

ENGINEERED CROP TOLERANCE TO GLYPHOSATE AND ITS IMPACT ON THE USE OF THE HERBICIDE

Ken Pallett from Chelmsford, Essex, UK reviews the history and background of the introduction of crops engineered to be tolerant to glyphosate, briefly addresses the impact of these crops on the use of glyphosate and the future perspectives for this weed management technology

Keywords: engineered tolerance to glyphosate, genes, herbicide tolerant (HT) crops, glyphosate use



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Introduction

The agricultural importance and particularly the consequences of the use of glyphosate in crops engineered to be tolerant to this non-selective herbicide is discussed in some of the other articles in this special issue of *Outlooks on Pest Management*. However, a specific article reviewing the science and magnitude of what can be considered as a major scientific development in plant science is justified and is the most important aspect of the success of this herbicide (Duke & Powles, 2008).

The background to the identification of tolerance genes for glyphosate

Today, engineering plants by inserting genes to give a range of traits, such as tolerance to herbicides, resistance to insects and a broad range of quality traits, is now a relatively standard and routine process for plant molecular and cell biologists. In the early 1990s, however, the development and commercialisation of the first engineered crops was a major technological breakthrough for tolerance to what was then, and still is, a highly effective broad-spectrum herbicide.

The discovery of enoyl pyruvyl shikimate phosphate synthase (EPSPS) as the molecular target of glyphosate (Steinrücken & Amrhein, 1980) prompted extensive studies to improve understanding of the catalytic mechanism and the structure and function relationship of this enzyme. This underpinned the challenge to engineer tolerance to glyphosate so that it could be used selectively. Initial attempts focussed on selective evolution, site-directed mutagenesis and microbial screening but these were not successful. This was because

phospho-enol pyruvate (PEP), one of the substrates of EPSPS and glyphosate bind at the same site in EPSPS protein and any alteration of the enzyme to reduce the binding of glyphosate had a similar impact on PEP, reducing the fitness of EPSPS (Pollegioni *et al*, 2011).

Further research identified that EPSPS from different organisms can be divided into two classes according to intrinsic glyphosate sensitivity. Class I enzymes, found in all plants and in many Gram-negative bacteria are inhibited at low micromolar glyphosate concentrations. Class II enzymes retain their catalytic activity in the presence of high glyphosate concentrations and were initially identified in naturally occurring glyphosate tolerant microbes such as *Agrobacterium* sp. strain CP4, *Achromobacter* sp. strain LBAA, and *Pseudomonas* sp. strain PG2982 (Pollegioni *et al*, 2011).

The EPSPS gene from *Agrobacterium* sp. strain CP4, isolated from a waste-fed column at a glyphosate production facility, proved to have sufficient glyphosate-resistance and kinetically efficient EPSPS for the production of the first glyphosate tolerant crops such as soybean. EPSPS mutagenesis studies, based on a greater understanding of EPSPS structure and function, resulted in lower sensitivity to glyphosate, particularly multi-site mutations of class 1 EPSPSs, such as that from maize where a double mutation led to the GA21 corn event (Lebrun *et al*, 2003)

The alternative approach for engineering tolerance to herbicides and proven to be successful with herbicides such as glufosinate and bromoxynil, is detoxification. Two approaches have proved successful for tolerance to glyphosate, glyphosate oxidoreductase (GOX) and glyphosate acetyltransferase (GLYAT or GAT).

GOX was isolated from a strain of bacterium identified in a glyphosate waste stream treatment facility. An LBAA strain of *Ochrobactrum* (formerly *Achromobacter*) *anthropi* strain has the ability to degrade glyphosate and using it as a phosphorous source forming aminomethylphosphonic acid (AMPA) and glyoxylate (Barry & Kishore, 1995). GLYAT catalyses the acetylation of glyphosate to non-herbicidal *N*-acetylglyphosate and was isolated from *Bacillus licheniformis* strains (Castle *et al*, 2004). Gene shuffling techniques led to the discovery of variants of GLYAT providing commercial levels of tolerance to glyphosate in soybean and canola (Siehl *et al*, 2007)

The current list of genes conferring tolerance to glyphosate and the numbers of crops for which approvals have been sought globally, are summarised in Tables 1 and 2.

The International Service for the Acquisition of Agri-Biotech Applications (ISAAA) database for GM approvals

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Table 1. Genes leading to glyphosate tolerance.

Gene	Gene Source	Description
2mepsps	<i>Zea mays</i>	5-enolpyruvyl shikimate-3-phosphate synthase enzyme (double mutant version)
cp4 epsps (aroA:CP4)	<i>Agrobacterium tumefaciens</i> strain CP4	herbicide tolerant form of 5-enolpyruvylshikimate-3-phosphate synthase enzyme
epsps (Ag)	<i>Arthrobacter globiformis</i>	5-enolpyruvylshikimate-3-phosphate-synthase enzyme
epsps grg23ace5	synthetic gene; similar to epsps grg23 gene from <i>Arthrobacter globiformis</i>	modified 5-enolpyruvylshikimate-3-phosphate synthase protein
gat4601	<i>Bacillus licheniformis</i>	glyphosate N-acetyltransferase enzyme
gat4621	<i>Bacillus licheniformis</i>	glyphosate N-acetyltransferase enzyme
goxv247	<i>Ochrobactrum</i> (formerly <i>Achromobacter anthropi</i> strain LBAA)	glyphosate oxidase
mepsps	<i>Zea mays</i>	modified 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme

Prepared from the GM Approval Database (www.isaaa.org)

Table 2. Numbers of events and gene distribution in glyphosate tolerant crops.

Crop	Events*	Genes
Maize	140	cp4 epsps; mepsps; 2mepsps; grg25ace5 epsps; gat4621; goxv247
Cotton	22	cp4 epsps; 2mepsps
Soybean	22	cp4 epsps; 2mepsps; gat4601
Argentinian Canola	15	cp4 epsps; gat4601; gat4621; goxv247
Alfalfa	4	cp4 epsps;
Potato	4	cp4 epsps;
Polish Canola	3	cp4 epsps; goxv247
Sugar beet	2	cp4 epsps; goxv247
Creeping Bentgrass	1	cp4 epsps;
Wheat	1	cp4 epsps;

*an event is defined by the insertion of DNA into the plant genome as a result of a single transformation process

Prepared from the GM Approval Database (www.isaaa.org)

(www.isaaa.org) currently lists 504 different events from 30 species, including trees, flowers and vegetables; of which 336 contain herbicide tolerance (HT) traits and 212 of which are tolerance to glyphosate. In addition to glyphosate, there are 7 other HT traits in the GM approval process, e.g. bromoxynil (no longer commercialised), 2,4-D, dicamba, glufosinate, isoxaflutole, mesotrione and the sulfonylureas, some of which are being combined or stacked with glyphosate tolerance, however GM crops tolerant to glyphosate and to a lesser extent glufosinate dominate HT crops commercially cultivated globally, to date.

Impact of glyphosate tolerant crops on use of glyphosate

The agricultural use of glyphosate in broad-acre crops in the first 20 years of its commercialisation in 1971 was restricted to

pre-plant applications, limited directed applications between crops avoiding contact with emerged crops and pre-harvest applications to desiccate the crop (Duke & Powles 2008; Benbrook 2016a).

In 1996, the first genetically modified (GM) soybean, maize, and cotton varieties tolerant to glyphosate were approved for cultivation in the USA. As a result, it became possible to utilize this herbicide as a broadcast, post-emergence herbicide, thereby extending the time period during which glyphosate-based herbicides could be applied in these crops. More recently, commercial production of alfalfa and sugar beet engineered to tolerate glyphosate began in 2011 (alfalfa) and 2012 (sugar beet) in the USA.

Glyphosate-tolerant crops have been both a technological and commercial success and, at least initially, simplified weed management in maize, soybean and cotton crops both in the USA and worldwide specifically providing environmental benefits such as reducing soil erosion via minimum tillage linked to the cultivation of HT crops (Duke & Powles 2008; Benbrook 2016a). It was rapidly adopted by growers and was seen as a safe, economical and simple way to control problem weeds (Green & Owen, 2011).

The magnitude of the take-up of the technology is illustrated by the proportion of soybean grown globally that has been engineered or modified. In 2017, 77% of the global soybean acreage was genetically engineered or modified, with the vast majority containing HT traits (ISAAA, 2017). In 2014, soybean containing HT traits in the major cultivation countries of USA, Argentina and Brazil was 94%, 100% and 93% of the total cultivated area, respectively. GM soybean now accounts for 50% of the total global cultivated area of GM crops, i.e. 94.1 million ha (ISAAA, 2017). The percentage of cultivated area in 2014 for the four principle GM crops containing HT traits is summarised for in Table 3. Whilst tolerance to glyphosate is the major HT trait, tolerance to glufosinate would also be included in these data. In 2017, the proportion of the global acreage of canola, cotton and maize was 30%, 80% and 32%, respectively (ISAAA, 2017). Unlike soybean, traits additional to HT are more dominant in these three crops. Following approval in 2011, glyphosate tolerant

Table 3. Percentage of the global acreage in 2014 of the four principle GM crops containing HT traits*.

Crop	USA		Rest of the World	
	Percentage of total crop area	Area planted with HT traits (mio ha)	Percentage of total crop area	Area planted with HT traits (mio ha)
Canola	61%	0.4	23%	7.0
Cotton	91%	4.0	6%	1.5
Maize	89%	32.7	12%	15.0
Soybean	94%	32.0	80%	52.5

From Benbrook 2016b (suppl. data S20); *HT not limited to glyphosate tolerance.

or RR alfalfa was cultivated on 1.14 million hectares in 2017 in the USA (ISAAA, 2017).

Glyphosate use in these HT crops has clearly increased compared to the non-selective uses alone prior to the commercialisation of crops tolerant to this herbicide. The magnitude of this is illustrated by Benbrook (2016a) who has compiled the use data for glyphosate both in the USA and globally. In 2014, virtually all cotton and soybean acreage in the USA was treated with glyphosate as was 77% of maize acreage (Table 4).

The introduction of the HT lines of the principle GM crops in the USA resulted in a 15-fold increase in the use of glyphosate in soybean; a 26-fold increase in maize; a 19-fold increase in cotton; a 19-fold increase in alfalfa and a 25-fold increase in sugar beet (Table 5). There have also been significant increases in glyphosate use in many non-HT crops (Table

3 in Benbrook, 2016a), due to extensive pre-planting, pre-emergence, directed and crop desiccation applications of glyphosate.

At the global level, prior to the commercialisation of HT GM crops in 1995, glyphosate use for pre-planting, crop desiccation and other non-selective uses was estimated at 51.3 million kg. Since then its use has increased continually year-on-year and in 2014 was estimated at 747 million kg (Table 6). From 2010 onwards, 50% or more of glyphosate used globally has been applied in HT crops (see suppl. data S20 in Benbrook, 2016b). In 2014, 378.4 million kg of glyphosate was applied globally to HT crops, representing 50.6% of the estimated global applications of glyphosate in agriculture. A further 79.2 million kg of glyphosate was used for non-agricultural purposes in 2014 (Benbrook 2016b). 285.4 million kgs of glyphosate were applied on HT soybeans, or about 75% of the total amount glyphosate applied on all HT crops, and 38% of total amount of glyphosate used in global agriculture (Table 6). The importance of glyphosate in non-GM settings with a breakdown of its non-GM uses in the USA is provided by Gaines (2018) in this issue.

Table 4. The percentage of cotton, maize and soybean acreage in the USA treated with glyphosate in 1996 and 2014.

Crop	1996	2014
Cotton	13%	99%
Maize	4%	77%
Soybean	25%	98%

From Benbrook 2016b (suppl. data S1-S3).

Table 5. Glyphosate active ingredient applied in the USA (million kg)

Crop (year of commercial production)	Glyphosate use prior to the commercialisation of HT lines	Glyphosate use in after the commercialisation
Soybean (1996)	3.463 in 1995	51.728 in 2012
Maize (1996)	1.189 in 1995	31.427 in 2012
Cotton (1996)	0.554 in 1995	8.967 in 2012
Alfalfa (2011)	0.213 in 2005	4.024 in 2014
Sugar beet (2012)	0.054 in 2005	1.254 in 2014

Adapted from Benbrook 2016a & b (Table 3 and suppl. data S17).

Table 6. The estimated total use of glyphosate in the 4 principle HT crops compared to total global agricultural crop use in 2014.

Crop	Estimated use of glyphosate (million kg)	
	USA	Rest of the World
Canola	0.5	15.0
Cotton	6.3	2.9
Maize	36.4	31.9
Soybean	51.9	233.5
Total use in HT crops		378.4
Total use in all crops*		747.0
% use in HT crops**		50.6

*a further 79.2 million kg or 9.6% of total glyphosate use was for non-agricultural applications in 2014.

** decrease from 56% in 2012 although total use has increased by 15 million kg over the use in 2012.

(from Benbrook 2016a & b and suppl. data S23 & S24).

Table 7. Other HT traits listed in the GM approval database September 2018

Herbicide	Gene	Mechanism of tolerance	Crop
Glufosinate	phosphinothricin acetyltransferase (pat) (<i>Streptomyces viridochromogenes</i>)	detoxification	maize, canola, soybean
Glufosinate	bialaphos resistance (bar) (<i>Streptomyces hygroscopicus</i>)	detoxification	cotton
Isoxaflutole	hydroxyphenylpyruvate deoxygenase (hppd) (<i>Pseudomonas fluorescens</i>)	insensitive target site	soybean
Mesotrione	hppd (<i>Avena sativa</i>)	insensitive target site	soybean
Sulfonylureas	acetolactate synthase (als) (various sources)	insensitive target site	soybean, flax, cotton, carnation, maize
Dicamba	dicamba mono-oxygenase (dmo) (from <i>Stenotrophomonas maltophilia</i>)	detoxification	cotton, maize, soybean
2,4-D	aryloxyalkanoate dioxygenase I (aad-I) (from <i>Sphingobium herbicidovorans</i>) & aad-I2 (from <i>Delftia acidovorans</i>)	detoxification	maize (aad-I), soybean and cotton (aad-I2)

Prepared from the GM Approval Database (www.isaaa.org)

Other impacts of glyphosate use in HT crops

In addition to lack of any crop damage by the use of post-emergence applications of glyphosate in HT crops, the herbicide gave a level of weed control not usually seen in conventional soybean, maize and cotton by selective herbicides traditionally used prior to the development of HT lines. Many of the conventional selective herbicides often had incomplete weed spectrum and also could lead to crop damage. The reasons for the rapid adoption by growers were; cost savings, better weed management and simplicity (Duke & Powles, 2009). The high initial efficacy of glyphosate has declined with repeated use, as weeds adapted to the use of only one herbicide to control weeds, described as the perfect storm for the development of weed resistance (Green & Owen 2011). By 2010, 19 weed species were identified as being resistant to glyphosate, 50% of which were stated as having evolved due to glyphosate use in HT crops. In 2018, there are now 42 species resistant to glyphosate (Heap, 2018). Greater detail of the extent of weed resistance to glyphosate is covered by Duke, *et al* (2018) in this issue.

The introduction of crops tolerant to glyphosate with the high initial efficacy of glyphosate lead to a decline in the use of the older more conventional selective herbicide options. This decline led to a gradual loss of many of the conventional selective herbicides and many were withdrawn from the market. The burden of re-registration costs to maintain them in the market could not be justified with reduced market size and profitability. The use of glyphosate in HT crops also reduced the attractiveness of the crop protection industry to invest in R&D for new herbicide active ingredients. (Duke, 2012; Pallett, 2016).

Future perspectives for HT crops tolerant to glyphosate

The challenge for crops tolerant to glyphosate is clearly the spread of weed resistance leading to a loss of effective weed

control due to the heavy reliance on just this one herbicide (see Duke, *et al* (2018) in this issue). Glyphosate has not lost all utility; it still controls more weeds more effectively than other current herbicides, however it can no longer be applied alone on any weed anywhere (Green & Owen, 2011). Growers using HT crops need to change to a more diverse array of herbicidal, mechanical, and cultural practices (herbicide rotations, sequences, combinations of robust rates and different herbicide modes of action as well as non-herbicidal weed control approaches) (Duke & Powles, 2009). If this diversity in weed management systems can be maintained weed control by glyphosate can be sustainable in the future (Duke & Powles, 2009). The issue is that diversification of weed control will not be as simple as the sole use of glyphosate, so grower education will be necessary to convince growers to switch to less attractive and more complex traditional weed control options (Green & Owen, 2011)

The introduction of new herbicides with novel modes of action would clearly help increase diversity of herbicide options however, the decline in new herbicide introductions in recent years would need to be reversed (Duke, 2012; Pallett, 2016). If this can occur, given the time needed for discovery, development and regulatory studies, this is not a rapid option for increased diversity in the short-term, particularly if crop tolerance of these new molecules needs to be engineered, rather than relying on natural crop selectivity of these molecules or the use of safeners (in maize and other monocotyledonous crops), as was the case before HT crop development.

The short- or medium-term option for HT technology would be the combination or stacking of HT traits from established herbicides, either by breeding of lines with different single HT traits or by combining the desired tolerance genes in a single transformation vector (Dill, *et al.* 2008). A number of HT traits are at various stages of the approval process in a range of crops (Table 7). These traits can be used alone or combined or stacked via breeding or as a molecular stack with glyphosate tolerance as one strategy, to reduce the development of weed resistance.

Engineered crops with tolerance to glufosinate, 2,4-D, dicamba, ALS inhibitors, HPPD inhibitors (isoxaflutole and mesotrione), are at various stages of approval according to the GM Approval Database (www.isaaa.org). With the exception of glufosinate the other herbicides do not have the same broad non-selective spectrum as glyphosate. However, in the short-term, these offer the best solution to provide herbicide diversity with glyphosate tolerant crops as they will enable options with soil residual activity or different MOAs to control key glyphosate resistant weeds.

The introduction of diverse weed management practices by growers will preserve the utility of HT traits and herbicide technologies and help maintain profitable and environmentally sustainable crop production systems for future generations. They will work better for the prevention of weeds becoming resistant to glyphosate rather than their control (Green & Owen, 2011)

In areas of the world that have not yet adopted glyphosate tolerant crops, growers can learn from the experiences of weed resistance in North and South America. Growers must not wait, but implement best management practices as soon as new HT trait and herbicide technologies are available.

Conclusions

The development of crops engineered to be tolerant to glyphosate via genes coding for insensitive target site and/or herbicide detoxification represents one of the major technological breakthroughs in the plant sciences in the last 30 years. The use of this herbicide in these HT crops has received broad acceptance as it provides the growers with simple and effective weed management options. HT crops are now treated with 50% of the glyphosate applied globally. The over-reliance on the one herbicide for these HT crops has led to the development of resistance to glyphosate in major weeds. To maintain the utility of glyphosate in these HT crops more diverse weed management strategies will be necessary. The development of new herbicides with different modes of actions is desirable, but a long-term option. Short- or medium-term options to introduce diversity in weed management will include the development of crops engineered with tolerance to established herbicides combined with tolerance to glyphosate either by breeding of lines with different single HT traits or by combining the desired tolerance genes in a new event.

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