Weedy and Invasive Plant Genomics

1 Why Should Weed Scientists Care About Genomics?

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Genomics To A Weed Scientist

Genomics does not provide any information that cannot be obtained by more traditional genetic approaches. However, traditional approaches analyze one or a few genes at a time. Among other things, genomics seeks to examine the response of the entire genome to a given stimuli —in one of the most pertinent cases in weed science, an herbicide. A better understanding and use of these technologies potentially allows the weed scientist to find new herbicides and herbicide mechanisms-of-action and extend the use of current herbicide mechanisms-of-action by overcoming weed resistance, developing crop resistance, or making them more efficacious.

Weed scientists and those interested in controlling invasive plants face many challenges concerning available control techniques. When examining chemical control of weeds, there are three major issues facing weed scientists: (1) resistance of weeds to existing herbicide mechanisms-of-action, (2) loss of older herbicides, and especially specific herbicide mechanisms-of-action (MOA) through regulatory or economic means, and (3) lack of new herbicides, and especially herbicides with novel mechanisms-of-action.

When we examine the past decade in weed science, we see a revolution in weed control through the introduction of herbicide-resistant crops. Currently, in the U.S., between 50% and 75% of the major grain, oilseed, and fiber crops have either an herbicide resistance trait or an insecticide trait, or in some cases, both (Dill *et al.* 2008). The rapid adoption of herbicide-resistant crops has had many positive impacts on weed management, but it has also led to some troubling trends. The widespread reliance on a few herbicides for weed control in the major row crops has led to downward price pressure on other herbicides, which has contributed to industry consolidation. The lower return on investment of newer herbicides has been a contributing factor in fewer herbicide introductions and the lack of new herbicide mechanisms-of-action since 1993 (Kraehmer *et al.* 2007).

In some major row crops, such as soybeans and cotton, there has been an overreliance on one herbicide for weed control that has created high selection pressure for resistance development. The conundrum is thus: if widespread resistance occurs to the most commonly used herbicides and we have fewer older herbicides available because of regulatory issues and economic reasons, and there are fewer herbicides and new herbicide mechanisms-of-action in the pipeline, are we far from having a scenario in which we have no herbicides available for certain crops? Furthermore this scenario is building at a time of increasing demand because of population growth, more affluence in the developing world with its modernization of agriculture, and biofuel demand. For a weed scientist, it is obvious that many of the technologies such as screening thousands of organic compounds a year to discover a potential herbicide, which has provided new herbicides and herbicide mechanisms-of-action in the past, might not be viable in the future. Many genomic technologies could provide methods of obtaining the new herbicides and even new classes of herbicides that are the cornerstone of modern weed control.

Resistance

The first case of herbicide resistance in a weed was documented in the late 1960s, when common groundsel (*Senecio vulgaris* L.) was found to be resistant to triazine herbicides (Heap 2008). Herbicide resistance in weeds has grown dramatically; there are now 319 cases of herbicide-resistant biotypes in 185 species covering all herbicide mechanisms-of-action (Heap 2008). See Chapters 9 and 10 for more information on herbicide resistance.

Herbicide resistance in weeds has had a major impact on herbicide use patterns. As a result, the loss of effective herbicides for weed control has the potential to negatively impact the production of certain crops in some areas where the prospect of having no available herbicides available for weed control is very real. One example is cotton in the southeastern United States, where glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) has been confirmed in twenty-nine Georgia counties since 2005 (Stanley Culpepper, personal communication). In addition, acetolactate synthase (ALS) herbicide resistance to Palmer amaranth is present in sixty-one Georgia counties. There is sizable overlap in these same counties and there have been observations of double-resistant Palmer amaranth biotypes to both glyphosate- and ALS-inhibiting herbicides (Stanley Culpepper, personal communication). The presence of ALS- and glyphosate-resistant Palmer amaranth will leave cotton growers with few options for control. The current practice of using protoporphyrinogen oxidase (PPO or PROTOX) -inhibiting herbicides comes with the concern that if PPO-resistant Palmer amaranth develops, there would be no available herbicides for controlling Palmer amaranth in cotton.

The consolidation process in the agrochemical industry (Copping 2003) has severely reduced overall research and development expenditures. In 2005, there were only eleven companies with significant efforts in crop protection research and development, compared with thirty-five companies in 1985 (Rüegg 2007). Coupled with a loss of herbicide MOA to regulatory action (e.g., organic arsenicals in the U.S.; substituted ureas in Europe), widespread resistance to ALS herbicides (Heap 2008), and large increases in resistance to glyphosate, we are facing a crisis of herbicide availability. To some extent, weed scientists are the victims of their own success. In a survey of growers in Indiana conducted by Johnson and Gibson (2006), 65% of growers reported that they were not concerned about glyphosate resistance problems (now in the future) because new herbicide products would be introduced to replace glyphosate when it was no longer effective because of resistant weeds.

The intensive use of a single herbicide such as glyphosate in glyphosate-resistant crops is likely to accelerate the evolution of herbicide resistance. This is especially true if a single herbicide is used in various crops grown in the same rotation, as is currently the case with glyphosate in herbicide-resistant crops in the U.S. (Duke and Powles 2008). In addition, regulatory requirements are increasing worldwide (Rüegg *et al.* 2007). This has encouraged industry to focus development in "safe herbicide harbors," or those chemistries that have proven records of positive environmental and toxicological profiles to make the registration process easier, such as ALS or acetyl-CoA carboxylase (ACCase) inhibitors (Rüegg *et al.* 2007). This compounds the problem when resistance to these chemistries becomes widespread. This can partly explain small variations in chemistries and MOA among recently launched herbicides.

Better Understanding of Resistance

Herbicide resistance can occur via an altered target site (see Chapter 9) or non-target site resistance such as enhanced metabolism, or an exclusion mechanism such as decreased foliar

uptake or translocation out of treated leaves (see Chapter 10). There are several cases of non-target herbicide resistance such as glyphosate resistance in horseweed (*Conyza canadensis*). One particularly intriguing case in which genomics could be effective in characterizing glyphosate resistance is Palmer amaranth. It appears that the EPSPS gene coding for a sensitive enzyme has been duplicated, perhaps over 100 times, leading to very high levels of EPSPS enzyme and resistance (Gaines *et al.* 2009). In these cases, genomic tools could be powerful in elucidating genes and proteins responsible for resistance and altered translocation, and possibly finding ways to overcome the resistance mechanism to restore utility to the herbicide.

Many of the non-target site herbicide resistance cases have been established using enzyme assays and metabolite analysis, but few resistance genes have been cloned and characterized from weeds (Basu *et al.* 2004). Many important questions regarding the mechanisms of non-target herbicide resistance have not been answered. For instance, does resistance result from gene transcriptional regulation, an increase in enzyme affinity, altered substrate specificity, or combinations thereof? Does increased enzyme activity involve a site mutation? A functional genomics approach has recently been successfully applied in herbicide resistance studies and led to the identification of several resistance genes (Gachon *et al.* 2005; Zhen and Singh 2001). In a review, Yuan *et al.* (2007) proposed an integrated functional genomics approach to identify genes involved with non-target herbicide resistance in weed species. Cytochrome P450, glutathione S-transferase, and ABC transporter gene families have been implicated in non-target herbicide resistance.

Genomic technologies might allow the identification of weed taxa with propensity for resistance so growers might be advised to use alternative weed management strategies or agronomic practices (Weller *et al.* 2001).

New Herbicides And Herbicide Mechanisms-of-action?

In 1960, the number of compounds that had to be screened to yield one single product was 10,000; by 2000 the number had increased to 140,000 (Stenzel 2004). In the 1980s, around 10,000 compounds could be screened to yield a compound showing activity in greenhouse assays. This number increased to 30,000 in the 1990s and reached 100,000 in 1998. Since 1991, when sulcotrione, a 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD) -inhibiting herbicide, was introduced in the marketplace, no new herbicide mechanism-of-action has been commercialized (Rüegg 2007). In contrast, between 1970 and 1985, ten new herbicide mechanisms-of-action were introduced in Europe and the U.S.

Since the discovery of the auxinic herbicides in the late 1940s, empirical screening has led to the commercialization of nearly 270 active ingredients, representing seventeen mechanisms-of-action (Lein *et al.* 2004). Of these, approximately 50% act on one of three target sites: photosystem II, ALS, and protoporphyrinogen oxidase PPO. Ten of the 270 active ingredients account for 45% of total market value (Lein *et al.* 2004) and glyphosate accounts for 30% of herbicide sales worldwide and 20% of all pesticide sales. An overreliance on a few herbicides has led to an explosive growth in herbicide resistance worldwide. Agrochemical companies have shifted to a strategy that is driven by *in vitro* testing rather than whole plant screening of herbicide candidates. Most of the known herbicide MOA involve enzyme inhibition and only a handful disrupt other process such auxin response or cell division.

Approximately 20% of the genes in *Arabadopsis* and rice code for enzymes. Does this mean herbicide targets are restricted to a small subset of plant genes or have previous approaches simply favored their discovery (Lein *et al.* 2004)? Since the early 1990s, agrochemical

companies have shifted from whole plant screening to more target-based approaches. Initially, other enzymes of existing herbicide targets were examined with limited success (Abell 1996). Researchers have examined "key" or "limiting" proteins in essential plant processes, also with limited success.

Another approach that is useful in herbicide discovery is to provide evidence that the gene that encodes the target protein is essential to plant growth and development. Abell (1996) suggested that a protein is a suitable target site if inhibition of 60% to 80% of its activity leads to severe growth reduction. The accumulation of large amounts of sequence information from the late 1990s onward from expressed sequence tag (EST) sequencing and full genome sequencing made it possible to use unbiased and genome-wide strategies to identify targets. Unfortunately, the function of more than 30% of genes from completely sequenced species is still unknown or incomplete. Jun *et al.* (2002) initiated a study in *Arabadopsis* in which 1,000 antisense lines were created using cDNAs that had been randomly selected. This study indicated that 1% to 2% of *Arabidopsis* genes (say, a few hundred genes) encode potential herbicide targets. However, the numbers of genes identified were too small to allow any firm conclusions about their distribution in different functional categories. In addition, *Arabidopsis* is not a weedy species (see Chapter 3) and so perhaps an examination of truly weedy species would reveal some potential targets as well.

Lein *et al.* (2004) created a normalized cDNA library from tobacco, sequenced it, excluded redundant clones, transformed 20,000 randomly selected cDNAs in sense or antisense configuration in tobacco, scored plants for visual phenotypes, and carried out retransformation to confirm the result. As of 2004, about 10,000 genes had been put through the process, resulting in forty-six potential herbicide targets. Genes whose partial inhibition leads to chlorosis, necrosis, and concomitant growth defects have been discovered in this process. They contain known herbicide targets (e.g. glutamine synthetase) and genes for which antisense (lack of expression) has already been reported to mimic herbicide phenotypes (e.g., Rubisco and ferredoxin:NADP oxidoreductase [Stitt 1999 and Palatnik *et al.* 2003]). About half of these genes identified as encoding herbicide targets are annotated as enzymes. The remaining genes have an extremely imprecise annotation, including a quarter of which with no known function.

This finding indicates that current herbicide targets found by traditional approaches only represent a small percentage of potential targets. More recently, some groups have initiated programs to create large numbers of RNAi lines. RNAi produces a partial inhibition of gene expression that generally leads to higher suppression compared with antisense methods. Virus-induced gene silencing methods have the potential to speed up the genetic identification of potential herbicide targets. Within a few years, lists of hundreds of potential herbicide targets might be formulated. There will likely be a premium on the speed and effectiveness with which the next two stages of agrochemical discovery pipeline (role of the protein and development of high-throughput assay) will be developed.

Genomics has allowed for the discovery of many genes with unknown functions. Herbicide research could possibly contribute to elucidating the function of these gene products while possibly providing new active ingredients for the marketplace. There are historical parallels in which herbicide research led to much of what we know about photosynthetic function through tracers, inhibitors, and resistant plant species. Bioinformatics tools will allow the common metabolic response (Ott *et al.* 2003) of the plant to be profiled and compared with known herbicide MOA so that enzymes that are targeted by potential herbicides can be viewed by known pathways and biological processes (Thimm *et al.* 2004). Genomics should allow high-throughput testing of target-based screening based on genes that are affected by a test compound.

Currently, most known herbicides interfere with the synthesis of an essential compound by inhibiting a rate-limiting step in a biosynthetic pathway. The use of genomic technologies

could allow the discovery of target sites that do not have enzymatic function or any known function at all. These possible target sites could be regulatory proteins or components of signal transduction pathways.

Genomics has the potential to increase the efficiency of discovering new herbicide mechanisms-of-action. This can be achieved by two general strategies: (1) reverse genetics in which genes are knocked out in a model organism resulting in a reduced-function or non-functional target site leading to a detectable and relevant phenotype, and (2) forward genetics in which a model organism is treated with an active compound with an unknown mechanism-of-action and the molecular target is subsequently uncovered (Stenzel 2004; Egner *et al.* 2005). To identify a target by reverse genetics, it is necessary to generate knock-out mutants that display mimicking effects or lethal phenotypes similar to an herbicide treatment. Such mutants can be generated via multiple genomics methods such as chemical mutagenesis, transposon mutagenesis, antisense down regulation, sense-cosuppression, ribozymes, or RNAi technology (Stenzel 2004). Death of the plant from the disabling of a specific protein might confirm a potential herbicide target site. The ideal target site needs to fulfill at least the criteria of what Stenzel (2004) calls (1) essentiality or proven by genomic knockout in the model organism, (2) druggability or discovery of small molecules binding to the target protein, (3) lethality proven by *in vivo* activity in a subsequent *in vivo* screen, and (4) proof by commercial success.

Klaus Grossmann (2005) described a physiognomic approach to herbicide discovery in which test compounds are compared to known herbicide mechanisms-of-action on several whole plant levels, including functional gene identification, gene expression, protein profiling, histochemistry, and analysis via metabolite profiling.

Gene expression profiling (genomics) and metabolic profiling (metabolomics) can allow fast and reliable detection of known herbicides' mechanisms-of-action and clear identification and classification of herbicides with an unknown mechanism-of-action (Ott *et al.* 2003). Artificial neural networks analysis of ^{1}H NMR spectra was used to determine changes in the metabolic profile (or metabolome) of maize caused by herbicide application. Ott *et al.* (2003) used this method to classify nineteen distinct herbicide mechanisms-of-action in maize. Genomic, metabolomic, and proteomic technology can also be used to analyze potential changes in crop plants from genetic transformation. This could be used to allay consumer fears over genetically modified crops in regard to the nutritional content or allergenicity of a modified crop (Wheelock and Miyagawa 2006).

Approaches for mining and exploiting genomic information that rely solely on genetic or molecular techniques typically do not provide sufficient confidence that a potential site of interest can be effectively modulated by chemical intervention. For example, the effect of a genetic knockout of a gene may have more impact than the impact of a chemical inhibitor of the protein. Conversely, genetic redundancy may underestimate the potential effects on an inhibitor that can interact with two or more members of a target encoded by a gene family. The design of new chemistry that interacts with a novel site of interest predicted from genetic evidence requires significant resources and a level of risk that is typically not taken by companies interested in pesticide discovery. There is a great need for shortcuts in this discovery process that can take advantage of genomic information while simultaneously providing insights into chemistry that can effectively interact with new sites-of-action. A hybrid or chemical genetic approach may be the most practical route (Walsh 2007).

Chemical genetics can be defined as the use of small molecules to mimic the effect of genetic mutations in a biological system of interest (Stockwell 2000), allowing the production of a specific phenotype in a treated organism or cell that can be investigated in much the same way as a genetic mutant. This approach allows for compounds to be applied and removed at specific times and tissues to rapidly produce their effects, with their effects being readily titratable in

a dose response. The use of chemistry to interrupt or modulate key biological processes can produce "phenotypes" with distinct physiological impairment or lethality. In this manner, the principles of forward genetic screening for distinct and desired phenotypes can be readily used to organize a chemical genetic approach for pesticide discovery that takes advantage of both chemical screening and genomic resources.

Genomic screening in model organisms can be used to identify phenotypes of interest that might allow the discovery of potential novel target sites. However, no obvious chemical starting points are available now. Validation that a target has the potential to be chemically modulated can be difficult to achieve and might require considerable resources with little chance of return of a commercial product. In addition, the barrier of translating *in vitro* results to *in vivo* activity can be difficult to overcome. A chemical genetic approach combines the use of an organized chemical library with phenotype screens and robust target identification to produce novel targets of interest coupled with interacting chemistry (Grossmann 2005). This approach requires more upstream tools than other approaches. There are three components of a chemical genetic process to uncover novel sites of herbicide action: chemical libraries, phenotype screens, and target site identification.

Better Use Of Existing Herbicides

Herbicide Safeners

Herbicide safeners are chemicals that reduce herbicide toxicity to crop plants via a physiological mechanism, usually by enhancing herbicide metabolism. They can be used to examine systemwide effects of an herbicide application on a target species. For example, Castro *et al.* (2005) treated grapevine with flumioxazin and found that thirty-three distinct proteins had altered synthesis patterns compared with untreated plants. These proteins included a diverse range of functions including photosynthesis-related proteins and antioxidant systems, allowing an overview of the systemic effects of the herbicide application. Zhang and Reichers (2004) used a similar approach to examine the influence of the herbicide safener fluxofenim on the chloroacetamide herbicide dimethenamid in wheat. They found that the safener caused eighteen proteins to be induced, including fifteen glutathione-S-transferase (GST) subunits and three proteins with known roles in glycolysis and the Krebs cycle. Herbicide safeners were shown to induce GSTs and glucosyltransferases in maize and *Arabidopsis* (Edwards *et al.* 2005). This could be used to differentiate safener use in a wide range of crop species.

Surfactants

Pesticide surfactants are chemicals that improve the emulsifying, dispersing, spreading, and wetting properties of herbicides, improving their foliar uptake. Madhou *et al.* (2006) examined the role of surfactants on plant gene expression in *Arabidopsis*. The expression of 169 genes were altered within one hour after plants were treated with 0.2% volume/volume of surfactant NUK1026. Functional category analysis of these genes revealed that the largest categories included metabolism, physiological processes, transport, protein metabolism, response to stimulus, and transcription. Genes coding for cytochrome P450 and GST proteins were unregulated as were enzymes involved in 1-aminocyclopropane-1-carboxylate synthase genes for ethylene production.

Summary

Herbicide resistance in a growing number of weed species coupled with a lack of new herbicides has brought traditional chemical weed control programs to a crossroads. In the near future there could be several weed species without adequate chemical control in major row crops. However, new genomic technologies could potentially provide weed scientists with more herbicides with novel mechanisms-of-action and a better understanding of herbicide resistance, and provide techniques to improve the efficacy and crop safety of current herbicides. Thus, weed scientists are in a unique position to collaborate with genomicists in discovery research that could lead to better weed management.

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