Chapter 7

Selection for Herbicide Resistance at the Whole-Plant Level

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We have isolated single, nuclear, dominant mutations in whole plants of the small crucifer Arabidopsis thaliana which confer a high level of resistance to a sulfonylurea herbicide. The ease and rapidity with which such mutations can be isolated using A.thaliana suggests that the approach may be of broad utility for studies of herbicide resistance and mode of action.

The growing dependence of modern agriculture on herbicidal compounds has led to the development of hundreds of commercial herbicides which inhibit a wide variety of plant processes (1). By analogy with bacterial antibiotics, the primary target of many herbicides is a specific step in a biochemical or physiological pathway and may involve only a single protein (2,3). It is not surprising, therefore, that single mutations in genes encoding such proteins, can increase the resistance of a plant to a herbicide (2,4-7). These mutations may cause a loss of function by decreasing herbicide uptake or decreasing the activity of an enzyme that normally metabolizes the herbicide to a toxic form. Herbicide resistance can also occur by a gain-of-function mutation which increases catabolism of the herbicide to a nontoxic form, overproduces a component affected by the herbicide, or alters the structure of a protein thereby decreasing protein-herbicide interaction.

Herbicide-resistant plant varieties have proven to be valuable experimental tools in determining the molecular mode of action of herbicides (2,8). In addition, such varieties are likely to be an important source of selectable markers for use in plant molecular genetics and in the engineering of resistant crop species (9). The earliest herbicide-resistant biotypes described arose spontaneously from weed populations which had been repeatedly exposed to a herbicide (10,11). More recently, mutagenesis and selection on defined media have been used to isolate herbicide-resistant or herbicide-tolerant mutants of higher plants from populations of cells in tissue culture (4,5,7,12). While the use of plant tissue culture has proven useful for isolation of some types of mutants, the technique has several drawbacks: [1] regeneration of the mutants may be difficult or impossible, [2] mutations expressed in culture may be unstable or may not be expressed at the whole plant level, [3] some herbicides may not be lethal at the cell culture level, and [4] the time-scale for mutant isolation and plant regeneration can be relatively long.

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Although direct selection at the whole plant level has been successfully used to isolate auxin-resistant mutants of Arabidopsis thaliana (6), the approach has not been widely used. We describe here the selection at the whole plant level of rare mutants of A. thaliana resistant to the herbicide chlorsulfuron. We suggest that the approach described here can be easily and inexpensively applied to the isolation of mutants resistant to any one of a wide range of herbicidal compounds. After producing M2 seed it requires only a few hours of work to carry out the manual manipulations and the result is available in less than two weeks. The selection is rapid, simple, requires relatively little space and has none of the drawbacks associated with tissue culture. In principle, the technique can be easily applied to any plant which is easily mutagenized and has a small seed size. However, the use of A. thaliana with its small genome, short life cycle, ease of handling and well-defined genetics (for recent reviews see 13,14,15) increases the probability of isolating a specific mutation and facilitates gene isolation and subsequent genetic and molecular analysis.

Chlorsulfuron

Chlorsulfuron (2-chloro-N[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl]benzenesulfonamide) is a potent sulfonylurea herbicide produced by DuPont. Several lines of evidence strongly support the idea that sulfonylurea herbicides act by specifically inhibiting acetolactate synthase (ALS), the first enzyme of the branched-chain amino acid biosynthetic pathway (Fig-1). First, effects of the herbicides are reversed by exogenously supplied isoleucine and valine, the end products of the branched-chain amino acid biosynthetic pathway (16-18). Second, ALS activity is inhibited in vitro by low concentrations of the sulfonylurea herbicides (8,16,18,19). Finally, sulfonylurea-resistant mutants of Escherichia coli, (20), Salmonella typhimurium (16), yeast (19) and tobacco (4) have been isolated. In each case the dominant herbicide resistant phenotype cosegregates with a sulfonylurea-resistant ALS activity. The mutations conferring resistance in enteric bacteria and yeast are known to map to the ALS structural genes ilvG and ILV2, respectively (16,19,20).

A. thaliana is rapidly killed by low concentrations of chlorsulfuron. When seeds were placed on a defined agar medium (21) containing concentrations of herbicide exceeding 28 nM (10 ppb), seedling emergence was completely inhibited. As expected, addition of 1.0 mM isoleucine and valine together, but not separately, allowed germination even in the presence of 280 nM chlorsulfuron. Thus, it appears that the only target in A. thaliana for the herbicide is branched-chain amino acid biosynthesis. Although it is convenient to know beforehand that there is a single mode of toxicity, it is not a prerequisite to the isolation of resistant mutants.

Mutagenesis

Mutagenesis is used to increase the mutation frequency in A. thaliana over 1000-fold (22), thereby making feasible the isolation of very rare mutants. Although a wide range of radiation and chemical treatments are effective in mutagenizing A. thaliana, a simple procedure involving the exposure of seed to the mutagen ethyl methane sulfonate (EMS) is commonly used (21). This compound is believed to cause mutations by alkylating the N-7 or the O-6 positions of guanine (23). The fate of N-7 alkylated guanine is uncertain. However the O-6 methylation appears to cause primarily GC to AT transitions. This possibility of specificity implies that not all possible mutations will be represented in an EMS-mutagenized population irrespective of the population size. Thus, the use of another mutagen with different specificity may be advisable under some circumstances.

Mutagenized seed of A. thaliana gives rise to M1 plants chimeric for a large number of heterozygous mutations. Spontaneous self-fertilization of M1 plants produces M2 seed carrying those mutations induced in one of the two embryonic germline cells (24). The M2 rather than the M1 generation is used for mutant isolation because M2 plants are no longer chimeras and nuclear mutations have segregated to produce homozygous seed.

Redei (25) has calculated the necessary size of the M1 population and the number of bulked M2 plants that need to be screened in order to have a high probability of recovering a recessive loss-of-function mutation. Although a recessive loss-of-function mutation can result from any one of a number of nucleotide substitutions, additions or deletions within a specific gene, a dominant gain-of-function mutation is likely to be caused by only one or a few changes within the genome. For example, varietal resistance to Atrazine appears to be conferred by a relatively few specific changes within the gene for the 32kd protein (26,27). Because many mutations causing herbicide resistance are a result of relatively rare gain-of-function mutations, it is important to determine the size of the M1 and M2 populations needed to recover any mutation within the genome. Under our standard conditions for EMS mutagenesis, the frequency of recovery of simple loss-of-function mutants for any one of several different enzymatic activities is approximately 1 per 2000 M2 plants (28-30). Using this number as an estimate of the average frequency of loss-of-function mutants and some broad assumptions, we can calculate the probability of saturating the genome with mutations under our standard mutagenesis conditions. To do this we first need to estimate the number of different EMS mutational events which can give rise to a loss-of-function mutant. The percentage of GC to AT transitions which result in an amino acid substitution or chain termination is 60.4% and 5.2%, respectively. If we assume that there is no bias in codon usage, then 65.6 represents the maximum percentage of EMS induced changes which could result in a loss of function. However, many amino acid substitutions have a neutral effect on the function of a protein. It has been estimated (31) that

only 42% of amino acid substitutions affect the lac repressor. Using the lac repressor as a model it can be estimated that (42% x 60.4%) + 5.2% or 31% of EMS-induced changes result in a loss-of-function.

If we assume that on average the coding region of a gene is 1500 bp and that the GC content of the A. thaliana chromosome is 41.4% (32), then the possible number of EMS induced changes per gene is 1500 x 0.414 or 621. A given loss of function mutant could have any one of 31% x 621 = 193 different mutations. Therefore, the frequency of inducing a mutation in a specific nucleotide is 1/[2000 x 193] = 1 mutation in 386,000 M2 plants. With this number as an estimate, the size of an M2 population required to represent every possible EMS-induced homozygous mutation (excepting gamete or embryonic lethals) with a given probability can be calculated using the binomial equation:

$$N = \frac{\ln(1 - P)}{\ln(1 - X)}$$

Where P = probability that every mutation is represented, N = the number of plants in the population and X = the frequency of occurrence of a specific mutation in the population. Using the values derived above and a probability of 95%, this gives a value of:

$$N = \frac{\ln([1-.95]}{\ln[1-1/386000]}$$

Where $N = 9.99 \times 10^5$ plants and it is assumed that the M1 population size is not limiting. The number of viable fertile M1 plants that are needed to generate the necessary M2 diversity is then calculated by the formula:

$$\frac{\text{\# M2 plants needed to cover all mutations}}{\text{ploidy factor x GECN}}$$

In A. thaliana the genetically effective cell number (GECN) is two (24) and the ploidy factor is four (a mutation heterozygous in the M1 parent has a 25% chance of being homozygous in a given M2 progeny). Thus, approximately $9.99 \times 10^5/8 = 125{,}000$ M1 plants are needed.

Our M1 populations are, generally 100,000 to 200,000 plants in size. Thus, the calculations above suggest that we routinely saturate the genome with all possible EMS-induced mutations. Furthermore, the high seed yield per plant and the small size of A. thaliana seed make mutant selections using 1.0×10^6 M2 seed feasible.

One additional calculation can be derived from the discussion above. The size of the A. thaliana genome is 70,000 kb (32). The total number of possible EMS-induced changes is $7 \times 10^7 \times 0.414 = 2.9 \times 10^7$. Thus, if any given mutation occurs at a frequency of 1/386000 plants then there must be 2.9×10^7 homozygous mutations per 3.86×10^5 plants or 73 homozygous mutations per M2 plant; 219 including heterozygous mutations. Many of these mutations will not affect the function of any gene because they cause a neutral change or are not within the coding region of a gene. Nevertheless, such a calculation

underscores the need to decrease the number of background mutations in a newly isolated mutant by repeated backcrossing to a wild type strain.

Selection of Herbicide Resistant Mutants

Herbicide resistant mutants are selected on a solid sterile selection medium prepared by autoclaving a minimal salts nutrient solution (21) with 0.7% agar. After cooling to 50C the herbicide is added and the medium is distributed to 9 cm petri plates. Initially a range of herbicide concentrations should be tested to determine the lowest concentration of herbicide that kills germinating wild-type seedlings effectively. This concentration is then used for the large-scale selection of M2 seed.

Seed is sterilized for 15 minutes in a solution containing 5% sodium hypochlorite (30% laundry bleach) and 0.02% Triton-X100 at a concentration of 1000 seed/ml. Following treatment, seeds are rinsed 5 to 10 times with sterile distilled water. Large numbers of sterilized seed can be distributed onto plates by rotating plates on a bacteriological turntable and delivering a spiral of seed onto the surface of the spinning plate from a pasteur pipet (Fig. 2). A piece of cheesecloth placed on the surface of the agar prevents clumping of the seed. Using this technique we have distributed up to 10,000 M2 seed onto a single petri plate. Such a high plating density is only practical for selection purposes if the herbicide kills at a very early stage of development, as is the case for chlorsulfuron. By contrast, A. thaliana seedlings develop past the cotyledon stage on even high (1 mM) concentrations of glyphosate (Haughn and Moffatt, unpublished). At plating densities higher than 2000 seed/plate, mutants resistant to glyphosate would not be easily distinguished from sensitive seedlings due to loss of visual resolution.

Selection plates with M2 seed are incubated at 21 to 25C under continuous fluorescent illumination at about 80 uE m^{-2} s^{-1}. Herbicide resistant mutants should be easily recognizable after 5 to 10 days. Putative resistant plants are transplanted to soil and retested for resistance in the subsequent generations.

Chlorsulfuron Resistant Mutants

Three hundred thousand A. thaliana M2 seed were placed on 30 petri plates containing 0.2 uM (75 ppb) chlorsulfuron. Four putative chlorsulfuron resistant mutants were readily identified against a background of non-germinated seeds. When progeny from these plants were retested in the M3 generation, three of the four lines proved to be resistant to chlorsulfuron. One of the mutant lines, designated GH50, was tested in the M4 generation for its level of resistance. Six day old seedlings were transferred to plates containing a range of concentrations of the herbicide and incubated at 24C in continuous illumination for an additional two weeks. This line showed a level of resistance 300 to 1000-fold higher than that of the wild type (Fig. 3).

Genetic analysis has shown that of the three resistant plants

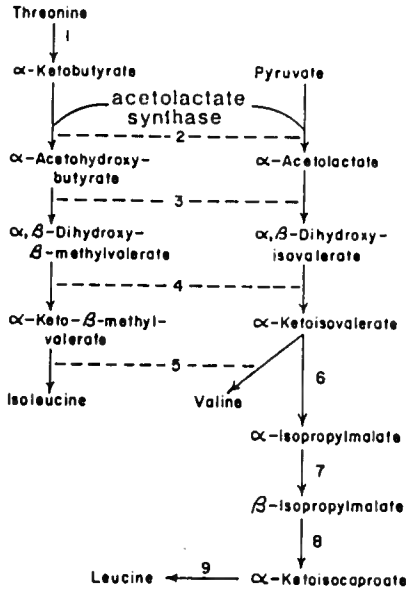

Figure 1. The branched-chain amino acid biosynthetic pathways of enteric bacteria (35). The biosynthetic step catalyzed by acetolactate synthase is step 2.

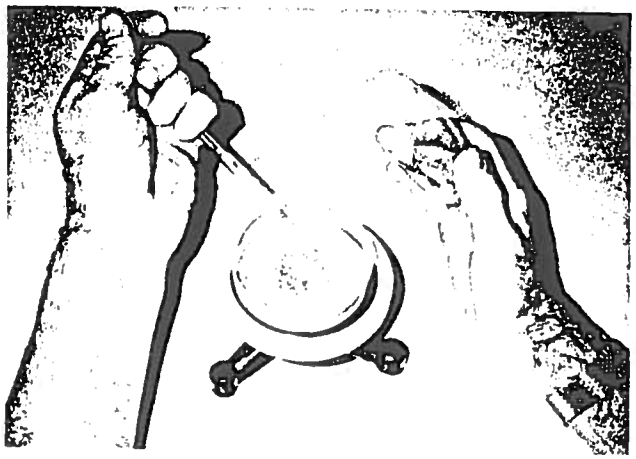

Figure 2. Illustration of the technique used to distribute high numbers of A. thaliana seed onto petri plates.

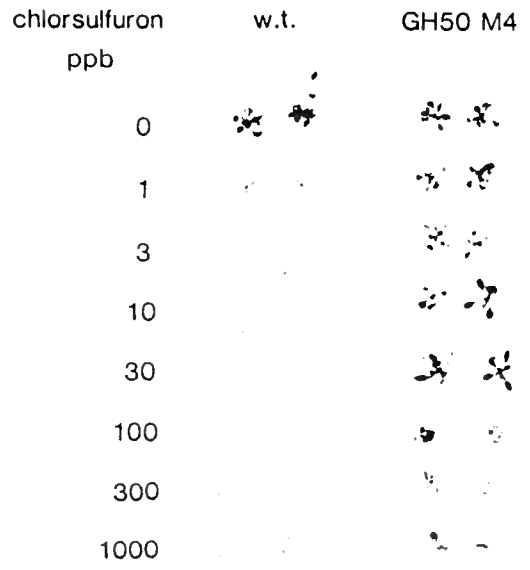

Figure 3. The effect of increasing concentrations of chlorsulfuron on the growth of wild type and resistant (GH50) lines of A. thaliana.

originally selected, one was heterozygous and two were homozygous for a single nuclear mutation at a locus we have designated csr (Haughn and Somerville, in preparation). Thus the frequency of a homozygous mutant in the M2 population was no higher than 1 in 1.5 x 10^3, or approximately 75-fold lower than the frequency of a simple loss-of-function mutation. If 193 EMS-induced mutations/gene can give a loss of function (see section on mutagenesis), then it is likely that only 1 to 3 nucleotides/genome can be altered by EMS to give resistance to 0.2 uM chlorsulfuron. This result is expected of a dominant gain-of-function mutation.

The frequency of the chlorsulfuron-resistance mutations has some practical implications with respect to the frequency with which herbicide-resistant weed biotypes may be expected to arise following agricultural use of the herbicide. Our estimate is that a spontaneous mutant comparable to those described here should arise at a frequency of about 1 per 10^9 plants (ie., 1 per 150000 for a mutagenized population in which the frequency was increased 5000-fold). If we assume that a typical number of viable weed seeds per hectare is about 0.13 billion (33) then we may expect to find about 1 chlorsulfuron-resistant weed per 8 hectares.

Our preliminary results indicate that, like the chlorsulfuron-resistant tobacco mutant, the A. thaliana line GH50 has chlorsulfuron-resistant ALS activity in vitro (34). It is possible, therefore, that the chlorsulfuron-resistance mutation of GH50 lies within the structural gene for ALS. In the absence of chlorsulfuron the mutants do not grow as quickly as the wild-type. However, it is not yet clear whether this is related to the herbicide resistance or to the many other mutations which are induced during mutagenesis.

Discussion

We have described methods which allow the selection of relatively rare chlorsulfuron-resistant mutants in A. thaliana at the whole plant level. We suggest that the approach is of broad utility for the investigation of herbicide mode of action because of simplicity and speed. The approach is so simple that it can be used as a diagnostic tool to determine the relative frequency with which spontaneous mutants resistant to a given herbicide arise - an important criterion in determining the usefulness of a newly developed herbicide.

Assuming that the chlorsulfuron resistance in the A. thaliana mutants described here is due to a mutation within the ALS structural locus, it may be possible to clone the resistant allele. Such a resistance gene should provide a very useful selectable marker for plant transformation studies. Indeed, transfer of the gene to other species may be useful in extending the agronomic utility of the herbicide.

The approach described here is not restricted to sulfonylureas. Our laboratory has successfully applied these techniques in isolating mutants resistant to the herbicides 2,4-dichlorophenoxyacetic acid (Estelle and Somerville, in preparation) and an Imazidolinone

herbicide (Haughn and Somerville, in preparation). By contrast, we have not yet been able to identify mutations which confer high-level resistance to glyphosate. This is particularly intriguing in view of the fact that single gene resistance to this herbicide has been established in microorganisms. It is possible that in the case of glyphosate resistance in A. thaliana the required amino acid substitution cannot occur by way of a GC to AT transition.

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