

**Physical mapping of *EPSPS* gene copies in glyphosate resistant
Italian ryegrass (*Lolium perenne* ssp. *multiflorum*)**

by

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Abstract

Italian ryegrass (*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot), one of the problem weeds of the US, evolved resistance to multiple herbicides including glyphosate due to selection in Arkansas (AR). Glyphosate is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor and amplification of *EPSPS* gene, the molecular target of glyphosate confers resistance to this herbicide in several weed species, including Italian ryegrass from AR. The objective of this study was to determine the expression of *EPSPS* gene and protein as well as distribution of *EPSPS* copies on the genome of glyphosate-resistant Italian ryegrass (ARR) using a known susceptible Italian ryegrass (ARS) from AR. *EPSPS* gene copies and expression of ARR and ARS were determined using quantitative PCR with appropriate endogenous controls. EPSPS protein expression was determined using Western blot analysis. Fluorescence *in situ* hybridization (FISH) was performed on somatic metaphase chromosomes to determine the location of *EPSPS* copies. Based on qPCR analysis, ARR plants showed a wide range of 12 to 118 *EPSPS* copies compared to a single copy in ARS. *EPSPS* gene expression correlated with the gene copy number in both ARR and ARS. Individuals with high *EPSPS* copies showed high protein expression in Western blot analysis. FISH analysis showed presence of brighter *EPSPS* signals, distributed randomly throughout the genome of ARR individuals compared to a faint signal in ARS plants. Random distribution of *EPSPS* copies was previously reported in glyphosate-resistant Palmer amaranth. Overall, the results of this study will help understand the origin and mechanism of *EPSPS* gene amplification in Italian ryegrass.

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Dedication

To my mother for her love and support who could not see this thesis completed.

Chapter 1 - Review of Literature

Italian Ryegrass – Habitat, Biology & Reproduction

Italian ryegrass (*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot) belongs to the family Poaceae. It is native to Southern Europe and the continent of Africa. It was introduced throughout the world including the United States as a forage grass aiding to its high palatability and nutritious value as animal feed (Lamp et al., 1990). It is a cool season annual grass, which prefers to grow in moist surroundings (Romani et al., 2002). Italian ryegrass can also occur as a biennial or perennial depending on the climatic and seasonal variations. Grass species usually exhibit high phenotypic plasticity and they readily adapt to their environment (Casler and Duncan, 2003). Italian ryegrass can be found growing in pastures, roadsides, crop fields and open fields. This species grows generally in areas with high rainfall and soils with high to medium fertility. Extreme growth conditions like drought and excessive moisture do not favor the establishment of this species. Italian ryegrass grows best in soils with an optimum pH ranging from 6-7 and up to 8. This species is also well adapted to grow in a wide range of soil types and can tolerate occasional flooding in well-drained soils (<http://www.cabi.org/isc/datasheet/31165>, Accessed February 3rd, 2017). Italian ryegrass cannot persist in hot and dry weather and also to freezing temperatures (Beddows, 1973).

Italian ryegrass is a diploid ($2n=14$) (Beddows, 1973) and grows to about 3 feet tall and stems grow as individual stalks or tillers and have rounded to flattened appearance (Figure 1.1). Italian ryegrass has a fine fibrous-adventitious root system and can grow over 3 feet even on non-irrigated sites. Leaf blades are dark green with flat and hairless upper surface. It has small well-developed auricle with white and membranous ligule, leaves have a length of up to 40 cm and width of about 5-12 mm (Lamp et al., 2001). The inflorescence consists of a spike of about 30 cm length and spikelets are arranged alternate, stalkless and produce about 10 to 20 florets. Italian

ryegrass can be distinguished from other ryegrass species by their needlelike awns and a higher number of florets clustered per spikelet (Lamp et al., 2001).

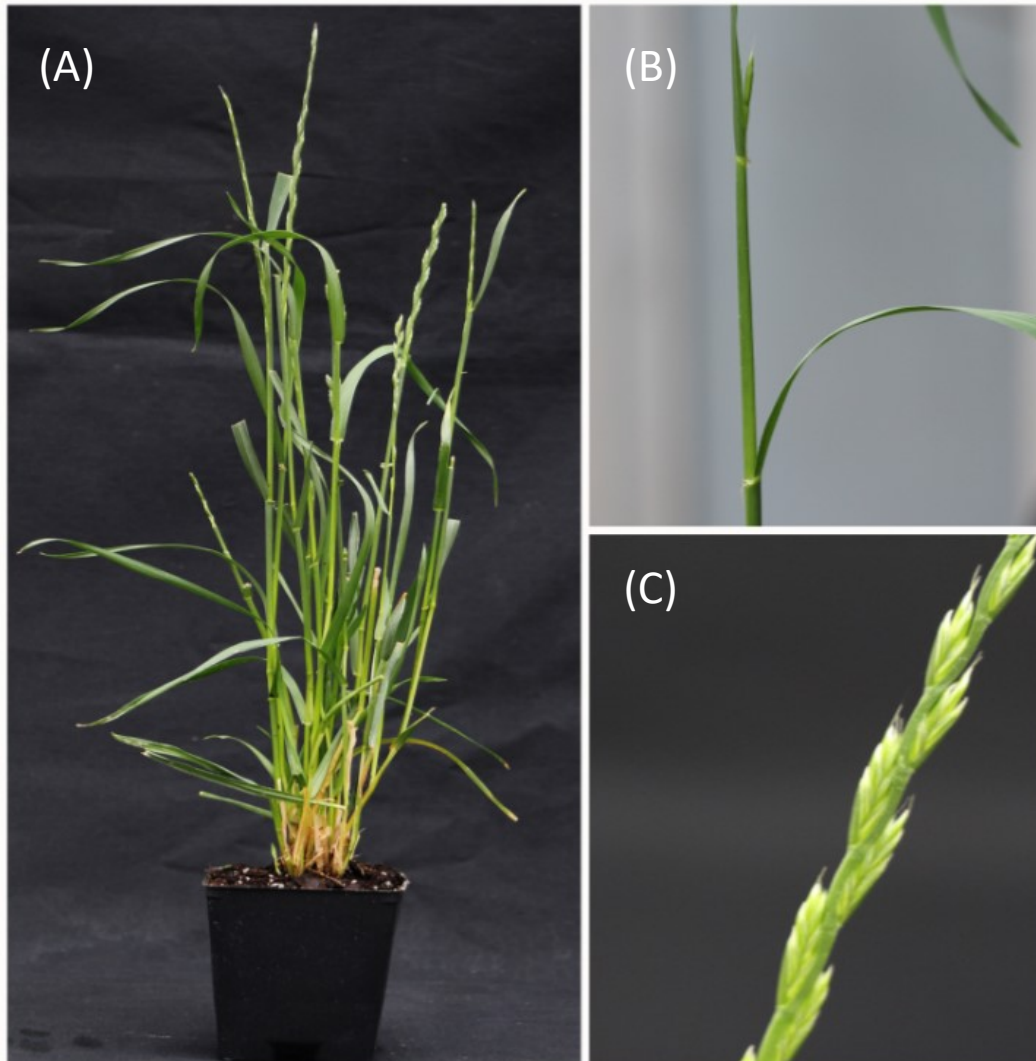


Figure 1.1 Morphology of Italian ryegrass plant. Italian ryegrass plant with tillers (A). Structure of the Italian ryegrass stem (B). Inflorescence of Italian ryegrass (C).

Italian ryegrass is self-incompatible, hence an obligate outcrosser and seeds are usually dispersed by animals and the wind due to their flattened structure (Kannenbergh and Allard, 1967). Italian ryegrass exhibits a self-incompatibility system (SZ), similar to perennial ryegrass, which is

governed by two alleles. Selfing is prevented to avoid inbreeding depression when both alleles controlling the gametophytic incompatibility are matched to those in style (Fearon et al., 1983). Flowering usually occurs from April through September and reproduction occurs solely through seed. Ryegrass species can also spread through tillering (Najda, 2004). This species can also grow in relatively high salt stress conditions as opposed to perennial ryegrass (Marcar, 1987). Italian ryegrass is a highly competitive species and can produce huge quantities of seed in a short time. A single Italian ryegrass plant can produce up to 45,000 seed (<http://www.deltafarmpress.com/soybeans/italian-ryegrass-nearly-perfect-winter-weed>, Accessed February 3rd, 2017). Italian ryegrass seed is large, lack well-defined dormancy mechanisms and can germinate at varying temperatures (Thompson and Grime, 1979). Under controlled conditions, i.e. 10 °C and 95% relative humidity, Italian ryegrass seed can maintain viability for up to 5 years (Rutledge and McLendon, 1998). It can readily hybridize with perennial ryegrass (*Lolium perenne* L.) and the offspring are hard to distinguish from perennial ryegrass (Carey, 1995). Hybrids between Italian ryegrass and perennial ryegrass are capable of interbreeding. It has also been shown that Italian ryegrass can produce fertile hybrids with both *Lolium* sp and *Festuca* sp (Jacobs et al., 2008). This ability to outcross with a variety of different species allows it to pass its genetic background and produce high heterotic individuals.

Italian Ryegrass – Impact on Agriculture

Italian ryegrass is primarily grown as a forage grass throughout the temperate regions of North, South America, New Zealand, Japan and Europe (Rios et al., 2015) but persists as a weed in many grain crops and orchards. The characteristics such as adaptability to diverse environments, the ability to outcross with other forage species, quick growth rate and high seed production toughen

its position as a prominent weed in several cropping systems. It is a problem weed especially in winter wheat (*Triticum aestivum* L.) in the US and can reduce the wheat yield significantly (Stone et al., 1999). Italian ryegrass and winter wheat have similar maturity stages, which interfere with the harvest (Rex and Worsham, 1987). The perseverance of Italian ryegrass at harvest can cause contamination of crop grain and severe price dockage in net returns (Justice et al., 1994). Weeds are generally considered to be less effective at utilizing the available nutrients but highly efficient in removing nutrients from the soil (Vengris et al., 1955). It has been documented that Italian ryegrass has twice the net uptake of NO_3^- and K^+ than wheat in greenhouse conditions (Rex and Worsham, 1987). Yield losses of up to 4100 kg/ha were reported in wheat when competing with Italian ryegrass density at 93 plants/m² (Appleby et al., 1976). Similarly, Italian ryegrass when present at a density of 10 plants/m² in competition with wheat can reduce the yield by 4.2% (Rex and Worsham, 1987). One study has reported that Italian ryegrass competes for more below ground resources with wheat than showing above ground interference due to the presence of fine fibrous roots (Martin et al., 1998). Winter wheat populations received 68% less sunlight due to the greater leaf area indices of Italian ryegrass in the top canopy (Hashem et al., 1998).

Significant reduction of corn yield was reported when in competition with Italian ryegrass. Importantly, herbicide-resistant populations of Italian ryegrass can reduce the yield of corn (*Zea mays* L.) even more than the susceptible ryegrass populations (Nandula, 2014). Competition of Italian ryegrass with major row crops like corn, cotton (*Gossypium hirsutum* L.), and soybean (*Glycine max* (L.) Merr.) resulted in 65, 85 and 37% yield losses, respectively (https://ag.purdue.edu/btny/weedscience/Documents/50737_12_TA_FactSheet_ItalianRyegrass_V3_LR.pdf, Accessed February 6th, 2017). Italian ryegrass densities of 600 to 1000 plants m⁻¹ per a row of broccoli (*Brassica oleracea* var. *Italica*) resulted in 100% yield loss (Bell, 1995). Apart

from direct interference, Italian ryegrass can also disturb the ecosystem by harboring pathogens that can infect crop species, such as *Pythium arrhenomanes* (causes root rot disease in sugarcane (*Saccharum officinarum* (L.)) (Dissanayake et al., 1997), and *Pseudomonas glumae* and *Pseudomonas plantarii* (important pathogens of rice) (Miyagawa et al., 1988).

Glyphosate – Development and Mode of Action

Glyphosate [N-(phosphonomethyl) glycine] is the most extensively used broad spectrum herbicide worldwide. It is considered as an environmentally and toxicologically safe compound (Giesy et al., 2000). Glyphosate as a compound was first developed in 1950 by Swiss chemist Dr. Henri Martin at Cilag AG pharmaceuticals (Franz et al., 1997). Cilag became part of the Johnson and Johnson in 1959 and in the later years, glyphosate ended up in the Inorganic Division of Monsanto (Nandula, 2010). Dr. John E. Franz and his team synthesized a more potent form of glyphosate and in 1970, herbicidal properties of glyphosate were tested on perennial weed species in the greenhouse (Franz et al., 1997). Later in 1971, Monsanto Inc. first released the commercial formulation of glyphosate under the trade name Roundup® for weed control (Baird et al., 1971).

The molecular formula for glyphosate is $C_3H_8NO_5P$. The chemical structure of glyphosate is comprised of one basic amino group and three ionizable acidic sites (Figure 1.2). Acidic formulation of glyphosate has a high water solubility of 15,700 mg/L in pH 7 water at 25° C. Detailed molecular and crystal structure of glyphosate was reviewed in 1979 (Knuutila and Knuutila, 1979). Glyphosate is an inhibitor of EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme, which plays a crucial role in catalyzing the transfer of enolpyruvyl moiety of phosphoenolpyruvate (PEP) to S3P (shikimate-3-phosphate) in the shikimic acid pathway. This pathway is crucial for the synthesis of essential aromatic amino acids like tryptophan, phenylalanine, and tyrosine (Amrhein et al., 1980; Duke and Powles, 2008). These amino acids

will be ultimately used for the production of key hormones and plant metabolites including flavonoids and lignins (Dill, 2005). About 20% of fixed carbon is passed through the shikimate pathway in plants and the EPSPS enzyme is present only in plants, bacteria and fungi but not animals (Alibhai and Stallings, 2001; Kishore and Shah, 1988). This quality makes it less toxic to non-target species. The complete interaction of glyphosate with EPSPS enzyme in atomic detail is well understood (Schönbrunn et al., 2001). Glyphosate competitively binds to the binding site of PEP and forms a stable EPSPS:S3P complex, which inhibits the Shikimic acid pathway resulting in plant mortality (Duke and Powles, 2008). Glyphosate is a non-volatile compound and it has a short half-life in soil due to microbial degradation (Duke and Powles, 2008; Giesy et al., 2000) and also a slow acting herbicide (Baylis, 2000).

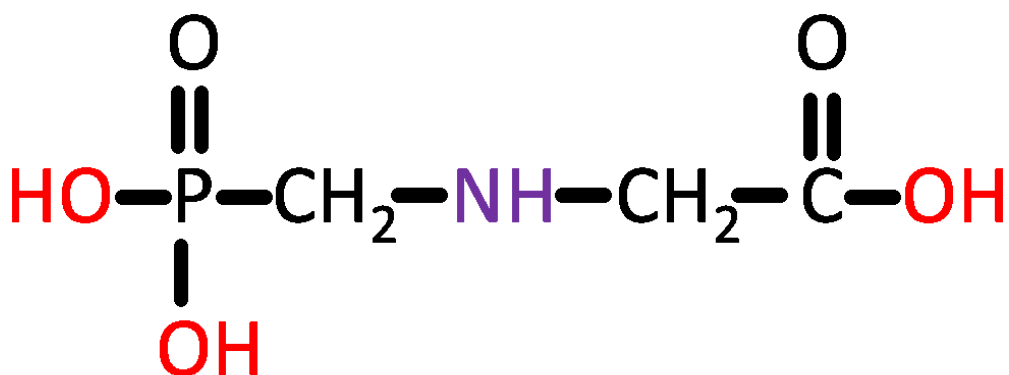


Figure 1.2 Structure of glyphosate with one basic amino site (purple) and three acidic sites (red).

Glyphosate is a phloem-mobile herbicide that can diffuse through leaf cuticle and accumulate in the meristematic regions of the plant. The rate and efficiency of translocation depend on the environmental factors as well as plant species (Caseley and Coupland, 1985). Some species like sugarbeet (*Beta vulgaris* subsp. *vulgaris*) has a rapid translocation of glyphosate i.e. herbicide moves out of the plant before it starts its activity thereby limiting its efficiency (Geiger et al.,

1999). Inhibition of EPSPS enzyme by glyphosate leads to accumulation of Shikimic acid, which can be used as a method to determine glyphosate toxicity (Shaner et al., 2005; Singh and Shaner, 1998). Generally, grasses are more susceptible to glyphosate injury than broad-leaved weeds but species-specific tolerance is not uncommon (Baylis, 2000). Some weed species such as field bindweed (*Convolvulus arvensis* L.) and birdsfoot trefoil (*Lotus corniculatus* L.) are naturally tolerant to glyphosate (Boerboom et al., 1990; DeGennaro and Weller, 1984). Other species like common lambsquarters (*Chenopodium album* L.), velvetleaf (*Abutilon theophrasti* Medik.) and wild buckwheat (*Polygonum convolvulus* L.) have become increasingly difficult to control with glyphosate (Owen and Zelaya, 2005).

Glyphosate –Resistant Crops and Weeds

The introduction of glyphosate-resistant soybean, in 1996 marked the beginning of glyphosate-resistant (GR) crop technology. In the same year Roundup® Ready corn and soybean varieties were also released for planting in the U.S. Currently, we have six different GR crops commercially available, i.e. soybeans, corn, cotton, canola (*Brassica napus* L.), alfalfa (*Medicago sativa* L.) and sugar beet (*Beta vulgaris* L.) (Green, 2016) (Figure 1.3. Biotechnological and tissue culture strategies were tried for developing GR crops (Duke and Powles, 2008). Some of the approaches include overexpression of the native *EPSPS* gene, detoxification of glyphosate molecule in the plant by introducing glyphosate oxidase (*GOX*) gene. These methods were not very successful either due to poor growth of plants or insufficient level of glyphosate tolerance (Dill, 2005). To date, the most successful method of development of GR crops was through the transgenic insertion of *CP4* gene of *Agrobacterium* sp., which encodes for an insensitive form of EPSPS (Padgett et al., 1996). The *CP4* gene coupled with an altered promoter in the genome of GR crops showed high levels of glyphosate resistance. A commercial variety of GR maize was developed through

site-directed mutagenesis which resulted in a missense mutation that showed acceptable levels of glyphosate resistance (Lebrun et al., 2003).

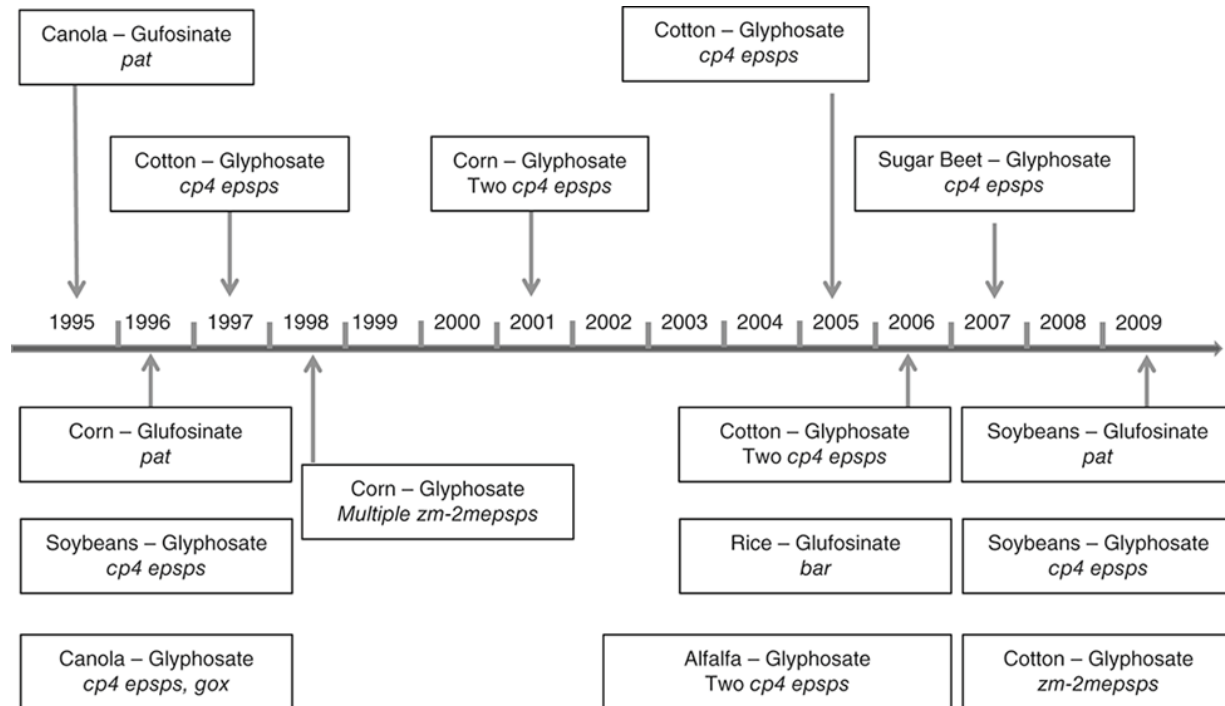


Figure 1.3 Commercial transgenic crop traits introduced between 1995 and 2009. Image adapted from Green, 2016.

GR crop technology has immensely benefited glyphosate sales by increasing the area of application. This technology was deemed to be one of the fastest adopted technologies, especially in the U.S., GR trait technology alone represents more than half of the total biotech crop market. Glyphosate use rate per crop, as well as the number of applications per acre, have rapidly increased since the introduction of GR crops (Benbrook, 2016). Out of the 120 million ha of GM crops grown worldwide, ~80% include GR technology. By 2002, the number of herbicides used on 10% of U.S. soybean reduced to just glyphosate from 11 modes of action of herbicides previously used (Duke and Powles, 2009). About 56% of total glyphosate used globally (~8.6 billion kg) is applied in GR

crops (Benbrook, 2016). Wide adoption of GR crops coupled with glyphosate use has revolutionized the agricultural practices in the U.S. (Green, 2016).

In 1994, after 20 years of glyphosate use, there were no reports of the evolution of GR weeds (Powles, 2008). However, upon introduction of GR crops in 1996, glyphosate use increased significantly (Figure 1.4). Initial cell and tissue culture studies on several plant species had shown that it is unlikely for the weed species to develop glyphosate resistance under field conditions as it was very hard to get reproducible GR regenerates that are overexpressing EPSPS to tolerate field used dose of glyphosate. Also, the unique chemical structure of glyphosate, its metabolism route and poor residual activity of glyphosate in the soil also supported this hypothesis (Bradshaw et al., 1997). Nonetheless, the first case of glyphosate resistance was reported in a rigid ryegrass (*Lolium rigidum* Gaudin) population from the orchards of Australia, which showed 7-11 fold resistance compared to a known susceptible population (Powles et al., 1998). Currently, there are 17 monocot and 20 dicot species that are resistant to glyphosate in 26 countries (Heap, 2017). Several species of *Conyza* and *Lolium* have evolved glyphosate resistance in many scenarios such as vineyard, orchard and even roadsides due to persistent use of glyphosate (Powles, 2008). Due to the intensive and extensive use of glyphosate in Australia, large areas of crop land have been infested with GR rigid ryegrass (Preston, 2007). As of 2016 at least 678, 102 sites for glyphosate-resistant annual ryegrass (*Lolium rigidum* Gaudin) and barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) were reported in Australia alone, which determines the severity of the problem (glyphosate resistance org, 2016).

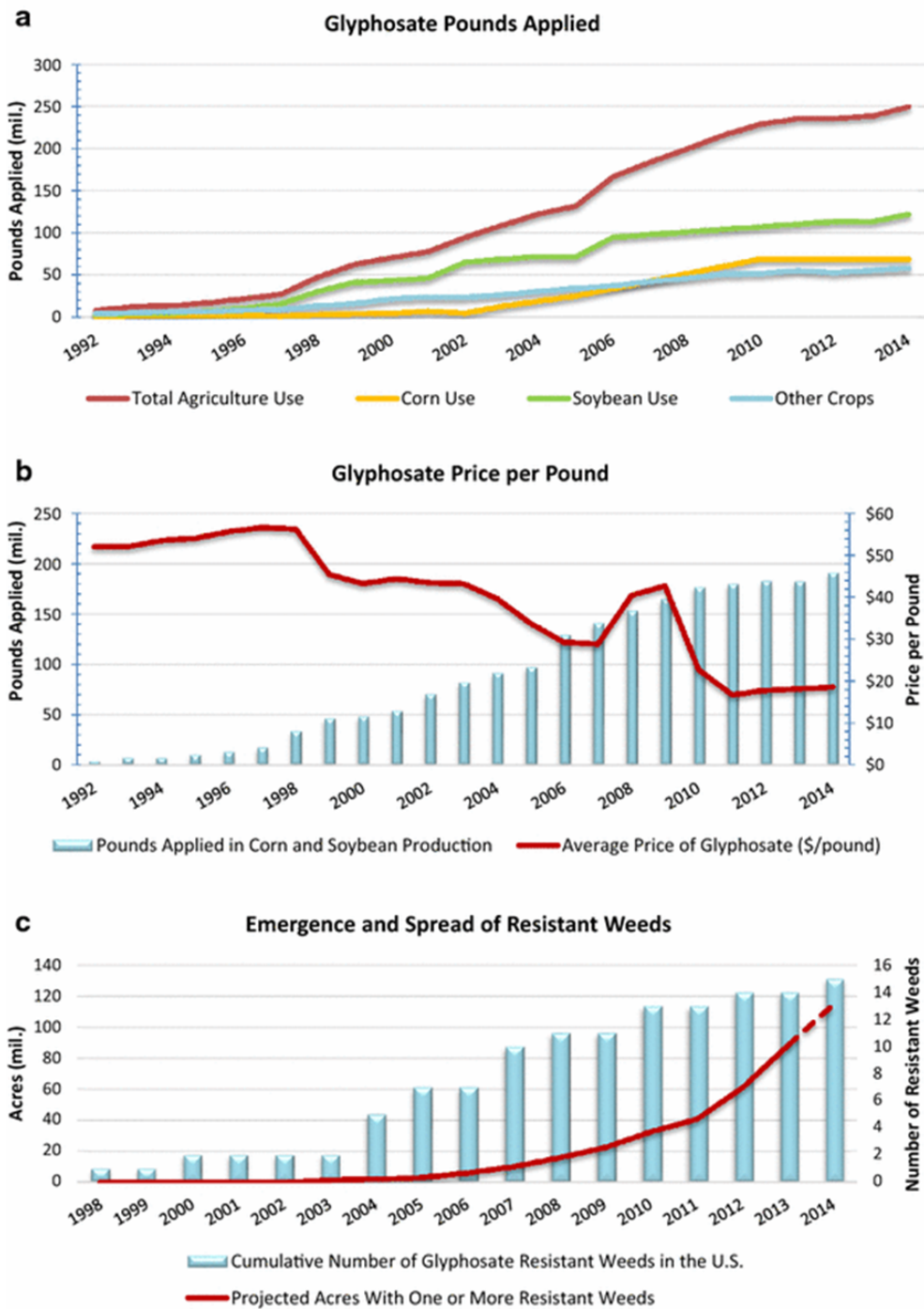


Figure 1.4 Use of glyphosate in corn and soybean cropping systems and evolution of resistant weeds in this scenario. Image adapted from Benbrook, 2016.

Some of the examples of important GR weeds include jungle rice (*Echinochloa colona* L. Link) (Goh et al., 2016), hairy fleabane (*Conyza bonariensis* (L.) Cronq.) (Dinelli et al., 2008; Urbano et al., 2007), horseweed (*Conyza canadensis* (L.) Cronq.) (Koger and Reddy, 2005; Mueller et al., 2003) (VanGessel, 2001; Zelaya et al., 2004), rigid ryegrass (*Lolium rigidum* Gaudin) (Feng et al., 1999; Lorraine-Colwill et al., 2002; Powles et al., 1998; Pratley et al., 1999; Wakelin and Preston, 2006; Yu et al., 2007), Italian ryegrass (*Lolium perenne* L. *ssp. multiflorum* (Lam.) Husnot) (Jasieniuk et al., 2008; Nandula et al., 2008; Perez-Jones et al., 2007; Perez-Jones et al., 2005; Perez et al., 2004; Perez and Kogan, 2003; Salas et al., 2012), Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Culpepper et al., 2006; Gaines et al., 2011; Norsworthy et al., 2008; Steckel et al., 2008), Kochia (*Kochia scoparia* (L.) Schrad.) (Beckie et al., 2013; Jugulam et al., 2014; Kumar et al., 2014; Waite et al., 2013; Wiersma et al., 2015) and Common waterhemp (*Amaranthus rudis* Sauer) (Legleiter et al., 2008; Patzoldt et al., 2002). More recently two populations of GR ripgut brome (*Bromus diandrus* Roth) that were at least 5 fold more resistant to glyphosate than susceptible plants were reported in pastures of southern Australia (Malone et al., 2016). GR Italian ryegrass was found in 9 countries globally with the highest number of cases being reported in the U.S. (Heap, 2017). Several populations of GR Italian ryegrass were reported in the U.S. (Jasieniuk et al., 2008; Nandula et al., 2007; Nandula et al., 2008; Ngo et al., 2017; Perez-Jones et al., 2005; Salas et al., 2012).

Italian Ryegrass – Herbicide Resistance and Management

According to Weed Science Society of America (WSSA), herbicide resistance is defined as “the inherited ability of the plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type”. Traditionally, acetyl-CoA carboxylase (ACCase), or acetolactate

synthase (ALS)-inhibitors have been used to control Italian ryegrass. Most of the herbicides belonging to these two classes were registered for use in several cropping systems. Extensive and intensive use of these herbicides resulted in the selection of Italian ryegrass populations resistant to these compounds. In 1987, the first case of diclofop-methyl (an ACCase-inhibitor)-resistant Italian ryegrass population was reported in a wheat field in Oregon (Charles and Appleby, 1989). In the following year i.e. 1990, diclofop-methyl-resistant populations were identified in both North and South Carolina (Heap, 2017). To date, Italian ryegrass has developed resistance to five herbicide sites-of-action: acetyl-CoA carboxylase (ACCase), acetolactate synthase (ALS), EPSPS synthase, glutamine synthetase (GS)-inhibitors, and long chain fatty acid inhibitors in the U.S. It is not uncommon to find Italian ryegrass resistant to multiple sites of action of herbicides in the U.S.

Glyphosate has been used as a viable option since its release in 1974 to control Italian ryegrass infestations in roadsides, vineyards and orchards (Nandula, 2014). The first case of GR Italian ryegrass was found in the Chilean orchards of San Bernardo and Oliver in 2003. Later, the first case of GR Italian ryegrass in the US was reported in the orchards of Oregon (Perez-Jones et al., 2005). The introduction of GR crops in 1996 accelerated the use of glyphosate worldwide. The rapid adoption of GR crops like soybean, corn, canola and cotton encouraged farmers to spray glyphosate as a single weed control option. As a result, GR Italian ryegrass populations were found in at least 7 states in the US and 12 countries globally (Heap, 2017).

Italian ryegrass infestation severely hinders the pre-plant burndown operations especially in reduced or no-tillage situations (Nandula, 2014). Use of herbicide mixtures and herbicide application at the right phenological stage can possibly give better control of GR Italian ryegrass (Christoffoleti et al., 2005). Herbicides like atrazine were used to control Italian ryegrass infestations in wheat aiding to its selectivity. Wheat has much lesser accumulation and persistence

of atrazine in the plant due to higher activity of herbicide detoxifying enzymes like glutathione S-transferase (GST) (Del Buono et al., 2011). More recently, one study has reported soil-applied pyroxasulfone had good control of Italian ryegrass in winter wheat (Hulting et al., 2012). Herbicides like clomazone, pyroxasulfone, and *S*-metolachlor provide growers with residual control and application in November maximizes the control of GR Italian ryegrass (Bond et al., 2014). Herbicide rotation with different modes-of-action and other integrated weed management approaches can help in delay evolution of resistance.

Mechanism of Glyphosate Resistance in Italian Ryegrass

Mechanism of resistance in weeds can be broadly classified into target-site and non-target-site-based. Target-site resistance can be due to an altered target-site of the herbicide, which can occur due to a mutation in the target gene resulting in a change in its amino acid sequence (substitution, or deletion) or as a result of overexpression of the target enzyme (gene amplification or changes in promoter binding site) (Powles and Yu, 2010). On the other hand, non-target-site resistance can be as a result of decreased absorption/translocation or metabolism of herbicide (Délye, 2013) (Yuan et al., 2007). This mechanism can occur either in one or many combinations (Powles and Yu, 2010).

Both target site- and non-target site-based resistance to glyphosate have been documented in many GR weeds (Sammons and Gaines, 2014). Glyphosate resistance in *Lolium rigidum* Gaudin was shown to be inherited as a nuclear encoded single semi-dominant or incompletely dominant trait (Lorraine-Colwill et al., 2002; Lorraine-Colwill et al., 2001). Nuclear-encoded resistance can spread rapidly as it can be passed on both via seed and pollen (Mithila and Godar, 2013). Translocation of glyphosate is substantially reduced in GR giant ragweed (*Ambrosia trifida* L.)

compared to susceptible due to rapid necrosis and also reduced translocation of herbicide from mature leaves to meristematic regions (Robertson, 2010). There are several studies that have shown reduced foliar uptake and translocation as a mechanism of glyphosate resistance in Italian ryegrass (González-Torralva et al., 2012; Michitte et al., 2007; Nandula et al., 2008; Perez-Jones et al., 2007; Perez et al., 2004). Also, it has been shown that GR Johnson grass (*Sorghum halepense* (L.) Pers.) at 19 degrees C and *Lolium rigidum* at 9 degrees C exhibit more susceptibility as opposed to 30 degrees C and 19 degrees C respectively (Vila-Aiub et al., 2013).

The first case of target-site-resistance to glyphosate was reported in goose grass (*Eleusine indica* (L.) Gaertn.) which had a mutation in *EPSPS* gene resulting in proline -106-Serine substitution (Baerson et al., 2002). This population was 2-4 fold resistant to glyphosate compared to a susceptible population (Baerson et al., 2002). Later, a proline to alanine and threonine substitutions at amino acid position 106 were also identified in *Lolium* species (*Lolium rigidum* Gaudin, *Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot) and goose grass (*Eleusine indica* (L.) Gaertn.) (Kaundun et al., 2008; Powles and Yu, 2010). Six species were reported to have a mutation in *EPSPS* gene endowing resistance to glyphosate (Sammons and Gaines, 2014). Several populations of GR Italian ryegrass were shown to have mutations in *EPSPS* gene conferring resistance to glyphosate (González-Torralva et al., 2012; Jasieniuk et al., 2008; Perez-Jones et al., 2007). Another important mechanism of target site-based resistance to glyphosate is due to amplification of *EPSPS* gene, which is discussed in detail in the next section.

Gene Amplification as a Mechanism of Glyphosate Resistance

Gene amplification is a mechanism through which a part of genomic region gets duplicated resulting in increased copies of the same gene. The role of gene duplication in driving evolution

and speciation has been well documented in many studies (Kondrashov, 2012; Kubo et al., 2015; Ohno, 2013; Taylor et al., 2001; Zhang, 2003). Gene duplications can arise through several events, namely, via a) unequal crossing over between two homologous sister chromatids, b) retroposition or c) chromosomal duplication (Zhang, 2003) (Figure 1.5). Unequal crossing over between two homologous sister chromatids results in tandem gene duplication. Depending on the recombination region, the duplicated product may contain one to several genes and are generally believed to be linked (Zhang, 2003). This recombination can give rise to even deletions or inversions of cross over region (Hurles, 2004). During retrotransposition the mature RNA is reverse transcribed to cDNA (complementary DNA) and gets integrated randomly into the genome, this commonly results in duplicated genes, which lack introns and contain poly-A tails. Gene duplication through retrotransposition results in unlinked genes (Hurles, 2004; Zhang, 2003). One exception to this might be possible if duplicated genes are present in a single operon (Zhang, 2003). Most of the duplicated genes that arise through such retrotransposition lack expression due to random integration of genes in the genome that lack a driving promoter but an insertion downstream of a promoter construct can result in stable expression (Zhang, 2003). Another important mechanism of gene duplication is chromosomal or segmental duplication, it is believed to occur due to the disjunction between daughter chromosomes or more precisely due to increase in duplication breakpoints at replication termination sites (Hurles, 2004; Zhang, 2003). The exact mechanism of such kind of duplication is unclear but is mainly attributed to non-homologous recombination events (Hurles, 2004).

It is now widely accepted that application of pesticides and insecticides over a period of time promotes adaptive duplication of genes or enzymes (Devonshire and Field, 1991; Gaines et al., 2011; Jugulam et al., 2014; Powles, 2010; Terriere, 1983). Bass and Field (2011) documented

that for three families of detoxifying enzymes (esterases, glutathione *S*-transferases and cytochrome P450 monooxygenases), gene amplification of the structural gene is the reason for enhanced metabolism of many insecticides (Bass and Field, 2011). This has been well studied in case of insect species like *Myzus persicae*, *Schizaphis graminum*, *Nilaparvata lugens*, *Culex pipiens*, *Culex quinquefasciatus*, *Culex tarsalis* and *Culex tritaeniorhynchus* (Bass and Field, 2011). Two duplicated genes, *EF4* and *FE4* were found to be amplified in insecticide resistant peach potato aphid (*Myzus persicae*) populations with an increase of upto four fold in gene copies as compared to the wild aphids (Field et al., 1999). Fluorescent *in situ* hybridization (FISH) analysis of the *EF4* gene linked the amplification of this gene to a chromosomal translocation at a single heterozygous location (Blackman et al., 1995). Studies on Organophosphate resistant *Culex pipiens* mosquitoes have shown that amplification of either single or allelic pairs of esterase genes can result in resistance (Buckingham et al., 2005; Hemingway, 2000). Esterase genes such as *Est-3*, *Est-2* or allelic pairs of genes (*esta2-estβ2*, *esta4-estβ4*, *esta5-estβ5*, *esta8-estβ8*) are responsible for insecticide resistance (Buckingham et al., 2005; Hemingway, 2000). One of the most common allele attributed to insecticide resistance is *esta2-estβ2* coamplicon which is present in most of the insect-resistant species and is believed to confer a fitness advantage over other allelic groups (Hemingway, 2000).

Earlier, it has been reported that cell suspension cultures of tobacco under continuous glyphosate selection showed amplification of *EPSPS* gene resulting in resistance to glyphosate (Widholm et al., 2001). However, the natural evolution of glyphosate resistance via *EPSPS* gene amplification has been documented in seven weed species as a result of continuous selection. Examples of the GR weed species with gene amplification as mechanism of resistance include Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Culpepper et al., 2006; Gaines et al., 2011;

Ribeiro et al., 2014), Spiny amaranth (*Amaranthus spinosus* L.) (Nandula et al., 2014), Common waterhemp (*Amaranthus tamariscinus* auct. non Nutt.) (Chatham et al., 2015), Ripgut brome (*Bromus diandrus* Roth) (Malone et al., 2016), Kochia (*Kochia scoparia* (L.) Schrad.) (Jugulam et al., 2014; Wiersma et al., 2015), Italian ryegrass (*Lolium multiflorum*) (Salas et al., 2012; Salas et al., 2015) and Goose grass (*Eleusine indica*) (Chen et al., 2015).

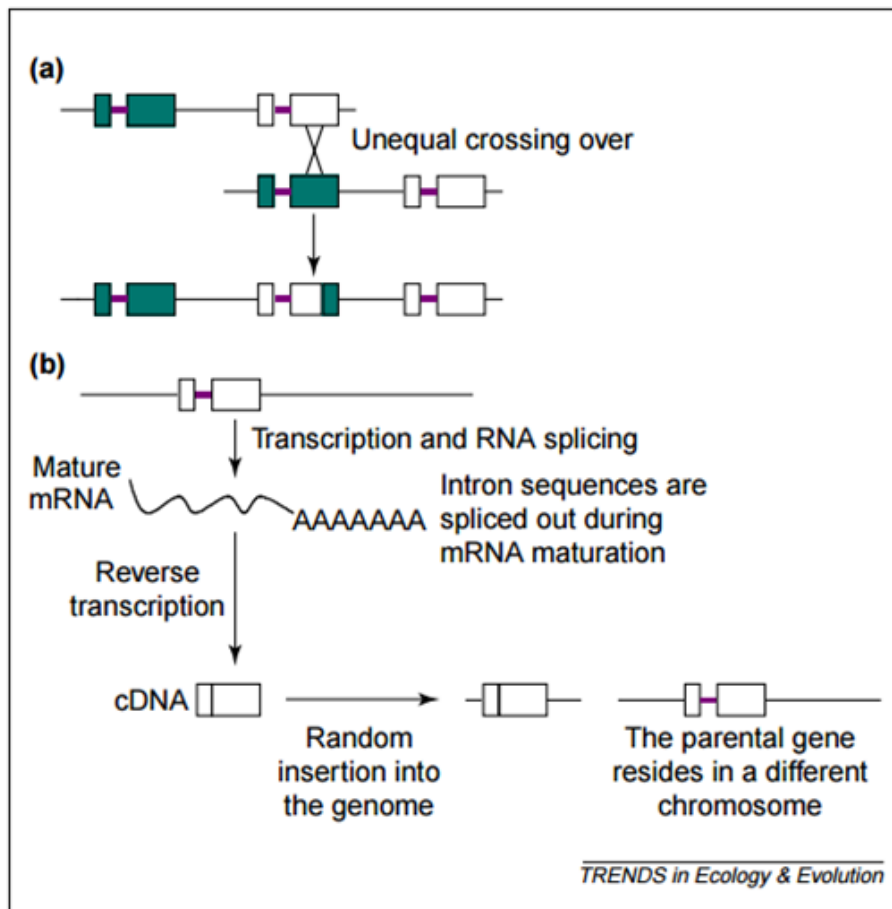


Figure 1.5 Most frequently reported possible mechanisms of gene duplication or gene amplification. Image adapted from Zhang, 2003.

Tandem amplification of chromosomal region containing *EPSPS* gene copies in GR *K. scoparia* has been reported recently suggesting a possible role of unequal crossing over in gene duplication (Jugulam et al., 2014). In GR *A. palmeri* the mechanism for gene duplication was

shown to be possibly due to transposon-mediated mechanism (Gaines et al., 2010). Another study by this group reported that the gene duplication might arise due to DNA transposon-mediated replication showing the involvement of miniature inverted-repeat transposable elements (MITEs) next to *EPSPS* gene copies in Palmer amaranth showing the involvement of miniature inverted-repeat transposable elements (MITEs) next to *EPSPS* gene copies (Gaines et al., 2013).

More recently, physical mapping of distribution of amplified *EPSPS* gene copies on GR *A. rudis* suggested that the amplified copies in resistant individuals were present closer to the pericentromeric region of two homologous chromosomes. Additionally, some GR individuals also had an extra chromosome with *EPSPS* copies (Dillon et al., 2016). They proposed the pericentromeric localization of *EPSPS* copies may involve segmental duplication and/or followed by other mechanisms of gene duplication (Dillon et al., 2016).

Studies on rodents and humans with anti-cancer drugs like methotrexate in culture have shown that the resistance to these drugs endowed by gene amplification dissipated once the selection pressure was removed (Gressel, 2015; Sharma and Schimke, 1994). General opinion is that in real-time, resistance dissipation might take longer in case of weeds and insects as opposed to bacteria (Gressel, 2015). Any alteration to target-site either as a result of gene mutation or amplification may incur fitness cost in the organism. However, there are no studies, especially in the case of gene duplication to prove this scenario. This is due to the complications involved in studying of copy-number variants and also due to the well agreed on belief that duplications lead to novel functions which outweigh the short-term implications like fitness penalties (Kondrashov, 2012). However, no fitness penalty has been reported in GR *A. palmeri* conferring resistance via *EPSPS* gene amplification (Vila-Aiub et al., 2014).

Previous studies elucidating the target-site resistance to glyphosate in Italian ryegrass suggest that the mechanism is either due to mutations or amplification of *EPSPS* gene. In this dissertation, a population of GR Italian ryegrass from Arkansas was used. This population showed 7-19 fold more resistance to glyphosate compared to a known susceptible population. The resistance to glyphosate was determined by *EPSPS* gene amplification (Salas et al., 2012; Salas et al., 2015). Furthermore, no difference in ^{14}C glyphosate uptake or translocation was found between GR and susceptible plants (Salas et al., 2015). *EPSPS* gene sequencing results showed no mutation associated with resistance to glyphosate in this population. The GR Italian ryegrass individuals had *EPSPS* gene copy numbers ranging between 11 to 151 compared to susceptible plants, which had a single copy of *EPSPS* (Salas et al., 2015). A minimum number of *EPSPS* gene copies to survive the field dose of glyphosate was found out to be 10. *EPSPS* copy number, enzyme activity, the level of glyphosate resistance were all positively correlated (Salas et al., 2012; Salas et al., 2015). However, no information is available on the location of amplified *EPSPS* copies on the genome of Italian ryegrass. Such information would help understand the initial event contributing to gene amplification and also potential rapid increase in *EPSPS* copies thereby increase in glyphosate resistance. Therefore, the main objective of this research is to physically map the distribution of amplified *EPSPS* gene copies on the genome of GR Italian ryegrass as well as to determine the relationship between *EPSPS* gene copies, *EPSPS* gene expression and EPSPS protein expression.

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Chapter 2 - Determination of Genomic Copy Number, Protein Expression and Physical Mapping of *EPSPS* Gene in Glyphosate-Resistant Italian Ryegrass

Abstract

Italian ryegrass (*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot), one of the problem weeds of the US, evolved resistance to multiple herbicides including glyphosate due to selection in Arkansas (AR). Glyphosate is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor and amplification of *EPSPS* gene, the molecular target of this herbicide contributes to resistance in several weed species, including Italian ryegrass population from AR. The objective of this study was to determine the expression of *EPSPS* gene and protein as well as distribution of *EPSPS* copies on the genome of glyphosate-resistant Italian ryegrass (ARR) using a known susceptible Italian ryegrass (ARS) from AR. *EPSPS* gene copies and expression of ARR and ARS were determined using quantitative PCR with appropriate endogenous controls. EPSPS protein expression was determined using Western blot analysis. Fluorescence *in situ* hybridization (FISH) was performed on somatic metaphase chromosomes to determine the location of *EPSPS* copies. Based on the qPCR analysis, ARR plants showed 12 to 118 *EPSPS* copies compared to a single copy in ARS. *EPSPS* gene expression correlated with the gene copy number in both ARR and ARS. Individuals with high *EPSPS* copies showed high protein expression in Western blot analysis. FISH analysis showed the presence of brighter *EPSPS* signals, distributed randomly throughout the genome of ARR individuals compared to a faint signal in ARS plants. The random distribution of *EPSPS* copies was previously reported in glyphosate-resistant Palmer amaranth. Overall, the results of

this study will help understand the origin and mechanism of *EPSPS* gene amplification in Italian ryegrass.

Introduction

Italian ryegrass is a monocot weed native to Southern Europe, which was introduced throughout the world including the United States as a forage grass. It is a self-incompatible species and does not cross with cultivated annual ryegrass and other ryegrass species (Jacobs et al. 2008). Italian ryegrass has excellent palatability and seedling vigor. Recently, it has been reported that this species has potential to be a good source for biofuel production (Yasuda et al. 2015). Despite the useful characteristics of the ryegrass in certain aspects its position in the field as a noxious weed far outweighs the benefits. Italian ryegrass is allelopathic and is shown to inhibit the growth of other species in the proximity (Ferreira 2011). Italian ryegrass has been reported to show interference with many crops affecting their growth by outcompeting them for resources (Liebl & Worsham 1987; Stone et al. 1998; Nandula 2014). Italian ryegrass populations if left uncontrolled in the field can cause substantial yield losses to crops and they can also harbor certain plant pathogens that can infect crop species (Ikley et al. 2015). Traditionally Acetyl-CoA Carboxylase (ACCase) herbicides like diclofop-methyl, quizalofop, clodinafop etc. and Acetolactate Synthase (ALS) inhibitors like chlorosulfuron, iodosulfuron-methyl etc. were used for the control of ryegrass populations. Extensive use of these herbicides selected for Italian ryegrass populations resistant to these herbicides.

Since the introduction of glyphosate in 1974, as a non-selective herbicide it was widely used to control many grass and broadleaf weeds mainly due to its efficacy and low mammalian toxicity (Henderson et al. 2010). Later in 1996, the development of glyphosate-resistant (GR) crops further intensified the use of glyphosate (Powles 2008). Glyphosate belongs to the chemical

family of glycine and is a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitor. EPSPS enzyme is required for the regulation of shikimate pathway in plants which in turn is responsible for producing essential aromatic amino acids (Maeda & Dudareva 2012). Glyphosate shares structural similarity with phosphoenol pyruvate and therefore blocks the substrate binding site of EPSPS enzyme. Intensive use of glyphosate over the past two decades resulted in the evolution of glyphosate resistance in a number of weed species, including Italian ryegrass. First case of GR Italian ryegrass was reported in 2001 in Chilean fruit and orchards (Perez & Kogan 2003). In the United States, GR Italian ryegrass biotype was first reported in 2004 in the orchards of Oregon (Perez-Jones et al. 2005). Till today, GR Italian ryegrass populations with multiple herbicide resistance have been reported in 9 countries.

Mechanisms of resistance to herbicides can broadly be grouped into two categories: a) target site resistance where the target is altered, thus the herbicide will not have toxic effect; b) non-target resistance, primarily mediated by metabolism of herbicides (Ma et al. 2013); or impaired uptake/translocation, and/or avoidance (Bowler et al. 1992). Non-target-site resistance is much more complex than target site based and can involve many genes and can result in cross-resistance to several herbicides (Délye 2013). There were several reports of target site resistance with a mutation in *EPSPS* gene leading to Pro106Ser substitution resulting in 5 fold resistance in Italian ryegrass compared to susceptible populations (Jasieniuk et al. 2008; González-Torralva et al. 2012; Perez-Jones et al. 2007). GR Italian ryegrass populations with impaired translocation were also reported recently (González-Torralva et al. 2012). Another important and novel mechanism of glyphosate resistance reported in five GR species so far is, target gene amplification or duplication (Sammons and Gaines, 2014) where a single copy of *EPSPS* gene is amplified resulting in the production of multiple copies of this gene. The increase in *EPSPS* copies also

showed a positive correlation to the level of glyphosate resistance in Italian ryegrass (Salas et al. 2012), kochia (Jugulam et al. 2014) and common waterhemp (Dillon et al. 2016).

Previously, the importance of gene duplication as a prominent event driving evolution has been reported in many instances (Soukup 1974; Taylor & Raes 2004; Kubo et al. 2015). Gene duplications can arise through several events namely unequal crossing over between two homologous sister chromatids, retroposition or chromosomal duplication (Zhang 2003). Tandem amplification of the chromosomal region containing *EPSPS* gene copies in GR kochia has been reported recently suggesting a possible role of unequal crossing over resulting in gene duplication (Jugulam et al. 2014). On the other hand, in GR *A. palmeri*, a possible role of transposons in mediating *EPSPS* gene amplification was reported (Gaines et al. 2010). Subsequently, it was also suggested that in *A. palmeri* the gene duplication might have resulted due to DNA transposon-mediated replication with the involvement of miniature inverted-repeat transposable elements (MITEs) adjacent to *EPSPS* gene (Gaines et al. 2013). However, no fitness penalty has been reported for GR *A. palmeri* populations endowed through *EPSPS* gene amplification (Vila-Aiub et al. 2014). Fitness costs associated with gene amplification is important to study as it would determine the time for penetrance of the resistant trait in the individuals and also the persistence of glyphosate resistance in the population which could have long-term implications for the sustainable use of glyphosate (Giacomini et al. 2014).

EPSPS gene amplification resulting in the evolution of resistance to glyphosate in Italian ryegrass populations from Arkansas (ARR) was previously reported (Salas et al. 2015; Salas et al. 2012). The ARR populations showed a 7-19 fold resistance (with up to 25 *EPSPS* copies) to glyphosate compared to a known susceptible population (ARS) with a single copy. Furthermore, a positive correlation between enzyme activity, copy number and level of glyphosate resistance

was also found (Salas et al. 2015). However the mechanism of *EPSPS* gene duplication in this weed species is elusive. Currently, there are several molecular cytogenetic techniques available to study the basis of gene amplification in herbicide resistant weed species. Some of the techniques that can be employed are fluorescence *in situ* hybridization (FISH), and high-resolution FISH on stretched DNA (fiber-FISH). These techniques help in visualizing the physical location of amplified genes or small DNA fragments of interest on chromosomes by using multi-colored probes. Here in this research, the FISH technique was used to illustrate the location and distribution of amplified *EPSPS* copies on the chromosome of ARR Italian ryegrass. These insights will help understand the basis of gene amplification in the evolution of glyphosate resistance in this species.

Research Hypothesis and Objectives

This research was conducted based on the hypothesis that *EPSPS* gene amplification in ARR Italian ryegrass may have evolved as a result of unequal crossing over or transposon-mediated mechanism. The overall objective of this dissertation is to determine the distribution of *EPSPS* copies in ARR Italian ryegrass as an initial step to uncover the mechanism of *EPSPS* gene amplification.

The specific objectives of this study were, using a known Italian ryegrass population susceptible to glyphosate from AR (ARS) to determine:

1. the *EPSPS* gene copy number in the ARR Italian ryegrass.
2. the level of *EPSPS* gene expression in ARR Italian ryegrass.
3. whether *EPSPS* gene expression is translated to EPSPS protein expression.
4. the distribution of duplicated *EPSPS* gene copies on the genome of ARR Italian ryegrass.

Materials and Methods

Plant Material and Growth Conditions

ARS and ARR Italian ryegrass seed were obtained from AR and used in this research. The ARR seed were collected from a field in Desha County, AR. The seeds were treated with discriminating dose of glyphosate and survivors were bulked in the greenhouse (Salas et al., 2012). Bulked seed were germinated on moist filter paper in small Petri dishes at room temperature in dark room. After establishing 2-3 cm roots, seedlings were transferred to individual pots (4 cm x 4 cm) containing Miracle-Gro potting mix (Scotts Miracle-Gro Company, Marysville, OH, USA) and grown in greenhouse with appropriate growth conditions (temperature of 25/20 °C with photoperiod 16/8 h light day/night and light intensity of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ supplemented by sodium vapor lamps). Relative humidity in the greenhouse was maintained at around 65%. Plants were fertilized with Miracle-Gro water-soluble all-purpose plant food (Scotts Miracle-Gro Company., OH) every two weeks. A total of 23 ARS and 60 ARR plants were grown until one tiller stage and tillers were separated to make one clone for each plant respectively.

Herbicide treatment

When ARS and ARR plants started tillering (Fig. 1), one vegetative clone was made for each individual plant by separating the tillers and grown in separate pots (4 cm x4 cm). A total of 23 ARS and 60 ARR Italian ryegrass clones derived from separate ARS and ARR plants were treated with a discriminating dose (1X) of glyphosate along with appropriate adjuvant (Roundup Weathermax[®] @ 840 g ae ha⁻¹ in 2% (v/v) ammonium sulfate (AMS)) at three-leaf stage. The herbicide was applied using a track sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing., MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., IL) delivering 168 L ha⁻¹ at 222 kPa in a single run at 4.8 km h⁻¹. An untreated check of 3-4

plants of both ARS and ARR were included. Sprayed plants were maintained at the same growth conditions as mentioned above. Plants were assessed for percent survival 28 days after treatment (DAT).

Genomic DNA extraction and sequencing

EPSPS gene was sequenced to check for any non-synonymous amino acid changes that may confer resistance to glyphosate in the Italian ryegrass populations. Fresh leaf tissue (~100 mg) was collected in 50 ml falcon tubes, flash frozen in liquid nitrogen and stored at -80 °C for genomic DNA extraction. Genomic DNA was extracted using Qiagen DNEasy Plant Mini Kit (Qiagen Inc., CA) following the manufacturer's provided protocol. DNA quantification was done using a Nano Drop 1000 spectrophotometer (Thermoscientific., DE). Genomic DNA was amplified in a 50 µl reaction using a Forward primer EPSPS LOLF2 (5'-AGAGCTGTAGTCGTTGGCTGT-3') and reverse primer EPSPS LOLR2 (5'-TAGGTCGCTCCCTCATTCTTG-3'), which amplifies a 350 bp fragment. This fragment would cover the previously reported Proline 106 amino acid position on the *EPSPS*, which is reported to endow glyphosate resistance. The reaction mixture contains 25 µl Taq 2X Master mix (Promega., WI), 10 µl nuclease free water 5 µl of each primer (5 µM) and 5 µl of genomic DNA. Reaction conditions for the Polymerase Chain Reaction (PCR) include an initial denaturation step of 95 °C for 3 min followed by an additional denaturation step at 95 °C for 30 sec (40 cycles), primer annealing step at 56 °C for 45 sec, product elongation step at 72 °C for 1 min and a final elongation step at 72 °C for 5 min . PCR was set up in a thermocycler (Biorad., CA). The amplified PCR product was analyzed on a 0.8% agarose gel with ethidium bromide staining and a 100 bp ladder (MidSci., MO) as a marker. DNA bands were visualized in a UV Transilluminator (Fotodyne Inc., WI). PCR product was purified using QIAquick PCR purification

kit (Qiagen Inc., CA) following the manufacturer guidelines. Samples were sequenced at the DNA sequencing and genotyping facility in the Plant Pathology department at Kansas State University. Sequence chromatograms were analyzed using SnapGene software (GSL Biotech LLC., IL) and sequences were aligned using Multalin software (Corpet, 1988).

Total RNA isolation and cDNA Synthesis

Fresh leaf tissue (50-100 mg) was collected from the same above plants in 50 ml falcon tubes and flash frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Tissue was homogenized using a pre-chilled/flash frozen mortar and pestle and transferred to 2 ml pre-chilled Eppendorf tubes. RNA was extracted using TRIzol reagent method (Invitrogen., MA) following the manufacturer's protocol with few modifications. Isolated RNA was analyzed on a 0.8% agarose gel and Nano drop spectrophotometer. Starting concentration of 1 µg of RNA was used for cDNA synthesis. cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific., MA) following manufacturer's recommendations. The final product is about 20 µl and was diluted 5 times with nuclease free water (+80 µl) for direct use in gene expression studies.

***EPSPS* gene amplification and expression**

Real-time quantitative PCR was used to determine the relative genomic copy number and gene expression between the ARS and ARR Italian ryegrass. Genomic DNA (gDNA) was isolated as described above. gDNA was used for *EPSPS* copy number determination in a Quantitative PCR (StepOnePlus™ real-time detection system, Thermo Fisher Scientific) using *β-tubulin* (Godar et al., 2015) as a reference gene. Diluted cDNA was used for *EPSPS* expression studies with *actin* as an endogenous control. Reaction volume is set at 14 µl which includes 8 µl of *Power* SYBR Green (Life Technologies corp., NY), 2 µl of each primer (5 mMol) and 2 µl of genomic DNA normalized to 20 ng/µl or 2 µl diluted cDNA as described previously in cDNA synthesis method. At least three

replicates were used for each individual plant. Reaction conditions were initial denaturation at 94 °C for 10 min followed by denaturation at 94 °C for 15 seconds and annealing at 60 °C for 30 sec repeated for 39 cycles. Melt curve profile was included at the end of the cycle to determine the specificity of the reaction. Single curves were generated for both the *EPSPS* and *β-tubulin* primers. Primers used for determining the *EPSPS* copy number were EPSPS LOLF (5'-CTGATGGCTGCTCCTTTAGCTC-3') and EPSPS LOLR (5'-CCCAGCTATCAGAATGCTCTGC-3') (Salas et al. 2012) that will amplify a 136 bp fragment, Beta Tub F (5'-ATGTGGGATGCCAAGAACATGATGTG-3') and Beta Tub R (5'-TCCACTCCACAAAGTAGGAAGAGTTCT-3') that will amplify a fragment of 157 bp. Additionally, for determining the *EPSPS* gene expression *actin* was used as an endogenous control instead of *β-tubulin* for its stable gene expression. Primer sequences for *actin* are LM Actin F (5'-CTGACTGAGGCACCCCTGAA-3') and LM Actin R (5'-GCTGACACCATCACCAGAATCCAAC-3') that will amplify a fragment of 168 bp. Both copy number and gene expression were determined using the manufacturer provided Real-time PCR system software version 2.3 (Applied Biosystems., MA).

Statistical analysis

The relative fold in copy number and gene expression between the ARS and ARR plants was determined by the comparative Ct method (as $2^{-\Delta Ct}$) where $\Delta Ct = [Ct \text{ target gene} - Ct \text{ reference gene}]$. *β-tubulin* and *actin* were used as endogenous controls (reference gene) for normalizing the copy number and gene expression data. At least one glyphosate susceptible sample is used as a calibrator sample to represent 1x copy number or gene expression. Gene copy number and expression data were analyzed using one-way ANOVA in SigmaPlot (version 12.3). Means were

separated using Fisher's LSD ($p < 0.05$) and standard errors (SE) were calculated based on three technical replicates ($n = 3$).

Leaf-Protein Extraction, SDS-PAGE, and Western Blotting

Fresh leaf tissue (2 g) from ARS and ARR Italian ryegrass was homogenized in liquid nitrogen and added to 20 mL extraction buffer (50mM Tris-HCl, pH 8, 50 mM NaCl, 1mM EDTA, 1 mM MgCl₂ and 0.038 g PMSF, 1 tablet of Pierce Protease Inhibitor (Thermoscientific), 1g insoluble PVPP). Both the protein extraction and purification procedures employed here were developed with minor modifications to the existing methods (Wang et al., 2006, Wu et al., 2014). Homogenized leaf tissue samples were centrifuged at 4 °C, 10 min, 16000 rpm (Beckman J2-HC centrifuge., USA) and the supernatant was collected. The resulting supernatant (20 ml) was split into two tubes of 10 ml each. One ml of 100% Trichloro acetic acid (TCA) was added to each 10 ml supernatant tubes and incubated for 60 min at 4 °C. After incubation, the tubes were centrifuged again at 4 °C for 10 min at 16000 rpm. The resulting supernatant was carefully discarded without removing the pellet. Pellet was resuspended in 2 ml of 100% methanol by vortexing vigorously for 60 seconds. Tubes were centrifuged again at conditions (4 °C, 10 min, 16000 rpm). The resulting supernatant was discarded and the pellet was washed with 2 ml of 80% acetone by vortexing vigorously. Tubes were centrifuged again at conditions (4 °C, 10 min, 16000 rpm) and resulting supernatant was discarded. The remaining pellet was completely air dried to remove the remaining traces of acetone. After air drying, the pellet was suspended in 2 ml phenol (equilibrated with Tris-HCL, pH 8.0, Sigma-Aldrich) by vortexing vigorously. Tubes were centrifuged at the following conditions (4 °C, 10 min, 16000 rpm) and the supernatant was collected. Protein precipitation was done by incubating samples overnight at -20 °C after adding 2 mL ammonium acetate (0.1 M in methanol). Samples were subjected to one final centrifugation at 4 °C for 10 min

at 16000 rpm and supernatant was discarded. Pellet was washed twice once with methanol (100%) followed by acetone (80%) and finally air dried. Samples were re-suspended in 200 μ L SDS-Sample buffer and protein concentration was measured using the manufacturer's protocol (RED 660TM Protein Assay., G-Biosciences).

Protein samples were resolved by SDS gel electrophoresis. Samples were incubated at 90-95 °C for 5-7 minutes. At least 50 μ g total protein was resolved on an 11% polyacrylamide gel running at 120 V for 90 min. Samples on the SDS gel were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) by resolving them at 150 V for 60 min or at 30 V overnight (4 °C) preferably. 5% non-fat dry milk was used for blocking the PVDF membrane by gentle stirring at room temperature for 30 min. The membrane was washed at least three times in TBST buffer. The membrane was incubated with EPSPS primary antibody (Monsanto., MO) at a dilution of 1:000. After several washes in TBST buffer, the membrane was incubated with donkey anti-rabbit HRP conjugated polyclonal antibody (Jackson Immuno Research Laboratories Inc., dilution 1:50,000) at room temperature for 60 min. After three more washes, the membrane was exposed to HRP (Horse-radish peroxidase) substrate solution (LuminataTM., Millipore) to produce fluorescence. Bands were visualized using a G-BOX image detector from Syngene.

Fluorescence *in situ* hybridization (FISH) procedure

FISH was performed by nitrous oxide-enzymatic maceration method described in the protocol (Kato, 1999; Kato et al., 2004, 2006). The procedure is divided into three sections, which are explained below

Somatic metaphase spread preparation from young roots

Newly formed 1 cm long root tips (3-5 tips) were excised from germinated seeds of ARS and ARR grown on moist filter paper and also young plants grown in soil. The root tips were immediately placed in a moist 1.5 ml microcentrifuge tube with a hole punched to its cap and placed on ice until nitrous oxide treatment. The root tips were treated in a pressurized nitrous oxide chamber for 2.5 hours to arrest chromosomes at metaphase stage. The root tips were fixed in ice-cold 90% glacial acetic acid for 20 min and transferred to 70% ethanol and stored at -20 °C until use. Suspension dropping method as described by Kato (1999) was used to prepared somatic metaphase chromosome spreads with few modifications. The fixed root tips were washed with ice-cold 1X HCl buffer (pH 4.0) or 1X citric acid buffer (pH 4.0) dried on filter paper and opaque (actively dividing) region of about 2 mm is excised with its root cap separated. The actively dividing region of the root tip was treated with 20 µl enzyme solution (4% cellulose Onuzuka R-10 (Yakult., Tokyo), 1% pectolyase Y23) in Kcl buffer (pH 4.0) for 40-45 min in water bath at 37 °C. The enzyme mixture was transferred on to the ice and treated with 500ul ice cold 1X TE buffer for 5 min. TE buffer was carefully removed and washed 3 times with 100% ice-cold ethanol solution. The mixture was centrifuged briefly to remove any liquid content. 30 µl of freshly prepared 90% glacial acetic acid and 10% methanol were added to the reaction in a microcentrifuge tube. Root sections were homogenized gently with a dissecting needle 6 µl of the solution was dropped onto labeled microscope slides in a humid chamber and chromatin to slide was crosslinked with a UV crosslinker (GE Healthcare Bio-Sciences., MA).

***EPSPS* probe preparation for FISH analysis**

Primers were designed with OligoAnalyzer 3.1 (<https://www.idtdna.com/calc/analyzer>) using Italian ryegrass sequence information from Genbank (Accession number: DQ153168.2) (National Center for Biotechnology Information., MD). Both forward primer *EPSPS* LOLF1 (5'-TGCAGCCCATCAAGGAGATCT-3') and reverse primer *EPSPS* LOLR2 (5'-ACGAAGGTGCTTAGCACGTCAAA-3') were designed to amplify a 1255 bp fragment of *EPSPS* gene. Genomic DNA was extracted by Qiagen DNEasy Plant Mini Kit (Qiagen Inc., CA) from susceptible Italian ryegrass population which was used as a template. The PCR product was purified using QIAquick PCR purification kit (Qiagen Inc., CA) following the manufacturer guidelines. PCR product was gel extracted using the Qiagen Gel Extraction kit (Qiagen Inc., CA) and was re-amplified using the same primers as before. The final cleaned PCR product was sequenced at the DNA sequencing and genotyping facility at Kansas State University. After confirming the *EPSPS* gene sequence, PCR product was cloned using a TOPO XL PCR cloning kit (Invitrogen., MA) following the manufacturer recommendations. Several (3-4) PCR products were tested and the best reactions were pooled to use as a FISH *EPSPS* gene probe.

Chromosome, probe DNA denaturation and hybridization

Probe cocktail was prepared in 2X SSC-1X TE buffer with recommended concentration (Kato et al., 2006) at a volume of 5 μ l for each slide. All the reactions were performed at low light conditions. Five μ l of probe mixture with DNA concentration greater than 250 ng/ μ l was used for labeling by nick translation with dig-11-dUTP; 5 μ l of denatured salmon sperm DNA (140 ng/ μ l, 2XSSC, 1X TE) was added to the center of the slide and sealed with a 22 x 22 mm coverslip. The chromosomes and probe DNA cocktail were denatured by heating in a water bath at 100 °C for 5 min. Coverslips were removed and 5 μ l of pre-chilled probe cocktail was pipetted onto the slides

and sealed with a coverslip. Slides were stored in a humid chamber at 55 °C for hybridization overnight. Slides were washed in a Coplin jar with 2X SSC for 5 min. Coverslips were removed and washed for another 20 min in 2X SSC at 55 °C. Slides were counterstained in Vectashield antifade solution with 4',6-diamidino-2-phenylindole (DAPI). They can be stored at 4 °C for short term storage or -20 °C for longer storage. Probe signals on the chromosome were visualized using a Zeiss Axioplan 2 microscope fixed with a cooled CCD camera Cool SNAP HQ2 (Photometrics) and AxioVision 4.8 software (Zeiss). The final contrast of the images was processed using Adobe Photoshop CS5 software (Adobe Systems Incorporated, CA) package.

Results

Whole-Plant Response to Glyphosate

A total of 23 ARS and 60 ARR clones were treated with glyphosate. Results showed that there were no survivors of ARS individuals treated with glyphosate indicating a homogenous glyphosate susceptible population (Figure 2.1 A). On the other hand, the response of ARR plants showed a variation in the level of resistance to glyphosate indicating that this population was segregating for glyphosate resistance and susceptibility (Figure 2.1 B). Out of the 60 ARR plants treated with 1X glyphosate dose, only 24 plants survived (Table 2.1).

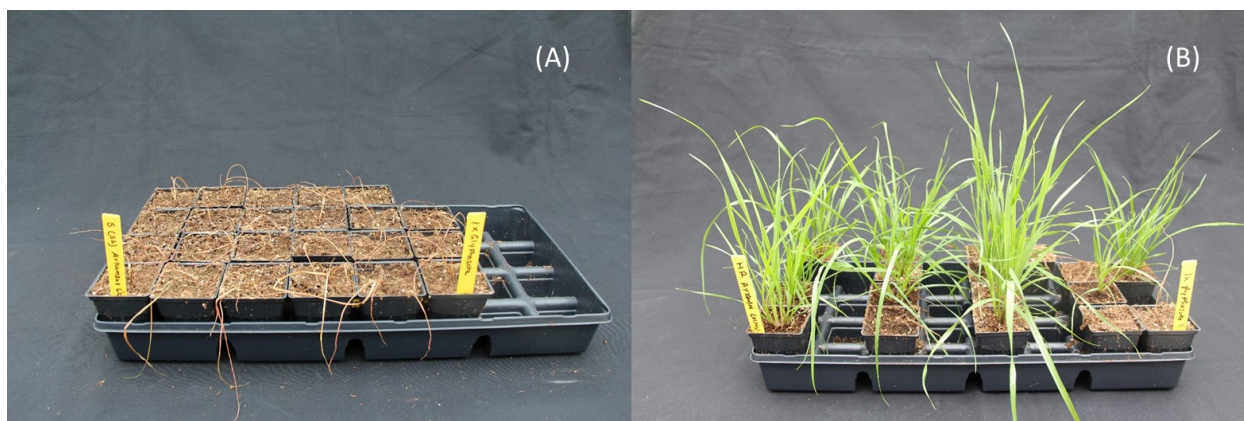


Figure 2.1 Response of ARS (A) and ARR (B) Italian ryegrass populations to glyphosate [840 g ae ha⁻¹ in 2% (v/v) ammonium sulfate (AMS)] application 28 days after treatment (DAT).

Table 2.1 The percent survival of ARS and ARR Italian ryegrass populations from Arkansas, 28 days after treatment (DAT) with glyphosate [840 g ae ha⁻¹ in 2% (v/v) ammonium sulfate (AMS)].

Glyphosate (840 g ae ha ⁻¹) 28 DAT				
Populations	Total # of plants treated	Alive	Dead	%survival
ARS	23	0	23	0
ARR	60	24	36	40

***EPSPS* Gene Sequencing**

Previously, a point mutation at amino acid position Proline 106 causing a single amino acid substitution from proline to serine/threonine/leucine and alanine has been reported to endow glyphosate resistance in Italian ryegrass by preventing binding of glyphosate to target (González-Torralva et al., 2012; Jasieniuk et al., 2008; Perez-Jones et al., 2007). Italian ryegrass *EPSPS* gene covering the known mutations at P106 was sequenced to assess if such mutations are present in

ARR populations resulting in glyphosate resistance. The sequences are also checked for amino acid position 102 for the presence of TIPS (Threonine 102 Isoleucine + Proline 106 Serine) mutation, which is reported to endow >2000 fold glyphosate resistance in *E.indica* (Yu et al. 2015). None of the ARR plants sequenced showed any substitution at the amino acid position 106 (Figure 2.2). This confirms that the basis of glyphosate resistance in these populations is not due to mutations in the target site.

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ARS1 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARS2 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR1 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR2 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR3 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR4 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR5 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR6 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR7 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR8 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR9 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
ARR10 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGCCATTGACGGC
13R5 GCTCTTCTTGGGGAACGCTGGAACTTGCGATGCGGACATTGACGGC

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Threonine 102 Proline 106

Figure 2.2 *EPSPS* gene sequences of Italian ryegrass covering the previously reported Proline 106 amino acid position. Glyphosate susceptible plants were marked in red (S1, S2) and glyphosate resistant plants were marked in black. Amino bases highlighted in yellow represent the wild type/no mutation (CCA-proline, ACT-threonine) at amino acid position 106 and 102 respectively. 13R5 is the control i.e. known Italian ryegrass biotype with single base pair change at amino acid position 106 (ACA- Threonine).

***EPSPS* Genomic Copy Number and Gene Expression Analysis**

Seventeen ARR plants that survived the treatment of 1X glyphosate were tested for *EPSPS* genomic copy number along with 2 ARS individuals. The relative *EPSPS*: β -*tubulin* genomic copy number was determined with an ARS plant as a control. Both the susceptible plants (S2.2, S2.1) showed a relative *EPSPS*: β -*tubulin* copy number around one (0.99 and 1.17) but for simplicity,

the values were rounded to 1 with the rest of the samples being normalized to this control sample (Figure 2.3). The relative *EPSPS:β-tubulin* gene copy number for resistant plants ranged between 12.8 and 118.2 (Table 2.2). Based on the number of *EPSPS* copies possessed, the ARR plants were grouped into three categories, i.e. plants that had low (LR with 10-20); medium (MR with 20-40) and high (HR with >40) *EPSPS* copies. The non-survivors of ARR populations had <10 *EPSPS* copies, which is not sufficient to bestow resistance to glyphosate at a field used dose.

Similarly, the 17 ARR plants used for *EPSPS* genomic copy number analysis were also tested for relative *EPSPS:actin* gene expression. Additionally, the two ARS (S1, S2) were also used as controls and two other plants that failed to survive the 1X glyphosate were also used to check for *EPSPS* gene expression (Figure 2.4). *Actin* was used as an endogenous control in this study because of its stable expression. Both the susceptible plants (S1, S2) showed a relative *EPSPS:actin* cDNA transcript abundance around one (0.99 and 1.07) but for simplicity, the values were rounded to 1 with the rest of the samples being normalized to this control sample. The relative *EPSPS:actin* cDNA transcript abundance for resistant plants ranged between 12.4 and 205.0 (Table 2.2). Relative *EPSPS* copy number and gene expression between individuals differ significantly ($p < 0.01$) (Table 2.2).

Further analysis of *EPSPS:β-tubulin* genomic copy number and *EPSPS:actin* cDNA transcript abundance indicate a strong positive correlation (Figure 2.5) with a coefficient of correlation close to 1 ($r = 0.832463$). These results indicate that relative *EPSPS:actin* cDNA transcript abundance increased with an increase in *EPSPS:β-tubulin* genomic copy number.

Table 2.2 Summary statistics of ARS and ARR susceptible Italian ryegrass individuals and their respective relative *EPSPS: β -tubulin* gene copy number and relative *EPSPS: actin* gene expression. The values in the parenthesis indicate the standard error of means (n=3). Means followed by the same letter in the same column are equal at 1% level (LSD). NA stands for missing data.

	Biotypes	Relative <i>EPSPS</i> Copy Number	Relative <i>EPSPS</i> Gene Expression
Susceptible	S1	1.177 (0.103)m	1.080 (0.332)m
	S2	1.000 (0.060)m	1.000 (0.107)m
Resistant	LR1	12.821 (2.754)jklm	59.064 (14.944)de
	LR2	17.015 (3.059)ijkl	35.427 (4.974)fg
	LR3	18.677 (4.485)hijk	12.583 (2.249)klm
	MR1	22.405 (2.500)ghij	12.443 (0.845)klm
	MR2	24.506 (5.703)ghij	30.882 (7.651)ghij
	MR3	24.959 (1.528)fghij	16.428 (1.595)jkl
	MR4	28.123 (5.029)fghi	63.496 (14.036)d
	MR5	29.482 (5.946)fghi	28.933 (8.674)hij
	MR6	31.862 (2.263)efgh	44.742 (11.316)efg
	MR7	35.825 (6.797)defg	17.917 (0.968)jk
	MR8	39.391 (7.603)cdef	24.076 (8.424)ijk
	HR1	44.784 (3.214)cde	121.647 (13.307)b
	HR2	48.047 (16.473)cd	48.963 (9.268)ef
	HR3	48.220 (1.940)cd	29.556 (3.013)hij
	HR4	51.215 (10.899)c	43.140 (8.598)fgh
	HR5	67.957 (13.437)b	81.567 (10.770)c
	HR6	118.243 (17.402)a	205.057 (7.253)a

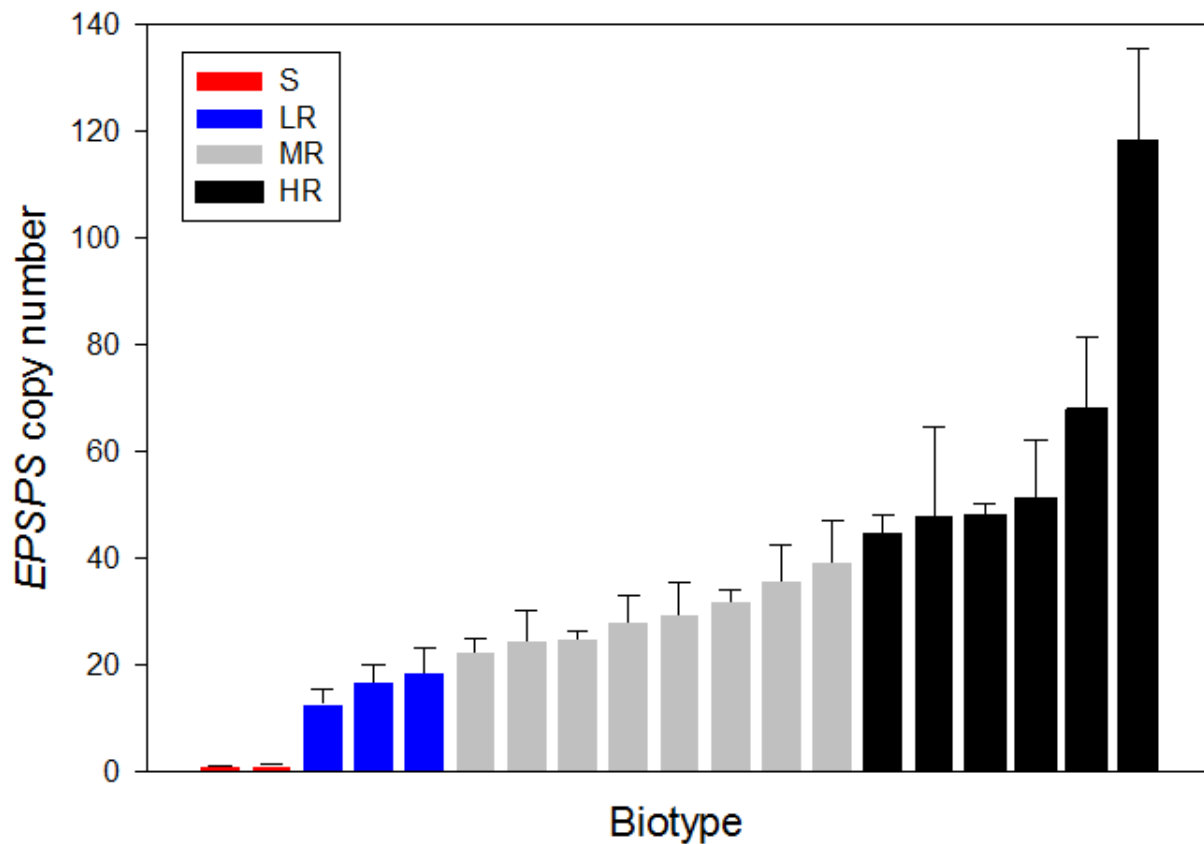


Figure 2.3 Bar chart showing the relative *EPSPS* gene copies in Italian ryegrass. The x-axis represents the individual plants of ARS and ARR populations and Y-axis represents the relative *EPSPS* genomic copy number. Error bars represent the standard error of means. S represents glyphosate susceptible individuals and LR, MR, HR represent ARR plants with low (10-20), medium (20-40) and high (>40) *EPSPS* copies.

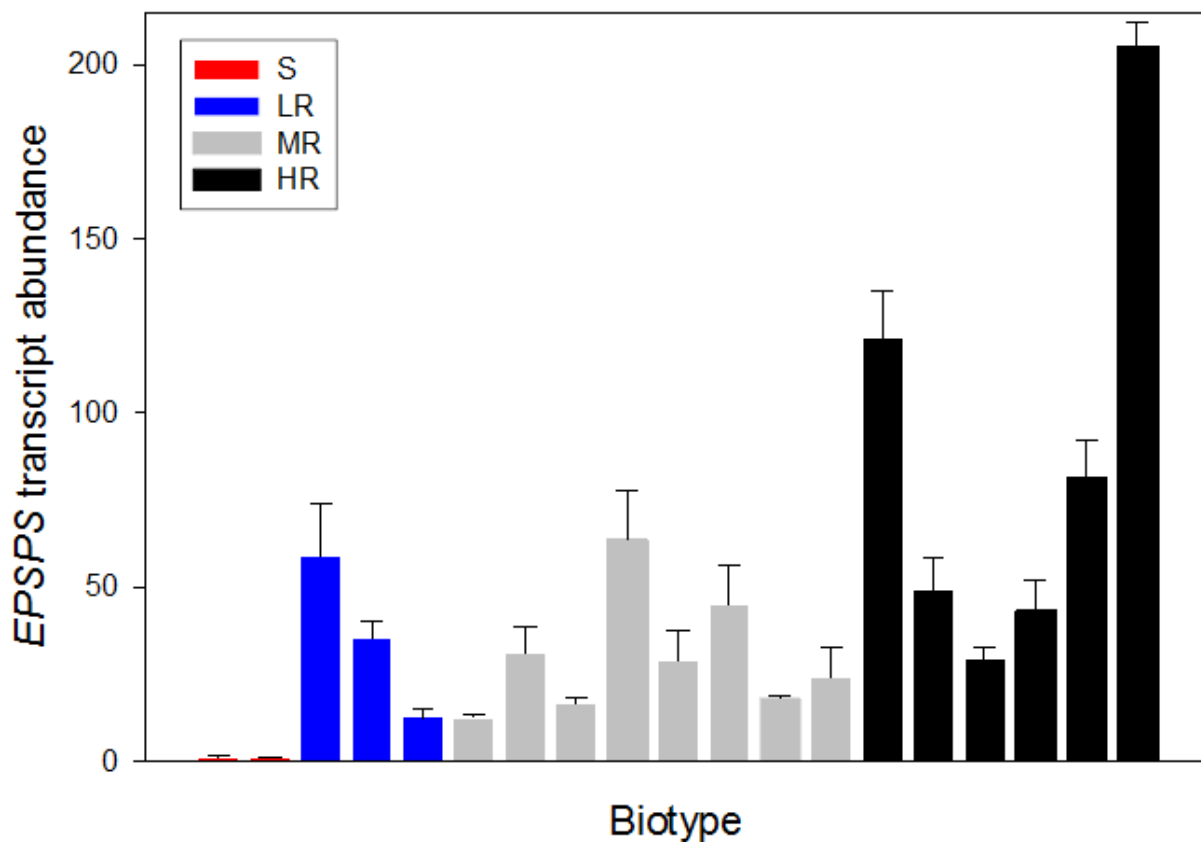


Figure 2.4 Bar chart showing the relative *EPSPS* gene expression in ARS and ARR Italian ryegrass. X- axis represent the individual plants and Y-axis represents the relative *EPSPS* transcript abundance. Error bars represent the standard error of means. S represents glyphosate susceptible and LR, MR, HR represent glyphosate-resistant transcript plants.

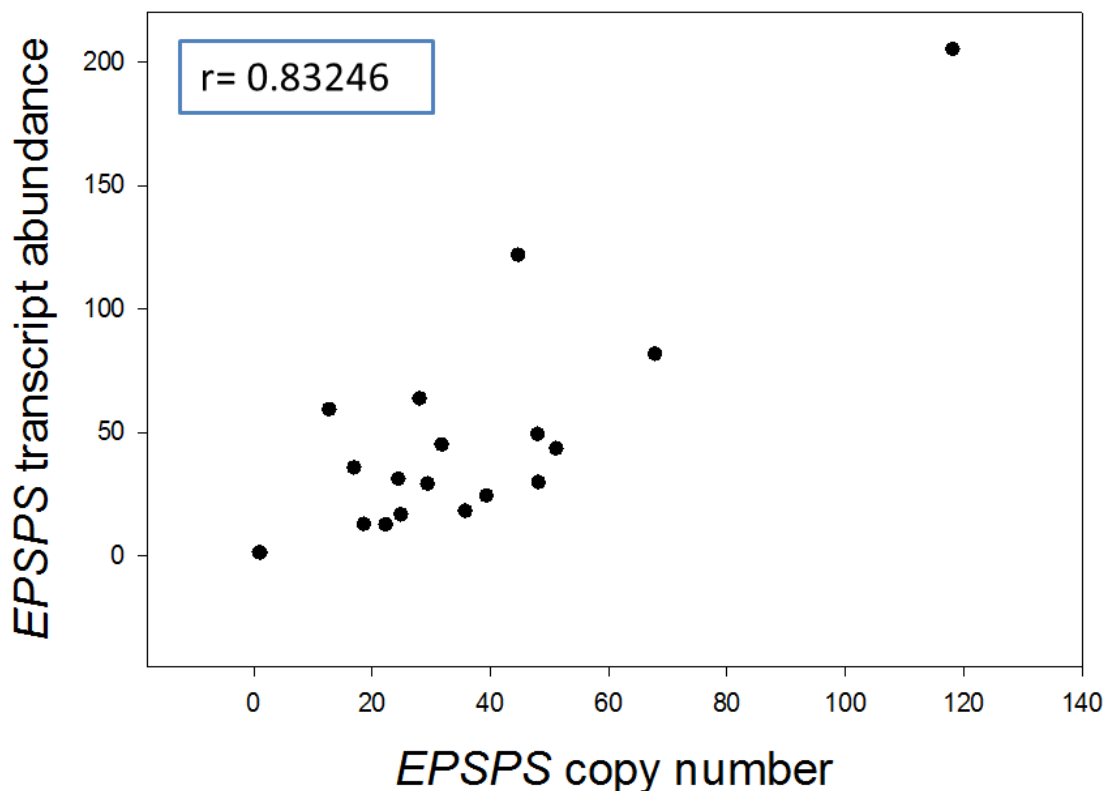


Figure 2.5 Scatter plot shows the relationship between relative *EPSPS* gene copy number (X-axis) and relative *EPSPS* transcript abundance (Y-axis). *r* is the correlation coefficient.

EPSPS protein expression

EPSPS protein quantity was determined using immunoblotting assay. Three ARR plants (LR1, HR4, HR6) with varying *EPSPS* copy number and gene expression were chosen along with an ARS for comparison (S1). The plants selected for this study had a relative *EPSPS*: β -*tubulin* genomic copy number of ~ 1.17 (S1), 12.82 (LR1), 51.21 (HR4) and 118.24 (HR6), respectively. Also, these plants (S1, LR1, HR4, HR6) had a relative *EPSPS*:*actin* cDNA transcript abundance of ~ 1.07, 43.14, 59.06 and 205.05, respectively. Fifty μ g total soluble protein resolved on 11%

polyacrylamide gel showed differences among the samples (Figure 2.6). EPSPS protein had a size of approximately 45.6 kDa. Susceptible sample (S1) appeared to be the faintest band whereas resistant sample with high *EPSPS* gene expression (HR6-205.05) appeared to be the brightest band (Figure 2.6). Other resistant samples (LR1 and HR4) showed subtle differences between the intensity of the band, however, HR4 having an *EPSPS* gene expression of 59.06 appeared to have more band intensity than LR with *EPSPS* gene expression of 43.14. Overall, these results indicate that the *EPSPS* gene expression was translated to EPSPS protein expression.

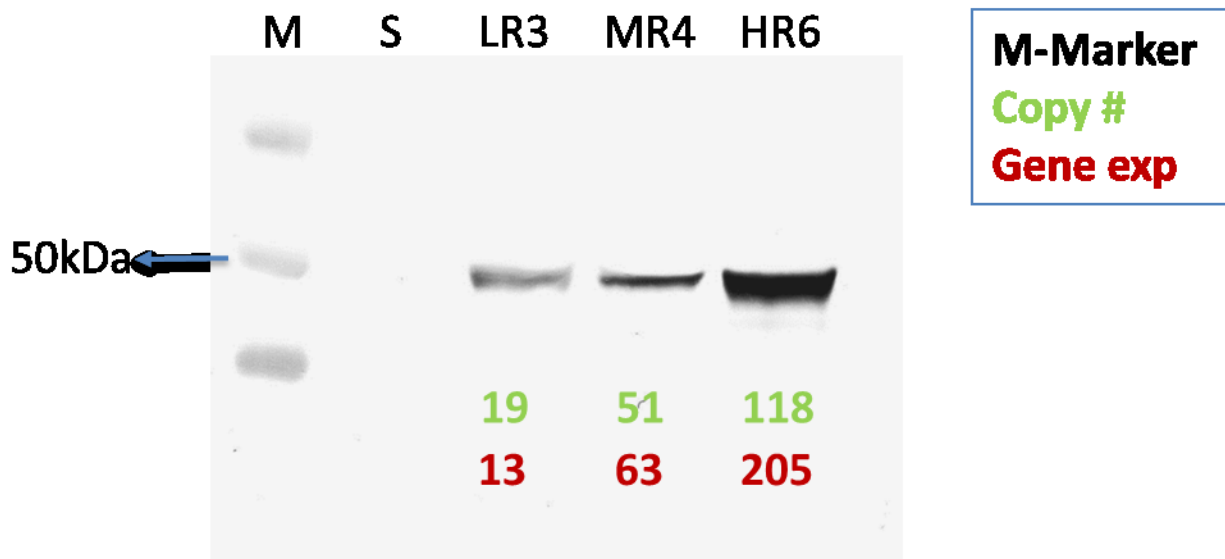


Figure 2.6 EPSPS protein expression in GR Italian ryegrass. S1 is glyphosate susceptible; LR3, MR2 and HR6 are glyphosate-resistant plants. M represents the Marker (ladder). Copy # represents the relative *EPSPS*: β -*tubulin* copy number in green. Gene exp represents the relative *EPSPS*:*actin* gene expression. Approximate size of the EPSPS protein is around 50 kDa (Kilo-daltons).

Distribution of amplified *EPSPS* gene copies on genome of Italian ryegrass

Distribution of amplified copies of *EPSPS* gene on the genome of Italian ryegrass plants was assessed using FISH. The ARR plants with varying relative *EPSPS*: β -*tubulin* genomic copy

number (MR3 ~ 25, HR4 ~ 51 and HR6 ~ 118) and an ARS plant (S1~1) was used as a control for comparison. Somatic metaphase spreads of these samples revealed only two *EPSPS* signals on the susceptible whereas resistant Italian ryegrass plant with relative *EPSPS: β -tubulin* genomic copy number (HR6 ~ 118) showed several bright *EPSPS* signals dispersed throughout the genome on all chromosomes (Figure 2.7). The other two resistant individuals (HR4 ~ 51, MR3~ 25) also showed bright *EPSPS* signals distributed throughout the genome of Italian ryegrass plants but the signals were lesser in number compared to HR6 [Figure 2.7 (B, C, D)]. It is important to note that it is hard to distinguish between the background and the actual *EPSPS* signals. Nonetheless, the results of FISH analysis still show a clear difference in *EPSPS* signal strength between the glyphosate-susceptible (S1), and -resistant (MR3, HR3 and HR6) Italian ryegrass. Also, these results show that the amplified *EPSPS* copies are not located at the telomeric region as in *K. scoparia* (Jugulam et al., 2014) nor they were present at the pericentric regions of two homologous chromosomes in as in glyphosate-resistant *A. tuberculatus* (Dillon et al., 2016). Instead, the *EPSPS* signals in glyphosate-resistant Italian ryegrass are randomly distributed throughout the genome as seen in glyphosate resistant *A. palmeri*, which is likely mediated by transposable elements (Gaines et al., 2010, 2013).

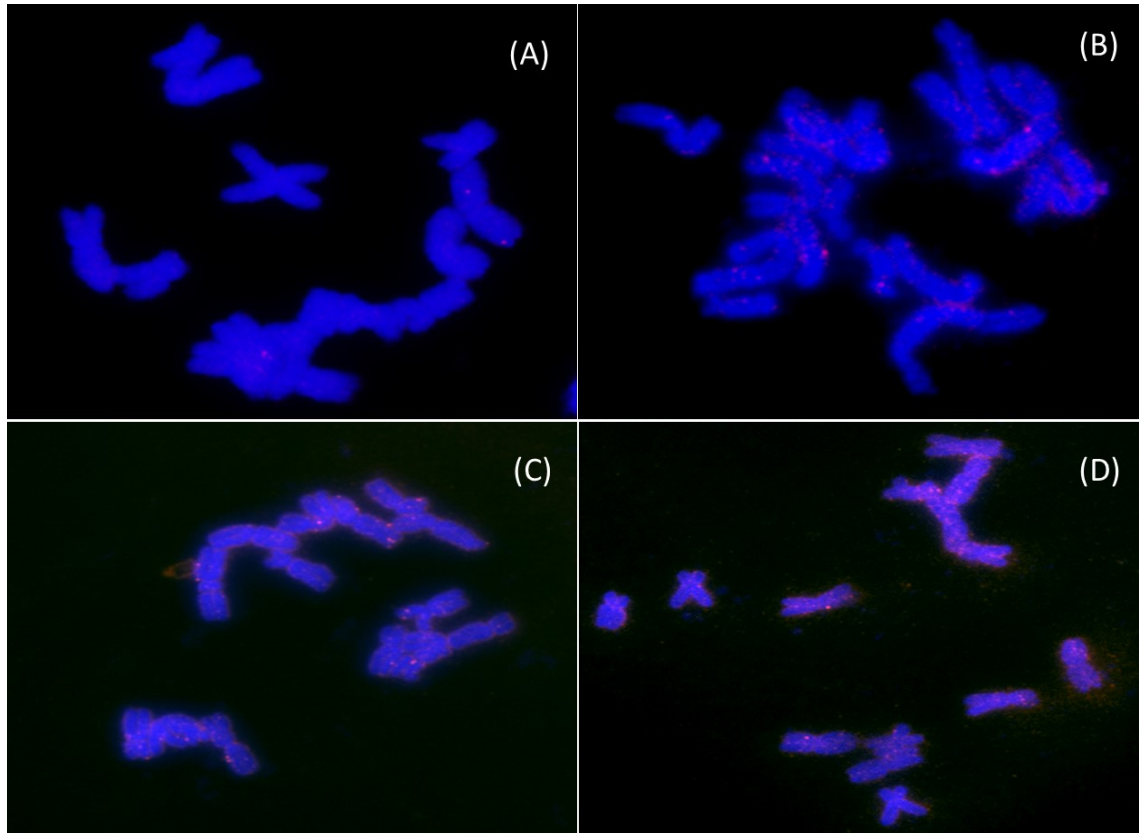


Figure 2.7 Fluorescence *in situ* hybridization (FISH) mapping of *EPSPS* gene on somatic metaphase chromosomes of Italian ryegrass. Image A shows a glyphosate susceptible Italian ryegrass (S1). Images B, C and D illustrate glyphosate-resistant Italian ryegrass individuals with 25, 51, and 118 *EPSPS* gene copies, respectively. Bright pink signals represent the physical location of amplified *EPSPS* copies.

Discussion

EPSPS gene copy number and gene expression analyses, indicate that the glyphosate resistance in ARR Italian ryegrass is conferred as a result of increased *EPSPS* copy number and expression as previously reported (Salas et al. 2012). Previously, several studies have shown *EPSPS* gene amplification as the mechanism of glyphosate resistance in a variety of weed species. Some examples include *A. palmeri*, *K. scoparia*, *A.tuberculatus*, *L. multiflorum* (Dillon et al., 2016; Gaines et al., 2011; Ribeiro et al., 2014; Chatham et al., 2015; Jugulam et al., 2014; Wiersma et al., 2015; Salas et al., 2012; Salas et al., 2015). Salas et al (2012) found that GR Italian ryegrass requires at least 10 copies to survive field recommended dose (840 g ae/ha) of glyphosate. But in

the case of GR *A. palmeri*, *A. tuberculatus* and *K. scoparia*, at least 30-50, >3, >3 copies of *EPSPS* were needed, respectively, to survive the same dose of glyphosate (Gaines et al., 2010; Chatham et al., 2015; Jugulam et al., 2014). The data of *EPSPS* genomic copy number and response to glyphosate treatment in this study suggest that the Italian ryegrass individuals with less than 12 copies did not survive the 1X dose of glyphosate, which is in agreement with previous reports (Salas et al., 2012; Salas et al., 2015). Hence, GR Italian ryegrass falls in between GR *A. tuberculatus*, *K. scoparia* and GR *A. palmeri*, thus needs to have a minimum threshold of *EPSPS* copies (10 copies) to withstand the field recommended dose of glyphosate.

Both relative *EPSPS* gene copy number and gene expression showed a strong correlation indicating that the amplified copies were translated to cDNA transcripts. Some of the GR Italian ryegrass individuals with low copy number (LR1 ~ 13 copies) showed 5 times more *EPSPS* gene expression (~ 59 cDNA transcripts), while other individuals with relatively high copy number (HR3 ~ 48, MR7 ~ 36 copies) showed a similar or lower gene expression (HR3 ~ 30, MR7 ~ 18 cDNA transcripts) respectively) (Table 2.2). This is possibly because gene copy number vs gene expression at different cellular conditions may or may not correlate for the same gene (Bussey et al., 2006). However, for the most part, a strong correlation between genomic copy number and gene expression or herbicide response (in this case) can suggest that the gene is subjected to selection pressure (Bussey et al., 2006).

Our study on *EPSPS* protein expression determined that the *EPSPS* gene expression is translated into functional protein. Because the *EPSPS* gene copy number and gene expression show a strong correlation and *EPSPS* protein expression, which is the actual measure of increased *EPSPS* enzyme activity, there can also be an additive effect with additional *EPSPS* copies. This additive effect of increased *EPSPS* copies was also reported in GR *A. palmeri* (Gaines et al., 2010).

FISH analysis on somatic metaphase spreads of ARS and ARR Italian ryegrass revealed the presence of amplified *EPSPS* copies distributed throughout the genome (Figure 2.7) similar to GR *A. palmeri* population from Georgia (Gaines et al., 2010). These authors reported that the likely mechanism of gene amplification in GR *A. palmeri* is by miniature inverted-repeat transposable elements (MITES) associated with *EPSPS* copies (Gaines et al., 2010; Gaines et al., 2013). This is in contrast with the results of FISH analysis on GR *K. scoparia* from Kansas where the amplified *EPSPS* copies are arranged in tandem in the telomeric regions on one chromosome pair, likely because of unequal crossing over of homologous chromosomes (Jugulam et al., 2014). Recent FISH study on GR common *A. tuberculatus* revealed that the amplified *EPSPS* copies were present in the pericentric region in one chromosome pair and in an additional chromosome (2n=33) harboring several *EPSPS* copies (Dillon et al., 2016). However this study suggested that the presence of an additional chromosome is not due to interspecific hybridization from GR *A. palmeri* but due to an initial event of unequal crossover followed by other mechanisms of gene amplification, such as chromosomal rearrangement or centromere-specific segmental duplication (Dillon et al., 2016).

It would be an interesting question to answer whether the gene amplification in GR Italian ryegrass has evolved as a result of one or more mechanisms of gene amplification. Also, this information would be valuable to understand whether glyphosate resistance through gene amplification occurred independently or spread via pollen from different locations. Insights from this study can also be used to better understand gene amplification as a mechanism for crop improvement in glyphosate-resistant crops.

As discussed earlier in chapter-1, glyphosate is a valuable tool to manage weeds. But due to a widespread increase in the number of GR weeds, management of weeds particularly in fields

with glyphosate-tolerant crops has become challenging. Although several mechanisms for glyphosate resistance in weeds were reported, gene amplification seems to be the most prevalent mechanism for glyphosate resistance in weeds. Therefore, knowledge on the basis of gene amplification in the evolution of glyphosate resistance will provide us with valuable insights for managing this weed. Also, this is the first report to show the physical mapping of amplified *EPSPS* copies in the genome of Italian ryegrass. Such valuable information could help us manage the herbicide as well as weed better in the near future.

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