed as previously described, except that 80 ng D416S0 was used as a probe for all northern blots.

Protein extraction

Frozen shoot tissue (1 g fresh weight) 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 extraction buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 mM sodium borate, 50 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM DTT, 15 μ l antifoam A, and 0.2 g polyvinylpolypyrrolidone (PVPP) by homogenization with a Polytron® tissue homogenizer (Brinkmann Co.). Samples were filtered through 4 layers of cheesecloth and one layer of Miracloth® (Calbiochem-Novabiochem Corp.) and centrifuged at 10,000g and 4°C for 10 min. The supernatant was transferred to a new centrifuge tube and precipitated by adding 8 volumes of -20 °C acetone. Protein was recovered by centrifugation at 10,000g and 4°C for 15 min, and the pellet was redissolved in 0.5 ml sample 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 using the Bradford method (Bradford, 1976) using BSA as a standard. Prior to storage at -20 °C, samples were diluted 1:1 with 2X 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 μ g/ml bromophenol blue), boiled for 5 min, centrifuged at 14,000g for 5 min at room temperature, and the supernatant

transferred to a new 1.5 ml centrifuge tube.

Western blot analysis

Unless otherwise noted, 15 µg total protein from each sample was separated by SDS-PAGE on 12% acrylamide gels following the procedures of Davis (1964). Samples were electrophoresed at 50 volts constant voltage for 16 hours and separation verified by Coomassie blue staining. For western analysis, proteins electrophoresed as above were 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.

Detection of CMO protein was performed by adding 4 µl mouse polyclonal antibody raised against the *Spinacia* 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.). Intensity of CMO protein bands was quantified by scanning densitometry of blots using 1D Molecular Imaging® software (v. 3.0.1, Kodak Digital Sciences).

Glycine betaine extraction

GB was extracted from *K. scoparia* plants using a method modified from Fan et al. (1993). Frozen shoot tissue (1 g fresh weight) was pulverized to a powder under liquid nitrogen using a prechilled mortar and pestle, transferred to a 15-ml centrifuge tube, and extracted with 3 ml 5% (v/v) perchloric acid in water at 0°C for 60 min. Twenty μl of Antifoam A (Sigma Chemical Co.) was added and the extracts adjusted to pH 7.0 by adding 165 μl of 5 M K_2CO_3 . The extracts were centrifuged at 10,000g and 4°C for 20 min and 1 ml of the supernatant was lyophilized and redissolved in 0.5 ml deuterium dioxide. An internal NMR standard of alanine (10 mg) dissolved in deuterium dioxide was added to each sample just prior to NMR analysis.

^1H NMR

Nuclear Magnetic Resonance used to determine GB levels in *K. scoparia* was performed by Dr. Scott Busse, Interim NMR Director in the Department of Chemistry and Biochemistry at Montana State University. Because of the free rotation of the methyl group in GB, the resulting peaks were very sharp due to the relatively long relaxation time and the peak areas were

large due to the presence of six equivalent protons that gave rise to the signal. All NMR spectra were collected on a Bruker DRX500 NMR spectrometer operating at a proton frequency of 500.13 MHz. The time domain data was collected with a spectral width of 6000 Hz and 32k complex points. A total of 16 scans were averaged for each spectrum. The proton pulse length was 8 μ sec and a 7.7 sec recycle was used. The spectra were zero filled and Fourier transformed to give a final frequency domain size of 16k 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 D416S0 partial-length cDNA yielded a PCR product 1.2 Kb in size, and was designated as WCMO. DNA sequencing revealed a 870-bp open reading frame potentially coding 290 amino acids and a 311-bp 3' untranslated region (Figure 29). PILEUP sequence analysis indicated that the deduced amino acid sequence had strong similarity (>80% identical, >90% similar) to choline monooxygenase sequences from *Spinacia*, *Atriplex*, *Beta*, and *Amaranthus* (Figure 30), and weaker identity (55%) to a hypothetical protein from *Arabidopsis*. The deduced amino acid sequence contains several motifs present in CMOs from other plants and related prokaryotic genes encoding proteins with oxygenase functions. First, the cysteine/histidine consensus sequence for a Rieske-type [2Fe-2S] cluster (Cys-X-His-X₁₅₋₁₇-Cys-X₂-His) is present in the *K. scoparia* CMO reported here, as it is in all CMOs isolated to date (Figure 30). Secondly, CMO cDNAs from *Beta* and *Spinacia* (Russell et al., 1998), *Amaranthus*, *Kochia*, and *Atriplex* (Figure 30), as well as numerous bacterial monooxygenases contain a proposed consensus sequence for coordination sites of mononuclear, nonheme iron (Glu/Asp-X₃₋₄-Asp-X₂-His-X₄₋₅-His; Jiang et al., 1996). The same motif is present in plant CMOs except that the central asparagine and histidine residues are three residues apart instead of two. Further evidence that WCMO encodes a stromal enzyme could be gained by

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2  tgccgagctggcggaagggaagtgcatgcatttcacaacgtttgtactcatcgcttctcg
   C R A G E G K V H A F H N V C T H R A S
62  attctcgcttgtggaagtggcaaaaagtcctgctttgtgtgcccttaccatggatggggtt
   I L A C G S G K K S C F V C P Y H G W V
122 tttggcatgaatggagatctcacaaaagcaaccaagctgaaactcaaactttttgatgct
   F G M N G D L T K A T Q A E T Q T F D A
182 aaggaacttgggctagtggccctaaagggttgacgtatggggaccattcggttctgatcagc
   K E L G L V A L K V A V W G P F V L I S
242 ttggacaaaactctccctgaaactgatgttggcactgaatggccttggcaaatctgctgaa
   L D K T L P E T D V G T E W L G K S A E
302 gatgtcaaggcccatgcctttgatccctccctccaattcatccataggagtgaattcccc
   D V K A H A F D P S L Q F I H R S E F P
362 atggagtgcatttgaagggttttctgtgacaactatctggatagctcataccatgtccct
   M E C N W K V F C D N Y L D S S Y H V P
422 tatgcacacaaataactacgcaactgaactcgactttgacacatacgacactcaaattgatt
   Y A H K Y Y A T E L D F D T Y D T Q M I
482 gagaatgttgtgattcaaagggttggaggtaacaaaaacaagactgatgggatcgataga
   E N V V I Q R V G G N K N K T D G I D R
542 cttggaaatcaagcattctatgcctttgcttatcccaactttgctattgaaagggtatggc
   L G N Q A F Y A F A Y P N F A I E R Y G
602 ccttggatgaccacaatgcattgttcaaccattgggactaaggaaatgcaagcttgttgtt
   P W M T T M H V Q P L G L R K C K L V V
662 gactattatattgaagactctaagttggaggacaaggactacatagagaaagggtatcgca
   D Y Y I E D S K L E D K D Y I E K G I A
722 atcaacgataacgtacagagtgaagataagggtgttatgtgaaagtgtccaaaggggggtta
   I N D N V Q S E D K V L C E S V Q R G L
782 gagactccatcatacagcacagggagatatgtgatgccaattgagaaaggaatccaccac
   E T P S Y S T G R Y V M P I E K G I H H
842 ttccactgctggttgaccaaagtgttgacgtgattttcatagatctcttccatgggatc
   F H C W L H Q V L Q *
902 aattacgacgcataattaaaatgtggttaagcataatgtgtgtctgctttgcttaattgt
962 gtacttgagtgtgaagggtgctaataatgactattataataaaacaccatggcacccttc
1022 ccctaaatagttttcaaataattttgggggggttaagcgaagaatagatcgcttatgaatgt
1082 tttatctagtcatgtatggttttcattagtttaagtacatgaggaaaacattattgtgtgt
1142 tgaatacagcatattaataacaacatattatcagttgtagtataaaaaaaaaaaaaaagtac
1202 tagtcgacgcgtggccaa 1219

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Figure 29. WCMO 3' RACE product. DNA (lower case) and deduced amino acid sequence (upper case) of the WCMO partial-length cDNA. The stop codon is indicated by an asterisk and the putative polyadenylation signal is underlined.

	1				50
<i>Kochia</i>	CRAGEGKVHA	FHNVCTHRAS	ILACGSGKKS	CFVCPYHG WV	FGMNGDLTKA
<i>Spinacia</i>	SRDGEQKVHA	FHNVCTHRAS	ILACGSGKKS	CFVCPYHG WV	YGMGDSLAKA
<i>Atriplex</i>	CRDGEQKVHA	FHNVCTHRAS	ILACGSGKKS	CFVCPYHG WV	YGMNGSLTKA
<i>Beta</i>	SRDGQGEQVHA	FHNVCTHRAS	ILACGSGKKS	CFVCPYHG WV	YGLDGSILAKA
<i>Amaranthus</i>	CRDGQGEQVHA	FHNVCTHRAS	ILACGTGKKS	CFVCPYHG WV	FGLDGSILMKA
		^ ^		^ ^	
	51				100
<i>Kochia</i>	TQA.ETQTFD	AKELGLVALK	VAWGPFFVLI	SLDKTLPE.T	.DVGTEWLK
<i>Spinacia</i>	SKAKPEQNL	PKELGLVPLK	VAWGPFFVLI	SLDRSLEE.G	GDVGTEWLGT
<i>Atriplex</i>	SKATPEQSLN	PDELGLVPLK	VAWGPFFILI	SLDRSSRE.V	GDVGSEWLGS
<i>Beta</i>	SKATETQNL	PKELGLAPLK	VAEWGPFFILI	SLDRSLDA.N	ADVGTIEWIGK
<i>Amaranthus</i>	TK.TENQVFD	PKELGLVTLK	VAIWGPFFVLI	SLDRSGSEGT	EDVGKEWIGS
	101				150
<i>Kochia</i>	SAEDVKAHAF	DPSLQFIHRS	EFPMESNWKV	FCDNYLDSSY	HVPYAHKYIA
<i>Spinacia</i>	SAEDVKAHAF	DPSLQFIHRS	EFPMESNWKI	FSDNYLDSSY	HVPYAHKYIA
<i>Atriplex</i>	CAEDVKAHAF	DPNLQFINRS	EFPIESNWKI	FSDNYLDSSY	HVPYAHKYIA
<i>Beta</i>	SAEDVKAHAF	DPNLKFTHRS	EFPMESNWKV	FCDNYLDSSY	HVPYAHKYIA
<i>Amaranthus</i>	CAEEVKKHAF	DPSLQFINRS	EFPMESNWKV	FCDNYLDSAY	HVPYAHKYIA
				♦ ♦ ♦ ♦	
	151				200
<i>Kochia</i>	TELDFTYDT	QMIENVVIQR	VGGNKNKTDG	IDRLGNQAFY	AFAYPNFAIE
<i>Spinacia</i>	TELNFTYDT	QMIENVTIQR	VEGSSNKP	FDVVGIQAFY	AFAYPNFAVE
<i>Atriplex</i>	TELDFTYQT	DMVGNVTIQR	VAGTSN..NG	FNRLGTQAFY	AFAYPNFAVE
<i>Beta</i>	AELDFDYNT	EMIEKCVIQR	VGSSSNKP	FDRLGTEAFY	AFIYPNFAVE
<i>Amaranthus</i>	AELDFDYKT	DLLEKVVQR	VASSSNKPNG	FDRLGSEAFY	AFIYPNFAVE
	201				250
<i>Kochia</i>	RYGPWMTTMH	VQPLGLRKCK	LVVDYYIEDS	KLEDKDYIEK	GIAINDNVQS
<i>Spinacia</i>	RYGPWMTTMH	IHPLGPRKCK	LVVDYYIENS	MLDDKDYIEK	GIAINDNVQR
<i>Atriplex</i>	RYGPWMTTMH	IVPLGPRKCK	LVVDYYIEKS	KLDDKDYIEK	GIAINDNVQK
<i>Beta</i>	RYGTWMTTMH	VVPMGQRKCK	LVVDYYLEKA	MLDDKAYIDK	GIAINDNVQK
<i>Amaranthus</i>	RYGPWMTTMH	IGPLGPRKCK	LVVDYYLENA	MMNDKPYIEK	SIMINDNVQK
	251				293
<i>Kochia</i>	EDKVLCEVQ	RGLETPSYST	GRYVMPIEK	IHHFHCWLHQ	VLQ*
<i>Spinacia</i>	EDVVLCEVQ	RGLETPAYRS	GRYVMPIEK	IHHFHCWLQQ	TLK*
<i>Atriplex</i>	EDVVLCEVQ	KGLETPAYRS	GRYVMPIEK	IHHFHCWLHQ	VLK*
<i>Beta</i>	EDKVLCEVQ	RGLETPAYRS	GRYVMPIEK	IHHFHCWLHE	TLQ*
<i>Amaranthus</i>	EDVVLCEVQ	RGLETPAYRS	GRYVMPIEK	IHHFHCWLHQ	TLN*

Figure 30. PILEUP comparisons of WCMO and similar amino acid sequences as recovered by BLAST. Amino acid sequence identities among the cDNAs are in bold and the stop codons are indicated by asterisks. Conserved cysteine/histidine pairs involved in the putative Rieske-type cluster are indicated by a ^ . Conserved asparagine/histidine residues involved in mononuclear iron binding are underscored by a ♦ .

isolating the remainder of the cDNA and checking for chloroplast stroma targeting sequences that are poorly conserved but rich in polar amino acid residues (Cline and Henry, 1996). Experiments are underway to isolate the 5' end of the *K. scoparia* CMO cDNA.

CMO expression in response to NaCl stress

GB production in response to osmotic stress is widespread among members of the Chenopodiaceae (Russell et al., 1998), although the molecular details of induction are unknown. To date, induction of CMO mRNA or protein in response to osmotic stress has been documented in *Spinacia* (Rasinasabapathi et al., 1997), *Beta* (Russell et al., 1998), and *Amaranthus* (Wang et al., 1999). In *K. scoparia*, osmotic stress similarly induced expression of CMO mRNA, protein, and GB (Figures 31 and 32). Basal CMO mRNA levels were moderately abundant as they could be detected with overnight film exposures of northern blots (Figure 31A). This finding is consistent with reports that some members of the Chenopodiaceae accumulate moderate levels of GB regardless of stress conditions (Weretilnyk and Hanson, 1990). The radiolabeled D416S0 probe recognized a 1.9-Kb message that was relatively abundant in unsalinized plants, and increased greatly (>20-fold in S) after plants had been treated with 500 mM NaCl for 2 days (Figure 31B). In R plants, the CMO mRNA was induced more slowly and mRNA levels increased by a maximum of about 12-fold.

Mouse polyclonal antibodies raised against *Spinacia* CMO (Russell et al.,

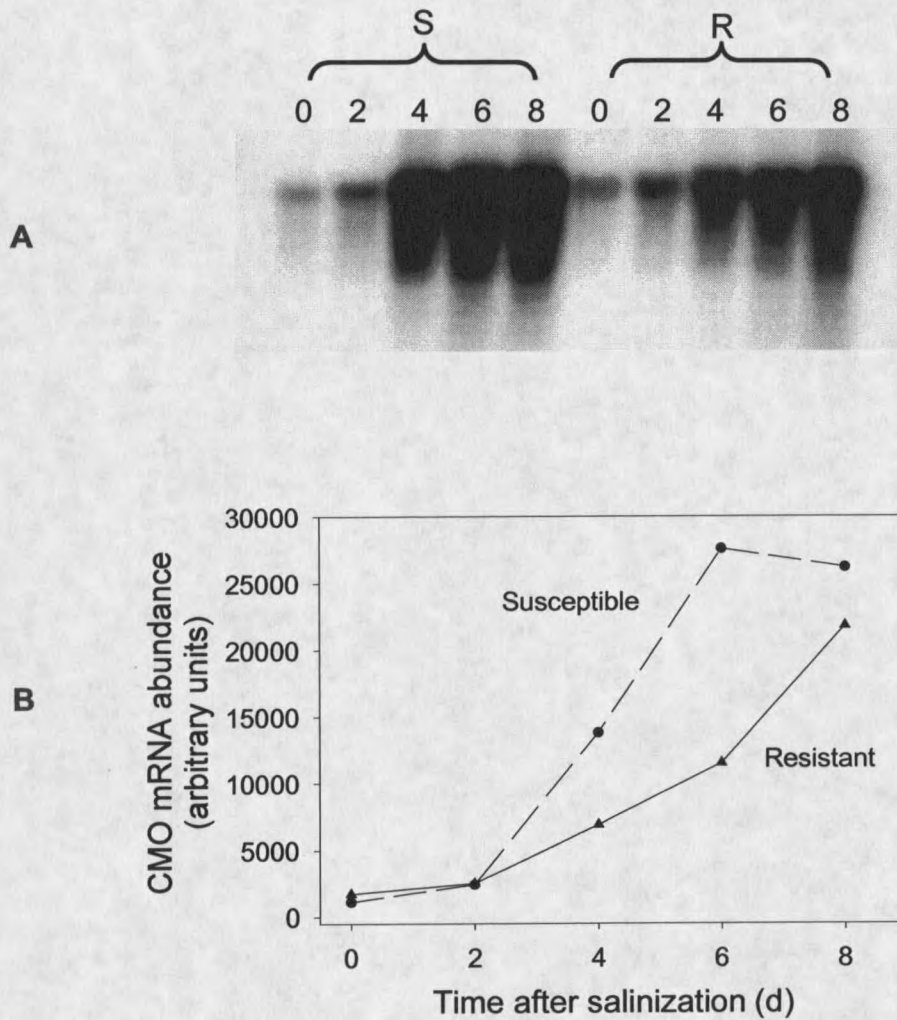


Figure 31. Expression of CMO mRNA in response to salt stress. (A) RNA isolated 0, 2, 4, 6, and 8 days after salinization treatments began and probed with the radiolabeled D416S0 cDNA. (B) Phosphorimager analysis of message abundance from blot (A).

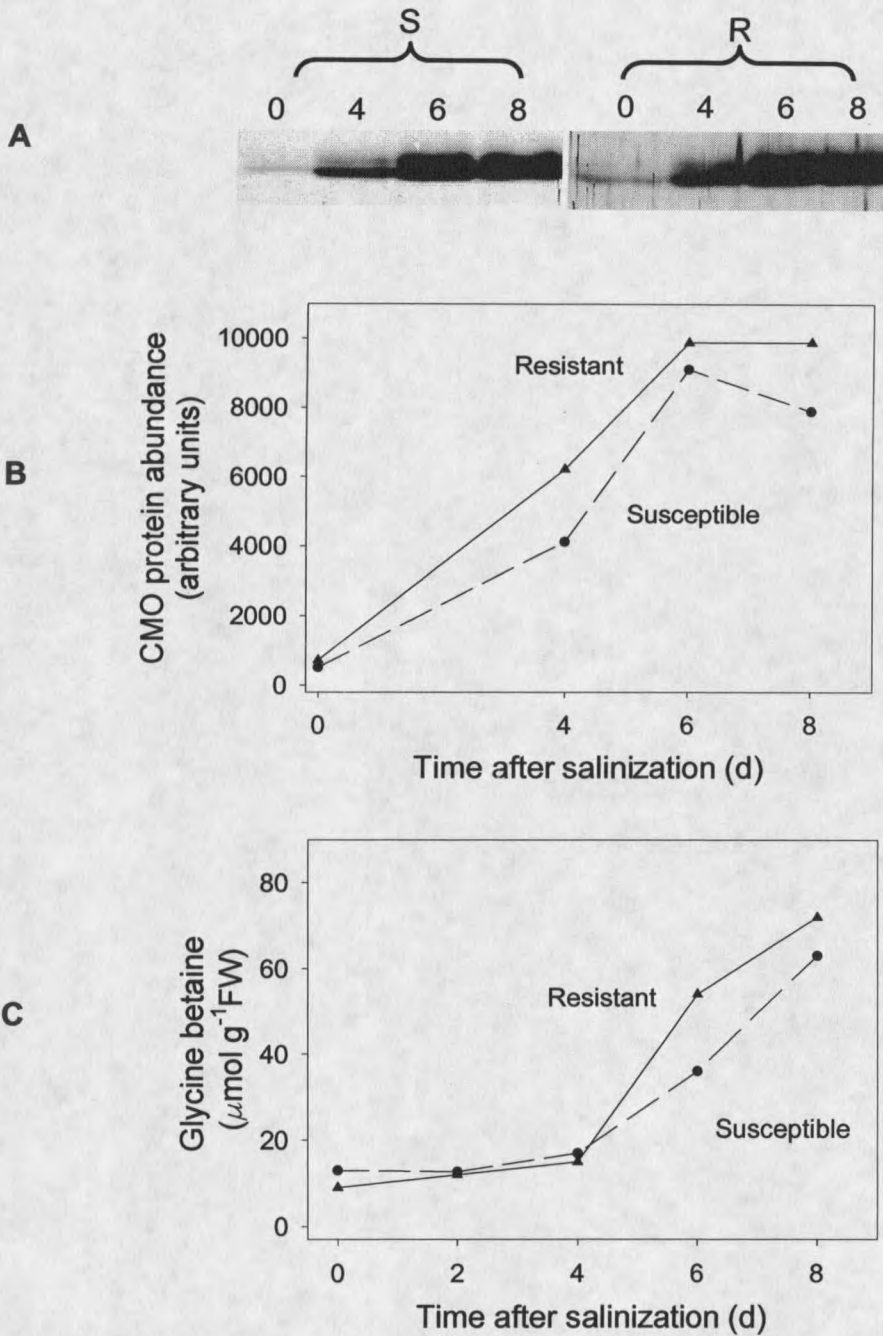


Figure 32. Accumulation of CMO protein and glycine betaine in response to salt stress. (A) CMO protein as detected with mouse anti-CMO antibodies 0, 4, 6, and 8 days after salinization treatments began. (B) Densitometry analysis of protein bands in (A). (C) Corresponding GB levels in shoot tissues used in (A).

1998) recognized two protein bands of about 42 and 43 Kd in protein extracts from *K. scoparia* (Figure 32A). CMO enzyme levels followed the pattern of mRNA induction, and the enzyme accumulated more slowly as expected. By 4 days after treatment, S and R plants had about 8- and 9-fold more enzyme than untreated plants, respectively. After 8 days, CMO levels in R and S plants were about 15 times their original levels. CMO protein levels increased similarly in salt-stressed *Amaranthus tricolor* (Wang et al., 1999), *Beta vulgaris*, and *Amaranthus caudatus* (Russell et al., 1998) plants by 4 days after treatment with 300 mM NaCl. However, it is evident that *K. scoparia* has the ability to continue accumulating CMO protein under conditions of more severe osmotic stress (750 mM NaCl).

Other studies using SDS-PAGE and western blotting have detected a single CMO protein of about 45 Kd in *Beta vulgaris* and *Amaranthus caudatus* (Russell et al., 1998), as well as in *Amaranthus tricolor* (Wang et al., 1999). While it is possible that the presence of two CMO bands in *K. scoparia* is a degradation artifact, it is also reasonable to speculate that the two bands represent products of two different genes that may form a dimeric holoenzyme *in vivo*. Consistent with this idea, Southern blotting indicated that there are two to four CMO genes in the *K. scoparia* genome (data not shown).

Glycine betaine accumulated in both biotypes in response to salt treatment (Figure 32C). Prior to salinization, GB was present at about 10 $\mu\text{mol g}^{-1}$ fresh weight, a finding consistent with the idea that GB is a constitutive

chemical component of the Chenopodiaceae (Grieve and Maas, 1984). After 8 days of NaCl treatment, GB levels increased about 6-fold to more than 60 $\mu\text{mol g}^{-1}$ fresh weight, and were apparently still increasing. Treatment of *Amaranthus tricolor* with 300 mM NaCl caused GB values to rise to about 30 $\mu\text{mol g}^{-1}$ fresh weight (Wang et al., 1999), the highest value reported so far in the literature.

The increases in CMO mRNA, protein, and GB content in response to salt stress are consistent with the natural drought tolerance of *K. scoparia*, which grows very well in xeric environments. In Montana, *K. scoparia* is often the only plant found growing in saline seeps, and the plant is especially well-adapted to the dry climate of the western U.S. where it infests millions of hectares of cropland and rangeland (Eberlein and Fore, 1984). Even given the extreme NaCl stress in these experiments, neither *K. scoparia* biotype displayed any outward symptoms of drought stress (such as wilting), although growth essentially stopped. One cause of this stunting may be due to the metabolic costs of GB production. At the end of these experiments, GB had accumulated to 2 to 3% of the total plant dry weight (data not shown), and thus likely represents a significant carbon and nitrogen sink.

CMO expression in response to dicamba treatment in R and S *K. scoparia*

Initial differential display results indicated that the message for clone D416S0 was attenuated rapidly after dicamba treatment (page 59). To more thoroughly investigate the kinetics of this apparent down-regulation, CMO mRNA, protein, and GB levels were determined at several times after dicamba treatment of R and S *K. scoparia*. These experiments were designed to determine the effect of dicamba treatment on CMO expression in plants that had not been previously subjected to NaCl stress.

As noted before, steady-state CMO mRNA levels were moderately high in untreated plants (Figure 33). Treatment of R and S plants with 70 g ai ha⁻¹ of dicamba resulted in a rapid attenuation of the CMO message, with the apparent down-regulation occurring somewhat more rapidly in R than in S. By 90 min after treatment, both biotypes exhibited a >10-fold reduction in the CMO mRNA, which remained low in S plants through the course of this experiment, but recovered slightly in R by 6 and 24 hr after treatment. The difference in CMO mRNA levels at 24 hr after treatment may be associated with the different physiological responses to dicamba treatment in R and S plants. By this time, S plants began to show injury symptoms associated with dicamba treatment such as severe 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.

Western analysis indicated that CMO enzyme levels closely followed the kinetics of mRNA abundance (Figure 34). Basal protein levels were low, and became nearly undetectable by 6 hr after treatment, even when 25 µg total

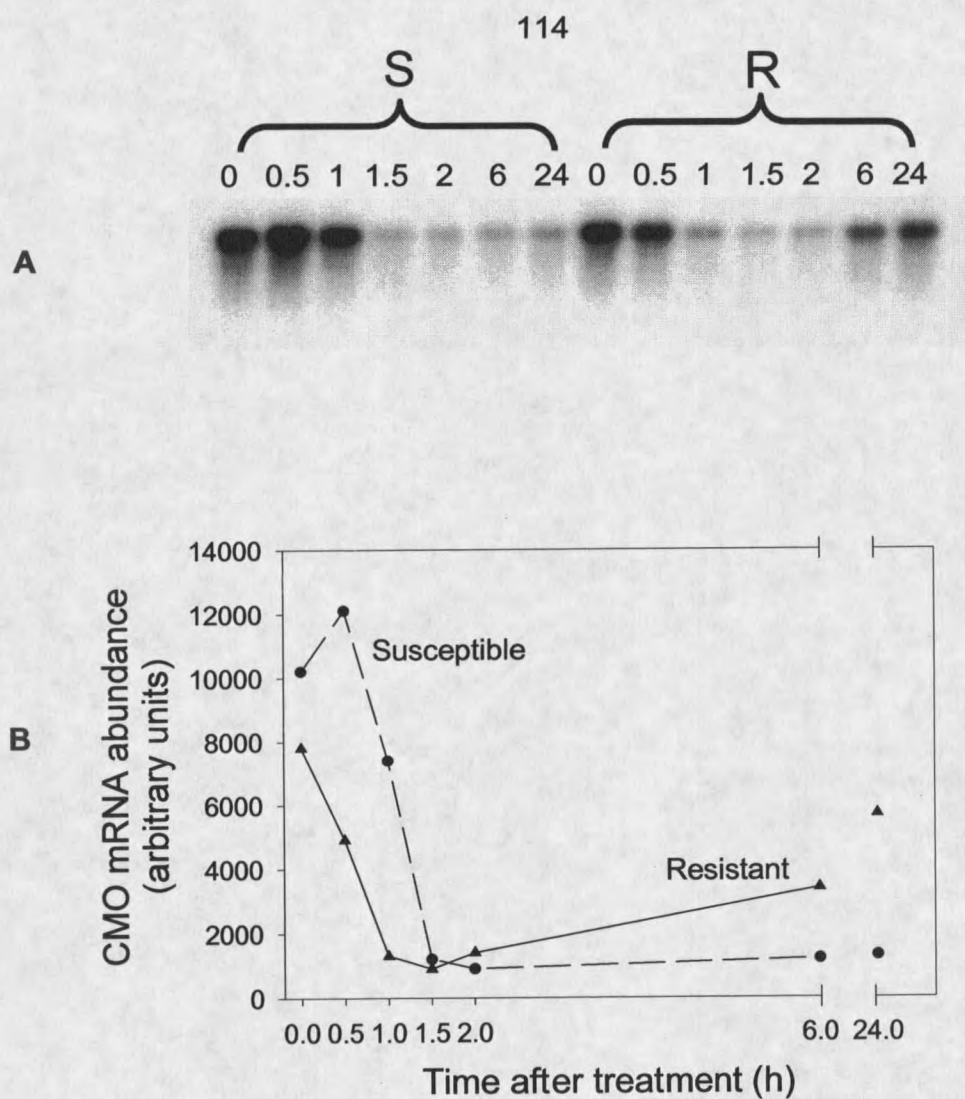


Figure 33. Expression of CMO mRNA after dicamba treatment of unstressed plants. (A) RNA isolated 0, 0.5, 1, 1.5, 2, 6, and 24 hours after dicamba treatment and probed with the radiolabeled D416S0 cDNA. (B) Phosphorimager analysis of message abundance from blot (A).

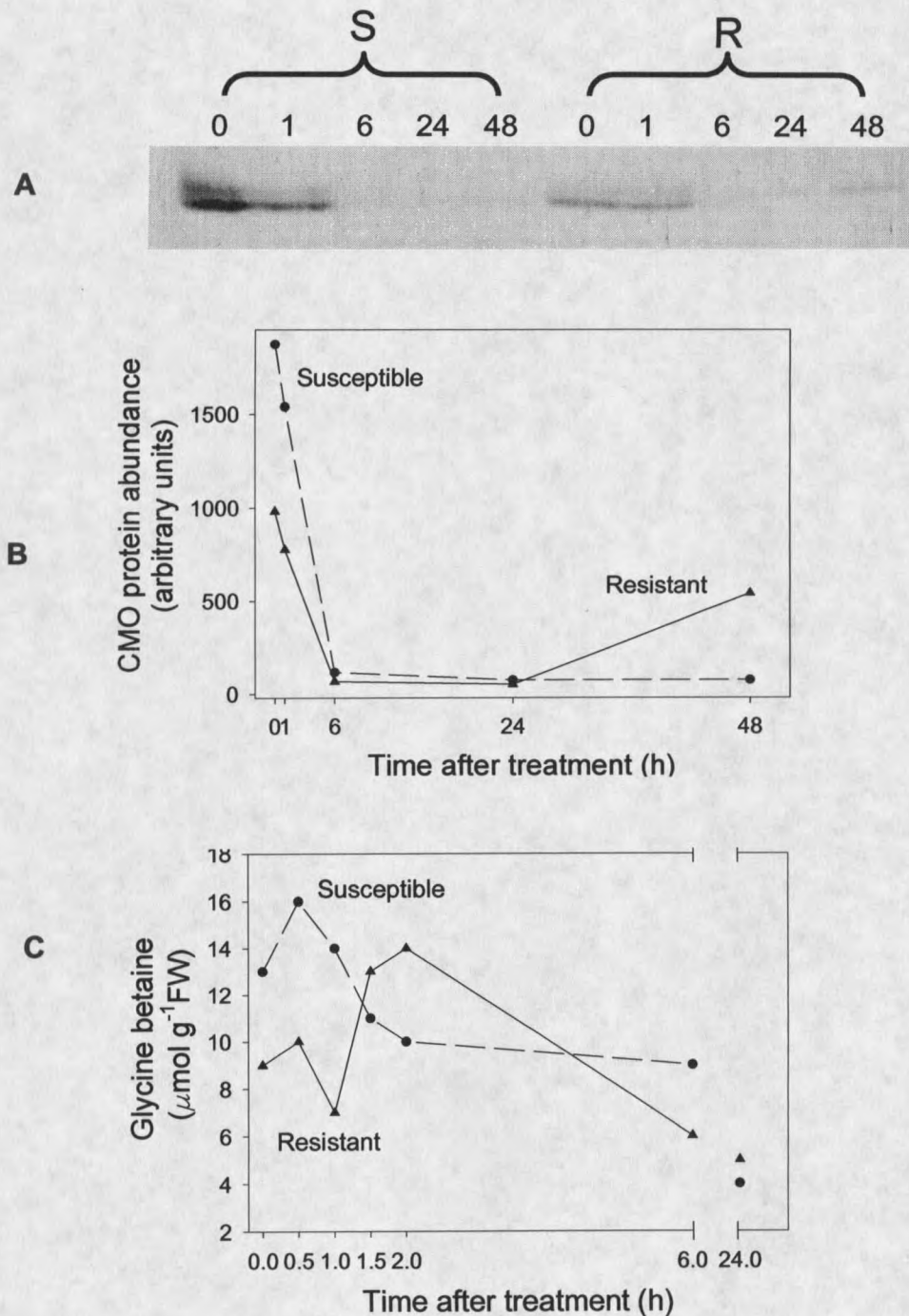


Figure 34. Reduction of CMO protein and glycine betaine levels in unstressed plants after dicamba treatment. (A) Western blots of CMO protein isolated 0, 1, 6, 24, and 48 hours after dicamba treatment. (B) Densitometry analysis of protein bands in (A). (C) GB levels in tissue used in (A).

protein was used for SDS-PAGE as in Figure 34. Enzyme levels remained low in S plants up to 48 hours after treatment, whereas levels increased slightly in R plants by this timepoint. It is evident that levels of CMO protein decline rapidly in response to dicamba treatment in both R and S *K. scoparia*, although these studies do not address the molecular mechanism of attenuation. A similar decline in the CMO protein levels was reported in *Beta* after removal of drought stress (Russell et al., 1998). However, it is unknown whether the mechanism of CMO mRNA and protein decline seen in response to dicamba treatment is similar to that caused by release from drought stress.

If the value for R plants at 1 hr after treatment is disregarded, GB levels in unstressed tissues increased transiently following dicamba treatment, and then steadily declined to about one-half their initial levels after 24 hr. GB is metabolized very slowly, if at all, in members of the Gramineae that produce low levels ($< 5 \mu\text{mol g}^{-1}$ fresh weight) in response to drought or salt stress (Grieve and Maas, 1984; Nakamura et al., 1996). However, some GB catabolism was noted in *Beta vulgaris* (Russell et al., 1998) and *Sorghum bicolor* (Wood et al., 1996), but tissue GB levels remained higher than in unstressed plants, even several days after release from osmotic stress. These findings suggest that GB may also be catabolized slowly in *K. scoparia*.

CMO expression in response to dicamba treatment in salt-stressed *K. scoparia*

Since the auxinic herbicides dicamba and 2,4-D (data not shown) cause a rapid decline in CMO mRNA and protein levels and GB content in unstressed plants, experiments were conducted to determine whether these herbicides had similar effects on plants acclimated to severe osmotic stress. Herbicide efficacy commonly declines when target plants are stressed by low temperatures or water deficit, probably because of reduced herbicide uptake and translocation (Ashton and Crafts, 1981). When acclimated to 750 mM NaCl, *K. scoparia* plants showed no external symptoms of osmotic stress but essentially ceased growth. These experiments were conducted to determine if the attenuation caused by dicamba treatment would also be observed in plants under severe NaCl stress. For the results reported here, plants were treated with 750 mM NaCl for 4 days prior to dicamba treatment.

CMO mRNA levels declined rapidly in salinized R *K. scoparia* plants by 1 hr after dicamba treatment, dropping about 7-fold (Figure 35). mRNA levels remained low until 2 hr after treatment, but then rapidly increased. By 24 hr after treatment, CMO mRNA levels had risen to levels higher than in unsprayed, NaCl-treated plants, possibly as a result of increased osmotic stress due to a concomitant decline in GB levels (Figure 36C). The transient nature of this attenuation is markedly similar to the decline and subsequent recovery of CMO mRNA seen in unstressed, dicamba-treated R plants on page 114, and likely represents a recovery from dicamba treatment. It is tempting to attribute the

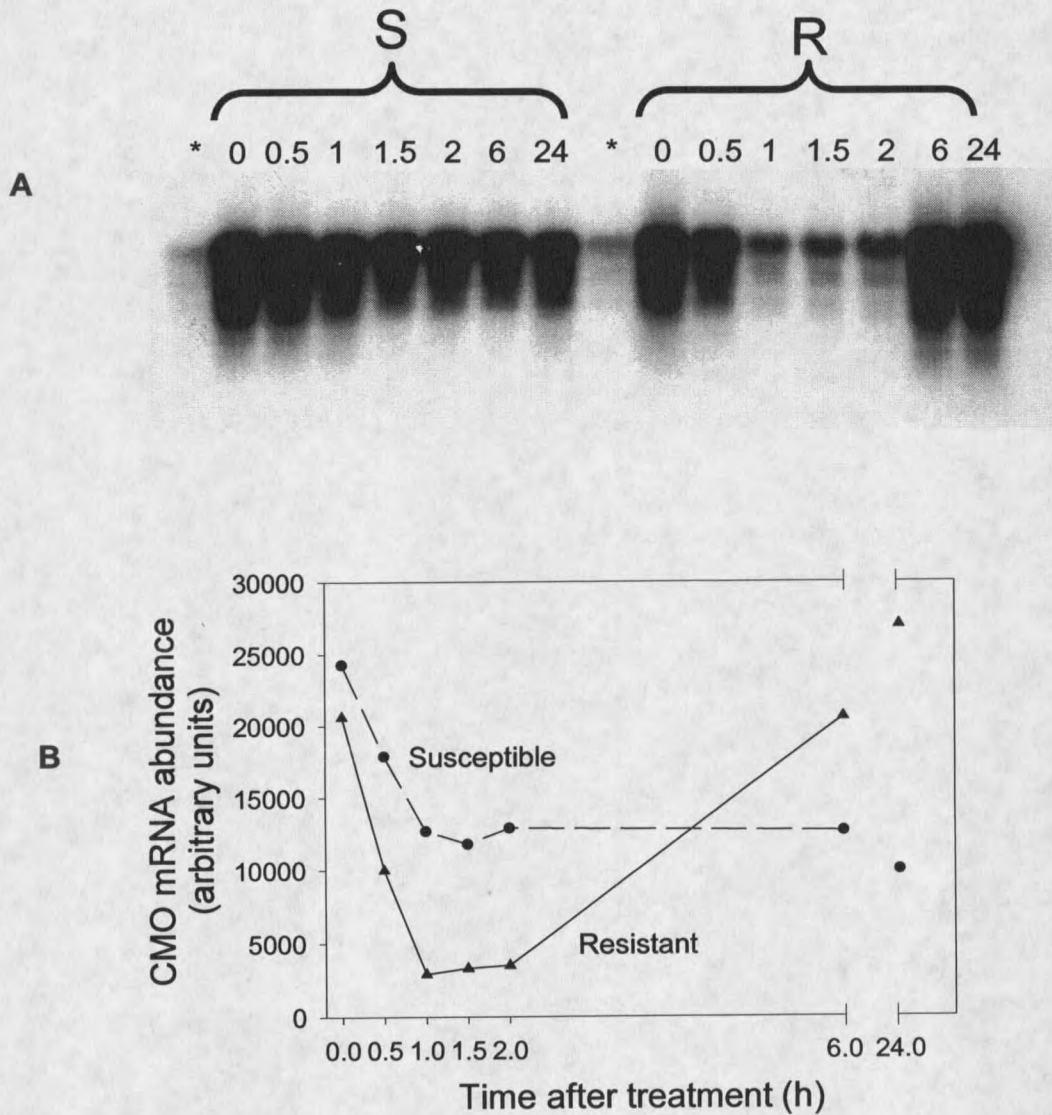


Figure 35. Expression of CMO mRNA after dicamba treatment in salt-stressed plants. (A) RNA isolated 0, 0.5, 1, 1.5, 2, 6, and 24 hours after dicamba treatment and probed with the radiolabeled D416S0 cDNA. RNA isolated from unsprayed, unstressed S and R control plants is indicated with an asterisk. (B) Phosphorimager analysis of message abundance from blot (A).

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 et al., 2001). In contrast to the mRNA attenuation seen in R plants, NaCl-stressed S plants showed less response to dicamba treatment, and CMO mRNA levels declined only about 2-fold by 24 hr after treatment. Consistent with visual observations that NaCl-stressed S plants did not show injury symptoms after dicamba treatment, these results may be explained by a reduction in herbicide absorption or translocation as a result of salt stress. However, the continued responsiveness to dicamba seen in NaCl-stressed R plants represents a fundamental difference between S and R plants and warrants further investigation.

Changes in CMO protein levels were noted after dicamba treatment and closely paralleled the changes in mRNA abundance. Protein abundance remained relatively uniform in S plants after dicamba treatment, declining only slightly (Figure 36). In contrast, the CMO protein declined rapidly in R plants, dropping about 3-fold before beginning to recover by 48 hr after dicamba treatment. GB levels declined slowly after herbicide treatment of both R and S salt-stressed plants (Figure 36C), consistent with other reports that GB is catabolized slowly (Ishatani et al., 1995, Wood et al., 1996).

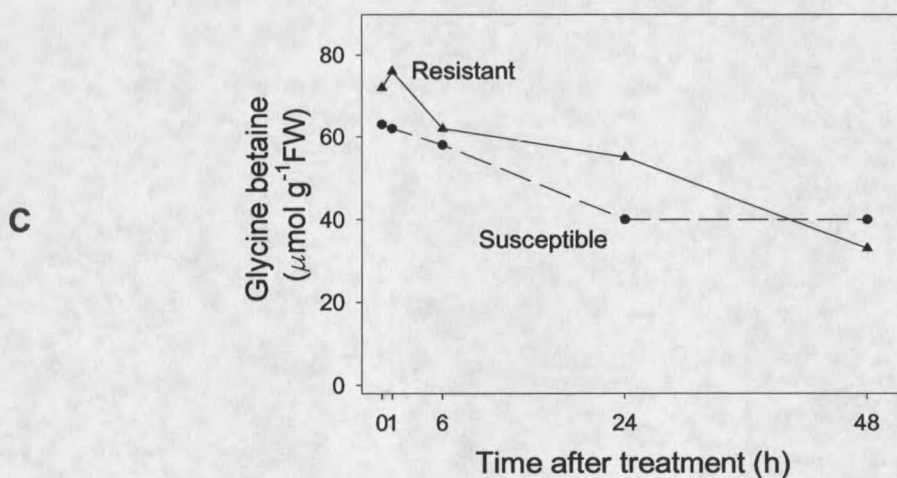
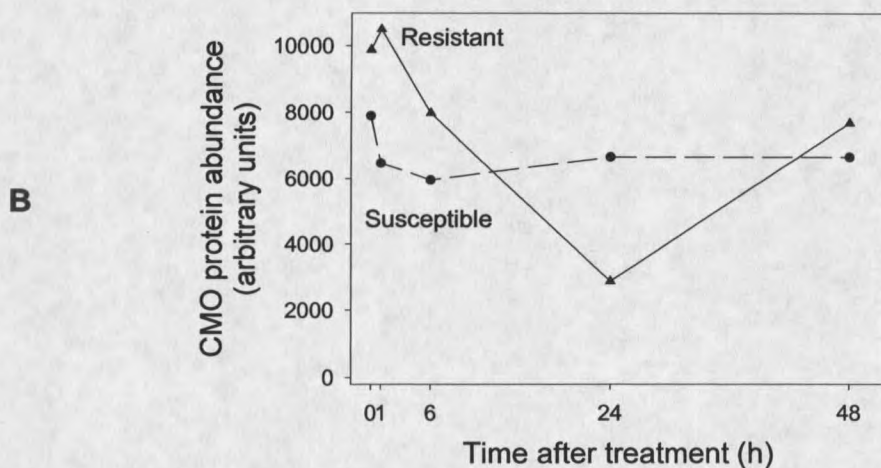
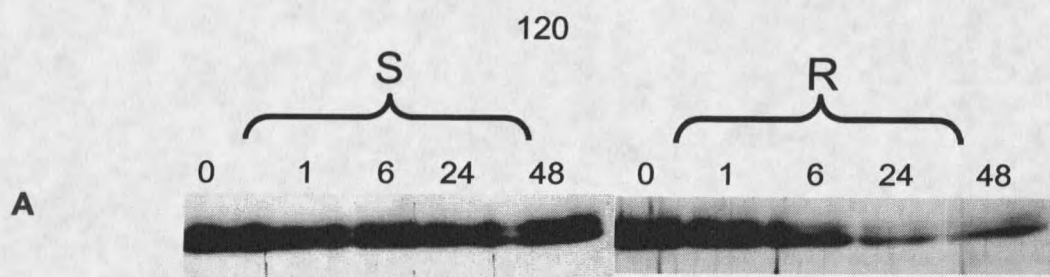


Figure 36. Levels of CMO protein and glycine betaine in salt stressed plants after dicamba treatment. (A) Western blots of CMO protein levels isolated 0, 1, 6, 24, and 48 hours after dicamba treatment. (B) Densitometry analysis of protein bands in (A). (C) GB levels in tissue used in (A).

With the exception of salt-stressed *K. scoparia*, the rapid decline of CMO mRNA and enzyme levels following dicamba treatment was similar in R and S plants. The most likely explanation for the relative unresponsiveness of CMO expression to dicamba treatment in NaCl-stressed S plants is a reduction in herbicide uptake and translocation, caused by osmotic stress. Even though R plants have slightly higher levels of CMO enzyme and GB than S plants in response to NaCl stress, the differences do not seem to be of sufficient magnitude to protect R plants from NaCl stress. Indeed, growth of both biotypes ceased during salinization. Little is known about the mechanism(s) of auxinic herbicide perception, but it is clear that there is a fundamental difference in the effects of osmotic stress on dicamba perception (as measured by CMO expression) between R and S *K. scoparia*. In light of this, logical follow-up experiments may study dicamba absorption and translocation in NaCl-stressed R and S plants.

This is the first report documenting a rapid change in CMO expression in response to treatment with auxin or an auxin mimic. The few auxin down-regulated genes isolated to date generally encode proteins of unknown functions such as the Glycine ADR gene family (Datta et al., 1993). Other down-regulated genes encode enzymes involved in plant defense (Ozeki et al., 1990; Pasquali et al., 1992) and are induced by ABA. Although the studies reported here did not investigate the effect of ABA treatment on CMO gene expression, it is possible that CMO provides another example of a gene induced by ABA and repressed by auxin. The ABA-induced expression of GAD1 and GAD2 mRNAs become

insensitive to ABA treatment after pretreatment with auxin (Jacobsen and Olszewski, 1996), and the gene for the dessication-related LEA protein EDP31 does not respond to ABA after auxin pretreatment (Kiyosue et al., 1992). One well-known effect of osmotic stresses similar to those reported here is an overall increase in ABA levels, leading to physiological responses consistent with reducing evaporative water loss (Jensen et al., 1996). Expression of CMO may thus exemplify the opposite effects of ABA and auxin.

There is a considerable body of literature reporting the effects of auxin treatment on gene expression, yet there is no consensus on how these changes ultimately affect the physiological processes unique to plants. The findings of this study suggest that dicamba is perceived similarly to auxin at the level of gene expression, and provide verification of several genes previously reported to be responsive to auxin treatment. These studies also document other genes, some of which remain unidentified, that have not previously been shown to respond to auxin treatment. One of these genes encodes choline monooxygenase, an enzyme that has received attention as a target for genetic modification of crops to improve drought and salt tolerance. Genetic characterization of this enzyme is essential if it is to be pursued as such a target, and these studies suggest that its expression is regulated by a possibly complex interaction of hormones and osmotic stress.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

Screening for genes responsive to dicamba treatment

One of the initial goals of this dissertation research was to isolate and characterize genes that may be involved in the mechanism of dicamba resistance in *K. scoparia*. Although none of the genes described here, with the possible exception of C08R60-2, appear to fulfill these criteria, several genes were isolated whose expression levels changed in response to dicamba treatment, and thus represent the first such genes to be reported. Several of these genes were previously reported to be responsive to auxin treatment, such as ACC synthase, xyloglucan endotransglycosylase, and a DnaJ protein. In addition to these, genes not previously shown to be responsive to auxin or auxinic herbicides are described here. Further, their expression patterns are consistent with auxin-mediated responses. For example, the apparent down-regulation of C03S0, putatively encoding 5,10-methylenetetrahydrofolate reductase, may increase the available pool of methyl group donors involved in thymidylate synthesis, probably in response to auxinic herbicide-mediated induction of mitotic cell division. Similarly, the induction of C07S120, putatively encoding a chloride channel protein, may serve to increase chloride ion uptake,

as previously noted after auxin treatment.

The auxinic herbicides are thought to mimic auxin primarily because of their effects on plant physiological processes. Relatively few studies have provided molecular evidence supporting this idea, and this research adds credibility to the notion that the auxinic herbicides affect gene expression in ways similar to the natural plant hormone. Still, several side projects not reported in this dissertation suggest that there may be important differences between auxin- and auxinic herbicide-mediated effects on gene expression. Several clones of known auxin induced genes from *Arabidopsis* were obtained from different research groups, and northern blot analysis indicated none of them were induced by dicamba treatment in *K. scoparia*. In addition, expression levels of several of the dicamba-responsive genes reported here were unaffected by treatment with 2,4-D or auxin. These disparities suggest that the auxinic herbicides may not be completely accurate mimics of auxin, which is a significant finding because many of the studies in the primary literature equate the auxinic herbicides with auxin. To help understand these differences at the molecular level, our group is beginning studies to investigate the effects of different auxinic herbicides on gene expression. We plan to use many of the clones isolated in this research, coupled with known auxin-induced genes from other research groups, to gain a more complete understanding of the differences between auxin- and auxinic herbicide-mediated changes in gene expression in *K. scoparia*.

One question that can be raised is whether differential display (DD) is the best technique to isolate genes responding to auxinic herbicide treatment. DD

has been extensively utilized for isolating genes differentially expressed in response to various treatments, and is the most widely used method for this purpose. Since its introduction in 1992, over two-thirds of published papers utilized DD as the method of choice, and more papers using DD have been published than all combined research using subtractive hybridization, serial analysis of gene expression, arbitrarily-primed RNA PCR, representational difference analysis, and DNA microarray analysis (see www.genhunter.com). However, despite its broad acceptance, DD is well known to be a somewhat inefficient method, largely due to the high occurrence of false positives, as was seen in this report. Another drawback of DD is the high percentage of 3' untranslated region in most clones, making it more difficult to assign putative functions based on nucleotide or inferred amino acid sequence.

If these studies were to begin anew, I believe that microarray analysis would be faster, more useful, and a more rigorous method to isolate genes responsive to dicamba treatment. However, the studies could not be done in *K. scoparia*, but would rather have to be completed in a species with a large EST library such as *Arabidopsis*, and therefore could not address the mechanism of dicamba resistance. Essentially, there are three possible approaches utilizing array technology to address this research question. The first would be to obtain a large number (>1000) of random EST clones from the species of choice, amplifying the inserts using PCR, and then spotting the array. Next, the microarray would be simultaneously probed with differentially-labeled fluorescent RNAs isolated from two different treatments, such as dicamba-treated and

untreated *Arabidopsis* shoots. Differentially expressed clones are then identified by scanning the array and determining which clones hybridized preferentially to RNA from either treatment, potentially yielding both induced and down-regulated clones. The primary drawback of this approach is the cost of producing or purchasing EST clones and the time associated with PCR amplification and array preparation.

A second approach to screening *Arabidopsis* EST libraries to isolate dicamba-responsive genes would be to use a commercially-available *Arabidopsis* microarray. Such arrays were offered to the public sector during 2001 through the *Arabidopsis* Functional Genome Consortium (<http://afgc.stanford.edu/>), and will be offered through the Genomic *Arabidopsis* Resource Network during the summer of 2002 (<http://www.york.ac.uk/res/garnet/garnet.htm>), but array availability is still quite limited and highly competitive. As of this writing, the only source for pre-made *Arabidopsis* microarrays is Affymetrix, a private company specializing in microarray technology. The Affymetrix systems require specialized array reading equipment which costs \$220,000 (<http://www.affymetrix.com>).

A third approach would be to create and screen a DNA macroarray. Briefly, RNA is isolated from tissue, reverse transcribed, and used to make a cDNA library. Random cDNAs are isolated, amplified by PCR, and immobilized onto a nylon membrane. Macroarray analysis is conceptually similar to microarrays, but utilizes two identical arrays probed separately with radiolabeled cDNA reverse transcribed from mRNAs from two different treatments, such as

dicamba-treated and untreated *K. scoparia* shoot tissue. There are two major benefits to this approach. The first is that a researcher can preferentially find induced genes by making a cDNA library from dicamba-treated tissue and using this library to create the macroarray. This will skew the cDNA makeup of the library, and thus the cDNAs immobilized onto the array towards dicamba-induced genes. A similar DNA macroarray approach was successfully used to isolate genes induced by pathogen infection in *Capsicum* (Jung and Hwang, 2000). Of 282 random cDNAs used as interrogation sequences on the array, 36 (13%) were differentially expressed between infected and uninfected leaves as a result of *Xanthomonas* infection. Not surprisingly, all were induced and several were genes previously known to be induced by pathogens. A second major benefit that would be gained by utilizing macroarray technology is that *K. scoparia* RNA could be used, thus more directly addressing the issue of dicamba resistance. In addition, the use of radioactive cDNA populations prevents the need for specialized array reading equipment. The major drawback of using a random cDNA macroarray is that this approach severely discriminates against rare or down-regulated messages.

While array technology probably holds more promise than DD for isolating genes whose expression patterns are affected by dicamba treatment, it cannot be used to elucidate the mechanism of dicamba resistance unless the resistance mechanism is correlated with changes in gene expression. Mutations in auxin binding proteins or proteins involved in herbicide transport, for instance, could theoretically confer resistance, and a better system to screen for such mutations

would be to isolate a dicamba-resistant *Arabidopsis* mutant. Because the *Arabidopsis* genome is saturated with molecular markers and has been sequenced, it would be relatively easy to use genetic tools to map the mutation. Once mapped, potential candidate genes can be identified and sequenced, allowing for putative identification of the genetic lesion responsible for the resistance trait. Such studies are now underway in the Dyer lab. The major drawback of using this model system is that a dicamba-resistant *Arabidopsis* mutant has not yet been isolated and may not have the same mutation as dicamba-resistant *K. scoparia*. Nonetheless, these studies could provide interesting insights into one possible mechanism of resistance to auxinic herbicides and could provide a valuable complement to my results.

Analysis of choline monooxygenase expression

These studies provide the first evidence that choline monooxygenase (CMO) mRNA, protein, and product (glycine betaine, GB) are down-regulated in response to treatment with an auxinic herbicide, and suggest that *in vivo* auxin levels are an important indicator of plant stress. Because CMO has only been shown to be present in certain members of the Chenopodiaceae and Amaranthaceae, and because *K. scoparia* is highly tolerant to drought stress, this species provides an excellent model for characterizing this gene. Further, this approach may provide useful genetic information for future experiments using CMO to genetically modify crop plants to increase their ability to withstand water deficit. One such characterization that is still underway in our lab is to

identify *cis*-acting promoter elements within the CMO gene that are involved in auxin-mediated downregulation of CMO mRNA. While there has been considerable work investigating promoter elements of auxin-induced genes (Guilfoyle et al., 1998; Hong et al., 1995; Sakai et al., 1996; Gil and Green, 1997; Plesch et al., 1997), only a few reports have characterized the promoter elements from auxin-repressible genes. Different regions of the *Nicotiana* β -1,3-glucanase promoter have been shown to be essential for auxin-mediated mRNA attenuation, although it was noted that this promoter is most responsive to auxin-mediated increases in ethylene production and thus the changes are not attributable to auxin treatment *per se* (Vögeli-Lange et al., 1994). Even though auxin-responsive promoter elements probably also play a role in auxin-mediated gene attenuation, it is possible that other factors are involved in the reduction of cytoplasmic mRNA levels. Datta et al. (1993), using nuclear run-on transcription assays of the ADR genes, noted that the rapid reduction in ADR mRNA levels could not be attributed solely to a decrease in rates of transcription and suggested that post-transcriptional processes were more important in reducing mRNA levels in response to auxin treatment.

The paucity of studies characterizing *cis*-acting elements of auxin down-regulated genes, coupled with the excellent example of the auxin-repressible mRNA reported here, have prompted continued experiments to isolate and characterize the auxin-responsive CMO promoter elements from *K. scoparia*. A genomic library has been created and screened, and a 6.5-Kb clone has been isolated that likely contains regulatory regions. Future experiments will include

characterization of protein-binding regions in the CMO promoter using gel mobility shift assays, as well as performing run-on transcription assays to determine if translational control is the primary level at which the CMO message is attenuated in response to dicamba treatment.

LITERATURE CITED

- Abel, S., M.D. Nguyen, W. Chow, and A. Theologis. 1995a. *ASC4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. *J. Biol. Chem.* 270:19093-19099.
- Abel, S., M.D. Nguyen, and A. Theologis. 1995b. The *PS-IAA4/5*-like family of early auxin-inducible mRNAs in *Arabidopsis thaliana*. *J. Mol. Biol.* 251:533-549.
- Abel, S., and A. Theologis. 1994. Early auxin-induced genes encode short-lived nuclear proteins. *PNAS* 91:326-330.
- Abel, S., and A. Theologis. 1996. Update on signal transduction. Early genes and auxin action. *Plant Physiol.* 111:9-17.
- Abels, F.B. 1973. Ethylene in plant biology. Academic Press, New York.
- Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson. 1989. Molecular biology of the cell. Garland Publishing Inc., New York.
- Allard, F., M. Houde, M. Kröl, A. Ivanov, N.P.A. Huner, and F. Sarhan. 1998. Betaine improves freezing tolerance in wheat. *Plant Cell Physiol.* 39:1194-1202.
- Alia, H. Hayashi, A. Sakamoto and N. Murata. 1998a. Enhancement of the tolerance of *Arabidopsis* to high temperatures by genetic engineering of the synthesis of glycine betaine. *Plant J.* 16:155-161.
- Alia, H. Hayashi, T.H.H. Chen, and N. Murata. 1998b. Transformation with a gene for choline oxidase enhances the cold tolerance of *Arabidopsis* during germination and early growth. *Plant Cell Environ.* 21:232-239.
- Alia, Y. Kondo, A. Sakamoto, H. Nonaka, H. Hayashi, P. Pardha, T.H.H. Chen, and N. Murata. 1999. Enhanced tolerance to light stress of transgenic *Arabidopsis* plants that express the *codA* gene for a bacterial choline oxidase. *Plant Mol. Biol.* 40:279-288.
- Alliotte, T., C. Tiré, G. Engler, J. Peleman, A. Caplan, M. van Montagu, and D. Inzé. 1989. An auxin-regulated gene of *Arabidopsis thaliana* encodes a DNA-binding protein. *Plant Physiol.* 89:743-752.

- Altschul, S.F., T.L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Antosiewicz, D.M., D.H. Polisensky, and J. Braam. 1995. Cellular localisation of the Ca^{2+} binding TCH3 protein of *Arabidopsis*. *Plant J.* 8:623-636.
- Arakawa, K., Mizuno, K., Kishitani, S., and Takabe, T. 1992. Immunological studies of betaine aldehyde dehydrogenase in barley. *Plant Cell Physiol.* 33:833-840.
- Arrowsmith, D.A. and J. de Silva. 1995. Characterization of two tomato fruit-expressed cDNAs encoding xyloglucan *endo*-transglycosylase. *Plant Mol. Biol.* 28:391-403.
- Arteca, J.M. and R.N. Arteca. 1999. A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (ACS6) in mature *Arabidopsis* leaves. *Plant Mol. Biol.* 39:209-219.
- Ashton, F.M. and A.S. Crafts. 1981. Mode of action of herbicides. John Wiley & Sons, New York.
- Assman, S.M. 1993. The guard cell-environment connection. *Plant Physiol.* 102:711-715.
- Babourina, O., S. Shabala, and I. Newman. 1998. Auxin stimulates Cl^- uptake by oat coleoptiles. *Ann. Bot.* 82:331-336.
- Baima, S., F. Nobili, S. Sessa, S. Luccetti, I. Ruberti, and G. Morelli. 1995. The expression of the *Athb-8* homeobox gene is restricted to provascular cells in *Arabidopsis thaliana*. *Development* 121:4171-4182.
- Ball, M.C. 1988. Salinity tolerance in the mangroves *Aegiceras corniculatum* and *Avicennia marina*. I. Water use in relation to growth, carbon partitioning, and salt balance. *Aust. J. Plant Physiol.* 15:447-464.
- Barthakur, S., V. Babu, and K.C. Bansal. 2001. Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. *J. Plant Biochem. Biotechnol.* 10:31-37.
- Bennett, M.J., A. Marchant, H.G. Green, S.T. May, S.P. Ward, P.A. Millner, A.R. Walker, B. Schulz, and K.A. Feldmann. 1996. *Arabidopsis* AUX1 gene: A permease-like regulator of root gravitropism. *Science* 273:948-950.

- Bilang, J., and A. Sturm. 1995. Cloning and characterization of a glutathione S-transferase that can be photolabeled with 5-azido-indole-3-acetic acid. *Plant Physiol.* 109:253-260.
- Bishop, T., S.B. Powles, and G. Cornic. 1987. Mechanism of paraquat resistance in *Hordeum glaucum*. II. Paraquat uptake and translocation. *Aust. J. Plant Physiol.* 14:539-547.
- Blackshaw, R.E., J.T. O'Donovan, M.P. Sharma, K.N. Harker, and D. Maurice. Response of triallate-resistant wild oat (*Avena fatua*) to alternative herbicides. *Weed Tech.* 10:258-262.
- Blumberg, H. and P.A. Silver. 1991. A homologue of the bacterial heat-shock gene DnaJ that alters protein sorting in yeast. *Nature* 349:627-629.
- Boerjan, W., M.T. Cervera, M. Delarue, T. Beeckman, W. Dewitte, C. Bellini, M. Caboche, H. van Onckelen, M. van Montagu, D. Inze, H. van Onckelen, and M. van Montagu. 1995. Superroot, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *Plant Cell* 7:1405-1419.
- Bonner, K. I., A. Rahman, T. James, K. Nicholson, and D. Wardle. 1998. Relative intra-species competitive ability of nodding thistle biotypes with varying resistance to the herbicide 2,4-D. *New Zealand J. Agric. Res.* 41: 291-297.
- Botella, J.R., J.M. Arteca, C.D. Schlagnhauer, R.N. Arteca, and A.T. Phillips. 1992. Identification and characterization of a full-length cDNA encoding for an auxin-induced 1-aminocyclopropane-1-carboxylate synthase from etiolated mug bean hypocotyl segments and expression of its mRNA in response to indole-3-acetic acid. *Plant Mol. Biol.* 20:425-436.
- Botella, J.R., and R.N. Arteca. 1994. Differential stimulation of two calmodulin genes in response to physical and chemical stimuli. *Plant Mol. Biol.* 24:757-766.
- Botella, J.R., J.M. Arteca, M. Somodevilla, and R.N. Arteca. 1996. Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mugbean (*Vigna radiata*). *Plant Mol. Biol.* 30:1129-1137.
- Bourgeois, L., N.C. Kenkel, and I.N. Morrison. 1997. Characterization of cross-resistance patterns in acetyl-CoA carboxylase inhibitor resistant wild oat (*Avena fatua*). *Weed Sci.* 45:750-755.

- Bourot, S., O. Sire, A. Trautwetter, T. Touzé, L.F. Wu, C. Blanco, and T. Bernard. 2000. Glycine betaine-assisted protein folding in a *lysA* mutant of *Escherichia coli*. *J. Biol. Chem.* 275:1050-1056.
- Boyer, J.S. 1982. Plant productivity and environment. *Science* 218:443-448.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem* 72:248-254.
- Brown, H.M. 1991. Mode of action, crop selectivity, and soil relations of the sulfonylurea herbicides. *Pestic. Sci.* 29:263-281.
- Burnet, M., P.J. Lafontaine, and A.D. Hanson. 1995. Assay, purification, and partial characterization of choline monooxygenase from spinach. *Plant Physiol.* 108:581-588.
- Caplan, A.J. and M.G. Douglas. 1991. Characterization of YDJ1: a yeast homologue of the bacterial DnaJ protein. *J. Cell Biol.* 114:609-621.
- Cheeseman, J.M. 1988. Mechanisms of salinity tolerance in plants. *Plant Physiol.* 87:547-550.
- Chen, J., D. Wu, F.H. Witham, C.W. Heuser, and R.N. Arteca. 1996. Molecular cloning and characterization of auxin-regulated genes from mugbean hypocotyls during adventitious root formation. *J. Am. Soc. Hortic. Sci.* 121:393-398.
- Choi, S.W. and J.B. Mason. 2001. Folate and carcinogenesis: an integrated scheme. *J. Nutr.* 130:129-132.
- Cline, K. and R. Henry. 1996. Import and routing of nucleus-encoded chloroplast proteins. *Ann. Rev. Cell Dev. Biol.* 12:1-26.
- Colmer, T.D., E. Epstein, and J. Dvorak. 1995. Differential solute regulation in leaf blades of various ages in salt-sensitive wheat and salt-tolerant wheat *X Lophopyrum elongatum* (Host.) A. Love amphiploid. *Plant Physiol.* 108:1715-1724.
- Conner, T.W., V.H. Goekijian, P.R. LaFayette, and J.L. Key. 1990. Structure and expression of two auxin-inducible genes from *Arabidopsis*. *Plant Mol. Biol.* 15:623-632.

- Coupland, D., D. T. Cooke, and C. S. James. 1991. Effects of 4-chloro-2-methylphenoxypropionate (an auxin analogue) on plasma membrane ATPase activity in herbicide-resistant and herbicide-susceptible biotypes of *Stellaria media* L. J. Exp. Bot. 42: 1065-1071.
- Coupland, D. and M.B. Jackson. 1991. Effects of mecoprop (an auxin analogue) on ethylene evolution and epinasty in two biotypes of *Stellaria media*. Ann. Bot. 68:167-172.
- Cox, K., D. Robertson, and R. Fites. 1999. Mapping and expression of a bifunctional thymidylate synthase, dihydrofolate reductase gene from maize. Plant Mol. Biol. 41:733-739.
- Cranston, H.J., A.J. Kern, J.L. Hackett, E.K. Miller, B.D. Maxwell, and W.E. Dyer. 2001. Dicamba resistance in *Kochia*. Weed Sci. 49:164-170.
- Crowell, D.N., and R.M. Amasino. 1991. Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. Plant Physiol. 15:711-715.
- Cyr, D.M., T. Langer, and M.G. Douglas. 1994. DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. Trends Biochem. Sci. 19:176-181.
- Dargeviciute, A., C. Roux, A. Decreux, F. Sitbon, and C. Perrot-Rechenmann. 1998. Molecular cloning and expression of the early auxin-responsive Aux/IAA gene family in *Nicotiana tabacum*. Plant Cell Physiol. 39:993-1002.
- Darley, C.P., O.C.M. van Wuytswinkel, K. van der Woude, W.H. Mager, and A.H. de Boer. 2000. *Arabidopsis thaliana* and *Saccharomyces cerevisiae* NHX1 genes encode amiloride sensitive electroneutral Na^+/H^+ exchangers. Biochem. J. 351:241-249.
- Datta, N., P.R. LaFayette, P.A. Kroner, R.T. Nagao, and J. Key. 1993. Isolation and characterization of three families of auxin down-regulated cDNA clones. Plant Mol. Biol. 21:859-869.
- Davenport, R.J. and M. Tester. 2000. A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat. Plant Physiol. 122:823-834.

- Dean, J.V., J.W. Gronwald, and M.P. Anderson. 1991. Glutathione-S-transferase activity in nontreated and CGA-154281-treated maize shoots. *Z. Naturforsch.* 46c:850-855.
- Delarue, M., E. Prinsen, H. van Onckelen, M. Caboche, C. Bellini, and H. van Onckelen. 1998. Sur2 mutations of *Arabidopsis thaliana* define a new locus involved in the control of auxin homeostasis. *Plant J.* 14:603-611.
- Deleu, C., M. Coustant, M.F. Niogret and F. Larher. 1999. Three new osmotic stress-regulated cDNAs identified by differential display polymerase chain reaction in rapeseed leaf discs. *Plant Cell Environ.* 22:979-988.
- de Pater, B.S., F. van der mark, S. Rueb, F. Katagiri, N.H. Chua, R.A. Schilperoort, and L.A.M. Hensgens. 1992. The promoter of the rice gene GOS2 is active in various different monocot tissues and binds rice nuclear factor ASF-1. *Plant J.* 2:837-844.
- Devine, M., S.O. Duke, and C. Fedtke. 1993. *Physiology of herbicide action.* Eaglewood Cliffs, N.J: Prentice Hall.
- DeWald, D.B., A. Sadaka, and J.E. Mullet. 1994. Sucrose modulation of soybean *vsp* gene expression is inhibited by auxin. *Plant Physiol.* 104:439-444.
- Ebener, W., T.J. Fowler, H. Suzuki, J. Shaver, and M.L. Tierney. 1993. Expression of DcPRP1 is linked to carrot storage root formation and is induced by wounding and auxin treatment. *Plant Physiol.* 101:259-265.
- Eberlein, C.V. and Z.Q. Fore. 1984. *Kochia Biology.* *Weeds Today* 15:5-6.
- Esaka, M., K. Fujisawa, M. Goto, and Y. Kisu. 1992. Regulation of ascorbate oxidase expression in pumpkin by auxin and copper. *Plant Physiol.* 100:231-237.
- Faulkner, J.S. 1976. A paraquat resistant variety of *Lolium perenne* under field conditions. Pp. 485-490 *In* Brighton Crop Protection Conference-Weeds. Farnham, U.K.
- Fogelfors, H. 1979. Changes in the Flora of Farmland. Swed. Univ. Agric. Sci. Dept, Ecol. Environ. Res. Rep. No. 5, Uppsala. pp 66.
- Franks, P.J., and G.D. Farquhar. 2001. The effect of exogenous abscisic acid on stomatal development, stomatal mechanics, and leaf gas exchange in *Tradescantia virginiana*. *Plant Physiol.* 125:935-942.

- Frugis, G., G. Mele, D. Giannino, and D. Mariotti. 1999. MsJ1, an alfalfa DnaJ-like gene, is tissue-specific and transcriptionally regulated during cell cycle. *Plant-mol-biol.* 40:397-408.
- Fry, S.C., R.C. Smith, K.F. Renwick, D.J. Martin, S.K. Hodge, and K.J. Matthews. 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* 282:821-828.
- Fuerst, E.P., N.Y. Nakatani, A.D. Dodge, D. Penner, and C.J. Arntzen. 1985. Paraquat resistance in *Conyza*. *Plant Physiol.* 77:984-989.
- Fuerst, E. P., T. M. Sterling, M. A. Norman, T. S. Prather, G. P. Irzyk, Y. Wu, N. K. Lownds, and R. H. Callihan. 1996. Physiological characterization of picloram resistance in yellow starthistle. *Pestic. Biochem. Physiol.* 56: 149-161.
- Galweiler, L., C. Guan, A. Mueller, E. Wisman, K. Mendgen, A. Yephremov, and K. Palme. 1998. Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* 282:2226-2230.
- Gao, M., A. Sakamoto, K. Miura, N. Murata, A. Sugiura, and R. Tao. 2000. Transformation of Japanese persimmon (*Diospyros khaki* Thunb.) With a bacterial gene for choline oxidase. *Mol. Breed.* 6:501-510.
- Gaxiola, R.A., R. Rao, A. Sherman, P. Grisafi, S.L. Alper, and G.R. Fink. 1999. The *Arabidopsis thaliana* proton transporters, *AtNhx1* and *Avp1*, can function in cation detoxification in yeast. *PNAS* 96:1480-1485.
- Gehring, C., H.R. Irving, and R.W. Parish. 1990. Effects of auxin and abscisic acid on cytosolic calcium and pH in plant cells. *PNAS* 87:9645-9649.
- Gil, P. and P.J. Green. 1997. Regulatory activity exerted by the *SAUR-AC1* promoter region in transgenic plants. *Plant Mol. Biol.* 34:803-808.
- Gimmler, H., L. Schneider, and R. Kaaden. 1989. The plasma membrane ATPase of *Dunaliella parva*. *Z. Natur.* 44:128-138.
- Goddijn, O.J.M., R.J. de Kam, A. Zanetti, R.A. Schilperoort, and J.H.C. Hoge. 1992. Auxin rapidly down-regulates transcription of the tryptophan decarboxylase gene from *Catharanthus roseus*. *Plant Mol. Biol.* 18:1113-1120.
- Gonneau, M., B. Pasquette, F. Cabanne, and R. Scalla. 1988. Metabolism of chlortoluron in tolerant species: possible role of cytochrome P-450 monooxygenases. *Weed Res.* 28:19-25.

- Gorham, J. 1995. Betaines in higher plants—biosynthesis and role in stress metabolism. *In: Amino acids and their derivatives in higher plants.* Cambridge University Press, Cambridge, U.K.
- Gray, W.M., J.C. del Ponzo, L. Walker, L. Hobbie, E. Risseuw, T. Banks, W.L. Crosby, Y. Mang, M. Hong, M. Estelle, M. Yang, and H. Ma. 1999. Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes and Devel.* 13:1678-1691.
- Grieve, C.M. and E.V. Maas. 1984. Betaine accumulation in salt-stressed sorghum. *Physiol. Plant.* 61:167-171.
- Gronwald, J. W. 1994. Resistance to photosystem II inhibiting herbicides. *In* Powles, S.B. and Holtum, J.A.M., eds., *Herbicide Resistance in Plants- Biology and Biochemistry.* Lewis Publishers, London.
- Gronwald, J.W., R.N. Andersen, and C. Yee. 1989. Atrazine resistance in velvetleaf (*Abutilon theophrasti*) due to enhanced atrazine detoxification. *Pestic. Biochem. Physiol.* 34:149-163.
- Grossman, K. 1998. Quinclorac belongs to a new class of highly selective auxin herbicides. *Weed Sci.* 46:707-716.
- Grossmann, K. 2000. Mode of action of auxin herbicides: a new ending to a long, drawn out story. *Trends Plant Sci.* 5:506-508.
- Gruwel, M.L.H., V.L. Rauw, M. Loewen, and S.R. Abrams. 2001. Effects of sodium chloride on plant cells: a ^{31}P and ^{23}Na NMR system to study salt tolerance. *Plant Sci.* 160:785-794.
- Guilfoyle, T., G. Hagen, T. Ulmasov, and J. Murfett. 1998. How does auxin turn on genes? *Plant Physiol.* 118:341-347.
- Hagen, G. and T. Guilfoyle. 1985. Rapid induction of selective transcription by auxins. *Mol. Cell Biol.* 5:1197-1203.
- Hajouj, T., R. Michelis, and S. Gepstein. 2000. Cloning and characterization of a receptor-like protein kinase gene associated with senescence. *Plant Physiol.* 124:1305-1314.
- Hall, L.M., J.A.M. Holtum, and S.B. Powles. 1994. Mechanisms responsible for cross resistance and multiple resistance. pp. 243-261 *In* Powles, S.B. and Holtum, J.A.M., eds., *Herbicide Resistance in Plants- Biology and Biochemistry.* Lewis Publishers, London.

- Hall, J.C., S.M.M. Alam, and D.P. Murr. 1993. Ethylene biosynthesis following foliar application of picloram to biotypes of wild mustard (*Sinapis arvensis* L.) susceptible or resistant to the auxinic herbicides. *Pestic. Biochem. Physiol.* 47:36-43.
- Hall, J.C. and M.L. Romano. 1995. Morphological and physiological differences between susceptible (S) and resistant (R) wild mustard (*Sinapis arvensis*) biotypes. *Pestic. Biochem. Physiol.* 52:149-155.
- Hanson, A.D., D.A. Gage, and Y. Shachar-Hill. 2000. Plant one-carbon metabolism and its engineering. *Trends Plant Sci.* 5:206-213.
- Harling, H., I. Czaja, J. Schell, and R. Walden. 1997. A plant cation-chloride co-transporter promoting auxin-independent tobacco protoplast division. *EMBO J.* 19:5855-5866.
- Hayashi, N., Alia, L. Mustardy, P. Deshniem, M. Ida, and N. Murata. 1997. Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. *Plant J.* 12:133-142.
- Heap, I. 2001. International survey of herbicide-resistant weeds. Available at <http://www.weedscience.org/>
- Hemerly, A.S., P. Ferreira, J. A. Engler, M. Van Montagu, G. Engler, and D. Inez. 1993. *cdc2a* expression in *Arabidopsis* is linked with competence for cell division. *Plant Cell* 5:1711-1723.
- Henskens, J.A.M., G.J.A. Rouwendal, A.T. have and E.J. Woltering. 1994. Molecular cloning of two different ACC synthase PCR fragments in carnation flowers and organ-specific expression of the corresponding genes. *Plant Mol. Biol.* 26:453-458.
- Hibino, T., Y.L. Meng, Y. Kawamitsu, N. Uehara, N. Matsuda, Y. Tanaka, H. Ishikawa, S. Baba, T. Takabe, K. Wada, T. Ishii, T. Takabe, and Y.L. Meng. Molecular cloning and functional characterization of two kinds of betaine-aldehyde dehydrogenase in betaine-accumulating mangrove *Avicennia marina* (Forsk.) Vierh. *Plant Mol. Biol.* 45:353-363.
- Hilton, H.W. 1957. Herbicide tolerant strains of weeds, Hawaiian Sugar Plant. Assoc. Annu. Rep.
- Hirt, H., A. Pay, L. Bogre, I. Meskiene, and B.E. Heberle. 1993. *cdc2MsB*, a cognate *cdc2* gene from alfalfa, complements the G1/S but not the G2/M transition of budding yeast *cdc28* mutants. *Plant J.* 4:61-69.

- Hong, J.C., Y.H. Cheong, R.T. Nagao, J.D. Bahk, J.L. Key, and M.J. Cho. 1995. Isolation of two soybean G-box binding factors which interact with a G-box sequence of an auxin-responsive gene. *Plant J.* 8:199-211.
- Huang, J., R. Hirji, L. Adam, K.L. Rozwadowski, J.K. Hammerlindl, W.A. Keller, and G. Selvaraj. 2000. Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiol.* 122:747-756.
- Hull, A.K., R. Vij, and J.L. Celenza. 2000. Arabidopsis cytochrome P450s that catalyse the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. *PNAS* 97:2379-2384.
- Hurkman, W.J., and C.K. Tanaka. 1996. Effect of salt stress on germin gene expression in barley roots. *Plant Physiol.* 110:971-977.
- Hutchison, K.W., P.B. Singer, S. McInnis, S.C. Diaz, and M.S. Greenwood. 1999. Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin. *Plant Physiol.* 120:827-831.
- Ikuta, S., S. Mamura, H. Misaki, and Y. Horiuti. 1977. Purification and characterization of choline oxidase from *Arthrobacter globiformis*. *J. Biochem.* 82:1741-1749.
- Irving, H.R., C. Gehring, and R.W. Parish. 1992. Changes in cytosolic pH and calcium of guard cells precede stomatal movements. *PNAS* 89:1790-1794.
- Ishida, S., Y. Takahashi, and T. Nagata. 1993. Isolation of cDNA of an auxin-regulated gene encoding a G protein β -subunit-like protein from tobacco BY-2 cells. *PNAS* 90:11152-11156.
- Ishida, S., Y. Takahashi, and T. Nagata. 1996. The mode of expression and promoter analysis of the *arcA* gene, an auxin-regulated gene in tobacco BY-2 cells. *Plant Cell Physiol.* 37:439-448.
- Ishitani, M., T. Nakamura, S.Y. Han and T. Takabe. 1995. Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol. Biol.* 307-315.
- Iwahara, M., T. Saito, S. Ishida, Y. Takahashi, and T. Nagata. Isolation and characterization of a cytokinin up-regulated gene from tobacco mesophyll protoplasts. *Plant Cell Physiol.* 39:859-864.

- Jacobsen, S.E., and N.E. Olszewski. 1996. Gibberellins regulate the abundance of RNAs with sequence similarity to proteinase inhibitors, dioxygenases, and dehydrogenases. *Planta* 198:78-86.
- Jasieniuk, M., A.L. Brule-Babel, and I.N. Morrison. 1994. Inheritance of trifluralin resistance in green foxtail (*Setaria viridis*). *Weed Sci.* 42:123-127.
- Jena, P.K., A.S. Reddy, and B.W. Poovaiah. 1989. Molecular cloning and sequencing of a cDNA for plant calmodulin: Signal-induced changes in the expression of calmodulin. *PNAS* 86:3644-3648.
- Jensen, A.B., P.K. Busk, M. Figueras, M.M. Alba, G. Peracchia, R. Messeguer, A. Goday, M. Pages, M. M. Alba, and E. Belhassen. 1996. Drought signal transduction in plants. *J. Plant Growth Reg.* 20:105-110.
- Jiang, H., R.E. Parales, N.A. Lynch, and D.T. Gibson. 1996. Site-directed mutagenesis of conserved amino acids in the alpha subunit of toluene dioxygenase: potential mononuclear non-heme iron coordination sites. *J. Bacteriol.* 178:3133-3139.
- Jiban-Mitra and J. Mitra. 2001. Genetics and genetic improvement of drought resistance in crop plants. *Current Sci.* 80:758-763.
- John, P.C., K. Zhang, C. Dong, L. Diederich, and F. Wightman. p34^{cdc2} related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development, and stimulation of division by auxin and cytokinin. *Aust. J. Plant Physiol.* 20:503-526.
- Jolivet, Y., F. Larher, and J. Hamelin. 1982. Osmoregulation in halophytic higher plants: the protective effect of glycinebetaine against the heat destabilization of membranes. *Plant Sci. Lett.* 25:193-201.
- Jones, M.L. and W.R. Woodson. 1999. Differential expression of three members of the 1-aminocyclopropane-1-carboxylate synthase gene family in carnation. *Plant Physiol.* 119:755-764.
- Jung, H.W. and B.K. Hwang. 2000. Isolation, partial sequencing, and expression of pathogenesis-related cDNA genes from pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria*. *Plant Mol. Microbe Interactions* 13:136-142.
- Kapazoglou, A., F. Sagliocco, and L. Dure III. 1995. PSII-T, a new nuclear encoded luminal protein from photosystem II. *J. Biol. Chem.* 270:12197-12202.

- Kapros, T., L. Bögre, K. Németh, L. Bakó, J. Györgyey, S.c. Wu, and D. Dudits. 1992. Differential expression of histone H3 gene variants during cell cycle and somatic embryogenesis in alfalfa. *Plant Physiol.* 98:621-625.
- Keller, C.P. and E. van Volkenburgh. 1996. The electrical response of *Avena* coleoptiles to auxins: Evidence *in vivo* for activation of a Cl⁻ conductance. *Planta* 198:404-412.
- Kende, H. 1989. Enzymes of ethylene biosynthesis. *Plant Physiol.* 91:1-4.
- Kerk, N.M. and L.J. Feldman. 1995. A biochemical model for the initiation and maintenance of the quiescent center: Implications for the organization of root meristems. *Development* 121:2825-2833.
- Kern, A.J. and W.E. Dyer. 1998a. Compartmental analysis of herbicide efflux in susceptible and difenzoquat-resistant *Avena fatua* L. suspension cells. *Pestic. Biochem. Physiol.* 61:27-37.
- Kern, A.J., B.E. Murray, M.A. Jasieniuk, B.D. Maxwell, and W.E. Dyer. 2002. Triallate resistance in wild oats (*Avena fatua* L.) is conferred by two recessive nuclear genes. *J. Heredity* (In press).
- Kern, A.J., D.M. Peterson, E.K. Miller, C.C. Colliver, and W.E. Dyer. 1996. Triallate resistance in *Avena fatua* L. is due to reduced herbicide activation. *Pestic. Biochem. Physiol.* 56:163-173.
- Kern, A.J., D.M. Peterson, T.M. Myers, J.L. Wright, E.K. Miller, C.C. Colliver, M.A. Jasieniuk, B.D. Maxwell, L.L. Jackson, and W.E. Dyer. 1998b. Reduced rates of herbicide metabolism confer triallate resistance in *Avena fatua*. *Pestic. Sci.* 52:191-193.
- Kernan, A., and R.W. Thornburg. 1989. Auxin levels regulate the expression of a wound-inducible proteinase inhibitor-chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. *Plant Physiol.* 91:73-78.
- Kim, W.T., A. Silverstone, W.K. Yip, J.G. Dong, and S.F. Yang. 1992. Induction of 1-aminocyclopropane-1-carboxylate synthase mRNA by auxin in mug bean hypocotyls and cultured apple shoots. *Plant Physiol.* 98:465-471.
- Kishitani, S., T. Takanami, M. Suzuki, M. Oikawa, S. Yokoi, M. Ishitani, A.M. Alvarez-Nakase, T. Takabe and T. Takabe. 2000. Compatibility of glycinebetaine in rice plants: evaluation using transgenic rice plants with a gene for peroxisomal betaine aldehyde dehydrogenase from barley. *Plant, Cell and Environ.* 23:107-114.

- Kitamiya, E., S. Suzuki, T. Sano, and T. Nagata. 2000. Isolation of two genes that were induced upon the initiation of somatic embryogenesis on carrot hypocotyls by high concentrations of 2,4-D. *Plant Cell Rep.* 19: 551-557.
- Kiyosue, T., S. Satoh, H. Kamada, and H. Harada. 1992. Endogenous levels of abscisic acid in embryogenic cells, non-embryogenic cells and somatic embryos of carrot (*Daucus carota* L.). *Biochem. Physiol. Pflanzen* 188:343-347.
- Kiyosue, T., J.K. Beetham, F. Pinot, B.D. Hammock, Y. Shinozaki, and K. Shinozaki. 1994. Characterization of an *Arabidopsis* cDNA for a soluble epoxide hydrolase gene that is inducible by auxin and water stress. *Plant J.* 6:259-269.
- Klotz, K.L. and L.M. Lagrimini. 1996. Phytohormone control of tobacco anionic peroxidase promoter. *Plant Mol. Biol.* 31:565-573.
- Kroczyńska, B., R. Zhu, W. Clifford, and J.A. Miernyk. 1996. AtJ1, a mitochondrial homologue of the *Escherichia coli* DnaJ protein. *Plant Mol. Biol.* 31:619-629.
- Labavitch, J.M. and P.M. Ray. 1974. Turnover of cell wall polysaccharides in elongating pea stem segments. *Plant Physiol.* 53:669-673.
- Landfald, B. and A.R. Strøm. 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *J. Bacteriol.* 165:849-855.
- Last, R.L., P.H. Bissinger, D.J. Mahoney, E.R. Radwanski, and G.R. Fink. 1991. Tryptophan mutants in *Arabidopsis*: the consequences of duplicated tryptophan synthase beta genes. *Plant cell* 3:345:358.
- Legaria, J., R. Rajisbaum, R.A. Munoz-Clares, N. Villegas-Sepulveda, J. Simpson, and G. Iturriaga. 1998. Molecular characterization of two genes encoding betaine-aldehyde dehydrogenase from amaranth. Expression in leaves under short-term exposure to osmotic stress or abscisic acid. *Gene* 218:69-76.
- Lerma, C., P.J. Rich, G.C. Ju, W.J. Yang, A.D. Hanson, and D. Rhodes. 1991. Betaine deficiency in maize. Complementation tests and metabolic basis. *Plant Physiol.* 95:1113-1119.
- Leyser, H.M.O., C. A. Lincoln, C. Timpte, D. Lammer, J. Turner and M. Estelle. 1993. *Arabidopsis* auxin-resistance gene AXR1 encodes a protein related to ubiquitin-activating enzyme E1. *Nature* 364:161-164.

- LeRudulier, D., A.R. Strøm, A.M. Dandekar, L.T. Smith, and R.C. Valentaine. 1984. Molecular biology of osmoregulation. *Science* 224:1064-1068.
- Leyser, O. 1997. Auxins: Lessons from a mutant weed. *Physiol. Plant.* 100:407-414
- Liang, P., and A.B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-971.
- Lincoln, C., J.H. Britton, and M. Estelle. 1990. Growth and development of the *axr1* mutants of *Arabidopsis*. *Plant Cell* 2:1071-1080.
- Lobler, M. and Klambt. 1985. Auxin-binding protein from coleoptile membranes of corn (*Zea mays* L.). *J. Biol. Chem.* 260:9948-9853.
- Lomax, T., G. Muday, and P. Rubery. 1995. Auxin transport. In Davies, P.J., ed. *Plant Hormones: Physiology, Biochemistry, and Molecular Biology*, Kluwer Academic Publ., London, pp 509-530.
- Luke, M.M., A. Sutton, and K. Arndt. 1991. Characterization of SIS1, a *Saccharomyces cerevisiae* homologue of bacterial DnaJ proteins. *J. Cell. Biol.* 114:623-638.
- Luschnig, C., R.A. Gaxiola, P. Grisafi, and G.R. Fink. 1998. EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes Dev.* 12:2175-2187.
- Maher, E.P. and S. J. B. Martindale. 1980. Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. *Biochem. Genet.* 18:1041-1053.
- Marchant, A., J. Kargul, S.T. May, P. Muller, A. Delbarre, C. Perrot-Rechenmann, and M.J. Bennett. 1999. AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO-J.* 18:2066-2073.
- Marcum, K.B. 1999. Salinity tolerance mechanisms of grasses in the subfamily Chloridoideae. *Crop Sci.* 39:1153-1160.
- Marshall, G., R.C. Kirkwood, and D.J. Martin. 1987. Studies on the mode of action of asulam, aminotriazole and glyphosate in *Equisetum arvense* L. (Field Horsetail). II. The metabolism of [^{14}C]asulam, [^{14}C]aminotriazole and [^{14}C]glyphosate. *Pestic. Sci.* 18:65-77.

- Martinez, M.C., J.E. Jørgensen, M.A. Lawton, C.J. Lamb, and P. Doerner. 1992. Spatial pattern of *cdc2* expression in relation to meristem activity and cell proliferation during plant development. *PNAS* 89:7360-7364.
- Marty, I., C. Douat, L. Tichit, K. Jungsup, T. Leustek, and G. Albagnac. 2000. The cystathione- γ -synthase gene involved in methionine biosynthesis is highly expressed and auxin-repressed during wild strawberry (*Fragaria vesca* L.) fruit ripening. *Theor. Appl. Genet.* 100:1129-1136.
- Matsumoto, T., F. Sakai, and T. Hayashi. 1997. A xyloglucan-specific *endo*-1,4- β -glucanase isolated from auxin-treated pea stems. *Plant Physiol.* 114:661-667.
- Maxwell, B.D. and A.M. Mortimer. 1994. Selection for herbicide resistance. pp 1-25 *In* Powles, S.B. and Holtum, J.A.M., eds., *Herbicide Resistance in Plants- Biology and Biochemistry*. Lewis Publishers, London.
- McCann, M.C., B. Wells, and K. Roberts. 1992. Complexity in the spatial localization and length distribution of plant cell-wall matrix polysaccharides. *J. Microsc.* 166:123-136.
- McClure, B., R. Wright, and T. Guilfoyle. 1989. Auxin-induced expression of the soybean SAUR locus in transgenic petunia. *J. Plant Growth Reg.* 8:283-291.
- McClure, B.A. and T. Guilfoyle. 1987. Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. *Plant Mol. Biol.* 9:611-623.
- McCue, K.F. and A.D. Hanson. 1992. Salt-inducible betaine aldehyde dehydrogenase from sugar beet: cDNA cloning and expression. *Plant Mol. Biol.* 18:1-11.
- McNeil, S.D., M.L. Nuccio, and A.D. Hanson. 1999. Betaines and related osmoprotectants: targets for metabolic engineering of stress resistance. *Plant Physiol.* 120:945-949.
- McNeil, S.D., D. Rhodes, B. Russell, M. Nuccio, Y. Shachar-Hill, and A.D. Hanson. 2000. Metabolic modeling identifies key constraints on an engineered glycine betaine synthesis pathway in tobacco. *Plant Physiol.* 124:153-162.
- Meyer, A.M. and F. Muller. 1989. Triasulfuron and its selective behaviour in wheat and *Lolium perenne*. Pp. 441-448 *In* Brighton Crop Protection Conference-Weeds. Farnham, U.K.

- Merritt, F., A. Kemper, and G. Tallman. 2001. Inhibitors of ethylene synthesis inhibit auxin-induced stomatal opening in epidermis detached from leaves of *Vicia faba* L. *Plant Cell Physiol.* 42:223-230.
- Miao, G.H., Z. Hong, and D.P.S. Verma. 1993. Two functional soybean genes encoding p34^{cdc2} protein kinases are regulated by different plant developmental pathways. *PNAS* 90:943-947.
- Mito, N., and A.B. Bennett. 1995. The *diageotropica* mutation and synthetic auxins differentially affect the expression of auxin-regulated genes in tomato. *Plant Physiol.* 109:293-297.
- Mohnen, d., H. Shinshi, G. Felix, and J. Meins. 1985. Hormonal regulation of β -1,4-glucanase messenger RNA levels in cultured tobacco tissues. *EMBO J.* 4:1631-1635.
- Mudge, L.C., B.J. Gossett, and T.R. Myrphy. 1984. Resistance of goosegrass (*Eleusine indica*) to dinitroaniline herbicides. *Weed Sci.* 32:591-594.
- Mulugeta, D., B.D. Maxwell, P.K. Fay, and W.E. Dyer. 1994. Kochia (*Kochia scoparia*) pollen dispersion, viability and germination. *Weed Sci.* 42:548-552.
- Meurer, J., A. Berger, and P. Westhoff. 1996. A nuclear mutant of Arabidopsis with impaired stability on distinct transcripts of the plastid psbB, psbD/C, ndhH, and ndhC operons. *Plant Cell* 8:1193-1207.
- Nagata, T., Y. Takahashi, J.C. Omrod, and D. Francis. 1993. Auxin-mediated activation of DNA synthesis via par genes in tobacco mesophyll protoplasts. *Mol. Cell Biol. Plant Cell Cycle.* Kluwer Academic Press, Netherlands.
- Nakagawa, N., H. Mori, K. Yamazaki, and H. Imaseki. 1991. Cloning of a complementary DNA for auxin-induced 1-aminocyclopropane-1-carboxylate synthase and differential expression of the gene by auxin and wounding. *Plant Cell Physiol.* 32:1153-1163.
- Nakamura, T., M. Ishitani, P. harinasut, M. Nomura, T. Takabe, and T. Takabe. 1996. Distribution of glycinebetaine in old and young leaf blades of salt-stressed barley plants. *Plant Cell Physiol.* 37:873-877.
- Nakamura, T., S. Yokota, Y. Muramoto, K. Tsutsui, Y. Oguri, K. Fukui, and T. Takabe. 1997. Expression of a betaine aldehyde dehydrogenase gene in rice, a glycinebetaine nonaccumulator, and possible localization of its protein in peroxisomes. *Plant J.* 11:1115-1120.

- Neale, A.D., C.K. Bomstedt, P. Bronson, T.N. Le, K. Guthridge, J. Evans, D.F. Gaff, and J.D. Hamill. 2000. The isolation of genes from the resurrection grass *Sporobolus stapfianus* which are induced during severe drought stress. *Plant Cell Environ.* 23:265-277.
- Nishitani, K. and Y. Masuda. 1982. Auxin-induced changes in the cell wall xyloglucans: Effects of auxin on the two different subfractions of xyloglucans in the epicotyl cell wall of *Vigna angularis*. *Plant Cell Physiol.* 24:345-356.
- Norman, M.A. and E.P. Fuerst. 1997. Interactions of cations with paraquat in leaf sections of resistant and sensitive biotypes of *Conyza bonariensis*. *Pestic. Biochem. Physiol.* 57:181-191.
- Normanly, J., J.D. Cohen, and G.R. Fink. 1993. *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. *PNAS* 90:10355-10359.
- Normanly, J., P. Grisafi, G.R. Fink, and B. Bartel. 1997. *Arabidopsis* mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene. *Plant Cell* 9:1781-1790.
- Nuccio, M.L., B.L. Russell, K.D. Nolte, B. Bathinasabapathi, D.A. Gage, and A.D. Hanson. 1998. The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. *Plant J.* 16:487-496.
- Okamoto, H., Y. Tanaka, and S. Sakai. 1995. Molecular cloning of the cDNA of an auxin-regulated calmodulin gene. *Plant Cell Physiol.* 36:1531-1539.
- Olson, D.C., J.H. Oetiker, and S.F. Yang. 1995. Analysis of *LE-ACS3*, a 1-aminocyclopropane-1-carboxylic acid synthase gene expressed during flooding in the roots of tomato plants. *J. Biol. Chem.* 270:14056-14061.
- Ozeki, Y., A. Komamine, and Y. Tanaka. 1990. Induction and repression of phenylalanine ammonia-lyase and chalcone synthase enzyme proteins and mRNA in carrot cell suspension cultures regulated by 2,4-D. *Physiol. Plant.* 78:400-408.
- Palme, K. and L. Galweiler. 1999. PIN-pointing the molecular basis of auxin transport. *Cur. Op. Plant Biol.* 2:375-381.
- Papageorgiou, G.C. and N. Murata. 1995. The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. *Photosynthesis Res.* 44:243-252.

- Pappas, D.L. and M. Hampsey. 2000. Functional interaction between Ssu72 and the Rpb2 subunit of RNA polymerase II in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 20:8343-8351.
- Pasquali, G., O.J.M. Goddijn, A. de Waal, R. Verpoorte, R.A. Schilperoort, H.J.C. Hoge, and J. Memelink. 1992. Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. *Plant Mol. Biol.* 18:1121-1131.
- Peck, S.C. and H. Kende. 1995. Sequential induction of ethylene biosynthetic enzymes by indole-3-acetic acid in etiolated pea. *Plant Mol. Biol.* 28:293-301.
- Peniuk, M.G., M.L. Romano, and J.C. Hall. 1993. Physiological investigations into the resistance of a wild mustard (*Sinapis arvensis* L.) Biotype to auxinic herbicides. *Weed Res.* 33:431-436.
- Perez-Amador, M.A., J. Carbonell, and A. Granell. 1995. Expression of arginine decarboxylase is induced during early fruit development and in young tissues of *Pisum sativum* (L.). *Plant Mol. Biol.* 28:997-1009.
- Pickett, F.B., A. K. Wilson and M. Estelle. 1990. The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance, *Plant Physiol.* 94:1462-1466.
- Plesch, G., K. Störmann, J.T. Torres, R. Walden, and I.E. Somssich. 1997. Developmental and auxin-induced expression of the *Arabidopsis prha* homeobox gene. *Plant J.* 12:635-647.
- Prasad, K.V.S.K., P. Sharmila, and P. P. Saradhi. 2000a. Enhanced tolerance of transgenic *Brassica juncea* to choline confirms successful expression of the bacterial *codA* gene. *Plant Sci.* 159:233-242.
- Prasad, K.V.S.K., P. Sharmila, P.A. Kumar, and P.P. Saradhi. 2000b. Transformation of *Brassica juncea* (L.) Czern with a bacterial *codA* gene enhances its tolerance to salt stress. *Mol. Breed.* 6:489-499.
- Rajendrakumar, C.S.V., T. Suryanarayana, and A.R. Reddy. 1997. DNA helix destabilization by proline and betaine: possible role in the salinity tolerance process. *FEBS Lett.* 410:201-205
- Rashotte, A.M., A. DeLong, and G.K. Muday. 2001. Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. *Plant Cell* 13:1683-1697.

- Ratcliffe, O.J., J.L. Riechmann, and J.Z. Zhang. 2000. INTERFASCICULAR FIBERLESS1 is the same gene as REVOLUTA. *Plant Cell* 12:315-317.
- Rathinasabapathi, B., K.F. McCue, D.A. Gage, and A.D. Hanson. 1994. Metabolic engineering of glycine betaine synthesis: plant betaine aldehyde dehydrogenases lacking typical transit peptides are targeted to tobacco chloroplasts where they confer betaine aldehyde resistance. *Planta* 193:155-162.
- Rathinasabapathi, B., M. Burnet, B.L. Russel, D.A. Gage, P.C. Liao, G.J. Nye, P. Scott, J.H. Golbeck, and A.D. hanson. 1997. Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of glycine betaine synthesis in plants: prosthetic group characterization and cDNA cloning. *PNAS* 94:3454-3458.
- Rayle, D.L. and R.E. Cleland. 1992. The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* 99:1271-1274.
- Reddy, A.N., P.K. Jena, S.K. Mukherjee, and B.W. Poovaiah. 1990. Molecular cloning of cDNAs for auxin-induced mRNAs and developmental expression of the auxin-inducible genes. *Plant Mol. Biol.* 14:643-653.
- Reddy, A.N. and B.W. Poovaiah. 1990. Molecular cloning and sequencing of a cDNA for an auxin-repressed mRNA: correlation between fruit growth and repression of the auxin-regulated gene. *Plant Mol. Biol.* 14:127-136.
- Revilla, G., and I. Zarra. 1987. Changes in the molecular weight distribution of the hemicellulosic polysaccharides from rice coleoptiles growing under different conditions. *J. Exp. Bot.* 38:1818-1825.
- Rhodes, D. and A.D. Hanson. 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 44:357-384.
- Roje, S., W. Hong, S.D. McNeil, R.K. Raymond, D.R. Appling, Y. Shachar-Hill, H.J. Bohnert, A.D. hanson, and H. Wang. 1999. Isolation, characterization, and functional expression of cDNAs encoding NADH-dependent methylenetetrahydrofolate reductase from higher plants. *J. Biol. Chem.* 274:36089-36096.
- Rottmann, W.H., G.F. Peter, P.W. Oeller, J.A. Keller, N.F. Shen, B.P. Nagy, L.P. Taylor, A.D. Campbell, and A. Theologis. 1991. 1-Aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. *J. Mol. Biol.* 222:937-961.

- Roux, C., J. Bilang, B.H. Theunissen, and C. Perrot-Rechenmann. 1998. Identification of new early auxin markers in tobacco by mRNA differential display. *Plant Mol. Biol.* 37:385-389.
- Rubinstein, B. and E.N. Light. 1973. Indoleacetic-acid-enhanced chloride uptake into coleoptile cells. *Planta* 110:43-56.
- Ruegger, M., E. Dewey, L. Hobbie, D. Brown, P. Bernasconi, J. Turner, G. Muday, and M. Estelle. 1997. Reduced naphthylphthalamic acid binding in the *tir3* mutant of *Arabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. *Plant Cell* 9:745-757.
- Russell, B.L., B. Rathinasabapathi, and A.D. Hanson. 1998. Osmotic stress induces expression of choline monooxygenase in sugar beet and amaranth. *Plant Physiol.* 116:859-865.
- Ryan, G.F. 1970. Resistance of Common Groundsel to Simazine and Atrazine. *Weed Sci.* 18:614-616
- Sabba, R. P., T. Sterling M., and N. Lownds K. 1998. Effect of picloram on resistant and susceptible yellow starthistle (*Centaurea solstitialis*): the role of ethylene. *Weed Sci.* 46: 297-300.
- Sakai, T., Y. Takahashi, and T. Nagata. 1996. Analysis of the promoter of the auxin-inducible gene, *parC*, of tobacco. *Plant Cell Physiol.* 37:906-913.
- Sakamoto, A., Alia, and N. Murata. 1998. Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. *Plant Mol. Biol.* 38:1011-1019.
- Sakamoto, A. and N. Murata. 2000. Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. *J. Exp. Bot.* 51:81-88.
- Sakamoto, A. and N. Murata. 2001. The use of bacterial choline oxidase, a glycinebetaine synthesizing enzyme, to create stress-resistant transgenic plants. *Plant Physiol.* 125:180-188.
- Salisbury, F.B. and C.W. Ross. 1992. *Plant Physiology*. Belmont, CA: Wadsworth, Inc.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

- Schachtman, D. and W.H. Liu. 1999. Molecular pieces to the puzzle of the interaction between potassium and sodium uptake in plants. *Trends Plant Sci.* 4:281-287.
- Schlicher, T. and J. Soll. 1997. Chloroplastic isoforms of DnaJ and GrpE in pea. *Plant Mol. Biol.* 33:181-185.
- Schmitt, R., and H. Sandermann. 1982. Specific localisation of β -D-glucoside conjugates of 2,4-dichlorophenoxyacetic acid in soybean vacuoles. *Z. Naturforsch* 37:772-777.
- Schroeder, J.I. 1995. Anion channels as central mechanisms for signal transduction in guard cells and putative functions in roots for plant-soil interactions. *Plant Mol. Biol.* 28:353-361.
- Sedbrook, J.C., C.R. Jin, P.H. Masson, and R.J. Chen. 1999. ARG1 (altered response to gravity) encodes a DnaJ-like protein that potentially interacts with the cytoskeleton. *PNAS* 96:1140-1145.
- Shen, Y. and S. Chen. 2001. Direct submission to GenBank. Accession #AF270651
- Shimomura, S., S. Watanabe, and H. Ichikawa. 1999. Characterization of auxin-binding protein 1 from tobacco: content, localization and auxin-binding activity. *Planta* 209:118-125.
- Shinshi, H., D. Mohnen, and F. Meins. 1987. Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and actinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *PNAS* 84:89-93.
- Sitbon, F., and C. Perrot-Rechenmann. 1997. Expression of auxin-regulated genes. *Physiol. Plant.* 100:443-455.
- Sitrit, Y. and A.B. Bennett. 1998. Regulation of tomato fruit polygalacturonase mRNA accumulation by ethylene: a reexamination. *Plant Physiol.* 116:1145-1150.
- Sivakumaran, K. 1992. Characteristics of differential herbicide response in sulfonylurea-resistant *Kochia scoparia* accessions. M.S. thesis. Montana State University-Bozeman.
- Slakeski, N., and G.B. Fincher. 1992. Barley (1,3,1,4)- β -glucanase isozyme E1 gene expression is mediated by auxin and gibberellic acid. *FEBS Lett.* 306:98-102.

- Snow, A.A., P.P. Moran, L.H. Riesenbert, A. Wszelaki, and G.J. Seiler. 1998. Fecundity, phenology, and seed dormancy of F1 wild-crop hybrids in sunflower (*Helianthus annuus*, Asteraceae). *Am. J. Bot.* 85: 794-801.
- Solymosi, P., Z. Kostyal, and A. Gimesi. 1987. Resistance of *Cirsium arvense* (L.) Scop. to phenoxyacetic acid derivatives. *Novenyvedelem.* 23: 301-305.
- Somssich, I.E., and K. Hahlbrock. 1998. Pathogen defence in plants - a paradigm of biological complexity. *Trends Plant Sci.* 3:86-90.
- Stafstrom, J.P. and I.M. Sussex. 1992. Expression of a ribosomal protein gene in axillary buds of pea seedlings. *Plant Physiol.* 100:1494-1502.
- Sterling, T.M. and J.C. Hall. 1997. Mechanism of action of natural auxins and the auxinic herbicides. Pages 111-141 in R.M. Roe, J.d. Burton, and R.J. Kuhr, eds. *Herbicide Activity: Toxicology, Biochemistry, and Molecular Biology*. Amsterdam: IOS Press.
- Strizhov, N., E. Abraham, L. Okresz, S. Blickling, A. Zilberstein, J. Schell, C. Koncz, and L. Szabados. 1997. Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. *Plant J.* 12:557-569.
- Summers, P.S. and E.A. Weretilnyk. 1993. Choline synthesis in spinach in relation to salt stress. *Plant Physiol.* 103:1269-1276.
- Suzuki, H., T. Wagner, and M.L. Tierney. 1993. Differential expression of two proline-rich soybean genes (*Glycine max* L.) after wounding. *Plant Physiol.* 101:1283-1287.
- Taha, R., D. Mills, Y. Heimer, and M. Tal. 2000. The relation between low K^+/Na^+ ratio and salt tolerance in the wild tomato *Lycopersicon pennellii*. *Plant Physiol.* 157:59-64.
- Takahashi, Y., M. Kusaba, Y. Hiraoka, and T. Nagata. 1991. Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. *Plant J.* 1:327-332.
- Takahashi, Y., and T. Nagata. 1992. *parB*: An auxin-regulated gene encoding glutathione S-transferase. *PNAS* 89:56-59.

- Tamminen, I., P. Makela, P. Heino, and E.T. Palva. 2001. Ectopic expression of ABI3 gene enhances freezing tolerance in response to abscisic acid and low temperature in *Arabidopsis thaliana*. *Plant J.* 25:1-8.
- Tanaka, Y., H. Chisaka, and H. Saka. 1986. Movement of paraquat in resistant and susceptible biotypes of *Erigeron philadelphicus* and *E. canadensis*. *Physiol. Plant.* 66:605-608.
- Theologis, A., T.V. Huynh, and R.W. Davis. 1985. Rapid induction of specific mRNA by auxin in pea epicotyl tissue. *J. Mol. Biol.* 183:53-68.
- Thomine, S., F. Levievre, M. Boufflet, J. Guern, and H. Barbier-Brygoo. 1997. Anion-channel blockers interfere with auxin responses in dark-grown *Arabidopsis* hypocotyls. *Plant Physiol.* 115:533-542.
- Thornburg, R.W., and X. Li. 1990. Wounding *Nicotiana tabacum* leaves causes a decline in endogenous indole-3-acetic acid. *Plant Physiol.* 96:802-805.
- Tillmann, U., G. Viola, B. Kayser, G. Siemeister, T. Hesse, K. Palme, M. Löbler, and D. Klämbt. 1989. cDNA clones of the auxin-binding protein from corn coleoptiles (*Zea mays* L.): isolation and characterization by immunological methods. *EMBO J.* 8:2463-2467.
- Timmerman, K.P. 1989. Molecular characterization of corn glutathione S-transferase isozymes involved in herbicide detoxication. *Physiol. Plant.* 77:465-471.
- Trebitsh, T., J.E. Staub, and S.D. O'Neill. 1997. Identification of a 1-aminocyclopropane-1-carboxylic acid synthase gene linked to the *Female* (*F*) locus that enhances female sex expression in cucumber. *Plant Physiol.* 113:987-995.
- Troughton, J., and L.A. Donaldson. 1972. *Probing Plant Structure*. McGraw-Hill, New York.
- Tucker, M.L., r. Sexton, E. del Campillo, and L.N. Lewis. 1988. Bean abscission cellulase. Characterization of a cDNA clone and regulation of gene expression by ethylene and auxin. *Plant Physiol.* 88:1257-1262.
- Tyerman, S.D. 1992. Anion channels in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 43:351-373.
- Utsuno, K., T. Shikanai, Y. Yamada, and T. Hashimoto. 1998. *AGR*, an *Agravitropic* locus of *Arabidopsis thaliana*, encodes a novel membrane protein family member. *Plant Cell Physiol.* 39:1111-1118.

- van der Straeten, D., R.A. Rodrigues-Pousada, R. Villarroel, S. Hanley, H.M. Goodman, and M. van Montagu. 1992. Cloning, genetic mapping, and expression analysis of an *Arabidopsis thaliana* gene that encodes 1-aminocyclopropane-1-carboxylate synthase. PNAS 89:9969-9973.
- van der Zaal, E.J., J. Memelink, A.M. Mennes, A. Quint, and K.R. Libbenga. 1987. Auxin-induced mRNA species in tobacco cell cultures. Plant Mol. Biol. 10:145-157.
- van der Zaal, E.J., F.N. Droog, C.J.M. Boot, L.A. Hensgens, J.H. Hogé, R.A. Schilperoort, and K. R. Libbenga. 1991. Promoters of auxin-induced genes from tobacco can lead to auxin-inducible and root tip-specific expression. Plant Mol. Biol. 16:983-998.
- Vera, P., C. Lamb, and P.W. Doerner. 1994. Cell-cycle regulation of hydroxyproline-rich glycoprotein *HRGPnt3* gene expression during the initiation of lateral root meristems. Plant J. 6:717-727.
- Vera-Estrella, R., B.J. Barkla, H.J. Bohnert, and O. Pantoja. 1999. Salt stress in *Mesembryanthemum crystallinum* L. cell suspensions activates adaptive mechanisms similar to those observed in the whole plant. Planta 207:426-435.
- Verhoeff, B.J., M.D. Trip, M.H. Prins, J.J.P. Kastelein, and P.H. Reitsma. 1998. The effect of a common methylenetetrahydrofolate reductase mutation on levels of homocysteine, folate, vitamin B12 and on the risk of premature atherosclerosis. Atherosclerosis 141:161-166.
- Vermass, W.F.J., and C.J. Arntzen. 1983. Synthetic quinones influencing herbicide binding and photosystem II electron transport: the effects of triazine-resistance on quinone binding properties in thylakoid membranes. Biochem. Biophys. Acta 725:483-491.
- Vögelie-Lange, R., C. Fründt, C.M. Hart, F. Nagy, and F. Meins. 1994. Developmental, hormonal, and pathogenesis-related regulation of the tobacco class I β -1,3-glucanase B promoter. Plant Mol. Biol. 25:299-311.
- Wang, T.W. and R.N. Artica. 1995. Identification and characterization of cDNAs encoding ethylene biosynthetic enzymes from *Pelargonium x hortorum* cv Snow Mass leaves. Plant Physiol. 109:627-636.
- Wang, Y., Y.L. Meng, H. Ishikawa, T. Hibino, Y. Tanaka, N. Nii, and T. Takabe. 1999. Photosynthetic adaptation to salt stress in three-color leaves of a C_4 plant *Amaranthus tricolor*. Plant Cell Physiol. 40:668-674.

- Watillon, B., R. Kettmann, A. Arredouani, J.F. Hecquet, P. Boxus, and A. Burny. 1998. Apple messenger RNAs related to bacterial lignostilbene dioxygenase and plant SAUR genes are preferentially expressed in flowers. *Plant Mol. Biol.* 36:909-915.
- Webb, S.R. and J.C. Hall. 1995. Auxinic-herbicide resistant and susceptible wild mustard (*Sinapis arvensis* L.) biotypes: effect of auxinic herbicides on seedling growth and auxin binding activity. *Pestic. Biochem. Physiol.* 52:137-148.
- Wei, Y.D., H.G. Zheng, and J.C. Hall. 2000. Role of auxinic herbicide-induced ethylene on hypocotyl elongation and root/hypocotyl radial expansion. *Pest. Manag. Sci.* 56:377-383.
- Weretilnyk, E.A. and A.D. Hanson. 1990. Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. *PNAS* 87:2745-2749.
- Weretilnyk, E.A., D.D. Smith, G.A. Wilch, and P.S. Summers. 1995. Enzymes of choline synthesis in spinach. Response of phospho-base *N*-methyltransferase activities to light and salinity. *Plant Physiol.* 109:1085-1091.
- Weretilnyk, E.A., K.J. Alexander, M. Drebenstedt, J.D. Snider, P.S. Summers, and B.A. Moffatt. 2001. Maintaining methylation activities during salt stress. The involvement of adenosine kinase. *Plant Physiol.* 125:856-865.
- Whitehead, C. W., and C. M. Switzer. 1963. The differential response of strains of wild carrot to 2,4-D and related herbicides. *Can. J. Plant Sci.* 43 (3) : 255-262
- Wolf, O., Munns, R., M.L. Tonnet, and W.D. Jeschke. 1991. The role of the stem in the partitioning of Na⁺ and K⁺ in salt-treated barley. *J. Exp. Bot.* 42:697-704.
- Wood, A.J., H. Saneoka, D. Rhodes, R.J. Joly, and P.B. Goldsbrough. 1996. Betaine aldehyde dehydrogenase in sorghum. *Plant Physiol.* 110:1301-1308.
- Wu, S.C., J.M. Blumer, A. Darvill, and P. Albersheim. 1996. Characterization of an endo- β -1,4-glycanase gene induced by auxin in elongating pea epicotyls. *Plant Physiol.* 110:163-170.

- Xie, Qi., G. Frugis, D. Colgan, C. NamHai, Q. Xie, and N.H. Chua. Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes and Develop.* 14:3024-3036.
- Xu, W., M.M. Purugganan, D.H. Polisensky, D. Antosiewicz, S.C. Fry, and J. Braam. 1995. Arabidopsis *TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *Plant Cell* 7:1555-1567.
- Xu, W., P. Campbell, A.K. Vargheese, and J. Braam. 1996. The *Arabidopsis* *XET*-related family: Environmental and hormonal regulation of expression. *Plant J.* 9:879-889.
- Yamamoto, M., H. Mori, and H. Imaseki. 1992. Novel mRNA sequences induced by indole-3-acetic acid in sections of elongating hypocotyls of mung bean (*Vigna radiata*). *Plant Cell Physiol.* 33:13-20.
- Yamamoto, M. and K.T. Yamamoto. 1998. Differential effects of 1-naphthaleneacetic acid, indole-3-acetic acid, and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin-resistant mutant of Arabidopsis, *aux1*. *Plant Cell Physiol.* 39:660-664.
- Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus, and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. *Science* 217:1214-1222.
- Yancey, P.H. 1994. *In* Cellular and molecular physiology of cell volume regulation. CRC Press, Boca Raton, FL.
- Yoon, I.S., D.H. Park, H. Mori, H. Imaseki, and B.G. Kang. 1992. Characterization of an auxin-inducible 1-aminocyclopropane-1-carboxylate synthase gene, VR-ACS6, of mungbean (*Vigna radiata* (L.) Wilczek) and hormonal interactions on the promoter activity in transgenic tobacco. *Plant Cell Physiol.* 40:431-438.
- Yoon, I.S., H. Mori, J.H. Kim, B.G. Kang, and H. Imaseki. 1997. VR-ACS6 is an auxin-inducible 1-aminocyclopropane-1-carboxylate synthase gene in mugbean (*Vigna radiata*). *Plant Cell Physiol.* 38:217-224.
- Yoshida, Y., T. Kiyosue, T. Katagiri, H. Ueda, T. Mizoguchi, S.K. Yamaguchi, K. Wada, Y. Harada, and K. Shinozaki. 1995. Correlation between the induction of a gene for Δ -1-pyrroline-5-carboxylate synthetase and the accumulation of proline in Arabidopsis thaliana under osmotic stress. *Plant J.* 7:751-760.

- Yoshida, S., M. Ito, I. Nishida, and A. Watanabe. 2001. Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in *Arabidopsis thaliana*. *Plant Cell. Physiol.* 42:170-178.
- Zegzouti, H., C. Marty, B. Jones, T. Bouquin, A. Latche, J.C. Pech, and M. Bouzayen. 1997. Improved screening of cDNAs generated by mRNA differential display enables the selection of true positives and the isolation of weakly expressed messages. *Plant Mol. Biol. Rep.* 15:236-245.
- Zegzouti, H., B. Jones, P. Frasse, C. Marty, B. Maitre, A. Latche, J.C. Pech, and M. Bouzayen. 1999. Ethylene-regulated gene expression in tomato fruit: characterization of novel ethylene-responsive and ripening-related genes isolated by differential display. *Plant J.* 18:589-600.
- Zhong, R., J.J. Taylor, and Z.H. Ye. 1997. Disruption of interfascicular fiber differentiation in an *Arabidopsis* mutant. *Plant Cell* 9:2159-2170.
- Zhong, R., J.J. Taylor, and Z.H. Ye. 1999. Transformation of the collateral bundles into amphivasal vascular bundles in an *Arabidopsis* mutant. *Plant Physiol.* 120:53-64.
- Zhong, R. and Z.H. Ye. 2001. Alteration of auxin polar transport in the *Arabidopsis ifl1* Mutants. *Plant Physiol.* 126:549-563.

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