

Physiological, genetic, and molecular characterization of differential sensitivity of wheat
(*Triticum aestivum*) to triketone herbicides

by

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B.Sc., Avinashilingam Institute for Home Science and Higher Education for Women, 2014
M.Sc., Bharathiar University, 2016

AN ABSTRACT OF A DISSERTATION

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Abstract

Wheat (*Triticum aestivum*) is a major cash crop grown worldwide for food, feed, biofuel production, etc. Global wheat production is hindered by various biotic stresses, including weed infestation. Post-emergence (POST) application of herbicides is effective for weed management in wheat. Broadening the POST herbicide options is essential to combat the evolution of herbicide-resistant weeds. Triketone herbicides (mesotrione and tembotrione) belong to 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (Group 27) and are widely used for broad-spectrum weed management in corn, which is naturally tolerant to triketones via cytochrome P450 (P450) enzyme-mediated metabolism; however not registered for use in wheat due to crop injury. The objectives of this dissertation were to a) characterize the response of a wide collection of wheat genotypes/lines to triketone herbicide treatment (Chapter 2), b) investigate the genetic basis of differential sensitivity of wheat to triketone herbicides (Chapter 3), and c) functional validation of genes responsible for wheat sensitivity to triketones via genetic engineering (Chapter 4). All experiments were conducted either in the greenhouse, controlled environmental growth chambers or in laboratory conditions. The results indicate that winter wheat genotypes, WW-1, WW-2, and Jagger were least sensitive to these herbicides compared to the most sensitive WW-24, mutant lines (J38 and J327), and spring wheat genotypes (Chapter 2). Importantly, high-performance liquid chromatography analyses suggested that WW-1, WW-2, and Jagger metabolize ~ 70 to 85% of ^{14}C -tembotrione faster than the sensitive genotypes/lines. Further, the application of malathion (P450- inhibitor) followed by triketone herbicide treatment resulted in increased sensitivity of WW-1, WW-2, and Jagger, suggesting that similar to corn, P450 enzymes possibly are involved in metabolizing triketones in wheat as well (Chapter 2). In Chapter 3, the F₁ and F₂ progenies generated by direct and

reciprocal crosses between WW-24 and WW-1 or WW-2 were evaluated for their response to different doses of mesotrione and tembotrione. The results indicate that the reduced sensitivity of WW-1 and WW-2 to these herbicides is a partially dominant, polygenic trait (Chapter 3). In Chapter 4, two genes of interest (GOI), i.e., wheat *CYP81Q32-like* (metabolic gene) and *HPPD* (the target site of these herbicides) were overexpressed in a sensitive spring wheat cultivar via genetic transformation. A total of 216 and 224 transgenic events were generated after bombarding with constructs containing the *CYP81Q32-like* or *HPPD* genes separately. The putative-transformed plants were screened for the presence of GOI. T₀ plants expressing the GOI were selected to generate T₁ plants. Upon phenotypic and genotypic analyses, a few T₁ plants were found to exhibit reduced sensitivity to mesotrione with high transcript expression of the GOI (Chapter 4). Taken together, the outcome of this dissertation provides valuable information that, a) rapid metabolism of triketone herbicides likely mediated by P450 activity, is a partially-dominant polygenic trait that bestows reduced sensitivity of WW-1 and WW-2 to these herbicides, and b) overexpression of *CYP81Q32-like* or *HPPD* genes can reduce the sensitivity of spring wheat to triketones. Importantly, these results also open up opportunities to develop triketone herbicide-resistant wheat cultivars, that can help broad-spectrum weed control without injury to the crop.

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Table of Contents

List of Figures	xii
List of Tables	xvii
Acknowledgements	xx
Dedication	xxii
Chapter 1 - Introduction and Literature review	1
1.1 Importance of Wheat	1
1.2 Weeds: Major Players in Wheat Yield Loss	3
1.3 Herbicide-resistant Technologies in Wheat	5
1.3.1 Clearfield® wheat	6
1.3.2 CoAXium® wheat	7
1.4 Mechanisms of Herbicide Resistance	8
1.4.1 Target site mechanisms	8
1.4.2 Non-target site mechanisms	9
1.5 HPPD-inhibitors	11
1.5.1 HPPD-inhibitor-resistant weeds	13
1.5.2 HPPD-inhibitor-resistant crops and crop technologies	13
1.6 Objectives of This Dissertation	14
Chapter 2 - Characterization of Winter, Spring, and Mutant Lines of Wheat (<i>Triticum aestivum</i>)	
Response to 4-Hydroxyphenylpyruvate Dioxygenase (HPPD)-Inhibitors	17
2.1 Abstract	17
2.2 Introduction	19
2.3 Materials and methods	23
2.3.1 Plant materials	23
2.3.2 Generation of EMS lines of AM2003:	23
2.3.3 Response of winter, spring genotypes and EMS mutant lines of wheat to mesotrione or tembotrione application	24
2.3.4 Mesotrione and tembotrione dose response assay	25
2.3.5 Physiological characterization of wheat response to tembotrione	26
2.3.6 Absorption and translocation of [¹⁴ C]tembotrione	26

2.3.7 Metabolism of [¹⁴ C]tembotrione.....	27
2.3.8 Cytochrome P450- inhibitor assay	28
2.3.9. Target gene (<i>HPPD</i>) expression analysis	29
2.3.10 Statistical analysis	30
2.4 Results.....	31
2.4.1 Variable response of wheat to mesotrione and tembotrione application	31
2.4.2 Mesotrione and tembotrione dose-response assays	32
2.4.3 Absorption and translocation of [¹⁴ C]tembotrione.....	36
2.4.4 Metabolism of [¹⁴ C]tembotrione.....	39
2.4.5 P450 inhibitor assay	42
2.4.6 Target gene (<i>HPPD</i>) expression	44
2.6 Discussion.....	44
2.7 Conclusions.....	49
Chapter 3 - Reduced Sensitivity of Winter Wheat (<i>Triticum aestivum</i>) to Triketone Herbicides is a Partially Dominant, Polygenic Trait	50
3.1 Abstract.....	50
3.2 Introduction.....	52
3.3 Materials and Methods.....	54
3.3.1 Plant materials and growth conditions.....	54
3.3.2 Reciprocal crosses and generation of F ₁ and F ₂ progenies	55
3.3.3 Mesotrione and tembotrione dose-response of WW-1, WW-2, WW-24 and F ₁ progenies	57
3.3.4 Evaluation of segregation of F ₂ progenies	58
3.3.5 Data Analysis	58
3.4 Results.....	60
3.4.1 Phenotypic response of the parents and F ₁ progeny to the dose-response experiments	60
3.4.2 Mesotrione dose-response of WW-1, WW-2, WW-24, and F ₁ progenies.....	63
3.4.3 Tembotrione dose-response of WW-1, WW-24, and F ₁ progenies	66
3.4.4 Segregation of reduced or increased sensitivity to mesotrione of F ₂ progenies.....	69
3.4.5 Segregation of reduced or increased sensitivity to tembotrione of F ₂ progenies	70

3.5 Discussion	78
3.6 Conclusions	81
Chapter 4 - Overexpression of <i>TaHPPD</i> or <i>CYP81Q32-like</i> genes in wheat (<i>Triticum aestivum</i>)	
reduces sensitivity to triketone herbicides	82
4.1 Abstract	82
4.2 Introduction	84
4.3 Materials and method	86
4.3.1 Designing constructs for transformation	86
4.3.2 Cloning plasmids into DH5 α competent cells and colony PCR of recombinant clones	87
4.3.3 Biolistic transformation of wheat	89
4.3.4 Identification of positive transgenic plants	93
4.3.5 Analysis of the positive T ₀ plants for the presence of the GOI	94
4.3.6 Identification of transgenic T ₁ plants and their response to mesotrione application ...	95
4.3.7 Relative expression of the PCR-positive T ₁ plants	97
4.3.8 Design of single guide RNA (sgRNA) targeting <i>CYP81Q32-like</i> gene	98
4.3.9. Restriction digestion of Cas9 vector	100
4.3.10. Synthesis of oligo duplex sgRNAs and ligation into digested vector	102
4.4. Result	102
4.4.1 Colony PCR	102
4.4.2 Plasmid isolation and PCR amplification of the vectors before bombardment	104
4.4.3 Identification of the positive transgenic plants	104
4.4.4 Analysis of the positive T ₀ plants for the presence of the gene of interest	107
4.4.5. Identification of T ₁ transgenic plants	108
4.4.6 The response of the T ₁ transgenic plants to mesotrione application	113
4.4.7. Relative transcript expression of GOI in T ₁ transgenic plants	119
4.4.8. Cloning of the guide RNAs into the vector	121
4.4.9 Whole vector sequencing	121
4.5 Discussion	122
4.6 Conclusion	125
Chapter 5 - General Summary and Conclusions	126

References or Bibliography	131
Appendix A - Supplementary material	144
Appendix B - Supplementary material-II	151

List of Figures

Figure 1.1 Planted acres of wheat by class throughout the United States in 2022 (USDA, 2023a).	2
Figure 1.2 Number of herbicide-resistant weed species by crop (top 10). Graph source: International herbicide-resistant weed database (Heap, 2023).	5
Figure 1.3. Possible mechanisms of herbicide resistance in plants. (A) Target site mutations alter the active site of the protein, which prevents the herbicide from binding successfully; (B) target gene overexpression confers resistance to the herbicide; (C) metabolism (non-target site mechanism) involves various enzymes in the detoxification process; the picture adapted and modified from (Edwards & Onkokesung, 2020). Created using biorender.com.	10
Figure 1.4. Biosynthesis pathways of carotenoids and plastoquinone. Source: (Shino et al., 2021). Created using Biorender. Com.....	12
Figure 2.1 A) Response of winter wheat (WW) genotypes to 6X dose of mesotrione (630 g ai ha ⁻¹ and tembotrione (552 g ai ha ⁻¹) at 2 WAT. B) Response of spring wheat (SW) genotypes to 4X dose of mesotrione (368 g ai ha ⁻¹) and tembotrione (420 g ai ha ⁻¹) at 2WAT.	32
Figure 2.2 A) Mesotrione dose-response of the least sensitive genotypes WW-1, WW-2 along with the most sensitive genotype WW-24 based on the aboveground biomass reduction at 4 WAT. B) Tembotrione dose-response of the least sensitive genotypes WW-1, WW-2 along with the most sensitive genotype WW-24 based on the aboveground biomass reduction at 4 WAT.	33
Figure 2.3. A) Mesotrione dose-response of the least sensitive genotype Jagger along with the most sensitive lines J38, J327 based on aboveground dry biomass reduction at 4WAT. B) Tembotrione dose-response of the least sensitive genotype Jagger along with the most sensitive lines J38, J327 based on aboveground dry biomass reduction at 4WAT.	35
Figure 2.4. A) [¹⁴ C] Tembotrione absorption in corn, Jagger, J38, J327. A rectangular hyperbola was utilized to analyze percent [¹⁴ C] Tembotrione absorption at 6, 24, and 48 HAT. B) [¹⁴ C] Tembotrione translocation in corn, Jagger, J38 & J327 at 6, 24 & 48 HAT.	39

Figure 2.5. A) HPLC chromatographs depict resolved [^{14}C] tembotrione parent compound (11.4 min) and its metabolites M1 and M2 at 24 HAT. B) Percent parent compound remaining at 24 and 48 HAT in WW-1, WW-2, and WW-24.	40
Figure 2.6. HPLC chromatographs depict resolved [^{14}C] tembotrione parent compound (11.4 min) and its metabolites M2 and M3 at 24 HAT in corn.	41
Figure 2.7. A) HPLC chromatographs depicting resolved [^{14}C] tembotrione parent compound (11.4 min) and its metabolites M1 and M2 at 48 HAT. B) Percent parent compound remaining at 6, 24, and 48 HAT in Jagger, J327, and J38.	41
Figure 2.8. A, B) Aboveground dry biomass of Jagger, J38, and J327 when pre-treated with tembotrione or mesotrione and herbicide plus malathion at 4 WAT. The error bars represent the standard error; different alphabets indicate a significant difference between treatments ($p \leq 0.01$) (left); Response of Jagger, J38, J327 to 6X doses of tembotrione (TT) or mesotrione (MT), malathion at 2000 (T1) and 4000 g ai ha $^{-1}$ (T2) prior to herbicide treatment at 3 WAT (right). C, D) Above-ground dry biomass of WW-1, WW-2, and WW-24 when pre-treated with tembotrione or mesotrione, herbicide plus malathion at 4 WAT. The error bars represent the standard error; different alphabets indicate a significant difference between treatments ($p \leq 0.01$) (left); Response of WW-24, WW-1, and WW-2 to 6X doses of tembotrione (TT) or mesotrione (MT), malathion at 2000 (T1) and 4000 g ai ha $^{-1}$ (T2) prior to herbicide treatment at 3 WAT (right).	43
Figure 2.9. Target gene (<i>HPPD</i>) expression in WW-1 and WW-2 relative to WW-24. Bars represent mean $\pm$ SE of 3-4 biological replicates.	44
Figure 3.1. Schematic diagram illustrating the generation of F $_1$ and F $_2$ families in a crossing program using WW-1, WW-2 (less sensitive), and WW-24 (more sensitive) winter wheat [Created with BioRender.com].	56
Figure 3.2. The response of A) winter wheat genotypes (WW-1, WW-2, and WW-24) and F $_1$ (F $_1$ -1, F $_1$ -2, F $_1$ -3, and F $_1$ -4) progenies to different doses of mesotrione at 2 weeks of treatment. B) winter wheat genotypes and F $_1$ progenies to different doses of mesotrione at 4 weeks of treatment.	61
Figure 3.3. The response of A) winter wheat genotypes (WW-1, WW-2, and WW-24) and F $_1$ (F $_1$ -1, F $_1$ -2, F $_1$ -3, and F $_1$ -4) progenies to different doses of tembotrione at 2 weeks of treatment.	

B) winter wheat genotypes and F ₁ progenies to different doses of tembotrione at 4 weeks of treatment.	62
Figure 3.4. A) Mesotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-1, WW-24, F ₁ -1, and F ₁ -2 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight. B) Mesotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-2, WW-24, F ₁ -3, and F ₁ -4 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight.	64
Figure 3.5. A) Tembotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-1, WW-24, F ₁ -1, and F ₁ -2 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight. B) Tembotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-2, WW-24, F ₁ -3, and F ₁ -4 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight.	67
Figure 3.6. Distribution of cumulative dry biomass accumulation of WW-24 (most sensitive), WW-1 (least sensitive), F ₂ -1, and F ₂ -2 progenies in response to 6X dose of mesotrione (A & B) and tembotrione (C & D) <i>via</i> density plots. The relative dry weight reduction of samples was converted into bins and plotted on the X-axis. The Y-axis was plotted by calculating density which is the proportion of total plants in each bin. The dotted lines represent the mean dry weight of WW-1, WW-24, F ₂ -1, and F ₂ -2.	77
Figure 3.7. Distribution of cumulative dry weight reduction of WW-24 (most sensitive), WW-2 (less sensitive), F ₂ -3, and F ₂ -4 progenies in response to 6X dose of mesotrione (A & B) and tembotrione (C & D) <i>via</i> density plots. The relative dry weight reduction of samples was converted into bins and plotted on the X-axis. The Y-axis was plotted by calculating density which is the proportion of total plants in each bin. The dotted lines represent the mean dry weight of WW-2, WW-24, F ₂ -1, and F ₂ -2.	78
Figure 4.1. Schematic representation of the vector containing the gene of interest (gene of interest synthesized, codon-optimized, and cloned in vector at GenScript).	87
Figure 4.2. Steps involved in biolistic transformation followed by <i>in vitro</i> regeneration.	92

Figure 4.3. Liberty painting process of the putative transgenic plants.	94
Figure 4.4. The schematic representation of screening for positive plants with GOI and response of T ₁ plants to 6X mesotrione application.	97
Figure 4.5. The highlighted regions of the cDNA of the <i>CYP81Q32-like</i> gene indicates the target region of sgRNA 1 (green) and sgRNA 2 (blue).	99
Figure 4.6. The <i>Bsa</i> I recognition sequence and the enzyme recognition region in the vector backbone. The blue crossed-off portion is the fragment that has been removed from the vector after restriction digestion with the enzyme <i>Bsa</i> I. Thus, 5' AGCG 3' and 5' AAAC 3' are added on the top and bottom strands of the sgRNAs so that they are complementary to the overhangs on the vector backbone, which would allow ligation of oligo duplex at this site.	100
Figure 4.7. Physical map of p0390sgCas9 vector used in the CRISPR/Cas9 experiment for cloning sgRNA1 and sgRNA 2.	101
Figure 4.8. The gel image showing the digested plasmid p0390sgCas9 as the bright band and the lighter band at ~1200 bp is the released fragment from the vector backbone. The bright band was extracted from the gel for further analyses.	101
Figure 4.9. The gel image shows the PCR amplification of the colonies containing the vector PA17_TaHPPD with primers UbiABF and PA17_TaHPPD RE1.	103
Figure 4.10. The gel image showing the PCR amplification of the colonies containing the vector PA17_CYP81Q32 with primers PA17_CYP81Q32 FW1 and RE1.	104
Figure 4.11. Agarose gel images to illustrate the presence of plasmids. The gel picture on the left shows the plasmids (PA17_TaHPPD and PA17_CYP81Q32 isolated from the colonies. The gel picture on the right shows the PCR amplification of the isolated plasmids.	104
Figure 4.12. Results of Liberty® painting on T ₀ plants. A) The plant that contained the <i>bar</i> gene was resistant to the Liberty herbicide and did not show burning symptoms. B) The plant that did not contain the <i>bar</i> gene was susceptible to the Liberty herbicide and showed burning symptoms.	105
Figure 4.13. The gel image illustrating the presence of the <i>bar</i> gene in transgenic plants T ₀ -HP-1 to 10 bombarded with the PA17_TaHPPD plasmid.	106
Figure 4.14. The gel image illustrating the presence of the <i>bar</i> gene in transgenic plants T ₀ -P-1 to 10 bombarded with PA17_CYP81Q32 plasmid.	106

Figure 4.15. The gel image illustrating the PCR amplification and presence of the GOI (<i>TaHPPD</i>) in transgenic plants T ₀ -HP-6, T ₀ -HP-9, and T ₀ -HP-10.	107
Figure 4.16. The gel image illustrating the PCR amplification and presence of the GOI (<i>CYP81Q32-like</i>) in transgenic plants T ₀ -P-4, T ₀ -P-5, T ₀ -P-7, and T ₀ -HP-8.	108
Figure 4.17. PCR analysis to detect the presence of A) the transgene <i>TaHPPD</i> in the T ₁ plants HP-6-1 to HP-6-15 and HP-9-1 to HP-9-15, B) The <i>bar</i> gene in the T ₁ plants HP-6-1 to HP-6-15 and HP-9-1 to HP-9-15, C) <i>TaHPPD</i> in the T ₁ plants HP-10-1 to HP-10-221, and D) The <i>bar</i> gene in the T ₁ plants HP-10-1 to HP-10-28.	109
Figure 4.18. PCR analysis to detect the presence of A) the transgene <i>CYP81Q32-like</i> in the T ₁ plants P-8-1 to HP-8-17 and P-7-1 to P-7-14 and B) the <i>bar</i> gene in the T ₁ plants P-8-1 to HP-8-17 and P-7-1 to P-7-14.	113
Figure 4.19. A) The response of the HP-6 transgenic T ₁ lines to 6X dose of mesotrione at 3 weeks after treatment. B, C) The recovery of the HP-6 transgenic at 4 weeks after treatment.	115
Figure 4.20. A) The response of the HP-9 transgenic T ₁ lines to 6X dose of mesotrione at 3 weeks after treatment. B, C) The recovery of the HP-9 transgenic T ₁ lines at 4 weeks after treatment.	116
Figure 4.21. A, B) The response of the HP-10 transgenic T ₁ lines to 6X dose of mesotrione at 3 weeks after treatment. C, D) The recovery of the HP-10 transgenic T ₁ lines at 4 weeks after treatment.	117
Figure 4.22. A, B) The response of the P-8 transgenic T ₁ lines to 6X dose of mesotrione at 3 weeks after treatment. C, D) The recovery of the P-8 transgenic T ₁ lines at 4 weeks after treatment.	118
Figure 4.23. Relative expression of transgene <i>TaHPPD</i> in T ₁ plants. Relative expression was estimated using the $\Delta\Delta C_t$ method, with actin as the reference gene.	120
Figure 4.24. Relative expression of transgene <i>CYP81Q32-like</i> in T ₁ plants. Relative expression was estimated using the $\Delta\Delta C_t$ method, with actin as the reference gene.	120
Figure 4.25. The gel image illustrating the PCR amplification of the positive colonies containing the guide RNA (sgRNA 1) cloned into the p0390sgCas9 vector.	121

List of Tables

Table 2-1. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1, WW-2 (least sensitive) winter wheat genotypes aboveground dry biomass to different rates of mesotrione at 4 weeks after treatment (WAT).	33
Table 2-2. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1, WW-2 (least sensitive) winter wheat genotypes aboveground dry biomass to different rates of tembotrione at 4 weeks after treatment (WAT).	34
Table 2-3. Summary of regression parameters describing the response of Jagger (least sensitive), Jagger-EMS-38, and Jagger-EMS-327 (most sensitive) lines aboveground dry biomass to different rates of mesotrione at 4 weeks after treatment (WAT).	35
Table 2-4. Summary of regression parameters describing the response of Jagger (least sensitive), J38, and J327 (most sensitive) lines aboveground dry biomass to different rates of tembotrione at 4 weeks after treatment (WAT).	36
Table 2-5. Regression parameters of [¹⁴ C]tembotrione absorption in WW-24 (most sensitive), WW-1, and WW-2 (least sensitive) genotypes.	37
Table 2-6. Comparison of p-values of A _{max} and A ₉₀ in WW-24 (most sensitive), WW-1, and WW-2 (least sensitive) lines.	37
Table 2-7. Regression parameters of [¹⁴ C]tembotrione absorption in Jagger (least sensitive), Jagger-EMS-38, and Jagger-EMS-327 (most sensitive) lines.	38
Table 2-8. Comparison of p-values of A _{max} and A ₉₀ in Jagger (least sensitive), Jagger-EMS-38, and Jagger-EMS-327 (most sensitive) lines	38
Table 3-1. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1 (least sensitive), F ₁ -1, and F ₁ -2 aboveground dry biomass in response to different doses of mesotrione at 4 weeks after treatment (WAT). (GR ₅₀ : dose required for 50% growth reduction; SE: standard error).	65
Table 3-2. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-2 (least sensitive), F ₁ -3, and F ₁ -4 aboveground dry biomass to different doses of mesotrione at 4 weeks after treatment (WAT). (GR ₅₀ : dose required for 50% growth reduction; SE: standard error).....	66

Table 3-3. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1 (least sensitive), F ₁ -1, and F ₁ -2 aboveground dry biomass to different doses of tembotrione at 4 weeks after treatment (WAT). (GR ₅₀ : dose required for 50% growth reduction; SE: standard error).....	68
Table 3-4. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-2 (least sensitive), F ₁ -3, and F ₁ -4 aboveground dry biomass to different rates of tembotrione at 4 weeks after treatment (WAT). (GR ₅₀ : dose required for 50% growth reduction; SE: standard error).....	69
Table 3-5. Chi-square test for goodness of fit of the observed segregation of plants as least (10% to 30%) or most (31% to 50%) sensitive to mesotrione for a single-locus model in F ₂ -1 and F ₂ -2 families of winter wheat. The expected survival frequency for each F ₂ family was calculated and compared with the observed frequency of differential sensitivity to mesotrione.	71
Table 3-6. Chi-square test for goodness of fit of the observed segregation of plants as least sensitive (10% to 30%) or most sensitive (31% to 50%) to mesotrione to the expected frequency for a single-locus model in F ₂ -3 and F ₂ -4 families of winter wheat. The expected survival frequency for each F ₂ family was calculated and compared with the observed frequency of differential sensitivity to mesotrione.	72
Table 3-7. Chi-square test for goodness of fit of the observed segregation of plants as less sensitive (5% to 20%) or most sensitive (21% to 40%) to tembotrione to the expected frequency for a single-locus model in F ₂ -1 and F ₂ -2 families of winter wheat. The expected survival frequency for each F ₂ family was calculated and compared with the observed frequency of differential sensitivity to tembotrione.....	73
Table 3-8. Chi-square test for goodness of fit of the observed segregation of plants as less sensitive (5% to 20%) or most sensitive (21% to 40%) to tembotrione the expected frequency for a single-locus model in F ₂ -3 and F ₂ -4 families of winter wheat. The expected survival frequency for each F ₂ family was calculated and compared with the observed frequency of differential sensitivity to mesotrione.	75
Table 4-1. The description of primer sets used for the detection of the presence of the transgene	90

Table 4-2. Media composition used in the plant tissue culture. A brief description of components used in the tissue culture media was obtained from Tian et al., 2019.	91
Table 4-3. The primer sets used for the quantitative expression (RT-qPCR) of GOI.	96
Table 4-4. The oligo sequence of the top and bottom strands of the sgRNAs	99
Table 4-5. A brief description of whether the T ₁ plants HP-6-1 to HP-6-15 contain the vectors PA17_TaHPPD (GOI) and PAHC20 (<i>bar</i> gene). (+ = presence of the vector and - = absence of the vector)	110
Table 4-6. A brief description of whether the T ₁ plants HP-9-1 to HP-9-15 contain the vectors PA17_TaHPPD and pAHC20. (+ = presence of the vector and - = absence of the vector)	110
Table 4-7. A brief description of whether the T ₁ plants HP-10-1 to HP-10-21 contain the vectors PA17_TaHPPD and pAHC20. (+ = presence of the vector and - = absence of the vector)	111
Table 4-8. brief description of whether the T ₁ plants P-8-1 to P-8-17 contain the vectors PA17_TaHPPD (GOI) and pAHC20 (<i>bar</i> gene). (+ = presence of the vector and - = absence of the vector)	112

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Dedication

I dedicate this dissertation to my mother, Mrs. S. Chitra, my father, Dr. D. Sudhakar, and my sister, Shreya for their love, support, prayers, and sacrifices.

Chapter 1 - Introduction and Literature review

1.1 Importance of Wheat

Wheat is one of the three major cereal crops grown worldwide along with maize and rice (Shewry, 2009). Total wheat production in 2023/24 is estimated to be ~800.2 million metric tons (MMT) compared with 1214.5 million tonnes of maize and 520.9 million tons (milled basis) of rice. It also ranks third in the United States after corn and soybean in terms of planted acreage, gross farm receipts, and with an annual production of ~1.81 billion bushels (USDA, 2023a, b). Kansas is the largest winter wheat-producing state in the US with a production of 191 million bushels (USDA, 2023a). About 95% of the total cultivated wheat in the US is hexaploid bread wheat (*Triticum aestivum*) and the remaining 5% is tetraploid durum wheat (*Triticum durum*) or pasta wheat (Laghetti et al., 2009).

Wheat is a temperate crop primarily grown for human consumption and livestock feed, nonetheless, it is also used for ethanol production, brewing beer, cosmetics, wheat proteins in meat substitutes, etc. (Shewry, 2009). Approximately 90% of the total wheat produced is used for human consumption (Igrejas et al., 2020). Almost one-third of the world's population receives calories and proteins from wheat compared to other cereal crops (Kumar et al., 2011). Moreover, it is also considered a good source of minerals, vitamin B complex, beneficial phytochemicals, dietary fiber, and macro- and micro-nutrients (Shewry, 2007).

Wheat belongs to the family Poaceae which evolved around 50 million years ago (Kellogg, 2001). About 10,000 years ago, the domestication of wheat first started in the fertile crescent (Middle East region) (Carver, 2009). Wild emmer wheat (*Triticum dicoccoides*, $2n=4x=28$) containing the genome AABB was initially the cultivated species, and eventually was hybridized with goatgrass (*Aegilops tauschii*, $2n=2x=14$) containing the genome DD to produce

hexaploid (AABBDD) wheat (Feldman & Kislev, 2007). Wheat is a model species for the evolutionary theory of allopolyploid speciation and adaptation in plants (Carver, 2009).

Wheat is classified into two types- winter wheat and spring wheat based on the season they are grown (Entz & Fowler, 1991). Winter wheat is planted in late fall and harvested in early summer, whereas spring wheat is planted in the spring and harvested in late summer or fall (Weiss et al., 2003). The total harvested area of wheat including winter, spring, and durum in 2023 was 37.3 million acres (USDA, 2023a). The different classes of wheat grown across the US in 2022 are shown in Figure 1. The protein content and purpose of usage vary depending on the type of wheat. Spring wheat is higher in protein content which makes it desirable for baking whereas spring wheat has high yield potential (Koppel & Ingver, 2008).

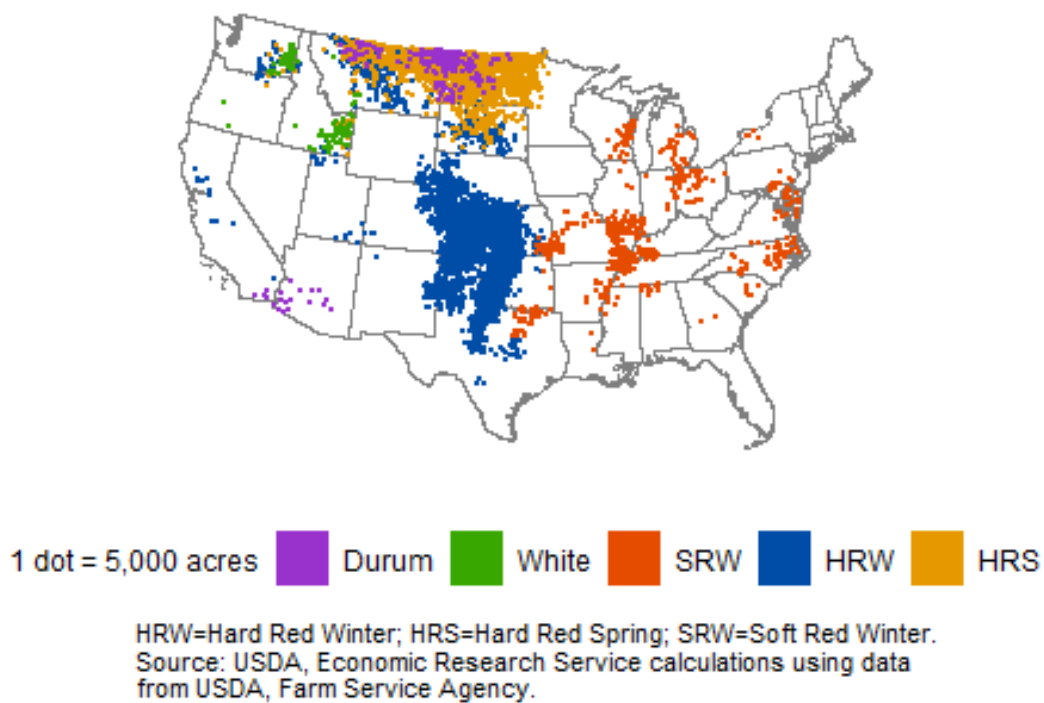


Figure 1.1 Planted acres of wheat by class throughout the United States in 2022 (USDA, 2023a).

1.2 Weeds: Major Players in Wheat Yield Loss

Crop production in agriculture has been under constant threat due to the lack of availability of arable lands along with biotic and abiotic stresses. Numerous biotic and abiotic stress factors pose a challenge to crop productivity. Abiotic stresses such as drought, heat, cold, and salinity can reduce crop yields by up to 51% to 82% (Oshunsanya et al., 2019; Suzuki et al., 2014). Further, biotic factors such as pathogens, insects, and weeds have also a negative impact on crop production (Pandey et al., 2017). Among the various biotic stresses, weeds are the most troublesome as they compete with crops for nutrition, space, water, sunlight, etc., (Oerke, 2006). Weed infestation is estimated to cause a significant amount of yield up to 23% in wheat (Oerke, 2006). About 23.4% of yield loss was projected in winter wheat in the US and Canada combined, whereas 19.5% was estimated in spring wheat (Flessner et al., 2021). The potential annual loss in value for spring wheat and winter wheat due to weeds was estimated to be \$2.19 and \$1.14 billion, respectively (Flessner et al., 2021)

Common weed species found in wheat include jointed goatgrass (*Aegilops cylindrica*), cereal rye (*Secale cereale* L.), Japanese brome (*Bromus japonicus*), downy brome (*Bromus tectorum* L.), foxtail (*Setaria spp.*) *Lolium perenne* spp., Henbit (*Lamium amplexicaule*), kochia (*Kochia scoparia*), Russian thistle (*Salsola tragus* L.), mustard *spp.* etc., (Appleby, 2005; Conley & Bradley, 2005; Ogg & Jackson, 2001; Reddy et al., 2013; Rydrych, 1974; Swan, 1971). The application of post-emergence herbicides was reported to be effective for weed control in wheat (Flessner et al., 2021). The commonly used herbicides in wheat cropping systems include synthetic auxin herbicides, acetolactate synthase (ALS), microtubule-, photosystem II (PS-II)-, and very long-chain fatty acid synthesis (VLCFA)-inhibitors (Flessner, et al., 2021). Herbicides are the most widely used pesticides in wheat; about 61, 96, and 93% of planted acres of winter

wheat, spring wheat, and durum wheat are sprayed with herbicides across the US annually (USDA-NASS, 2017). However, the repeated usage of herbicides has resulted in selection pressure eventually leading to the evolution of herbicide-resistant weeds.

Several weed species have developed resistance to herbicides that are commonly used in wheat. Downy brome has evolved resistance to multiple herbicides, but most commonly to the triazine herbicides, aryloxy phenoxy propionate (APP) herbicides, cyclohexanedione (CHD) herbicides, and ALS- inhibitors (Ball et al., 2007; Kumar & Jha., 2017; Park & Mallory-Smith, 2005). Japanese brome (*Bromus japonicus*) has developed resistance to the ALS-inhibiting herbicide flucarbazone-sodium; the resistant population exhibited ~120-fold resistance to this herbicide (Lan et al., 2022). A study showed that henbit could tolerate higher doses of ALS-inhibiting herbicides such as chlorsulfuron, imazamox, and propoxycarbazone (Varanasi et al., 2016). Mustard spp. has evolved resistance to several auxinic herbicides, ALS inhibitors, and triazines (Dang et al., 2017; Rosario et al., 2011; Zheng & Hall., 2001). A kocio population from Nebraska was found to be resistant to auxinic herbicides such as 2,4-D, dicamba, and fluroxypyr (LeClere et al., 2018). Further, another population from Illinois was resistant to PS II- and ALS inhibitors belonging to the chemical families triazine, imidazolinone, and sulfonyleurea (Foes et al., 1999). Shortawn foxtail (*Alopecurus aequalis*) populations that were moderately resistant to sulfonyleureas and triazolopyrimidines, and highly resistant to ACCase inhibitors were identified (Xia et al., 2015). *Lolium* spp has been reported to have bestowed with resistance to multiple herbicides including ACCase-, microtubule-, PS-II-, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)-, glutamine synthetase-, very-long-chain fatty acid (VLCFA)-, and photosystem I (PS-I)- inhibitors (Han et al., 2021). Herbicide-resistant weeds are not only common in wheat but have also been reported in 99 different crops worldwide (Heap, 2023). The

number of herbicide-resistant weed species by crop is depicted in Figure 1.2. About 269 weed species have developed resistance to 21 different herbicide target sites (Heap, 2023). This issue threatens the use of the available herbicide options in crops. Therefore, diversifying the herbicide options in crops, especially in wheat, is necessary. Additionally, developing herbicide-resistant-wheat technologies that would facilitate the use of alternate herbicide options to control herbicide-resistant weeds efficiently is crucial.

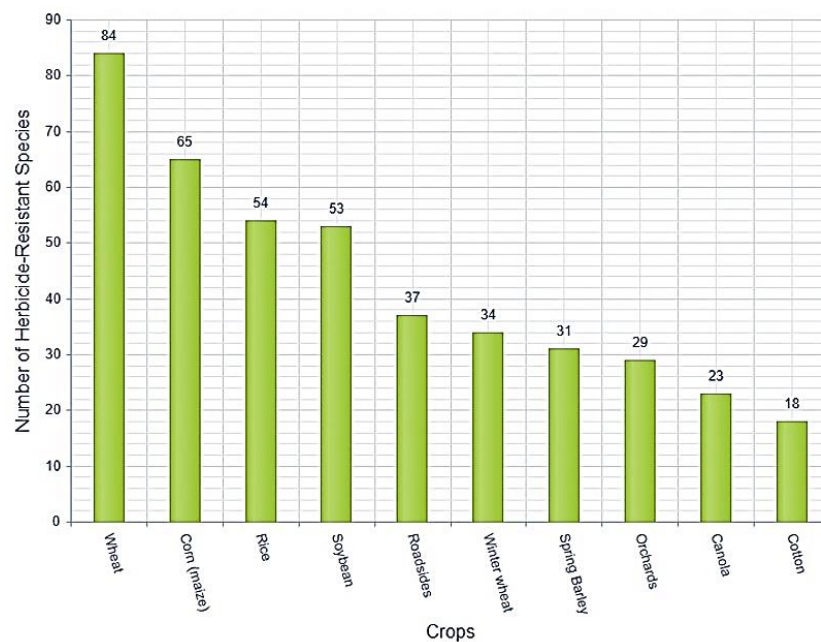


Figure 1.2 Number of herbicide-resistant weed species by crop (top 10). Graph source: International herbicide-resistant weed database (Heap, 2023).

1.3 Herbicide-resistant Technologies in Wheat

Herbicide-resistant (HR) crop technologies developed *via* non-transgenic methods such as seed mutagenesis, pollen mutagenesis, tissue culture, cell selection, transfer of genes from a weedy relative, etc., and transgenic methods *via* genetic engineering have been promising in

weed management in several crops (Duke, 2005; Jhala et al., 2021). Among these, seed mutagenesis involving ethyl methane sulfonate (EMS) treatment is the most common method to develop HR crops. EMS is a chemical mutagen that causes random (non-targeted) point mutations throughout the DNA sequence (Chen et al., 2023); the mutations can sometimes confer resistance to herbicides. This method has been used to successfully develop HR wheat cultivars. The two wheat technologies currently available in the market are 1) the Clearfield® production system and 2) the CoAXium® production system (Nakka et al., 2019).

1.3.1 Clearfield® wheat

Clearfield® technology was first developed in corn (*Zea mays*) by BASF in the 1990s. This technology was eventually used in other crops such as sunflowers, rice, canola, and wheat. The Clearfield® wheat cultivars are resistant to the herbicide imazamox (Beyond), which belongs to the chemical family imidazolinone of ALS-inhibitors. The amino acid substitution serine653asparagine in the ALS gene confers resistance to this herbicide in wheat (Sudianto et al., 2013). The ALS enzyme, is also referred to as acetohydroxyacid synthase (AHAS), plays a major role in the biosynthesis of branched-chain amino acids such as isoleucine, valine, and leucine (Tranel & Wright., 2002). ALS inhibition due to the interference of the herbicides could lead to plant death. Clearfield® wheat being resistant to imazamox is unaffected, but the grass and the broad-leaf weeds are controlled effectively. The herbicide imazamox controls weeds such as downy brome, feral rye (cereal rye), jointed goatgrass, mustards, and volunteer wheat. Further, the Clearfield®plus wheat containing the point mutations in two genomes has also been developed (Nakka et al., 2019).

1.3.2 CoAXium® wheat

CoAXium® wheat was developed by the collaborative effort of the Wheat Research Foundation, Colorado State University (CSU), Albaugh LLC, and Limagrain Cereal Seeds. These wheat cultivars are resistant to the herbicide quizalofop-p-ethyl (Aggressor). This herbicide is an ACCase inhibitor belonging to the chemical family aryloxyphenoxypropionates. A point mutation resulting in the amino acid substitution alanine²⁰⁰⁴valine confers resistance to this herbicide (<http://www.coaxiumwps.com/stewardship/>). ACCase enzyme plays a major role in lipid biosynthesis; it catalyzes the conversion of acetyl co-enzyme A to malonyl co-enzyme A, the first step in fatty acid synthesis. The interference of ACCase inhibitors with fatty acid synthesis results in the inability to synthesize cell membranes leading to the death of the plant (Sasaki & Nagano., 2004; Secor & Cseke., 1988). CoAXium® wheat contains mutations in two genomes and is highly tolerant to quizalofop-p-ethyl. This system allows the use of the herbicide Aggressor to control many grass weeds such as downy brome, jointed goatgrass, rye, etc., effectively (Nakka et al., 2019).

Currently, transgenic wheat is not available commercially. Roundup Ready® wheat resistant to glyphosate was developed *via* a genetic engineering technique by Monsanto (Duke, 2018). The MON 71800 event were developed by introducing the *CP4EPSP* gene of *Agrobacterium* to the spring wheat cultivar ‘Bobwhite’. The *CP4EPSPS* was insensitive to glyphosate and could tolerate higher doses of glyphosate. Even though the use of this transgenic wheat would have helped control several weed species, it was terminated for commercial use due to various reasons such as the risk of outcrossing into non-GMO wheat, gene flow to wild species, high seed cost, consumer rejection, etc., (Nakka et al., 2019; Ogg & Isakson, 2001; Van Acker et al., 2014). The lack of HR wheat systems hinders the use of a broad range of herbicides.

Therefore, developing HR wheat by exploring different herbicide targets can mitigate this issue. The HR wheat discussed so far has been manipulated (mutation) at the target gene. Future technologies involving the manipulation of herbicide non-target genes, such as those that are involved in the degradation (metabolism) of herbicides will also be beneficial. An accurate understanding of HR mechanisms would help in the application of this knowledge in developing HR crop technology.

1.4 Mechanisms of Herbicide Resistance

Over the years, due to the repeated use of herbicides, weeds have evolved multiple mechanisms to cope with herbicide stress (Gressel & Segel., 1978). The two major mechanisms include alteration to the target site or those that are not the target for herbicides. These mechanisms are not only applicable to HR weeds, but they can also prevail in any plant. They offer advantages to the plants to evade herbicide-related stress by making them less sensitive to the herbicide (Gaines et al., 2020).

1.4.1 Target site mechanisms

Herbicide target sites are primarily enzymes or proteins that play a major role in a plant's growth and development. Target site modifications include point mutations, deletions, increased expression, and amplification of the gene coding for the herbicide target proteins (Gaines et al., 2020; Nakka et al., 2019; Nandula, 2010; Patzoldt et al., 2006). Alterations such as point mutation and deletion in the molecular target decrease the affinity of the enzyme for the herbicide whereas increased expression or copy number would require higher doses of herbicide to achieve a lethal effect (Laforest et al., 2017). For instance, scientists have discovered 29 amino acid changes at eight different locations in the *ALS* gene that confer resistance to ALS inhibitors. Target site mutations Ile-2041 and Gly-2096 in the ACCase gene are known to confer resistance

to herbicides belonging to the FOP chemistry in some weed species (Délye., 2005). In the 5-enolpyruvylshikimate 3-phosphate synthase (*EPSPS*) gene, double mutations are known to show a better resistance than single mutations; a Thr-102-Ile mutation along with Pro-106-Ser decreases the plants' sensitivity to glyphosate (Heap & Duke., 2018).

1.4.2 Non-target site mechanisms

Non-target site resistances involve all mechanisms that do not involve the target site but rather affect physiological processes hindering herbicide reaching the target site (Jugulam & Shyam., 2019). These include altered herbicide uptake or translocation, enhanced metabolism, or sequestration into vacuoles (Rigon et al., 2020). Most crops and weed species are tolerant to herbicides *via* metabolism imparted by enzymes such as cytochrome P450s (P450) and glutathione-S-transferases (GSTs) (Nakka et al., 2017a). They detoxify any xenobiotic substances including herbicides that enter the plant system. The members of P450 are a family of enzymes responsible for Phase I metabolism in plants; they mediate the hydroxylation or oxidation of the parent herbicide, rendering them less toxic (Siminszky, 2006). The Phase II metabolism, majorly imparted by GSTs, increases the polarity of the metabolites; this occurs through the conjugation of glucose, amino acids, or glutamyl-cysteinyl glycine to the Phase I products (Zhang & Yang., 2021). Further, this leads to Phase III metabolism, where the conjugates are transported to the vacuole (Riechers et al., 2005). The *CYP81A10v7* gene in the weed rigid ryegrass (*Lolium rigidum*) is known to impart resistance to a broad spectrum of herbicides belonging to ACCase-, ALS-, PSII-, microtubule-, and hydroxyphenylpyruvate dioxygenase HPPD-inhibitor (Han et al., 2021). In corn, the *CYP81A9* (*Nsf1*) gene confers tolerance to several herbicides belonging to at least four modes of action such as nicosulfuron, primisulfuron, mesotrione, dicamba+diflufenzopyr, carfentrazone, and bentazone (Choe &

Williams, 2020). In rice, the overexpression of the GST gene, *OsGSTL2* conferred resistance to glyphosate and chlorsulfuron (Hu., 2014). Several studies have shown that expressing a P450 from an unrelated species conferred resistance to herbicides; transgenic potatoes that co-expressed three human P450 genes *CYP1A1*, *CYP2B6*, and *CYP2C19* showed resistance to various herbicides (Inui & Ohkawa., 2005). Mutations in P450 genes conferring resistance to herbicides have been reported but need to be further explored (León et al., 2008). As these genes make up about 1% of the plant genome, studying and comparing the gene sequences of the resistant and susceptible plants will facilitate the possibility of targeting them for developing HR crops *via* gene editing or genetic engineering (Ghosh, 2017). P450 inhibitors such as malathion, piperonyl butoxide (PBO), tetcyclasis (TCT), etc., have been routinely used to assess the metabolic resistances to herbicides in several crop/weed species (Pandian et al., 2020a; Shyam et al., 2021a). Inhibitor treatment before herbicide application would competitively reduce the P450 activity eventually decreasing the herbicide metabolism (Siminszky, 2006). The mechanisms of target and non-target resistances are depicted in Figure 1.3.

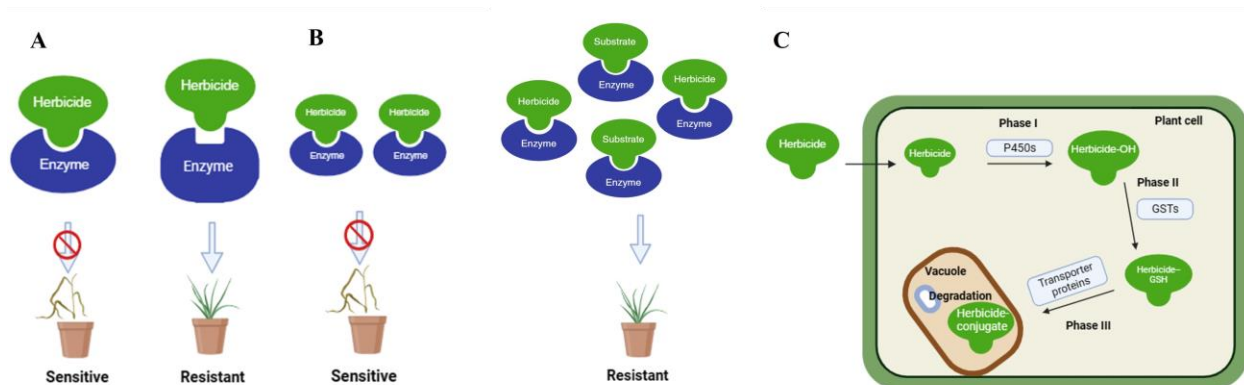


Figure 1.3. Possible mechanisms of herbicide resistance in plants. (A) Target site mutations alter the active site of the protein, which prevents the herbicide from binding successfully; (B) target gene overexpression confers resistance to the herbicide; (C) metabolism (non-target site

mechanism) involves various enzymes in the detoxification process; the picture adapted and modified from Edwards & Onkokesung (2020). Created using biorender.com.

1.5 HPPD-inhibitors

The chemical leptospermone was discovered in 1977 when scientists accidentally discovered bleaching symptoms in plants growing under the bottle brush tree (*Calistemon citrinus*). The naturally occurring syncarpic acid of leptospermone was combined with the benzoyl moiety to develop the triketone chemical of HPPD-inhibitors with high herbicidal activity (Jhala et al., 2023; Knudsen et al., 2000). This herbicide was later introduced in the late 1990s to control a wide variety of weed species. HPPD-inhibitors belong to Group 27 inhibitors. These inhibitors prevent the conversion of 4-hydroxyphenylpyruvic acid (HPPA) into homogentisic acid (HGA) (Lindblad et al., 1958) which is a precursor in the formation of Plastoquinone, tocopherols, and tocopherolquinones (Whistance & Threlfall, 1970).

Plastoquinone plays a major role in electron shuttling between the two photosystems in the photosynthetic apparatus and can inhibit lipid membrane oxidation by quenching singlet oxygen ($^1\text{O}_2$) when present in its reduced state (Ksas et al., 2018). It also serves as a co-factor of phytoene desaturase, an enzyme responsible for the biosynthesis of carotenoids (Norris et al., 1995). Carotenoids are tetraterpenoid pigments present in the hydrophobic domain of membrane proteins in the lipid membrane matrix of the photosynthetic membranes (Siefermann-Harms, 1985; Sun et al., 2018). They play a major role in light harvesting by absorbing light energy and transferring the excitation to the reaction center *via* chlorophyll molecules. They also provide photoprotection to chlorophyll by quenching chlorophyll triplet states and scavenging singlet oxygen (Frank & Brudvig, 2004). Thus, the depletion of carotenoids due to herbicidal activity causes bleaching symptoms in the emerging foliar tissues and eventually death of the plant at

higher doses (Matringe et al., 2005). The biosynthetic pathway of carotenoids and plastoquinone is depicted in Figure 1.4.

HPPD-inhibitors are classified into three major chemical families: isoxazoles (e.g., isoxaflutole and pyrasulfotole), pyrazolones (e.g., topramezone), and triketones (e.g., mesotrione and tembotrione) based on their chemical structure (Van Almsick, 2009). These herbicides are widely used in crops such as corn and sugarcane (*Saccharum officinarum* L.). Mesotrione and tembotrione are used for pre-emergence (PRE) and post-emergence (POST) options in the corn fields as they are tolerant to these herbicides (Jhala et al., 2023). Mesotrione is used for PRE options in sorghum (*Sorghum bicolor*) but not for POST (Pandian et al., 2020b). Pyrazolate is registered for use in rice (*Oryza sativa*) as they are tolerant. HPPD-inhibitors are not registered as stand-alone herbicides in wheat; A herbicide Huskie® containing pyrasulfotole of the family pyrazoles and bromoxynil (PS-II-inhibitor) has been used in the US.

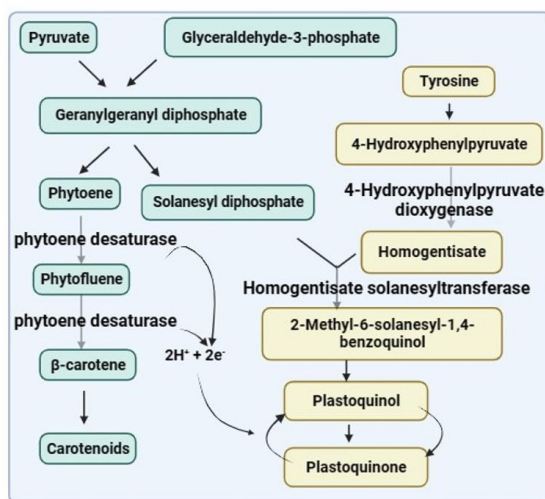


Figure 1.4. Biosynthesis pathways of carotenoids and plastoquinone. Source: (Shino et al., 2021).

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1.5.1 HPPD-inhibitor-resistant weeds

Till today, only three weed species have been identified to be resistant to HPPD-inhibitors: 1) Palmer amaranth, 2) waterhemp (*Amaranthus tuberculatus*), and 3) wild radish (*Raphanus raphanistrum*). In 2009, the first case of HPPD resistance was reported in a waterhemp population from Iowa. Waterhemp is resistant to the herbicides mesotrione, tembotrione, and topramezone. Palmer amaranth is resistant to mesotrione, tembotrione, topramezone and pyrasulfotole. Wild radish resistance is reported only in Australia and is resistant to mesotrione, tembotrione, isoxaflutole, pyrasulfotole, and topremazone (Heap, 2023). All these weeds were found to confer resistance to these herbicides by rapid metabolism of herbicides by P450 activity. Target site mutations conferring resistance to these herbicides have not been documented so far in weed species. However, target gene (HPPD) overexpression leading to HPPD-inhibitor resistance is observed in Palmer amaranth (Nakka et al., 2017b).

1.5.2 HPPD-inhibitor-resistant crops and crop technologies

Corn is naturally tolerant to HPPD-inhibitors *via* P450 metabolic activity regulated by the gene, *CYP81A9* (Williams & Pataky., 2010). Recent studies have identified sorghum genotypes resistant to tembotrione and mesotrione *via* enhanced P450 metabolic activity (Pandian et al., 2020b, 2023). A non-target gene *HIS1* conferencing tolerance to HPPD-inhibitors in rice was identified; *Arabidopsis thaliana* genetically engineered with this gene showed less sensitivity to HPPD-inhibitors (Maeda et al., 2019). Target site mechanisms conferring resistance to HPPD-inhibitors have also been documented in crops. Soybean has been genetically engineered with an *HPPD* gene in oat (*Avena sativa*); this gene harbors a point mutation which makes it less sensitive to HPPD-inhibitors (Kramer et al., 2014). A *Pseudomonas fluorescens* *HPPD* gene containing a G336W mutation has also been used to develop transgenic soybean (Dreesen et al.,

2018). These transgenic soyabeans are available for commercial use. Studies involving gene editing *via* CRISPR technology of HPPD, which may ultimately confer resistance to these herbicides, have not been conducted so far in wheat or any other crop. However, several crops with resistance to ALS, EPSPS, and ACCase inhibitors have been developed *via* gene editing technologies including base editing and prime editing methods. As mutations in the *HPPD* gene leading to HPPD-inhibitor resistance are not common, a deep investigation of the proteins *via* molecular docking is essential. Even though the development of HPPD-resistant wheat might be painstaking, it will be promising for effective weed control.

1.6 Objectives of This Dissertation

As discussed above, wheat is an agronomically important crop worldwide. The production of wheat can be increased by effective weed management. Herbicide usage is a commonly used weed management strategy in wheat in the US. Due to selection, many weed species found in wheat have evolved resistance to herbicides of different modes of action. This drives the necessity to evaluate alternate herbicide options for effective weed control in wheat. As HPPD-inhibitors are widely used to control broad-leaf and grass weeds, they are a good alternative option for controlling weeds in wheat fields. Developing HPPD-inhibitor-resistant wheat is also necessary for the successful use of herbicides such as mesotrione and tembotrione; conventional breeding, genetic engineering, and gene editing *via* CRISPR/Cas technology are a few methods that can be adopted to achieve this. This dissertation unravels the physiological, genetic, and molecular basis for HPPD-inhibitor resistance in wheat, with the following objectives of each chapter of the dissertation.

Chapter 2: Characterization of Winter, Spring, and Mutant Lines of Wheat (*Triticum aestivum*)

Response to 4-Hydroxyphenylpyruvate Dioxygenase (HPPD)-Inhibitors

- a) Determine the response of the wheat genotypes/lines to mesotrione or tembotrione treatment.
- b) Investigate the physiological basis of the wheat response to mesotrione or tembotrione.

Chapter 3: Reduced Sensitivity of Winter Wheat (*Triticum aestivum*) to Triketone Herbicides is a Partially Dominant, Polygenic Trait

- a) Generate F₁ progenies by performing reciprocal crosses between the least sensitive (WW-1 and WW-2) and the most sensitive (WW-24) winter wheat genotypes and evaluate their response to mesotrione and tembotrione application to determine the dominant or recessive nature of the reduced sensitivity.
- b) Generate F₂ progenies and assess their response to mesotrione and tembotrione to determine the number of genes involved in the reduced sensitivity to these herbicides.

Chapter 4: Chapter 4 - Overexpression of *TaHPPD* or *CYP81Q32-like* genes in wheat (*Triticum aestivum*) reduces sensitivity to triketone herbicides.

- a) Develop transgenic lines overexpressing the wheat *TaHPPD* gene and assess their response to mesotrione.
- b) Develop transgenic lines overexpressing the *CYP81Q32-like* gene and assess their response to mesotrione.

- c) Downregulate the *CYP81Q32-like* gene *via* CRISPR/Cas and assess the mutants for increased mesotrione sensitivity.

Chapter 5: General summary/conclusions of the dissertation and future directions

Note: There is some repetition of information as the chapters were written in the format of manuscripts for publication in peer-reviewed journals.

Chapter 2 - Characterization of Winter, Spring, and Mutant Lines

of Wheat (*Triticum aestivum*) Response to 4-

Hydroxyphenylpyruvate Dioxygenase (HPPD)-Inhibitors

2.1 Abstract

Weed management is crucial in agronomically important crops like wheat (*Triticum aestivum*). The use of herbicides is the most reliable weed control strategy. Unfortunately, the repeated use of herbicides registered for use in wheat has led to the evolution of herbicide-resistant weeds. Therefore, farmers need new herbicide options with different target sites to manage weeds in this crop. 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors such as mesotrione and tembotrione are widely used in maize to control broad leaf and grass weeds. But these herbicides are not registered for use in wheat due to crop injury. These herbicides inhibit the enzyme, HPPD which plays a major role in carotenoid biosynthesis; the depletion of carotenoids causes bleaching symptoms followed by necrosis and death in plants. We have identified wheat genotypes surviving more than the field recommended dose of mesotrione and tembotrione. So, we hypothesized that wheat genotypes can withstand HPPD-inhibitor application *via* cytochrome P450 (P450) enzyme activity, similar to maize. The objectives of this study were to a) determine the response of the wheat genotypes/lines to mesotrione and tembotrione treatment and b) investigate the physiological basis of wheat's response to mesotrione or tembotrione.

Upon screening 24 winter wheat genotypes (WW-1 to WW-24) with 6X dose of mesotrione and tembotrione, two genotypes (WW-1 and WW-2) were found least sensitive to these herbicides compared to the most sensitive genotype (WW-24). Screening 15 spring wheat genotypes with

4X doses of mesotrione and tembotrione showed that SW-I was least sensitive to both the herbicides, whereas SW-7 and SW-15 were found most sensitive to tembotrione and mesotrione, respectively. The dose-response data showed that the even most sensitive genotype (WW-24) and the most sensitive mutant lines (J-38 and J-327) of winter wheat had GR_{50} values higher than the field-recommended doses conveying wheat can survive more than field recommended dose of these herbicides. Further, high-performance liquid chromatography profiles indicated that WW-1 and WW-2 metabolized ~ 70 to 75% of ^{14}C -tembotrione, whereas WW-24 metabolized ~ 60% at 24 hours after treatment. Jagger, the parent genotypes, metabolized ~95% of ^{14}C -tembotrione compared to ~ 80 to 85% in the mutant lines at 48 hours after treatment (HAT). Additionally, the use of a P450 inhibitor (e.g., malathion) along with mesotrione and tembotrione resulted in increased sensitivity of WW1, WW-2, and Jagger suggesting that P450 enzymes are involved in metabolizing tembotrione in wheat. Overall, the data suggest that WW-1, WW-2, and Jagger exhibit less sensitivity to tembotrione because of the metabolism of this herbicide *via* P450 activity. These results suggest that the wheat genotypes can be improved for HPPD-inhibitor resistance through breeding programs which will eventually benefit wheat growers.

2.2 Introduction

Wheat is the second most consumed crop in the world (FAO, 2023) with approximately 60 to 80% of the world population depending on it for calories and protein (Chaves et al., 2013). It is also used as a) feed grain for livestock, b) forage crop, and c) for biofuel production and various other industries (Diekmann, 2009). In 2023, the global wheat production was around 800.2 million tonnes after maize with 1214.5 million tonnes (USDA, 2023a, b). The total production of wheat was 1.81 billion bushels in 2023 making it the third major crop after corn (*Zea mays* L.) and soybean (*Glycine max*) in the US (USDA, 2023a, b). Approximately 70% of the total wheat production in the US constitutes winter wheat, and the remaining 25% and 5% constitute spring and durum wheat (Laghetta et al., 2009).

Biotic and abiotic stresses can significantly affect wheat production (Shiferaw et al., 2013). Among the biotic stresses, weed infestation can result in a significant yield loss of up to 23% in wheat (Oerke, 2006). Common weed species found in wheat include jointed goatgrass (*Aegilops cylindrica*), Japanese brome (*Bromus japonicus*), downy brome (*Bromus tectorum*), *Lolium perenne* spp., Henbit (*Lamium amplexicaule*), kochia (*Bassia scoparia*), Russian thistle (*Salsola tragus*), mustard spp. (*Brassica* spp. L), etc, (Appleby et al., 1976; Ogg & Seefeldt, 1997; Swan, 1971; Rydrych, 1974; Conley & Bradley, 2005; Reddy et al., 2013). Post-emergence (POST) application of herbicides was reported to be effective for weed management in wheat (Flessner et al., 2021). The commonly used herbicides in wheat cropping systems include acetolactate synthase (ALS), microtubule-, photosystem II (PS-II), and very long-chain fatty acid synthesis (VLCFA)- inhibitors. Wheat is naturally tolerant to these herbicides as it can metabolize the active form of herbicides into non-toxic compounds (Mougin et al., 1991; Rojano-Delgado et al., 2015). Repeated application of these herbicides results in selection

pressure leading to the development of herbicide resistance in weeds. Downy brome has evolved resistance to multiple herbicides, but most commonly to the triazine herbicides, aryloxy phenoxy propionate (APP) herbicides, cyclohexanedione (CHD) herbicides, and ALS- inhibitors (Ball et al., 2007; Kumar & Jha, 2017; Park & Mallory-Smith, 2005). A study showed that henbit can tolerate higher doses of ALS-inhibiting herbicides such as chlorsulfuron, imazamox, and propoxycarbazone (Varanasi et al., 2016). Whereas Mustard *spp.* has evolved resistance to several auxinic herbicides, ALS-inhibitors, and triazines (Dang et al., 2017; Rosario et al., 2011; Zheng & Hall, 2001). Therefore, more herbicide options are essential for the growers to control these weeds.

Herbicide resistance in plants is imparted by two major mechanisms: 1) target site resistance (TSR) involves alterations to the herbicide target as a result of either mutations or duplication/overexpression of the target gene (Devine & Shukla, 2000). and 2) non-target site resistance (NTSR), which involves reduced absorption, translocation, or rapid metabolism of herbicide by the activity of enzymes such as cytochrome P450 (P450), glutathione-s-transferases (GSTs), glucosyl, and other transferases (Jugulam & Shyam, 2019). The P450 monooxygenases superfamily is known to detoxify harmful compounds and herbicides in plants *via* Phase I metabolism (Siminszky, 2006).

4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors are group 27 herbicides, widely used for broad-spectrum weed management. This group of herbicides has been classified into three major chemical families: 1) isoxazoles (e.g., isoxaflutole and pyrasulfotole), 2) pyrazolones (e.g., topramezone), and 3) triketones (e.g., mesotrione and tembotrione) based on their chemical structure. These inhibitors prevent the conversion of 4-hydroxyphenyl pyruvic acid (HPPA) into homogentisic acid (HGA), which is a precursor in the formation of

plastoquinone, tocopherols, and tocopherols (Lindblad et al., 1958; Whistance & Threlfall, 1970). Plastoquinone, a major electron transporter between the photosystems in the photosynthetic apparatus, serves as an important component in carotenoid biosynthesis (Ksas et al., 2018; Norris et al., 1995). Carotenoids, present in the lipid membrane matrix of the photosynthetic membranes, play a major role in 1) light harvesting and 2) protecting the chlorophyll by quenching chlorophyll triplet states and scavenging singlet oxygen (Frank & Brudvig, 2004; Siefermann-Harms, 1985; Sun et al., 2018). Thus, the depletion of carotenoids due to herbicidal activity causes bleaching symptoms in the emerging foliar tissues and eventually death of the plant at higher doses (Matringe et al., 2005).

Mesotrione and tembotrione belonging to triketones are widely used in corn to control a broad spectrum of weeds due to their ability to metabolize these herbicides efficiently (Williams & Pataky., 2010). Among other cereal crops, mesotrione is registered for pre-emergence (PRE) use in sorghum, whereas tembotrione is not registered as PRE or POST use in sorghum due to crop injury (Pandian et al., 2020b). A recent study identified two sorghum genotypes conferring resistance to tembotrione and mesotrione *via* P450-mediated metabolism (Pandian et al., 2020b, 2023). Also, both these triketone herbicides are not registered for use in wheat either as PRE or POST; nonetheless, wheat has been reported to be tolerant to pyrazolones in China (Ndikuryayo et al., 2017; Wang et al., 2020). In corn, one of the P450 enzymes encoded by the *Nsf1* gene was reported to be responsible for the metabolism of HPPD inhibitors and other herbicides (Bollman et al., 2008; Pataky et al., 2008). The activity of the P450 enzymes was found to be inhibited by several chemicals such as malathion, tetcyclacis (TET), piperonyl butoxide (PBO), etc. (Busi et al., 2017). It has been shown in several crop and weed species that the treatment with P450-

inhibitors prior to the application of herbicides can reverse the resistance thereby imparting sensitivity to the herbicides (Barrett, 1995).

The development of herbicide-resistant wheat is crucial to broaden the herbicide options for effective weed control. Currently, target gene alterations conferring herbicide resistance in wheat have been developed and are commercially available (Duke, 2005). In 2001, the Clearfield production system was developed *via* EMS mutagenesis with base alterations in the wheat ALS gene. The Clearfield wheat is resistant to imidazolinones herbicides which can control grass and broadleaf weeds (Rojano-Delgado et al., 2015). In 2015, the CoAXium wheat production system was developed with base alterations in the acetyl-CoA carboxylase (ACCase) gene conferring resistance to aryloxyphenoxypropionate herbicides. These herbicides are primarily used to control monocot weeds (www.coaxiumwps.com). However, several weed species have already evolved resistance to both ALS and ACCase-inhibitors. Therefore, new herbicide-resistant wheat technologies are warranted to manage a broad spectrum of weeds including herbicide-resistant biotypes.

In this research, we characterized the response of winter wheat, spring wheat, and ethyl methanesulfonate (EMS) derived lines to the triketone chemical family, i.e., mesotrione and tembotrione of HPPD-inhibitors. We hypothesize that similar to corn, wheat may exhibit natural resistance to these herbicides, via P450 enzyme-mediated metabolism. The objectives of this study were to a) determine the response of the wheat genotypes/lines to mesotrione and tembotrione treatment and b) investigate the physiological basis of the wheat response to mesotrione or tembotrione.

2.3 Materials and methods

2.3.1 Plant materials

Twenty-four winter wheat, fifteen spring wheat genotypes, as well as ethyl methanesulfonate (EMS) derived mutant lines derived from the genotypes AM2003 (spring wheat), and Jagger (winter wheat) were used in this study along with a corn hybrid (DKC59-82RIB) for comparison in some experiments. The EMS mutant lines of AM2003 were generated as described below.

2.3.2 Generation of EMS lines of AM2003:

The spring wheat cultivar AM2003 was used for generating a mutant population. To optimize the procedure to generate EMS mutant lines, a total of 50,000 seeds were subdivided into 25 subsets. Each subset contained approximately 2,000 seeds. The subsets of seeds were placed in mesh bags and soaked in ddH₂O dissolved with the chemical EMS concentrations ranging from 0.6 to 1%. The seeds were left overnight under these conditions with aeration. The resulting M₁ (mutant generation 1) seeds were rinsed with ddH₂O three to four times until the chemical residues were washed off completely. Each seed was planted in plug trays containing a commercial potting mixture (ProMix Ultimate; Premier Tech Horticulture, Mississauga, Ontario, Canada) and maintained in a greenhouse at 25/18° C Day/night (d/n) and 16 h of photoperiod. Light in the greenhouse was supplemented with 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination by sodium vapor lamps and natural light. The batch that received 0.6% of EMS had 50% germination, while the seeds treated with higher doses failed to germinate. The M₁ plants that were phenotypically different (i.e., stunted growth, reduced tillers) from the non-EMS plants were selected and transplanted into bigger pots. Before anthesis, each panicle of wheat was covered with a pollination bag to prevent cross-pollination. At maturity, the M₂ seeds were harvested from each

plant separately and threshed. Seeds from each plant represent a different mutant line. Five hundred mutant lines were developed by the end of the process.

2.3.3 Response of winter, spring genotypes and EMS mutant lines of wheat to mesotrione or tembotrione application

The seed of twenty-four winter (WW-1 to WW-24); fifteen spring wheat (SW-1 to SW-15) genotypes as well as EMS-derived lines of Jagger, and AM2003 were used. The seeds were planted in a germination sheet soaked in water and kept in dark at 4°C for 2 to 3 days to break the seed dormancy. The seeds were then exposed to light at 25°C. After the seeds germinated, they were transferred into pots (6x6x6 cm) filled with a potting mixture (ProMix Ultimate; Premier Tech Horticulture, Mississauga, Ontario, Canada) and were grown in a greenhouse-maintained condition at 18/15°C (d/n) and 16 h of photoperiod supplemented with 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination by sodium vapor lamps and natural light. When the plants reached the 3-5 leaf stage, they were treated with 6X (630 g ai ha⁻¹; 1X is the field recommended dose which is 105 g ai ha⁻¹) dose of mesotrione (Callisto, Syngenta crop protection, LLC, Research Triangle Park, NC, US) along with 1% crop oil concentrate (COC, Agridex, Collierville, TN, US) and 2.5% of ammonium sulphate (AMS; Liquid N-Pak; Winfield, MN, US); or 6X (552 g ai ha⁻¹; 1X= 92 g ai ha⁻¹) dose tembotrione (Laudis, Bayer CropScience St. Louis, MO, US) along with 1% methylated soy oil (Destiny; Winfield United, US) and 1% AMS. The 6X dose was used as a discriminating dose to identify wheat genotypes that were most or least sensitive to mesotrione or tembotrione. A bench-top track spray chamber (Generation III; De Vries Manufacturing, Hollandale, MN, US) equipped with a single flat-fan nozzle (80015LP TeeJet tip; Spraying Systems, Wheaton, IL, US) delivering 187 L ha⁻¹ was used for herbicide application. Four to

five replications were maintained, and the experiment was repeated. Visible injury symptoms were recorded using an injury rating scale of 0 to 100 (0 = no injury, 100% = plant mortality).

2.3.4 Mesotrione and tembotrione dose response assay

In the above experiment, overall, winter wheat genotypes were found to be least sensitive to either mesotrione or tembotrione compared to spring wheat or mutant lines. Among the winter wheat genotypes WW-1 and WW-2 were found to be least sensitive, whereas WW-24 was most sensitive to both chemicals. The WW-1, WW-2, and WW-24 genotypes were further subjected to mesotrione or tembotrione dose response assays. Among the Jagger mutant lines, Jagger-EMS-38 (J38) and Jagger-EMS-327 (J327) were found to be most sensitive compared to Jagger parent and were selected for dose-response assays, as follows. The seeds of WW-1, WW-2, WW-24, Jagger, J38, J327 were planted and grown as described previously. When the plants reached the 3-5 leaf stage, they were treated with several doses of mesotrione or tembotrione. For the WW genotypes, mesotrione doses used were 0, 420, 630, 840, 1050 g ai ha⁻¹ along with 1% crop oil concentrate (COC, Agridex, TN, US) and 2.5% of ammonium sulphate (AMS, Liquid N-Pak; Winfield), whereas the doses of 0, 368, 552, 736, 920 g ai ha⁻¹ of tembotrione along with methylated seed oil and ammonium sulfate (AMS, Liquid N-Pak; Winfield, MN, US) at 1% v/v and 1% w/v, respectively, were used. For Jagger and mutants, the mesotrione dose used were 0, 105, 210, 420, 630, 840, 1050 g ai ha⁻¹ and the tembotrione does applied were 0, 92, 184, 368, 552, 736, 920 g ai ha⁻¹. The same procedure as described above was followed for herbicide applications. The plants were transferred to the same greenhouse after treatment with herbicides. The treatments were arranged in a completely randomized design with five replications and the experiment was conducted twice. The above-ground biomass was collected at 4 weeks after

treatment (WAT). The harvested plants were stored in paper bags and oven dried at 60°C for a week and the dry biomass was weighed.

2.3.5 Physiological characterization of wheat response to tembotrione

Because of the non-availability of radiolabeled mesotrione, experiments were conducted using only radiolabeled tembotrione, i.e., [^{14}C]tembotrione. The spring wheat genotypes were not used in this experiment as they were found more sensitive to tembotrione compared to Jagger and winter wheat varieties. The Jagger and mutant lines of J38 and J237 were used in both tembotrione absorption, translocation, and metabolism experiments. Whereas WW-1, WW-2, and WW-24 were used only in tembotrione metabolism experiments, because there was no difference in tembotrione uptake or translocation in the wheat lines tested (Jagger, J38 and J327).

2.3.6 Absorption and translocation of [^{14}C]tembotrione

Wheat plants of Jagger, J38, and J327 at the 5-leaf stage were transferred to the growth chambers maintained at 18/15°C and 18/6 h (d/n) photoperiod, supplemented with 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination to acclimatize. A cocktail mixture containing [phenyl-UL- ^{14}C]-tembotrione (Bayer AG) with a specific activity of 3.38 MBq/mg and unlabeled (commercial) tembotrione (Laudis, Bayer CropScience St. Louis, MO, US) was prepared at recommended field rate 92 g ai ha^{-1} in a carrier volume of 187 L ha^{-1} with appropriate adjuvants such as 1% v/v and 1% w/v of methylated soy oil (MSO) and ammonium sulphate (AMS, Agrilience), respectively, to enhance droplet-to-leaf surface contact. The plants were then treated with 10 μl (10,000 dpm/ μl) of [^{14}C]tembotrione on the upper surface of the fourth-youngest leaf. After 20 min the plants were placed back in the growth chamber. Plants parts were harvested at 6, 24, and 48 h after treatment (HAT) and separated into treated (TL), tissue above the treated leaf (ATL), and below the treated leaf (BTL). The treated leaves were then washed with 5 ml of wash solution (containing 10% ethanol

and 0.05% Tween 20) for 1 minute in a 20 ml scintillation vial to remove unabsorbed herbicide. The rinsate was measured for radioactivity using liquid scintillation spectrometry (LSS; Beckman Coulter LS6500 Liquid Scintillation Counter, Beckman Coulter Inc., Fullerton, CT, US). Trisected plant sections (ATL, TL, & BTL) were wrapped in tissue papers and dried at 60 °C for 2 to 3 days in an oven. The radioactivity absorbed in each plant part was quantified by combusting using a biological oxidizer (OX-501, RJ Harvey Instrument, Tappan, NY, US) followed by estimating the radioactivity by LSS. Five replications were included in each treatment and the experiment was repeated once.

The total absorbed [^{14}C]-tembotrione was determined as follows:

% absorption tembotrione absorbed = $(\text{total R applied} - \text{R of rinsate}) \times 100 / \text{total R applied}$; where R = radioactivity.

The percent tembotrione translocated (T) was determined using the following equation:

%T = $100 - [^{14}\text{C al} / (^{14}\text{C al} + ^{14}\text{C ot}) \times 100]$, where, $^{14}\text{C al}$ is the amount of ^{14}C measured in the treated leaf, and $^{14}\text{C ot}$ is the amount of ^{14}C detected in other untreated tissues of the plant.

2.3.7 Metabolism of [^{14}C]tembotrione

To evaluate if the metabolism of tembotrione is providing reduced sensitivity of wheat to this herbicide, WW-1, WW-2, WW-24, Jagger, J38, and J327 plants were used. Corn was used as a positive control for this experiment. The amount of parent ^{14}C tembotrione and its metabolites found after treatment were assessed using HPLC. The plants were grown in the greenhouse under the previously mentioned conditions. Once the plants reached the 5-leaf stage, they were transferred to the growth chambers maintained at 18/15°C and 18/6 h (d/n) photoperiod, supplemented with 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination to acclimatize. The fourth youngest leaf was treated with 10 μL of [phenyl-UL- ^{14}C]tembotrione. At 6, 24, and 48 HAT, the

treated leaves were harvested and rinsed with 5 mL of wash solution (10% v/v ethanol and 0.5% v/v Tween 20, ThermoFisher Scientific) in a 20 mL scintillation vial for 1 minute twice to remove the unabsorbed [phenyl-UL-¹⁴C] tembotrione. Washed and treated leaves were flash-frozen and ground in liquid nitrogen. [phenyl-UL-¹⁴C]tembotrione and metabolites were extracted by incubating in 15 mL of 90% acetone (HPLC grade, ThermoFisher Scientific,) at 4 °C overnight (16 h). The samples were centrifuged at 4 °C at 7000 rpm for 10 min. The supernatant was collected and evaporated using a vacuum concentrator (Centrivap, Labconco) for 2 - 2.5 h at 45 °C. The supernatant was concentrated until a volume of 500 µl was reached and centrifuged at 13, 000 rpm for 10 minutes at room temperature to remove the debris. The total extractable radioactivity [¹⁴C] compounds in each sample was measured by liquid scintillation spectrometry and normalized to 3000 dpm/50 µL using acetonitrile (HPLC grade, ThermoFisher Scientific).

Total extractable radioactivity in 50 µL was resolved into parent compound [¹⁴C]tembotrione and/or its polar metabolites by reverse phase high-performance liquid chromatography (HPLC; Agilent Technologies, 1260 Infinity II) with Zorbax SB-C18 column (4.6 mm × 250 mm, 5-µm particle size; Agilent Technologies, Santa Clara, CA) at a flow dose of 1 mL min⁻¹ and radio flow detector LB 5009 (Berthold Technologies, Oak Ridge, TN) following the protocol optimized previously in our laboratory. The tembotrione metabolism experiment was repeated once.

2.3.8 Cytochrome P450- inhibitor assay

To determine if P450s are responsible for metabolizing tembotrione and mesotrione in wheat, experiments were conducted using two known P450-inhibitors, malathion and PBO. The WW-1, WW-2, WW-24, Jagger, J38, and J327 genotypes were grown in the greenhouse, as

described before. Malathion (spectracide malathion insect spray concentrate; Spectrum brand) was applied at 0, 2000, 4000 g ai ha⁻¹ along with 0.25% non-ionic surfactant (NIS). PBO (Thermo Fisher Scientific, Waltham, MA, United States) was used at 2100 g ai ha⁻¹ along with 7.5% acetone and 0.25% NIS as an adjuvant. After one hour of inhibitor treatment, tembotrione or mesotrione was applied at 6X dose with appropriate adjuvants. Based on the protocols established in our laboratory (Shyam et al., 2021a; Pandian et al., 2020b), soil drenching of 5 mM malathion at 24 h after the primary application was provided as a booster dose; and no soil drenching was performed for PBO treatment. At 4 WAT, the biomass was collected and dried at 60°C for 2 to 3 days and weighed.

2.3.9. Target gene (*HPPD*) expression analysis

Leaf samples were collected from WW-24, WW-1, and WW-24 and flash frozen in liquid nitrogen and stored at -80°C for RNA isolation. The frozen tissue was homogenized in liquid nitrogen using a pre-chilled mortar and pestle to prevent thawing and transferred 100 mg tissue into a 1.5 mL microcentrifuge tube. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The quality and quantity of total RNA were determined using agarose gel (1%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific) and RNA was stored at -80°C. Further, the isolated RNA was treated with DNase I enzyme (Thermo Scientific, Waltham, MA, USA) to remove any genomic DNA. cDNA was synthesized from 1 µg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR/real-time PCR (qPCR) was used to determine *HPPD* gene expression in all samples. The qPCR reaction mix consisted of 7 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™), 0.25 µL each of forward (5'-AGGTCTCATGGAGCAAAAGA-3') and reverse primers (5'-GTTCATGTGTGAGCCGAG-3') (10 µM), and 20 ng cDNA to make the

total reaction volume of 14 μ L. HPPD gene expression was normalized using actin as a reference gene. The reaction was carried out in triplicates with PCR cycling conditions set as 50°C for 2 min, 95°C for 5 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min as per the manufacturer's instruction of the master mix (Applied Biosystems™). The relative quantifications were performed using the $\Delta\Delta$ CT method. qPCR experiments were carried out with 3 to 4 biological replicates of WW-1, WW-2, and WW-24. The primer sequence for the HPPD gene and actin gene was designed by retrieving the cDNA sequence from the National Center for Biotechnology Information (NCBI) database. (primer efficiency test is yet to be conducted).

2.3.10 Statistical analysis

The dose-response data (% of untreated control) were analyzed using 'drc' package in R 4.1.2. All the data derived from drc experiments were subjected to non-linear regression analysis using a three or four-parameter log-logistic model. The three-parameter and four-parameter regression model had the following formulas:

$$Y = d + \exp [b (\log x - \log e)] \text{ and } Y = c + \{d-c/1+\exp[b[\log(x) - \log(\text{GR}_{50})]\},$$

respectively, where Y is the response (dry biomass) expressed as percentage of the untreated control, d is asymptotic value of Y at upper limit, b is the slope of the curve around e and e is GR_{50} (amount of mesotrione or tembotrione required to reduce shoot dry weight by 50%), and x is the herbicide rate. Using the function 'compParm' in 'drc' package, estimated GR_{50} of winter wheat genotypes were further tested for difference *via* t-test. Absorption (% of applied), translocation (% of absorbed) and metabolism data were analyzed using one-way ANOVA in R 4.1.2 and the means were compared using Tukey's HSD test. The absorption data used the rectangular hyperbolic model ($\text{absorption} = (\text{Amax} \times t) / [(10/90) \times t_{90} + t]$). Where, absorption is

the percentage of absorbed herbicide at time t , A_{max} is the upper limit (maximum) for absorption of herbicide, and t_{90} is the time required to achieve 90% of the maximum absorption. The effect of malathion on mesotrione or tembotrione metabolism was analyzed using a factorial analysis of variance (ANOVA) in R program and contrast comparisons were adjusted by the Tukey's HSD test.

2.4 Results

2.4.1 Variable response of wheat to mesotrione and tembotrione application

The response of all wheat lines evaluated in this study at field-recommended rates or lower doses of mesotrione and tembotrione was similar, with less visual injury (data not shown). However, at higher doses i.e., 4X and 6X of these herbicide treatments, there were differences in response among the wheat genotypes/lines and hence these were used as discriminatory doses to determine the sensitivity. Based on winter response to 6X dose of mesotrione or tembotrione, two varieties i.e., WW-1 and WW-2 were found least sensitive with < 10% visual injury whereas, WW-24 was most sensitive with > 40% injury (Figure 2.1 A). Similar variable sensitivity was recorded in spring wheat genotypes as well. SW-1 was least sensitive with ~15% visual injury whereas, SW-7 showed >50% injury to 4X mesotrione application (Figure 2.1 B). In response to 4X tembotrione, SW-1 was least sensitive with ~25% visual injury and SW-15 showed ~80% injury (Figure 2.1 B). The AM2003 and the 10 mutant lines derived from EMS treatment of AM2003 showed a similar response and were found sensitive to mesotrione and tembotrione at 4X dose (data not shown). Among the 500 EMS-derived lines of the Jagger genotype, J38 and J327 were found most sensitive compared to Jagger. The least and most sensitive genotypes of winter wheat (WW-1, WW-2, and WW-24) and Jagger, J38, and J327 were used in other experiments.

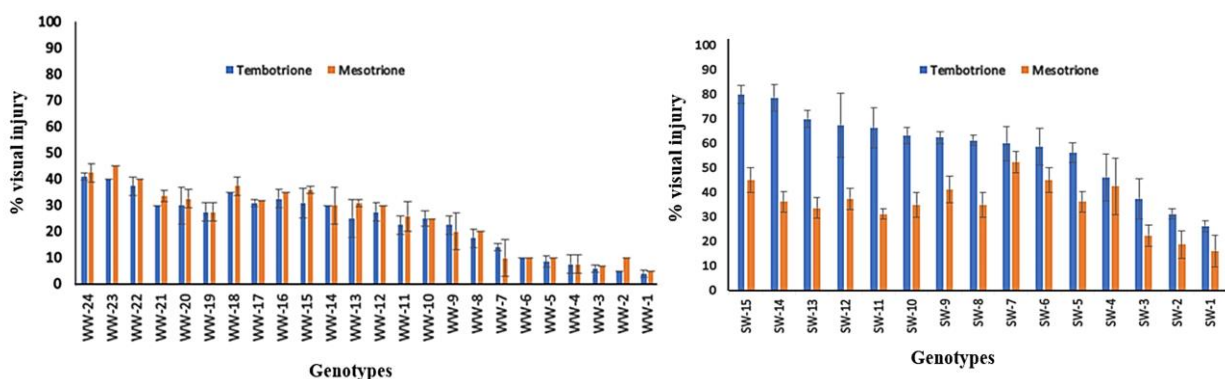


Figure 2.1 A) Response of winter wheat (WW) genotypes to 6X dose of mesotrione (630 g ai ha⁻¹) and tembotrione (552 g ai ha⁻¹) at 2 WAT. B) Response of spring wheat (SW) genotypes to 4X dose of mesotrione (368 g ai ha⁻¹) and tembotrione (420 g ai ha⁻¹) at 2WAT.

2.4.2 Mesotrione and tembotrione dose-response assays

Winter wheat genotypes

The analyses of results of mesotrione dose-response assay suggests that the GR₅₀ of WW-24 at 4 WAT was 208.9 g ai ha⁻¹, whereas GR₅₀ of WW-1 and WW-2 were 558.6 g ai ha⁻¹ and 476.0 g ai ha⁻¹, respectively (Figure 2.2 A, Table 2-1). WW-24 required ~ 2 times the field recommended rate of mesotrione for 50% growth reduction. The analysis of tembotrione dose-response data suggests that the GR₅₀ of WW-24 was 452.914 g ai ha⁻¹ compared to 746.5 g ai ha⁻¹ and 568.9 g ai ha⁻¹ for WW-1 and WW-2, respectively (Figure 2.2 B, Table 2-2). WW-24 required ~ 5 times the field recommended rate (1X=92 g ai ha⁻¹) for 50% growth reduction, suggesting that even the most sensitive genotype can withstand field-recommended doses of mesotrione or tembotrione.

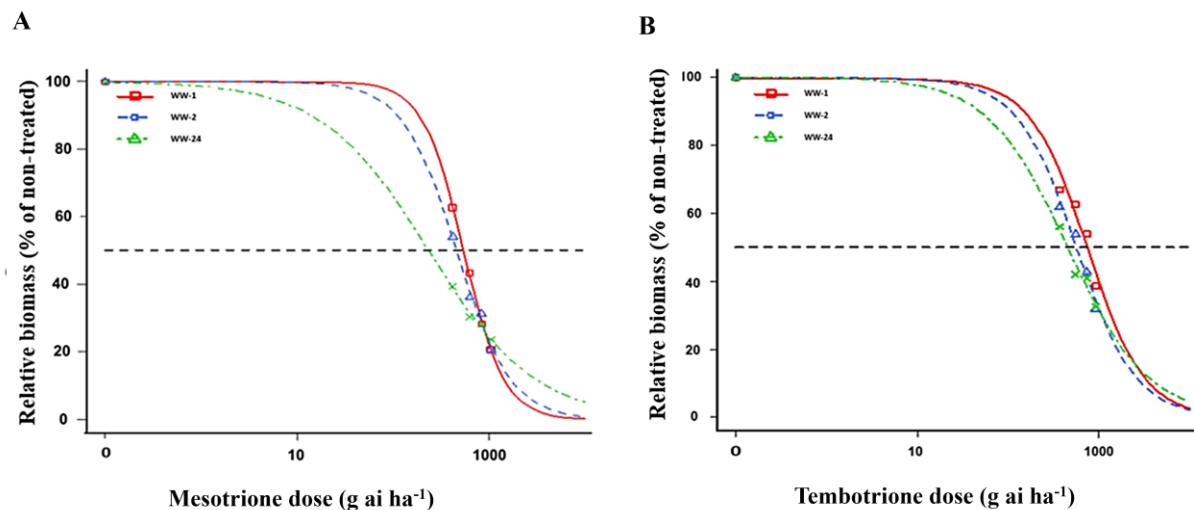


Figure 2.2 A) Mesotrione dose-response of the least sensitive genotypes WW-1, WW-2 along with the most sensitive genotype WW-24 based on the aboveground biomass reduction at 4 WAT. B) Tembotrione dose-response of the least sensitive genotypes WW-1, WW-2 along with the most sensitive genotype WW-24 based on the aboveground biomass reduction at 4 WAT.

Table 2-1. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1, WW-2 (least sensitive) winter wheat genotypes aboveground dry biomass to different rates of mesotrione at 4 weeks after treatment (WAT).

Genotype	Regression Parameters		GR ₅₀
	b (SE)	d (SE)	g ai ha ⁻¹ (SE)
WW-1	1.98 (0.87)	99.97 (3.52)	558.6 (175.96) ^{NS}
WW-2	1.47 (1.29)	100.01 (3.51)	476 (338.76) ^{NS}
WW-24	1.40 (2.61)	100 (3.51)	208.9 (101.35)

^aGR₅₀: dose required for 50% growth reduction; SE: standard error.

^bNS: significantly not different from WW-24 at $P \leq 0.1$.

Table 2-2. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1, WW-2 (least sensitive) winter wheat genotypes aboveground dry biomass to different rates of tembotrione at 4 weeks after treatment (WAT).

Genotype	Regression Parameters		GR ₅₀ g ai ha ⁻¹ (SE)
	b (SE)	d (SE)	
WW-1	1.39 (0.52)	99.64 (6.17)	746.5 (103.15) ^{NS}
WW-2	1.33 (0.49)	99.82 (6.14)	568.9 (79.38) ^{NS}
WW-24	0.98 (0.38)	100.02 (6.12)	452.9 (95.38)

^aGR₅₀: dose required for 50% growth reduction; SE: standard error.

^bNS: significantly not different from WW-24 at $P \leq 0.1$.

Jagger, Jagger-EMS-38, and Jagger-EMS-327

The least sensitive Jagger parent and the most sensitive mutant lines, J38 and J327 were used in dose-response experiments. The mesotrione dose response assay shows that the GR₅₀ at 4 WAT was ~ 301.75 g ai ha⁻¹ for Jagger compared to 173.98 g ai ha⁻¹ and 162.18 g ai ha⁻¹ for J38 and J327, respectively (Figure 2.3 A, Table 2-3). The tembotrione dose-response assay suggests that the GR₅₀ at 4 WAT for Jagger was 303.13 g ai ha⁻¹, whereas for the mutant lines they were 204.83 g ai ha⁻¹ and 100.75 g ai ha⁻¹, respectively (Figure 2.3 B, Table 2-4). The GR₅₀ values of the most sensitive mutant lines suggest that they can survive more than the field recommended rates of mesotrione and tembotrione.

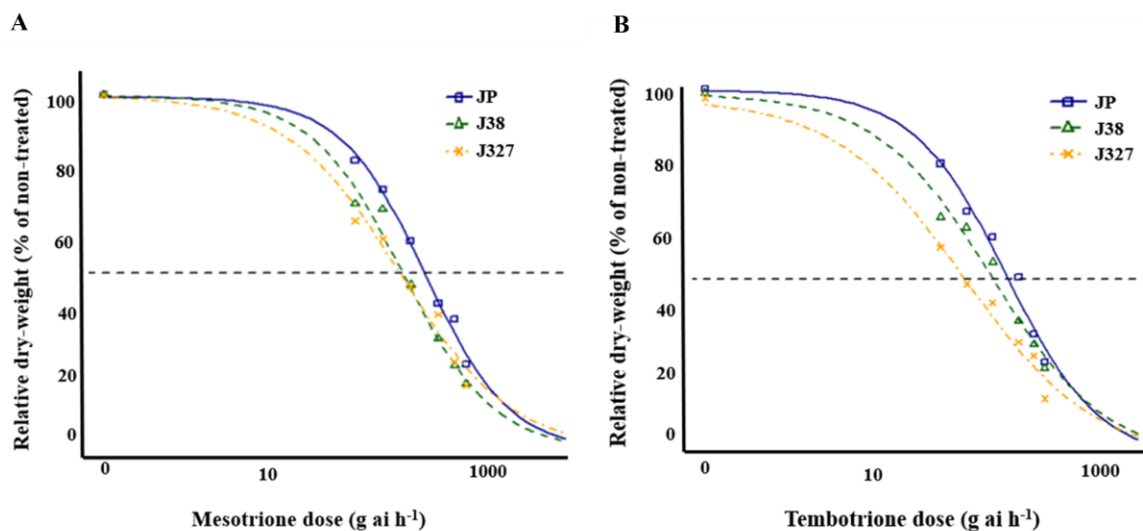


Figure 2.3. A) Mesotrione dose-response of the least sensitive genotype Jagger along with the most sensitive lines J38, J327 based on aboveground dry biomass reduction at 4WAT. B) Tembotrione dose-response of the least sensitive genotype Jagger along with the most sensitive lines J38, J327 based on aboveground dry biomass reduction at 4WAT.

Table 2-3. Summary of regression parameters describing the response of Jagger (least sensitive), Jagger-EMS-38, and Jagger-EMS-327 (most sensitive) lines aboveground dry biomass to different rates of mesotrione at 4 weeks after treatment (WAT).

Genotype	Regression Parameters		GR ₅₀
	b (SE)	d (SE)	g ai ha ⁻¹ (SE)
Jagger (JP)	0.95 (0.08)	99.27 (2.97)	301.7 (29.51)
J38	0.87 (0.07)	99.69 (2.05)	173.9 (17.43)***
J327	0.71 (0.07)	99.54 (3.03)	162.1 (22.22)***

^aGR₅₀: dose required for 50% growth reduction; SE: standard error.

^bsignificantly different from JP at *** $p < 0.001$

Table 2-4. Summary of regression parameters describing the response of Jagger (least sensitive), J38, and J327 (most sensitive) lines aboveground dry biomass to different rates of tembotrione at 4 weeks after treatment (WAT).

Genotype	Regression Parameters		GR ₅₀ gai ha ⁻¹ (SE)
	b (SE)	d (SE)	
Jagger (JP)	0.81 (0.07)	102.06 (2.76)	303.1 (32.39)
J38	0.65 (0.07)	101.13 (2.43)	204.8 (25.92)***
J327	0.57 (0.07)	99.92 (3.91)	100.7 (19.17)***

^aGR₅₀: dose required for 50% growth reduction; SE: standard error.

^bsignificantly different from JP at *** $p < 0.001$

2.4.3 Absorption and translocation of [¹⁴C]tembotrione

The tembotrione absorption data showed that corn, WW-1, WW-2, WW-24, absorbed ($A_{\max}$) ~ 95% of the [¹⁴C]-tembotrione applied (Table 2-5, 2-6). Similar results were observed in Jagger, J38, and J327 (Table 2-7, 2-8). The time taken for 90% maximum absorption (A_{90}) of tembotrione was ~ 2 to 3 hours (Figure 2.4 A). Similar results were observed in corn, WW-24, WW-1, and WW-2 without any significant difference in the amount of tembotrione absorption. As HPPD-inhibitors are systemic herbicides that can translocate *via* xylem and phloem; we observed 24 to 27% translocation at 48 HAT without any significant difference between Jagger and the mutant lines (Figure 2.4 B). The translocation of tembotrione was not determined, in WW-24, WW-1, and WW-2, as there was no difference in tembotrione translocation in Jagger and its mutant lines.

Table 2-5. Regression parameters of [¹⁴C]tembotrione absorption in WW-24 (most sensitive), WW-1, and WW-2 (least sensitive) genotypes.

Genotype	A_{max} (SE)	A₉₀ (SE)
Corn	97.49 (1.10)	1.04 (2.62)
WW-1	98.76 (0.94)	5.97 (2.94)
WW-2	98.72 (1.22)	3.16 (2.74)
WW-24	97.99 (0.91)	4.23 (3.81)

A_{max}: is the maximum herbicide absorbed, A₉₀: Time taken to reach 90% of maximum absorption, and SE: Standard error.

Table 2-6. Comparison of p-values of A_{max} and A₉₀ in WW-24 (most sensitive), WW-1, and WW-2 (least sensitive) lines.

WW-1, WW-2, and WW-24 comparison (p-value)			
	WW-1 vs WW-2	WW-1 vs WW-24	WW-2 vs WW-24
A _{max}	0.96 ^{NS}	0.56 ^{NS}	0.59 ^{NS}
A ₉₀	0.64 ^{NS}	0.50 ^{NS}	0.68 ^{NS}

A_{max}: is the maximum herbicide absorbed, A₉₀: Time taken to reach 90% of maximum absorption, SE: Standard error, NS: Not significant at $p \leq 0.1$.

Table 2-7. Regression parameters of [¹⁴C]tembotrione absorption in Jagger (least sensitive), Jagger-EMS-38, and Jagger-EMS-327 (most sensitive) lines.

Line	A _{max} (SE)	A ₉₀ (SE)
Corn	97.95 (0.6)	0.75 (2.0)
Jagger	96.95 (0.5)	2.05 (0.56)
J38	98.24 (0.54)	2.31 (1.05)
J327	97.90 (0.5)	3.20 (1.5)

A_{max}: is the maximum herbicide absorbed, A₉₀: Time taken to reach 90% of maximum absorption, and SE: Standard error.

Table 2-8. Comparison of p-values of A_{max} and A₉₀ in Jagger (least sensitive), Jagger-EMS-38, and Jagger-EMS-327 (most sensitive) lines

Jagger, J38, and J327 comparison (p-value)			
	JP vs J38	JP vs J327	J38 vs J327
A _{max}	0.18 ^{NS}	0.95 ^{NS}	0.18 ^{NS}
A ₉₀	0.73 ^{NS}	0.10 ^{NS}	0.22 ^{NS}

A_{max}: is the maximum herbicide absorbed, A₉₀: Time taken to reach 90% of maximum absorption, SE: Standard error, NS: Not significant at $p \leq 0.1$.

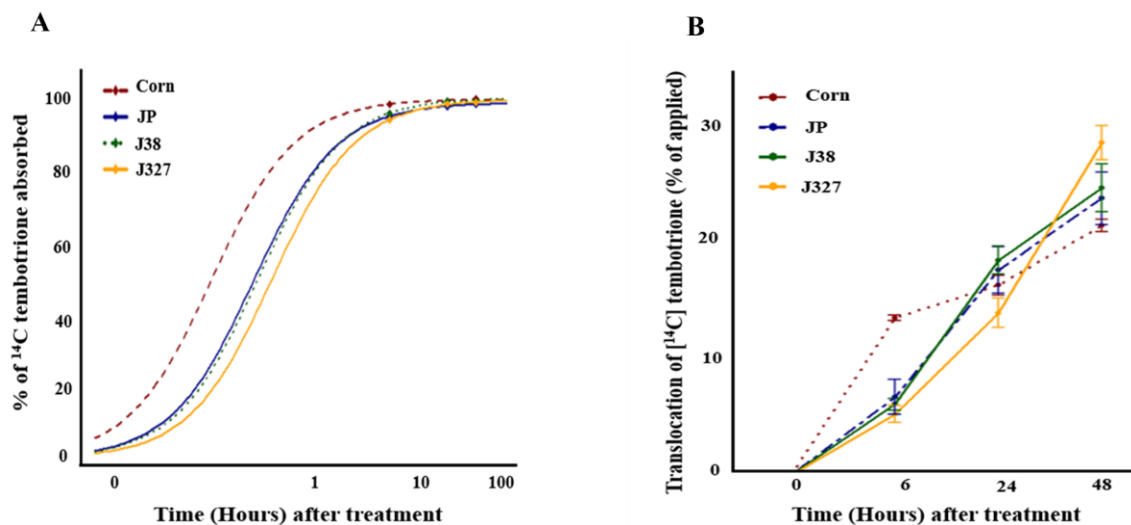


Figure 2.4. A) [^{14}C] Tembotrione absorption in corn, Jagger, J38, J327. A rectangular hyperbola was utilized to analyze percent [^{14}C] Tembotrione absorption at 6, 24, and 48 HAT. B) [^{14}C] Tembotrione translocation in corn, Jagger, J38 & J327 at 6, 24 & 48 HAT.

2.4.4 Metabolism of [^{14}C]tembotrione

The parental compound of ^{14}C -tembotrione and its metabolites formed were identified from the chromatograph images using HPLC. The chromatographs indicated a retention time of ^{14}C tembotrione (parent compound) at 11.4 and its metabolites at 10 (M1), 9 (M2), and 6.5 (M3) min (Figure 2.5 A). WW-24 metabolized 60% of the parent compound compared to 70 and 75% in WW-2 and WW-1, respectively at 24 HAT (Figure 2.5 B). There was a significant difference in the rate of metabolism between WW-1 and WW-24 at 24 HAT ($p < 0.001$) (Figure 2.5 A, B). WW-24, WW-1, and WW-2 retained ~ 5 to 7% of the parent compound at 48 HAT suggesting degradation (metabolism) of majority of applied ^{14}C tembotrione. As corn is known to rapidly metabolize tembotrione, it was used as a positive control in this experiment. Corn metabolized the parent compound completely and no peak of the parent compound was found at 11.4 min either at 6 HAT (data not shown) or 24 HAT. Further, the two major metabolites M1 and M3

were found at 24 HAT in corn (Figure 2.6). The metabolism profiles of Jagger indicated that 45% of parent tembotrione was retained at 24 HAT compared to only 30 to 35% in the mutants, J38 and J327, respectively. At 48 HAT, Jagger retained 5% of the parent compound, whereas the mutants had 15 to 20% with ($p < 0.001$) (Figure 2.7 A, B). The difference in the rate of metabolism at 24 HAT in WW genotypes, and 48 HAT in Jagger and its mutants are most likely to be the basis for the differential sensitivity of these genotypes/lines of wheat to tembotrione.

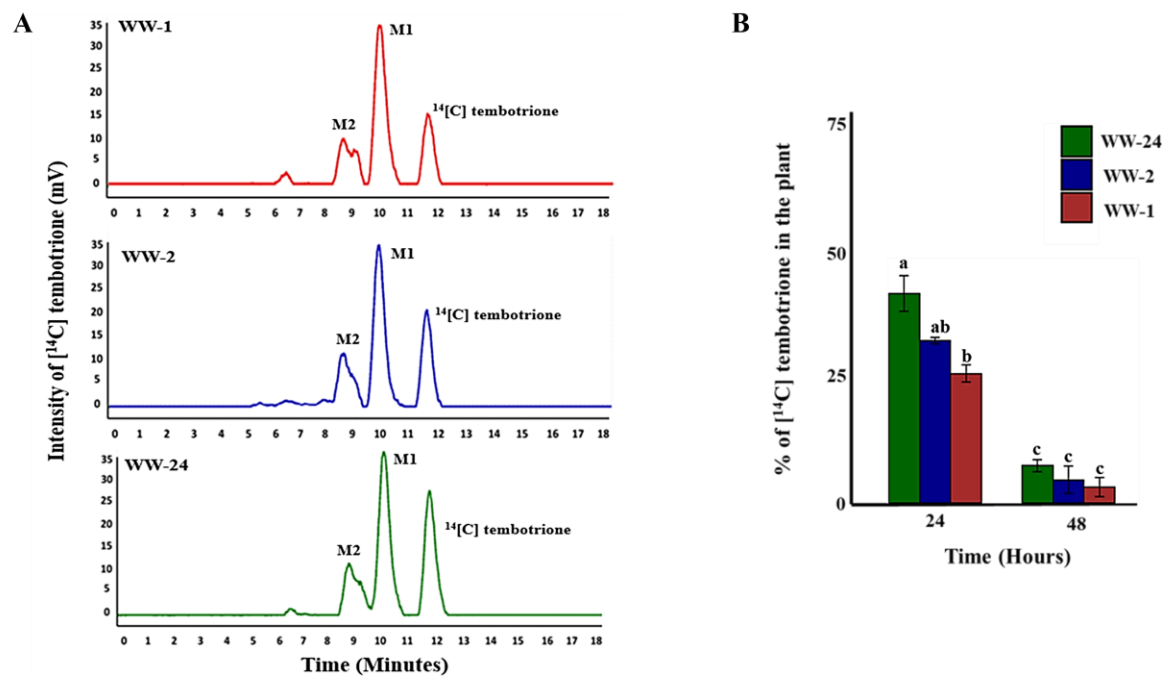


Figure 2.5. A) HPLC chromatographs depict resolved [¹⁴C] tembotrione parent compound (11.4 min) and its metabolites M1 and M2 at 24 HAT. B) Percent parent compound remaining at 24 and 48 HAT in WW-1, WW-2, and WW-24.

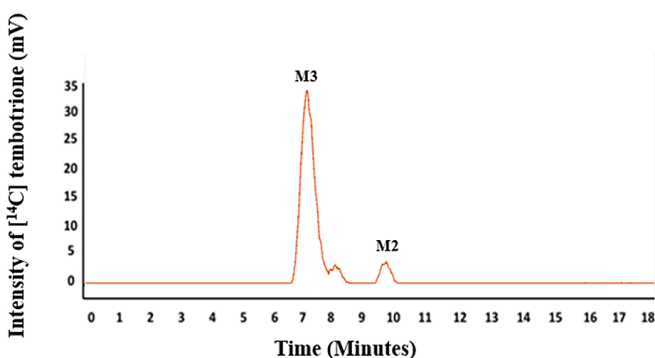


Figure 2.6. HPLC chromatographs depict resolved $[^{14}\text{C}]$ tembotrione parent compound (11.4 min) and its metabolites M2 and M3 at 24 HAT in corn.

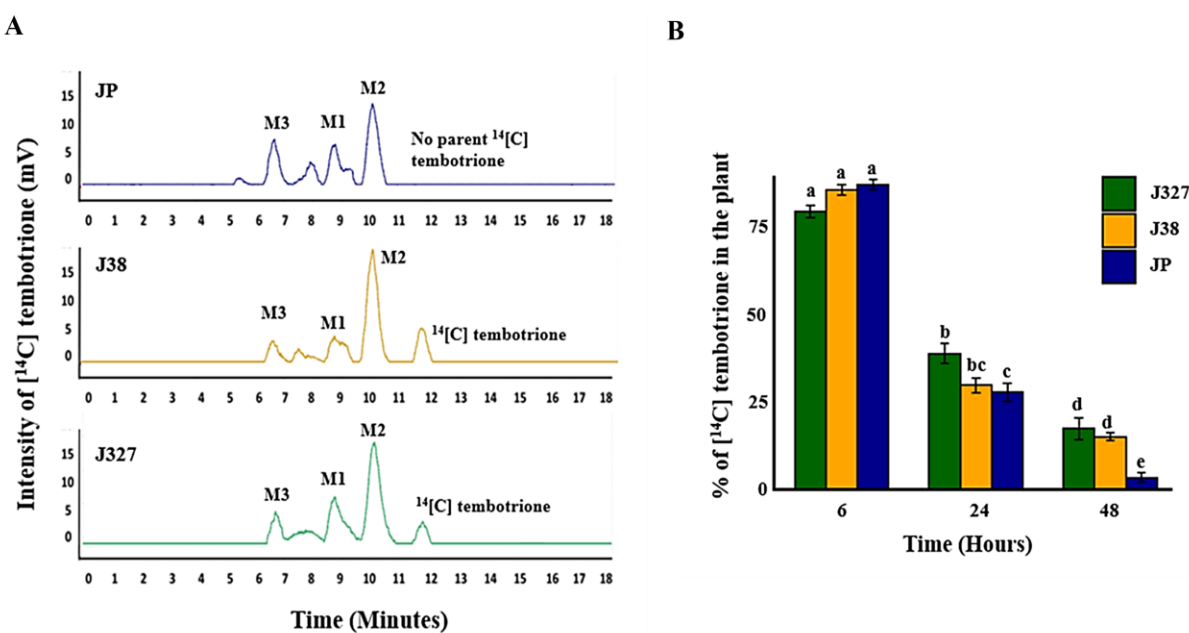
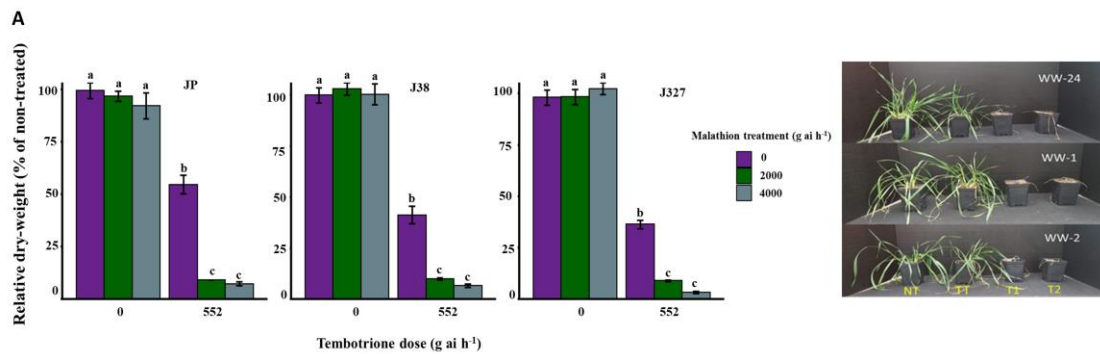


Figure 2.7. A) HPLC chromatographs depicting resolved $[^{14}\text{C}]$ tembotrione parent compound (11.4 min) and its metabolites M1 and M2 at 48 HAT. B) Percent parent compound remaining at 6, 24, and 48 HAT in Jagger, J327, and J38.

2.4.5 P450 inhibitor assay

Jagger, J38, and J327 treated with 6X dose of tembotrione had a biomass accumulation of about ~ 55%, ~ 45%, and ~ 35% when treated with tembotrione alone (Figure 2.8A). The treatment with tembotrione (6X), followed by malathion application (2000 g ai ha⁻¹ or 4000 g ai ha⁻¹) resulted in the complete death of the plants with a significant difference in biomass accumulation ($p < 0.01$) (Figure 2.8A). Similarly, a biomass accumulation of ~ 45%, ~35%, and ~30% respectively, was observed when treated with mesotrione alone (Figure 2.8B). The treatment with mesotrione (6X) followed by malathion application resulted in the complete death of both the least and most sensitive plants with a significant difference in biomass accumulation ($p < 0.01$) (Figure 2.8B). Similar results were seen in WW-1, WW-2, and WW-24 with malathion followed by tembotrione (Figure 2.8C) or mesotrione (Figure 2.8D) treatment. On the other hand, when the genotypes were treated with PBO plus tembotrione or mesotrione showed no difference in biomass accumulation compared to the herbicide treatment alone (data not shown).



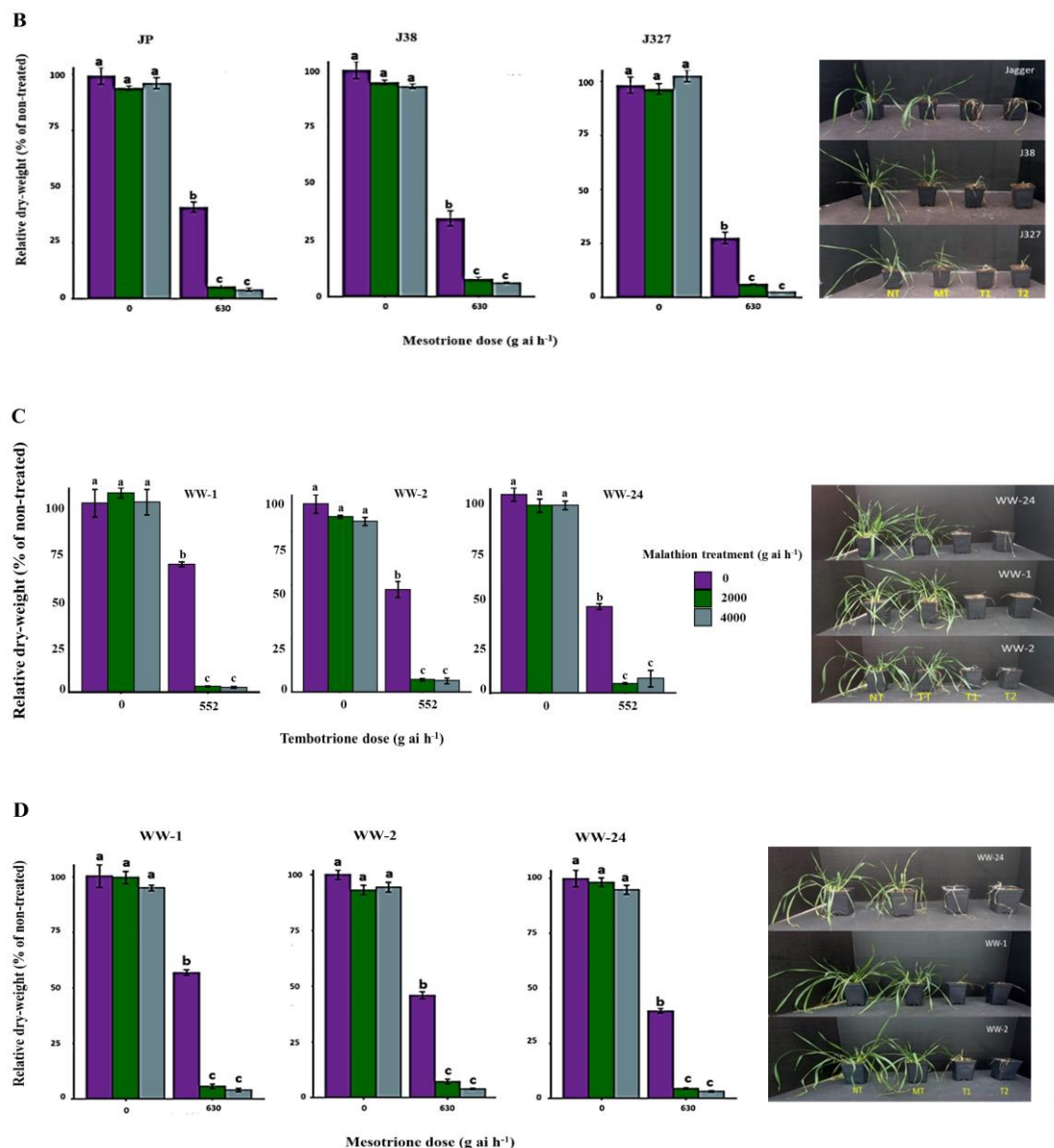


Figure 2.8. A, B) Aboveground dry biomass of Jagger, J38, and J327 when pre-treated with tembotrione or mesotrione and herbicide plus malathion at 4 WAT. The error bars represent the standard error; different alphabets indicate a significant difference between treatments ($p \leq 0.01$) (left); Response of Jagger, J38, J327 to 6X doses of tembotrione (TT) or mesotrione (MT), malathion at 2000 (T1) and 4000 g ai ha⁻¹ (T2) prior to herbicide treatment at 3 WAT (right). C, D) Above-ground dry biomass of WW-1, WW-2, and WW-24 when pre-treated with tembotrione or mesotrione, herbicide plus malathion at 4 WAT. The error bars represent the

standard error; different alphabets indicate a significant difference between treatments ($p \leq 0.01$) (left); Response of WW-24, WW-1, and WW-2 to 6X doses of tembotrione (TT) or mesotrione (MT), malathion at 2000 (T1) and 4000 g ai ha⁻¹ (T2) prior to herbicide treatment at 3 WAT (right).

2.4.6 Target gene (*HPPD*) expression

Real-time PCR analysis was performed to study whether increased expression of the *HPPD* gene imparted less sensitivity to mesotrione and tembotrione in WW-1 and WW-2 compared to WW-24. The results showed that there was no difference in the expression level of the *HPPD* gene among the genotypes. The mRNA transcript abundance was found to be similar in all the genotypes (Figure 2.9).

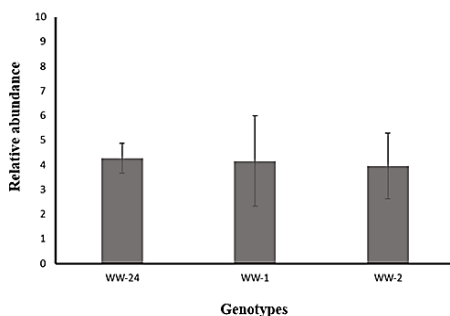


Figure 2.9. Target gene (*HPPD*) expression in WW-1 and WW-2 relative to WW-24. Bars represent mean $\pm$ SE of 3-4 biological replicates.

2.6 Discussion

The discovery of the chemical leptospermone from bottlebrush plants (*Callistemon citrinus*) in the 1980s, and further combining the benzoyl moiety to the syncarpic unit led to the development of triketone family of HPPD-inhibitors with a broad spectrum weed control

(Hamprecht et al., 2011). Triketones are toxic to plants but not mammals; they not only interfere with tyrosine catabolism but also inhibit the biosynthesis of tocopherols and plastoquinone, which is unique to the plant system (Yang et al., 2004). Moreover, HPPD-inhibitors exhibit selectivity and are environment-friendly (Cho et al., 2013; Matringe et al., 2005). Corn can detoxify HPPD-inhibitors by hydroxylation *via* the activity of the P450 enzyme coded by the *CYP81A9* gene (*nsf1*) (Brazier-Hicks et al., 2022; Williams & Pataky, 2010). Topramezone (pyrazolone family of HPPD-inhibitor) is detoxified by P450 enzymes *via* N-demethylation (Grossmann & Ehrhardt, 2007). Isoxaflutole, a pre-emergence herbicide of the family diketonitrile, gets converted into an inactive benzoic acid derivative in corn (Governa et al., 2022). In Sorghum, we have found genotypes G-200 and G-350, as well as G-1 and G-10 confer metabolic resistance to tembotrione and mesotrione, respectively (Pandian et al., 2020b, 2023). Further, HPLC results also showed that G-1 and G-10 can metabolize [¹⁴C]-mesotrione two times faster than the susceptible genotype Pioneer 84G62 (Pandian et al., 2023).

Till date, only three weed species, have been documented to have evolved resistance in HPPD-inhibitors (Heap, 2023). The first case of resistance to HPPD-inhibitors was reported in a waterhemp (*Amaranthus tuberculatus*) biotype in IL, USA (Hausman et al., 2011). Subsequently, resistance in Palmer amaranth (*Amaranthus palmeri*), and wild radish (*Raphanus raphanistrum*) has also been documented (Heap, 2023). All these weeds confer resistance to HPPD-inhibitor *via* P450-mediated metabolism (Kramer et al., 2014; Lu et al., 2020; Ma et al., 2013; Nakka et al., 2017b).

Apart from the P450-mediated HPPD-inhibitor metabolism, there have been a vary few cases where an alteration in the target site (i.e., HPPD gene) has been found to confer resistance to these herbicides. For example, soybean (*Glycine max*) has been genetically engineered with

avhppd-03, a gene isolated from oat (*Avena Sativa*) (Dreesen et al., 2018). The transgenic soybean showed low sensitivity to mesotrione compared to the wild-type (Kramer et al., 2014). Furthermore, transgenic soybean has also been developed with a *HPPD* gene of *Pseudomonas fluorescens* which had a G336W mutation (Dreesen et al., 2018). Rice (*Oryza sativa*) is naturally tolerant to pyrazolate and is registered for use in rice fields (Matsumoto et al., 2002). Recently, a rice gene *HIS1* conferring resistance to several triketone herbicides such as benzobicyclon, sulcotrione, tefuryltrione, mesotrione, and tembotrione was identified. Further, *Arabidopsis thaliana* engineered with this gene also showed resistance to these herbicides (Maeda et al., 2023).

Recent reports from China suggested that wheat cultivars could tolerate high doses of bipyrazone (up to 750 g a.i. ha⁻¹) (Wang et al., 2020). Also, a new HPPD-inhibiting chemical, QYM21 has been registered for use in winter wheat fields in China (Zhang et al., 2019). Pyrasulfutole, a chemical belonging to the family pyrazoles along with bromoxynil (photosystem II inhibitor) under the trade name Huskie® has been registered for post-emergence use in wheat in the U.S. But no HPPD inhibitor is registered as a stand-alone herbicide in wheat in the US. In this research, we characterized the differential sensitivity of wheat to mesotrione and tembotrione. Our results suggest that the most sensitive genotype (WW-24) survived up to 8 times the field recommended dose of mesotrione and tembotrione with GR₅₀ values of 209 g ai ha⁻¹ and 452.9g ai ha⁻¹, respectively for these herbicides (Table 2-1 and 2-2) Similarly, the mutant line J327 was found less sensitive to these herbicides with GR₅₀ of 162.18 g ai ha⁻¹ for mesotrione (Table 2-3) and 100.75 g ai ha⁻¹ for tembotrione (Table 2-4). Overall, these results indicate that winter wheat is less sensitive to mesotrione or tembotrione and can survive >6X

doses of these herbicides. These results are encouraging to further improve the resistance of genotype, possibly by breeding.

Non-target site mechanisms such as absorption and translocation did not show any differences among all the genotypes assessed (Figures 2.4A, B) and hence, cannot be attributed to the differential sensitivity to tembotrione in wheat. Similarly, no significant difference between ^{14}C -mesotrione absorption and translocation was found between the susceptible and resistant sorghum genotypes (Pandian et al., 2023). Likewise, in HPPD-inhibitor resistant Palmer amaranth, there was no difference in amount of mesotrione or tembotrione absorbed or translocated (Nakka et al., 2017b; Aarthy et al., 2022).

In this research, the least sensitive winter wheat genotypes (WW-1, WW-2) showed the metabolism of tembotrione *via* the formation of two major metabolites M1 and M2 at 24 HAT (Figure 2.5A). The same metabolites (M1 and M2) were also found in Jagger, J38, and J327, although at 48 HAT (Figure 2.7A). Whereas in corn, similar to our previous report (Aarthy et al., 2022) the M2 and M3 were found as two major metabolites of tembotrione formed at 24 HAT, with M3 being more predominant metabolite in this research as well (Figure 2.6). However, M1, which was a major metabolite in wheat (Figure 2.5A) was not found in corn plants (Figure 2.6). These results suggest that corn can rapidly metabolize tembotrione *via* the formation of a different type of metabolite, M3 that was absent in wheat at 24 HAT. Corn is known to detoxify triketones *via* Phase I hydroxylation and dealkylation mediated by the P450s, specifically by *CYP81A9*. Interestingly, at 48 HAT, the winter wheat genotype Jagger and its mutants also showed the formation of M3 as a minor metabolite along with M1 and M2 metabolites (Figure 2.7). Additionally, at 48 HAT, the percent of parent tembotrione (toxic form) present among the winter wheat genotypes was different. While the least sensitive genotypes, WW1, WW2, and

Jagger had <5% of parent ^{14}C tembotrione (Figure 2.5B), there was still ~ 20% of the toxic form of tembotrione present in the most sensitive winter wheat mutant lines J327 and J38 (Figure 2.7B). These differences can be attributed to the differential sensitivity of wheat to tembotrione.

Our data also suggests the possible role of P450 enzymes in metabolizing mesotrione or tembotrione in wheat. The plants treated with malathion followed by mesotrione or tembotrione died at 3 WAT (Figure 2.8). Similarly, HPPD-resistant sorghum or weeds species also showed a reversal of resistance when treated with malathion followed by herbicides (Pandian, et al., 2020b; 2023; Ma et al., 2013; Nakka et al., 2017b). In this study the *HPPD* gene expression analysis denies the possible role of target overexpression in reduced sensitivity of the least sensitive genotypes (Figure 2.9).

The P450s are widely present across plant species, occupying about 1% of the total plant genome; this prevalence has been beneficial for plants to acquire new metabolic pathways during evolution (Mizutani, 2012). They have several functions, such as biosynthesis of terpenoids and alkaloids, biosynthesis and catabolism of plant hormones, detoxification of xenobiotics, and major factors in protecting plants against various biotic and abiotic stresses (Mizutani, 2012; Pandian et al., 2020a). Several P450 genes have been characterized to confer herbicide resistance in crops and weeds. For example, P450 genes, *CYP72A31* and *CYP749A16* were found responsible for ALS-inhibitor resistance in rice and cotton (*Gossypium hirsutum*), respectively (Choe and Williams, 2020). Tobacco genes *CYP71A11* and *CYP81B2* were reported to be involved in imparting resistance to chlorsulfuron, an ALS-inhibitor (Yamada et al., 2000). In corn, the *CYP81A9* gene has been characterized to confer resistance to mesotrione, tembotrione, nicosulfuron, and other ALS inhibitors (Choe & Williams, 2020). Recently, we identified overexpression of *CYP81E* gene responsible for tembotrione resistance in Palmer amaranth

(Aarthy et al., 2022). Interestingly in rigid ryegrass, (*Lolium rigidum*) overexpression of *CYP81A10v7* gene was found to bestow resistance to multiple herbicides including ACCase, ALS, HPPD, photosystem-II, and tubulin inhibitors (Han et al., 2021). Transgenic soybean expressing *P450-N-Z1*, a P450 gene from Bermuda grass (*Cynodon dactylon*), exhibiting resistance to nicosulfuron, flazasulfuron, and 2,4-dichlorophenoxyacetic acid (2,4-D) was developed (Zheng et al., 2022). Previously, transgenic potato co-expressing three human P450 genes *CYP1A1*, *CYP2B6*, and *CYP2C19* showing resistance to herbicides was also reported (Inui & Ohkawa., 2005). Overall, there is considerable evidence suggesting that HPPD-inhibitors may be metabolized by P450 enzyme activity, however, identifying a specific P450 gene involved in such metabolism is critical to decipher the basis of metabolism of these herbicides, which could be species specific.

2.7 Conclusions

The outcome of this research indicates that the differential metabolism of tembotrione was responsible for the variable sensitivity of winter wheat to this herbicide. We also observed the possible role of P450 enzymes in tembotrione and mesotrione metabolism in wheat. Inheritance studies will help identify whether the tembotrione and mesotrione resistance is a monogenic or polygenic trait (Chapter 3). Further, identifying specific P450s involved in metabolism would help understand the molecular basis of the metabolism of HPPD-inhibitors in wheat (Chapter 4).

Chapter 3 - Reduced Sensitivity of Winter Wheat (*Triticum aestivum*) to Triketone Herbicides is a Partially Dominant, Polygenic Trait

3.1 Abstract

Wheat is an important cash crop and its production is affected by several stresses including weed competition. Repeated use of herbicides has resulted in the evolution of herbicide-resistant weeds in wheat. Therefore, exploring herbicide options with different modes of action groups for POST application in wheat is warranted. Herbicides such as mesotrione and tembotrione, belonging to the triketone family of 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors, are widely used in corn but not registered for use in wheat fields. The results of Chapter 2 identified winter wheat genotypes with differential sensitivity to mesotrione and tembotrione, i.e., genotypes, WW-1 and WW-2 being least sensitive and WW-24 as the most sensitive to these herbicides, due to metabolism of these herbicides likely mediated by cytochrome P450 (P450) activity. Understanding the inheritance of differential sensitivity of winter wheat to triketones can help in the introgression via breeding program, which was investigated in this Chapter. F₁ progenies were generated by performing reciprocal crosses between WW-1 or WW-2 and WW-24. Further, F₂ progenies were also generated by self-pollinating F₁ plants. Mesotrione and tembotrione dose-response of F₁ progenies showed an intermediate response to both the herbicides compared to their parents, a characteristic of a partially dominant trait. Further, the similar response of F₁ progenies derived from both the direct and the reciprocal crosses implies that the reduced sensitivity to triketones in wheat is

conferred by a nuclear trait. The response of F₂ progenies to mesotrione (630 g ai h⁻¹) or tembotrione (552 g ai h⁻¹) treatment did not follow the Mendelian segregation for a single gene inheritance pattern. This suggests that the reduced sensitivity of WW-1 and WW-2 winter wheat to triketone herbicides is a polygenic trait, a characteristic inheritance pattern of traits bestowing metabolism of herbicides *via* P450 activity.

3.2 Introduction

Wheat is one of the major cereal crops grown worldwide (Shewry, 2009) and ranks third among the field crops grown in planted acreage, production, and gross farm returns in the US (USDA, 2023a). It is an important crop in temperate regions and is primarily used for human consumption and livestock feed. Wheat is also grown for ethanol production, brewing beer, cosmetics, and in meat substitutes, etc., (Diekmann, 2009). It is also a good source of minerals, vitamin B complex, beneficial phytochemicals, and dietary fiber (Shewry, 2007). Wheat production is hindered by weed infestations which could potentially cause up to 23% yield losses (Oerke, 2006). Herbicides are the most widely used pesticides in wheat with ~61 to 96% of planted acres of winter and spring wheat sprayed with herbicides across the US annually for weed management (USDA-NASS, 2017). Extensive and often exclusive use of herbicides with the same mode of action groups resulted in the selection of herbicide-resistant weed species (Gaines et al., 2020). Therefore, exploring herbicide options with different modes of action groups for weed management is vital. Triketone herbicides that inhibit 4-hydroxyphenyl pyruvate dioxygenase (HPPD) enzyme (Group 27/F2) such as mesotrione and tembotrione are widely used to control broadleaf and grass weeds in corn (Williams & Pataky., 2010) but are registered for use in wheat due to crop injury. Corn is naturally tolerant to these herbicides due to rapid metabolism mediated by cytochrome P450 enzyme (P450) activity (Brazier-Hicks et al., 2022). Triketone herbicides inhibit the HPPD enzyme, which is important for the conversion of 4-hydroxyphenyl pyruvate to homogentisate, an intermediate in the plastoquinone and the tocopherol biosynthesis pathway in plants (Lee et al., 1998). Plastoquinone plays a major role in the biosynthesis of carotenoids, which are involved in light harvesting and protecting

chlorophyll. Depletion of plastoquinone causes bleaching symptoms and the eventual death of sensitive plants (Ksas et al., 2018; Siefermann-Harms., 1985).

Two winter wheat genotypes (WW-1 and WW-2) with reduced sensitivity to mesotrione and tembotrione compared to the most sensitive WW-24 genotype were identified and characterized in this dissertation (Chapter 2). The genotypes, WW-1 and WW-2 were found to metabolize triketone herbicides possibly *via* P450 activity (Chapter 2). Generally, in plant species metabolic resistance to herbicides, including HPPD-inhibitors is bestowed by polygenic traits (Ahrens et al., 2013; Jugulam & Shyam., 2019). Genetic factors including the degree of dominance, and/or the number of alleles controlling the trait will determine how the trait will be inherited (Jasieniuk et al., 1996). For example, resistance conferred by a dominant gene will be expressed in both the homozygous and heterozygous conditions and therefore, they are easily inherited, whereas recessive traits are expressed only in a homozygous condition. If herbicide resistance is governed by multiple genes, several recombination events are needed for accumulation of all the alleles (Menalled et al., 2016). In a multiple herbicide-resistant population of Palmer amaranth from Kansas, the metabolic resistance to mesotrione was found to be imparted by multiple nuclear alleles (Shyam et al., 2021b). Likewise, mesotrione resistance in several waterhemp populations was also inherited as a partially dominant polygenic trait (Huffman et al., 2015; Kohlhase et al., 2018). In crop species such as sorghum, tembotrione resistance is a partially dominant polygenic trait, whereas mesotrione resistance is conferred by a single dominant gene (Pandian et al., 2023; Pandian et al., 2020b). The information on the inheritance of traits will be crucial in a breeding program to introgress agronomically valuable traits into elite germplasm.

The inheritance of reduced sensitivity of WW-1 and WW-2 winter wheat to mesotrione or tembotrione is not known. We hypothesize that this reduced sensitivity is possibly bestowed by multiple genes. The overall objectives of this study were to: 1) generate F₁ progenies by performing reciprocal crosses between the least sensitive (WW-1 and WW-2) and the most sensitive (WW-24) winter wheat genotypes and evaluate their response to mesotrione and tembotrione application to determine the dominant or recessive nature of the reduced sensitivity, and ii) generate F₂ progenies and assess their response to mesotrione and tembotrione to determine the number of genes involved in the reduced sensitivity to these herbicides.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

The same winter wheat genotypes that were found least sensitive to mesotrione and tembotrione i.e., WW-1 and WW-2, and the most sensitive genotype i.e., WW-24 (Chapter 2) were used in this study. Experiments to generate F₁ and F₂ progenies and to characterize their response to mesotrione and tembotrione were conducted either in a greenhouse or controlled environmental growth chambers. Greenhouse was maintained with the following conditions: 18/15°C (d/n) and 16 h of photoperiod supplemented with natural light at 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination by sodium vapor lamps and with natural light. The growth chambers were maintained at 22/18°C (d/n) and 16 h of photoperiod supplemented with 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination by incandescent and fluorescent lamps. The winter wheat seeds were planted in a germination sheet soaked in water and kept in the dark at 4°C for 2 to 3 days to break the seed dormancy. The seeds were then removed from the dark and maintained at 25°C. After seed germination, the seedlings were transferred into single pots (6x6x6 cm) filled with a potting

mixture (ProMix Ultimate; Premier Tech Horticulture, Mississauga, Ontario, Canada) and were grown in the greenhouse.

3.3.2 Reciprocal crosses and generation of F₁ and F₂ progenies

The winter wheat seedlings (2-3 leaf stage) were transferred to individual pots (4.5" diameter x 4" tall). Once they reached 5 leaf stage, they were transferred to the vernalization chamber for 8 weeks which was maintained at 4°C. Vernalization is essential for winter wheat for initiation of the reproductive stage and produce seeds. At flowering, direct and reciprocal crosses were performed between WW-1 or WW-2 and the WW-24 genotypes. For the direct cross, WW-1 or WW-2 was designated as a female parent and WW-24 as a male parent; and vice-versa for reciprocal crosses. When the spikes fully emerged, emasculation was performed. Spikes with small and green anthers were chosen to prevent self-pollination. For emasculation, the spikelets at the tip and base were removed as they are generally sterile and asynchronous compared to the rest (Wells & Caffeyz., 1956). Further, the florets in the center of each spikelet were also removed as they develop a bit later compared to the lateral florets (Miller, 1980; Wells & Caffeyz., 1956). Using a pair of scissors, an incision was made across each selected floret, for easy anther removal and pollination. The anthers were removed using forceps. The plants were labeled, and spikes were covered with a pollination bag (7" length X 2" width), to avoid pollen contamination. After three to four days of emasculation, the stigmas were receptive to pollination. The mature anthers of WW-24, that were ready to shed the pollen were chosen and gently taped inside the florets of the female plant (WW-1 or WW-2). Once all the selected spikes were pollinated, they were covered with new pollination bags and labeled appropriately. The reciprocal crosses were performed following the same procedure. Upon maturity, the F₁ seeds

were collected. The F₁ seed derived from the crosses (WW-1 X WW-24), (WW-24 X WW-1), (WW-2 X WW-24), and (WW-24 X WW-2) were labelled as F₁-1, F₁-2, F₁-3, and F₁-4, respectively (Figure 1). The F₂ progenies were generated by self-pollinating the F₁ plants which were grown in the greenhouse as described previously. Once the ear heads of F₁ plants emerged from the flag leaves, they were covered with pollination bags for selfing. The seeds produced from these plants were grouped into F₂ families as shown in Figure 3.1.

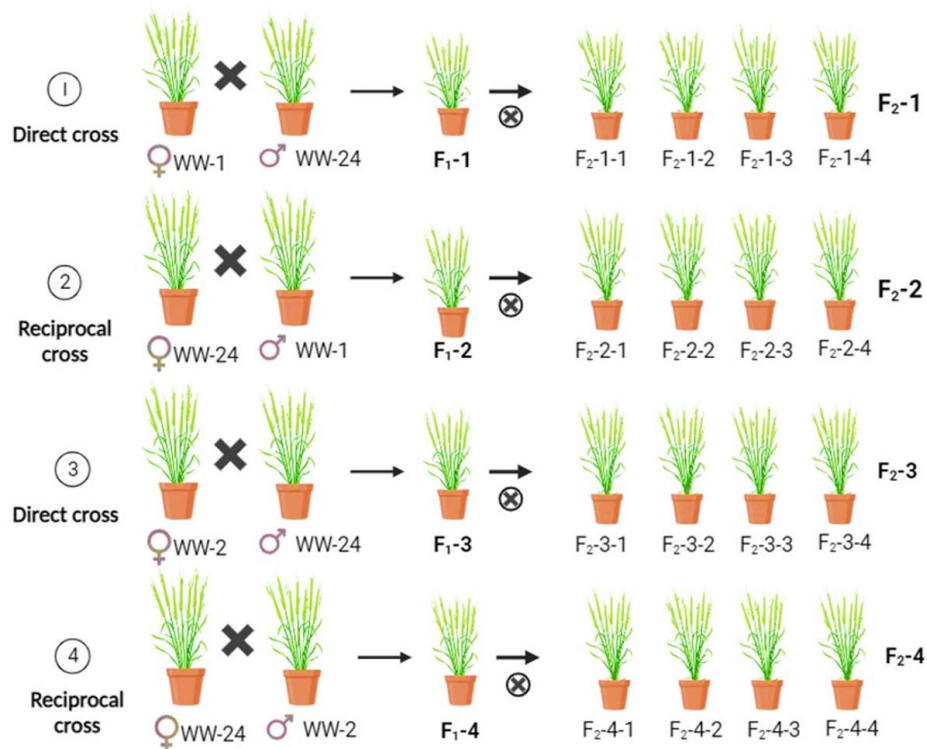


Figure 3.1. Schematic diagram illustrating the generation of F₁ and F₂ families in a crossing program using WW-1, WW-2 (less sensitive), and WW-24 (more sensitive) winter wheat [Created with BioRender.com].

3.3.3 Mesotrione and tembotrione dose-response of WW-1, WW-2, WW-24 and F₁ progenies

Whole-plant dose-response experiments were conducted using parental WW-1, WW-2, WW-24, and F₁ families (F₁-1, F₁-2, F₁-3, F₁-4). The experiments were conducted in a completely randomized design in growth chambers. The plants were grown as previously described. When the plants reached the 3-5 leaf stage, they were treated with different doses (1 to 8X) of mesotrione (1X = 105 g ai h⁻¹) along with 1% crop oil concentrate (COC, Agridex) and 2.5% of ammonium sulfate (AMS, Liquid N-Pak; Winfield). A separate set of parents and F₁ progenies were also treated with different doses (1 to 8X) of tembotrione (1X = 92 g ai h⁻¹) along with methylated seed oil and ammonium sulfate (AMS, Liquid N-Pak; Winfield) at 1% v/v and 1% w/v, respectively, were used. A bench-top track spray chamber (Generation III; De Vries Manufacturing, Hollandale, MN, US) equipped with a single flat-fan nozzle (80015LP TeeJet tip; Spraying Systems, Wheaton, IL, US) delivering 187 L ha⁻¹ was used for herbicide application. Four to five replications were maintained, and the experiment was repeated. The above-ground biomass was collected at 4 weeks after treatment (WAT). The harvested plants were stored in paper bags and oven-dried at 60°C for a week and the dry biomass was weighed. Dry weight was measured and converted into relative dry weight (% of non-treated) as described below:

$$\text{RDW (\%)} = [(\text{DW} \times 100) / \text{ADW}]$$

where RDW is the relative dry weight (% of non-treated control), DW is the dry weight of the sample and ADW is the average dry weight of the non-treated control.

3.3.4 Evaluation of segregation of F₂ progenies

The seeds of the F₂ along with parents, WW-1, WW-2 and WW-24 were grown in the greenhouse as described previously. Based on the response (least or most sensitive) of the parental populations (Chapter 2), i.e., WW-1, WW-2, and WW-24, the 6X dose was identified as a discriminatory dose for mesotrione (630 g ai h⁻¹) and tembotrione (552 g ai h⁻¹) to determine the segregation of F₂ progenies into least sensitive or most sensitive to these herbicides. Once the F₂ plants from several families (described in Figure 1) reached the 3 to 5-leaf stage, they were treated with a 6X dose of mesotrione or tembotrione. Two weeks after treatment (WAT), the visual injury of each plant was recorded using an injury rating scale of 0 to 100 (0 = no injury, 100% = plant mortality). Plants that had a visual injury of < 30% were grouped as less sensitive and those with >30% injury were considered most sensitive to mesotrione. Similarly, for tembotrione-treated plants, a visual injury rating from <20% was considered less sensitive and plants with >20 injury were considered sensitive; because tembotrione-treated plants had less visual injury compared to mesotrione at the 6X dose. In response to 6X mesotrione treatment, the genotypes, WW-1 and WW-2 exhibited a visual injury of ~10%, while WW-24 showed ~45 to 50% injury. Whereas the WW-1 or WW-2 had a visual injury of ~5%, and WW-24 showed ~40% injury to 6x tembotrione treatment. Therefore, these injury scores were used as the lower and upper limit while grouping the F₂ plants upon treatment with mesotrione or tembotrione. At 4 WAT, the above-ground biomass was collected, dried, and weighed as described above. The experiment was repeated.

3.3.5 Data Analysis

Relative dry biomass data from two runs of mesotrione and tembotrione dose-response experiments of each F₁ family were tested using Levene's test ($\alpha = 0.05$) in R (version 4.1.2, R

Core Team, 2020) for homogeneity of variance. The dose-response data (% of untreated control) were analyzed using the drc package in R. All the data derived from drc experiments were subjected to non-linear regression analysis using a three-parameter regression model. The model had the following formula:

$$Y = d + \exp [b (\log x - \log e)]$$

where Y is the response variable, x is the herbicide dose, d is the upper limit, b is the relative slope around e and e is GR₅₀ (amount of herbicide required to reduce plant dry weight by 50%). The GR₅₀ values of different F₁ families and populations were further tested *via* t-test using the function ‘compParm’ in ‘drc’ package.

The observed segregation ratio of the F₂ plants after herbicide treatment was compared to the expected segregation number using the chi-square test. The expected segregation of mesotrione and tembotrione phenotypes was determined based on the assumption of the Mendelian single-locus segregation model, that the mesotrione and tembotrione resistance in winter wheat is controlled by a single gene. The following equation was used:

$$\text{Exp. } F_2 = (1 \times \text{Obs. WW-1 or WW-2} + 2 \times \text{Obs. } F_1 + 1 \times \text{Obs. WW-24})$$

In this equation Exp. F₂ is the expected segregation frequency of the F₂ progenies and Obs. is the observed segregation frequency of WW-1 or WW-2, F₁, and WW-24 phenotypes. The chi-square goodness of fit test (χ^2) was used to analyze the data of the plant segregation by comparing the observed segregation frequency with the expected. The null hypothesis (H₀) for the test was that the segregation of mesotrione and tembotrione resistance in F₂ progenies would follow the 3:1 ratio. The p-value from each F₂ family was compared with the significance level of $\alpha = 0.05$. The following is the formula used for the test:

$$\chi^2 = (E-O)^2 / E$$

where χ^2 is the chi-square goodness of fit-value; O and E are the observed and expected segregation frequencies, respectively, of progenies in each F₂ family. H₀ was rejected if the obtained p-value was < 0.05. The cumulative relative weight reduction data of the F₂ progenies were also illustrated as a density plot.

3.4 Results

3.4.1 Phenotypic response of the parents and F₁ progeny to the dose-response experiments

Mesotrione and tembotrione dose responses of the parents and the F₁ progenies are shown in Figures 3.2 and 3.3. The WW-24 genotype exhibited more injury to these herbicides compared to WW-1 and WW-2. The F₁ progenies from each cross showed an intermediate injury compared to the parent genotypes at 2 WAT, although the plants recovered from the injury at 4 WAT (Figures 3.2 and 3.3). The biomass accumulation data at the 4 WAT was used to determine the GR₅₀ values in the mesotrione and tembotrione dose-response experiments.

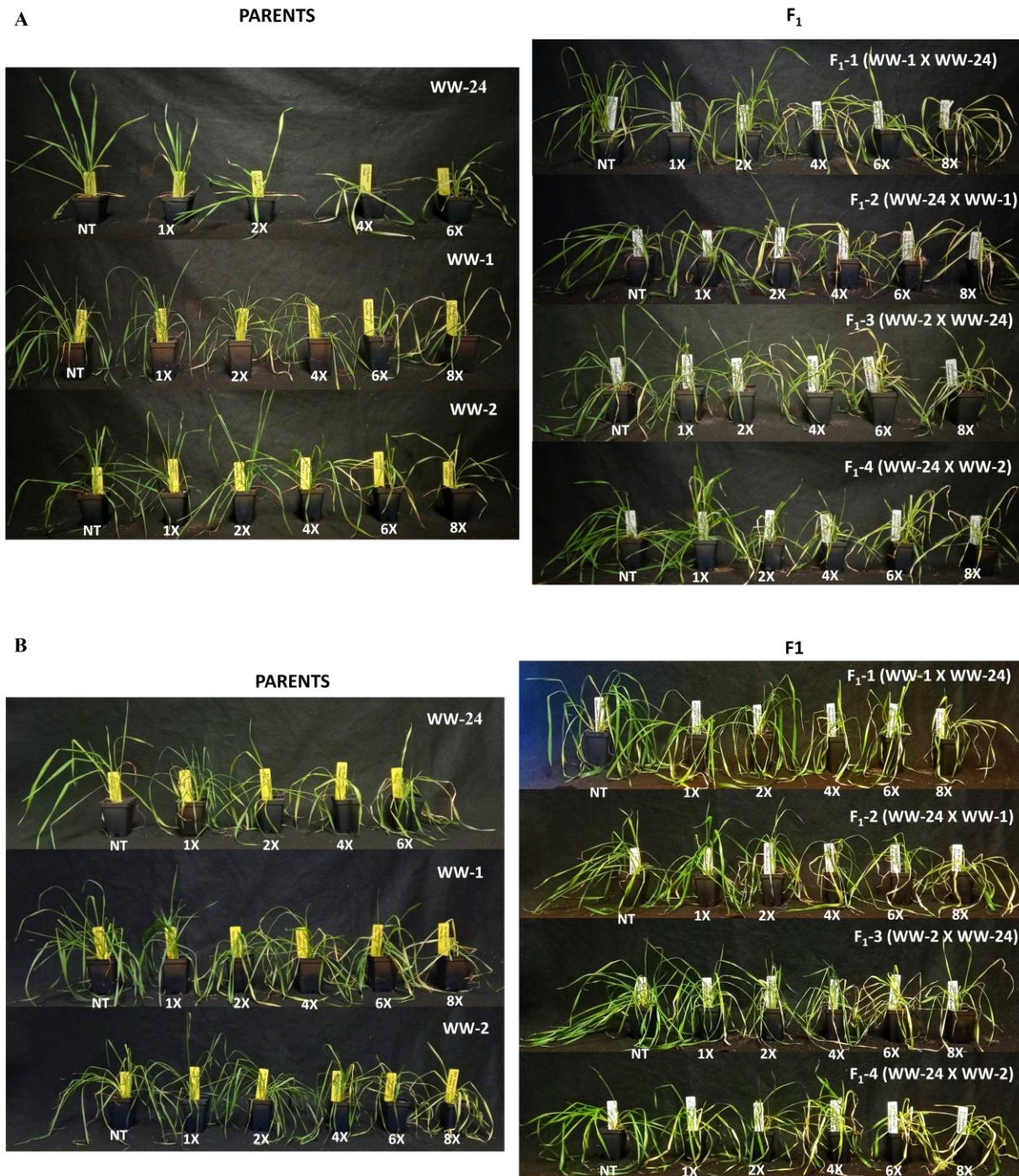


Figure 3.2. The response of A) winter wheat genotypes (WW-1, WW-2, and WW-24) and F₁ (F₁-1, F₁-2, F₁-3, and F₁-4) progenies to different doses of mesotrione at 2 weeks of treatment. B) winter wheat genotypes and F₁ progenies to different doses of mesotrione at 4 weeks of treatment.

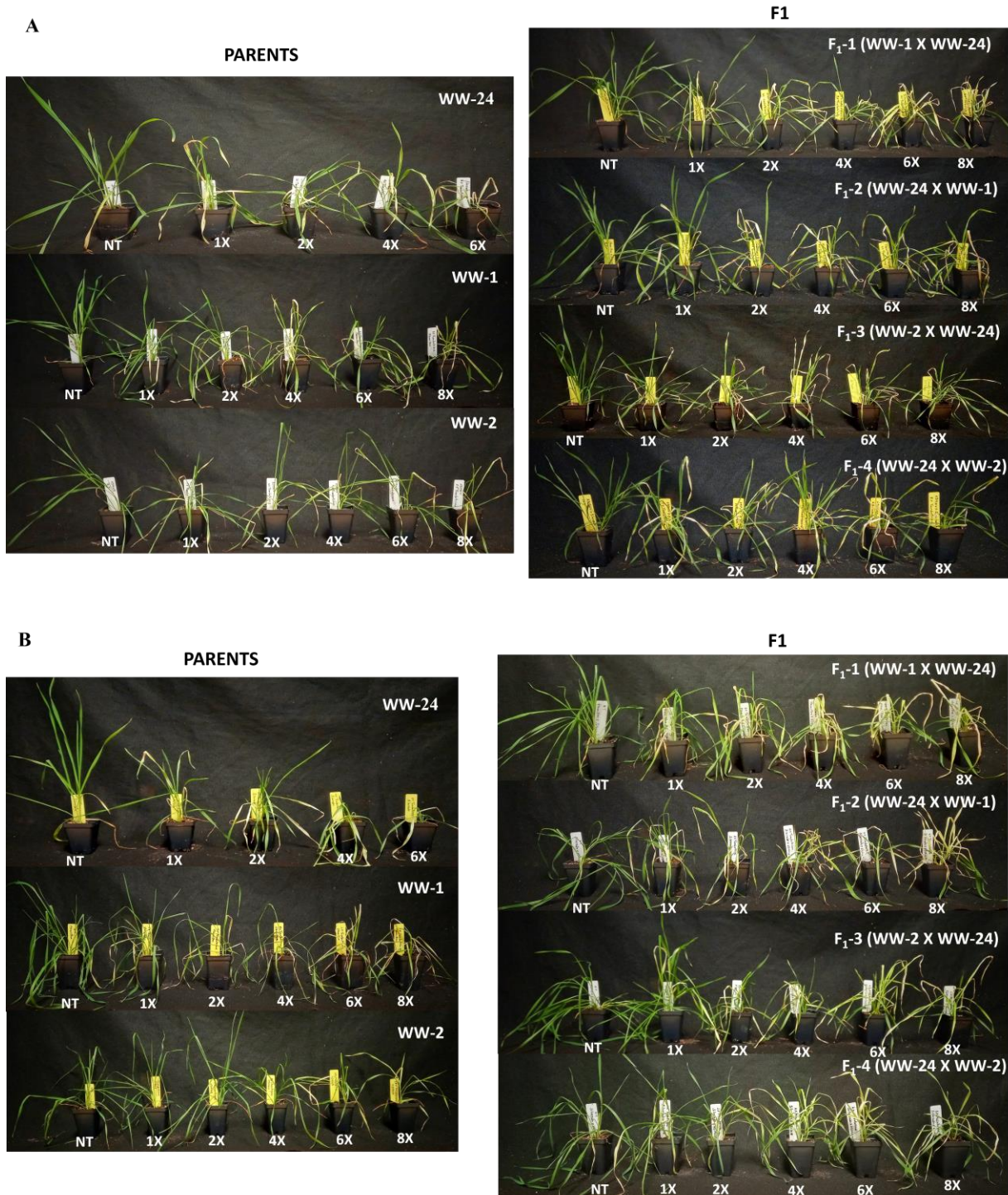


Figure 3.3. The response of A) winter wheat genotypes (WW-1, WW-2, and WW-24) and F₁ (F₁-1, F₁-2, F₁-3, and F₁-4) progenies to different doses of tembotrione at 2 weeks of treatment. B)

winter wheat genotypes and F₁ progenies to different doses of tembotrione at 4 weeks of treatment.

3.4.2 Mesotrione dose-response of WW-1, WW-2, WW-24, and F₁ progenies

The response of F₁-1, F₁-2, F₁-3, and F₁-4 progenies and parents were assessed *via* dose-response experiments. The GR₅₀ values of F₁-1 and F₁-2 were 303.08 and 343.81 g ai h⁻¹, whereas WW-1 and WW-24 were 428.27 and 191.50 g ai h⁻¹ (Figure 3.4A, Table 3-1). The GR₅₀ of F₁-1 and F₁-2 were not significantly different at $p \leq 0.05$. Similarly, The GR₅₀ of F₁-3 and F₁-4 were 245.035 and 233.488 g ai h⁻¹, while WW-2 and WW-24 had values of 328.50 and 176.77 (Figure 3.4B, Table 3-2). Further, The GR₅₀ of F₁-3 and F₁-4 were not significantly different at $p \leq 0.05$. Both the direct (F₁-1 and F₁-3) and the reciprocal cross-derived progenies (F₁-2 and F₁-4) showed a similar response to mesotrione treatment, implying that the reduced sensitivity to mesotrione is conferred by a nuclear trait. Additionally, the F₁ progenies showed an intermediate response to mesotrione treatment (Figure 3.4 and Table 3-1, 3-2) compared to the parents suggesting that reduced sensitivity to mesotrione in WW-1 and WW-2 is an incompletely dominant trait.

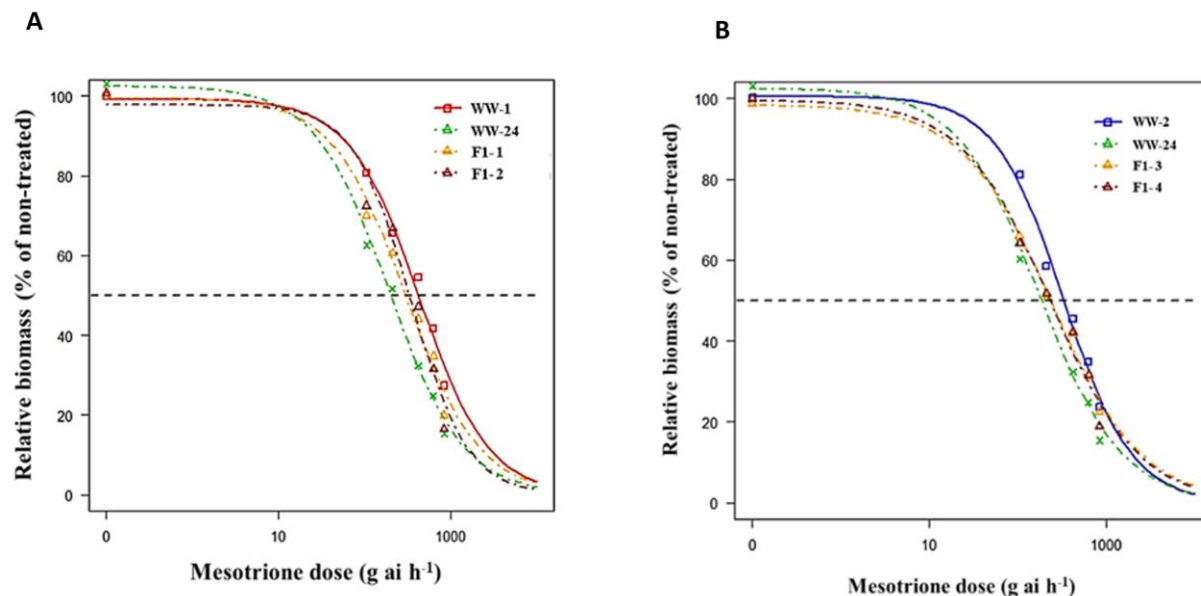


Figure 3.4. A) Mesotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-1, WW-24, F₁-1, and F₁-2 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight. B) Mesotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-2, WW-24, F₁-3, and F₁-4 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight.

Table 3-1. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1 (least sensitive), F₁-1, and F₁-2 aboveground dry biomass in response to different doses of mesotrione at 4 weeks after treatment (WAT). (GR₅₀: dose required for 50% growth reduction; SE: standard error).

Genotype	Regression parameter		GR ₅₀ (g ai h ⁻¹)
	b (SE)	d (SE)	
WW-24	1.00 (0.06)	102.49 (2.49)	191.50 (14.39)
WW-1	1.07 (0.06)	99.27 (2.48)	428.27 (28.4)***
F ₁ -1	1.02 (0.07)	99.51 (2.51)	303.08 (14.39)***
F ₁ -2	1.30 (0.10)	97.78 (2.59)	343.81 (21.7)***

***Significantly different from WW-24 at $p \leq 0.001$

Table 3-2. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-2 (least sensitive), F₁-3, and F₁-4 aboveground dry biomass to different doses of mesotrione at 4 weeks after treatment (WAT). (GR₅₀: dose required for 50% growth reduction; SE: standard error).

Genotype	Regression parameter		GR ₅₀ (g ai h ⁻¹)
	b (SE)	d (SE)	
WW-24	0.93 (0.06)	100.50 (2.28)	176.77 (13.43)
WW-2	1.12 (0.05)	100.52 (2.35)	328.50 (19.92)***
F ₁ -3	0.84 (0.06)	98.40 (2.33)	245.03 (20.19)**
F ₁ -4	0.86 (0.64)	99.53 (2.35)	233.48 (19.09)***

***, ** Significantly different from WW-24 at $p \leq 0.001$ and $p \leq 0.01$

3.4.3 Tembotrione dose-response of WW-1, WW-24, and F₁ progenies

The analyses of data of tembotrione dose-response assay suggested the GR₅₀ of F₁-1 and F₁-2 as 467.82 and 472.82 g ai h⁻¹; whereas the parents, WW-24 and WW-1 were 346.06 and 472.82 g ai h⁻¹ (Figure 3.5A, Table 3-3). The F₁-3 and F₁-4 progenies had GR₅₀ values of 405.19 and 390.31 g ai h⁻¹, respectively, and WW-2 and WW-24 had 444.40 g and 345.99 g ai h⁻¹ (Figure 3.5B, Table 3-4). Further, the GR₅₀ of the direct and reciprocal crosses were not significantly different at $p \leq 0.05$ implying that reduced sensitivity to tembotrione in WW-1 and WW-2 is controlled by a nuclear trait. In addition, the F₁ progenies showed an intermediate response to tembotrione (Figure 3.5 and Table 3-3, 3-4) compared to the parents suggesting that

reduced sensitivity to tembotrione in winter wheat (WW-1 and WW-2) is conferred by an incompletely dominant trait.

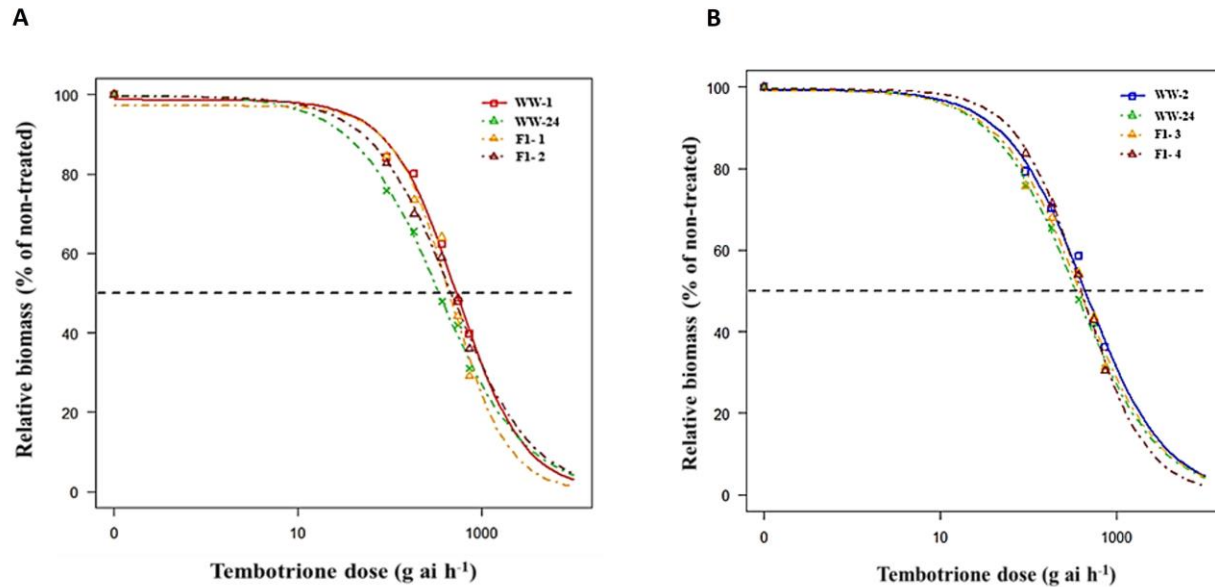


Figure 3.5. A) Tembotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-1, WW-24, F₁-1, and F₁-2 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight. B) Tembotrione dose-response curves representing the relative dry weight (% of non-treated) of WW-2, WW-24, F₁-3, and F₁-4 progenies using the three-parameter log-logistic regression model at 4 WAT. The dotted arrow at the center of the plot represents 50% of the relative dry weight.

Table 3-3. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-1 (least sensitive), F₁-1, and F₁-2 aboveground dry biomass to different doses of tembotrione at 4 weeks after treatment (WAT). (GR₅₀: dose required for 50% growth reduction; SE: standard error).

Genotype	Regression parameter		GR ₅₀ (g ai h ⁻¹) (SE)
	b (SE)	d (SE)	
WW-24	1.19 (0.14)	99.69 (2.74)	346.06 (30.40)
WW-1	0.93 (0.08)	98.62 (2.84)	543.38 (38.90)**
F ₁ -1	1.00 (0.10)	97.13 (2.87)	465.82 (31.20)
F ₁ -2	1.40 (0.14)	99.49 (2.76)	472.82 (38.78)*

**, * Significantly different from WW-24 at $p \leq 0.01$ and $p \leq 0.05$

Table 3-4. Summary of regression parameters describing the response of WW-24 (most sensitive), WW-2 (least sensitive), F₁-3, and F₁-4 aboveground dry biomass to different rates of tembotrione at 4 weeks after treatment (WAT). (GR₅₀: dose required for 50% growth reduction; SE: standard error).

Genotype	Regression parameter		GR ₅₀ (g ai h ⁻¹) (SE)
	b (SE)	d (SE)	
WW-24	0.93 (0.07)	99.69 (2.23)	345.99 (26.47)
WW-2	0.97 (0.09)	99.29 (2.41)	444.40 (34.01)*
F ₁ -3	0.95 (0.11)	99.06 (2.42)	405.19 (25.35)
F ₁ -4	1.19 (0.09)	99.38 (2.36)	390.31 (29.66)

* Significantly different from WW-24 $p \leq 0.05$

3.4.4 Segregation of reduced or increased sensitivity to mesotrione of F₂ progenies

The response of the F₂ families along with the parents were evaluated by treating with mesotrione at a dose of 630 g ai h⁻¹. At 2 WAT, WW-1 (least sensitive) and WW-24 (most sensitive) showed visual scores of ~ 10% and ~ 45%, respectively (data not shown). The F₂-1 and F₂-2 progenies showed a varied response ranging from 10% to 50%. The plants that showed a visual injury of 10% to 30% were grouped as least sensitive and those with 31% to 50% as most sensitive to mesotrione. Based on Chi-square test analyses, segregation of F₂-1 and F₂-2 plants as most and least sensitive plants, deviated from the 3:1 for a single gene inheritance model (Table 3-5). Similar results were also found for F₂-3 and F₂-4 progenies; with deviation from the 3:1 segregation ratio for a single gene-dominant trait (Table 3-6). Overall, based on the

segregation of F₂ progenies, the reduced sensitivity of WW-1 and WW-2 to mesotrione is likely controlled by multiple genes.

3.4.5 Segregation of reduced or increased sensitivity to tembotrione of F₂ progenies

The response of the F₂ families along with the parents to a tembotrione dose of 552 g ai h⁻¹ was evaluated. At 2 WAT, WW-1 (least sensitive) and WW-24 (most sensitive) showed visual injury scores of ~ 5% and ~ 35%, respectively (data not shown); whereas the F₂-1 and F₂-2 progenies showed varied responses ranging from 5% to 40%. The plants that showed a visual injury of 5% to 20% were grouped as least sensitive and those with 21% to 40% as most sensitive. The segregation of F₂-1 and F₂-2 as well as F₂-3 and F₂-4 progenies deviated from the expected 3:1 segregation ratio for a single gene-dominant trait (Table 3-7, 3-8), suggesting that the reduced sensitivity to tembotrione in WW-1 and WW-2 is also likely controlled by multiple genes.

Furthermore, the distribution of the relative dry biomass accumulation of the F₂ progenies was illustrated through a density plot. The data showed widespread biomass accumulation bins representing plant-to-plant variation in response to mesotrione and tembotrione, as expected for a multi-genic trait (Figures 3.6 and 3.7).

Table 3-5. Chi-square test for goodness of fit of the observed segregation of plants as least (10% to 30%) or most (31% to 50%) sensitive to mesotrione for a single-locus model in F₂-1 and F₂-2 families of winter wheat. The expected survival frequency for each F₂ family was calculated and compared with the observed frequency of differential sensitivity to mesotrione.

Run	F ₂ Family/ Population	Total plants	Observed		Expected		P-value
			10% -	31% -	10% -	31% -	
			30%	50%	30%	50%	
1	F ₂ -1-1	32	18	14	24	8	0.01431
	F ₂ -1-2	30	15	15	22.5	7.5	0.001565
	F ₂ -1-3	22	12	10	16.5	5.5	0.02672
	F ₂ -1-4	20	5	15	15	5	<0.00001
	Total (F ₂ -1)	104	50	54	78	26	<0.00001
	F ₂ -2-1	22	10	12	16.5	5.5	0.001373
	F ₂ -2-2	15	10	5	11.25	3.75	0.4561
	F ₂ -2-3	20	10	10	15	5	0.00986
	F ₂ -2-4	25	5	20	18.75	6.25	<0.00001
	Total (F ₂ -2)	82	35	47	61.5	20.5	<0.00001
2	F ₂ -1-1	15	5	10	11.25	3.75	0.000194
	F ₂ -1-2	20	10	10	15	5	0.00986
	F ₂ -1-3	12	2	10	9	3	<0.00001
	F ₂ -1-4	20	10	10	15	5	0.00986
	Total (F ₂ -1)	67	27	40	50.25	16.75	<0.00001
	F ₂ -2-1	20	12	8	15	5	0.1213

	F ₂ -2-2	10	3	7	7.5	2.5	0.001015
	F ₂ -2-3	22	12	10	16.5	5.5	0.02672
	F ₂ -2-4	14	7	7	10.5	3.5	0.03077
	Total (F ₂ -2)	66	34	32	49.5	16.5	<0.0001
1 & 2	F₂-1	171	77	94	128.25	42.75	<0.00001
Com bined	F₂-2	148	69	79	111	37	<0.00001

Table 3-6. Chi-square test for goodness of fit of the observed segregation of plants as least sensitive (10% to 30%) or most sensitive (31% to 50%) to mesotrione to the expected frequency for a single-locus model in F₂-3 and F₂-4 families of winter wheat. The expected survival frequency for each F₂ family was calculated and compared with the observed frequency of differential sensitivity to mesotrione.

Run	F₂ Family/ Population	Total plants	Observed		Expected		P-value
			10% 30%	- 50%	10% 30%	- 50%	
1	F ₂ -3-1	22	20	2	16.5	5.5	0.08535
	F ₂ -3-2	15	5	10	11.25	3.75	0.000194
	F ₂ -3-3	20	10	10	15	5	0.009827
	F ₂ -3-4	12	5	7	9	3	0.007661
	Total (F ₂ -3)	69	40	29	51.75	17.25	0.001089
	F ₂ -4-1	30	10	20	22.5	7.5	<0.0001

	F ₂ -4-2	17	9	8	12.75	4.25	0.03571
	F ₂ -4-3	22	10	12	16.5	5.5	0.001374
	F ₂ -4-4	23	13	10	17.25	5.75	0.0407
	Total (F ₂ -4)	92	42	50	69	23	<0.0001
2	F ₂ -3-1	25	5	20	18.75	6.25	<0.0001
	F ₂ -3-2	22	10	12	16.5	5.5	0.001374
	F ₂ -3-3	10	10	0	7.5	2.5	0.06803
	F ₂ -3-4	23	13	10	17.25	5.75	0.0409
	Total (F ₂ -3)	80	38	42	60	20	<0.0001
	F ₂ -4-1	24	12	12	18	6	0.004678
	F ₂ -4-2	18	3	15	13.5	4.5	<0.0001
	F ₂ -4-3	19	10	9	14.25	4.75	0.02434
	F ₂ -4-4	27	14	13	20.25	6.75	0.005473
	Total (F ₂ -4)	88	39	49	66	22	<0.0001
1 & 2 combined	F₂-3	149	78	71	111.75	37.25	<0.0001
	F₂-4	180	81	99	135	45	<0.0001

Table 3-7. Chi-square test for goodness of fit of the observed segregation of plants as less sensitive (5% to 20%) or most sensitive (21% to 40%) to tembotrione to the expected frequency for a single-locus model in F₂-1 and F₂-2 families of winter wheat. The expected survival

frequency for each F₂ family was calculated and compared with the observed frequency of differential sensitivity to tembotrione.

Run	F ₂ Family/ Population	Total plants	Observed		Expected		P-value
			5% 20%	- 40%	5% 20%	- 40%	
1	F ₂ -1-1	30	15	15	22.5	7.5	0.001565
	F ₂ -1-2	15	3	12	11.25	3.75	<0.00001
	F ₂ -1-3	20	10	10	15	5	0.00986
	F ₂ -1-4	25	5	20	18.75	6.25	<0.00001
	Total (F ₂ -1)	90	33	57	67.5	22.5	<0.00001
	F ₂ -2-1	20	10	10	15	5	0.00986
	F ₂ -2-2	25	10	15	18.75	6.25	<0.0001
	F ₂ -2-3	22	12	10	16.5	5.5	0.02672
	F ₂ -2-4	15	5	10	11.25	3.75	0.0001
	Total (F ₂ -2)	82	37	45	61.5	20.5	<0.00001
2	F ₂ -1-1	30	5	25	22.5	7.5	<0.00001
	F ₂ -1-2	24	10	14	18	6	0.0001
	F ₂ -1-3	22	12	10	16.5	5.5	0.02672
	F ₂ -1-4	12	12	0	9	3	0.0455
	Total (F ₂ -1)	88	39	49	66	22	<0.00001
	F ₂ -2-1	22	12	10	16.5	5.5	0.02672
	F ₂ -2-2	15	7	8	11.25	3.75	0.01127

	F ₂ -2-3	22	12	10	16.5	5.5	0.02672
	F ₂ -2-4	24	14	10	18	6	0.05935
	Total (F ₂ -2)	83	45	38	62.25	20.75	<0.0001
1 & 2	F₂-1	178	72	106	133.5	44.5	<0.0001
combined	F₂-2	165	82	83	123.75	41.25	<0.0001

Table 3-8. Chi-square test for goodness of fit of the observed segregation of plants as less sensitive (5% to 20%) or most sensitive (21% to 40%) to tembotrione the expected frequency for a single-locus model in F₂-3 and F₂-4 families of winter wheat. The expected survival frequency for each F₂ family was calculated and compared with the observed frequency of differential sensitivity to mesotrione.

Run	F ₂ Family/ Population	Total plants	Observed		Expected		P-value
			5% 20%	- 40%	5% 20%	- 40%	
1	F ₂ -3-1	20	10	10	15	5	0.00986
	F ₂ -3-2	25	15	10	18.75	6.25	0.08326
	F ₂ -3-3	15	5	10	11.25	3.75	0.000195
	F ₂ -3-4	22	11	11	16.5	5.5	0.00677
	Total (F ₂ -3)	82	41	41	61.5	20.5	<0.0001

	F ₂ -4-1	22	12	10	16.5	5.5	0.02672
	F ₂ -4-2	23	13	10	17.25	5.75	0.0407
	F ₂ -4-3	20	10	10	15	5	0.009827
	F ₂ -4-4	25	14	11	18.75	6.25	0.02824
	Total (F ₂ -4)	90	49	41	67.5	22.5	<0.0001
2	F ₂ -3-1	20	5	15	15	5	<0.0001
	F ₂ -3-2	22	21	1	16.5	5.5	0.02686
	F ₂ -3-3	18	10	8	13.5	4.5	0.05678
	F ₂ -3-4	15	2	13	11.25	3.75	<0.0001
	Total (F ₂ -3)	75	38	37	56.25	18.75	<0.0001
	F ₂ -4-1	23	13	10	17.25	5.75	<0.0001
	F ₂ -4-2	19	10	9	14.25	4.75	0.02434
	F ₂ -4-3	20	10	10	15	5	0.009823
	F ₂ -4-4	24	10	14	18	6	0.000163
	Total (F ₂ -4)	86	43	43	64.5	21.5	<0.0001
1 & 2	F ₂ -3	157	79	78	117.75	39.25	<0.0001
combin	F ₂ -4	176	92	84	132	44	<0.0001

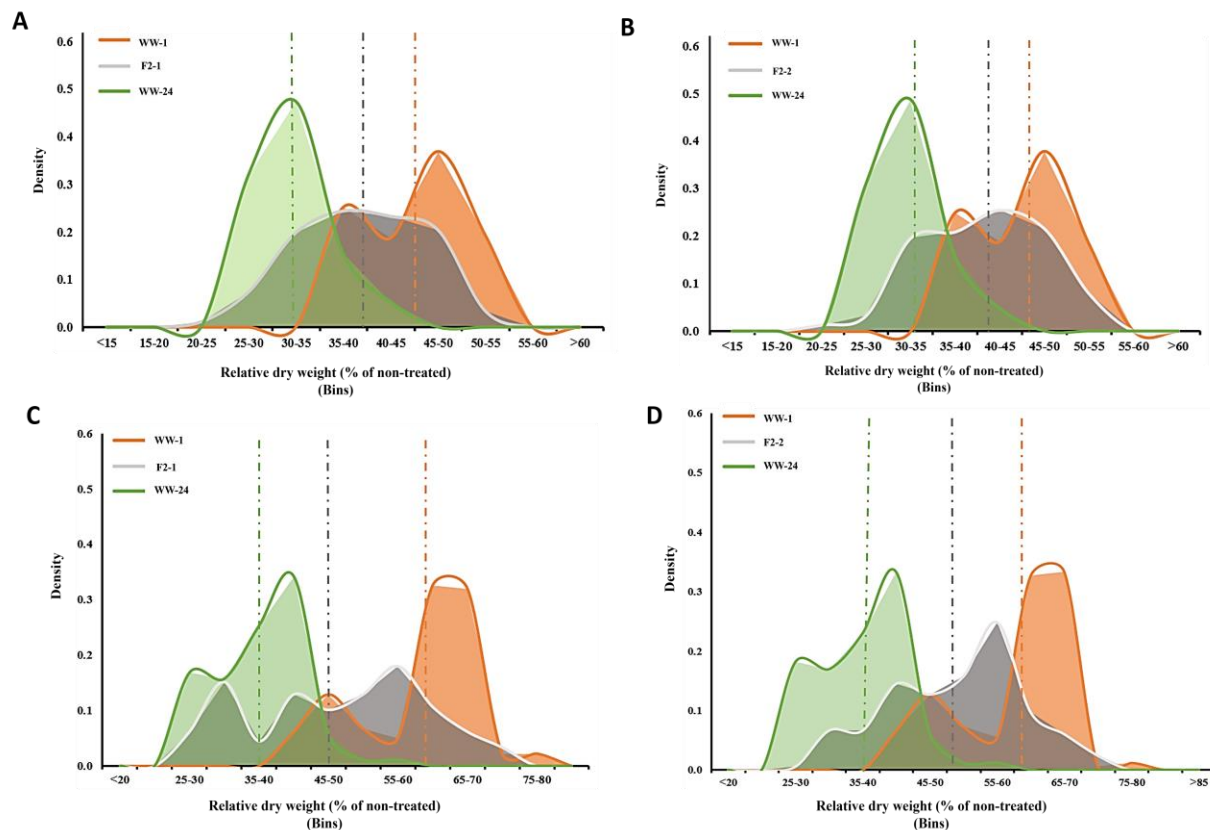


Figure 3.6. Distribution of cumulative dry biomass accumulation of WW-24 (most sensitive), WW-1 (least sensitive), F₂-1, and F₂-2 progenies in response to 6X dose of mesotrione (A & B) and tembotrione (C & D) *via* density plots. The relative dry weight reduction of samples was converted into bins and plotted on the X-axis. The Y-axis was plotted by calculating density which is the proportion of total plants in each bin. The dotted lines represent the mean dry weight of WW-1, WW-24, F₂-1, and F₂-2.

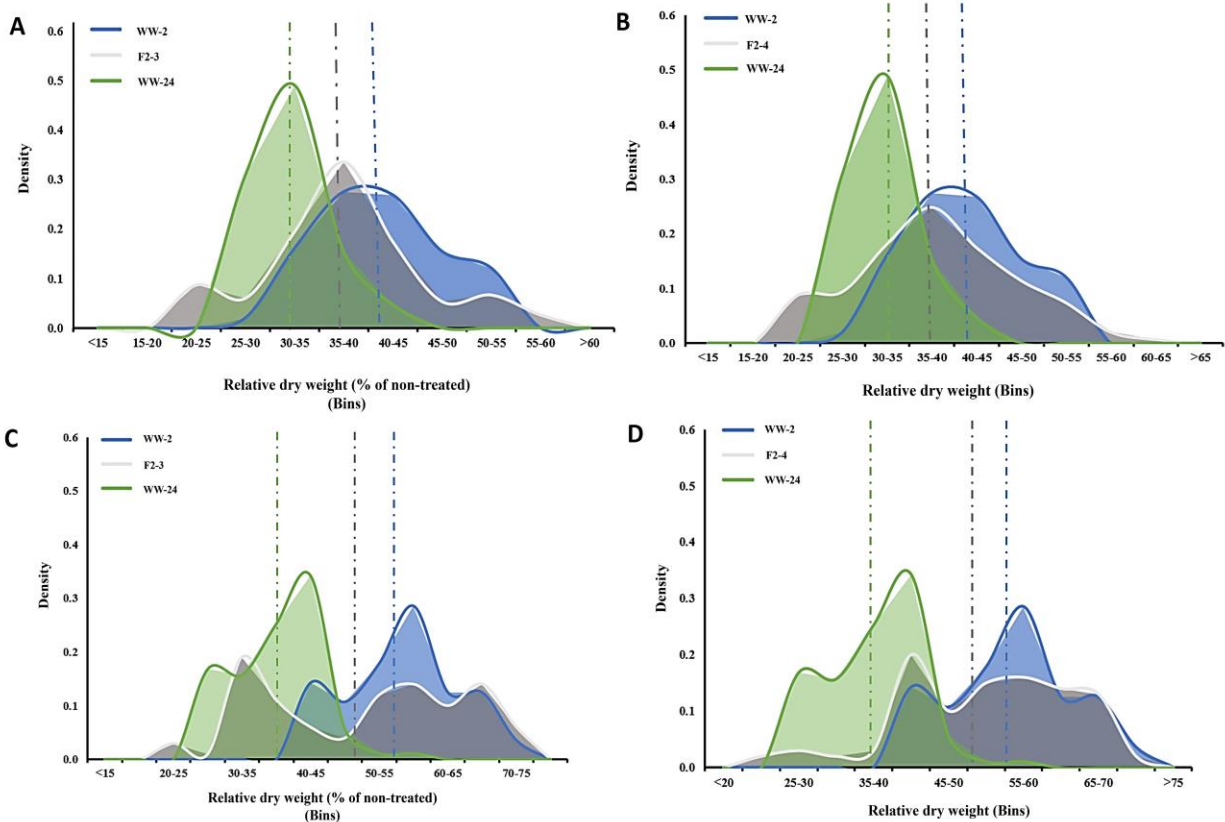


Figure 3.7. Distribution of cumulative dry weight reduction of WW-24 (most sensitive), WW-2 (less sensitive), F₂-3, and F₂-4 progenies in response to 6X dose of mesotrione (A & B) and tembotrione (C & D) *via* density plots. The relative dry weight reduction of samples was converted into bins and plotted on the X-axis. The Y-axis was plotted by calculating density which is the proportion of total plants in each bin. The dotted lines represent the mean dry weight of WW-2, WW-24, F₂-1, and F₂-2.

3.5 Discussion

In response to the treatment with multiple doses of mesotrione or tembotrione, the F₁ progenies derived from direct or reciprocal crosses (F₁-1 and F₁-2 as well as F₁-3 and F₁-4) showed an intermediate response compared to their parents, WW-1 and WW-24 (Figure 3.2A, B; Table 3.1, 3.2; Figure 3.3A, B; Table 3-3, 3-4), suggesting that the differential sensitivity to both

mesotrione and tembotrione in WW-1 and WW-2 is controlled by partially-dominant traits. F₁ progenies exhibit intermediate responses compared to parents to triketones as well as other herbicide resistances in crops and weeds. In sorghum, the response of F₁ progeny to tembotrione treatment suggested that the tembotrione resistance is a partially dominant trait (Pandian et al., 2020b). However, in some cases, HPPD-inhibitor resistance was also found to be an incompletely recessive trait (Huffman et al., 2015). On the other hand, in a waterhemp population from Nebraska, the HPPD-inhibitor resistance showed varied patterns of incomplete recessive to incomplete dominance based on the response of F₁ progeny (Oliveira et al., 2018).

In this study, the F₁ progenies derived from both direct and reciprocal crosses show similar responses to either mesotrione or tembotrione indicating that differential sensitivity of winter wheat to triketones is a nuclear trait. Studies so far have shown that 95% of herbicide-resistant alleles are nuclear inherited and only 5% as maternal (Ghanizadeh et al., 2019). Resistance to acetolactate synthase (ALS)-inhibitor, acetyl-coenzyme A carboxylase (ACCase)-inhibitors, glyphosate, auxinic herbicides, dinitroanilines, protoporphyrinogen oxidase (PPO)-inhibitors in several weed species were reported to be nuclear traits, which can spread both *via* pollen and seeds (Ghanizadeh et al., 2019). If a trait is maternally inherited, seeds rather than pollen would be the primary vehicle for spreading the resistant allele (Gronwald, 1997).

The four families of F₂-1 (F₂-1-1, F₂-1-2, F₂-1-3, F₂-1-4), F₂-2 (F₂-2-1, F₂-2-2, F₂-2-3, F₂-2-4), F₂-3 (F₂-3-1, F₂-3-2, F₂-3-3, F₂-3-4), and F₂-4 (F₂-4-1, F₂-4-2, F₂-4-3, F₂-4-4) were evaluated with the discriminatory doses of mesotrione and tembotrione separately and the response of these progenies did not follow a typical Mendelian segregation for a single gene inheritance (Table 3-5, 3-6, 3-7, 3-8), suggesting that the reduced sensitivity to mesotrione and tembotrione in WW-1 and WW-2 is a polygenic trait. Further, the variability represented in the density plot showed no left-

skewed distribution, characteristic of a single gene dominant trait (Figure 3.6, 3.7). The results of Chapter 2 suggested that the metabolism of triketone herbicides is likely mediated by P450 enzymes confer reduced sensitivity in WW-1 and WW-2 winter wheat. Mesotrione resistance in waterhemp was also possibly bestowed by P450s (Ma et al., 2013). Similarly, mesotrione resistance in Palmer amaranth is conferred primarily by metabolism possibly *via* P450 enzyme activity although, overexpression of the *HPPD* gene, the target site of mesotrione was also found as an additional mechanism (Nakka et al., 2017b). Furthermore, overexpression of P450 genes, *CYP81E8* was implicated in the rapid metabolism of tembotrione in a Palmer amaranth population (Aarthy et al., 2022). In sorghum, the metabolism of mesotrione and tembotrione likely *via* P450 activity was reported (Pandian et al., 2023; Pandian et al., 2020b). Natural tolerance to triketone herbicides in corn is also attributed to P450 activity (Brazier-Hicks et al., 2022).

Metabolic resistance to HPPD-inhibitors is controlled by multiple genes in several species. In sorghum, the metabolic resistance to tembotrione was a polygenic trait (Pandian et al., 2020b). Similarly, in several Palmer amaranth and waterhemp populations, triketone herbicide resistance is inherited by multiple genes (Huffman et al., 2015; Oliveira et al., 2018; Shyam et al., 2021b). However, HPPD-inhibitor resistance in plants has not always been a polygenic trait. Genetic analysis of sweet corn inbred lines suggests that tembotrione sensitivity is controlled by a single recessive allele (Williams & Pataky, 2008). The mesotrione resistance in sorghum is also controlled by a single gene (Pandian et al., 2023).

The metabolism of herbicides is a complex process mediated *via* various biochemical reactions primarily in three Phases, i.e., (I) herbicide inactivation (II) conjugation, and (III) transporting or compartmentalizing of degraded molecules into the vacuole of a plant cell. The Phase I process is carried out by the P450 monooxygenases by adding a functional group to the

herbicide *via* oxidation, reduction, or hydroxylation (Gaines et al., 2020; Siminszky, 2006). Apart from the P450 genes, metabolic resistance can also involve conjugation and transportation processes. Additionally, genes involved in stress response and plant recovery may also play a major role in imparting less sensitivity including herbicide stress. Therefore, a thorough analysis of the genes imparting less sensitivity in WW-1 and WW-2 is warranted. An RNA-seq analysis followed by validation of gene expression would help to identify the candidate genes involved in reduced sensitivity of WW-1 and WW-2 to triketone herbicides.

3.6 Conclusions

In summary, the F₁ data suggest that the reduced sensitivity of WW-1 and WW-2 winter wheat is a partially dominant nuclear trait. The response of the F₂ progenies suggests that multiple genes impart reduced sensitivity. Since the triketone herbicides are likely metabolized by P450s, an RNA-seq analysis will help identify the genes involved in reduced sensitivity. Further, identifying QTLs associated with the reduced sensitivity to triketones will be useful for future breeding programs.

Chapter 4 - Overexpression of *TaHPPD* or *CYP81Q32-like* genes in wheat (*Triticum aestivum*) reduces sensitivity to triketone herbicides

4.1 Abstract

Effective weed control is essential for the sustainable production of agronomically important crops like wheat (*Triticum aestivum*). The repeated use of herbicides for weed control in the wheat fields led to the evolution of herbicide-resistant weeds. Development of new herbicide-resistant technologies in wheat will provide alternate herbicide options for managing herbicide-resistant weeds. Triketone herbicide such as mesotrione is widely used in corn but not registered for use in wheat due to crop injury. Corn is naturally tolerant to triketones due to its ability to rapidly metabolize the herbicides via P450 enzyme, *CYP81A9*. Two winter wheat genotypes, WW-1 and WW-2 were found least sensitive to these herbicides, possibly via P450-mediated metabolism (Chapters 2). We found a *CYP81Q32-like* gene in wheat with ~83% sequence similarity with the corn *CYP81A9* gene. Therefore, we hypothesized that overexpressing the *CYP81Q32-like* gene in a known, triketone herbicide-sensitive, spring wheat cultivar Bobwhite, may demonstrate the function of this gene in reduced sensitivity of WW-1 and WW-2 to these herbicides. Additionally, previous reports suggest alteration of the molecular target of these herbicides, i.e., the *HPPD* gene provided resistance to triketones in other plants. Thus, we also hypothesized that overexpressing the native *TaHPPD* gene may also reduce the sensitivity of wheat to these herbicides. The overall objective of this Chapter was to develop transgenic lines expressing the *CYP81Q32-like* or *TaHPPD* genes of wheat and assess their response to mesotrione. Transformation via particle bombardment method was used. Callus-

derived T₀ plants were generated. Molecular analysis of the transgenic T₁ plants showed that a few lines expressed relatively higher levels of mRNA transcripts. Further, phenotypic analysis of these lines also correlated with less sensitivity to mesotrione. Therefore, the outcome of this research suggests that overexpression of these genes reduce mesotrione sensitivity in wheat.

4.2 Introduction

Wheat (*Triticum aestivum*) is an important cash crop consumed worldwide (Shewry, 2009). One of the major factors affecting global wheat production is weed infestation. Uncontrolled weed infestation may potentially reduce wheat yield by up to 23% (Oerke, 2006). As a result, an effective weed management program is warranted. Chemical weed control is the most widely used strategy in wheat; about 61% and 96% of winter wheat and spring wheat fields are sprayed with herbicides (Flessner et al., 2021). The evolution of herbicide-resistant weeds due to the repeated usage of herbicides needs to be addressed by diversifying the herbicide options in wheat. Currently, there are only two herbicide-resistant wheat technologies commercially available: 1) the Clearfield production system harbors mutation Serine653Asparagine in the acetolactate synthase (ALS) gene which confers resistance to the herbicide Imazamox (Beyond) (Rojano-Delgado et al., 2015; Sudianto et al., 2013) and 2) the CoAXium production system is resistant to Quizalofop-ethyl (Aggressor) herbicide due an Alanine2004Valine mutation in the acetyl-CoA carboxylase (ACCase) gene (www.coaxiumwps.com). These two production systems were developed by mutagenesis and marketed as non-GMO-based technologies (Nakka et al., 2019). Currently, there is no transgenic wheat available in the market. To broaden the herbicide options in wheat, developing herbicide-resistant technologies targeting different herbicide mode of action groups is necessary.

4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors such as mesotrione and tembotrione belong to the triketone family of herbicides (Lee et al., 1998). These herbicides are widely used in corn fields for broad-spectrum control of weeds but are not registered for use in wheat. A cytochrome P450 (P450) gene, *CYP81A9* also known as *Nsf1* is responsible for the natural tolerance to these herbicides in corn (Bollman et al., 2008; Brazier-Hicks et al., 2022).

Among triketone herbicides, mesotrione is registered only for pre-emergence (PRE) use in sorghum, whereas tembotrione is not registered due to crop injury (Pandian et al., 2020b). Soybean is naturally sensitive to HPPD-inhibitors; recently, HPPD-resistant transgenic soybean containing modified HPPD gene from *Pseudomonas fluorescens* and *Avena sativa* was by Bayer Crop Science and Syngenta, respectively, for commercial use (Kramer et al., 2014; Nandula, 2019).

Studies have shown that HPPD-inhibitor resistance in weed species is mainly due to the rapid metabolism of triketones likely mediated by the P450s (Lu et al., 2020; Ma et al., 2013; Nakka et al., 2017b). However, increased expression of *HPPD* (target gene) as a secondary mechanism of resistance was also reported in a Palmer amaranth population (Nakka, et al., 2017b). Moreover, target site mutation in wild oat (*Avena sativa*) *avhppd-3* gene conferring resistance to HPPD-inhibitors is also known (Kramer et al., 2014).

Upon screening and assessing the physiological basis, two winter wheat genotypes, WW-1 and WW-2 were found to be least sensitive to triketone herbicides likely mediated by P450 metabolism (Chapter 2). As described above, HPPD-inhibitor resistance could be bestowed by various mechanisms; and one of those possibly be conferring less sensitivity of WW-1 and WW-2 to these herbicides. The main goal of this study was the functional validation of genes responsible for triketone sensitivity. We hypothesized that overexpressing the wheat *TaHPPD* (molecular target of these herbicides) and *CYP81Q32-like* gene in a known, triketone herbicide-sensitive, spring wheat cultivar ‘Bobwhite’, may reduce its sensitivity to triketones. The objectives were to 1) develop transgenic lines expressing the wheat *TaHPPD* gene and assess their response to mesotrione, 2) develop transgenic lines expressing the *CYP81Q32-like* gene and

assess their response to mesotrione and 3) downregulate the *CYP81Q32-like* gene via CRISPR/Cas and assess the mutants for increased mesotrione sensitivity.

4.3 Materials and method

4.3.1 Designing constructs for transformation

The *TaHPPD* gene and *CYP81Q32-like* genes were retrieved from the National Center of Biological Information (NCBI) database. The nucleotide sequence of the *CYP81A9* gene of corn was blasted against the wheat genome to find the CYP gene that shares sequence similarity. The *CYP81Q32-like* (XM_044543291.1) gene was found to be approximately 83% similar to that of the corn gene. Further *TaHPPD* (XM_044555889.1) gene and *CYP81Q32-like* (XM_044543291.1) genes were blasted against at least 14 varieties of wheat genome (both winter wheat and spring wheat included) in the Ensembl Plant database. The *TaHPPD* gene and *CYP81Q32-like* gene sequences were found to belong to the B and D genomes, respectively. Upon aligning all the 14 nucleotide sequences using a multiple sequence alignment tool, clustal omega; the sequence of the genes was found to be similar across the wheat varieties. Therefore, these gene sequences were used. The codon-optimized genes were synthesized and cloned into the pAHC17 vector by GenScript (Piscataway, NJ, US). The genes were cloned under the control of the maize Ubiquitin1 (Ubi-1) promoter and Nopaline Synthase terminator (tNOS). The plasmid containing the *TaHPPD* gene was named PA17_HPPD and the *CYP81Q32-like* gene was named PA17_CYP81Q32. These plasmids were bombarded separately into the embryos of wheat cultivar Bobwhite, via the biolistic method (Tian et al., 2019) which is described later. The plasmids were co-bombarded with pAHC20, which contains the *bar* gene under the control of the Ubi-1 promoter (Christensen & Quail, 1996). The *bar* gene imparts resistance to the

herbicide glufosinate, which is used as a plant selectable marker to detect transgenic plants. The schematic representation shows the gene of interest cloned into the plasmid (Figure 4.1).

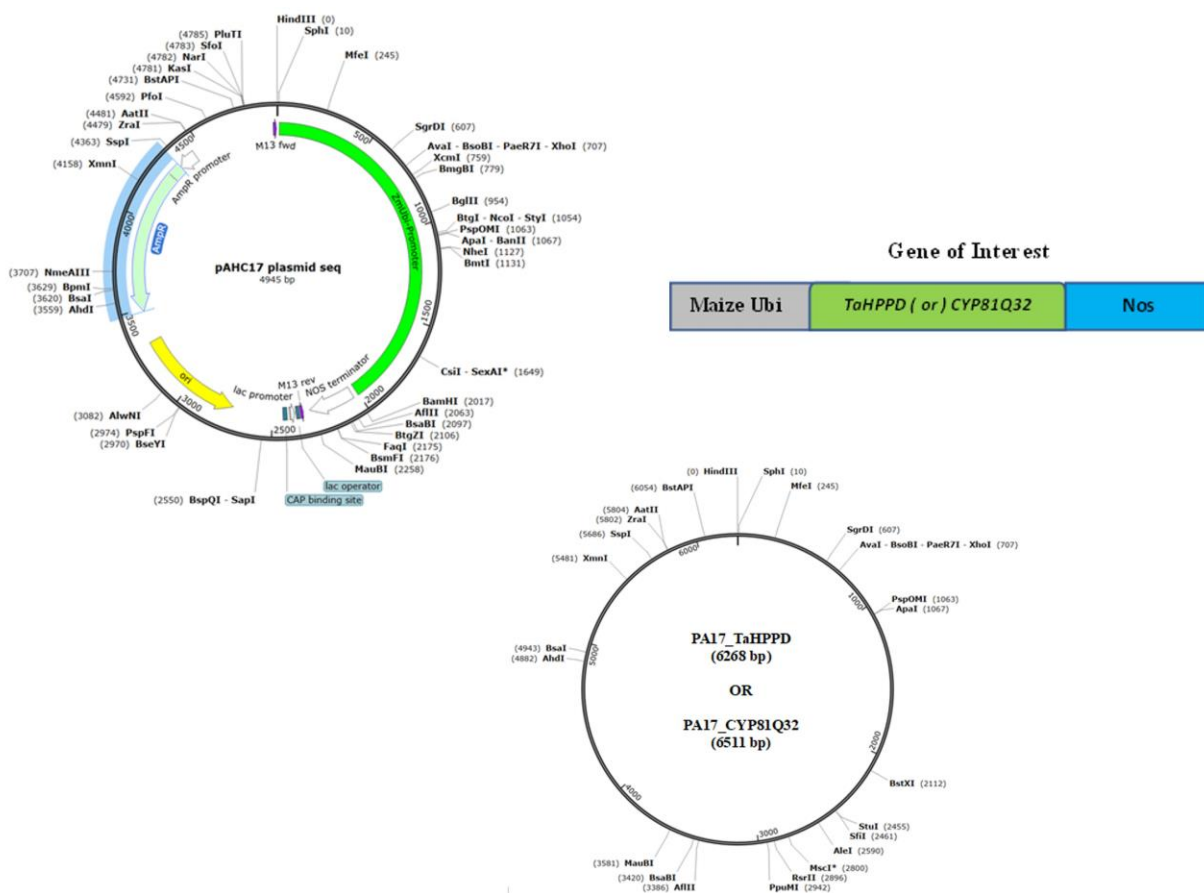


Figure 4.1. Schematic representation of the vector containing the gene of interest (gene of interest synthesized, codon-optimized, and cloned in vector at GenScript).

4.3.2 Cloning plasmids into DH5 α competent cells and colony PCR of recombinant clones

Once the plasmids were received, they were transformed into DH5 α -competent cells separately (Invitrogen, MA, US) by heat shock method. About 400 to 500 ng of plasmid was added to 150 μ l of DH5 α competent cells under sterile conditions. Then the mixture was kept on ice for 30 min, followed by heat shock treatment at 42 $^{\circ}$ C for 90 sec, and then on ice for 20 min. One milliliter of LB broth was then added, and the cells were incubated in a 37 $^{\circ}$ C shaker at 180

rpm for an hour and a half. Finally, the cells were centrifuged at 4000 rpm for 2 min, after which the pellet was resuspended in 75 µl of supernatant (LB broth) and plated on LB medium supplemented with ampicillin 100 mg l⁻¹. The plates were incubated for 12 to 16 hours at 37 °C. Putative recombinant colonies that had grown were randomly selected for colony PCR analysis with primers designed to detect the construct. The primer sequences were designed in such a way that the forward primer falls in the ubiquitin promoter region and the reverse primer in the codon-optimized region of the gene of interest (GOI). Primers were synthesized at IDT (Integrated DNA Technologies, Coralville, Iowa) and the sequences are shown in Table 4.1. UbiABF was used as the forward primer and PA17_TaHPPD RE1 and R2 were used for the detection of the presence of the construct PA17_TaHPPD. UbiABF was used as the forward primer and PA17_CYP81Q32 RE1 and R2 were used for the detection of the presence of the construct PA17_CYP81Q32. The PCR reaction mixture consisted of 1 µl culture, 10 µl of 2X GoTaq® G2 Green Master Mix (Promega™, Madison, Wisconsin, US), 1 µl each of 10 pmol µl⁻¹ forward and reverse primers and 7 µl sterile water. PCR amplification was done using the following PCR cycling conditions, initial denaturation: 95 °C for 5 min, followed by 34 cycles of denaturation: 94 °C for 30 s, annealing 60 °C for 45 s, extension: 72 °C for 45 s, and final extension: 72 °C for 7 mins. The PCR products were analyzed in 1.0% agarose gel to confirm the targeted amplicon size. Further, the colonies that were found positive were cultured in 5 ml LB broth overnight at 37°C and the plasmid was isolated using the Plasmid DNA isolation mini kit (Thermo Fisher Scientific, Waltham, Massachusetts, US) following the manufacturer's protocol. Finally, the plasmids were sequenced by the Sanger sequencing service provided by Agenta Life Sciences, LLC (New Jersey, South Plainfield, US). The sequences were aligned using the Clustal

Omega multiple sequence alignment tool (EMBL-EBI) to check the correct orientation of the gene of interest in the construct.

4.3.3 Biolistic transformation of wheat

The spring wheat cultivar Bobwhite (BW; CM33203; released in 1984 by CIMMYT) was used for transformation experiments. Embryonic calli were used for the transformation process and the protocol was followed as described by Tian et al. (2019). The wheat spikes were collected 10 to 14 days post-anthesis from the wheat plants maintained in a growth chamber under the following conditions: 20°C/18°C day/night temperature, a 16-hour photoperiod, with a light intensity of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 50 to 60% relative humidity. The immature seeds were isolated and surface sterilization was performed with 20% (v/v) bleach and 0.1% (v/v) Tween-20 for 20 minutes under continuous shaking in a laminar air flow hood. The seeds were rinsed with sterile double-distilled water (ddH₂O) five times to remove the remnant solution. The immature embryos were excised from the seeds and placed on plates containing the callus induction medium CM4, with the embryo axis faced down in contact with the medium. The plates were incubated in the dark at room temperature (room temperature, 22 $\pm$ 1 °C) for 7 to 10 days. Calli regenerated from the scutellum were extracted, placed on brand-new plates with CM4 media, and then allowed to regenerate. Around 25 calli were placed in the center of the plate, in a 5 x 5 arrangement, and kept in the dark for 2 to 3 days. The calli were air-dried in a laminar flow chamber for 20 min on the day of transformation. Tungsten particles were washed 3 times with ddH₂O by centrifugation at 10000 rpm for 1 min and were resuspended in 500 μL of ddH₂O followed by keeping them on ice. Particles were coated by mixing 25 μL of the particles with 2 μL plasmids (PA17_HPPD or PA17_CYP81Q32) and 2 μL of the plasmid pAHC20 (Christensen & Quail, 1996). Tungsten particles and plasmids were mixed by vortexing and

incubating at room temperature for 1 min. After incubation, 25 µL of 2.5 M CaCl₂ and 10 µL of 0.1 M spermidine were added. The solution was mixed in a vortex and incubated in ice for 4 min. After the particles settled, 50 µL of the supernatant was removed and discarded. The remaining coated-particle solution was kept on ice, the solution was mixed by vortexing before each shot, using 2 µL per shot. Particle bombardment was done using the biolistic system described by Finer et al. (1992), with a helium pressure of 60 PSI. After bombardment, embryogenic calli in CM4 plates were incubated for 3 to 4 days in the dark at room temperature. This biolistic method and the remaining plant tissue culture process were followed as per the protocol established by Tian et al., 2019. A brief description of the plant tissue culture and selection media is represented in the form of a table (Table 4-2). The steps involved in the biolistic transformation followed by the *in-vitro* regeneration/plant tissue culture process are represented with pictures (Figure 4-2).

Table 4-1. The description of primer sets used for the detection of the presence of the transgene

Primer Name	Sequence (5' - 3')	PCR product size (bp)	Description
UbiABF PA17_TaHPPD RE1 PA17_TaHPPD RE2	CCTGCCTTCATACGCTATTTATTTG AGGAAGGTCTGGATTGGCTG GTGGACTGGCTCGTTGAGTG	959 924	PCR – <i>TaHPPD</i> detection
UbiABF PA17_CYP81Q32RE PA17_CYP81Q32RE	CCTGCCTTCATACGCTATTTATTTG TGAGTTGGACCCTAGCAGCG GCCTAGCCATAGCCCTCACC	649 589	PCR – <i>TaCYP81Q32</i> <i>-like</i>
UbiABF BarR	CCTGCCTTCATACGCTATTTATTTG CTTCAGCAGGTGGGTGTAGAGCGT	453	PCR – <i>bar</i> detection

Table 4-2. Media composition used in the plant tissue culture. A brief description of components used in the tissue culture media was obtained from Tian et al., 2019.

CM4		MSP		MSE	
Components	Quantity (1 L)	Components	Quantity	Components	Quantity
MS Sulfates	10 ml	MS FeEDTA	10 ml	MS FeEDTA	10 ml
MS Halides	10 ml	MS Sulfates	10 ml	MS Sulfates	10 ml
MS P, B, Mo	10 ml	MS Halides	10 ml	MS Halides	10 ml
B5 Vitamins	10 ml	MS P, B, Mo	10 ml	MS P, B, Mo	10 ml
NH ₄ NO ₃	1.65 g	B5 Vitamins	10 ml	B5 Vitamins	10 ml
KNO ₃	1.90 g	NH ₄ NO ₃	1.65 g	NH ₄ NO ₃	1.65 g
2,4-D	0.5 ml	KNO ₃	1.90 g	KNO ₃	1.90 g
Picloram (1mg/ml)	2.2 ml	MES	1.90 g	MES	1.90 g
Maltose	40 g	Ascorbic acid	100 mg	Ascorbic acid	100 mg
pH	5.7	2,4-D (1mg/ml)	0.5 ml	Picloram	2.2 ml
Gelrite (into bottle)	2.0 g	Picloram (1mg/ml)	2.2 ml	Maltose	40 g
		Maltose	40 g	pH	5.7
		pH	5.7	Gelrite (into	2.0 g
		Gelrite (into bottle)	2.0 g		
CM4+5G		MSP+10G		MSE+5G	
Filter sterilized (stock 10mg/ml)		Filter sterilized (stock 10mg/ml)		Filter sterilized (stock 10mg/ml)	
Glufosinate Amm – 500ul		Glufosinate Amm – 1 ml		Glufosinate Amm – 500ul	
CM4+10G					
CM4 medium: wheat initiation, proliferation, and selection medium					
MSP: wheat shoot production medium					
MSE: wheat shoot elongation & rooting medium					

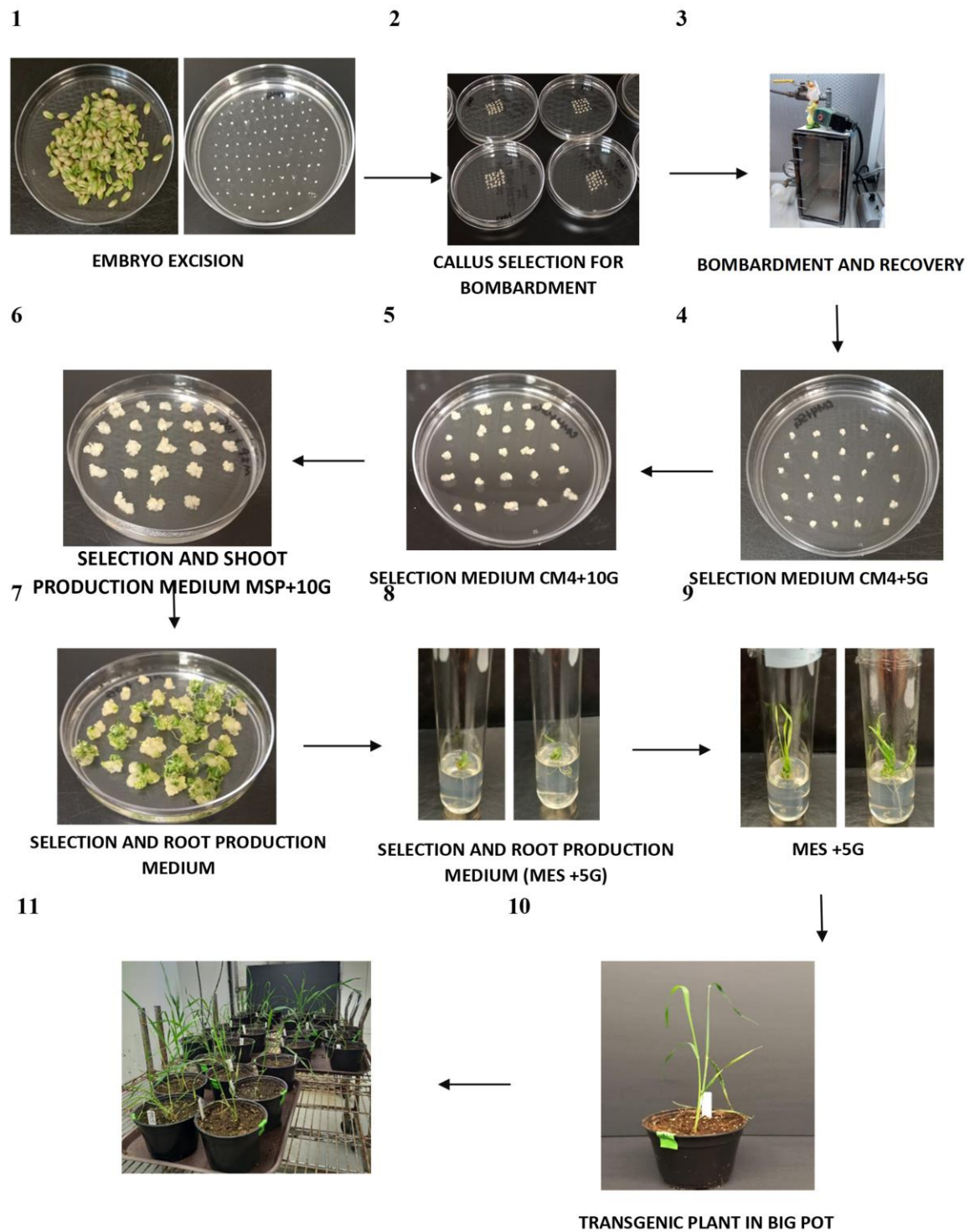


Figure 4.2. Steps involved in biolistic transformation followed by *in vitro* regeneration.

4.3.4 Identification of positive transgenic plants

The presence of the *bar* gene was identified using the “liberty painting” method described by Tian et al. (2019). Once the plants reached the three to four-leaf stage, one leaf from each tiller was chosen and was painted with a 0.2% Liberty® solution (Liberty® 280 SL herbicide, Bayer CropScience, Raleigh, NC, USA) using a cotton ball (Figure 4.3). The T₀ plants were maintained in a growth chamber and the response to the herbicide, glufosinate was evaluated 7 to 10 days after treatment. The growth chamber conditions were maintained at 22/18° C Day/night (d/n) and 16 h of photoperiod supplemented with 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination. As Liberty is a contact herbicide, the symptoms appear as leaf burning and necrosis at the place of herbicide contact after treatment. Therefore, the tillers that did not show necrosis on the painted leaf were considered positive for the presence of the *bar* gene. The genomic DNA of the T₀ plants that were positive was extracted using the cetyltrimethylammonium bromide (CTAB) method. Further, PCR analysis was performed with primers designed (Table 4-1) to amplify the *bar* gene. The PCR amplifications were performed with the following conditions: initial denaturation: 95 °C for 5 min, followed by 34 cycles of denaturation: 95 °C for 30 s, annealing 60 °C for 45 s, extension: 72 °C for 45 s, and final extension: 72 °C 7 mins. The PCR products were analyzed on 1.0% agarose gel to confirm the targeted amplicon size. The T₀ plants that showed the presence of the *bar* gene were further tested for the presence of the gene of interest.



Figure 4.3. Liberty painting process of the putative transgenic plants.

4.3.5 Analysis of the positive T₀ plants for the presence of the GOI

The bar gene-positive T₀ plants were further evaluated for the presence of the GOI. The primer sequences UbiABF and PA17_TaHPPD RE were used to identify the presence of the *TaHPPD* gene. The primer sequences UbiABF and PA17_CYP81Q32 RE were used for the identification of the presence of the *CYP81Q32-like* gene. The PCR amplifications were performed with the following conditions: initial denaturation: 95 °C for 5 min, followed by 34 cycles of denaturation: 95 °C for 30 s, annealing 60°C for 45 s, extension: 72 °C for 45 s, and final extension: 72 °C 7 mins. The PCR products were analyzed on 1.0% agarose gel to confirm the targeted amplicon size. The plants that showed the presence of the GOI were selected and grown in growth chambers under the above-mentioned conditions. Once the plants reached maturity, the T₁ seeds were harvested and subjected to further analysis.

4.3.6 Identification of transgenic T₁ plants and their response to mesotrione application

The T₁ seeds were threshed, cleaned and stored. The T₁ seeds from three T₀ plants transformed with PA17_HPPD and two T₀ plants transformed with PA17_CYP81Q32 were selected for further analysis. About 15 to 20 seeds were selected from each plant and were planted in a germination sheet soaked in water and kept in the dark at 4°C for 2 to 3 days to break the seed dormancy. The seeds were then removed from the dark and maintained at 25°C. After the seeds germinated, they were transferred into pots (6x6x6 cm) filled with potting mixture (ProMix Ultimate; Premier Tech Horticulture, Mississauga, Ontario, Canada) and were grown in a greenhouse. Greenhouse was maintained in the following conditions: 18/15°C (d/n) and 16 h of photoperiod supplemented with natural light and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ illumination by sodium vapor lamps. Once they reached the 3 to 4-leaf stage, plant tissues were collected to detect the presence of the transgene in T₁ plants which were expected to segregate for presence or absence of GOI. Further, additional leaf samples were collected, and flash-frozen in liquid nitrogen, and stored at -80 °C for RNA isolation to study the transcript levels of the GOI. After leaf sample collection, the plants were left to recover for 4 to 5 days and were treated with 6X dose of mesotrione (x= labeled dose of herbicide; which is 105 g ai h⁻¹) herbicide along with 1% crop oil concentrate (COC, Agridex) and 2.5% of ammonium sulfate (AMS, Liquid N-Pak; Winfield). A bench-top track spray chamber (Generation III; De Vries Manufacturing, Hollandale, MN, US) equipped with a single flat-fan nozzle (80015LP TeeJet tip; Spraying Systems, Wheaton, IL, US) delivering 187 L ha⁻¹ was used for herbicide application. The visual injury of each transgenic plant was recorded using an injury rating scale ranging from 0 to 100 (0 = no injury, 100% = plant mortality), at 3 to 4 weeks after the herbicide treatment by comparing

to the non-treated wild-type plants. Further genomic DNA was isolated from these plants to detect the presence of the *bar* gene and GOI by PCR. Finally, the plants that tested positive *via* PCR for the GOI were subjected to RT-qPCR analysis to study the transcript level of GOI. The schematic representation describing the screening for positive plants with GOI and analysis of the T₁ plants is given in Figure 4.4.

Table 4-3. The primer sets used for the quantitative expression (RT-qPCR) of GOI.

Primer Name	Sequence (5' - 3')	Description
Actin 3F Actin 3R	GCATGAGCAAAGAGATCACGGCCC CGGGACCAGACTCATCGTACTCCG	Housekeeping gene
HPPD_3-RT FW HPPD_3-RT FW	CCAAATCAAGGAGTGCCAAGAG ATCATCTCCAGGAAGAGGGTTG	Relative expression of <i>TaHPPD</i>
CYP_11-RT FW CYP_11-RT RE	GACGAGATCATCCCACACATC CGATGAGCCTGAGCATGAA	Relative expression of <i>CYP81Q32-like</i>

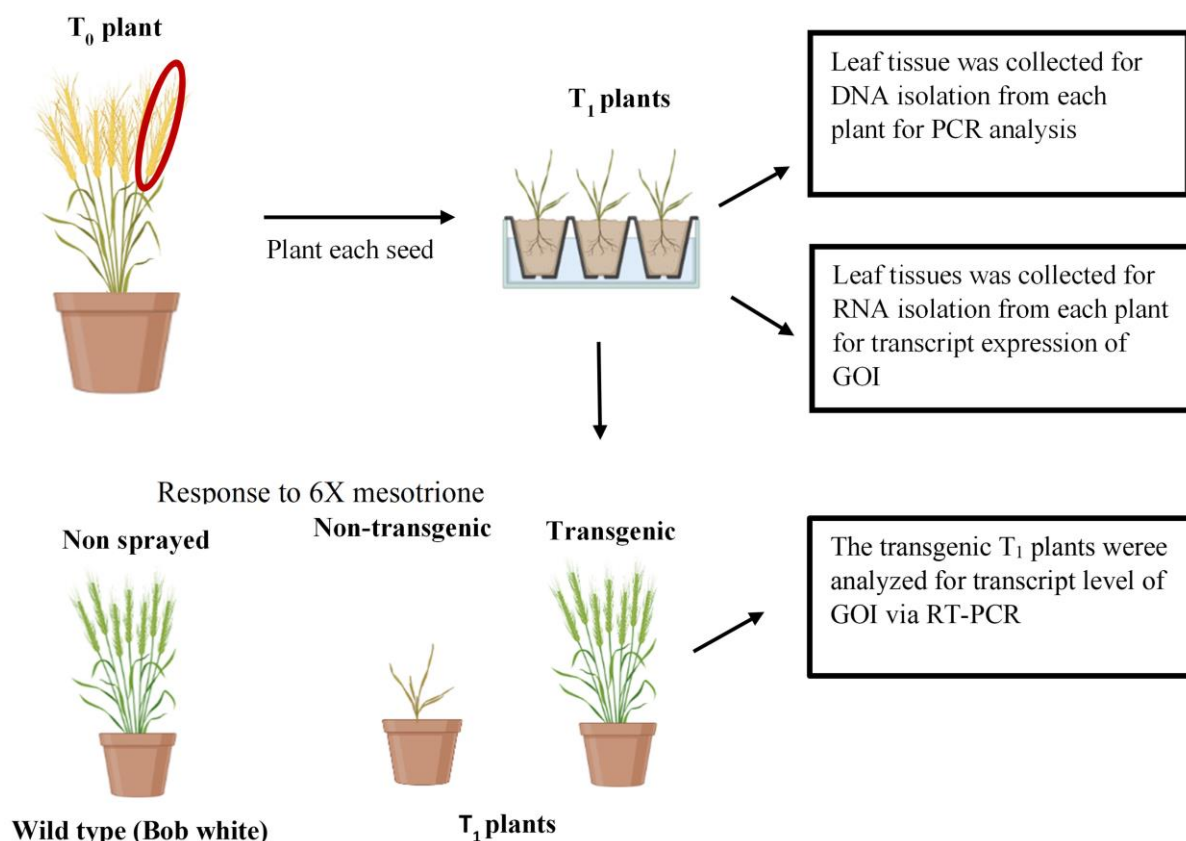


Figure 4.4. The schematic representation of screening for positive plants with GOI and response of T₁ plants to 6X mesotrione application.

4.3.7 Relative expression of the PCR-positive T₁ plants

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The quality and quantity of total RNA were determined using agarose gel (1%) electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific). Further, the isolated RNA was treated with DNase I enzyme (Thermo Scientific, Waltham, MA, USA) to remove any genomic DNA contamination. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR/real-time PCR (qRT PCR) was used to determine the expression levels of GOIs *TaHPPD* and *CYP81Q32-like* in selected transgenic plants. The qRT PCR was performed with primers designed separately for gene expression analysis (Table 4-3).

The gene expression was normalized using a housekeeping gene, actin as a reference gene. The reaction was carried out in triplicates with following the PCR conditions: 50°C for 2 min, 95°C for 5 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min as per the manufacturer's instruction of the master mix (Applied Biosystems™). The reaction mixture consisted of 7 mL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™), 1 mL each of forward and reverse primers (10 mM) and 1:10 diluted cDNA.. The relative quantifications were performed using the $\Delta\Delta CT$ method.

4.3.8 Design of single guide RNA (sgRNA) targeting *CYP81Q32-like* gene

The gene sequence of the *CYP81Q32-like* gene was retrieved from the NCBI database. For sequence validation, the retrieved sequence in FASTA format was blasted against various wheat genotypes in EnsemblPlants. This sequence was used to design sgRNAs using CRISPR-P 2.0 (<http://crispr.hzau.edu.cn>) (Liu et al., 2017), a freely available web-based tool. Two sgRNAs were shortlisted, namely sgRNAs 1 and 2 from the negative strand of the first exon of the gene. BsaI adaptors with 5' AGCG 3' in the top strand and 5' AAAC 3' in the bottom strand were added to the 5' end of the designed sgRNAs for DNA oligomer synthesis. These two sgRNAs were used in the CRISPR/Cas9-mediated genome editing experiment. The 2 regions in the *CYP81Q32-like* gene where the sgRNAs were designed are shown below in Figure 4.5. The guide RNA with adapter sequence is shown in Table 4-4. The adapter sequence added was complementary to the overhangs on the vector backbone as shown in Figure 4.6.

cDNA of *CYP81Q32-like* gene |

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>CACAACACACATCCAATCAACGCACATCACTTCCCTTATGACACAGTTAACCATGGATAAGGCATATATCGCCAT
CCTCTCCTTCGCCTTCTCTTCTGCTCCACTACATTCTAGGCAAGGTGAGCAATGGCAGGCGCAGCAAGGGCGCC
GTCCAGCTGCCGCCGAGCCCCAGGCCATCCCGTTCTCGGCACCTCCACCTCTGGAGAAGCCGTTCCACGCCG
CTCTGTGCCGCTCGCCGAGCGCCTCGGCCGGTCTTCTCGCTCGCCTCGGCTCGCGCCGCCGCGTGGTCTCTC
CTCGGCCGAGTGCGCCAGGGAGTGCTTCACGGAGCAGCAGCTGATCTTCGCCGACCGGCCCAAGTCCCTCGCA
GCTGCTCGTGTCTTCCAGCGCATCGCGCTCTCCAGTCCAGCTACGGCCGCACTGGCGCAACCTCCGCCGCTC
GCCGCCGTGCAGCTGCTCTGCGCACCGCGCTCGCTGCAATGTCGGCGTGATCGGGGGCGAGGTGCGCGCCATG
GCGCGCCGGCTCTTCCGCGCCGCCGCGCTCCCCGGTGGGGACGGCGCCGCCGGTCCAGCTGAAGCGGAG
GCTCTTCGAGCTCTCCCTCAGCGTGCTCATGGAACCATCGCCAGACCAAGGGGACCGGTGGAGGCCGACGC
CGACACGGACATGTCCGTGGAGGCCAGGAGTTCAAGAAGGTGGTGACGAGATCATCCGCACATCGGGGCCG
CCAACTGTGGGACTACCTGCCGGTGTCTCGGTGGTTCGACGTGTTGGAGTGAGGAACAAGATCCTTGCCGCCG
TGAGCAGGAGGGACGCTTCATGCTGCGTCTCATCGACGCCGAGCGCCGAGGGTTGACGGCGGCCGCAAGGC
GACAAGAAGAGCATGATGCCGTGTTGCTCACTCTGAGAAGACGGAGCCGAGGTGTACACCGATACTATGATC
ATGGCTCTCTGTGCGAATTTGTTGGGGCTGGGACGGAGACCACGTGACGACGAGCGAGTGGGCGATGTCGT
GCTGCTGAACACCCGGCGGCGCTCAAAAAGGCGCAGGCCGAGATCGACGCGCGGTGGGCACCTCTCGGCTGG
TGACGCCGACGACGTGCGCGGCTCGCTACCTGAGTGATCGTGAGCGAGACGCTGCGGCTGTACCCGGCG
GCGCCGCTGCTGCTGCCGACCACTCTCGCGGAGTGAAGGTGGGCGGCTACAACGTGCCGAGCGGCACGAT
GCTGATGGTGAACGCTACGCCATCCACCGGACCCGGCGGCTGGGAGCGGCCGTGGAGTTCGTCGCCGGAGC
GGTTCGAGGACGGGAAGGCCGAGGGCGGTTATGATCCCGTTCGGGATGGGCCGCGCGGTGCCCGGGGA
GACGCTGGCGCTGCGGACCATCGGCATGGTGTGGCGACGCTGGTGCACTGCTTCGACTGGGAGCGCGTGAGCG
GTGCGGAGGTGGACATGACGGAGAGCGCGGCTCACCATCCCAAGGCCGTGCGGCTCGAGGCCGTGTGCA
GCCGCGCACGGCATGCGCGACGTCTTCAAGGCTCTGATGATCAACTTCGTTCTTGCAATCAAAATGCATG
GACTGGTGGCGTGTGAAATAGTAGTAAATAAACTTGAGCAGAGTCAAGCTAGCTATCATCATATGTAATTAC
AGGTACGTGTGAGGTTTGTAAAGAACTAGCTAAGCTATCTAGGTAGCTAGCTCGTAGTACTACCAACAGCAAT
TTGTAATTTAGCTTGTATCAAGAAATGTTTGTATGGAGTAATTTATATCATCTCTCTAAAATTGCAATAAAA
AAATGTCGGGAACGAACGTTATGGTTGGGCTTACACCGATCTGGACACGCGAGGGAAGGGCAGCTTCACTTAG
AATCTGAGACTTCGAATCAAAAATAATAATCA

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Figure 4.5. The highlighted regions of the cDNA of the *CYP81Q32-like* gene indicates the target region of sgRNA 1 (green) and sgRNA 2 (blue).

Table 4-4. The oligo sequence of the top and bottom strands of the sgRNAs

gRNA	5' TO 3' sgRNA SEQUENCE	sgRNA SEQUENCE with adaptors 5' to 3'
gRNA 1 Top	ACGACGTGATCTTCGCCGAC	AGCG ACGACGTGATCTTCGCCGAC
gRNA 1 Bottom	GTCGGCAAGATCACGTCGT	AAAC GTCCGGCAAGATCACGTCGT
gRNA 2 Top	AGGCCATCCCGTTCCTCGGC	AGCG AGGCCATCCCGTTCCTCGGC
gRNA 2 Bottom	GCCGAGGAACGGGATGGCCT	AAAC GCCGAGGAACGGGATGGCCT

The bold letters show the *BsaI* adaptors 5'AGCG 3' in the top strand and 5'AAAC 3' in the bottom strand added for synthesis of sgRNAs 1 and 2.

***Bsa*I recognition sequence:**

5'...GGTCTC(N)1...3'

3'...CCAGAG(N)5...5'

Enzyme recognition region in the vector backbone:

5' ACCAAGAAGCGTGAG 3'
3' TGGTTCTTCGCACTC 5'

5' GGTCTCGGTTTTACT 3'
3' CCAGAGCCAAAATGA 5'

Figure 4.6. The *Bsa*I recognition sequence and the enzyme recognition region in the vector backbone. The blue crossed-off portion is the fragment that has been removed from the vector after restriction digestion with the enzyme *Bsa*I. Thus, 5' AGCG 3' and 5'AAAC 3' are added on the top and bottom strands of the sgRNAs so that they are complementary to the overhangs on the vector backbone, which would allow ligation of oligo duplex at this site.

4.3.9. Restriction digestion of Cas9 vector

The binary vector chosen for cloning the sgRNAs was p0390sgCas9. The physical map of the plasmid is shown below in Figure 4.7. This plasmid consists of two *Bsa*I restriction sites which facilitates cloning of the sgRNA duplex. The plasmid was isolated from 5 ml overnight grown cultures in LB broth supplemented with kanamycin 50 mg l⁻¹ at 37 °C using a plasmid DNA Miniprep Kit. For this plasmid, a single digestion reaction was set up using the following components: 4µl of plasmid (250 ng l⁻¹), 1.5 µl of *Bsa*I restriction enzyme (20 U µl⁻¹) (NEB; catalog no. R3733S), 4 µl of 10X CutSmart buffer, 30.5 µl of nuclease-free water making a total reaction of 40 µl. This reaction mixture was incubated at 37 °C for an hour for restriction

digestion to take place. Then the reaction mixture was loaded into a 0.8% agarose gel to check the presence of the digested fragments. The 1200 bp length of the DNA fragment released from the vector is shown in the gel picture in Figure 4.8. The digested vector was excised from the gel and gel purification was performed using GeneJET Gel Extraction Kit (Thermo Scientific, K0691), following manufacturer's protocol.

Figure 4.7. Physical map of p0390sgCas9 vector used in the CRISPR/Cas9 experiment for cloning sgRNA1 and sgRNA 2.

Figure 4.8. The gel image showing the digested plasmid p0390sgCas9 as the bright band and the lighter band at ~1200 bp is the released fragment from the vector backbone. The bright band was extracted from the gel for further analyses.

4.3.10. Synthesis of oligo duplex sgRNAs and ligation into digested vector

The oligo duplex of the sgRNAs were synthesized with the top and bottom strand in a thermocycler maintained at 55 °C for 30 mins. The reaction mixture consisted of the following: 9 µl of the top strand (10 µM) of the gRNA, 9 µl of the bottom strand (10 µM), 2 µl of 10X ligase buffer. Then ligation reaction was performed with the oligo duplex and the plasmid at 25°C for 3 hours. The ligation mixture consists of the following components: 50 ng of linearized vector, 3 µl of the annealed oligos, 1 µl of T4 DNA ligase enzyme (5 units µl⁻¹), 2 µl 10X ligase buffer, and 12 µl sterile water. Once the ligation process was completed, they were transformed into DH5α cells as mentioned earlier. The positive colonies were confirmed with colony PCR and the plasmid was isolated. Finally, sequencing of recombinant clones was performed to ensure perfect ligation of the oligo duplex at the expected *BsaI* site of the vector.

4.4. Result

4.4.1 Colony PCR

The colony PCR performed with the primers (PA17_TaHPPD FW-1 and RE-1) to detect the presence of the vector PA17_TaHPPD showed the presence of positive colonies. Out of the six colonies picked for analysis, 5 of them were positive containing the vector, with an expected amplicon size of 959 bp (Figure 4.9). Several negative controls including genomic DNA, pAHC17, and water were used in this experiment and they did not amplify at the required fragment length, or no amplification was identified. Therefore, confirming that the PA17_TaHPPD was cloned into the DH5α cells successfully. The amplification performed with the second set of primers (PA17_TaHPPD FW-2 and RE-2) is given in Appendix A (Figure A.1)

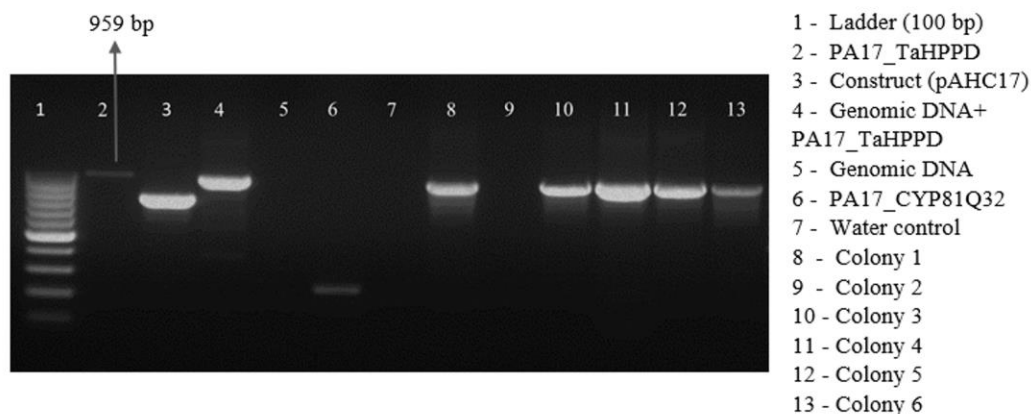


Figure 4.9. The gel image shows the PCR amplification of the colonies containing the vector PA17_TaHPPD with primers UbiABF and PA17_TaHPPD RE1.

Similarly, The colony PCR performed with the primers (PA17_CYP81Q32 FW-1 and RE-1) to detect the presence of the vector PA17_CYPQ32 showed the presence of positive colonies (Figure 4.10). All the six colonies selected for analysis showed the presence of the vector. The desired amplification at 649 bp was found in all the colonies. The negative control did not show any amplification. These results confirmed successful cloning of the PA17_CYP81Q32 into the DH5a cells. The amplification performed with the second set of primers (UbiABF and PA17_CYP81Q32 RE-2) is given in Appendix A (Figure A.1).

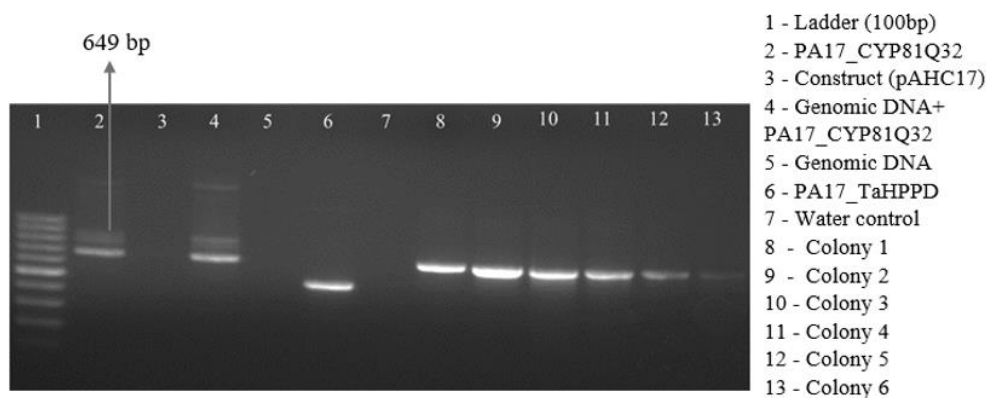


Figure 4.10. The gel image showing the PCR amplification of the colonies containing the vector PA17_CYP81Q32 with primers PA17_CYP81Q32 FW1 and RE1.

4.4.2 Plasmid isolation and PCR amplification of the vectors before bombardment

The PA17_TaHPPD plasmid from the 1st colony was isolated and reconfirmed by PCR with the primers PA17_TaHPPD FW2 and RE2 (Figure 4.11). Similarly, the PA17_CYP81Q32 plasmid from the 2nd colony was isolated and reconfirmed with the PCR amplification with the primers PA17_CYP81Q32 FW2 and RE2 (Figure 4.11). Further, the sequencing results showed that the GOI was synthesized and cloned into the vector in the desired region and orientation. The sequence results are provided in Appendix A (Figure A.2).

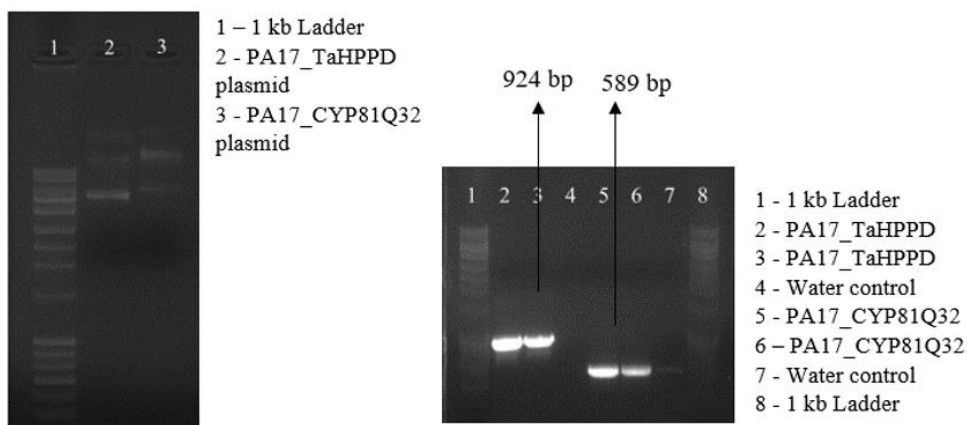


Figure 4.11. Agarose gel images to illustrate the presence of plasmids. The gel picture on the left shows the plasmids (PA17_TaHPPD and PA17_CYP81Q32) isolated from the colonies. The gel picture on the right shows the PCR amplification of the isolated plasmids.

4.4.3 Identification of the positive transgenic plants

The two plasmids PA17_TaHPPD and PA17_CYP81Q32 were bombarded separately in two different experiments. The total number of plants regenerated *via* callus culture after

bombardment with the PA17_TaHPPD was 224. The Liberty painting experiment showed that a total of 71 plants were positive for the presence of the *bar* gene. The *bar* gene-positive plants did not show burning symptoms of the leaf (Figure 4.12 A) whereas, the plants that were devoid of the *bar* gene showed burning symptoms (Figure 4.12 B). Further, the presence of the *bar* gene was confirmed by PCR analysis in T₀-HP-1 to 10 (Figure 4.13). The PCR analysis of more T₀ plants is represented in Appendix A (Figure A.3).

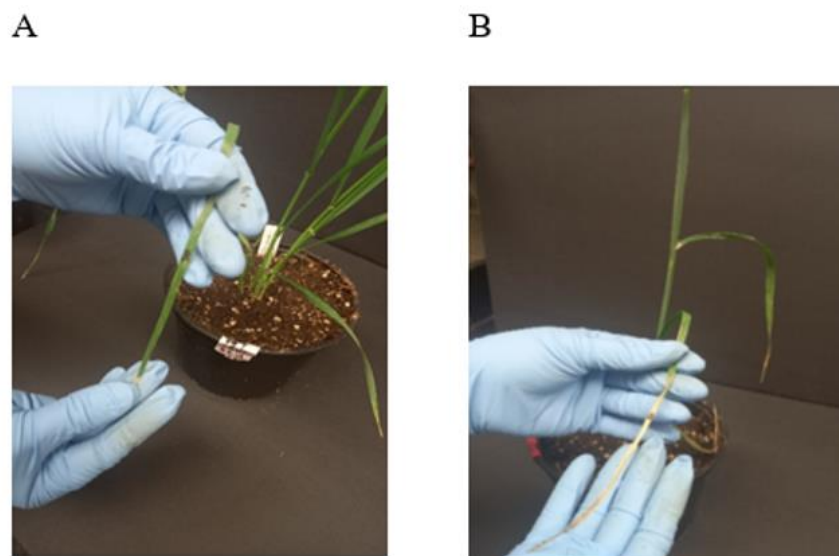


Figure 4.12. Results of Liberty® painting on T₀ plants. A) The plant that contained the *bar* gene was resistant to the Liberty herbicide and did not show burning symptoms. B) The plant that did not contain the *bar* gene was susceptible to the Liberty herbicide and showed burning symptoms.

Similarly, a total of 216 plants were regenerated after bombarding the callus with the PA17_CYP81Q32 plasmid. The Liberty painting experiment resulted in a total of 63 plants that were positive for the presence of the *bar* gene. The presence of the *bar* gene was confirmed by PCR analysis as well; the putative transgenic plants T₀-P-1 to 10 were shown in the gel picture

(Figure 4.14). The PCR analysis of a few more T₀ plants is represented in Appendix A (Figure A.4).

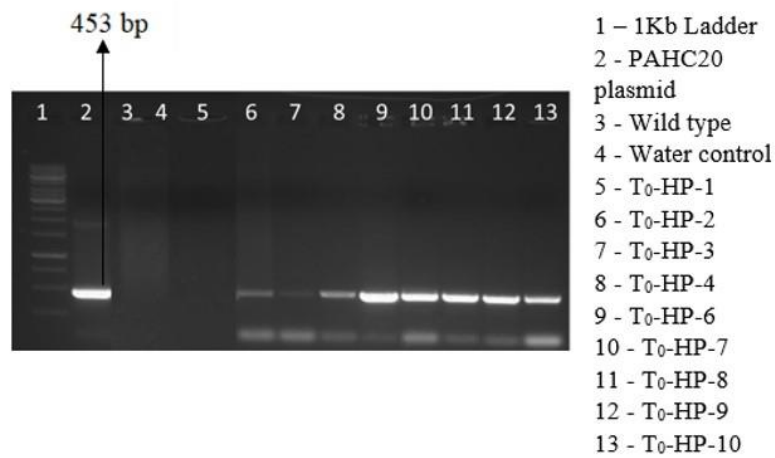


Figure 4.13. The gel image illustrating the presence of the *bar* gene in transgenic plants T₀-HP-1 to 10 bombarded with the PA17_TaHPPD plasmid.

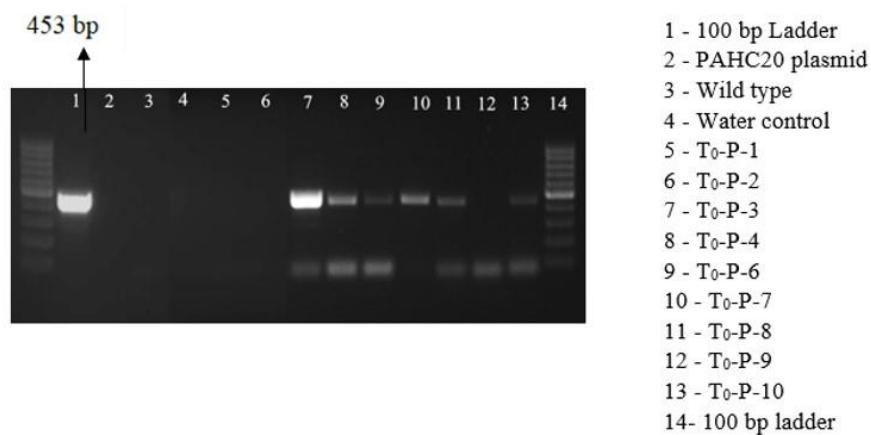


Figure 4.14. The gel image illustrating the presence of the *bar* gene in transgenic plants T₀-P-1 to 10 bombarded with PA17_CYP81Q32 plasmid.

4.4.4 Analysis of the positive T₀ plants for the presence of the gene of interest

T₀- PA17_ TaHPPD plant analyses:

Upon PCR analysis of the plants T₀-HP-1 to 10 to identify the presence of the transgene, the plants T₀-HP-6, T₀-HP-9, and T₀-HP-10 showed amplification at the expected fragment size of 959 bp. Even though the other plants showed amplification at the expected size, they had non-specific amplification as well (Figure 4.15). Therefore, the plants that showed specific amplification were only chosen for future experimentation. Upon analysis of all the T₀ plants up to T₀-HP-71, a total of 20 plants were found to be transgenic with the presence of GOI (*TaHPPD*). The gel pictures of more transgenic T₁ plants are given in Appendix A (Figure A.4).

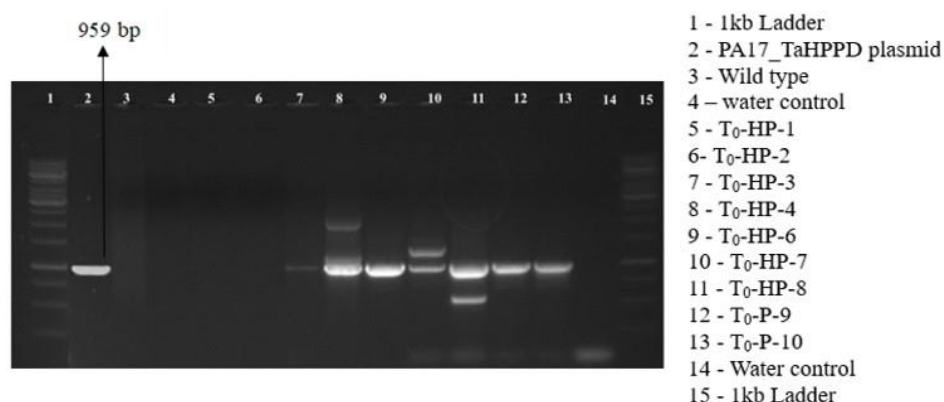


Figure 4.15. The gel image illustrating the PCR amplification and presence of the GOI (*TaHPPD*) in transgenic plants T₀-HP-6, T₀-HP-9, and T₀-HP-10.

T₀- PA17_ CYP81Q32 plant analyses:

The PCR analysis to identify the presence of the transgene in T₀-P-1 to 10 plants showed amplification of expected fragment size of 528 bp only in T₀-P-3, T₀-P-4, T₀-HP-7, and T₀-HP-8 plants (Figure 4.16). Therefore, these plants were chosen to generate T₁ plants. Upon analysis of all the T₀ plants expressing PA17_CYP81Q32, a total of 17 plants were found to be transgenic

with the presence of GOI (*CYP81Q32-like*). The gel pictures are shown in Appendix A (Figure A.4).

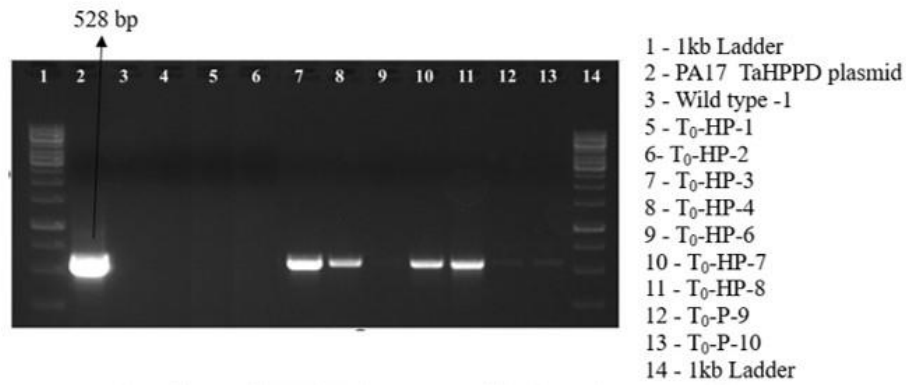


Figure 4.16. The gel image illustrating the PCR amplification and presence of the GOI (*CYP81Q32-like*) in transgenic plants T₀-P-4, T₀-P-5, T₀-P-7, and T₀-HP-8.

4.4.5. Identification of T₁ transgenic plants

GOI (*TaHPPD*): The T₁ seeds from the plants T₀-HP-6, T₀-HP-9, and T₀-HP-10 were used in the further molecular analyses of the transgenic plants. About 15 T₁ plants (HP-6-1 to HP-6-15) derived from T₀-HP-6 were analyzed *via* PCR amplifications for the presence of the GOI (*TaHPPD*) and *bar* gene. Eight plants were found to be transgenic containing both the GOI and the *bar* gene (Figure 4.17 A, B; Table 4-5). Similarly, T₁ plants (HP-9-1 to HP-9-15) derived from T₀-HP-9 were analyzed and 7 plants were found to be transgenic (Figure 4.17 A, B; Table 4-6). Upon the analysis of 21 T₁ plants (HP-10-1 to HP-10-21) derived from the T₀-HP-10, 11 plants were found to be transgenic (Figure 4.17C, D Table 4-7).

GOI (*CYP81Q32-like*): The T₁ seeds from the plants T₀-P-7 and T₀-P-8 were used in the further molecular analyses of the transgenic plants. Seventeen T₁ plants (P-8-1 to P-8-17) derived from T₀-P-8 were analyzed *via* PCR amplifications for the presence of the GOI (*CYP81Q32-like*) and *bar* gene. Nine plants were found to be transgenic (Figure 4.18 A, B; Table 4-8). The T₁ seeds

from the plants T₀-P-7 did not show the presence of the GOI (*CYP81Q32-like*) or the *bar* gene. None of the 14 plants tested were found to be transgenic even though the parent plant of the T₀ generation (T₀-P-7) showed amplifications in PCR analysis. Therefore, this line was excluded from future analysis.

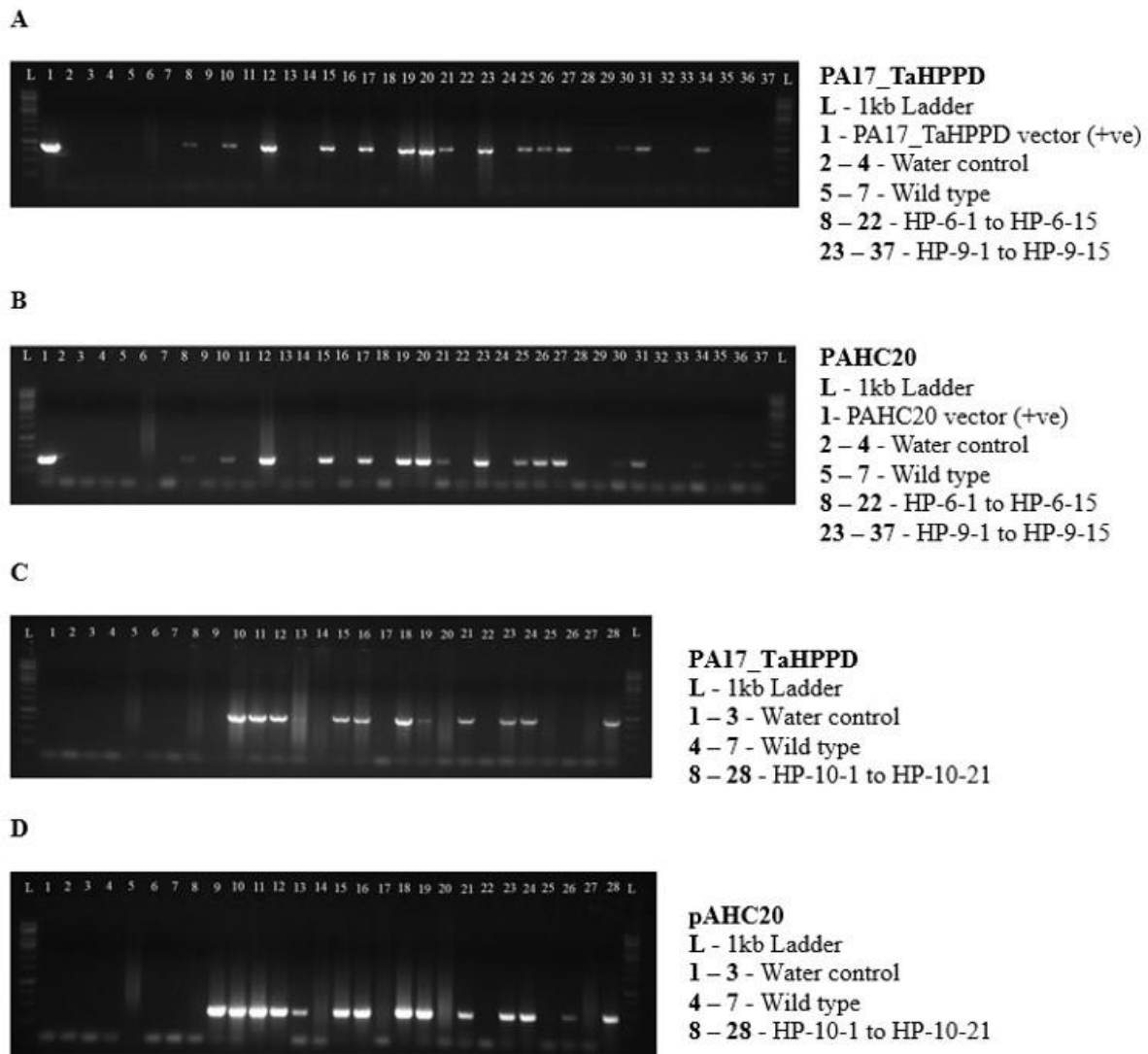


Figure 4.17. PCR analysis to detect the presence of A) the transgene *TaHPPD* in the T₁ plants HP-6-1 to HP-6-15 and HP-9-1 to HP-9-15, B) The *bar* gene in the T₁ plants HP-6-1 to HP-6-15 and HP-9-1 to HP-9-15, C) *TaHPPD* in the T₁ plants HP-10-1 to HP-10-221, and D) The *bar* gene in the T₁ plants HP-10-1 to HP-10-28.

Table 4-5. A brief description of whether the T₁ plants HP-6-1 to HP-6-15 contain the vectors PA17_TaHPPD (GOI) and PAHC20 (*bar* gene). (+ = presence of the vector and - = absence of the vector)

T ₁ plants	HP-6-1	HP-6-2	HP-6-3	HP-6-4	HP-6-5	HP-6-6	HP-6-7	HP-6-8	HP-6-9	HP-6-10	HP-6-11	HP-6-12	HP-6-13	HP-6-14	HP-6-15
PA17_TaHPPD	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-
pAHC20	+	-	+	-	+	-	-	+	-	+	-	+	+	+	-

Table 4-6. A brief description of whether the T₁ plants HP-9-1 to HP-9-15 contain the vectors PA17_TaHPPD and pAHC20. (+ = presence of the vector and - = absence of the vector)

T ₁ plants	HP-9-1	HP-9-2	HP-9-3	HP-9-4	HP-9-5	HP-9-6	HP-9-7	HP-9-8	HP-9-9	HP-9-10	HP-9-11	HP-9-12	HP-9-13	HP-9-14	HP-9-15
PA17_TaHPPD	+	-	+	+	+	-	-	+	+	-	-	+	-	-	-
pAHC20	+	-	+	+	+	-	-	+	+	-	-	+	-	+	+

Table 4-7. A brief description of whether the T₁ plants HP-10-1 to HP-10-21 contain the vectors PA17_TaHPPD and pAHC20. (+ = presence of the vector and - = absence of the vector)

T ₁ plants	HP- 10-1	HP- 10-2	HP- 10-3	HP- 10-4	HP- 10-5	HP- 10-6	HP- 10-7	HP- 10-8	HP- 10-9	HP- 10-10	HP- 10-11	HP- 10-12
PA17_TaHPPD	-	-	+	+	+	-	-	+	+	-	+	+
pAHC20	-	+	+	+	+	+	-	+	+	-	+	+
T ₁ plants	HP- 10-13	HP- 10-14	HP- 10-15	HP- 10-16	HP- 10-17	HP- 10-18	HP- 10-19	HP- 10-20	HP- 10-21			
PA17_TaHPPD	-	+	-	+	+	-	-	-	+			
pAHC20	-	+	-	+	+	-	+	-	+			

Table 4-8. brief description of whether the T₁ plants P-8-1 to P-8-17 contain the vectors PA17_TaHPPD (GOI) and pAHC20 (*bar* gene). (+ = presence of the vector and - = absence of the vector)

T ₁ plants	P-8-1	P-8-2	P-8-3	P-8-4	P-8-5	P-8-6	P-8-7	P-8-8	P-8-9	P-8-11	P-8-12	P-8-13
PA17_CYP81Q32	-	-	+	+	+	-	+	+	-	+	-	+
pAHC20	-	-	+	+	+	-	+	+	-	+	-	+
T ₁ plants	P-8-14	P-8-15	P-8-16	P-8-17								
PA17_CYP81Q32	+	-	-	+								
pAHC20	+	-	-	-								

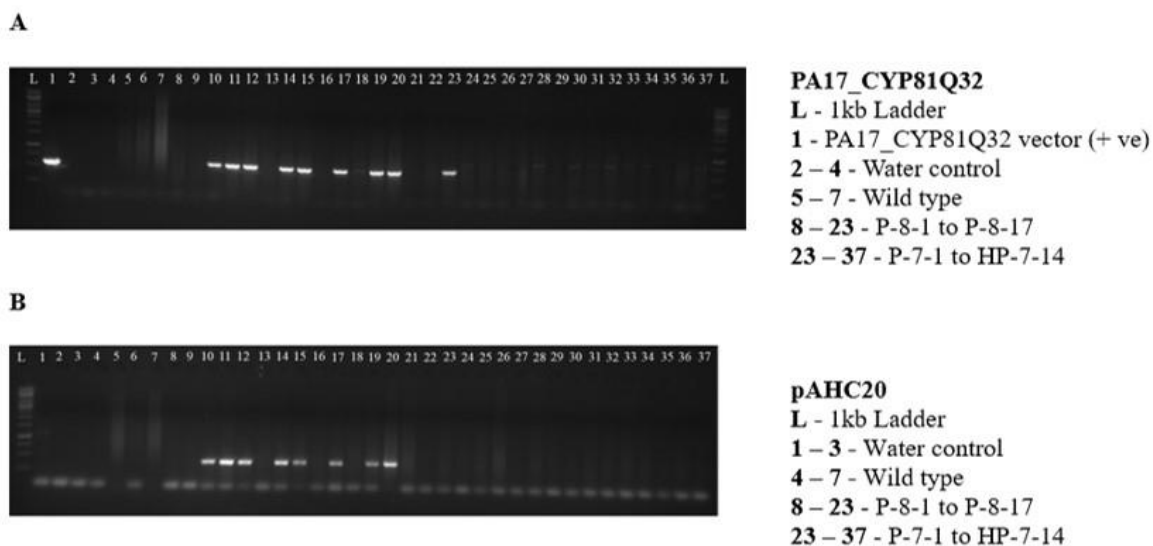


Figure 4.18. PCR analysis to detect the presence of A) the transgene *CYP81Q32-like* in the T₁ plants P-8-1 to HP-8-17 and P-7-1 to P-7-14 and B) the *bar* gene in the T₁ plants P-8-1 to HP-8-17 and P-7-1 to P-7-14.

4.4.6 The response of the T₁ transgenic plants to mesotrione application

GOI TaHPPD: The T₁ plants of HP-6 transgenic lines were scored for mesotrione injury 3 weeks after treatment (WAT). The plants exhibited differential sensitivity to mesotrione. The visual injury scoring was based on comparison with the non-treated wild type for a) bleaching symptoms, b) plant height, c) number of tillers, d) number of leaves, and e) number of spikes. The scoring scale ranged from 0% to 100% where 0% denoted no visual injury and 100% denoted the complete death of the plant. The plants that had the same percentage of visual injury were grouped together. The T₁ transgenic lines HP-6-1 and HP-6-5 showed 60% visual injury, whereas HP-6-3, HP-6-8, and HP-6-10 had 50% injury. HP-6-12, HP-6-13, and HP-6-14 showed less visual injury compared to the other HP-6 lines with a score of 40% (Figure 4.19 A). At 4 WAT, all the T₁ transgenic lines recovered from mesotrione injury. In particular, HP-6-13, and HP-6-14 recovered faster compared to others with a score of 30% (Figure 4.19 B, C). The T₁

transgenic lines HP-9-1, HP-9-5, and HP-9-12 showed 65% visual injury, whereas HP-9-3 and HP-9-4 had an injury of 55%. Compared to the other HP-9 transgenic lines, HP-9-8 and HP-9-9 were less sensitive to mesotrione (Figure 4.20 A). At 4 WAT, the HP-9-9 and HP-9-8 recovered with a visual injury of 40% (Figure 4.20 B, C). The HP-10-3 line showed more visual injury than the herbicide-treated wild-type. The HP-10-9 and HP-10-5 showed 60% visual injury similar to the herbicide-treated wild-type whereas the lines HP-10-8, HP-10-4, HP-10-14, HP-10-21, HP-10-12, and HP-10-17 showed ~ 40 to 45% injury (Figure 4.21 A, B). The least injured HP-10 lines were HP-10-11 and HP-10-16 with a score of 35%. At 4 WAT, these two lines had a score of 37% and 25%, respectively. Among all the tested HP lines, HP-10-16 was the least injured (Figure 4.21 C, D).

GOI (CYP81Q32-like): The three P-8 lines, i.e., P-8-3, P-8-8, and P-8-5 showed the highest injury symptoms with a score of 50%. However, the plants, P-8-11 and P-8-4 showed about 35% injury whereas P-8-17, P-8-14, and P-8-13 were the least injured with 30% (Figure 4.22 A, B). At 4 WAT, the four lines P-8-4, P-8-17, P-8-14, and P-8-13 recovered and had a visual injury of 10% (Figure 4.22 C, D).

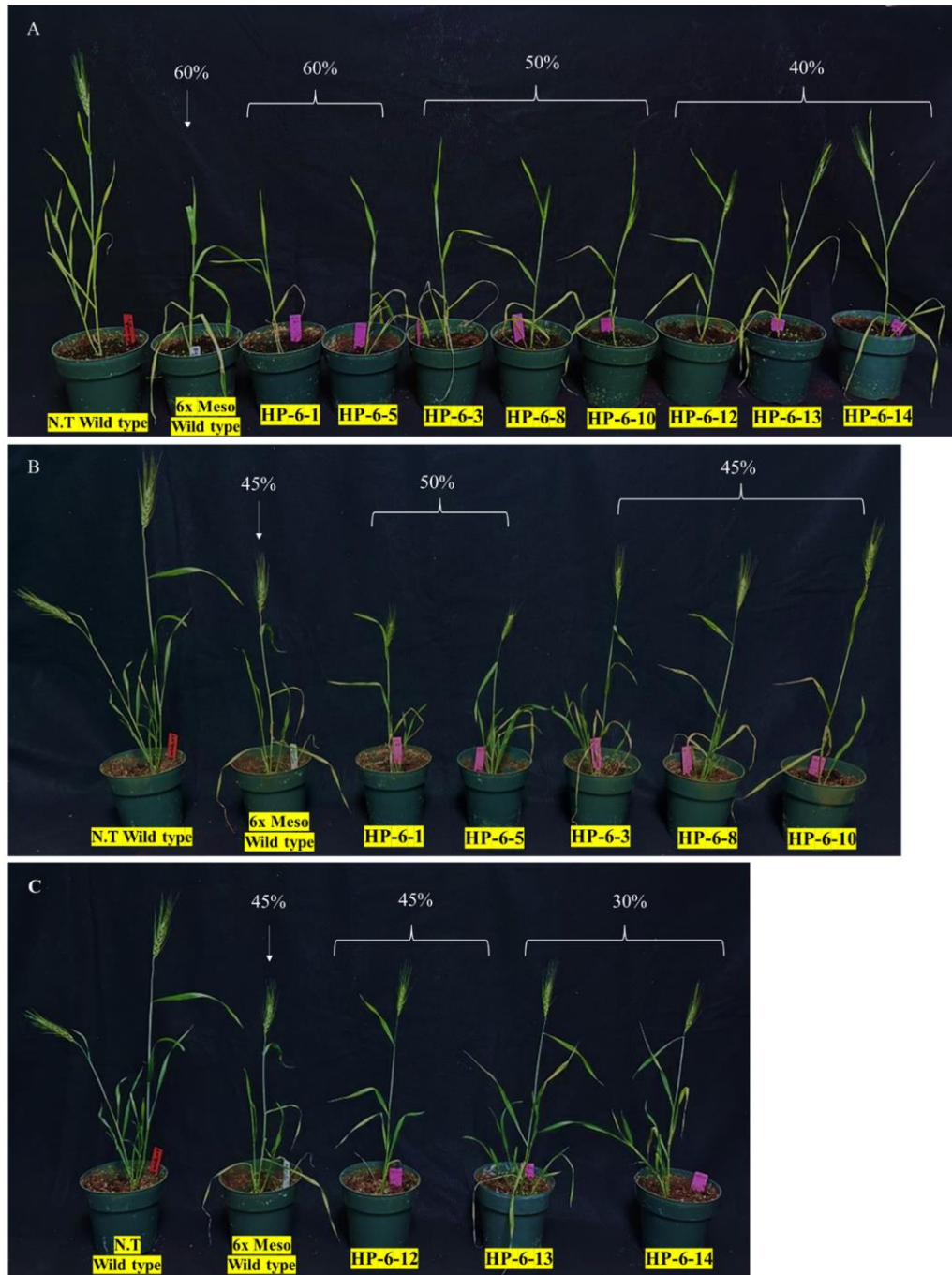


Figure 4.19. A) The response of the HP-6 transgenic T₁ lines to 6X dose of mesotrione at 3 weeks after treatment. B, C) The recovery of the HP-6 transgenic at 4 weeks after treatment.

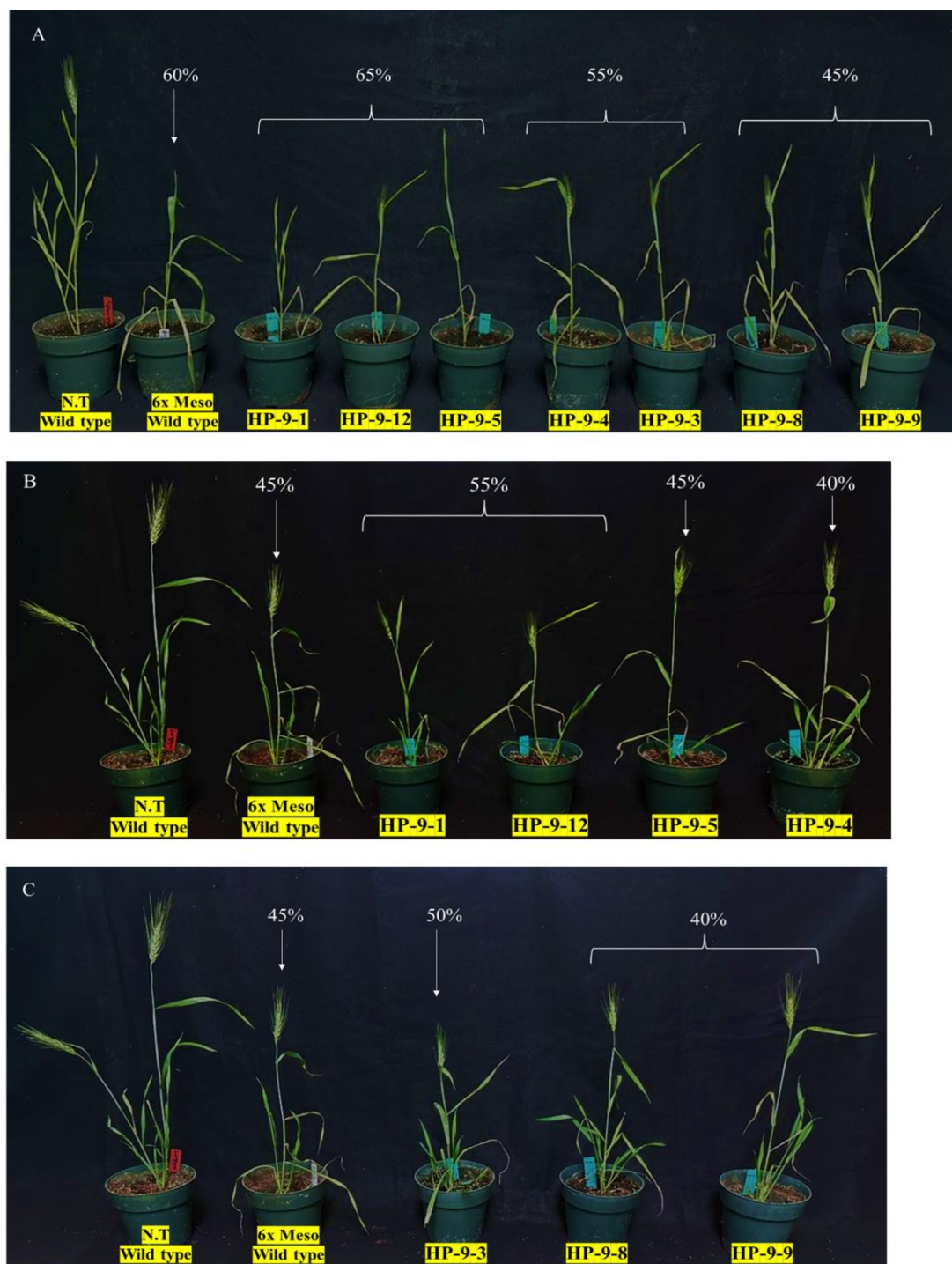


Figure 4.20. A) The response of the HP-9 transgenic T₁ lines to 6X dose of mesotrione at 3 weeks after treatment. B, C) The recovery of the HP-9 transgenic T₁ lines at 4 weeks after treatment.

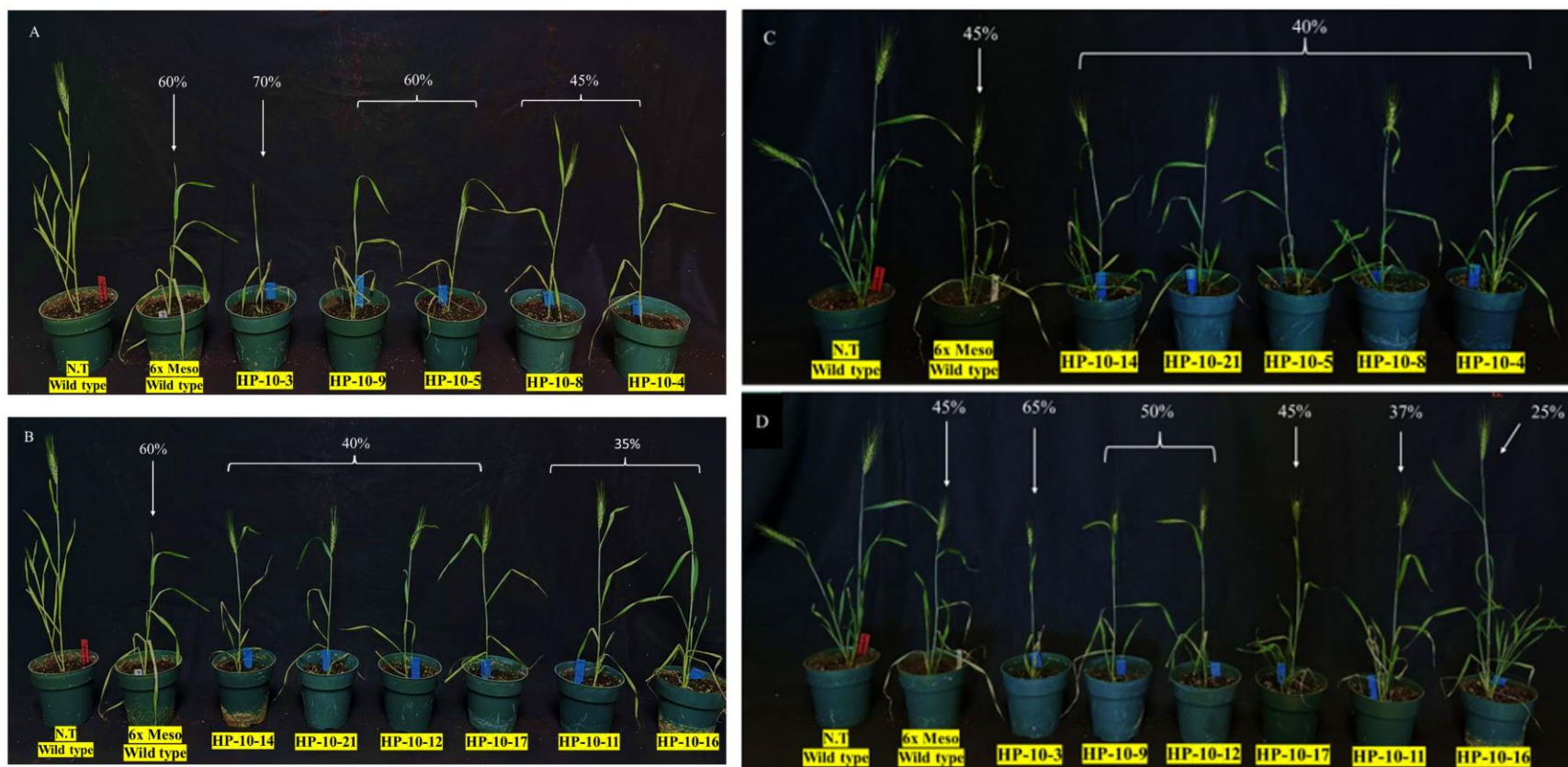


Figure 4.21. A, B) The response of the HP-10 transgenic T₁ lines to 6X dose of mesotrione at 3 weeks after treatment. C, D) The recovery of the HP-10 transgenic T₁ lines at 4 weeks after treatment.

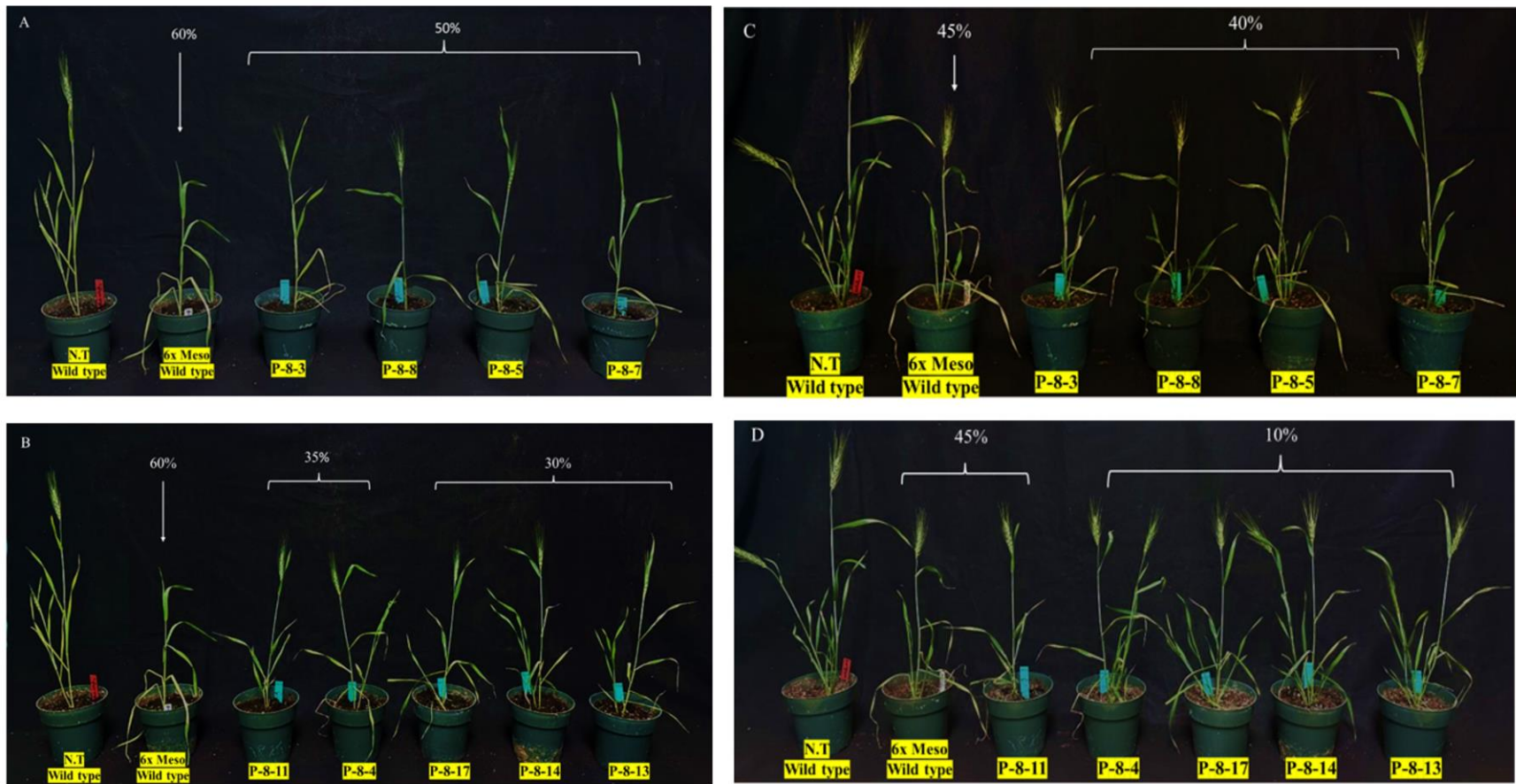


Figure 4.22. A, B) The response of the P-8 transgenic T₁ lines to 6X dose of mesotrione at 3 weeks after treatment. C, D) The recovery of the P-8 transgenic T₁ lines at 4 weeks after treatment.

4.4.7. Relative transcript expression of GOI in T₁ transgenic plants

GOI *TaHPPD*: Based on the visual injury data, plants at 3 WAT were categorized as least injured, intermediate- injured, and most injured. One representative plant from each category was chosen to study the relative expression of *TaHPPD*. HP-6-1 and HP-9-1 were chosen as the representative lines of the most injured category as they had a visual injury score of 60 to 65% (Figure 4.19, 4.20). The lines HP-6-3, HP-9-5, HP-10-14, HP-9-8, and HP-10-12 were chosen to represent the intermediate category as they had a visual injury score of 40 to 50% (Figure 4.19, 4.20, 4.21). And, HP-6-14 and HP-10-16 were chosen as the least injured with 30 to 35%. A high level of expression of the transgene *TaHPPD* was found in the HP-10-16, which also had the least injury and recovered quickly at 4 WAT (Figure 4.23, 4.21). This implies that the reduced sensitivity to mesotrione in HP-10-16 is possibly due to the overexpression of the transgene *TaHPPD*. Even though HP-10-12 had a relatively higher expression of the GOI compared to the other lines, it did not show reduced symptoms in response to mesotrione treatment (Figure 4.21, 4.23). HP-6-14 had less injury but the expression level was also very low (Figure 4.19, 4.23). Therefore, the best candidate to forward to the T₂ generation is HP-10-16. This plant accumulated more biomass and recovered faster compared to the other HP lines.

GOI *CYP81Q32-like*: To study the relative expression of the transgene *CYP81Q32-like* in T₁ plants, P-8-3 was chosen to represent the most injured category, and the lines, P-8-14 and P-8-13 were chosen as least injured plants (Figure 4.22). The relative expressions of P-8-14 and P-8-13 were higher than P-8-3 (Figure 4.24). This shows that the least injured plants were able to metabolize mesotrione possibly because of the overexpression of the transgene *CYP81Q32-like*.

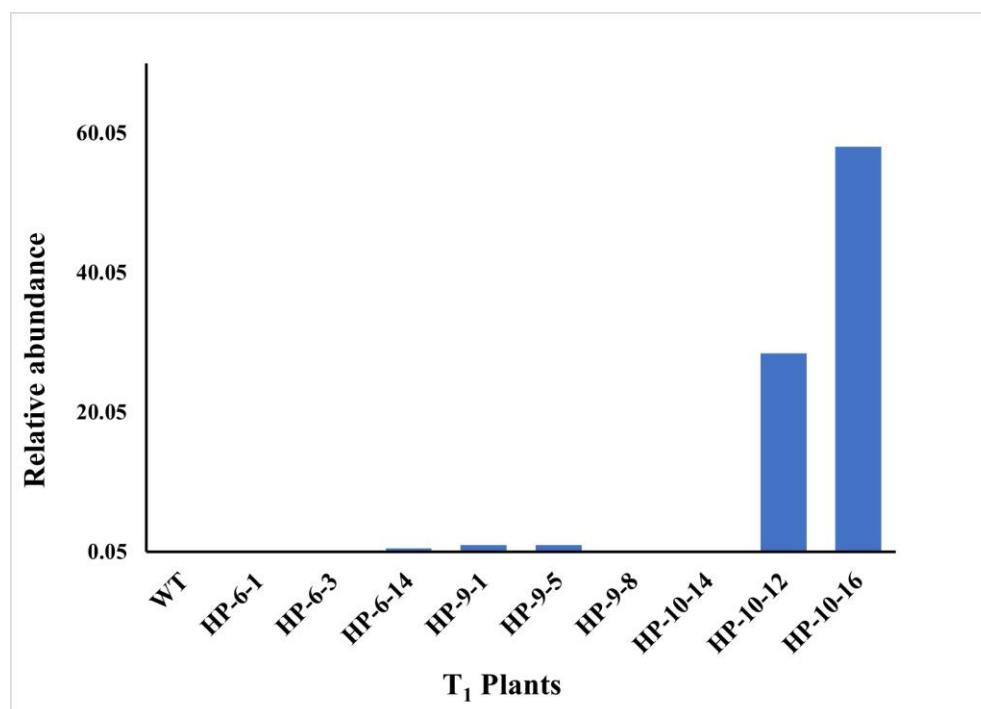


Figure 4.23. Relative expression of transgene *TaHPPD* in T₁ plants. Relative expression was estimated using the $\Delta\Delta C_t$ method, with actin as the reference gene.

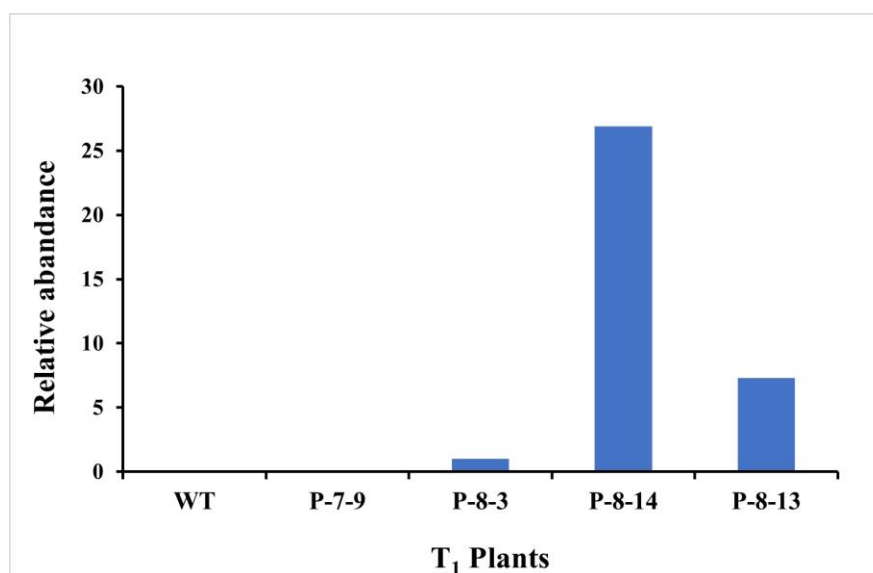


Figure 4.24. Relative expression of transgene *CYP81Q32-like* in T₁ plants. Relative expression was estimated using the $\Delta\Delta C_t$ method, with actin as the reference gene.

4.4.8. Cloning of the guide RNAs into the vector

The colony PCR was performed with a primer from the vector sequence as forward primer (5' GTCGTGCAAAATTGCCTG 3') and the bottom sequence of guide RNA 1 to detect the successful cloning of sgRNA1 into the vector. Out of the eight colonies picked for analysis, all of them were positive for the guide RNA. The amplicon size was 452 bp and all the positive colonies showed amplification at that size (Figure 4.25). p0390sgCas9 vector without the guide RNA was used as a negative control along with water control and no amplification was identified. These results suggest that the sgRNA1 was successfully cloned into the vector and transformed into DH5a cells. Similarly, PCR amplification was performed with the same forward primer and the bottom sequence of guide RNA 2. All eight colonies picked for analysis were positive (data not shown).

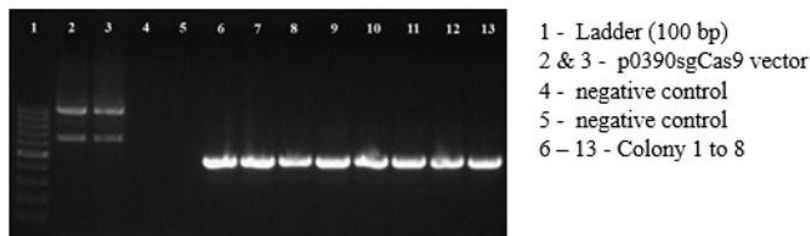


Figure 4.25. The gel image illustrating the PCR amplification of the positive colonies containing the guide RNA (sgRNA 1) cloned into the p0390sgCas9 vector.

4.4.9 Whole vector sequencing

The plasmids were isolated from the positive colonies containing sgRNA1 and sgRNA2. The whole plasmid was then sent to Eurofins genomics (Louisville, Kentucky). The whole plasmid sequencing results showed the successful cloning of both the guide RNAs in the expected region. The sequence is shown in Appendix B.

4.5 Discussion

The two types of herbicide resistance mechanisms such as the target site (TS) and the non-target site (NTS) play a major role in determining how a plant withstands herbicide applications and grows normally. The TS mechanisms involve mutations in the target gene that reduce its affinity to the herbicide or an increased expression of the target gene (Murphy & Tranel., 2019). The NTS mechanisms involve reduced absorption/translocation, or increased metabolism of herbicides possibly mediated by enzymes such as P450s and Glutathione-s-transferases (GSTs) (Jugulam & Shyam., 2019).

So far, the HPPD-inhibitor resistance has been reported in three weed species: Palmer amaranth (*Amaranthus palmeri*), waterhemp (*Amaranthus tuberculatus*), and wild radish (*Raphanus raphanistrum*). Metabolism-based resistance to HPPD-inhibitors *via* P450 enzyme activity was found in these weed species (Lu et al., 2020; Ma et al., 2013; Nakka et al., 2017b). However, a Palmer amaranth population (KCTR) showed metabolism-based resistance *via* P450s along with a 4 to 12-fold increase in HPPD gene expression (Nakka et al., 2017b). In an RNA-seq experiment, about 29 P450 genes including *CYP72A15* were found to be up-regulated in a HPPD-resistant waterhemp compared to the susceptible population (Kohlhase et al., 2018). Moreover, a specific P450 gene *CYP81E8* was found to be overexpressed in the KCTR Palmar amaranth that was resistant to tembotrione compared to the susceptible KSS population (Aarthy et al., 2022). In ryegrass (*Lolium rigidum*), the overexpression of a gene *CYP81A10v7* conferring resistance to mesotrione and other herbicides was reported (Han et al., 2021).

In crops, corn is naturally tolerant to mesotrione and tembotrione *via* P450-based metabolism specifically carried out by the *CYP81A9* (*Nsf 1*) gene (Bollman et al., 2008; Williams & Pataky., 2010). In rice, the, *HIS1* (HPPD inhibitor sensitive 1) conferring resistance to triketones was

found (Maeda et al., 2019). Mesotrione-resistant sorghum genotypes, G-1 and G-10, rapidly metabolized mesotrione compared to the susceptible genotype *via* P450 activity (Pandian et al., 2023). A similar mechanism was also found in the tembotrione-resistant genotypes of sorghum, G-200 and G-350 as well (Pandian et al., 2020b). In Chapter 2 of this dissertation, winter wheat genotypes, WW-1 and WW-2 were identified least sensitive to triketone herbicides due to metabolism likely mediated by P450 activity. In this study, we overexpressed the wheat P450 gene *CYP81Q32-like* in a known HPPD-sensitive spring wheat genotype to study its response to mesotrione application. The wheat *CYP81Q32-like* gene and protein shared approximately 83% and 78% sequence similarity with the corn *CYP81A9* gene; Therefore, *CYP81Q32-like* is an ideal candidate for functional validation.

The T₁ transgenic lines P-8-14 and P-8-13 showed relatively higher expression of the *CYP81Q32-like* gene and were also less sensitive to mesotrione treatment at 3 WAT and 4 WAT compared to the other T₁ lines. From this, we can imply that the overexpression of the *CYP81Q32-like* gene has also increased the metabolic activity resulting in reduced mesotrione sensitivity in the plants. This data also suggests that the least sensitive differential sensitivity of the winter wheat genotypes found in Chapter 2 might possibly be due to the variable expression of *CYP81Q32-like* leading to differential rate of triketone herbicide metabolism. However, an RNA-seq study is essential to identify the potential candidates responsible for the differential sensitivity in winter wheat. Moreover, an investigation involving whether the downregulation of the *CYP81Q32-like* gene (*via* CRISPR) also helps to confirm its function in increased sensitivity to triketone herbicides (work in progress). The *CYP81A9* gene in corn shows cross-sensitivity to ALS-, HPPD-, protoporphyrinogen oxidase (PPO)-, and photosystem II (PSII)-inhibitors

(Pataky et al., 2008). Similarly, the possibility of the cross-sensitivity to other herbicides needs to be assessed in future studies.

Amidst the plethora of metabolic-resistant mechanisms found in HPPD-inhibitor-resistant plants, a few cases of target site alterations conferring resistance are also reported. For e.g., the HPPD-inhibitor resistance in wild oats is due to the gene mutations in the gene *avhppd-03*. Transgenic soybeans have been developed with the introduction of the gene *avhppd-03* isolated from wild oats (Dreesen et al., 2014). Also, transgenic soybean consisting of an expression cassette with an insensitive shuffled variant of corn fused to soybean native 86 amino-terminal showed resistance to mesotrione, tembotrione, and isoxaflutole (Siehl et al., 2014). Transgenic soybeans have also been developed with a *Pseudomonas fluorescens* *HPPD* gene harboring a G336W mutation (Dreesen et al., 2007). More recently, random mutagenesis of the *Sphingobium* *sp.* *HPPD* gene, resulting in single point mutations E322R and G327C with improved topramezone resistance was reported (Wang et al., 2022). In this study, we overexpressed the target *TaHPPD* gene to determine whether the increased expression eventually reduces the mesotrione sensitivity. The T₁ line HP-10-16 showed relatively high expression of the *TaHPPD* gene compared to the other T₁ lines (Figure 4.21). This line performed the best phenotypically with the least sensitivity to mesotrione. However, other lines that were less sensitive to mesotrione such as HP-9-8 had low gene expression compared to the other HP-9 lines (Figure 4.23). Sometimes less mRNA transcript abundance can result in increased protein levels. Conversely, the HP-10-12 lines showed about 50% visual injury at 4 WAT but had relatively higher gene expression (Figure 4.21, 4.23). In this case, HPPD mRNA transcript abundance might have not correlated with increased HPPD protein levels. Therefore, analysis of protein

levels *via* the western blotting method will help correlate whether increased expression always translates to increased protein production.

The transgenic T₁ lines P-8-14, P-8-13, and HP-10-16 showed less sensitivity to mesotrione application due to the increased expression of the GOIs (Figure 4.21, 4.22, 4.32, 4.44). Therefore, analyzing these homozygous plants in the T₂ generation will be valuable. Further, functional validation of the *CYP81Q32-like* gene by downregulating via CRISPR/Cas is also under progress. Overall, the outcome of this research provides evidence that the sensitivity to triketone herbicides in spring wheat can be reduced by increasing the expression levels of the *CYP81Q32-like* or *HPPD* gene. These results also indirectly provide a molecular basis for the differential sensitivity of winter wheat (Chapter 2) to these herbicides.

4.6 Conclusion

This study suggests that overexpression of the target gene *TaHPPD* reduces the sensitivity of wheat to mesotrione. The possible function of the P450 gene *CYP81Q32-like* in imparting reduced sensitivity to HPPD-inhibitors was also assessed. In particular, the transgenic T₁ lines P-8-14, P-8-13, and HP-10-16 confirm that the increased expression of the GOIs (*TaHPPD* and *CYP81Q32-like*) reduces herbicide sensitivity to mesotrione. Further, RNA-seq analysis and gene functional validation studies are warranted to identify and confirm the roles of genes responsible for the differential sensitivity of wheat to triketone herbicides.

Chapter 5 - General Summary and Conclusions

Weed management is necessary for agriculturally important crops like wheat. In the US, chemical weed control is a commonly used weed management strategy in wheat fields. The most commonly used herbicides are ALS-, PS-II- inhibitors, and synthetic auxinic herbicides (Flessner et al., 2021). Unfortunately, the repeated use of herbicides as a sole method of weed control resulted in the evolution of ~84 weeds species resistant to herbicides in wheat (Heap, 2023). Therefore, there is a need for alternate herbicide options in wheat. Currently, there are only two herbicide-resistant technologies available in wheat, viz., Clearfield® and COAxiom® wheat that confer resistance to herbicides of the class ALS-inhibitors and ACCase-inhibitors, respectively (Nakka et al., 2019). Therefore, developing new herbicide-resistant wheat technologies is crucial to using alternate herbicide options for weed management in wheat. The HPPD-inhibiting herbicides such as mesotrione and tembotrione are widely used in corn but, not in wheat due to crop injury. Identification and characterization of wheat response to HPPD-inhibitors will help in the development of HPPD-inhibitor-resistant wheat.

The HPPD-inhibitors can be a viable options for weed control in wheat due to the following reasons: 1) these herbicides are known to effectively control broad-leaf and grass weeds. 2) only three weed species namely, Palmer amaranth, waterhemp, and wild radish have been recorded to have evolved resistance to these herbicides worldwide. In this dissertation, a wide collection of wheat genotypes was screened to evaluate their response to triketone herbicides, e.g., mesotrione and tembotrione (Chapter 2). Further, the physiological basis of the wheat response to HPPD-inhibitors was also studied in Chapter 2. The inhibition of response to HPPD-inhibitors were investigated in Chapter 3), and finally, the focus of Chapter 4 was to

understand the molecular basis of wheat response to triketone herbicides. The outcome of the research of each Chapter is summarized below.

In Chapter 2, wheat germplasm including 24 winter, 15 spring wheat genotypes, ~ 500 EMS-derived lines of wheat of Jagger (winter wheat), and ~ 15 EMS-derived lines of wheat of AM2003 (spring wheat) were evaluated for their response to mesotrione or tembotrione. Overall, variable levels of sensitivity to mesotrione and tembotrione were found. Spring wheat genotypes were more sensitive to these herbicides compared to winter wheat. Two winter wheat genotypes i.e., WW-1 and WW-2 were found to be the least sensitive compared to the most sensitive genotype WW-24. Further, the Jagger (parent of mutant lines) was the least sensitive and the mutant lines i.e., J38 and J327 were found most sensitive. Importantly, the least sensitive genotypes of winter wheat, WW-1 and WW-2 survived more than six times the field-recommended doses of mesotrione or tembotrione. The physiological characterization of these genotypes indicated that WW-1 and WW-2 can metabolize ~ 70 to 75% of ^{14}C -tembotrione, whereas WW-24 metabolized ~ 60% possibly *via* Phase I biochemical reactions, at 24 hours after treatment. Also, Jagger was found to metabolize ~95% of ^{14}C -tembotrione compared to ~ 80 to 85% in the mutant lines at 48 hours after treatment. Further, a possible role of P450 enzymes in the metabolism of mesotrione or tembotrione. In plants, the P450s and GSTs play a major role in the metabolism/degradation process (Zhang & Yang., 2021). Weed species such as waterhemp, Palmer amaranth, and wild radish are also known to metabolize these herbicides *via* Phase I metabolism. Previous studies suggest metabolism of mesotrione or tembotrione confers resistance in sorghum *via* P450 activity (Pandian et al., 2023; Pandian et al., 2020b). The P450s are a family of enzymes which make up to 1% of the total plant genome (Ghosh, 2017). Therefore, it is valuable to identify specific P450 genes in wheat responsible for reduced

sensitivity to HPPD inhibitors in wheat. The *CYP81A9* gene in corn, *CYP81E8* gene in Palmer amaranth, and *CYP81A10v7* gene in *Lolium rigidum* were found to regulating the metabolic resistance to these herbicides (Aarthy et al., 2022; Han et al., 2021).

To introduce the herbicide-resistant trait into high-yielding cultivars *via* breeding programs, it is important to understand the inheritance of the trait. The Chapter 3 of this dissertation investigated this aspect and F₁ progenies were generated by performing direct and reciprocal crosses between WW-1 or WW-2 (least sensitive) and WW-24 (most sensitive) winter wheat genotypes. Upon treatment with mesotrione or tembotrione, the F₁ progenies from both direct and reciprocal crosses showed an intermediate responses compared to the parents, suggesting that the reduced sensitivity in WW-1 and WW-2 was a partially dominant trait nuclear trait. The F₂ progenies were derived by self-pollinating the F₁ progenies. After treatment with mesotrione (630 g ai h⁻¹) or tembotrione (552 g ai h⁻¹), the F₂ progenies did not follow the typical Mendelian segregation ratio of 3:1 for a single gene inheritance. Thus, the reduced sensitivity to mesotrione or tembotrione in WW-1 and WW-2 is likely polygenic trait. Generally, the P450-mediated metabolism is controlled by either a single gene or by multiple genes. Future studies using RNA-seq analyses will possibly help identify potential candidate genes and quantitative trait loci (QTLs) associated with triketone herbicide sensitivity in wheat.

In Chapter 4, the functional validations of genes responsible for mesotrione sensitivity were analyzed. The wheat gene *CYP81Q32-like* (Non-target gene) was found to share about 83% sequence similarity to the *CYP81A9* gene in corn, which is known to involve in metabolism of triketone herbicides. We hypothesized the overexpression of structurally similar gene *CYP81Q32-like* may confer less sensitivity to mesotrione in wheat. Additionally, as there is evidence of target site alterations conferring resistance to HPPD-inhibitor in wild oats and

Palmer amaranth (Kramer et al., 2014; Nakka et al., 2017b), the *TaHPPD* (gene), the molecular target of triketone herbicides, was also overexpressed in a mesotrione-sensitive spring wheat cultivar ‘Bobwhite’. The wheat transformation was performed *via* gene gun method/particle bombardment. The transgenic plants regenerated were screened for the presence of genes of interest (GOI, i.e., *TaHPPD* and *CYP81Q32-like*) in the T₀ generation. The T₀ plants that were found positive for the GOI were further subjected to phenotypic and genotypic analysis in the T₁ generation. The T₁ lines P-8-14, P-8-13, and HP-10-16 were found to have increased expression of the GOIs (*via* RT-qPCR analysis) and also had reduced sensitivity to mesotrione, upon treatment with at six times, field recommended dose of mesotrione. Therefore, it appears that increased expression of the *CYP81Q32-like* may have also increased the metabolic activity of the enzyme to degrade mesotrione which has eventually resulted in reduced sensitivity to this herbicide. The overexpression of the *TaHPPD* gene has also reduced mesotrione sensitivity, possibly with over production of HPPD enzyme. Overall, the outcome of these experiments suggests that the differential sensitivity of WW-1, WW-2, and WW-24 genotypes to triketone herbicides may possibly be bestowed because of differential metabolic rate of P450 enzyme, coded by *CYP81Q32-like* gene. To validate the function of the *CYP81Q32-like* gene for less sensitivity to mesotrione, two sgRNAs that would target the first exonic region of the P450 gene were designed. The guide RNAs were cloned into the Cas9 vectors. In the future these vectors can be used to perform the CRISPR/Cas9 based gene editing, which is in progress.

As next steps, it is important to map the precise genomic locations that are responsible for imparting resistance to triketone herbicides in wheat. As the reduced sensitivity to HPPD-inhibitor in wheat is likely to be mediated by P450 activity, development of genetic markers and identification of quantitative trait locus (QTL) linked to reduced sensitivity *via* bi-parental

mapping and bulk segregant analysis will be valuable. These markers can be used in introgression of the trait *via* conventional breeding programs. Further RNA-seq analysis followed by validation will help identify potential candidate genes responsible for reduced sensitivity to triketones. These future studies will assist in the development of HPPD-resistant wheat *via* genetic engineering techniques as well. Finally, molecular docking of the *HPPD* gene or the non-target genes help determine if they can be subjected to gene editing to develop triketone-resistant wheat. As no point mutations leading to HPPD-inhibitor resistance (except *Avena sativa* and *Pseudomonas fluorescens*) are found, a thorough understanding of the target and non-target genes is essential, if considered for genome editing.

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Appendix A - Supplementary material

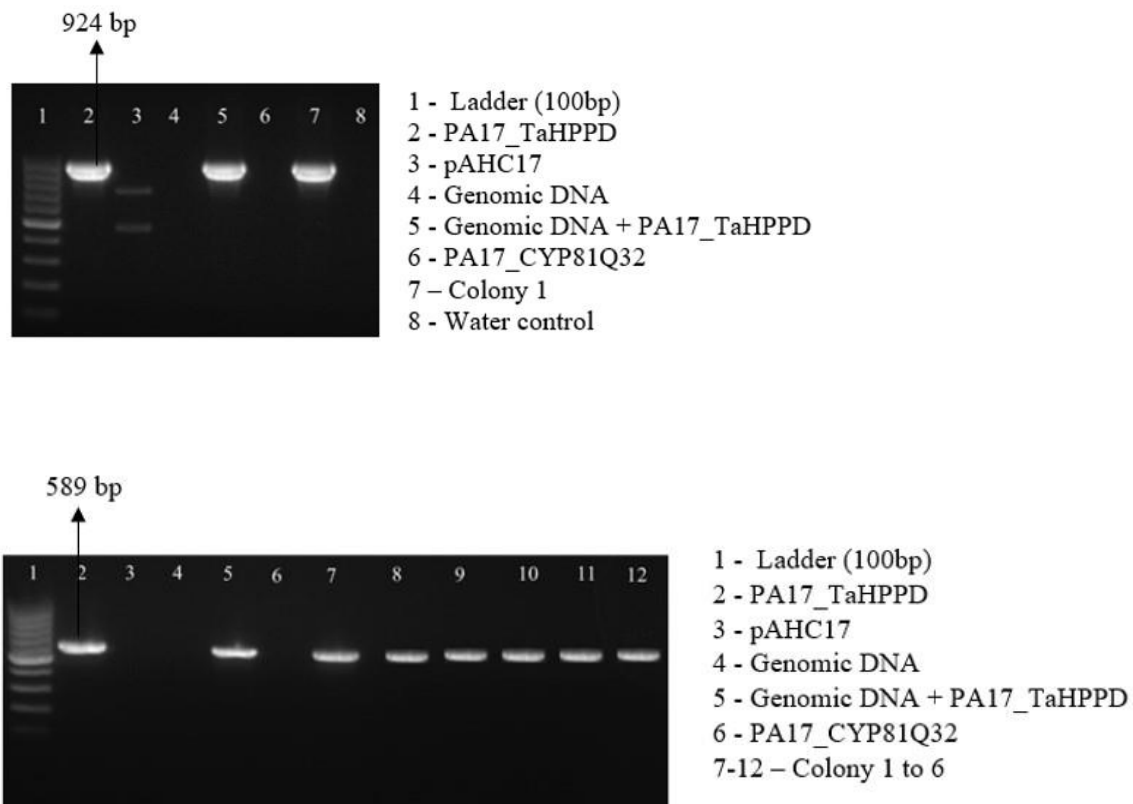


Figure A. 1 (a) The gel image shows the PCR amplification of the colonies containing the vector PA17_TaHPPD with primers UbiABF and PA17_TaHPPD RE2. (b) The gel image shows the PCR amplification of the colonies containing the vector PA17_TaHPPD with primers UbiABF and PA17_TaHPPD RE2.

A

```

HPPD ----- 0
CD GTTTAAGATGGATGGAATATCGATCTAGGATAGGTATACATGTTGATGCGGTTTACT 60

HPPD ----- 0
CD GATGCATATACATGATGGCATATGCAGCATCTATTTCATATGCTCTAACCTTGAGTACCTA 120

HPPD ----- 0
CD TCTATTATAATAACAAGTATGTTTTATAATTATTTGATCTTGATATACCTGGATGATG 180

HPPD ----- 0
CD GCATATGCAGCAGCTATATGTGGATTTTTTATGCCCTGCCTCATACGCTATTATTATTC 240

HPPD ---NNNNNNNNNTTTTGTGATGCTCCCTGTTGTTGGTGTACTTCTGCAGGTGCA 57
CD TTGGTACTGTTTCTTTTGTGATGCTCACCTGTTGTTGGTGTACTTCTGCAGGTGCA 300
*****

HPPD CTCTAGAGGATCCGCCACCATGCTCCGACGCCGACACCCAGCCGCCACCGGGCCGC 117
CD CTCTAGAGGATCCGCCACCATGCTCCGACGCCGACACCCAGCCGCCACCGGGCCGC 360
*****

HPPD CGCTACCGCCGCCGTTACGCTGAGCAGCAGCAGCCAGAGAATGGTCAAGTTCAACCC 177
CD CGCTACCGCCGCCGTTACGCTGAGCAGCAGCAGCCAGAGAATGGTCAAGTTCAACCC 420
*****

HPPD AAGGTCCGACCGCTTCCACACCTCGCCTTCCACACGTGGAGTTCTGSGCGCTGACGC 237
CD AAGGTCCGACCGCTTCCACACCTCGCCTTCCACACGTGGAGTTCTGSGCGCTGACGC 480
*****

HPPD TGCTAGCGCTGCTGGCAGGTTGCGCTTCGCCCTCGCGGCCCACTGCGCGCCGCTCCGA 297
CD TGCTAGCGCTGCTGGCAGGTTGCGCTTCGCCCTCGCGGCCCACTGCGCGCCGCTCCGA 540
*****

HPPD CCTGAGCACCAGCACTCCGTCACGCTAGCCTCTGAGGTCCGCAACCTCGCCTT 357
CD CCTGAGCACCAGCACTCCGTCACGCTAGCCTCTGAGGTCCGCAACCTCGCCTT 600
*****

HPPD CCTGTTACCGCCCATACGCCAACGGCTGCGACGCTGCTACCGCTAGCTGCGCTCTT 417
CD CCTGTTACCGCCCATACGCCAACGGCTGCGACGCTGCTACCGCTAGCTGCGCTCTT 660
*****

HPPD CAGCGCTGACGCTGCTAGGCGCTTCTCCGCCGACACCGCCTCGCCCTCAGGAGCATCG 477
CD CAGCGCTGACGCTGCTAGGCGCTTCTCCGCCGACACCGCCTCGCCCTCAGGAGCATCG 720
*****

HPPD TCTGAGGTTGGCTGACGCTGCTGAGGCTTTCAGGCTTCCGTTGAGCAGGCGCCGCC 537
CD TCTGAGGTTGGCTGACGCTGCTGAGGCTTTCAGGCTTCCGTTGAGCAGGCGCCGCC 780
*****

HPPD AGCCTTCAGCCCAAGTGGACCTCGGCAAGGCTTCCGCTTCCGTTGAGTGGAGCTTACGG 597
CD AGCCTTCAGCCCAAGTGGACCTCGGCAAGGCTTCCGCTTCCGTTGAGTGGAGCTTACGG 840
*****

HPPD CGACGTGCTCTGCGCTTCTGCTCCACCCAGACGACACCGAGTGCATTCTGCGCAGG 657
CD CGACGTGCTCTGCGCTTCTGCTCCACCCAGACGACACCGAGTGCATTCTGCGCAGG 900
*****

HPPD CTTGAGGGCGTCAACACCCAGACGCCGTGACTACGGCTCACCAGTTTCAGCCAGT 717
CD CTTGAGGGCGTCAACACCCAGACGCCGTGACTACGGCTCACCAGTTTCAGCCAGT 960
*****

HPPD GGTGGGCAACGTGCCAGAGCTCGCTCCAGCTGCTACGTGCTGCTTACCGGCTT 777
CD GGTGGGCAACGTGCCAGAGCTCGCTCCAGCTGCTACGTGCTGCTTACCGGCTT 1020
*****

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B

```

HPPF ----- 0
CD TCTATTATAATAACAAGTATGTTTTATAATTATTTGATCTTGATATACCTGGATGATG 180

HPPF ----ANGCAGCAGCTATATGTGGATTTTTTAGECCCTGCCTTNCATACGCTATTATTG 56
CD GCATATGCAGCAGCTATATGTGGATTTTTTAGECCCT-GCCCTCATACGCTATTATTG 239
*****

HPPF CTGGTACTGTTTCTTTTGTGATGCTGACCTGTTGTTGGTGTACTTCTGCAGGTGCG 116
CD CTGGTACTGTTTCTTTTGTGATGCTGACCTGTTGTTGGTGTACTTCTGCAGGTGCG 299
*****

HPPF ACTCTAGAGGATCCGCCACCATGCTTCGACGCCGACACCCAGCCGCCACCGGGCCGC 176
CD ACTCTAGAGGATCCGCCACCATGCTTCGACGCCGACACCCAGCCGCCACCGGGCCGC 359
*****

HPPF CCGCTACCGCCGCCGT TACGCTGAGCAGCCAGACCCAGAGAATGGTCAAGTTCAACC 236
CD CCGCTACCGCCGCCGT TACGCTGAGCAGCCAGACCCAGAGAATGGTCAAGTTCAACC 419
*****

HPPF CAAGGTCCGACCGCTTCCACACCTCGCCTTCCACACGTGGAGTTCTGGTGGCTGACG 296
CD CAAGGTCCGACCGCTTCCACACCTCGCCTTCCACACGTGGAGTTCTGGTGGCTGACG 479
*****

HPPF CTGTAGEGCTGCTGGCAGGTTCCGCTTCGCCCTCGCGGCCCACTGGCCGCCGCTCCG 356
CD CTGTAGEGCTGCTGGCAGGTTCCGCTTCGCCCTCGCGGCCCACTGGCCGCCGCTCCG 539
*****

HPPF ACCTGAGCACCAGCACTCCGTCACGCTAGCAGCTCTGAGGTCCGCAACCTCGCCT 416
CD ACCTGAGCACCAGCACTCCGTCACGCTAGCAGCTCTGAGGTCCGCAACCTCGCCT 599
*****

HPPF TCTGTTTACCGCCCATACGCCAACGGCTGCGACGCTGCTACCGCTAGCTGCGCTCT 476
CD TCTGTTTACCGCCCATACGCCAACGGCTGCGACGCTGCTACCGCTAGCTGCGCTCT 659
*****

HPPF TCAGCGCTGACGCTGCTAGGCGCTTCTCCGCCGACACGGCTCGCCCTCAGGAGCATCG 536
CD TCAGCGCTGACGCTGCTAGGCGCTTCTCCGCCGACACGGCTCGCCCTCAGGAGCATCG 719
*****

HPPF CTCTGAGGTTGGCTGAGGCTGCTGAGGCTTTCAGGCTTCCGTTGAGCAGGCGCCGCC 596
CD CTCTGAGGTTGGCTGAGGCTGCTGAGGCTTTCAGGCTTCCGTTGAGCAGGCGCCGCC 779
*****

HPPF CAGCCTTCAGCCCAAGTGGACCTCGGCAAGGCTTCCGCTTCCGTTGAGTGGAGCTTACG 656
CD CAGCCTTCAGCCCAAGTGGACCTCGGCAAGGCTTCCGCTTCCGTTGAGTGGAGCTTACG 839
*****

HPPF GCGACGTGGTCTCGGCTTCTGCTTCCACCCAGACGACACCGAGCTGCCATTCTGCCAG 716
CD GCGACGTGGTCTCGGCTTCTGCTTCCACCCAGACGACACCGAGCTGCCATTCTGCCAG 899
*****

HPPF GCCTCAGAGGCGCTCAGCAACCCAGACGCCGTGGACTACGGCTCACCAGTTTCAGCAGC 776
CD GCCTCAGAGGCGCTCAGCAACCCAGACGCCGTGGACTACGGCTCACCAGTTTCAGCAGC 959
*****

HPPF TGGTGGGCAACGTGCCAGAGCTCGCTCAGCTGCTGCCATGCTGCTTCAACGGCT 836
CD TGGTGGGCAACGTGCCAGAGCTCGCTCAGCTGCTGCCATGCTGCTTCAACGGCT 1019
*****

HPPF TCCACAGATTCCGCGAGTTCAACACCCAGAGAGCTGGGACCGCTGAGTCCGGCTCAACA 896
CD TCCACAGATTCCGCGAGTTCAACACCCAGAGAGCTGGGACCGCTGAGTCCGGCTCAACA 1079
*****

HPPF GCANGGTNNNNCAACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 925
CD GCATGGTCTTGGCCCAACACCTCCGAGGGCGTGTCTTGGCCATCAACGAGCCAGTCCAGC 1139
*****

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Figure A.2 (a) A part of the sequence results of PA17_TaHPPD after aligning with the expected sequence.

A

```

HPPD ----- 0
CD GTTTAAGATGGATGGAATATCGATCTAGGATAGGTATACATGTTGATGGGTTTACT 60

HPPD ----- 0
CD GATGCATATACATGATGGCATATGCAGCATCTATTATATGCTTAACCTTGAGTACCTA 120

HPPD ----- 0
CD TCTATTATAATAAACAGTATGTTTATAATTATTTTATGCTTATGATATCTGGATGATG 180

HPPD ----- 0
CD GCATATGCAGCAGCTATATGTGGATTTTTTTATGCTTGCCTTATACGCTATTATTATTC 240

HPPD ---NNNNNNNNHTTTTTCGATGCTCNCCTGTTGTTGGTGTACTTCTGCAGGTGCA 57
CD TTGGTACTGTTCTTTTTCGATGCTCACCCTGTTGTTGGTGTACTTCTGCAGGTGCA 300
*****

HPPD CTCTAGAGGATCCGACCATGCTCCGACGCGACACCCAGCCGACACGGGGCCG 117
CD CTCTAGAGGATCCGACCATGCTCCGACGCGACACCCAGCCGACACGGGGCCG 360
*****

HPPD CGCTACCGCCGCGTTACGCTGAGCAGCGCAGACCCAGAAGATGTCAGGTTCACCC 177
CD CGCTACCGCCGCGTTACGCTGAGCAGCGCAGACCCAGAAGATGTCAGGTTCACCC 420
*****

HPPD AAGGTCCGACCGCTTCCACACCTCGCTTCCACACGTGGAGTTCTGTGCGCTGACGC 237
CD AAGGTCCGACCGCTTCCACACCTCGCTTCCACACGTGGAGTTCTGTGCGCTGACGC 480
*****

HPPD TGCTAGCGCTGCTGGCAGGTTGCGCTTCGCCCTCGGCGCCCACTGGCGCCGCTCCGA 297
CD TGCTAGCGCTGCTGGCAGGTTGCGCTTCGCCCTCGGCGCCCACTGGCGCCGCTCCGA 540
*****

HPPD CCTGAGCACCGGCAACTCCGTCACGCTAGCCTTCTGAGGTCGGCAACTCGCCTT 357
CD CCTGAGCACCGGCAACTCCGTCACGCTAGCCTTCTGAGGTCGGCAACTCGCCTT 600
*****

HPPD CCTGTTACCGCCCATACGCAACGGCTGCGACGCTGCTACGCTAGCTGCGCTCTT 417
CD CCTGTTACCGCCCATACGCAACGGCTGCGACGCTGCTACGCTAGCTGCGCTCTT 660
*****

HPPD CAGCGCTGACGCTGCTAGCGCTTCTCCGCGACACGCGCTCGCGCTCAGGAGCATCG 477
CD CAGCGCTGACGCTGCTAGCGCTTCTCCGCGACACGCGCTCGCGCTCAGGAGCATCG 720
*****

HPPD TCTGAGGTTGGCTGACGCTGCTGAGGCTTTCAGGCTTCCGTTGACGCGCGCGCCGCC 537
CD TCTGAGGTTGGCTGACGCTGCTGAGGCTTTCAGGCTTCCGTTGACGCGCGCGCCGCC 780
*****

HPPD AGCCTTACGCCCAGTGGACCTCGGACGGGCTTCGGCTTCGCTGAGGTGGAGCTCTACGG 597
CD AGCCTTACGCCCAGTGGACCTCGGACGGGCTTCGGCTTCGCTGAGGTGGAGCTCTACGG 840
*****

HPPD CGACGTGGTCTGCGCTTCGCTTCCACCCAGACGACACGAGTGCATTCTGCGAGG 657
CD CGACGTGGTCTGCGCTTCGCTTCCACCCAGACGACACGAGTGCATTCTGCGAGG 900
*****

HPPD CTTGAGGGCGTCAGCAACCCAGACGCCGTGGACTACGCGCTCACCAGTTTCAGCCAGT 717
CD CTTGAGGGCGTCAGCAACCCAGACGCCGTGGACTACGCGCTCACCAGTTTCAGCCAGT 960
*****

HPPD GGTGGGCAACGTGCCAGAGCTCGCTCCAGCTGCTGCTACGTCGCTGAGTTTACCAGGCTT 777
CD GGTGGGCAACGTGCCAGAGCTCGCTCCAGCTGCTGCTACGTCGCTGAGTTTACCAGGCTT 1020
*****

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B

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HPPF ----- 0
CD TCTATTATAATAAACAGTATGTTTATAATTATTTTATCTTATATACCTTGGATGATG 180

HPPF ---ANGCAGCAGCTATATGGAATTTTTTASGCTTGCCTTNCATACGCTATTATTG 56
CD GCATATGCAGCAGCTATATGGAATTTTTTASGCTTGCCTTNCATACGCTATTATTG 239
*****

HPPF CTGGTACTGTTTCTTTTTCGATGCTCACCCTGTTGTTGGTGTACTTCTGCAGGTGCG 116
CD CTGGTACTGTTTCTTTTTCGATGCTCACCCTGTTGTTGGTGTACTTCTGCAGGTGCG 299
*****

HPPF ACTCTAGAGGATCCGACCATGCTTCGACGCCGACACCCAGCCGACACGGGGCCG 176
CD ACTCTAGAGGATCCGACCATGCTTCGACGCCGACACCCAGCCGACACGGGGCCG 359
*****

HPPF CCGCTACGCGCGCGTTACGCTGAGCAGCGCAGACCCAGAAGATGGTCAGGTTCACCC 236
CD CCGCTACGCGCGCGTTACGCTGAGCAGCGCAGACCCAGAAGATGGTCAGGTTCACCC 419
*****

HPPF CAAGGTCCGACCGCTTCCACACCTCGCTTCCACACGTGGAGTTCTGGTGGCTGACG 296
CD CAAGGTCCGACCGCTTCCACACCTCGCTTCCACACGTGGAGTTCTGGTGGCTGACG 479
*****

HPPF CTGCTAGCGCTGCTGGCAGGTTGCGCTTCGCCCTCGGCGCCCACTGGCGCCGCTCGG 356
CD CTGCTAGCGCTGCTGGCAGGTTGCGCTTCGCCCTCGGCGCCCACTGGCGCCGCTCGG 539
*****

HPPF ACCTGAGCAGCGGCAACTTCCGTCACGCTAGCCTTCCAGGTCGGGCAACTTCGCT 416
CD ACCTGAGCAGCGGCAACTTCCGTCACGCTAGCCTTCCAGGTCGGGCAACTTCGCT 599
*****

HPPF TCTGTTTACCAGCCCATACGCAACGGCTGCGACGCTGCTACCGCTAGCTGCGCTCT 476
CD TCTGTTTACCAGCCCATACGCAACGGCTGCGACGCTGCTACCGCTAGCTGCGCTCT 659
*****

HPPF TCAGCGCTGACGCTGCTAGGCGCTTCTCCGCGACACGCGCTCGCGCTCAGGAGCATCG 536
CD TCAGCGCTGACGCTGCTAGGCGCTTCTCCGCGACACGCGCTCGCGCTCAGGAGCATCG 719
*****

HPPF CTCTGAGGGTGGCTGACGCTGCTGAGGCTTTCAGGGCTTCCGTTGAGGTCGGCGCCGCC 596
CD CTCTGAGGGTGGCTGACGCTGCTGAGGCTTTCAGGGCTTCCGTTGAGGTCGGCGCCGCC 779
*****

HPPF CAGCCTTACGCCCAGTGGACCTCGGACGGGCTTCGGCTTCTGCTGAGGTGGAGCTCTACG 656
CD CAGCCTTACGCCCAGTGGACCTCGGACGGGCTTCGGCTTCTGCTGAGGTGGAGCTCTACG 839
*****

HPPF GCGACGTGGTCTGCGCTTCTGCTTCCACCCAGACGACGAGCTGCGATTCTTGCAG 716
CD GCGACGTGGTCTGCGCTTCTGCTTCCACCCAGACGACGAGCTGCGATTCTTGCAG 899
*****

HPPF GCTTCAGGGGCTCAGCAACCCAGACGCCGTGGACTACGGCTCACCAGGTTTCAGCCAGG 776
CD GCTTCAGGGGCTCAGCAACCCAGACGCCGTGGACTACGGCTCACCAGGTTTCAGCCAGG 959
*****

HPPF TGGTGGGCAACGTGCCAGAGCTCGCTCAGCTGCTGCTTACGTCGCTGGCTTACCAGGCT 836
CD TGGTGGGCAACGTGCCAGAGCTCGCTCAGCTGCTGCTTACGTCGCTGGCTTACCAGGCT 1019
*****

HPPF TCACAGGTTTCGCGAGTTTACACACGAGGAGCTGGGACGCTGAGTTCGGGCTTCAACA 896
CD TCACAGGTTTCGCGAGTTTACACACGAGGAGCTGGGACGCTGAGTTCGGGCTTCAACA 1079
*****

HPPF GCANGGTNNNNCCACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 925
CD GCATGGTCTTGGCCCAACCTCCGAGGGGCTGCTCTGCGACTCAACGAGCGAGTCCAGG 1139
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Figure A. 2 (b) A part of the sequence results of PA17_CYP81Q32 after aligning with the expected sequence

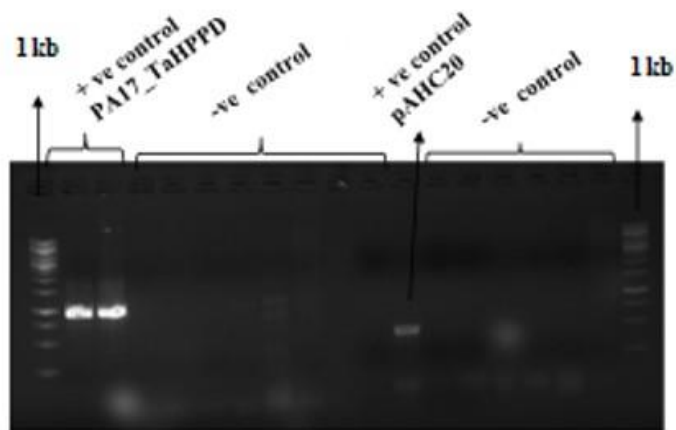


Figure A. 3 (a) Positive control and negative control for testing the presence of *TaHPPD* gene and *bar* gene.

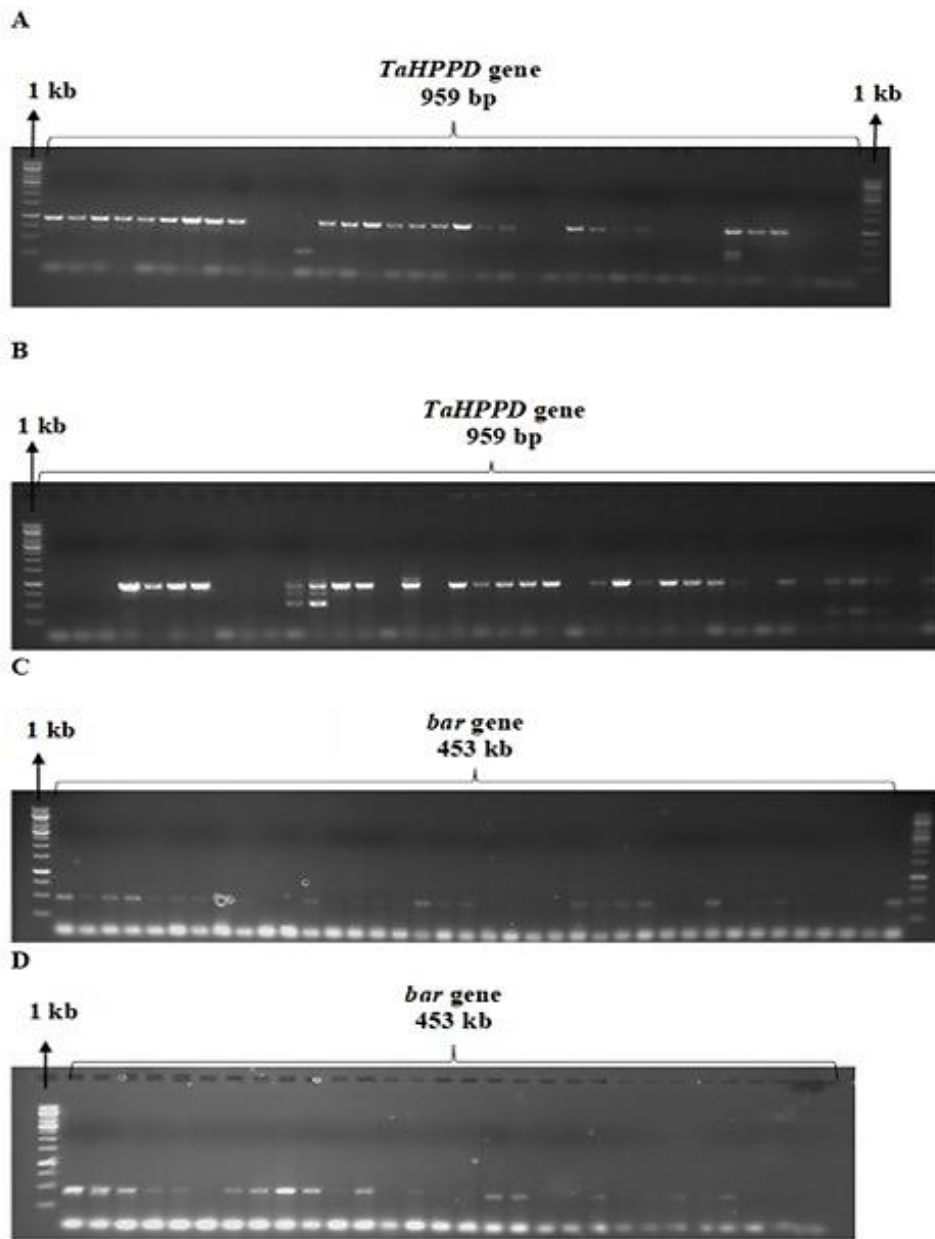


Figure A. 3 (b) A, B) Gel picture showing amplification showing the presence of the *TaHPPD* gene in T₀ plants T₀-HP-11 to T₀-P-36. C, D) presence of *bar* gene in T₀ plants T₀-HP-11 to T₀-HP-36. Almost all the tillers were tested.

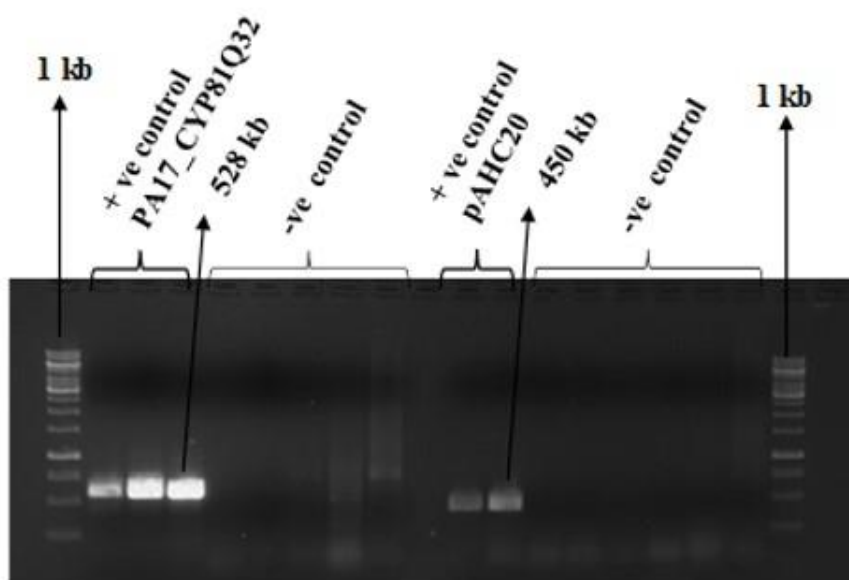


Figure A. 4 (a) Positive control and negative control for testing the presence of *CYP81Q32-like* gene and *bar* gene.

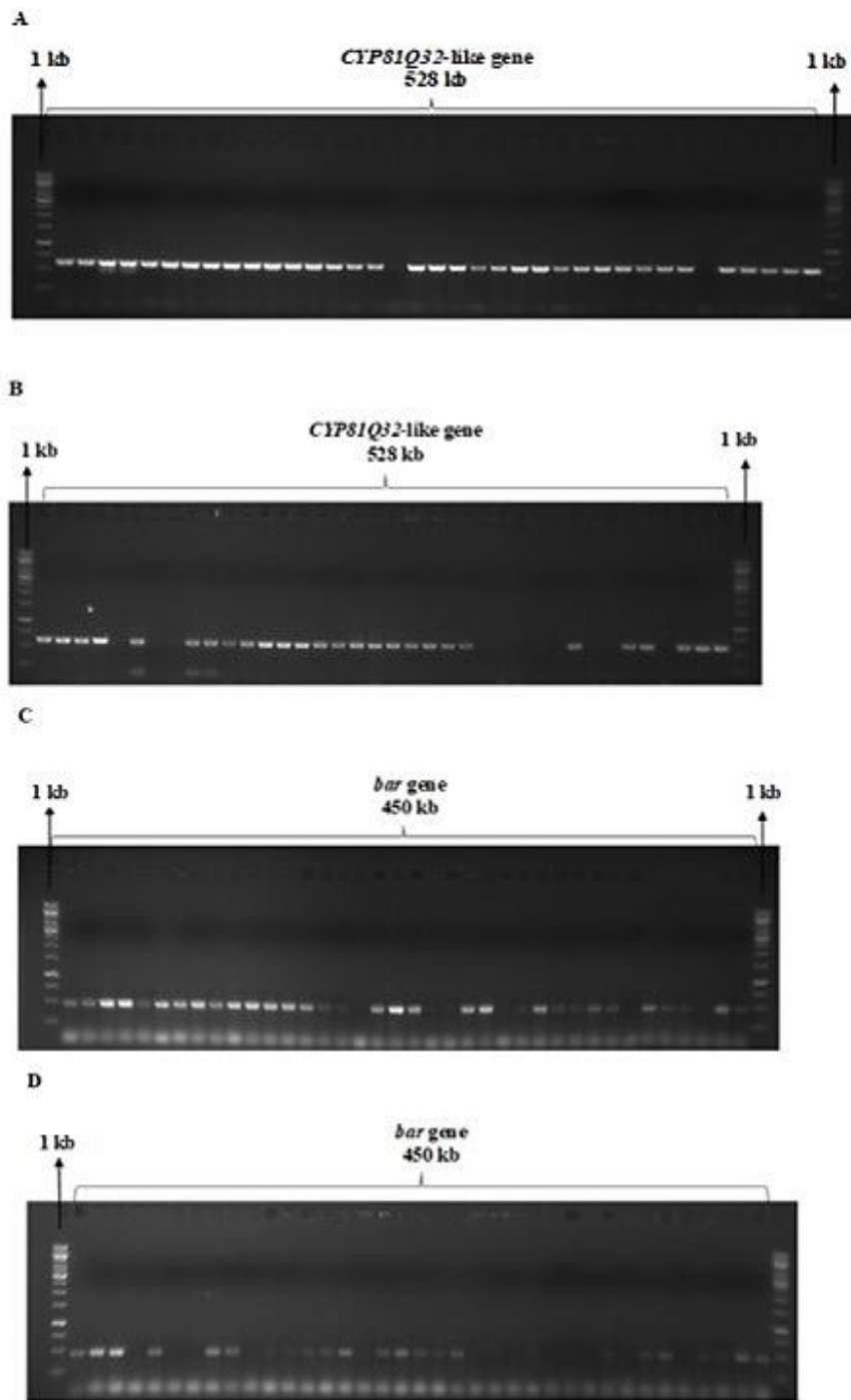


Figure A. 4 (b) A,B) Gel picture showing amplification showing the presence of the *CYP81Q32-like* gene in T₀ plants T₀-P-11 to T₀-P-36. C, D) presence of bar gene in T₀ plants T₀-P-11 to T₀-P-36. Almost all the tillers were tested.

Appendix B - Supplementary material-II

The vector sequence of the plasmid containing sgRNA1. The yellow highlighted region shows the sgRNA 1 region.

```

1  AGTACTTCTGAAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTAC 6526
2  AGTACTTCTGAAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTAC 5789
  *****

1  TTCTGAAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTG 6586
2  TTCTGAAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTG 5849
  *****

1  AAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTGAAAAA 6646
2  AAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTGAAAAA 5909
  *****

1  AGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGCACCGACTCGGTGCCACTTTTT 6706
2  AGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGCACCGACTCGGTGCCACTTTTT 5969
  *****

1  CAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA----- 6755
2  CAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTCGGCG 6029
  *****

1  -----CGCTTCTTGGTGCCGCGCCTCCGCTTATAAGCTGGTTCTGCTCCCTG 6802
2  AAGATCACGTCGTCGCTTCTTGGTGCCGCGCCTCCGCTTATAAGCTGGTTCTGCTCCCTG 6089
  *****

1  TAAACTGGCGAGGTGGGACTAATGTTAGACGTGCTGCTGCACAGCAGCGCCAGCGCCCCT 6862
2  TAAACTGGCGAGGTGGGACTAATGTTAGACGTGCTGCTGCACAGCAGCGCCAGCGCCCCT 6149
  *****

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1	CGGCGGGAGCTTTTAGAAGGACTCGTAATTGCTGGCCCGTGAGGTTGGTGGGCTGCTGCC	6922
2	CGGCGGGAGCTTTTAGAAGGACTCGTAATTGCTGGCCCGTGAGGTTGGTGGGCTGCTGCC	6209

1	TGCTAGATAGACAGATCCCGGCCCATCTGAGAGACATCAGTCTCGTATGGCTTTTTCTTT	6982
2	TGCTAGATAGACAGATCCCGGCCCATCTGAGAGACATCAGTCTCGTATGGCTTTTTCTTT	6269

1	TCCTACCTAAAAAAAAGTATGGCTTTTTCTTTCCTTTTTCCGTGCATCAGCTTTCATTT	7042
2	TCCTACCTAAAAAAAAGTATGGCTTTTTCTTTCCTTTTTCCGTGCATCAGCTTTCATTT	6329

1	TTGTTACTAATAACAGTTCAGAAGCTCTTCTCAAATGTCAGTATCTACTTGTCAATTATT	7102
2	TTGTTACTAATAACAGTTCAGAAGCTCTTCTCAAATGTCAGTATCTACTTGTCAATTATT	6389

The vector sequence of the plasmid containing sgRNA2. The yellow highlighted region shows the sgRNA 2 region.

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1  TTCTGAAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTG 6586
2  TTCTGAAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTG 5383
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1  AAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTGAAAAA 6646
2  AAAAAAGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGATGTTGTACTTCTGAAAAA 5443
*****

1  AGAGACTGACTAAAAAAACAGAAGGAC-AAAAAAAAAAGCACCGACTCGGTGCCACTTTT 6705
2  AGAGACTGACTAAAAAAACAGAAGGACAAAAAAAAGCACCGACTCGGTGCCACTTTT 5503
*****

1  TCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTA----- 6755
2  TCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAACGCCGAG 5563
*****

1  -----CGCTTCTTGGTGCCGCGCCTCCGCTTATAAGCTGGTTCTGCTCCCT 6801
2  GAACGGGATGGCCTCGCTTCTTGGTGCCGCGCCTCCGCTTATAAGCTGGTTCTGCTCCCT 5623
*****

1  GTAAACTGGCGAGGTGGGACTAATGTTAGACGTGCTGCTGCACAGCAGCGCCAGCGCCCC 6861
2  GTAAACTGGCGAGGTGGGACTAATGTTAGACGTGCTGCTGCACAGCAGCGCCAGCGCCCC 5683
*****

1  TCGGCGGGAGCTTTTAGAAGGACTCGTAATTGCTGGCCCGTGAGGTTGGTGGGCTGCTGC 6921
2  TCGGCGGGAGCTTTTAGAAGGACTCGTAATTGCTGGCCCGTGAGGTTGGTGGGCTGCTGC 5743
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1	CTGCTAGATAGACAGATCCCGGCCCATCTGAGAGACATCAGTCTCGTATGGCTTTTTCTT	6981
2	CTGCTAGATAGACAGATCCCGGCCCATCTGAGAGACATCAGTCTCGTATGGCTTTTTCTT	5803

1	TTCCTACCTAAAAAAAAGTATGGCTTTTTCTTTCCCTTTTCCGTGCATCAGCTTTCCATT	7041
2	TTCCTACCTAAAAAAAAGTATGGCTTTTTCTTTCCCTTTTCCGTGCATCAGCTTTCCATT	5863

1	TTTGTTACTAATAACAGTTCAGAAGCTCTTCTCAAATGTCAGTATCTACTTGTCAATTAT	7101
2	TTTGTTACTAATAACAGTTCAGAAGCTCTTCTCAAATGTCAGTATCTACTTGTCAATTAT	5923
