DISSERTATION

MOLECULAR MECHANISMS OF HERBICIDE RESISTANCE IN RICE AND KOCHIA

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2024

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ABSTRACT

MOLECULAR MECHANISMS OF HERBICIDE RESISTANCE IN RICE AND KOCHIA

Herbicide stress is an important challenge in agriculture and understanding how plants respond to herbicide exposure is crucial for developing effective weed management strategies. Transcription factors (TFs) play a pivotal role in regulating gene expression and mediating plant responses to various environmental stimuli, including herbicide stress.

This dissertation aimed to elucidate the role of TFs in herbicide tolerance and sensitivity across plant species. A brief introduction was provided in Chapter 1. Subsequently, by analyzing transcriptomic data from different studies, we identified key TFs involved in herbicide responses. Our findings in Chapter 2 revealed distinct TF signatures, including bZIP, NAC, WRKY, and ERF, that were consistently upregulated in herbicide-tolerant plants. associated with herbicide tolerance or sensitivity, suggesting potential regulatory mechanisms in metabolic pathways and downstream signaling. These results underscore the importance of complex interplay between herbicide class, treatment duration, and plant species on TF expression patterns.

In Chapter 3, we focused on herbicide resistance in rice, a critical staple crop. Transcriptomic analysis revealed upregulation of key detoxification genes, including glutathione S-transferase (GST) and cytochrome P450 (CYP450), in the NTSR mutant, suggesting their involvement in herbicide metabolism. Functional characterization confirmed increased glutathione S-transferase activity in the NTSR genotype. Additionally, computational studies identified a novel transcription factor, ZOS-1-16, with a potential role in regulating herbicide response. We investigated a novel non-target site resistance (NTSR) mechanism conferred by a mutation in the transcription factor ZOS-1-16. Our findings demonstrated that ZOS-1-16 upregulates genes like GSTs and CYPs involved in herbicide detoxification, leading to increased resistance

to the herbicide quizalofop-p-ethyl (QPE). This study highlights the potential of targeting TFs for developing herbicide-resistant rice varieties. Finally, Chapter 4 explored glyphosate resistance in the invasive species *Bassia scoparia* (kochia). We investigated the inheritance of glyphosate resistance in kochia populations and found that it is primarily due to an increase in the copy number of the EPSPS (5-enolpyruvyl-3-shikimate phosphate synthase) gene. Additionally, we estimated the outcrossing rate of kochia under field conditions and found a high level of outcrossing, which contributes to the rapid spread of glyphosate-resistant biotypes.

Overall, this dissertation provides valuable insights into the role of TFs in herbicide responses and highlights the potential for developing novel strategies to enhance herbicide tolerance and manage herbicide-resistant weeds.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the USDA National Institute of Food and Agriculture, Hatch Projects 7005022/ COL00785A (FED) and COL00783 (TAG), Nutrien Ag Solutions, and RiceTec Inc. for their financial support, which made this research possible.

A tremendous thank you to my committee members, Dr. Todd A. Gaines, Dr. Anireddy Reddy, and Dr. Vipan Kumar, for their encouragement and guidance throughout my PhD journey. I also extend my heartfelt appreciation to Dr. Frank Dayan and Renee Dayan for their consistent support.

I am extremely thankful to the weed lab cohort and my friends in India for their invaluable technical advice and support in various experiments. I would like to thank Crystal Sparks and Naveen BK for their guidance with transcriptomic and in-silico analysis. Special thanks to Caleb Knepper and Emily Luna for their help with rice mutagenesis study and seed germination respectively.

Finally, I owe my deepest gratitude to Naina Gupta, Vijay Kumar Gupta, Smriti, and Pushkar Gupta, who have always been my greatest cheerleaders, offering unwavering support and helping me stay grounded during the most critical moments. My dreams are incomplete without my family back in India, and I am profoundly thankful to each one of them.

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CHAPTER 1: INTRODUCTION

Herbicides have proven vital in worldwide agriculture, considerably boosting weed management and crop output. However, their broad and continued usage has led in the development of herbicide resistance, which poses a serious danger to sustainable agricultural production. Resistance mechanisms can be categorized into target-site and NTSR, with NTSR emerging as an important topic of research. Unlike target-site resistance, which requires mutations at the herbicide's binding site, NTSR is more complex and encompasses modifications in the plant's metabolic pathways, including transcriptional reprogramming and control of gene expression.

The world's most important staple crop, rice (*Oryza sativa*), is especially susceptible to herbicide-resistant weeds such as barnyard grass, jungle rice, weed rice etc. Therefore, studying the molecular mechanisms underlying herbicide resistance in rice may help develop novel approaches to controlling this resistance in other major crops. This thesis focuses on two main areas: first, a thorough meta-analysis of TF responses to herbicide-induced stress; and second, a detailed investigation of the or zinc finger of *Oryza sativa* (*ZOS1-16*) mutation and its function in conferring NTSR in rice.

1.1 Transcription Factors in Herbicide Stress: Insights from a Meta-Analysis

Important regulators of plant responses to abiotic stressors, such as exposure to herbicides, are transcription factors (TFs) (Latchman, 1997a). They control intricate networks of gene expression that allow plants to endure, adjust, and occasionally fend against the effects of herbicides (Singh *et al.*, 2002, Zhang *et al.*, 2022). Significant. Important trends in TF-mediated responses are shown in this context by a meta-analysis of transcriptome data that has already been collected from a variety of plant species exposed to herbicides.

Several research studies on TF expression patterns under herbicide stress are included in this meta-analysis, which sheds light on the important regulatory nodes that control plant survival tactics. Notably, responses to oxidative stress and detoxification pathways are regularly associated with families including

MYB, WRKY, and bZIP; these pathways are important components of herbicide resistance mechanisms. It is believed that these TF families control the expression of downstream genes that are involved in metabolic modifications, cell wall reinforcement, and xenobiotic detoxification.

Moreover, the meta-analysis shows that TF responses are specific to particular herbicides, implying that the mode of action of a herbicide determines which TFs are activated specifically. This research emphasizes how intricate herbicide resistance may be, particularly when plants show cross-resistance to several different herbicide classes. Transcriptional variables, then, offer attractive targets for the creation of innovative herbicide compositions or biotechnological treatments meant to lessen resistance.

1.2 The ZOS1-16 Mutation: A Novel Mechanism of Non-Target Site Herbicide Resistance in Rice

A new mutation in the rice *ZOS1-16* gene that gives non-target-site herbicide resistance is the focus of this thesis, which builds on the knowledge gained from the larger landscape of TF responses. Zinc finger transcription factor ZOS1-16 has been linked to plant stress responses in the past (refs), but its direct involvement in herbicide resistance has not yet been thoroughly investigated. Experimental investigations revealed that the *zos1-16* mutation had no effect on the herbicide's major target location ACCase. It increases the plant's resistance to oxidative damage caused by herbicides by altering the expression of genes involved in metabolic detoxification pathways. This mutation sets off a series of defense responses, including alterations in cellular redox states, the activation of xenobiotic transporters, and the overexpression of antioxidant enzymes. Together, these reactions lessen the buildup of harmful reactive oxygen species (ROS), which are usually produced when exposed to herbicides.

Our knowledge of NTSR has advanced with the identification of the *ZOS1-16* mutation. In contrast to conventional target-site mutations, which directly disrupt herbicide binding, *ZOS1-16* modifies more extensive stress response pathways, providing a more widespread type of resistance that may enable cross-resistance to several herbicides. In addition to advancing our understanding of the mechanisms underlying herbicide resistance, this discovery creates opportunities for the development of herbicide-resistant rice

cultivars independent of genetic alterations that impact major target sites.

There are still a number of unresolved issues despite tremendous progress in our knowledge of the function of TFs in plant stress responses, including the following:

- Identifying novel TFs involved in herbicide stress responses.
- Understanding the molecular mechanisms by which herbicides activate TFs.
- Investigating the interactions between TFs and other signaling molecules involved in herbicide stress responses.
- Exploring the potential of manipulating TF expression to improve herbicide tolerance or resistance in crops.
- Developing novel herbicide control strategies based on our understanding of TF-mediated stress responses.

By addressing these questions, we can gain a deeper understanding of the complex interplay between herbicides and plant regulatory networks, leading to the development of more sust ainable and effective weed management practices.

1.3 Growing Threat of Glyphosate-Resistant Kochia: The Genetic Basis of Glyphosate Resistance in *Bassia scoparia*

A common broad-spectrum herbicide in agriculture, glyphosate has proven to be quite successful at suppressing weeds. Crop productivity is seriously threatened by glyphosate-resistant weeds, which have evolved as a result of the herbicide's extensive use. The glyphosate-resistant weed *Bassia scoparia*, also referred to as kochia, is one of the most troublesome. An extremely versatile and invasive species, Kochia may spread quickly and compete with crops for resources. Glyphosate-resistant Kochia has become more challenging to manage, increasing crop losses and raising weed management expenses. Developing ways to lessen the impact of glyphosate resistance on agriculture requires an understanding of the genetic basis of this resistance in Kochia.

One prevalent mechanism of herbicide resistance is gene amplification (refs). Herbicide resistance can

be conferred via gene amplification, which increases the expression of a gene involved in herbicide detoxification or target site modification. Numerous studies have demonstrated that one of the main mechanisms of glyphosate resistance in a variety of weed species, including Kochia, is gene amplification. The *EPSPS* gene, which codes for the glyphosate-targeting enzyme 5-enolpyruvylshikimate-3-phosphate synthase, is usually the amplified gene in glyphosate-resistant Kochia.
Both genetic and environmental factors can have an impact on the complicated inheritance of glyphosate resistance in Kochia. Predicting the spread and persistence of resistant biotypes requires an understanding of the glyphosate resistance inheritance pattern. Glyphosate resistance in Kochia is frequently inherited as a dominant feature, according to earlier research (refs). This indicates that resistance can be conferred with just one copy of the amplified?? resistance gene. However, other elements including the plant's genetic heritage and recombination in the duplicated gene region can also impact inheritance pattern.
The spread of glyphosate resistance in Kochia can be influenced by outcrossing, which is the interchange of genetic material between various individuals of the same species. Outcrossing can produce new resistant biotypes that might be more tolerant of various environmental circumstances by introducing resistance genes into novel genetic backgrounds. A number of variables, including the density of the plant population, the presence of reproductive barriers, and the availability of pollinators, might affect the pace at which Kochia outcross. Within a Kochia population, glyphosate resistance can spread quickly if there is a high outcrossing rate.

The thesis is organized into three main chapters:

- Chapter 2 presents the results of the meta-analysis of transcription factor responses to herbicide stress in various plant species.
- Chapter 3 focuses on the characterization of the ZOS1-16 mutation in rice and its role in non-target-site herbicide resistance.
- Chapter 4 discusses the broader implications of these findings, including their relevance to glyphosate resistance in *Bassia scoparia* (kochia), and proposes future research directions for integrated weed management.

CHAPTER 2: RNA-SEQ META ANALYSIS UNVEILS PLANT TRANSCRIPTION FACTOR RESPONSES TO HERBICIDE STRESS

OVERVIEW

Transcription factor responses to herbicide stress are poorly understood. Transcriptomic data of several available were analyzed to identify key transcription factors (TFs) responding to herbicide exposure across plant species. Our findings reveal cues on TF signatures linked to herbicide tolerance or sensitivity, suggesting potential control points in metabolic pathways and downstream signaling. This work highlights the need for properly designed experiments to fully explore the interplay between herbicide class, treatment duration, and plant species on TF expression patterns.

2.1 CHAPTER INTRODUCTION

Weeds pose a difficult challenge to agricultural productivity (Basu and Rao, 2020). Their competition for light, water, and nutrients can reduce crop quality and yield, while also increasing harvesting costs (Maqsood *et al.*, 2020) This threat arises from both native weed populations and invasive plant species introduced into new environments (Oerke and Dehne, 2004). While the mechanisms of action of herbicides are well-documented, their interaction with broader plant signaling pathways remains unclear. Notably, herbicides not only disrupt physiological and metabolic processes, but also trigger a cascade of secondary stress responses that alter the expression of a myriad of genes.

TFs are key regulators of plant physiology, controlling gene expression (Meshi and Iwabuchi, 1995, Jores *et al.*, 2023). Plants rely on TFs to orchestrate a multitude of processes, including responses to stress, growth, and development (Yanagisawa, 1998). While the relationship between herbicide application and TF expression is unclear, TF-mediated pathways likely contribute to the plant's stress response. Understanding these interactions could advance knowledge of plant regulatory mechanisms, particularly TF-DNA interactions. TFs are proteins that bind specific DNA sequences in target gene promoters, regulating transcription initiation. A typical TF has two domains: a DNA-binding domain (DBD) for high-affinity binding and a transactivation domain (TAD) for initiating transcription (Fig. 1A). Some TFs have

a combined DBD-TAD domain. Additionally, nuclear localization signals (NLS) ensure proper targeting to the nucleus. Notably, DBDs are crucial for plant TFs, enabling specific promoter recognition and transcription initiation. These domains also facilitate DNA site recognition and mediate interactions with other proteins.

Plants encode a remarkably diverse repertoire of TF families, exceeding that of any other eukaryotic lineages, likely due to lineage-specific expansions (Latchman, 1997b). To respond to various kinds of biotic and abiotic stresses, plants have 58 TF families (Ng *et al.*, 2018). The plant TF database (https://planttfdb.gao-lab.org/) has an extensive collection of TF families expressed in 165 species with high annotation and regulatory mapping analysis (Jin *et al.*, 2016, Tian *et al.*, 2019). Microarray analyses have revealed six major TF families – basic helix-loop-helix (bHLH), ethylene-responsive element binding factors (ERF), WRKY, basic-leucine zipper (bZIP), myeloblastosis (MYB), and cup shaped cotyledon (CUC2)/NAC – that are prominently upregulated under stress conditions (Singh *et al.*, 2002, Seo *et al.*, 2015, Jin *et al.*, 2016). Altered expression of these TF families triggers a cascade of responses, including signal transduction pathways, phytohormone activation, and the deployment of defense mechanisms.

This meta-analysis of RNA-sequencing (RNA-Seq) aims to identify trends in the recruitment of TF families upon exposure to different herbicides. Deciphering TF-herbicide interactions will increase our understanding of plant responses to herbicide stress and open exciting new possibilities toward the development of novel tools for weed management.

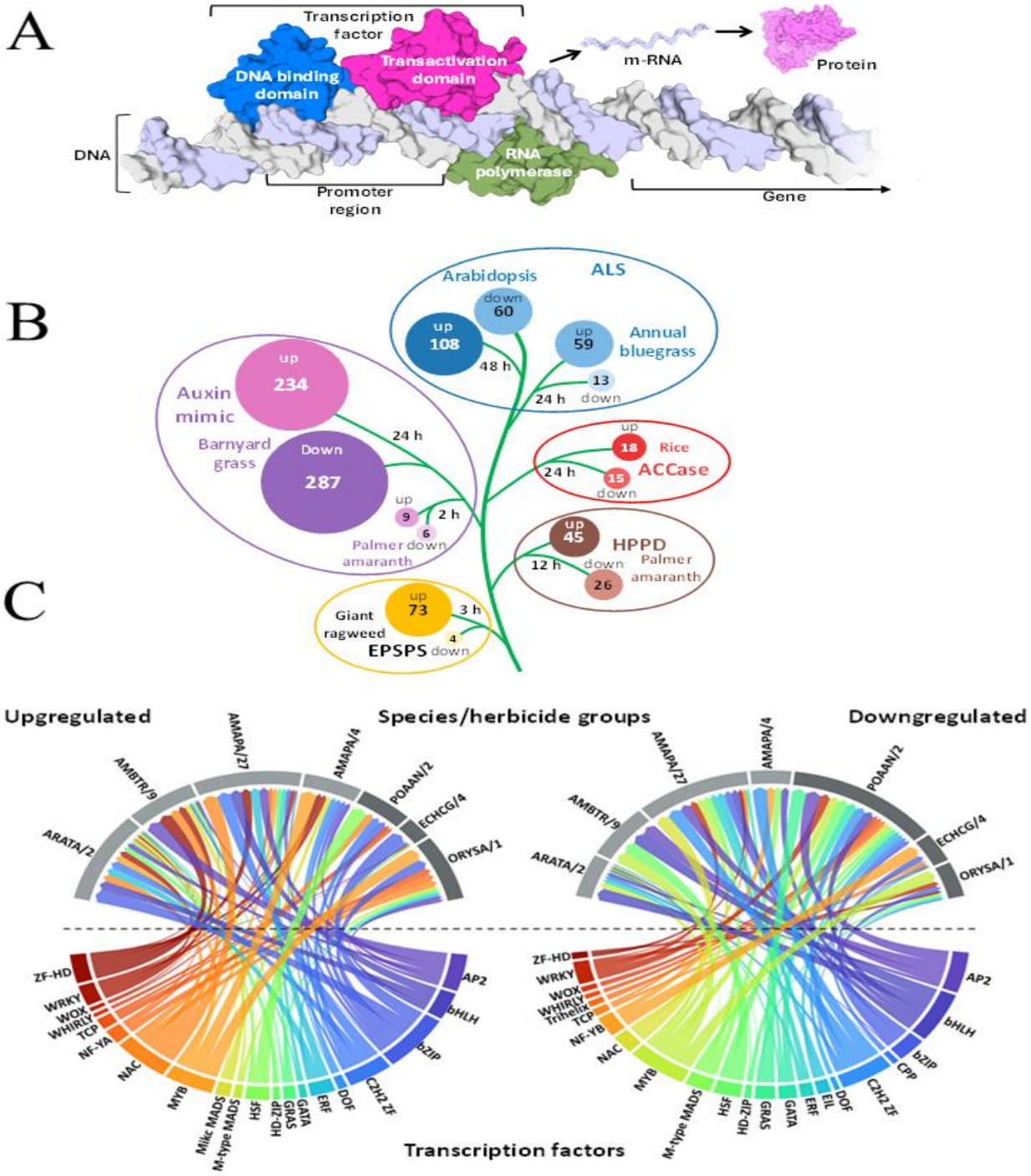

Figure 2.1. An illustration of TF control of gene expression

(A). The DNA binding and transactivation domains of TF are shown in blue and pink, respectively, during the interaction with the promoter region of a gene. This drives the activity of RNA polymerase (shown in green), resulting in the transcription of the DNA into m-RNA and translation into protein synthesis. (B) Summary of changes in transcription factor gene expression in plant species exposed to herbicides with different modes of action over different lengths of time. (C). Up and down-regulated TF families in plants treated with herbicides. The colors for the species are light gray for dicot and dark gray for monocot

2.2 MATERIALS AND METHODS

2.2.1 Plant culture, treatment and RNA sequencing (RNA-Seq)

RNA-sequencing dataset from six plant species viz. rice *(Oryza sativa L.* subsp. japonica), annual bluegrass (*Poa annua* L.), Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), Palmer amaranth (*Amaranthus palmeri S.* Watson), barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.), and giant ragweed (*Ambrosia trifida* L.) were used. RNA-seq data of rice was generated by us. Briefly, three-week-old rice plants were treated with quizalofop-p-ethyl at a field rate of 77 g ha^{-1}. A single leaf was collected 24 HAT for RNA-seq analysis (flash-frozen in liquid N2). Total RNA was extracted using Trizol reagent. RNA quality was assessed using a Bioanalyzer 2100 (RIN number > 7.0). Library construction and sequencing were performed by LC Sciences in collaboration with RiceTec, Inc. RNA was fragmented under elevated temperatures, and divalent cations were used to fragment poly(A) or poly(A)+ RNA fractions. These fragments were reverse transcribed to create a cDNA library. The transcriptome assembly was annotated using Nipponbare as a reference genome from the Rice Genome Annotation Project (https://www.weedgenomics.org/).

Laforest et al. (Laforest *et al.*, 2021) treated a susceptible annual bluegrass biotype with trifloxysulfuron at a label rate of 27.8 g ha^{-1} and RNA was collected at 0 and 12 h HAT. Sequencing and library preparation was performed at University of Texas-Austin genomic sequencing and analysis support facility. Sequences were aligned to the reference assembly of annual bluegrass transcriptome GenBank accession GCZY01000000 (Laforest *et al.*, 2021).

RNA-seq data of Arabidopsis plants treated with imazethapyr at a rate of 10.6 g ha^{-1} and samples were collected 48 HAT (RNA-seq library preparation and analysis performed earlier (Markus *et al.*, 2021). The libraires were mapped to reference genome Arabidopsis TAIR10 (Lamesch *et al.*, 2012).

RNA-seq data of Palmer amaranth was generated from F2 generations of sensitive populations collected from Nebraska, treated with 77 g a.i. ha^{-1} tembotrione (Küpper *et al.*, 2018). The library were annotated to reference genome (Montgomery *et al.*, 2020). The RNA-seq data is available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under accession number GSE243128.

RNA-seq data of barnyardgrass was collected from 3-4 leaf stages of the vale verde-01 biotype treated with florpyrauxifen at rate 30 g ha^{-1} (data unpublished). The authors performed the RNA sequencing at Novogene and analysis was performed using the reference genome report earlier. (Wu *et al.*, 2022)

RNA-seq data of giant ragweed was obtained from mature leaves of plants from Ontario, Canada (Sparks, 2024). The leaves were treated with Roundup PowerMAX® at a dose of 0.84 kg ae ha^{-1} (mRNA library preparations and sequencing performed by Fasteris DNA sequencing services, Geneva, Switzerland). The authors used the reference genome from the Internal Weed Genomics Consortium (IWGC, https://www.weedgenomics.org/) for annotation.

Transcription factor expression values were normalized to a scale of 0 to 1 and chord diagrams were generated using the following libraries (tidyverse, viridis, patchwork, hrbrthemes, circlize and chorddiag) in RStudio (2024.04.2 Build 764). Within the chord diagram, TF family names were arranged alphabetically, and the width of each chord represents the relative abundance of differentially expressed TFs within each family.

2.2.2 Transcription factors identification

Transcription factors (TFs) expression with log2 fold change (FC) greater than 2 and -2 were considered upregulated and downregulated, respectively (Feng *et al.*, 2012). Putative TF classification was determined from BLAST searches (Mount, 2007) against a curated weed genomics plant transcription factor database (https://www.weedgenomics.org/) (Montgomery *et al.*, 2024).

2.2.3 Normalization and chord diagram generation

Transcription factor expression values were normalized to a scale of 0 to 1 and chord diagrams were generated using the following libraries (tidyverse, viridis, patchwork, hrbrthemes, circlize and chorddiag) in RStudio (2024.04.2 Build 764). Within the chord diagram, TF family names were arranged alphabetically, and the width of each chord represents the relative abundance of differentially expressed TFs within each family.

2.2.4 Data availability

The RNA-seq data is available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) for Palmer amaranth under accession number GSE243128 and annual bluegrass under accession number GCZY01000000. Data for rice species can be provided by us upon request. Data for other species can be obtained upon request from the corresponding authors of the referenced studies.

2.3 RESULTS AND DISCUSSION

Engineering TFs, such as WRKY, bHLH, NAC, and ERF, has emerged as a promising strategy for enhancing plant tolerance to abiotic stresses (Rabara *et al.*, 2014). Studies in rice (*Oryza sativa* japonica), Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), and barnyardgrass (*Echinochloa crus-galli* (L.) P. Beauv.) have demonstrated the induction of MYB, bHLH, and bZIP TFs upon the abiotic stress caused by herbicide exposure (Rabara *et al.*, 2014). In a recent study, it was observed that the up-regulation of the bZIP88 transcription factor is associated with resistance to multiple herbicides, including ALS inhibitors, ACCase inhibitors, and auxin-like herbicides, in the japonica rice species, *Echinochloa crus-galli* and *E. glabrescens* (Zhang *et al.*, 2022).

A comprehensive overview of major plant TF families and their associated DNA-binding domains and their biological function is provided in Supplementary Table 1. Since our aim was to identify trends in TF expression patterns associated with herbicide treatment, we conducted a meta-analysis of these datasets, investigating the relationships between differentially expressed genes and associated biological processes targeted by herbicides (Table 2.1).

Table 2.1. Species information and treatment used in the study:

Species	Code[a]	Herbicide[b]	Group/target[c]	Ploidy (n)	Time[d]
Monocot					
Annual bluegrass	POAAN	Trifloxysulfuron	2/ALS	Tetraploid n=14	24

Barnyardgrass	ECHGH	Florpyrauxifen	4/Auxin	Hexaploid n=27	24
Rice	ORYSA	Quizalofop-p-ethyl	1/ACCASE	Diploid n=12	24
Dicot					
Arabidopsis	ARATA	Imazethapyr	2/ALS	Diploid n=5	48
Giant ragweed	AMBTR	Glyphosate	9/EPSPS	Diploid n=12	3
Palmer amaranth	AMAPA	Tembotrione	27/HPPD	Diploid n =17	12
Palmer amaranth	AMAPA	Dicamba	4/Auxin	Diploid n =17	2

[a]European and Mediterranean Plant Protection Organization (EPPO) plant code

[b]Common name of active ingredient

[c]Herbicide Resistance Action Committee herbicide group followed by mode of action. ALS, acetolactate synthase; HPPD, hydroxyphenylpyruvate dioxygenase; ACCase, acetyl-CoA carboxylase; EPSPS, enolpyruvyl shikimate phosphate synthase; Auxin, auxin-mimic herbicides.

[d]Time after herbicide application when RNA was collected.

As would be expected, herbicides alter TF expression in treated plants. However, the types of herbicides, the timing of application, the ploidy of species tested and the time after treatment when RNA was collected affect the TF response to different degrees (Fig. 2.1B).

At a high level, the number of differentially expressed TF genes is dependent on time of exposure. Auxin mimic herbicides (group 4 - deregulation of the auxin response pathway) triggered a much greater change in barnyardgrass after 24 h than in Palmer amaranth (*Amaranthus palmeri* S. Watson) after 2 h. Similarly, a more pronounced change in TF expression was observed in arabidopsis treated with herbicides targeting branched chain amino acid synthesis (group 2 - acetolactate synthase or ALS) after 48 h than in annual bluegrass (*Poa Annua* L.) after 24 h exposure (Fig. 1B). As well, the type of herbicide applied can trigger different degrees of change in TF expression. For example, after 24 h, herbicides disrupting fatty acid synthesis (group 1 – acetyl-CoA carboxylase or ACCase) induced a small change in TF expression in

rice compared to a larger change in in response to an inhibitor of branched chain amino acid synthesis (ALS) caused more change in annual bluegrass, and altering the functions of auxins in barnyardgrass caused the strongest TF response (Fig. 1B). Consequently, it is not advisable to compare RNA-Seq datasets from studies that either use different herbicides or different time of exposure. This is because the plant's response to herbicides is rapid, usually occurring within 2 h of application. Moreover, extended exposure to herbicides causes the accumulation of secondary signals and reactive oxygen species (ROS), subsequently resulting in increased gene expression. For example, Palmer amaranth treated with an inhibitor of plastoquinone synthesis (group 27 – hydroxyphenylpyruvate dioxygenase (HPPD)) for 12 h had fewer TF families responding than giant ragweed (*Ambrosia trifida* L.) exposed to glyphosate (group 9 - enolpyruvyl shikimate phosphate synthase (EPSPS)) for 3 h (Fig. 1B).

Finally, the ploidy of the species also adds a layer of complexity because polyploid species will have several homeologs of each of the TF. The number of TF families varied significantly when comparing hexaploid barnyardgrass, tetraploid annual bluegrass, and diploid rice at a constant exposure of 24 h. As the ploidy increased, there was a corresponding increase in the expression response of TFs. It is also likely that the smaller change in TF expression between plants treated with an ALS inhibitor may be affected by the ploidy between the tetraploid annual bluegrass and the diploid arabidopsis (Fig. 1B). Other factors affecting plant response to herbicides and the subsequent pattern of TF expression may include whether the species were monocot or dicot or whether they have C3 or C4 forms of carbon assimilation.

At a more granular level, comparative RNA-Seq analysis revealed a distinct expression pattern of different TF families in response to herbicide stress. All six prominent TF families responding to abiotic stresses also responded to the herbicide treatments (Singh *et al.*, 2002, Seo *et al.*, 2015, Jin *et al.*, 2016). The most upregulated TF families were, in decreasing order, bZIP, NAC, C2H2 ZF, MYB, AP2, bHLH, ZF-HD, WRKY and ERF, whereas the most downregulated TF families were bHLH, MYB, AP2, C2H2 ZF, WRKY, bZIP, NF-YB, NAC, HSF and M-type MADS (Fig. 1C). Most of these TF families had members that were either upregulated or downregulated in response to herbicides across analyses (Fig 1C). This functional heterogeneity within these families highlights the complex interplay between different TF

families and their response to herbicides.

The bZIP family, typically involved in plant stress responses such as drought and wounding, was downregulated after herbicide treatment, suggesting a suppression of stress pathways and increased plant susceptibility. Conversely, the ERF family, associated with oxidative stress and drought responses, was upregulated, indicating an ethylene-mediated defense response. The ZF-HD family, related to root development and auxin signaling, also showed altered expression, suggesting a role in maintaining root function under herbicide stress. Collectively, the upregulation of these transcription factor families (bZIP, ERF, NAC, WRKY) suggests their involvement in activating stress signaling pathways, possibly through interactions with the mitogen activated protein kinase (MAPK) cascade, cytosolic calcium changes, or stress and hormonal signaling crosstalk, highlighting potential overlaps in abiotic stress and herbicide response mechanisms. The bZIP family, known for its involvement in plant responses to various stresses like drought and wounding (Yu *et al.*, 2020), was downregulated upon herbicide treatment. This downregulation suggests a potential suppression of stress response pathways, potentially increasing plant susceptibility to the herbicide. Conversely, the ERF family, associated with responses to oxidative stress and drought (Ma *et al.*, 2024), exhibited upregulation. This upregulation could be part of an ethylene-mediated defense response triggered by the herbicide, as ERF plays a role in the ethylene signaling pathway (Guo and Ecker, 2004). Interestingly, the ZF-HD family, involved in root development and auxin signaling (Niu *et al.*, 2021), showed altered expression in response to herbicides. While its precise role remains unclear, its known function in root development suggests a potential involvement in maintaining root function under herbicide stress (Chandra and Leon, 2022).

These families, including bZIP, ERF, NAC, and WRKY, are well-known for their roles in plant responses to various stresses such as drought (Rabara *et al.*, 2014), salinity (Singh *et al.*, 2002), and pathogens (Niu *et al.*, 2021). Their upregulation in response to herbicides suggests their potential involvement in activating stress signaling pathways within the plant (Yanagisawa, 1998). This activation could be mediated through interactions with the MAPK cascade (He *et al.*, 2020), changes in cytosolic

calcium levels, or crosstalk between stress and hormonal signaling pathways (Ku *et al.*, 2018). Notably, all these TF families have been previously studied in abiotic stress responses, suggesting potential overlap in the signaling mechanisms triggered by herbicides (Fig. 2.1B).

While this study provides some insights into the influence of different herbicide classes, treatment duration, and plant species on TF expression patterns, it also exposes the limitations of relying on data derived from different experimental designs. In this case, the divergence in plant species treated with different herbicides and variable length of exposure prevents us from fully capturing the interplay between herbicides and their effects on plant TF. Future studies employing a single plant species grown under identical conditions, treated with various herbicide groups at the same stage of growth, at standardized time points, along with consistent RNA-seq sequencing depths all the treatments, would be crucial for robust comparative analyses. Alternatively, one could also test one herbicide on a number of different plants species but processed in an identical manner. Another factor to consider is the primary response to the presence of herbicide by collecting RNA within the first few hours. If the collection is delayed beyond that time frame, TF will begin responding to the injury caused by the herbicide. By overcoming the experimental limitations currently existing by using dataset from different and uncoordinated studies, it will be possible to dissect the TF network responding to herbicide stress and compare gene regulation patterns across plant subcategories. This approach will provide deeper understanding of the interplay between herbicide mode of action, plant characteristics, and gene expression, ultimately paving the way for the development of more effective and targeted weed control strategies.

2.4 CHAPTER CONCLUSION

This transcriptomic meta-analysis reveals key insights into the role of transcription factors (TFs) in plant responses to herbicide stress. We identified changes in the expression of major TF families, such as bZIP, NAC, WRKY, and ERF, which are involved in plant stress responses. These families were either upregulated or downregulated depending on the type of herbicide, treatment duration, and species. For example, bZIP was often downregulated, indicating suppression of certain stress pathways, while ERF

upregulation suggested activation of ethylene-mediated defense mechanisms. The variability in TF expression across different studies highlights the complexity of herbicide-induced transcriptomic responses. Factors like herbicide mode of action, dosage, ploidy levels, and sampling times influence TF activity. Extended herbicide exposure triggers secondary stress responses, such as reactive oxygen species (ROS) production, complicating the interpretation of TF-mediated responses. Thus, transcriptomic changes following herbicide application cannot be easily generalized across species or treatments without accounting for these variables. Future studies should standardize experimental conditions, including uniform species and treatment protocols, to enable more robust comparisons of herbicide responses. Focusing on early transcriptional changes could offer a clearer view of primary TF-mediated responses before secondary stress signals interfere. This approach will enhance our understanding of TF networks involved in herbicide tolerance or sensitivity, potentially guiding the development of crops with improved herbicide resistance and more effective weed management strategies.

CHAPTER 3: MUTATION IN TRANSCRIPTION FACTOR IMPARTS TOLERANCE TO QUIZALOFOP IN RICE

3.1 CHAPTER INTRODUCTION

Rice, one of the most important staple grain in the world, sustains billions and provides income for millions of farmers cultivating over 165 million hectares (Akbar et al., 2011). Sustaining rice productivity is an important component of global food security. However, numerous weed species like bigpod sesbania (*Sesbania exaltata*), blue mudplantin (*Heteranthera limosa*) barnyardgrass (*Echinochloa crus-galli*), and red rice (*Oryza* spp.) (Roma-Burgos et al., 2021), hinder rice productivity and degrade seed quality (Smith, 1968, Qiong et al., 2019, Ding et al., 2023). Weed competition can result in yield losses of 30–40% (Dawson, 1964, Burgos et al., 2014).While farmers heavily rely on pesticides (with herbicides accounting for 40% of the $6 billion global rice pesticide market) to control weeds, repeated use leads to herbicide resistance in weeds, jeopardizing yields. . Genetic engineering, coupled with plant breeding, has revolutionized rice production, increasing yields by over 50% and generating disease-resistant varieties (Hanafiah et al., 2020). This approach holds immense potential for addressing the weed challenge. One promising approach is herbicide resistant (HR) rice varieties (Singh et al., 2013, Osei et al., 2014, Varshney et al., 2018, Hanafiah et al., 2020).

QPE, a graminicide herbicide targeting acetyl-coA carboxylase (ACCase), exemplifies this approach. Although rice is typically QPE-sensitive, HR-rice varieties like MAX-ACE can tolerate post-emergence application at rates ranging from 100 to 138 g ai ha-1 (Lancaster et al., 2018). This selectivity stems from a target-site mutation (TSR) within the ACCase CT domain, most commonly the Ile1781Leu substitution, conferring high-level resistance (Amaro-Blanco et al., 2021). ACCase is a key enzyme that converts acetyl-CoA into malonyl-CoA in fatty acid biosynthesis (Sasaki and Nagano, 2004). Fatty acids are crucial for various cellular functions, including cell membrane formation and energy storage (Rajasekharan and Nachiappan, 2010). Mutations in the ACCase gene can render the enzyme less

susceptible to binding by certain herbicides. These herbicides act as an ACCase inhibitor, work by blocking ACCase activity, thereby disrupting fatty acid production and ultimately killing the plant. Rice breeders can leverage this by developing rice varieties with specific mutations that confer resistance to ACCase-inhibiting herbicides. This allows for targeted weed control in rice fields without harming the rice crop itself. However, overuse of such herbicides can lead to the evolution of resistance in weeds, posing a challenge for weed management. Therefore, the long-term sustainability of TSR-based HR rice is a concern. Furthermore, weeds can evolve resistance mechanisms beyond TSR, such as non-target site resistance (NTSR) involving enhanced herbicide metabolism. NTSR offers an alternative resistance pathway for weeds. Unlike TSR, it doesn't involve changes in the herbicide's target site (ACCase). Instead, NTSR employs other mechanisms that include enhancing the activity of genes involved in herbicide metabolism. Interestingly, mutations in transcription factors (TF) can also disrupt this control, leading to abnormal regulation of genes involved in herbicide metabolism. TF are proteins that act like molecular switches, binding to DNA near genes and controlling their activity by turning the genes 'on' or 'off' (Pan et al., 2010). In the case of NTSR, a mutation in a TF could increase the activity of genes responsible for herbicide breakdown, essentially flipping the 'off' switch to 'on'. This enhanced breakdown grants the plant a level of resistance to the herbicide. By understanding how TF mutations influence metabolic genes, we can develop more effective and sustainable weed control strategies for rice. Understanding this novel NTSR mechanism is crucial for developing more sustainable weed control strategies in rice. Herein, we address this gap by investigating a novel, unrelated mutation identified during the mutagenesis screen for QPE resistance, employing DNA Affinity Purification coupled with Sequencing (DAP-seq) to identify its interaction partners.

During a mutagenesis screen aimed at identifying TSR mutations in acetyl-CoA carboxylase (ACCase) conferring QPE tolerance, we serendipitously discovered an additional, unlinked mutation that also conferred a moderate level of QPE resistance in *Oryza sativa Japonica* rice. This mutation, mapped to a transcription factor (ZOS-1-16) on chromosome 1, appears to confer moderate QPE resistance through an

NTSR mechanism. We investigated the role of ZOS-1-16, a member of the GRAS family of transcription factors, in NTSR to QPE. The GRAS family, named after its founding members Gibberellin acid insensitive (GAI), Repressor of GA1 (RGA), and SCARECROW, plays a crucial role in various biological processes including gibberellic acid (GA) signaling, plant growth and development, and responses to abiotic stresses (Ikeda et al., 2001, Zanetti et al., 2002, Guo et al., 2019). Our findings demonstrate that the ZOS-1-16 mutation enhances the expression of genes typically associated with NTSR, such as glutathione S-transferases (GSTs) and cytochrome P450 monooxygenases (P450s). This upregulation of metabolic enzymes leads to increased QPE degradation, contributing to the observed resistance phenotype. Functional validation experiments confirmed that inhibiting these metabolic enzymes abolished the QPE resistance conferred by the ZOS-1-16 mutation. These findings establish ZOS-1-16 as a novel regulator of NTSR in rice, offering valuable insights for developing more durable herbicide resistance strategies.

3.2 MATERIALS AND METHODS

3.2.1 Source of plant material

Four rice genotypes – NTI (wildtype), TSR (target site mutant), NTSR (non-target site mutant) and TSR+NTSR (target site + non target site mutant) were obtained from RiceTec, Inc. (Bernacchi et al., 2018). The mutagenized lines were developed by exposing 10,000 rice seeds in a 1 L flask to 325 mL of 1 mM sodium azide in sodium phosphate buffer (pH 3.5) for 3 h at room temperature. After rinsing thrice with 400 mL ultrapure water, seeds were then soaked in ultrapure water overnight. These seeds were then treated with 400 mL of 15 mM N-methyl-N-nitrosourea (MNU) and again incubated for 3 h at room temperature with occasional mixing. Lines with resistance to quizalop-p ethyl were identified by spraying 77 g ha-1 HighcardTM quizalofop-p ethyl over seedlings grown in small field plots.

3.2.2 Growth conditions for greenhouse studies

A soil mixture was prepared by mixing 1:1 pro-mix 'BX' mycorise and fritted clay and was used to fill 18-insert flats. The soil mixture was watered to saturation before planting 1 seed per insert. The soil was kept moist until seedling is established. Plants were fertilized twice a week with Peters Excel 13-2-13 granular fertilizer at 300 ppm N and were treated with iron chelate micronutrient (SPRINT 330, BASF Corporation, Florham Park, NJ) 3-week post cotyledon emergence. All the plants were maintained at about 25±2°C with 60% humidity. Trays were covered with a plastic dome to maintain high humidity until plants were at 2nd leaf stage. Plants were treated with quizalofop-p ethyl when at their 4th leaf stage.

3.2.3 Dose-response experiments

All four rice genotypes NTI, TSR, NTSR and TSR+NTSR were sprayed with doses of quizalofop-p ethyl ranging from 4.81 to 2,464 g ai ha-1 using a single nozzle overhead track sprayer (Generation III Research Sprayer: DeVries, Hollandale, MN, USA) with XRTEEJET nozzle with a spray volume of 180 L ha-1. All spray solutions included 0.25% non-ionic surfactant. Injury symptoms were recorded after every 6 d, and fresh weight was measured 28 DAT to graph a dose response curve. Plant samples were kept in the oven for 48 h and weighed again. A full dose response is plotted using the fresh weight to the dose applied. A dose response was conducted with R studio (version 2022.07.2 Build 576) with the drc module (3.0-1). To model the effect of herbicide on plant growth, fresh weight data was first normalized to percent of control means. A three-parameter log-logistic regression was fit using the LL.3 [drc] function.

3.2.4 Herbicide metabolism

Rice lines were treated with 77 g ai ha-1 (field rate) of quizalofop-p ethyl. Areal portions of the rice plants (including meristems) were collected 2, 4 and 8 d after treatment (DAT). Three replicates of each dose were taken and weighed to normalize the tissue for all replicates. These leaves were washed in

20% acetone v/v in water twice in a 50 mL falcon tube to remove unabsorbed quizalofop-p ethyl and were patted dry. Samples were kept at -80°C until extraction and analysis.

To determine the effect of the GST inhibitor, all varieties were treated with 0.25% of 4-chloro-7-nitrobenzoxadiazole (NBD) dissolved in dimethyl sulfoxide 2 d before spraying the plants with quizalofop. A full dose response is conducted by spraying all the doses from 1/16X to 8X along with the application of NBD at 7 d intervals.

3.2.5 Quizalofop-p-ethyl extraction

Quizalofop-p ethyl extraction was adapted from the QuEChERS protocol (Sack et al., 2015). Rice leaves (approx. 2.5 g) were powderized in liquid N2 and homogenized in 20 mL extraction solvent (1:1 water and acetonitrile) for 30 s (Power Gen 125, Fischer Scientific, Waltham, MA). The extracts were shaken at 45°C for 20 min at room temperature and centrifuged for 10 min at 4,700 g (Sorvall Legend X1R (Thermo Scientific, Waltham, MA). Supernatants were transferred to fresh tubes, 4 g of QuEChERS salt was added to the supernatants, and the tubes were vortexed for 15-30 s. Clean extracts obtained by centrifugation were filtered through 0.2 µm PTFE filter into LCMS vials. These extracts were then analyzed using LC-MS/MS.

3.2.6 LC-MS/MS analysis

Quizalofop acid was determined by LC-MS/MS using a Nexera X2 UPLC system (Shimadzu, Columbia, MD) equipped with dual LC-30AD pumps, a SIL-30AC MP autosampler, a DGU-20A5 Prominence degasser, a CTO-30A column oven, and SPD-M30A diode array detector coupled to an 8040 quadrupole mass-spectrometer (Shimadzu, Columbia MD). For positive-ion mode MRM detection of quizalofop acid (m/z 345 – 299) , the dwell time was 100 ms with Q1 and Q3 pre-bias of -24.0 V and –21.0 V respectively and a collision energy of -21.0 V. Chromatographic separation was achieved on a

Kinetix 2.6 µm biphenyl column (100 × 4.6 mm; Phenomenex, Torrance, CA) maintained at 40°C. Mobile phases A and B were 0.1% formic acid in water methanol, respectively. A linear gradient from 5% to 95% B over 10 min at a flow rate of 0.4 mL min^{-1} was employed. Injection volume was 10 µL.

3.2.7 Glutathione *S*-Transferase assay

Rice leaves were flash-frozen in liquid N_2 and ground to a fine powder. The resulting powder was extracted with 10 mL extraction buffer (50 mM Tris-HCl, containing 20 mM β-mercaptoethanol, 0.1% isoascorbic acid and 10% polyvinylpyrrolidone, pH 7.8) (Chen *et al.*, 2011). After centrifugation at 12,000 g × g for 20 min at 4°C, soluble proteins were precipitated with ammonium sulfate (30-80% saturation). The precipitate was collected by ultracentrifugation at 12,000 × g for 20 min at 4 °C (L-70, Beckman Coulter, Brea CA). The pellet was resuspended in 2.5 mL of extraction buffer with 20% glycerol. Desalting is performed using spin desalting columns (Zeba™, Thermo Fischer Scientific, Waltham, MA) and protein concentrations was determined using bovine serum albumin as a standard (Bio-Rad, Hercules, CA).

GST activity was measured using a commercially available kit (MAK453, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions (Burns *et al.*, 2017). Briefly, the reaction mixture contained 980 µL of Dulbecco's phosphate buffered saline (DBPS, 980 µL), reduced L-glutathione (10 µL), and 1-chloro-2,4-dinitrobenzene (CDNB, 10 µL) with protein concentrations ranging from 5 to 20 µL. A GST standard (MilliporeSigma, St. Louis MO) was included as a positive control. The reaction was monitored at 340 nm for 10 mins at 30 °C on a microplate reader (Synergy H1, Agilent Technologies Inc., Santa Clara, CA).

3.2.8 Homology modelling of transcription factor ZOS1-16

The three-dimensional (3D) structure of wild-type ZOS1 from Nipponbare (LOC_Os01g62460) was modelled using AlphaFold web server (Jumper *et al.*, 2021, Varadi *et al.*, 2022) . The full-length

sequence (820 amino acids) was submitted, and AlphaFold identified 6KPD, 6KPB, 5B3H, 5B3G, and 5HYZ as primary templates for modeling. Due to the lack of structural templates in databases for the first 440 residues, only the region from amino acid 440 to 820 was considered for further analysis, as this region exhibited sequence homology with the identified templates based on AlphaFold's multiple sequence alignment report (Supplementary Figure). A 16-bp optimized nucleotide sequence (AAAACTGAAAGGGAGA), representing the SCL9 protein binding site, was used for docking studies alongside the wild-type and mutant ZOS1 protein structures (Hess *et al.*, 2008). Protein-nucleotide complex conformations were generated using the NPDOCK and HDOCK web servers (Yan *et al.*, 2017).

3.2.11 Total RNA isolation and qPCR

Total RNA was extracted from leaf tissues 24 h after quizalofop-p ethyl treatment using TRIzol reagent (Invitrogen, Waltham, MA) and subjected to DNase I (Promega, Madison, WI) digestion to eliminate genomic DNA contamination. cDNA synthesis was synthesized from 1 µg DNase-treated RNA using ProtoScript II reverse transcriptase (New England Biolabs, MA) according to manufacturer's protocol and diluted to 100 ng μL^{-1} with nuclease free water. Quantitative real time PCR (RT-qPCR) was performed using a thermocycler (Biorad, Hercules, CA) with SYBR green-I Master Mix and gene-specific primers (10 µmol L^{-1} each). ACTIN served as an endogenous control. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$. All experiments were performed with three biological replicates.

3.2.12 Library construction and RNA sequencing

Four-leaf stage rice plants were treated with Highcard™ quizalofop-p-ethyl at the field rate in flooded condition for 24 h. Total RNA was extracted from treated samples and quantified on a Bioanalyzer2100 with an RNA 6000 Nano LabChip Kit (Agilent, CA, USA). Samples with RNA Integrity Numbers (RINs) >7.0 were used for subsequent analysis. Poly (A) mRNA was isolated from approximately

10 µg of total RNA using poly-T oligo-attached magnetic beads (Invitrogen, Waltham, MA). Purified mRNA was fragmented into small pieces using divalent cations at elevated temperature. Strand-specific cDNA libraries were prepared using the mRNA-Seq sample preparation kit (Illumina, San Diego, CA, USA), with an average insert size of 300 ± 50 bp. Paired-end sequencing was performed on an Illumina HiSeq 4000 platform (LC-Bio, China).

3.2.13 Transcriptomic analysis

Raw FastQ files for each sample were subjected to quality control using FastQC. Adapter contamination, low quality bases, and undetermined nucleotides were removed with Cutadapt and Trimmomati. Cleaned reads were aligned to the *Oryza sativa japonica* genome using HISAT2. StringTie was used to assemble mapped reads into transcripts for each sample, followed by a merged transcriptome construction using custom Perl scripts and gffcompare. Transcripts expression levels were quantified using StringTie and Ballgown. RSEM software normalized mRNA expression levels to fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed mRNAs were identified using Ballgown with a log2 fold change threshold of ±1 and an adjusted p-value < 0.05. Enrichment analysis of differentially expressed genes was performed using the KEGG pathway database in R, with a p-value cutoff of 0.05.

3.3 RESULTS AND DISCUSSION

3.3.1 NTSR mutation increases tolerance to QPE in rice

Wild-type rice (NTI) was sensitive to QPE, with a GR_{50} of 12.85 g ha^{-1}. In contrast, NTSR rice, harboring an ACCase mutation, exhibited QPE resistance (GR_{50} = 66.54 g ha^{-1}). To further investigate this phenotype, four-leaf-stage plants were treated with a QPE dose range of 2.425–2,000 g ai ha^{-1}. Wild-type plants displayed pronounced injury symptoms at 21 d after treatment (DAT), while NTSR plants had higher tolerance. By 28 DAT, wild-type plants treated with >77 g ai ha^{-1} were completely dead, whereas NTSR survived up to 616 g ai ha^{-1}. Both TSR and NTSR exhibited similar injury symptoms at higher doses and

were 6.77- and 5.2-fold more tolerant to QPE than NTI, respectively (Fig. 1A–C). The TSR+NTSR double mutant demonstrated a 59.3-fold increase in QPE tolerance compared with NTI (Fig. 1D), suggesting that the A529T amino acid substitution in NTSR enhances herbicide resistance. This enhanced resistance in the double mutant indicates a combined effect of target-site (ACCase) and non-target-site resistance mechanisms. The resistance factor for QPE was statistically significant in the chi-square test ($\alpha = 0.05$) (Fig. 3.1E).

Table 3.3. Relative herbicide effective doses (GR_{50} in g ai ha^{-1}) on NTI, TSR, NTSR and TSR+NTSR for 50% reduction.

Rice line	Trait	GR_{50}	R/S
WT	None	12.85±3.16	1
TSR	Mutation in ACCase	87.34±16.4	6.77
NTSR	Mutation in ZOS1	66.54±13.57	5.2
TSR+NTSR	Mutations in both ACCase and ZOS1	762±101.5	59.3

[a]R/S = resistance factor

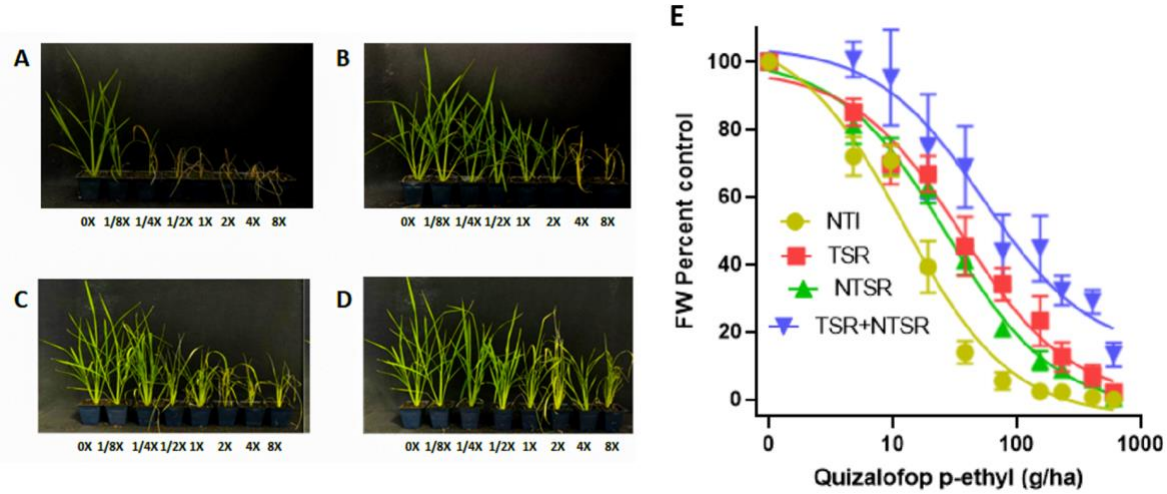

Figure 3.1:Plant growth rate under different doses of QPE
A) NTI, B) TSR, C) NTSR, and D) NTSR+TSR double mutant plant growth rate under different doses of QPE. E) Dose response curve used to estimate the GR_{50}s (doses of QPE required to reduce fresh weight biomass growth by 50%). GR_{50} ranges from 12.08 to 116.6 g ae ha^{-1}. The dose response is modelled with a 3- parametric log-logistic model. The mutant variants TSR, NTSR, and TSR+NTSR show 6.77, 5.2, and 59.3-folds improved tolerance to QPE, respectively, relative to the wild type.

3.3.2 Transcriptomic response to rice carrying NTSR mutation

Non-target site resistance (NTSR) is a complex trait influenced by the differential expression of genes in response to herbicide stress. Previous studies have implicated cytochrome P450s, glutathione *S*-transferases, glycosyltransferases, esterases, ABC transporters, and peroxidases in herbicide detoxification and NTSR development (Rigon et al., 2020). Our transcriptomic analysis revealed higher expression of glutathione *S*-transferase F1, cytochrome P450 A1-like, peroxidase, and heat shock proteins in both NTSR and TSR+NTSR mutant lines compared to wild-type and TSR controls (Table 3.2). This upregulation was observed under both induced and constitutive conditions. Enhanced expression of these genes is likely to contribute to NTSR by increasing herbicide detoxification and reducing the amount of herbicide reaching the target site.

3.3.4 DEGS analysis of common responsive genes between TSR, TSR+NTSR rice varieties

Transcriptome analysis of sprayed TSR and TSR+NTSR rice identified 2,043 differentially expressed genes (DEGs) with expression ratios >2.000 or <0. 050.Untreated wild-type and mutant plants were analyzed to investigate basal gene expression. A total of 243 DEGs associated with transcription and translation were identified (Fig. 3.2A). These DEGs were further categorized as shown in (Fig. 3.2B).

Table 3.4. Statistics of annotation results, all of the genes were compared to the sequences available in public databases. TSR+NTSR sprayed shows higher relative gene expression of cytochrome p450s, GSTs and peroxidases as compared to TSR+NTSR unsprayed.

		NTI	TSR	TSR+ NTSR	NTI	TSR	TSR+NTSR
Locus	**Description**	**Sprayed**			**Unsprayed**		
Os03g55240	Putative Cytochrome P450	20.06	28.21	**238.77**	30.53	22.29	**144.28**
Os01g55830	putative GST	103.19	6.59	**215.51**	138.78	127.40	**163.46**
Os03g50130	Putative Microsomal GST3	42.87	57.06	**172.93**	51.83	13.81	**67.81**
Os04g46960	glutathione peroxidase domain	57.05	41.65	**127.32**	86.29	56.81	**83.34**
Os10g38610	Putative GST	15.96	21.89	**123.24**	17.81	23.77	**92.84**
Os03g17690	Cytosolic ascorbate peroxidase	19.28	27.75	**83.33**	24.96	16.69	**19.84**
Os01g43710	Putative cytochrome P450 72A1	11.61	14.79	**62.40**	17.50	22.16	**24.89**
Os02g30110	putative cytochrome P450	6.12	15.34	**57.03**	18.34	13.69	**37.26**
Os03g57200	putative GST	0	0.02	**52.09**	0.27	0.13	**0**
Os12g10720	putative GST	4.40	7.31	**39.38**	6.27	8.41	**25.20**
Os03g25490	Putative cytochrome P450 72A1	0.22	0.74	**38.79**	0.44	0.06	**28.46**
Os02g30100	Putative Cytochrome P450	6.06	2.62	**30.02**	2.50	1.67	**11.44**
Os01g12740	Putative Cytochrome P450	12.03	0.94	**25.78**	18.45	11.74	**14.25**
Os10g38740	putative GST	0.51	1.21	**23.98**	0.93	0.73	**0**
Os01g43844	Putative cytochrome P450 72A1	9.55	4.65	**22.72**	9.42	8.29	**12.42**
Os10g38489	putative GST GSTU6	0.71	1.46	**19.72**	1.74	2.91	**13.99**
Os03g22020	putative peroxidase precursor	0	0	**9.41**	0.03	0	**7.22**
Os10g38110	Putative Cytochrome P450	6.61	2.04	**8.41**	1.75	2.40	**3.53**
Os10g08474	Putative Cytochrome P450	0.86	2.43	**7.39**	3.33	5.61	**3.52**
Os07g44140	Putative cytochrome P450 72A1	0	0.19	**6.71**	0.03	0.15	**5.07**
Os02g57290	Putative Cytochrome P450	2.16	1.97	**6.03**	3.13	8.84	**2.72**
Os06g43430	Putative Cytochrome P450	2.48	2.23	**6.01**	3.51	4.21	**1.23**
Os07g07320	putative GST	0	0	**5.15**	0	0	**0**
Os01g52790	Putative cytochrome P450 72A1	1.95	0.55	**4.51**	2.46	0.87	**2.60**

Os09g36750	Putative L-ascorbate peroxidase 4	1.32	0	**3.50**	2.44	0	**1.04**
Os07g02440	putative peroxidase precursor	1.18	0.23	**3.38**	1.65	2.33	**1.03**
Os08g02110	putative peroxidase precursor	0	0	**2.86**	0	0	**0**
Os12g16720	putative cytochrome P450 71A1	0.19	0.95	**2.24**	1.07	0.89	**1.76**
Os03g04250	putative GST	0.83	0.46	**1.32**	0.40	0.84	**0.48**
Os07g44130	Putative cytochrome P450 72A1	1.06	0.09	**0.90**	0.60	0.10	**0.38**
Os05g12040	Putative Cytochrome P450 51	0.23	0.13	**0.78**	0.38	0.04	**0.55**
Os02g06630	putative peroxidase precursor	0.40	0.30	**0.77**	0.16	0.18	**0.31**
Os10g36740	Putative Cytochrome P450	0.20	0.07	**0.66**	0.03	0	**0.20**
Os10g38160	putative GST	0	0.06	**0.63**	0.54	0.16	**0.25**
Os10g38350	putative GST	0	0.14	**0.60**	0	0	**0.68**
Os10g08540	putative cytochrome P450	0	0.07	**0.56**	0	0	**0.27**
Os10g26340	putative cytochrome P450	0.40	0.09	**0.51**	0.09	0.77	**0.02**
Os02g09390	putative cytochrome P450	0.26	0.13	**0.42**	0.24	0.04	**0.35**
Os09g26980	putative cytochrome P450	0.42	0.11	**0.42**	0.13	0	**0.32**
Os06g22340	putative cytochrome P450	0.27	0.07	**0.39**	0.07	0.04	**0.26**
Os05g14260	putative peroxidase precursor	0	0.15	**0.35**	0	0	**0.07**
Os02g09240	putative cytochrome P450 71D8	0.22	0.09	**0.20**	0	0.17	**0.66**

3.3.5 The networking analysis of genes responsive to quizalofop treatment

Pathway enrichment analysis was performed to identify biological processes associated with differentially expressed genes (DEGs). Gene Ontology (GO) enrichment analysis revealed significant overrepresentation of pathways at a p-value threshold of 0.05. To further explore the functional implications of DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted for both untreated and treated wild-type and double mutant samples. Fourteen pathways, primarily involved in cellular, biosynthetic, and biological processes, were significantly enriched. A more

detailed KEGG analysis of DEGs in all four experimental groups (Fig. 3.2 A, B) identified enrichment in stress response, cellular processes, mRNA surveillance, and amino acid biosynthesis pathways. mRNA surveillance, and amino acid biosynthesis pathways. The top 25 enriched KEGG pathways for each comparison are presented in Fig. 3.2 C and 3.2 D

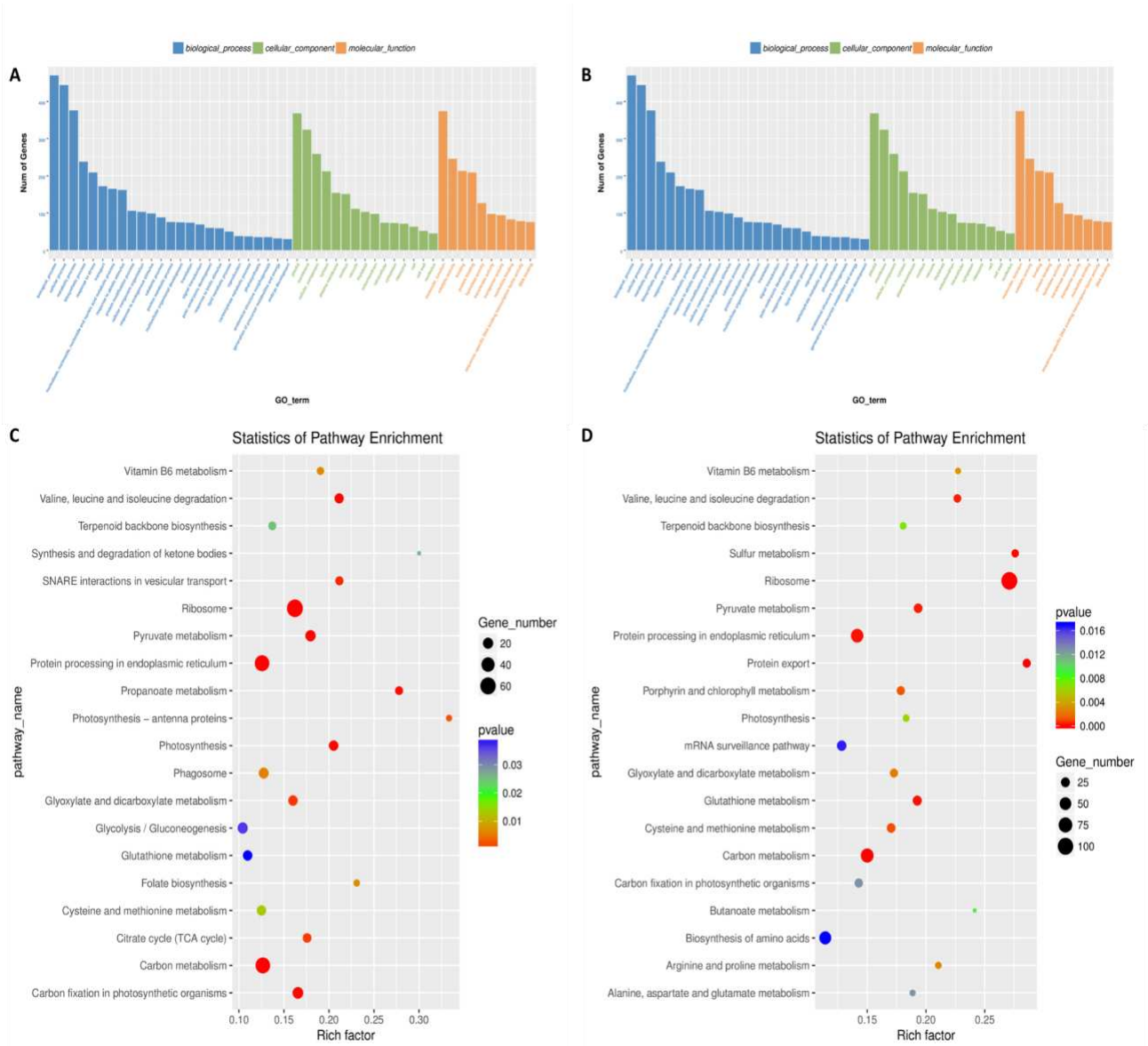

Figure 3.2. KEGG enrichment analysis of differentially expressed genes (DEGs).

(A) & B). TSR+NTSR, TSR sprayed, TSR+NTSR, TSR unsprayed – KEGG enrichment analysis of differentially expressed genes (DEGs) in wild type and mutants respectively. The DEGs were summarized in three main KEGG categories (biological process, molecular function, and cellular component). C), D)

Scatter plot of KEGG pathway enrichment statistics in untreated and treated TSR, TSR+NTSR respectively. Rich Factor is the ratio of differentially expressed gene numbers annotated in this pathway term to all gene numbers annotated in this pathway term. Greater Rich Factor means greater intensiveness. Q-value is corrected P-value ranging from 0 ~ 1, and its less value means greater intensiveness. The top 25 pathway terms enriched by KEGG database are displayed.

3.3.6 Enhanced metabolism in mutant line

Quizalofop-p-ethyl is rapidly converted to its active form, quizalofop acid (QZA), by plant esterases. Wild-type (NTI) rice is sensitive to QPE due to its limited ability to degrade QZA into inactive metabolites (Figure 3.3). Consequently, over 50% of QZA persisted in NTI aerial tissues under greenhouse conditions. TSR rice, harboring an ACCase mutation, exhibits QPE resistance. Although initial QZA metabolism in TSR resembled NTI, the plant's health allowed for complete QZA degradation within 8 d.

The ZOS-1-16 transcription factor mutant displayed increased QPE tolerance attributed to accelerated QZA metabolism compared to NTI and TSR. This metabolic enhancement was further amplified in the double mutant due to the combined effects of target-site and metabolic resistance. Previous studies have implicated non-target site resistance (NTSR) genes, including glutathione *S*-transferases and cytochrome P450s, in herbicide detoxification. Our RNA sequencing data reveal elevated NTSR gene expression in comparison to wild-type, aligning with its observed increased resistance.

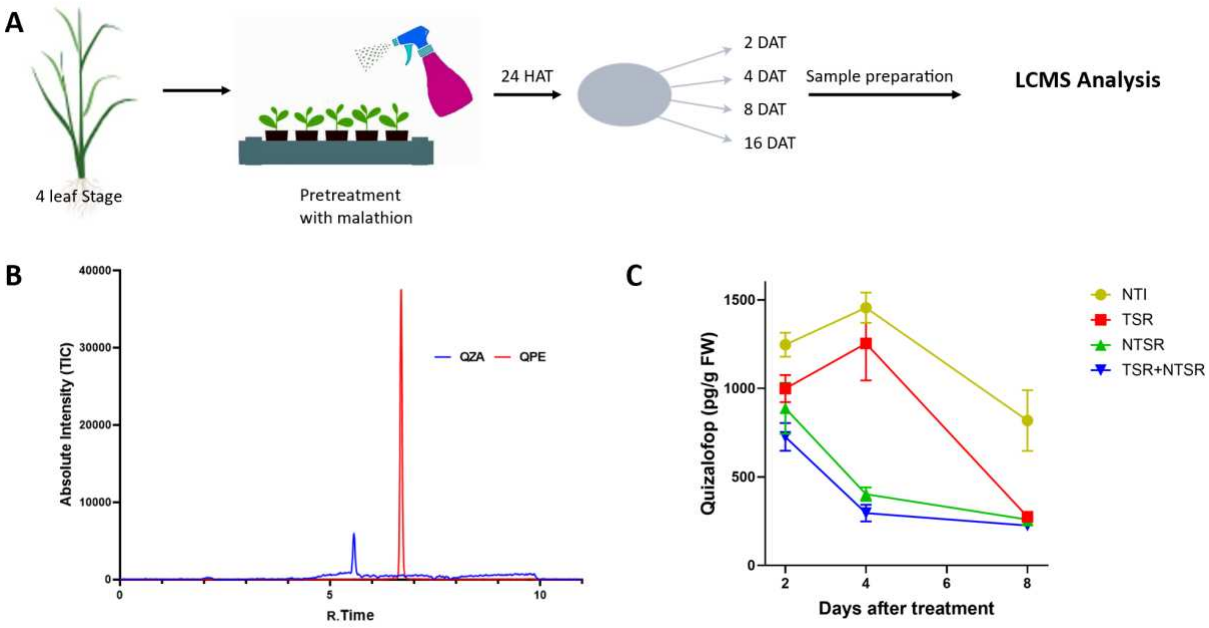

Figure 3.3. Metabolism studies
A) workflow to conduct metabolism studies, B) Chromatogram indicating QPE and QZA peaks at around 5.7 and 6.8 min, respectively and C) metabolism of QZA in all four rice varieties, the NTSR rice variety with mutation on transcription factor shows faster metabolism as compared to the target site ACCase mutant TSR, the double mutant line TSR+NTSR shows fastest metabolism

3.3.7 Glutathione *S*-transferase activity in rice lines

Protein extracts were prepared from NTI and NTSR genotypes for glutathione *S*-transferase (GST) activity analysis. Protein concentrations, as determined by the Bradford assay, were 0.9 mg and 1.2 mg for wild-type and mutant, respectively. GST activity increased proportionally with enzyme volume in both genotypes. Notably, NTSR exhibited higher specific GST activity compared to NTI under both untreated and treated conditions (Fig. 3.4 A). A dose-response curve, correlating fresh weight with herbicide dose at 21 d post-treatment, is presented in Fig. 3.4 B

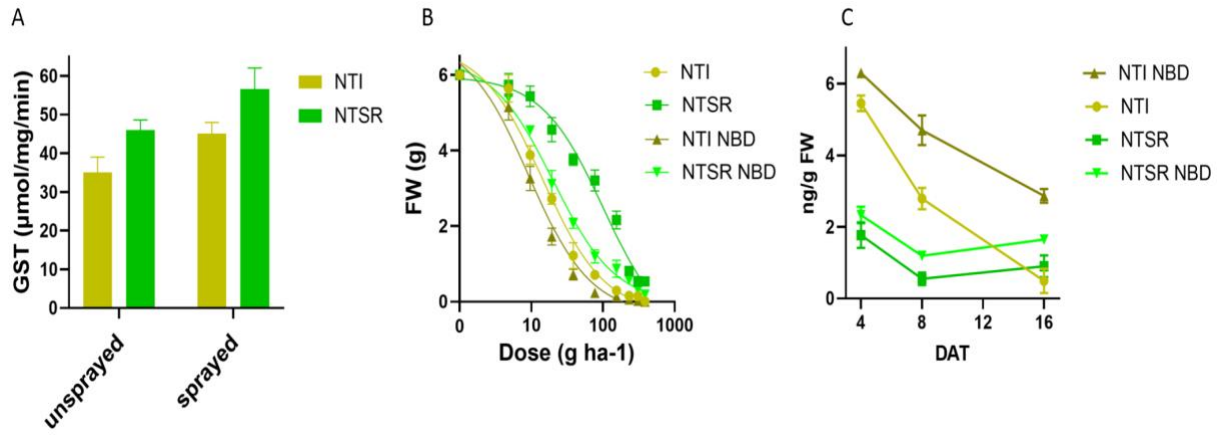

Figure 3.4. GST response of NTI and NTSR genotypes in unsprayed and sprayed condition
(A) GST enzyme in NTI and NTSR genotypes in unsprayed and sprayed condition. B) Four parametric log-logistic models of NTI, NTSR with QPE and QPE+NBD sprayed. Chloro-7-nitrobenzoxadiazole [1, nitro-benzoxadiazole (NBD)-Cl lowers tolerance of both genotypes to herbicide, GR_{50} of fresh weight of NTSR QPE+NBD and NTSR QPE (FW) alters from 20.03 g to 78.11g ha^{-1}. C) Metabolism of QPZ 4, 8, 12 and 16 DAT showing NTI, NTSR with NBD becomes more sensitive to QPE applied when compared to NTI, NTSR without NBD.

3.3.8 Cytochrome P-450 activity in rice lines

Malathion, by targeting cytochrome P450 genes, can counteract the effects of QPE on rice plants. NTSR rice, carrying an ACCase mutation, exhibits resistance to QPE with a GR_{50} of 66.54 g ha^{-1}. When four-leaf-stage plants were exposed to a QPE dose range of 2.425 to 2,000 g ai ha^{-1}, distinct phenotypic responses were observed between wild-type and NTSR rice. Wild-type plants displayed severe injury symptoms 21 DAT, while NTSR plants showed remarkable tolerance. By 28 DAT, complete mortality occurred in wild-type plants treated with doses exceeding 77 g ai ha^{-1}, whereas NTSR rice survived up to 616 g ai ha^{-1} as shown in Fig 3.5.

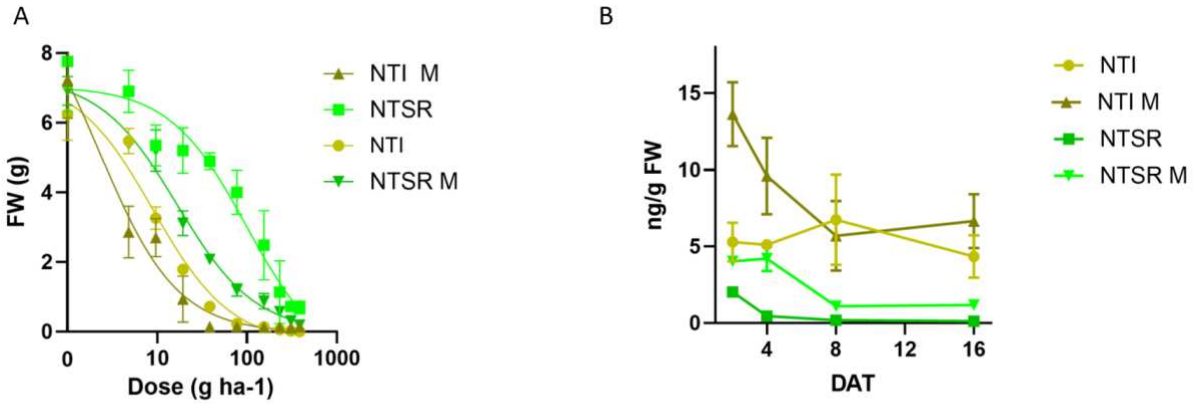

Figure 3.5. Cytochrome P-450 activity in NTI and NTSR genotypes in unsprayed and sprayed condition

A) Four parametric log-logistic models of NTI, NTSR with QPE and QPE+malathion sprayed. B) Metabolism graph after 4, 8, 12 and 16 DAT showing NTI, NTSR with malathion becomes more sensitive to QPE applied when compared to NTI, NTSR without malathion.

3.3.9 Computational studies to delineate the role of mutation ZOS1 16 TF

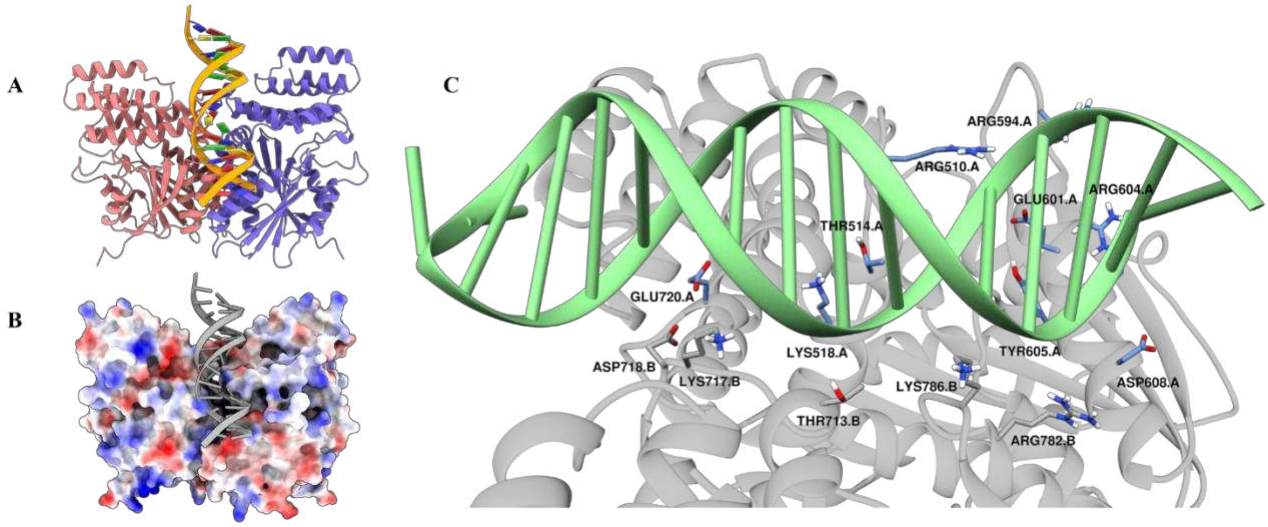

Figure 3.6. Molecular docking studies.

(A) the conformation of protein-DNA obtained from Molecular docking studies, B) electrostatic analysis of protein where the charged residues are highlighted, C) the interacting amino acids with DNA molecule. The molecular docking studies were obtained from HDock web server where the binding energy was -180.78 Kcal mol-1. The electrostatic interactions involve positively charged amino acids (in blue) and negatively charged amino acids (in red), and neutral amino acids (in white). The DNA molecule requires positive interactions for its stabilization, the interface of the protein contains Arg594, Arg510, Arg604, Arg782, Lys786, Lys518, and Lys717 on the surface which will interact with DNA to stabilize it.

BLASTp analysis revealed 100% identity and query coverage with SCARECROW (SCR)-LIKE9 protein, a known regulator of HDAC19 activity (Altschul *et al.*, 1990, Altschul *et al.*, 1997). This transcription factor belongs to the GRAS family, characterized by a dimeric structure that enhances DNA binding. ZOS-1-16 protein, approximately 820 amino acids in length, exhibited a highly flexible N-terminus (residues 1-442) that precluded structural determination. Subsequent analysis focused on the structurally conserved core (residues 443-820), which showed similarity to GRAS family transcription factor structures (PDB IDs: 6KPD, 6KPB, 5B3H, 5B3G, and 5HYZ) (Li *et al.*, 2016). Structural validity was confirmed by pLDDT scores and Ramachandran plot analysis (Fig. 3.7 B, C).

To predict protein-nucleotide (PN) complexes, HDock was employed, generating multiple conformations ranked by binding energy. The optimal PN complex exhibited a binding energy of -188 kcal/mol. Given the negative charge of nucleotides, interactions with positively charged residues were anticipated. Analysis revealed Lys518, Arg594, Arg604, Gln611, Arg782, His785, and Lys786 as potential nucleotide binding sites, located on the protein surface within the dimeric interface. As these residues are charged residues, they mainly interact with the DNA due to their positive charge. Ala519thr facilitates hydrogen bond instead of hydrophobic interaction.

The DNA binding region was mapped to amino acid residues 440-820 (Fig. 3.7A). Alignment scores for the modeled monomeric (Fig. 3.7A) and dimeric (Fig. 3.7B) structures are shown. AlphaFold modeling of the full-length protein (residues 1-820) yielded extended loop and helical regions for the N-terminal domain (residues 1-439), compromising structural accuracy. In contrast, the C-terminal domain (residues 440-820) exhibited higher alignment scores and improved structural fidelity. As the template structures (6KPD, 6KPB, 5B3H, 5B3G, and 5HYZ) adopted dimeric conformations essential for DNA binding, the ZOS-1-16 protein was modeled as a dimer.

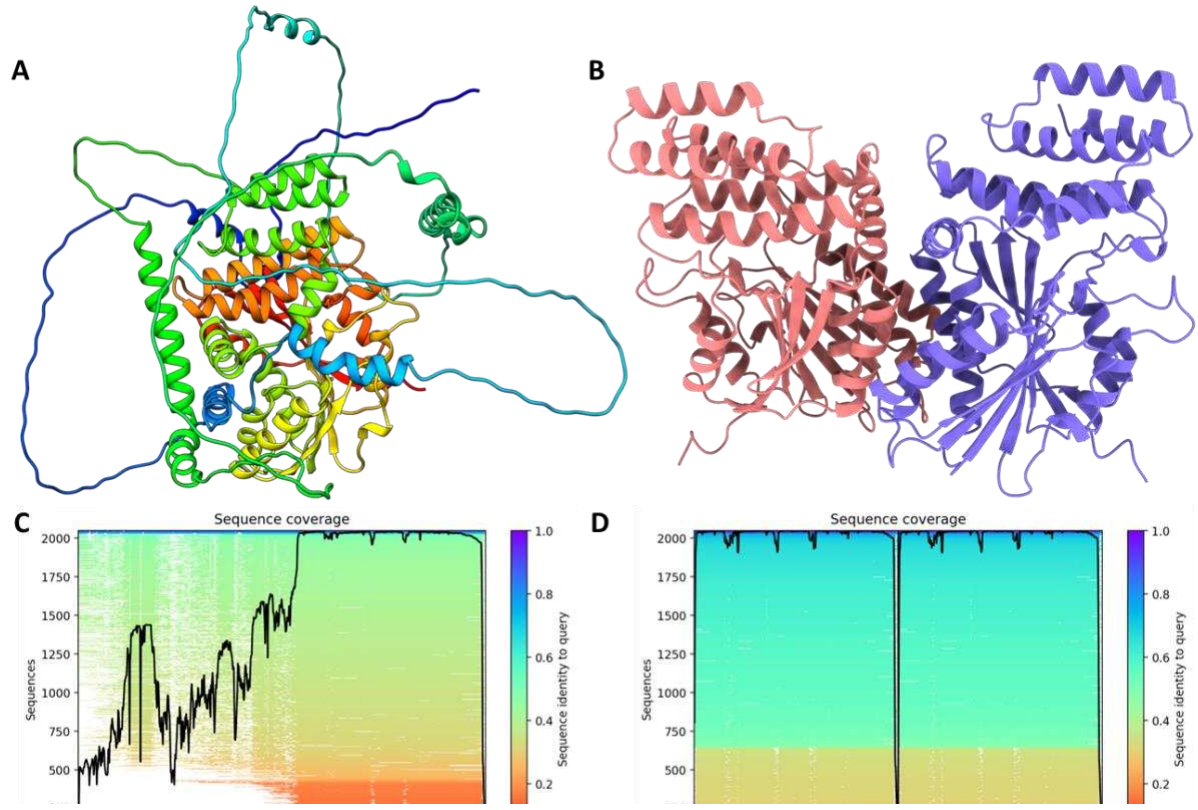

Figure 3.7. AlphaFold Modelled structure of SCARECROW (SCR)-LIKE9 protein.
(A) The modelled structure of SCARECROW (SCR)-LIKE9 protein having loosely modelled regions having loops and long helices (amino acid residues 1-439), B) dimeric form structures are shown. AlphaFold modeling of the full-length protein (residues 1-820) yielded extended loop and helical regions for the N-terminal domain (residues 1-439), compromising structural accuracy. In contrast, the C-terminal domain (residues 440-820) exhibited higher alignment scores and improved structural fidelity. As the template structures (6KPD, 6KPB, 5B3H, 5B3G, and 5HYZ) adopted dimeric conformations essential for DNA binding, the ZOS-1-16 protein was modeled as a dimer.

3.4 CHAPTER CONCLUSION

This study demonstrates that the NTSR mutation in rice confers enhanced tolerance to the herbicide QPE. The NTSR mutant exhibited significantly higher QPE tolerance compared to the wild type, as evidenced by reduced injury symptoms and increased GR_{50} values. The double mutant, combining both target-site and non-target-site resistance mechanisms, displayed the highest level of QPE tolerance. Transcriptomic analysis revealed upregulation of key detoxification genes, including glutathione *S*-transferase and cytochrome P450, in the NTSR mutant, suggesting their involvement in herbicide metabolism. Functional characterization confirmed increased glutathione *S*-transferase activity in the NTSR genotype. Additionally, computational studies identified a novel transcription factor, ZOS-1-16, with a potential role in regulating herbicide response. These findings collectively highlight the complex mechanisms underlying QPE tolerance in rice and provide insights into potential strategies for developing herbicide-resistant crop varieties. Further research is warranted to elucidate the precise molecular interactions between the identified genes and proteins in conferring QPE tolerance.

CHAPTER 4: INHERITANCE OF GLYPHOSATE RESISTANCE AND CROSS-POLLINATION RATES UNDER FIELD CONDITIONS IN KOCHIA (*Bassia Scoparia*)

Overview:

Bassia scoparia (kochia) is an invasive species in the high plains of the United States that poses formidable management challenges in agricultural systems, primarily due to its evolution of resistance to glyphosate. Resistance is due to a transposon-associated increase in 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS) gene copy number relative to the sensitive biotype. Factors behind the rapid spread of glyphosate-resistant biotypes are likely associated with certain aspects of kochia biology, such as a protogynous flower morphology producing large amounts of pollen, that encourages outcrossing and favors high genetic diversity. Furthermore, its ability to tumble over long distances ensures a rapid spread of the resistance trait. Herein, we explore glyphosate resistance in kochia in eastern Colorado. There was no difference in EPSPS gene (Type I, Type II) and FAR1 copy numbers between parent and progeny kochia populations across multiple years (2018, 2020, and 2022), suggesting stable inheritance of glyphosate resistance. Further, the inheritance of glyphosate resistance was investigated using three specific microsatellites or simple sequence repeat (SSR) markers viz 2656, 2896, and 1792. SSR marker analysis revealed an outcrossing rate of 78% and a selfing rate of 22% in kochia progeny. By investigating the complex interplay between kochia's biology and genetics, this study investigates the inheritance of glyphosate resistance in kochia, estimates the outcrossing rate under field conditions, and underscores the importance of developing effective management strategies to mitigate its impact on agricultural ecosystems.

4.1 CHAPTER INTRODUCTION

Kochia (Bassia scoparia) is a resilient and adaptable plant species that has garnered attention for its ecological and agricultural impact (Qadir et al., 2008, Kumar et al., 2019, Geddes and Sharpe, 2022). Native to Eurasia, kochia has become widespread across North America where it is a troublesome weed

(Fig 4.1). Historically, kochia has been utilized for various purposes, including erosion control, forage production, and ornamental landscaping. However, its prolific seed production, rapid growth, and ability to thrive in diverse environmental conditions have contributed to its status as a major problematic weed in agricultural as well as non-crop settings.

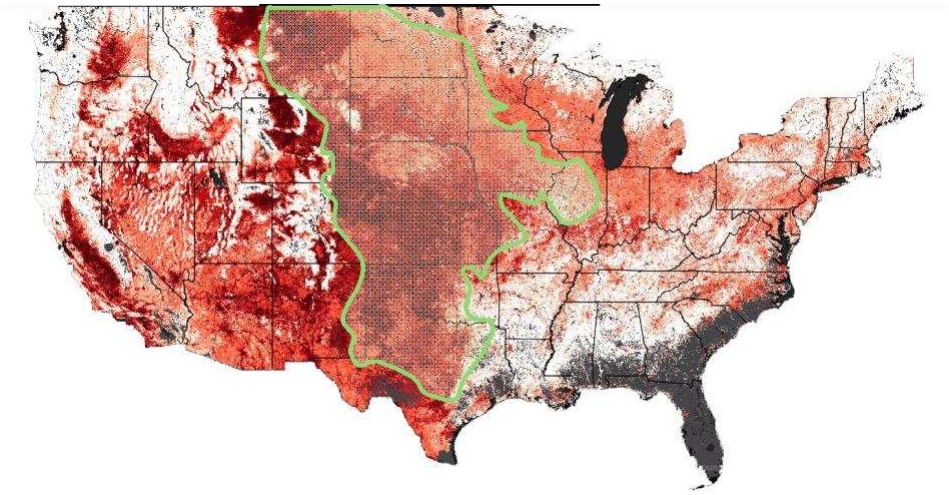

Figure 4.1. Map of kochia in the United States based on the Invasive Species Habitat Tool (INHABIT).
The INHABIT tool is designed to display outputs from models of exotic species as completed by USGS FORT utilizing the Software for Assisted Habitat Modeling (SAHM) following the methods described elsewhere (Young *et al.*, 2020, Engelstad *et al.*, 2022) (https://gis.usgs.gov/inhabit/). The green outline indicates the geographical location of the Great Plains in the United States according to the Audubon Society. Shading on the map represents a gradient of kochia resistance, with darker shades of red indicating high kochia resistance population and gradient to black indicating decreasing kochia resistance.

A key adaptation for kochia's success is its C4 photosynthetic pathway, coupled with a unique emergence and flowering strategy. As a C4 plant, kochia thrives in hot and dry environments due to efficient water use. This is particularly advantageous when it emerges early in spring, capitalizing on cool, moist conditions for initial growth while tolerating late spring time frosts (Friesen *et al.*, 2009). By delaying flowering until the hottest part of summer, kochia avoids the combined stress of high temperature and water demand during its critical reproductive stage. This flexibility makes it a successful competitor in harsh conditions (Friesen *et al.*, 2009). Genetic studies have revealed the existence of multiple genetic lineages within kochia populations, reflecting both natural selection and human-mediated gene flow (Martin *et al.*, 2020). This genetic diversity enables acclimation in response to environmental

cues such as tolerance to high and low temperatures, low moisture, poor soils with high salt content, and nutrient availability (Fig. 4.2 A and B) (Kumar *et al.*, 2019, Yadav *et al.*, 2023).

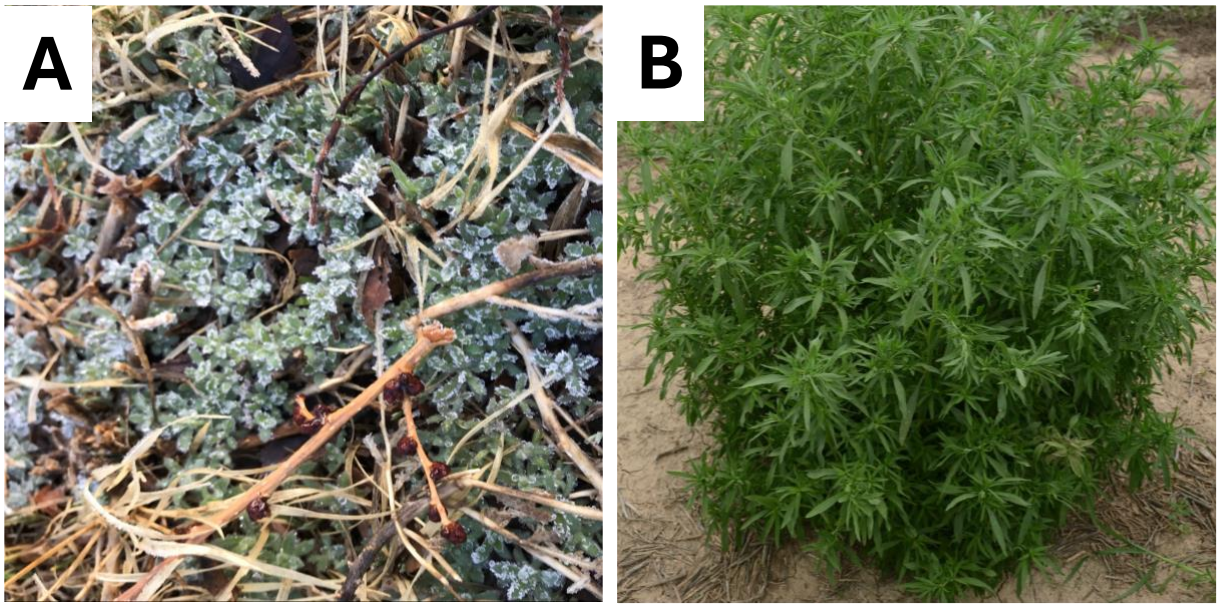

Figure 4.2. Examples of kochia growing in Colorado,
A) Seedlings can withstand exposure to freezing temperatures during spring cold snap and B) resume growth without major injury (photos by Phil Westra).

Kochia has been a problematic weed in the high plains of the United States for years, but its ability to evolve resistance to herbicides has complicated its management. Kochia has currently evolved resistance to synthetic auxins, photosystem II, acetolactate synthase inhibitors, and glyphosate (Varanasi *et al.*, 2015, Kumar *et al.*, 2019). With respect to glyphosate resistance, most plant species, including kochia, normally have a single copy of 5-enolpyruvyl-3-shikimate phosphate synthase (*EPSPS*), but glyphosate-resistant (GR) kochia populations have a transposon-driven copy number variation (CNV) of *EPSPS (Jugulam et al., 2014, Wiersma et al., 2015)*, with some plants having as many as 20 *EPSPS* copies (Godar *et al.*, 2015).

As with other weeds, effective management of GR kochia requires integrated weed management strategies that incorporate diverse approaches, such as herbicide rotation, use of alternative herbicides with different modes of action, adoption of cultural practices, and development of non-chemical control methods (Kumar *et al.*, 2019). Proactive monitoring of herbicide resistance and early detection of

resistant populations are essential for implementing timely and targeted management interventions to mitigate the spread of resistance and preserve the efficacy of herbicides for weed control in agricultural systems (Torbiak *et al.*, 2024).

Herein, we report a survey for the frequency of glyphosate resistance in field-collected kochia populations; the inheritance pattern of gene copy number for total *EPSPS,* the two types of *EPSPS* CNV, and the associated transposon in a field study; and the frequency of outcrossing in a field study.

4.2 MATERIALS AND METHODS

4.2.1 Pollen Scanning Electron Microscopy

Naturally dried pollen was separated from mature flowers and prepared for scanning electron microscope (SEM) observation according to Lynch and Webster (1975) (Lynch and Webster, 1975). The grains were sprinkled evenly on double-sided conductive adhesive on gold–palladium metallic stubs and coated with a gold sputter coater (Vacuum Desk II Gold Sputter Coater, Denton North America, Moorestown, NJ). The pollen grains were observed using a field emission scanning electron microscope (JSM-6500, JEOL USA, Inc., Peabody, MA) at the Center for Imaging and Surface Science of Colorado State university.

4.2.2 Geographic Localization of GR Kochia and Wind Analysis

The location and date of report of GR kochia was obtained from the International Herbicide-Resistant Weed Database (Heap, 2024) accessed July 2024. 20-Year (January 2001 - December 2020) average monthly wind speed and direction were obtained from the National Aeronautics and Space Administration (NASA) Langley Research Center (LaRC) Prediction of Worldwide Energy Resource (POWER) Project funded through the NASA Earth Science/Applied Science Program (https://power.larc.nasa.gov/).

4.2.3 Plant Material for Field Survey and Treatment

A field survey was conducted in 2015 to assess glyphosate resistance in kochia (*Bassia scoparia*) across Colorado. Kochia seeds were collected in autumn (October-November) from field and roadside locations along transects, with a minimum distance of 16 km between sites. Within crop fields, seeds were harvested from individual kochia plants that had survived the entire growing season. To create composite samples for each location, seeds from 5-20 plants were pooled. Seeds were then transferred to the Colorado State University greenhouse for screening and herbicide application as described in Westra *et al* (Westra *et al.*, 2019). Seedlings were treated with glyphosate (RoundUp WeatherMax®, Monsanto, St. Louis, MO) at a rate of 840 g ae ha^{-1} (grams of acid equivalent per hectare) with 20 g L^{-1} ammonium sulfate (AMS) using a moving overhead single-nozzle sprayer (DeVries Manufacturing, Hollandale, MN) calibrated to deliver 187 l ha^{-1}.

Kochia accessions were classified as susceptible (≥20% survival) or resistant (<20% survival) based on their response to the discriminating glyphosate rate (Westra *et al.*, 2019). Geo-referenced collection sites were mapped using Arc Catalogue and ArcMap (version 10.2.1) to visualize spatial patterns of glyphosate resistance across Colorado and facilitate comparisons over time (Khater *et al.*, 2022).

4.2.4 Plant Material for Inheritance Study

Survivor plant samples were collected from Roundup Ready sugar beet fields that had been treated with a post- emergence commercial rate of glyphosate in Colorado over three years (2018, 2020, 2022) during autumn. For 2018, parental tissues were randomly collected randomly from a field at coordinates 40° 37'N×105° 01'W in Northern Colorado and seeds were brought to Colorado State University. Collection for 2020 was performed in a more targeted fashion at 40° 36'N×105° 01'W. Samples 1-11 were collected from the west side of the field, samples 12-22 were clusters of survivors on the east side of the same field, samples 23-31 were isolated from the central part of the field, and samples 32-44 were gathered from the northwest part of the field. For 2022, healthy plants that survived multiple doses of glyphosate applied

throughout the growing season were collected from the sugar beet field at 40º36'N×105º01'W, transplanted from the field into large pots (22 L), and transported to CSU where they were grown to maturity for seed collection reproductive stage. Samples 1-5 were collected from the west side of the field whereas samples 6-10 were collected from the east side. All seeds were placed in 81.3 cm Miracle-Gro® Moisture Control® Potting Mix with Lambert LM-GPS, Miracle- Gro, US until reaching the seedling stage in the greenhouse, where plants were watered once a day to maintain field capacity. The harvested populations were designated as resistant due to their survival following exposure to 900 g ae/ha glyphosate throughout the growing season in field. For evaluating gene copy number variation, the M32, a resistant population with high *EPSPS* copy number collected during a herbicide resistance survey in Colorado (Westra *et al.*, 2019) and 7710, a glyphosate-susceptible inbred population were selected as controls (Preston *et al.*, 2009, Pettinga *et al.*, 2018, Patterson *et al.*, 2019).

4.2.5 Extraction of Genomic DNA

Two young rosette leaves were collected from each individual plant at the 2.5 cm seedling height growth stage. Samples were immediately flash-frozen in liquid nitrogen and stored at -80 °C until DNA extraction. Three biological replicates were used for DNA extraction from both parental and progeny lines. DNA extraction was carried out using the cetyl trimethylammonium bromide (CTAB) method (Doyle, 1991), followed by quantification using a Thermo ND-1000 model Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA).

4.2.6 Quantitative Real-Time PCR (qRT-PCR) for *EPSPS* Copy Number Determination

Quantitative real-time PCR (qRT-PCR) was employed to determine the *EPSPS* copy number variation. Ten nanograms per microliter (ng/µL) of genomic DNA from three biological replicates of each parental and progeny line were used for the qRT-PCR reactions. Additionally, three technical replicates were

performed for each biological replicate to account for variation during the sample preparation for qRT-PCR. The reactions were performed using a Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) on a Bio-Rad CFX Real-time PCR System (Bio-Rad Technologies Pvt Ltd, USA). The thermal cycling conditions included an initial 3 min denaturation step at 95°C, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 s and a combined annealing/extension step at 70°C for 30 s, as described elsewhere (Wiersma *et al.*, 2015, Patterson *et al.*, 2019).

Specific primer sets targeted the full-length Type I *EPSPS* segment (56.1 kb), the shorter Type II segment (32.7 kb), and a flanking mobile genetic element, *FAR1* (15 kb) (Table 4.1) (Patterson *et al.*, 2019, Ravet *et al.*, 2021). The acetolactate synthase (*ALS*) reference gene was used to normalize *EPSPS* copy number using the ΔΔCt method (Wiersma *et al.*, 2015, Gaines *et al.*, 2016). Briefly, the relative expression ratio (R) was calculated as $R = 2^{-(\Delta Ct(sample) - \Delta Ct(calibrator))}$ (Schmittgen and Livak, 2008), where ΔCt(sample) represents the difference in threshold cycle (Ct) values between the target gene (*EPSPS*) and the reference gene (*ALS*) for a given sample, and ΔCt(calibrator) represents the average ΔCt value for the parental line used as the calibrator. The technical replicates were averaged first; and then biological replicates were averaged for the analysis, followed by calculation of standard deviation to assess variation within each treatment group.

Table 4.1. Forward and reverse primer sequences for ALS (control), EPSPS, Type I, Type II repeats and mobile genetic element (FAR1)

Repeats	Forward primer	Reverse primer
ALS	CCAGAAAAGGCTGCGATG	CTGACTC GCTCTGATTCCA
EPSPS	CGCTATATGTTGGATGCTCTAAG	CACTCCTATTCTCTTTACCAGC
Type I	GACGGAAATACCCTCAATATAGA CA	ACGCCCAA GATGTACATTGATA
Type II	GACGGAAATACCCTCAATATAGA CA	CATGCCTTTGATGT CCAAGTTT
FAR1	GAAGATAGCGAGACGTTT GAG	CGGCTTGATCGGTTAAGATAC

4.2.7 SSR Genotyping using Bio-analyzer

Simple sequence repeats (SSRs) were used to study inheritance in selected GR kochia populations. From the set of 11 SSR primers mentioned in a study by Ravet et al. (Ravet *et al.*, 2021), three specific markers (SSRs 2656, 2896, and 1792) were selected for further analysis (Table 4.2).

Genomic DNA was diluted to a concentration of 5 ng/µL for PCR with EconoTaq PLUS master mix for the PCR. The process starts with a 2 min denaturation step at 94°C, followed by denaturation at 94°C for 30 s, a 30 s annealing step at 58°C, a 45 s extension step at 72°C, and a final extension of 2 min at 72°C. The PCR products were then multiplexed using the three SSR markers (Table 2) and processed for fragment analysis using a 2100 bioanalyzer (Agilent technologies) at the biotechnology core facilities at the University of Nebraska. A high sensitivity DNA kit for the sizing and quantitation of fragmented DNA was utilized for sample preparation prior to fragment analysis.

Table 4.2. List of SSR primers used for genotyping

SSR name	Forward primer sequence (5′–3′)	T*m*	Reverse primer sequence (5′–3′)	T*m*	Amplicon size (bp)	Motif repeat	Annealing temperature (°C)
1792	AACTAGTCGGATCGAGCCTT	58	AATCACACAACTCCGCAAGT	58.2	174	(CCCAA)n	57
2656	AACCAAACCGCACTAAACTG	57.8	GCACAATAGAGAGGGCAAAA	58	277	(TGGTT)n	62
2895	GTCATAGCCATCCCTTACCC	58.3	TATTGCCCTGTTCTTCAGGA	58.3	267	(AGTTC)n	62

4.2.8 Statistical analysis

Statistical significance of gene copy numbers was determined by Analysis of Variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test ((Williams and Abdi, 2010)W at the α=0.05 level using module agricolae in RStudio (2024.04.2 Build 764) and R version 4.3.1 (2023-06-16 ucrt).

The homogeneity of the gene copy number variance across years for parental and progeny EPSPS was assessed with the Bartlett's test (Bartlett and Fowler, 1937) using the stats module in R. This test was used

to determine whether the data from the various collection years could be pooled. The null hypothesis (H0) assumes equal variances across groups, while the alternative hypothesis (Ha) states that at least two groups have unequal variances. Bartlett's test revealed no statistically significant difference in variance for EPSPS copy number across the three collection years (2018, 2020, and 2022) in the parental lines (Bartlett's K-squared = 1.948, df = 2, p-value = 0.3776) and progenies (Bartlett's K-squared = 0.45504, df = 2, p-value = 0.7965).

4.3 RESULTS AND DISCUSSION

4.3.1 Pollination Dynamics and Inheritance

As part of the Amaranthaceae family, kochia is characterized by its small, inconspicuous greenish white to pinkish flowers lacking showy petals or sepals arranged in dense clusters along slender stems (Fig. 4.3A) (Friesen *et al.*, 2009). Additionally, it is considered an outcrossing species because its flower are protogynous where stigmas are receptive one week prior to dehiscence of anthers on same flower (Friesen *et al.*, 2009). Furthermore, its pollen grains are spheroidal with a diameter of 20-40 µm having a granular surface due to 100-130 pores (Stallings *et al.*, 1995b) (Fig. 4.3B), which is typical for a anemophilous pollen, compared to pollen from biotically-pollinated plants that are more than 200 µm (Harder, 1998). These small spherical and dimpled pollen grains have structural similarities to a golf ball whose structure reduces air resistance and increases lift, maximizing their ability to remain suspended in the air (Ackerman, 2000).

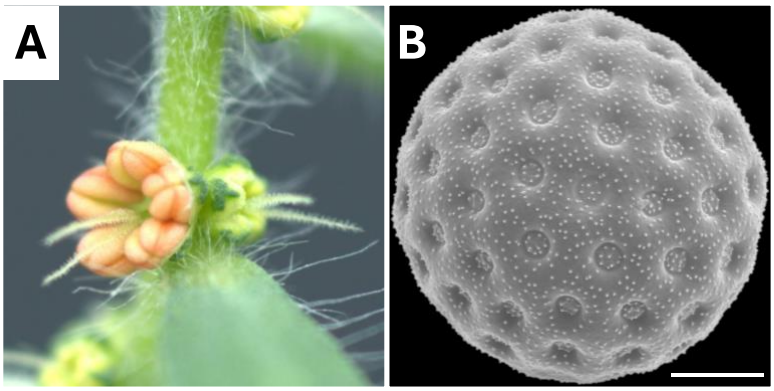

Figure 4.3. Kochia flower and pollen grain
A) Photograph of a kochia flower and B) scanning electron micrograph of a kochia pollen grain observed. Micrograph was obtained at 3,000× magnification and with 5 kV (line = 10 µm).

Being prone to wind-pollination, kochia relies on abiotic factors such as wind speed and direction for pollen dispersal and pollination (Chen *et al.*, 2020). The absence of showy petals or nectar rewards suggests that kochia is not dependent on insect pollinators for reproduction (Gill, 2004). Wind pollination enables pollen transfer over long distances, facilitating widespread dispersal and colonization of new habitats. Being prone to wind-pollination, kochia relies on abiotic factors such as wind speed and direction for pollen dispersal and pollination (Chen *et al.*, 2020).

4.3.2 Glyphosate Resistance Survey

Dispersal of herbicide-resistant alleles via pollen-mediated and/or seed-mediated means can drive the movement of herbicide-resistant populations across an agroecoregion. GR kochia was first reported in Kansas in 2007 and is now causing problems in 10 western states (Fig. 4.4) (Waite *et al.*, 2013, Heap, 2024). This weed is highly fecund, releasing abundant amounts of pollen and produces large numbers of seeds that can be dispersed by wind as the mature plants tumble across a field. Earlier studies demonstrated that pollen-mediated dispersal is strongly dependent on prevailing wind direction during pollination. Similarly, tumbling mature kochia plants can dropped 100,000 or more seeds or more over 1 km with wind speed as low as 0.3 m/s (Beckie *et al.*, 2016). These combined methods of dispersion is

expected to lead to a rapid expansion of the resistance trait without a reduction in genetic variation (Martin *et al.*, 2020).

Interestingly, GR kochia populations have expanded primarily westward and against the predominant wind direction in this region of the USA (Fig. 4.4 and Supplemental Table 4.1). Indeed, analysis of the wind pattern from 2001 to 2020 reveals that this region received wind from the west or southwest with an average wind speed around 5 m/s (Supplemental Table 4.1). Therefore, the westward movement of GR kochia over long distances may not be solely the result of wind dispersal. On the other hand, the lack of eastward expansion may be due to the wetter Midwest conditions that are not conducive to good kochia survival and expansion. Consequently, the emergence of GR kochia in other states may be the result of seed transport by farming equipment or birds, or the result of independent selection of new resistant biotypes (Kumar and Jha, 2015b).

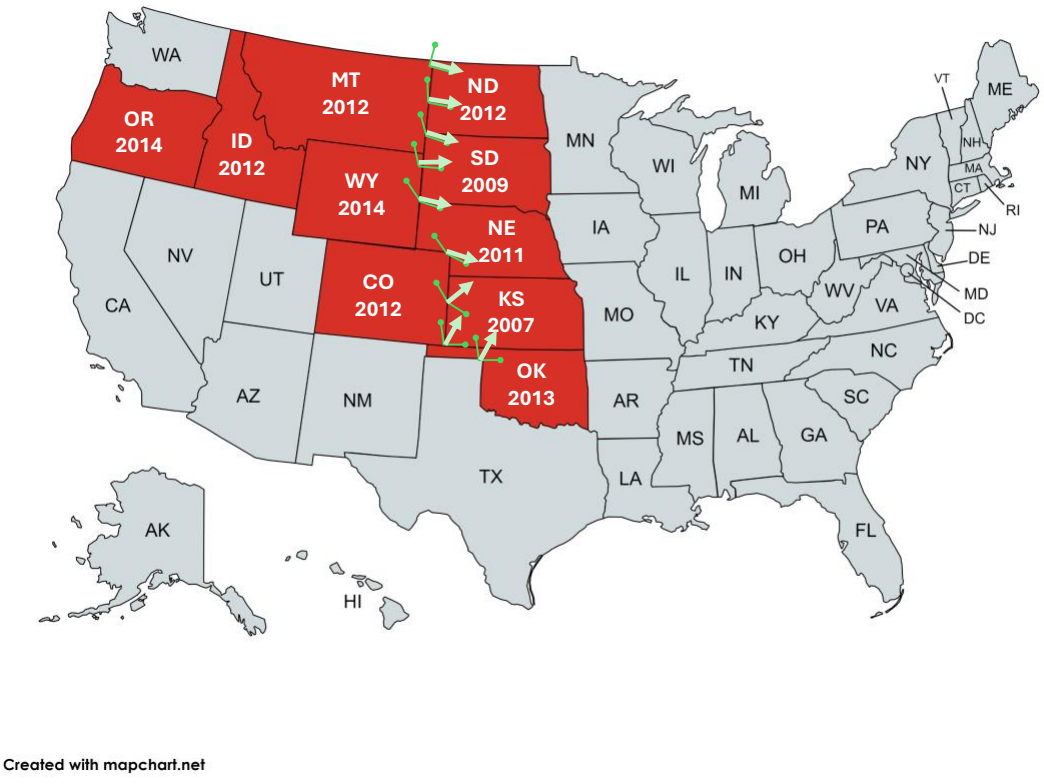

Figure 4.4. States with reported GR kochia populations (and year of first report) based on data from Heap (2024).

The map was generated with MapChart (https://www.mapchart.net/usa.html). Light green arrows indicate 20-year (January 2001 - December 2020) dominant wind direction and small lines represent the range of wind direction during that period (data from NASA power).

GR kochia poses a great challenge to agricultural weed management strategies, particularly in regions where this weed species has become widespread (Kumar *et al.*, 2019) (Fig. 4.5A). GR kochia seeds can spread during harvesting or while tumbling across large areas resulting in movement of GR kochia in the field and beyond (Fig. 4.5B).

Figure 4.5. Impact of kochia on agroecosystem
(A) GR kochia growing in a Colorado sugarbeet field (photo André Araujo). B) Path taken by a GR kochia tumbling across a field, dropping seeds that later on emerge and grow as GR weeds (photo by Philip Westra).

A total of 51 kochia samples were collected from eastern Colorado and assessed for glyphosate resistance frequency (Supplemental Table 4.1). Most of the samples were collected from the fallow phase

of wheat or corn cropping systems. However, some samples were also from roadsides, field margins and a few from alfalfa fields. Due to the reliance on glyphosate for weed management in no-till fallow, 60 – 65% of the kochia plants were resistant to glyphosate.

Overall, glyphosate resistance frequency ranged from 0 to 63%. Mapping the kochia resistance survey data on 51 accessions collected in 2015 and screened in 2016 indicates GR kochia georeferenced clustering within an 80 km radius of this survey (Fig. 4.6). A 80 km radius was selected as a practical limit for sample collection logistics. Identifying GR kochia clusters is important to focus weed control efforts in areas with the highest GR kochia pressure. Resources can be directed towards these areas to prevent further spread and ensure effective weed management.

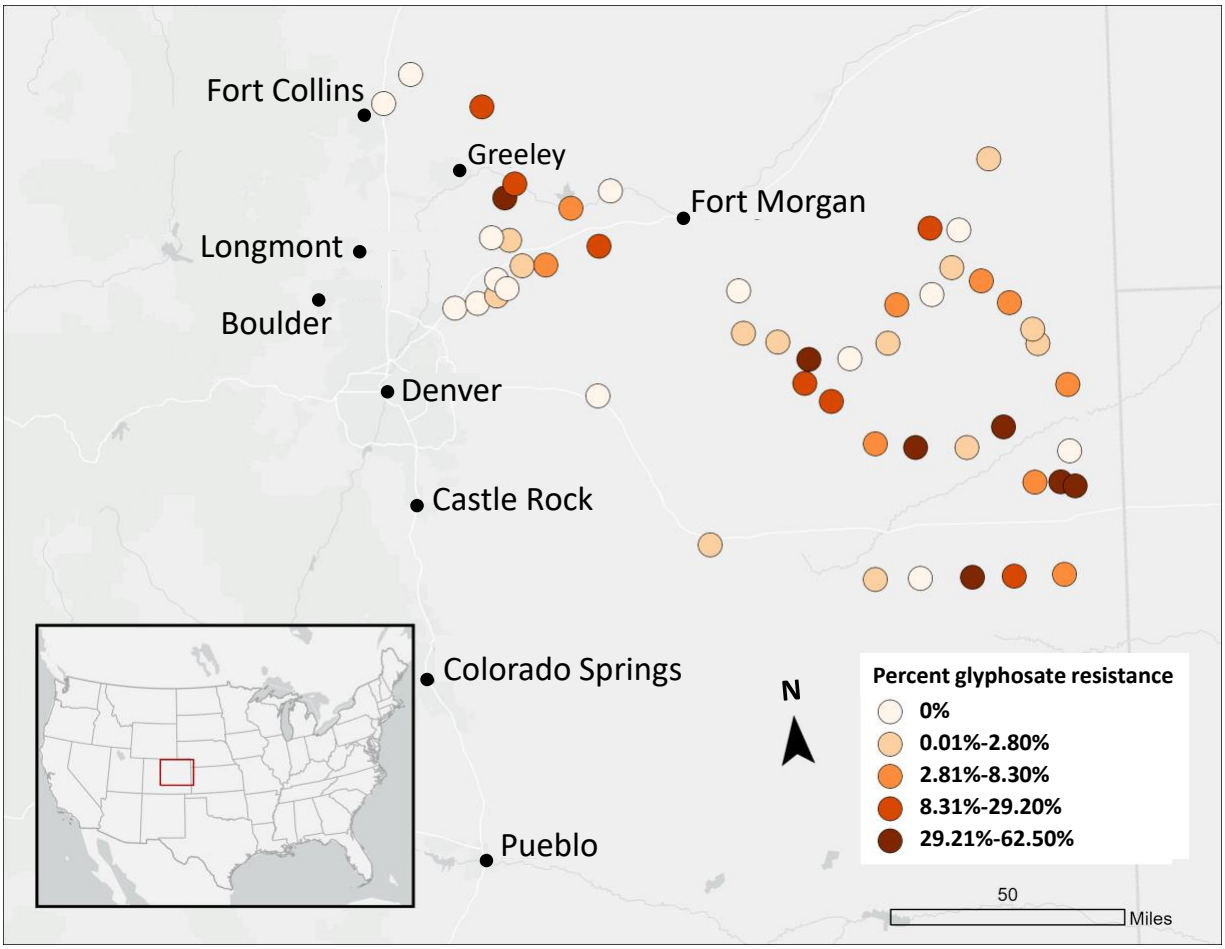

Figure 4.6. Geo-referenced kochia collection sites.
Each collection site was mapped using Arc Catalogue and ArcGis (Maguire, 2008) (version 10.2.1) to visualize spatial patterns of GR across Colorado and facilitate comparisons over time.

4.3.3 Glyphosate Resistance in Kochia

The mechanism of glyphosate resistance in kochia is associated with copy number variation (CNV) of *EPSPS* gene (Jugulam *et al.*, 2014, Godar *et al.*, 2015, Gaines *et al.*, 2016). While sensitive kochia individuals have a single *EPSPS* copy, GR kochia plants with multiple copies of *EPSPS* have reduced sensitivity to glyphosate and can survive and reproduce in glyphosate-treated fields, leading to the proliferation of resistant populations and exacerbating weed management challenges (Lim *et al.*, 2021).

The origin of the *EPSPS* gene duplication event and the evolution of glyphosate resistance are attributed to a mobile genetic element (MGE) containing an FHY3/*FAR1*-like gene. Amplified *EPSPS* copies are typically positioned in tandem in the GR kochia genome. Jugulam et al., have performed FISH analysis to study these tandem repeats and found the sizes of repeat about ~45kb and ~66kb (Jugulam *et al.*, 2014).

There are two dominant repeats up and downstream boundaries of CNV known as Type I having a full length 56.1-kb repeat and Type II having a smaller 32.9-kb repeat, as well as a large MGE ~15kb interspersed in the repeat structure (Patterson *et al.*, 2019). A typical MGE containing AR1 DNA binding, zinc finger, and SWIM domains is present both upstream and downstream of the CNV repeats in GR populations. This MGE causes various unequal alignments and recombination, leading to subsequent crossing-over events. A recently discovered Muntjac, a mutator Don-Robertson transposase that has transcription factor-like activity, provides perspectives of GR kochia via transduplication processes (Dupeyron *et al.*, 2019, Hall *et al.*, 2023). In kochia, the larger Type I *EPSPS* locus contains seven other co-duplicated genes whereas the smaller Type II EPSPS (32.7kb) repeat contains only four genes (Patterson *et al.*, 2019). Since the mechanism underlying GR in kochia involves a transposable element, there can be variations in the number of EPSPS gene copies inherited by progeny relative to their parents, even though GR remains a heritable trait.

4.3.4 *EPSPS* and FAR1 Copy Number Variation:

The homogeneity of the gene copy number variance across years for parental and progeny *EPSPS* was assessed with the Bartlett's test. This test was used to determine whether the data from the various collection years could be pooled. The null hypothesis (H_0) assumes equal variances across groups, while the alternative hypothesis (H_a) states that at least two groups have unequal variances. Bartlett's test revealed no statistically significant difference in variance for *EPSPS* copy number across the three collection years (2018, 2020, and 2022) in the parental lines (Bartlett's K-squared = 1.948, d*f* = 2, *p*-value = 0.3776) and progenies (Bartlett's K-squared = 0.45504, d*f* = 2, *p*-value = 0.7965). The pooled data had normal distribution in both parents and progeny (Fig. 4.7), with an average 12-13 *EPSPS* copy numbers in both parents and progenies. While the number of copies ranged from 7-19 and 6-20 in the parent and progeny, respectively, both populations include 95% of the distribution.

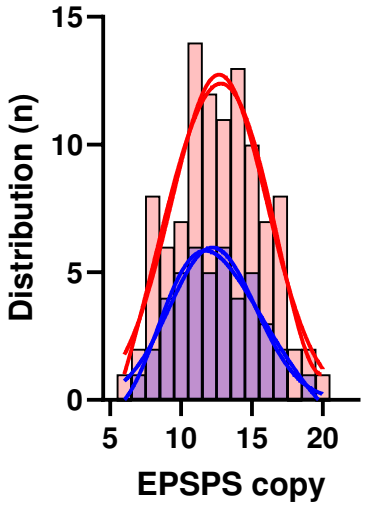

Figure 4.7. Distribution of EPSPS copy number in parent (blue) and progeny (red) samples

There was no difference (*p*-value = 0.769) between the *EPSPS* copy number of parental and progeny populations (Fig. 4.8A). This suggests that the *EPSPS* copy number distribution was constant in the parental and the progeny populations over the 2018-2022 study period (Fig. 4.8).

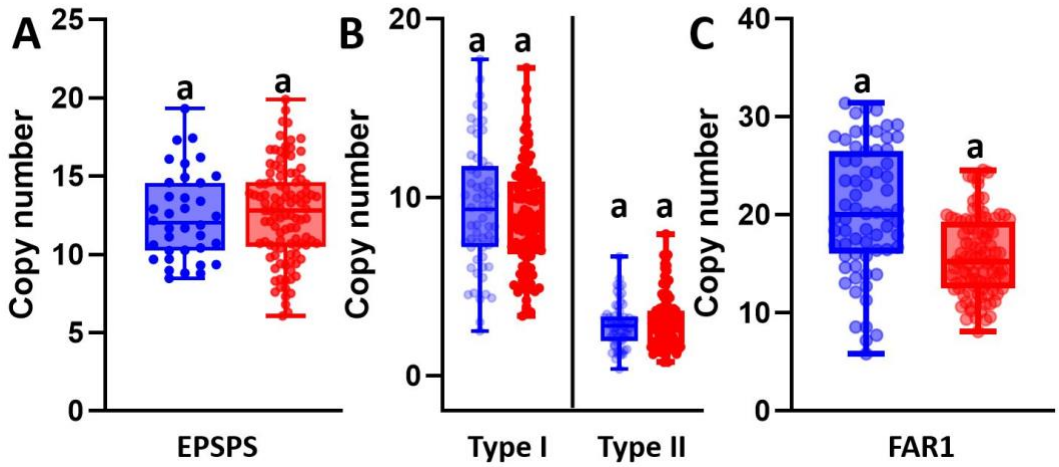

Figure 4.8. *EPSPS* gene copy number.
A) Total EPSPS copy number comparison between parents (blue) and progeny (red) for the years 2018, 2020 and 2022. B), C) Pooled parent and pooled progeny Type I, Type II EPSPS and Far1 copy numbers. Each data point represents individual samples. Means with same letters are not statistically different at the α=0.05 level, using Fisher's protected LSD test.

The copy number variations of the Type I and Type II *EPSPS* segments, and the MGE/*FAR1*

transposable element were investigated. The range in copy numbers of Type I *EPSPS* was similar in the parental and progeny lines (Fig. 4.8B). While the same is true with Type II *EPSPS*, this smaller duplication event is not as common as the Type I duplication (Fig. 4.8B), which accounts for most of the copies of *EPSPS*. This provides evidence for a stable parental *EPSPS* copy number, that at a population level is passed down to the progenies in the same proportion. Finally, there were more *FAR1* copy numbers than the total EPSPS copy numbers (Fig. 4.8A and C), suggesting that *FAR1* is also present in other parts of the genome (Hall *et al.*, 2023). Interestingly, based on qPCR markers, Ravet et al. defined three genotypes of EPSPS gene duplication. Our samples from all three years fall into the category of genotype I, characterized by an increase in EPSPS, Type I and II repeats, and MGE copy numbers corresponding to over 10 *EPSPS* copies (Ravet *et al.*, 2021).

The frequency of self- and cross-pollination was investigated using three specific markers SSRs (2656, 2896, and 1792) across the parent and progeny from the samples collected in 2018, 2020, and 2022. SSR marker analysis requires some interpretation of the peaks. In this study, peaks within ±5 bp from an SSR marker were considered to match that marker, whereas those with a size beyond the ±5 bp threshold were considered different (as illustrated in Fig. 4.9). Plant samples with amplicon size outside this threshold were considered as outcrossing events. For example, the parent (4) had amplicons at 170, 177, 267 and 278. One of its progenies (4a) had amplicons at 169, 177, 263, 272, 278 and 303 which indicates an outcrossing event, another progeny (4c) had 177, 263, 272 and 278 amplicons, which indicates a self-pollinating event (defined as the absence of alleles not found in the parent).

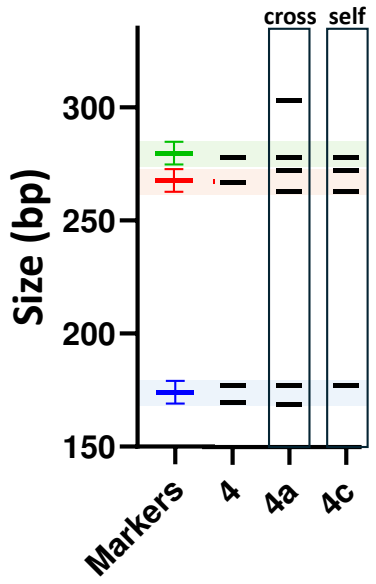

Figure 4.9. Example of an SSR analysis for assessing whether progenies were the result of a self-pollinating or outcrossing event.
The amplicon sizes for the selected SSR markers were 174, 267 and 277. The range considered as matching the markers was ± 5 bp as shown by bars.

Based on the 58 progenies from GR kochia parent lines collected between 2018 and 2022, the outcrossing rate was approximately 78% whereas the estimated self-pollinating rate was 22% (Fig. 4.10). However, this rate of self-pollinating may be an overestimation, as it may combine true self-pollinating events as well as outcrossing events that yielded progenies reporting the same markers as the parents for the three SSR markers evaluated.

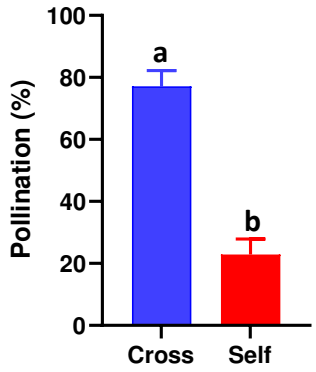

Figure 4.10. Percentages of progeny resulting from outcrossing (blue) and self-pollinating (red) events.
Error bars are ±1 SE of the mean, $n = 58$. Means with different letters are statistically different at the $\alpha=0.05$ level, using Fisher's protected LSD test.

GR kochia biotypes have a transposon-associated CNV of *EPSPS*. On average, parents and progenies have 12-13 copies of *EPSPS*, and most of these duplicated genes have the larger Type I genetic structure. Inheritance of the resistant trait is stable, with the number of copies being similar in the parents and progenies over years, suggesting that populations have sufficient resistance to glyphosate and there has not been selection for greater copy numbers. The wide range in the distribution of the number of *EPSPS* copies can be accounted for by the fact that kochia favors outcrossing, which is encouraged by its protogynous flower structure with stigmas being receptive several days before anthers release their pollen. Additionally, the open configuration of the flower promotes wind dispersal of kochia's small spheroidal anemophilous pollen grains. The high fecundity and wind dispersal of the glyphosate-resistant trait through pollen likely promote the local spread of GR kochia plants (Geddes and Pittman, 2022). However, the westward spread of GR kochia from Kansas to Oregon is against prevailing wind, suggesting that these seeds moved by other means pose significant challenges for weed management in agricultural systems. Strong winds can easily spread kochia seeds in multiple directions, highlighting the pattern of seed dispersal from tumbling mother plants (Stallings *et al.*, 1995a). Effective monitoring of pollen movement could be beneficial in controlling the distribution of resistant traits across fields.

Unchecked seed dispersal of this tumbling weed has potential of contributing to further herbicide resistance development. Integrating herbicides with different modes of actions especially effective soil residual preemergence herbicides, crop rotation, and crop rotation may prevent seed deposition and infestation of GR kochia in soil (Kumar and Jha, 2015a, Kumar and Jha, 2015b).

Table 4.1S. 20-year Meteorological and Annual Climatologies (January 2001 - December 2020) for selected locations.

Location		Range		Dominant	Speed
Latitude	Longitude	Degrees		Degrees	m/s
36.5125	-100.0339	167	275	197	5.39
37.0124	-102.0818	173	290	209	5.20
39.051	-102.0598	150	310	231	4.95
41.0124	-102.0906	145	301	288	4.93
43.0169	-104.0593	146	289	282	4.85
44.2729	-104.0637	168	277	269	4.23
45.9475	-104.0373	165	289	287	5.44
47.4245	-104.0549	178	289	279	5.17
48.9941	-104.0286	245	294	284	5.09

Table 4.2S. Percentage survival of *Kochia scoparia* plants collected across Colorado to assess glyphosate resistance. Kochia seeds were collected in autumn (October-November) from field and roadside locations along transects, with a minimum distance of 16 km between sites. Seeds were harvested and assessed for glyphosate resistance frequency

Smaple #	Lat	Long	#(no. of plants	Site Description	Glyphosate
1	-104.70	39.99	15	Hay/Grazing	0.0
2	-104.62	40.00	10	w wheat	0.0
3	-104.55	40.02	21	wheat fallow	2.8
4	-104.55	40.07	25	w wheat	0.0
5	-104.49	40.20	18	rangeland	1.4
6	-104.57	40.20	21	w wheat	0.0
7	-104.51	40.33	17	irrigated corn	36.1
8	-104.47	40.37	16	irrigated corn, w wheat, oil well	26.4
9	-104.24	40.29	12	irrigated corn	8.3
10	-104.08	40.35	15	irrigated alfalfa	0.0
11	-104.13	40.17	13	w wheat, alfalfa, pasture	15.3
12	-104.35	40.12	23	w wheat	6.9
13	-104.44	40.12	20	irrigated corn	2.8
14	-103.31	39.74	20	dryland corn	29.2
15	-103.21	39.68	20	fallow, dryland corn	29.2
16	-103.03	39.55	20	w wheat	4.2
17	-102.87	39.53	15	grain sorghum	30.6
18	-102.67	39.53	14	dryland corn	1.4
19	-102.52	39.59	18	w wheat	62.5
20	-102.40	39.42	16	irrigated corn	8.3
21	-102.30	39.42	14	irrigated corn	36.1
22	-102.24	39.40	10	fallw, wheat	34.7
23	-102.26	39.51	20	irrigated corn	0.0
24	-102.26	39.72	20	w wheat, corn	4.2
25	-102.37	39.85	30	rangeland, sand hills	2.8
26	-102.39	39.89	25	dryland corn	1.4
27	-102.48	39.98	35	dryland corn	4.2
28	-102.59	40.05	30	irrigated corn	6.9
29	-102.71	40.09	20	w wheat	1.4
30	-102.79	40.01	30	w wheat irrigated	0.0
31	-102.94	39.98	15	w wheat, irrigated corn	8.3
32	-102.97	39.86	20	wheat fallow	2.8
33	-103.13	39.81	10	wheat fallow	0.0
34	-103.29	39.81	18	wheat fallow	56.9
35	-103.42	39.87	20	w wheat	2.8
36	-103.55	39.90	16	w wheat	1.4
37	-103.57	40.03	20	wheat fallow	0.0
38	-105.00	40.62	20	sugar beets	0.0
39	-104.89	40.71	20	wheat fallow/ organic	0.0
40	-104.60	40.61	20	irrigated alfalfa, sugar beets	29.2
41	-104.50	40.05	20	grass hay	0.0
42	-104.14	39.71	20	wheat fallow	0.0
43	-103.70	39.24	30	alfalfa	1.4
44	-103.05	39.13	20	dryland corn	2.8
45	-102.87	39.13	20	wheat fallow	0.0
46	-102.66	39.13	20	w wheat / irrgated corn	61.1
47	-102.49	39.13	20	w fallow/ dryland corn	27.8
48	-102.29	39.13	20	w wheat	4.2
49	-102.79	40.21	24	w wheat	27.8
50	-102.68	40.21	20	irrigated corn	0.0
51	-102.55	40.42	20	wheat fallow	2.8

4.4 CHAPTER CONCLUSION

This research offers valuable insights into the inheritance of glyphosate resistance in Bassia scoparia (kochia), an invasive weed that poses a significant threat to agricultural systems in the high plains of the

United States. By analyzing gene copy number variation (CNV) and outcrossing rates within field populations, we demonstrate the stability of glyphosate resistance across generations, underscoring the resilient and adaptable nature of this species. Our examination of EPSPS gene duplication, particularly the consistent inheritance of Type I and Type II EPSPS gene copies in both parental and progeny populations, indicates that resistance is reliably transmitted, contributing to the persistence of glyphosate-resistant (GR) biotypes in affected areas.

Additionally, our findings reveal that kochia's biological traits, particularly its high outcrossing rate (78%) and its capacity to produce and disperse large amounts of pollen through wind, significantly facilitate the spread of resistance. The combination of pollen-mediated gene flow and the tumbleweed dispersal mechanism enables resistant alleles to proliferate rapidly throughout agroecosystems. This elevated gene flow rate, along with the genetic diversity promoted by outcrossing, presents ongoing challenges for managing glyphosate resistance.

Given the widespread occurrence of GR kochia and its capacity to thrive across various cropping systems and non-crop environments, integrated weed management strategies are essential. As herbicide resistance continues to evolve and create challenges, effective and sustainable weed management will depend on a comprehensive understanding of the mechanisms of driving resistance, as well as the development of innovative strategies to outpace the evolutionary adaptations of troublesome weeds like kochia.

5.1 Summary

This dissertation looks at the molecular and genetic mechanisms underpinning herbicide resistance in plants, with a focus on transcription factor responses and particular resistance mutations in crops and weeds. The second chapter contains a detailed meta-analysis of the role of TFs in plant responses to herbicide-

induced stress. Transcription factors are key regulatory proteins that control the expression of genes involved in a variety of physiological processes. This meta-analysis brings together data from a variety of research on plant species, revealing both general trends and species-specific variances in transcriptional control. Notable transcription factor families, such as WRKY, MYB, and AP2/ERF, consistently play a role in herbicide stress responses by regulating genes involved in detoxification, antioxidant defense, and stress signaling. This chapter provides useful insights into prospective molecular targets for herbicide resistance management by identifying transcription factors and gene networks that are often activated across numerous studies.

Unlike target-site resistance, which alters the herbicide's target directly, NTSR uses indirect mechanisms such as increased detoxification, sequestration, or metabolic alterations to prevent the herbicide from reaching its intended target.

Chapter 3 finds the ZOS1-16 mutation as a unique mechanism that gives modest resistance to the herbicide QPE. The study found that the mutant transcription factor alters the expression of genes involved in detoxification processes and cellular defense systems, lowering the herbicide's efficiency. This mutation causes detoxification enzymes, such as cytochrome P450s and GSTs, to become more active, which is essential for herbicide metabolism and degradation. The findings show that altering transcriptional regulators such as ZOS1-16 could be a more sustainable approach to building herbicide resistance in crops.

Chapter 4 focuses on glyphosate resistance in Kochia, indicating increasing copy number of the EPSPS gene as a significant component contributing to glyphosate resistance in Kochia populations. It underlines the stable inheritance of this enhanced EPSPS gene copy number, which improves glyphosate tolerance and allows for the rapid development of resistance through outcrossing. This study emphasizes the importance of integrated weed management tactics in controlling such highly adaptable resistance characteristics in weeds.

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