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Source: Weed Science, 58(3) : 340-350

Published By: Weed Science Society of America

URL: https://doi.org/10.1614/WS-09-092.1

Weed Science 2010 58:340–350

Herbicides as Probes in Plant Biology

Franck E. Dayan, Stephen O. Duke, and Klaus Grossmann*

Herbicides are small molecules that inhibit specific molecular target sites within plant biochemical pathways and/or physiological processes. Inhibition of these sites often has catastrophic consequences that are lethal to plants. The affinity of these compounds for their respective target sites makes them useful tools to study and dissect the intricacies of plant biochemical and physiological processes. For instance, elucidation of the photosynthetic electron transport chain was achieved in part by the use of herbicides, such as terbutryn and paraquat, which act on photosystem II and I, respectively, as physiological probes. Work stemming from the discovery of the binding site of PS II–inhibiting herbicides was ultimately awarded the Nobel Prize in 1988. Although not as prestigious as the seminal work on photosynthesis, our knowledge of many other plant processes expanded significantly through the ingenious use of inhibitors as molecular probes. Examples highlight the critical role played by herbicides in expanding our understanding of the fundamental aspects of the synthesis of porphyrins and the nonmevalonate pathway, the evolution of acetyl-coenzyme A carboxylase, cell wall physiology, the functions of microtubules and the cell cycle, the role of auxin and cyanide, the importance of subcellular protein targeting, and the development of selectable markers.

Key words: Molecular probe, herbicides as tools, chemical genetics, proteomics, metabolomics, genomics, mode of action.

Herbicides are small molecules (molecular mass 700 Da or less) that normally do not have intrinsic toxicities. That is, in most cases, these molecules do not cause injury to plants by simply being present in the tissues, such as would be the case with photodynamic compounds (e.g., rose bengal) or radioactive molecules. Instead, herbicidal compounds inhibit specific molecular target sites within critical plant biochemical and/or physiological pathways, and those inhibitions often have catastrophic and lethal consequences. Consequently, most herbicide target sites are enzymes involved in primary metabolic pathways (i.e., processes that are necessary for the growth and development of an organism) or proteins carrying out essential physiological functions. In contrast, inhibition of target sites involved in secondary metabolism is less likely to be lethal because, by definition, those processes are not necessary for sustaining life, at least during the period that a herbicide is expected to work. Nevertheless, the destabilization of primary metabolism caused by herbicides often has downstream repercussions on secondary metabolism, which has been useful for probing some of these pathways.

Generally, herbicides and other toxins have been identified because of their high, specific affinity for their respective target sites. Thus, herbicides have been classified based on their mode of action, which includes at least 16 main characterized mechanisms that encompass all major plant functions, such as photosynthesis; fatty acid, amino acid, protein, and pigment synthesis; the hormone system; and the cell cycle and cell wall formation. The specific nature of the interaction between herbicides and their respective target sites makes the judicious use of these molecules an important tool for studying and dissecting the intricacies of plant biochemical and physiological processes. Because of the scope of the topic and the wealth of information in the literature, this review will only highlight some notable examples of the use of herbicides as molecular probes in plant biology. However, one could readily argue that herbicides have provided unique insights to most, if not all, areas in plant science.

DOI: 10.1614/WS-09-092.1

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Dissecting the Photosynthetic Electron Transport

Photosynthesis, the process of converting light energy to chemical energy, is divided into two broad phases: the light reaction (Hill reaction) and the dark reaction (Calvin cycle). Although no inhibitor of the Calvin cycle has been developed into a herbicide (which is, in itself, interesting), many inhibitors of the light reaction have been commercialized as herbicides. As a result, photosynthesis and herbicide research have had a long, common history.

The light reaction is compartmentalized within the lipid environment of the thylakoid membranes of the chloroplasts. Photosystem II (PS II) catalyzes the energy-demanding, light-driven splitting of water, which releases O_2 and provides the reducing equivalents (electrons) required for the conversion of CO_2 into chemical energy (Figure 1). The reaction center of purple bacteria (*Rhodobacter sphaeroides*), which consists of a large, dimeric complex (700 kDa) consisting of 19 subunits and at least 57 different cofactors, is a functional and structural homolog to higher plant PS II. Crystallization of the bacterial homolog was elemental to the elucidation of the structure of PS II (Michel and Deisenhofer 1988). However, the judicious use of PS II–inhibiting herbicides played a key role in expanding our understanding of the photosynthetic electron transport (Draber et al. 1991; Trebst 2007). In fact, Michel and Deisenhofer were awarded the Noble Prize in 1988 for their studies of the photosynthetic electron transport system in purple bacteria, which were dependent on research on the binding site of PS II–inhibiting herbicides (Michel and Deisenhofer 1988).

In other seminal studies, radiolabeled azido derivatives of PS II–inhibiting herbicides, used as photoaffinity labeling probes, led to the discovery that a 32-kDa protein in isolated photosynthetic membranes was part of PS II (Pfister et al. 1981). Proteolysis of the azidotriazine-labeled protein determined that atrazine binds to Met 214 of the D1 protein. In a similar experiment, sequencing of the D1 protein after azidomonuron or azidocyanophenol photo labeling demon-

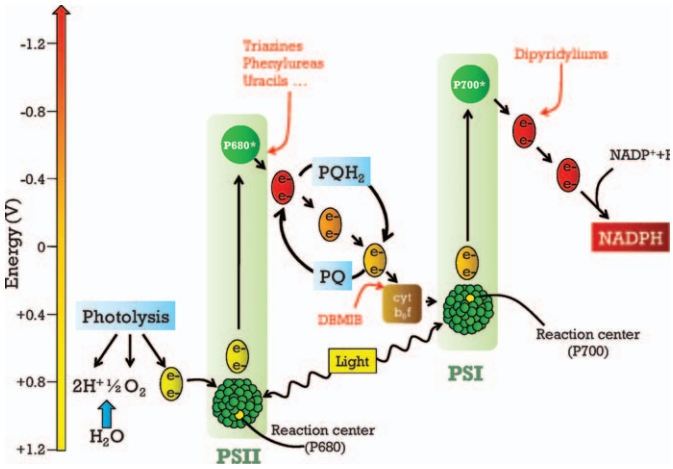

Figure 1. Diagram of the Z-scheme describing the Hill reaction. The sites of herbicide interactions are indicated with the red arrows. The color of the electrons is indicative of their relative energy in the pathway, based on the reference arrow on the left of the figure.

strated that Tyr 237 and 254 and Val 246 were other critical amino acids in the plant Q_B binding pocket (Dostatni et al. 1988; Oettmeier et al. 1989). Herbicides were also used to probe the plastoquinone binding site on PS II (Ohad and Hirschberg 1992). All of these observations were subsequently confirmed in various crystal structures of bacterial PS II complexed with herbicides (Lancaster and Michel 1999).

The highly specific interaction of herbicides with their binding site also led to the discovery that quinones were the primary electron acceptors involved in electron transfer from PS II to the cytochrome b6f complex in a cyclic redox of the plastoquinone/hydroplastoquinone pool (Witt 1973). Herbicides were essential for the understanding of the features of a photosystem, such as the elucidation of the pathway of light excitation of PS II from varying antennae surrounding the reaction centers (Trebst 2007).

Two experimental herbicides, dibromothymoquinone and the dinitrophenyl ether of iodonitrothymol, are potent quinone antagonists that prevent plastohydroquinone oxidation (Draber et al. 1970; Trebst et al. 1978). These specific inhibitors of the cytochrome b6f complex were instrumental in establishing that there are two energy-conserving sites in the electron-flow system (two proton transduction sites through the membrane that couple to adenosine triphosphate [ATP] synthesis) and that there is artificial proton pumping through the membrane possibly coupled to ATP formation, thereby proving the existence of electron flow and validating the chemiosmotic theory (Draber et al. 1991).

In addition to providing important information in the characterization of PS II, herbicides also played a key role in identifying PS I reactions. PS II inhibitors specifically block electron transfer from the Q_B binding site of PS II to plastoquinone, but they have no direct effect on PS I. Therefore, these herbicides enabled the study of the properties of PS I independent of the function of PS II. PS I consists of an assembly of more than 10 Psa proteins, with PsaA and PsaB proteins forming the core of the complex. Electrons transferred from plastoquinone to plastocyanin are accepted by four redox factors in the core protein complex of PS I (Figure 1). Two iron–sulfur clusters embedded within PsaC act as electron carriers to ferredoxin (Brittle 1997; Chitnis 1996). Although the interaction between bipyridylium herbicides, such as paraquat and diquat, and PS I is known to be in the proximity of the PsaC protein, the precise location where these compounds divert electrons from the normal photosynthetic route is still unresolved.

Unlike PS II inhibitors, which directly block the electron transfer by competing for the binding site of the natural electron acceptor (plastoquinone), bipyridylium herbicides interfere with photosynthesis by diverting electrons from PS I, thereby preventing nicotinamide adenine dinucleotide phosphate (NADP) reduction (Hess 2000; Trebst 2007). Also, bipyridyliums have a more intimate participation in the development of phytotoxic symptoms than PS II inhibitors. Bipyridilium herbicides are dication molecules that become highly reactive free radicals upon acceptance of electrons from PS I. These unstable free radicals undergo autoxidation back to the parent ion. The subsequent generation of superoxide and hydroxyl radicals and hydrogen peroxide resulting from the bipyridilium cycle overwhelms the plant's natural quenching mechanisms (Hess 2000). The rapid cycling between the native herbicide ion and its reactive radical, and the high flow rate of electrons in PS I, accounts for the rapid desiccation of foliar tissues treated with such inhibitors (Hess 2000). The ability of bipyridilium herbicides to divert electrons from PS I has been a useful tool to probe the energy levels of the electrons as they are excited by light in PS I.

Isoprenoid Synthesis through the Nonmevalonate Pathway

Terpenoids account for most of the plant secondary metabolites (Gershenzon and Croteau 1991). All terpenoids are derived from the assembly of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate building blocks (Eisenreich et al. 2004), and it was assumed for many years that these C5 precursors originate exclusively from the ubiquitous mevalonic acid pathway (Porter and Spurgeon 1981). However, that paradigm was challenged by several observations made following the discovery of the herbicide clomazone. For instance, clomazone inhibited carotenoid synthesis, but unlike what happens with phytoene desaturase inhibitors, no phytoene or phytofluene accumulated in treated tissues (Duke et al. 1985; Duke et al. 1991). Neither synthesis of protochlorophyllide (PClide) nor phototransformation of PClide to chlorophyllide was affected by clomazone. However, the shift in the light absorption maximum from the phytylation of chlorophyllide to form chlorophyll was greatly inhibited. This striking effect was assumed to be due to inhibition of phytol synthesis (Duke et al. 1985; Duke et al. 1991), something that no other herbicide was reported to do. The possibility that clomazone inhibited the conversion of IPP into geranyl, farnesyl, and geranylgeranyl pyrophosphate was also considered (Croteau 1992). However, the sesquiterpenoids hemigossypol and the dimeric sesquiterpenoid gossypol accumulated in greater amounts in clomazone-treated than in control cotton tissues (*Gossypium hirsutum* L.; Duke et al. 1991). These effects were puzzling because all terpenoids were thought to be produced by the mevalonate pathway at the time this work was conducted.

This mystery began to unravel when a new pathway to IPP synthesis via the 1-deoxy-D-xylulose phosphate (DOXP) was discovered in higher plants (Schwarz 1994). This biosynthetic

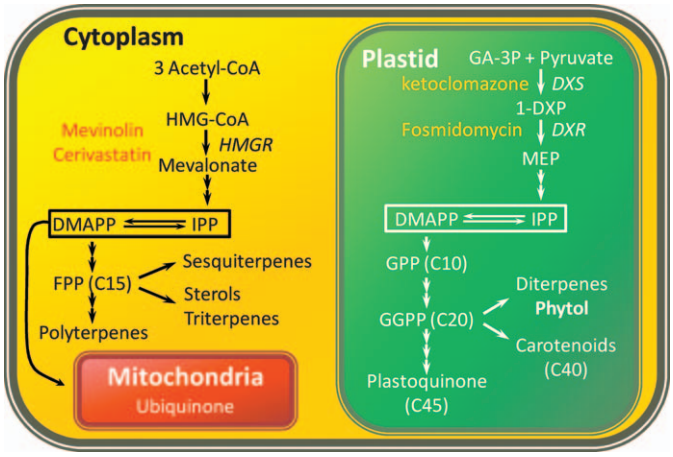

Figure 2. Schematic of the mevalonic acid (MVA) pathway localized in the cytoplasm and the 1-deoxy-D-xylulose 5-phosphate (DOXP) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway compartmentalized in the plastid.

route is now called the DOXP or the methyl-erythritol phosphate (MEP) pathway, in reference to the early pentose intermediates, 1-deoxy-D-xylulose 5-phosphate and 2-C-methyl-D-erythritol 4-phosphate (Eisenreich et al. 1998, 2001, 2004; Rohmer 1999).

This discovery of these two parallel, yet, for the most part, separate, pathways to generate IPP could fully account for the disturbance in the pattern of isoprenoid biosynthesis observed in plants treated with clomazone. Indeed, clomazone is a specific inhibitor of the MEP isoprenoid pathway, which is known to be compartmentalized in plastids (Eisenreich et al. 2004). As a result, levels of plastid-synthesized isoprenoids, such as carotenoids and phytol, are reduced, but the production of cytosol-synthesized secondary terpenoids (e.g., gossypol) is not affected (Figure 2).

Clomazone is the only commercial herbicide to affect the MEP pathway, although it does so indirectly. Clomazone is a proherbicide that must be metabolized in planta to form 5-ketoclomazone. This metabolite is a potent inhibitor of the first step in the plastid-specific, MEP terpenoid pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXS; Ferhatoglu and Barrett 2006; Mueller et al. 2000; Figure 2). Fosmidomycin has a similar effect on the biosynthesis of isoprenoids as clomazone does, but it inhibits the second enzyme in the pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; Kuzuyama et al. 1998; Zeidler et al. 1998).

Ketoclomazone and fosmydomycin are excellent tools to manipulate the MEP pathway. For example, they have been used to probe the biosynthesis pathways involved in the production of various terpenoids in peppermint (*Mentha* × *piperita* L.) oil glands (Lange et al. 2001) and to prove that brassinolide was a product of the nonmevalonate pathway in spotless watermeal [*Wolffia arrhiza* (L.) Horkel ex Wimm.] and *Chlorella vulgaris* (Beijerinck) (Bajguz and Asami 2004, 2005). Clomazone has also been used to study the kinase that catalyzes phosphorylation of 1-deoxy-D-xylulose (Hemmerlin et al. 2006).

Regulation of Porphyrin Synthesis

Porphyrins are essential precursors in the synthesis of chlorophylls and heme. In plants, porphyrins are synthesized in the plastid, mitochondrion, and the cytoplasm, before being chelated with metals (either Mg or Fe), and are subsequently associated with proteins to function in plant metabolism. Considering the absolute requirement of porphyrins and their derivatives to maintain vital plant functions (e.g., photosynthesis, respiration, light sensing), this pathway has been considered a good target for developing new herbicides (Rebeiz et al. 1994).

Most of the enzymatic steps involved in porphyrin biosynthesis proved to be poor target for herbicides, with the notable exception the enzyme protoporphyrinogen oxidase (Protox). This chloroplast, membrane-localized enzyme has proved to be an excellent target site for a structurally diverse group of herbicides (reviewed by Dayan and Duke 2003). The mode of action of Protox inhibitors is unique because inhibition of this enzymatic oxidation of protoporphyrinogen IX (Protogen) into protoporphyrin IX (Proto) results in a dramatic extraplastidic accumulation of the product of the reaction (Dayan and Duke 1997). Accumulation of the highly photodynamic Proto, especially at the plasma membrane (Lehnen et al. 1990), leads to rapid and severe membrane damage when exposed to sunlight. Protox inhibitors have been valuable assets in understanding the regulation of several processes in plants and animals.

For example, the Protox-inhibiting herbicide acifluorfen was used to block porphyrin synthesis, causing Proto to accumulate in darkness (Becerril et al. 1992). A flash of light to transform the small amount of protochlorophyllide to chlorophyllide in these tissues greatly enhanced Proto accumulation, demonstrating that protochlorophyllide is a feedback inhibitor of the chlorophyll pathway. This experiment would not have been possible without a Protox inhibitor to inhibit accumulation of the feedback inhibitor because it allowed Proto to accumulate, demonstrating the amount of carbon flow in the pathway. Before this work, phytochrome was considered the most important photoreceptor for regulation of this pathway.

The unexpected accumulation of Proto resulting from the in vivo inhibition of Protox provided valuable proof that Protox and the next step in porphyrin synthesis (Mg chelatase in the case of chlorophyll, and Fe chelatase in the case of heme) are intimately connected, so that breaking this connection causes the loss of Protogen from the pathway. Protogen is apparently synthesized in the plastid stroma and moves to the plastid envelope where Protox resides. Without conversion to Proto by Protox, Protogen apparently moves on to the cytoplasm, where it is converted to Proto that is not linked to a porphyrin pathway (Lee et al. 1993, 1994). Accumulation of Proto in the plasma membrane in Protox inhibitor–treated plant cells can be seen with fluorescence microscopy (Lehnen et al. 1990).

Protox-inhibiting herbicides are competitive inhibitors and have been useful in understanding the substrate-binding pocket of Protox from both plants and animals (Corradi et al. 2006; Dayan et al. 2009; Koch et al. 2004). Some porphyrias are caused by inadequate Protox and accumulation of toxic levels of porphyrins, including Proto. Because mitochondrial Protox of both plants and animals are inhibited by herbicidal Protox inhibitors, these compounds have been useful in simulating genetic porphyria in animal systems (e.g., Krijt et al. 2003).

It should be noted that although most of the compounds discovered to inhibit other enzymatic steps in the porphyrin

biosynthesis pathway were insufficiently active to be developed into viable commercial herbicides, many of them have been excellent probes to study various steps in the biosynthesis of porphyrins (see Duke and Duke 1997).

Acetyl-Coenzyme A Carboxylase Inhibitors and Evolutionary Biology

Acetyl-CoA carboxylase (ACCase) is a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. The reaction involves the concerted activity of a biotin carboxylase and a carboxyltransferase. ACCase is the first committed step of lipid biosynthesis, providing the malonyl-CoA starter unit in the synthesis of fatty acids. It had been postulated that plants possessed two forms of ACCases: a large multifunctional eukaryotic protein in the cytoplasm, and a multisubunit prokaryotic form in the plastids (Kannanga and Stumpf 1972). However, that hypothesis was abandoned because the prokaryotic form of the enzyme was never identified in plants. Around the same time, in the 1970s, several classes of herbicides (e.g., aryloxyphenoxypropionate and cyclohexanedione) were developed to control grass weeds in dicotyledonous crops. However, the basis for selectivity between grasses and other plants remained unexplained for several years. These two mysteries were solved when the forms of acetyl-CoA carboxylase present in plants were surveyed by detecting the biotinylated subunits of ACCase in 28 plant families (Konishi et al. 1996). All the species studied contained the large (220 kDa), biotinylated, eukaryotic ACCase polypeptide. All plants also contained a small (ca. 35 kDa), biotinylated subunit of the prokaryotic ACCase in the plastids, with the exception of the grass species, which possessed only the eukaryotic form in both the cytoplasm and the plastids (Figure 3A).

The cytoplasmic, multifunctional eukaryotic form of ACCase is sensitive to herbicides, whereas the plastidic, multisubunit prokaryotic form is herbicide resistant. Therefore, these herbicides specifically control grasses because these plants only have the herbicide-sensitive, multifunctional eukaryotic form in both the cytoplasm and the plastids (Alban et al. 1994; Konishi and Sasaki 1994; Figure 3B).

This very unusual situation may be the first example of substitution of a plastid gene by a nuclear gene for a nonribosomal component (Konishi et al. 1996). The replacement of the prokaryotic ACCase gene (accD) by the eukaryotic ACCase in plastids provides new evidence relevant to the evolution of plastid genomes. Plastid genomes seem to have been under selective pressure to be compact and to lose genes. Thus, many proteins from the ancestral plastid genome are now encoded by nuclear genes. This seems to be the case for the deletion of accD in the plastid genome of Gramineae. However, its replacement by a nuclear gene may be the first example of such replacement in the case of a gene for a nonribosomal component.

Finally, a recent study (Délye et al. 2004), investigating the nucleotide variability of the ACCase gene in blackgrass (*Alopecurus myosuroides* Huds.) populations, identified, for the first time, the evolutionary signature of herbicide selection in natural populations. Most nonsynonymous substitutions observed in blackgrass were related to resistance to ACCase-inhibiting herbicides, suggesting that the selection pressure exerted by these herbicides had a measurable effect on ACCase nucleotide diversity (Délye et al. 2004). As such, these herbicides can also be used to probe evolutionary processes.

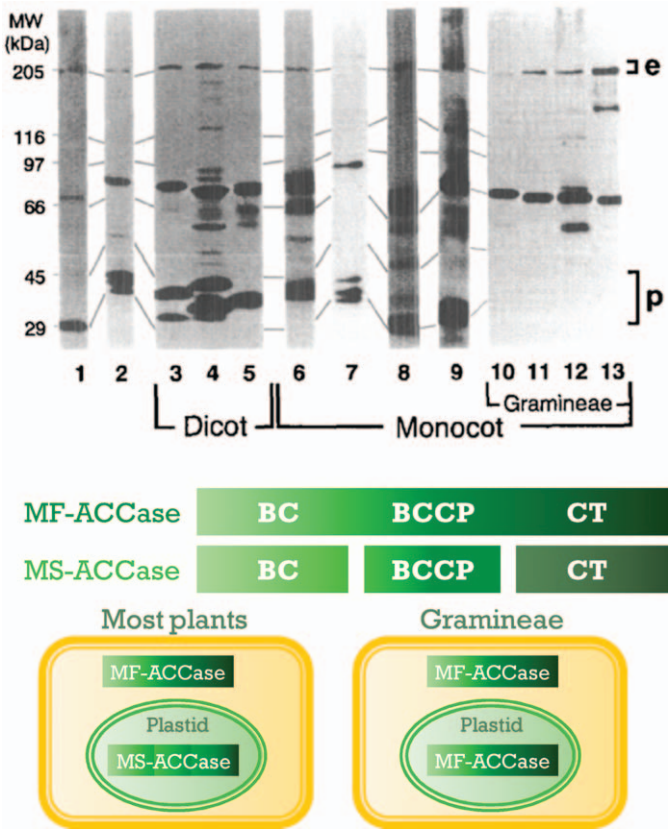

Figure 3. Top panel: Detection of biotinylated polypeptides in the protein extracts of various plants (from Konishi et al. 1996). Bottom panel: Comparison of the forms of ACCase occurring in plants. A multifunctional structure (MF-ACCase) is encoded in a single, large polypeptide, whereas a multisubunit structure (MS-ACCase) consists of three or more subunits that form a large complex. The MF-ACCase is believed to occur in the cytosol of all plants and the plastid of graminaceous plants. The MS-ACCase occurs in the plastids of most other plants (from Ohlrogge and Browse 1995).

Deciphering the Interaction between Microtubules and Cellulose Synthesis

Plant cells are surrounded by a cell wall. This fiber–laminate, outer structure provides support and tensile strength to cells and performs many important biological functions, such as regulation of cell expansion, control of tissue cohesion, ion exchange, and defense against biotic invasions. Cell walls are generally composed of a middle lamella and both a primary and a secondary cell wall, but the presence and composition of each of these layers varies greatly between cell types and among plant families (Popper 2008).

Cell walls are composed primarily of cellulose microfibrils, large, β-1,4-linked glucan biopolymers, synthesized on the outside of the plasma membrane by cellulose synthase (CESA) complexes. This process can be disrupted either by directly inhibiting the synthesis of cellulose or by perturbing the self-organization of the cortical microtubules responsible for guiding the deposition of cellulose in elongating cells.

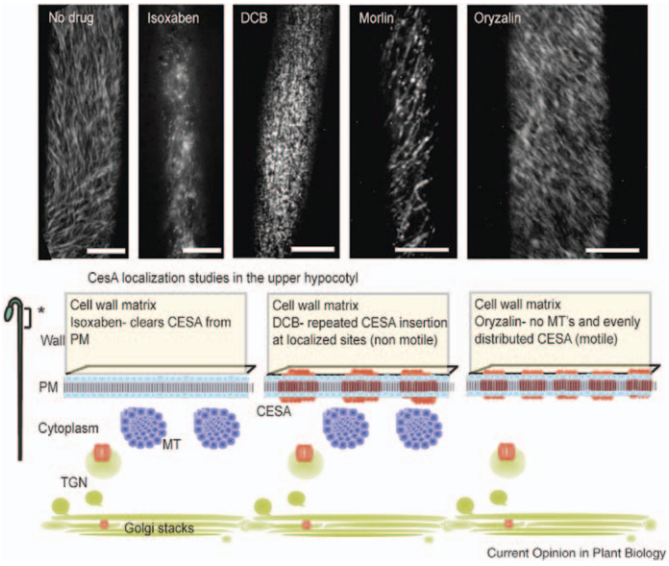

Figure 4. Probing cell wall formation with herbicides. Upper panel: Time average images of YFP-CESA6 in rapidly expanding cells in etiolated hypocotyls. Lower panel; Summary of observations during the different drug treatments. Abbreviations: DCB, 2,6-dichlorobenzonitrile; MT, microtubule; PM, plasma membrane. Scale bars = 10 μm. The figures are reproduced from DeBolt et al. (2007a) and Mutwil et al. (2008).

Dichlobenil and isoxaben are the most common chemical probes used to study cellulose biosynthesis without having a direct effect on microtubule function (Sabba and Vaughn 1999). Isoxaben stops the activity of the CESA complexes by interfering with the incorporation of sugars into the cellulosic cell wall components (Figure 4; Mutwil et al. 2008; Scheible et al. 2001). Because isoxaben also prevents callose formation, this molecular probe probably inhibits an early step in the pathway, such as the biosynthesis of uridine diphosphate–glucose from sucrose (Sabba and Vaughn 1999). This herbicide is much more active than dichlobenil; therefore, isoxaben is an ideal probe for perturbing the mechanical properties of the primary cell wall. It was recently demonstrated that although isoxaben did not depolymerize cortical microtubules, its inhibitory action on CESA nevertheless affected the skeletal microtubule organization, which suggests that cellulose synthesis may affect microtubule assembly (Fisher and Cyr 1998). However, the exact nature of this bidirectional recognition has not yet been determined.

Dichlobenil, on the other hand, inhibits cellulose synthesis but increases the callose content in cell walls. This suggests that it targets a later step in cellulose biosynthesis than isoxaben does (Figure 4), but the precise mode of action of dichlobenil is still unknown. However, it is now known to bind to MAP20, a protein involved in secondary cell wall synthesis (Rajangam et al. 2008). Additionally, the effects of dichlobenil on CESA make this herbicide a useful probe for elucidating the mechanism of CESA mobility and its insertion at the plasma membrane. Because dichlobenil causes an enrichment of CESA complexes, it may also be a useful tool for future efforts to purify this important, yet poorly understood, enzyme complex (DeBolt et al. 2007b).

Inhibitors of microtubule assembly are often used to investigate the relationship between the organization of the cytoskeleton and the process of cellulose deposition at the plasma membrane–cell wall interface. Microtubules are dynamic proteins that provide the overall cytoskeletal structure to plant cells. These linear biopolymers form an ordered cortical array against the plasmalemma that guides the movement of the CESA complexes (Paredez et al. 2006). Oryzalin inhibits microtubule polymerization, and it is often used to probe the effect of cytoskeleton distribution and its role in the movement of the CESA complexes in the membrane. Studies using oryzalin confirmed that microtubules serve as tracks directing cellulose deposition (Paredez et al. 2006), and there is a dynamic feedback regulation between cellulose synthesis, microtubule orientation and organization, and microtubule-associated proteins (Lloyd and Chan 2008; Mutwil et al. 2008).

Oxaziclomefone is a new herbicide that inhibits cell expansion without affecting the turgor of plant cells. Unlike inhibitors of CESA activity, it did not affect incorporation from D-glucose or *trans*-cinnamate into the cell wall. Oxaziclomefone decreased wall extensibility without influencing the synthesis or postsynthetic modification of major architectural wall components or the redox environment of the apoplast. Therefore, this herbicide is an interesting new molecular probe for exploring aspects of cell-wall physiology related to wall loosening and tightening (O'Looney and Fry 2005).

Manipulating the Cell Cycles in Plants

The ability to synchronize cell division is a powerful tool for investigating the biochemical events of the cell cycle in plants. In fact, most of our knowledge on the cell cycle has been obtained from plant cell suspensions that were synchronized by applying herbicides or other chemical agents that arrest the cell cycle at various stages. Furthermore, most of these herbicides are reversible inhibitors, which make them useful probes for manipulating the cell cycle (Planchais et al. 2000).

Many commercial herbicides are mitotic inhibitors that act either directly or indirectly on microtubule assembly and organization. As mentioned in the preceding section, microtubules are one of the components of the cytoskeleton. These large and hollow biopolymers have a diameter of 25 nm and can reach several microns in length. Microtubules serve as structural components within cells and guide the deposition of cellulose, but they are also involved in many other cellular processes, including vesicular transport, cytokinesis, and mitosis.

Mitotic inhibitors have proved to be useful tools in investigations of the essential functions of microtubules in plant cytoskeletal arrays. These inhibitors have been grouped into three divisions based on the stages of the cell cycle they affect (i.e., those that cause arrested prometaphase figures, those that disrupt spindle microtubule organization, and those that disrupt phragmoplast microtubule organization (Vaughn and Lehnen 1991).

Dinitroaniline herbicides (e.g., trifluralin and oryzalin) have played a critical role in revealing the fundamental properties of tubulin gene expression and microtubule structure (Anthony and Hussey 1999). In particular, the elucidation that a point mutation in α-tubulin (a major microtubule cytoskeletal protein) was the molecular basis for dinitroaniline resistance has provided new insight into the mechanism of tubulin assembly. Microtubules are formed by

the self-assembly of heterodimeric tubulin units that polymerize in a head-to-tail fashion. Upon binding to α-tubulin, dinitroaniline herbicides prevent the polymerization of free tubulin subunits into microtubule. The subsequent loss of spindle apparatus stops the movement of the chromosomes to the poles during mitosis, and the cell cycle is arrested in a prometaphase configuration. The effects of the phosphoric amide herbicides are similar to that of the dinitroanilines, although they appear to interact differently with tubulin.

Other microtubule inhibitors, such as the *N*-phenyl carbamate herbicides (e.g., barban, propham, and chlorpropham), alter the organization of the spindle microtubules so that multiple spindles are formed. The cell cycle can be arrested at the metaphase or the anaphase stage, and they tend to accumulate multiple centers of radiating microtubules. As a result, chromosomes move to many poles resulting in the formation of multiple nuclei. Although little is known about the exact target site probed by these herbicides, they have a direct effect on microtubule polymerization (Morejohn and Fosket 1991).

Additionally, terbutol induces star anaphase configurations in which the chromosomes are drawn into an area at the poles in a star-like aggregation, and the phthalic acid herbicide DCPA causes multiple, branched, and curved phragmoplast microtubule arrays, which provided new insight into the organization of the new cell plate after mitosis.

Finally, a large number of natural phytotoxins have been used to probe other aspects of the cell cycle (Planchais et al. 2000). However, the use of these natural products as molecular probes is beyond the scope of this review.

Auxin Herbicides and the Regulation of Plant Growth

Indole-3-acetic acid (IAA), the principal natural auxin in higher plants, influences virtually every aspect of plant growth and development and acts as a "master hormone" in the complex network of interactions with other phytohormones (Ross et al. 2001). An array of synthetic IAA derivatives, including naphthalene acids (e.g., 1-naphthalene acetic acid [1-NAA]), phenoxycarboxylic acids (e.g., MCPA, 2,4-D), benzoic acids (e.g., dicamba), pyridinecarboxylic acids (e.g., picloram), and quinolinecarboxylic acids (quinmerac, quinclorac) elicit strong auxin-like plant responses with the advantage of being more stable in planta than the natural phytohormones (Woodward and Bartel 2005). Consequently, these synthetic analogues have gained considerable importance for probing the complex functions of auxin in plants.

Synthetic auxins mimic the deformative and growth-inhibiting effects caused by supraoptimal endogenous concentrations of IAA. The physiological and biochemical events associated with increasing in planta concentrations (gradient development) of auxin herbicides or IAA have been divided into a stimulation phase, an inhibitory phase, and a decay phase (see Grossmann 2003b for more details).

The recent identification of transport inhibitor response 1 (TIR1)-type receptors for auxin perception (Dharmasiri et al. 2005; Kepinski and Leyser 2005) and the discovery of a new hormone interaction in signaling between auxin, ethylene, and the up-regulation of 9-*cis*-epoxycarotenoid dioxygenase (NCED) in abscisic acid (ABA) biosynthesis (Kraft et al. 2007) have accounted for a large part of the repertoire of auxin-mediated responses (Figure 5). IAA binds to the base of the

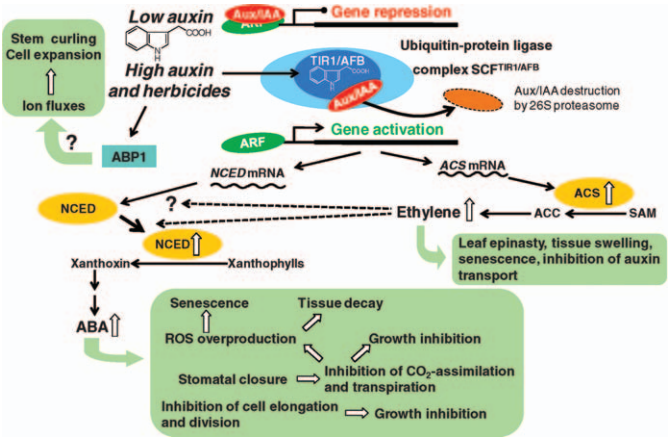

Figure 5. Proposed mechanism of action of auxinic herbicides and supraoptimal concentrations of indole-3-acetic acid (IAA). See text for detailed description of the mechanism of action. TIR1/AFB, auxin receptors; Aux/IAA, Aux/IAA transcriptional repressor proteins; ARF, auxin response factors; ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ABP1, auxin-binding protein 1; ABA, abscisic acid; ROS, reactive oxygen species.

same TIR1 pocket that docks an Aux/IAA transcriptional repressor protein on top of IAA, which occupies the rest of the TIR1 pocket (Tan et al. 2007). In this respect, IAA functions as a "molecular glue" to enhance TIR1–Aux/IAA protein interaction. TIR1 also responds to 2,4-D and 1-NAA because they interact with the partially promiscuous IAA binding site at the base of the TIR1 receptor pocket (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Tan et al. 2007). Ultimately, binding of IAA (or synthetic auxins) to TIR1 stabilizes the interaction between the Aux/IAA repressor and the receptor, causing the E3 ubiquitin ligase SCF^{TIR1} complex to covalently bind ubiquitin to Aux/IAA protein, which marks it as substrate for degradation by the 26S proteasome (Figure 5). The loss of Aux/IAA repressor proteins leads to derepression of preexisting auxin response factors (ARFs). These DNA-binding transcriptional activator proteins continuously activate transcription of auxin-responsive genes, including those for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in ethylene biosynthesis and Aux/IAA repressors for feedback inhibition, as long as auxin concentrations in the tissue remain high (Guilfoyle 2007; Hagen and Guilfoyle 2002).

Plants have multiple TIR1 homologs, including auxin-binding F-box proteins (AFBs), which respond differently to 2,4-D and the pyridinecarboxylic acid-type auxin herbicides (Walsh et al. 2006), indicating that these herbicides can be used to probe the different auxin receptor proteins. The diversity and tissue specificity of both the Aux/IAA proteins and ARFs can explain the plethora of auxin-specific responses (Badescu and Napier 2006).

In the case of rapid auxin responses, such as auxin-induced ion fluxes in cell expansion, an additional signaling pathway, possibly via the membrane-bound, auxin-binding protein 1 (ABP1) and related proteins may be involved (Badescu and Napier 2006; Vanneste and Friml 2009). ABP1 may act as a coordinator of cell division and expansion, with local auxin levels that influence ABP1 effectiveness (Braun et al. 2008; Figure 5).

The newly discovered TIR1/AFB receptors link binding of auxin herbicides and supraoptimal concentrations of IAA to transcription factor abundance and overexpression of auxin-

responsive genes responsible for the sequential biochemical and physiological events associated with herbicide action (Kelley and Riechers 2007; Figure 5). In this context, excessive stimulation of ACC and ethylene formation through induced ACC synthase activity is a well-known, early, and ubiquitous response to auxins in sensitive species (Argueso et al. 2007; Grossmann 2003a; Sterling and Hall 1997; see next section).

Auxin herbicides are also useful tools to trigger de novo ABA biosynthesis (Grossmann 2000a, 2003a; Scheltrup and Grossmann 1995). Auxin-stimulated ABA biosynthesis is induced exclusively in the shoot tissue by increasing xanthophyll cleavage, which releases the ABA precursor xanthoxin (Figure 5; Hansen and Grossmann 2000). This key regulated step in the pathway is catalyzed by the plastid enzyme NCED (Schwartz et al. 2003; Taylor et al. 2005), which is triggered by auxin-stimulated ethylene (Grossmann 2003a; Hansen and Grossmann 2000). Exemplified for the highly auxin-sensitive dicot *Galium aparine*, IAA and auxin herbicides of different chemical classes (e.g., quinmerac, dicamba, and picloram) cause transient increases in Ga*NCED1* mRNA levels exclusively in the shoot tissue within 1 h of treatment and a subsequent accumulation of ABA. Interestingly, increases in *GaNCED1* transcript levels preceded those in ACC synthase activity, ACC and ethylene production.

Consequently, auxin is the trigger for *NCED* gene expression, whereas ethylene appears to enhance ABA biosynthesis by up-regulation of NCED posttranscriptionally (Kraft et al. 2007). However, it is still an open question about whether auxin signaling in *NCED* gene expression also involves $SCF^{TIR/AFB}$ mediated degradation of Aux/IAA transcriptional repressors, as proposed in Figure 5.

Many questions regarding the functions and mechanism of action of auxin remain to be clarified, and auxin herbicides will continue to play important roles in dissecting the principles of auxin perception, hormone interactions, and the up-regulation of ethylene and ABA biosynthesis in plant growth regulation.

Role of Cyanide, Derived from Ethylene Synthesis, in Cell Death

HCN is a potent phytotoxic agent produced by more than 2,500 plant species via the degradation of cyanogenic glycosides, or from pathways associated with histidine, hydroxylamine, and glyoxylate metabolism (Siegien and Bogatek 2006). HCN is also an equimolar product of the enzymatic conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by ACC oxidase (Figure 6; Grossmann 1996; Yang and Hoffman 1984; Yip and Yang 1998); questions have been raised about the physiological significance of HCN in plants (Grossmann 2003a). However, under normal circumstances, HCN is rapidly detoxified by the sequential action of β-cyanoalanine synthase (β-CAS) and β-cyanoalanine hydrolase, leading to the formation of asparagine (Figure 6; Blumenthal et al. 1968; Grossmann 1996; Miller and Conn 1980; Siegien and Bogatek 2006). Ethylene can regulate its own biosynthesis (and that of HCN) up (autostimulation) or down (autoinhibition; see dotted arrows on Figure 6) by modulating ACC synthase and ACC oxidase activities at the transcriptional and posttranscriptional level and is able to induce de novo synthesis of β-CAS (Argueso et al. 2007; Grossmann 1996; Yip and Yang 1998).

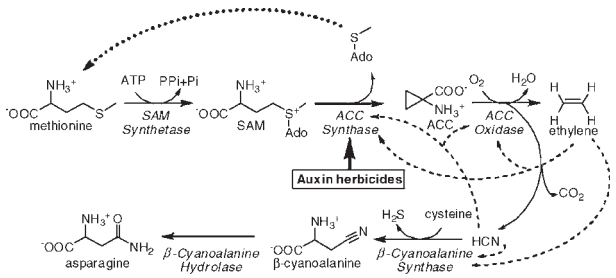

Figure 6. Biosynthetic pathway of ethylene and cyanide. See text for detailed description. The names of the enzymes are in italics and capitalized. SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; HCN, hydrogen cyanide. Small dotted arrows indicate steps stimulated by ethylene or HCN. Bold dashed arrow indicates the reincorporation of methylthioadenosine into methionine. Modified from Yang and Hoffman (1984).

HCN also up-regulates ACC synthase (*ACS6*) gene expression (McMahon et al. 2000) and can modulate ACC oxidase and β-CAS (Liang 2003; Ververidis and Dilley 1995).

ACC oxidase is an exclusively cytosolic enzyme, which is where ethylene and HCN are initially released (Reinhardt et al. 1994). In contrast, β-CAS is compartmentalized in the mitochondria (Wurtele et al. 1985). In spite of the physiological separation between the site of HCN synthesis and degradation, plants normally do not exhibit phytotoxic responses to HCN because the high β-CAS activity is usually sufficient to detoxify HCN formed during ethylene biosynthesis (Yip and Yang 1998). Consequently, the possibility of a metabolic and physiological role for HCN in plants was neglected for many years until studies with auxin herbicides were used to reexamine this question.

A potential involvement of HCN in the mode of action of auxin herbicides was first suggested with 2,4-D (Tittle et al. 1990), but the phytotoxic action of HCN in grass plants was elucidated primarily by probing this pathway with quinclorac (Grossmann 1998; Grossmann 2000b, 2003a; Abdallah et al. 2006). The principal site of quinclorac action is localized in the root tissue, but endogenous levels of ACC, ethylene formation, and HCN accumulation increased predominantly in the shoot, where the phytotoxic symptoms develop (Grossmann 1998; Grossmann and Kwiatkowski 1993, 1995, 2000; Grossmann and Scheltrup 1997). Quinclorac induces ACC synthase activity specifically in the root tissue and the excess ACC is translocated to the shoot, where it is converted to high levels of ethylene and HCN by ACC oxidase. Under these circumstances, compartmentalization of β-CAS in the mitochondria prevents it from successfully detoxifying the large amount of HCN released in the cytoplasm. The combined effects of the high ethylene and HCN levels are responsible for the metabolic and physiological responses observed in sensitive plants.

Glyphosate Effects on Plastid Targeting and Plant Disease

The enzymes of the shikimate pathway responsible for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) are nuclear-coded, translated in the cytoplasm, and subsequently imported into the plastid, where the functional pathway operates. Nuclear-coded, plastid-localized proteins are translated with transit peptide extensions that tag them for import into plastids. Normally, the transit peptides are cleaved after the proteins enter the plastids, releasing the

Table 1. Herbicides used as selectable markers.[a]

Group	Herbicide	Enzyme	Gene	Source
K_3	Acetochlor	Cytochrome P450	*CYP1A1* *CYP2B6* *CYP2C19*	*Homo sapiens*
E	Butafenacil	Protoporphyrinogen oxidase	*Ppx*	*Myxococcus xanthus*
	Cyanamide	Cyanamide hydratase	*Cah*	*Myrothecium verrucaria*
G	Glyphosate	5-Enolpyruvylshikimate-3-phosphate synthase	*Epsps*	*Petunia hybrid, Zea mays*
			aroA	*Salmonella typhimurium* *Escherichia coli*
			cp4 epsps	*Agrobacterium tumefaciens*
		Glyphosate oxidoreductase	*Gox*	*Ochrobactrum anthropi*
B	Imidazolinones	Acetolactate synthase	*csr1-2*	*Arabidopsis thaliana*
H	Phosphinothricin Glufosinate	Phosphinothricin acetyl transferase	*bar, pat*	*Streptomyces hygroscopicus* *Streptomyces viridochromogenes*
F_1	Norflurazon Fluridone	Phytoene desaturase	*Pds*	*Hydrilla verticillata*
C_3	Bromoxynil	Bromoxynil nitrilase	*Bnx*	*Klebsiella pneumoniae* subsp. *Ozaenae*
B	Sulfonylureas	Acetolactate synthase	*csr1-1*	*Arabidopsis thaliana*
K_1	Trifluralin	α-Tubulin	*TUAm*	*Eleusine indica*

[a] Data from Sundar and Sakthivel (2008).

mature functional proteins. It was assumed that no nuclear-coded plastid proenzyme (enzyme still possessing its transit peptide) was active in the cytoplasm. However, this paradigm was challenged when work with glyphosate and its target site, 5-enolpyruvyl-3-phosphoshikimate synthase (EPSPS) demonstrated that proEPSPS was enzymatically functional before cleavage of the transit peptide (Della-Cioppa et al. 1986). Furthermore, the activities of both proEPSPS and mature EPSPS were sensitive to glyphosate inhibition. Finally, binding of glyphosate to the proenzyme prevented import of the proEPSPS into the plastids (Della-Cioppa and Kishore 1988).

Initial attempts by Calgene Inc. (Creve Coeur, MO) to generate glyphosate-resistant transgenic plants were not entirely successful because the bacterial EPSPS gene (which is naturally resistant to glyphosate) lacked the transit peptide that would allow the enzyme to enter the plastids to participate in the shikimate pathway (Comai et al. 1983). Scientists at Monsanto (Creve Coeur, MO) subsequently generated transgenic plants with much higher resistance to glyphosate by fusing a gene for a bacterial resistant EPSPS with the plant *N*-terminal transit peptide from petunia (*Petunia* sp. Juss.) that directed the proEPSPS to the plastids (Della-Cioppa et al. 1987). This chimeric enzyme, produced by in vitro transcription and translation of the gene fusion, was imported into plastids, and proteolytically processed to give a stable, plastid-localized, glyphosate-resistant enzyme, enhancing resistance of the plant to glyphosate.

Glyphosate has also been a powerful tool for improving understanding about the role of phytoalexins in disease resistance. Many phytoalexins are products of the shikimic pathway. Numerous studies have shown that sublethal treatment of plants with glyphosate reduces phytoalexin production and thereby enhances disease symptoms (e.g., Holliday and Keen 1982; Latunde-Dada and Lucas 1985; Liu et al. 1997; Sharon et al. 1992). Finally, studies on glyphosate resistance have substantially enhanced our understanding of the mode of action of glyphosate, the shikimate pathway, and protein sorting within plant cells, as well as developmental and tissue specific expression of genes in plants (Kishore et al. 1992).

Herbicides as Selectable Markers

Selectable marker genes and their companion inhibitors have played critical roles in the development of plant transformation protocols. These systems enable the identification of cells expressing the cloned DNA and the selection of the transformed progenies. This is particularly critical because, in most situations, only a small portion of the cells is transformed, and the transgenic lines could not be recovered without the selection pressure exerted by the inhibitor.

Selectable markers usually are genes that confer resistance to biocides. Classical selectable marker technologies were based on antibiotics and their antibiotic resistance genes (i.e., kanamycin/kanamycin phosphotransferase, *aphA2* or hygromycin/hygromycin phosphotransferase, *hpt*). However, there are some limitations in the efficacy of these markers in plant systems. For example, plants transformed with *hpt* selectable marker do not always withstand the strong selection pressure imposed by hygromycin (Nazakawa and Matsui 2003).

Herbicides are relatively inexpensive and potent molecules that inhibit very specific plant molecular target sites. They are, therefore, ideally suited to be used in conjunction with their respective resistance genes for the selection of transformant plant lines. For example, phytoene desaturase genes were discovered in herbicide-resistant *Hydrilla verticillata* biotypes (Michel et al. 2004). Manipulation of these genes generated a phytoene desaturase with high resistance to norflurazon and fluridone, which is a particularly good selectable marker (Arias et al. 2006). The array of selectable markers currently available for creating transgenic plants has been recently reviewed (Miki and McHugh 2004; Sundar and Sakthivel 2008), and Table 1 summarizes those systems that are based on herbicides. These selectable markers consist either of genes encoding for herbicide-resistant molecular target sites or genes for enzymes that rapidly deactivate the herbicides.

Chemical Genetics to Probe Plant Biological Pathways

Strategies for herbicide discovery have evolved from the testing of random chemicals for efficacy on whole plants to

the use of high-throughput biochemical, genomic, and proteomic assays. Combinatorial high-throughput screens have enabled the testing of large libraries of compounds on specific molecular target sites, but leads identified in these in vitro assays often had little in vivo herbicidal activity. Similarly, microarray technology has had limited success because the effects of herbicides on their molecular target site are often so catastrophic that there are many downstream repercussions on secondary metabolism and unrelated pathways, often masking the primary target site. As well, genetic screens using knockout mutants have not been very successful either because the phenotype is the result of the complete inhibition of a key gene, whereas most herbicides do not exert 100% inhibition of the target sites to be phytotoxic.

Novel and more promising approaches using antisense RNA suppression of enzyme translation to determine the extent of inhibition required for toxicity (Abell 1996), and metabolomics (metabolic fingerprinting) to visualize the molecular organization of complex organisms (Hall 2006), are being developed.

Chemical genetics, the systematic exploration of plant biological pathways with small molecule probes, is becoming increasingly popular (Armstrong 2007; Blackwell and Zhao 2003). Chemical genetics couples the diverse chemistry of nature and the large combinatorial libraries available to agrochemical industries with the strengths of molecular genetics to dissect signaling pathways in complex organisms, such as plants. It has broadened our understanding of genetic signaling pathways that may usher new pest management approaches and has shown some promises in the area of herbicide target discovery and the generation of lead compounds. (Cole et al. 2000; Walsh 2007).

A chemical genetics approach to compounds that cause altered cell morphology resulting in swollen root phenotype in *Arabidopsis* led to the discovery of morlin (7-ethoxy-4-methyl chromen-2-one) in a screen of 20,000 small molecules. Morlin acts on the cortical microtubules and alters the movement of CESA, causing novel cytoskeletal defects, characterized by cortical array reorientation and compromised rates of both microtubule elongation and shrinking. Morlin appears to be a useful new probe of the mechanisms that regulate microtubule cortical array organization and its functional interaction with CESA (DeBolt et al. 2007a).

Similarly, 13 auxinic compounds were discovered in a screen of 10,000 molecules for auxin-like activity in *Arabidopsis* roots. WH7 (2-(4-chloro-2-methylphenoxy)-*N*-(4-*H*-1,2,4-triazol-3-yl)acetamide) was one of the most active substances identified. Therefore, 20 analogues of WH7 were used to provide detailed information about the structure–activity relationship, based on their efficacy at inhibiting and stimulating root and shoot growth. These small molecules will be useful for studying the signaling events that follow alteration of the auxin perception site by inhibitors (Christian et al. 2008).

Acknowledgment

The senior author is thankful to have had the opportunity to present this paper at a symposium on the Non-Herbicide Uses of Herbicides during the 49th annual meeting of the Weed Science Society of North America (2009).

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Received June 3, 2009, and approved July 27, 2009.