Agriculture: The selector of improbable mutations

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Crop domestication took place mainly through the selection for mutations that are advantageous for agriculture. In parallel, an unconscious process of selection for undesirable mutants is taking place, such as the selection for pests (weeds, insects, and pathogens) and more recently for pests that are resistant to pesticides. Experiments for selection of exceedingly rare events cannot be performed in the laboratory due to time and space constraints. Farmers, who enjoy larger facilities than scientists, are demonstrating time and again that they are capable of selecting for mutations that researchers cannot find. We are used to reports of single-nucleotide point mutations that change an amino acid, but in the report by Patzoldt *et al.* (1) in this issue of PNAS, we are regaled with a mutant that has lost a whole amino acid in the target protein of a xenobiotic herbicide as well as lost the gene encoding an isozyme of the target of the herbicide. People have easily made artificial deletion mutations in the laboratory by using DNA synthesizers, but naturally occurring mutations with internal deleted amino acids have not been reported. Single-nucleotide point mutations typically occur at a frequency of approximately one in a million per gene and approximately one per billion per specific nucleotide. It is thus conceivable to obtain herbicide resistance due to a single nucleotide substitution. If two independent mutations are necessary to obtain the resistance phenotype, e.g., in both alleles for recessive mutations or if two amino acids must be substituted, the frequencies go lower and jump to the square (e.g., 10^{-18} instead of 10^{-9} per nucleotide). Taking into account the possibility of gene conversion, or of partial resistance of the single mutant, the actual frequency for a double mutant might be higher. Still, these frequencies are extremely low and are probably in a range where the odds for such mutations in any mammalian species are almost nil.

Evolution of Other Rare Resistances

Agriculture can do the trick, and has, especially with weeds and herbicides (2). Herbicides apply intense selection pressure and weed seeds are ubiquitous. A species such as *Amaranthus* (pigweeds and waterhemp) can have seed banks with >600 million seeds per hectare if uncontrolled, and 200 million seeds per hectare if continuously treated with conventional herbicides (3). Some herbicides are used on over a million hectares per year, so it is possible that 10^{14} seeds of one species could be treated. Thus, weeds are more ubiquitous than insects (but not fungi), and herbicides are applied to much larger areas than insecticides and fungicides; in 2002, 66% of pesticide use in the United States was of herbicides (4). The selection pressure is highest when herbicides with high residual activity (season-long weed control) are used. Few currently used insecticides and fungicides have such residual activities, and thus they have not exerted the selection pressures that led to selection of rare mutations. It was widely proposed that current agricultural practices could lead to selection of insect resistance to *Bacillus thurengiensis* (Bt) toxins in transgenic crops, and widely ignored (by farmers) models were formulated to delay this (5), and resistance did not evolve in the field. It is now known that more than one gene mutation would be required to confer resistance to the Bt toxin levels in transgenic crops (see ref. 6), and the models were probably highly incorrect due to wrong assumptions, and resistance may be a long way off (6); this might not be the case if we were dealing with weeds with two gene mutations required for resistance. At the seed densities found, such a double mutation might be found in a million hectares.

More than one mutation would be required to confer resistance to the *Bacillus thurengiensis* toxin levels.

Thus, the repeated use of a herbicide on millions of hectares annually can do what we are unable to do in the laboratory with higher plants. For example, the manufacturers of glyphosate performed experiments to ascertain the frequency of glyphosate resistance in EMS mutagenized plants. There were no glyphosate-resistant mutants among one million M_2 progeny of 250,000 *Arabidopsis thaliana* M_1 lines, but resistance to acetolactate synthase inhibiting herbicides appeared at frequencies of 3.2×10^{-5} (7). Such results allowed them to declare that it was highly unlikely that resistance would occur in the field (8), but nature is wiser and resistance has appeared in the field (www.weedscience.org), and at least in some cases is due to mutations in two genes (9, †). The high prevalence of many cases of concurrent resistance in particular species may be that one of the two genes is already present and/or there may be mutator gene(s) active in that weed (2). When farmers use low herbicide doses, or if the weed germinates after much of the pesticide has dissipated, it is possible to select for one of the two genes, if each provides a modicum of resistance. The second gene can be then selected for by the higher dose (2).

The occurrences of herbicide resistance in the field have led to important basic research with implications far beyond agriculture. Farmers using triazine herbicides selected for resistance based on a point mutation in the chloroplast *psbA* gene. Leaf cells each contain many chloroplasts, and each chloroplast has many copies of DNA. When the wild-type gene product is present with the herbicide, and a plant is in the sunny field, active forms of oxygen are formed that fry the cells. Thus, for a plant to live, all of the chloroplast DNA molecules in all of the cells must have the mutant *psbA.* Thus, the frequency of individuals with resistance must be exceedingly low, too low to even guess a number, but theoretically far less than 10^{-15}. We are still not clear how this can happen, but it has happened enough times over the four decades that these herbicides have been used, such that millions of hectares have triazine-resistant weeds, often with the offspring of a single mutational event spreading slowly over thousands of square kilometers (for example, see ref. 10). Such findings led Michel and Diesenhofer (11, 12) to generate triazine-resistant mutations in purple bacteria where there is but a single copy of DNA, crystallize and compare wild-type and mutant proteins, and look at the binding by x-ray crystallography. Their Nobel prize-winning work on herbicide nonbinding in the mutants is the basis for much of our understanding of drug binding and drug resistance and is used for drug design, all far from the farmers' fields.

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How Could Protox Deletion Resistance Have Evolved?

To get the resistance protoporphyrinogen oxidase (protox) inhibiting herbicides reported by Patzoldt *et al.* (1), two things seemed to have happened: the mutant protox had to have a very low binding affinity to the herbicide and the mutant protox had to be targeted to both the chloroplasts and the mitochondria. A blockage of protox in either organelle would result in a build up of photodynamically toxic protoporphyrin IX. They found that their mutant has a hitherto unknown "one size fits all" transit peptide that targets products to both chloroplasts and mitochondria. They consider that the loss of the gene encoding the isozyme may be an evolutionary afterthought, occurring after the second gene was made redundant, but it could be that resistance could only evolve in plants lacking this isozyme. The resistance they found took decades to evolve clearly because of the rarity of the event but also because of the fact that protox-inhibiting herbicides are not nearly as widely used as other types. Resistant populations probably would have evolved earlier if broader use had been achieved.

Mutant protoxes have been generated in the laboratory that confer a modicum of resistance due to one or two mutations within the protox gene (13–15) but never a deletion. How can one obtain the reported mutation where a whole 3-nt codon is missing from a gene resulting in a whole amino acid lacking in the protein, as reported by Patzoldt *et al.* (1)? A few possibilities come to mind.

The most common mutations are point mutations, resulting from a nucleotide misincorporation during DNA replication, or from error-prone repair of damaged DNA. The frequency for such mutations that might alter amino acid composition, or create stop codons, is in the 10^{-9} range. Obviously, these classical mutations cannot explain the loss of a nucleotide triplet. Such deletion could occur through the slippage of DNA polymerase in an array of microsatellites consisting of repeats of a triplet, as for example in the case of the p(CCG)n repeat responsible for the fragile site in the fragile X syndrome (16). In the current manuscript, the single codon deletion appeared in the context of two overlapping trinucleotide repeats [$(TGG)_2$ or $(GTG)_2$] as shown in Fig. 1. It is generally established that the longer a repeat array, the less stable it is (17). However, the stability of very short arrays is not known in plants. In other organisms, there are conflicting data on the fate of such "nanosatellites." Some studies suggest that ditrinucleotide repeats are below the threshold of slippage (17). Other studies in mammals suggest that single codon deletions are ~5-fold more frequent than expected in the context of two trinucleotide repeats, suggesting that nanosatellites are more prone to slippage-like events than nonrepeat sequences (18). In addition to the single codon deletion responsible for herbicide resistance, Patzoldt *et al.* report an additional trinucleotide polymorphism (unrelated to resistance) between the resistant and the sensitive PPO allele, also in the context of a di-trinucleotide. These two instances of single-codon deletion suggest that nanosatellites might also be unstable in plants, a possibility that deserves genome-wide analysis. Other mutators, such as transposons are notorious for leaving footprints following their excision. However, although they can cause small deletions, they usually leave parts of the host duplication they generated upon insertion (19). We would thus have to assume transposon insertion (a rare event by itself) followed by untypical excision; this is possible, but unlikely. Another more likely mechanism for generating an indel is through DNA double-strand break repair. In plants, it has been shown that the repair of double-strand breaks occurs essentially by end-joining and that this process is error-prone, generating a wide array of deletions or insertions at the site of the break (20). According to such a scenario, a double-strand break occurred at the site of the deletion and was repaired in a way that left 3 nt missing. Interestingly, such an event would have occurred a few times independently.

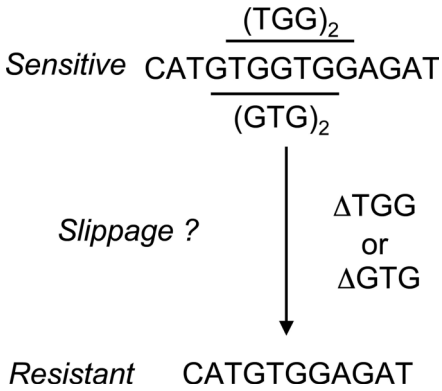

Fig. 1. Single-codon deletion through a slippage-like mechanism. The deletion leading to the deletion of glycine G210 in the PPX2L gene occurred in the genomic context of two overlapping trinucleotide repeats, $(TGG)_2$ or $(GTG)_2$, the deleted triplet unit is either a TGG or a GTG. In both cases, the deletion corresponds to a triplet unit and thus might have been lost through a slippage-like mechanism.

Another intriguing finding of the work by Patzoldt *et al.* (1) is that the independent R biotypes had, in addition to the glycine 210 deletion in protox PP2XL, a deletion of the whole protox PP2X gene. Although the bacterial complementation data suggest that the glycine 210 deletion is sufficient to confer resistance to lactofen, it remains highly surprising that the codon deletion was associated with the loss of another homologous (protox PP2X) gene in all cases assayed. It is very difficult to assume that the gene loss and the amino acid loss (both of which are very rare events) have both occurred by chance in independent mutants and that this is not related to the herbicide resistance phenotype. Further studies will be needed to address this issue.

Thus, we have learned of a way that nature has of evolving. Surely, this mechanism will be used in developing further generations of transgenic herbicide-resistant crops (the most widespread transgenic crops presently used) and for many other uses. It also tells us to be on the lookout for such deletion mutations in drug-resistant cells and in many other situations.

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