THESIS

REGIONAL WHOLE PLANT AND MOLECULAR RESPONSE OF *KOCHIA SCOPARIA* TO GLYPHOSATE

Submitted by

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ABSTRACT

REGIONAL WHOLE PLANT AND MOLECULAR RESPONSE OF *KOCHIA SCOPARIA* TO GLYPHOSATE

Globally, glyphosate (Roundup®) resistant weeds pose a serious challenge to modern agricultural practices that utilize glyphosate for weed control, including Roundup Ready® cropping regimes. Locally, glyphosate resistant *K. scoparia* have been identified throughout the central Great Plains, and the infested range is expanding rapidly. Glyphosate and Roundup Ready® crops form the foundation of no-till technology, which has considerably reduced water use and soil loss in arid to semi-arid regions of North America. Unfortunately, the continued spread of glyphosate-resistant *K. scoparia* will jeopardize the utility of glyphosate and the sustainability of no-till agricultural practices. In an effort to suppress glyphosate-resistant *K. scoparia*, more needs to be known about 1) the spread of resistance, 2) the level of resistance, and 3) the mechanism responsible for glyphosate resistance in *K. scoparia*.

Suspected glyphosate-resistant *K. scoparia* accessions were collected from Kansas, Colorado, North Dakota, South Dakota, and Alberta. Whole plant glyphosate dose response and shikimate assays were used to confirm resistance and assess the level of resistance. Then PCR, quantitative PCR, sequencing, and immunoblotting techniques were used to determine the mechanism responsible for glyphosate resistance. Sequence of the *EPSPS* binding site proline confirmed that amino acid substitution at that residue was not responsible for resistance in *K. scoparia*. However, quantitative PCR estimates of *EPSPS* copy number revealed increased copy number in all glyphosate-resistant individual ─ranging from 3 to 9 *EPSPS* copies relative to the

reference *ALS* gene. Furthermore, increased *EPSPS* copy number was correlated to increased transcript and protein abundance. Based on these finding, I confirm resistance for all tested accessions throughout the North American central Great Plains, and conclude that increased glyphosate rates will have little effect in controlling glyphosate-resistant *K. scoparia*. Furthermore, I suggest that *EPSPS* gene amplification may be the mechanism responsible for glyphosate resistance in *K. scoparia*, and that lower level increases in EPSPS expression (as compared to *A. palmeri*) are sufficient for glyphosate resistance. Moreover, this research, again, demonstrates the adaptability of plants and foreshadows the need for diversifying weed management practices.

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CHAPTER ONE: INTRODUCTION

Introduction

In 2005, farmers and land managers that traditionally relied on glyphosate for broad spectrum weed management began noticing a strange pattern in fields throughout the US central Great Plains. After applying the labeled, reportedly lethal dose of glyphosate to their fields, some weeds did not die. More surprising, these surviving weeds were often arranged in neat meandering trails throughout the field. Upon closer analysis, it became clear that the weeds in question were *Kochia scoparia* tumbleweeds that had developed resistance to glyphosate (more commonly known as Roundup®), and plants growing in streak patterns were the resistant progeny of a resistant parent that had released its seeds while tumbling in the wind during the previous fall (Fig.1).

Beginning in 2009, the weed science group at Colorado State University (CSU) began research on this local agricultural problem. Fields with putative glyphosate-resistant *K. scoparia* were assessed by weed scientists and seed was collected and stored in a seed repository at CSU where further phenotypic and molecular analysis could be done. Using these materials as a base, I developed the following research objectives: 1) to monitor the spread of glyphosate-resistant *K. scoparia*; 2) to characterize the level of glyphosate-resistance in geographically isolated accession of *K. scoparia*; and 3) to determine the mechanism of glyphosate-resistance in *K. scoparia* accessions collected throughout the central Great Plains.

Figure 1. Glyphosate-resistant *K. scoparia* field streak pattern. Glyphosate-resistant *K. scoparia* persist after glyphosate field treatment.

Kochia scoparia

The broadleaf annual weed *Kochia scoparia* (L.) Schrad (synonym: *Bassia scoparia* (L.) A. J. Scott) can be found in nearly all of North America but has the most economic impact in the western United States and the central Great Plains. *K. scoparia* is a plant native to Eurasia that was introduced to North America in the mid- to late 1800s. Evidence suggests that *K. scoparia* was originally introduced as an ornamental, but then escaped and invaded arid to semi-arid regions of North America. While this plant has nutritional qualities that make it marginally desirable for cattle grazing, *K. scoparia* is generally regarded as a problematic weed in other agricultural scenarios. It utilizes heat, cold, salinity, and drought tolerances to successfully colonize new areas, often forming dense monocultures. *K. scoparia* has been listed as one of the

five most troublesome annual weeds in Colorado, Montana, Nebraska, and Wyoming (Friesen *et.al.* 2008).

Another factor contributing to *K. scoparia's* invasive success is its ability to adapt rapidly to stress. A good example of this is the development of herbicide resistance. Given time, *K. scoparia* has adapted to nearly every herbicide used to control it. To date, *K. scoparia* populations have developed resistance to multiple herbicide modes of action, which include: synthetic auxins, *ALS*-inhibiting herbicides, and photosystem II-inhibiting herbicides. The same was true for the fourth herbicide mode of action group called glycines which includes glyphosate (Heap, 2012). In 2007, the first glyphosate-resistant *K. scoparia* was formally identified in western Kansas. By 2010, many accessions from the same area were confirmed resistant. In subsequent years, the number of glyphosate-resistant *K. scoparia* cases has multiplied, and putative resistance has been reported in numerous locations in Kansas, Colorado, Nebraska, North Dakota, South Dakota, Montana, and Alberta (Heap 2012, personal communication).

Glyphosate

Over the past three decades, the broad-spectrum, postemergent, systemic herbicide glyphosate has revolutionized modern agriculture. Glyphosate is used to control annual and perennial weeds in numerous agricultural and non-agricultural settings. Because of its qualities such as affordability, effectiveness, and application flexibility, growers adopted glyphosate use at a rapid rate. The subsequent parallel development of glyphosate-resistant (Roundup Ready®) crop varieties ultimately led to large scale shifts in modern agricultural practice (Powles *et. al.* 2006, Bradshaw *et. al.* 1997).

Glyphosate (*N*-(phosphonomethyl)glycine) kills plants by interfering with the shikimate pathway, which is responsible for production of the aromatic amino acids: tryptophan,

phenylalanine, and tyrosine -and consequently numerous secondary metabolites. Glyphosate competitively inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by occupying the binding site for phosphoenolpyruvate (PEP). This inhibition results in a metabolic roadblock where upstream substrates accumulate while the production of essential downstream products is blocked. Furthermore, feedback loops drive an increased flow of carbon through the shikimate pathway, thereby exacerbating the problem. Ultimately, the lack of essential plant compounds and the loss of carbon lead to plant death (Powles *et. al.* 2010, Schonbrunn *et. al.* 2001).

Initially glyphosate was primarily used in non-crop, orchard, and vineyard settings. However, the 1996 development of glyphosate-resistant (Roundup Ready®) crop varieties greatly expanded its utility and use (Nandula *et. al* 2010). Glyphosate-resistant crops were initially engineered using the glyphosate-insensitive CP4 EPSPS enzyme. Then, by manipulating the expression pattern of CP4 EPSPS, or by pairing it with a glyphosate-deactivating enzyme such as glyphosate oxioreductase (GOX) or glyphosate acetyltransferase (GAT), higher levels of resistance were achieved (Bradshaw *et. al.* 1997, Nandula *et. al.* 2010). The success of these glyphosate-resistant crop varieties is evident in the widespread adoption of the technology. In 2002, estimates of glyphosate use were as high as 50 lb per square mile in some parts of the US, and much of that glyphosate was used in conjunction with Roundup Ready® crops (United States Geological Survey 2004).

Currently, glyphosate is being used as a stand-alone weed control method on several million hectares of crop land (Shaner *et. al.* 2011). This wide spread adoption and often exclusive reliance on glyphosate does not bode well for the long term utility of glyphosate. Rather, such massive selection pressure is likely to result in widespread weed adaptation to what has been a very effective herbicide. To date, at least 23 weed species have evolved glyphosate

resistance (Heap 2012). Glyphosate-resistant weeds now pose a serious challenge to modern agriculture practices and are likely to increase the cost of production, and complicate weed control.

Possible ramifications of glyphosate-resistant *Kochia scoparia*

While the risks are numerous, glyphosate-resistant *K. scoparia* poses the most serious threat to no-tillage (no-till) practices in the arid and semi-arid west. Modern no-till practices are inextricably bound to glyphosate and Roundup Ready® cropping systems (Givens *et. al.* 2009). Therefore, if glyphosate loses effectiveness on *K. scoparia*, Roundup Ready® cropping in the central Great Plains could lose its advantage -placing no-till agriculture in jeopardy. The most concerning aspect of this scenario is that currently, no-till crop production is the primary form of soil and water conservation in the arid and semi-arid west (Gersmehl 1978).

Tillage is a mechanical alternative to chemical weed control. By agitating the soil using a variety of mechanical means, weeds are buried or uprooted while simultaneously creating a prepared soil bed for planting crops. While tillage was the standard weed control method for centuries, recent advances in no-till technology, such as Roundup Ready® cropping, have shifted agricultural practices toward no-till or limited tillage. In a field planted with Roundup Ready® crops, glyphosate can be used to control weeds, rather than mechanical tillage (Givens *et. al.* 2009). While other advantages exist, the primary advantages of no-till are two-fold in the US central Great Plains. First, tillage loosens soil aggregates and disrupts crop residues resulting in a soil surface that is more susceptible to wind and/or water erosion. Second, valuable soil moisture under the soil surface is exposed and quickly evaporates rather than being used by crops (Gersmehl 1978).

As glyphosate-resistant *K. scoparia* spreads, farmers and land managers are faced with a serious decision: what to do if glyphosate doesn't control weeds. While alternative weed control options vary among crops and cropping regimes, tillage is frequently at the top of the list. In a Roundup Ready® sugar beet field infested with glyphosate-resistant *K. scoparia*, one farmer opted to cultivate (till) between crop rows. Although weed control was achieved to a moderate degree, it was more labor intensive and resulted in the loss of soil and soil moisture. Besides having to invest extra time, finances, and effort into tilling his field, after every few passes with the cultivator, the blades needed to be cleaned to dislodge weeds from the machinery. Furthermore, cultivation loosened soil and increased the surface area of exposed soil -thereby increasing the likelihood of wind and/or water erosion. Lastly, the loss of soil moisture was evident based on the color of the soil. In uncultivated sections, the soil had a light tan hue whereas recently cultivated sections of soil were shades darker (Fig.2). At a small scale, these issues may seem negligible; however, when spread across vast tracts of land where labor, soil quality, and water availability are limited, these issues will present substantial challenges to agriculture throughout the affected region.

Figure 2. Intercrop tillage as a method to control glyphosate-resistant *K. scoparia*. Tillage results in the loss of soil moisture, and leaves soil more prone to wind and water erosion.

CHAPTER TWO:

WEED ADAPTATION AND MECHANISMS OF GLYPHOSATE RESISTANCE

Weed adaptation

When selection pressure is relentless, weeds evolve. While my main focus will be on glyphosate-resistant weed evolution, the above statement could apply to any selection pressure. To solidify this point, I will first draw your attention to a non-herbicidal example of weed evolution. In many areas of the world that cultivate rice, the preferred method of weed control is hand weeding. After hand weeding for thousands of years, varieties of barnyard grass (*Echinochloa crus-galli var. oryzicola*) have evolved to resemble rice. After analyzing 15 morphological and growth characteristics during the first 30 days of growth, few differences were observed between rice and mimetic barnyard grasses compared to non-mimetic barnyard grasses. The only distinguishable difference between rice and mimetic barnyard grasses was the presence of a ligule in rice. Alternatively, mimetic barnyard grasses had an erect habit and dark green culm and leaf bases which closely resembled rice, while non-mimetic barnyard grasses were less erect and had a pink to dark red culm and leaf bases (Barret 1983). In this case, relentless hand weeding led to barnyard grass vavilovian mimicry of rice -to the extent that perceptive eyes could barely distinguish the two. Owing in part to this adaptation, barnyard grass continues to be a problematic weed in global rice production.

Similarly, the use of herbicides over recent decades has also led to weed adaptation. Even though herbicides are diverse, generally each herbicide targets a specific enzyme. As a result, evolved herbicide resistance falls into one of two categories: target-site resistance, or non-target-site resistance. Target-site resistance includes changes to the targeted enzyme such as amino acid

substitutions that reduce the binding affinity of the herbicide, or changes in expression that overwhelms the herbicide. Conversely, non-target-site resistance includes anything that limits the amount of herbicide reaching a target enzyme such as: reduced herbicide absorption or translocation, or increased herbicide sequestration or metabolism (Powles *et. al.* 2010). While most weed herbicide resistance mechanisms fall neatly into the above mentioned categories, the variety of forms these mechanisms of resistance take in reality is astounding. A combination of strong herbicidal selection and millions of weeds worldwide has unveiled the true potential for plant adaptation.

Weed evolved mechanisms of glyphosate resistance

Globally, glyphosate-resistant weeds are receiving increased attention, and a detailed understanding of the mechanisms of resistance is needed to inform future weed management practices. Observed mechanisms of glyphosate resistance fall into three main categories: 1) alteration of the glyphosate binding site, 2) altered mobility of glyphosate, and 3) increased expression of the glyphosate-targeted enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Over the past decade, mechanisms of glyphosate resistance have been characterized for a handful of weeds, and the following is a brief review.

Alteration of the EPSPS glyphosate binding site has been reported for three glyphosate-resistant weed species: *Lolium multiflorum*, *Lolium rigidum*, and *Eleusine indica* (Nandula *et. al* 2010). In every case, an amino acid substitution of alanine, serine, or threonine was identified at the same binding site, proline 106 residue (numbered according to *Petunia*). However, this mutation only confers a modest degree of resistance because glyphosate occupies the binding site for phosphoenolpyruvate (PEP), and most mutations that reduce the binding affinity for glyphosate also reduce the affinity for PEP. The mutations responsible for glyphosate-insensitive

EPSPS found in transgenic glyphosate-resistant crops all occur in sets of at least two, and have not yet been observed in field evolved glyphosate-resistant weeds. In comparison to other herbicides, few mutations confer glyphosate-resistance because glyphosate binds within a highly conserved functional region of the target enzyme (Powles *et. al.* 2010).

Altered glyphosate mobility has been reported for four glyphosate-resistant plant species: *Lolium multiflorum*, *Lolium rigidum*, *Conyza canadensis,* and *Conyza bonariensis* (Nandula *et. al* 2010). In these species, glyphosate is excluded from meristematic tissues by limited or reduced movement. Glyphosate is a systemic herbicide which normally follows photoassimilate from source to sink. Based on studies using ^{14}C labeled glyphosate, glyphosate seems to be trapped in treated leaves and leaf tips of resistant plants (Powles *et. al.* 2010). What is unclear is how this occurs at the cellular level. To address this question, ^{31}P nuclear magnetic resonance was used to monitor the localization of glyphosate within the cell during pulse-chase glyphosate treatment. Using this technique, strong evidence was found for vacuolar sequestration of glyphosate in resistant *C. canadensis* (Ge *et. al.* 2010). While further research is needed to characterize this resistance mechanism at the cellular level in other plant species, the basic hypothesis is consistent -limited glyphosate mobility within the plant protects newly expanding tissues, and allows continued plant growth.

The third mechanism of glyphosate-resistance, increased expression of the glyphosate-targeted enzyme EPSPS, was discovered more recently. In 2010, glyphosate-resistant *Amaranthus palmeri* was shown to have increased *EPSPS* copy number ranging from 4-160 copies relative to the reference *ALS* gene. Increased copy number was correlated with increased transcription and translation of the enzyme (Gaines *et. al.* 2010). Hypothetically, increased EPSPS expression results in an abundance of uninhibited EPSPS that maintains metabolic

function (Powles 2010). *EPSPS* gene amplification and overexpression have been implicated as the resistance mechanism in a number of other weed species; however, in every other case, sufficient experimental replication is lacking and vital data were not obtained. Lastly, this mechanism of glyphosate-resistance stands out because it is the only example of gene amplification and overexpression of a target enzyme that is thought to confer herbicide resistance (Powles *et. al.* 2010).

Based on this brief review of glyphosate-resistance mechanisms, weeds utilize a diversity of molecular and genetic means to obtain resistance. That being said, I have only discussed comprehensive mechanistic research done on 6 of the 23 glyphosate-resistant weeds (Heap, 2012). As for the others, while preliminary evidence points mostly to known mechanisms of resistance, chances are that novel mechanisms of resistance still exist.

CHAPTER THREE: GENE AMPLIFICATION OF EPSP SYNTHASE IN GLYPHOSATE RESISTANT *KOCHIA SCOPARIA*

Summaries:

The confirmation of glyphosate-resistant *Kochia scoparia* across the US central Great Plains in 2007 has raised concerns about the long-term usefulness of the herbicide. Accessions of *K. scoparia* plants were collected from fields with detected glyphosate resistance in Kansas, Colorado, North Dakota, and South Dakota, and susceptible and resistant germplasm within the accessions were identified using glyphosate dose response. Sequence analysis confirmed that there was no mutation of the *EPSPS* binding site proline in glyphosate-resistant *K. scorpia*. *EPSPS* copy number and transcript abundance, however, were elevated in the resistant relative to susceptible plants. Glyphosate-resistant plants with increased relative *EPSPS* copy numbers had consistently lower shikimate accumulation in leaf disks treated with 100 μM glyphosate. Compared to glyphosate susceptible plants, EPSPS enzyme accumulation is higher in glyphosate resistant plants with increased gene copy number. These results are consistent with a model attributing increased EPSPS expression as a mechanism for glyphosate resistance in *K. scoparia*.

Introduction:

Glyphosate (*N*-(phosphonomethyl)glycine) is arguably the most important herbicide worldwide because of its widespread use in cropping systems including Roundup Ready® crops (Shaner *et. al.* 2011). Glyphosate is a broad-spectrum systemic herbicide that kills plants by disrupting the synthesis of aromatic amino acids. It inhibits 5-Enolpyruvylshikimate-3-Phosphate synthase (EPSPS) by occupying the binding site for phosphoenolpyruvate, which leads to accumulation of upstream metabolites rather than downstream products such as phenylalanine,

tyrosine, and tryptophan (Steinrucken *et. al.* 1980, Nandula 2010). In part, the advent of glyphosate-tolerant (i.e. Roundup Ready ®) cropping systems led to the widespread adoption of glyphosate (Shaner *et. al.* 2011, Funke *et. al.* 2006). The utility of this cropping system is that crops are capable of surviving a lethal dose of glyphosate while surrounding weeds are effectively eliminated. This simple and reliable form of weed control revolutionized modern agricultural by increasing application flexibility and reducing labor demand (Gersmehl 1978).

An unfortunate consequence of continuous stand-alone use of glyphosate is a strong selection pressure for glyphosate-resistant weeds (Shaner *et. al.* 2011, Nandula 2010). Globally, by 2011, 23 weed species had developed resistance to glyphosate (Heap 2012). Of these, 13 glyphosate-resistant weed species are found in the United States. The impact of glyphosate-resistant weeds is profound, and in some cases the last lines of defense against these plants is hand weeding or relapse to tillage practices (Sprague 2012). As the number of glyphosate-resistant weeds increases, the utility of glyphosate-tolerant crops will diminish and confront modern agriculture with serious challenges.

The broadleaf annual weed *Kochia scoparia* (L.) schrad. (synonymous with *Bassia scoparia* (L.) A. J. Scott.) has a huge economic impact in the central Great Plains states where it infests irrigated and non-irrigated wheat, corn, sorghum, sugar beet, alfalfa, pastures, rangeland, waste areas, ditch banks, and roadsides (Friesen *et. al.* 2008). *K. scoparia* is problematic because it proliferates and adapts rapidly. Up to 30,000 seeds per plant are produced and disseminated over large distances because of a tumbleweed mode of seed dispersal (Mengistu *et. al.* 2002, Friesen *et. al.* 2008). *K. scoparia* is well adapted to high salinity soils, high temperature, and low water availability (Friesen *et. al.* 2008). Protogynous flowers and wind pollination facilitate gene transfer and, in theory, could expedite genetic adaptation (Mengistu *et. al.* 2002, Stallings *et. al.*

1995). As a testament to this weed's adaptability, *K. scoparia* has developed resistance to numerous herbicides from four modes of action which include: acetolactate synthase inhibitors, photosystem II inhibitors, synthetic auxins, and now a glycine -glyphosate (Friesen *et. al.* 2008).

K. scoparia is the first weed in the central Great Plains to develop resistance to glyphosate. The first reports of crop failures caused by suspected glyphosate-resistant *K. scoparia* were from western Kansas in 2005. Glyphosate-resistant *K. scoparia* were identified in 2007 and confirmed in 2010 (Heap 2012). Glyphosate-resistant *K. scoparia* are now reported to occur in Colorado, Nebraska, North Dakota, South Dakota, Montana, and Alberta (Heap 2012, Monsanto Canada Inc. 2012).

As glyphosate-resistant *K. scoparia* begin to reduce the usefulness of glyphosate in the central Great Plains, one consequence stands out above the rest: the loss of no-till agricultural practices. Roundup Ready® cropping is critical to no-till farming as it is now practiced. As farmers and land managers lose control of glyphosate-resistant *K. scoparia*, the next best option is often tillage. Unfortunately, tillage results in the loss of soil moisture and exposes soil to wind and water erosion (Warkentin 2001). The adoption of glyphosate and glyphosate-tolerant crops shaped a setting in which reduced and no-tillage practices could be integrated into water and soil conservation efforts (Unger *et. al.* 1991, Carpenter *et. al.* 1999). If glyphosate loses its effectiveness, the west could again be faced with the challenge of preserving soil and soil moisture.

Thus far, three mechanisms of glyphosate resistance have been identified in weedy plant species: 1) alteration of the EPSPS binding site, 2) altered mobility of glyphosate, and 3) increased EPSPS expression. Alteration of the *EPSPS* binding site at the proline-106 codon (numbered according to *Petunia*) results in a lower level of glyphosate resistance (between 2 and

3 fold compared to a susceptible counterpart) (Sammons 2006). Nonsynonymous substitution at this site (Pro_{106} to Ser, Ala, or Thr) has been identified in glyphosate resistant *Eleusine indica*, *Lolium rigidum*, and *Lolium multiflorum* (Baerson *et. al.* 2002, Simarmata *et. al.* 2008, Perez-Jones *et. al.* 2007). Altered mobility of glyphosate has also been listed as a glyphosate resistance mechanism. By trapping glyphosate in leaves and leaf tips, resistant plants reduced damage to young meristematic tissue (Shaner *et. al.* 2011). While underlying changes in cellular glyphosate movement are subtle, recent NMR data suggest that vacuolar sequestration could be responsible for resistance in one weed (Shaner *et. al.* 2011). Lastly, elevated EPSPS expression, achieved by *EPSPS* gene amplification, was shown to confer glyphosate resistance to *A. palmeri* (Gaines *et. al.* 2010). In this case, *EPSPS* copy numbers as high as 160, relative to a reference gene, produced proportional amounts of enzyme which was predicted to result in an abundant supply of uninhibited EPSPS after glyphosate treatment (Powles *et. al.* 2010). Hypothetically, uninhibited EPSPS maintain regular function and alleviate the metabolic bottleneck caused by glyphosate.

Until recently, EPSPS overexpression alone was not recognized as a viable means of developing glyphosate resistance in higher plant species. Up until the report of *EPSPS* gene amplification in glyphosate-resistant *A. palmeri* (Gaines *et al.*, 2010), EPSPS overexpression and gene amplification had only been observed in plant and bacterial cell cultures that had been slowly adapted to tolerate glyphosate (Widholm *et. al.* 2001). *EPSPS* gene amplification has now been reported in two other weedy plant species -*Amaranthus tuberculatus (syn. rudis)* and *Lolium multiflorum* (Salas *et. al.* 2012, Bell *et. al.* 2009, Tranel *et. al.* 2011). In *A. tuberculatus*, it is unclear to what extent gene amplification contributes to glyphosate resistance (Shaner *et. al.* 2011, Tranel *et. al.* 2011). On the other hand, gene amplification in *L. multiflorum* is suggested

to contribute to glyphosate resistance (Salas *et. al.* 2012). *L. multiflorum* plants with between 1 and 25 relative *EPSPS* gene copies were tested for glyphosate resistance, and high *EPSPS* copy number was found to correlate with both high GR_{50} values and high EPSPS activity. In this case, EPSPS expression was not quantified (Salas *et. al.* 2012). Preliminary evidence shows slight increases in *EPSPS* transcript abundance in glyphosate-resistant *Conyza Canadensis*, *Conyza bonariensis* and *Lolium ridigum* biotypes (Shaner *et. al.* 2011, Dinelli *et. al.* 2006, Dinelli *et. al.* 2008, Baerson *et. al.* 2002). Thus, increasing evidence suggests that EPSPS overexpression contributes to glyphosate resistance in a number of plant species, but more robust experimentation needs to be done to elucidate the effect of lower level increases in EPSPS expression (Nandula *et. al.* 2010).

To better inform weed management practices in the central Great Plains and to expand the body of knowledge surrounding weed evolved glyphosate-resistance mechanisms, it was important to determine the mechanism of glyphosate-resistance in *K. scoparia*. Previous work showed no differences in the absorption and translocation of ^{14}C labeled glyphosate in glyphosate-susceptible and -resistant *K. scoparia* (Waite 2010). As a result, I focused my efforts on testing the two other known mechanisms of glyphosate resistance: alteration of the *EPSPS* binding site, and increased expression of EPSPS. My aim was to identify the resistance mechanism(s) in *K. scoparia* plants that were confirmed resistant from a wide geographical range spanning the US central Great Plains.

Methods and materials:

Plant collection

In 2011, seed was collected from individual *K. scoparia* plants from geographically isolated fields throughout the central Great Plains. Collections sites were variable and included

pastures, soybean, and wheat fields. Plants suspected to be glyphosate-resistant were identified either by roadside survey, or based on problematic weed reports from farmers. At each site where *K. scoparia* was suspected resistant, weed specialists looked for plants that had survived glyphosate treatment, and ensured that surrounding weeds had been eliminated. Glyphosate-susceptible accessions were collected from two locations in Kansas, while glyphosate-resistant accessions were collected from nine locations throughout Kansas, Colorado, North Dakota, and South Dakota (Table 1). For the purpose of this paper, I define accession as the first generation progeny from seed of a single plant isolated from a geographically distinct field. In this way, plants from a given accession are maternally related, and paternal inheritance is unknown because of field wind pollination. The county of origin was recorded, and an accession ID was assigned to each location (Table 1). Each accession ID consists of the abbreviated state of origin, its designation as resistant or susceptible, and a unique identifying number (ie. KS-S1 = Kansas susceptible accession 1).

Growing conditions, plant treatment and assessment

Seeds from each accession were planted in germination flats. After emergence, seedlings were transplanted into 18-insert (8 cm x 8 cm pots) flats containing Farfard® custom mix potting soil, and were grown at 23 ˚C under a 14 h light/10 h dark regime. When plants reached 8 cm tall, 3 to 4 wks after planting, they were sprayed with Roundup WeatherMax® at the following rates: 0, 0.28, 0.42, 0.56, 0.84, 1.40, 1.68, 3.36, and 6.73 kg ae ha^{-1}. With two exceptions, six or more plants from each accession were treated at each rate. Because seed was limited, only four plants from KS-R6 and SD-R1 were treated at each rate, and SD-R1 was only treated with 0, 0.84, 1.40, and 6.73 kg ae ha^{-1} (Table 1). To improve glyphosate absorption into leaves, ammonium sulfate (16.3 g/100ml) was added to each treatment (Mueninghoff *et. al.* 2001). Glyphosate applications

were made in a controlled laboratory using a moving flat-nozzle (teejet 8002EVS) industrial spray chamber. At 3 wks after treatment (WAT), plant survival at each rate was assessed. According to the Roundup WeatherMax (Monsanto Co.) label, a rate of 0.84 kg ae ha^{-1} is accepted as a rate lethal to glyphosate-susceptible *K. scoparia* less than 12 inches tall, and I consider plants that survive that rate to be glyphosate-resistant. Following survival assessment, three plants from each accession were selected for further molecular analysis and biological replication. Plants were selected from the highest rates survived by each accession, so long as enough living tissue was available for further molecular analysis.

Extraction of nucleic acids and cDNA synthesis

For DNA extractions, 100 mg of plant tissue were ground to a fine powder under liquid nitrogen using a 1.5 ml tube as a mortar and a plastic drill bit powered by a handheld electric drill as a pestle. DNA was extracted using the Qiagen Dneasy® Plant Mini Kit following the manufacturer's protocol (Qiagen Handbooks and Protocols, 2012). Genomic DNA was eluted into 120 µL of Qiagen® AE buffer, and the quality and concentration were determined spectrophotometrically using a Nanodrop® 1000. Genomic DNA was stored at -20 ˚C when not in use.

RNA was extracted from 60 mg of finely ground plant tissue using the Qiagen RNeasy® Plant Mini Kit following manufacturer's protocol (Qiagen Handbooks and Protocols, 2012). Buffer RLT, containing both ß-mercaptoethanol and a high concentration of guanidine isothiocycanate, was immediately added to frozen tissue to inhibit RNases. After elution of total RNA in HPLC pure water, DNase I digestion was done in solution based on the protocol outlined in the Qiagen Rneasy® handbook (Qiagen Handbooks and Protocols, 2012). To remove Dnase I contaminant, the Qiagen Rneasy® cleanup was done according to protocol. The quality

and concentration of RNA was determined spectrophotometrically using a Nanodrop® 1000 and by running RNA on a 1% RNase-free agarose gel stained with ethidium bromide.

RNA concentrations were standardized, and SuperScript™ III First-Strand Synthesis System was used to reverse transcribe and amplify complimentary DNA (cDNA) using oligo(dT) primers. Three no- reverse transcriptase (RT) controls were included (one glyphosate-susceptible and two -resistant samples). RNA and cDNA were stored at -80 ˚C when not in use.

Sequencing the binding site proline

To amplify and sequence a roughly 200 bp PCR product encoding the EPSPS binding site (at the proline-106 position), primers designed for *A. palmeri* sequence were used (Gaines *et. al.* 2010). The sequences of the forward and reverse primers were 5' ATGTTGGACGCTCTCAGAACT 3' and 5' TGAATTTCCTCCAGCAACGGC 3', respectively. Each reaction contained 0.4 µL of dNTPs [10 mM], 1 µL of each primer [5 µM], and 0.2 µL of Phusion® high-fidelity DNA polymerase with 1x Phusion® HF buffer (New England Biolabs®). Twenty ng of gDNA template was added to each reaction individually. The initial PCR denaturation step was held at 98 ˚C for 30 sec, which was followed by 30 cycles of denaturation at 98 ˚C for 10 sec, primer-template annealing at 50 ˚C for 30 sec, and product extension at 72 ˚C for 45 sec. A final 7 min extension cycle at 72 ˚C was included, and the reaction was terminated and held at 4 ˚C. The PCR product was separated on 1% agarose gel stained with ethidium bromide and bands were detected and excised. The PCR product was then isolated from the agarose gel piece using the GENECLEAN® II Kit (MP Biochemical™) by following the manufacturer's protocol for the glassmilk slurry procedure. Sanger sequencing was done at CSU on an ABI™ 3130xL Genetic Analyzer. The same primers used for PCR were used for sequencing, and samples were prepared with ABI's BigDye® Terminator v3.1 sequencing

chemistry. The sequence reads were analyzed using CLC genomics workbench software. *EPSPS* binding site sequence from glyphosate-susceptible and -resistant *K. scoparia* accessions was aligned to a reference glyphosate-susceptible *A. palmeri* sequence to search for Proline-106 substitutions in glyphosate-resistant *K. scoparia*.

Copy number determination on genomic DNA

To estimate the relative *EPSPS* copy number in *K. scoparia*, quantitative PCR (qPCR) was done on gDNA. To control for variation among DNA preparations, *EPSPS* was normalized to a reference gene *ALS* (encoding for acetolactate synthase); *ALS* was selected as a reference gene because the *ALS* copy number is not expected to vary across *K. scoparia* biotypes. Although the number of *ALS* loci in *K. scoparia* is unknown, copy number is expected to be a low (Gaines *et. al.* 2010). As clarification, by calculating copy number using a reference gene approach, the relative *EPSPS:ALS* gene copy number is a ratio of *EPSPS* to *ALS* PCR product fluorescence; because of minor variation in amplicon size, qPCR conditions, and fluorescence detection, the values reported are estimates of relative gene copy number.

Relative *EPSPS*:*ALS* gene copy number was estimated using qPCR on gDNA template in the following manner. Primers specific to *K. scoparia EPSPS* sequence were designed from binding sight sequence obtained as described above. The EPSPS forward and reverse primer sequences were 5' GGCCAAAAGGGCAATCGTGGAG 3' and 5' CATTGCCGTTCCCGCGTTTCC 3', respectively. These EPSPS primers produce a 102 bp product. Reference primers specific to *K. scoparia ALS* sequence were designed from sequence obtained from NCBI (accession: EU517498.1). The ALS forward and reverse primer sequences were 5' ATGCAGACAATGTTGGATAC 3' and 5' TCAACCATCGATACGAACAT 3', respectively. The ALS primers produced a 159 bp product. Dissociation curves were produced at

the end of each qPCR experiment to assess the amplicon specificity. Three standard curves were produced for each primer pair, and primer efficiency was calculated to be 98% to 102% for *EPSPS* and 96% to 101% for *ALS* according to MIQE guidelines (data not shown). The qPCR master mixes contained the following components for each reaction: 6.25ul of Absolute^{TM} Fast qPCR mix (Thermo Scientific®), and 0.5ul of each primer [5uM]. Sixteen ng of gDNA template was added to each reaction individually. The Applied Biosystems^{TM} PRISM 7000 Sequence Detection System thermocycler was used for all qPCR reactions. The initial denaturation step was held at 95 ˚C for 15 min, which was followed by 40 cycles of denaturation at 95 ˚C for 30 sec, and a combined annealing/extension step at 62 ˚C for 1 min. Fluorescence was measured at the end of each annealing/extension step. On each qPCR plate, negative controls were included for each primer pair, the same glyphosate susceptible gDNA was used, and each reaction was done in triplicate.

The point at which PCR amplification curves crossed the threshold was recorded (C_T), and gene copy number was calculated in Microsoft© Excel using the ΔC_T method ($2^{-\Delta C_T}$ = relative gene copy number). *EPSPS* copy number was normalized to a reference gene (*ALS*) that remained constant across *K. scoparia* accessions ($\Delta C_T = C_T^{EPSPS} - C_T^{ALS}$) (Gaines *et. al.* 2010).

Copy number determination on complementary DNA

qPCR was done on complimentary DNA (cDNA) as described above for gDNA. In addition to the controls used on each gDNA qPCR plate, three no-RT controls were included to validate the effectiveness of DNase digestion, and two *K. scoparia* gDNA samples (one glyphosate-susceptible and one -resistant) were included as a positive control. Complementary DNA gene copy number (transcript abundance) was calculated in the same manner previously described for gDNA.

Shikimate accumulation assay

The shikimate assay is a biochemical assay that can be used to indirectly measure EPSPS inhibition by glyphosate. Glyphosate inhibition of EPSPS causes a metabolic bottleneck and leads to the buildup of shikimate-3-phosphate (a substrate of EPSPS) and its dephosphorylated state-shikimate (Shaner *et. al.* 2005). Following the protocol below, shikimate accumulation was estimated for an untreated subset of glyphosate-susceptible and -resistant *K. scoparia* from which seed was readily available. Specific accessions included in this analysis were KS-S2 a glyphosate-susceptible accession and 5 glyphosate-resistant accessions: KS-R2, KS-R3, KS-R4, KS-R5, and KS-R6 (Fig. 7). The shikimate assay was done on six individual plants from each accession. Then follow up analysis was done on a subset of the same plants to elucidate the relationship between shikimate accumulation and *EPSPS* copy number (Fig. 8). *EPSPS* gene copy numbers were estimated in the same manner described above. The aim was to see if low shikimate accumulation was a good predictor of *EPSPS* gene amplification.

Leaf disk shikimate accumulation was measured as outlined by Shaner (Shaner *et. al.* 2005). Three 6-mm diameter leaf disks from a single plant were analyzed at 100 μM glyphosate while another three leaf disks from the same plant were analyzed at 0 μM glyphosate. The leaf disks were excised from expanding *K. scoparia* leaves of equal size, and placed adaxial side up into the wells of a microtiter plate containing 0.6902 g ammonium phosphate dissolved into 600 ml diH_2O and molecular grade glyphosate (0 μM glyphosate wells did not contain glyphosate). Plates were covered with plastic wrap to minimize evaporation, and incubated under lights for 16 h at ambient temperature. Plates were then frozen (-20 ˚C) and thawed (60 ˚C), and then subjected to 1.25 N HCl treatment (25 μL/well) for 50 min at 20 ˚C. A 25 μL aliquot of solution was transferred to a second microtiter plate containing reaction buffer (periodic acid (0.25% v/v)/

meta-periodate (0.25% v/v)) (100 μL per well). The reaction was allowed to run for 90 min at room temperature, at which point a quenching buffer (0.6 M sodium hydroxide/0.22 M sodium sulfite) was added (100 μL per well). Shikimate levels were then determined spectrophotometrically at OD_{380} using the BioTek Synergy™ 2 spectrophotometer.

A standard shikimate concentration curve was generated to facilitate the conversion of optical density to ng shikimate μL^{-1} in test wells (Shaner *et. al.* 2005). Replicate wells were averaged and standard deviation (sd) calculated. By subtracting wells with 0 μM glyphosate from those with 100 μM glyphosate, shikimate accumulation could be reported as Δ ng shikimate μL^{-1}. In this case, my interest is in measuring shikimate accumulation after glyphosate treatment, rather than total shikimate.

Protein Extraction and Immunoblot analysis

Leaf tissue (100 mg) from *K. scoparia* was flash-frozen and ground in liquid nitrogen. Protein was isolated by mixing tissue with 4 volumes of Laemmli buffer (10% β-mercaptoethanol, 60% SDS (10% w/v), 20% Glycerol). Proteins were denatured by boiling the samples for 5 min. After centrifugation at 13,500 rpm, the supernatant was loaded in 10 μL aliquots and resolved by 10% SDS-PAGE at 20 mA per gel for approximately 1.5 h. Proteins were transferred to nitrocellulose membranes for 1 h at 300 mA, and the membranes were then blocked overnight at 4˚C in TBST (20 mM Tris base [pH 7.5], 0.5 M NaCl, 0.05% Tween-20) containing 5% milk powder. Membranes were incubated for 1 h at room temperature with primary EPSPS antibody (Gaines *et. al.* 2010) that had been diluted 1:2000 in TBST milk solution. The membranes were rinsed three times in TBST, and incubated at room temperature for 1 h in the secondary goat: rabbit antibody conjugated with horseradish peroxidase (Thermo Scientific®) at a dilution of 1:5000 in TBST milk solution. After three rinses in TBST, activator

solution was applied to membranes to initiate chemiluminescence. Film was exposed for 2 and 30 min and immediately developed using a Kodak film processor to visualize bands. EPSPS is expected to be 48 kDa. Protein concentrations for each sample were compared in duplicate gels stained using Coomassie Brilliant Blue. Four positive controls were included from *A. palmeri* representing low and high EPSPS expression.

Results:

Identification and characterization of glyphosate-resistant K. scoparia

Putative glyphosate-resistant *K. scoparia* were identified in locations scattered throughout the US central Great Plains. In an effort to characterize glyphosate resistance, *K. scoparia* accessions from Kansas, Colorado, North Dakota, and South Dakota were included in this study. Thus far, resistance has been most problematic in western Kansas. Therefore, most of the *K. scoparia* accessions included were from that area, and a map of the Kansas collection sites has been included (Fig. 3). Also, the geographic distribution of plants analyzed in this study reflects the approximate distribution of glyphosate-resistant *K. scoparia* in the year 2011.

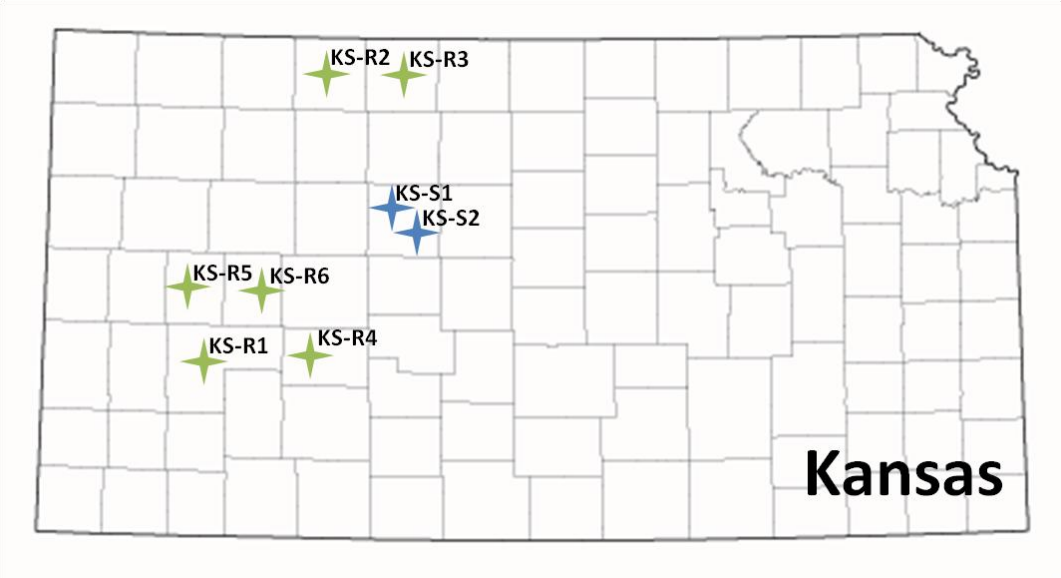

Figure 3. Geographical distribution of glyphosate-susceptible and -resistant *K. scoparia* accessions in western Kansas. Blue (susceptible) and green (resistant) stars indicate the counties where *K. scoparia* accessions were collected.

One field level pattern that was consistently observed at glyphosate-resistant *K. scoparia* collection sites was trails of resistant plants in fields treated with glyphosate (Fig. 1). Because *K. scoparia* utilizes the "wind-driven tumbleweed" mechanism for seed dissemination, it is probable that an individual field streak pattern is the glyphosate-resistant progeny of a single glyphosate-resistant parent. The path traveled by the parent is revealed when progeny survive glyphosate treatment.

While strong evidence for glyphosate resistance was observed in the field, further characterization of glyphosate-resistant *K. scoparia* was needed to confirm resistance. For the purpose of this paper, glyphosate resistance is defined as any plant that survives the prescribed application rate of glyphosate (Weed Technology Notes 1998). Dose response results for *K. scoparia* accessions treated with glyphosate are presented in Table 1. Although plant response to glyphosate treatment was variable, plant survival was clear based on new growth from plant nodes and the presence of living green tissue (Fig. 4). Consistently, early signs of glyphosate-susceptibility in *K. scoparia* included chlorosis, and the loss of turgor pressure. Eventually, all glyphosate-susceptible plants would become completely necrotic. The response of glyphosate-resistant *K. scoparia* was less severe, and signs of chlorosis and necrosis were localized to leaf tips and growing points. By the third week after treatment, recovering glyphosate-resistant *K. scoparia* could be easily differentiated from -susceptible plants (Fig. 4).

Figure 4. Representative glyphosate-susceptible and -resistant *K. scoparia* at 3 weeks after treatment. Glyphosate-susceptible (left) *K. scoparia* could be clearly differentiated from -resistant (right) plants based on whole plant response to glyphosate treatment.

K. scoparia response to glyphosate was also evaluated at the accession level. The percentage of plants within each accession that survived treatment at each rate was calculated and recorded (Table 1). All glyphosate-susceptible plants treated with 0.84 kg ae ha^{-1} died, and confirmed that this rate was ideal for distinguishing glyphosate-susceptible from -resistant plants. Conversely, between 75 and 100% of glyphosate-resistant plants survived a glyphosate treatment rate of 0.84 kg ae ha^{-1}. Furthermore, many glyphosate-resistant plants survived rates as high as 3.36 kg ae ha^{-1}, which is a rate four times that of the prescribed treatment rate. In cases where less than 100% of glyphosate-resistant plants survived treatment at 0.84 kg ae ha^{-1}, it is probable that accessions are still segregating for resistance because of field wind pollination (Table 1).

After evaluating plant survival, representative glyphosate-susceptible and -resistant *K. scoparia* needed to be selected for further molecular analysis. To select the most resistant plants from glyphosate-resistant accessions, plants that survived the highest dose and that had enough healthy tissue for nucleic acid and protein extraction were used for further analysis. All

glyphosate-resistant plants selected had survived glyphosate treatment rates ranging from 1.40 to 3.36 kg ae ha^{-1}. Glyphosate-susceptible plants were selected from the 0 kg ae ha^{-1} glyphosate treatment control (Table 1).

Table 1. Dose response and individual plant selection of glyphosate-susceptible and -resistant *K. scoparia*.

Location	Accession code	n	Percent survival									Glyphosate treatment (kg ae ha^{-1}) of individually selected plants		
			Glyphosate treatment (kg ae ha^{-1})									Plant number		
			0	0.28	0.42	0.56	0.84	1.40	1.68	3.36	6.73	1	2	3
Ellis, KS	KS-S1	10	100	90	60	20	0	0	0	0	0	0	0	0
Ellis, KS	KS-S2	6	100	83	67	33	0	0	0	0	0	0	0	0
Finney, KS	KS-R1	16	100	100	100	100	100	100	94	56	0	1.40	1.40	1.40
Norton, KS	KS-R2	6	100	100	100	100	83	83	83	33	17	3.36	1.68	1.68
Phillips, KS	KS-R3	6	100	100	100	100	100	100	50	83	33	3.36	3.36	3.36
Hodgeman, KS	KS-R4	6	100	100	100	100	83	83	83	17	0	1.68	1.68	1.68
Scott, KS	KS-R5	6	100	100	83	100	100	67	100	67	0	3.36	3.36	3.36
Lane, KS	KS-R6	4	100	100	100	100	100	50	75	50	0	1.68	1.68	1.68
Burleigh, ND	ND-R1	7	100	100	100	100	100	100	100	100	14	3.36	3.36	3.36
Sulley, SD	SD-R1	4	100	N/A	N/A	N/A	75	100	N/A	N/A	25	1.40	1.40	1.40
Logan, CO	CO-R1	18	100	100	83	83	78	72	61	17	0	1.68	1.68	1.68

Note: Glyphosate-susceptible (blue) and -resistant (green) accessions were treated with multiple rates of glyphosate, and the percentages of surviving plants were recorded for each accession at each rate. Glyphosate treatment rates to the right of the dashed red line are ≥ the prescribed rate (0.84 kg ae ha^{-1}). Three representative plants that exhibited higher levels of resistance were selected for further molecular analysis, and the glyphosate rate that each plant survived was recorded. Accession locations are the county and state of origin. Accession codes are the state of origin, its resistant or susceptible designation, and a unique identifying number. The sample sizes (n) of plants used for glyphosate dose response was included.

Sequencing the EPSPS binding site

Figure 5 displays the binding site sequence from glyphosate-susceptible and -resistant *K. scoparia* aligned to a reference glyphosate-susceptible *A. palmeri* sequence. No SNPs of the binding site proline were identified among the *K. scoparia* accessions. Furthermore, only synonymous SNPs were identified in the comparison of *K. scoparia* and *A. palmeri* binding site sequence. Based on this alignment, glyphosate-resistance in *K. scoparia* is likely not because of *EPSPS* proline-106 mutation. However, because *K. scoparia* sequence was generated from PCR product, higher frequency amplicons could mask those of lower frequency.

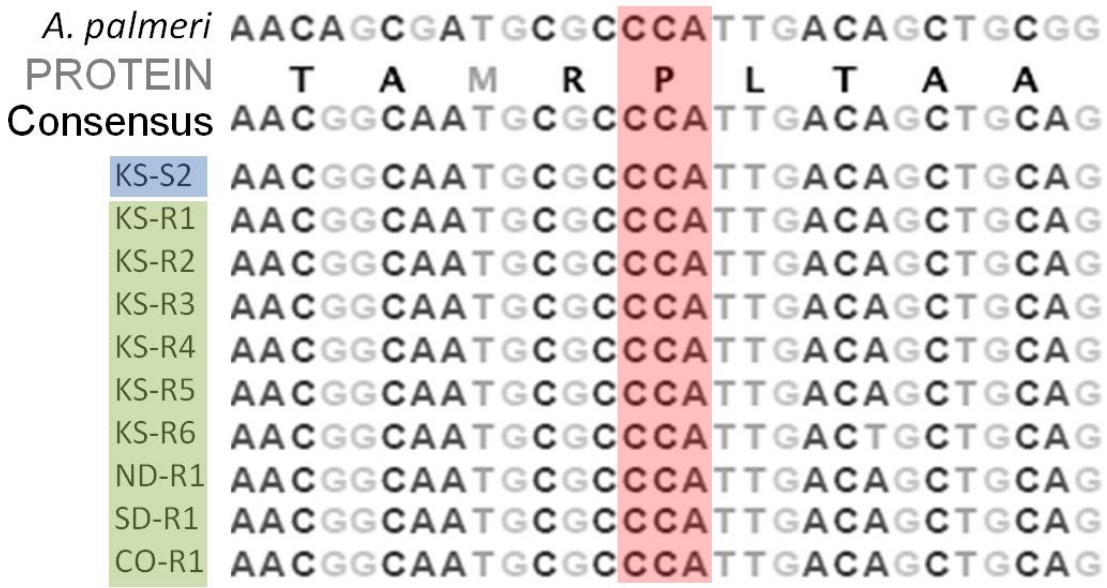

Figure 5. *EPSPS* binding site sequence alignment. *EPSPS* binding site sequence alignment confirm no proline 106 mutations (red shading) in glyphosate-susceptible *A. palmeri*, and representative glyphosate-susceptible (blue) and –resistant (green) *K. scoparia* (10 accessions). Protein alignment and consensus of *K. scoparia* sequence was included.

EPSPS gene amplification in glyphosate-resistant K. scoparia

Relative *EPSPS* gene copy numbers for glyphosate-susceptible and -resistant *K. scoparia* are shown in Figure 6. *EPSPS* copy number remained constant in glyphosate-susceptible individuals at approximately one copy relative to *ALS*, with little variation. The average relative *EPSPS:ALS* gene copy number across glyphosate-susceptible accessions was 0.70, and ranged

from 0.63 to 0.74. Conversely, glyphosate resistant *K. scoparia* had increased *EPSPS* copy number across all plants and accessions. The average relative *EPSPS:ALS* gene copy number across glyphosate-resistant accessions was 5.75, and ranged from 3.08 to 8.48. *EPSPS* copy number also varied between siblings and half-siblings from the same accession (Fig. 6). Field wind pollination and unknown paternal inheritance is suspected to be the likely the source of this variation. In conclusion, glyphosate-susceptible plants had consistently low *EPSPS* copy number, while *EPSPS* gene amplification was found in all glyphosate-resistant individuals.

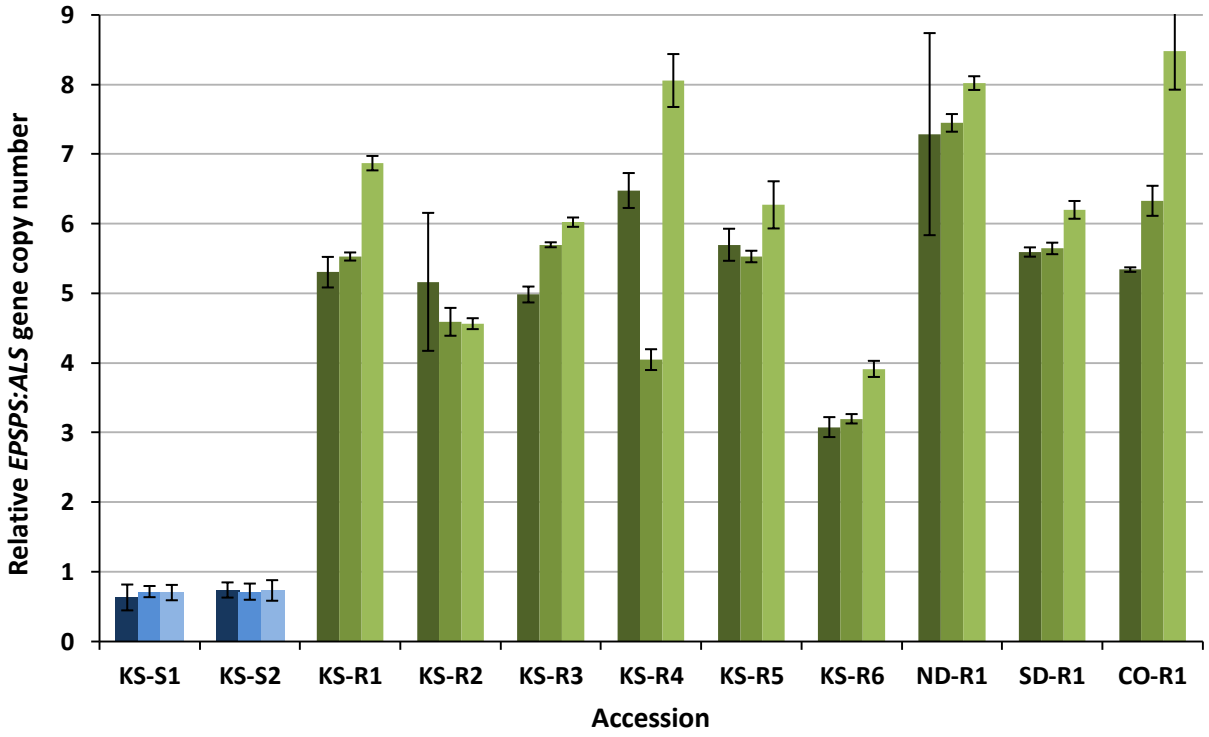

Figure 6. Relative *EPSPS:ALS* gene copy number in glyphosate-susceptible and -resistant *K. scoparia*. *EPSPS* copy number estimates for two glyphosate-susceptible accessions (blue) and nine -resistant accessions (green). Three plants measured per each accession (dark, medium, light shading). Error bars indicate the standard error of six technical replicates, three for *EPSPS* reactions and three for *ALS*.

Reduced shikimate accumulation in plants with elevated EPSPS gene copy number

Leaf disk shikimate accumulation was measured in glyphosate-susceptible and –resistant *K. scoparia*, and levels are recorded in Figure 7. Shikimate accumulated for all glyphosate-

susceptible *K. scoparia* and levels ranged from 13.2 to 27.5 Δ ng shikimate μL^{-1}. Conversely, glyphosate-resistant plants generally accumulated little to no shikimate and levels ranged from -0.5 to 10.9 Δ ng shikimate μL^{-1}. Shikimate accumulation in glyphosate susceptible plants was significantly higher $t(5.38) = 9.11$, $p<0.0001$ (assuming unequal variance). Leaf disks from some of the plants suspected to be resistant accumulated a modest amount of shikimate (Fig. 7: KS-R2(4), KS-R4(5), and KSR5(7)). Elevated levels of accumulated shikimate in these individuals indicates that accessions are segregating for resistance—an interpretation that is consistent with dose response data (Table 1).

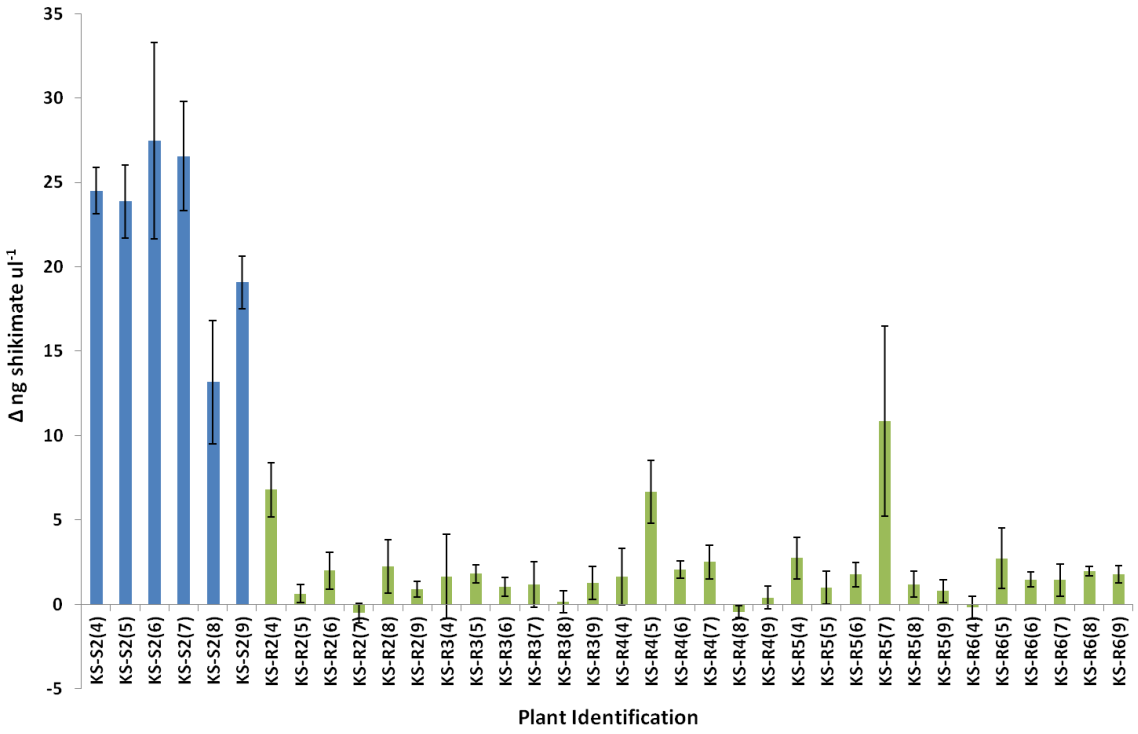

Figure 7. Leaf disk shikimate accumulation after glyphosate treatment. Shikimate accumulation measurements on glyphosate-susceptible (blue) and -resistant (green) plants are reported as the difference in shikimate level between three untreated and three treated leaf disks from the same plant. Error bars indicate the standard error between biological leaf disk replicates.

In Figure 8, *K. scoparia* shikimate accumulation levels are plotted against respective *EPSPS* gene copy numbers. Glyphosate-susceptible plants with high shikimate accumulation had

a single relative *EPSPS* gene copy. Conversely, glyphosate-resistant plants with low shikimate accumulation all had elevated *EPSPS* gene copy numbers ranging from 2.9 to 5.6 relative to *ALS*. Based on this analysis, I suggest that low shikimate accumulation is a good predictor of increased *EPSPS* gene copy number. Also, elevated *EPSPS* gene copy numbers may express enough EPSPS to alleviate the metabolic bottleneck created by glyphosate—as indicated by lower levels of accumulated shikimate.

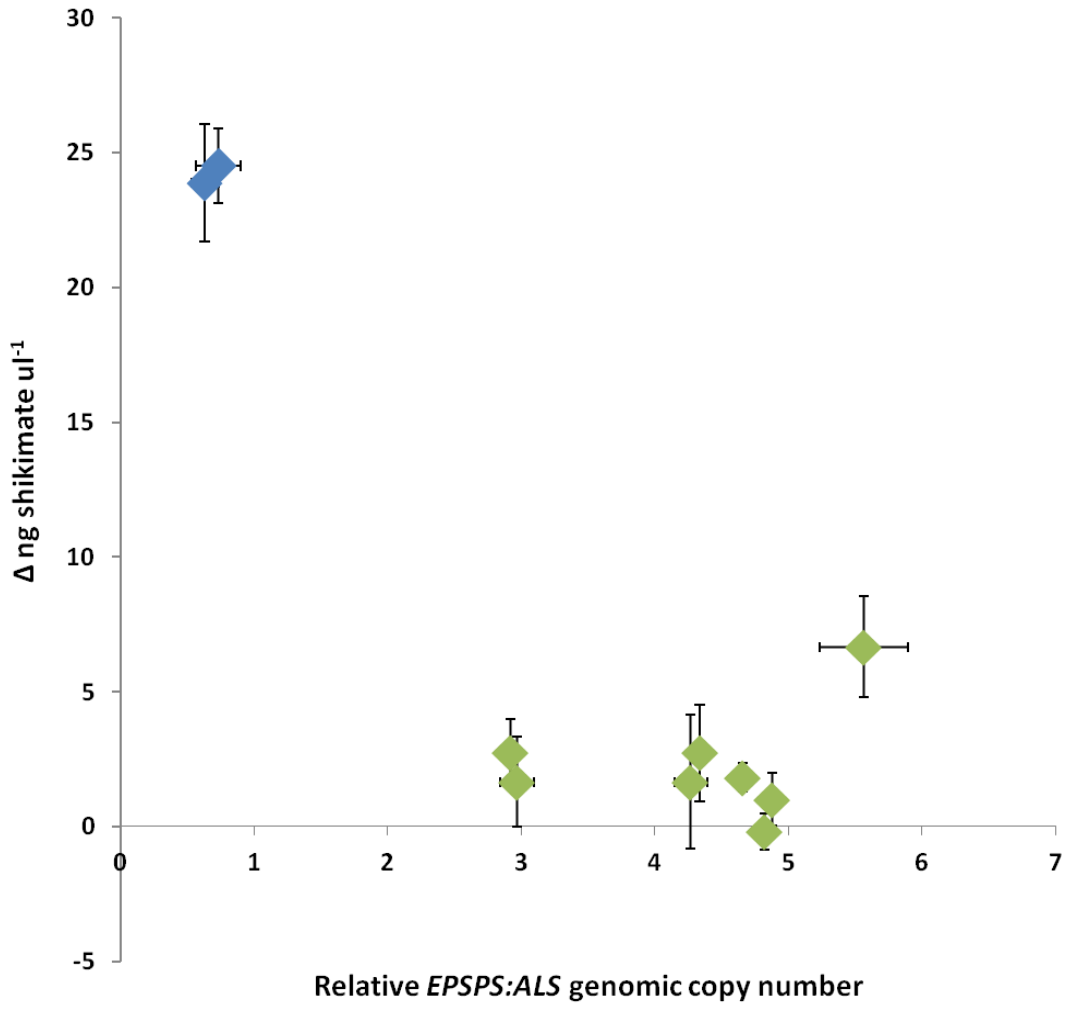

Figure 8. Increased *EPSPS* copy number versus reduced shikimate accumulation. Shikimate accumulation in glyphosate-susceptible (blue) and -resistant (green) *K. scoparia* leaf disks with known *EPSPS* gene copy number. Shikimate accumulates to a lesser degree in *K. scoparia* with increased *EPSPS* copy number. Error bars indicate standard error and were previously described.

Transcription and translation of amplified EPSPS gene copies

EPSPS transcript abundance correlates with *EPSPS* copy number

To ensure that increased *EPSPS* transcript abundance accompanied increases in *EPSPS* gene copy number, reverse transcriptase (RT) qPCR was used to quantify relative amounts of *EPSPS* transcript in plants with known copy number (Fig. 9). The plants assessed were a subset of those used for copy number estimation in Fig. 6. By plotting relative *EPSPS:ALS* transcript abundance against relative *EPSPS:ALS* gene copy number, a linear correlation was observed (R^2 = 0.854). Glyphosate-susceptible *K. scoparia* with a single *EPSPS* gene copy, had low *EPSPS* transcript abundance relative to *ALS*. In contrast, glyphosate-resistant plants with increased *EPSPS* gene copy numbers had roughly proportional increases in relative *EPSPS* transcript abundance (Fig.9). Based on this evidence, it is likely that increases in *EPSPS* transcription result from elevated *EPSPS* gene copy number.

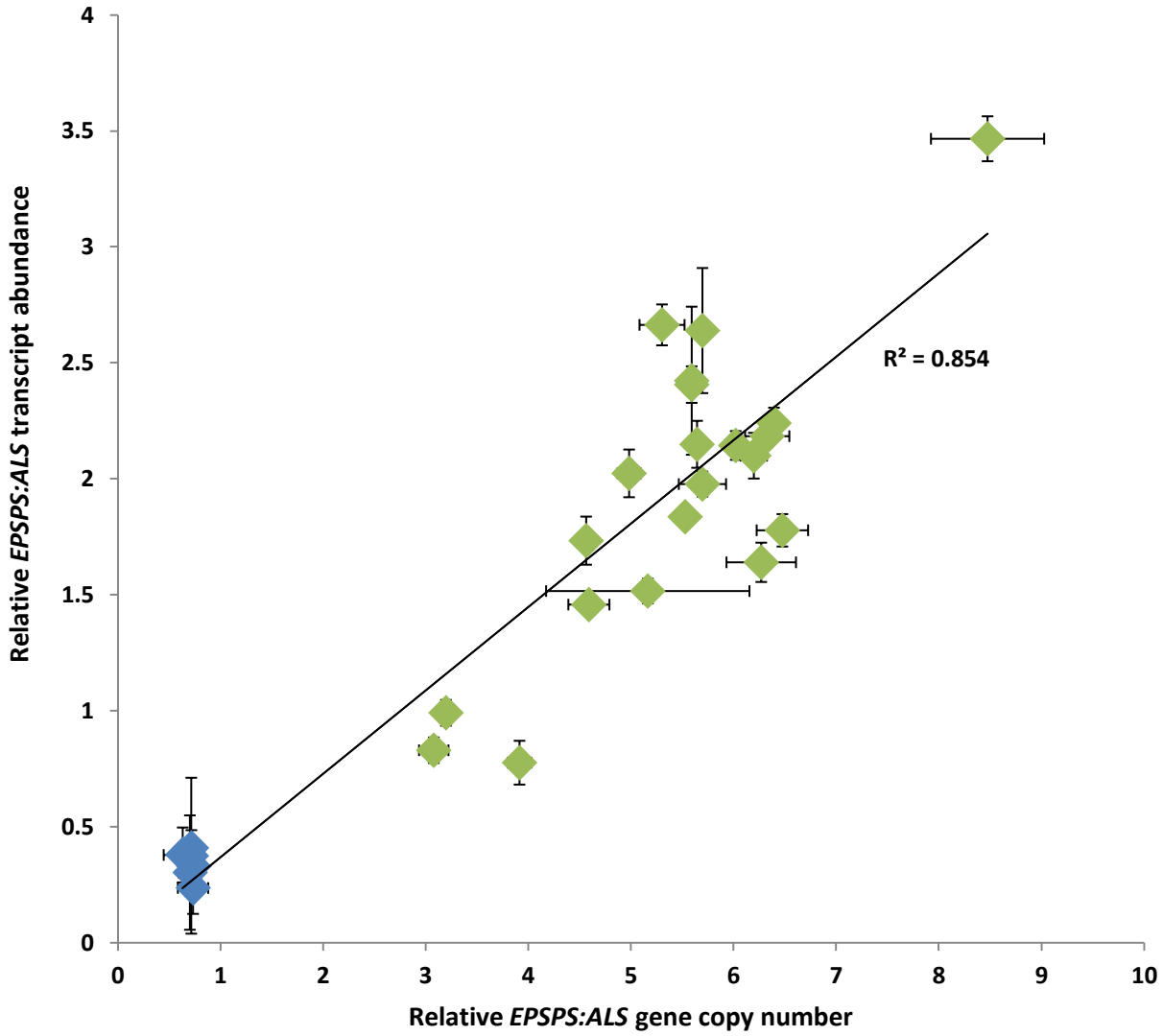

Figure 9. Increased *EPSPS* copy number correlates with increased *EPSPS* transcript abundance. *EPSPS* transcript abundance was estimated for glyphosate-susceptible (blue) and –resistant (green) plants with known *EPSPS* gene copy number. Transcript abundance was found to be linearly correlated to copy number ($R^2 = 0.854$). Error bars for transcript abundance indicate the standard error of six technical replicates, three for *EPSPS* reactions and three for *ALS*.

<u>High EPSPS protein abundance in glyphosate-resistant *K. scoparia*</u>

Anti-EPSPS immunoblotting was used to estimate amounts of EPSPS protein in *K. scoparia* with known relative copy number and transcript abundance (Fig. 10). The *K. scoparia* assessed were a subset of those used for *EPSPS* transcript estimation in Fig. 9. For comparison, glyphosate-susceptible and -resistant *A. palmeri* were included as positive controls for EPSPS

expression. In glyphosate-susceptible *A. palmeri* with a single relative *EPSPS:ALS* gene copy, EPSPS protein could not be detected. Conversely, in glyphosate-resistant *A. palmeri* with *EPSPS* copy number estimates of 63 and 51 relative to *ALS*, EPSPS signal saturated the radiological film at a molecular weight of 48 kDa. Similarly, no EPSPS signal was detected from five glyphosate-susceptible *K. scoparia*, while EPSPS signal from 13 glyphosate-resistant plants saturated the film at 48 kDa. In most lanes containing *K. scoparia* protein extract, a nonspecific or cross-hybridizing band was detected at about 38 kDa (Fig. 10). In other cases, another nonspecific or cross-hybridizing band was also detected only in *K. scoparia* samples at roughly 115 kDa (data not shown in figure). Because glyphosate-resistant EPSPS signal saturated the radiological film, the quantity of EPSPS protein could not be estimated accurately. Nonetheless, EPSPS signal was consistently detected in glyphosate-resistant *K. scoparia* with increased relative *EPSPS* gene copy number and transcript abundance, while signal could not be detected in -susceptible plants with a single *EPSPS* copy and low transcript abundance. Based on this evidence, it is likely that *EPSPS* gene amplification leads to elevated EPSPS expression in glyphosate-resistant *K. scoparia*.

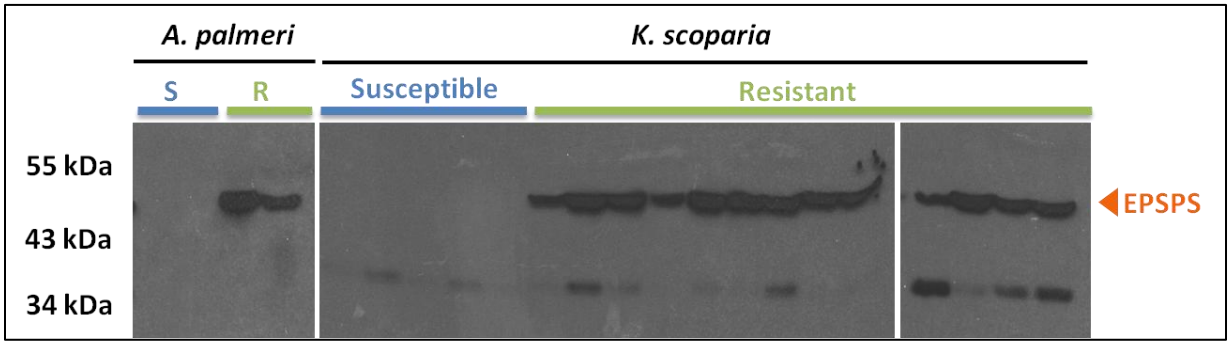

Figure 10. Increased EPSPS abundance in glyphosate-resistant *A. palmeri* and *K. scoparia*. Immunoblotting was used to detect EPSPS protein (48 kDa) in glyphosate-susceptible (blue), and resistant (green) *A. palmeri*, and *K. scoparia*. EPSPS was not detected in glyphosate-susceptible individuals, but EPSPS signal saturated in -resistant individuals. A nonspecific or cross hybridizing band was detected in most *K. scoparia* lanes at roughly 38 kDa. Coomasie stain not pictured.

Discussion:

Basis of glyphosate resistance in K. scoparia

To elucidate the mechanism responsible for glyphosate resistance in *K. scoparia* from the US central Great Plains, three known mechanisms of glyphosate resistance were considered: 1) altered absorption and translocation of glyphosate, 2) alteration of the *EPSPS* active site, and 3) overexpression of EPSPS. Alignment of *EPSPS* binding site sequence from glyphosate-susceptible and -resistant individuals revealed complete conservation of the binding site proline in *K. scoparia*. This is a likely indication that amino acid substitution at that position did not prohibit or reduce interaction with glyphosate. Conversely, all glyphosate-resistant *K. scoparia* had increased relative *EPSPS* gene copy numbers, and copy number was linearly correlated with relative transcript abundance. Furthermore, EPSPS protein was reliably detected in glyphosate-resistant individuals, and could not be detected in -susceptible individuals. Thus, glyphosate resistance in *K. scoparia* is likely caused by *EPSPS* gene amplification which results in the overproduction of EPSPS. Based on this reasoning, I hypothesize that resistant plants are able to survive treatment because uninhibited EPSPS are available to maintain metabolic function.

Small increase in EPSPS copy number may be sufficient for resistance across plant species

By comparing *K. scoparia* results with published data for two other glyphosate-resistant plant species, relative *EPSPS* copy number as low as three may be sufficient for glyphosate resistance across plant species. A range of roughly 3 to 9 relative *EPSPS* gene copies was observed in glyphosate-resistant *K. scoparia* that survived rates of 1.40 to 3.36 kg ae ha^{-1} (Fig. 6 and Table 1). Furthermore, *K. scoparia* with additional *EPSPS* gene copies did not accumulate shikimate to the same extent as susceptible plants with a single *EPSPS* copy (Fig. 8). Similarly, as low as 5 relative *EPSPS:ALS* gene copies were detected in glyphosate-resistant *A. palmeri*,

and shikimate did not accumulate in plants with elevated *EPSPS* gene copy number (Fig. 1 and 2, Gaines *et. al.* 2010). Although glyphosate rates were not equivalent and species respond differently to treatment, it is noteworthy that a similar relationship between *EPSPS* gene copy number and shikimate accumulation levels persists across glyphosate-resistant *K. scoparia* and *A. palmeri*. Furthermore, three relative *EPSPS* gene copies were detected in *Lolium perenne* ssp *multiflorum* plants deemed to have intermediate levels of glyphosate resistance (Salas *et. al.* 2012). Accordingly, comparing glyphosate resistance across *K. scoparia*, *A. palmeri*, and *L. multiflorum*, suggests that relative *EPSPS* gene copy number as low as three may be sufficient for resistance.

If a few copies of *EPSPS* are sufficient for glyphosate-resistance, then smaller changes in EPSPS expression could confer resistance in a number of plant species. Such changes in expression might be introduced in a variety of ways other than *EPSPS* gene amplification. For instance, alteration of the *EPSPS* promoter or increased transcription factor activity could lead to increases in EPSPS expression sufficient to confer glyphosate resistance (Nandula 2010). These more subtle changes in EPSPS expression should be considered for future glyphosate resistance research.

Genetic mediation of EPSPS gene amplification

While similarities exist, the mechanism mediating *EPSPS* gene amplification in glyphosate-resistant *K. scoparia* is likely different from *A. palmeri*. Glyphosate-resistant *K. scoparia* had between 3 and 9 relative *EPSPS* gene copies and there was little variation among maternally related plants and geographically distinct accessions. Alternatively, glyphosate-resistant *A. palmeri* had a broad range of 5-160 *EPSPS* copies, and amplified *EPSPS* copies were distributed across the genome. Furthermore, copy number varied from one to greater than the

sum of copy numbers from both parents (Gaines *et. al.* 2010). In *A. palmeri*, a non-Mendelian inheritance pattern was immediately clear and mobile genetic elements are suspected to mediate *EPSPS* gene amplification (Gaines *et. al.* 2010). In *K. scoparia*, the inheritance of amplified *EPSPS* genes is unknown. Nonetheless, based on the narrow range and little variation of *EPSPS* copy number within and between accessions, a Mendelian inheritance pattern remains a possibility.

If the mechanism mediating *EPSPS* gene amplification in *K. scoparia* is the same as *A. palmeri*, then gene amplification in *K. scoparia* was likely detected at an early stage in the development of resistance in *K. scoparia* populations. However, in respect to this possibility, I suggest that fitness advantages will eventually plateau with further *EPSPS* gene amplification. Furthermore, I have shown that low copy number, as low as three, may be sufficient for *K. scoparia* survival when treated with a lethal dose of glyphosate. Thus, I propose that there is little likelihood of *K. scoparia* further amplifying *EPSPS* copies to levels observed in *A. palmeri*. Alternatively, I postulate that extremely high *EPSPS* copy numbers (up to 160) reported in *A. palmeri* are simply a byproduct of the genetic mechanism that mediated gene amplification. If that is the case, unequal crossing-over or rolling circle replication-based mechanisms of gene amplification could explain the lower *EPSPS* copy number in *K. scoparia*.

Geographic origin(s) of glyphosate-resistant K. scoparia

The geographic origin(s) of glyphosate-resistant *K. scoparia* is primarily important for reasons of agricultural weed control. Knowing the origin(s) of resistance could focus mitigation efforts, and may even point to weed management practices that select for glyphosate resistance. If glyphosate-resistant *K. scoparia* with elevated *EPSPS* copy number were identified from multiple and diverse geographic regions, then attempts to quarantine resistance would be

ineffective because relatively frequent gene amplification events would select for new individuals with elevated copy number. On the other hand, if glyphosate-resistant *K. scoparia* originated in one location and spread, there would be more incentive to eradicate isolated populations of glyphosate resistant plants. Moreover, if gene amplification events are relatively infrequent, growers and land managers may be more encouraged to adopt weed management practices that will reduce the overall likelihood of evolving herbicide resistance. These better management practices, such as diversifying weed management tactics and simultaneously utilizing multiple herbicide modes of action, will likely preserve the effectiveness of herbicides and herbicide-tolerant cropping systems (Norsworthy *et. al.* 2012).

Theoretical and practical implications

Confirmed cases of glyphosate-resistant *K. scoparia* in Kansas, Colorado, North Dakota, and South Dakota likely foreshadow the further spread of resistance throughout the region. Already, there are suspected cases of glyphosate-resistant *K. scoparia* in Nebraska, Montana, and southern Alberta (Monsanto Canada Inc. 2012). Many factors including high selection pressure, wind pollination, tumble weed and anthropogenic modes of seed dissemination, and the ability of a single plant to produce upwards of 30,000 seeds will continue to favor the spread of resistance (Friesen 2008). Also, the widespread distribution of non-resistant *K. scoparia* across much of North America, could lead to glyphosate-resistant *K. scoparia* infestation in areas where *K. scoparia* was not a problem in the past (Wiersma *et. al.* 2012). In any case, the continued monitoring of resistance is an important aspect of future research, and the use of a molecular resistance diagnostics could prove to be an invaluable resource.

Finally, this research fits into the larger framework of biological processes driving glyphosate resistance evolution. The discovery of glyphosate-resistant *A. palmeri, L.*

multiflorum, and *K. scoparia* with increased *EPSPS* gene copy number, suggests that increased EPSPS expression may be a mechanism of glyphosate resistance common to other plant species too. Just as *EPSPS* binding-site mutations and altered translocation of glyphosate have been implicated in other cases of glyphosate resistance, elevated expression of EPSPS should also be considered in cases of weed-evolved glyphosate resistance.

CHAPTER FOUR: CONCLUSIONS AND FUTURE PERSPECTIVES

The three research objectives listed were 1) to monitor the spread of glyphosate-resistant *K. scoparia*, 2) to assess the level of resistance, and 3) to determine the mechanism of resistance. Taking into account all the evidence included in chapter 3 and appendix 1, *K. scoparia* accessions from 27 geographically isolated fields spanning four US states and one Canadian province were confirmed resistant using glyphosate dose response and shikimate assays (Table 1 and 2, Fig. 7 and 12). The combined linear distance between glyphosate-resistant *K. scoparia* sites was roughly equal to 2,000 kilometers. Irrespective of the origin(s), the range of glyphosate-resistant *K. scoparia* is expanding rapidly. As for the level of glyphosate resistance in *K. scoparia* two things stand out. First, when treated with a labeled rate of glyphosate, high survival percentages were observed for nearly all glyphosate-resistant *K. scoparia* accessions. Second, individuals from many glyphosate-resistant *K. scoparia* accessions survived glyphosate treatment rates as high as 3.36 kg ae ha^{-1} (Tables 1 and 2). Together, these observations confirm that the level of glyphosate resistance in *K. scoparia* is high enough to render the labeled glyphosate rate ineffective, and that attempts to overcome resistance with higher rates will simply select for higher levels of resistance. Lastly, because all glyphosate-resistant *K. scoparia* analyzed had increased *EPSPS* copy number (between 3 and 9 copies relative to *ALS*), I suggest that *EPSPS* gene amplification along with a proportional increase in EPSPS expression is the likely mechanism of glyphosate resistance in *K. scoparia* (Fig. 6, 8, 9, 10, and 13).

While the glyphosate resistance mechanism in *K. scoparia* is similar to the resistance mechanism in *A. palmeri*, future research should address notable differences between the two weed species. The obvious difference between *K. scoparia* and *A. palmeri* is the drastic

difference in relative *EPSPS* copy number. Future research efforts should focus on how *EPSPS* copy number affects resistance across plant species. More specifically, are 3 relative *EPSPS* copies sufficient for resistance in plant species other than *K. scoparia*? Does the selective advantage of increased copy number plateau in *K. scoparia* or other plant species? Or, more fundamentally, could there be more to glyphosate resistance in *K. scoparia* that allows lower *EPSPS* copy number to be sufficient for resistance? Lastly, is lower *EPSPS* copy number in *K. scoparia* a result of shorter time since the onset of resistance development, or the genetic mediation of gene amplification? As is the case with any basic research, new findings always lead to new questions.

In an effort to convey the impact that glyphosate-resistant *K. scoparia* could have on agriculture in the central Great Plains, let's briefly revisit the glyphosate-resistant *K. scoparia* field streak pattern (Fig.1). Imagine all the glyphosate-resistant *K. scoparia* in that field streak mobilizing in the wind and releasing glyphosate-resistant seed as they tumble. In a year, one streak has the potential to become hundreds of streaks. While glyphosate may still be effective on other weeds in the central Great Plains, its days of controlling *K. scoparia* could be coming to an end. As herbicide companies consider the next best options for *K. scoparia* control, many of the alternative options could already be ineffective. One option under consideration is the herbicide dicamba. Unfortunately, dicamba-resistant *K. scoparia* was documented as early as 1995 (Heap 2012). Moreover, random screening at CSU in 2012 may have already identified a wild *K. scoparia* accession with combined glyphosate and dicamba resistance. By shifting from complete reliance on one herbicide to complete reliance on another, weed management will always be a losing battle. A better option is to diversify weed control, and contain areas with glyphosate-resistant *K. scoparia* in an attempt to temporarily preserve glyphosate's utility.

However, two pieces of information are critical for success. First, alternative weed control options need to be identified, and should include chemical, mechanical, and cultural methods. Second, a detailed analysis of the origin or origins of resistance needs to be done to inform management strategies. If glyphosate-resistant *K. scoparia* are all related to a common ancestor, a rigorous weed management strategy could eradicate isolated populations of resistant plants. On the other hand, if *K. scoparia* evolved resistance in numerous locations, containment of resistance could be difficult and efforts should focus mainly on the development of new and better management strategies.

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APPENDIX I: FURTHER CHARACTERIZATION OF GLYPHOSATE-RESISTANT *KOCHIA SCOPARIA*

Introduction:

In 2011 and 2012, numerous reports of putative glyphosate-resistant *K. scoparia* reached CSU, and uncharacterized germplasm in our seed repository continued to build up. Local farmers wanted to know if the *K. scoparia* they sent us was resistant, and collaborators wanted to know if plants from their region had increased *EPSPS* copy number. In response, I planted 19 accessions of *K. scoparia* collected from Kansas, Colorado, North Dakota, and Alberta, and characterized resistance using whole plant glyphosate dose response, shikimate assays, and qPCR to measure relative *EPSPS* copy number.

While at first this research may seem redundant with the research described in chapter 3, it instead compliments past research in a number of ways. It provides essential information to those attempting to manage glyphosate-resistant *K. scoparia* in their own region. In addition, it replicates past results, and displays surprising phenotypic consistency across glyphosate-resistant accessions from an even more extensive range. This research confirms the further distribution of glyphosate-resistant *K. scoparia*, and demonstrates the sensitivity our system has to detect resistance. Lastly, results obtained from this research provide more evidence that 3 relative *EPSPS* gene copies are sufficient for glyphosate resistance in *K. scoparia*.

Methods and materials:

Plant identification and collection

K. scoparia plants or seed were collected from geographically isolated fields throughout Kansas, Colorado, North Dakota, and Alberta in the following manner. Of the 19 total accessions

collected, 18 were suspected to be glyphosate-resistant and 1 was suspected to be glyphosate-susceptible. The five accessions collected throughout Kansas were isolated from counties that had not been characterized before, and the geographic distribution of these accessions is depicted in Figure 11. Accessions collected throughout Colorado were isolated from four sites. One site, from Sterling, had already been characterized (Chapter 3), and was included as a positive control. The other three sites had not been previously characterized, and the geographic distribution of Colorado accessions is also depicted in Figure 11. The only accession included from North Dakota arrived with very little information detailing its history and origin. On the other hand, the nine accessions from Alberta arrived with a detailed report that is described in the following paragraph.

In 2010, collaborator in southern Alberta identified three fields with apparent glyphosate-resistant *K. scoparia*. Telltale signs of glyphosate resistance, including streak patterns, were present in each of the fields. After growing plants in a greenhouse setting, resistance was confirmed using conventional glyphosate treatment. The following year, a comprehensive survey was done to monitor the spread of resistance within that area. *Kochia scoparia* seed was collected from multiple locations within a 20 kilometer radius of the original three sites. Greenhouse screening confirmed glyphosate resistance at seven new sites located to the East and Southeast of the original three. Researchers involved suggest that wind and anthropogenic effects contributed to the spread of resistance in that area. Shortly thereafter, seed from the original three sites and six of the survey sites (confirmed to be resistant) was sent to CSU for further whole plant and molecular characterization.

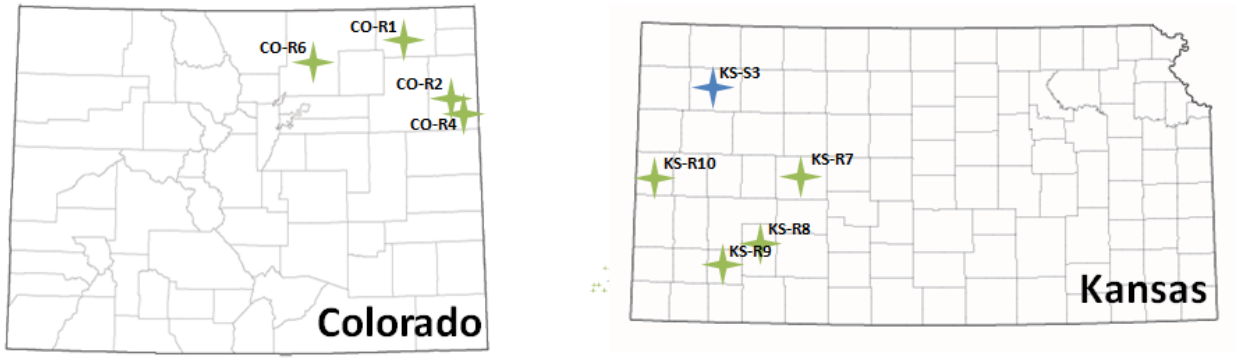

Figure 11. Geographical distribution of glyphosate-susceptible and -resistant *K. scoparia* accessions in Colorado and Kansas. Blue (susceptible) and green (resistant) stars indicate the counties where *K. scoparia* accessions were collected.

Growing conditions, plant treatment and assessment

Accessions used for this study were grown from either seed collected off a single parent plant, or aggregate seed from a suspect field. Planting and growth conditions were the same as previously described (Chapter 3).

Plant treatment using Roundup WeatherMax® at the following rates: 0, 0.28, 0.42, 0.56, 0.84, 1.12, 1.68, 2.24, 3.36, and 4.48 kg ae ha^{-1} was done in the same manner previously described (Chapter 3). The only accession that received every treatment rate was the glyphosate-susceptible accession KS-S3. The number of plants available from each accession limited how many could be treated at each rate. However, plants from every accession were treated with four glyphosate rates: 0, 0.42, 0.84, 1.68, and 4.48 kg ae ha^{-1}.

At 4 WAT, two plants from each accession were selected for further molecular analysis. One plant from each accession was selected from the 0 kg ae ha^{-1} glyphosate rate, while the other plant was selected from the 0.84 kg ae ha^{-1} glyphosate rate.

Shikimate accumulation assay

Leaf disk shikimate accumulation was estimated in the same manner previously described (Chapter 3). Shikimate assays were done on five untreated plants from each accession.

Six leaf disks were excised from each plant. Three leaf disks were treated with 100 μM glyphosate, and three leaf disks were not treated. By subtracting shikimate levels in treated leaf disks from ambient shikimate levels in untreated leaf disks, shikimate accumulation could be estimated for each plant.

Extraction of nucleic acids

Nucleic acid extraction and follow up quantity and quality control was done in the same manner previously described (Chapter 3).

Copy number determination on genomic DNA

Estimation of relative *EPSPS* copy number was done in the same manner previously described (Chapter 3). Relative *EPSPS:ALS* gene copy number was estimated for 2 plants from each glyphosate-resistant accession, and 16 glyphosate-susceptible plants. One plant from each glyphosate-resistant accession had survived glyphosate treatment at 0.84 kg ae ha^{-1}, and the other had never received glyphosate treatment. In this way, copy number was measured in plants that were known to be glyphosate-resistant, and in plants without a resistant or susceptible designation. All copy number estimates for the glyphosate-susceptible accession were done on plants that had never received glyphosate treatment.

Results:

Confirmation of glyphosate resistance and susceptibility

Whole plant glyphosate dose response was used to confirm glyphosate resistance or susceptibility (Table 2). As expected, glyphosate-resistant *K. scoparia* accessions generally had high survival rates at the prescribed treatment rate (0.84 kg ae ha^{-1}). Furthermore, many individual plants from glyphosate-resistant accessions survived rates as high as 3.36 kg ae ha^{-1}. Unexpectedly, the accession suspected to be glyphosate-susceptible had 4% survival at 1.12 kg

ae ha^{-1}, a rate that should have been lethal (Table 2). Based on these results, all accessions appear to be segregating for glyphosate resistance, including the glyphosate-susceptible accession.

Table 2. Dose response of glyphosate-susceptible and -resistant *K. scoparia*

Accession code	Percent survival									
	Glyphosate treatment (kg ae ha^{-1})									
	0	0.28	0.42	0.56	0.84	1.12	1.68	2.24	3.36	4.48
KS-S3	100	85	7	7	0	4	0	0	0	0
KS-R7	100		75		83		0			
KS-R8	100		100		89		43			
KS-R9	100		89		94		68	39	28	22
KS-R10	100		100		89		53		0	
AL-R1	100		100		100		78	78	65	
AL-R2	100		94		100		56	78	71	
AL-R3	100		100		100		67		39	
AL-R4	100		94		100		94		72	
AL-R5	100		44		89		39		0	
AL-R6	100		100		100		72		35	
AL-R7	100		28		15		11		6	
AL-R8	100		100		100		94		67	
AL-R9	100		89		50		0		11	
ND-R2	100		67		44		11		11	
CO-R1	100	100	100	89	100		89	78	44	17
CO-R2	100		89		67		63	56	17	
CO-R4	100		94		100		70	67	39	
CO-R6	100		78		78		56			

Note: Glyphosate-susceptible (blue) and -resistant (green) accessions were treated with multiple rates of glyphosate, and the percentages of surviving plants were recorded for each accession at each rate. Glyphosate treatment rates to the right of the dashed red line are ≥ the prescribed rate (0.84 kg ae ha^{-1}). Accession codes are the state of origin, its resistant or susceptible designation, and a unique identifying number.

Shikimate accumulation assays were also used to confirm glyphosate resistance or susceptibility, and results were found to be consistent with whole plant glyphosate dose response (Fig. 12). Shikimate accumulation was variable, but generally lower in glyphosate-resistant accessions. In the glyphosate-susceptible accession, shikimate accumulation was high in four individuals, but low in one. Alternatively, most glyphosate-resistant plants accumulated low levels of shikimate, but a fraction of the resistant plants accumulated shikimate to the same level

as -susceptible plants (Fig. 12). These results are further confirmation that all *K. scoparia* accessions included in this study are segregating for glyphosate resistance, including the accession with a glyphosate-susceptible designation.

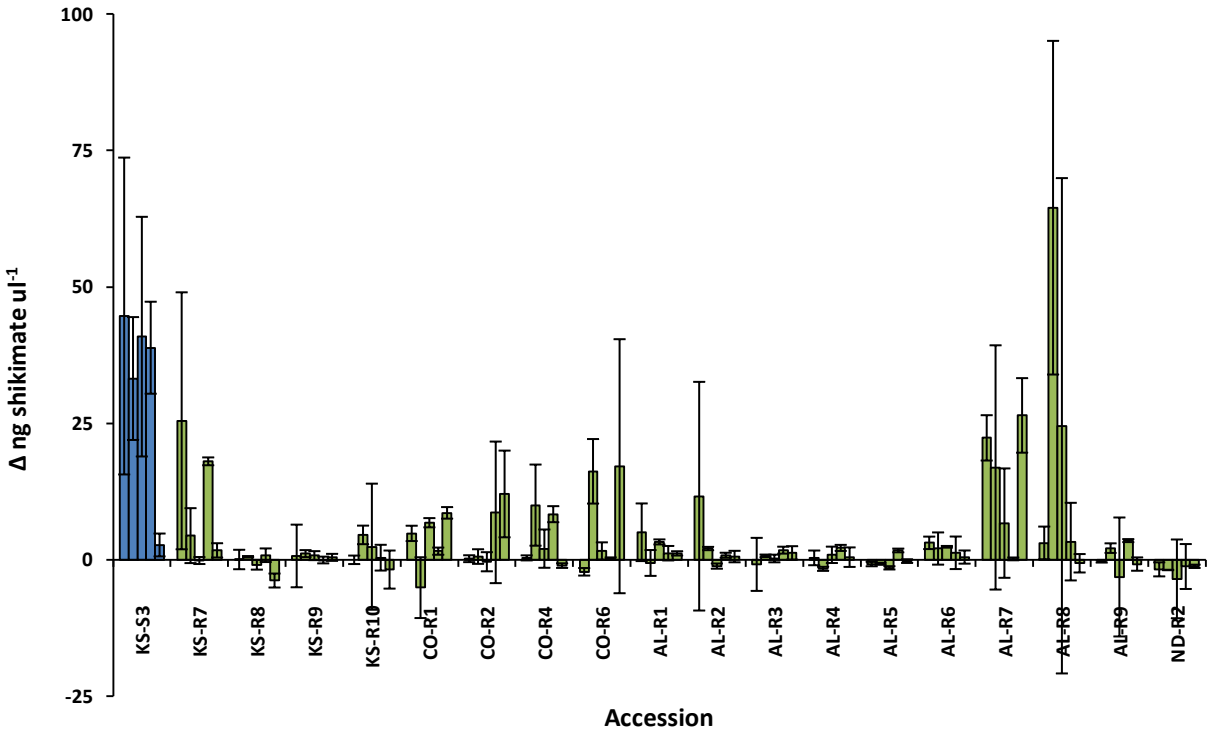

Figure 12. Leaf disk shikimate accumulation after glyphosate treatment (2). Shikimate accumulation measurements on glyphosate-susceptible (blue) and –resistant (green) plants are reported as the difference in shikimate level between three untreated and three treated leaf disks from the same plant. Error bars indicate the standard error between biological leaf disk replicates.

EPSPS gene amplification in glyphosate-resistant K. scoparia

Relative *EPSPS:ALS* gene copy number was estimated for *K. scoparia* that had never been treated with glyphosate, and plants that had survived a prescribed glyphosate treatment rate (Fig. 13). Glyphosate-resistant plants that had survived treatment with a labeled rate of glyphosate all had between 3 and 9 relative *EPSPS:ALS* gene copies (Fig. 13 -dark green columns). Glyphosate-resistant plants that had never been treated with glyphosate had between 1 and 9 relative *EPSPS:ALS* gene copies (Fig. 13 -light green columns). Glyphosate-susceptible

plants generally had a single relative *EPSPS* copy, but in two cases had relative gene copy numbers closer to 3 (Fig. 13 -blue columns). Based on whole plant glyphosate dose response and shikimate assays, all the accessions included were segregating for resistance. Consistent with this observation, two untreated plants designated glyphosate-susceptible had increased copy number, and two untreated plants designated glyphosate-resistant had no amplified *EPSPS* copies.

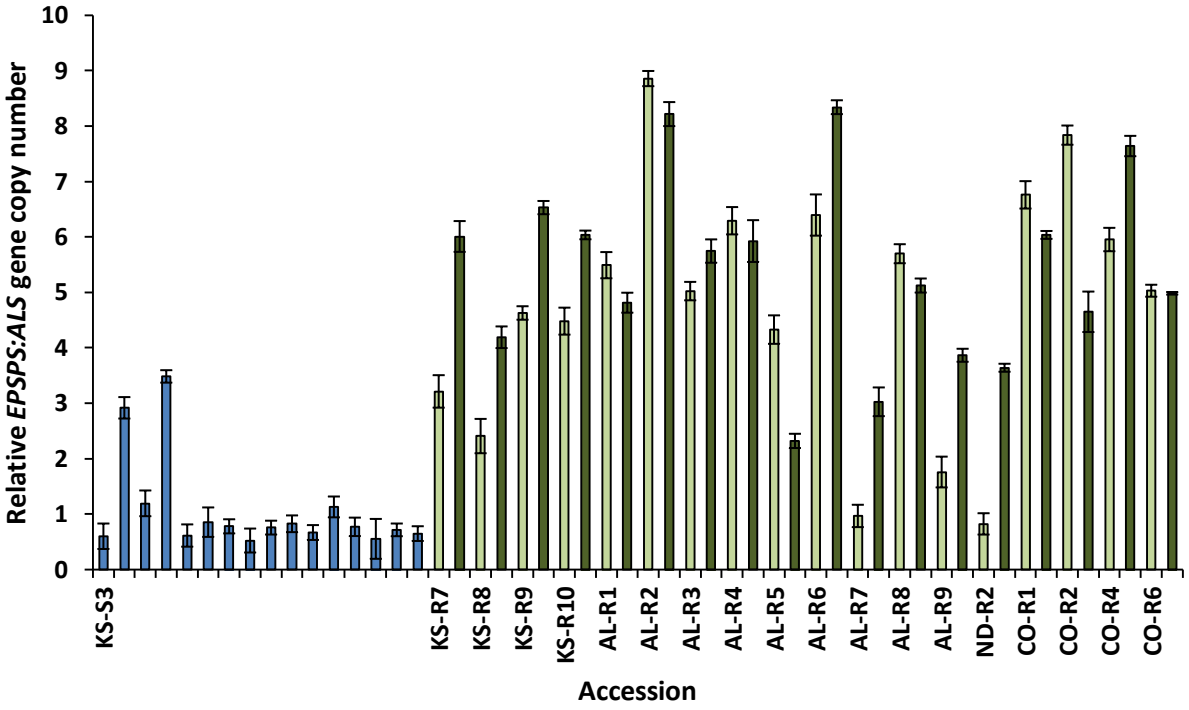

Figure 13. Relative *EPSPS:ALS* gene copy number in glyphosate-susceptible and -resistant *K. scoparia* (2). *EPSPS* copy number estimates for glyphosate-susceptible (blue) and -resistant (green) accessions. Accession names are positioned at the base of the column furthest to the left of the columns it represents. Light green columns represent untreated individuals, while dark green columns represent plants that survived 0.84 kg ae ha^{-1} glyphosate treatment. Error bars indicate the standard error of six technical replicates, three for *EPSPS* reactions and three for *ALS*.

Discussion:

This research is further validation of the results and hypotheses presented in chapter 3. First, our system was capable of detecting glyphosate resistance in an accession that was thought

to be glyphosate-susceptible. Even though collaborators sent seed in a bag labeled susceptible, whole plant dose response, and shikimate assays pointed to the same conclusion -this accession was in fact segregating for glyphosate resistance. Second, these results complement those presented in chapter 3. Even without a homogeneous glyphosate-susceptible accession, I suggest that an absolute correlation between increased *EPSPS* copy number and resistance exists. After, concluding that some glyphosate-susceptible plants were actually segregating for resistance, *EPSPS* copy number followed the same pattern: two plants out of 16 designated glyphosate-susceptible had increased *EPSPS* copy number. Furthermore, every plant that survived a lethal treatment of glyphosate had increased *EPSPS* copy number. Consistently, 3 relative *EPSPS* copies is the lowest copy number reported for confirmed glyphosate-resistant *K. scoparia* plants, and this observation is consistent with the reasoning laid out in chapter 3.

On a more practical level, this research influences weed management decisions of farmers and land managers. Glyphosate dose response and shikimate assays confirm resistance in *K. scoparia* accessions collected throughout Kansas, Colorado, North Dakota, and Alberta. This information is important because it signals the need for action in these areas. Glyphosate-resistant *K. scoparia* should be contained to protect from further infestation. Lastly, these findings need to be communicated back to the farmers and land managers that deal with glyphosate resistance problems at the ground level.