# Chemical Genetic Approaches to Plant Biology[1]

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Synthetic chemistry and plant biology intersect in myriad ways. To date, however, the overlap of research methods between these two fields is limited. Outside of the agrochemical industry, most synthetic chemists simply view plants as sources of diverse and structurally complex organic molecules (i.e. natural products), frequently with exquisite biological activities. Little knowledge of plant biology is required to extract the desired natural products out of the plant tissues, in fact, pharmacognostic endeavors with natural products date back thousands of years (Parascandola, 1980; McTavish, 1987; Travis, 1989; Akerele, 1993). Similarly, most plant biologists rarely rely on synthetic chemistry to address day-to-day questions in their research. In other areas of the biological sciences, however, the synthetic chemistry/biology interface is substantially more developed. For example, the iterative synthesis and evaluation of collections of non-natural compounds is one of the cornerstones of the pharmaceutical sciences (Spencer, 1998). With the advent of combinatorial chemistry (Balkenhohl et al., 1996; Oliver and Abell, 1999) and high-throughput screening techniques over the past decade (Oldenburg, 1998; Engels and Venkatarangan, 2001), significantly larger synthetic compound libraries have been generated, and the pace of screening has dramatically escalated. Such techniques are now accessible to academic research labs (Tan et al., 1999) and have become central tools in the burgeoning field of chemical biology (Wells, 1999).

Taking cues from the pharmaceutical industry, the agrochemical industry has also implemented combinatorial chemistry and high-throughput screening to uncover new herbicides and pesticides (Ridley et al., 1998). However, the systematic exploration of plant biological pathways with small molecule probes (molecular mass $< 700$ D) is an approach still in its infancy. This general approach to biology, today called "chemical genetics," is centered around the tenet that small organic molecules can be used like mutations in classical genetics to modulate protein functions and to assist in the delineation of biological pathways (Stockwell, 2000; Alaimo et al., 2001; Shogren-Knaak et al., 2001). Herein, we give a general introduction to chemical genetics, and through analysis of representative examples from the recent literature and our own laboratories, we highlight its potential as a wide-ranging tool for plant biology research.

## CHEMICAL GENETICS: A BRIEF OVERVIEW

To our knowledge, the term chemical genetics was first used in the scientific literature in relation to plant biology. In 1935, von Euler et al. published work on the "chemical genetics of chlorophyll-mutating barley strains" obtained through spectroscopic analyses of metabolites of selected barley (*Hordeum vulgare*) strains (von Euler et al., 1935). From then until the late 1980s (Beadle, 1947, 1948; DeBusk, 1956; Allen, 1967; Macey and Barber, 1969, 1970; Avato, 1987), the term chemical genetics was used mainly to describe studies of the differences in chemical constitution between mutant strains of various organisms, the majority of which were plants. Chemical genetics reemerged on the scientific landscape with force in the middle to late 1990s, when Schreiber (1998) and Mitchison (1994) recognized the potential power of the systematic use of small molecules to address questions in cell biology. They termed this general approach to biology chemical genetics, of which the goal is the delineation and characterization of signaling pathways for particular gene products using cell-permeable small molecules as modulating ligands. Here, in contrast to earlier incarnations of the term, the chemicals were not generated by the organism being studied, but rather were generated by synthetic chemists using combinatorial chemistry techniques.

Although the ad hoc use of organic compounds as probes to modulate biological pathways is not new, the systematic design, synthesis, and screening of collections of structurally unbiased small molecules (i.e. not directed at a protein target) for those that illicit novel biological effects represents a new approach for both chemists and biologists (Lokey, 2003). An extremely ambitious goal set at the outset for chemical genetics was the eventual discovery of a small molecule modulator for every known protein. With such a molecule in hand, the activity of a pro-

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tein (or any associated with it) could be suppressed or enhanced theoretically on demand (Mitchison, 1994; Schreiber, 1998). Although this goal is far from being met, significant progress has been made toward using small molecules as general tools to explore biology. First, a suite of highly evolved combinatorial chemistry techniques has been developed (Blackwell et al., 2001), along with a planning algorithm for the synthesis of functionally and conformationally diverse molecules that mimic the complexity of natural products (Schreiber, 2000). Second, standardized methods have been developed for formatting these synthetic compounds for high-throughput in vitro and in vivo screening (Clemons et al., 2001). These technologies were all designed to be readily exportable and accessible to other academic labs. Chemical genetics has in part driven the expansion of the chemical biology field over the past decade, and this approach is now seeing increasing use in diverse areas of biology, including applications in bacteriology (Eggert et al., 2001), cancer biology (Torrance et al., 2001), vertebrate development (Peterson et al., 2000), and neurobiology (Stockwell, 2002).

## FORWARD AND REVERSE CHEMICAL GENETICS

In direct analogy to classical genetics, both forward and reverse chemical genetic approaches are possible. In forward chemical genetics (Fig. 1a), large collections of structurally unbiased compounds are screened in whole organisms or cells for those that induce specific phenotypic outcomes. These screens are thus analogous to classical forward genetic screens in model organisms that have been subjected to random mutagenesis. In the forward chemical genetic screen, specificity is not necessarily a requirement at the outset. If the compound induces an interesting and reproducible phenotype in a given system, it can prove useful as a biological probe as long as its other effects are minimal or at least are in nonoverlapping pathways. However, target identification is often an ultimate goal of forward chemical genetic approaches, especially if the target is believed to be novel, and the development of new target identification methods is an active area of research (King, 1999). In general, target identification can be achieved through a biochemical approach, because often the small molecule can be derivatized readily and incorporated into a support matrix for affinity chromatography applications (Taunton et al., 1996). Targets can also be identified with a genetic approach by isolating mutants insensitive to the compound if the organism of interest has a tractable genetic system. Finally, effects of small molecules on global gene expression can be examined using now readily available DNA microarrays (Southern, 2001). Such expression profiles can assist in target identification under certain circumstances; for example, comparing an expression profile of the small molecule with expression profiles obtained after deleting candidate genes can expose whether the molecule is targeting one or more proteins (Marton et al., 1998; Kuruvilla et al., 2002).

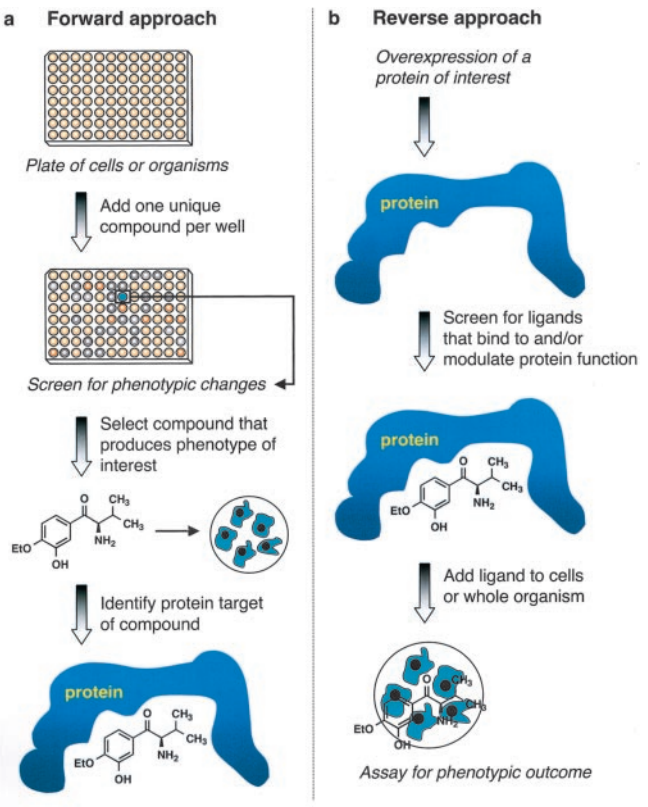


**Figure 1.** General schematic of the chemical genetic process. a, Forward chemical genetics involves the screening of synthetic molecules in cells or organisms for phenotypic changes, the selection of a molecule that induces a phenotype of interest, and the eventual identification of the protein target(s) of the small molecule. b, Reverse chemical genetics involves the overexpression of a protein target of interest, the screening of compound libraries for a ligand that modulates the function of the protein, and using the ligand to determine the phenotypic consequences of altering the function of the target protein in a cellular or organismal context. The blue cells and the small molecule in this figure represent a generic phenotypic change and a generic library member, respectively.

In contrast to forward chemical genetics, reverse chemical genetic approaches require a known protein target, which is subjected to binding or functional assays to identify a small molecule partner (Fig. 1b). This small molecule then can be used to elucidate the phenotypic consequences of inhibiting the target protein in vitro or in vivo. This approach has direct parallels to the use of "knock-outs" in classical genetics, where a mutation is introduced into a gene of interest and the phenotypic consequences of the mutation are studied in a cellular or organismal context. However, in contrast to classical genetic techniques, the addition of the small molecule to a system results in a temporary and spatial perturbation of the normal, wild-type (WT) state. Thus, using this strategy,

it is possible to identify reagents that behave in a manner similar to conditional mutations without the disadvantages associated with, for example, temperature-sensitive alleles, where the required temperature change may compromise the system. The effect of small molecules in biological systems is most often (a) rapid, (b) reversible, allowing temporal and spatial control of protein function, and (c) tunable, enabling gradient phenotypes to be observed. These attributes make chemical genetic approaches especially attractive for the study of biological processes that are regulated on a millisecond to hour timescale, processes requiring spatial and temporal control, and/or processes involving proteins for which the functions can be compensated by other related gene products.

## CHEMICAL GENETICS IN PLANTS

The exquisite temporal and spatial control possible with small molecule modulators make chemical genetic techniques uniquely suited for the study of developmental processes. However, there are also challenges in using chemical genetic approaches, including problems associated with the permeability, transport, and potential metabolism of small molecules. Furthermore, identification and characterization of small molecule targets and their action mechanisms can be difficult. In light of these challenges, we believe that chemical genetic techniques will be especially powerful for the dissection of biological pathways in plant development, because plant systems provide abundant opportunities and the necessary tools for efficient elucidation of the action mechanisms of identified compounds. First, all known plant growth regulators are small molecules, and their effects have been studied extensively by classical genetic and biochemical approaches (Roberts and Hooley, 1988). Therefore, the experimental protocols for analyzing plant growth regulators are well defined and can be easily adapted to support unbiased screens for compounds that modulate any process of interest. Second, the genome of the most common reference organism for plant biology, Arabidopsis, is now fully sequenced (Arabidopsis Genome Initiative, 2000), and a variety of classical genetic tools are available to the scientific community. Such a tractable genetic system should greatly facilitate the target identification process for the identified compounds. The feasibility of identification of targets (receptors) for small molecules by classical genetic approaches has been proven in Arabidopsis because both the brassinolide receptor (Li and Chory, 1997) and ethylene receptors (Chang et al., 1993; Hua et al., 1995) were identified in genetics screens for mutants insensitive to brassinolide and ethylene, respectively. We have also identified a target for a non-natural small molecule, sirtinol, using a classical genetic approach (see below; Zhao et al., 2003). Third, the plant root system has evolved for the efficient uptake of minerals and nutrients and thus provides an excellent route for the uptake of small molecules.

Finally, a chemical genetics approach can greatly facilitate assignment of specific functions to each identified gene and thus dissection of complex pathways in plants. As discussed above, chemical genetics can be extremely useful for delineating functions of essential genes and redundant gene families, because it directly targets proteins instead of DNA with tunability and reversibility. Genetic redundancy is even more common in plants than in other systems. Among the 25,000+ genes in Arabidopsis, only about one-third are single copy, and two-thirds have at least one homolog within the genome (Arabidopsis Genome Initiative, 2000). More than one-third (37.4%) of the predicted Arabidopsis proteins belong to families of more than five members. Because it is highly unlikely to obtain mutants in which all copies of a particular gene and its homologs have been functionally compromised by random mutagenesis, the use of classical loss-of-function genetic screens to examine the roles of any genes that are members of gene families or which have functionally redundant homologs will have limited success. In contrast, a small molecule that inactivates a particular protein most likely will be able to inactivate all of the members of a closely related family, provided that they are truly redundant and that they operate by a similar mechanism. Therefore, small molecules can be used to generate what is effectively a "chemical knock-out" of an entire gene family in Arabidopsis, and this can be used to examine loss-of-function phenotypes of redundant genes. To this end, we believe that chemical genetic approaches will enable functional genomics in Arabidopsis and other plant species in the future.

## RECENT EXAMPLES OF CHEMICAL GENETIC SCREENS IN ARABIDOPSIS

Despite the potential utility of small molecule screens in plants, there have been only limited reports of systematic chemical genetic plant screens over the past few years. Two approaches to chemical genetic plant screens are shown schematically in Figure 2. Selected pertinent examples from the recent literature and our laboratories are outlined here.

Forward chemical genetic screens (Fig. 2a) of natural product or fully synthetic libraries in Arabidopsis are the predominant approach of more chemically oriented laboratories. For example, in an attempt to discover small molecule inhibitors of the plant growth regulator auxin in Arabidopsis, fermentation-derived natural products from the soil microorganism *Streptomyces diastatochromogenes* were screened for compounds that inhibit auxin-responsive gene expression (Kirst et al., 1995; Hayashi et al., 2001). The researchers used transgenic Arabidopsis harbor-

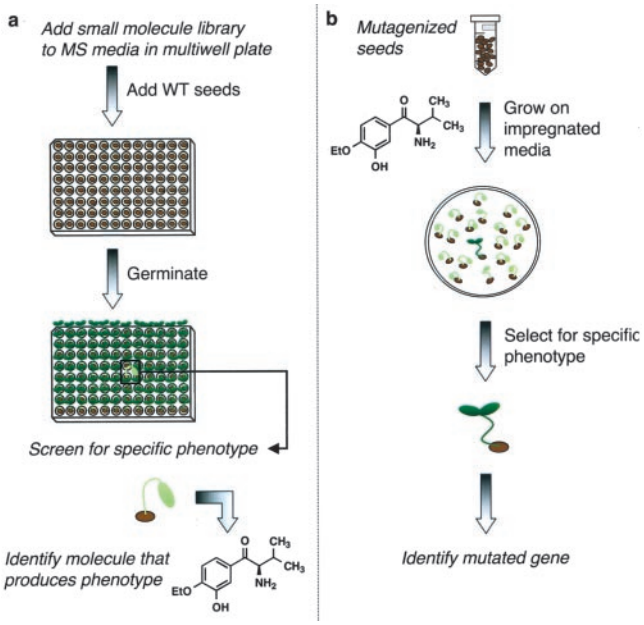


**Figure 2.** Chemical genetic applications in plant biology. a, Schematic of a forward chemical genetic screen using Arabidopsis. One unique compound is added per well to media in a multiwell plate. WT seeds are added to each well, and the germinated seedlings are monitored for phenotypic changes after a certain period of time. Molecules are then identified that induce phenotypes of interest. The pale green seedling and small molecule shown in the figure represent a generic phenotypic change and a generic library member, respectively. b, Schematic of a genetic screen for Arabidopsis mutants resistant to the effects of a small molecule. EMS mutagenized M2 seeds of Arabidopsis are grown on media impregnated with the small molecule. Mutants are selected that do not exhibit the pale green phenotype of interest. The mutated gene is then identified using traditional cloning techniques.

ing β-glucuronidase (GUS) under the control of an auxin-inducible reporter and screened for compounds that inhibited GUS expression. This screen yielded two potent compounds, yokonolides A and B (shown in Fig. 3a), which could become useful bioprobes for exploration of auxin signal transduction in Arabidopsis. However, it is worthwhile to point out that these highly complex spiro-ketal natural products are neither easily isolated from natural sources nor straightforward to synthesize, and this could limit their use as general tools in plant biology.

Whereas work on yokonolides A and B represents an example of forward chemical genetics in plants, recent work from Min et al. (1999) has demonstrated the power of reverse chemical genetics in plant biology research. A fully synthetic, as opposed to naturally derived, library of compounds was screened in rice (*Oryza sativa*) and cress (*Lepidium sativum*) for those that inhibit cytochrome P450s involved in brassinosteroid biosynthesis. This "mini" library of 10 compounds was based on a triazole structural motif, because triazole-derived compounds have been shown previously to be potent inhibitors of cytochrome P450s involved in other biosynthetic pathways (Sakurai and Fujioka, 1997; Yokota, 1997). One inhibitor was isolated from this targeted screen and termed brassinazole (shown in Fig. 3a). Further biochemical studies of the effect of brassinazole on Arabidopsis revealed that it specifically inhibits the protein DWF4, a cytochrome P450 monooxygenase involved in brassinosteroid biosynthesis (Asami et al., 2000, 2001). The simple structure of brassinazole and its straightforward synthesis should facilitate further structure-function studies of this class of molecules in Arabidopsis (Min et al., 1999). In fact, brassinazole has already been widely used in brassinosteroid research, and its use has led to the identification of a key brassinosteroid-signaling component, *bzr1* (Wang et al., 2002).

In our own laboratories, we have initiated a joint program involving the forward chemical genetic screening of fully synthetic libraries prepared via combinatorial chemistry for compounds that induce phenotypic changes in Arabidopsis. Specifically, we are looking to uncover compounds that can modulate light responses or auxin biosynthesis/signaling processes. We anticipate that screening highly diverse sets of compounds could lead to the discovery of molecules with unique and highly specific effects on these processes in Arabidopsis.

As an initial test of this approach, a small library of biaryl-derived molecules was screened for effects on germination in Arabidopsis (Spring et al., 2002). Although this test library was only 57 members in size, these compounds were of particular interest because the axially dissymmetric cyclic biaryl motif is a common structural element of numerous biologically active compounds (Quideau and Feldman, 1996; Nico-

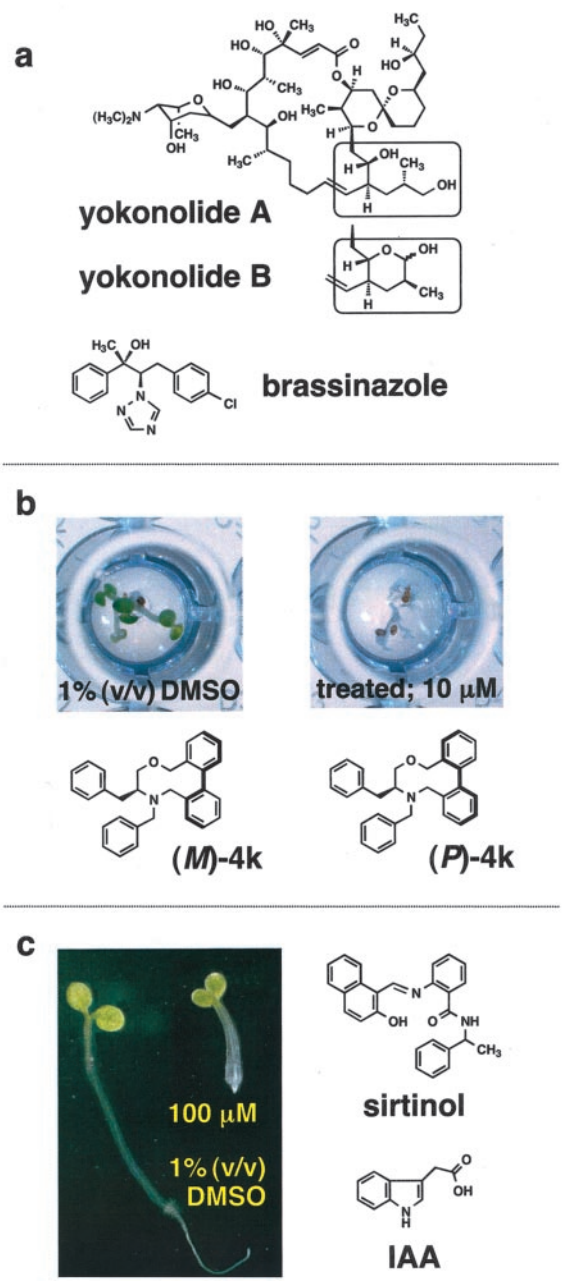


**Figure 3.** Recent examples of small molecules identified through chemical genetic screens in plants. a, The chemical structures of naturally occurring auxin inhibitors, yokonolides A and B, and the synthetic brassinosteroid biosynthesis inhibitor, brassinazole. b, Forward chemical genetic screen of a library of cyclic biaryl compounds in Arabidopsis. Top left, Seven-d-old WT Arabidopsis seedlings germinated on Murashige and Skoog medium with 1% (v/v) dimethylsulfoxide (DMSO) in a 96-well plate. Top right, Colorless phenotype observed in 7-d-old WT Arabidopsis seedlings germinated on Murashige and Skoog medium impregnated with 10 $\mu$M of cyclic biaryl compound (*P*)-4k with 1% (v/v) DMSO. Bottom, The chemical structures of cyclic biaryl compound (*P*)-4k and its atropisomer (*M*)-4k. c, Effects of sirtinol on development in Arabidopsis. Left, Phenotypes induced in 4-d-old WT Arabidopsis seedlings upon germination on Murashige and Skoog media impregnated with either 1% (v/v) DMSO or 100 $\mu$M of sirtinol with 1% (v/v) DMSO. Right, The chemical structures of sirtinol and indole-3-acetic acid (IAA).

laou et al., 1999). Moreover, biaryl compounds that differed only in the stereochemistry of the biaryl linkage (i.e. atropisomers) were contained within this library, allowing us to evaluate the activity of different stereoisomers simultaneously. WT seeds were germinated and grown on agar impregnated with 1% (v/v) DMSO and 10 mM small molecule in 96-well plates. One biaryl compound, (*P*)-4k, was observed to cause stunted development of Arabidopsis seedlings, eventually leading to loss of pigmentation by d 4 after germination, and death by d 7 (shown in Fig. 3b). Interestingly, the opposite atropisomer, (*M*)-4k, was only very weakly active in this assay, and treated plants appeared relatively unchanged (Fig. 3b). Although the protein target(s) of compound (*P*)-4k remain unknown, this work indicated that screening unbiased libraries of synthetic molecules, even of modest size, could yield compounds inducing interesting phenotypic effects in Arabidopsis. Subsequent screening of an approximately 500 member library of small molecules derived from selected, structurally unrelated combinatorial libraries has resulted in the discovery of five additional compounds inducing unique, reversible phenotypic changes in 7-d-old Arabidopsis seedlings (H.E. Blackwell, unpublished data).

It should be pointed out again that although screening for compounds that modulate a particular process is straightforward, interpretation and further analysis of the action mechanisms of candidate compounds are more challenging. In many cases, before the compound can become a useful tool, it is necessary to determine the specificity of the compound for a given biological process. In general, the specificity of a particular compound can be determined if: (a) the compound regulates a set of genes known to be involved in the process of interest (e.g. this can be assessed often by a DNA microarray experiment), (b) the compound causes any known developmental phenotypes related to the process of interest, and/or (c) known mutants involved in the process respond to the compound as predicted. All of these experiments are feasible in Arabidopsis, because many processes have been extensively studied and numerous mutants are available from Arabidopsis stock centers and the Arabidopsis community.

Our recent work with the non-natural small molecule sirtinol and its use to identify a key auxin-signaling component can serve as a model for applying a chemical genetic approach to elucidate biological problems in plants (Zhao et al., 2003). We carried out forward chemical genetic screens for compounds that can alter the expression pattern and/or levels of an auxin reporter line, DR5-GUS (Sabatini et al., 1999), and/or can suppress/enhance phenotypes of an auxin overproduction mutant, *yucca* (Zhao et al., 2001). Sirtinol (Fig. 3c) was originally identified as an inhibitor of the Sirtuin family of NAD-dependent deacetylases in Brewer's yeast (*Sac-*

*charomyces cerevisiae*) and later found to affect root and vascular tissue development in Arabidopsis, suggesting that sirtinol affects an auxin-related process (Grozinger et al., 2001). We then conducted a series of physiological and molecular experiments to conclude that sirtinol constitutively activates auxin signal transduction. First, sirtinol specifically activates many auxin-inducible genes, because the gene expression profile of sirtinol-treated plants is similar to that of auxin-treated plants: More than 65% of the genes induced by sirtinol were also induced by auxin. Second, sirtinol promotes several auxin-related developmental phenotypes including stimulation of adventitious root growth and inhibition of primary root elongation (Fig. 3c). Third, all known auxin-signaling mutants were resistant to sirtinol, further supporting the thought that sirtinol specifically regulates auxin signaling. Finally, we found that sirtinol treatment, like auxin treatment, led to a rapid degradation of the auxin/indole-3-acetic acid (AUX/IAA) proteins that is a hallmark of auxin signaling, indicating that sirtinol-induced gene expression could also arise from regulated protein degradation.

Because sirtinol and IAA (Fig. 3c) are not structurally related but cause similar outputs, we hypothesized that sirtinol and IAA most likely target different components in auxin signaling and that sirtinol targets should be key components in auxin signaling. We took advantage of the well-developed genetic system of Arabidopsis and undertook a genetic screen for mutants that were insensitive to the effects of sirtinol in an attempt to isolate sirtinol targets or downstream components. We point out that several classes of mutants could come out of such a genetic screen, including targets for the compound, downstream components, and genes involved in the chemical conversion of the compound to an active form. Although targets for the compound are of most interest, other types of mutants are also informative. For example, although extensive auxin and abscisic acid (ABA)-resistant mutant screens have so far failed to identify receptors for auxin and ABA, these studies have shaped our current understanding of auxin- and ABA-signaling mechanisms through the identification of other key components in these complex signaling pathways (Koornneef et al., 1998; Swarup et al., 2002).

Many alleles of *axr1* and *axr2* were identified in our sirtinol-resistant mutant screen as we expected (Y. Zhao, unpublished data). A novel mutant, however, was also identified, sirtinol resistant 1 (*sir1*), which exhibited elongated primary roots, elongated hypocotyls, and normal cotyledons in the presence of 25 $\mu$M sirtinol. In contrast to *axr* mutants that are less sensitive to both auxin and sirtinol, *sir1* was hypersensitive to exogenous auxin in a root elongation assay, indicating that SIR1 may negatively regulate auxin signaling and SIR1 functions upstream of *axr* mutants, which also provides one explanation for why *sir1* did not come out of previous auxin-resistant mutant screens. *SIR1* encodes a protein composed of a ubiquitin-activating-enzyme E1-like domain and a Rhodanese-like domain homologous to that of a prolyl isomerase, providing important clues on how SIR1 may participate in auxin signaling. In our current model, we propose that SIR1 and a prolyl isomerase associate via their related Rhodanese domains, and this complex could act to regulate the conformation of a critical Pro residue that is known to be essential for AUX/IAA protein stability. This conformational change could then be translated to a signal for protein degradation, most likely via the ubiquitin-activating-enzyme E1-like activity of SIR1. On-going work is directed at further examination of the role of SIR1 in auxin signaling, and we anticipate further application of forward and reverse chemical genetic techniques in these studies.

## SUMMARY AND FUTURE IMPACT

The term "chemical genetics" was initially used in the context of plants, and we believe that the examples highlighted herein demonstrate the feasibility and power of the latest incarnation of the chemical genetic approach in plants. Because small molecules can be used with exquisite spatial and temporal control, chemical genetics is uniquely poised to assist in the study of developmental processes. Furthermore, plants are especially suited to a chemical genetic approach because they provide a system for efficient identification of both candidate compounds and their targets. Arabidopsis has proven to be an outstanding model system for initial chemical genetic studies, but we predict that other organisms could be valuable in the future, e.g. cyanobacteria for study of photosynthetic and carbon metabolism. As in other areas of biology, we anticipate that it will require chemists working together with plant biologists, and vice versa to realize fully the power of the chemical genetic approach in plants. On-going collaborative work in our labs, along with interdisciplinary work at the Center for Plant Cell Biology at the University of California (Riverside), the Salk Institute (La Jolla, CA), and the Carnegie Institute (Stanford, CA), is focused on uniting plant biology with the tools of synthetic chemistry through chemical genetic analyses. The advent of accessible combinational chemistry techniques (Blackwell et al., 2001; Clemons et al., 2001) along with affordable commercial compound collections and advances in high-throughput plant-based screening (Ridley et al., 1998) should dramatically accelerate progress toward this goal.

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