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


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 NaCI 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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Anthony John Kern

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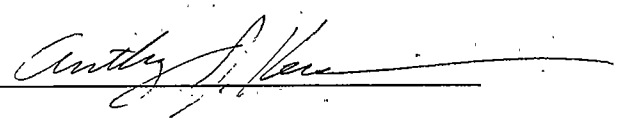
Signature 

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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*-phosophonomethyl 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) phenoxyl] 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).

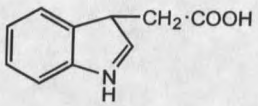
Under conditions of extensive herbicide selection pressure, the evolution and increase of herbicide-resistant plants is highly likely (Maxwell and Mortimer, 1994). In most cases, inheritance of resistance can be explained by the action of a single dominant gene, although recessive (loss of function) 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

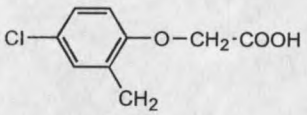
Auxinic herbicides have long played a key role in controlling dicot weed species in grass crops. Although the precise mode of action of the individual auxinic herbicides is unknown, it is widely thought that they act as synthetic versions of the phytohormone auxin (indole-3-acetic acid, IAA; Sterling 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 phenoxycarboxylic 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-

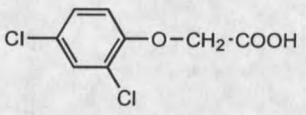



indole-3-acetic acid

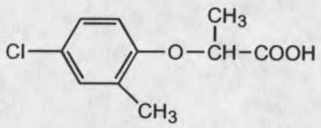
**Phenoxycarboxylic acids**



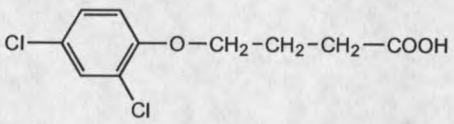

MCPA




2,4-D

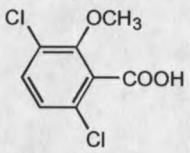



mecoprop

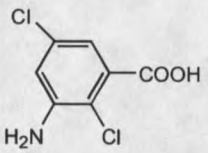



2,4-DB

**Benzoic acid derivatives**

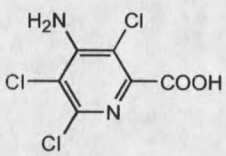



dicamba

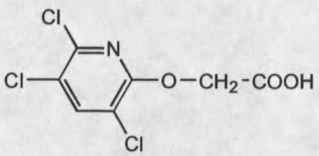



chloramben

**Pyridine carboxylic acids**

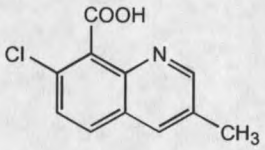



picloram

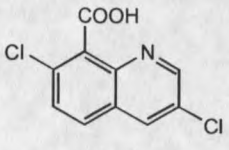



triclopyr

**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 $^{3}$H-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 (Löbler 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-napthaleneacetic 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 permutations 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-napthylphtalamic 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 *interfasicular 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 |
|---|---|---|---|---|---|
| Genes encoding DNA binding proteins | | | | | |
| *PS/IAA4/5* | transcription factor | *Pisum* hypocotyls | 20 µM IAA, NAA, 2,4-D | 10 min | Theologis et al., 1985 |
| *DBP* | histone | Arabidopsis peduncles | 1 µM NAA | <4 hr | Aliotte et al., 1989 |
| *H3C-11* | Histone | *Medicago* callus | 100 µM 2,4-D | <1 hr | Kapros et al., 1992 |
| *AT-IAA1* | transcription factor | Arabidopsis seedlings | 20 µM IAA, NAA, 2,4-D | 4 min | Abel et al., 1995 |
| *SGBF-2* | G-box factor | *Glycine* hypocotyls | 23 µM 2,4-D | <2 hr | Hong et al., 1995 |
| *ATHB-8* | homeobox protein | Arabidopsis leaves | 10 µM IAA, NAA, 2,4-D | <1 hr | Baima et al., 1996 |
| Genes encoding calcium-modulated proteins | | | | | |
| *PCM-1* | calmodulin | *Fragaria* fruit | 1 mM NAA | 12 hr | Jena et al., 1989 |
| *MBCAM-1* | calmodulin | *Vicia* leaves | 500 µM IAA | <3 hr | Botella and Ateca, 1994 |
| *TCH3* | calcium-binding protein | Arabidopsis leaves | 1 µM IAA | <30 min | Antosiewicz et al., 1995 |
| *ARCAM* | calmodulin | *Vicia* hypocotyls | 1 µM IAA | <1 hr | Okamoto et al., 1995 |
| *VR-CDPK1* | Ca-dependent kinase | *Vicia* seedlings | 500 µM IAA | 3 hr | Botella et al., 1996 |
| Genes encoding cell cycle-associated proteins | | | | | |
| *CDC2* | CDC2 protein kinase | Arabidopsis roots | 0.1 µM IAA | <2 d | Martinez et al., 1990 |
| *CDC2MS* | CDC2 protein kinase | *Medicago* cell culture | 100 µM 2,4-D (pulse) | >8 hr | Hirt et al., 1993 |
| *P34CDC2* | CDC2 protein kinase | *Pisum* roots | 50 µM IAA | <1 hr | John et al., 1993 |
| *CDC2-S5, CDC2-S6* | CDC2 protein kinase | *Glycine* roots | 0.5 mM NAA | <24 hr | Miao et al., 1993 |
| Genes encoding cell wall-associated protiens and hydrolytic enzymes | | | | | |
| *EII* | β-glycanase | *Hordeum* alurone | 5 µM IAA | <20 hr | Slakeski and Fincher, 1992 |
| *DCPRP1* | proline-rich protein | *Daucus* roots | 10 µM IAA, NAA, 2,4-D | 3-24 hr | Ebener et al., 1993 |
| *SBPRP2* | proline-rich protein | *Glycine* roots | 10 µM IAA, NAA, 2,4-D | <20 hr | Suzuki et al., 1993 |
| *HGRPNT3* | hydroxyproline-rich GP | *Nicotiana* roots | 10 µM IAA | 1-2 hr | Vera et al., 1994 |
| *TCH4* | xyloglucan transglycosylase | Arabidopsis seedlings | 1 µM IAA | <10 min | Xu et al., 1995 |
| *EXT* | xyloglucan transglycosylase | Arabidopsis seedlings | 1 µM IAA | 10-30 min | Xu et al., 1996 |
| *EGL1* | β-1,4 glucanase | *Pisum* hypocotyls | 5 µM 2,4-D | <5 hr | Wu et al., 1996 |
| *DD21.4-1* | α-expansin | *Pinus* hypocotyls | 10 µM 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 |
|---|---|---|---|---|---|
| Genes encoding hydrolitic enzymes | | | | | |
| *ATSEH* | epoxide hydrolase | Arabidopsis plants | 100 µM 2,4-D, IAA | <1 hr | Kiyosue et al., 1994 |
| *VR-ACS6* | ACC synthase | *Vigna* hypocotyls | 500 µM IAA | <4 hr | Yoon et al., 1997 |
| *AT-ACS4* | ACC synthase | Arabidopsis seedlings | 20 µM IAA, NAA, 2,4-D | 20 min | Abel et al., 1995 |
| *ADC* | arginine decarboxylase | *Pisum* ovaries | 45 µM 2,4-D | <12 hr | Perez-Armador et al., 1995 |
| Genes encoding oxidative enzymes | | | | | |
| *AOP1* | ascorbate oxidase | *Cucurbita* fruit | 4.5 µM 2,4-D | 24 hr | Esaka et al., 1992 |
| *AAO* | ascorbate oxidase | *Zea* roots | 5 µM 2,4-D | <48 hr | Kerk and Feldman, 1995 |
| *PS-ACO* | ACC oxidase | *Pisum* seedlings | 100 µM IAA | <4 hr | Peck and Kende, 1995 |
| *GERMIN* | oxalate oxidase | *Hordeum* seedlings | 10 µM IAA | <4 hr | Hurkman and Tanaka, 1996 |
| Genes encoding GSTs | | | | | |
| *GH2/4* | GST | *Glycine* hypocotyls | 2.2 µM 2,4-D, NAA, IAA | 15 min | van der Zaal et al., 1991 |
| *CNT103* | GST | *Nicotiana* cell culture | 2.2 µM 2,4-D, NAA, IAA | 15 min | van der Zaal et al., 1987 |
| *PARA* | GST | *Nicotiana* protoplasts | 4.5 µM 2,4-D, NAA, IAA | 10 min | Takahashi et al., 1991 |
| *PARB* | GST | *Nicotiana* protoplasts | 4.5 µM 2,4-D | <20 min | Takahashi and Nagata, 1992 |
| *HMGST-1* | GST | *Hyoscyamum* culture | 10 µM 2,4-D, 2,4,5-T | <24 hr | Bilang and Sturm, 1995 |
| Genes encoding proteins of unknown function | | | | | |
| *SAUR* | | *Glycine* hypocotyls | 100 µM 2,4-D, IAA | 3 min | McClure and Guilfoyle, 1987 |
| *GH3* | | *Glycine* hypocotyls | 100 µM 2,4-D, IAA | <15 min | Hagen et al., 1985 |
| *ARCA* | | *Nicotiana* cell culture | 0.9 µM 2,4-D, NAA | 2 hr | Ishida et al., 1993, 1996 |
| *SAR1, SAR2* | | *Fragaria* fruit | 1 mM NAA | 2 hr | Reddy et al., 1990 |
| *MII-3* | | *Vicia* leaves | 500 µM IAA | <2 hr | Chen et al., 1996 |
| *GO15-13* | | *Nicotiana* seedlings | 1 µM 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 uM

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