
THE UNIVERSITY OF WESTERN AUSTRALIA

# Identification of biochemical and molecular mechanisms of resistance to glufosinate and glyphosate in *Eleusine indica*

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# Abstract

Herbicides are important tools in agriculture. They allow for a simple and effective method of weed control for growers in order to meet the global food demand. Unfortunately, intensive herbicide usage with diminishing diversity of weed control methods has resulted in weeds evolving resistance to herbicides. Herbicide resistance is now a major issue and challenge for growers globally. One of the problematic weed species, especially in the tropical and warm climate regions is *Eleusine indica*. *Eleusine indica* is a pernicious weed that is prone to evolve resistance to herbicides. Currently, global incidences of evolved resistance in *E. indica* include eight different herbicide sites of action, including glufosinate.

Working with a glufosinate-resistant *E. indica* population from Malaysia, the resistance profile was further characterised and assessed for multiple resistance. Glufosinate resistance was confirmed in the *E. indica* population, with the $GR_{50}$ (rate required to reduce the growth by 50%) and $LD_{50}$ (rate required to kill 50% of the population) R/S ratios being 5- and 14-fold, respectively. More importantly, multiple resistance was observed, with the selected glufosinate-resistant sub-population (R*) exhibiting a very high level of glyphosate resistance. The $GR_{50}$ and $LD_{50}$ R/S ratios obtained were 12- and 144-fold, respectively for glyphosate. This population had also evolved resistance to paraquat, albeit at a low level ($GR_{50}$ and $LD_{50}$ R/S ratios 2 to 3-fold, respectively). This species is the first to be reported to have evolved resistance to all three non-selective herbicides. Additionally, resistance to several ACCase-inhibiting herbicides, namely fluazifop-*p*-butyl, haloxyfop-*p*-methyl and butroxydim, was caused by a Trp-2027-Cys substitution in the ACCase protein sequence.

In order to investigate the glufosinate resistance mechanism(s) in the R* population, activity of glutamine synthetase (GS) (the target-site of glufosinate) was compared in the S and R* populations. No difference in enzyme sensitivity towards glufosinate was observed. Specific GS activity was also similar between S and R*. Differences in foliar uptake and translocation of [$^{14}$C]-glufosinate were not significant between the two populations. HPLC analysis of glufosinate metabolism did not detect any metabolites in S or R* plants. Consequently, the resistance mechanism to glufosinate is not due to an insensitive target-site, target-site over production,

differential glufosinate uptake and translocation, nor enhanced glufosinate metabolism, and remains to be determined.

Sequencing of the glyphosate target gene, EPSPS, in the highly glyphosate resistant *E. indica* population revealed that a double mutation in the EPSPS gene, i.e. Thr-102-Ile and Pro-106-Ser (TIPS), was responsible for the high level glyphosate resistance. Importantly, this double mutation is similar to the first commercialised transgenic, glyphosate-tolerant EPSPS in maize, but has never been reported to occur naturally. Dose-response experiments showed that the naturally evolved TIPS mutants are 180-fold ($LD_{50}$ based) more resistant to glyphosate than the wild type (WT) *E. indica* plants, and 32-fold more resistant than the Pro-106-Ser (P106S) ($LD_{50}$ based) mutants. EPSPS inhibition assays also revealed similar results, with the TIPS EPSPS enzyme activity showing very high glyphosate resistance relative to wild type (WT) EPSPS (2600-fold) and P106S EPSPS (600-fold). Interestingly, the highly resistant TIPS mutant exhibited a resistance cost in terms of vegetative growth and seed production, while no resistance cost was observed for plants with the P106S mutation. Plants with the TIPS mutation had a higher basal shikimic acid (the substrate for EPSPS) level and lower tryptophan (a downstream product) levels than WT and P106S plants. The evolution of the TIPS double mutation is likely a sequential event, with the P106S mutation being selected first, followed by the T102I mutation, creating the highly glyphosate resistant TIPS EPSPS.

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# Statement of candidate contribution

**DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION**

This thesis contains published work and/or work prepared for publication, **some of which has been co-authored**. The bibliographical details of the work and where it appears in the thesis are outlined below.

**Chapter 3**: Jalaludin, A., Yu, Q., & Powles, S. B. (2014). Multiple resistance across glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population. *Weed Research, 55*, 82-89.

The work associated with the production of this manuscript in this thesis is my own. The contribution of the different co-authors is associated with the initial research directions and editorial input in various versions of the drafts of the manuscript.

**Chapter 5**: Yu, Q., Jalaludin, A., Han, H., Chen, M., Sammons, R. D., & Powles, S. B. (2015). Evolution of a double amino acid substitution in the EPSP synthase in *Eleusine indica* conferring high level glyphosate resistance. *Plant Physiology*, *167*(4), 1440-1447.

For Chapter 5, I did all the experiments involved in the published manuscript except for 2 experiments done by our collaborators, Chen, M. and Sammons, R.D. from Monsanto Company, St Louis, USA. Han, H. helped with the cDNA cloning. Yu, Q. was the main liaison person between AHRI and Monsanto. She also co-authored the paper with me. Powles, S. B. was the corresponding author for the published manuscript.

Student Signature

Coordinating Supervisor Signature

# Chapter 1

## General introduction

Chapter 1

# General introduction

## 1.1 Herbicides in agriculture

*History of agricultural weed control*

Around 10,000 years ago, in a few independent regions of the world, the human population shifted from a hunter-gatherer society towards a domesticated society dependent on farmed grain crops, e.g. wheat, rice, etc. Humans were finally able to produce more food than needed, and store it for longer periods of time. Grains were also easily transportable. This achievement of food security allowed the human population to grow and expand. Much later, following the Industrial Revolution, advancement in technology and healthcare improvement meant longer lifespan and allowed for population growth. From 2 billion people in the 1930s, the world population has grown to more than 7 billion people in 2015 and is projected to continue growing at a rate of 80 million people per year (Worldometers, 2015).

In order to feed the increasing population, food production and specifically, world grain production, needs to increase in a productive and sustainable manner. However, the effort to increase grain yield is constantly being threatened by pests (insects, mites, nematodes, etc.), plant pathogens (viruses, bacteria, fungi, etc.) and weeds (competitive plants) (Oerke, 2006). Among these threats, weeds have been proven to be the most problematic pest, causing the greatest limitation in crop production (Green, 2014). Potential crop loss by weeds has been estimated to reach up to 34%, nearly double that of animal pests and pathogens (18% and 16% respectively) (Oerke, 2006). In the United States, the cost of crop losses to weeds was estimated to be $US 26 billion annually (Pimentel et al., 2000). Since the dawn of agriculture, growers have been combating weeds using various forms of human, animal and mechanical weed controls. Early methods (e.g. hand weeding, tillage) were only moderately successful, and were labour intensive, time consuming and not economically viable (Oerke, 2006).

All that changed after the Second World War. The introduction of the first modern, synthetic herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) in 1945 ushered in a new era for agriculture. Herbicides revolutionized agricultural practices. The use of herbicides replaced labour-intensive weed control methods. Herbicides are efficient

tools, allowing larger cropping areas to be treated in shorter amount of time. Furthermore, they are far more efficient than all previous weed control methods, killing more than 90% of the weeds targeted (Foster et al., 1993). The efficiency and effectiveness of herbicides made them economical for growers to use. Without a doubt, herbicides contributed tremendously in meeting the global demand for food.

Since the introduction of 2,4-D (Peterson, 1967), there are now a plethora of herbicides for growers to choose from; more than 20 different sites of action ("World of Herbicides Map," 2010). The golden age of herbicide discovery (during the 1970s to 1980s) (Figure 1.1) saw several new herbicide sites of action discovered and introduced to the market. This includes a variety of selective herbicides such as the acetyl coenzyme A carboxylase (ACCase) inhibitors (for control of perennial weeds in dicot crops and several grass weeds in cereals), acetolactate synthase (ALS) inhibitors (effective control of weeds at very low dose rates per hectare), hydroxyphenylpyruvatedioxygenase (HPPD) inhibitors and protoporphyrinogen oxidase (PPO) inhibitors, and two key non-selective herbicides, the glycine herbicide glyphosate and the glutamine synthetase inhibitor glufosinate (Kraehmer, 2012; Green, 2014).

Herbicides clearly have become indispensable tools in weed management practices. Worldwide, herbicides make up 39% of the world pesticide market ("2006-2007 Pesticide Market Estimates," 2015). Herbicide sales in the USA alone are about $17 billion annually (Kraehmer, 2012) and are expected to increase by another 11% by 2016. Even in developing countries such as Malaysia, herbicide usage far exceeds other pesticides such as insecticides and fungicides. In 2010 alone, Malaysia consumed more than 36000 tonnes of herbicides, compared to 21000 tonnes for insecticides and about 2100 tonnes for fungicides (2015).

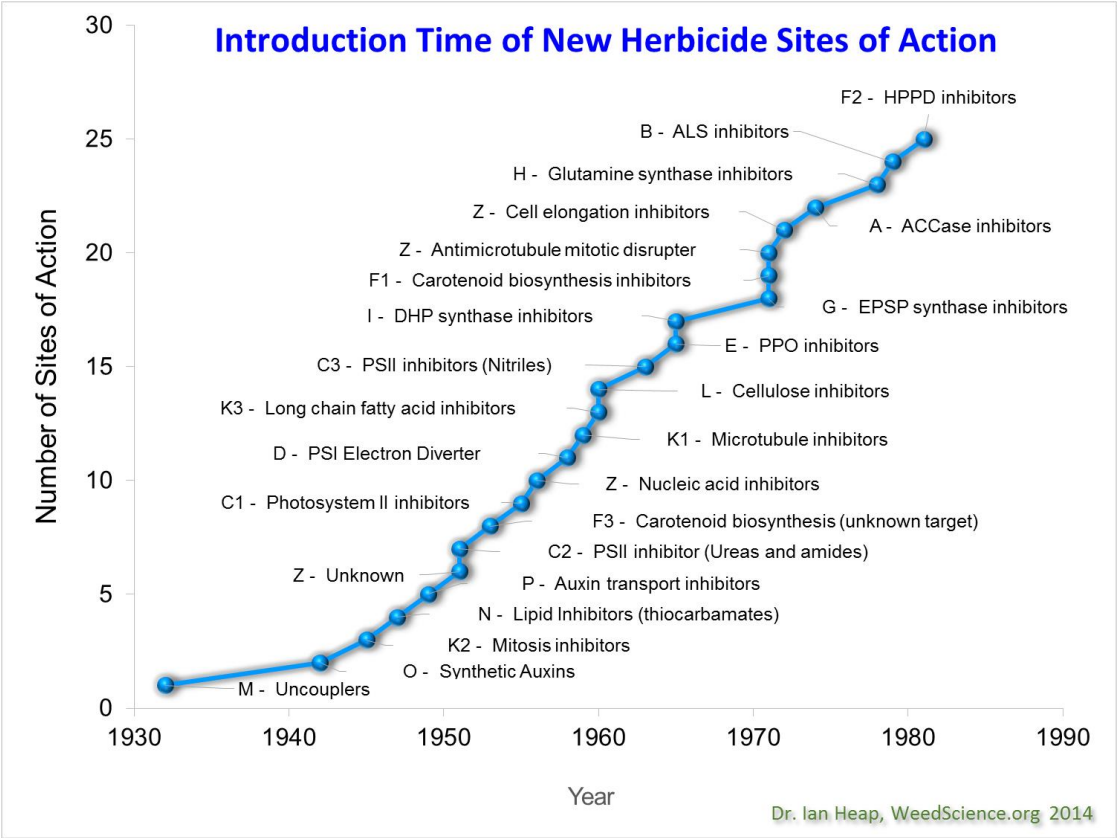


Figure 1.1 Timeline showing the introduction of new herbicide sites of action (reproduced from Heap, 2015).

*The evolution of herbicide resistance*

Over reliance on herbicides with diminishing diversity of non-chemical weed control methods imposed heavy selection pressure on weeds. Prior to herbicide introduction in 1945, there were already an increasing number of insecticide resistance cases (Taylor & Georghiou, 1979). Observing these trends, Blackman (1950) was the first to predict that weeds would consequently evolve resistance to herbicides. Surprisingly, within a year after that prediction, the first resistance cases to 2,4-D herbicide were reported in *Commelina diffusa* (Hilton, 1957) and *Daucus carota* (Switzer, 1957). However, it was not until 1970 when the first well-documented case of triazine resistant *Senecio vulgaris* initiated serious attention from weed scientists (Ryan, 1970).

Sixty years after the first herbicide resistance case, evolution of herbicide resistant weeds has now become a global problem, with more than 448 unique cases (weed species × herbicide site of action) of herbicide resistance worldwide (as of Feb 9

2015) (Heap, 2015). While this a global occurrence, the impact of herbicide resistant weeds is greatest in the great grain exporting nations such as the United States (144 resistant weed cases), Australia (62 resistant weed cases) and Canada (59 resistant weed cases) (Heap, 2014). These developed countries practice broad-acre farming and rely heavily on herbicides, with low diversity of other weed management practices. Such intense selection pressure selects for herbicide resistant weeds. In the past two decades, developing countries such as Brazil and Malaysia have adopted herbicide as the principal weed control measure. This has contributed an increase in the number of herbicide resistant cases in those countries.

During the herbicide discovery age, there was a huge shift of weed management practices from mechanical and chemical practices to chemical practices only (Green, 2014). However, some element of chemical diversity was still present as growers were relying on several different herbicide sites of action in their weed management program. The introduction of transgenic glyphosate-resistant (GR) crops in 1996 allowed growers, for the first time, to use only a single herbicide in cropping systems (reviewed by Powles, 2008). The benefits of using this new technology, such as its simplicity, effectiveness and cost efficiency, has led to rapid adoption by growers and now GR crops have became the dominant variant in soybean, cotton, maize and sugar beet fields in the United States (reviewed by Green, 2012). However, the lack of diversity (i.e. usage of a single, broad-spectrum herbicide on millions of hectares) only further intensifies herbicide selection pressure, resulting in selection of many resistant weed species. There are now sixteen weed species that have evolved resistance to glyphosate from GR cropping system fields in the United States alone (Heap, 2014).

In weed populations, herbicide resistance genes are pre-existing (Maxwell & Mortimer, 1994), occurring naturally in very few individuals (between 1 in a million to 1 in a billion individuals), before exposure to herbicide treatments (Délye et al., 2013a). Under relentless herbicide selection on a large weed population, these initially rare resistance genes (enabling plant survival and reproduction) are selected and consequently enriched in the population. Over time, the resistant individuals dominate the population, making a herbicide no longer effective. Herbicide resistance in weeds is an evolutionary process, driven by a number of factors, such as the genetics of a particular weed species (initial frequency of the resistance gene, its dominance and associated fitness costs of the resistance gene), its biological characteristics (sexual reproduction, i.e. allogamous vs. autogamous, seed production, dormancy and

movement), herbicide related issues (site of action, dosage, residual activity, herbicide mixture or rotation) and agro-ecosystem factors (crop rotation, nonchemical weed management practices, etc.) (Maxwell & Mortimer, 1994; Jasieniuk et al., 1996; Powles & Yu, 2010; Yu et al., 2012).

*Mechanisms of herbicide resistance: an overview*

Herbicide resistance can be target-site based (TSR) or non-target site based (NTSR). One of the major target-site resistance mechanisms involves target-site gene mutation(s), effectively reducing or preventing herbicide binding to its target enzyme (reviewed by Tranel & Wright, 2002; Délye, 2005; Powles & Yu, 2010; Beckie & Tardif, 2012). Another target-site based mechanism is gene amplification and/or gene overexpression (Gaines et al., 2010). This mechanism increases the production of the target enzyme, requiring higher herbicide concentrations to inhibit the site-of-action and damage/kill the plant.

When herbicide is applied to a plant, it needs to enter the plant through foliar or root uptake and then translocates to the target site at a lethal concentration, inhibiting the target enzyme and causing plant death. Therefore, NTSR works through various means by reducing the amount of herbicide that reaches the target site. These can include reduced herbicide uptake and/or herbicide translocation, increased vacuolar sequestration, or enhanced herbicide metabolism(s) (reviewed by Shaner, 2009; Yu & Powles, 2014) . Confirming and studying NTSR mechanisms is relatively difficult compared to target-site based mechanisms. However, non target-site based metabolic resistance gene discovery has progressed in recent years and research in this area is expected to gain more prominence in the near future (reviewed by Délye et al., 2013; Gaines et al., 2014; Iwakami et al., 2014; Yu & Powles, 2014) .

Cross resistance occurs when resistance to two or more herbicides is conferred by a single mechanism (Heap, 2014). This mechanism could be target-site based (alteration to target enzyme/site-of-action) or non target-site based (e.g., enhanced metabolism) (Hall et al., 1994). Often, if the mechanism is target-site based, cross resistance is to herbicides with the same site of action, and if non-target site based cross resistance can be extended to different herbicide sites of action (reviewed by Hall et al., 1994; reviewed by Beckie & Tardif, 2012; reviewed by Yu & Powles, 2014) . On the

other hand, if an individual plant (or population) possesses more than one resistance mechanism, it causes multiple resistance. Multiple resistance results from accumulation of resistance genes from gene/pollen exchange or sequential selection by different herbicide sites of action (reviewed by Beckie & Tardif, 2012; Heap, 2014).

## 1.2 Herbicide-resistant *Eleusine indica*

*Eleusine indica* is a highly self-pollinated noxious C4 grass weed. It is an annual weed that thrives in warm climates, including tropical countries such as Malaysia and the Philippines. It grows mostly in open areas with little to no shading, and once established, is very difficult to control. *E. indica* is a problematic weed in many parts of the world, especially in food and/or cash cropping systems such as vegetable farms, rice fields, fruit orchards, palm oil nurseries, cotton fields, etc. Compounding this problem is that *E. indica* is prone to herbicide resistance evolution. Worldwide, there is documented resistance to seven herbicide sites-of-action. These include resistance to the dinitroaniline herbicides (Mudge et al., 1984), the ACCase-inhibiting herbicides (Leach et al., 1993; Osuna et al., 2012), the ALS-inhibiting herbicide imazapyr (Valverde et al., 1993), glyphosate (Lee & Ngim, 2000), the bipyridilium herbicide paraquat (Buker et al., 2002), the photosystem II inhibitors (Brosnan et al., 2008) and glufosinate (Chuah et al., 2010; Jalaludin et al., 2010) (also see Chapter 3).

Under optimal growing conditions (see Chapter 2) and high herbicide selection pressure (4 to 7 applications of a single herbicide per year), *E. indica* could evolve resistance in less than 5 years (Lee & Ngim, 2000; Kaundun et al., 2008). The *E. indica* population used in this PhD study originated from Malaysia, where the author previously reported glufosinate resistance in a preliminary study (Jalaludin et al., 2010). In Malaysia, *E. indica* alone accounts for 5 of the 19 reported cases of herbicide resistant weeds, with 3 cases being multiple resistance. Herbicide resistance in Malaysian *E. indica* populations is occurring for several of the world's most widely used sites of action, including ACCase-inhibiting herbicides, paraquat, glyphosate and glufosinate (Leach et al., 1995; Lee & Ngim, 2000; Chuah et al., 2010; Jalaludin et al., 2010).

## 1.3 Research objectives

The aim of this PhD research was to understand the biochemical and genetic basis of multiple herbicide resistance in *Eleusine indica* to glufosinate and glyphosate (and other herbicides). The specific objectives were to:

1. Quantify resistance to glufosinate and glyphosate in a resistant *E. indica* population and examine for multiple resistance to any other herbicides.
2. Investigate glufosinate resistance mechanism(s).
3. Elucidate the biochemical and molecular basis of glyphosate resistance and assess the resistance cost of resistance alleles.

## 1.4 Thesis outline

This thesis is presented based on (but not identical to) three scientific papers, in agreement with the Postgraduate and Research Scholarship regulation 1.3.1.33(1) of the University of Western Australia. Two manuscripts have been published and are included in the Appendices as PDFs. Another manuscript is at an advanced stage of preparation for submission. All the data presented has resulted from the work done towards this thesis. There are six main chapters in this thesis; a General Introduction, Literature Review, three Experimental Chapters and a Summary and Future Directions. The General Introduction covers the background for the work presented in the thesis in order to justify the research objectives stated above. The Literature Review offers a more focused background of the components that are featured throughout the thesis, such as herbicides (history, chemistry, site of actions, etc.), and *Eleusine indica* (biology, morphology, etc.) and other information that is related to the topics in the thesis. The three Experimental Chapters are presented in the format of scientific papers that can be read individually or as a part of the thesis. Each Experimental Chapter includes the following sections: Abstract, Introduction, Materials and Methods, Results, Discussions, Acknowledgements and References. This thesis-as-a-series-of-papers result in some unavoidable repetition, especially in the Introduction sections of Chapter 3, 4 and 5.

**Chapter 3** highlights the multiple resistance of *E. indica* across three different non-selective herbicides (glufosinate, glyphosate and paraquat) and selective ACCase-inhibiting herbicides. It also reports on the high-level glyphosate resistance observed in the dose-response experiment. Identification of an ACCase target-site mutation endowing resistance to ACCase inhibitors is also included in this chapter. **Chapter 4**

focuses on examining glufosinate resistance mechanisms in *E. indica*. **Chapter 5** is committed to detailed studies on the evolution of a novel glyphosate resistance mechanism. In this chapter, the evolution of a double (T102I + P106S) (TIPS) mutation in the EPSPS gene conferring high level glyphosate resistance is revealed for the first time. This chapter also provides preliminary insights into the resistance cost of the homozygous TIPS mutants. Lastly, the findings from all chapters are summarised and the implications and future research directions are discussed in **Chapter 6**.

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# Chapter 2

## Literature review

Chapter 2

# Literature Review

## 2.1 Introduction

Glufosinate, glyphosate and paraquat are three globally important non-selective herbicides. Together with the selective ACCase-inhibiting herbicides, these four different herbicide sites of action are widely and intensively used, especially so the glyphosate and ACCase inhibitor herbicides. As a consequence, numerous incidences of evolved resistance in weeds to these herbicides have been reported, including in the grass weed *Eleusine indica* (Heap, 2015).

This review aims to give a brief introduction to these herbicides and to *Eleusine indica*. It also aims to summarize literature relevant to this PhD research, such as history of the herbicides, mode of action, resistance mechanisms and other related issues, for example, the transgenic herbicide resistant crops A brief note on the resistance cost in plants evolving herbicide resistance is included.

## 2.2 Glufosinate-ammonium

Glufosinate-ammonium (henceforth referred to as glufosinate) is a non-selective, post-emergence, contact herbicide used to control a wide range of annual and perennial broadleaf and grass weeds. First produced by Hoechst AG (now Bayer CropScience), it has several commercial names such as Basta® and Liberty™. Commercial glufosinate have the active ingredient in its ammonium salt form ($C_5H_{15}N_2O_4P$, Fig 2.1). The history of glufosinate began when two *Streptomyces* strains (*Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*) (Bayer et al., 1972) that produce the phytotoxin (Jansen et al., 2000), were discovered in the late 1960s/early 1970s. This microbial phytotoxin is a tripeptide consisting of two alanine molecules and an unusual amino acid containing a phosphino group. The tripeptide was later known as bialaphos and the amino acid with phosphino group was named L-phosphinothricin (L-PPT)(Donn, 2012). Eventually, L-phosphinothricin was recognised as a glutamate analogue and is an irreversible inhibitor of wheat glutamine synthetase (Manderscheid & Wild, 1986), opening up its potential as an herbicide. Since then, the chemically synthesized racemic mixture of D,L-phosphinothricin (called glufosinate) (Lea &

Ridley, 1989), was commercialised and has become an important herbicide for post emergence weed control. Later in the 1990s the same *Streptomyces* strains were used to create transgenic herbicide-resistant crops (see Section 2.2.5) (D'Halluin et al., 1992; Dröge-Laser et al., 1994; CaJacob et al., 2007).

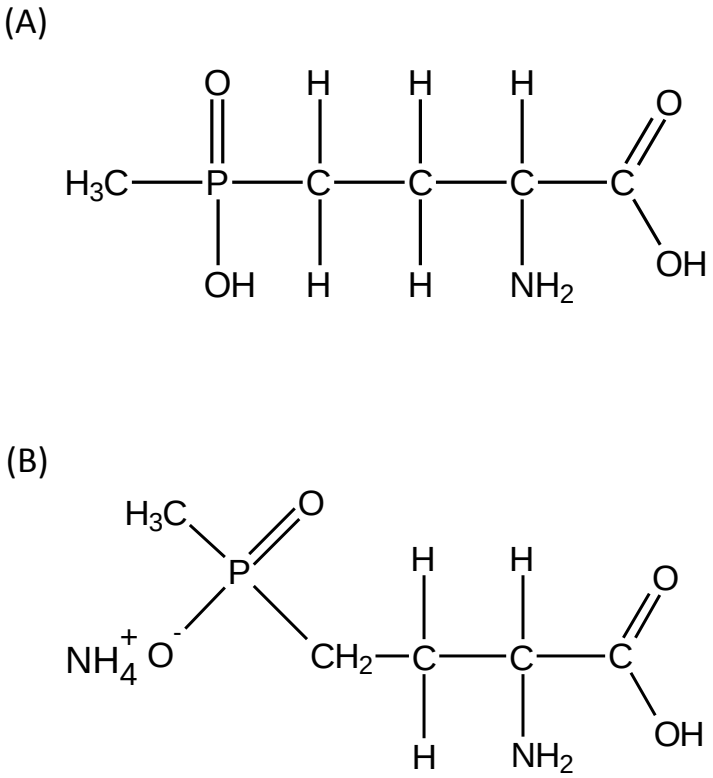


Figure 2.1. Chemical structures of glufosinate acid (A) and glufosinate ammonium (B).

Due to its nonselective nature, glufosinate was initially used in non-crop systems or minimum tillage systems, orchards, vineyards, palm oil nurseries, chemical fallows, and as pre-harvest desiccant in cropping systems (Mersey et al., 1990). The introduction of glufosinate-resistant crops in the 1990s saw an increase in glufosinate usage in cropping systems, mainly in the United States and Canada (Rasche & Gadsby, 1997).

### 2.2.1 Glufosinate mode of action

Glufosinate works by inhibiting glutamine synthetase (GS), an enzyme that is essential for plant nitrogen metabolism (see Section 2.2.2). Inhibition of glutamine

synthetase causes a rapid buildup of ammonia in the plants, reduced concentrations of several amino acids, and photosynthesis inhibition.

Using commercial glufosinate sprayed on intact plants, Coetzer and Al-Khatib (2001) observed that ammonium accumulation occurred at a faster rate than photosynthesis inhibition. The level of ammonium accumulation observed exceeded the amount known to cause suppression of photosynthesis by uncoupling of photophosphorylation (Krogmann et al., 1959). High concentrations of ammonia can also bind to the water-splitting complex in photosystem II (PSII), causing the inhibition of photosynthesis (Izawa, 1977). It was suggested that the rapid accumulation of ammonium contributed to photosynthesis inhibition (Coetzer & Al-Khatib, 2001). They also observed decreased stomatal conductance in their study, which they believe also contributed to the photosynthetic inhibition. This is in agreement with the earlier findings of Ullrich et al. (1990), where glufosinate caused membrane depolarization and a loss of $K^+$ within *Lemna* (duckweed) cells. The losses of $K^+$ from guard cells are known to cause reduced stomatal opening which leads to membrane perturbations, causing an efflux of $K^+$ (Ullrich et al., 1990).

Another factor that can be attributed to photosynthesis inhibition is the inhibition of the photorespiratory pathway. The inhibition of GS following glufosinate treatment causes a reduction in glutamine production. This depletion of glutamine in plants leads to glyoxylate accumulation in the photorespiratory cycle (Wendler et al., 1990). Toxic levels of glyoxylate inhibit Rubisco and consequently diminish $CO_2$ assimilation. Disruptions in the photorespiratory cycle also cause a deficiency in the Calvin cycle (Wendler et al., 1992).

The combined effects of these disruptions following glufosinate treatment, i.e. GS inhibition, ammonia build up, inhibition of photorespiratory pathway, etc., leads to damage within the chloroplast, causing the termination of photosynthetic activity. Eventually, this results in necrosis of the tissue and plant death (Wendler et al., 1990; Pline et al., 1999; Coetzer & Al-Khatib, 2001).

### 2.2.2 Glutamine synthetase

The target enzyme for glufosinate, glutamine synthetase (GS) (E.C 6.3.1.2), is the main assimilatory enzyme for ammonia in plants. It can have eight, ten or twelve

subunits, and can either be homomeric or heteromeric (Miflin & Habash, 2002). Glutamine synthetase catalyzes the formation of the amino acid glutamine from glutamate and ammonia. Glutamine synthetase is an important enzyme in plants because of its function in ammonia assimilation, nitrogen metabolism and to a certain extent, maintaining the carbon balance in plants via its interaction with the glutamine-2-oxo-glutarate aminotransferase (GOGAT) and glutamate dehydrogenase (GDH) pathways (Fig. 2.2) (Donn et al., 1984; Miflin & Habash, 2002). Glutamine synthetase assimilates ammonia produced from nitrogen fixation and nitrate or ammonia nutrition. Ammonia from photorespiration, and breakdowns of nitrogen transport compounds and proteins are also reassimilated via GS (Miflin & Habash, 2002).

There are two isoforms of glutamine synthetase: cytosolic and plastidic GS. The cytosolic glutamine synthetase (GS1), the major isoform found in roots and non-leaf tissue (Avila-Garcia et al., 2012), is encoded by three to five major genes in a single gene family. GS1 is responsible for recycling ammonia during the photorespiratory nitrogen cycle as well as ammonia assimilation in the dark (Hirel & Gadal, 1982). The plastidic glutamine synthetase (GS2) is encoded by a single gene (Bernard et al., 2008) and is predominant in the chloroplasts (Avila-Garcia et al., 2012) but is also found in the plastids of roots and other non-green tissues. However, this distribution appears to differ between species and with respect to plastid subtypes (Tobin & Yamaya, 2001). Hirel and Gadal (1980) in their study of rice GS found that GS2 activity is light-dependent and regulated by $H^+$, $Mg^{2+}$ and ATP stromal concentrations. The primary role of GS2 is reassimilating ammonia generated in photorespiration (Miflin & Habash, 2002).

GS is generally distributed in the leaves and roots (McNally et al., 1983). However, the ratios of the different GS isoforms present in plants differ between species and can be broadly classified into four categories as follows (McNally et al., 1983):

1) Plants with only cytosolic GS1 (e.g *Lathraea squamosa, Orobanche ramosa*)
2) Plants with only plastidic GS2 (e.g *Petunia grandiflora, Spinacia oleracea*)
3) Plants with higher GS1 than GS2 (e.g *Sorghum vulgare, Zea mays*)
4) Plants with higher GS2 than GS1 (e.g *Hordeum vulgare, Pisum sativum)*

However, studies have shown that the GS distribution in plants is more sophisticated than expected, as it is also found in specialist tissues and organs that are associated with the generation and transport of reduced nitrogen (reviewed by Miflin & Habash 2002).

### 2.2.3 Glufosinate uptake and translocation

Glufosinate uptake and translocation varies among plant species. Some glufosinate treated plant species, e.g. *Senna obtusifolia* (L.) Irwin & Barneby, absorbed more than 90% of applied glufosinate (Everman et al., 2009), while others, e.g. *Commelina diffusa* Burm. f., absorbed very little (less than 5% of applied glufosinate) (Neto et al., 2000). Glufosinate uptake by plants is generally rapid in the first 24 hours but increases little afterwards before reaching a plateau (Steckel et al., 1997; Everman et al., 2009).

Although glufosinate has the physicochemical properties for phloem mobility (it is a weak acid)(Beriault et al., 1999), it is not translocated extensively in plants (Bromilow et al., 1993). Beriault et al. (1999) hypothesized that the low phloem mobility of glufosinate is caused by the rapid action of the herbicide at the site of application. In a way, glufosinate action limits itself from translocating to other parts of the plant. This 'self-limitation' phenomenon has been observed in other herbicides such as glyphosate, chlorsulfuron and paraquat (Beriault et al., 1999).

In an experiment involving four different weed species, Steckel et al. (1997) observed that more than 85% of applied glufosinate remained in the treated leaf, 72 hours after treatment (HAT). Everman et al. (2009) also observed similar results, where more than 90% of absorbed glufosinate remained in the treated leaf of *Eleusine indica* (L.) Gaertn., *Digitaria sanguinalis* (L.) Scop. and *Senna obtusifolia* (L.) Irwin & Barneby. Glufosinate is predominantly translocated towards the leaf tips, with minimum translocation in the basipetal direction (Kumaratilake et al., 2002). This is in agreement with injury symptoms observed in glufosinate-treated plants, where the injury symptoms extend from the leaf tips to the leaf base (Mersey et al., 1990). However, basipetal movement of glufosinate does occur, albeit at different amounts, as plants treated with radiolabelled [$^{14}$C]-glufosinate have shown glufosinate to be present in roots (Mersey et al., 1990; Steckel et al., 1997; Pline et al., 1999; Everman et al., 2009). Environmental factors such as temperature and relative humidity (RH) have been found to affect glufosinate translocation, but not uptake. Higher temperature and RH increased glufosinate translocation within plants, and correlate with better control following glufosinate treatment (Coetzer et al., 2001; Kumaratilake & Preston, 2005).

Although glufosinate is a non-selective herbicide, its efficacy varies between plant species. Differential glufosinate uptake and translocation were attributed as the main reasons for the varying tolerance to glufosinate observed in plant species (Ridley & McNally, 1985; Mersey et al., 1990; Steckel et al., 1997; Pline et al., 1999). However, other factors such as glufosinate metabolism (Everman et al., 2009), plant growth stage, herbicide formulation and application rate also contribute to the differential tolerance to glufosinate (Jansen et al., 2000) (also see Chapter 4).

### 2.2.4 Glufosinate metabolism

Glufosinate degrades rapidly in soil and this has been extensively studied (Behrendt et al., 1990; Tebbe & Reber, 1991; Gallina & Stephenson, 1992). Soil microorganisms metabolize glufosinate into several metabolites, such as 4-methylphosphinyl-2-oxobutanoic acid (PPO), 3-methylphosphinylpropionic acid (MPP), 2-methylphosphinylacetic acid (MPA) and 4-methylphosphinylbutanoic acid (MPB). Currently, it is not known if any of these metabolites have herbicidal activity.

Glufosinate metabolism in plants has also been studied with cell cultures (Müller et al., 2001; Ruhland et al., 2002) and in whole plants (Dröge et al., 1992; Dröge-Laser et al., 1994; Jansen et al., 2000; Ruhland et al., 2004), often via comparing cell cultures/whole plants transformed with the bacterial *pat* or *bar* genes (see Section 2.2.5) that are able to metabolize glufosinate with untransformed plants. Generally, plants do not metabolize glufosinate (Jansen et al., 2000). However, some plants do have a very low level of glufosinate metabolism (Jansen et al., 2000). In the latter, glufosinate undergoes stepwise degradation involving deamination into PPO and subsequent decarboxylation steps with the final metabolites being MPP and 4-methylphosphinyl-2-hydroxybutanoic acid (MHB) (Dröge-Laser et al., 1994; Jansen et al., 2000) (Fig. 2.3).

In transgenic glufosinate-resistant cell cultures and plants, glufosinate is metabolized into the non-phytotoxic N-acetyl-glufosinate (NAG) via acetylation by the N-acetyl transferase enzyme (Fig. 2.3) (Dröge et al., 1992; Dröge-Laser et al., 1994; Müller et al., 2001; Ruhland et al., 2004). Initially it was thought that MPP and MHB are the final stable products of glufosinate degradation in untransformed plants (Dröge-Laser et al., 1994). However, it was found that transgenic plants also underwent these processes. In a metabolism study using transgenic glufosinate-resistant maize and canola, Ruhland et al. (2004) found that NAG was the main metabolite along with

several other forms of methylphosphinyl fatty acids, namely PPO, MPB, MHB and traces of MPA. Comparisons of glufosinate metabolism in transgenic, glufosinate-resistant wheat versus conventional wheat found that both had glufosinate, PPO and MHB, with the resistant wheat having NAG, MPB and MPP as additional metabolites (Rojano-Delgado et al., 2013).

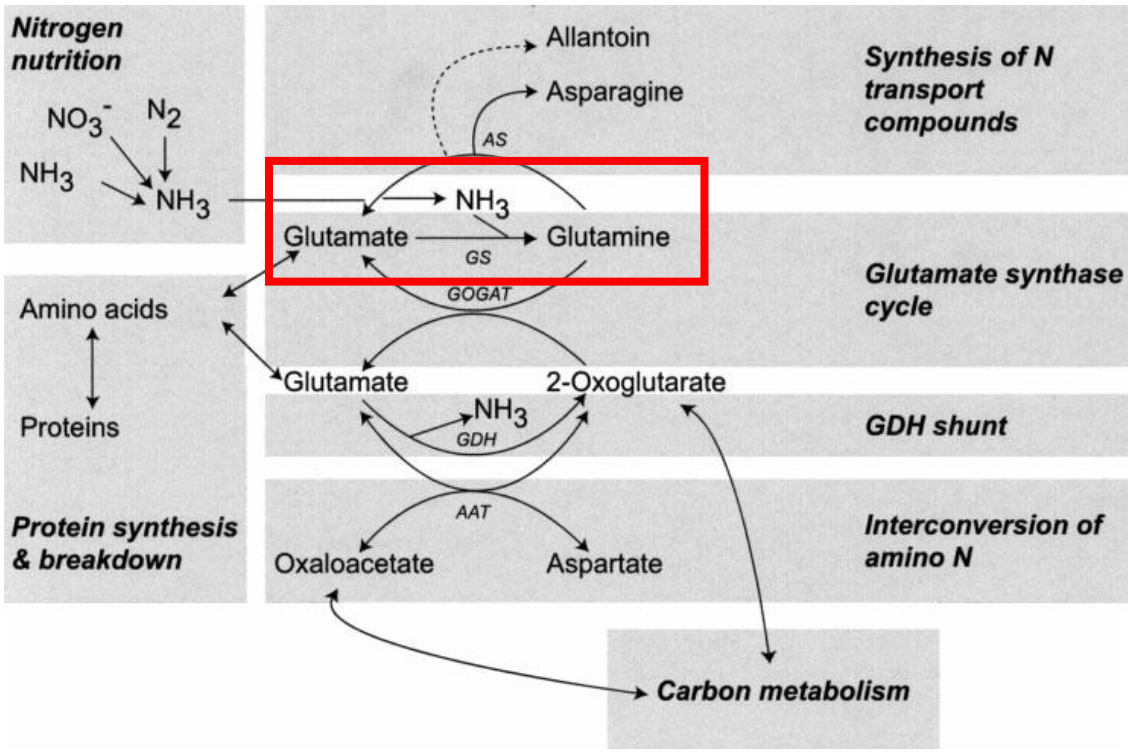


Figure 2.2. Examples of enzyme pathways that involve GS (red box) with other enzymes in maintaining carbon and nitrogen balance in plants. This image was taken from Miflin and Habash (2002).

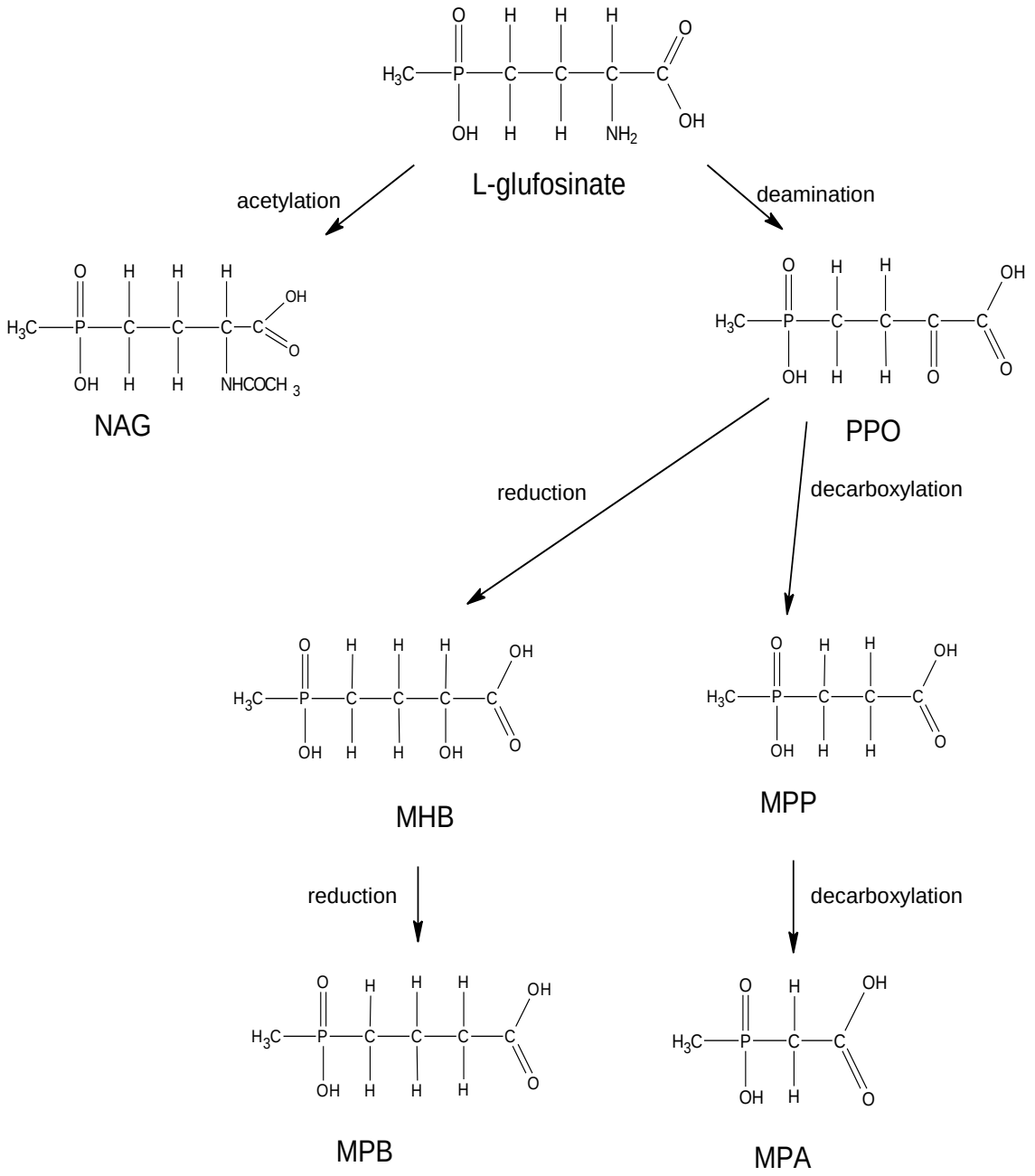


Figure 2.3. Glufosinate metabolism pathway in transgenic and non-transgenic plants (adapted from Ruhland et al., 2004); N-acetyl glufosinate (NAG), 4-methylphosphinyl-2-oxobutanoic acid (PPO), 3-methylphosphinylpropionic acid (MPP), 2-methylphosphinylacetic acid (MPA), 4-methylphosphinyl-2-hydroxybutanoic acid (MHB) and 4-methylphosphinylbutanoic acid (MPB).

### 2.2.5 Glufosinate resistant crops

Glufosinate resistance in crops was achieved by expressing an enzyme that metabolizes glufosinate. The microorganisms (i.e. *S. hygroscopicus* and *S. viridochromogenes* ) from which glufosinate was first isolated respectively produce the enzymes called bialaphos acetyltransferase (BAR) and phosphinothricin acetyltransferase (PAT) that detoxify glufosinate by acetylating the free $NH_2$ group of glufosinate to form N-acetyl glufosinate (NAG), rendering it non-toxic (Fig. 2.2) (Green, 2009). In *S. hygroscopicus*, the BAR enzyme is encoded by the *bar* gene (Deblock et al., 1987) while in *S. viridochromogenes*, the similar PAT enzyme with the same glufosinate-metabolizing function is encoded by the *pat* gene (Dröge et al., 1992). Both microbial genes have significant sequence homology and were isolated and expressed in plants, producing glufosinate-resistant crops. Crops with glufosinate-resistant gene traits are designated under the LibertyLink® brand. Currently, registered LibertyLink® crops include soybean, cotton, canola and maize (Green, 2009; "LibertyLink," 2015).

### 2.2.6 Glufosinate resistance and mechanisms

To date, there are only four documented cases of field-evolved glufosinate resistance in two weed species (*Eleusine indica* and *Lolium perenne* ssp. *multiflorum)* (Chuah et al., 2010; Jalaludin et al., 2010; Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia et al., 2012). Currently, the resistance mechanisms in resistant *E. indica* biotypes are unknown while the resistance mechanism for resistant biotypes of *Lolium* ssp. is discussed in Chapter 4.

## 2.3 Glyphosate

Glyphosate (*N*-[phosphonomethyl]glycine), is no doubt, the most important herbicide in the world (Duke & Powles, 2008). Since its introduction in 1974, glyphosate has become the most-used global herbicide. Its unique properties, along with technological innovations such as glyphosate-resistant transgenic crops, helped propel glyphosate into becoming the most widely used ‘once-in-a-century herbicide’ in the world (Duke & Powles, 2008).

. The story of glyphosate can be traced back to 1950. A Swiss chemist named Dr. Henri Martin who worked for a small pharmaceutical company called Cilag, was the first to discover glyphosate (Franz et al., 1997). However, the compound was found to not have any pharmaceutical application and thus, the discovery was not reported in the literature (at that time). Around 1970, the Monsanto Company was developing compounds for water-softening agents. When these compounds were screened for herbicidal activity, only two showed sufficient unit activity for further development. These two compounds were then passed to a Monsanto chemist, Dr. John E Franz. He was tasked with improving the herbicidal activity of the compounds via making analogs and derivatives. After nearly giving up on the project, Dr. Franz succeeded and became the first person to synthesize and test glyphosate as a herbicide (reviewed by Dill et al., 2010). In 1971, glyphosate herbicidal activity was for the first time described (Baird et al., 1971).

Glyphosate has limited solubility in the acid form (Fig. 2.4A). Because of this, it is formulated and marketed as an isopropylamine salt (Fig. 2.4B)(reviewed by Dill et al., 2010). Commercially, glyphosate was marketed as Roundup. As an agrichemical, glyphosate is a non-selective, broad spectrum, systemic herbicide. Glyphosate controls a wide range of annual broadleaf and perennial weeds. Initially, glyphosate was used in non-crop situations, as weed control around container nursery stock and groundcover removal in plantations. It also found wide usage in fallow and industrial weed control initiatives. In broad acre farming systems, glyphosate was used to remove weeds prior to seeding. Glyphosate effectiveness is complemented by its unique properties. It is a non-residual and environmentally benign herbicide (Comai et al., 1983). It binds to soil rapidly, reducing the risk of leaching into groundwater. It also undergoes rapid biodegradation (Pline-Srnic, 2006). However, the most unique aspect of glyphosate lies with its enzyme target site, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This enzyme exists in plants, fungi and bacteria but not in animals (Eschenburg et al., 2002), therefore glyphosate has very low toxicity to humans, birds, fish and frogs (Pline-Srnic, 2006).

### 2.3.1 Glyphosate mode of action

Glyphosate inhibits the enzyme 5-enoloyruvylshikimate-3-phosphate synthase (EPSPS. E.2.5.2.19). This enzyme catalyzes the conversion of phosphoenolpyruvate

(PEP) and shikimate-3-phosphate (S3P) into 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Fig. 2.5). This is a crucial step for the production of the aromatic amino acids phenylalanine, tyrosine and tryptophan in the shikimate pathway (Fig 2.6) (Dill et al., 2010). EPSPS catalyses the reaction via the transfer of an enolpyruvyl moiety from PEP to S3P, condensing them into EPSP (Fig. 2.5) (Stallings et al., 1991; Schönbrunn et al., 2001). The EPSPS active site in higher plants is highly conserved (Dill, 2005). The glyphosate binding site overlaps with the PEP binding site, making glyphosate a competitive inhibitor of PEP but uncompetitive to S3P. Glyphosate inhibits EPSPS in a slow reversible reaction, and the glyphosate:EPSPS:S3P complex is not only very stable, but also mimics the transition state of the ternary enzyme-substrate complex (Schönbrunn et al., 2001; Eschenburg et al., 2002; Dill, 2005).

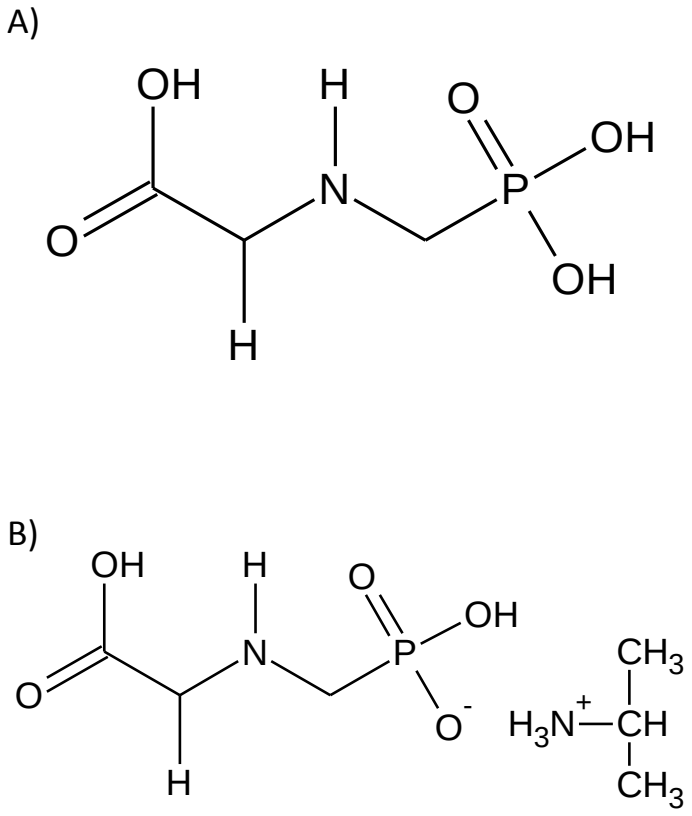


Figure 2.4. The structure of glyphosate acid (A) and glyphosate isopropylamine salt (B).

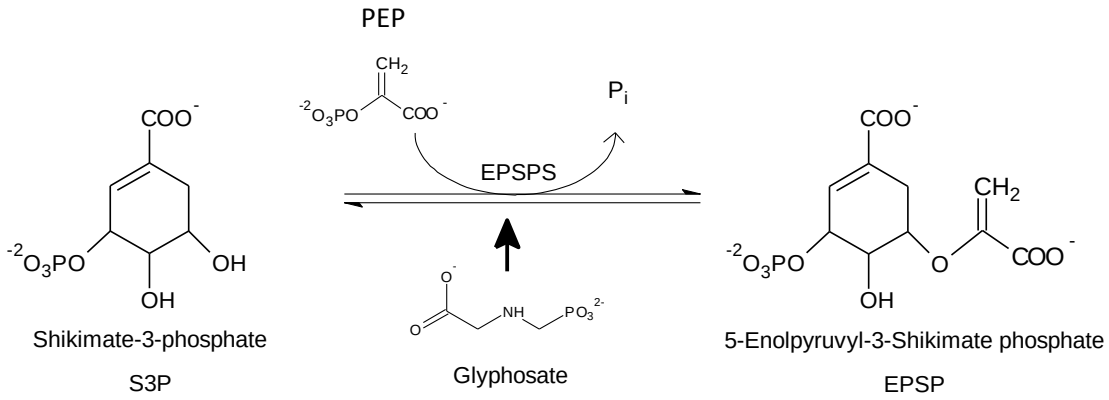


Figure 2.5. Conversion of S3P and phosphoenolpyruvate (PEP) into EPSP and inorganic phosphate by EPSPS, showing the inhibition site of glyphosate.

Inhibition of EPSPS causes a diversion of the carbon flow to shikimate-3-phosphate, which is then converted to shikimate resulting in its accumulation in high levels (Duke, 1988). It also blocks the production of threonine, tyrosine and tryptophan. These aromatic amino acids are important building blocks for protein synthesis, hormones, plant metabolites, lignin, flavonoids, phenolic compounds and biosynthetic pathways for plant growth (Ng et al., 2004; Dill et al., 2010). The accumulation of shikimate causes shortages of carbon for other essential plant biochemical pathways . Over time, the combined effects cause reduction in photosynthesis and degradation of chlorophyll (Cole, 1985) resulting in glyphosate phytotoxicity such as chlorosis, pigmentation, stunting and reduction of apical dominance (Ng et al., 2003), followed by plant death.

### 2.3.2 Glyphosate resistance

When glyphosate was first introduced, some considered that resistance to glyphosate would not evolve (see Chapter 5). Several factors contributed to this view, namely, 1) the transition state mimicry that glyphosate forms upon EPSPS inhibition, 2) plants lack the metabolic capability to metabolize glyphosate, 3) the absence of known active transporters for glyphosate in plants, 4) the limited success of resistance selection to glyphosate using whole plant, tissue/cell culture and mutagenesis techniques (Bradshaw et al., 1997). This was indeed true for the first 25 years of glyphosate use.

However, since the first case of evolved glyphosate resistance was reported (Powles et al., 1998), there are now more than 30 weed species with reported glyphosate resistance in the world (Heap, 2015).

### 2.3.3 Glyphosate resistance mechanisms

Evolved resistance to glyphosate in weed species can be due to target-site and/or non-target site mechanisms. Various target-site EPSPS mutations at proline-106 (Pro-106-Ser/Thr/Ala/Leu) endow glyphosate resistance (Baerson et al., 2002; Ng et al., 2003; Yu et al., 2007a; Kaundun et al., 2011) (Table 2.1). Glyphosate resistance due to EPSPS gene amplification has also been reported in a few weed species (Gaines et al., 2010; reviewed by Sammons & Gaines, 2014)(Table 2.1). Non-target-site glyphosate resistance mechanisms include reduced glyphosate uptake (Michitte et al., 2009) and restricted glyphosate translocation (Lorraine-Colwill et al., 2003; Yu et al., 2007a) or enhanced vacuolar sequestration (Ge et al., 2010) (Table 2.1). Most of these mechanisms confer low to moderate levels of resistance to glyphosate (Table 2.1).

### 2.3.4 Glyphosate resistant crops

The introduction of the first glyphosate resistant crop in 1996 marked the beginning of a new era in agriculture. Glyphosate-resistant crops (alfalfa, canola, cotton, maize, soybean and sugarbeet) (Green, 2009) have now been adopted in various countries including the USA, Australia, Canada, Brazil and Argentina, to name a few. Glyphosate tolerance was achieved by introducing a glyphosate insensitive form of EPSPS. There are two resistant gene traits that are currently used in commercial glyphosate resistant crops; 1) the double mutant Thr102Ile/Pro106Ser (referred to as the TIPS mutation) modified from endogenous maize EPSPS (also known as the GA21 event), and 2) the CP4-EPSPS isolated from *Agrobacterium* sp. strain CP4 (Spencer et al., 2000; Lebrun et al., 2003; Dill, 2005; Green, 2009; Alibhai et al., 2010). Another mechanism for glyphosate tolerance was achieved via detoxification using glyphosate oxidoreductase (GOX). The gene that expresses this enzyme was isolated and cloned from *Achromobacter* sp. strain LBAA (Barry et al., 1992). However, while this method provided glyphosate tolerance, the level did not meet commercial requirements. As such, the GOX gene is ‘stacked’ with the CP4-EPSPS gene (reviewed by CaJacob et al., 2004).

Creating a glyphosate insensitive form of EPSPS that still favorably binds PEP was difficult, primarily due to the overlapping of the glyphosate and PEP binding sites (Dill, 2005). Indeed, various single and multiple point mutations of plant and bacterial EPSPS have resulted in unfavorable enzyme kinetics (Table 2.2). Eventually, the TIPS EPSPS mutant was found to have favorable characteristics, i.e. insensitive to glyphosate but with high affinity for PEP (Lebrun et al., 2003; Funke et al., 2009). It was eventually introduced into maize and became the first commercialized transgenic glyphosate resistant maize variety.

The CP4-EPSPS, isolated from *Agrobacterium* sp. strain CP4, was discovered at a glyphosate production facility, in a waste column (Funke et al., 2006). Due to its high catalytic efficiency in the presence of high glyphosate concentrations (Table 2.1) and considerable difference in sequence homology with plant (~50% similar and ~23% identical to maize EPSPS) and several bacterial (such as *Escherichia coli* and *Salmonella typhimurium*) EPSPS enzymes, it was classified as a Class II EPSPS (Barry et al., 1997; Dill, 2005; Funke et al., 2009). However, its substrate and glyphosate binding site is identical to that found in the EPSPS of the majority of plant species (Dill, 2005). The high glyphosate resistance in CP4-EPSPS was attributed to Ala100, whose methyl side chain protrudes into the glyphosate binding site, making glyphosate unable to bind (Schönbrunn et al., 2001). Interestingly, Ala100 in CP4-EPSPS is equivalent to Gly96 in *E. coli* and Gly101 in plants. However, the same mutation to Ala in *E. coli* (Gly96Ala) and plants (Gly101Ala) caused a huge decrease in PEP affinity despite the mutated enzyme being highly resistant to glyphosate (Table 2.1) (Funke et al., 2006). Due to its high glyphosate resistance, the CP4-EPSPS gene trait is currently employed in almost all of the available commercial glyphosate resistant crops.

Table 2.1. Examples of glyphosate resistance mechanisms in weed species (adapted from Sammons & Gaines, 2014).

| Resistance mechanism | Weed species | Resistance fold | Additional resistance mechanisms? | References |
|---|---|---|---|---|
| Target site mutation | | | | |
| Pro-106-Ser | *Eleusine indica* | 2-4 | No | Baerson et al. (2002) |
| Pro-106-Thr | *E. indica* | 3 | No | Ng et al. (2003) |
| Pro-106-Ala | *Lolium rigidum* | 14 | Yes, reduced translocation | Yu et al. (2007a) |
| Pro-106-Leu | *L. rigidum* | 1.7 | Yes, unknown mechanism | Kaundun et al. (2011) |
| Target-site gene amplification | *Amaranthus palmeri* | 40 | No | Gaines et al. (2011) |
| | *Lolium perenne* ssp. *multiflorum* | 7-13 | No | Salas et al. (2012) |
| | *Kochia scoparia* | 4-8 | No | Wiersma et al. (2015) |

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| | | | | |
|---|---|---|---|---|
| Non-target-site reduced uptake | *Lolium multiflorum* | not determined | Yes, reduced translocation | Michitte et al. (2009) |
| Non-target-site restricted translocation | *L. rigidum* | 4-6 | No | Lorraine-Colwill et al. (2003); Wakelin et al. (2004) |
| | *L. rigidum* | 14 | Yes, Pro-106-Ala mutation | Yu et al. (2007a) |
| Non-target-site vacuolar sequestration | *Lolium* spp. | 4.8-14 | Yes, Pro-106 mutation for some populations | Ge et al. (2012) |

Table 2.2. Kinetic properties for selected EPSPS enzymes (based on Dill, 2005; Alibhai et al., 2010).

| Mutation(s) | $K_m$ for PEP (μM) | $K_i$ for glyphosate (μM) | $K_i/K_m$ |
|---|---|---|---|
| Theoretical ideal | <15 | ~1500 | 100 |
| Petunia (wild type) | 5.0 | 0.4 | 0.08 |
| Petunia Gly101Ala | 200 | 2000 | 10.0 |
| Petunia Gly101Ala/Ala192Thr | 54 | 683 | 12.6 |
| Maize wild type | 27 | 0.5 | 0.02 |
| Maize Thr102Ile | 233 | 148.6 | 0.6 |
| Maize Pro106Ser | 17.1 | 1.0 | 0.06 |
| Maize Thr102Ile/Pro106Ser | 10.6 | 58.0 | 5.5 |
| *Agrobacterium* spp. CP4 | 14.4 | 5100 | 354 |

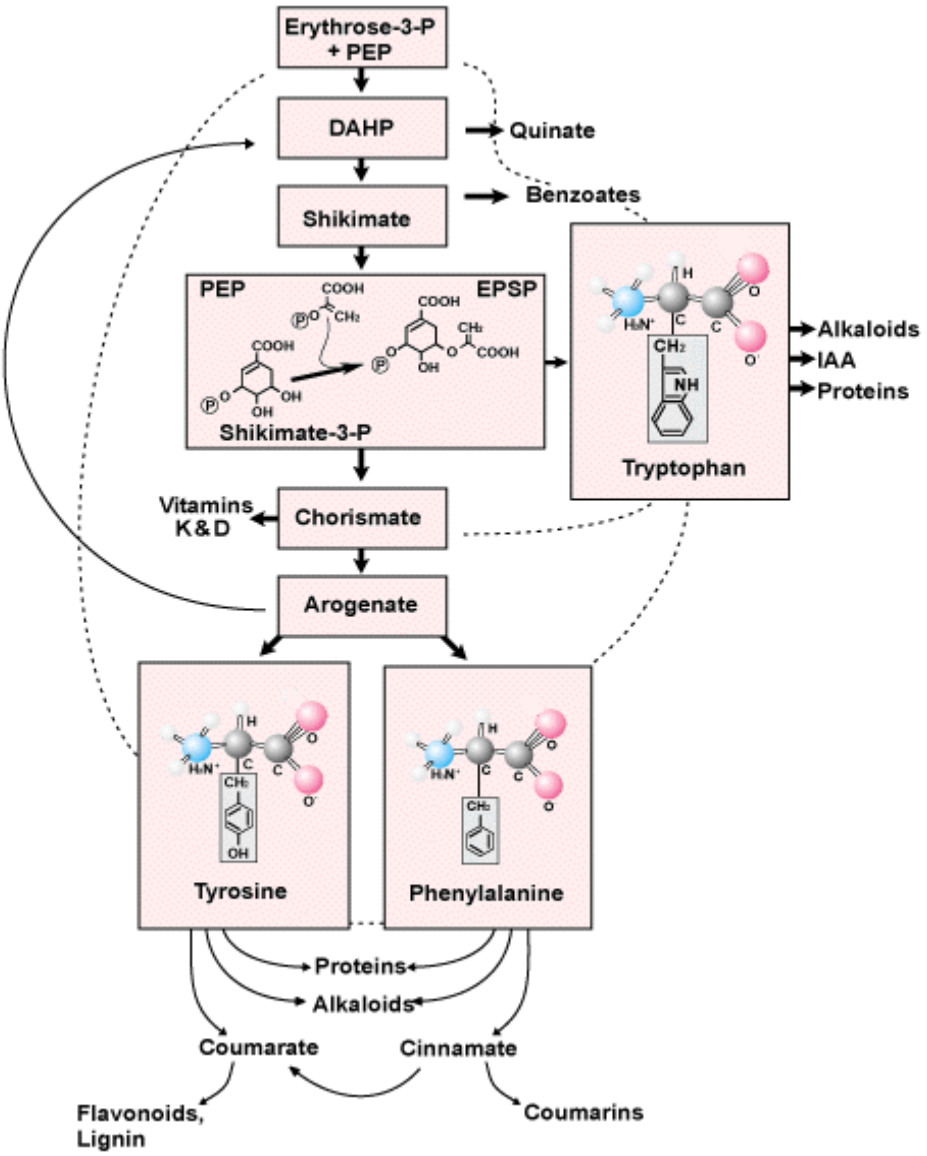



Figure 2.6. The shikimate pathway (image taken from "Shikimate Pathway," 2015) . DAHP: 3-deoxy-D-arabino-hept-2-ulosonate-7-phosphate; IAA: indole acetic acid.

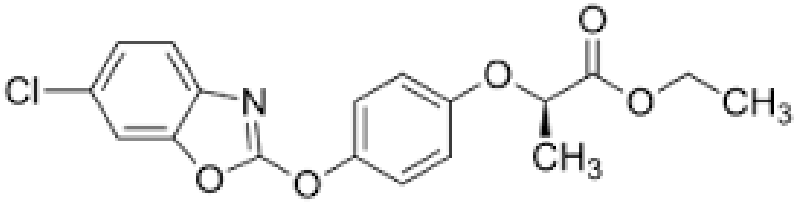
## 2.4 ACCase-inhibiting herbicides

Acetyl coenzyme A carboxylase (ACCase, EC 6.4.1.2) inhibiting herbicides (hereafter referred to as ACCase herbicides) are post-emergence, selective herbicides comprising three chemical classes based on their chemical structure (Kaundun, 2014), namely the aryloxyphenoxypropionates (APP or fops) and cyclohexanediones (CHD or dims) classes (Fig. 2.7), which have been on the market since the 1970s (Devine & Shimabukuro, 1994), and a chemical group, the phenylpyrazolines (PPZ or dens) (Hofer et al., 2006).The ACCase herbicides are mainly used to control grass weeds in dicot crops, but there are cereal crops selective ACCase herbicides (reviewed by Kaundun, 2014).

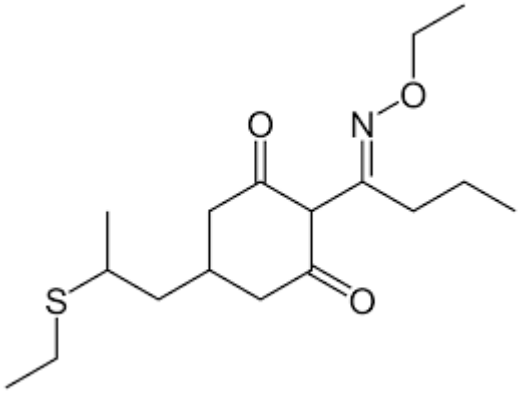
The ACCase herbicides inhibit the enzyme acetyl coenzyme A carboxylase, an important enzyme in fatty acid synthesis. It catalyses the formation of malonyl-CoA from the carboxylation of acetyl-CoA in an ATP-dependent reaction using $HCO_3^-$ as a carboxyl donor. Malonyl-CoA is an important substrate for plastidic *de novo* fatty acid biosynthesis, elongation of very-long-chain-fatty-acids (VLCFAs) and for secondary metabolites including flavonoids and anthocyanins (reviewed in Délye, 2005; Kaundun, 2014).

Plants have two ACCase isoforms; cytosolic and plastidic ACCase. The plastidic ACCase accounts for the majority of total plant ACCase activity and is the target of ACCase herbicides (Délye, 2005). Both isoforms are made up of 3 functional domains; the biotin carboxyl carrier (BCC), the biotin carboxylase (BC) and carboxyl transferase (CT), which is further divided into α- and β-subunits. The plastidic ACCase isoform in the Poaceae (grass family) is homomeric, with all three functional domains (BCC, BC and CT) occurring on a single polypeptide. In contrast, plastidic ACCase is heteromeric in the majority of other plant species, with the subunits encoded by different genes (reviewed by Délye, 2005; reviewed by Kaundun, 2014).

A)




B)




C)

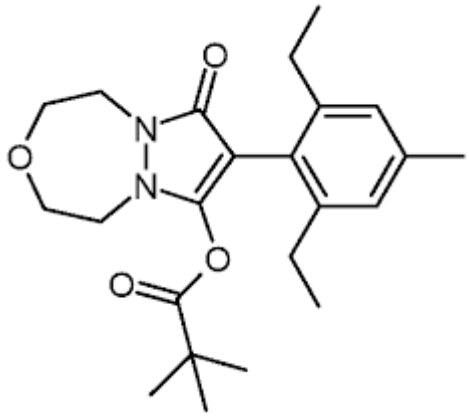



Figure 2.7. An example of Acetyl CoA carboxylase herbicides; A) fenoxaprop-*p*-ethyl, B) sethoxydim and C) pinoxaden (adapted from "World of Herbicides Map," 2010).

Selectivity of ACCase herbicides is based on the differing sensitivities of the monocot homomeric (generally sensitive) and dicot heteromeric (generally insensitive) plastidic ACCase. Because of this, ACCase herbicides can be used in dicot crops to control grass weeds. However, some fops (e.g. fenoxaprop and diclofop) and dims (e.g. tralkoxydim) are used to selectively control weeds in grass crops such as maize, wheat and rice, which have the same sensitive homomeric plastidic ACCase but have the ability to metabolize the fops and dims into non-toxic compounds (reviewed by Délye, 2005).

### 2.4.1 Resistance to ACCase herbicides and mechanisms

Extensive and repetitive use of ACCase herbicides, in some cases without diversity of other herbicide sites of action or weed control methods has resulted in rapid resistance evolution. Resistance to ACCase herbicides is widespread globally, with 46 different weed species having evolved resistance (Heap 2015). Mechanisms of resistance to ACCase herbicides include target-site ACCase gene mutations, enhanced metabolism of ACCase herbicide (non-target site) or both (Délye, 2005; Kaundun, 2014; Han et al., 2015).

The target-site mechanism for ACCase herbicide resistance consists of changes in specific amino acids in the CT domain of the plastidic ACCase of resistant weeds. Until 2014, there were a total of 8 different amino acid substitutions with various multiple allelic variants reported to cause ACCase resistance, involving positions Ile-1781, Trp-1999-, Trp-2027, Ile-2041, Asp-2078, Cys-2088 and Gly-2096 (reviewed by Powles & Yu, 2010; reviewed by Beckie & Tardif, 2012) (Table 2.3). Recently, a new substitution involving Asn-2097-Asp was reported, believed to contribute to high-level ACCase resistance in an *E. indica* population from Malaysia (Cha et al., 2014) (Table 2.3).

Over the years, research has shown that that the resistance level and cross-resistance pattern endowed by each ACCase target-site mutation are influenced by a number of factors such as the specific amino acid changes involved, homozygosity/heterozygosity of plants for the mutation, herbicide dose used for evaluation, etc. (Yu et al., 2007b; reviewed in Powles & Yu, 2010; Kaundun, 2014). Furthermore, some of the mutations have been reported to cause a resistance/fitness cost, while others do not. In fact, the Ile-1781-Leu mutation even displayed a fitness

advantage (Wang et al., 2010). A more detailed review on ACCase mutations and associated plant fitness/resistance cost is available in Vila-Aiub et al. (2009).

Enhanced rates of ACCase metabolism (hereafter also referred as metabolic resistance) have long been documented to contribute to herbicide resistance. Back in the mid 1980s, the first documented diclofop-methyl resistant *L. rigidum* in Australia showed cross-resistance to chlosulfuron to which the resistant population had not yet been exposed (Heap & Knight, 1986). Later on, it was established that the cross-resistance was due to enhanced rates of herbicide metabolism (reviewed by Preston, 2004; reviewed by Yu & Powles, 2014a). Now, metabolic resistance to selective herbicides is common in many species.

Various studies involving ACCase-resistant *L. rigidum* and *Alopecurus myosuroides* have indicated several enzymes that are involved in enhanced ACCase metabolism. These enzymes include the cytochrome P450s monooxygenases (P450s), glutathione-S-transferases (GSTs) and glucosyl transferases (GT) (Cummins et al., 1999; Preston, 2004; reviewed by Yu & Powles, 2014a). Recent advances in next generation sequencing technology have made it possible to identify candidate genes involved in metabolic resistance in resistant *L. rigidum* population (Gaines et al., 2014).

There has been a recent increase in the occurrence of resistant populations with ACCase target-site mutations plus enhanced ACCase metabolism (Kaundun et al., 2013a; Kaundun et al., 2013b; Han et al., 2015). As metabolic resistance can confer resistance to different herbicide sites of action including new herbicides that have not been commercialized (Yu & Powles, 2014a; Yu & Powles, 2014b), it calls for major research and management efforts.

Table 2.3. Update on identified amino acid substitutions in ACCase resistant grass weed species (adapted from Powles & Yu (2010) and Beckie & Tardif (2012)).

| Amino acid position[a] | Substitution | Grass weed species | Resistance spectrum[b] | | | References |
|---|---|---|---|---|---|---|
| | | | APP | CHD | PPZ | |
| Ile-1781 | Leu | *Alopecurus myosuroides* | R | R | R | Petit et al. (2010) |
| | | *Avena fatua* | R | R | R | Christoffers and Pederson (2007) |
| | | *Avena sterilis* | R | R | - | Liu et al. (2007) |
| | | *Lolium multiflorum* | - | R | - | White et al. (2005) |
| | | *Lolium. rigidum* | R | R | R | Zhang and Powles (2006a); Yu et al. (2007b) |
| | | *Setaria viridis* | R | R | - | Délye et al. (2005); Délye et al. (2008) |
| | Thr | *A. myosuroides* | S | r[c] | S | Kaundun et al. (2013b) |
| | Val | *Phalaris paradoxa* | r | R | r | Collavo et al. (2011) |
| | Ala | *Digitaria sanguinalis* | - | - | - | Heckart et al. (2008) |
| Trp-1999 | Cys | *A. sterilis* | R[d]/S | S | - | Petit et al. (2010) |
| | | *A. fatua* | - | - | - | Beckie and Tardif (2012) |
| | Leu | *Lolium spp.* | - | - | - | Scarabel et al. (2011) |
| | Ser | *L. multiflorum* | R/r[e] | r/S | R | Kaundun et al. (2013a) |

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| | | | | | | |
|---|---|---|---|---|---|---|
| Trp-2027 | Cys | *A. myosuroides* | R | r | R | Petit et al. (2010) |
| | | *A. sterilis* | R/r | r | - | Liu et al. (2007) |
| | | *L. rigidum* | nd | r | - | Yu et al. (2007b) |
| Ile-2041 | Asn | *A. myosuroides* | R | S | r | Petit et al. (2010) |
| | | *A. sterilis* | R | r | - | Liu et al. (2007) |
| | | *P. paradoxa* | - | - | - | Hochberg et al. (2009) |
| | | *L. rigidum* | R | r/S | - | Zhang and Powles (2006b); Yu et al. (2007b) |
| | | *A. fatua* | - | - | - | Cruz-Hipolito et al. (2011); Beckie and Tardif (2012) |
| | Val | *L. rigidum* | S/R | S | - | Délye (2005); Délye et al. (2008) |
| | Asp | *L. rigidum* | - | - | - | Malone et al. (2013) |
| | Thr | *L. rigidum* | - | - | - | Malone et al. (2013) |
| Asp-2078 | Gly | *A. myosuroides* | R | R | R | Petit et al. (2010) |
| | | *A. sterilis* | R | R | - | Liu et al. (2007) |
| | | *L. multiflorum* | R | R | R | Kaundun (2010) |
| | | *L. rigidum* | R | R | R | Yu et al. (2007b) |
| | | *P. paradoxa* | R | R | R | Hochberg et al. (2009) |
| Cys-2088 | Arg | *L. rigidum* | R | R | R | Yu et al. (2007b); Malone et al. (2013) |
| | Phe | *L. rigidum* | | | | Malone et al. (2013) |

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| | | | | | | |
|---|---|---|---|---|---|---|
| Gly-2096 | Ala | *A. myosuroides* | R | r/S | S | Petit et al. (2010) |
| | Ser | *A. fatua* | - | - | - | Beckie and Tardif (2012) |
| Asn-2097 | Asp | *Eleusine indica* | R | - | - | Cha et al. (2014) |

Abbreviations: APP, aryloxyphenoxypropionate; CHD, cyclohexanedione; PPZ, phenylpyrazolin (pinoxaden).

[a]Amino acid positions correspond to the full-length plastidic ACCase in *A. myosuroides*.

[b]R: resistant; S: susceptible; r: low to moderate level resistance; dash: not determined.

[c]Resistant to cycloxydim only.

[d]Resistant to fenoxaprop-*P*-ethyl only.

[e]r to haloxyfop-*P*-methyl

## 2.5 Paraquat resistance and mechanisms

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) (Fig. 2.8) was the first non-selective herbicide, introduced to the market in the 1960s. It is a contact herbicide that acts rapidly in a light-dependent manner (Preston et al., 1994). Its rapid uptake into plants (making it rain fast) makes it a favorable herbicide, especially in situations where rain is prevalent. Paraquat inhibits Photosystem 1 (PS1) by diverting the electrons to oxygen molecules, producing superoxide and other free radicals (Fig. 2.9). These free radicals then peroxidate the fatty acid side chains of membrane lipids, causing loss of membrane integrity, cell death and ultimately, desiccation of the plant tissue (Preston et al., 1994; reviewed by Hawkes, 2014). Symptoms of initial paraquat injury are visible wilting and interveinal chlorosis in the first few hours after treatment (under warm and bright conditions) followed by brown, chlorotic leaf tissue and ensuing leaf desiccation. The rapid action of paraquat in sunlight makes it self-limiting, as the rapid leaf necrosis limits its translocation within plants (Smith & Sagar, 1966).

To date, paraquat resistance has been reported in 31 species worldwide (Heap, 2015). Currently, the molecular basis of paraquat resistance remains unknown. However, various research involving resistant weeds, especially *Conyza* and *Hordeum* species have provided possible insights into the resistance mechanisms (reviewed by Fuerst & Vaughn, 1990; Preston et al., 1994; reviewed by Hawkes, 2014). Two major hypotheses are 1) prevention of paraquat from reaching PS1 in the chloroplast via sequestration, and 2) detoxification of free radicals by the Halliwell-Asada cycle (see below).

Reduced herbicide translocation has been observed in paraquat resistant *Hordeum glaucum*, *Arctotheca calendula* and *L. rigidum* (Bishop et al., 1987; Preston et al., 1994; Yu et al., 2004). Isolation of paraquat-treated, intact leaf protoplasts showed that protoplasts from resistant *L. rigidum* plants contained a higher amount of paraquat than the S protoplasts (Yu et al., 2010), suggesting that paraquat is kept away from entering the chloroplasts likely via sequestration into the vacuole. In addition, other lines of indirect evidence in *Conyza bonariensis* and *C. canadensis* also support the sequestration hypothesis (Fuerst et al., 1985; Norman et al., 1993; Norman et al., 1994; Jóri et al., 2007).

The second hypothesis involves detoxification of free radicals by protective enzymes (SOD, APX, catalase, peroxidase, glutathione reductase, dehydroascorbate

reductase) and the soluble antioxidants ascorbate and glutathione. These are all available in the chloroplast, and are collectively known as the Halliwell-Asada system (Fuerst & Vaughn 1990). Various studies with paraquat resistant *Conyza* species from Egypt suggested that the resistance mechanism was due to enhanced antioxidative capacity via expression/activity of Halliwell-Asada system enzymes (reviewed by Hawkes 2014). However, attempts to overexpress chloroplastic SOD in tobacco and thylakoid APX in *A. thaliana* only shifted the paraquat tolerance level less than 2-fold (Tepperman & Dunsmuir, 1990; Gupta et al., 1993; Murgia et al., 2004). This indicates that enhanced antioxidant capacity alone may not be sufficient for paraquat resistance. Another possible explanation is that the mechanism only worked for that particular *Conyza* biotype.

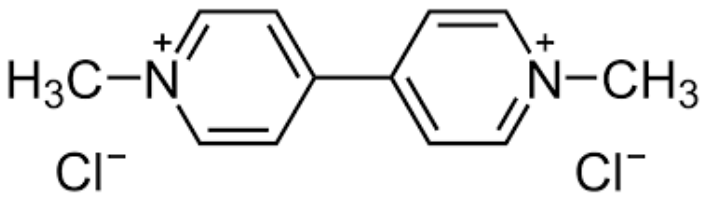


Figure 2.8. Structure of paraquat dichloride (adapted from Vencill, 2002).

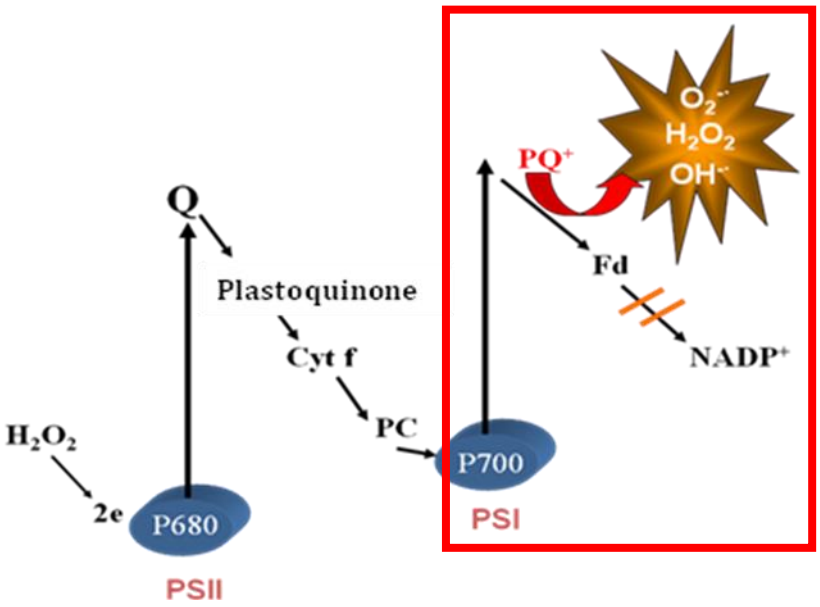


Figure 2.9. A cartoon depicting paraquat diverting electrons from PS1 (in red box). $H_2O_2$: hydrogen peroxide; e: electron; Q: quinine; Cyt f: cytochrome f; PC: plastocyanin; $PQ^+$: paraquat; Fd: ferrodoxin; $O_2^-$: oxygen radical; $OH^-$: hydroxyl radical; $NADP^+$: nicotinamide adenine dinucleotide phosphate.

## 2.6 Resistance cost

The general dogma of evolutionary genetics dictates that adaptation to a new environment often results in negative pleiotropic effects on fitness compared to the original environment, i.e. adaptation cost (Strauss et al., 2002). Herbicide resistance in weeds is an evolutionary process where selection and enrichment of resistance traits or alleles enables plant survival under a herbicide challenge (Vila-Aiub et al., 2005). If the adaptive traits turn out to be disadvantageous to other plant functions, the plant with the resistant allele could suffer a reduction in its fitness as compared to its wild type relative in the absence of herbicide selection. This phenomenon is termed a physiological cost or direct cost of resistance, i.e. resistance cost (Strauss et al., 2002).

Resistance cost can be attributed to several factors. Firstly, target site resistance due to mutation of the target enzyme could interfere with the normal function of the target enzyme (Gronwald, 1994; Purrington & Bergelson, 1999; Berticat et al., 2002). In the case of triazine-resistant weeds, a serine-264-glycine (Ser-264-Gly) substitution caused by the mutation of the chloroplastic *psbA* gene enabled weeds to survive triazine

herbicides. However, it also reduced the photosynthetic capacity of the plants, due to the inefficiency of the electron transfer in the PSII complex (Jansen & Pfister, 1990). In turn, plants that possess this mutation exhibit significant reduction in growth rates, resources competitiveness and sexual reproduction (reviewed by Holt & Thill, 1994). In *Amaranthus powellii* resistant to acetolactate synthase (ALS) inhibiting herbicides, resistant plants containing the tryptophan-574 (Trp-574) mutation in the ALS gene produced 67% less biomass than their susceptible counterparts (Tardif et al., 2006).

Secondly, plants with adaptive resistance alleles could divert resources intended for growth or reproduction to increase or produce detoxification enzymes in cases of non-target-site mechanisms such as enhanced metabolic resistance (Coley et al., 1985; Herms & Mattson, 1992). Indeed, this was the case observed in ACCase-resistant *Lolium rigidum* with enhanced cytochrome P450 resistance mechanism. The resistant biotype produced less above-ground biomass than the susceptible biotype. The P450-based herbicide metabolism was shown to be associated with physiological resistance cost (Vila-Aiub et al., 2005).

Thirdly, resistance cost could also originate as a result from altered ecological interactions (Vila-Aiub et al., 2009). Plants that adapted resistance allele could have pleiotropic phenotypic affects that make the plants become less attractive to pollinators or more susceptible to diseases (Strauss, 1997; Agrawal et al., 1999).

Resistance cost can be evaluated at the enzyme level and whole plant level (at both the vegetative and reproductive stage). Among other factors, control of genetic background and knowledge of the molecular basis of the resistance mechanism is important (Vila-Aiub et al., 2009).

## 2.7 *Eleusine indica*

*Eleusine indica* (L.) Gaertn. (Fig. 2.10) or goosegrass is an annual, monocot grass weed within the Poaceae family. The debate on its origin remains inconclusive, with early records coming from China, India, Japan, Malaysia, Africa and Tahiti (Holm et al., 1977). However in recent years Africa is seen as the place of origin, replacing India (Waterhouse, 1994). *Eleusine indica* is a diploid ($2n = 18$) and is believed to be the maternal ancestor of the allotetraploid *Eleusine* species (i.e. *Eleusine coracana*, *Eleusine africana* and *Eleusine kigeziensis*) (Liu et al., 2011).

*Eleusine indica* stems are often erect but can also be prostrate, geniculate and branching. Its leaf blade is 5 to 20 cm long and 3 to 8 mm wide. *Eleusine indica* flowers are bisexual and are grouped together in a terminal spikelet. Spikelets are arranged subdigitately (anywhere from 3 to 10 spikes) forming the inflorescence (Fig. 2.11a). Each spikelet has 3 to 9 fertile flowers (Fig. 2.11b). One raceme may be inserted about 1 cm below the others (Fig. 2.11c). The rachis is narrow (1 mm wide) and has two dense rows of spikelets. The grain or seed of *E. indica* is oblong shaped and dark red to dark brown in colour when mature (Figure 2.12) (Teng & Teo, 1999; "*Eleusine indica*," 2013; "*Eleusine indica* (goosegrass)," 2013; "*Eleusine indica* (L.) Gaertn.-POACEAE-Monocotyledon," 2013).

*Eleusine indica* is widely distributed throughout the tropics, subtropics and temperate regions (up to 50 degrees latitude), extending all the way from South Africa to Japan and the northern border of the United States (Waterhouse, 1994; "*Eleusine indica* (goosegrass)," 2013). Its habitat includes disturbed lands, waste places, in fields of rotation crops and perennial crops and grassland (Waterhouse, 1994). Extremely competitive, it is a serious weed in warmer climate areas of the world, especially in the tropics (Waterhouse, 1994), where it can grow and flower all year round when moisture is sufficient. A typical example is Malaysia, where *E. indica* can have 3 to 5 generations per year. In four-season countries, *E. indica* normally grows in spring and flowers in summer.

One of the reasons that *E. indica* grows well in warmer climates is because *E. indica* is a $C_4$ plant (Waterhouse, 1994). In photosynthesis, light energy is transformed into chemical energy. Most plants undergo either $C_3$ or $C_4$ photosynthesis, depending on the primary carboxylator of the photosynthesis process. In $C_3$ plants the primary carboxylator is ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) and its first stable product is a 3 carbon acid, 3-phosphoglycerate (reviewed by Cobb & Reade, 2011). In $C_4$ plants, the first carboxylator is phosphoenolyruvate carboxylase (PEPC) (Cobb & Reade, 2011). The first detectable products for $C_4$ plants are oxaloacetate, which is then converted to a more stable compound in the form of malate or aspartate, all of which are composed of four carbon atoms (Cobb & Reade, 2011). The oxygenase activity of Rubisco increases more rapidly with increasing temperature than the carboxylase activity, resulting in wasteful photorespiration. The process of $C_4$ photosynthesis (shuttling high concentrations of $C_4$ compounds to Rubisco in the bundle-sheath cells) evolved from $C_3$ photosynthesis as a way to adapt to high light

intensities, high temperatures and dryness (Gowik & Westhoff, 2011). As a result, plants with $C_4$ photosynthesis are more efficient at capturing carbon dioxide and have better water-use efficiency than $C_3$ plants, allowing them to grow better and more competitively in the warmer climates of the tropical and subtropical regions (Edwards et al., 2010). Thus, a $C_4$ weed such as *E. indica* infesting a $C_3$ crop can be very competitive. In fact, it is noted that eight out of the world's top ten worst weeds are $C_4$ plants (Holm et al., 1977).

A single isolated *E. indica* plant growing without competition in good conditions can produce as many as 140 000 seeds (Chin, 1979), though the mean production of a population is 40,000 seeds per plant (Holm et al., 1977). *Eleusine indica* can germinate and grow in nearly all soil types and prefers temperature above 20 but below 40°C (Nishimoto & McCarty, 1997). *Eleusine indica* seeds can remain viable even if buried up to 20 cm deep in the soil for 2 years (Chuah et al., 2004). In farming systems in warm regions with a lack of shading, *E. indica* can easily become the dominant weed, due to its vigorous growth and rampant seeding. At a high density, it can cause staggering yield loss to crops. In work done in the Philippines where *E. indica* is the second most abundant weed in upland rice, a yield loss of up to 80% was observed (Lourens et al., 1989). A density of 20 *E. indica* plants per maize plant caused 15% yield reduction (Eke & Okereke, 1990). A study involving *E. indica* growing in groundnuts showed that 16 *E. indica* plants per 10 m row reduced the yield by 19% (McCarty, 1983).

Given its tremendous capability for reproduction and its prevalence and competitiveness, it is no surprise that *E. indica* is listed as one of the world's worst weeds (Holm et al., 1977). Its adaptability to different conditions allows it to grow and infest almost any crop type. In the 1970s, it was already considered as one of the most serious weeds in cotton (11 countries), maize (10 countries), upland rice (8 countries), sweet potatoes (4 countries) and sugarcane (3 countries) (Table 2.4). *Eleusine indica* also occurs in a wide range of other crops such as banana, cassava, pineapple, rape, jute, soybean, pawpaw, abaca, cowpea, millet, mango, cacao, sorghum, tobacco, wheat and many vegetable crops (Holm et al., 1977).

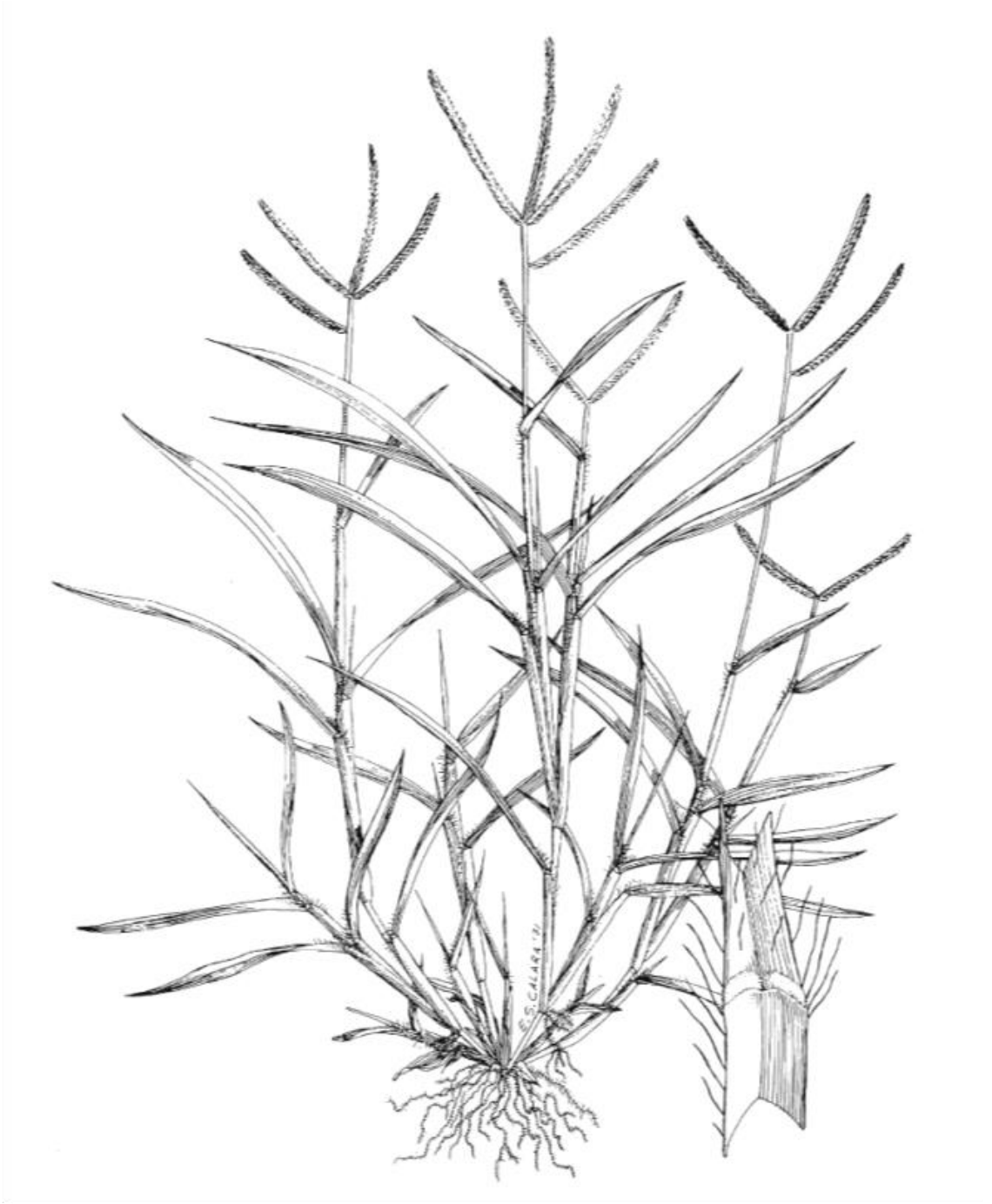

Figure 2.10. Illustration of *Eleusine indica* (Holm et al., 1977).

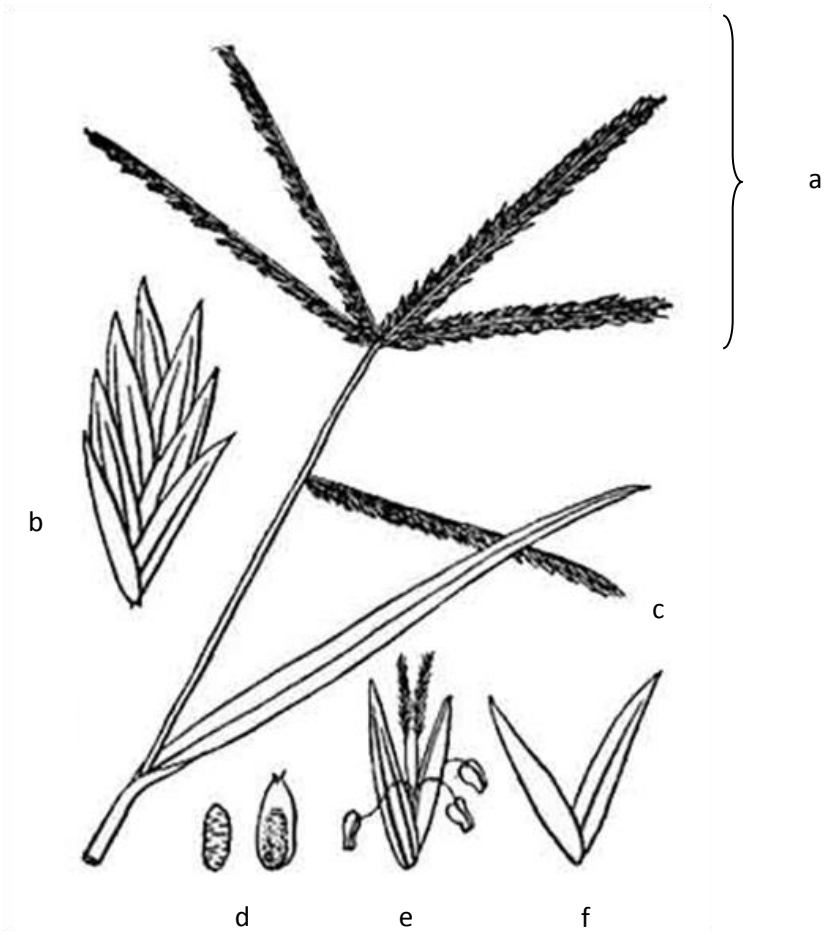


Figure 2.11. Various parts of the *Eleusine indica* shoot. Inflorescence, a; spikelet, b; raceme, c; seeds, d; flower with anther and stigma, e; and leaf blade, f (image taken from "Philippine Medicinal Plants," 2013).


Figure 2.12. *Eleusine indica* seeds with (top) and without (bottom) seed coat (image taken from "*Eleusine indica* (L.) Gaertn.," 2014).

Table 2.4. List and location of crops with *E. indica* as the most serious weed (Holm et al., 1977).

| Crop | Countries |
|---|---|
| Cotton | India |
| | Kenya |
| | Mozambique |
| | Nicaragua |
| | Nigeria |
| | Tanzania |
| | Thailand |
| | Uganda |
| | United States of America |
| | Zambia |
| | Zimbabwe |
| Maize | Angola |
| | Malaysia |
| | Philippines |
| | Taiwan |
| | Venezuela |
| | Zambia |
| Upland rice | Brazil |
| | India |
| | Indonesia |
| | Thailand |
| Sweet potatoes | Hawaii |
| | Japan |
| | Malaysia |
| | Taiwan |
| Sugarcane | Indonesia |
| | Taiwan |
| | Tanzania |

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# Chapter 3

## Multiple resistance to glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population

**Preface to Chapter Three**

This chapter is highly similar to the following publication: Jalaludin, A., Yu, Q., & Powles, S. B. (2014). Multiple resistance across glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population. *Weed Research*, *55*, 82-89. However, a part of this chapter, namely, the ACCase gene sequencing, is not available in the published manuscript.

Chapter 3

# Multiple resistance to glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population

## 3.1 Abstract

An *Eleusine indica* population from Malaysia was reported as the world's first case of field-evolved glufosinate resistance. Here we further characterised this population and found that this population displayed multiple resistance to glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides. Dose-response experiments with glufosinate showed that the resistant population is 5-fold and 14-fold resistant ($GR_{50}$ 156 g $ha^{-1}$; $LD_{50}$ 820 g $ha^{-1}$) relative to the susceptible population ($GR_{50}$ 31 g $ha^{-1}$; $LD_{50}$ 58 g $ha^{-1}$), based on $GR_{50}$ and $LD_{50}$ R/S ratio, respectively. The selected glufosinate-resistant sub-population also displayed a high level resistance to glyphosate, with the respective $GR_{50}$ and $LD_{50}$ R/S ratios being 12- and 144-fold. In addition, the sub population also displayed a level of resistance to paraquat (2- to 3-fold; $GR_{50}$ 105 g $ha^{-1}$ and $LD_{50}$ 292 g $ha^{-1}$, respectively), and ACCase-inhibiting herbicides fluazifop-*p*-butyl, haloxyfop-*p*-methyl and butroxydim. ACCase gene sequencing revealed that the Trp-2027-Cys mutation is likely responsible for resistance to the ACCase inhibitors examined. Here we confirm glufosinate resistance and importantly, we find very high level glyphosate resistance, as well as resistance to paraquat and ACCase inhibiting herbicides. This is the first confirmed report of a weed species that has evolved multiple resistance across all the three commonly used non-selective herbicides, glufosinate, glyphosate and paraquat.

## 3.2 Introduction

*Eleusine indica*, one of the world's worst weeds (Holm et al., 1977), is a very competitive and cosmopolitan species. *Eleusine indica* is suited to a wide range of soils and temperature conditions (20 to 40°C) (Nishimoto & McCarty, 1997), producing as many as 140,000 seeds per plant (Chin, 1979). This adaptive capacity allows *E. indica* to grow and infest a wide range of crops including cotton, maize, upland rice, sweet

potatoes, sugarcane and many fruit and vegetable orchards (Holm et al., 1977), resulting in large reductions in crop yield (McCarty, 1983; Eke & Okereke, 1990).

In a tropical climate as in Malaysia, *E. indica* infests field crops, areas including fruit and vegetable orchards, nurseries and young palm oil plantations. At high densities, *E. indica* greatly affects crop growth, resulting in yield loss and increased incidence of plant disease, including *Phytophtora* spp. (Chee et al., 1990; Teng & Teo, 1999). As a result of its widespread presence, competitiveness and fecundity, growers in Malaysia and other countries have become reliant upon herbicides for *E. indica* control. However, overreliance on herbicides has resulted in resistance evolution in *E. indica* in at least eight countries (Heap, 2015). Since the first case of dinitroaniline herbicide resistance was reported in *E. indica* in 1973, herbicide resistance has been documented to seven herbicide sites-of-action, including resistance to the dinitroaniline herbicides (Mudge et al., 1984), acetyl coA carboxylase (ACCase)-inhibiting herbicides (Leach et al., 1993; Osuna et al., 2012), the acetolactate synthase (ALS)-inhibiting herbicide imazapyr (Valverde et al., 1993), the EPSPS-inhibiting herbicide glyphosate (Lee & Ngim, 2000), the bipyridylium herbicide paraquat (Buker et al., 2002), photosystem II-inhibiting herbicides (Brosnan et al., 2008) and most recently the glutamine synthetase-inhibiting herbicide glufosinate (Chuah et al., 2010; Jalaludin et al., 2010).

Glyphosate, and its widely used alternative, glufosinate, are globally important herbicides. Glyphosate was widely used in Malaysia to control large *E. indica* populations in fallow, nurseries and plantation maintenance. Glyphosate allowed growers to reduce costs and save time in weed control. However, persistent use was a heavy selection pressure on *E. indica* populations. Following just 3 years of use, glyphosate resistance evolved in *E. indica* (Lee and Ngim, 2000). This rapid resistance evolution is partly because *E. indica* can have a few (3-5) generations treated per year (see Section 2.6, Chapter 2). Now many *E indica* populations have been identified to be glyphosate resistant (Ng et al., 2003; Ng et al., 2004; Kaundun et al., 2008). The rapid increase in glyphosate-resistant *E. indica* cases lead to growers adapting glufosinate in order to combat glyphosate-resistant weeds.

In 2010 the first case of glufosinate resistance was reported in a Malaysian *E. indica* population (Jalaludin et al., 2010). Prior to glufosinate usage, this population had a field history of paraquat, fluazifop-*p*-butyl and glyphosate use. At the same time, a different Malaysian *E. indica* population was reported to be resistant to glufosinate and

paraquat (Chuah et al., 2010). Subsequently, two other cases of glufosinate resistance and multiple-resistance to glufosinate and glyphosate have been reported in *Lolium perenne* in Oregon, United States (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia et al., 2012). Working with the known glufosinate-resistant population (Jerantut population) identified by the author (Jalaludin et al., 2010), this study aims to quantify the level of resistance to glufosinate and evaluate any efficacy of other herbicide sites of action.

## 3.3 Materials and methods

The glufosinate resistant (R) *E. indica* (Jerantut population) population used in this study was previously reported (Jalaludin et al., 2010). A glufosinate-susceptible *E. indica* population provided by Chuah, T. S., and a subset of this population that has been confirmed to be susceptible to all herbicides, was used as an herbicide-susceptible (S) population.

### 3.3.1 Glufosinate dose-response

*Eleusine indica* seeds were germinated on 0.6% agar containing 0.2% potassium nitrate ($KNO_3$) (Ismail et al., 2002) in a glasshouse during summer (Jan to Mar) with average temperatures of 30/20°C (day/night) with a 15 h photoperiod of natural sunlight. After 4 – 7 d, the seedlings were transplanted into pots (18 cm diameter with 15-20 seedlings per pot) containing potting mix (25% peat moss, 25% sand, 50% mulched pine bark) and fertilized (weekly/daily) with 2g Scotts polyfeed™ soluble fertilizer (N 15% [urea 11.6%, ammonium 1.4%, nitrate 2%], P 2.2%, K 12.4%, Ca 5%, Mg 1.8%, S 3.8%, Fe 120 mg $kg^{-1}$, Mn 60 mg $kg^{-1}$, Zn 15 mg $kg^{-1}$, Cu 15 mg $kg^{-1}$, B 20 mg $kg^{-1}$, Mo 10 mg $kg^{-1}$). Once the seedlings reached the 3- to 5-leaf stage, the seedlings were treated with glufosinate at 0, 20, 41, 83, 124, 248, 495, 1485, 1980, 3960 and 7920 g $ha^{-1}$ (Basta, 200 g a.i. $L^{-1}$, SC; Bayer CropScience) (hereafter all herbicide doses are expressed as g $ha^{-1}$), using a custom-built, dual nozzle cabinet sprayer delivering herbicide at 118 L $ha^{-1}$ at 210 kPa, with a speed of 1 m $s^{-1}$. Following herbicide treatment, all pots were arranged in a completely randomized block design in a glasshouse with at least three replicate pots per herbicide rate. Visual assessment for resistance (R) and susceptibility (S) were made 21 d after treatment (DAT). Plants were considered to be R if they were actively growing whilst the S plants desiccated and

died. Above-ground biomass was harvested and dried in an oven (65ºC) for 3 d before weighing.

Six plants that survived 1485 and 1980 g $ha^{-1}$ glufosinate were bulked together to produce a glufosinate-selected progeny sub population (referred as R*). This R* population was tested again for glufosinate resistance, and used for glyphosate, paraquat and ACCase-inhibiting herbicide resistance screening experiments.

### 3.3.2 Glyphosate and paraquat dose-response

Seed germination and establishment was conducted as described above. At the 3- to 5-leaf stage, *E. indica* plants from the S and R* populations were treated with glyphosate at rates of 0, 34, 68, 100, 135, 170, 200, 540, 1080, 4320, 8640, 12960, 17280 and 25920 g a.e. $ha^{-1}$ (Roundup Attack with IQ inside, 570 g a.e. $L^{-1}$, SL; Nufarm Australia) (hereafter glyphosate concentration is referred as g $ha^{-1}$) and paraquat at rates of 0, 47, 94, 188, 375, 750, 1500 and 3000 g $ha^{-1}$ (Gramoxone, 250 g a.i. $L^{-1}$, SL; Syngenta Crop Protection).Visual assessments were made at 21 d after treatment as described above. The paraquat dose-response experiment was conducted in a controlled environment room with alternating temperatures of 30/25°C (day/night), 75% humidity and a 12 h photoperiod with light intensity of 400 µmol $m^{-2}$ $s^{-1}$.

### 3.3.3 Herbicide single-rate test

In order to check for multiple resistance, label rates of the ACCase-inhibiting herbicides fluazifop-*p*-butyl (210 g $ha^{-1}$) (Fusilade Forte, 128 g a.i. $L^{-1}$, EC, Syngenta Crop Protection Pty Ltd), haloxyfop-*p*-methyl (60 g $ha^{-1}$) (Verdict 520, 520 g a.i. $L^{-1}$, EC, Dow Agrosciences Australia Ltd), clethodim (100 g $ha^{-1}$) (Select, 240 g a.i. $L^{-1}$, EC, Sumitomo Chemical Australia Pty Ltd), butroxydim (100 g $ha^{-1}$) (Falcon, 250 g a.i. $kg^{-1}$, WG, Nufarm Australia Ltd) and sethoxydim (230 kg $ha^{-1}$) (Sertin 186, EC, 186 g a.i. $L^{-1}$, EC, Bayer CropScience Pty Ltd), and the ALS-inhibiting herbicide imazapyr (50 g $ha^{-1}$) (Arsenal, 250 g a.i. $L^{-1}$, SC, Nufarm Australia Ltd) were applied to S and R* plants of *E. indica* at the 3- to 5-leaf stage. Plants were established in trays (50-60 per tray with two to four replicate trays per herbicide treatment). Following treatment all populations were maintained in a temperature controlled glasshouse with day/night temperature of 30/25°C under natural sunlight.

### 3.3.4 Statistics

The herbicide rate causing 50% mortality ($LD_{50}$) or reduction in growth ($GR_{50}$) was estimated by non-linear regression analysis using Sigma Plot ® software (version 12.0, SPSS Inc. 233 South Wacker Drive, Chicago, IL). The data were fitted to the three parameter logistic curve model:

$$y = \frac{a}{1 + (\frac{x}{ED_{50}})^b}$$

where $y$ denotes plant survival or biomass, $a$ = upper limit, $ED_{50}$ = estimated dose causing 50% response and $b$ = slope around $ED_{50}$. A t-test at 5% probability was used to test the significance of the regression parameters. The $LD_{50}$ and $GR_{50}$ values of the S and R(*) biotypes were used to calculate the R/S ratio of the resistant populations. Several pilot trials were conducted prior to final herbicide dose-response experiments which contained at least three replicate pots per herbicide rate. Each dose-response experiment was repeated at least twice with similar results, and therefore, only results from a single experiment were presented.

### 3.3.5 ACCase gene sequencing

Genomic DNA was extracted from the leaf tissue of R* and S plants according to Yu et al. (2008). Published primers (Osuna et al. 2012) were used to amplify two plastidic ACCase gene fragments in which point mutations known to confer herbicide resistance in plants have been identified (reviewed by Délye 2005; Powles and Yu 2010; Beckie and Tardif 2012). The PCR was conducted in a 25 µl volume that consisted of 1-2 µl of genomic DNA, 0.5 µM of each primer, and 12.5 µl of 2× GoTaq Green Master Mix® (Promega). The PCR was run with the following profile: 94ºC for 4 min; 40 cycles of 94ºC for 30s, 58ºC (annealing temperature) for 30s, and 72ºC for 1 min; followed by a final extension step of 7 min at 72°C.The PCR product was purified from agarose gel with the Wizard® SV Gel and PCR Clean-up System (Promega Co., Madison, WI USA) and sequenced by a commercial service. All sequence chromatograms were visually checked for quality and consistency before sequences were assembled and aligned. Sequences of the plastidic ACCase gene from the R and S individuals were aligned and compared using the Clustal Omega Multiple Sequence

Alignment programme available at the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/clustalo/) to detect any nucleotide differences.

## 3.4 Results

### 3.4.1 Glufosinate resistance

The results of this study confirm that the R *E. indica* population was glufosinate-resistant with approximately 78% of R plants surviving the field rate (495 g $ha^{-1}$), which completely controlled the S population. Following treatment all plants showed symptoms, dark grey (almost burnt-looking) from the middle of the leaves to the leaf tip, with slight wilting, 24 h after treatment. The damaged area then extended in the basipetal direction, developing necrosis over 14 d, turning wilted leaves from yellow into brown. While the S plants died, the resistant (R and R*) plants were observed to recover and grow again two weeks after treatment. Following full dose-response screening, the R population displayed an $LD_{50}$ of 820 g $ha^{-1}$, compared to 58 g $ha^{-1}$ for the S population (Table 3.1), resulting in a R/S ratio (based on the $LD_{50}$) of 14. This is higher than the previously reported $LD_{50}$ R/S ratio of 7.6 (Jalaludin et al. (2010). The difference could be attributed to different susceptible populations and experimental conditions being used in the two studies. For plant growth, the R population $GR_{50}$ rate was found to be 5-fold greater (156 g $ha^{-1}$) than for the S population (Table 3.2). Glufosinate resistance in the R population was found to be heritable with the glufosinate $LD_{50}$ R/S ratio increasing to 22 when comparing the glufosinate-selected progeny R* and the S population (Fig. 3.2A; Table 3.1 and 3.2). However the R* population was only about 2-fold more resistant to glufosinate compared to the basal R population, indicating the glufosinate-resistant sub population is still segregating. Despite having a high R/S ratio based on survival and plant biomass, resistant *E. indica* was still satisfactorily controlled at 1485 g $ha^{-1}$ and above (Fig. 3.1).

Table 3.1. Parameter estimates for logistic analysis of glufosinate, glyphosate and paraquat dose-response survival data for the susceptible (S) and resistant (R) *E. indica* populations.

| Population | a | b | $x_0 = LD_{50}$ (g $ha^{-1}$) | $R^2$ (coefficient) | R/S ratio of $LD_{50}$ |
|---|---|---|---|---|---|
| Glufosinate dose-response | | | | | |
| S | 100.00 (0) | 5.71 (0.23) | 58 (0.81) | 0.99 | |
| R | 100.00 (0) | 2.42 (0.37) | 820 (85.6) | 0.93 | 14 |
| [c]R* | 100.00 (0) | 2.3 (0.25 | 1278 (63.9) | 0.99 | 22 |
| Glyphosate dose-response | | | | | |
| S | 100.00 (0) | 15.28 (1.71) | 148 (1.81) | 0.98 | |
| R* | 100.00 (0) | 0.99 (0.1) | 21274 (1773) | 0.98 | 144 |
| Paraquat dose-response | | | | | |
| S | 100.00 (0) | 3.76 (0.66) | 98 (23.6) | 0.97 | |
| R* | 100.00 (0) | 1.5 (0.2) | 292 (27.9) | 0.94 | 3 |

[c] R* refers to the selected glufosinate-resistant sub population.

Standard errors are in parentheses

Table 3.2. Parameter estimates for logistic analysis of glufosinate, glyphosate and paraquat dose-response biomass data for the susceptible (S) and resistant (R) *E. indica* populations.

| Population | a | b | $x_0 = GR_{50}$ (g $ha^{-1}$) | $R^2$ (coefficient) | R/S ratio of $GR_{50}$ |
|---|---|---|---|---|---|
| Glufosinate dose-response | | | | | |
| S | 100.00 (0) | 2.23 (0.36) | 31 (2.3) | 0.94 | |
| R | 100.00 (0) | 1.36(0.17) | 156 (17.4) | 0.98 | 5 |
| [c]R* | 100.00 (0) | 1.25 (0.25) | 325 (37.1) | 0.98 | 11 |
| Glyphosate dose-response | | | | | |
| S | 100.00 (0) | 1.7 (0.22) | 41 (3.6) | 0.92 | |
| R* | 100.00 (0) | 0.88 (0.09) | 481 (55.6) | 0.95 | 11.8 |
| Paraquat dose-response | | | | | |
| S | 100.00 (0) | 3.22 (0.72) | 52 (3.1) | 0.95 | |
| R* | 100.00 (0) | 1.84 (0.29) | 105 (8.4) | 0.96 | 2 |

[c] R* refers to the selected glufosinate-resistant sub population.

Standard errors are in parentheses

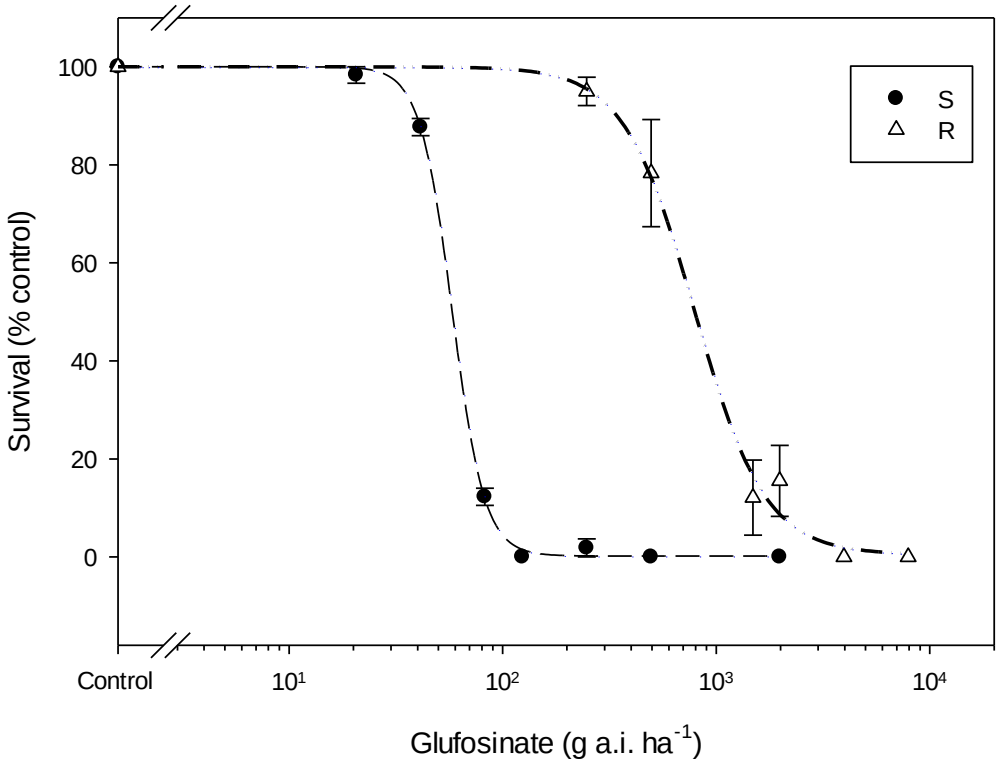


Figure 3.1. Glufosinate dose-response for survival of the susceptible (S) and resistant (R) populations of *Eleusine indica*. Data were collected at 21 DAT.

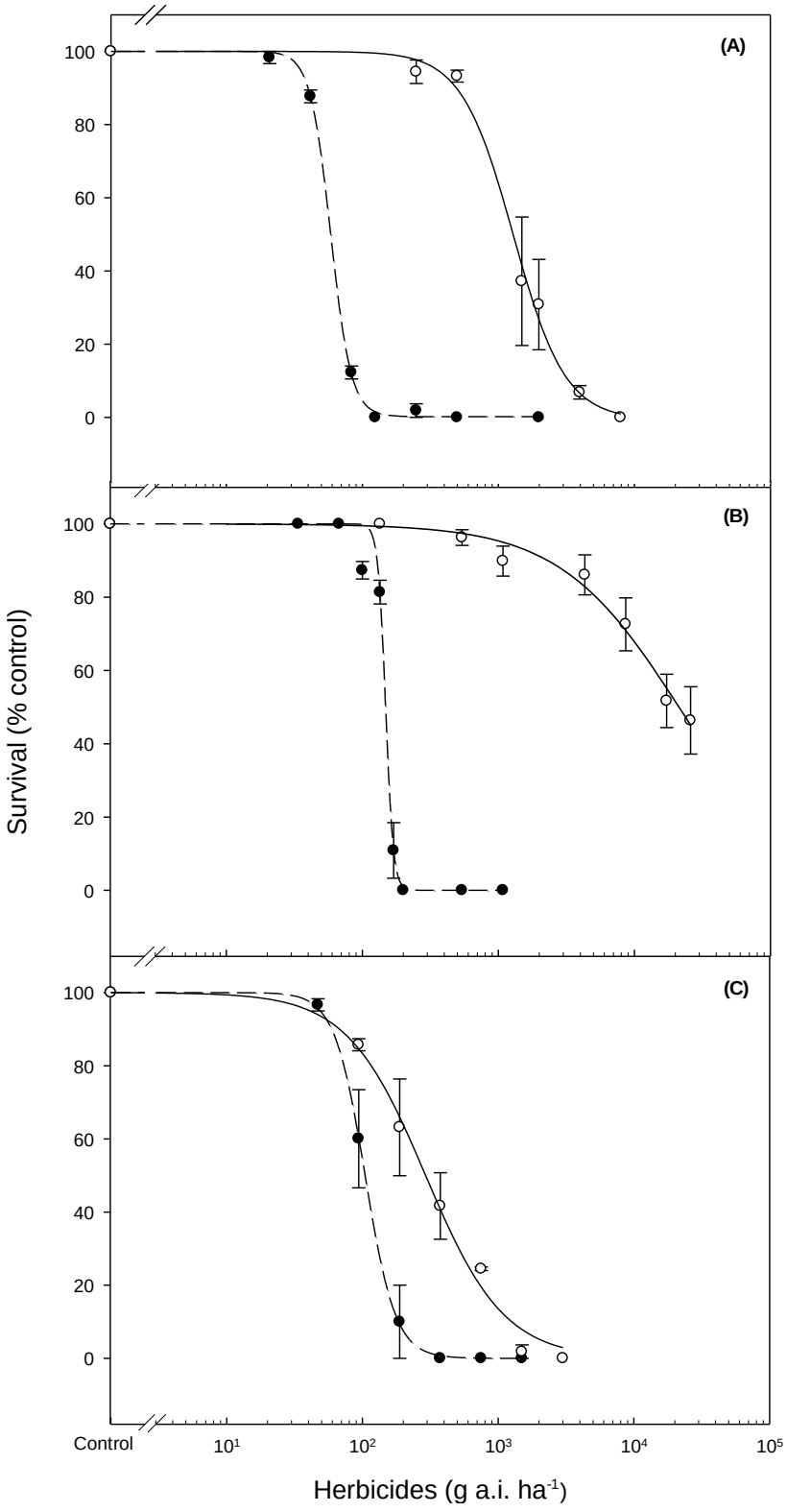


Figure 3.2. Survival response of the susceptible (closed circle; ●) and selected glufosinate-resistant (opened circle; ○) R* sub populations of *Eleusine indica* to glufosinate (A), glyphosate (B) and paraquat (C) treatment. Data were collected at 21 DAT. Glyphosate rates are in g a.e. ha$^{-1}$.

### 3.4.2 Glyphosate resistance

As expected the S population was very sensitive to glyphosate so that 200 g $ha^{-1}$ or higher controlled all S plants (Fig. 3.2B). However, the R* population was clearly glyphosate resistant (Fig. 3.2B). An extremely high rate (i.e. 25920 g $ha^{-1}$ glyphosate) was needed to inflict significant mortality in R* population (Fig. 3.2B). Based on the $LD_{50}$ R/S ratio, the R* population was more than 140-fold resistant to glyphosate (Table 3.1). While the resistant plants survived high glyphosate doses, plant growth was affected. The $GR_{50}$ for the S and R* population were 41 g $ha^{-1}$ and 481 g $ha^{-1}$, respectively, resulting in the R* population being 12-fold more resistant than the S biotype (Table 3.2). Therefore, in addition to glufosinate resistance, the R* population has high level resistance to glyphosate.

### 3.4.3 Paraquat resistance

The S population was very well controlled by paraquat. However, there was a clear difference R* sub-population (Fig. 3.2C) rate. Based on the $LD_{50}$ and $GR_{50}$ R/S ratios (Table 3.1 and 3.2), paraquat resistance in the selected glufosinate-resistant population is confirmed, albeit at a low level (2- to 3-fold). Both S and R* plants displayed rapid desiccation and necrosis following treatment. Similar to glufosinate-treated plants, the R* plants recovered two weeks after treatment while the S plants died.

### 3.4.4 Resistance to ACCase-inhibiting herbicides

All herbicides examined gave full control of the S population at the rates outlined in Table 3.3. However, 47%, 51% and 49% of the R* population survived fluazifop-*p*-butyl, haloxyfop-*p*-methyl and butroxydim respectively. In contrast, the population remained susceptible to sethoxydim, clethodim and imazapyr (Table 3.3).

### 3.4.5 ACCase gene sequencing

The plastidic ACCase gene sequences from a total of 9 plants surviving fluazifop or butroxydim were analyzed in comparison to susceptible plants. The primer pair ELEIN1781F/ELEIN1781R (Osuna et al., 2012) amplified a 600 bp DNA fragment

flanking the known mutation site 1781, and the primer pair ELEIN2027f/ELEIN2027r amplified an 832 bp fragment flanking the known mutation sites 1999, 2027, 2041, 2078, 2088 and 2096. Sequence alignment revealed an amino acid substitution of Trp-2027-Cys in R* individuals resulting from a G to T change at the third position of the Trp codon (TGG) (Fig. 3.3). No other single nucleotide polymorphism was found in the other known mutation sites in the plastidic ACCase gene (Fig. 3.3 and 3.4).

Table 3.3. Survival of the susceptible (S) and selected glufosinate-resistant (R*) sub populations of *E. indica* 21 days after treatment with various herbicides.

| Herbicide | Mean % survival | |
|---|---|---|
| | S | R* |
| ACCase inhibitor | | |
| Fluazifop-*p*-butyl (210 g $ha^{-1}$) | 0 | 47 |
| Haloxyfop-*p*-methyl (60 g $ha^{-1}$) | 0 | 51 |
| Sethoxydim (230 g $ha^{-1}$) | 0 | 0 |
| Clethodim (100 g $ha^{-1}$) | 0 | 0 |
| Butroxydim (100 g $ha^{-1}$) | 0 | 49 |
| ALS inhibitor | | |
| Imazapyr (50 g $ha^{-1}$) | 0 | 0 |

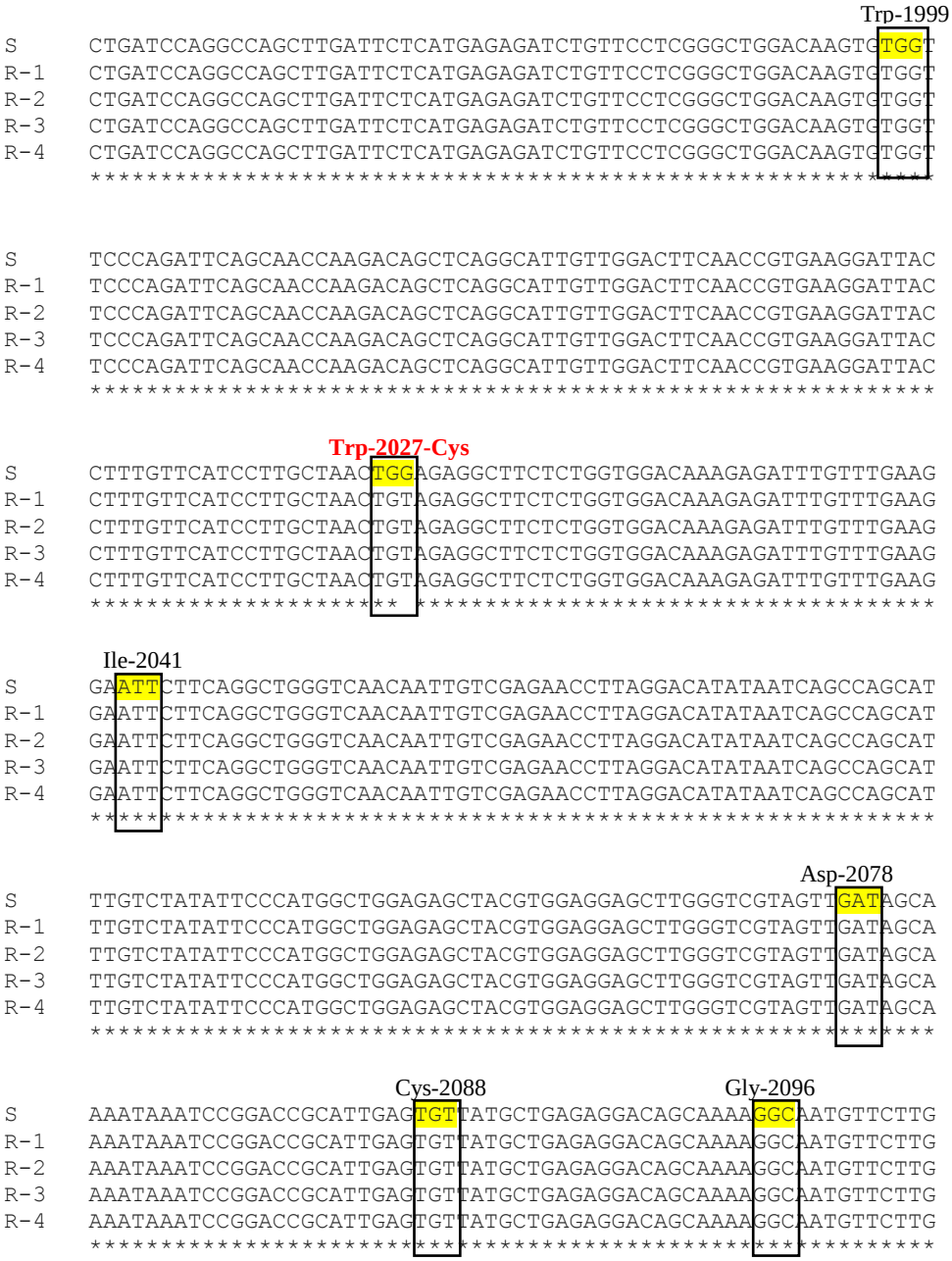


Figure 3.3. Multiple alignment of a clearly identified plastidic ACCase gene sequence covering the known mutation sites of Trp-1999, Cys-2027, Ile-2041, Asp-2078, Cys-2088 and Gly-2096 in the CT domain (in boxes) between the S and R samples. The reference codon (S) is highlighted in each box for comparison. A point mutation from G to T at the third position of the Trp-2027 codon resulted in an amino acid substitution from Trp to Cys. Results for R-5 to R-9 are the same as for R-1 to R-4 and are not shown.

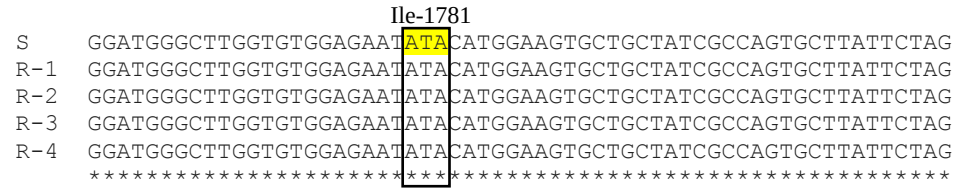


Figure 3.4. Multiple alignment of a clearly identified plastidic ACCase gene sequence covering the known mutation site Ile-1781 in the CT domain (in box) between the S and R samples. The reference codon (S) is highlighted in each box for comparison. Results for R-5 to R-9 are the same as for R-1 and R-4 and are not shown.

## 3.5 Discussion

In Malaysia, glufosinate has been used as an alternative to glyphosate for over 10 years; with glufosinate resistance first confirmed in 2010 (Chuah et al., 2010; Jalaludin et al., 2010). The current study confirmed the preliminary report on the evolution of resistance to glufosinate in a Malaysian *E. indica* population (Jalaludin et al., 2010). The level of resistance determined in this glufosinate-resistant population was modest (5- and 14-fold, based on $GR_{50}$ and $LD_{50}$, respectively) which is similar to those reported for the other *E. indica* populations (Chuah et al., 2010) and slightly higher than glufosinate-resistant *Lolium perenne* populations in Oregon, USA ($GR_{50}$ R/S ratios between 2.2 and 2.8) (Avila-Garcia & Mallory-Smith, 2011). The level of paraquat resistance in this population was also similar to that observed in a glufosinate- and paraquat-resistant Malaysian *E. indica* population (Chuah et al. 2010). It is worth noting that usually $GR_{50}$ R/S ratios are more variable than $LD_{50}$ ratios due to variations in growth conditions and especially, the length of experiments. In this sense, $LD_{50}$ R/S ratios would be the better option to compare results across research groups.

Globally glufosinate-resistance has thus far only been confirmed in *E. indica* (Chuah et al., 2010; Jalaludin et al., 2010) and *L. perenne* (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia et al., 2012). The resistance mechanism(s) endowing glufosinate resistance is not yet understood. In glufosinate-resistant *L. perenne*, the resistance mechanism in one population was speculated to be non-target-site based (Avila-Garcia and Mallory-Smith, 2011) while in another population it was reported to

be due to a target-site mutation in the glutamine synthetase gene (Avila-Garcia et al., 2012).

Importantly, in addition to glufosinate resistance, the *E indica* population characterised in the current study is highly resistant to glyphosate (Fig 3.2; Table 3.1). Despite surviving high glyphosate rates ($LD_{50}$ R/S 144), glyphosate application still resulted in a growth reduction ($GR_{50}$ R/S 12). However, the survival and plant biomass R/S ratios were both higher than previously reported in glyphosate-resistant populations of *E. indica* and other weed species (Lee & Ngim, 2000; Baerson et al., 2002; Culpepper et al., 2006; Mueller et al., 2011; Gaines et al., 2012). As is discussed above, we consider the $LD_{50}$ value is more accurate and meaningful in describing resistance levels because it is less affected by experimental conditions (e.g. harvest time, growth competition) as compared with the $GR_{50}$ value. Nevertheless, the large difference in the $LD_{50}$ and $GR_{50}$ R/S ratios obtained for glyphosate response in this *E. indica* population indicates that the glyphosate resistance mechanism(s) may incur a fitness cost in the presence of herbicide. The high level glyphosate resistance in this study requires further study. Potential mechanism(s) endowing such strong resistance may include (1) a new target-site EPSPS mutation, (2) multiple EPSPS mutations and (3) accumulation of several known glyphosate resistance mechanisms (e.g. EPSPS gene mutation or amplification, reduced glyphosate translocation or enhanced sequestration). Studies investigating the mechanistic basis of resistance in this population are outlined in Chapter 5.

The glufosinate and glyphosate and paraquat resistant population was also found to be resistant to ACCase-inhibiting herbicides (Table 3.3). Mutations in the ACCase gene that result in resistance to particular ACCase herbicides (e.g. APPs) could also confer cross-resistance to other chemically-dissimilar ACCase herbicide groups (e.g. CHDs and PPZ). The 1781-Leu and 2078-Gly mutant isoforms of *A. myosuroides* and *L. rigidum*, and 2088-Arg isoform in *L. rigidum* are resistant to not only APP herbicides, but also to CHD and PPZ herbicides (Yu et al., 2007; Petit et al., 2010; Powles & Yu, 2010). Other mutant isoforms such as the 2041 and 2096 are only resistant to APP herbicides (Délye, 2005), whilst the 1999 isoform is fenoxaprop-specific and remains susceptible to other APP herbicides (Liu et al., 2007). The ACCase resistance in this *E. indica* population is endowed by a resistant ACCase, specifically due to a point mutation at position 2027 of the ACCase sequence from Trp to Cys (Trp-2027-Cys)(Fig. 3.3).

The Trp-2027-Cys mutation identified in the ACCase gene of this *E. indica* population was recently identified in several fluazifop-resistant *E. indica* populations from Malaysia (Cha et al., 2014). This mutation is known to confer resistance to ACCase-inhibiting aryloxyphenoxypropionate (APP) herbicides (e.g. diclofop, fluazifop, haloxyfop) and to phenylpyrazoline (PPZ) herbicides. However, this mutation has not been found to endow resistance to cyclohexanedione (CHD) herbicides (e.g. clethodim, sethoxydim) (Délye 2005; Powles and Yu 2010). This study outlines the first case associating the Trp-2027-Cys mutation with butroxydim (a CHD herbicide) resistance. It must be noted that no investigations were made into non target-site resistance (NTSR) mechanisms conferring ACCase resistance, which remains a possibility.

The selection and frequency of resistant mutants in the ACCase gene are often heavily influenced by the herbicide selection intensity within the region (Kaundun, 2014). With the intensive use of fluazifop-*p*-butyl in Malaysia, Trp-2027-Cys mutants were found to be prevalent in *E. indica* populations. In Brazil, a resistant *E. indica* population that contained the Asp-2078-Gly mutation was selected by treatment with sethoxydim for the past 20 years (Osuna et al., 2012). This population also displayed cross-resistance to cyhalofop, fenoxaprop and tepraloxydim, as a result of the 2078 mutation. Other examples of predominant region-specific ACCase mutant variants include the Ile-1781-Leu in the United Kingdom and France, and Gly-2096-Ala in Germany (Kaundun, 2014).

It is interesting to note that Cha et al. (2014) found a new Asn-2097-Asp mutation in one of the highly fluazifop-*p*-butyl-resistant *E. indica* populations (150-fold based on $GR_{50}$ R/S ratio), which lacks the Trp-2027-Cys mutation. It was suggested that this new mutation and some other form of non-target-site mechanism may have contributed to the strong resistance to fluazifop (Cha et al. 2014). However, further research needs to be conducted in order to examine and verify this claim.

Prior to this report, multiple resistance in *E. indica* populations has previously been reported. These multiple resistance cases involved at most two different herbicide groups, e.g., fluazifop-*p*-butyl and glyphosate (Heap, 2015) or glufosinate and paraquat (Chuah et al., 2010). However, this study is the first to identify multiple resistance to four herbicidal sites of action (glufosinate, glyphosate, paraquat and ACCase inhibitor) in a single *E. indica* population. This is undoubtedly related to the herbicide selection

history of this R population. As resistance to glyphosate, paraquat and ACCase-inhibiting herbicides was detected from a selected glufosinate-resistant sub population, it is plausible (although not examined) that multiple resistance is evident at the individual-plant level. Multiple resistance to glyphosate, paraquat and ACCase-inhibiting herbicides in individual plants has been documented in *L. rigidum* due to the accumulation of multiple resistance mechanisms (Yu et al. 2007). In addition, multiple resistance to other herbicide classes has also been demonstrated in both cross- and self-pollinated grass and broad-leaf weed species (e.g. *Alopecurus myosuroide, Lolium spp, Echinochloa spp, Amaranthus spp*) (Heap, 2015).

In summary this study confirms the first case of multiple resistance to the non-selective herbicides glufosinate, glyphosate and paraquat in an *E. indica* population. The same population had target-site resistance to ACCase-inhibiting herbicides due to the Trp-2027-Cys mutation, which was identified here for the first time as potentially conferring resistance to a member of the CHD class of ACCase inhibitors. The evolution of resistance to herbicides of four different sites-of-action in this resistance-prone species is of concern as this *E. indica* population is not controlled by the world's most important herbicide (glyphosate) or its non-selective alternatives (glufosinate, paraquat), greatly reducing herbicidal control options for growers. Although other ACCase- or ALS-inhibiting herbicides (e.g. sethoxydim, clethodim, imazapyr) still provide effective control, additional selection is considered likely to result in resistance. Therefore, this study acts as a clear warning of the need for weed control diversity, and the need for the development of effective non-herbicidal control techniques. Non-chemical weed control options may include grazing by farm animals, cutting the grass for seed-set control or harvesting the seed for later treatment (known as harvest weed seed control, HWSC). If additional weed control diversity is added to the farming systems to reduce *E. indica* population size then the usefulness of effective herbicides could be maintained.

## 3.6 Acknowledgement

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# Chapter 4

## Glufosinate-resistance mechanism

Chapter 4

# Glufosinate-resistance mechanism(s)

## 4.1 Abstract

This research was undertaken to investigate glufosinate resistance mechanisms in *Eleusine indica* (sub population R*). Results show the target-site glutamine synthetase (GS) of S and R* was equally sensitive to glufosinate inhibition, with $IC_{50}$ at 0.85 mM and 0.99 mM for S and R*, respectively. The GS activity was also similar in S and R* samples. Foliar uptake of radiolabelled [$^{14}$C]-glufosinate did not differ in S and R* plants. Translocation of [$^{14}$C]-glufosinate into untreated shoots and root was also similar in both populations, with 44% to 47% of the herbicide translocated out from the treated leaf at 24 h after treatment. HPLC analysis of [$^{14}$C]-glufosinate metabolism revealed no major metabolites in S or R* plants. Therefore, glufosinate resistance in this resistant population is not due to an insensitive target-site, target-site over production or altered glufosinate uptake and translocation, nor enhanced glufosinate metabolism. The exact resistance mechanism(s) remain to be determined.

## 4.2 Introduction

Glufosinate is a post-emergence, non-selective herbicide that is globally used for broad spectrum control of grass and broadleaf weed species. It inhibits the activity of glutamine synthetase (GS), an enzyme that converts glutamate plus ammonia to glutamine (Beriault et al., 1999). Glutamine synthetase is a key enzyme for plant nitrogen metabolism. Irreversible inhibition of GS by glufosinate (Manderscheid, 1993) results in rapid accumulation of ammonia which inhibits photosynthesis activity and ultimately causes necrosis of the leaf tissue and plant death (Pline et al., 1999; Coetzer & Al-Khatib, 2001). Glutamine synthetase exists in two isoforms in higher plants, i.e. the cytosolic GS1 and the plastidic GS2 (Mann et al., 1979; Hirel & Gadal, 1982). Plants that undergo C4 carbon fixation generally have higher GS1 than GS2, while C3 plants mainly have more GS2 than GS1 (McNally et al., 1983; Avila-Garcia et al., 2012) . Differences in proportions of the two GS isoforms were not related to plant susceptibility to glufosinate, as both were found to be equally sensitive *in vitro* (Ridley & McNally, 1985).

Despite the non-selective nature of glufosinate, there is a varying degree of glufosinate sensitivity among plant species. In a study by Mersey et al. (1990), it was found that the amount of glufosinate required to reduce plant growth by 50% ($GR_{50}$) for green foxtail (*Setaria viridis*) was 65 g $ha^{-1}$, while the $GR_{50}$ for barley (*Hordeum vulgare*) was 500 g $ha^{-1}$. An experiment involving seven weed species found a 70-fold variation between the weed species tested (Ridley & McNally, 1985). This differential glufosinate sensitivity in plants was mainly species-dependent, and attributed to various factors, such as differential glufosinate uptake, translocation and metabolism, and is affected by herbicide rates, plant growth stages and environmental conditions (temperature, humidity, etc.) (Mersey et al., 1990; Anderson et al., 1993; Steckel et al., 1997a; Steckel et al., 1997b; Pline et al., 1999; Neto et al., 2000; Tsai et al., 2006; Everman et al., 2009).

Transgenic glufosinate-tolerant crops have been developed to allow glufosinate to be used as a selective herbicide. Crop plants were transformed with either the *bar* or *pat* gene, which encodes the enzyme phosphinothricin acetyl transferase. This enzyme detoxifies glufosinate by acetylating it into a non-toxic compound, *n*-acetyl-glufosinate (Deblock et al., 1987; Wohlleben et al., 1988; D'Halluin et al., 1992; Dröge et al., 1992; Dröge-Laser et al., 1994). The introduction and commercialization of glufosinate-tolerant crops allowed farmers to control weeds by in-season spraying and crop-topping without damaging crops. Currently, glufosinate-tolerant crops include canola (*Brassica napus* L.), maize (*Zea mays* L.), soybean (*Glycine max* L. Merr.) and cotton (Green, 2014) and are predominantly grown in the United States of America.

Glufosinate has been extensively used in minimum tillage systems, chemical fallows, as a pre-harvest desiccant in cropping systems and for burndown prior to crop emergence (Mersey et al., 1990; Green, 2014). Despite thirty years of glufosinate use, there have been only a few reported cases of evolved glufosinate resistance in two weed species, *Eleusine indica* (L.) Gaertn. and *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot (Chuah et al., 2010; Jalaludin et al., 2010; Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia et al., 2012; Jalaludin et al., 2014). Target-site GS gene mutation was reported to be responsible for glufosinate resistance in *L. perenne* ssp. *multiflorum* populations (Avila-Garcia et al., 2012). This work focuses on mechanisms endowing glufosinate resistance in *E. indica*.

## 4.3 Materials and methods

### 4.3.1 Plant material

A susceptible (S) *E. indica* sub-population and a selected glufosinate-resistant (R*) population (Jalaludin et al. 2014, see Chapter 3) were used in this study. Seeds were germinated on 0.6% (w/v) agar in a growth chamber under 30°/25° C (day/ night), 12 hours photoperiod with light intensity of 400 µmol $m^{-2}$ $s^{-1}$ and 75% humidity. After 4 – 7 days, the seedlings were transplanted into pots containing potting mix and grown in the same conditions.

### 4.3.2 Glutamine synthetase assay

The glutamine synthetase inhibition assay was carried out following the methods of D'Halluin et al. (1992) and Tsai et al. (2006) with modifications. When plants reached the 3- to 5-leaf stage (or about 7 cm height), leaf material was harvested, snap-frozen in liquid nitrogen, and kept in -80° C until further use. Leaf samples (4 g) were homogenized at 4 °C with a mortar and pestle in 12 ml 50 mM Tris-HCl (pH 7.5) extraction buffer containing 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM phenymethylsulfonylfluoride (PMSF), 0.5% polyvinylpyrrolidone (PVP-40) and 10% (v/v) glycerol. The homogenate was then filtered through two layers of Miracloth (Calbiochem) and centrifuged at 21,000 × *g* for 30 minutes at 4 °C. The supernatant was then precipitated with ammonium sulphate at 60% saturation at 4 °C and centrifuged again at 21,000 × *g* for 30 minutes at 4 °C. The pellet was collected and dissolved in 1 ml extraction buffer. The enzyme extract were desalted using a Sephadex G25 PD-10 column (GE Healthcare Life Sciences©) and used for the GS inhibition assay. An aliquot of 115 µl of glufosinate stock was added to a 935 µl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 6 mM ATP, 10 mM $MgSO_4$, 20 mM hydroxylamine, 3.3 mM cysteine and 65.2 mM glutamate, to give final glufosinate concentrations of 0, 0.001, 0.01, 0.1, 1, 10 or 100 mM. Then, 100 µl of the enzyme extract was mixed into the reaction mixture to start the reaction. The mixture was incubated at 37 °C for 20 minutes, and the reaction was terminated by adding 350 µl 10% (w/v) ferric chloride solution in 0.2 N HCl. For background control samples, the reaction was terminated immediately after the addition of the enzyme extract without incubation. After centrifugation at 3000 × *g* for 5 minutes, absorbance of the mixture was measured at

595 nm after factoring in absorbance of the background control. A standard curve using L-glutamic acid-γ-mono-hydroxamate as a standard was used as a reference. Protein concentration was determined following Bradford's method (Bradford, 1976) using bovine serum albumin as a standard. This experiment was conducted three times with two replicate samples each time. The basal GS specific activity (μmol $mg^{-1}$ $min^{-1}$) was determined in the absence of herbicide.

### 4.3.3 [$^{14}$C]-Glufosinate uptake and translocation

Seeds of the S and R* populations were germinated and grown as described earlier, with the exception that the temperature was changed to 25°/20° C (day/ night) to slow down chlorosis development following glufosinate treatment. At the 3- to 5-leaf stage, one droplet of [$^{14}$C]-glufosinate (Bayer CropScience, Frankfurt) diluted in commercially formulated glufosinate (Basta®) such that the total glufosinate concentration was 5 mM was applied to the midpoint of the adaxial surface of the youngest fully expanded leaf. This single droplet (1 μl) of glufosinate solution was at a concentration equivalent to 125 g $ha^{-1}$ of the commercial glufosinate and contained 0.98 kBq $^{14}$C. Previous dose-response experiments (Chapter 3, Fig. 3.1) have established that this is the lowest rate discriminating S and R individuals.

Treated plants were harvested (including roots) at 16, 24, 48 and 72 h after treatment (HAT), and differential visual symptoms (chlorosis) were observed for S and R* plants at 48 and 72 HAT. Unabsorbed radioactivity was determined by rinsing the treated leaf portion with 10 ml of 0.1% (v/v) Triton X-100. A 2 ml aliquot was taken from the leaf rinsate and mixed with 3 ml of liquid scintillation cocktail (IRGA Safe Plus) and the radioactivity present in the rinsate was quantified by liquid scintillation spectrometry (LSS) (Packard 1500, Tri-Carb®, USA). The plants were oven dried at 60°C for 72 hours and then exposed (pressed against) overnight to a phosphor imager plate (BS 2500, FujiFilm, Japan) at room temperature for visualization of glufosinate translocation, using a phosphor imager (Personal Molecular Imager™, Bio-Rad Laboratories, Inc., California, USA). After imaging, each individual plant was divided into three parts: roots, untreated leaf and treated leaf and combusted in a biological sample oxdiser (RJ Harvey Instrument Corporation, Hillsdale, NJ). Radioactive $CO_2$ produced was trapped in an absorbent mix of Carbosorb E and Permafluor E (1:1, v/v)

and radioactivity in oxidized samples was quantified using LSS. Glufosinate leaf uptake was calculated as a percentage of the total radioactivity recovered (radioactivity recovered from combustion plus radioactivity in leaf wash solution; average recovery was 88%). Glufosinate translocation was expressed as percentage of radioactivity absorbed (radioactivity recovered from combustion only). The experiment had 4 to 6 replicates per treatment.

### 4.3.4 Glufosinate metabolism

Plants were grown as described in section 4.3.1. Three- to five-leaf stage S and R* seedlings were first foliar-sprayed with 20 and 80 g $ha^{-1}$ glufosinate, respectively, and kept overnight in the growth chamber. The plants were then treated with a solution containing [$^{14}$C]-L-glufosinate-HCl (specific activity 3.9 kBq in 10 µl) with 0.3% (v/v) BioPower adjuvant (Bayer CropScience, Frankfurt), prepared as described earlier but with a final glufosinate concentration of 0.86 mM (concentration equivalent to 20 g $ha^{-1}$). The treatment solution was applied in 10 small droplets along the adaxial surface of the youngest fully expanded leaf. The treatment solution was allowed to dry before the plants were returned to the growth chamber. Transgenic glufosinate-tolerant tobacco plants (supplied by Bayer CropScience, Frankfurt) were also included in this treatment as a positive control.

Treated leaves were harvested at 24, 48 and 72 HAT, rinsed as described previously, blotted dry and stored in -80 °C until extraction and analysis. Six leaves of each population were pooled together as a replicate sample, with two replicate samples per time point per population. The samples were inserted in a 96-well round bottom plate. A metal bead and 600 µl of extraction buffer (90% water: 10% methanol) were added into each well containing the sample. The plate was then sealed with a rubber cap and homogenised for 10 minutes using a TissueLyser (Qiagen). Following homogenisation, the homogenate was centrifuged at 5700 × *g* for 10 minutes. The supernatant was transferred into a new 96-well square bottom plate and evaporated to dryness. The 96-well round bottom plate was refilled with 600 µl of extraction buffer, and extraction was repeated twice as described. In the final extraction the extraction buffer was replaced with 80% acetone. The samples in the 96-well square bottom plate were then resuspended in 200 µl extraction buffer, shaken and sonicated for five

minutes each. They were then transferred into a 96-well filtration plate and centrifuged at 780 × *g* for 10 minutes. The recovered radioactivity in the filtrate was determined using LSS (recovery was >80% with an average of 85%). A non-treated control sample, spiked with $^{14}C$-labelled glufosinate prior to extraction, was included.

The metabolism study was evaluated using HPLC with a strong anionic exchange (SAX) column (Phenomenex PhenoSphere 5u SAX 80A column, 250 × 4.6 mm) based on the method of Jansen et al. (2000). The chromatography was performed at a flow rate of 1.0 ml/min with a mobile phase consisting of 90% 10 mM $KH_2PO_4$ : 10% acetonitrile (pH 2.8) for 45 min (isocratic), followed by a 15 min linear gradient to 90% 50 mM $KH_2PO_4$ : 10% acetonitrile (pH 2.8), and isocratic 90% 50 mM $KH_2PO_4$ : 10% acetonitrile (pH 2.8) for 10 minutes. The system was then brought back to its initial conditions in a 15 min linear gradient and held for another 65 mins before the next run. An in-line radioactivity detector was used for radioactivity peak determination. Each sample was normalised to give about 1050 Bq per injection into the HPLC.

Parent herbicide [1-$^{14}C$]-glufosinate-HCl, its non-radiolabelled reference standard glufosinate-ammonium (GA) and its metabolite reference standards (non-radiolabelled) were injected both individually and as a mixture. The reference standards were 2-acetamido-4-methylbutanoic acid (NAG), 4-methylphospinyl-2-oxobutanoic acid (PPO), 3-methylphosphinylpropionic acid (MPP), 2-methylphosphinylacetic acid (MPA) and 4-methylphosphinylbutanoic acid (MPB). All standards were provided by Bayer CropScience. Detection of non-radiolabelled reference standards was carried out using an inline UV-Vis spectrophotometer at 210 nm to establish retention times. Preliminary experiments revealed that shifts in retention times occurred following injections. Thus a mixture of standards was analysed before and after each set of sample runs. This experiment was repeated.

Plants treated with non-radiolabelled glufosinate were also analysed using LC-MS with a HILIC column (Nucleodur HILIC 3µm, 150 × 4.6 mm column). Three- to five-leaf stage S and R seedlings were treated with 20 and 80 g $ha^{-1}$ glufosinate, respectively and kept overnight in the growth chamber. At 24, 48 and 72 HAT, the youngest fully expanded leaf on each plant was harvested and processed as described

previously. Samples were then sent to the Bayer CropScience Analytical Department (Frankfurt) for analysis using Bayer CropScience proprietary methods.

### 4.3.5 Statistical analysis

Glufosinate concentration causing 50% inhibition of GS activity was estimated by non-linear regression analysis using Sigma Plot ® software (version 12.0, SPSS Inc., 233 South Wacker Drive, Chicago, IL). The data were fitted to the three parameter logistic curve model:

$$y = \frac{a}{1 + (\frac{x}{ED_{50}})^b}$$

Where a = upper limit, $ED_{50}$ = estimated dose causing 50% response and b = slope around $ED_{50}$. Significant differences between R* and S populations in the concentration of herbicide that caused a 50% reduction in GS activity ($IC_{50}$), herbicide uptake, and herbicide translocation were determined by *t-test* (GraphPad Prism, version 5.0, GraphPad Software Inc., 7825 Fay Avenue, La Jolla, CA).

## 4.4 Results

### 4.4.1 Glutamine synthetase activity

Glutamine synthetase (GS) from S and R* plants was equally sensitive to glufosinate inhibition. At 0.1 mM glufosinate, GS activity of both S and R* samples was inhibited by less than 10%. However, GS activity was reduced to less than 50% of control values at 1 mM glufosinate. At the highest glufosinate concentration used (100 mM), GS activity was completely inhibited (Fig. 4.1) for both S and R* samples. The glufosinate $IC_{50}$ for the S and R* GS was similar (0.85 mM and 0.99 mM, respectively) (Table 1). Similarly, no significant differences were found in the specific activity of the S (0.065 **µ**$mol^{-1}$ $mg^{-1}$ protein $min^{-1}$) and R* (0.068 **µ**$mol^{-1}$ $mg^{-1}$ protein $min^{-1}$) GS in the absence of glufosinate (Table 4.1). This result indicates that glufosinate resistance in this *E. indica* population is unlikely to be target-site based.

### 4.4.2 Glufosinate uptake and translocation

Foliar uptake of [$^{14}$C]-glufosinate was similar in both S and R* plants, with about 28% [$^{14}$C]-glufosinate absorbed in the first 16 HAT (Table 4.2), which remained stable before reaching the maximum absorption of 49-57% at 72 HAT. At all sampling time points, no significant differences in [$^{14}$C]-glufosinate uptake rate was observed between the S and R*plants.

Images of S and R* plants showed typical visual symptoms of glufosinate damage such as chlorosis, with the damage extending from the leaf tip to the treated area. However damage in R* plants was visibly less severe than S plants, especially at 48 and 72 HAT (Fig. 4.2). In the R* plants, the damage was restricted to the herbicide application site and extended towards the leaf tip, whereas in S plants the damage extended beyond the application site both in an acropetal and basipetal direction (Fig. 4.2). Despite this, phosphor images showed similar glufosinate translocation patterns in the S and R* samples throughout the sampling time points (16 to 72 HAT). Quantification of [$^{14}$C]-glufosinate translocation showed that the majority of the absorbed [$^{14}$C] activity (between 65% and 79%) was retained in the treated leaf, even at 72 HAT. A similar amount of [$^{14}$C] was translocated to the roots and untreated shoots of the S and R* plants at all sampling time points. Translocation of [$^{14}$C] away from the application site was reduced in the S samples after 48 HAT, likely due to self-limitation caused by glufosinate damage (Fig. 4.2). Overall, except for significant but small (less than 1.8-fold) differences in [$^{14}$C] translocation at 24 and 72 HAT, no major differences were found in [$^{14}$C]-glufosinate translocation outside the treated leaf of S and R* plants (Table 4.2). Therefore, glufosinate resistance in this population is unlikely to be due to differential glufosinate foliar uptake and translocation.

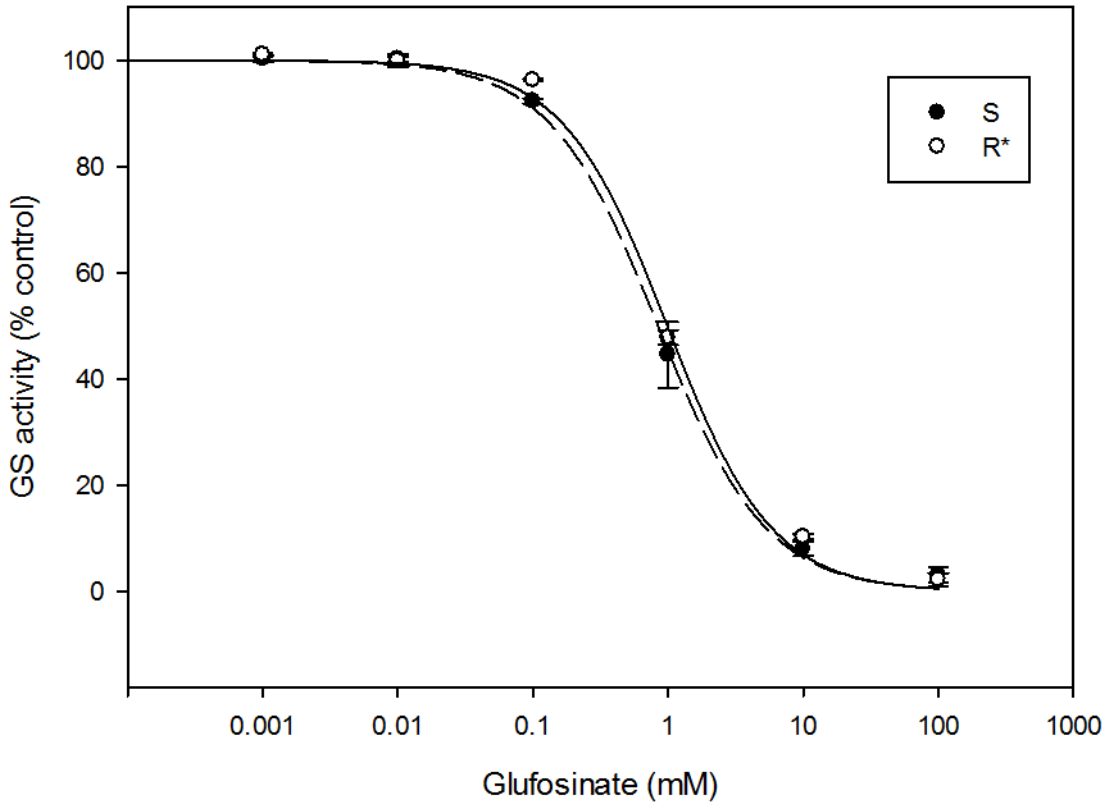


Figure 4.1. *In vitro* activity of glutamine synthetase (GS) from leaf extractions of *E. indica* S and R* populations in response to increasing glufosinate concentrations. GS specific activity without glufosinate inhibition was 0.065 and 0.068 $\mu mol^{-1}$ $mg^{-1}$ protein $min^{-1}$ for S and R*, respectively.

Table 4.1. Glutamine synthetase (GS) specific activity and parameters of the logistic analysis of glufosinate dose required to cause 50% inhibition of GS activity for the susceptible (S) and glufosinate-resistant (R*) populations. a = upper limit, $ED_{50}$ = estimated dose causing 50% response (in this case, 50% inhibition in the population, $IC_{50}$) and b = slope around $ED_{50}$.

| Population | GS specific activity ($\mu mol^{-1}$ $mg^{-1}$ protein $min^{-1}$) | a | b | $ED_{50} = IC_{50}$ (mM) | $R^2$ (coefficient) | $IC_{50}$ R/S ratio |
|---|---|---|---|---|---|---|
| S | 0.065 (0.09)[a] | 100 (0) | 1.09 (0.1) | 0.85 (0.06) [a] | 0.99 | n/a |
| R* | 0.068 (0.11)[a] | 100 (0) | 1.12 (0.07) | 0.99 (0.05) [a] | 0.99 | 1.16 |

Standard errors are in parentheses. Means with the same letter in a column are not significantly different ($\alpha = 0.05$) as determined by the *t-test*.

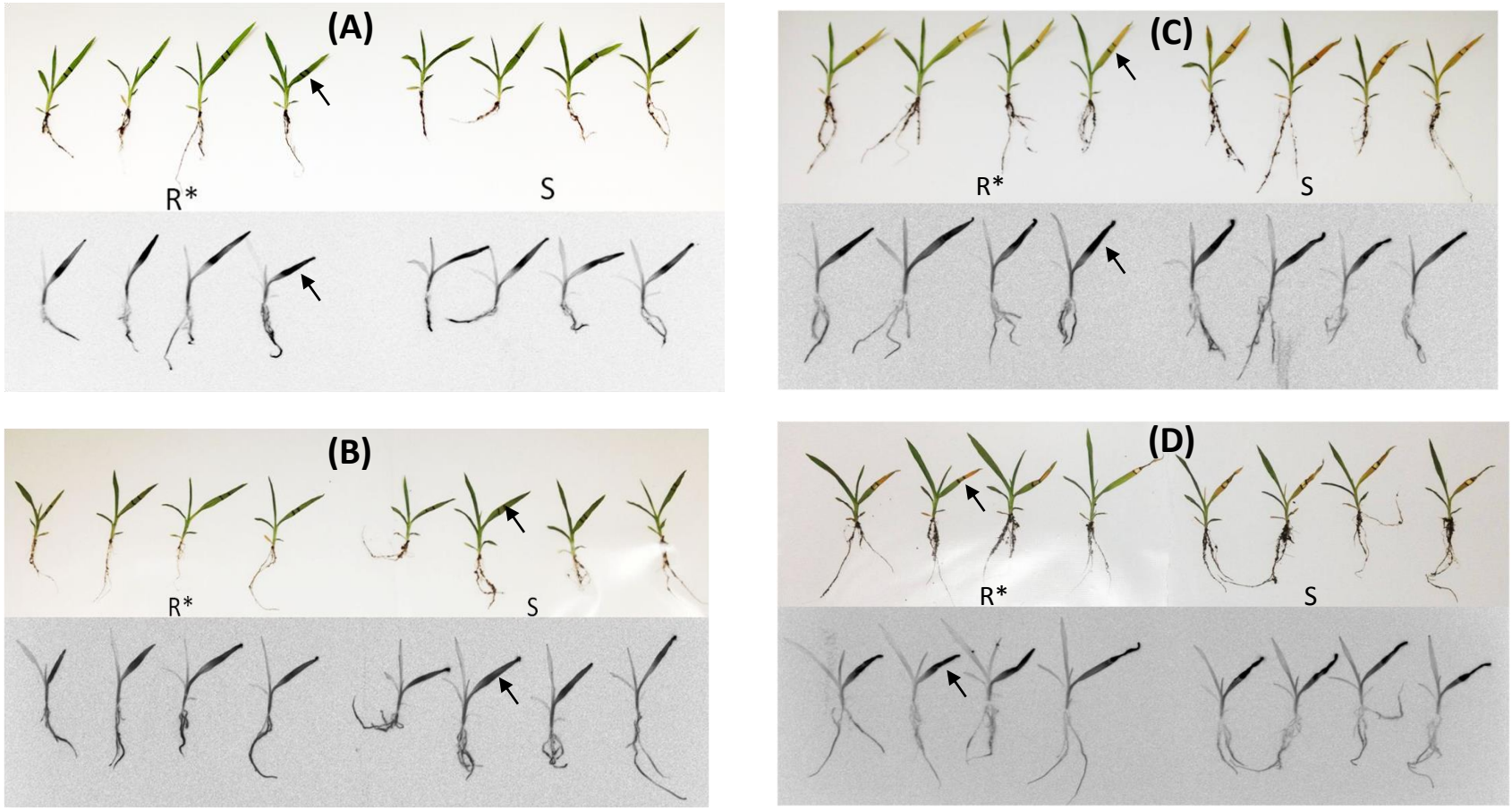


Figure 4.2. Normal and phosphor images of susceptible (S) and resistant (R*) *Eleusine indica* plants following [$^{14}$C]-glufosinate treatment at: (A) 16 HAT, (B) 24 HAT, (C) 48 HAT and (D) 72 HAT. The arrows indicate herbicide application site.

Table 4.2. Uptake and translocation of [$^{14}$C]-glufosinate (from treated leaf to root and untreated shoots) in susceptible (S) and resistant (R*) *Eleusine indica* plants at 16, 24, 48 and 72 h after treatment.

| Population | Foliar uptake (% of [$^{14}$C]-glufosinate recovered) | Translocation (% of absorbed [$^{14}$C]-glufosinate) | | |
|---|---|---|---|---|
| | | Root | Untreated shoots | Treated leaf |
| 16 h | | | | |
| S | 29.3 [a] | 26.8 (2.44)[a] | 17.4 (1.09)[a] | 55.8(4.7)[a] |
| R* | 27.6 [a] | 24.8 (3.72)[a] | 14.5 (1.93)[a] | 60.7 (4.7)[a] |
| 24 h | | | | |
| S | 25.9 (2.25)[a] | 26.8 (1.87)[a] | 20.5 (1.76)[a] | 52.7 (2.33)[a] |
| R* | 26.8 (0.98)[a] | 28.7 (3.24)[a] | 15.5 (1.18)[b] | 55. 9 (4.36)[a] |
| 48 h | | | | |
| S | 31.2 (2.20)[a] | 16.7 (1.47)[a] | 13.4 (1.35)[a] | 69.9 (1.94)[a] |
| R* | 29.1 (3.87)[a] | 18.6 (1.92)[a] | 15.3 (1.82)[a] | 65.9 (2.91)[a] |
| 72 h | | | | |
| S | 56.7 (4.05) [a] | 10.5 (1) [a] | 10.8 (0.69) [a] | 78.7 (1.35)[a] |
| R* | 49.9 (1.15) [a] | 18.8 (3.49) [b] | 15.3 (2.37) [a] | 65.9 (5.77)[b] |

Standard errors are in parentheses. Means with the same letter in a column for each paired S and R* sample at each time point are not significantly different ($\alpha = 0.05$) as determined by *t-test*.

### 4.4.3 Glufosinate metabolism

The retention time of glufosinate was resolved by HPLC (with UV-Vis detection) at 10.76 min for glufosinate-ammonium (GA), and the retention time for possible glufosinate metabolites was resolved at 16.64 min for MPB, 20.19 min for MPP, 22.39 min for MPA, 55.42 min for NAG and 72.88 min for PPO (Fig. 4.3A). The [$^{14}$C]-glufosinate HCl reference standard was resolved by HPLC (with radioactive detection) at 10.78 min (data not shown). Transgenic glufosinate-tolerant tobacco is known to metabolise glufosinate to non-toxic NAG which resolved by [$^{14}$C]-HPLC at 56.38 min (Fig. 4.3B), close to the non-radioactive NAG reference standard peak. Leaf extracts of [$^{14}$C]-glufosinate treated S and R* samples had only a single peak with the same retention time at about 10.65 min (Fig. 4.3C and D). This peak was believed to be un-metabolised glufosinate, as it corresponded to the retention time of [$^{14}$C]-glufosinate standard and the non-radiolabelled GA peak (peak a; Fig. 4.3A). None of the above glufosinate metabolites were detected from the S or R* leaf extracts at 24, 48 or 72 HAT.

Qualitative analysis done by technicians at Bayer CropScience (Frankfurt), using LC-MS on S and R* plants treated with non-radiolabelled glufosinate, also revealed a single peak corresponding to the retention time and mass of glufosinate from the S and R* leaf extracts (data not shown). No other metabolites were detected from the S and R* 24, 48 and 72 HAT leaf extracts. These results confirm that there is no major glufosinate metabolism in leaves of *E. indica*, and therefore, glufosinate metabolism is unlikely to play a role in resistance, at least in this resistant population.

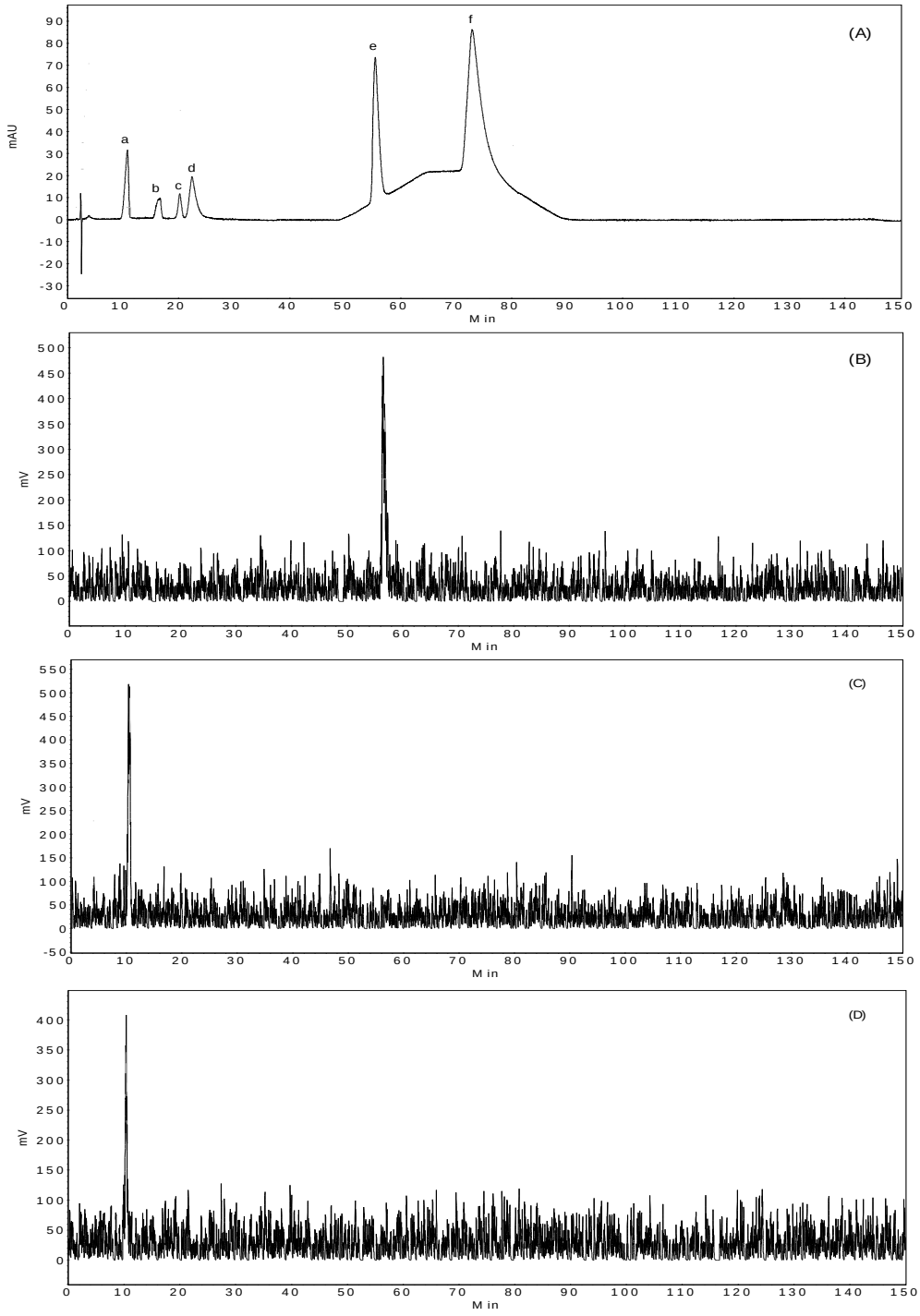


Figure 4.3. HPLC chromatogram of glufosinate (a) and its metabolite standards 4-methylphosphinylbutanoic acid (MPB) (b), 3-methylphosphinylpropionic acid (MPP) (c), 2-methylphosphinylacetic acid (MPA) (d), 2-acetamido-4-methylbutanoic acid (NAG) (e) and 4-methylphospinyl-2-oxobutanoic acid (PPO) (f) (A). The $^{14}$C-HPLC chromatogram of transgenic glufosinate-tolerant tobacco leaf extracts, showing resolution of the metabolite NAG (B). The $^{14}$C-HPLC chromatograms of S (C) and R* (D) *E. indica* leaf extracts at 24 HAT.

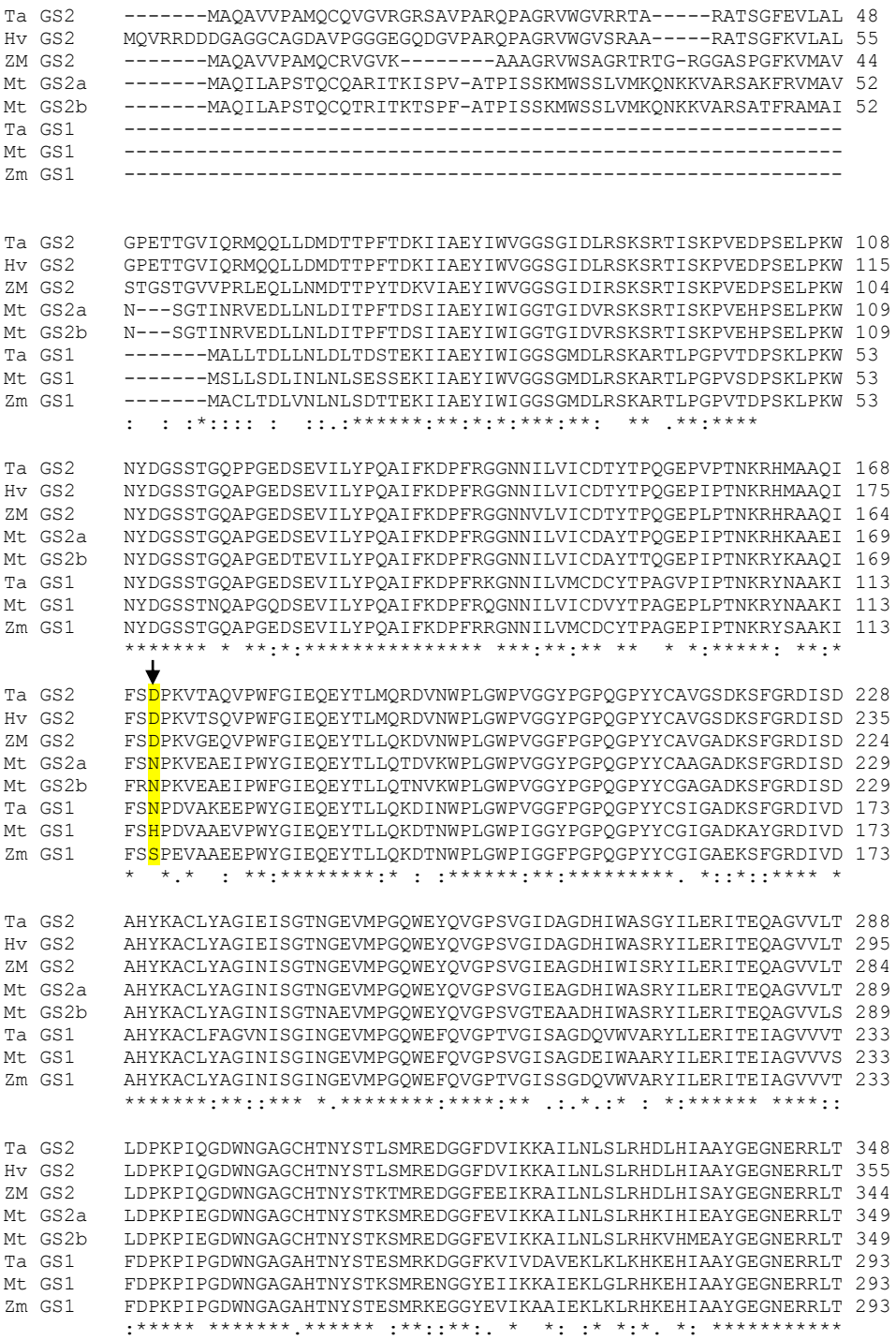


Figure 4.4. Multiple alignments of various plastidic GS2s and cytosolic GS1s (adapted from Torreira et al., 2014). Ta refers to *Triticum aestivum* (GS2, UniProt Q45NB4; GS1 UniProt Q45NB7), Hv, *Hordeum vulgare* (UniProt P13564), Zm, *Zea mays* (GS2, UniProt P25462; GS1, UniProt B9TSW5), Mt, *Medicago truncatula* (GS2a, UniProt Q84UC1; GS2b, UniProt E1ANG4; GS1, UniProt O04998). The glutamine synthetase amino acid sequence at 171 (based on wheat GS2 sequence) is highlighted in yellow with an arrow on top.

## 4.5 Discussion

Compared to field-evolved resistance to other major herbicides, resistance to glufosinate is still rare in crop weeds, involving only a few populations of two weed species (Heap, 2015). Here working with a glufosinate resistant (R*) Malaysian *E. indica* population (Jalaludin et al., 2014) we found that resistance in this population is unlikely to be due to a target-site based mechanism, for several reasons. Firstly, the total extractable enzyme activity (specific GS activity) was similar between the S and R* populations, indicating that resistance is not due to enhanced GS activity via GS gene amplification or overexpression. Secondly, glufosinate in-vitro inhibition assays showed that GS of S and R* populations is equally sensitive to glufosinate (Table 4.1). Thus, it is unlikely that the resistance is due to altered GS sensitivity caused by gene mutation(s). It is noted that the GS specific activity was lower than that obtained in other reports (0.1 to 40 $\mu mol^{-1}$ $mg^{-1}$ protein $min^{-1}$) (Hirel & Gadal, 1980; Pornprom et al., 2008; Pornprom et al., 2009). This could be due to method, species and sample differences between experiments. Interestingly, the $IC_{50}$ observed in this resistant population (0.99 mM) is comparable to that observed in resistant *Lolium* spp. ($IC_{50}$ 2.432 mM glufosinate) (Avila-Garcia et al., 2012).

Previous studies with lab selected glufosinate-resistant plant cell lines have shown that resistance can be due to gene amplification (Donn et al., 1984) or insensitive GS caused by point mutations resulting in amino acid substitutions (Chompoo & Pornprom, 2008; Pornprom et al., 2008; Pornprom et al., 2009). For instance, comparison between sensitive and resistant soybean cells showed eight amino acid substitutions in the GS sequence of a resistant cell line. Out of these eight amino acid substitutions, only one, the His-249-Tyr mutation, occurs at the GS substrate/inhibitor binding site (Pornprom et al., 2009). The role of other 7 amino acid substitutions on glufosinate resistance remains unclear.

In a field-evolved glufosinate-resistant *L. perenne* population, an amino acid substitution in the GS amino acid sequence of aspartic acid (D) to asparagine (N) at position 171 (based on the wheat GS2 amino acid sequence, UniProt Q45NB4) was identified and suggested to be the cause of reduced GS sensitivity to glufosinate ($IC_{50}$ R/S ratio of 79) (Avila-Garcia et al., 2012). Alignment of several GS sequences from monocot and dicot plants showed that amino acid 171 is not conserved (Fig. 4.4, also see Torreira et al., 2014). Notably, the glufosinate-sensitive GS2a and GS2b isozymes

of *Medicago truncatula* have N at 171 (Torreira et al., 2014) (Fig. 4.4), identical to the substituted amino acid reported in the glufosinate-resistant *L. perenne* population (Avila-Garcia et al., 2012). Conversely in maize, the GS1 and GS2 isoenzymes have similar sensitivity to glufosinate (Acaster & Weitzman, 1985) even though they are different in amino acid sequence at 171 (Fig. 4.4). Hence the amino acid residue at 171 may not be a determinant for glufosinate sensitivity. Instead, a few amino acids (Glu-131, Glu-192, Gly-245, His-249, Arg-291, Arg-311 and Arg-332) have been identified in GS that are possibly involved in glutamate/glufosinate binding (Unno et al., 2006) and therefore mutation(s) of these amino acids may result in herbicide resistance. Therefore, the involvement of Asp-171-Asn in glufosinate resistance remains to be confirmed by examining, for instance, co-segregation of the mutation and the resistance.

Differential glufosinate uptake and translocation usually contributes to species-dependent variations in glufosinate sensitivity (Mersey et al., 1990; Steckel et al., 1997a; Pline et al., 1999; Neto et al., 2000; Everman et al., 2009). In the current study, [$^{14}$C]-glufosinate foliar uptake was found to be similar between the S and R* with about 50% of total radioactivity recovered inside the plants at 72 HAT. The level of glufosinate absorption was similar to that observed in *E. indica* by Everman et al. (2009).

Glufosinate has the physicochemical characteristics for phloem mobility, although glufosinate translocation from the site of application is somewhat limited (Mersey et al., 1990; Steckel et al., 1997a; Neto et al., 2000; Everman et al., 2009), due to the rapid phytotoxicity of glufosinate at the source leaf tissue (Beriault et al., 1999). In our experiment, nearly half of the absorbed $^{14}$C-glufosinate had already been translocated out from the treated leaf in both S and R* plants at 24 HAT. Glufosinate phloem mobility was evident, with more than 20% of absorbed [$^{14}$C]-activity detected in roots at 16 and 24 HAT. At 48 and 72 HAT, however, translocation from the treated leaf became restricted in both S and R* plants, likely due to necrosis-induced self-limitation causing a higher amount of [$^{14}$C]-activity to be retained in the treated leaf. Due to this restricted translocation, phloem movement (downwards) of glufosinate was affected more than the upwards (acropetal) translocation of glufosinate, as evidenced by the decreased [$^{14}$C]-activity detected in the root. It was noticed that at 72 HAT, the S plants were more damaged than the R plants (Fig 4.3 D); hence significantly less

basipetal glufosinate translocation occurred in the S than R plants at this time point (Table 4.2).

Glufosinate metabolism in plants is low to non-existent, depending on the species (Jansen et al., 2000; Neto et al., 2000). In plants that do metabolise some glufosinate, the final stable metabolic products were identified as MPP, MHB or both (Dröge et al., 1992; Dröge-Laser et al., 1994; Jansen et al., 2000; Ruhland et al., 2004). Transgenic glufosinate-tolerant crops detoxify glufosinate by acetylating glufosinate into non-toxic *n*-acetyl-glufosinate (NAG) (Dröge et al., 1992). In treated leaves of both S and R* *E. indica*, glufosinate metabolism was not detected, and hence unlikely to be responsible for resistance in this R* population. In contrast, we were able to show that transgenic glufosinate-tolerant tobacco completely metabolises glufosinate into NAG. Our result contradicts the finding of Everman et al. (2009), where two glufosinate metabolites were detected in *E. indica*. It should be noted that Everman et al. (2009) used thin layer chromatography (TLC), and the nature of the two metabolites were not identified. In our preliminary TLC study using a solvent system similar to Everman et al. (2009), we obtained two bands, one co-migrating with [$^{14}$C]-glufosinate, and the other of an unknown nature (data not shown). However, HPLC analysis of the same sample only displayed a single radioactive peak corresponding to glufosinate. Efforts to scrape the unknown TLC band for further analysis were unsuccessful (data not shown). LC-MS analysis further confirmed that glufosinate metabolism in *E. indica* is negligible. Nevertheless, as we only examined glufosinate metabolism in herbicide treated leaves, the possibility of glufosinate metabolism in stem or roots cannot be excluded.

Despite much effort, the glufosinate resistance mechanism(s) in this R* population remains to be determined. What we know is that resistance is unlikely to be target-site based or metabolism based. Although the S and R* plants do not differ in their glufosinate uptake and translocation pattern at the whole plant level, we do not know whether glufosinate absorption and distribution differ in S and R* at the cellular level. For instance, could glufosinate uptake via the plasma membrane be reduced in R as compared to S plants? Could glufosinate be sequestered into vacuoles or confined in the cell wall or apoplast in the R* plants? Some of these strategies of reducing the herbicide concentration reaching the target-site have been demonstrated in glyphosate

resistant weed species (Ge et al., 2010; Ge et al., 2012; reviewed by Sammons & Gaines, 2014) and need further research for glufosinate resistance.

## 4.6 Acknowledgement

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# Chapter 5

# Evolution of a double amino acid substitution in the EPSP synthase in *Eleusine indica* conferring high level glyphosate resistance

**Preface to Chapter Five**

This chapter is highly similar to the following publication: Yu, Q., Jalaludin, A., Han, H., Chen, M., Sammons, R. D., & Powles, S. B. (2015). Evolution of a double amino acid substitution in the EPSP synthase in *Eleusine indica* conferring high level glyphosate resistance. *Plant Physiology*, *167*(4), 1440-1447. However, several parts included in this chapter are not available in the published manuscript as it was conducted after the manuscript was submitted for publication.

Chapter 5

# Evolution of a double amino acid substitution in the EPSP synthase in *Eleusine indica* conferring high level glyphosate resistance

## 5.1 Abstract

Glyphosate is the most important and widely used herbicide in world agriculture. Intensive glyphosate selection has resulted in the widespread evolution of glyphosate-resistant weed populations, threatening the sustainability of this valuable once-in-a-century agrochemical. Field-evolved glyphosate resistance level is generally low to modest. Here, working with a highly glyphosate-resistant *Eleusine indica* population, we identified a double amino acid substitution (T102I+P106S, known as TIPS) in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene in glyphosate resistant individuals. This TIPS mutation is the same as that in the biotechnology-engineered first generation commercial glyphosate-tolerant EPSPS in maize and, subsequently, in other crops. In *E. indica* plants sprayed with glyphosate, the naturally evolved TIPS mutants are highly (>180-fold) resistant to glyphosate compared to the wild type (WT), and more resistant (>32-fold) than the previously known P106S single mutants. The partially-purified *E. indica* TIPS EPSPS enzyme showed very high level (2600-fold) *in vitro* resistance to glyphosate relative to the WT, and was (600-fold) more resistant than the P106S variant. While exhibiting high level glyphosate resistance, plants homozygous for the TIPS mutation displayed a significant fitness cost (both vegetative growth and seed production). The evolution of the TIPS mutation in crop fields under glyphosate selection is likely a sequential event with the P106S mutation being selected first and fixed, followed by the T102I mutation to create the highly resistant TIPS EPSPS. The sequential evolution of the TIPS mutation endowing high level glyphosate resistance is an important real-time mechanism by which plants adapt to intense herbicide selection and is a dramatic example of evolution in action.

## 5.2 Introduction

Modern herbicides are vital to global food production by efficiently removing weeds whilst maintaining sustainable soil conservation practices. However, relentless

herbicide selection of huge weed numbers across vast areas has resulted in the widespread evolution of herbicide resistant weed populations (Powles & Yu, 2010). Worldwide, there are currently more than 448 unique cases of herbicide resistance, with on average about 11 new cases reported annually (Heap, 2015). Target-site resistance due to target gene mutation is one of the major mechanisms enabling resistance evolution (Gressel, 2002; Powles & Yu, 2010).

The most important and widely used herbicide in crop fields is glyphosate (Duke & Powles, 2008). Glyphosate disrupts the shikimate pathway (involved in aromatic amino acid biosynthesis) by specifically inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Steinrücken & Amrhein, 1980). Glyphosate resistance was initially considered to be unlikely to evolve in nature based on the facts that intentional selection for glyphosate tolerance using whole plants and cell/tissue culture was unsuccessful and laboratory-generated highly resistant EPSPS mutants displayed undesirable enzyme kinetics (Bradshaw et al., 1997; reviewed by Pline-Srnic, 2006; Shaner et al., 2011). This seemed to be true, as resistance was not found during the first 15 years of glyphosate use (primarily as a non-selective herbicide). However, unprecedented intensive glyphosate use for controlling large numbers of weeds over massive areas, especially after the introduction of glyphosate-resistant transgenic crops in 1996 imposed high selection pressure on weeds, resulting in the evolution of glyphosate resistance in populations of 32 weed species (Heap, 2015). Since the first identification of a resistance-endowing EPSPS point mutation, P106S, in a glyphosate-resistant *Eleusine indica* population (Baerson et al., 2002) several other resistance-endowing single amino acid substitutions at P106 (P106T, P106A, P106L) have been reported in glyphosate-resistant weeds (Yu et al., 2007; Kaundun et al., 2011; reviewed by Sammons & Gaines, 2014). These single-codon EPSPS resistance mutations only endow low level glyphosate resistance (survival at 2- to 3-fold of the recommended rate of glyphosate application). This is not surprising, because glyphosate is a competitor of the second substrate, phosphoenolpyruvate (PEP) (Boocock & Coggins, 1983) for binding to EPSPS and is considered a transition state mimic of the catalysed reaction course (Schönbrunn et al., 2001). Indeed, highly glyphosate-resistant EPSPS variants (e.g. mutants at G101 or T102) have a greatly increased $K_m$ (decreased affinity) for PEP when expressed in *E. coli* (Eschenburg et al., 2002; Funke et al., 2009; reviewed by Sammons & Gaines, 2014). In contrast, P106 substitutions confer weak glyphosate resistance but preserve adequate EPSPS functionality (Healy-Fried et al., 2007;

reviewed by Sammons & Gaines, 2014). Aside from P106 EPSPS gene mutations there are other glyphosate resistance mechanisms, including EPSPS gene amplification, and non-target-site reduced glyphosate translocation/increased vacuole sequestration (Lorraine-Colwill et al., 2003; reviewed by Powles & Preston, 2006; Shaner, 2009; Gaines et al., 2010; Ge et al., 2010; Ge et al., 2011). Generally, each of these mechanisms endows moderate level (survival at 4- to 8-fold the recommended rate) glyphosate resistance.

To date, there have been no published studies on the fitness cost of EPSPS gene mutations in evolved herbicide resistant weeds (Vila-Aiub et al., 2009). In a recent study conducted with EPSPS gene amplification based glyphosate resistant *Amaranthus palmeri* , the resistant biotype was found to have similar vegetative and reproductive growth with the susceptible biotype (Vila-Aiub et al., 2014). Similar results was found in another independent study involving the same species with the same resistance mechanism in the United States (Giacomini et al., 2014). In *Lolium rigidum* populations with reduced glyphosate translocation, the resistant biotype was shown to produce significantly fewer but larger seeds while having similar vegetative growth with the susceptible biotype under minimal competition intensity (Pedersen et al., 2007; Preston et al., 2009). Glyphosate resistant populations showed reduced (by 34%) frequency of resistant individuals in the field three years after relaxed glyphosate selection (Preston et al., 2009).

Low level glyphosate resistance due to the EPSPS P106 mutations was reported in populations of Malaysian *E. indica* (Baerson et al., 2002; Ng et al., 2004). Recently, we reported a highly (survival at >10 fold the recommended rates) glyphosate-resistant Malaysian *E. indica* population (Jalaludin et al., 2014). This paper investigates the mechanism of this high level glyphosate resistance in this population and reveals for the first time the sequential evolution of a double amino acid substitution in the EPSPS enzyme and the resistance cost associated with homozygous TIPS mutants.

## 5.3 Materials and methods

### 5.3.1 Plant material

The glyphosate-resistant and susceptible *E. indica* populations used in the current study are from Jalaludin et al. (2014). Seeds were germinated on 0.6% agar for

4-7 days and germinating seedlings transplanted into plastic pots (12-20 per pot) filled with potting mix (50% peat moss and 50% river sand) and grown in glasshouse during the summer growing season (Dec to March) with average day/night temperatures of 30/24C and 13-h photoperiod under natural sunlight. When seedlings reached the 3- to 4-leaf stage they were treated with various rates of commercial glyphosate using a cabinet sprayer with a spray volume of 112 L $ha^{-1}$ at a pressure of 200 kPa and a speed of 1 m $s^{-1}$. Visual assessment for mortality was made 3-4 weeks after treatment. Plants were recorded as alive if they were actively growing and tillering after herbicide treatment and as dead if there was little new growth and no new tiller formation.

### 5.3.2 EPSPS sequencing and cDNA cloning

Genomic DNA was extracted from the leaf tissue of resistant and susceptible plants and total RNA isolated using the Plant RNeasy Mini Kit (Qiagen). Genomic DNA contamination was removed using the TURBO-DNA free kit (Ambion). For EPSPS DNA partial sequencing a pair of published primers (Ng et al., 2003) was used to amplify a highly conserved region ($^{95}$LFLGNAGTAMRPL$^{107}$, refer to plant EPSPS numbering system) in which point mutations conferring glyphosate resistance in plants and bacteria have been found (Sammons & Gaines, 2014). The forward primer EleuEPSPS-F (5'-GCGGTAGTTGTTGGCTGTGGTG-3') and the reverse primer EleuEPSPS-R (5'-TCAATCCGACAACCAAGTCGC-3') (Ng et al., 2003) amplify a 301 bp DNA (includes 99 bp intron) fragment covering the potential mutation sites. Using the same primer pairs a 202 bp (without intron) cDNA fragment was amplified from pre-genotyped plants and cloned into the pGEM-T vector (Promega) and transformed into *E. coli*, using blue/white selection to identify successful cloning events. White colonies with putative inserts were used as templates for PCR re-amplification and sequencing of the 202 bp fragment. The PCR was conducted in a 25 µl volume that consisted of 1-2 µl of genomic DNA or cDNA, 0.5 µM of each primer, and 12.5 µl of 2× GoTaq Green Master Mix® (Promega). The PCR was run with the following profile: 94ºC for 4 min; 40 cycles of 94ºC for 30s, 57ºC (annealing temperature) for 30s, and 72ºC for 30-50s; followed by a final extension step of 7 min at 72°C. For EPSPS full cDNA sequencing, a 1338 bp cDNA was amplified with Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc.) using the primer pair EiRT1 and EiLFT1 as described in Baerson et al. (2002). The PCR was conducted in a 20 µl volume that consisted of 1µl (80 ng) of cDNA, 0.5 µM of each

primer, and 4 µl of 5× Phusion HF buffer, 200 µM dNTPs and 0.2 µl Phusion DNA Polymerase. The PCR was run with the following profile: 98$^{o}$C for 30 sec; 40 cycles of 98$^{o}$C for 10 sec, 72$^{o}$C for 50 sec; followed by a final extension step of 10 min at 72°C. The PCR product was purified from agarose gel with the Wizard® SV Gel and PCR Clean-up System (Promega Co., Madison, WI USA) and sequenced by commercial services (LotteryWest, State Biomedical Facility Genomics, Western Australia) using the primers EiRT1 and EiLFT1 and an internal forward primer (5'-CTCTTCTTGGGGAATGCTGGA-3', (Kaundun et al., 2008). All sequence chromatograms were visually checked for quality and consistency before sequences were assembled and aligned.

### 5.3.3 Derived cleaved amplified polymorphic sequence (dCAPS) marker development and genotyping

Based on the EPSPS sequence information obtained from the susceptible (20 plants) and resistant samples (at least 80), we developed dCAPS markers for detecting mutation(s) at amino acid position 102 and 106 using the web-based dCAPS finder 2.0 software (http://helix.wustl.edu/dcaps/dcaps.html). An A to T mismatch was introduced in the forward primer RsaIF (5'-TGCAGCTCTTCTTGGGGAATGCTGGTA-3') two nucleotide upstream of the 102 codon (i.e. N+2 position) to create a restriction site for R*sa*I (GT∧AC) in the WT sequence. Any nucleotide mutations resulting in substitution of the T102 would abolish the restriction site. Therefore, the primer pair RsaIF and EleuEPSPS-R was used to amplify a 234 bp fragment which was then digested with *Rsa*I. The WT sequence would generate a single digested 208 bp band, while the mutant sequence at 102 would generate an undigested 234 bp band (Fig 5.2A). A heterozygous sequence at 102 would produce both the 208 and 234 bp bands (Fig 5.2A). Similarly, a G to C mismatch was introduced in the forward primer Sau96IF (5'-CTCTTCTTGGGGAATGCTGGAACTGCAATGGGA-3') at the N+3 position of the 106 codon to create a restriction site for S*au96*I (G∧GNCC) in the WT sequence. Any mutations resulting in substitution of the P106 would abolish the restriction site. Therefore, the primer pair Sau96IF and EleuEPSPS-R was used to amplify a 233 bp fragment which was then digested with *Sau96I*. The WT sequence would produce a single digested 202 bp band, while the mutant sequence at 106 would produce an undigested 233 bp band (Fig 5.2B). A heterozygous sequence at 106 would produce both the 202 and 233 bp bands (Fig 5.2B) (note: no heterozygous sequences at 106 were detected). PCR conditions were similar to above except that the annealing temperature

was 62ºC. Restriction digestions were carried out according to the manufacturer's recommendations (New England BioLabs) and digestion patterns were viewed on 2% agarose gels (electrophoresis at 90-100 V for 50-80 min) stained with ethidium bromide. The accuracy of the two markers was confirmed by comparing sequencing and marker analysis results of over 40 samples.

It should be noted that the two dCAPS markers were designed to only detect mutant and WT sequences at the 102 and 106 codons without knowing the nature of the specific mutation. As we confirmed that the resistant population only possessed the T102I and P106S mutations (see Results), the two dCAPS markers could be used for genotyping in the population. If used in other uncharacterized *E. indica* populations, the specific mutations would have to be determined by sequencing.

### 5.3.4 Generation of purified sub-populations

Plants (7-12) that were confirmed by sequencing and marker analysis to be homozygous for the WT, P106S, or TIPS mutation were self-pollinated in isolation to bulk up seed stocks for the respective sub-populations. Progeny plants (10-12) from each of these purified sub-populations were randomly marker-analysed to confirm their genotype and homozygosity prior to use for subsequent experiments.

### 5.3.5 *E. coli* transformation

Total RNA was isolated from *E. indica* (abbreviated as Ei) P106S EPSPS mutant. cDNA was synthesized and mature P106S EiEPSPS cassette was PCR amplified using the primers pair 1 and 2 (Table 5.1, (Baerson et al., 2002). The PCR product was inserted into the pCR Blunt II TOPO vector and verified by sequencing. The P106S EiEPSPS was converted to WT (primer pair 3 and 4, Table 5.1) and the double mutant TIPS EiEPSPS (primer pair 5 and 6, Table S1) using the Phusion site-directed mutagenesis kit according to the manufacturer's instructions (Thermo Scientific). After sequencing verification, these 3 genes were PCR amplified using the primer pair 7 and 8 and the PCR products were digested by NdeI and then inserted into the NdeI site of the pET19-b vector to form an N-terminal His-Tag fusion to facilitate the downstream purification of the enzymes.

### 5.3.6 EPSPS purification and activity assay

The BL21 (DE3) cells (Invitrogen) harboring the EiEPSPS constructs in pET-19b vector were cultured in the MagicMedia *E. coli* Expression Medium (Invitrogen) according to the dual temperature protocol provided by the supplier. Soluble proteins were extracted from frozen cells using the B-per bacterium extraction reagent supplied with DNase I, lysozyme and Halt Protease inhibitor cocktail (Thermo Scientific). After centrifugation at 21,000 *g* for 5 min the supernatant fraction was used to purify His-Tagged EPSPS with HisPur Ni-NTA resin (Thermo Scientific). The binding buffer was made of 20 mM Tris (pH 8.0), 500 mM NaCl, 10 mM imidazole and 0.03% (v/v) Triton X-100; washing buffer contained 20 mM Tris (pH 8.0), 500 mM NaCl, 50 mM imidazole and 0.03% (v/v) Triton X-100; and elution buffer contained 20 mM Tris (pH 8.0), 500 mM NaCl and 500 mM imidazole. The eluted enzyme was kept in storage buffer (10 mM MOPS, 10% (v/v) glycerin, 0.5mM EDTA, 2.5 mM β-mercaptoethanol) after buffer exchange using Amicon Ultra 0.5 mL centrifugal filters (MWCO 30 kDa) 3 times 5 min at 14,000 *g*. The protein content was quantified using the Pierce 660 nm Protein Assay kit (Thermo Scientific) and purification results analyzed by SDS-PAGE electrophoresis.

Activity of purified EPSPS variants was measured by a coupled assay that measures continuous release of inorganic phosphate using the EnzChek Phosphate Assay Kit (E-6646, Invitrogen) (Gaines et al., 2010). For $IC_{50}$ measurement of EiEPSPS variants various concentrations of glyphosate were used (WT: 0, 0.1, 5, 10, 20, 50, 150, 500 μM; P106S: 0, 1, 10, 25, 50, 80, 150, 500, 1250 μM; TIPS: 0, 12.5, 1250, 15000, 25000, 35000, 50000, 70000, 100000 μM) holding shikimate 3-phospate (S3P) constant at 0.1 mM. To measure the $K_m$ (PEP) of the EiEPSPS variants, S3P concentration was fixed at 0.1 mM and various amounts of PEP (2.5, 5, 10, 15, 20, 25, 40, 60, 80 μM) were used.

**Disclaimer: Sections 5.3.5, *E. coli* transformation, and 5.3.6, EPSPS purification and activity assay were carried out in collaboration with Dr. Douglas Sammons's lab of Monsanto Company, St Louis, USA.**

### 5.3.7 Shikimic acid analysis

Analysis of shikimic acid in tissue extracts was adapted from Pedersen et al. (2011) using a Waters system consisting of a 600E dual head pump, 717 plus autosampler and a 996 photodiode array detector. In detail, separation was achieved with a Grace Davison (Deerfield, IL, USA) Prevail™ organic acid C18 column (250*4.6 mm I.D.) with 5 µm packing using a mobile phase of 100 mM $KH_2PO_4$ at pH 2.12 and a flow rate of 1 ml $min^{-1}$. Column temperature was 35 °C and typical sample injection volume was 5 µl. Gradient elution employing a mobile phase with 60% methanol every $5^{th}$ samples was used to reduce carry-over of more non-polar analytes and thus interference. Data acquisition with the photodiode array was from 195 to 400 nm to enable positive identification of shikimic acid by comparison of the retention time and PDA peak spectral information, including peak purity, of standards with those of the samples. Calibration curves for shikimic acid were generated from peak area at 210 nm vs the mass of acid injected. Data acquisition and processing were with Empower® chromatography software (Waters).

### 5.3.8 Aromatic amino acid analysis

Fresh material (300 mg) from the three different *Eleusine indica* genotypes (WT, P106S and TIPS) was harvested and snap-frozen in liquid nitrogen. They were then grounded with mortar and pestle and extracted (3 ml) in 80% methanol containing 50 µM γ-aminobutyric acid as an internal standard for extraction recovery. Extracts were transferred into 10 ml centrifuge tube and incubated on ice for 20 minutes. Following incubation, the extracts were centrifuged at 10 000 × *g* at 4 °C for 15 minutes. The supernatants were then transferred into fresh tubes and centrifuged again at the same speed, temperature and time, previously. Aliquots of 0.5 ml were taken and the samples were spin-dried under vacuum and stored at -80 °C until further use. Before analysis with HPLC, samples were redissolved by repeated vortexing with milli-q water (150 µl) containing 5.66 µM α-aminobutyric acid (HPLC internal standard) (Noctor et al., 2007; Vivancos et al., 2011).

Analysis of free aromatic amino acids Phenylalanine (Phe), Tyrosine (Tyr) and Tryptophan (Trp) in samples was undertaken using the Waters (Milford, MA, USA) AccQ.Tag® chemical package for High Performance Liquid Chromatography (HPLC) with a Waters system consisting of a 600E dual head pump, 717 plus autosampler, 996

photodiode array detector and a 470 scanning fluorescence detector. In detail, sample aliquots of 10 µL were derivitised by mixing with 70 µL of the borate buffer reagent and then 20 µL AccQ.Tag® reagent was added and solution immediately vortex mixed. Samples were then incubated at 55 °C for 10 minutes. Typical injection volume of the derivitised samples was 15 µL. Standards were prepared from the stock solutions provided with the kit and derivitised as described for the samples. Separation of amino acids was performed on a Nova-Pak C18 column (150 mm x 3.9 mm I.D.) with 4 µm particle size (Waters) held at 37°C with a flow rate of 1 ml $min^{-1}$. The mobile phase used for gradient elution comprised sodium acetate buffer pH 5.8 (Eluent A), acetonitrile (Eluent B) and milli-Q water (Eluent C), with all solvents vacuum filtered to 0.22 µm prior to use and were continually degassed with helium sparging. The gradient profile was as follows:

| Time (min) | Flow rate (mL/min) | % A | % B | % C | Curve |
|---|---|---|---|---|---|
| Initial | 1.0 | 100 | 0 | 0 | * |
| 0.5 | 1.0 | 99 | 1 | 0 | 11 |
| 18.0 | 1.0 | 95 | 5 | 0 | 6 |
| 19.0 | 1.0 | 91 | 9 | 0 | 6 |
| 29.5 | 1.0 | 83 | 17 | 0 | 6 |
| 36.0 | 1.0 | 0 | 60 | 40 | 11 |
| 39.0 | 1.0 | 100 | 0 | 0 | 11 |

All data were acquired and processed with Empower® chromatography software (Waters) with diode array data acquisition from 195 to 600 nm, using 250 nm for quantification and fluorescence detector settings of: Excitation 250 nm; Emission 395 nm, 0.5 filter and 100 gain. Quantification of amino acids was based on the internal standard method using α-aminobutyric acid from the response of the 470 fluorescence detector except for GABA and Trp (poor sensitivity) which were quantified based on photodiode array detector response. Comparison of calculated data for amino acid concentration using either detection system showed no difference in values.

### 5.3.9 Resistance cost study using plants homozygous for TIPS, P106S and WT in a non-competitive environment

Seeds of plants homozygous for TIPS, P106S and WT *E. indica* were germinated and grown as described earlier. The progeny plants of these purified sub-populations originated from a single population, thus having a similar genetic background. For vegetative growth assessment, individual seedlings of each genotype (WT, P106S and TIPS) were transplanted into single plastic pots (15 cm in diameter) containing potting mix (50% moss peat and 50% river sand) and grown in a glasshouse during January to February 2014, with average day/night temperatures of 30/24C and a 13-h photoperiod under natural sunlight. The pots were watered daily and fertilised as necessary. The pots were frequently rearranged to randomise environmental differences in the glasshouse. Aboveground biomass was harvested at 14, 21, 27 and 34 days after transplanting, with 25 replicate plants from each genotype for each harvest. Plants were oven-dried at 60°C for 3 days and weighed for dry biomass estimation (Yu et al., 2010).

For seed production assessment, 25 individual seedlings from each genotype were transplanted into single pots (30 cm in diameter) when the plants were at the two-leaf stage and arranged in a complete randomized design. Total resource allocation to vegetative biomass and reproductive traits were estimated at the end of the growth cycle in a number of plants (n = 25) of each genotype. Inflorescences produced by individuals from each genotype were collected at first maturity, up to 128 days after transplanting. Total aboveground biomass (together with inflorescences already harvested) was harvested at the end of the experiment and measured as described above. Inflorescences from each individual plant were threshed to separate the seed from seed coat, chaff and rachis material.

### 5.3.10 Statistics

The $LD_{50,}$ $GR_{50}$ or $IC_{50}$ was estimated by non-linear regression using the 3 parameter logistic curve model $y = a/[1+(X/X_0)^b]$ ,where a is the maximum plant response close to untreated controls, $X_0$ is the dose giving 50% response and b is the slope around $X_{0.}$ The estimates were obtained using the Sigmaplot software (version 12.3, Systat Software, Inc) and the test ($\alpha$=0.05) was used to test significance of the regression parameters. The $K_m$ (PEP) and $V_{max}$ were estimated by fitting the data to the Michaelis-Menten equation $v = V_{max}S/(K_m + S)$, where S is the concentration of the

substrate pyruvate, $v$ is the reaction velocity at any PEP concentration, and $V_{max}$ is the maximal reaction velocity. The kinetic values were obtained using GraFit (version 7.0.3, Erithacus Software Ltd) and the Chi-square ($X^2$) ($\alpha$=0.05) was used to test goodness of fit. *In vitro* glyphosate dose-response experiments were repeated at least twice with similar results so all data were pooled and evaluated for a composite line fit. Each EPSPS kinetic assay contained three technical replicates and three independent enzyme extracts were used for each assay set.

The unbiased formula proposed by Hoffmann and Poorter (2002) was used to determine plant relative growth rates (RGR). Variations in RGR are often positively correlated with variations in plant establishment ability and as such, RGR is a useful eco-physiological parameter in denoting the expression of herbicide resistance costs (Vila-Aiub et al., 2005). The variance ($V$) associated with RGR was estimated with Causton and Venus's formula (1981):

$$V(RGR) = \frac{V(\overline{\ln W_2}) + V(\overline{\ln W_1})}{(t_2 - t_1)^2}$$

Where $\ln W_2$ is the mean of the ln-transformed plant weights at harvest time 2; $\ln W_1$ is the mean of the ln-transformed plant weights at harvest time 1. The degree of freedom associated with *RGR* is $n$-2, where $n$ is the total number of plants in two harvest intervals. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was performed to compare RGR among WT, P106S and TIPS genotypes and across growth intervals.

The number of seeds produced per plant ($S_n$) was estimated as:

$$S_n = \frac{TS_w \times 100}{S_w}$$

($TS_w$ is the total seed weight per plant; $S_w$ is the mean weight of 100 seeds per plant; n=25). Individual seed weight was determined from the average seed weight of 100 seeds. Harvest index (HI) (%) per plant was calculated as the ratio of seed mass to total aboveground biomass (seed mass + vegetative biomass) (Vila-Aiub et al., 2005). The *E. indica* TIPS cDNA sequence information can be found in GenBank with an accession number KM078728.

## 5.4 Results

### 5.4.1 EPSPS gene sequencing revealed a double amino acid substitution in EPSPS

To identify the basis of the very high level glyphosate resistance observed in the *E. indica* population under investigation, a 301 bp EPSPS DNA fragment covering the highly conserved region ($^{95}$LFLGNAGTAMRPL$^{107}$) of the EPSPS gene was analysed from 43 resistant plants. These resistant individuals were found to have the known weak resistance mutation at codon 106 (CCA to TCA), but also display a very rare mutation at codon 102 (ACT to ATT) (Fig. 5.1 a and b versus c). Therefore, in this naturally evolved, highly glyphosate-resistant *E. indica* population, there are two resistance-endowing EPSPS amino acid substitutions, T102I and P106S. Cloning of the EPSPS cDNA fragment covering the 102 and 106 codons from resistant individuals revealed the two mutations were always present in the same EPSPS gene fragment, confirming the double amino acid substitution in a single EPSPS allele. This double amino acid substitution, T102I + P106S, is hereinafter referred to as the TIPS mutation.

Based on the sequence information obtained, dCAPS markers for the T102I and P106S mutations were developed (Fig. 5.2). Analysis (by sequencing and/or dCAPS markers) of 193 individuals (untreated) in the R population (Table 5.2) revealed that 84% of the individuals were resistant mutants and 16% were WT (Fig 5.1c). Among the mutant individuals, 1.6% were homozygous mutants for the TIPS mutation (referred to as RR, Fig 5.1a), 33.7% homozygous mutants solely for the P106S mutation (rr, Fig. 5.1d), and 48.7% were the resistant heterozygous mutants of Rr (Fig 5.1b). Interestingly, neither the single T102I mutants, or heterozygous P106S single mutants (r-WT), nor heterozygous TIPS mutants (R-WT) were found from the samples analyzed. Therefore, only three alleles (R, r and WT) were found in the samples examined and the frequency of mutant TIPS allele (R) is only half of the P106S allele (r) (Table 5.2). To better understand the resistance allele frequencies a more detailed analysis of the field population together with herbicide history is needed. The full EPSPS cDNA sequences (1338 bp) were compared among individuals of WT, P106S and TIPS mutant (*E. indica* TIPS cDNA, Genebank KM078728). Except for the single nucleotide polymorphism(s) (SNPs) at the 102 and 106 codons, there was only one SNP that resulted in an amino acid change, from a P381 in WT individuals to a L381 in mutant individuals. BLAST results showed that this amino acid residue is not conserved

in plants EPSPS, and the P381L mutation has been previously proven to be irrelevant to glyphosate resistance in *E. indica* (Baerson et al., 2002).

Table 5.1. Primers used in cloning of the EiEPSPS.

| Primer | Primer name or purpose | Sequence |
|---|---|---|
| 1 | EPSPS EiLFT1 | GCG GGC GCG GAG GAG GTG GT |
| 2 | EPSPS EiRT1 | TTA GTT CTT GAC GAA AGT GCT CAG CAC GTC GAA GTA GT |
| 3 | For P106S to wild type EPSPS conversion PCR primer R | 5'/5Phos/AAC TGC AAT GCG ACC ATT GAC AGC AGC CGT AAC TG |
| 4 | For P106S to wild type EPSPS conversion PCR primer L | 5'/5Phos/CCAGCATTCCCCAAGAAGAGCTGCACCT |
| 5 | For P106S to TIPS EPSPS conversion PCR primer R | 5'/5Phos/GGA ATG CTG GAA TTG CAA TGC GAT CAT TGA CAG CA |
| 6 | For P106S to TIPS EPSPS conversion PCR primer L | 5'/5Phos/CCA AGA AGA GCT GCA CCT CCT CTT TCG CAT C |
| 7 | pET19-bEiEPSPS-F; for cloning EPSPS into pET-19b vector primer L | ATATCATATG GCGGGCGCGGAGGAGGTG |
| 8 | pET19-bEiEPSPS-R; for cloning EPSPS into pET-19b vector primer R | ATATCATATGTCATTAGTTCTTGACGAAAGTGCTCAGCACGTCG |

### 5.4.2 Plants homozygous for the TIPS mutation displayed a high level of glyphosate resistance

To characterise the glyphosate-resistant genotypes, we produced from within the resistant population three purified sub-populations with individuals homozygous for WT, P106S and TIPS EPSPS, respectively, and conducted detailed glyphosate dose-response studies. To examine the possible involvement of any other glyphosate resistance mechanisms in these purified sub-populations, a herbicide susceptible (S) *E. indica* population was also included as a reference. The S and WT populations were identically fully susceptible to glyphosate (Table 5.3, Fig. 5.3A, 5.3B, Fig. 5.4) with 100% mortality at the recommended field rate of 1080 g $ha^{-1}$, indicating no major additional glyphosate resistance mechanisms present in the purified sub-populations. As expected, the P106S population was moderately resistant to glyphosate, with 30% survival at the recommended field rate (1080 g $ha^{-1}$). Conversely, homozygous TIPS mutant plants were highly glyphosate-resistant, such that a $LD_{50}$ could not be determined from the experimental data, and therefore was estimated to be greater than the highest glyphosate rate used (25,900 g $ha^{-1}$) (Fig. 5.3A). Based on the glyphosate $LD_{50}$ ratios (Table 5.3), homozygous TIPS mutants were highly (more than 182-fold) resistant, whereas, as expected, homozygous P106S mutant were only moderately (5.6-fold) resistant. The homozygous TIPS plants, therefore, can tolerate more than 20-fold the recommended glyphosate rate of 1080 g $ha^{-1}$. While the TIPS mutants survived high glyphosate doses, their growth was affected by glyphosate (Fig. 5.3B), resulting in a lower glyphosate $GR_{50}$ ratio than the $LD_{50}$ ratio (Table 5.3, Fig. 5.5).

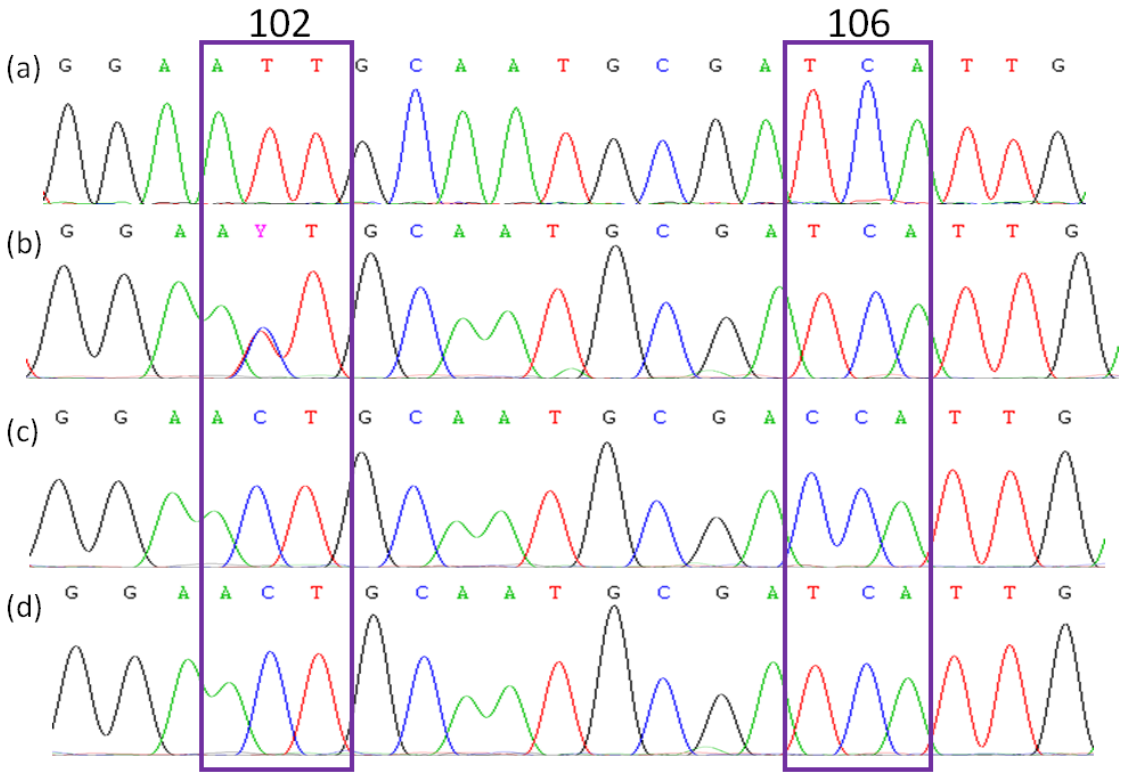
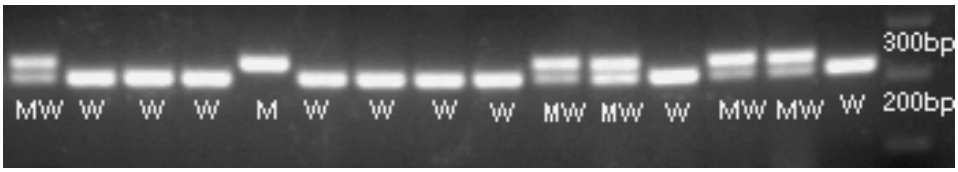



Figure 5.1. Chromatograms of the highly conserved region of the *E. indica* EPSPS sequence containing (in vertical boxes) the TIPS (a and b), the wild type (WT) (c), and the P106S sequences (d) The letter Y is an ambiguity code for mixed base position, in this case, Y = C + T.

**A. 102**




**B. 106**

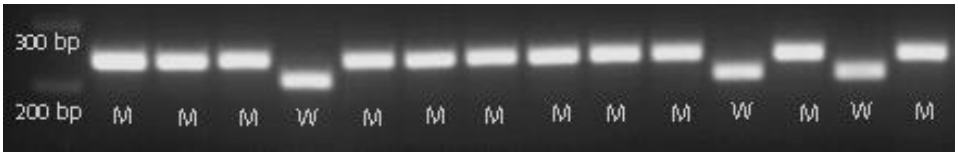



Figure 5.2. dCAPS markers developed for the T102I (A) and P106S (B) EPSPS mutations in *E. indica.* M refers to the mutant 102I (A) or 106S (B) allele and W refers to the wild type (WT) T102 (A) or P106 (B) allele. MW refers to heterozygote with both types of allele.

Table 5.2. Genotype and allele frequencies determined for 193 *Eleusine indica* individuals by the dCAPS method developed for the T102I and P106S mutations.

| Genotypes | No of individuals | Genotype frequency | Alleles | Allele frequency |
|---|---|---|---|---|
| RR | 3 | 1.6% | 102I-106S (R) | 26% |
| rr | 65 | 33.7% | T102-106S (r) | 58% |
| WT | 31 | 16% | T102-P106 (WT) | 16% |
| Rr | 94 | 48.7% | | |
| R-WT | 0 | 0 | | |
| r-WT | 0 | 0 | | |

Table 5.3. Parameter estimates of the non-linear regression analysis (the logistic 3 parameter model) of herbicide rates causing 50% plant mortality ($LD_{50}$) or growth reduction ($GR_{50}$) for glyphosate susceptible, EPSPS WT, and homozygous P106S and homozygous TIPS EPSPS *Eleusine indica* mutants. Standard error (SE) is in parentheses.

| Genotype | a | b | $X_0$ (g $ha^{-1}$) | *P* value for $X_0$ | Ratio to S of $X_0$ |
|---|---|---|---|---|---|
| | | | $LD_{50}$ | | |
| Susceptible (S) | 99.7 (0.22) | 8.71 (1.61) | 142 (1.3) | <0.0001 | 1 |
| WT | 99.7 (0.22) | 8.71 (1.61) | 142 (1.3) | <0.0001 | 1 |
| P106S | 101 (1.78) | 3.14 (0.78) | 798 (29) | <0.0001 | 5.6 |
| TIPS | | | >25900 | | >182 |
| | | | $GR_{50}$ | | |
| Susceptible (S) | 98.3 (8.42) | 1.65 (0.43) | 65 (8.0) | 0.0013 | 1 |
| WT | 97.8 (6.12) | 1.98 (0.37) | 57 (10.8) | 0.0063 | 0.88 |
| P106S | 100 (1.96) | 1.76 (0.12) | 173 (7.3) | <0.0001 | 2.67 |
| TIPS | 99.8 (3.87) | 0.85 (0.08) | 2023 (299) | 0.0005 | 31.1 |

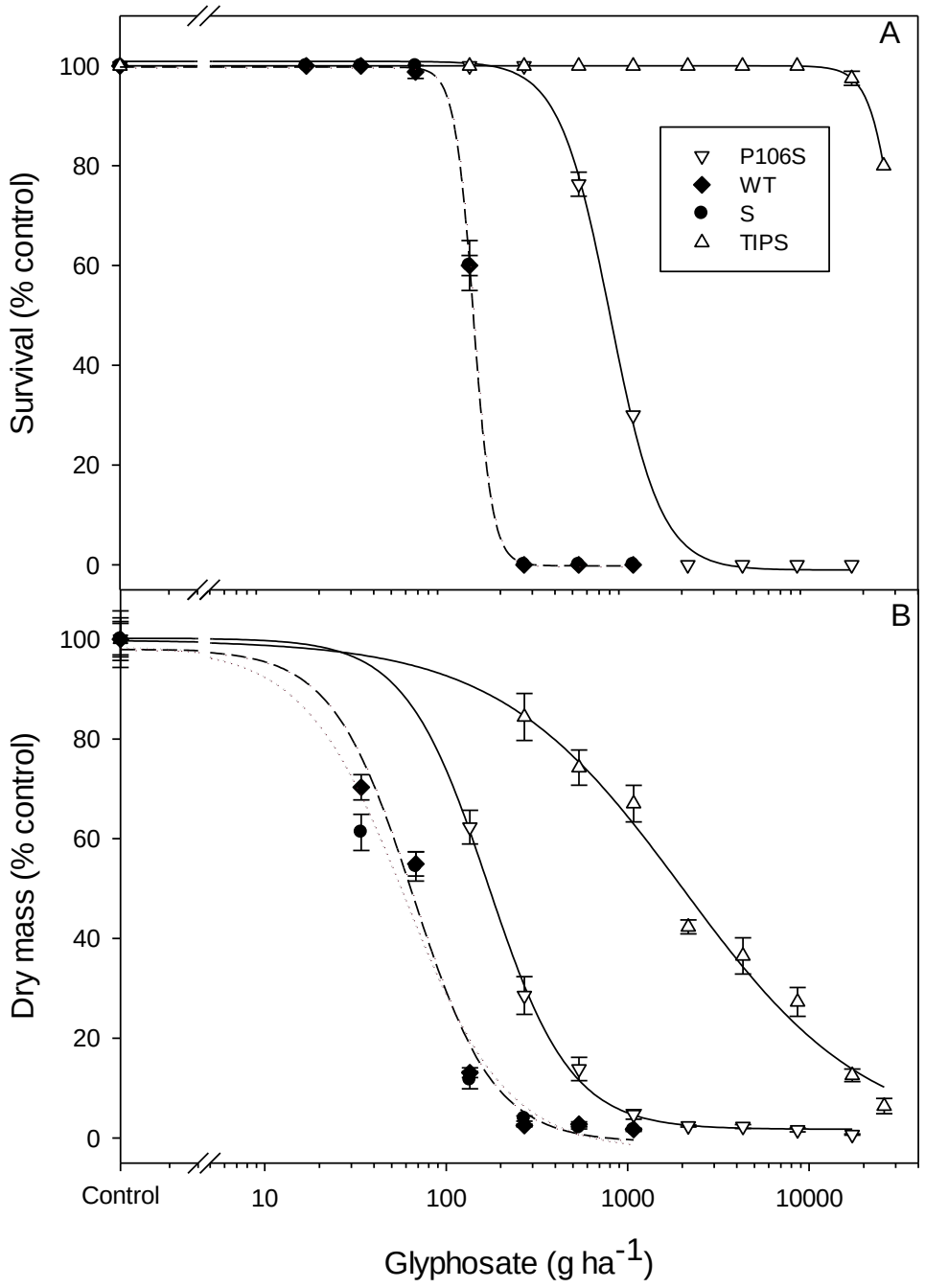



Figure 5.3. Glyphosate dose-response (A: mortality, B: dry mass) of susceptible (S), EPSPS WT, homozygous P106S and homozygous TIPS mutants of *E. indica*, 21 days after treatment.

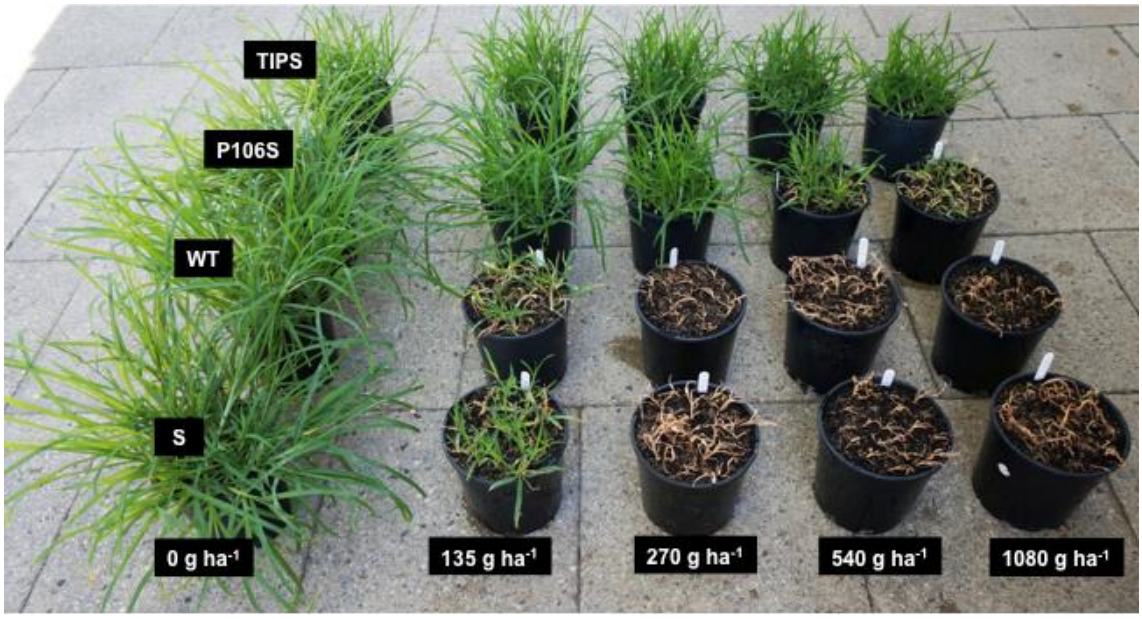



Figure 5.4. Glyphosate dose-response of susceptible (S), EPSPS WT, homozygous and P106S and homozygous TIPS mutants of *E. indica*, 21 days after treatment.

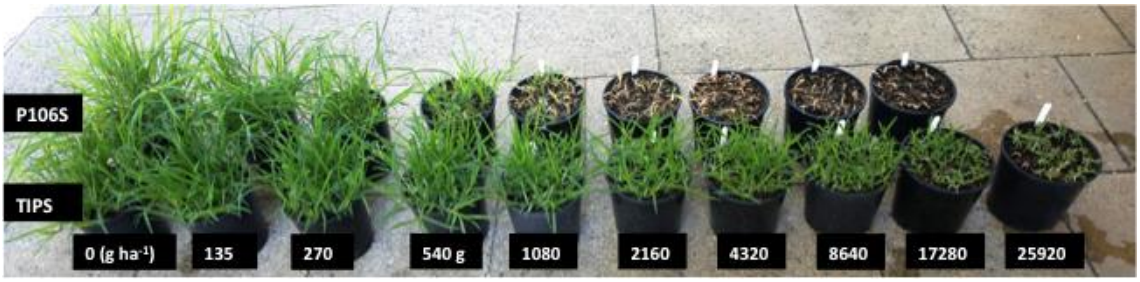



Figure 5.5. Glyphosate full dose-response of *E. indica* homozygous TIPS and homozygous P106S mutants, 21 days after treatment.

### 5.4.3 TIPS encodes a highly glyphosate-resistant EPSPS

To further characterise the TIPS mutation at the protein level, the WT, P106S and TIPS *E. indica* EPSPS (EiEPSPS) was expressed in *E. coli*, and the activity and $IC_{50}$ (herbicide dose causing 50% *in vitro* inhibition) of the His-tag purified recombinant EiEPSPS variants determined (Table 5.4 and 5.5). As expected, based on glyphosate $IC_{50}$ ratios, the *E. coli*-expressed P106S variant was moderately (4.3-fold) resistant to glyphosate, while the TIPS variant was highly (2647-fold) resistant (Table 5.4 and 5.5, Fig. 5.6). These results confirm that TIPS EiEPSPS is essentially insensitive to glyphosate, with an $IC_{50}$ value of 54 mM, and therefore, responsible for very high level glyphosate resistance as observed at the whole plant level (Fig. 5.3). Notably, while incurring no significant changes in the $K_m$ (PEP), the *E. coli*-expressed TIPS variant displayed an EPSPS activity dramatically (16-fold) lower than the WT (Table 5.5), indicating a resistance cost at the enzyme level due to reduced catalytic efficiency.

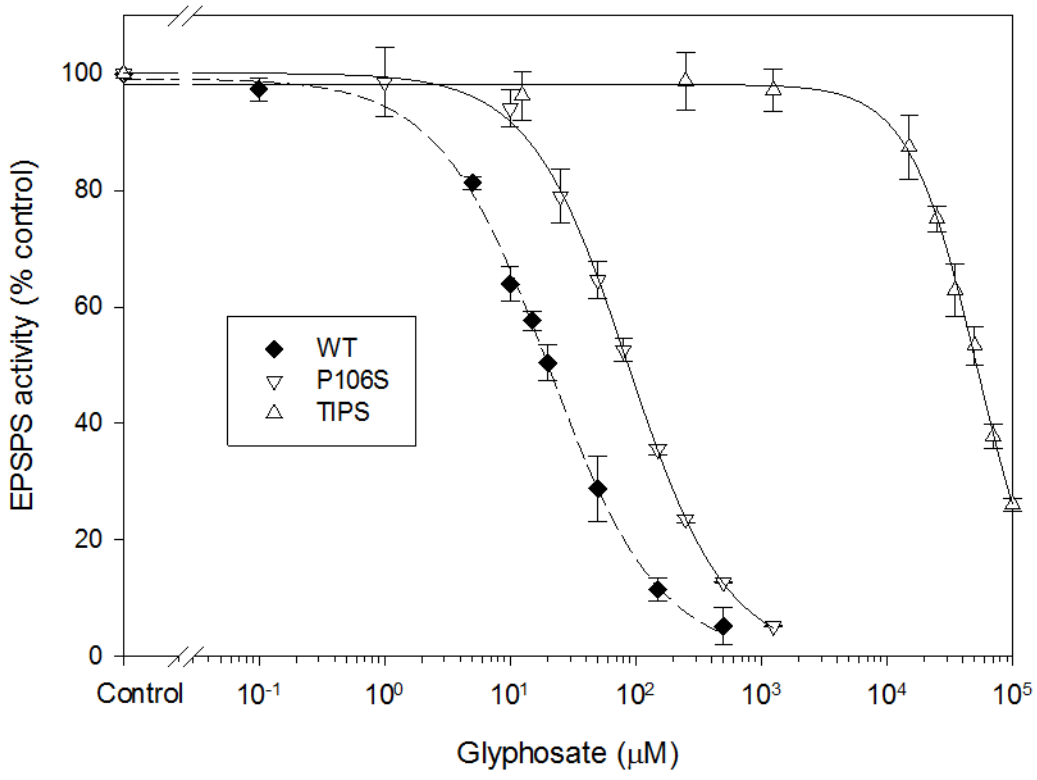



Figure 5.6. *In vitro* glyphosate dose-response of *E. coli*-expressed *E. indica* EPSPS variants.

Table 5.4. Parameter estimates of the non-linear regression analysis of herbicide rates causing 50% inhibition of *in vitro* enzyme activity ($IC_{50}$) of *E. coli*-expressed EIEPSPS variant. SE is in parentheses.

| Genotype | a | b | $X_0$ | *P* value |
|---|---|---|---|---|
| | | | $IC_{50}$ (µM) | |
| WT | 99.2 (1.10) | 0.99 (0.09) | 20 (0.84) | <0.0001 |
| P106S | 100 (0.69) | 1.11 (0.03) | 87 (2.15) | <0.0001 |
| TIPS | 98.2 (0.76) | 1.59 (0.07) | 52938 (1206) | <0.0001 |

Table 5.5. Glyphosate $IC_{50}$ (herbicide dose causing 50% inhibition of *in vitro* enzyme activity) and kinetic properties of *E. coli*-expressed EiEPSPS variants.

| Genotype | $IC_{50}$ (µM) | Ratio to WT | EPSPS $V_{max}$ (nmol Pi $\mu g^{-1}min^{-1}$) | Ratio to WT | $K_m$(PEP) (µM) | Ratio to WT |
|---|---|---|---|---|---|---|
| WT | 20 (0.84) | 1 | 28 (0.84) | 1 | 11.6 (1.1) | 1 |
| P106S | 87 (2.15) | 4.4 | 27.5 (0.55) | 1 | 10.0 (0.68) | 0.9 |
| TIPS | 52938 (1206) | 2647 | 1.8 (0.04) | 0.06 | 9.8 (0.72) | 0.8 |

### 5.4.4 TIPS plants accumulate a higher level of shikimic acid

As expected, in the absence of glyphosate, WT had very low level of shikimic acid, (Table 5.6). Shikimic acid level in the P106S plants were also similar to WT. Interestingly, even in the absence of glyphosate, the basal level of shikimate acid in TIPS plants were nearly 53-fold more than WT (Table 5.6). Tryptophan level was significantly (2- fold) lower in TIPS plants, with no significant difference in Tyrosine and Phenylalanine levels in WT, P106S and TIPS plants (Fig. 5.7).

### 5.4.5 Plants homozygous for the TIPS mutation express a significant resistance cost

Plants homozygous for the TIPS mutation produced significantly less aboveground biomass than WT and P106S at all harvest time points (Table 5.7). At the first harvest (14 DAT) TIPS plants had 3-fold less aboveground biomass than WT and P106S plants. At 27 DAT, TIPS plants had produced 6-fold less biomass than WT and P106S plants. By the final harvest (34 DAT), TIPS plants still had 3-fold less biomass than WT and P106S plants. No significant difference was observed between WT and P106S plants at 14, 27 or 34 DAT, but at 21 DAT P106S plants displayed growth significantly higher than WT (Table 5.7).

Irrespective of the genotype, all plants displayed a significantly higher RGR in the first growth interval ($1^{st}$-$2^{nd}$ harvest) than in the second growth interval ($2^{nd}$-$3^{rd}$ harvest) (Table 5.8). In the first growth interval, P106S plants had about the same RGR as the WT plants, while TIPS had a significantly lower RGR than the other two genotypes. A lower RGR was consistently observed for TIPS plants throughout the experiment.

Reproductive growth assessment showed that TIPS plants produced 2-fold less aboveground biomass (Fig. 5.8A), significantly less (63%) seed mass per plant (Fig. 5.8B) and fewer seed numbers (Fig. 5.8C) than WT and P106S plants. TIPS plants also allocated fewer (4% less) resources to reproduction, as indicated by the harvest index (Fig. 5.8E). No significant differences were observed between WT and P106S plants in the various reproductive traits assessed, except for individual seed mass, with mutant plants producing heavier seeds (Fig. 5.8D).

Overall, these results clearly indicate that the P106S mutation has no significant impact on plant growth and reproductive traits but the TIPS double mutation does when plants are homozygous for the mutation.

Table 5.6. Shikimic acid levels in WT, homozygous P106S and homozygous TIPS *E. indica*. Data are mean ($n$ = 6) with standard errors in parentheses. Means with different letters in a column indicate significant differences according to Tukey's multiple comparison test ($\alpha$ = 0.001).

| Genotype | Shikimic acid (μmol $g^{-1}$ FW) | Ratio to WT |
|---|---|---|
| WT | 0.0108(0.0005)a | 1 |
| P106S | 0.016 (0.002)a | 1.48 |
| TIPS | 0.567 (0.029)b | 52.5 |

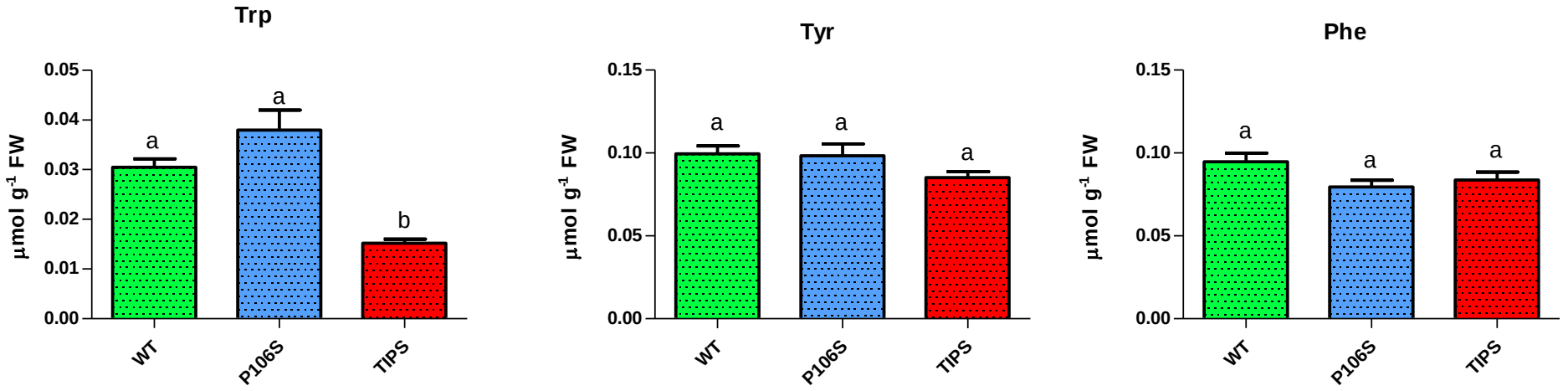



Figure 5.7. Aromatic amino acid levels in WT, homozygous P106S and homozygous TIPS *E. indica.* Data are mean (n = 6) with standard error. Means on top of column bars with different letters in a box graph indicate significant differences according to Tukey's multiple comparison test ($\alpha = 0.05$).

Table 5.7. Aboveground biomass of *E. indica* plants of WT, homozygous P106S, or homozygous TIPS. Data are mean ($n$ = 19-25) with standard errors in parentheses. Means with different letters in a column indicate significant differences according to Tukey's multiple comparison test ($\alpha = 0.001$). Plants were grown in a glasshouse during Jan-Feb 2014.

| Genotype | Aboveground dry biomass (g plant$^{-1}$) | | | |
|---|---|---|---|---|
| | 14 DAT | 21 DAT | 27 DAT | 34 DAT* |
| WT | 0.031 (0.0019)a | 0.306 (0.0164)a | 1.290 (0.0692)a | 4.141 (0.2437)a |
| P106S | 0.034 (0.0025)a | 0.358 (0.0201)b$^{\$}$ | 1.448 (0.0804)a | 4.059 (0.3427)a |
| TIPS | 0.011 (0.0007)b | 0.073 (0.0036)c | 0.226 (0.0139)b | 1.289 (0.0458)c |

*****Some plants started flowering.

$^{\$}$ Significant difference between a and b at 21 DAT is at 0.05 level.

Table 5.8. Relative growth rates (RGR) of *E. indica* plants of WT, homozygous P106S, or homozygous TIPS, purified from within the resistant population. Three plant harvests were performed 14, 21 and 27 days after transplanting. Data are mean RGR ($n$ = 25) with standard errors in parentheses estimated for the first (1$^{st}$-2$^{nd}$ harvest), second (2$^{nd}$-3$^{rd}$ harvest) and whole (1$^{st}$-3$^{rd}$ harvest) plant growing time intervals. Means with different letters in a column indicate significant differences according to Tukey's multiple comparison test ($\alpha = 0.001$). ANOVA analysis within each genotype showed significant differences ($p$ = 0.001) across the different growth intervals.

| Genotype | RGR (d$^{-1}$) | | |
|---|---|---|---|
| | 1$^{st}$-2$^{nd}$ harvest | 2$^{nd}$-3$^{rd}$ harvest | 1$^{st}$-3$^{rd}$ harvest |
| WT | 0.382 (0.0102) a | 0.288 (0.0112) a | 0.311 (0.0050) a |
| P106S | 0.400 (0.0115) a | 0.279 (0.0121) a | 0.316 (0.0059) a |
| TIPS | 0.313 (0.0089) b | 0.223 (0.0119) b | 0.249 (0.0048) b |

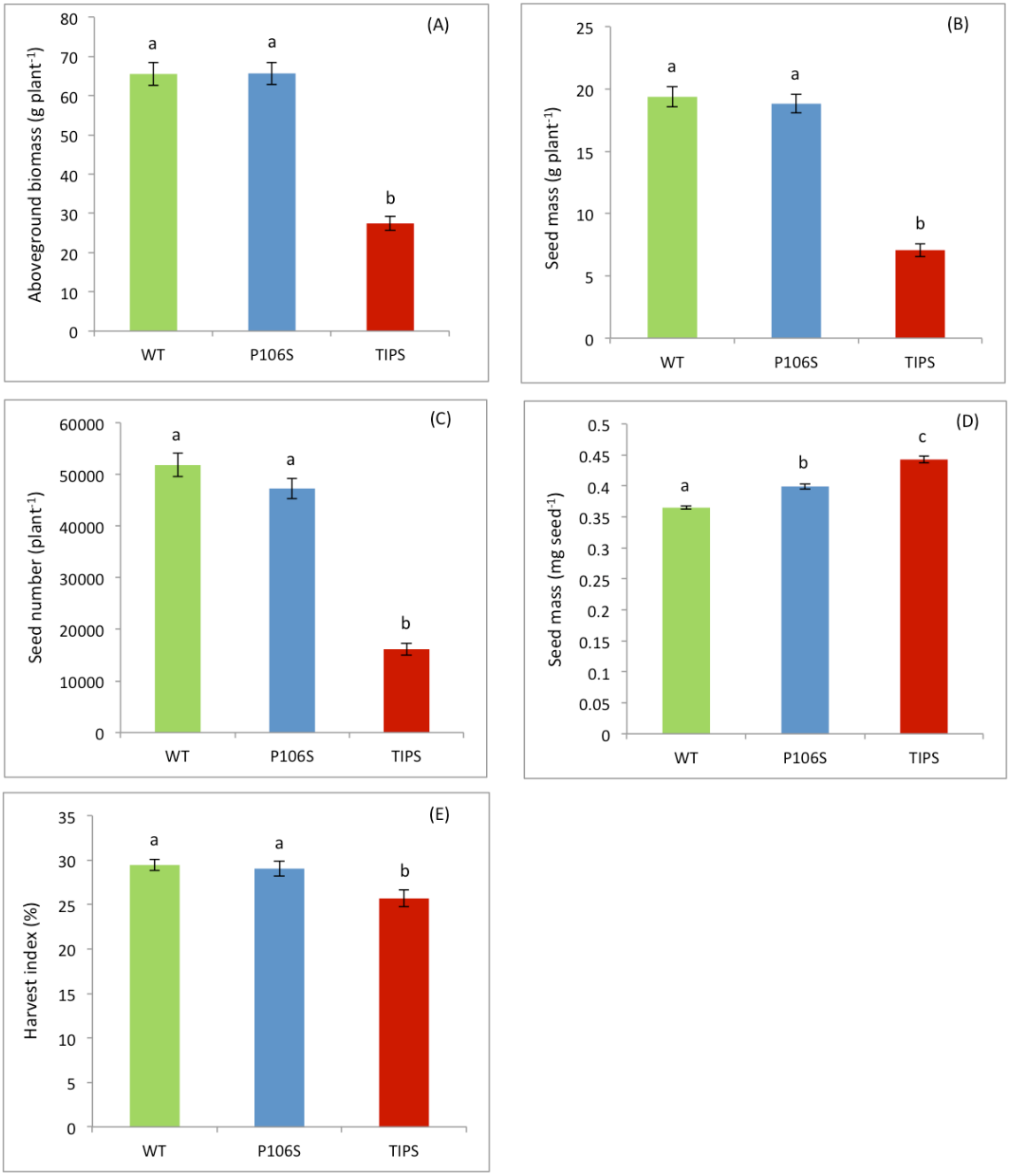



Figure 5.8. Reproductive traits of WT, homozygous P106S and homozygous TIPS *E. indica* for aboveground biomass (A), per plant seed mass (B), seed number (C), per seed mass (D) and harvest index (E) in the absence of crop competition. Plants were grown in a glasshouse during Jan-May 2014. Data are mean ($n$ = 24-25) with standard error. Means on top of column bars with different letters in a box graph indicate significant differences according to Tukey's multiple comparison test ($\alpha = 0.001$, except for the harvest index $\alpha = 0.05$).

## 5.5 Discussion

In the biotechnology search for glyphosate-tolerant crops, various EPSPS double mutations have been generated using site directed mutagenesis and expressed in *E. coli* and plants (Spencer et al., 2000; Howe et al., 2002; Lebrun et al., 2003; Kahrizi et al., 2007; Alibhai et al., 2010). The P106 and then later the TIPS mutation were first found empirically in a mutational screen in *Salmonella sp*. (Comai et al., 1983; Stalker et al., 1985). The TIPS mutation was engineered into tobacco (Arnaud et al., 1998) and field tested for glyphosate tolerance (Lebrun et al., 2003). Ultimately the TIPS mutation was used in the first generation of commercially successful glyphosate-tolerant transgenic maize (GA21) (Spencer et al., 2000). Here, we demonstrate that this TIPS mutation has now evolved in nature.

In target-site resistance evolution for acetolactate synthase (ALS) and acetyl coenzyme A carboxylase ACCase (ACCase)-inhibiting herbicides, highly resistant yet fit individuals with single target-site mutations are common (Vila-Aiub et al., 2009), as these herbicides have large binding sites in and adjacent to the enzyme catalytic site resulting in contacts with amino acids that are non-essential for structure or function (Sammons et al., 2007; Powles & Yu, 2010). The most dramatic examples are the multiple (more than 10) different amino acid substitutions identified at P197 in ALS (Tranel & Wright, 2002; Tranel et al., 2015). The transition state inhibitor designation for glyphosate as an inhibitor of EPSPS comes from the observation that glyphosate contacts 17 amino acids responsible for catalysis which necessarily prevents any substitutions of these essential amino acids (Schönbrunn et al., 2001). The P106S EPSPS provides relatively low glyphosate resistance (Arnaud et al., 1998), whereas the T102I EPSPS alone endows high level resistance, but with drastically decreased affinity for the second substrate PEP (Kishore et al., 1992; Funke et al., 2009). The concomitant mutations at the 106 and 102 codons are merely adjacent to the active site and together make very small (on the scale of a fraction of an angstrom) modifications structurally to the EPSPS active site, therefore selectively impacting glyphosate binding more than PEP (Funke et al., 2009) (Fig. 5.8). Therefore, the TIPS mutation endows high level glyphosate resistance with acceptable affinity for PEP.

Multiple mutations of a single pesticide target site gene are known in adaptive evolution of fungicide or insecticide resistance (Brunner et al., 2008; Karasov et al., 2010). Accumulation of multiple mutations in a single allele in insects and fungi can be

achieved via intragenic recombination between pre-existing resistant alleles in natural populations, in response to increased selective pressure (Mutero et al., 1994; Brunner et al., 2008). Double (or multiple) mutations in a single allele of a herbicide target gene may also occur in herbicide resistance by intragenic recombination between resistant alleles pre-existing in resistant populations. For example, it is common for cross-pollinated weed species (like black grass and annual ryegrass) to accumulate several ACCase or ALS target-site mutations in a single allele via intragenic recombination, as different ACCase or ALS resistance alleles have been found to pre-exist in weed populations and can be brought together in an individual through cross-pollination (Powles & Holtum, 1994; Délye et al., 2013; Yu & Powles, 2014). However, this is unlikely to occur in *E. indica*, as the single-codon mutation T102I was not detected in the resistant population, and was therefore unlikely to pre-exist in the population. Indeed the very poor kinetics of the T102I mutant enzyme (Alibhai et al., 2010) suggests this mutation would be unfit and even lethal when present alone. This severe resistance cost associated with the T102I mutation explains why this single mutation has not been observed in nature.

The notion that compensatory mutations may require a particular evolutionary trajectory to prevent lethal mutants is discussed by Weinreich et al. (2006) where a series of 5 amino acid point mutations providing 100,000-fold resistance (compared to susceptible counterparts) to β-lactamase in a matrix of combinations were studied to reveal a defined successful evolutionary pathway. Here, for the glyphosate-resistant *E. indica*, our data suggests that the TIPS evolved sequentially under intense glyphosate selection. First, the weak P106S mutation was selected, enriched and reached homozygosity. Indeed, many glyphosate resistant *E. indica* populations in Malaysia and other countries have been found to posses mutations at P106 (Baerson et al., 2002; Ng et al., 2003; Ng et al., 2004; Kaundun et al., 2008) and in Malaysia, glyphosate was used frequently (every month) and continually (5-10 years) at increased glyphosate rates (0.72-1.92 kg $ha^{-1}$) to control *E. indica* (Ng et al., 2004). Evolution of the P106S mutation would then have been followed by the T102I mutation, to create the highly resistant TIPS EPSPS. This TIPS EPSPS enables plants to survive high glyphosate rates and pass on the trait to their progeny.

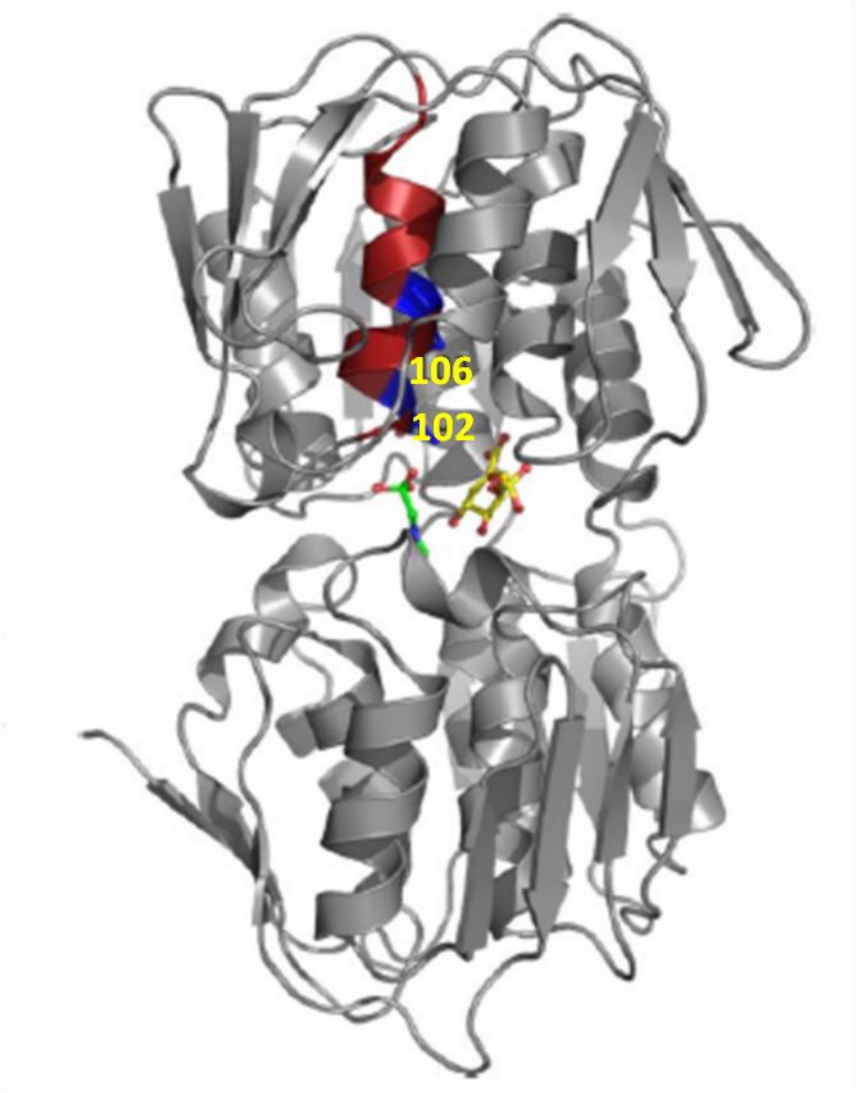



Figure 5.9. 5-Enolpyruvyl-3-shikimate phosphate synthase (EPSPS) structure with location of T102 and P106. The EPSPS is in a closed conformation in binding with S3P (yellow) and glyphosate (green). This picture is adapted from Funke et al. (2009) with slight modifications.

Will there be other EPSPS gene double mutations in nature? In addition to the TIPS mutant, various EPSPS double mutants at 102 and 106 were intentionally produced and the kinetics of *E. coli*-expressed EPSPS variants studied. Compared to the WT and T102I mutant alone, double mutants such as TIPA (T102I + P106A), TIPT (T102I +P106T) or TLPA (T102L + P106A) also show favourable kinetics comparable to or even better than TIPS (Alibhai et al., 2010; Sammons & Gaines, 2014). As various amino acid substitutions at P106 have been identified (e.g. P106A, P106S, P106T or P106L) in glyphosate-resistant weed species (reviewed by Sammons & Gaines, 2014), the evolution of other EPSPS double variants is also possible where glyphosate selection is intense.

The very low percentage (1.6%) of individuals homozygous for the TIPS genotype (RR) as compared to the higher percentage (~49%) of resistant individuals of Rr genotype (Table 5.2) suggest (1) the additional T102I mutation is a recent event (given that *E. indica* is a self-pollinating species, homozygosity at 102 can be increased in a few generations) and/ or (2) a significant fitness penalty is associated with homozygous TIPS mutants when glyphosate selection is relaxed. The latter correlates with the resistance cost (vegetative and reproductive traits) associated with homozygous TIPS genotypes (Table 5.7, Fig. 5.8). In the absence of glyphosate treatment and crop competition, a severe resistance cost (up to 69%) was identified for TIPS but not WT and P106S plants (Table 5.7, Fig. 5.8, Fig. 5.10). Consequently, homozygous (RR) TIPS mutants are outperformed overtime by Rr TIPS mutants which may suffer less or little resistance cost and therefore proliferate in the population. Due to predominate self-pollination in *E. indica*, a very low level of out-crossing (if any) between WT and homozygous P106S (rr) mutants may produce a small number of heterozygous P106S (r/WT) mutants. However, it is highly likely that these individuals would be unable to survive the field or higher glyphosate rates. This could explain why the r/WT mutants were not detected in the resistant population. Further fitness studies on the WT, P106S (rr) and TIPS (RR, Rr) EPSPS mutants under crop competition are underway, and we expect that homozygous TIPS mutants (RR) is a weaker competitor relative to WT and P106S genotypes in the absence of glyphosate selection. However, it is important to evaluate the resistance cost of the Rr TIPS genotype (Fig 5.1b), as this genotype was found to be prevalent in the resistant population.

The resistance cost of homozygous TIPS plants is likely due to disturbance of the shikimate pathway caused by greatly reduced catalytic efficiency of the TIPS

EPSPS (Table 5.5), as evidenced by accumulation of high level shikimate substrate and reduced production of the aromatic amino acid tryptophan in RR TIPS plants (Table 5.6; Fig 5.7). Tryptophan is a precursor of indole acetic acid (IAA), which is an important growth hormone. A lower level of tryptophan in TIPS plants may result in a lower level of IAA (Fig. 2.6 in Chapter 2), which may in part explain reduced growth of TIPS plants. A targeted metabolomics approach is proposed to investigate metabolic profile of the TIPS plants for the shikimate pathway in order to understand its impact on plant growth and reproductive production.

Significant fitness cost has been reported for the *psb*A (target site of group C herbicides) gene mutation S264G, the ALS gene mutation W574L and the α-tubulin (target site of group D herbicides) gene mutation T239I (reviewed by Gronwald, 1994; Tardif et al., 2006; reviewed by Vila-Aiub et al., 2009; Darmency et al., 2011). Menchari et al. (2008) demonstrated that the fitness cost incurred by the ACCase gene mutation D2078C in *Alopecurus myosuroides* was only associated with plants homozygous for this mutation in *Alopecurus myosuroides.* Should the resistance cost from decreased catalytic efficiency of TIPS be offset by, for instance, duplication of the TIPS gene as is required for commercial crops (CaJacob et al., 2004) then evolution of "Roundup Ready Like" *E. indica* may be expected in nature, especially in species exhibiting EPSPS gene amplification (reviewed by Sammons & Gaines, 2014) where the tandem repeat nature of the duplication (Jugulam et al., 2014) may facilitate incorporation of the necessary point mutations, and gene duplication is free of fitness cost (Vila-Aiub et al., 2014).

Therefore, the evolutionary recipe for high level glyphosate resistance in weedy plant species under glyphosate selection may have these primary components: (1) overexpression of EPSPS, as already reported in 4 weed species with gene duplication (Sammons & Gaines, 2014); (2) P106S (or T/A)-EPSPS, as documented in 6 weed species (Sammons & Gaines, 2014); (3) acquiring the second EPSPS T102I mutation, as described here for the first time, and (4) combining two or more glyphosate resistance-endowing mechanisms that would have additive impact on the resistance magnitude, as demonstrated in glyphosate resistant *L. rigidum* (Yu et al., 2007; Bostamam et al., 2012; Nandula et al., 2013).

In summary, this research reports for the first time the evolution of an EPSPS double mutation (TIPS) in a crop weed species. This TIPS mutation confers very high

level glyphosate resistance in *E. indica*, albeit expressing significant resistance cost when plants are homozygous resistant. This is a clear manifestation of the power of evolution in action and how nature responds and adapts to human-manipulated environmental stresses. This is also a very clear warning that herbicide sustainability demands much greater diversity in weed control tactics than sole reliance on a herbicide.

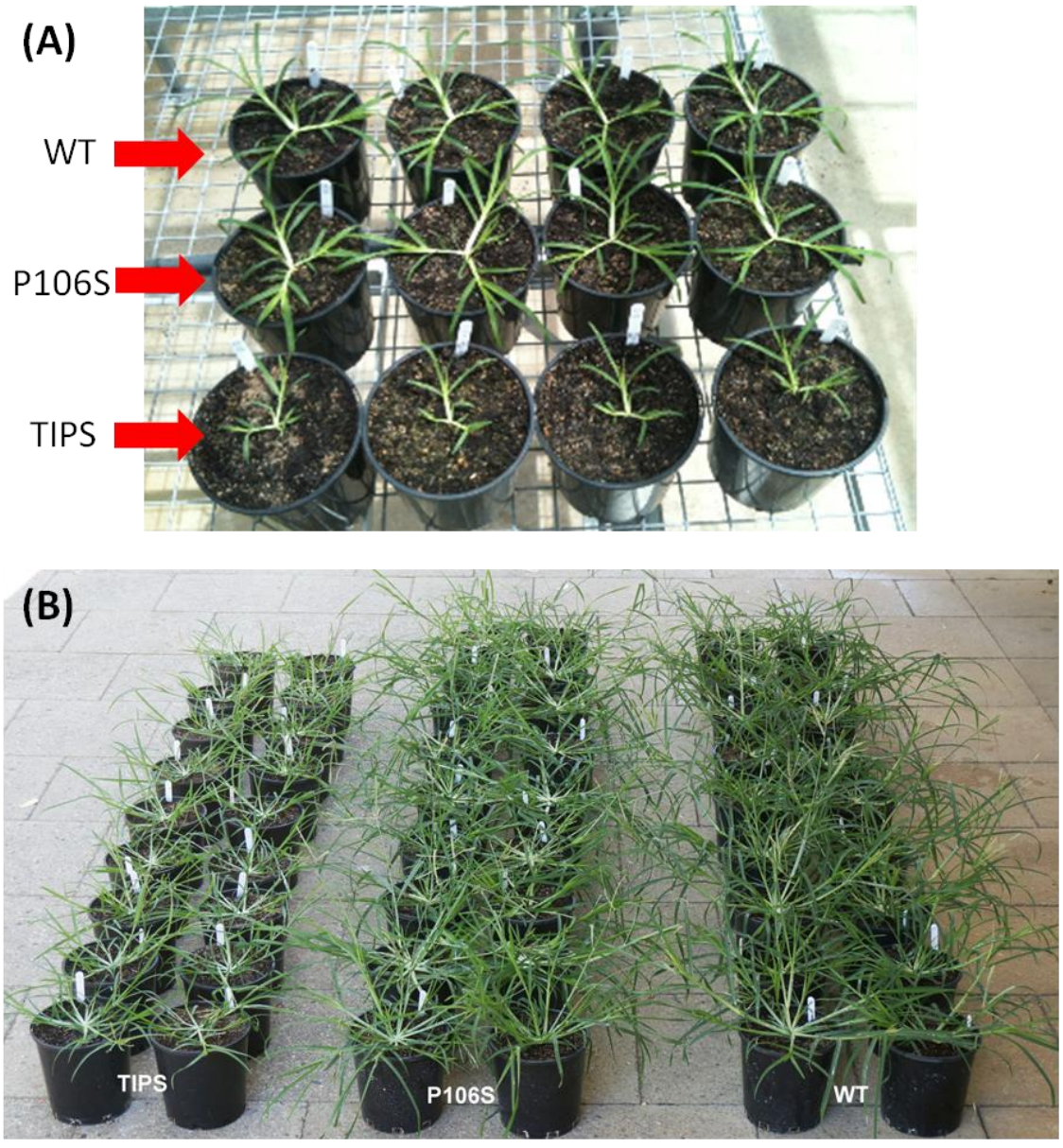



Figure 5.10. Reduced growth of the homozygous EPSPS TIPS mutants as compared to the WT and P106S mutants. *Eleusine indica* plants at three (A) and five (B) weeks after transplanting.

## 5.6 Acknowledgements

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# Chapter 6

## Summary and future directions

Chapter 6

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## 6.1 Introduction

Fundamental understandings of herbicide resistance evolution and resistance mechanisms in weed populations are important for better weed management. This PhD was undertaken to evaluate multiple resistance in *E. indica* to a number of important herbicides (e.g. glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides) and to investigate the biochemical and molecular basis of the resistance. This goal was achieved through glasshouse-based herbicide dose response experiments and biochemical and molecular experiments with the multiple resistant *E. indica* population.

## 6.2 Summary of key findings

### 6.2.1 Multiple resistance to glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *E. indica* population

Key findings:

1. Glufosinate-resistance was confirmed in two *E. indica* populations.
2. One glufosinate-resistant population was also found to be highly resistant to glyphosate, paraquat and several ACCase-inhibiting herbicides.
3. Resistance to ACCase inhibitors in this population is due to the Trp-2027-Cys mutation in the plastidic ACCase gene.

Overall, this study demonstrates the ability of the self-pollinating annual $C_4$ grass weed *E. indica* to evolve multiple resistance to various herbicides. Although chemical control remains an option, other weed control measures must be included to ensure sustainability of the remaining working herbicides.

### 6.2.2 Glufosinate resistance mechanism(s)

Key findings:

1. Glufosinate sensitivity of the target-site (glutamine synthetase) is similar in the resistant and susceptible populations, and therefore, the glufosinate resistance mechanism is unlikely to be target-site based.
2. Non-target-site differential uptake, translocation and metabolism of glufosinate are unlikely to be the resistance mechanisms.

Despite much effort, the glufosinate resistance mechanism(s) in this *E. indica* population remains to be determined. Cellular glufosinate uptake and sequestration needs to be examined.

### 6.2.3 Glyphosate resistance mechanism

Key findings:

1. High-level glyphosate resistance is due to a double mutation (T102I and P106S, referred to as TIPS) in the EPSPS gene.
2. Individuals possessing only the T102I mutation were not found, indicating sequential evolution of the double mutation (e.g. first the P106S then the T102I)
3. An *E. coli* expressed *E. indica* TIPS variant is essentially insensitive to glyphosate but had a greatly reduced enzyme catalytic activity.
4. At the whole plant level, homozygous TIPS mutants expressed a significant resistance cost with reduced aboveground biomass and fecundity, relative to WT and homozygous P106S mutants.
5. Resistance cost of the TIPS mutants is associated with an alteration in the shikimate pathway (e.g. accumulation of the substrate shikimate and reduced tryptophan production).

The discovery and characterization of the first field-evolved double (TIPS) mutation is a highlight of this PhD thesis. This resistance mechanism provides very high level glyphosate resistance, relative to any other known glyphosate resistance mechanism.

## 6.3 Future research directions

Based on the studies carried out in this research, several future research areas have been identified that could further improve understanding of glufosinate and glyphosate resistance and resistance evolution. They are discussed below.

1) **Target-site glufosinate resistance mechanisms**

In Chapter 4, total extractable GS activity and sensitivity was measured and compared between the R and S plants. As plants have cytosolic and chloroplastic GS isoforms, there is a small possibility that there may be subtle differences in sensitivity between these different GS isoforms. Thus, further investigation into GS sensitivity of the cytosolic and chloroplastic isoforms may be fruitful.

2) **Non-target-site glufosinate resistance (cellular uptake and vacuolar sequestration of glufosinate)**

Continuing the effort in elucidating the mechanism for glufosinate resistance, further work will be carried out to examine if there are any differences between S and R* in cellular glufosinate uptake and/or vacuolar sequestration, effectively reducing glufosinate entering into the cell and/or reaching the target site. Enhanced herbicide vacuolar sequestration has been indicated in paraquat resistant *Hordeum glaucum*, *Arctotheca calendula* and *Lolium rigidum* (Bishop et al., 1987; Preston et al., 1994; Yu et al., 2004; Yu et al., 2010). Similarly, vacuolar sequestration of glyphosate has been reported as one of the resistance mechanisms to glyphosate in *Conyza canadensis*. This mechanism reduces the glyphosate concentration reaching the target site to sub lethal levels, enabling plant survival (Ge et al., 2010; Ge et al., 2012; Ge et al., 2014).

3) **Genetic inheritance of glufosinate resistance**

Glufosinate resistance is a heritable trait, as demonstrated in Chapter 3. However, whether one or more genes is involved remains unknown. By crossing the glufosinate susceptible and resistant plants, analysing the $F_1$ phenotypic response, generating and further analysing the $F_2$ and $F_3$ populations, the dominance and number of genes for glufosinate resistance can be evaluated.

4) **Fitness studies for TIPS mutants under crop competition.**

Homozygous TIPS plants have been shown to express a fitness cost in the absence of crop competition in Chapter 5. Nevertheless, understanding the performance of TIPS plants (including homozygous (RR) and heterozygous (Rr) under crop competition is vital for better understanding of the evolutionary trajectory of the TIPS mutation, in terms of the proliferation and the frequency of the resistance gene into future generations. Therefore, further work should be

carried out to evaluate vegetative growth and fecundity in TIPS versus WT and P106S plants and in competition with crops. Additionally, investigation into the metabolic profile of the shikimate pathway in TIPS plants could reveal (any) connections between the low tryptophan level with the resistance cost.

5) **Survey for other EPSPS double mutations in Malaysia**

In Malaysia, glyphosate resistance in *E. indica* is widespread, with some populations having high resistance levels far exceeding the level conferred by a P106 mutation (Chuah, 2013). Additionally, the occurrence of another P106 mutation (P106T) in a Malaysian *E. indica* population has been reported before (Ng et al., 2004). Combining these two factors, it is possible that other double mutations, such as TIPT and/or TIPA could have evolved in other *E. indica* populations in Malaysia as a result of intense glyphosate selection pressure. A survey focusing on highly resistant *E. indica* populations in Malaysia may reveal the frequency of TIPS or other possible EPSPS double mutations.

## 6.4 Conclusion

In summary, this research has revealed the evolution in an *E. indica* population of multiple resistance across three non-selective herbicides (glufosinate, glyphosate and paraquat), and some ACCase-inhibiting herbicides (fluazifop-*p*-butyl, haloxyfop-*p*-methyl and butroxydim). This is a remarkable example of evolution in action in *E. indica* in response to unsustainable use of valuable herbicides. A breakthrough of this research is demonstration of the ability of *E. indica* to acquire high resistance to glyphosate by evolution of a TIPS double mutation in the EPSPS gene. Through comprehensive biochemical and molecular approaches, the study on glyphosate resistance provided insights into the evolution of the TIPS double mutation. This is a valuable addition to the literature on glyphosate resistance mechanisms. In practice, the knowledge gained in this research could help in future weed management in combating multiple resistance, especially in *E. indica*. With no likely introduction of new herbicide modes of action in the near future, it is essential to sustain precious herbicides by minimising selection pressure through diversification and integration of weed control tactics.

## 6.5 References


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# Appendices

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# Multiple resistance across glufosinate, glyphosate, paraquat and ACCase-inhibiting herbicides in an *Eleusine indica* population

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## Summary

An *Eleusine indica* population was previously reported as the first global case of field-evolved glufosinate resistance. This study re-examines glufosinate resistance and investigates multiple resistance to other herbicides in the population. Dose–response experiments with glufosinate showed that the resistant population is 5-fold and 14-fold resistant relative to the susceptible population, based on $GR_{50}$ and $LD_{50}$ *R*/*S* ratio respectively. The selected glufosinate-resistant subpopulation also displayed a high-level resistance to glyphosate, with the respective $GR_{50}$ and $LD_{50}$ *R*/*S* ratios being 12- and 144-fold. In addition, the subpopulation also displayed a level of resistance to paraquat and ACCase-inhibiting herbicides fluazifop-P-butyl, haloxyfop-P-methyl and butroxydim. ACCase gene sequencing revealed that the Trp-2027-Cys mutation is likely responsible for resistance to the ACCase inhibitors examined. Here, we confirm glufosinate resistance and importantly, we find very high-level glyphosate resistance, as well as resistance to paraquat and ACCase-inhibiting herbicides. This is the first confirmed report of a weed species that evolved multiple resistance across all the three non-selective global herbicides, glufosinate, glyphosate and paraquat.

**Keywords:** herbicide resistance, Indian goosegrass, non-selective herbicides, fluazifop-P-butyl.

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## Introduction

*Eleusine indica* (L.) Gaertn. (Indian goosegrass), one of the world's worst weeds (Holm *et al.*, 1977), is a very competitive and cosmopolitan species. *Eleusine indica* is fecund, found across a range of soils and temperatures (Nishimoto & McCarty, 1997) and infests a wide range of crops including cotton, maize, upland rice, sweet potatoes, sugarcane and many fruit and vegetable orchards (Holm *et al.*, 1977), causing major crop yield loss (Lourens *et al.*, 1989).

In tropical countries such as Malaysia, *E. indica* infestation occurs mostly in field crops areas, fruit and vegetable orchards, nurseries and young palm oil plantations. *Eleusine indica* has been shown to affect crop growth, cause yield loss and increase the incidence of plant disease such as *Phytophthora* spp. (Chee *et al.*, 1990; Teng & Teo, 1999). Control of *E. indica* is mainly with herbicides, but over-reliance on herbicides has resulted in resistance evolution in this species in at least eight countries (Heap, 2013). This includes resistance to dinitroaniline herbicides (Mudge *et al.*, 1984), acetyl coA carboxylase (ACCase)-inhibiting herbicides

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(Leach *et al.*, 1993; Osuna *et al.*, 2012), the acetolactate synthase (ALS)-inhibiting herbicide imazapyr (Valverde *et al.*, 1993), the glycine herbicide glyphosate (Lee & Ngim, 2000), the bipyridilium herbicide paraquat (Buker *et al.*, 2002), photosystem II inhibitors (Brosnan *et al.*, 2008), and most recently, the glutamine synthetase-inhibiting herbicide glufosinate (Chuah *et al.*, 2010; Jalaludin *et al.*, 2010).

Glyphosate and its alternative, glufosinate, are two of the most important herbicides globally. Glyphosate was initially used in Malaysia to control *E. indica* and other weeds in fallows, nurseries and to remove ground cover vegetation in plantations. Over-reliance on glyphosate was a strong selection pressure and glyphosate resistance in *E. indica* quickly evolved (Lee & Ngim, 2000). Now, many *E. indica* populations have been identified as glyphosate resistant (Ng *et al.*, 2003, 2004; Kaundun *et al.*, 2008). In response to glyphosate-resistant evolution in *E. indica*, high glufosinate usage has occurred. In 2010, the first case of glufosinate resistance was reported in a Malaysia *E. indica* population (Jalaludin *et al.*, 2010). Prior to glufosinate usage, this resistant population had a field history of paraquat, fluazifop-P-butyl and glyphosate treatment.

At the same time, another Malaysian *E. indica* population was reported to be resistant to glufosinate and paraquat (Chuah *et al.*, 2010). Subsequently, glufosinate resistance and multiple resistance to glufosinate and glyphosate have been reported in *Lolium perenne* L. populations in Oregon, USA (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia *et al.*, 2012). The objective of this study was to characterise the glufosinate-resistant population of *E. indica* following the preliminary study by Jalaludin *et al.* (2010) and evaluate for possible multiple resistance to a range of herbicides with different modes of action.

## Materials and methods

### *Plant material*

The glufosinate-resistant (*R*) *E. indica* population used in this study was preliminarily described (Jalaludin *et al.*, 2010). A glufosinate-susceptible population was originally provided by T S Chuah, and a subset of this population that was confirmed to be susceptible to all herbicides examined in this study was generated and used as the herbicide susceptible (*S*) population.

### *Glufosinate dose–response*

*Eleusine indica* seeds were germinated on water-solidified 0.6% agar containing 0.2% potassium nitrate ($KNO_3$) (Ismail *et al.*, 2002). After 4–7 days, seedlings were transplanted into pots (18 cm diameter with 15–20 seedlings per pot) and kept in a glasshouse during the normal summer growing months (January to March) with average temperatures of 30/20°C (day/night), and 15-h photoperiod under natural sunlight. At the 3–5 leaf stage, seedlings were treated at various rates of glufosinate (0, 20.6, 41.3, 82.5, 123.8, 247.5, 495, 1485, 1980, 3960 and 7920 g a.i. $ha^{-1}$) (Basta, 200 g a.i. $L^{-1}$, SC; Bayer CropScience), using a custom-built, dual nozzle cabinet sprayer delivering herbicide at 118 L $ha^{-1}$ at 210 kPa, with a speed of 1 m $s^{-1}$. After herbicide treatment, plants were returned to the glasshouse. The pots were arranged in a completely randomised block design with at least three replicate pots per herbicide rate. Visual assessment for resistance (*R*) and susceptibility (*S*) was made 21 days after treatment. Plants were considered as *R* if they are actively growing or tillering, while *S* plants were dead. Above-ground shoots were harvested and dried in oven (65°C) for 3 days for dry-weight measurements.

Additionally, six individual plants surviving 1485 and 1980 g a.i. $ha^{-1}$ of glufosinate were allowed to grow together to produce seeds (*E. indica* is a self-pollinated species), and the progeny was designated as selected glufosinate-resistant subpopulation (referred as *R**). This subpopulation was tested again for glufosinate resistance and used for subsequent experiments.

### *Glyphosate dose–response*

Seed germination and seedling growth were the same as described above for glufosinate experiments. Glyphosate rates at 0, 33.8, 67.5, 100, 135, 170, 200, 540, 1080, 4320, 8640, 12 960, 17 280 and 25 920 g a.e. $ha^{-1}$ (Roundup Attack with IQ inside, 570 g a.e. $L^{-1}$, SL; Nufarm Australia) were used.

### *Paraquat dose–response*

Seed germination was carried out as described earlier. After transplanting into pots, the seedlings were grown in a controlled environment room with alternating temperatures of 30/25°C (day/night), 12-h photoperiod with light intensity of 400 µmol $m^{-2}$ $s^{-1}$ and 75% humidity. At the 3–4 leaf stage, the plants were treated with paraquat at 0, 47, 94, 188, 375, 750, 1500 and 3000 g a.i. $ha^{-1}$ (Gramoxone, 250 g a.i. $L^{-1}$, SL; Syngenta Crop Protection).

### *Herbicide single-rate test*

In this experiment, germinating seedlings were transplanted to trays (50–60 seedlings per tray with two to

four trays per herbicide treatment) and kept in a glasshouse with day/night temperature of 30/25°C under natural sunlight. Single discriminating or label rates of ACCase-inhibiting herbicides fluazifop-P-butyl, 210 g a.i. $ha^{-1}$ (Fusilade Forte, 128 g a.i. $L^{-1}$, EC; Syngenta Crop Protection), haloxyfop-P-methyl, 60 g a.i. $ha^{-1}$ (Verdict 520, 520 g a.i. $L^{-1}$, EC; Dow Agrosciences Australia), clethodim, 100 g a.i. $ha^{-1}$ (Select, 240 g a.i. $L^{-1}$, EC; Sumitomo Chemical Australia), butroxydim, 100 g a.i. $ha^{-1}$ (Falcon, 250 g a.i. $kg^{-1}$, WG, Nufarm Australia) and sethoxydim, 230 g a.i. $ha^{-1}$ (Sertin 186 EC, 186 g a.i. $L^{-1}$, EC; Bayer CropScience), and the ALS-inhibiting herbicide imazapyr, 50 g a.i. $ha^{-1}$ (Arsenal, 250 g a.i. $L^{-1}$, SC; Nufarm Australia) were used for resistance screening.

### Statistics

The herbicide rate causing 50% mortality ($LD_{50}$) or reduction in growth ($GR_{50}$) was estimated by non-linear regression analysis using Sigma Plot ® software (version 12.0, SPSS, Chicago, IL, USA). The data were fitted to the three parameter logistic curve model:

$$y = \frac{a}{1 + (\frac{x}{ED_{50}})^b} \quad (1)$$

where $a$ = upper limit, $ED_{50}$ = estimated dose causing 50% response ($LD_{50}$ or $GR_{50}$) and $b$ = slope around $ED_{50}$. The $LD_{50}$ and $GR_{50}$ values of the susceptible and resistant biotypes were used to calculate the $R/S$ ratio of the resistant population. There were several pilot trials prior to final herbicide dose–response experiments, which contained at least three replicate pots per herbicide rate. Each dose–response experiment was repeated at least twice with similar results, and therefore, only results from a single experiment were presented for each dose–response.

### ACCase gene sequencing

Genomic DNA was extracted from the leaf tissue of surviving plants from $R^*$ population and susceptible plants from $S$ population according to Yu *et al.* (2008). Published primers (Osuna *et al.*, 2012) used to amplify two plastidic ACCase gene fragments in which point mutations known to confer ACCase herbicide resistance in plants have been identified (Délye & Michel, 2005; Powles & Yu, 2010; Beckie & Tardif, 2012). The PCR was conducted in a 25 µL volume that consisted of 1–2 µL containing 50–100 ng of genomic DNA, 0.5 µM of each primer and 12.5 µL of 2× GoTaq Green Master Mix® (Promega, Madison, WI, USA). The PCR was run with the following profile: 94°C for 4 min; 40 cycles of 94°C for 30 s, 58°C (annealing temperature) for 30 s and 72°C for 1 min; followed by a final extension step of 7 min at 72°C. The PCR product was purified from agarose gel with Wizard® SV Gel and PCR Clean-up System (Promega) and sequenced by commercial services. All sequence chromatograms were visually checked for quality and consistency before sequences were assembled and aligned.

## Results

### Glufosinate resistance

As expected, the $S$ plants were well controlled with glufosinate (Fig. 1). In contrast, much higher rates of glufosinate were required to cause substantial mortality for resistant ($R$ and $R^*$) plants. The plants became dark grey from the middle of the leaves to the leaf tip, almost burnt-like, with slight wilting, 24 h after treatment. The damaged area then extended in the basipetal direction, developing necrosis over 14 days, turning wilted leaves from yellow into brown. While the $S$ plants die, the $R$ and $R^*$ plants were observed to recover and grow again, 2 weeks after treatment. The glufosinate $LD_{50}$ for the $R$ population was 820 g $ha^{-1}$ as compared with 58 g $ha^{-1}$ for the $S$ population (Table 1), giving a $LD_{50}$ $R/S$ ratio of 14. This is slightly higher than the previously reported $LD_{50}$ $R/S$ ratio of 7.6 (Jalaludin *et al.*, 2010). The difference may be due to different susceptible populations and experimental conditions used in the two studies. The glufosinate $GR_{50}$ for $R$ population was found to be 156 g $ha^{-1}$, which was about 5-fold greater than for

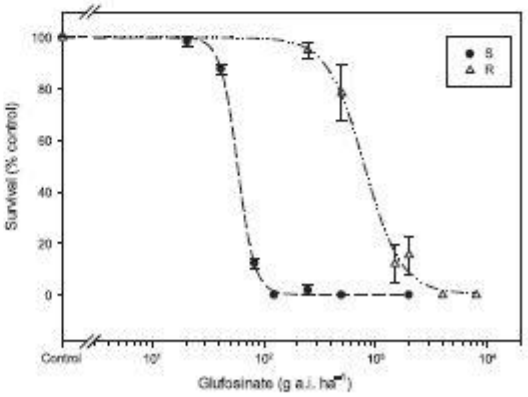


**Fig. 1** Glufosinate dose–response for survival of the susceptible ($S$) population and resistant ($R$) populations of *Eleusine indica*. Data were collected at 21 DAT.

**Table 1** Parameter estimates for logistic analysis of glufosinate, glyphosate and paraquat dose–response survival data for the susceptible (*S*) and resistant (*R*) *Eleusine indica* populations

| Population | *a* | *b* | $x_0$ – $LD_{50}$ (g a.i. $ha^{-1}$) | $R^2$ (coefficient) | *R*/*S* ratio of $LD_{50}$ |
|---|---|---|---|---|---|
| Glufosinate dose–response | | | | | |
| *S* | 100.00 (0) | 5.71 (0.23) | 58 (0.81) | 0.99 | |
| *R* | 100.00 (0) | 2.42 (0.37) | 820 (85.6) | 0.93 | 14 |
| †*R** | 100.00 (0) | 2.3 (0.25) | 1278 (63.9) | 0.99 | 22 |
| Glyphosate dose–response | | | | | |
| *S* | 100.00 (0) | 15.28 (1.71) | 148 (1.81) | 0.98 | |
| †‡*R** | 100.00 (0) | 0.99 (0.1) | 21 274 (1773) | 0.98 | 144 |
| Paraquat dose–response | | | | | |
| *S* | 100.00 (0) | 3.76 (0.66) | 98 (23.6) | 0.97 | |
| †*R** | 100.00 (0) | 1.5 (0.2) | 292 (27.9) | 0.94 | 3 |

Standard errors are in parentheses.
†*R** refers to the selected glufosinate-resistant subpopulation.
‡*R** Glyphosate $LD_{50}$ is in g a.e. $ha^{-1}$.

the *S* population (Table 2). The selected *R** population (the progeny of plants surviving glufosinate rates of 1485 and 1980 g $ha^{-1}$) was only about 2-fold more resistant to glufosinate relative to the original *R* population (Fig. 2A, Tables 1 and 2), indicating the glufosinate-resistant subpopulation is still segregating.

### *Glyphosate resistance*

As expected, the *S* population was susceptible to glyphosate, with 100% mortality at the glyphosate rate of 200 g $ha^{-1}$ (Fig. 2B). However, the glufosinate-resistant subpopulation *R** was found to be highly resistant to glyphosate, requiring an extremely high rate (25 920 g $ha^{-1}$) to cause substantial mortality (Fig. 2B). Based on the $LD_{50}$ *R*/*S* ratio, the *R** population was more than 144-fold resistant to glyphosate (Table 1). While the *R** plants survived high glyphosate doses, their growth was affected. The $GR_{50}$ for the *R** and *S* populations were 481 and 41 g $ha^{-1}$, respectively, resulting in the *R** population being 12-fold more resistant than the *S* population (Table 2). Therefore, in addition to glufosinate resistance, this *R** population had a high level of glyphosate resistance.

### *Paraquat resistance*

The *S* population was, as expected, well controlled by paraquat at 375 g $ha^{-1}$, whereas control of the *R* population required higher rates (Fig. 2C). Both *S* and *R** plants displayed rapid desiccation and necrosis following treatment. Similar to glufosinate-treated plants, the *R** plants recovered 2 weeks after treatment, while the *S* plants died. Based on the $LD_{50}$ or $GR_{50}$ *R*/*S* ratio (Tables 1 and 2), paraquat resistance in the purified glufosinate-resistant population was confirmed,

**Table 2** Parameter estimates for logistic analysis of glufosinate, glyphosate and paraquat dose–response biomass data for the susceptible (*S*) and resistant (*R*) *Eleusine indica* populations

| Population | *a* | *b* | $x_0$ – $GR_{50}$ (g a.i. $ha^{-1}$) | $R^2$ (coefficient) | *R*/*S* ratio of $GR_{50}$ |
|---|---|---|---|---|---|
| Glufosinate dose–response | | | | | |
| *S* | 100.00 (0) | 2.23 (0.36) | 31 (2.3) | 0.94 | |
| *R* | 100.00 (0) | 1.36 (0.17) | 156 (17.4) | 0.98 | 5 |
| †*R** | 100.00 (0) | 1.25 (0.25) | 325 (37.1) | 0.98 | 11 |
| Glyphosate dose–response | | | | | |
| *S* | 100.00 (0) | 1.7 (0.22) | 41 (3.6) | 0.92 | |
| †‡*R** | 100.00 (0) | 0.88 (0.09) | 481 (55.6) | 0.95 | 11.8 |
| Paraquat dose–response | | | | | |
| *S* | 100.00 (0) | 3.22 (0.72) | 52 (3.1) | 0.95 | |
| †*R** | 100.00 (0) | 1.84 (0.29) | 105 (8.4) | 0.96 | 2 |

Standard errors are in parentheses.
†*R** refers to the selected glufosinate-resistant subpopulation.
‡*R** Glyphosate $GR_{50}$ is in g a.e. $ha^{-1}$.

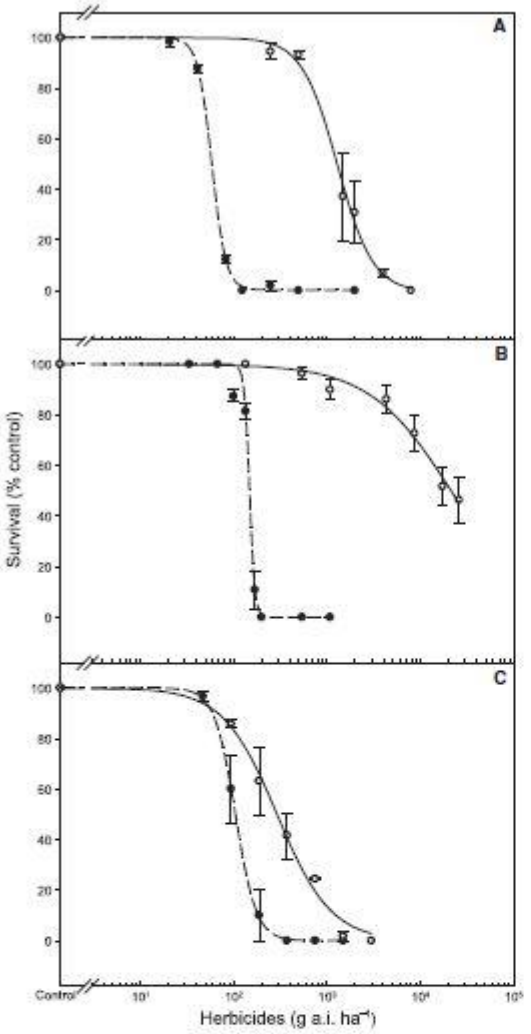


**Fig. 2** Survival response of the susceptible (closed circle; •) and selected glufosinate-resistant (opened circle; ○) *R** subpopulations of *Eleusine indica* to glufosinate (A), glyphosate (B) and paraquat (C) treatment. Data were collected at 21 DAT. Glyphosate rates are in g a.e. $ha^{-1}$.

albeit at a low level (2- to 3-fold in relation to the used rate).

### *Resistance to ACCase-inhibiting herbicides*

All ACCase herbicides examined (Table 3) caused 100% mortality in the *S* population at the respective rates used. However, there was about 50% of the *R** population surviving haloxyfop-P-methyl, fluazifop-P-butyl or butroxydim. In contrast, the *R** population remained susceptible to sethoxydim, clethodim and imazapyr (Table 3).

**Table 3** Percentage survival of the susceptible (*S*) and selected glufosinate-resistant (*R**) subpopulations of *Eleusine indica* 21 days after treatment with various herbicides

| | Mean % survival | |
|---|---|---|
| Herbicide | *S* | *R** |
| ACCase inhibitor | | |
| Fluazifop-P-butyl (210 g a.i. $ha^{-1}$) | 0 | 47 |
| Haloxyfop-P-methyl (60 g a.i. $ha^{-1}$) | 0 | 51 |
| Sethoxydim (230 g a.i. $ha^{-1}$) | 0 | 0 |
| Clethodim (100 g a.i. $ha^{-1}$) | 0 | 0 |
| Butroxydim (100 g a.i. $ha^{-1}$) | 0 | 49 |
| ALS inhibitor | | |
| Imazapyr (50 g a.i. $ha^{-1}$) | 0 | 0 |

### *ACCase gene sequencing*

The plastidic ACCase gene sequences from a total of nine individual plants surviving fluazifop-P-butyl or butroxydim were analysed in comparison with those of the susceptible plants. The primer pair ELEIN1781F/ELEIN1781R (Osuna *et al.*, 2012) amplified a 600 bp DNA fragment covering the known mutation site 1781, and the primer pair ELEIN2027f/ELEIN2027r amplified an 832 bp fragment with the known mutation sites 1999, 2027, 2041, 2078, 2088 and 2096. Sequence alignment revealed an amino acid substitution of Trp-2027-Cys in *R* individuals, resulting from a G to T change at the third position of the Trp codon (TGG). The same mutation was also recently found in several other fluazifop-resistant *E. indica* populations in Malaysia (Cha *et al.*, 2014). Generally, this mutation has been known to confer resistance to ACCase-inhibiting aryloxyphenoxypropionate herbicides (e.g. diclofop-methyl, fluazifop-P-butyl, haloxyfop-P-methyl) (Délye, 2005; Powles & Yu, 2010). However, it also confers resistance to ACCase-inhibiting cyclohexanedione herbicides, for example tralkoxydim in wild oats (Liu *et al.*, 2007). As the frequency of resistance to haloxyfop-P-methyl, fluazifop-P-butyl and butroxydim is close to each other (around 50%, Table 3), it is very likely that the Trp-2027-Cys mutation confers resistance to these three herbicides. Thus, this is the first case associating the Trp-2027-Cys mutation with butroxydim resistance at the rate used.

## Discussion

In this study, we confirmed the preliminary report on the evolution of resistance to glufosinate in a Malaysian *E. indica* population (Jalaludin *et al.*, 2010). The level of glufosinate resistance determined for this population was modest (5- and 14-fold, based on $GR_{50}$ and $LD_{50}$, respectively), which is similar to the

glufosinate-resistant *E. indica* population reported by Chuah *et al.* (2010) ($GR_{50}$ *R*/*S* ratio 3.4), and slightly higher than glufosinate-resistant *Lolium perenne* populations in Oregon, USA ($GR_{50}$ *R*/*S* ratios between 2.2–2.8) (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia *et al.*, 2012). The level of paraquat resistance in this population was also similar to that observed in a glufosinate- and paraquat-resistant Malaysian *E. indica* population (Chuah *et al.*, 2010). It is worth noting that usually $GR_{50}$ *R*/*S* ratios are more variable than $LD_{50}$ ratios, due to variations in growth conditions and especially, the length of experiments. In this sense, $LD_{50}$ *R*/*S* ratios would be the better option for comparing results across research groups.

Currently, documented glufosinate resistance evolution is confined to a few *E. indica* (Chuah *et al.*, 2010; Jalaludin *et al.*, 2010) and *L. perenne* populations (Avila-Garcia & Mallory-Smith, 2011; Avila-Garcia *et al.*, 2012), and all exhibit low to moderate levels of glufosinate resistance. Few resistance mechanisms studies have been undertaken. In resistant *L. perenne* populations, the resistance mechanism in one population was non-target-site based (Avila-Garcia & Mallory-Smith, 2011), while in another population it was due to a target-site mutation in the glutamine synthetase gene (Avila-Garcia *et al.*, 2012). We have commenced glufosinate resistance mechanism studies with this population.

Importantly, in addition to glufosinate resistance, individuals in this *E. indica* population were also highly resistant to glyphosate (Fig. 2B; Tables 1 and 2). Resistant plants survived very high glyphosate rates but suffered growth reduction, resulting in an *R*/*S* $LD_{50}$ ratio (144) much higher than the *R*/*S* $GR_{50}$ ratio (12). The *R*/*S* ratios based on survival and plant biomass were both higher than any previously reported evolved glyphosate resistance in any weed species (Lee & Ngim, 2000; Baerson *et al.*, 2002; Culpepper *et al.*, 2006; Mueller *et al.*, 2011; Gaines *et al.*, 2012). As is discussed above, we consider the $LD_{50}$ value is more accurate and meaningful in describing resistance levels, because it is less affected by experimental conditionals (e.g. harvest time, growth competition) as compared with the $GR_{50}$ value. Nevertheless, the large difference in the *R*/*S* $LD_{50}$ and $GR_{50}$ ratio obtained for glyphosate response in this *E. indica* population indicates that the potential glyphosate resistance mechanism(s) may incur fitness cost in the presence of herbicide. This unusually high-level glyphosate resistance needs investigation. A few possible mechanism(s) are (i) a new target-site EPSPS mutation, (ii) multiple EPSPS mutations and (iii) accumulation of several known glyphosate resistance mechanisms (e.g. EPSPS gene mutation or amplification, reduced glyphosate translocation or enhanced sequestration). We have initiated studies to reveal the mechanistic basis of this very high level of glyphosate resistance.

Multiple resistance in *E. indica* has been reported previously. These multiple resistance cases encompass at most, two different herbicide groups at any one time, for example fluazifop-P-butyl and glyphosate (Heap, 2013) or glufosinate and paraquat (Chuah *et al.*, 2010). However, the current study is the first case where multiple resistance across four dissimilar herbicide groups, glufosinate, glyphosate, paraquat and ACCase inhibitor herbicides, is present in a single *E. indica* population. This is likely related to the herbicide selection history of this population (involving application of at least paraquat, fluazifop-P-butyl, glyphosate and up to 12 glufosinate applications per year). As resistance to glyphosate, paraquat and ACCase-inhibiting herbicides was detected from a purified glufosinate-resistant subpopulation, it is very likely (although not examined) that multiple resistance is also displayed at the individual level. Multiple resistance to glyphosate, paraquat and ACCase-inhibiting herbicides in individual plants has been documented in *Lolium rigidum* L. due to accumulation of multiple resistance mechanisms (Yu *et al.*, 2007). This is the first global report of a weed species with evolved resistance across all three of the world's non-selective herbicides (glufosinate, glyphosate and paraquat). It is an unavoidable consequence of the selection pressures resulting from over-reliance on herbicides for weed control. Herbicides should be used wisely (e.g. in rotation or mixture) and in combination with other non-chemical control options.

In summary, we have confirmed in an *E. indica* population the first case of multiple resistance across the three non-selective herbicides, glufosinate, glyphosate and paraquat. The same population also showed target-site resistance to ACCase-inhibiting herbicides, likely due to the Trp-2027-Cys mutation. The evolution of multiple resistance to herbicides across four different modes of action in this resistance-prone species is worrying, as it threatens the world's most important herbicide (glyphosate) and its alternatives (glufosinate, paraquat) and results in greatly reduced herbicide control options for the grower. Although other ACCase-or ALS-inhibiting herbicides (e.g. sethoxydim, clethodim, imazapyr) still provide effective short-term control options, in the long run, additional diversity in weed control must be added, to limit seed set of resistant *E. indica* plants.

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# Evolution of a Double Amino Acid Substitution in the 5-Enolpyruvylshikimate-3-Phosphate Synthase in *Eleusine indica* Conferring High-Level Glyphosate Resistance[1]

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Glyphosate is the most important and widely used herbicide in world agriculture. Intensive glyphosate selection has resulted in the widespread evolution of glyphosate-resistant weed populations, threatening the sustainability of this valuable once-in-a-century agrochemical. Field-evolved glyphosate resistance due to known resistance mechanisms is generally low to modest. Here, working with a highly glyphosate-resistant *Eleusine indica* population, we identified a double amino acid substitution (T102I + P106S [TIPS]) in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene in glyphosate-resistant individuals. This TIPS mutation recreates the biotechnology-engineered commercial first generation glyphosate-tolerant EPSPS in corn (*Zea mays*) and now in other crops. In *E. indica*, the naturally evolved TIPS mutants are highly (more than 180-fold) resistant to glyphosate compared with the wild type and more resistant (more than 32-fold) than the previously known P106S mutants. The *E. indica* TIPS EPSPS showed very high-level (2,647-fold) in vitro resistance to glyphosate relative to the wild type and is more resistant (600-fold) than the P106S variant. The evolution of the TIPS mutation in crop fields under glyphosate selection is likely a sequential event, with the P106S mutation being selected first and fixed, followed by the T102I mutation to create the highly resistant TIPS EPSPS. The sequential evolution of the TIPS mutation endowing high-level glyphosate resistance is an important mechanism by which plants adapt to intense herbicide selection and a dramatic example of evolution in action.

Modern herbicides make major contributions to global food production by easily removing weeds while maintaining sustainable soil conservation practices. However, persistent herbicide selection of huge weed numbers across vast areas has resulted in the widespread evolution of herbicide-resistant weed populations. Worldwide, there are currently more than 449 unique cases of herbicide resistance, with about 11 new cases reported annually, on average (Heap, 2015). Target site resistance due to target gene mutation is one of the major mechanisms enabling resistance evolution (Gressel, 2002; Powles and Yu, 2010).

The most important and globally used herbicide in crop fields is glyphosate (Duke and Powles, 2008). Glyphosate disrupts the shikimate pathway by specifically inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; Steinrücken and Amrhein, 1980). Glyphosate resistance was initially considered to be unlikely to evolve in nature based on the facts that intentional selection for glyphosate tolerance using whole plants and cell/tissue culture was unsuccessful, and laboratory-generated highly resistant EPSPS mutants displayed undesirable enzyme kinetics (Bradshaw et al., 1997; for review, see Pline-Srnic, 2006). This seemed to be true, as resistance was not found during the first 15 years of glyphosate use, primarily as a nonselective herbicide. However, unprecedented intensive glyphosate use for controlling large numbers of weeds over massive areas, especially after the introduction of glyphosate-resistant transgenic crops, imposed high selection pressure on weeds, resulting in the evolution of glyphosate resistance in populations of 32 weed species (Heap, 2015). Since the first identification of a resistance-endowing EPSPS point mutation, P106S, in a glyphosate-resistant *Eleusine indica* population (Baerson et al., 2002), several other resistance-endowing single-amino acid substitutions at P106 (P106T, P106A, and P106L) have been reported in glyphosate-resistant weeds (e.g. Ng et al., 2004; Yu et al., 2007; Kaundun et al., 2011; for review, see Sammons and Gaines, 2014). These single-codon EPSPS resistance mutations only endow low-level glyphosate resistance (2- to 3-fold the recommended rates). This is not surprising, because glyphosate is a competitive inhibitor of the second substrate, phosphoenolpyruvate (PEP; Boocock and Coggins, 1983), and is considered a transition state mimic of the catalyzed reaction course (Schönbrunn et al., 2001). Indeed, highly glyphosate-resistant EPSPS variants (e.g. mutants at G101 or T102) have greatly increased $K_m$ values (decreased affinity) for PEP when expressed in *Escherichia coli* (Eschenburg et al., 2002; Funke et al., 2009; for review, see Sammons and

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Gaines, 2014). In contrast, P106 substitutions confer weak glyphosate resistance but preserve adequate EPSPS functionality (Healy-Fried et al., 2007; for review, see Sammons and Gaines, 2014). Aside from P106 EPSPS gene mutations, there are other glyphosate resistance mechanisms, including EPSPS gene amplification and nontarget-site reduced glyphosate translocation/nontarget-site increased vacuole sequestration (Lorraine-Colwill et al., 2002; Gaines et al., 2010; Ge et al., 2010; for review, see Powles and Preston, 2006; Shaner, 2009; Powles and Yu, 2010; Sammons and Gaines, 2014). Generally, each of these mechanisms endows moderate-level (4- to 8-fold the recommended rates) glyphosate resistance.

Low-level glyphosate resistance due to the EPSPS P106 mutations was reported in Malaysian *E. indica* (Baerson et al., 2002; Ng et al., 2004). Recently, we reported a highly (more than 10-fold the recommended rates) glyphosate-resistant Malaysian *E. indica* population (Jalaludin et al., 2015). This paper investigates the high-level glyphosate resistance in this population, and is, to our knowledge, the first to reveal the sequential evolution of a double amino acid substitution in EPSPS.

## RESULTS

### EPSPS Gene Sequencing Revealed a Double Amino Acid Substitution in EPSPS

To identify the basis of very high-level glyphosate resistance, a 301-bp EPSPS DNA fragment covering the highly conserved region ($^{95}$LFLGNAGTAMRPL$^{107}$) of the EPSPS gene was analyzed from 43 resistant plants. These resistant individuals were found to have the known weak resistance mutation at codon 106 (CCA to TCA), but importantly, also display a very rare mutation at codon 102 (ACT to ATT). Therefore, in this naturally evolved, highly glyphosate-resistant *E. indica* population, there are two resistance-endowing EPSPS amino acid substitutions, T102I and P106S. Cloning of the EPSPS complementary DNA (cDNA) fragment covering the 102 and 106 codons from resistant individuals revealed the two mutations were always present in the same EPSPS gene fragment, confirming the double amino acid substitution in a single EPSPS allele. This double amino acid substitution, T102I + P106S, is hereinafter referred to as the TIPS mutation. Based on the sequence information obtained, derived cleaved amplified polymorphic sequence (dCAPS) markers for the T102I and P106S mutations were developed (Supplemental Fig. S1). Analysis (by the dCAPS markers and sequencing) of 193 individuals (untreated) in the resistant population (Table I) revealed that 84% of the individuals are resistant mutants and 16% are the wild type. Only a very small percentage (1.6%) of the plants analyzed were homozygous mutants for the TIPS mutation (referred to as the RR genotype), about 30% were homozygous solely for the P106S mutation (rr), and nearly one-half were the resistant mutants of Rr. Importantly, neither the single T102I mutants, heterozygous P106S single mutants (r/wild type), nor heterozygous TIPS mutants (R/wild type) were found from the samples analyzed. Therefore, only three alleles (R, r, and the wild type) were found in the samples examined, and the frequency of the mutant TIPS allele (R) is only one-half of the P106S allele (r). To better understand the resistance allele frequencies, a more detailed analysis of the field population together with herbicide histories is needed. The full EPSPS cDNA sequences (1,338 bp) were compared among individuals of the wild type, P106S, and the TIPS mutant (GenBand accession no. KM078728). Except for the single-nucleotide polymorphisms at the 102 and 106 codons, there was only one single-nucleotide polymorphism that resulted in an amino acid change, a P381 in wild-type individuals, but an L381 in mutant individuals. However, blast results showed that this amino acid residue is not conserved in plant EPSPS, and the P381L mutation has been previously proven to be irrelevant to glyphosate resistance in *E. indica* (Baerson et al., 2002).

### Plants Homozygous for the TIPS Mutation Displayed High-Level Glyphosate Resistance

To characterize the glyphosate-resistant genotypes, from within the resistant population, we produced three purified subpopulations with individuals homozygous for the wild type, P106S, and TIPS EPSPS, respectively, and conducted detailed glyphosate dose response studies. To examine the possible involvement of any other glyphosate resistance mechanisms in these purified subpopulations, an herbicide-susceptible (S) *E. indica* population was also included as a further reference. The S and wild-type populations were found to be identically fully susceptible to glyphosate (Table II; Figs. 1, A and B and 2), indicating no major additional glyphosate resistance mechanisms present in the purified subpopulations.

**Table I.** *Genotype and allele frequencies determined for 193 E. indica individuals by the dCAPS method developed for the T102I and P106S mutations (see Supplemental Fig. S1)*

| Genotypes | No. of Individuals Detected | Genotype Frequency | Alleles | Allele Frequency |
|---|---|---|---|---|
| | | % | | % |
| RR | 3 | 1.6 | 102I-106S (R) | 26 |
| rr | 65 | 34 | T102-106S(r) | 58 |
| Wild type | 31 | 16 | T102-P106 (wild type) | 16 |
| Rr | 94 | 49 | | |
| R/wild type | 0 | 0 | | |
| r/wild type | 0 | 0 | | |

**Table II.** *Parameter estimates of the nonlinear regression analysis (the logistic 3-parameter model) of herbicide rates causing 50% plant mortality ($LD_{50}$) or growth reduction ($GR_{50}$) for glyphosate-susceptible (S), EPSPS wild-type, and homozygous P106S and TIPS EPSPS E. indica mutants*

SE is in parentheses.

| Genotype | *a* | *b* | $X_0$ g ha$^{-1}$ | *P* Value for $X_0$ | Ratio to S of $X_0$ |
|---|---|---|---|---|---|
| $LD_{50}$ | | | | | |
| S | 99.7 (0.22) | 8.71 (1.61) | 142 (1.3) | <0.0001 | 1 |
| Wild type | 99.7 (0.22) | 8.71 (1.61) | 142 (1.3) | <0.0001 | 1 |
| P106S | 101 (1.78) | 3.14 (0.78) | 798 (29) | <0.0001 | 5.6 |
| TIPS | | | >25,900 | | >182 |
| $GR_{50}$ | | | | | |
| S | 98.3 (8.42) | 1.65 (0.43) | 65 (8.0) | 0.0013 | 1 |
| Wild type | 97.8 (6.12) | 1.98 (0.37) | 57 (10.8) | 0.0063 | 0.88 |
| P106S | 100 (1.96) | 1.76 (0.12) | 173 (7.3) | <0.0001 | 2.67 |
| TIPS | 99.8 (3.87) | 0.85 (0.08) | 2,023 (299) | 0.0005 | 31.1 |

As expected, the P106S population is only moderately resistant to glyphosate, with 30% survival at the recommended field rate. Conversely, homozygous TIPS mutant plants were found to be highly glyphosate resistant, such that an $LD_{50}$ could not be determined and therefore must be greater than the highest glyphosate rate used (25,900 g ha$^{-1}$; Fig. 1A; Supplemental Fig. S2). Based on the glyphosate $LD_{50}$ ratios (Table II), homozygous TIPS mutants are highly (more than 180-fold) resistant, whereas, as expected, homozygous P106S mutants are only moderately (5.6-fold) resistant. The homozygous TIPS plants, therefore, can tolerate more than 20-fold the recommended glyphosate rate of 1,080 g ha$^{-1}$. Although the TIPS mutants survived high glyphosate doses, their growth was affected (Fig. 1B), resulting in a lower glyphosate $GR_{50}$ ratio than the $LD_{50}$ ratio (Table II).

### TIPS Encodes a Highly Glyphosate-Resistant EPSPS

To further characterize the TIPS mutation at the EPSPS level, the wild-type, P106S, and TIPS *E. indica* 5-enolpyruvylshikimate-3-phosphate synthase (EiEPSPS) were expressed in *E. coli*, and the activity and herbicide dose causing 50% in vitro inhibition ($IC_{50}$) of the His-tagged recombinant EiEPSPS variants were determined (Table III). As expected, based on glyphosate $IC_{50}$ ratios, the *E. coli*-expressed P106S variant is moderately (4.3-fold) resistant to glyphosate, whereas the TIPS variant is highly (2,647-fold) resistant (Fig. 3; Table III). These results confirm that TIPS EiEPSPS is essentially insensitive to glyphosate, with an $IC_{50}$ value of 54 mM, and is therefore responsible for very high-level glyphosate resistance as observed at the whole-plant level (Fig. 1). Notably, while incurring no significant changes in the $K_m$ (PEP), the *E. coli*-expressed TIPS variant displayed an EPSPS-specific activity greatly (16-fold) lower than the wild type (Table III), indicating a resistance cost at the enzyme level due to reduced catalytic efficiency.

## DISCUSSION

In the biotechnology search for glyphosate-tolerant crops, various EPSPS double mutations have been generated using site-directed mutagenesis and expressed in *E. coli* and plants (e.g. Spencer et al., 2000; Howe et al., 2002; Lebrun et al., 2003; Kahrizi et al., 2007; Alibhai et al., 2010). The P106 and then later the TIPS mutation were first found empirically in a mutational screen in *Salmonella* sp. (Comai et al., 1983; Stalker et al., 1985). The TIPS mutation was engineered into tobacco (*Nicotiana*

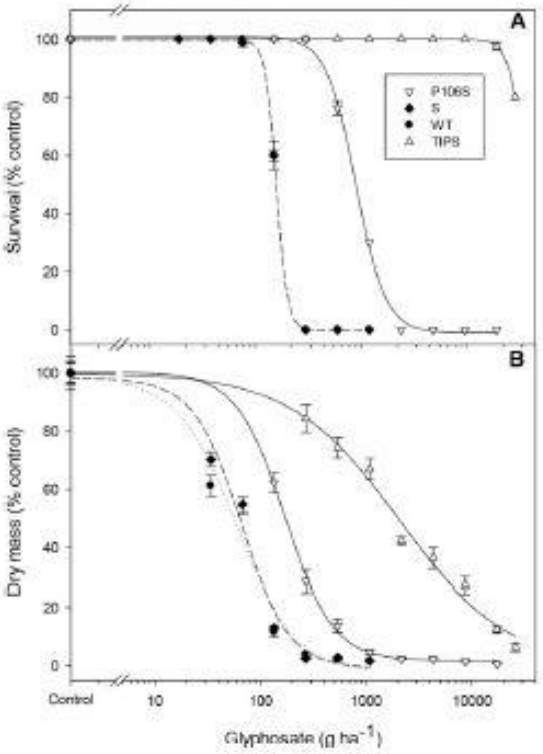


**Figure 1.** Glyphosate dose response: mortality (A) and dry mass (B) of susceptible (S), EPSPS wild type (WT), and homozygous P106S and TIPS mutant *E. indica*.

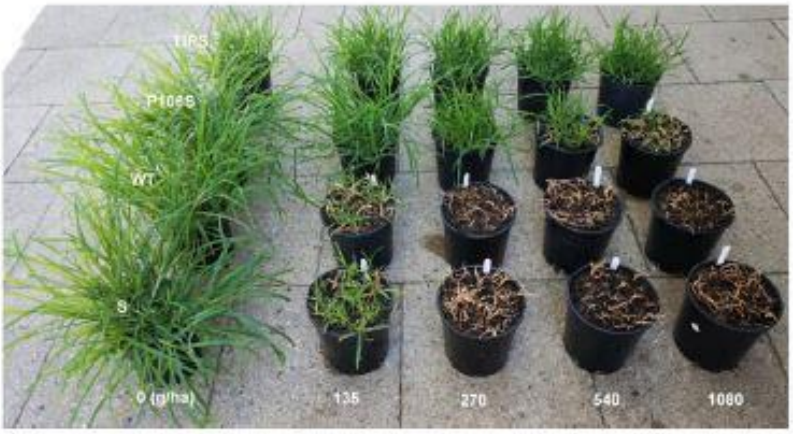


**Figure 2.** Glyphosate dose response of susceptible (S), EPSPS wild type (WT), homozygous P106S, and homozygous TIPS mutant *E. indica*.

*tabacum*; Arnaud et al., 1998) and field tested for glyphosate tolerance (Lebrun et al., 2003). The TIPS EPSPS was then used to produce the first generation commercially successful glyphosate-tolerant transgenic corn (GA21; Spencer et al., 2000). Here, we demonstrate that this TIPS mutation has now evolved in nature.

In target site resistance evolution for acetolactate synthase- and acetyl coenzyme A carboxylase-inhibiting herbicides, highly resistant yet fit individuals with single target site mutations are common (Vila-Aiub et al., 2009), as these herbicides have large binding sites in and adjacent to the enzyme catalytic site, resulting in contacts with amino acids that are nonessential for structure or function (Sammons et al., 2007; Powles and Yu, 2010). The most dramatic example is the multiple (more than 10) different amino acid substitutions at P197 in acetolactate synthase (Tranel and Wright, 2002; Tranel et al., 2015). The transition state inhibitor designation for glyphosate comes from the observation that PEP contacts 17 amino acids responsible for catalysis, which necessarily prevents any substitutions of these essential amino acids (Schönbrunn et al., 2001). The P106S EPSPS provides relatively low glyphosate resistance (Arnaud et al., 1998), whereas the T102I EPSPS alone endows high-level resistance, but with drastically decreased affinity for the second substrate, PEP (Kishore et al., 1992; Funke et al., 2009). The concomitant mutations at the 106 and 102 codons are merely adjacent to the active site and together make very small fractional Ångstrom modifications structurally to the EPSPS active site, therefore selectively impacting glyphosate binding more than PEP (Funke et al., 2009). Hence, the TIPS mutation endows high-level glyphosate resistance with acceptable affinity for PEP.

Multiple mutations of a single-pesticide target site gene are known in adaptive evolution of fungicide or insecticide resistance (Brunner et al., 2008; Karasov et al., 2010). Accumulation of multiple mutations in a single allele in insects and fungi can be achieved via intragenic recombination between preexisting resistant alleles in natural populations, in response to increased selective pressure (Mutero et al., 1994; Brunner et al., 2008). However, this is unlikely to occur in *E. indica*, as the single-codon mutation T102I was not detected in the resistant population, and is therefore unlikely to preexist in the population. Indeed, the very poor fitness of the kinetics of the T102I mutant enzyme (Alibhai et al., 2010) suggests this mutation would be unfit and even lethal when obtained alone. This lack of fitness of the T102I mutation explains why this single mutation has not been observed in nature.

The notion that compensatory mutations may require a particular evolution trajectory to prevent lethal mutants is discussed by Weinreich et al. (2006), where a series of five amino acid point mutations providing 100,000-fold resistance (compared with susceptible counterparts) to beta-lactamase in a matrix of combinations were studied

**Table III.** *Glyphosate $IC_{50}$ and EPSPS activity, $K_m$ (PEP) of E. coli-expressed EiEPSPS variants*

SE from the nonlinear regression analysis is in parentheses. The parameter estimates for $IC_{50}$ can be found in Supplemental Table S2. The $\chi^2$ for goodness of fit of the kinetic data ($V_{max}$ and $K_m$) is 0.8 ($\alpha = 0.05$).

| Genotype | $IC_{50}$ | Ratio to Wild Type | EPSPS $V_{max}$ | Ratio to Wild Type | $K_m$ (PEP) | Ratio to Wild Type |
|---|---|---|---|---|---|---|
| | μM | | nmol Pi $\mu g^{-1} min^{-1}$ | | μM | |
| Wild type | 20 (0.84) | 1 | 28.0 (0.84) | 1 | 11.6 (1.1) | 1 |
| P106S | 87 (2.15) | 4.3 | 27.5 (0.55) | 1 | 10.0 (0.68) | 0.9 |
| TIPS | 52,938 (1206) | 2,647 | 1.8 (0.04) | 0.06 | 9.8 (0.72) | 0.8 |

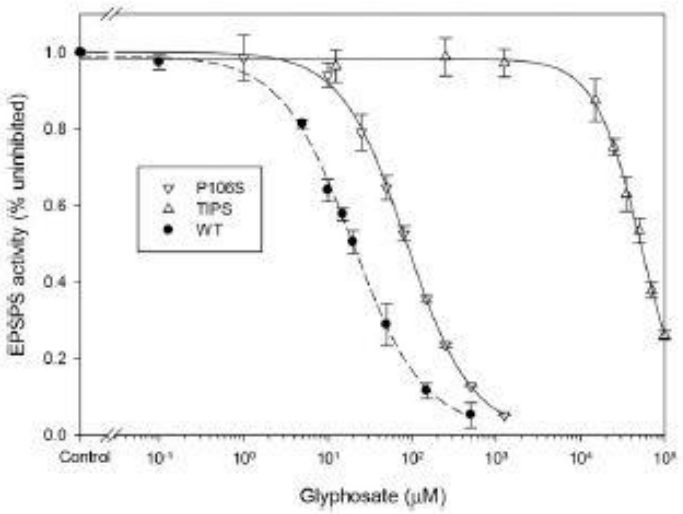


**Figure 3.** Glyphosate dose response of *E. coli*-expressed *E. indica* EPSPS variants.

to reveal a defined successful evolutionary pathway. Here, for the glyphosate-resistant *E. indica*, our data suggest that the TIPS evolved sequentially under intense glyphosate selection. First, the weak P106S mutation was selected, enriched, and reached homozygosity, and then was followed by the T102I mutation to create the highly resistant TIPS EPSPS. This TIPS EPSPS enables plants to survive high glyphosate rates. Indeed, many glyphosate-resistant *E. indica* populations in Malaysia and other countries have been found to possess mutations at P106 (Baerson et al., 2002; Ng et al., 2003, 2004; Kaundun et al., 2008), and in Malaysia, glyphosate was used frequently (every month) and continually (5–10 years) at increased glyphosate rates (0.72–1.92 kg $ha^{-1}$) to control *E. indica* (Ng et al., 2004). Therefore, evolution of at least the TIPS mutation can be expected from other glyphosate-resistant *E. indica* populations in Malaysia and other countries.

Will there be other EPSPS double mutations in nature? In addition to the TIPS mutant, various EPSPS double mutants at 102 and 106 were intentionally produced and the kinetics of *E. coli*-expressed EPSPS variants studied. Compared with the wild-type and T102I mutant alone, double mutants such as T102I + P106A, T102I + P106T, or T102L + P106A also show favorable kinetics comparable with or even better than TIPS (Alibhai et al., 2010; for review, see Sammons and Gaines, 2014). As various amino acid substitutions at P106 have been identified (e.g. P106A, P106S, P106T, or P106L) in glyphosate-resistant weed species (for review, see Sammons and Gaines, 2014), the evolution and selection for other EPSPS double variants is also possible where glyphosate selection is intense.

Does the decreased catalytic efficiency of TIPS EPSPS result in a whole-plant resistance cost due to possible impact on the shikimate pathway? The very low percentage


**Figure 4.** Reduced growth of the homozygous EPSPS TIPS mutants, as compared with the wild-type (WT) and homozygous P106S mutants. *E. indica* plants are 5 weeks after transplanting.

(1.6%) of resistant individuals homozygous for the TIPS EPSPS (RR) as compared with the higher percentage (49%) of resistant individuals of the Rr genotype (Table I) may suggest: (1) the additional T102I mutation is a recent event, and given that *E. indica* is a self-pollinated species, homozygosity at 102 can be increased in a few generations; and/or (2) a significant resistance cost is associated with homozygous TIPS mutants when glyphosate selection is relaxed. The latter correlates with the measured low catalytic turnover of the *E. coli*-expressed TIPS EiEPSPS ($V_{max}$ in Table III). This decreased catalytic efficiency then translates to the significantly reduced plant growth (Fig. 4, above-ground dry weight per plant of the wild type [4.14 ± 0.24 g], P106S [4.06 ± 0.34 g], and TIPS [1.29 ± 0.05 g; $n$ = 20–25]) and fecundity we have observed for the homozygous TIPS plants (data not shown). Consequently, RR TIPS mutants are outperformed over time by Rr TIPS mutants, which may suffer less or little fitness cost and therefore proliferate in the population. Due to predominant self pollination in *E. indica*, a very low level of outcrossing (if any) between wild-type and homozygous P106S (rr) mutants may produce a small number of heterozygous P106S (r/wild-type) mutants. However, as expected, these individuals are unable to survive the field or higher glyphosate rates, and hence are selected against. This would explain why the r/wild-type mutants were not detected in the resistant population. We have fitness studies underway with the wild-type, P106S (rr), and TIPS (RR, Rr) EPSPS mutants. However, if this fitness cost from decreased catalytic efficiency is offset, for instance, by gene duplication of the TIPS gene as required for commercial crops (CaJacob et al., 2004), then evolution of Roundup Ready-like *E. indica* may be expected in nature, especially in species exhibiting EPSPS gene amplification (for review, see Sammons and Gaines, 2014), where the tandem repeat nature of the duplication (Jugulam et al., 2014) may facilitate incorporation of the necessary point mutations, and gene duplication is free of fitness cost (Vila-Aiub et al., 2014).

Therefore, the evolutionary recipe to high-level glyphosate resistance in weedy plant species under glyphosate selection may have these primary components: (1) overexpression of EPSPS, as already reported in four weed species with gene duplication; (2) P106S (or T/A) EPSPS, as documented in six weed species (for review, see Sammons and Gaines, 2014); (3) acquiring the second EPSPS T102I mutation, as described here for the first time; and (4) combining with other glyphosate resistance-endowing mechanisms that would have an additive impact on the resistance magnitude, as demonstrated in glyphosate-resistant *Lolium rigidum* (Yu et al., 2007; Ge et al., 2012; Nandula et al., 2013).

In summary, this research is, to our knowledge, the first to report the evolution of an EPSPS double mutation (TIPS) conferring very high-level glyphosate resistance in crop fields. The TIPS mutation mimics the biotechnology-derived glyphosate-tolerant EPSPS, demonstrating that laboratory and field selection methods are linked. This is a dramatic manifestation of the power of evolution in action and how nature responds and adapts to manipulated environment stresses. This is also a very clear example that herbicide sustainability demands much greater diversity in weed control tactics than reliance on a single herbicide.

## MATERIALS AND METHODS

### Plant Material

The glyphosate-resistant *Eleusine indica* population was originally collected from several patches within a palm oil nursery in Jenantut, Malaysia (Jalaludin et al., 2010), and the resistant and susceptible populations used in the current study were further characterized by Jalaludin et al. (2015). Seeds were germinated on 0.6% (w/v) agar for 4 to 7 d, and germinating seedlings were transplanted into plastic pots (12–20 per pot) filled with potting mix (50% [v/v] moss peat and 50% [v/v] river sand) and grown in a greenhouse during the summer growing season (December–March) with an average day/night temperature of 30°C/24°C and a 13-h photoperiod under natural sunlight. When seedlings reached the three- to four-leaf stage, they were treated with various rates of commercial glyphosate using a cabinet sprayer with a spray volume of 112 L $ha^{-1}$ at a pressure of 200 kPa and a speed of 1 m $s^{-1}$. Each treatment contained at least three to four replicate pots. Visual assessment for mortality was made 3 to 4 weeks after treatment. Plants were recorded as alive if they were actively growing and tillering after herbicide treatment and as dead if there was little new growth and no new tiller formation.

### EPSPS Sequencing and cDNA Cloning

Genomic DNA was extracted from the leaf tissue of resistant and susceptible plants and total RNA isolated using the Plant RNeasy Mini Kit (Qiagen). Genomic DNA contamination was removed using the TURBO-DNA free kit (Ambion). For EPSPS DNA partial sequencing, a pair of published primers (Ng et al., 2003) was used to amplify a highly conserved region ($^{98}$LFLGNAGTAMRPL$^{107}$; refer to plant EPSPS numbering system) in which point mutations conferring glyphosate resistance in plants and bacteria have been found (Sammons and Gaines, 2014). The forward primer EleuEPSPS-F (5′-GCCGTAGTTGTTGGCTGTGTG-3′) and the reverse primer EleuEPSPS-R (5′-TCAATCCGACACCAAGTCGC-3′; Ng et al., 2003) amplify a 301-bp DNA (includes 99-bp intron) fragment covering the potential mutation sites. Using the same primer pairs, a 202-bp (without intron) cDNA fragment was amplified from pregenotyped plants and cloned into the pGEM-T vector (Promega) and transformed into *Escherichia coli*. White colonies with putative inserts were used as templates for PCR reamplification and sequencing of the 202-bp fragment. The PCR was conducted in a 25-μL volume that consisted of 1 to 2 μL of genomic DNA or cDNA, 0.5 μM each primer, and 12.5 μL of 2× GoTaq Green Master Mix (Promega). The PCR was run with the following profile: 94°C for 4 min; 40 cycles of 94°C for 30 s, 57°C (annealing temperature) for 30 s, and 72°C for 30 to 50 s; followed by a final extension step of 7 min at 72°C. For EPSPS full-cDNA sequencing, a 1,338-bp cDNA was amplified with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Inc.) using the primer pair (EiRT1 and EiLFT1; Baerson et al., 2002).

The PCR was conducted in a 20-μL volume that consisted of 1 μL (80 ng) of cDNA, 0.5 μM each primer, and 4 μL of 5× Phusion HF buffer, 200 μM deoxyribonucleotide triphosphate, and 0.2 μL of Phusion DNA Polymerase. The PCR was run with the following profile: 98°C for 30 s; 40 cycles of 98°C for 10 s, 72°C for 50 s; followed by a final extension step of 10 min at 72°C. The PCR product was purified from agarose gel as described earlier and sequenced with the primers (EiRT1 and EiLFT1) and an internal forward primer (5′-CTCTTCTTGGGGAATGCTGGA-3′; Kaundun et al., 2008).

The PCR product was purified from agarose gel with Wizard SV Gel and PCR Clean-up System (Promega) and sequenced by commercial services (LotteryWest, State Biomedical Facility Genomics). All sequence chromatograms were visually checked for quality and consistency before sequences were assembled and aligned.

### dCAPS Marker Development and Genotyping

Based on the EPSPS sequence information obtained from the susceptible (20 plants) and resistant samples (at least 80), we developed dCAPS markers for detecting mutation(s) at 102 and 106 using the Web-based dCAPS finder

version 2.0 software. The A-to-T mismatch was introduced in the forward primer *RsaIF* (5′-TGCAGCTCTTCTTGGGGAATGCTGCTA-3′) two nucleotides upstream of the 102 codon (i.e. N+2 position) to create a restriction site for *RsaI* (GTAC) in the wild-type sequence. Any nucleotide mutations resulting in substitution of the T102 would abolish the restriction site. Therefore, the primer pair *RsaIF* and *EluEPSPS-R* was used to amplify a 234-bp fragment that was then digested with *RsaI*. The wild-type sequence would generate a single digested 208-bp band, whereas the mutant sequence at 102 would generate an undigested 234-bp band (Supplemental Fig. S1. A heterozygous sequence at 102 would produce both the 208- and 234-bp bands (Supplemental Fig. S1). Similarly, a G-to-C mismatch was introduced in the forward primer *Sau96IF* (5′-CTCTTCTTGGGGAATGCTG-GAACTGCAATGGGA-3′) at the N+3 position of the 106 codon to create a restriction site for Sau96I (G↓GNCC) in the wild-type sequence. Any mutations resulting in substitution of the P106 would abolish the restriction site. Therefore, the primer pair *Sau96IF* and *EluEPSPS-R* was used to amplify a 233-bp fragment that was then digested with Sau96I. The wild-type sequence would produce a single digested 202-bp band, whereas the mutant sequence at 106 would produce an undigested 233-bp band (Supplemental Fig. S1). A heterozygous sequence at 106 would produce both the 202- and 233-bp bands (Supplemental Fig. S1; no heterozygous sequences at 106 were detected). PCR conditions were similar to those described earlier except that the annealing temperature was 62°C. Restriction digestions were carried out according to the manufacturer's recommendations (New England Biolabs), and digestion patterns were viewed on 2% (w/v) agarose gels (electrophoresis at 90–100 V for 50–80 min) stained with ethidium bromide. The accuracy of the two markers was confirmed by comparing sequencing and marker analysis results of over 40 samples.

It is noticed that the two dCAPS markers were designed to only detect mutant and wild-type sequences at the 102 and 106 codons without knowing the nature of the specific mutation. We confirmed that the resistant population only possessed the TIPS mutations, so the two dCAPS markers can be used for genotyping in the population. If used in other uncharacterized *E. indica* populations, the specific mutations have to be determined by sequencing.

## Generation of Purified Subpopulations

Plants (seven to 12) that were confirmed by sequencing and marker analysis to be homozygous for the wild type, P106S, or TIPS mutation were bulk selfed in isolation to produce seeds to enable respective subpopulations. Progeny plants (10–12) from each of these purified subpopulations were randomly marker analyzed to confirm their genotype and homozygosity prior to use for subsequent glyphosate dose response.

## *E. coli* Transformation

Total RNA was isolated from the *E. indica* (hereafter known as Ei) P106S EPSPS mutant cDNA was synthesized, and the mature P106S EiEPSPS cassette was PCR amplified using the primer pair 1 and 2 (Supplemental Table S1; Baerson et al., 2002). The PCR product was inserted into the pCR Blunt II TOPO vector and verified by sequencing. The P106S EiEPSPS was converted to the wild type (primer pair 3 and 4, Supplemental Table S1) and the double mutant TIPS EiEPSPS (primer pair 5 and 6, Supplemental Table S1) using the Phusion site-directed mutagenesis kit (Thermo Scientific). After sequencing verification, these three genes were PCR amplified using the primer pair 7 and 8, and the PCR products were digested by *NdeI* and then inserted into the *NdeI* site of pET19-b vector to form an N-terminal His-tag fusion to facilitate the downstream purification of the enzymes.

## EPSPS Purification and Activity Assay

The BL21 (DE3) cells (Invitrogen) harboring the EiEPSPS constructs in pET-19b vector were cultured in the MagicMedia *E. coli* Expression Medium (Invitrogen) according to the dual temperature protocol provided by the media supplier. Soluble proteins were extracted from frozen cells using the B-per bacterium extraction reagent supplied with DNase I, lysozyme, and Halt Protease inhibitor cocktail (Thermo Scientific). After centrifugation at 21,000*g* for 5 min, the supernatant fraction was used to purify His-tagged EPSPS with HisPur Ni-NTA resin (Thermo Scientific). The binding buffer was made of 20 mM Tris (pH 8.0), 500 mM NaCl, 10 mM imidazole, and 0.05% (v/v) Triton X-100; washing buffer contained 20 mM Tris (pH 8.0), 500 mM NaCl, 50 mM imidazole, and 0.05% Triton X-100; and elution buffer contained 20 mM Tris (pH 8.0), 500 mM NaCl, and 500 mM imidazole. The eluted enzyme was kept in storage buffer (10 mM MOPS, 10% [v/v] glycerin, 0.5 mM EDTA, 2.5 mM beta-mercaptoethanol) after buffer exchange using Amicon Ultra 0.5-mL centrifugal filters (MWCO 30 kD) three times for 5 min at 14,000*g*. The protein content was quantified using the Pierce 660 nm Protein Assay kit (Thermo Scientific), and purification results were analyzed by SDS-PAGE electrophoresis.

Activity of purified EPSPS variants was measured by the coupled assay that measures continuous release of inorganic phosphate using the EnzChek Phosphate Assay Kit (E-6646, Invitrogen; Gaines et al., 2010). For $IC_{50}$ measurement of EiEPSPS variants, various concentrations of glyphosate were used (wild type, 0, 0.1, 5, 10, 20, 50, 150, and 500 μM; P106S, 0, 1, 10, 25, 50, 80, 150, 500, and 1,250 μM; and TIPS, 0, 12.5, 1,250, 15,000, 25,000, 35,000, 50,000, 70,000, and 100,000 μM), holding shikimate 3-phosphate constant at 0.1 mM. To measure the $K_m$ (PEP) of the EiEPSPS variants, shikimate 3-phosphate concentration was fixed at 0.1 mM, and various amounts of PEP (2.5, 5, 10, 15, 20, 25, 40, 60, and 80 μM) were used.

## Statistics

The $LD_{50}$, $GR_{50}$, or $IC_{50}$ was estimated by nonlinear regression using the three-parameter logistic curve model $y = a/[1 + (X/X_0)^b]$, where $a$ is the maximum plant response close to untreated controls, $X_0$ is the dose giving 50% response, and $b$ is the slope around $X_0$. The estimates were obtained using the Sigmaplot software (version 12.3, Systat Software, Inc.), and the $t$ test ($\alpha = 0.05$) was used to test significance of the regression parameters. The $K_m$ (PEP) and $V_{max}$ were calculated by nonlinear fit of the data to the Michaelis-Menten equation, $v = VS/(K_m + S)$, where $S$ is the concentration of the substrate pyruvate, $v$ is the reaction velocity at any PEP concentration, and $V$ is the maximal reaction velocity. The kinetic values were obtained using GraFit (version 7.0.3, Erithacus Software Ltd.), and $\chi^2$ ($\alpha = 0.05$) was used to test goodness of fit. Glyphosate dose response experiments were repeated at least twice with similar results, so all data were pooled and evaluated for a composite line fit. Each EPSPS kinetic assay contained three technical replicates, and three independent enzyme extracts were used for each assay set.

The *E. indica* TIPS cDNA sequence information can be found in GenBank with an accession number KM078728.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** dCAPS markers for T102I and P106S.

**Supplemental Figure S2.** Survival of TIPS plants at very high glyphosate rates.

**Supplemental Table S1.** Primers used in cloning the EiEPSPS.

**Supplemental Table S2.** Parameter estimation of nonlinear regression of herbicide $IC_{50}$.

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**Supplementary data**

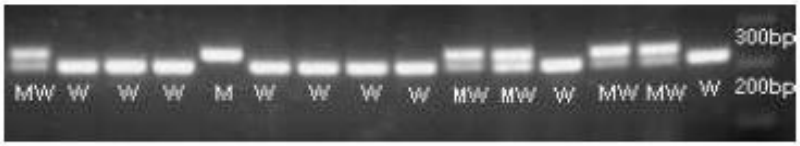
Table S1. Primers used in cloning the EiEPSPS gene.

| Primers | Primer name or purpose | Sequence |
|---|---|---|
| 1 | EPSPS EiLFT1 | GCG GGC GCG GAG GAG GTG GT |
| 2 | EPSPS EiRT1 | TTA GTT CTT GAC GAA AGT GCT CAG CAC GTC GAA GTA GT |
| 3 | For P106S to wild type EPSPS conversion PCR primer R | 5'/5Phos/AAC TGC AAT GCG ACC ATT GAC AGC AGC CGT AAC TG |
| 4 | For P106S to wild type EPSPS conversion PCR primer L | 5'/5Phos/CCAGCATTCCCCAAGAAGAGCTGCACCT |
| 5 | For P106S to TIPS EPSPS conversion PCR primer R | 5'/5Phos/GGA ATG CTG GAA TTG CAA TGC GAT CAT TGA CAG CA |
| 6 | For P106S to TIPS EPSPS conversion PCR primer L | 5'/5Phos/CCA AGA AGA GCT GCA CCT CCT CTT TCG CAT C |
| 7 | pET19-bEiEPSPS-F; for cloning EPSPS into pET-19b vector primer L | ATATCATATG GCGGGCGCGGAGGAGGTG |
| 8 | pET19-bEiEPSPS-R; for cloning EPSPS into pET-19b vector primer R | ATAT CATATG TCATTAGTTCTTGACGAAAGTGCTCAGCACGTCG |

Table S2. Parameter estimates of the non-linear regression analysis of herbicide rates causing 50% inhibition of in vitro enzyme activity ($IC_{50}$). SE is in parentheses.

| Genotype | a | b | $X_0$ | *P* value | Ratio to WT |
|---|---|---|---|---|---|
| | | | $IC_{50}$ (μM) | | |
| WT | 99.2 (1.10) | 0.99 (0.09) | 20 (0.84) | <0.0001 | 1 |
| P106S | 100 (0.69) | 1.11 (0.03) | 87 (2.15) | <0.0001 | 4.4 |
| TIPS | 98.2 (0.76) | 1.59 (0.07) | 52938 (1206) | <0.0001 | 2647 |

**A. 102**




**B. 106**

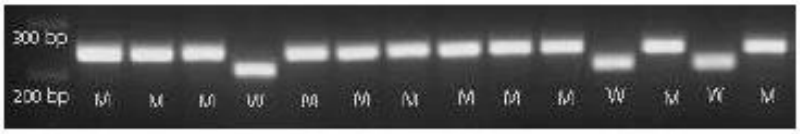



**Fig S1:** dCAPS markers developed for T102I (A) and P106S (B) EPSPS mutation in *Eleusine indica.* M refers to the mutant 102I (A) or 106S (B) allele and W refers to the wild type (WT) T102 (A) or P106 (B) allele.

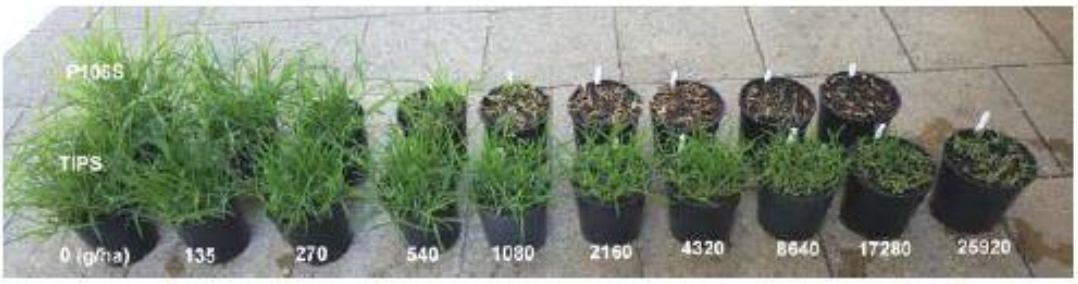



**Fig S2:** Very high glyphosate doses required to cause some mortality of the homozygousTIPS mutants as compared to the homozygous P106S mutant *Eleusine indica*.