Discovery and Directed Evolution of a Glyphosate Tolerance Gene

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The herbicide glyphosate is effectively detoxified by *N*-acetylation. We screened a collection of microbial isolates and discovered enzymes exhibiting glyphosate *N*-acetyltransferase (GAT) activity. Kinetic properties of the discovered enzymes were insufficient to confer glyphosate tolerance to transgenic organisms. Eleven iterations of DNA shuffling improved enzyme efficiency by nearly four orders of magnitude from 0.87 mM^{-1} min^{-1} to 8320 mM^{-1} min^{-1}. From the fifth iteration and beyond, GAT enzymes conferred increasing glyphosate tolerance to *Escherichia coli*, *Arabidopsis*, tobacco, and maize. Glyphosate acetylation provides an alternative strategy for supporting glyphosate use on crops.

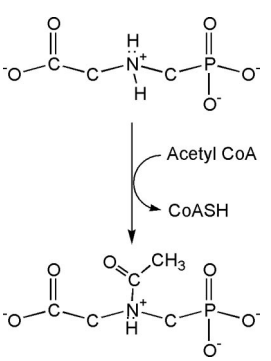
Herbicide tolerance is the most widely planted transgenic crop trait. Globally, about 75% of genetically modified crops are engineered for herbicide tolerance (*1*). Glyphosate-tolerant crops marketed as Roundup Ready occupy the greatest acreage. The popularity of glyphosate stems from its effectiveness, low cost, and low environmental impact (*2*). Glyphosate inhibits the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the plant chloroplast-localized pathway that leads to the biosynthesis of aromatic amino acids (*3*). Some microbial EPSPS enzyme variants are not inhibited by glyphosate and provide the only mode of action for today's commercial glyphosate-tolerant crops. However, the glyphosate remains in the plant and accumulates in meristems (*4*), where it may interfere with reproductive development and may lower crop yield (*5*). Tolerance is more commonly achieved through metabolic detoxification of herbicides by native plant or transgene-encoded enzymes. Commercial transgenic detoxification traits currently in use for other herbicides involve hydrolysis, acetylation, and oxidative cleavage (*6*–*9*). In each case, the relevant transgene is of microbial origin.

The advantage of glyphosate detoxification is the removal of herbicidal residue, which may result in more robust tolerance and allow spraying during reproductive development. *N*-Acetylglyphosate is not herbicidal (*10*) and is not an effective inhibitor of EPSPS (*11*). We sought a soluble enzyme capable of carrying out *N*-acetylation of glyphosate (Scheme 1). Acetyl coenzyme A (AcCoA) acts as an acetyl donor to a wide variety of substrates. Typically, hydroxyl groups and primary amines are the acceptors. Chemical acetylation of secondary amines is feasible, but enzymatic acetylation of compounds such as glyphosate appears to be uncommon although not unknown (*12*, *13*). We tested a variety of characterized and uncharacterized *N*-acetyltransferases for their ability to accept glyphosate as a substrate (*14*), but none were capable of acetylating the secondary amine.

To find a new enzyme exhibiting activity on glyphosate, we searched within our microbial collection. Because *Bacillus* species produce a wide variety of enzymes involved in secondary metabolism, we focused our search on bacilli isolated from non-extreme environments. We developed a mass spectrometry method (*11*) that detects <0.5 μM *N*-acetylglyphosate. Several hundred isolates were grown to stationary phase, permeabilized, and incubated with the substrates glyphosate and AcCoA. Supernatants were analyzed for *N*-acetylglyphosate. Many strains appeared to produce detectable product, but the *Bacillus licheniformis* strains catalyzed the greatest reproducible accumulation. *B. licheniformis* is a common benign saprophytic bacterium.

The activity was present in the soluble fraction of cell lysates and was heat labile, which suggests that the glyphosate *N*-acetyltransferase (GAT) was a soluble protein. To isolate the gene encoding GAT, we assayed recombinant *E. coli* expressing genomic DNA fragments from *B. licheniformis* by the mass spectrometry method (*11*). DNA sequences of multiple genomic fragments specifying GAT activity from *B. licheniformis* strains B6 and ST401 were determined. The region common to all active fragments contained a single open reading frame of 438 bp preceded by a consensus ribosome binding site (fig. S1). A polymerase chain reaction (PCR) survey of *B. licheniformis* strains revealed a third gene variant in isolate DS3 (*15*). GAT enzymes encoded by these genes (GenBank accessions AX543338, AX543339, and AX543340) are 94% identical. The amino acid diversity in these native GAT enzymes is shown in Fig. 1.

Position-specific iterated BLAST (PSI-BLAST) (*16*) search and analysis indicates that GAT shares similarity with enzymes of the GNAT or GCN5-like *N*-acetyltransferase superfamily (*17*, *18*). GNAT superfamily enzymes are found in mammals, plants, fungi, algae, and bacteria. Members often lack pairwise sequence similarity, although their tertiary structures are similar. Some GNAT proteins are involved in regulating sporulation, transcription, metabolism, or detoxification, but most of them remain to be characterized. Although the native function of GAT remains unknown, it is unusual among characterized acetyltransferases in that it acetylates a secondary amine (glyphosate).



Scheme 1. Enzymatic *N*-acetylation of glyphosate at pH 7.

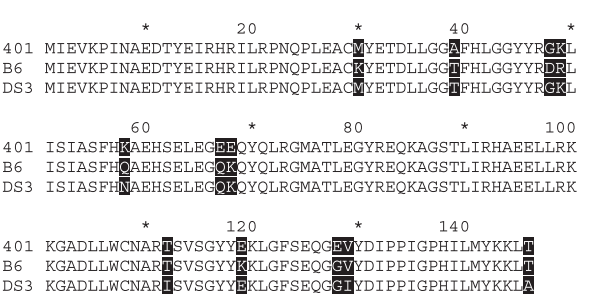


Fig. 1. Amino acid sequence of *B. licheniformis* GAT enzymes (*26*).

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The protein most closely related to GAT is YITI, a hypothetical *N*-acetyltransferase (NAT) protein predicted from the genomic sequence of *B. subtilis*. The *yitI* gene (GenBank accession Y09476) is 63% identical to *gat* and the hypothetical protein sequence is 59% identical and 66% similar to GAT. We used recombinant *E. coli* to produce YITI and found that it was capable of acetylating glyphosate but with lower efficiency than GAT. Similar hypothetical proteins (with GenBank accession numbers and the respective identity to *B. licheniformis* ST401 GAT) are encoded in *B. cereus* YITI (NP_830505, 49%); *B. anthracis* YITI (NP_654645, 48%); *Listeria inocua* NAT (CAC98097, 38%); *L. monocytogenes* NAT (AB1415, 39%); *Chloroflexus aurantiacus* NAT (ZP_00018467, 28%); *Vibrio parahaemolyticus* NAT (NP_799747, 26%); and *Zymomonas mobilis* NAT (CAA63806, 28%).

GAT enzymes are ~17 kD, are most active at pH 7, and require only an acetyl donor and glyphosate for activity. To evaluate GAT activity, we determined the k_{cat}, which is the rate constant of the catalytic reaction, and K_M, a measure of the affinity of the enzyme for the substrate. The K_M of GAT enzymes for AcCoA is 1 to 2 μM (*11*), which strongly suggests that this is the native acetyl substrate. This tight binding property enables enzyme purification by CoA-Agarose affinity chromatography, and subsequent Superdex-75 gel filtration results in protein preparations of >95% purity (*11*). The three parental GAT enzymes are similar and have k_{cat} of 1.0 to 1.7 min^{-1} and K_M for glyphosate of 1.2 to 1.8 mM at pH 6.8 and 21°C (*11*). The ratio of k_{cat}/K_M is a measure of enzyme efficiency and specificity. The average k_{cat}/K_M ratio of the GAT enzymes is 0.81 min^{-1} mM^{-1} (*11*) and was used as the benchmark for improvement using directed evolution.

Although the GAT enzymes from bacilli can acetylate glyphosate, they do so poorly. Production of the GAT proteins in *E. coli* did not enable the cells to grow on medium supplemented with inhibitory concentrations of glyphosate. Similarly, *gat* expression in transgenic tobacco and *Arabidopsis* did not confer herbicide tolerance (*19*). Therefore, we used ST401, B6, and DS3 *gat* genes as parents for fragmentation-based multigene shuffling (*20*, *21*) to create enzymes with higher efficiency and increased specificity for glyphosate with the goal of developing herbicide-tolerant plants.

Libraries of shuffled gene variants were created, expressed in *E. coli*, and screened (*11*). In a primary high-throughput screen, supernatants from permeabilized *E. coli* cells carrying shuffled gene variants were assayed for the quantity of *N*-acetylglyphosate formed. Shuffled variants that specified the accumulation of more product than the parental controls were selected for further analysis. At each iteration of DNA shuffling, we screened about 5000 shuffled gene variants and analyzed 24 to 48 purified enzymes. Typically, 3 to 12 improved variants with a high

Table 1. Summary of directed evolution iterations for improvement of glyphosphate *N*-acetyltransferase activity. The complexity of each library is determined by the number of enzyme sequences possible given the diversity encoded by the parental genes (not counting mutations that arise by PCR).

Shuffling iteration	No. of parental genes	Library complexity	No. of variants screened	Amino acid changes in hits from original parents
1	3	6,144	5000 unselected	1–9
2	10	32,768	5000 unselected	1–9
3	12	2,048	5000 unselected	5–13
4	8	8,192	5000 unselected	6–15
5	3*	1.3×10^8	5000 selected on 1 mM glyphosate	17–26
6	10	3.4×10^7	5000 selected on 1 mM glyphosate	20–30
7	7	131,000	5000 selected on 1 mM glyphosate	20–27
8	6*	4.3×10^6	5000 selected on 5 mM glyphosate	21–34
9	8	131,000	5000 selected on 5 mM glyphosate	27–35
10	9	38,000	5000 selected on 5 mM glyphosate	28–37
11	10	147,456	5000 selected on 5 mM glyphosate	30–35

*Synthetic libraries for incorporation of natural diversity.

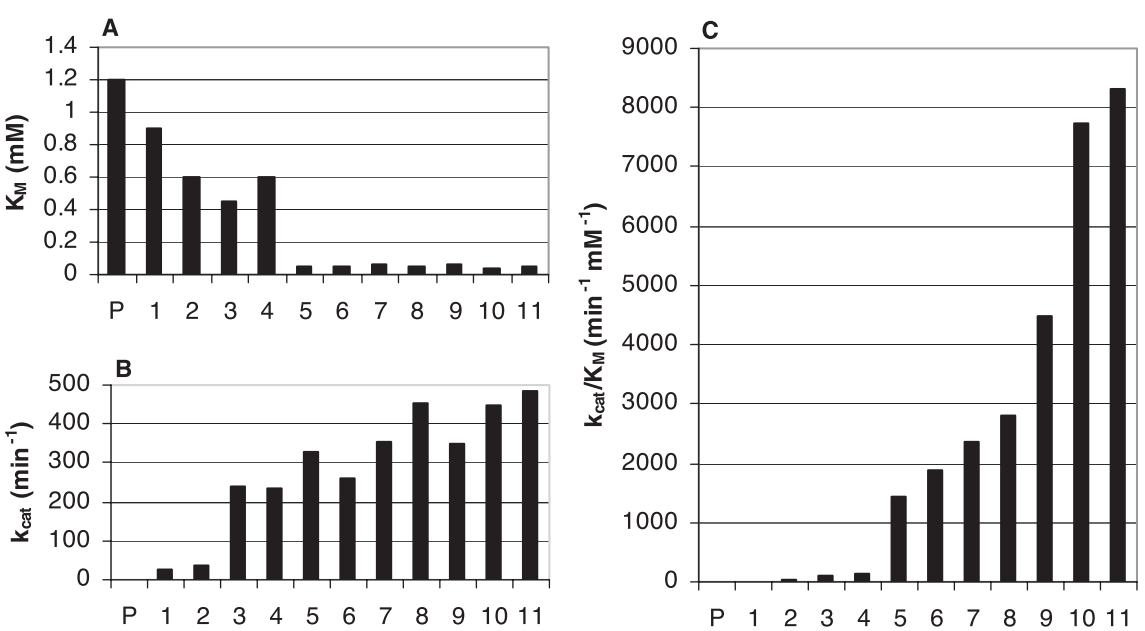

Fig. 2. Evolution of GAT activity. Parental and evolved GAT enzymes were purified and k_{cat}, K_M, and k_{cat}/K_M measured in 20 mM Hepes, pH 6.8, 10% ethylene glycol, 0.17 mM AcCoA, and ammonium glyphosate at 21°C (*11*). (**A**) The lowest K_M, (**B**) the highest k_{cat}, and (**C**) the highest k_{cat}/K_M ratios observed among the variants analyzed in each shuffling iteration. Although plateaus in k_{cat} and K_M occurred, the total catalytic efficiency and specificity of new GAT enzymes continued to increase (C).

k_{cat}, a low K_M, or a high ratio were chosen to be the parents for the next iteration (Table 1). Several GAT enzymes from the third iteration of gene shuffling showed ~100-fold improvement over the parent enzymes to $k_{cat}/K_M = 80\ min^{-1}mM^{-1}$, which was sufficient to allow growth of *E. coli* on 1 to 5 mM glyphosate in minimal agar medium (*11*). However, these gene variants were unable to confer glyphosate tolerance *in planta*.

Although we saw continuous improvement in k_{cat} values, the K_M appeared to plateau at 0.5 mM (Fig. 2). Efforts to elicit further decreases in K_M by random PCR mutagenesis of *gat* genes were unsuccessful. This impasse was addressed using two strategies: (i) a functional prescreen based on resistance of *gat*-expressing *E. coli* to glyphosate and (ii) a dramatic increase in the diversity available for recombination. The functional prescreen allowed the screening of more than 10^6 variants by eliminating those with low activity such that we picked only highly active variants to assay. To increase the genetic variance in our shuffling population, we used information from natural genetic variability in related hypothetical proteins. We constructed a synthetic library (*22*) using the best GAT variant from the fourth iteration as a template and incorporated diversity from the predicted *B. subtilis* and *B. cereus* YITI sequences [fig. S2A, Table 1 (*11*)]. To incorporate the entire diversity present in these three molecules, an unachievable 10^{33} molecules would have been required. To limit the library size, we allowed two residue choices at only 27 sites (*11*). The introduced residues were evenly scattered throughout the sequences. This is but one of many possible libraries.

In screening this fifth-iteration library, we found one enzyme variant with a K_M of 0.05 mM glyphosate. The low K_M was attributed to a mutation having Ala at position 28 replaced by Val. However, this change caused a corresponding decrease in k_{cat}. In later iterations, Val^{28} was excluded and we found new Ala^{28} variants with low K_M and high k_{cat}. No single residue was identified that could account for the improvement. Altering the context of the entire protein through introduction of many new residues at once resulted in a complex solution to the problem. At the sixth iteration, we moved from the product accumulation assay to a more informative and rapid spectrophotometric assay (*11*) that allowed us to select enzymes with specific k_{cat} and K_M combinations. Two iterations of recombining the new diversity resulted in isolation of variants with increasing k_{cat} while maintaining the low K_M.

With each iteration of selection, some residues (present in all chosen parents) were fixed, leading to a decrease in the diversity available for recombination (Table 1). To keep the library population from reaching another functional plateau, we incorporated into the eighth-iteration library additional amino acid diversity from the more distantly related putative proteins of *L. inocua* and *Z. mobilis* [fig. S2B (*11*)]. We completed an additional three iterations of multigene shuffling to recombine the new diversity.

After 11 iterations of directed molecular evolution, the most efficient GAT variant (Fig. 3) had a k_{cat} of 416 min^{-1} and K_M of 0.05 mM glyphosate, resulting in a k_{cat}/K_M ratio of 8320 min^{-1} mM^{-1}, nearly a 10,000-fold improvement over the parental enzymes. This protein is 76-79% identical to the original native GAT proteins. The evolved GAT enzyme is equally or more efficient than other characterized NAT enzymes (*11*, *23*, *24*). Over time, residues that had a positive effect on k_{cat} or K_M were selected; residues with negative effects were eliminated. Because of the number of varying sites in any given iteration of shuffling, it was not clear which residues accounted for the improvements. We substituted several amino acid changes present in improved variants into the parental or less active enzymes, but no simple combination of residues accounted for the efficiency of the improved variants. Our ability to improve enzyme activity by DNA shuffling and functional selection outpaced and outperformed attempts to predict sequence-function relations.

Our purpose in discovering and evolving the *gat* genes was to develop glyphosate-tolerant crop plants. To be effective, the GAT enzyme must convert the glyphosate to *N*-acetylglyphosate before cell death from inhibition of EPSPS occurs. Like glyphosate, *N*-acetylglyphosate is stable and is not metabolized in plants, but it is not herbicidal. The *gat* genes were introduced into *Arabidopsis* (*19*), tobacco (*11*), and maize (*11*, *25*). In all cases, transformants were selected on glyphosate, and the presence and activity of the *gat* gene were confirmed by PCR and Western analysis. The transgenes were introduced into the nuclear genome, and the pro-

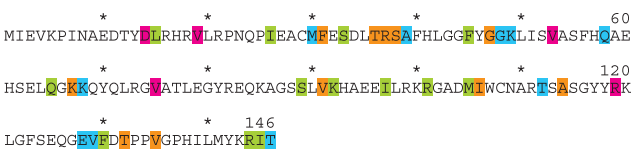

Fig. 3. Sequence analysis of the evolved 11th-iteration GAT enzyme with greatest k_{cat}/K_M for glyphosate. Blue residues represent the original diversity sites from the parental enzymes. Green residues were introduced in the fifth-iteration synthetic library and magenta residues in the eighth-iteration library. Orange residues arose by random mutagenesis as follows: V114A, 1st iteration; G37R, 2nd iteration; I135V, 2nd iteration: I132T, 3rd iteration; R47G, 5th iteration; Y31F, 6th iteration; I91V, 6th iteration; G38S, 9th iteration; L36T, 9th iteration; L106I, 10th iteration; Q67K, 11th iteration. The DNA sequence of this *gat* gene is given in GenBank AY597418.

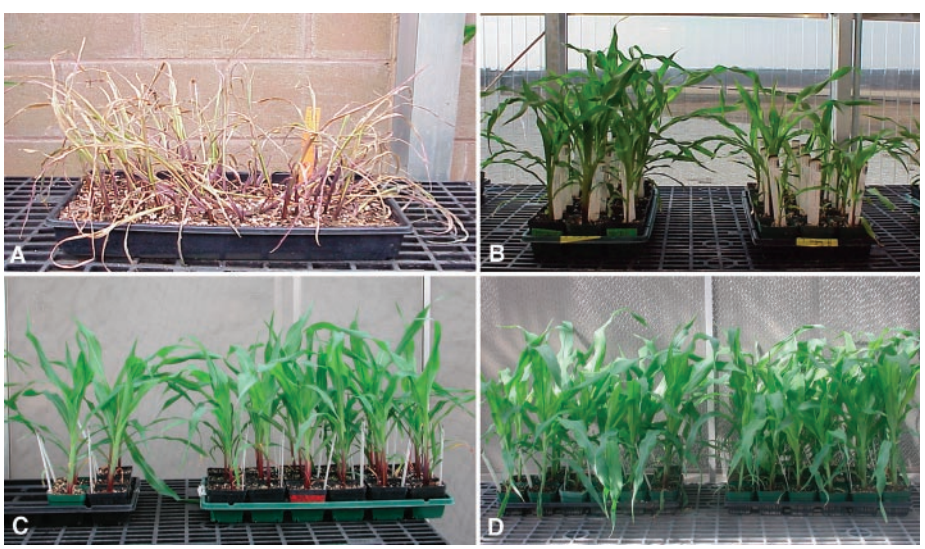

Fig. 4. Maize plants expressing GAT-encoding transgenes. (**A**) Untransformed maize regenerants 10 days after 1× glyphosate spray. (**B** to **D**) Plants on the right are shown 10 days after glyphosate spray application. On the left are untreated T_0 plants. (B) Transformed maize T_0 plants expressing a fifth-iteration *gat* variant. Although the plants were tolerant to 4× glyphosate, they showed a setback in growth. (C) Many events expressing a seventh-iteration *gat* variant showed no symptoms or growth setback at 4× glyphosate compared to unsprayed controls. (D) Maize plants expressing 10th- and 11th-iteration *gat* genes were tolerant to 6× glyphosate with no visual damage or growth setback. 1× glyphosate = 26 oz/ac Roundup UltraMAX.

teins were present in the cytosol. The specificity for glyphosate is high. Other common agrichemicals such as phosphinothricin, atrazine, and sulfonylureas are not acetylated by GAT (*11*).

Improved *gat* variants from the fifth iteration enabled the regeneration of glyphosate-tolerant transgenic tobacco plants. The plants were morphologically normal and fertile. To evaluate the tolerance profile of the tobacco plants, we selected T_1 plants that contained a segregating *gat* gene, using a glyphosate spray of 1 lb. acid equivalents per acre (ae/ac) and followed that with a dose-response regime using sprays of 2 to 24 lb. ae/ac. Untransformed plants showed severe symptoms or were killed by 1 lb. ae/ac (fig. S3A), whereas several transgenic GAT lines tolerated the highest dose sprayed (fig. S3B).

Fifth-iteration *gat* genes also allowed production of glyphosate-tolerant maize plants. T_0 plants were sprayed at the four-leaf stage with 104 oz./ac Roundup UltraMAX (4× field rate, equivalent to 3 lb. ae/ac glyphosate). The regenerants survived the treatment, but exhibited chlorotic banding and growth inhibition (Fig. 4B). Glyphosate tolerance improved with increases in the catalytic efficiency of GAT. With expression of seventh-iteration genes, nearly 50% of the maize regenerants showed no chlorotic banding and no growth inhibition (Fig. 4C). Most transformed plants expressing the best 10th and 11th round *gat* genes were tolerant to 6× glyphosate spray and showed no adverse symptoms (Fig. 4D). Efficacy trials of lines containing genes from several shuffling iterations are under way in the field to evaluate the commercial potential of this glyphosate tolerance trait.

Our approach shows that enzymes with useful yet insufficient activities can subsequently be improved by applying directed evolution until the desired activity is gained. DNA shuffling allowed us to develop crop plants exhibiting tolerance to the herbicide glyphosate, an important transgenic phenotype in global agriculture.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/304/5674/1151/DC1
Materials and Methods
Figs. S1 to S3
References

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Disulfide-Dependent Multimeric Assembly of Resistin Family Hormones

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Resistin, founding member of the resistin-like molecule (RELM) hormone family, is secreted selectively from adipocytes and induces liver-specific antagonism of insulin action, thus providing a potential molecular link between obesity and diabetes. Crystal structures of resistin and RELMβ reveal an unusual multimeric structure. Each protomer comprises a carboxy-terminal disulfide-rich β-sandwich "head" domain and an amino-terminal α-helical "tail" segment. The α-helical segments associate to form three-stranded coiled coils, and surface-exposed interchain disulfide linkages mediate the formation of tail-to-tail hexamers. Analysis of serum samples shows that resistin circulates in two distinct assembly states, likely corresponding to hexamers and trimers. Infusion of a resistin mutant, lacking the intertrimer disulfide bonds, in pancreatic-insulin clamp studies reveals substantially more potent effects on hepatic insulin sensitivity than those observed with wild-type resistin. This result suggests that processing of the intertrimer disulfide bonds may reflect an obligatory step toward activation.

The increasing prevalence of obesity in Western societies is a cause of great medical concern (*1*, *2*). Elevated body mass index correlates with susceptibility to disease states including type II diabetes, coronary artery disease, hypertension, and dyslipidemias (*3*). Type II diabetes currently affects 17 million Americans (*4*) and has become a major public health problem. Nonetheless, the molecular basis for the link between obesity and insulin resistance—the hallmark of type II diabetes—remains unclear (*5*).

Insulin resistance in type II diabetes, and in several animal models of obesity-associated insulin resistance, can often be improved systemically by treatment with agonists for

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