Physiological, genetic and genomic analyses of herbicide resistance in grain sorghum
(*Sorghum bicolor*)

by

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B.Sc., Tamil Nadu Agricultural University, 2012
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2020

Abstract

Grain sorghum [*Sorghum bicolor* (L.) Moench ssp. *bicolor*] is a versatile crop with multiple uses, including for food, feed, and fuel. Postemergence (POST) grass weed control continues to be a major challenge in grain sorghum, primarily due to a lack of herbicide options registered for POST use. The 4- hydroxyphenylpyruvate dioxygenase (HPPD)- (e.g., mesotrione or tembotrione) and acetolactate synthase (ALS)- inhibitor (e.g., chlorsulfuron) herbicides are used for POST control of a broad-spectrum of weeds including grasses in corn and wheat but not in sorghum, due to crop injury. The development of herbicide-resistant sorghum technology to facilitate broad-spectrum POST weed control can be an economical and viable solution. Previously we have identified four sorghum genotypes, two each resistant to mesotrione (G-1 and G-10), tembotrione (G-200 and G-350) and, one susceptible genotype (S-1) from the sorghum association panel. Further, we found that the genotype S-1 is highly resistant to chlorsulfuron. The objectives of this dissertation were to 1) investigate the inheritance, mechanism, and identify genetic loci conferring resistance to mesotrione and tembotrione, 2) characterize, and investigate the inheritance and mechanism of resistance to chlorsulfuron in grain sorghum. To understand the inheritance of the mesotrione and tembotrione resistance, F_1 and F_2 progeny were generated by performing crosses using S-1 and G-1, G-10, G-200, or G-350. The F_1 and F_2 progeny were evaluated for their response to various doses of mesotrione and tembotrione treatment. Likewise, chlorsulfuron dose-response experiments were conducted using S-1 along with BTx623, a susceptible check and also F_1 and F_2 progeny were generated by crossing S-1 and BTx623. The results of genetic analyses of the F_1 and F_2 progeny demonstrated that the mesotrione resistance in G-1 and G-10 is a single dominant trait, while the tembotrione resistance in G-200 and G-350 is a partially dominant polygenic trait. Further, sequencing of

HPPD gene, the molecular target of mesotrione and tembotrione in the resistant genotypes, revealed no mutations known to bestow resistance. Additionally, the role of cytochrome P450 (CYP) in metabolizing mesotrione and tembotrione, using CYP-inhibitors, malathion and piperonyl butoxide (PBO) was also assessed. The results indicated a significant reduction in biomass accumulation in sorghum plants pre-treated with malathion or PBO, suggesting the involvement of CYPs in the metabolism of mesotrione and tembotrione. Bulk segregation analysis combined with RNA-Seq (BSR-seq) was used to identify the genomic region associated with mesotrione resistance; however, the sequence analyses was unable to map the resistance gene within a smaller interval. Genotype-by-sequencing (GBS) based quantitative trait loci (QTL) mapping revealed three QTLs associated with tembotrione resistance in G-200. The results of the chlorsulfuron dose-response assay indicated that S-1 and F_1 progeny were ~20-fold, more resistant to chlorsulfuron relative to BTx623. Segregation of F_2 progeny into 3:1 (resistance: susceptibility), suggested that chlorsulfuron resistance in S-1 is a single dominant trait. Sequence analysis of the *ALS* gene, the molecular target of chlorsulfuron from S-1 revealed no mutations that confer resistance to chlorsulfuron; however, a significant reduction in biomass accumulation was found in plants pre-treated with malathion, indicating that the metabolism of chlorsulfuron contributes to resistance in S-1. Overall, the results of this dissertation provide opportunities to develop herbicide-resistant sorghum hybrids via introgression, which can help effective POST weed management.

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Acknowledgements

Dedication

I dedicate this dissertation to my teacher and mentor Prof. **Dr. S. Robin**, Rice Breeder and Former Dean, School of Post Graduate Studies, Tamil Nadu Agricultural University, Coimbatore, TN, India. Who's not here with us to see this completed.

Chapter 1 - Introduction and Literature Review

Grain Sorghum

Grain sorghum is the fifth most important cereal crop grown in the world and fourth-largest grain crop in the United States behind corn (*Zea mays*), soybean (*Glycine max*), and wheat (*Triticum aestivum*; USDA-FAS 2020). It stands out to be the most important crop for the future due to its high adaptability, versatility, water use efficiency and other multiple uses such as cattle feed, food, ethanol, starch, and plastics (Taylor et al., 2006). Grain sorghum is suitable for cultivation in semi-arid regions under low input conditions and performs better than corn in drought-prone regions. Grain sorghum is a staple food for millions of people in semi-arid tropics of Africa and Asia; Sorghum grain is rich in vitamins, minerals, fiber, antioxidants, protein, and also gluten-free. However, grain sorghum is mostly grown for animal feed and biofuel production in the US (Mundia et al., 2019). Approximately one-third of the US grain sorghum crop is used for ethanol production; sorghum dried distillers grains with soluble byproduct obtained after ethanol extraction from sorghum grain, can be used as livestock feed (Jenkin et al. 2007).

The world grain sorghum production in 2019 was 59.79 million metric tons (MMT) in which the US stands first producing 9.27 MMT followed by Nigeria, Sudan, and Ethiopia producing 6.72, 4.93, and 4.95 MMT, respectively (Fig 1; USDA-NASS 2020). In the US, grain sorghum cultivated primarily in the Great Plains, Midwest and Southwest. Approximately, 85% of the US grain sorghum is produced from Arkansas, Kansas, Missouri, Nebraska, South Dakota, and Texas (Dille et al., 2020). Kansas tops the grain sorghum production in the US with a total production of 6.8 million metric tons which is more than half of the sorghum grain produced in the United States (USDA-NASS, 2020).
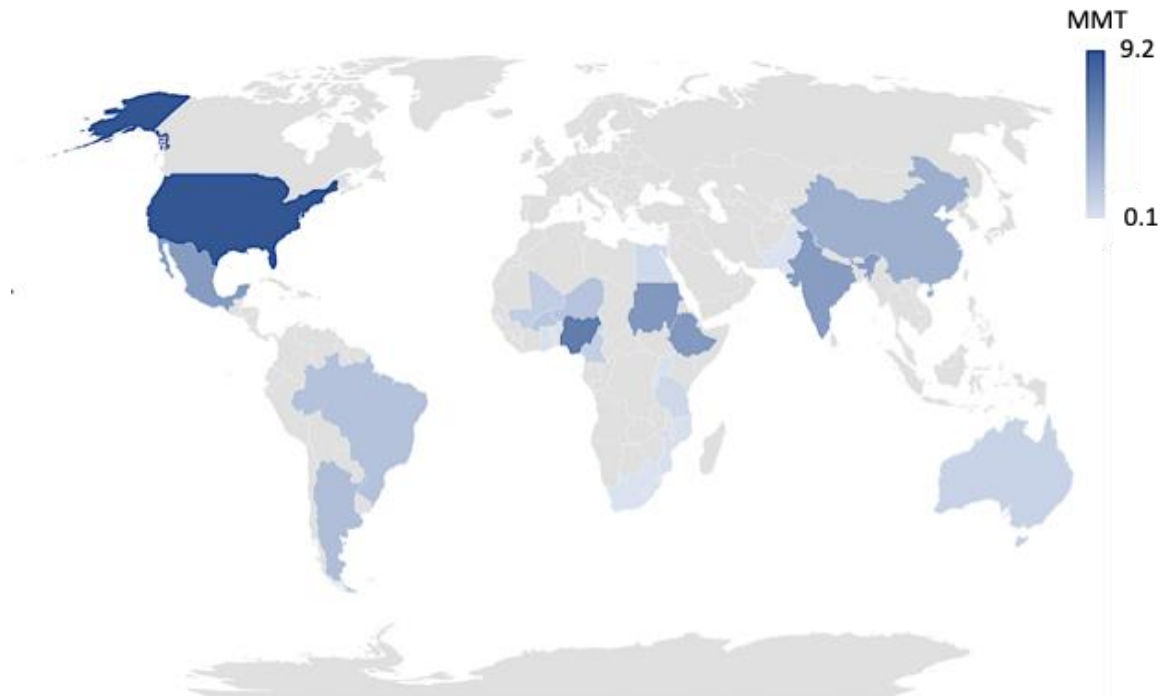
Figure 1.1. World map depicting the 2019 grain sorghum production in million metric tons (MMT); Data source: USDA-Foreign Agricultural Services (USDA-FAS 2020)

Challenges in Grain Sorghum Production

Despite the advantage of grain sorghum to perform well under limited resource conditions and ability to overcome several abiotic and biotic stresses, grain sorghum growers face numerous challenges to attain the maximum yield potential. Crop yields are adversely affected by infestation by several pests and more importantly competition from weeds cause extensive yield loss (Oerke, 2006). There was a plunge in grain sorghum cultivated area in the last two decades from 3.1 million acres in 2000 to 1.9 million ha in 2019 (Fig 2; USDA-NASS, 2020). There are several reasons for the shift from grain sorghum to other crops. The most important factor for this shift is lack of effective weed management options. Especially, weed infestation is a major problem in no-till sorghum due to the prevailing weed seeds and excess

moisture during early crop growth stages (Sreeram et al., 2016) and grain sorghum growers in the US find weed management as their primary concern in grain sorghum production (Tanya et al., 2017). The annual average yield loss without any weed control measure in grain sorghum production in the US has been estimated to be 47%, which is valued at approximately US $953 million (Dille et al., 2020).
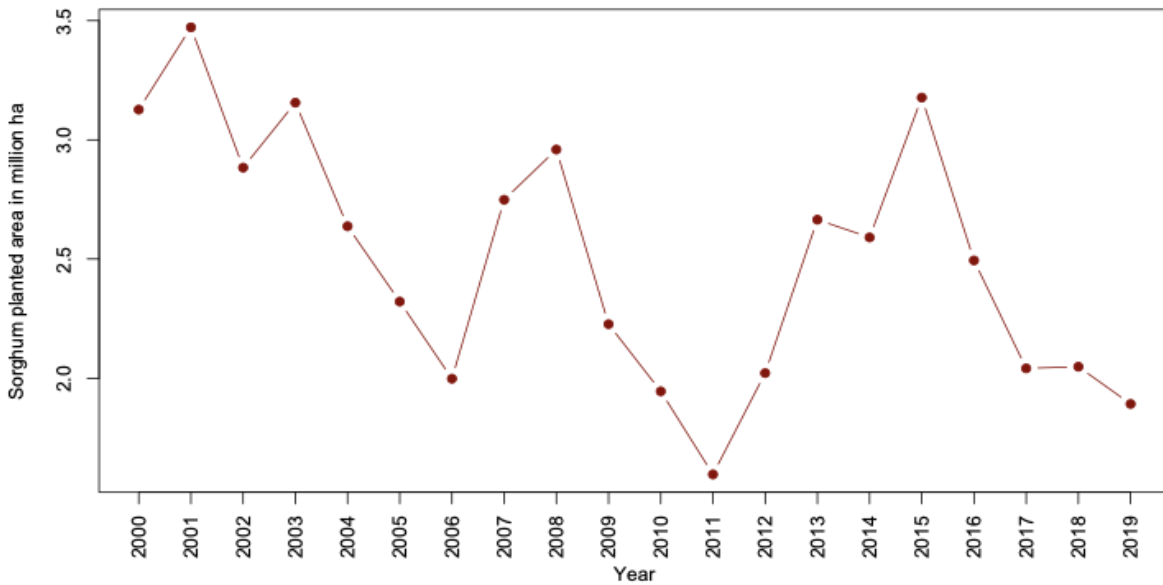
Figure 1.2 Annual planted area of grain sorghum in the US from 2000-2019; Data source: USDA- National Agricultural Statistical Services (USDA-NASS 2020)

Several broadleaf and grass weeds infest grain sorghum and create huge yield loss; Palmer amaranth (*Amaranthus palmeri*), waterhemp (*Amaranthus tuberculatus*), redroot pigweed (*Amaranthus retroflexus*), kochia (*Bassia scoparia*), Russian thistle (*Salsola kali*), Venice mallow (*Hibiscus trionum*), velvetleaf (*Abutilon theophrasti*), common cocklebur (*Xanthium strumarium*), morning glory (*Ipomoea spp*), Devil's claw (*Proboscidea louisianica*) are the major broadleaf weeds and shattercane (*Sorghum bicolor ssp. verticilliflorum*), Johnson grass (*Sorghum halapense*), large crabgrass (*Digitaria sanguinalis*), foxtail (*Setaria* spp.),

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longspine sandbur (*Cenchrus longispinus*), fall panicum (*Panicum dichotomiflorum*), and witchgrass (*Panicum capillare*) are the major grass weeds that infest sorghum in the US (Dille et al. 2020; Thompson et al. 2019).

Both preemergence (PRE) and postemergence (POST) herbicide options are limited in grain sorghum compared to corn or soybean. Although herbicide options for PRE and POST control of broadleaf weeds are available for control of broadleaf weeds, however, grass weed control in sorghum is completely dependent on PRE herbicides. Chloroacetamide group of long-chain fatty acid-inhibitors (Group 15) such as alachlor, acetochlor, S-metolachlor, dimethenamid-P, and 4-hydroxyphenylpyruvate dioxygenase (HPPD)- inhibitor mesotrione (Group 27) are major PRE herbicides used for grass weed control in grain sorghum. These herbicides can be effective on some of the annual grass species, but activation of PRE herbicides is dependent upon rainfall or irrigation. Moreover, short residual activity leads to poor control of weeds in the latter part of the growing season. Most of the commercial grain sorghum hybrids are susceptible to grass control herbicides such as HPPD-, acetolactate synthase (ALS)-, and acetyl-CoA carboxylase (ACCase)- Inhibitors. There are almost no herbicides registered for POST application in grain sorghum for grass weed control (Smith et al., 2010, Thompson et al., 2019) resulting in yield loss.

Table 1.1 Percent yield reduction caused by different grass weeds in grain sorghum; table

adapted and modified from Bean, 2020

Grass species	Percent Yield Reduction	Prevalence in US states
Johnsongrass (*Sorghum halapense)*	100	GA, IL, KS, MO, OK, TX
Shattercane (S. *bicolor ssp. verticilliflorum*)	80-96	IL, KS, MO, TX
Longspine Sandbur (*Cenchrus longispinus*)	42	KS, OK
Yellow Foxtail (*Setaria* spp.)	44	KS, NC, OK, DE
Barnyard grass (*Echinochloa crus-galli)*	42	OK
Texas and brown panicum (*Panicum* spp.)	80	GA, IL, NC, OK, TX

Potential Herbicide Options for POST-Grass Weed Control in Grain Sorghum

HPPD- Inhibitors

HPPD-inhibitors are a relatively new mode of action introduced in the 1980s. These herbicides biochemically inhibit HPPD, a key enzyme in tocopherol and plastoquinone biosynthesis (Lee et al., 1998). Plastoquinone acts as an important cofactor for phytoene desaturase, a key enzyme in the carotenoid biosynthesis pathway. Carotenoids are light-harvesting molecules and protect chlorophyll from photo-oxidation (Siefermann-Harms, 1987). Upon treatment with these herbicides, the susceptible plants exhibit leaf chlorosis also called bleaching as a result of the destruction of carotenoids. HPPD-inhibitors are broadly classified into three chemical families: isoxazoles, pyrazolones, and triketones (van Almsick, 2009). Mesotrione and tembotrione (triketones) are used in corn production for broad-spectrum weed control. Corn is naturally tolerant to mesotrione whereas tembotrione is applied with a safener,

isoxadifen-ethyl. Studies confirmed mesotrione and tembotrione were metabolized by cytochrome P450s (CYPs) in corn (Ahrens et al., 2013). HPPD-inhibitors can cause severe injury in grain sorghum; mesotrione is the only HPPD-inhibitor registered for PRE usage in grain sorghum (Abit et al., 2009). Currently, only two weed species, Palmer amaranth, and waterhemp have evolved resistance to HPPD-inhibitors.

ALS- Inhibitors

ALS also known as acetohydroxy acid synthase (AHAS), the target site of ALS-inhibitor herbicides, catalyzes the condensation of a 2-ketoacid with pyruvate as the first common step in the biosynthesis of the branched-chain amino acid (BCAA) such as valine, leucine, and isoleucine (Ray, 1982, Russel et al., 2002). BCAA are essential for many life forms but present only in microorganisms and plants. Inhibition of ALS enzyme leads to depletion of BCAAs levels in the cell leading to several phytotoxic processes and other secondary effects and plant death (Zhou et al., 2007). ALS-inhibiting herbicides include five chemical families viz., sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-thio benzoates (PTB), and sulfonyl-aminocarbonyl-triazolinone SCT; (Whitcomb, 1999). Although ALS-inhibitors are extremely popular and widely used for POST control of grasses in many crops such as wheat, corn, soybeans they are not registered for use in grain sorghum because of crop injury (Bararpour et al. 2019). Several crops such as wheat (Jabran et al., 2017), corn (Hinz and Owen, 1996), oats (*Avena sativa* L.; (Sweetser et al., 1982), and cotton (*Gossipium Sp.*; Kendig et al., 2007) are naturally tolerant to various ALS-inhibitor herbicides, primarily due to detoxification of these herbicides via CYPs (Yu and Powles, 2014).

Herbicide-Resistant Grain Sorghum Technology

Because of small acreage of sorghum production worldwide, (much smaller than corn and soybean) there is less focus on the development of herbicide-resistant sorghum technology by agrochemical and seed industry. Grain sorghum has not been a choice for the genetically modified (GM) herbicide-resistant hybrid development. Another major concern in the development of herbicide-resistant grain sorghum is the possible transfer of resistance genes into closely related wild species, such as shattercane or johnsongrass. Although latest research suggests that the chances of gene flow to wild species are minimal (Hodnett et al. 2019, Werle et al. 2017b), stewardship practices must be assessed to avoid gene flow and manage if the resistance trait escapes into these species. Essentially, the introduction of GM crops resistant to glyphosate in 1996 gained widespread acceptance as this technology facilitated effective weed management and encouraged no-till technology to conserve moisture and reduce soil erosion. Herbicide-resistant cropping traits are available for corn, soybean, cotton, wheat, rice, canola, sugarbeet, and alfalfa (Brookes and Barfoot, 2014). The development of herbicide-resistant grain sorghum has enormous potential to improve grass weed control.

The first-ever herbicide-resistant grain sorghum technology was initiated and developed by researchers at Kansas State University through transferring an altered *ALS* gene from an ALS-inhibitor-resistant shattercane biotype from Kansas by conventional breeding (Tuinstra and Al-Khatib, 2011). The trait has been branded originally by Dupont as "Inzen™" sorghum along with Zest™ herbicide, which is nicosulfuron an ALS-inhibitor herbicide belonging to the SU group. However, Inzen hybrids were not yet available in the market for commercial use. The ALS-inhibitor-resistant trait and the herbicide are now with Corteva AgroSciences. Advanta Inc., a seed company recently announced their new herbicide-resistant grain sorghum trait igrowth™

which is tolerant to IMI herbicides and is expected to be commercially available in the US in near future. This trait is an altered *ALS* gene developed by mutagenesis via ethyl methanesulfonate (EMS). The trait is commercially available in Argentina. The S&W seed company has announced their upcoming ACCase-inhibitor-resistant grain sorghum "Double Team[TM]", which was developed by Advanta in collaboration with the United Sorghum Checkoff Program.

Mechanisms of Herbicide Resistance

Herbicide resistance is the ability of a plant to survive and reproduce following herbicide application which is normally lethal to other individuals, whereas herbicide tolerance is the inherent ability of species to tolerate herbicide treatment (WSSA, 1998). Both the terms "tolerance and "resistance" have been used based on the context throughout the dissertation. Herbicide resistance evolution in weeds continues to be a great challenge in agriculture at the same time herbicide resistance or tolerance traits in crops are highly exploited in modern agriculture to enhance the use of herbicides to control weeds effectively without affecting the crop (Beckie et al., 2019). Herbicide resistance or tolerance mechanisms are classified into two types: 1) target-site based resistance (TSR) and b) non-target-site based resistance mechanism (NTSR) (Délye et al., 2015). The TSR is conferred by alteration in the herbicide target gene resulting in modifications in amino acid sequence, thereby protein configuration, gene over-expression, or amplification. Alteration of target site through point mutations resulting in deletion or substitution of one or more amino acids results in prevention of the herbicide from binding to the target-site. This is the most common mechanism of TSR to herbicides in weeds. This type of mechanism is prevalent for certain modes of action of herbicides, such as ALS-, ACCase-, protoporphyrinogen oxidase (PPO)-, photosystem (PS) II- inhibitors (Gaines et al.

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2020). Amplification of target site results in increased expression of the herbicide target gene, thus the plant can produce more target enzymes that cannot be inhibited by the recommended dose of herbicide. Amplification of the 5-enolpyruvylshikimate-3-phosphate synthase *(EPSPS)* gene (target site of glyphosate) confers high levels of resistance in many important weed species (Gaines et al., 2019). Increased constitutive expression of target-site through regulatory changes also confers resistance to herbicides (Nakka et al., 2017).

The NTSR mechanisms includes those that do not involve alterations in the target site of the herbicide. NTSR mechanisms include rapid metabolism, vacuolar sequestration, reduced absorption or translocation of herbicides and rapid necrosis (Yuan et al., 2007). Metabolism of herbicides before reaching the target enzyme will result in resistance, in plants two enzyme families i.e., glutathione-S-transferases (GSTs) and CYPs are known to play a key role in herbicide metabolism (Ohkawa et al., 1999). This is the most common mechanism in crops species that confer natural tolerance to herbicides. Several crops such as wheat (Koeppe et al., 1997), corn (Hinz and Owen 1996), soybean (Brown et al. 1990) are naturally tolerant to various ALS-inhibitors, primarily due to the detoxification of these herbicides via CYPs (Yu and Powles, 2014). CYPs are involved in metabolism of nicosulfuron, mesotrione, tembotrione in corn (Liu et al., 2019) trifloxysulfuron-sodium in cotton (Thyssen et al., 2018), and chlorsulfuron in wheat (Xiang et al., 2006) and several other crops.

Sources of Herbicide Resistance

Natural Variation

Natural genetic diversity in plant species is the primary source of variation utilized in breeding programs to develop high yielding, stress-resilient varieties that are better adapted to different agricultural systems worldwide (Glaszmann et al., 2010). Sorghum is one of the highly

diverse cereal crops consisting of five botanical races, *viz.*, Bicolor, Durra, Caudatum, Guinea and Kafir evolved by multiple domestication events (Harlan and De Wet, 1972). Grain sorghum germplasm exhibits diversity in plant height, grain quality, grain color, inflorescence, and several other traits that are important for biotic and abiotic stress resistance. A large collection of sorghum consisting of more than 41,860 accessions from 114 countries is maintained by the National Plant Germplasm System (NPGS) of the United States Department of Agriculture (USDA). The present-day grain sorghum breeding programs didn't fully exploit the genetic diversity available and several economically important traits harbored by the sorghum germplasm have not been ascertained by the researchers (Cuevas and Prom, 2020), and one such trait is, herbicide resistance. The genetic diversity available in grain sorghum can be utilized for identifying resistance to herbicides such as HPPD- or ALS-inhibitors, which have broad spectrum weed activity when applied POST. Most cultivated grain sorghum is highly susceptible to these herbicides. However, previously, upon screening wide collection of cultivated grain sorghum hybrids, variable levels of resistance to mesotrione and tembotrione was found (Abit et al., 2009; Cunha et al., 2016). The outcome of this dissertation resulted in identification and characterization of four grain sorghum genotypes, two each resistant to mesotrione or tembotrione which were identified upon screening ~900 sorghum genotypes from several germplasm collection including SAP and mutant lines (Pandian et al., unpublished). In addition to grain sorghum, utilizing natural variation for identifying herbicide-resistant traits has also been proven to be a successful in several other crops, e.g., imazapyr resistance in sugarcane has been identified by screening 64 breeding lines (Koch et al., 2009). Screening peanut mini-core collection resulted in the identification of a breeding line highly resistant to dicamba (Leon and Tillman, 2015). Screening a collection of advanced breeding lines revealed differential response

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to 2,4-D and metribuzin (Hartwig, 1987). The genotypes/lines possessing herbicide resistance traits can be used as parents to develop herbicide-resistant varieties.

Wild Relatives

The wild relatives of domesticated crops can also be source of desirable resistance alleles that have been purged out during the process of domestication. Those alleles can be useful for developing more resilient crop varieties as they potentially can harbor genes responsible for several biotic and abiotic stress and help breeders to improve adaptation and increase yield (Castañeda-Álvarez et al., 2016; Harlan, 1976). Two wild relatives of grain sorghum, e.g., shattercane and johnsongrass co-exist with the cultivated grain sorghum or in other crops as weeds in agriculture in North America. Continuous exposure of shattercane and johnsongrass to grass control herbicides such as ALS- and ACCase- inhibitors used in corn and soybean resulted in evolution of resistance to these herbicides in these weeds (Werle, 2016). Varying level of outcrossing has been reported between both the weedy sorghum species and cultivated grain sorghum (Hodnett et al., 2019; Schmidt et al., 2013). Researchers utilized this opportunity and transferred the resistant alleles from the weedy to cultivated sorghum to develop herbicide-resistant hybrids. A shattercane population from Kansas has been identified with a mutation, in the *ALS* gene resulting in Trp-574-Leu substitution, conferring resistance to SU as well as IMI herbicides. This altered *ALS* gene was transferred from the shattercane to grain sorghum using conventional breeding (Tuinstra and Al-Khatib, 2011). Similarly, another biotype of Sudangrass (*Sorghum bicolor* ssp. drummondii) from Bolivia that evolved resistance to ACCase-inhibitors by an altered *ACCase* gene resulting in Trp-2027-Cys substitution was also used to develop breeding lines resistant to aryloxyphenoxy propanoate (APP) family herbicides such as fluazifop-P and quizalofop-P (Kershner et al., 2012). Commercial hybrids were developed by

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transferring these genes to breeding lines by conventional breeding (Tuinstra and Al-Khatib, 2017). Similarly, in other crops, for example, an altered ALS-gene from common sunflower (*Helianthus annus* L.) was transferred to cultivated sunflower hybrids (Al-Khatib et al., 1998); resistance to SU herbicides was transferred from prickly lettuce to cultivated lettuce variety ID-BR1(Mallory-Smith et al., 1993). Also, IMI and SU resistant genes were identified in wild sunflower (*Helianthus praecox*) by phenotypic screening and these sources can be used transferring trait into cultivated sunflower via conventional breeding (Jacob et al., 2017).

Induced Mutagenesis

Strong selection for domestication-related traits for a long period of time created a genetic bottleneck reduced genetic variation of a population) in cultivated grain sorghum (Casa et al., 2005). Induced mutagenesis has been used as a popular method to enhance diversity in crops and several chemicals and radioactive materials were used as mutagens to create random variations in the DNA of plants (Forster et al., 2012). Using EMS as mutagen herbicide-resistant grain sorghum lines were identified. Specifically, mutations conferring a high level of resistance to ALS-inhibitors (Uriarte et al., 2019), and ACCase-inhibitors (Clement, 2013) have been patented and used to develop herbicide-resistant grain sorghum hybrids. Commercial varieties of Rice (Sudianto et al., 2013) and wheat (Ostlie et al., 2017) resistant to ALS- and ACCase-inhibitors respectively were developed by EMS mutagenesis were released under the trait name Clearfield and CoAxium. Similarly, treatment of three advanced lentil genotypes (LPP 11001, LPP 11100 and LPP 11116) with EMS produced 134 stable mutant plants resistant to SU herbicides (Rizwan et al., 2017). A rice mutant, HTM-N22 (HTM), tolerant to herbicide, imazethapyr was identified from an EMS-mutagenized population of approximately 100,000 plants in M2 generation of an upland rice variety, Nagina 22 (N22) (Shoba et al., 2017).

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Mapping Herbicide Resistance

An important step after identifying a resistant source is, deciphering the mechanism of resistance and map the precise genomic location of the gene(s) responsible for herbicide resistance. If the resistance is bestowed by TSR, such as mutation, increased expression, or amplification of the herbicide target gene, which can be identified by sequencing, RT-PCR, or qPCR, respectively. However, if there is NTSR mechanism involved in herbicide resistance, identifying genes involved is empirical to develop genetic markers which will be helpful to transfer the herbicide resistance into cultivated hybrids. Methods such as bi-parental quantitative trait mapping (QTL) mapping and bulk segregant analysis by RNA-Seq can be used to identify markers linked to herbicide resistance.

Bi-Parental QTL Mapping

This is a widely used trait mapping method in crops. This method uses mapping population such as F_2 individuals or recombinant inbred lines (RILs), generated from the cross between two parents contrasting for the trait of interest (Mackay et al., 2009). Two individuals with contrasting traits will be crossed and advanced to develop segregating (for the parental contrasting trait) population such as F_2 or F_3 can be used. Also, the recombination can be fixed by selfing them for several generations creating a set of RILs. This method is used in grain sorghum to map several biotic and abiotic stress resistance QTLs (Sanchez et al., 2002; Tao et al., 2003; Punnuri et al., 2013). The outcome of this dissertation identified six QTLs responsible for tembotrione resistance using linkage mapping technique (Pandian et al., 2020). A QTL associated with mesotrione and/or isoxazole herbicide resistance was identified in soybean (Bogner et al., 2018).

Bulk Segregant Analysis by RNA-Seq

Bulk segregant analysis (BSA) is a fine-mapping technique that uses biparental populations similar to QTL mapping. The DNA/RNA extracted separately contrasting individuals pooled together based on the phenotype. Traditionally BSA was performed using molecular markers such as simple-sequence repeats (SSRs) but the marker-based method is cumbersome, time-consuming, and not precise. The potential of bulk segregant analysis is further improved utilizing the next-generation sequencing capabilities, termed as bulk segregant RNA-Seq (BSR-Seq). In this method, RNA sequencing will be performed on susceptible and resistant gene pools to identify genomic regions associated with the trait (Liu et al., 2019). This method enables rapid and precise identification of causal genes in an effective and inexpensive way. Unlike Genome-wide association study (GWAS, a mapping is an approach used to identify the genetic basis of traits utilizing the naturally occurring genetic diversity) or QTL mapping, BSR-Seq can precisely identify the genomic location of the trait of focus in a short interval. BSR-Seq has been used to identify *CYP81A9* responsible for nicosulfuron resistance in corn (Liu et al., 2019).

Summary and Objectives of Research

Grain sorghum is one of the most versatile crops, which can produce high yield under limited water and other inputs. Post-emergent grass weed control continues to be a great challenge in grain sorghum due to lack of herbicide options, several grass control herbicides are not registered for use in grain sorghum because of crop injury. Unlike corn, wheat, or soybeans herbicide-resistant technology is not available for grain sorghum. The introduction of herbicide-resistant traits in many crops provided an option to control the wide spectrum of weeds effectively by avoiding crop injury. The development of herbicide-resistant grain sorghum has

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enormous potential to improve weed control. Recently, our group has identified grain sorghum genotypes resistant to mesotrione, tembotrione and chlorsulfuron herbicides known to control a wide spectrum of weeds. The overall goal of this dissertation was using these grain sorghum genotypes, investigate the genetic basis and inheritance of mesotrione, tembotrione and chlorsulfuron resistance in grain sorghum.

Chapter 2: a) Investigate the inheritance and mechanism of resistance to mesotrione

b) Identify genetic loci conferring mesotrione resistance

Chapter 3: a) Investigate the inheritance and mechanism of resistance to tembotrione

b) Identify genetic loci conferring tembotrione resistance

Chapter 4: a) Determine the level of resistance to chlorsulfuron

b) Investigate the inheritance and mechanism of resistance to chlorsulfuron

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 - Mechanism and Inheritance of Mesotrione Resistance

Abstract

Post-emergent (POST) grass weed control continues to be a challenge in grain sorghum production. Mesotrione is an effective option for POST control of broad-spectrum weeds, including grasses, but is not registered for use in sorghum. We identified two sorghum genotypes (G-1 and G-10) with resistance to mesotrione. The objectives of this study were to investigate the mechanism and inheritance of mesotrione resistance. The role of cytochrome P450 (CYP) in metabolizing mesotrione was tested using CYP-inhibitors such as malathion and piperonyl butoxide (PBO). Greenhouse experiments were conducted by pre-treating the G-1, G-10, and a mesotrione-susceptible (S-1) genotype with malathion or PBO followed by mesotrione application. The F_1 progeny were generated by crossing G-1, G-10 separately with S-1, and F_2 progeny were produced by self-pollination of F_1 plants. The F_1 progeny were evaluated in a mesotrione dose-response assay (0 to 8x of mesotrione; 1x=105 g ai ha-1), and the F_2 progeny were screened with 6x of mesotrione to determine the segregation of resistance or susceptibility. The results indicate a significant reduction in biomass accumulation in sorghum plants pre-treated with malathion and PBO, suggesting the involvement of CYPs in the metabolism of mesotrione. The F_1 progeny exhibited the same level of resistance to mesotrione as G-1 or G-10 and the F_2 progeny segregated 3:1(resistance: susceptibility); however, significant phenotype variability was found among the resistant pants of F_2 progeny implying that the mesotrione resistance in sorghum is controlled by a single dominant allele along with possibly other genes with minor effects. We have used bulk segregation analysis combined with RNA-Seq (BSR-seq) for mapping the genomic region associated with mesotrione resistance, leaf punches from thirty resistant and susceptible plants from the F_2 progeny of S-1xG-1 and S-1 x G-10 crosses were

pooled together for RNA extraction. The resistant and susceptible pools were sequenced and analyzed, however the genomic region responsible for mesotrione resistance was not able to map the gene within a smaller interval using the current method.

Introduction

Post-emergent (POST) grass weed control continues to be a challenge in grain sorghum (*Sorghum bicolor* (L.) Moench) production. Herbicide options for POST control grass weeds are very limited due to the susceptibility of grain sorghum grass control herbicides. The 4-hydroxyphenylpyruvate dioxygenase (HPPD)- provides useful and flexible options for both PRE and POST control of both broadleaves and grasses (Ahrens et al., 2013). Cereal crops such as corn (*Zea mays*) and wheat (*Triticum aestivum*) are naturally tolerant to some of the HPPD - inhibitor herbicides, this widened the herbicide options for POST broadleaf and grass weed control and became more potent herbicide tools for effective control of some of the most aggressive weeds (Sutton et al., 2002, Wang et al., 2020). Furthermore, use of these herbicides for both PRE and POST applications provides a flexible option for use in a wide range of herbicide mixtures that enable broad spectrum weed control throughout the season. Use of HPPD-inhibitors and in herbicide rotation is predominantly beneficial in controlling weeds that have evolved resistance to other herbicide modes of action (van Almsick, 2009).

Mesotrione belongs to the triketone family of HPPD-inhibitor that controls many annual broadleaves as well as grasses. Triketone herbicides have been developed based on the compound, leptospermone found in bottlebrush plants (*Callistemon citrinus*; Dayan et al., 2007). HPPD-inhibitors competitively inhibit the HPPD enzyme, a component of the tocopherol biosynthetic pathway (Mitchell et al., 2001). HPPD is required for the conversion of tyrosine to plastoquinone (PQ) which is essential for carotenoid biosynthesis, disruption of carotenoid biosynthesis inhibits pigment development, thereby causing bleached symptoms on the susceptible plants (Norris et al., 1995).

Previous research has demonstrated that some sorghum hybrids exhibited less injury to POST mesotrione applications compared to sensitive sorghum hybrids (Abit and Al-Khatib, 2009). Availability of mesotrione-resistance in grain sorghum will offer more options for weed management which will provide effective weed control during the critical periods of crop growth. Resistance to mesotrione will allow growers to manage resistant weeds that were difficult to control in the past, thereby delaying further spread of resistant weeds. Around 900 sorghum genotypes from the sorghum association panel (SAP), sorghum mini core collection and ethyl methanesulfonate (EMS) mutants were screened for mesotrione resistance under *in vitro* conditions. Two sorghum genotypes G-1 and G-10 with elevated resistance and one genotype S-1 highly susceptible to mesotrione were identified from the SAP. The identified genotypes were evaluated in a mesotrione dose response assay, based on the GR_{50} (dose of mesotrione required for 50% growth reduction); G-1 and G-10 found 26 and 17-fold more resistant to mesotrione compared to S-1. Further, analysis with radio labeled [^{14}C] mesotrione confirmed enhanced metabolism of mesotrione (Pandian et al., unpublished). Cytochrome P450 enzymes (CYPs) rapidly metabolizes HPPD-inhibitor herbicides including mesotrione in corn (Barrett, 1995). CYPs are enzyme superfamily having heme as a co-factor present in all living organisms such as bacteria, fungi, plants, and animals (Werck-Reichhart and Feyereisen, 2000). CYPs are involved in metabolizing xenobiotics which includes herbicides and insecticides through ring-methyl hydroxylation and N-demethylation, and N-dealkylation, HPPD-inhibiting herbicides are primarily metabolized through hydroxylation at the 4-position of the cyclohexanedione (Mitchell et al., 2001).

Most commonly used method is competitive inhibition of CYPs by several chemical compounds such 1-aminobenzo-triazole (ABT), tetcyclacis (TET), piperonyl butoxide (PBO),

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and organophosphate insecticides such as malathion and phorate (Busi et al., 2017). Pre-application of inhibitors before herbicide application will competitively inhibit CYPs and reduce the level of resistance. Each compound inhibits the CYPs through a unique mechanism, malathion an organophosphate insecticide release sulphur species that covalently binds to CYPs results in reduced metabolism of xenobiotics. PBO an insecticide synergist forms a metabolite inhibitory complex with the enzyme (Willoughby et al., 2007). Malathion and PBO are used to determine CYP-based metabolism of different herbicide classes in several crop and weed species such as ryegrass (Christopher et al. 1994), common waterhemp (Oliveira et al., 2018), late watergrass (Yun et al., 2005), wheat (Forthoffer et al., 2001).

We hypothesize that resistance to mesotrione in sorghum G-1 and G-10 is similar to associated with CYP-mediated metabolism corn. The specific objectives of this research were to investigate the inheritance and mechanism of resistance to mesotrione, and to identify genetic loci conferring mesotrione resistance.

Materials and Methods

Plant Material and Growth Conditions

Sorghum genotypes G-1, G-10 resistant and S-1 susceptible to mesotrione from the SAP were used in this study; corn inbred B73 (naturally tolerant to mesotrione) was used for comparison in the CYP-inhibitor study. Seeds were germinated and uniform seedlings at 2-3 leaf stage were transplanted in punnet pots ($6 \times 6 \times 6$ cm) containing commercial potting mixture (ProMix Ultimate; Premier Tech Horticulture, Mississauga, Ontario, Canada) in a greenhouse maintained at 25/20°C day/night, 60 ± 10 percent relative humidity, and 15/9 h day/night photoperiod supplemented with 750 µmol m^{-2} s^{-1} illumination provided with sodium vapor

lamps. Plants were watered as needed and fertilized weekly with MiracleGro (Scotts MiracleGro Products, Inc., Marysville, OH). All the herbicide treatments were given in 3 to 4 leaf stage.

Effect of CYP-Inhibitors on Mesotrione Resistant Genotypes

The experiment was conducted in factorial design with all possible combinations of factors and levels. Resistant genotypes G-1 and G-10 along with susceptible genotype S-1 and naturally tolerant B73 corn inbred were used in this experiment. All the experiments were conducted twice under the same conditions with four replications (four plants) of each genotype for each treatment. Malathion at 2000, or 4000 ai g ha^{-1} or PBO 4500 ai g ha^{-1} along with 0.25% NIS was applied separately on sorghum or corn genotypes one hour prior to mesotrione application. Additionally, soil drenching of 5mM malathion 24 hours after primary application as a booster dose was given only for the malathion treatments. Subsequently, the sorghum genotypes were treated with 105 (1x field recommended dose), 210 or 420 ai g ha^{-1} of mesotrione (Callisto®, Syngenta Corporation, Wilmington, DE, USA; https://www.syngenta.com) with 1% crop oil concentrate. Treatments were applied with a bench-type sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing, MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co, IL) delivering 187 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. The above-ground plant biomass was harvested three weeks after treatment (WAT) and dried in an oven at 60°C for 72 h and the weight of dried biomass was recorded for individual plants and subjected to statistical analysis (described separately in the section below).

Generation and Evaluation of F$_1$ and F$_2$ Progeny

To study the inheritance of mesotrione resistance, crosses were made using G-1 and G-10 and -sensitive S-1 genotypes in a crossing nursery at Kansas State University research farm,

Ashland bottoms, KS and the F_1 seeds were harvested from individual plants. The F_1 progenies of S-1 x G-1 and S-1 x G-10 along with the parents were grown under above said conditions and evaluated in a mesotrione dose-response assay consisting 0, 105, 210, 420 and 840 g ai ha^{-1} of mesotrione doses with 10 to 12 replications per cross. Except for the treatments, same experimental procedure mentioned above was followed. Three F_1 plants per cross survived 420 g ai ha^{-1} grown in the greenhouse and self-pollinated to generate F_2 seeds. Around 150 F_2 progenies of both cross (S1xG1 and S1xG10) were grown under above mentioned greenhouse conditions and treated with 630 g ai ha^{-1} along with parents to study the segregation of mesotrione resistance and BSR-Seq (described in a separate section).

HPPD-Gene Sequencing

The *HPPD* gene from G-1, G-10, and S-1 were sequenced to determine if any target site alterations confer resistance to mesotrione. Leaf tissue (3-4 leaf stage plants) was collected from three plants of each genotype grown in the greenhouse as described above and under similar growth conditions. The genomic DNA was extracted using GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific™, Waltham, Massachusetts, USA) following the manufacturer's instructions. The concentration of the DNA samples was quantified using NanoDrop™ (Thermo Scientific™, Waltham, Massachusetts, USA). The sorghum *HPPD* gene ~2kb was amplified using the primers Sg_HPPD F (5'GACACGATGAATGCCCATGC 3') and Sg_HPPD R (5' AGAGAGATGACAGTACAGTGTTGT 3') designed from Sobic.002G104200.1 in the sorghum reference genome V3.1. (McCormick et al., 2017). Polymerase Chain reaction (PCR) was performed using T100™ Thermal Cycler (Bio-Rad Inc., Hercules, California, USA). The PCR mixture contained 50-80 ng of gDNA, 0.5µM each of forward, reverse primer, and 1x of Promega™ PCR Master Mix (Promega™, Madison,

Wisconsin, USA). PCR amplification was done using the following PCR cycling conditions, initial denaturation: 94 °C for 5 min, followed by 35 cycles of denaturation: 94 °C for the 30 s, annealing 60 °C for 45 s and extension: 72 °C for 45 s and final extension: 72 °C 7 mins. The PCR products were analyzed in 1.5% agarose gel to confirm the targeted amplicon size and purified using GeneJET™ PCR Purification Kit (Thermo scientific™, Waltham, Massachusetts, USA) The PCR purified samples were sequenced by Sanger sequencing service provided by GENEWIZ, LLC., New Jersey, South Plainfield, USA. The sequences were aligned using Clustal Omega multiple sequence alignment tool (EMBL-EBI) to check for the mutations.

Bulk Segregant Analysis by RNA-Seq (BSR-Seq)

The F_2 progenies mentioned in the above experiment were individually numbered and leaf samples were collected in microcentrifuge tubes using leaf punching machine before herbicide treatment. Thirty resistant and susceptible plants were selected from F_2 progenies of both cross (S-1xG-1 and S-1xG-10). The leaf punches (three punch per plant) of thirty plants were pooled into four groups (resistant and susceptible plant pools of two crosses) and RNA was extracted using Trizol^{TM} (Thermo Fisher Scientific™, Waltham, Massachusetts, USA) based on the manufacturer guidelines. Libraries were constructed using TruSeq stranded mRNA library preparation kit and sequenced using Illumina Next -seq resulting in150bp PE reads at K-state Integrated Genomics Facility. Adapter regions and low-quality regions from the raw sequence reads were trimmed using trimmomatic 0.32 (Bolger et al., 2014). The trimmed reads were mapped to sorghum reference genome v3 from National Centre for Biotechnology Information (PRJNA13876) using spliced transcripts alignment to a reference (STAR) software (Dobin et al., 2013). Sequence variants (SNPs) were identified by the GATK pipeline and filtered to identify SNPs for BSR-Seq (McKenna et al., 2010). The SNPs were used for BSR-Seq analysis to

identify the chromosomal region associated with mesotrione using an in-house pipeline (Liu et al., 2012). The schematic representation of the experiment was given in Figure 1
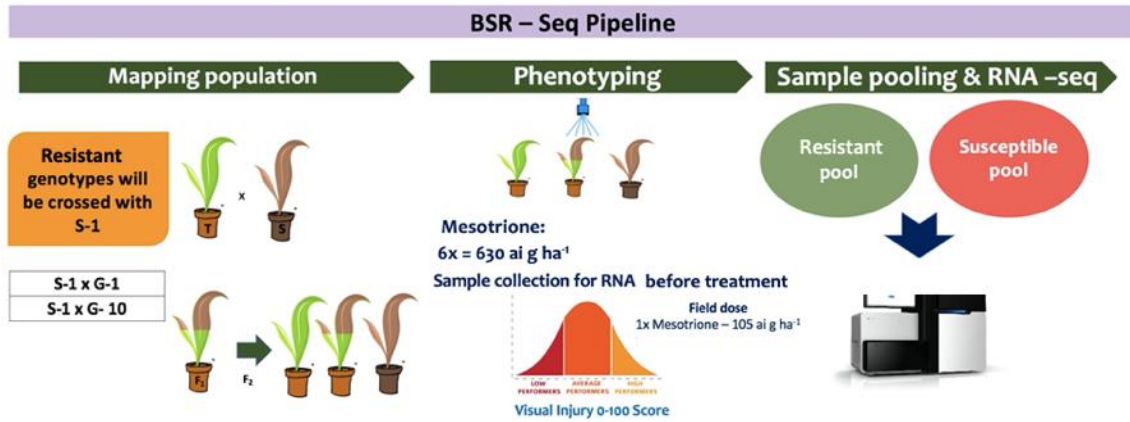
Figure 2.1 Schematic representation of bulk segregant RNA-Seq (BSR-Seq) experiment

Statistical Analysis

$$\text{Dry biomass (\% of non-treated)} = \frac{\text{Biomass of individual plant (g)}}{\text{Average biomass of the non-treated plants of the genotype (g)}} \times 100$$

Mesotrione dose-response data expressed as dry biomass (% of non-treated) or percent injury were subjected to non-linear regression analysis using a three or four-parameter log-logistic model using a 'drc' (Ritz et al., 2005) package in R (Development Core Team, 2013) following (Knezevic et al., 2007; Shyam et al., 2019) to estimate GR_{50} (dose required for 50% growth reduction) or ID_{50} (dose required for 50% visual injury). A "Lack-of-fit" test was performed using the "model fit" function of 'drc' to assess the fit of data to various regression models. Differences between the estimated GR_{50} values were tested with each other by t-test using the "compParm" function in the 'drc' package. The dose-response curves were generated using the 'plot' function in the 'drc' package. Analysis of variance (ANOVA) was performed following Fisher's LSD test to separate means and significance at $p \leq 0.05$ using the 'agricole'

package in R (de Mendiburu, 2014). The plots were generated using the 'R' package 'ggplot2' (Wickham and Wickham, 2007). A Chi-square ($\chi 2$) goodness of fit test (Cochran, 1952) was used to fit to a single dominant gene model by comparing the observed and expected segregation frequencies of mesotrione resistant or -susceptible plants.

Results

Mechanism of Mesotrione Resistance

No mutations were found in the coding region of the *HPPD* gene sequenced from G-1, G-10, and S-1 (Figure S2.1), suggesting that no target site alterations confer resistance to mesotrione in the resistant genotypes. In response to malathion or PBO followed by mesotrione treatments, G-1 and G-10 exhibited significant biomass reduction compared to those that were treated only with mesotrione. Malathion or PBO without mesotrione treatment did not affect the sorghum genotypes tested (Figures 2.2 and 2.3). Based on the percent dry biomass reduction, the corn inbred B73 did not show any significant biomass reduction at any dose of mesotrione application (Figure 2.2); however, pre-treatment with 4000 g ai ha^{-1} malathion followed by all doses of mesotrione caused a 40 to 80% reduction in biomass (Figure 2.2). The genotype G-1 and G-10 treated only with mesotrione did not show significant biomass reduction compared to non-treated; whereas, malathion followed by 105, 210, and 420 g ai ha-1 showed more than 60% reduction in biomass compared to mesotrione only treatments. There is a significant difference in biomass accumulation when treated with 2000 or 4000 g ai ha-1 of malathion (Figure 2.2). The susceptible genotype S-1, showed significant growth reduction at all doses of mesotrione or when pretreated with malathion (Figure 2.2).
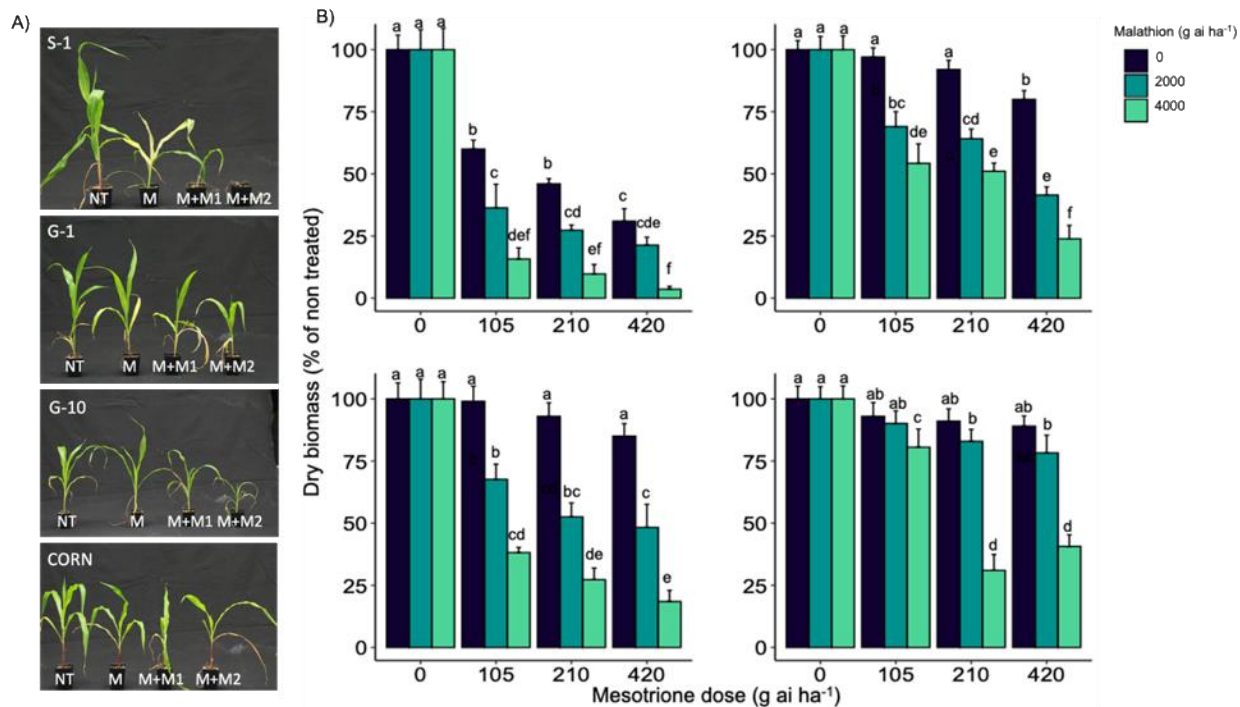
Figure 2.2 (A) The response of S-1 (susceptible), G-1, G-10 (resistant) and, corn to field recommended dose mesotrione (105 g ai ha^{-1}) (M) or malathion treatments (M1: 2000 g ai ha^{-1}; M2: 4000 g ai ha^{-1}) followed by field recommended dose mesotrione; NT: Non-treated; and (B) Aboveground dry biomass of sorghum genotypes when pre-treated with malathion or different doses of mesotrione. The error bars represent the standard error (n=8); different alphabets indicate a significant difference between treatments ($p \leq 0.05$)

Corn inbred, B73 didn't show any significant reduction for all the doses of mesotrione only treatments; but exhibited significant biomass reduction in response to PBO followed by 420 g ai ha-1 of mesotrione treatment (Figures 2.3). Both G-1 and G-10 genotypes showed a significant biomass reduction up to 75% when pretreated with PBO followed by different doses

of mesotrione. The S-1 exhibited gradual reduction in biomass with increasing dose of mesotrione, pre-treatment with PBO further contributed for growth reduction (Figure 2.3).
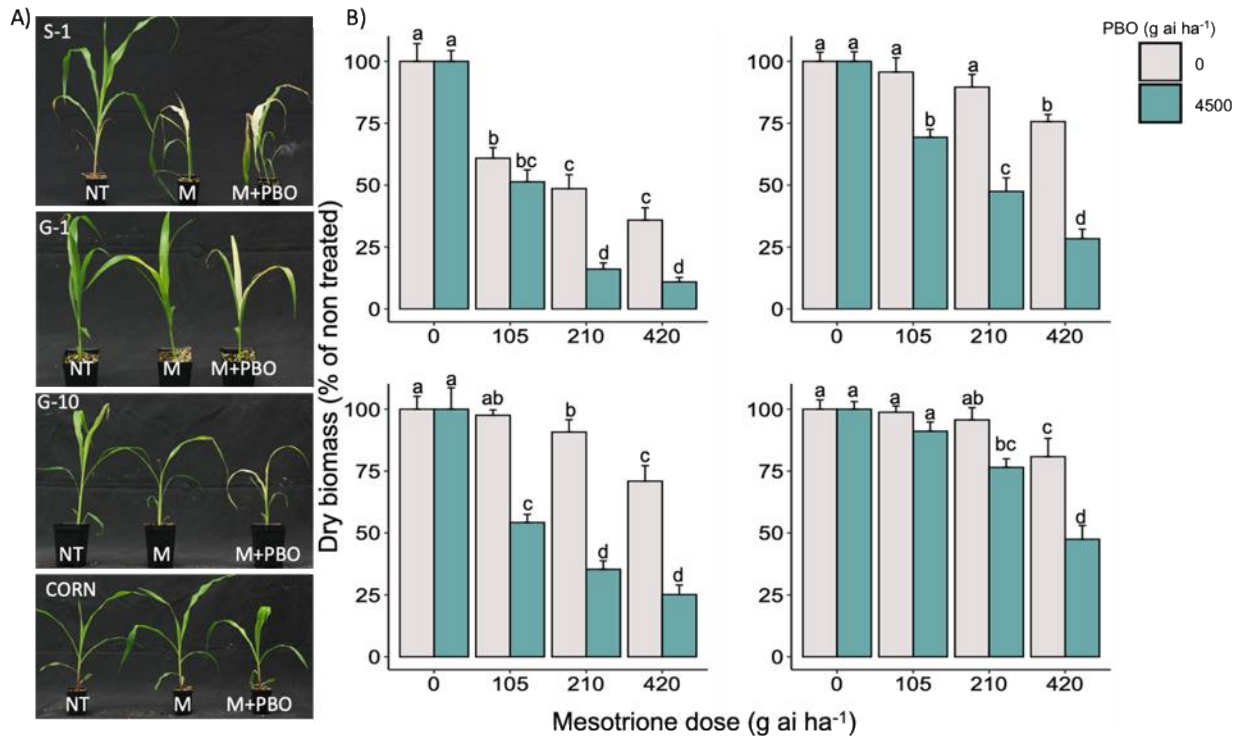
Figure 2.3 (A) The response of S-1 (susceptible), G-1, G-10 (resistant) and, corn to field recommended dose of mesotrione (105 g ai ha^{-1}) (M) or Piperonyl butoxide (PBO) treatment (M1: 4500 g ai ha^{-1}) followed by field recommended dose mesotrione; NT: Non-treated; and (B) Aboveground dry biomass of sorghum genotypes when pre-treated with PBO or different doses of mesotrione. The error bars represent the standard error (n=8); different alphabets indicate a significant difference between treatments ($p \leq 0.05$)

Inheritance of Mesotrione Resistance

The F_1 progeny of S-1 x G-1 and S-1 x G-10 exhibited the same level of resistance to mesotrione as G-1 or G-10. The GR_{50} of S-1 x G-1 and S-1 x G-10 were estimated as 1203 and

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1336, respectively, similar to the GR_{50} of G-1 (950) and G-10 (848) (Figure 2.4), suggesting that mesotrione resistance in G-1 and G-10 is controlled by a dominant gene (Table 2.1). The F_2 progeny segregated 3:1(resistance: susceptibility) following the single gene model of inheritance; however, significant phenotype variability was found among the resistant pants of F_2 progeny implying that the mesotrione resistance in sorghum is controlled by a single dominant allele along with possibly other genes with minor effects. The segregation experiments were conducted three times for each cross and observed similar trends (Table 2.2).

Table 2.1 Regression parameters describing the response of sorghum genotypes and their F_1 progeny to mesotrione under greenhouse conditions (GR_{50}: dose required for 50% growth reduction; SE: standard error; RI: Resistance index R/S; Resistant/Susceptible)

Genotype	GR_{50} (SE)	RI (R/S)
S-1	70 (8)	1.0
S-1 x G-1	1203 (155) **	17
S-1 x G-10	1336 (211) **	16
G-1	950 (33) **	13
G-10	848 (60) **	12

Significantly different from S-1 at *$p \leq 0.05$ **$p \leq 0.01$
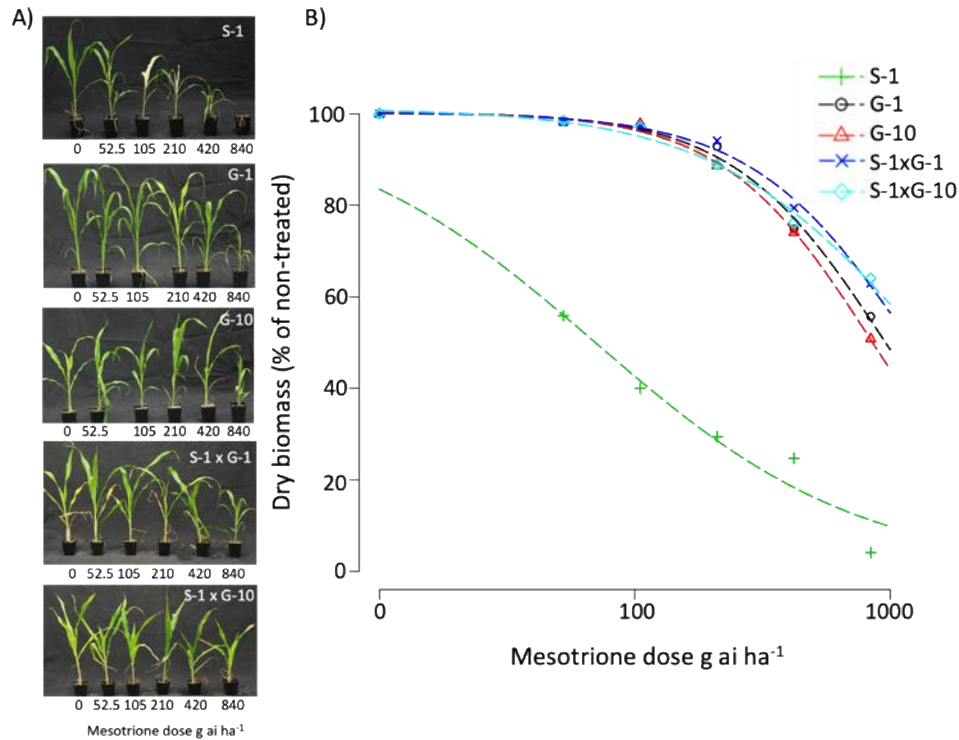
Figure 2.4 A) Mesotrione dose-response curves representing the above ground dry biomass of S-1 (susceptible), F_1 (S-1 x G-1), F_1 (S-1 x G-10) and G-1, G-10 (resistant) using the four-parameter log-logistic model. B) Response of parents S-1 (susceptible), G-1, G-10 (resistant), F_1 (S-1 x G-1), F_1 (S-1 x G-10) to different doses of mesotrione

Table 2.2 Chi-Square analysis of the segregation of mesotrione resistant (R) and- susceptible (S) phenotypes in sorghum F_2 progeny at three weeks after treatment

Cross	Experiment	Total	R	S	P-value
	1	170	133	37	0.373
S-1 x G-1	2	150	116	34	0.569
	3	280	210	70	0.167
	Combined	600	459	141	0.396

S-1 x G-10	1	200	155	45	0.414
	2	200	160	40	0.102
	3	150	115	35	0.702
	Combined	550	430	120	0.093

Mapping Mesotrione Resistance

A F_2 population of S-1 x G-10 was used to map the genomic region associated with mesotrione resistance in grain sorghum; a total of ~16Gb raw sequence data were obtained through RNA-seq. The SNPs were then identified between resistant and sensitive pools, a total of 38194 SNPs was obtained and analyzed, however no significant genomic region responsible for mesotrione resistance was detected in the analysis. Further, similar experiment was conducted in two independent F_2 populations, S-1x G-1 and S-1 x G-10 (Figure 2.5). Approximately 8Gb of raw bases were obtained from the resistant and sensitive bulked samples and a total of 33858 and 31692 SNPs were identified from S-1x G-1 and S-1 x G-10, respectively. A region associated with mesotrione resistance based BSR-Seq was located in sorghum chromosome 1 between 18.4-23.9 Mb in S-1x G-1, but no regions were identified in S-1 x G-10 (Figure 2.5). Further experiments were conducted to identify and validate region found in S-1x G-1 proved that it was a spurious association, the identified SNPs did not co-segregate with the phenotype (resistance or susceptibility). Interestingly some of the SNPs identified in the F_2 population were not found in either of the parents.
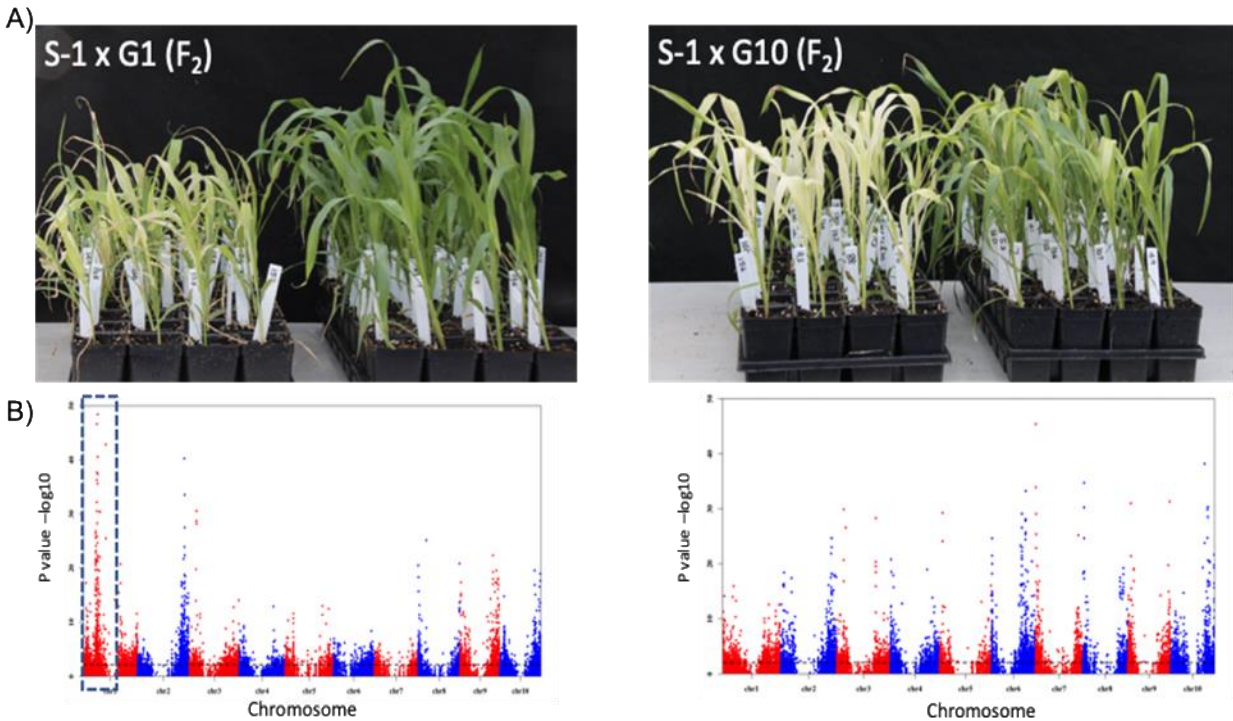
Figure 2.5 (A) Segregants showing contrasting response for mesotrione treatment at three weeks after treatment from F₂ progeny of S-1 xG-1 and S-1 x G-10 used for BSR-Seq B) Manhattan plot obtained by BSR-Seq analysis

Discussion

Base alterations in *HPPD* gene sequence was not found between the resistant (G-1 and G-10) or susceptible (S-1) genotype (Figure S2.1). Therefore, the natural resistance to mesotrione in G-1 and G-10 not conferred by any alteration to the molecular target of this herbicide, i.e. *HPPD* gene. Mutations in the *HPPD* gene that confer resistance to HPPD-inhibitors were not found in plants (Lu et al., 2020). However, soybean varieties resistant to HPPD-inhibitors were developed through transgenic technology by inserting an insensitive *HPPD* gene from *Pseudomonas fluorescens* (Dreesen et al., 2018). Experiments with [^{14}C] mesotrione suggests rapid metabolism of mesotrione in G-1 and G-10 (Pandian et al.

unpublished). Similarly, metabolism of mesotrione in sorghum hybrids has been reported previously (Abit and Al-Khatib 2009). The CYP enzymes metabolize mesotrione into 4-hydroxy-mesotrione (Alferness and Wiebe, 2002), and rapid metabolism of mesotrione has been previously reported in corn (Williams et al., 2008), Palmer amaranth (*Amarannthus palmeri*; Nakka et al., 2017) and common waterhemp (*Amaranthus tuberculatus*; Ma et al., 2013; Kaundun et al., 2017).

In this research, the genotypes, G-1, and G-10 exhibited significant biomass reduction in response to pre-treatment with CYP-inhibitors, malathion, or PBO followed by mesotrione application, suggesting possible involvement of CYPs in mesotrione metabolism (Figures 2.2 and 2.3). The use of malathion followed by mesotrione showed >20% more biomass reduction compared to mesotrione alone treatments in a mesotrione-resistant waterhemp population and corn. In corn, multiple CYPs located on *nsf1* locus were found to be involved in metabolism of tembotrione, mesotrione, as well as other herbicides (Williams and Pataky, 2010). Several *CYP* genes that metabolize mesotrione have been patented (Hawkes et al., 2012; Saika et al., 2018; Andrews et al., 2011). An RNA-seq study of mesotrione-treated common waterhemp populations revealed several CYPs that were upregulated in mesotrione-resistant populations compared to sensitive populations, i.e., CYP*72A15* and 10 other CYPs were upregulated at three and 24 hours after treatment (Kohlhase et al., 2019).

Based on the response of F_1 progeny (S-1 x G-1 and S-1 x G-10) to mesotrione treatment, we found that the mesotrione resistance in G-1 and G-10 is a dominant trait (Figures 2.4; Table 2.1). Furthermore, analysis of F_2 data indicated that this resistance is controlled by a single gene along with minor alleles (Table 2.2). A dominant-monogenic inheritance of mesotrione resistance has been reported in rice (*Oryza sativa*) (Lee et al., 2018), in contrast, dominant-

polygenic inheritance of mesotrione has been reported in corn (Williams et al., 2008). Also, several common waterhemp populations across the US Midwest resistant to mesotrione have been reported to be inherited by a partially dominant multiple genes (Huffman et al., 2015; Kohlhase et al., 2018; Oliveira et al., 2018).

BSR-Seq was used to map the mesotrione resistance using biparental F_2 populations derived from crossing S-1 with G-1 and G-10; However, we were not able to map the genomic regions associated with mesotrione resistance using this technique. The BSR-Seq has been used to successfully identify several traits such as brown mid rib (Tang et al., 2013), leaf glossiness (Li et al., 2013). Recently a *CYP81A9* responsible for nicosulfuron resistance in corn has been identified using this technique (Liu et al., 2019). BSR-Seq is more effective in mapping the traits that are controlled by a single major gene, mostly a mutated or altered variant of the gene that confers contrasting traits compared to the wild type gene (Liu et al., 2012). Based on the previous studies non-target site resistance mechanism of herbicide resistance in weeds and crops seems to be complex. A recent report, on RNA-seq study in common waterhemp population resistant to 2,4-D and mesotrione revealed that CYP genes and several other genes involved in resistance were co-localized and co-expressed in clusters and expression of these genes probably mediated by cis-acting genetic variation (Giacomini et al., 2020). Some of the SNP markers identified through BSR-Seq in S-1 x G-1 population were not found in the parents, this could be due to the residual heterozygosity or a possible mixture in the parents; nonetheless the parents didn't exhibit variation in the mesotrione resistance or any another visible phenotypic traits. A modified approach or a QTL mapping method with a standard mapping population need to be deployed to map the genomic regions associated with mesotrione resistance.

Conclusions

In conclusion, sorghum genotypes (G-1 and G-10) confer high level resistance to mesotrione possibly by CYP-mediated metabolism of mesotrione. Genetic analyses of F_1 and F_2 progeny demonstrated that the resistance is a dominant trait with monogenic inheritance, although effects of several minor genes have been identified. A monogenic trait can be precisely transferred to cultivated hybrids or breeding lines using marker assisted breeding. Furthermore, future research needs to be focused on the development of genetic markers linked to the trait and test the hybrid performance of elite breeding lines incorporated with the resistant trait.

Chapter 3 - Mechanism and Inheritance of Tembotrione Resistance

Abstract

The 4- hydroxyphenylpyruvate dioxygenase-inhibitor herbicides (e.g., mesotrione or tembotrione) can control broad-spectrum of weeds including grasses, which, however, is not registered for postemergence application in sorghum due to crop injury. In our previous research we have identified two tembotrione-resistant sorghum genotypes (G-200, G-350) and one susceptible genotype (S-1) by screening 317 sorghum lines from a sorghum association panel (SAP), the genotypes G-200 and G-350 exhibited 10- and 7-fold more resistance to tembotrione compared to S-1, respectively. The objectives of this research were to investigate the inheritance and mechanism of resistance to tembotrione, and to identify genetic loci conferring tembotrione resistance. To understand the inheritance of tembotrione-resistant trait, reciprocal crosses were performed using S-1 and G-200 or G-350 to generate F_1 and F_2 progeny. The F_1 and F_2 progeny were assessed for their response to tembotrione treatment. Genetic analyses of the F_1 and F_2 progeny demonstrated that the tembotrione resistance in G-200 and G-350 is a partially dominant polygenic trait. Furthermore, cytochrome P450 (CYP)-inhibitor assay using malathion and piperonyl butoxide suggested possible CYP-mediated metabolism of tembotrione in G-200 and G-350. Genotype-by-sequencing based quantitative trait loci (QTL) mapping revealed QTLs associated with tembotrione resistance in G-200 and G-350 genotypes Overall, the genotypes G-200 and G-350 confer a high level of metabolic resistance to tembotrione and controlled by a polygenic trait. There is an enormous potential to introgress the tembotrione resistance into breeding lines to develop agronomically desirable sorghum hybrids.

Introduction

Grain sorghum [*Sorghum bicolor* (L.) Moench ssp. *bicolor*] is one of the most versatile crops with multiple uses, including for food, feed, and fuel (Ciampitti et al., 2019). Sorghum performs better than corn (*Zea mays*) under rainfed and low input conditions (Valadabad et al., 2000; Staggenborg et al., 2008). The US is the largest producer of grain sorghum; and almost half of the US grain sorghum is produced in Kansas (USDA-NASS, 2019). Sorghum is primarily grown for cattle feed and ethanol production in the US, whereas it is a staple food for millions of people in Africa, India, and South America (Taylor et al., 2006; Dahlberg et al., 2012). Weed infestation, specifically grass weed species pose a major problem in sorghum production and can reduce the crop yields up to 60%, if left uncontrolled (Thompson et al., 2019; Dille et al., 2020). A wide range of postemergence (POST) herbicides are available to control broad-leaved weeds in sorghum. However, herbicide options for POST control of grasses are limited due to the susceptibility of sorghum to commonly used grass control herbicides (Thompson et al., 2019).

The 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors (e.g., mesotrione or tembotrione) are widely used to control broad-spectrum of weeds including grasses in corn because it can effectively metabolize HPPD-inhibitors (Williams and Pataky, 2010). However, these herbicides are not registered as POST in sorghum due to crop injury. Although these herbicides are widely used, till date only two weed species i.e., Palmer amaranth (*Amaranthus palmeri*) and common waterhemp (*Amaranthus tuberculatus*), have been documented to have evolved resistance to HPPD-inhibitors (Heap, 2020). These herbicides inhibit the HPPD enzyme, which is important for the conversion of 4-hydroxyphenyl pyruvate to homogentisate, an intermediate in plastoquinone and tocopherol biosynthesis pathway in plants (Lee et al., 1998). Plastoquinone is essential for the carotenoid biosynthesis, which protects the chlorophyll by

absorbing excited electrons released during photosynthesis. Depletion of carotenoids causes damage to the chlorophyll by photo-oxidation resulting in bleaching followed by necrosis and plant death (Dankov et al., 2009). HPPD-inhibitors include four chemical families isoxazole, pyrazole, pyrazolone, and triketones, and were introduced in the 1980s for weed control (van Almsick, 2009).

Herbicide resistance in plants can be conferred by two major mechanisms: a) target-site resistance (TSR): mutation(s) in the herbicide target gene leading to the reduced affinity of the target enzyme for herbicide binding or due to increased expression of target enzyme; and b) non-target site resistance (NTSR): increased metabolism or reduced absorption/translocation of herbicides (Gaines et al., 2020). Metabolism of HPPD-inhibitors by cytochrome P450 enzyme (CYPs) activity is the most common mechanism of resistance found in crops as well as weeds (Ahrens et al., 2013). Nonetheless, increased expression of *HPPD*-gene has also been reported in some biotypes of Palmer amaranth (Nakka et al., 2017). Recently, a modified *HPPD*-gene from *Pseudomonas fluorescens* and *Avena sativa* which is insensitive to HPPD-inhibitors was used to develop transgenic soybeans (*Glycine max*) resistant to HPPD-inhibitors by Bayer Crop Science (Matringe et al., 2005; Dreesen et al., 2018) and Syngenta (Hawkes et al., 2016), respectively. Dupont-Pioneer used an insensitive shuffled variant of corn *HPPD*-gene that confers a high level of resistance to HPPD-inhibitors in soybean (Siehl et al., 2014). CYPs are one of the largest enzyme families involved in xenobiotic metabolism in microorganisms, insects, plants, and humans imparting resistance, respectively, to antibiotics, insecticide, and herbicide, and drugs (Pandian et al., 2020). The activity of CYPs can be inhibited using several chemical compounds; 1-aminobenzo-triazole (ABT), tetcyclacis (TET), piperonyl butoxide (PBO), tridiphane, and organophosphate insecticides such as malathion, phorate (Siminszky, 2006; Busi et al., 2017).

Treatment with CYP-inhibitors before herbicide application will competitively reduce the CYP activity resulting in decreased metabolism of herbicide, thereby, reducing the level of resistance (Siminszky, 2006). CYP-inhibitors have been widely used to determine metabolic resistance to herbicides in several plant species. Specifically, malathion and PBO were used to demonstrate the inhibition of CYP activity and the reversal of crop tolerance to HPPD-inhibitors in corn (Ma et al., 2013; Oliveira et al., 2018). Development of sorghum hybrids resistant to HPPD-inhibitors will provide POST herbicide options to control grass weeds (Thompson et al., 2019). Tembotrione is a triketone herbicide which has broad-spectrum activity including grass weeds. Furthermore, the efficacy of tembotrione is high on grass weeds compared to other triketones (Ahrens et al., 2013). Mesotrione, a triketone herbicide similar to tembotrione is registered for preemergence (PRE) use in sorghum but not as POST; however, tembotrione is not registered for PRE or POST usage in sorghum. In our previous research we have identified two tembotrione-resistant sorghum genotypes (G-200, G-350) and one susceptible genotype (S-1) by screening 317 sorghum lines from a sorghum association panel (SAP), the genotypes G-200 and G-350 exhibited 10- and 7-fold more resistance to tembotrione compared to S-1, respectively (Pandian et al. 2020).We hypothesize that mechanism of resistance in G-200 and G-350 is associated with CYP-mediated metabolism similar to corn. The specific objectives of this research were to investigate the inheritance and mechanism of resistance to tembotrione, and to identify genetic loci conferring tembotrione resistance.

Materials and Methods

Plant Materials

Sorghum genotypes G-200, G-350 resistant and S-1 susceptible to tembotrione from the SAP were used in this study. A corn inbred B73 (naturally resistant to tembotrione) were also used for comparison.

Generation and Evaluation of F_1 and F_2 Progeny

To study the inheritance and mapping of tembotrione resistance, direct and reciprocal crosses were performed using tembotrione-resistant (G-200 and G-350) and -susceptible (S-1) genotypes in a crossing nursery at KSU research farm, Ashland Bottoms, KS. The crosses were made using the plastic bag method (Rakshit and Bellundagi, 2019). The F_1 seeds were harvested from individual plants.

The seeds of F_1 progeny from S-1 x G-200 and S-1 x G-350 were planted in square pots (15 x 15 x 15 cm) filled with a potting mixture (ProMix Ultimate, Premier Tech Horticulture, Mississauga, Ontario, Canada). The seedlings at 2-3 leaf stage (Roozeboom and Prasad, 2019), were transplanted in square pots (6 x 6 x 6 cm) and grown in a greenhouse maintained at 25/20℃, 15/9 h day/night photoperiod with a photosynthetic photon flux density of 750 μmol m^{-2} s^{-1} and relative humidity of 60 ± 10 percent. The plants were fertilized (Miracle GRO® All-purpose plant food, ScottsMiracle-Gro, Marysville, Ohio, USA) as needed. The sorghum seedlings at 4-5 leaf-stage (Roozeboom and Prasad, 2019) were treated with tembotrione (Laudis®, Bayer Crop Science, St.Louis, MO USA; https://www.cropscience.bayer.com) at 0, 23, 46, 92, 184, 368 g ai ha^{-1} with 0.25% methylated soy oil (Destiny®, WinField® United, https://www.winfieldunited.com/) using a bench-top track spray chamber (Generation III, De Vries Manufacturing, Hollandale, Minnesota, USA) equipped with a single flat-fan nozzle

(80015LP TeeJet tip, Spraying Systems Co., Wheaton, Illinois, USA) delivering 187 L ha^{-1}. Each plant was considered as an experimental unit, eight replications were used for each genotype. The response of sorghum genotypes to tembotrione treatment was evaluated by visual injury rating as described above (Abit et al., 2009). A total of 10 to 12 F_1 plants from each cross (S-1 x 200 and S-1 x 350) per dose were treated and the true F_1 plants were differentiated from the selfed plants by their response to tembotrione, hence the susceptible (S-1) was used as female parent, the plants that were derived by selfing would be killed at field recommend dose or higher. Additionally, the selfed plants survived at low doses were identified by parental phenotype/ vigor and were discarded. Each plant was considered as an experimental unit with 8 replications per dose. The above-ground plant biomass was harvested 3 weeks after treatment (WAT) and dried in an oven at 60°C for 72 h and the weight of dried biomass was recorded for individual plants.

Three F_1 plants per cross that exhibited resistance to tembotrione, were selected to generate F_2 seeds by self-pollination. The F_2 progeny were evaluated under greenhouse conditions with a single dose of tembotrione to determine the segregation of resistant and susceptible plants. Approximately 150 seedlings from a single F_2 family (up to two F_2 families) along with the parents were raised in the greenhouse (as described above under the same growth conditions). The seedlings (4-5 leaf stage) were treated with 276 g ai ha^{-1} of tembotrione following the same procedure as described above. The response of F_2 plants was assessed by visual injury rating (as described above) at two and three WAT (Abit et al., 2009). Further, plants were grouped as highly injured/dead (susceptible) or minor/no symptoms (resistant) at four WAT in comparison with the parental genotypes. In addition, total leaf chlorophyll index was estimated in parents and F_2 progeny on three and four WAT. Chlorophyll index was measured at three different spots on the leaf blade along the length of the youngest fully opened leaf using a

self-calibrating soil plant analysis development (SPAD) chlorophyll meter (Konica Minolta SPAD 502 Chlorophyll Meter, Chiyoda City, Tokyo, Japan). The chlorophyll index obtained from the three spots were averaged and considered as a total leaf chlorophyll index. However, the leaf chlorophyll index was recorded from the second run of S-1 x G-200 F_2 evaluation which was used for the quantitative trait loci (QTL) mapping experiment (described later in a separate section).

HPPD-Gene Sequencing

The *HPPD* gene from G-200, G-350, and S-1 were sequenced to determine if any target site alterations confer resistance to tembotrione. Leaf tissue (3-4 leaf stage plants) was collected from three plants of each genotype grown in the greenhouse as described above and under similar growth conditions. The genomic DNA was extracted using GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific™, Waltham, Massachusetts, USA) following the manufacturer's instructions. The concentration of the DNA samples were quantified using NanoDrop™ (Thermo Scientific™, Waltham, Massachusetts, USA). The sorghum *HPPD* gene ~2kb was amplified using the primers Sg_HPPD F (5'GACACGATGAATGCCCATGC 3') and Sg_HPPD R (5' AGAGAGATGACAGTACAGTGTTGT 3') designed from Sobic.002G104200.1 in the sorghum reference genome V3.1.1 (McCormick et al., 2017). Polymerase Chain reaction (PCR) was performed using T100™ Thermal Cycler (Bio-Rad Inc., Hercules, California, USA). The PCR mixture contained 50-80 ng of gDNA, 0.5µM each of forward, reverse primer, and 1x of GoTaq® G2 Green Master Mix (Promega™, Madison, Wisconsin, USA). PCR amplification was done using the following PCR cycling conditions, initial denaturation: 94 °C for 5 min, followed by 35 cycles of denaturation: 94 °C for the 30 s, annealing 60 °C for 45 s and extension: 72 °C for 45 s and final extension: 72 °C 7 mins. The

PCR products were analyzed in 1.5% agarose gel to confirm the targeted amplicon size and purified using GeneJET™ PCR Purification Kit (Thermo scientific™, Waltham, Massachusetts, USA) The PCR purified samples were sequenced by Sanger sequencing service provided by GENEWIZ, LLC., New Jersey, South Plainfield, USA. The sequences were aligned using Clustal Omega multiple sequence alignment tool (EMBL-EBI) to check for the mutations.

CYP-Inhibitor Study

To determine if CYP-mediated metabolism of tembotrione confers resistance in G-200 and G-350 genotypes, experiments were conducted using two CYP-inhibitors, malathion and PBO. The sorghum genotypes G-200, G-350, S-1, along with Pioneer 84G62 and a corn genotype B73, were grown in the greenhouse (as described above and under similar growth conditions). Malathion (Spectracide® malathion insect spray concentrate, Spectrum Brands, Inc, https://www.spectracide.com/) at 0, 2000, and 4000 g ai ha^{-1} or PBO (Fisher Scientific, Waltham, Massachusetts, USA) at 4500 g ai ha^{-1} along with 0.25% non-ionic surfactant (NIS) was applied one hour prior to tembotrione treatment. Soil drenching of 5mM malathion 24 hours after primary application as a booster dose was given only for the malathion treatments. Tembotrione was applied at 0, 92, 184, and 368 g ai ha^{-1} with 0.25% methylated soy oil. All the treatments were arranged in a factorial design. The same procedure, as mentioned in the above tembotrione dose-response assay, was followed for chemical treatments (malathion, PBO, and tembotrione) and data collection.

Genotyping-by-Sequencing (GBS)

Approximately 150 plants of a single F_2 family derived from S-1 x G-200 along with parents, were grown in the greenhouse as described above and under the same growth conditions. An equal amount (two 2 cm leaf bits; ~150 mg) of leaf tissue was collected from all plants in 96-

deep well plates. One 3.2 mm stainless steel bead was added to each well and the leaf tissue was ground for 3 min at 20 cycles per sec to obtain fine powder in a Mixer Mill (Retsch GmbH, Haan, North Rhine-Westphalia, Germany). Genomic DNA was extracted using the Cetyltrimethylammonium bromide (CTAB) method (Bai et al., 1999) with minor modifications. The DNA concentration in the extracted samples was quantified by FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Baden-Württemberg, Germany) using a Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Grand Island, New York, USA). Each sample was normalized to contain 10 ng/µl DNA using QIAgility Liquid Handling System (Qiagen, Germantown, Maryland, USA) for library construction. Approximately 150 ng of genomic DNA of each sample was used to construct a library following the tGBS® protocol (Ott et al., 2017) with modifications, and the DNA library was sequenced on the HiseqX 10 platform at Novogene Corporation Inc., Sacramento, California, USA. Sequencing reads were trimmed and de-barcoded using the pipeline described in the previous tGBS® study (Ott et al., 2017). Clean reads of each sample were aligned to *Sorghum bicolor* genome (Genbank accession GCA_000003195.3) (Paterson et al., 2009) using BWA v0.7.12-r1039 (Li and Durbin, 2009) and unique mapped reads were retained for variant discovery using HaplotypeCaller in GATK v4.1 (McKenna et al., 2010). GATK SelectVariants with parameters (-select-type SNP --restrict-alleles-to BIALLELIC -select 'QD ≥ 10.0' -select 'DP ≥ 200.0') was applied to filter variants. The SNPs were converted to ABH format ("A" represents resistant parent allele, "B" represents susceptible allele and "H" represents heterozygous allele) and only polymorphic SNPs between the R and S genotypes were retained using a custom-made Microsoft-Excel template. The filtered polymorphic SNPs were used for the construction of a linkage map and QTL analysis.

Linkage and QTL Mapping

The linkage map was obtained using QTL IciMapping (version 4.5). The grouping and ordering of 606 polymorphic SNP markers were carried out using the regression mapping algorithm RECORD (REcombination Counting and ORDering) based on recombination events between adjacent markers. Further, rippling was done for fine-tuning of the ordered markers on their respective chromosomes by the sum of adjacent recombination fractions (SARF) algorithm with a default window size. The QTL mapping for recovery (RE) and visual injury (VI) was performed using the inclusive composite interval mapping (ICIM) method with additive assumption was performed using the QTL IciMapping (version 4.5) (Meng et al., 2015). The logarithm of the odds (LOD) significance thresholds ($P < 0.05$) were determined by running 1000 permutations (Churchill and Doerge, 1994). Previously reported QTLs for similar regions were obtained from sorghum QTL Atlas (Mace et al., 2019). The QTLs (q) were named based on the trait abbreviation followed by the chromosome number.

Statistical Analysis

Dry biomass (% of non-treated) was calculated following the formula:

$$\text{Dry biomass (\% of non-treated)} = \frac{\text{Biomass of individual plant (g)}}{\text{Average biomass of the non-treated plants of the genotype (g)}} \times 100$$

Tembotrione dose-response data expressed as dry biomass (% of non-treated) or percent injury were subjected to non-linear regression analysis using a three or four-parameter log-logistic model using a 'drc' (Ritz et al., 2005) package in R (Development Core Team, 2013) following (Knezevic et al., 2007; Shyam et al., 2019) to estimate GR_{50} (dose required for 50% growth reduction) or ID_{50} (dose required for 50% visual injury). A "Lack-of-fit" test was

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performed using the "model fit" function of 'drc' to assess the fit of data to various regression models. Differences between the estimated GR_{50} or ID_{50} values were tested with each other by t-test using the "compParm" function in the 'drc' package. The dose-response curves were generated using the 'plot' function in the 'drc' package.Analysis of variance (ANOVA) was performed following Fisher's LSD test to separate means and significance at $p \leq 0.05$ using the 'agricole' package in R (de Mendiburu, 2014). The plots were generated using the 'R' package 'ggplot2' (Wickham and Wickham, 2007). A Chi-square ($\chi 2$) goodness of fit test (Cochran, 1952) was used to fit to a single dominant gene model by comparing the observed and expected segregation frequencies of tembotrione resistant or -susceptible plants.

Results

Mechanism of Tembotrione Resistance

Upon sequencing the *HPPD* gene from G-200, G-350, and S-1, no mutations were identified in the coding region of the *HPPD* gene (Figure S3.1), suggesting that no target site alterations confer resistance to tembotrione in G-200 or G-350. In response to malathion or PBO followed by tembotrione treatments, G-200 and G-350 exhibited significant biomass reduction compared to plants treated with tembotrione alone. Malathion or PBO without tembotrione treatment had no effect on the sorghum genotypes tested (Figures 3.1 and 3.2). The corn inbred line B73 (known to be resistant to tembotrione) did not show any significant biomass reduction at 92, or 184 g ai ha^{-1} of tembotrione application (Figure 3.1); however, exhibited a significant reduction in biomass in response to pre-treatment with malathion followed by 184 and 368 g ai ha^{-1} of tembotrione (Figure 3.1). The genotype G-200 treated with malathion followed by 92, 184, and 368 g ai ha^{-1} showed more than 50% reduction in biomass compared to plants treated only with tembotrione. There is no significant difference in biomass accumulation when treated

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with 2000 or 4000 g ai ha^{-1} of malathion, except in malathion followed by 368 g ai ha^{-1} tembotrione treatment (Figure 3.1). Whereas, G-350 showed significant biomass reduction only when treated with malathion followed by 92 g ai ha^{-1} tembotrione. The susceptible genotype, S-1, showed significant growth reduction at all doses of tembotrione or when pre-treated with malathion (Figure 3.1). The corn B73 exhibited significant biomass reduction in response to PBO, followed by 92, 184, and 368 g ai ha^{-1} of tembotrione treatment (Figures 3.2). Both G-200 and G-350 genotypes also showed a significant reduction in biomass when pre-treated with PBO followed by all doses of tembotrione. The S-1 was susceptible to all treatments applied (Figure 3.2).
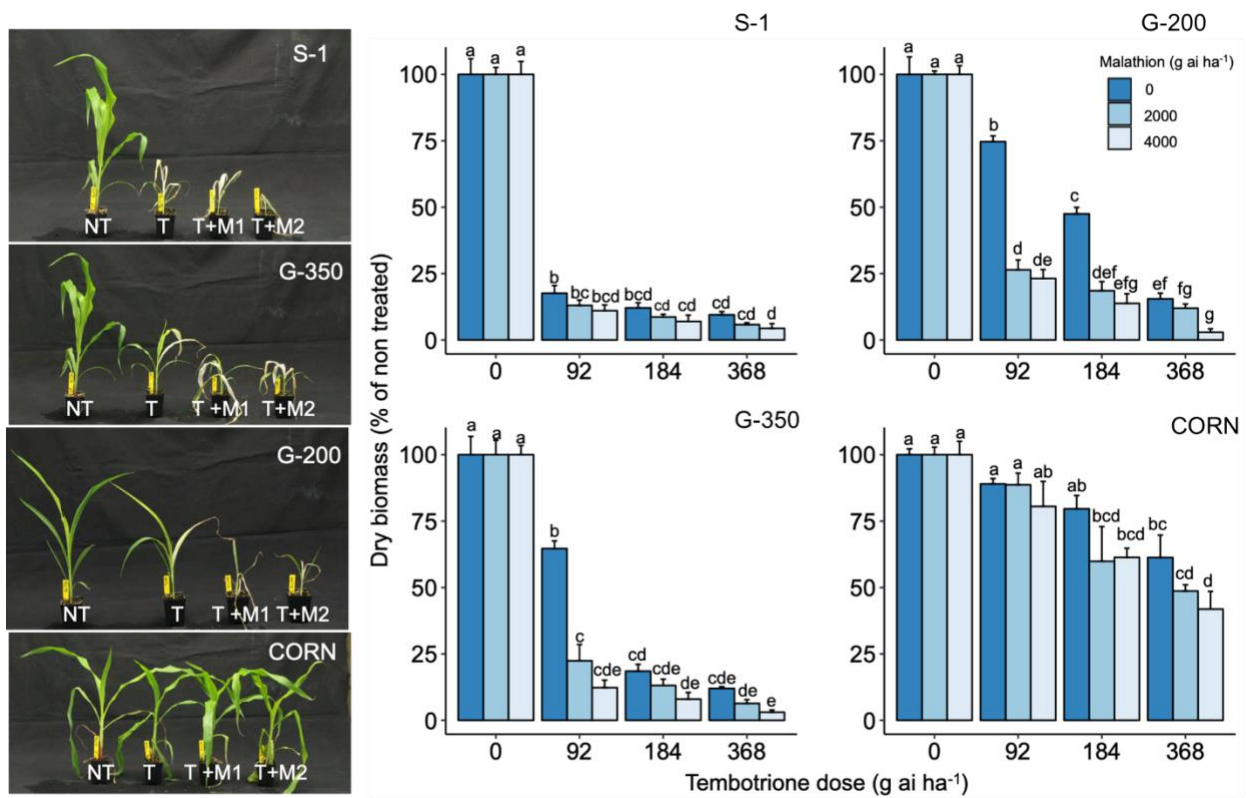
Figure 3.1 (A) The response of S-1 (susceptible), G-200, G-350 (resistant) and, corn to field recommended dose tembotrione (92 g ai ha^{-1}) (T) or malathion treatments (M1: 2000 g ai ha^{-1};

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M2: 4000 g ai ha^{-1}) followed by field recommended dose tembotrione; NT: Non-treated; and (B)

Aboveground dry biomass of sorghum genotypes when pre-treated with malathion or different

doses of tembotrione. The error bars represent the standard error (n=8); different alphabets

indicate a significant difference between treatments (p ≤ 0.05)
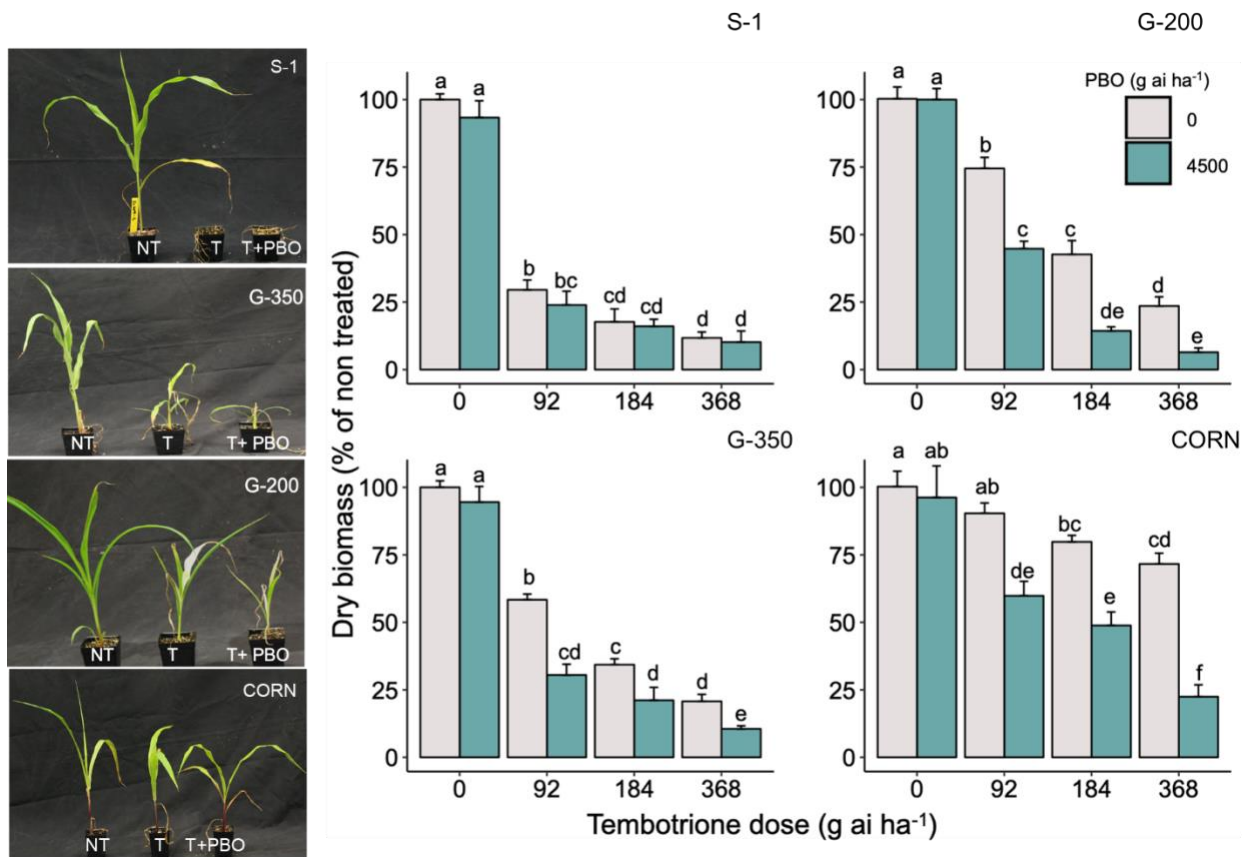
Figure 3.2 (A) The response of S-1 (susceptible), G-200, G-350 (resistant) and, corn to field

recommended dose of tembotrione (92 g ai ha^{-1}) (T) or Piperonyl butoxide (PBO) treatment (M1:

4500 g ai ha^{-1}) followed by field recommended dose tembotrione; NT: Non-treated; and (B)

Aboveground dry biomass of sorghum genotypes when pre-treated with PBO or different doses

of tembotrione. The error bars represent the standard error (n=8); different alphabets indicate a

significant difference between treatments (p ≤ 0.05)

Inheritance of Tembotrione Resistance

The F_1 progeny of S-1 x G-200 and S-1 x G-350 showed an intermediate response relative to parents when treated with several doses of tembotrione. The GR_{50} of S-1 x G-200 and S-1 x G-350 were estimated at 104 and 117 g ai ha^{-1}, respectively, which were less than their respective tembotrione-resistant parents, i.e., G-200 (218 g ai ha^{-1}) and G-350 (172 g ai ha^{-1}) (Figures 3.3; Table 3.1), suggesting that tembotrione resistance is a partially-dominant trait. The F_2 progeny exhibited a continuous variation for tembotrione injury and recovery. Therefore, to perform a chi-square test frequency of segregation of tembotrione resistance or susceptibility in F_2 progeny, the plants that had more than 80% tembotrione injury were grouped as susceptible and others as resistant. The observed segregation of resistant: susceptible (R:S) ratios from both the crosses did not comply with the expected ratios of 3:1 (R:S) for a single gene inherited trait, indicating that more than one gene is involved in tembotrione resistance in G-200 or G-350 genotypes of sorghum (Table 3.2).
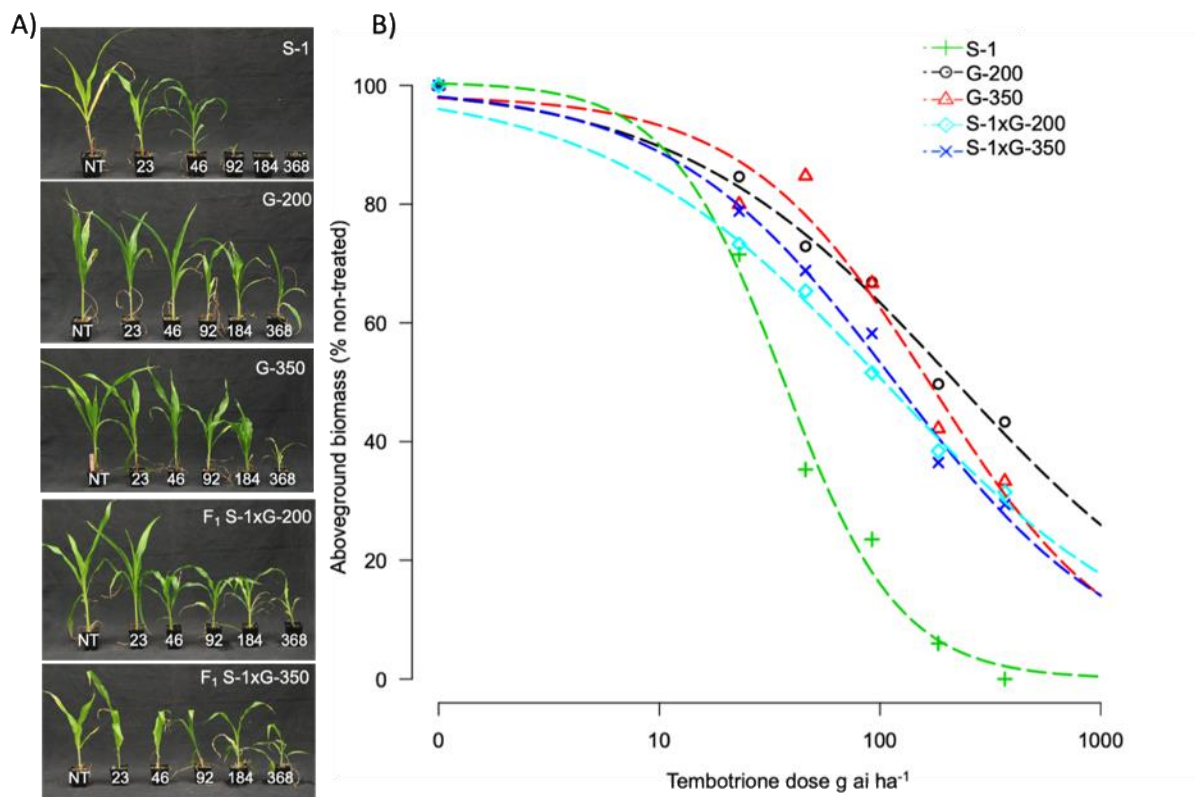
Figure 3.3 (A) Tembotrione dose-response curves representing the above ground dry biomass of S-1 (susceptible), F_1 (S-1 x G-200), F_1 (S-1 x G-350) and G-200, G-350 (resistant) using the four-parameter log-logistic model. B) Response of parents S-1 (susceptible), G-200, G-350 (resistant), F_1 (S-1 x G-200), F_1 (S-1 x G-350) to different doses of tembotrione

Table 3.1 Regression parameters describing the response of sorghum genotypes and their F_1 progeny to tembotrione under greenhouse conditions (GR_{50}: dose required for 50% growth reduction; SE: standard error; RI: Resistance index R/S; Resistant/Susceptible)

Genotype	GR_{50} (SE)	RI (R/S)
S-1	36 (4)	1.0
S-1 x G-200	104 (27) **	2.8
S-1 x G-350	117 (25) **	3.2

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G-350		172 (33) **			4.0
G-200		218 (60) **			6.0

Significantly different from S-1 at *p ≤ 0.05 **p ≤ 0.01

Table 3.2 Chi-Square analysis of the segregation of tembotrione resistant (R) and- susceptible (S) phenotypes in sorghum F_2 progeny at four weeks after treatment

Cross	Run	Total	R	S	P-value
S-1 x G-200	1	200	168	32	0.00329**
	2	150	124	26	0.0372*
	Run 1and 2 Combined	350	292	58	0.00033**
S-1 x G-350	1	220	197	23	0.00001**

Significantly different at **≤ 0.01 *p ≤ 0.05

Mapping Tembotrione Resistance

To map the genomic loci controlling tembotrione resistance, a total of 208, 376 SNPs were obtained using GBS from 150 F_2 progeny (S-1 x G-200) and parents (S-1, G-200). A subset of 1,954 SNP markers polymorphic to both parents with less than 30% missing values were retained. Further, filtering for missing rate (>90%), strong segregation distortion, marker distribution and redundant markers resulted in a total of 696 markers that were used for construction of a linkage map. The map of 1,021 cM was prepared which had an average distance of 1.7cM between two adjacent markers (Figure S3.2). A total of three QTLs on chromosomes 2, 4, and 8 were mapped with a high LOD score (LOD>2) (Figure 3.4; Table 3.3) for two traits, RE i.e. difference between leaf chlorophyll index at 2 and 4 WAT, and visual scoring at 3 WAT VI obtained from 150 F_2 plants (Figure S3.3). The LOD score of detected

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QTLs ranged from 3.0 to 6.0 and the phenotypic variations explained (PVE) values ranged from 9 to 44%.
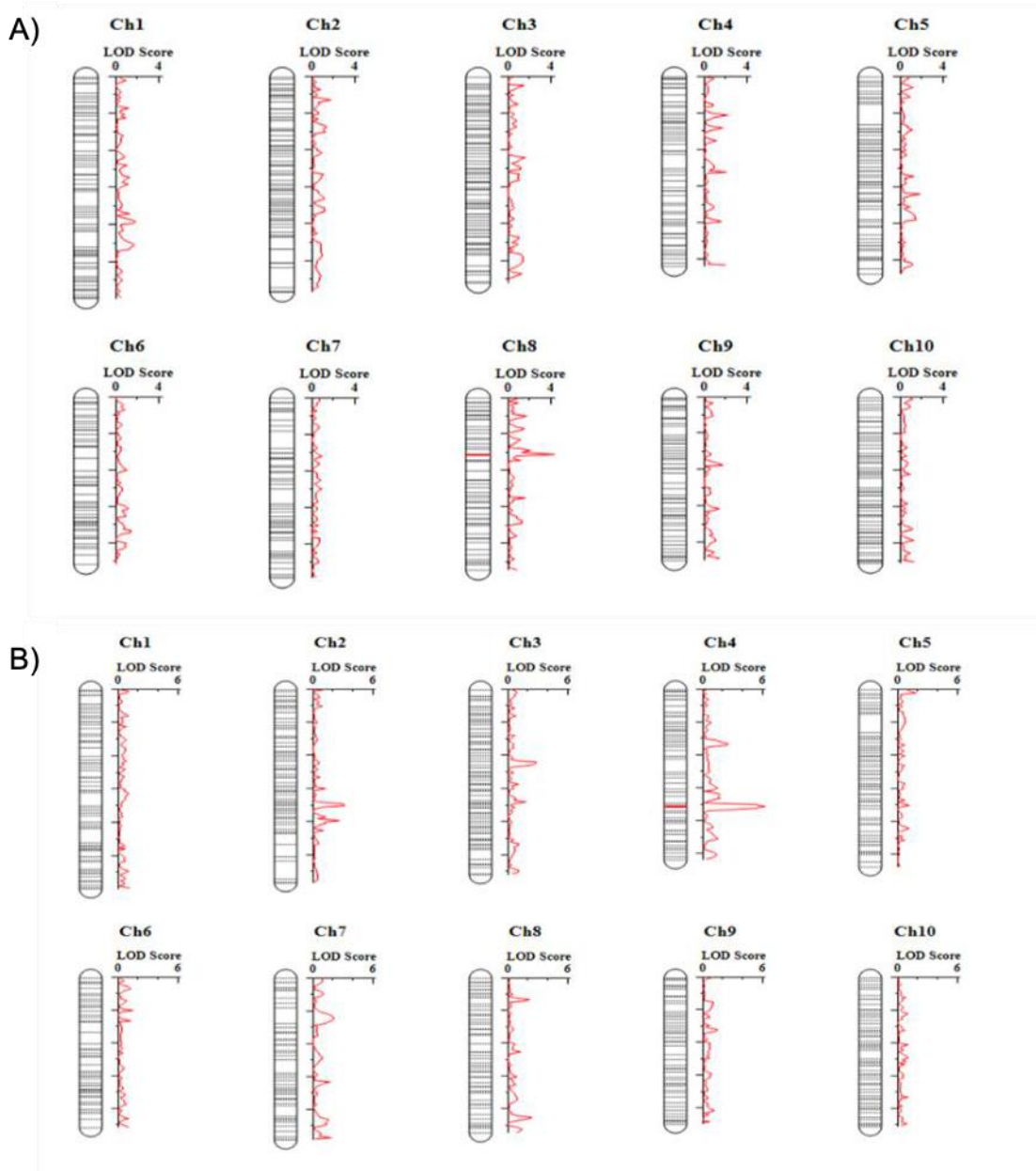
Figure 3.4 Quantitative trait loci (QTLs) detected from analysis of 150 plants from a single F_2 family of S-1 x G-200 for different traits: (A) Recovery (RE); and (B) visual injury 21 days after treatment (VI)

Table 3.3 Quantitative trait loci (QTLs) detected for the recovery (RE) and visual injury 3 weeks after treatment (VI) along with

logarithm of the odds (LOD) and phenotypic variation explained (PVE) explained by QTLs

Trait	QTL	Left Marker	Right Marker	LOD	PVE (%)	Add	Previously Reported Trait	Reference
RE	qRE8.1	Ch8_20217087	Ch8_20519857	4.32	44.35	-1.97	Efficiency of PSII reaction centers, Chlorophyll fluorescence	Ortiz et al., 2017, Fiedler et al., 2014
VI	qVI4.0	Ch4_46023941	Ch4_48400958	6.20	21.23	17.18	-	-
VI	qVI2.1	Ch2_45821038	Ch2_46847104	3.15	9.71	11.79	-	-

Discussion

Mechanism of Tembotrione Resistance

The natural resistance to tembotrione in G-200 and G-350 appears to be not conferred by any alteration to the molecular target of this herbicide, i.e. *HPPD* gene, because no difference in *HPPD* gene sequence was found between the resistant (G-200 and G-300) or susceptible (S-1) genotype (Data S3.1). Likewise, no naturally evolved mutations in the *HPPD* gene that confer resistance to HPPD-inhibitors were found in plants (Lu et al., 2020). Nonetheless, recently, soybean varieties resistant to HPPD-inhibitors were developed through transgenic technology by inserting an insensitive *HPPD* gene (Siehl et al., 2014; Dreesen et al., 2018). The CYP enzymes are known to metabolize HPPD-inhibitors, such as mesotrione (Ma et al., 2013; Nakka et al., 2017), tembotrione (Küpper et al., 2018), or topramezone (Elmore et al., 2015) in naturally evolved resistant weed biotypes. In this research, the genotypes, G-200, and G-350 exhibited significant biomass reduction in response to pre-treatment with CYP-inhibitors, malathion, or PBO followed by tembotrione application, suggesting that tembotrione is metabolized by CYP activity (Figures 3.1 and 3.2). Similarly, the use of these inhibitors, followed by tembotrione showed ~10% more biomass reduction compared to tembotrione alone treatments in a tembotrione-resistant common waterhemp population (Oliveira et al., 2018). Metabolism of tembotrione by hydroxylation followed by glycosylation, catalyzed by CYPs, has also been reported in a tembotrione-resistant Palmer amaranth biotype (Küpper et al., 2018). Furthermore, RNA-Seq analysis revealed differential expression of several CYP genes, for example, 3-4-fold upregulation of *CYP72A219, CYP81E8*, respectively, was found in the same above tembotrione-resistant Palmer amaranth biotype (Küpper, 2018). In corn, multiple CYPs located in *nsf1* locus were found to metabolize tembotrione, mesotrione, as well as other herbicides (Williams and Pataky, 2010).

Inheritance of Tembotrione Resistance in Sorghum

Based on the response of F_1 progeny (S-1 x G-200 and S-1 x G-350) to tembotrione treatment, we found that the tembotrione resistance in G-200 and G-350 is a partially dominant trait (Figures 3.1; Table 3.1). Furthermore, F_2 data demonstrated that this resistance is controlled by multiple genes (Table 3.2). Genetic analyses of sweet corn inbred lines revealed a single recessive allele controlling the sensitivity to tembotrione (Williams and Pataky, 2008). The genetic basis of tembotrione resistance is not extensively studied in plants; however, mesotrione (another widely used HPPD-inhibitor) resistance in several common waterhemp populations across US Midwest (Huffman et al., 2015; Kohlhase et al., 2018; Oliveira et al., 2018) was found to be inherited by a partially-dominant polygenic trait.

QTL Mapping

We mapped three QTLs associated with tembotrione resistance on chromosomes 2, , 4, and 8 using the sequence data from 150 F_2 plants from S-1 x G-200 cross (Figure 3.4). To our knowledge, this is the first report of QTLs associated with tembotrione resistance in grain sorghum. The QTL mapped using RE was previously reported for other traits in sorghum related to chlorophyll fluorescence (Fiedler et al., 2014), photochemical quenching (Ortiz et al., 2017) (Table 3.3); the QTLs mapped on chromosome 2 and 4 using VI, were novel and not previously reported for any other trait. These QTLs need to be tested in multiple environments with more number of F_2 plants and markers to improve the estimation accuracy Experiments are in progress to further fine map and identify the precise location of the gene(s) responsible for tembotrione resistance in grain sorghum. As mentioned earlier, our data indicate that the tembotrione resistance is a polygenic trait, and such traits can express differently in different genetic backgrounds. Therefore, tembotrione resistance can potentially be

improved by crossing G-200 and G-350 or with other commercial genetic backgrounds. Such work has been reported to enhance the performance of quantitative traits in different genetic backgrounds and environmental conditions such as drought (Reddy et al., 2009), stay green (Subudhi et al., 2000), cold tolerance (Knoll and Ejeta, 2008), and yield (Nagaraja-Reddy et al., 2013) in grain sorghum. Therefore, there is enormous potential for improving tembotrione resistance by testing the expression of this trait in different genetic backgrounds and for the development of tembotrione-resistant sorghum varieties. Since sorghum can outcross with closely related wild and weedy species, such as johnsongrass or shattercane, one of the major concerns of the development of herbicide-resistant sorghum varieties has been a natural transfer of such resistance into these weed species (Ohadi et al., 2017). However, recent reports suggest that the outcrossing rate of sorghum with johnsongrass was as low as ~1% under controlled conditions (Hodnett et al., 2019) and 2-16% with shattercane under field conditions (Schmidt et al., 2013). Although the possibility of outcrossing is minimal, if an herbicide resistance trait escapes into the wild species, necessary stewardship practices must be developed and integrated into sorghum weed management practices.

Conclusion

In conclusion, we have identified sorghum genotypes (G-200 and G-350) with natural resistance to tembotrione from the sorghum association panel, which can potentially be used to introgress the tembotrione resistance into breeding lines by conventional or marker-assisted breeding methods. CYP-inhibitor assay suggested CYP-mediated metabolism of tembotrione in the resistant genotypes. Genetic analyses of F_1 and F_2 progeny demonstrated that the resistance is a partially dominant polygenic trait. Furthermore, GBS-based QTL mapping revealed three QTLs associated with tembotrione resistance in grain sorghum. Future research needs to be focused on incorporating the resistant trait with elite

breeding varieties, testing the hybrid performance, and improving herbicide resistance in high yielding and stress tolerant hybrids.

Chapter 4 - Identification and Characterization of Chlorsulfuron Resistance

Abstract

Chlorsulfuron, an acetolactate synthase (ALS)-inhibitor effectively controls post-emergence grass and broadleaf weeds but not registered for use in sorghum because of crop injury. The objectives of this study were to characterize and investigate the inheritance and mechanism of chlorsulfuron resistance in the sorghum genotype S-1. Chlorsulfuron dose-response experiments were conducted using S-1 along with BTx623 (susceptible check), and Pioneer 84G62 (commercial sorghum hybrid). The F_1 and F_2 progeny were generated by crossing S-1 with BTx623. To assess if the target site alterations bestow resistance, the *ALS* gene, the molecular target of chlorsulfuron, was sequenced from S-1. The role of cytochrome P450 (CYP) in metabolizing chlorsulfuron, using malathion, a CYP-inhibitor was tested. The results of the chlorsulfuron dose-response assay indicated that S-1 and F_1 progeny were ~20-fold, more resistant to chlorsulfuron relative to BTx623. The F_2 progenies segregated 3:1 (resistance: susceptibility) suggesting that chlorsulfuron resistance in S-1 is a single dominant trait. No mutations in the *ALS* gene were detected in the S-1; however, a significant reduction in biomass accumulation was found in plants pre-treated with malathion indicating that metabolism of chlorsulfuron contributes to resistance in S-1. Also, S-1 is highly susceptible to other herbicides (e.g. mesotrione and tembotrione) compared to Pioneer 84G62, suggesting the existence of s negative cross-resistance in S-1. Overall, these results confirm a high-level of metabolic resistance to chlorsulfuron inherited by a single dominant allele in S-1 sorghum. These results have potential for developing chlorsulfuron-resistant sorghum hybrids, with the ability to improve post-emergence weed control.

Introduction

Grain sorghum (*Sorghum bicolor* (L.) Monech ssp. *bicolor*) is the fifth-largest cereal crop in the world and is suitable for cultivation in semi-arid and arid conditions (Ciampitti and Prasad 2019). The US is the largest producer of grain sorghum in the world, followed by Nigeria, Mexico, Sudan, and India (Visarada and Aruna 2019). Despite its ability to perform under adverse conditions, the cultivated area of sorghum has been declining in recent years across the US (USDA-NASS, 2020). There are several reasons for the decline of sorghum cultivation, nonetheless, lack of herbicide options for grass weed control tops the list. Weed infestation can reduce the sorghum yield up to ~60% (Dille et al., 2020, Stahlman et al., 2000, Thompson et al., 2019). Although herbicide options are available to control broadleaf weeds, no herbicides are currently registered for postemergence (POST) control of grass weeds in grain sorghum (Smith et al., 2010). Acetolactate synthase (ALS)-inhibiting herbicides are widely used pre-emergence (PRE) or POST emergence for effective control of a broad spectrum of weeds, including grasses and broadleaf weeds in many cropping systems.

ALS, the target site of ALS-inhibitors, is one of the key enzymes in the biosynthesis of branched-chain amino acids (BCAAs) such as valine, leucine, and isoleucine (Ray, 1984, Russel et al., 2002). BCAAs play a critical role in protein synthesis, and inhibition of ALS enzyme leads to depletion of BCAAs levels in the cell leading to plant death. However, the phytotoxic processes and other secondary effects caused by the inhibition of the ALS enzyme is unclear (Tranel and Wright, 2002, Zhou et al., 2007).ALS-inhibiting herbicides include five chemical families *viz.*, sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-thio benzoates (PTB) and sulfonyl-aminocarbonyl-triazolinone (SCT). Sulfonylurea is one of the largest herbicide families with ~27 registered active ingredients (e.g. chlorsulfuron, nicosulfuron) across the world (Russel et al., 2002).

Two major mechanisms known to confer resistance to ALS-inhibitors in plants (Tranel and

Wright, 2002) are a) mutation(s) in the *ALS* gene (target site resistance) leading to the altered affinity of

the ALS enzyme for herbicide binding; and b) as a result of metabolism of ALS-inhibitors (non-target

resistance) via cytochrome P450 (CYP) enzyme activity. Mutations in the *ALS* gene resulting in seven

amino acid substitutions confer resistance to different chemical families of ALS inhibitors (Heap,

2020). Enhanced metabolism of ALS-inhibitors, also known to confer resistance to these herbicides in

crops and weeds (Jugulam and Shyam, 2019). Several crops [e.g., wheat (*Triticum aestivum*), corn (*Zea*

mays), oats (*Avena sativa*), and cotton (*Gossipium hirsutum*)] are naturally tolerant to various SU

herbicides, primarily due to detoxification of these herbicides via CYP enzyme activity (Yu and Powles

2014). For example, rapid metabolism of nicosulfuron in corn (Liu et al., 2019), trifloxysulfuron

sodium in cotton (Thyssen et al., 2018), and chlorsulfuron in wheat (Xiang et al., 2006) were attributed

to CYP activity.

The metabolism-based resistance to ALS-inhibitors is commonly assessed by the use of

competitive inhibitors of CYP enzymes. Several chemical compounds that can inhibit CYP activity

have been identified; the most commonly used ones are, 1-aminobenzo-triazole (ABT), tetcyclacis

(TET), piperonyl butoxide (PBO), tridiphane, and malathion. Pre-treatment of CYP-inhibitors before

herbicide (i.e. ALS-inhibitors) application will competitively inhibit CYP activity resulting in reduced

metabolism of herbicide, thereby, reducing the level of resistance (Siminszky, 2006). Specifically,

malathion, an organophosphate insecticide is widely used to determine metabolic resistance to ALS-

inhibitors in several crop and weed species such as corn (Kreuz and Fonné-Pfister, 1992), rice (*Oryza*

sativa; (Li et al., 2015), sunflower (*Helianthus annuus*; (Kaspar et al., 2011), rigid ryegrass (*Lolium*

rigidum; (Christopher et al., 1994), and common waterhemp (*Amaranthus tuberculatus*; (Oliveira et al.,

2018). Malathion reacts with CYP and release atomic sulfur that covalently binds to CYPs resulting in reduced activity to metabolize herbicide (Werck-Reichhart and Feyereisen, 2000).

Although ALS- inhibitors are extremely popular and widely used for POST control of grasses in many crops, they are not registered for use in grain sorghum because of crop injury. Development of grain sorghum hybrids resistant to ALS-inhibitors has enormous potential to improve grass weed control in grain sorghum. Researchers have been exploiting the naturally available genetic diversity in sorghum and wild relatives of sorghum to identify sources with resistance to herbicides (Gaines et al., 2020). In this effort, sorghum hybrids resistant to nicosulfuron were developed by introgressing the nicosulfuron-resistant trait from shattercane (*Sorghum bicolor* ssp. drummondii) a grass weed closely related to cultivated sorghum. The nicosulfuron resistance in these hybrids was conferred target-site resistance as a result of mutation (Trp-574-Leu) in the *ALS* gene (Tuinstra and Al-Khatib, 2011). The Trp-574-Leu mutation confers resistance to SU as well as IMI herbicides. However, these hybrids are not commercially available yet and are expected to be made available in the near future. Recently, our group has also identified sorghum genotypes resistant to hydroxyphenylpyruate dioxygenase (HPPD)-inhibitor herbicides known to control a wide spectrum of grass weeds (Pandian et al., 2020).

One of the major concerns in the development of herbicide-resistant technology in crops, especially in crops such as sorghum is the possible transfer of resistance genes into closely related wild species, such as shattercane or johnsongrass (*Sorghum halepense*) leading to the evolution of herbicide resistance in these weed species (Ohadi et al., 2017). Although, the possibility maybe minimal, nonetheless, we must assess all available options for the management of wild species if and when the resistance trait escapes into these species (Werle et al., 2017a). A plant genotype/biotype that confers resistance to one herbicide or herbicide family but exhibits sensitivity to a different herbicide is termed

as negative cross-resistance (Gressel and Segel, 1990). Such negative cross-resistance can help in the management of weed species if they inherit the resistance trait by natural crossing.

The overall goal of this research was to identify and characterize chlorsulfuron resistance in grain sorghum. Chlorsulfuron (Glean®) is a SU herbicide registered for use in only some cereal crops, such as wheat, barley, and oats. This herbicide is widely used to control a broad spectrum of weeds at a very low chemical dosage (Ray, 1984). However, sorghum is susceptible to chlorsulfuron and hence not registered for use on this crop. In our previous research we have identified four sorghum genotypes from the sorghum association panel (Casa et al., 2008) two each with high level of resistance and a genotype, highly susceptible to mesotrione or tembotrione, belonging to 4-hydroxyphenypyruvate dioxygenase (HPPD)-inhibitor group of herbicides (Pandian et al., 2020). Upon testing the response of these five genotypes to herbicides we found the sorghum genotype, S-1, susceptible to HPPD-inhibitors, but surviving chlorsulfuron treatment (data not shown). This research was conducted based on the hypothesis that the metabolism of chlorsulfuron via CYP activity confers chlorsulfuron resistance in S-1 genotype. The specific objectives of this research were to a) determine the level of resistance of S-1 to chlorsulfuron, b) assess the possibility of the response of S-1 to other groups of herbicides, and c) investigate the inheritance and mechanism of resistance to chlorsulfuron in S-1.

Materials and Methods

Plant Materials

The sorghum genotypes, S-1 was used i) in chlorsulfuron-dose response assay and response to other herbicide groups, ii) to investigate the inheritance of chlorsulfuron resistance and iii) to confer if target- or non-target resistance mechanism bestow resistance. The other sorghum genotypes, *viz*.,

BTx623, a chlorsulfuron susceptible genotype (commonly used breeding line) and/or Pioneer 84G62, a commercial hybrid was used for comparison.

Chlorsulfuron Dose-Response Assay

The seeds of sorghum genotypes S-1, BTx623, and Pioneer 84G62, were planted in pots (15x15 cm) filled with a potting mixture (Peat-based PRO-MIX Premium Potting Mix, Premier horticulture Inc., Quakertown, PA, USA). Upon emergence, the seedlings at 2-3 leaf stage, were transplanted in punnet pots ($6 \times 6 \times 6$ cm) and grown in the greenhouse maintained at 25/20° C day/night, 15/9 hours photoperiod with a photosynthetic photon flux density of 750 µmol m^{-2} s^{-1} and relative humidity of 60 ± 10 percent. The plants were fertilized (Miracle GRO® All-purpose plant food) as needed. When sorghum seedlings were at 4-5 leaf-stage, chlorsulfuron (Glean® XP) was applied at 0, 18 (1x field recommended dose), 36, 72, 144, 288 and, 576 g ai ha^{-1} with 0.25% non-ionic surfactant (NIS) using bench-top track spray chamber (Generation III, De Vries Manufacturing, RR 1 Box 184, Hollandale, MN, USA) equipped with a single flat-fan nozzle (80015LP TeeJet tip, Spraying Systems Co., Wheaton, IL, USA) delivering 187 L ha^{-1}. The experiment was conducted in a completely randomized design (CRD) with four replications and repeated maintaining the same growth conditions and treatments as described above. Each plant was considered as an experimental unit, four replications were kept for each genotype. The aboveground plant biomass was harvested at three weeks after treatment (WAT) in paper bags and dried in an oven at 60°C for 72 h. The dry weight of the biomass was recorded and subjected to statistical analysis (described separately in the section below).

Response of S-1 to HPPD-Inhibitors

In a preliminary study, the S-1 genotype was found to be susceptible to HPPD-inhibitors (e.g. mesotrione and tembotrione), one of the widely used herbicides for broad-spectrum weed control in

crops such as corn. The response of S-1 to mesotrione and tembotrione was tested using Pioneer 84G62 (known to be susceptible to these herbicides) for comparison. The plants of S-1 and Pioneer 84G62 were grown in the greenhouse (as described above). When the seedlings were at 4-5 leaf stage, they were treated with mesotrione (Callisto^{TM}) at 0, 26.25, 52.5, 105 (1x field recommended dose), 210, 420, 840,1680 g ai ha^{-1} with 1% crop oil concentrate (COC) or tembotrione (Laudis^{TM}) at 0, 5.75, 11.5, 23, 46, 92 (1x field recommended dose), 184, 368, 736 g ai ha^{-1} 0.25% Destiny$^®$ (methylated soy oil). The experiments were conducted following the same procedure mentioned above for chlorsulfuron dose-response assay.

Generation of F_1 and F_2 Progeny

To investigate the inheritance of chlorsulfuron resistance, crosses were performed between S-1 and BTx623. Sorghum genotypes S-1 and BTx623 were grown in 33 x 23 cm pots in the greenhouse (above-mentioned growth conditions). The genotype, BTx623 was designated as the female parent and emasculated by the plastic bag method (Rakshit and Bellundagi, 2019). The bags were removed after flowering, and anthers were removed by head tapping, the pollen collected from S-1 plants were used to pollinate the emasculated 623 plants. Upon maturity, the F_1 seeds were collected from BTx623 plants and used in chlorsulfuron dose-response assay (as described in chlorsulfuron dose-response assay). About five F_1 plants that survived 72 g ai ha^{-1} of chlorsulfuron treatment were allowed to grow, and fully emerged panicles were covered with a paper bag to ensure complete self-pollination to produce F_2 seeds (Reddy and Kumar, 2008).

Evaluation of F_2 Response to Chlorsulfuron

A total of 227 seedlings from a single F_2 family along with the parents were raised in the greenhouse (as described above and under similar growth conditions). The seedlings (4-5 leaf stage)

were treated with a high dose of chlorsulfuron ($4x=72$ g ai ha^{-1}, following the same procedure as described above). The response of F$_2$ plants was rated as highly injured/dead (susceptible) or minor/no symptoms (resistant) at four WAT in comparison with the parents. Specifically, the plants that exhibited interveinal chlorosis and stunting were grouped as susceptible and those with fully formed leaves, new growth, and without the above symptoms were grouped as resistant (Hennigh et al., 2010).

ALS Gene Sequencing

To determine any target site alterations, confer resistance to chlorsulfuron, the genotypes S-1, and BTx623 were grown in the greenhouse (as described above and under similar growth conditions). Leaf tissue (3-4 leaf stage plants) was collected from three plants of each genotype, and genomic DNA (gDNA) was extracted using GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific™) following manufacturer's instructions. The quality of DNA samples was checked by resolving the DNA in 0.8% agarose gel, and the concentration of the DNA samples were quantified using NanoDrop™ (Thermo Scientific™). The full-length sorghum *ALS* gene (~2300 bp) was amplified using the primers Sg_ALS1 F (5'TTGCTAGTCCCATTCCCATC3'; Forward primer) and Sg_ALS1 R (5'CAACAGTGATACCTGGGTTGG3'; reverse primer) designed based on *ALS* gene large sub-unit (Sobic.004G155800.2) from the sorghum V3.1.1 reference genome (McCormick et al., 2018). Polymerase Chain reaction (PCR) was performed using T100™ Thermal Cycler (Bio-Rad Inc., Hercules, CA, USA). The PCR mixture contains 50-80 ng of gDNA, 0.5µM each of forward, reverse primer, and 1x of GoTaq® G2 Green Master Mix (Promega™). PCR amplification was done using the following PCR cycling conditions, initial denaturation: 94 °C for 5 min, followed by 35 cycles of denaturation: 94 °C for the 30s, annealing 60°C for 45s and extension: 72°C for 45s and final extension: 72°C 7 mins. The PCR products were analyzed in 1.5% agarose gel to confirm the targeted

amplicon size and purified using GeneJET™ PCR Purification Kit (Thermo scientific™) The PCR

purified samples were sequenced by Sanger sequencing service provided by GENEWIZ, LLC., NJ,

USA. The sequences obtained by Sanger sequencing were aligned using Clustal Omega multiple

sequence alignment tool (EMBL-EBI) to check for the mutations.

Response of S-1 to CYP-Inhibitor

To determine the presence of non-target resistance via CYP metabolism of chlorsulfuron in S-1,

experiments were conducted using CYP-inhibitor, malathion. The sorghum genotypes S-1, BTx623,

and Pioneer 84G62 were grown in the greenhouse (as described above and under similar growth

conditions). The seedlings at a four to five leaf stage was used in this experiment. The experiment was

conducted in a complete randomized design (CRD) with four replications; each plant was considered as

an experimental unit. The treatment includes malathion (Spectracide™ malathion insect spray

concentrate) and chlorsulfuron (Glean®XP). Malathion was applied at 0, 1500 g ai ha^{-1} with 0.25% NIS

one hour prior to chlorsulfuron application. Chlorsulfuron was applied at 0, 18, 72, and 144 g ai ha^{-1}.

All the treatments were arranged in a factorial design to ensure all possible combinations. Chemical

treatments (malathion and chlorsulfuron) and data collection were performed as mentioned in the

chlorsulfuron dose-response assay.

Statistical Analysis

The biomass data were used to calculate percent dry biomass, relative to the non-treated control

of respective genotypes.

$$\text{Dry biomass (\% of non-treated)} = \frac{\text{Biomass of individual plant (g)}}{\text{Average biomass of the non-treated plants of the genotype (g)}} \times 100$$

The dose-response expressed as a percentage of the non-treated control was subjected to non-linear regression analysis using a three-parameter log-logistic model (Eq.1) using a 'drc' (Ritz et al. 2005) package in R (Development Core Team, 2013) to detect the relationship between herbicide dose and biomass. All the analyses were performed as per the method given by (Knezevic et al., 2007, Shyam et al. 2019). To assess the fit of data to various regression models, a "Lack-of-fit" test was performed using the "model fit" function of 'drc'.

Eq.1

$$Y = \{ \frac{d}{1} + \exp [b \ (logX - loge)]\}$$

In the equation above Y is the response, and b is the doses where d is the upper limit, b is the slope of the curve, and e is GR_{50} which is the herbicide dose required for 50% reduction in plant biomass (Knezevic et al. 2007). Differences between the estimated GR_{50} values were tested with each other by t-test using the "compParm" function in the 'drc' package. The dose-response curves were generated using the 'plot' function in the 'drc' package. The resistant index was calculated using the formula GR_{50} of resistant / GR_{50} of susceptible genotypes based on (Nakka et al., 2017). To estimate the significant differences between treatments on various traits in the CYP-inhibitor study, analysis of variance (ANOVA) was performed following Fisher's LSD test used to separate means insignificant treatments at $p \leq 0.05$ using the 'agricole' package in R (de Mendiburu, 2014). The bar plots with error bars representing the standard error were generated using the 'R' package 'ggplot2' (Wickham and Wickham, 2007). A Chi-square ($\chi 2$) goodness of fit (Cochran, 1952) was used to compare the observed and expected segregation ratio for chlorsulfuron resistance or susceptibility according to a single dominant gene model.

Results

Chlorsulfuron Dose-Response Assay to Determine the Level of Resistance

No significant differences were found between two runs of dose-response assays, and hence the data were pooled for statistical analysis ($p<0.05$). Based on the resistance index calculated from the chlorsulfuron dose-response of S-1, F_1, BTx623, and Pioneer 84G62 confirmed that S-1 and F_1, progeny were ~20-fold more resistant to chlorsulfuron compared to either BTx623 or Pioneer 84G62 (Table 4.1). The S-1 genotype exhibited minimal injury at even high doses of 16x or 32x ($x = 18$ g ai ha^{-1}; field recommended dose). Similarly, the F_1 plants also exhibited minimal injury (Fig. 1). The GR$_{50}$ values of S-1 and F_1 were significantly different from the susceptible genotypes BTx623 ($p\leq0.01$) and Pioneer 84G62 ($p\leq0.05$) (Table 4.1; Figure 4.2). The BTx623 and Pioneer 84G62 genotypes exhibited severe injury symptoms even at a 1x dose. (Fig. 1). However, there was no significant difference between the GR$_{50}$ value of BTx623 and Pioneer 84G62 ($p= 0.15$). In the susceptible genotypes, typical chlorsulfuron injury symptoms, such as interveinal chlorosis at one WAT, followed by stunted growth, at two, and three WAT were observed.

Table 4.1 Regression parameters describing the response of sorghum genotypes to chlorsulfuron (GR$_{50}$: dose required for 50% growth reduction; SE: standard error; RI: Resistance index (GR$_{50}$ of Resistant /GR$_{50}$ of Susceptible)

Genotype	GR$_{50}$ (SE)	RI
BTx623	30.61 (9.7) *	-
Pioneer 84G62	49.71 (14.84) *	1.6
F_1 (BTx623 x S-1)	577.95 (136.4)	18.8

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| S-1 | 645.02 (117.5) | 21.5 |
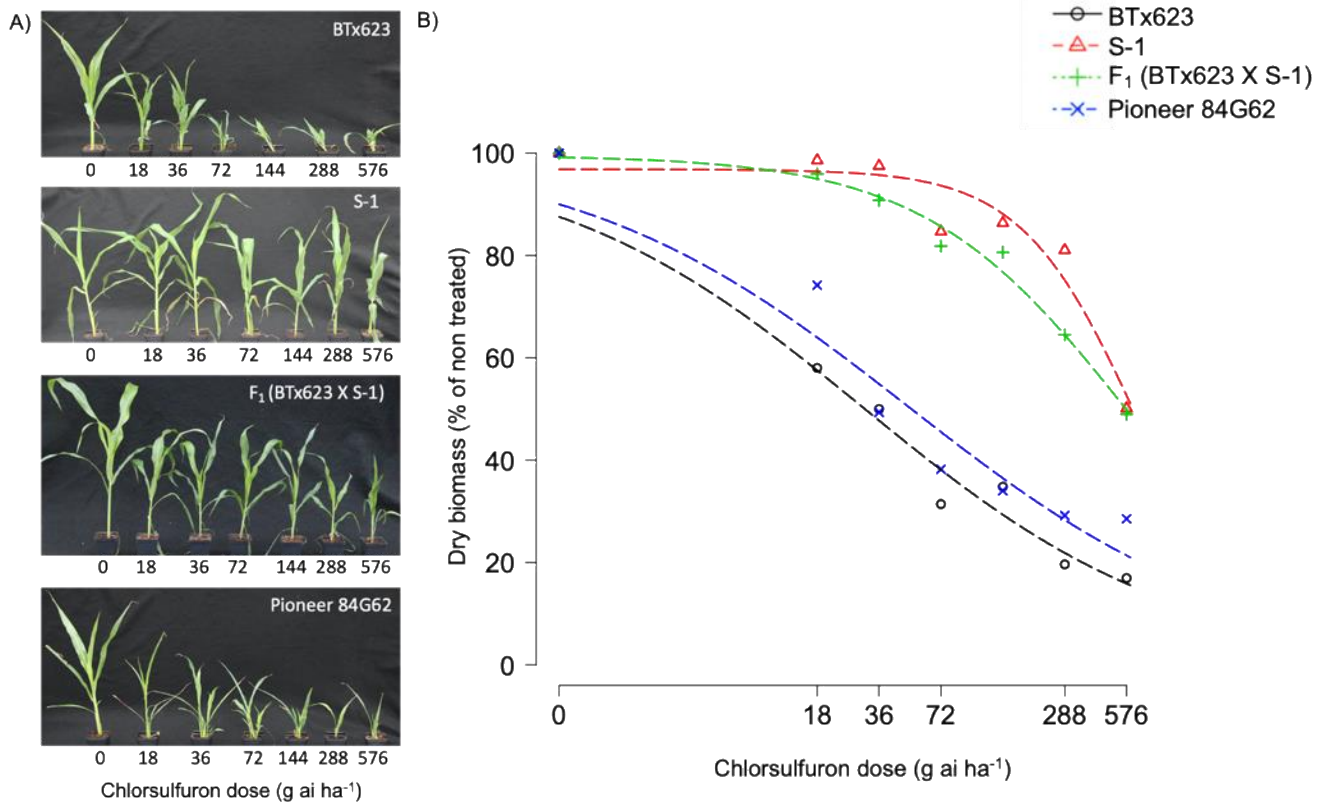
Figure 4.1 A) Response of genotypes BTx623 (susceptible), Pioneer 84G62 (commercial hybrid), F_1 (BTx623 x S-1), and S-1 (resistant) to multiple doses of chlorsulfuron at three weeks after treatment. B) Chlorsulfuron dose-response curves of BTx623 (susceptible), Pioneer 84G62 (commercial hybrid), F_1 (BTx623 x S-1), and S-1 (resistant) using the three-parameter log-logistic model $Y = \{\frac{d}{1} + \exp[b(logX\text{-}loge)]\}$

Response of S-1 Sorghum Genotype to HPPD-inhibitors

The results of the mesotrione or tembotrione-dose-response assay suggested that S-1 is highly susceptible to these herbicides (Fig. 2 A, B). On the basis of resistance index the S-1 was found to be ~4-fold more susceptible to mesotrione, than Pioneer 84G62 (Table 4.2; Figure 4.3A). Whereas, S-1 was ~2-fold more susceptible to tembotrione than the Pioneer 84G62 (Table 4.2; Figure 4.3B). These results indicate the extreme sensitivity of S-1 to HPPD-inhibitors.
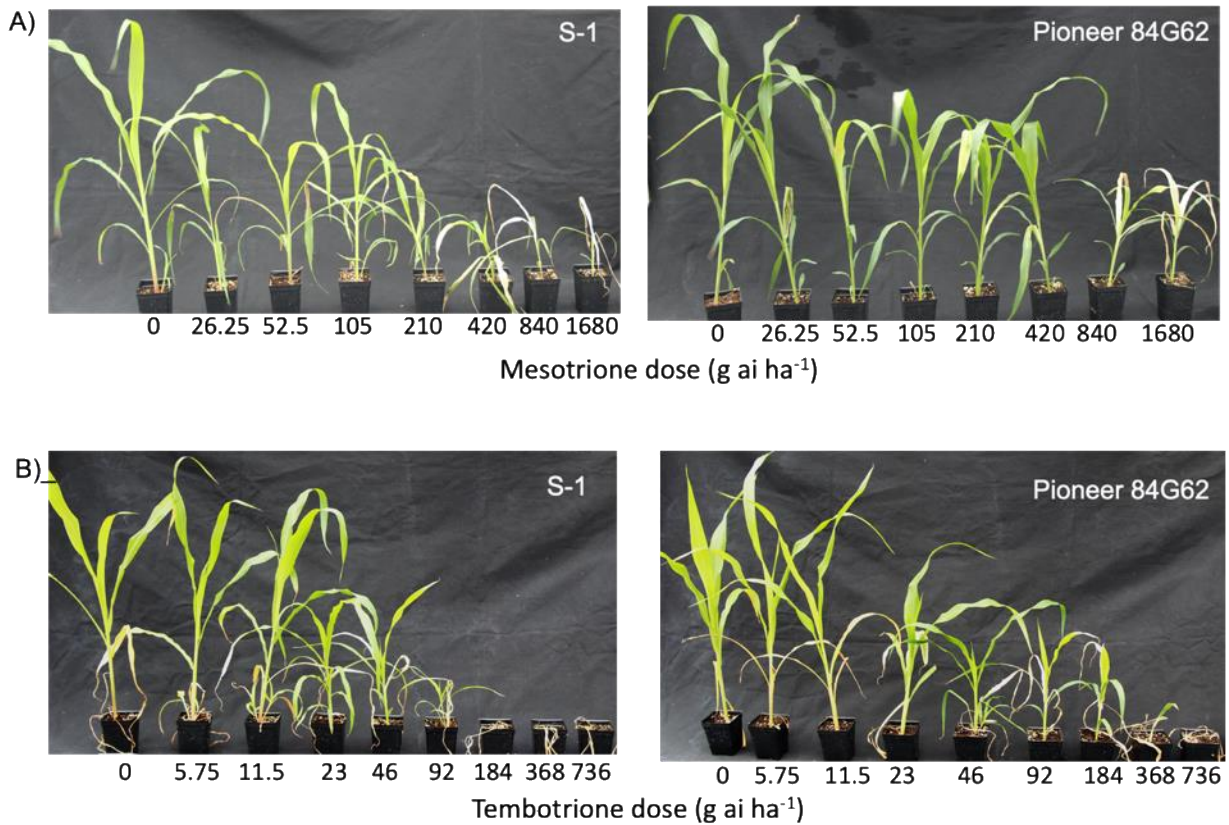
Figure 4.2 Response of genotypes S-1 (resistant) and Pioneer 84G62 (commercial hybrid) to different doses of A) mesotrione, B) tembotrione at three weeks after treatment.

Table 4.2 Regression parameters describing the response of sorghum genotypes to mesotrione and tembotrione (GR_{50}: dose required for 50% growth reduction; SE: standard error)

Genotype	GR$_{50}$ (SE)	
	Mesotrione	Tembotrione
S-1	101.4 (17)	24.3 (4)
Pioneer 84G62	376.4 (79) *	38.6 (5)*

*Significantly different from S-1 at $p \leq 0.05$
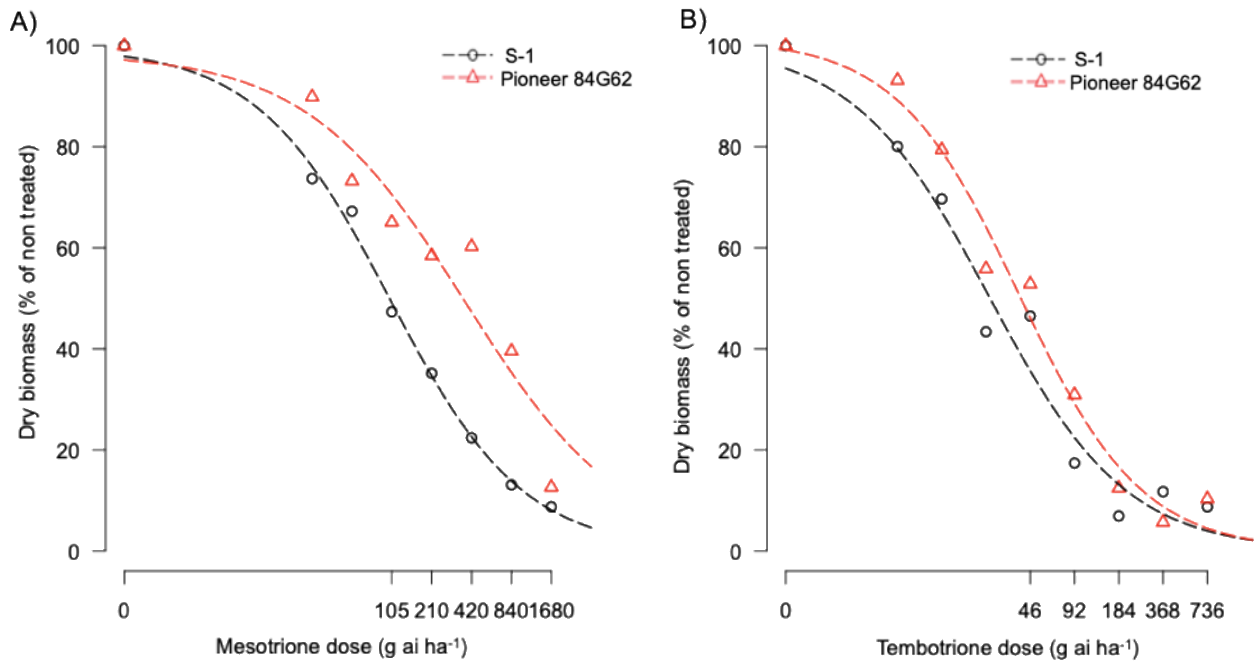
Figure 4.3 Dose-response curves obtained by nonlinear regression analysis of above-ground dry biomass of S-1 (resistant) and Pioneer 84G62 (commercial hybrid) at different doses of A) mesotrione B) tembotrione

Inheritance of Chlorsulfuron Resistance

As described above, the F$_1$ progeny exhibited a similar response to chlorsulfuron treatment as that of the resistant parent S-1 in the dose-response assay (Table 4.1), suggesting that the chlorsulfuron

resistance in S-1 is a dominant trait. The F_2 progeny segregated 3:1 for resistance (R): susceptible (S) at

four WAT with 4x dose of chlorsulfuron (Table 4.3), which is expected for a single dominant gene

model. Chi-square tests performed for goodness of fit to a 3:1 segregation (resistant: susceptible)

suggests that the observed frequencies (resistant or susceptible) after chlorsulfuron treatment were in

accordance with expected frequencies for a 3:1 (resistant: susceptible) segregation ratio (Table 4.3).

The response of F_1 progeny and the segregation of resistance and susceptible plants observed in the F_2

progeny confirms that the chlorsulfuron resistance in S-1 is a single dominant trait.

Table 4.3 Chi-Square analysis of chlorsulfuron resistance at three and four weeks after treatment (R:

Resistant and S: Susceptible)

Genotype	Weeks after treatment	Expected		Observed		χ^2 value	p-value
		R	S	R	S		
BTx623 x S-1	4	170	57	167	60	0.211	0.64

Assessment of Target-Site Resistance to Chlorsulfuron

To determine the presence of any mutations in the ALS gene conferring target-site resistance to

chlorsulfuron in S-1, the whole sequence of the *ALS* gene from S-1, BTx623 was amplified using PCR

and sequenced. The sequence alignment of the *ALS* gene did not reveal any mutations conferring amino

acid substitutions at positions (Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574) previously

known to confer resistance to sulfonylurea herbicides. Also, previously reported mutations known to

confer resistance to other classes of ALS-inhibitors were not found (Heap, 2020). Further, no other

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synonymous or non-synonymous mutations were observed in the exon region of the *ALS* gene (Figure S4. 1). These results confirm that no alterations in *ALS* gene confer resistance to chlorsulfuron in S-1.

Investigation of Metabolic Resistance to Chlorsulfuron

No significant differences were found between two runs of CYP-inhibitor study, and hence the data were pooled for statistical analysis (p<0.05). In response to pre-treatment with malathion followed by chlorsulfuron treatment, the S-1 genotype exhibited significant biomass reduction (Figure 4.4). Malathion treatment at 1500 g ai ha^{-1} (without chlorsulfuron) had no effect on the dry biomass of all sorghum genotypes tested (Figure 4.4). The S-1 plants treated with 18, 72 and 144 g ai ha^{-1} of chlorsulfuron did not show a significant reduction in biomass relative to non-treated plants; whereas, a significant reduction in biomass was observed in S-1 plants treated with malathion prior to chlorsulfuron treatment (p≤0.05) (Figure 4.4). S-1 plants exhibited up to 40% reduction in above ground biomass compared to chlorsulfuron only treatments (Figure 4.4). Whereas, the susceptible genotypes treated with chlorsulfuron showed significant growth reduction (Figure 4.4). However, pre-treatment with malathion did not affect the response of susceptible genotypes, BTx623, and Pioneer 84G62 to chlorsulfuron treatment (Figure 4.4). These results suggest non-target, metabolic resistance to chlorsulfuron via CYP activity in S-1 sorghum.
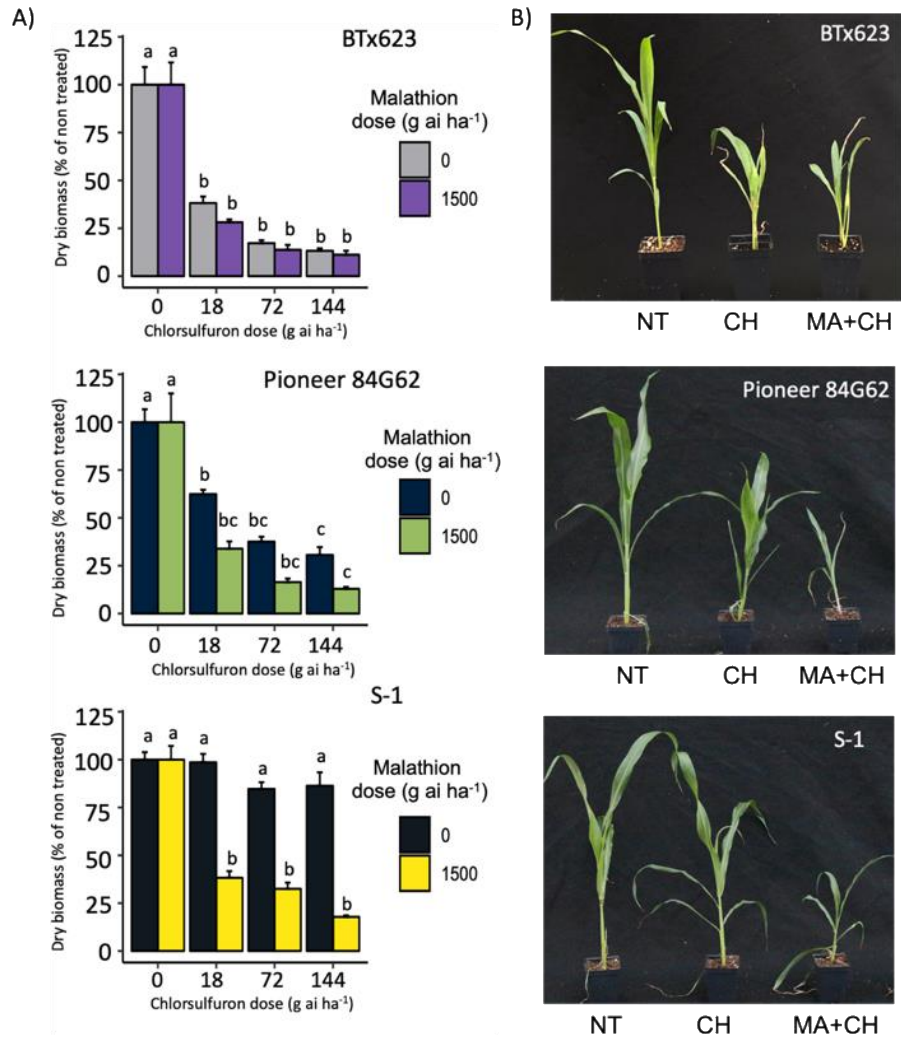
Figure 4.4 (A) Response of genotypes BTx623 (susceptible), Pioneer 84G62 (commercial hybrid), and S-1 (resistant) to field recommended dose chlorsulfuron (CH) or malathion followed by field recommended dose chlorsulfuron (MA+CH); NT: Non-treated at three weeks after treatment. B) Aboveground dry biomass of a: BTx623, b: Pioneer 84G62, and c: S-1, and when pre-treated with malathion or different doses chlorsulfuron. The error bars represent the standard error (n=8); different alphabets indicate a significant difference between treatments ($p \leq 0.05$)

Discussion

Herbicide options are limited for POST control of grass weeds in grain sorghum due to its susceptibility to commonly used grass control herbicides (Smith et al., 2010, Thompson et al., 2019). Chlorsulfuron is effective in controlling a broad spectrum of weeds in wheat, barley, and oats but is not registered for use in sorghum (Hageman and Behrens, 1981). POST application of chlorsulfuron was reported to reduce the shoot biomass, plant height, and panicle size of grain sorghum (Abusin et al., 2017; Hatzios, 1984). Chlorsulfuron residues in soils severely affect the growth, development, and yield of grain sorghum following a wheat crop (Leetch, 1985, Peterson and Eugene, 1986, Sharma et al., 2002). In this research, the results of chlorsulfuron dose-response experiments demonstrate that the sorghum genotype S-1 is ~20-fold more resistant to chlorsulfuron compared to the susceptible genotype BTx623 and commercial hybrid Pioneer 84G62 (Table 4.1). A sorghum hybrid that confers a high level of resistance to SU and IMI classes of ALS-inhibitors was developed previously by introgressing an altered *ALS*-gene from shattercane (Tuinstra and Al-Khatib, 2011). Further, they reported >100-fold resistance to nicosulfuron (SU) and imazethapyr (IMI) in ALS-resistant sorghum hybrid compared to BTx623 (Kershner, 2010). Generally, alterations in the *ALS* gene confer a very high level of resistance to ALS-inhibitors in plants (Devine and Shukla, 2000). Our results suggest no modifications in the *ALS* gene of S-1 sorghum (Fig. S1), conferring resistance to chlorsulfuron (Figure 1).

Response of S-1 Sorghum to HPPD-Inhibitor Treatment

Our data suggest that the S-1 is highly susceptible to mesotrione and tembotrione compared to cultivated hybrid Pioneer 84G62 (Table 4.2; Figure 4.2) nonetheless, highly resistant to chlorsulfuron (Figure 1), suggesting that the existence of this negative cross-resistance in S-1. Several cases of the evolution of such negative-cross resistance have been reported in weeds. Negative-cross resistance to

mesotrione and carfentrazone (protoporphyrinogen oxidase (PPO)-inhibitor) has been reported in kochia (*Bassia scoparia*) population with Trp-574 mutation that confer resistance to several ALS-inhibitor herbicides (Beckie and Tardif, 2012). A fluridone- (phytoene desaturase (PDS)- inhibitor)-resistant aquatic weed, hydrilla (*Hydrilla verticillata*) excreted negative cross-resistance to HPPD-inhibitors mesotrione and topramezone (Puri et al. 2009). Similarly, flixweed (*Descurainia sophia*) biotype with Pro-197-Ser mutation in *ALS*-gene conferring a high level of resistance to SU (halosulfuron, triasulfuron) and TP (flumetsulam, and penoxsulam) herbicides showed negative cross-resistance to IMI herbicide imazethapyr. The SU and TP resistant biotype exhibited ~2.5x - more sensitivity to imazethapyr compared to the biotype susceptible to all classes of ALS-inhibitor herbicides (Deng et al., 2014). Barnyard grass (*Echinochloa crus-galli*) population resistant to Photosystem II (PS II) -inhibitor herbicide atrazine exhibited ~1.5x more- sensitivity to chlorsulfuron relative to a biotype, susceptible to PS II- and ALS-inhibitors (Gadamski et al., 2000). Several weed biotypes resistant to atrazine exhibit negative cross-resistance to herbicides with different modes of action (De Prado et al., 1992, Gressel and Segel, 1990, Lopez-Martinez et al., 1997).

Inheritance of Chlorsulfuron Resistance

The F_1 and F_2 progeny response to chlorsulfuron in this study, suggest that a single dominant allele controls chlorsulfuron resistance in S-1 (Figure 4.1; Table 4.3). Both single and multigenic inheritance of chlorsulfuron was reported in crops and weeds. A single dominant allele controlling chlorsulfuron resistance was reported in kochia (*Bassia scoparia*; (Thompson et al., 1994). Single and two gene-mediated resistance was reported in rigid ryegrass (Busi et al., 2011). Nonetheless, multiple gene-mediated inheritance of chlorsulfuron resistance has also been reported in corn (Landi et al., 1990), rigid ryegrass (Han et al., 2014) , corn poppy (*Papaver rhoeas*; (Scarabel et al., 2004),

blackgrass (*Alopecurus myosuroides*; (Petit et al., 2010), oriental mustard (*Sisymbrium orientale*; (Preston and Malone, 2015). Based on F_2 phenotypes, it is clear that the chlorsulfuron resistance in S-1 is a dominant trait.

Assessment of Target Site or Non-Target Site Resistance to Chlorsulfuron

Mutations in the *ALS* gene conferring to chlorsulfuron and other ALS-inhibitors in weeds is documented (Nakka et al., 2017, Yu and Powles, 2014). However, our results did not reveal any previously reported mutations in the *ALS*-gene of S-1 sorghum (Fig. S1). The two weed species, shattercane, and johnsongrass, closely related to cultivated sorghum evolved natural resistance to ALS-inhibitors with alterations in the *ALS* gene resulting Trp-574-Leu substitution, which confers high levels of resistance to SU and IMI and SU herbicides (Werle et al., 2017a). Crops resistant to ALS-inhibitors were developed by artificially inducing genetic mutations in the *ALS* gene by random mutagenesis by ethyl methanesulfonate (EMS), somatic cell mutations, and site-directed mutagenesis (Shimizu et al., 2005).

The ability to metabolize chlorsulfuron by CYP enzyme activity has been documented in naturally tolerant crop species such as wheat, oats, and barley (Sweetser et al., 1982). Enhanced metabolism of herbicides by CYPs is one of the common non-target site resistance mechanisms in several crop and weed species (Werck-Reichhart et al., 2000; Siminszky, 2006; Jugulam and Shyam, 2019). CYP enzymes are involved in phase I metabolism of herbicides and other xenobiotic compounds in plants (Bolwell et al. 1994, Siminszky 2006). Several chemical compounds such as ABT, TET, PBO, tridiphane, and malathion can inhibit CYP activity; malathion, an organophosphate insecticide has been reported to cause highest level of CYP inhibition and reverse chlorsulfuron resistance compared to other CYP-inhibitors (Christopher et al., 1994, Tardif and Powles, 1999). Pre-treatment of

chlorsulfuron-resistant plants with malathion before herbicide treatment will partially or fully reverse the level of resistance (Baerg et al., 1996, Christopher et al., 1994, Kreuz and Fonné-Pfister, 1992). Reversal of resistance by CYP inhibitor treatment provides indirect evidence for metabolic resistance mediated by CYP activity. The S-1 genotype exhibited significant biomass reduction in response to pre-treatment with malathion followed by chlorsulfuron (Figure 4), suggesting that the chlorsulfuron is metabolized by CYPs in S-1. Similar results were reported in wheat, when field application of malathion tank-mixed with chlorsulfuron, resulted in the reversal of natural tolerance and caused chlorosis, stunting in early stages and reduced the yield up to 15 to 20% (Ferreira et al., 1990). In a different study, the application of chlorsulfuron within 72 h of malathion treatment significantly reduced the photosynthetic rate of wheat (Cink, 1986). Further, studies revealed that wheat CYP enzyme also metabolizes several SU herbicides, including chlorsulfuron by hydroxylation (Wen-Sheng et al., 2006, Xiang et al., 2006). In addition to crops, CYP-mediated resistance to chlorsulfuron was also found to be reversed with malathion treatment in several weed species. Malathion followed by chlorsulfuron treatment reduced resistance level up to ~70%, 56%, and 90% in rigid ryegrass (Christopher et al. 1994), Palmer amaranth (*Amaranthus palmeri*) (Nakka et al., 2017) and V*ulpia bromoides* (Yu et al., 2004).

Conclusion

In conclusion, the sorghum genotype S-1 confers a high level of metabolic resistance to chlorsulfuron controlled by a single dominant allele. There is an enormous potential to introgress the chlorsulfuron-resistance trait into agronomically desirable sorghum hybrids. The major advantage of using S-1 as a donor parent in an introgression breeding program is its susceptibility to other

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herbicides such as HPPD-inhibitors. Because in the event a commercial hybrid is developed using S-1

as a donor parent, even if there is a natural escape of the resistance trait into closely related weed

species of sorghum (e.g. shattercane or Sudangrassthe use of other herbicides such as HPPD-inhibitors

can control those weed species. Also, the chlorsulfuron-resistant trait can be effectively used to

develop hybrids/varieties with stacked resistance by combining traits such as resistance to acetyl-

coenzyme A carboxylase (ACCase)-inhibitors.

Chapter 5 - General Discussion, Conclusions, and Future Direction

Grain Sorghum is considered as one of the most resilient grain crops; however, POST grass weed control continues to be a major challenge, primarily due to lack of herbicide options. Several broadleaf and grass weeds infest grain sorghum and can cause huge yield loss. Although herbicides options for PRE and POST control of broadleaf weeds are available, most of the commercial grain sorghum hybrids are susceptible to POST grass control herbicides such as 4-Hydroxyphenylpyruvate dioxygenase (HPPD)-, acetolactate synthase (ALS)-, and acetyl-CoA carboxylase (ACCase)- inhibitors, (Smith et al., 2010, Thompson et al., 2019). Hence, grass weed control in sorghum is completely dependent on application of PRE herbicides. These PRE herbicides can be effective during the beginning of the season, but short soil residual will lead to poor control later in the growing season and cause significant yield loss (Table 1). Natural genetic diversity in plant species is the primary source of variation utilized in breeding varieties better adapted to different agricultural landscape. Sorghum is one of the highly diverse cereal crops (Glaszmann et al., 2010; Harlan and De Wet, 1972) with availability of extensive germplasm. Previously, varied levels of natural resistance to herbicides in sorghum genotypes, specifically resistance to mesotrione (Abit et al., 2009) and tembotrione (Dan et al., 2010) has been reported in grain sorghum.

The development of herbicide-resistant sorghum technology to facilitate broad-spectrum postemergence weed control can be an economical solution for the growers. Four sorghum genotypes two of each resistant to mesotrione (G-1, G-10) or tembotrione (G-200, G-350) and a susceptible genotype S-1 have been identified by screening ~900 sorghum genotypes from the sorghum association panel, sorghum mini-core collection, and Ethyl methanesulfonate (EMS) mutants (Varanasi, 2015).

Further investigation of the genotypes revealed that the susceptible genotype S-1 is highly resistant to ALS-inhibitor, chlorsulfuron (Pandian et al., in review).

In this dissertation, the mechanism and inheritance of resistance to HPPD- and ALS-inhibitors was investigated. Upon sequencing the *HPPD* and *ALS* gene, from sorghum genotypes resistant to mesotrione, tembotrione, or chlorsulfuron, no mutations that can alter the herbicide target protein were identified. The sorghum genotypes treated with cytochrome P450 (CYP) inhibitors malathion or PBO prior to mesotrione, tembotrione, or chlorsulfuron application exhibited a reduction in biomass accumulation suggesting CYP mediated metabolism of these herbicides. Even though alterations in the herbicide targets predominantly confer resistance in weeds, they are not naturally evolved in crops, potentially due to lack of herbicide selection pressure. Metabolism of herbicides is the most common mechanism by which certain crop species withstand herbicide applications (e.g. corn tolerance to HPPD- and ALS-inhibitors). In plants two enzyme families i.e., glutathione S-transferases (GSTs) and CYPs are known to play a key role in herbicide metabolism (Ohkawa et al., 1999). For example, metabolism of mesotrione has been reported in both crops and weeds such as corn (Meyer et al. 2010), Palmer amaranth (Nakka et al., 2017), and waterhemp (Oliveira et al., 2018); similarly tembotrione in corn (Paporisch and Rubin, 2017), and Palmer amaranth (Küpper et al., 2018) and waterhemp(Oliveira et al., 2018). CYP mediated metabolism of chlorsulfuron is the basis of selectivity in wheat (Wen-Sheng et al., 2006), the same has been reported in weeds such as ryegrass (Busi et al., 2011).

The CYP-mediated metabolism of herbicides can be controlled by both single (monogenic) or multiple (polygenic) genes. In this research, genetic analyses of F_1 and F_2 progenies developed by crossing herbicide-resistant and -susceptible genotypes revealed that resistance to mesotrione in G-1, G-10, and chlorsulfuron in S-1 is a dominant- monogenic trait. Whereas, tembotrione metabolism in G-200 and G-350 was controlled by a semi-dominant polygenic trait. Similarly, dominant-monogenic

inheritance of mesotrione resistance has been reported in rice (Lee et al., 2018). In contrast, in corn dominant-polygenic inheritance of mesotrione resistance was reported (Williams et al., 2008), whereas tembotrione resistance is controlled by a single recessive gene (Williams and Pataky, 2008). However, partially dominant polygenic inheritance was reported for both mesotrione and tembotrione resistance in waterhemp (Oliveira et al., 2018; Huffman et al., 2015). Both monogenic and polygenic inheritance of chlorsulfuron resistance were reported. Single and two gene inheritance of chlorsulfuron resistance was reported in kochia (Thompson et al., 1994) and ryegrass (Busi et al., 2011), respectively. Nonetheless, the polygenic inheritance of chlorsulfuron resistance has also been reported in corn (Landi et al., 1990), rigid ryegrass (Han et al., 2014), corn poppy (*Papaver rhoeas*) (Scarabel et al., 2004), blackgrass (*Alopecurus myosuroides*) (Petit et al. 2010), oriental mustard (*Sisymbrium orientale*) (Preston and Malone, 2015).

Bulk segregant analysis (BSA) was used to map the mesotrione resistance gene using biparental F_2 populations derived from crossing S-1 with G-1 and G-10. However, the sequence analyses did not precisely map any genomic region involved in resistance. Previously, BSR-Seq has been used to precisely identify CYP81A9 responsible for nicosulfuron resistance in corn (Liu et al., 2019). A Genotyping by sequencing (GBS)-based quantitative trait loci (QTL) mapping was used to map six QTLs associated with tembotrione resistance in G-200. This is a widely used trait mapping method in crops. Using GBS, QTLs involved in several biotic and abiotic stress resistance were mapped QTLs (Sanchez et al., 2002; Tao et al., 2003; Punnuri et al., 2013). A QTL associated with mesotrione and/or isoxazole herbicide resistance was also identified in soybean (Bogner et al., 2018).

In conclusion, the CYP-inhibitor assay suggested the CYP-mediated metabolism of mesotrione, tembotrione, and chlorsulfuron in the sorghum genotypes resistant to these herbicides. Genetic analyses of F_1 and F_2 progeny demonstrated that the mesotrione and chlorsulfuron resistance is controlled by a

single major gene whereas tembotrione resistance is a partially dominant polygenic trait. Furthermore, BSR-Seq was not successful in identifying the genetic basis of mesotrione resistance, however, the GBS-based QTL mapping revealed three QTLs associated with tembotrione resistance in grain sorghum.

Future research needs to be focused on identifying the precise genomic region associated with mesotrione resistance using a GBS-based mapping strategy; further, specific gene(s) involved in mesotrione resistance will be identified by deploying fine mapping techniques. The identified QTLs regions responsible for tembotrione resistance needs to be validated and tested in multiple environments with more number of F_2 plants and markers to improve the estimation accuracy. Similar GBS-based mapping technique will be administered to map the chlorsulfuron resistance gene in S-1. Upon identifying the precise genomic regions markers need to be developed to enable introgression of the resistant trait into elite breeding lines through marker assisted breeding; the elite breeding lines can be used to generate high yielding hybrids with herbicide resistance. Based on the results from this research, there is an enormous potential to introgress the mesotrione, tembotrione, and chlorsulfuron-resistance trait into agronomically desirable sorghum hybrids. Also, these traits can be effectively used to develop hybrids/varieties with stacked resistance by combining traits such as resistance to ACCase-inhibitors.

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Appendix A - Supplementary Material

Figure S2.1. Sequence alignment of 4-Hydroxyphenylpyruvate dioxygenase (*HPPD*) gene from resistant genotypes G-1, G-10, susceptible genotype S-1

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Sobic.002G104200    TAACCATACATATATAACACAGTTTCACAAGCTCATCAACTGCTGAAACCTGCAGACACG    60
G-1                 TAACCATACATATATAACACAGTTTCACAAGCTCATCAACTGCTGAAACCTGCAGACACG    60
G-10                TAACCATACATATATAACACAGTTTCACAAGCTCATCAACTGCTGAAACCTGCAGACACG    60
S-1                 TAACCATACATATATAACACAGTTTCACAAGCTCATCAACTGCTGAAACCTGCAGACACG    60
                    ************************************************************

Sobic.002G104200    ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA   120
G-1                 ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA   120
G-10                ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA   120
S-1                 ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA   120
                    ************************************************************

Sobic.002G104200    AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG   180
G-1                 AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG   180
G-10                AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG   180
S-1                 AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG   180
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Sobic.002G104200    CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA   240
G-1                 CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA   240
G-10                CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA   240
S-1                 CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA   240
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Sobic.002G104200    ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC   300
G-1                 ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC   300
G-10                ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC   300
S-1                 ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC   300
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Sobic.002G104200    ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC   360
G-1                 ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC   360
G-10                ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC   360
S-1                 ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC   360
                    ************************************************************

Sobic.002G104200    GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC   420
G-1                 GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC   420
G-10                GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC   420
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97

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S-1              GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC      420
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Sobic.002G104200 CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC      480
G-1              CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC      480
G-10             CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC      480
S-1              CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC      480
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Sobic.002G104200 GCGCTCCTCTTCACCGCCCCGTACGCGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA      540
G-1              GCGCTCCTCTTCACCGCCCCGTACGCGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA      540
G-10             GCGCTCCTCTTCACCGCCCCGTACGCGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA      540
S-1              GCGCTCCTCTTCACCGCCCCGTACGCGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA      540
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Sobic.002G104200 ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC      600
G-1              ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC      600
G-10             ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC      600
S-1              ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC      600
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Sobic.002G104200 GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      660
G-1              GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      660
G-10             GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      660
S-1              GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      660
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Sobic.002G104200 GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      720
G-1              GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      720
G-10             GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      720
S-1              GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      720
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Sobic.002G104200 CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      780
G-1              CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      780
G-10             CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      780
S-1              CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      780
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Sobic.002G104200 TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      840
G-1              TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      840
G-10             TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      840
S-1              TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      840
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Sobic.002G104200 TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      900
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98

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G-1                    TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      900
G-10                   TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      900
S-1                    TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      900
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Sobic.002G104200       CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      960
G-1                    CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      960
G-10                   CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      960
S-1                    CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      960
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Sobic.002G104200       AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG     1020
G-1                    AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG     1020
G-10                   AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG     1020
S-1                    AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG     1020
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Sobic.002G104200       GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG     1080
G-1                    GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG     1080
G-10                   GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG     1080
S-1                    GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG     1080
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Sobic.002G104200       AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG     1140
G-1                    AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG     1140
G-10                   AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG     1140
S-1                    AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG     1140
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Sobic.002G104200       ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC     1200
G-1                    ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC     1200
G-10                   ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC     1200
S-1                    ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC     1200
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Sobic.002G104200       TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG     1260
G-1                    TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG     1260
G-10                   TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG     1260
S-1                    TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG     1260
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Sobic.002G104200       GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG     1320
G-1                    GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG     1320
G-10                   GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG     1320
S-1                    GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG     1320
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99

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Sobic.002G104200      GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGTAATAA      1380
G-1                   GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGTAATAA      1380
G-10                  GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGTAATAA      1380
S-1                   GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGTAATAA      1380
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Sobic.002G104200      TAATCGCGTCCTTTGCATGCATGTTTCTGTTTTGACAATATAAATCGTCGACTAATCCCA      1440
G-1                   TAATCGCGTCCTTTGCATGCATGTTTCTGTTTTGACAATATAAATCGTCGACTAATCCCA      1440
G-10                  TAATCGCGTCCTTTGCATGCATGTTTCTGTTTTGACAATATAAATCGTCGACTAATCCCA      1440
S-1                   TAATCGCGTCCTTTGCATGCATGTTTCTGTTTTGACAATATAAATCGTCGACTAATCCCA      1440
                      ************************************************************


Sobic.002G104200      TCCATGTTCGCCTACTATAAAATATATAAACAACCTACTGCAGTACAAAAGAATCTATCA      1500
G-1                   TCCATGTTCGCCTACTATAAAATATATAAACAACCTACTGCAGTACAAAAGAATCTATCA      1500
G-10                  TCCATGTTCGCCTACTATAAAATATATAAACAACCTACTGCAGTACAAAAGAATCTATCA      1500
S-1                   TCCATGTTCGCCTACTATAAAATATATAAACAACCTACTGCAGTACAAAAGAATCTATCA      1500
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Sobic.002G104200      TTTATAATTTTTCTTTTCTTTGCGGGTATTCATTATTTCTCATAAAAAAAAGCTATAACA      1560
G-1                   TTTATAATTTTTCTTTTCTTTGCGGGTATTCATTATTTCTCATAAAAAAAAGCTATAACA      1560
G-10                  TTTATAATTTTTCTTTTCTTTGCGGGTATTCATTATTTCTCATAAAAAAAAGCTATAACA      1560
S-1                   TTTATAATTTTTCTTTTCTTTGCGGGTATTCATTATTTCTCATAAAAAAAAGCTATAACA      1560
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Sobic.002G104200      ACGAAACGTGTTACATATGCATGTTGACTTTAATCTTAACTGAGAATCTCGAAAAAAATA      1620
G-1                   ACGAAACGTGTTACATATGCATGTTGACTTTAATCTTAACTGAGAATCTCGAAAAAAATA      1620
G-10                  ACGAAACGTGTTACATATGCATGTTGACTTTAATCTTAACTGAGAATCTCGAAAAAAATA      1620
S-1                   ACGAAACGTGTTACATATGCATGTTGACTTTAATCTTAACTGAGAATCTCGAAAAAAATA      1620
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Sobic.002G104200      GCTAAAAAGACTAGCCAAATTTATTAATTTAGATATTTTATAAATTAAAATTTTTGATAA      1680
G-1                   GCTAAAAAGACTAGCCAAATTTATTAATTTAGATATTTTATAAATTAAAATTTTTGATAA      1680
G-10                  GCTAAAAAGACTAGCCAAATTTATTAATTTAGATATTTTATAAATTAAAATTTTTGATAA      1680
S-1                   GCTAAAAAGACTAGCCAAATTTATTAATTTAGATATTTTATAAATTAAAATTTTTGATAA      1680
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Sobic.002G104200      AAAAAATACTAGGCTACTCGAACAGCTATAAAACCAAGAGATTGAATGTAGCTTATCCAA      1740
G-1                   AAAAAATACTAGGCTACTCGAACAGCTATAAAACCAAGAGATTGAATGTAGCTTATCCAA      1740
G-10                  AAAAAATACTAGGCTACTCGAACAGCTATAAAACCAAGAGATTGAATGTAGCTTATCCAA      1740
S-1                   AAAAAATACTAGGCTACTCGAACAGCTATAAAACCAAGAGATTGAATGTAGCTTATCCAA      1740
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Sobic.002G104200      ACACAGAGAATAAATAAAATGAGATAGAAAGCTACTAAATTTAGCTAATAAATAATATAG      1800
G-1                   ACACAGAGAATAAATAAAATGAGATAGAAAGCTACTAAATTTAGCTAATAAATAATATAG      1800
G-10                  ACACAGAGAATAAATAAAATGAGATAGAAAGCTACTAAATTTAGCTAATAAATAATATAG      1800
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100

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S-1                    ACACAGAGAATAAATAAAATGAGATAGAAAGCTACTAAATTTAGCTAATAAATAATATAG        1800
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Sobic.002G104200       AAAATAATTTAGCTAATTTCTTGAAGATGCTTTTACATCCCTATTCTCTATTCTAGTACT        1860
G-1                    AAAATAATTTAGCTAATTTCTTGAAGATGCTTTTACATCCCTATTCTCTATTCTAGTACT        1860
G-10                   AAAATAATTTAGCTAATTTCTTGAAGATGCTTTTACATCCCTATTCTCTATTCTAGTACT        1860
S-1                    AAAATAATTTAGCTAATTTCTTGAAGATGCTTTTACATCCCTATTCTCTATTCTAGTACT        1860
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Sobic.002G104200       ACAAGAACACAAGGTACTACTCTTTCATTCTAAATTATAATAATTTTGTTTAAACATCTT        1920
G-1                    ACAAGAACACAAGGTACTACTCTTTCATTCTAAATTATAATAATTTTGTTTAAACATCTT        1920
G-10                   ACAAGAACACAAGGTACTACTCTTTCATTCTAAATTATAATAATTTTGTTTAAACATCTT        1920
S-1                    ACAAGAACACAAGGTACTACTCTTTCATTCTAAATTATAATAATTTTGTTTAAACATCTT        1920
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Sobic.002G104200       GACATAGTATATATTTAAATGTATAATAAAAATAATGTAAACCTAAATTTATAATTCGAA        1980
G-1                    GACATAGTATATATTTAAATGTATAATAAAAATAATGTAAACCTAAATTTATAATTCGAA        1980
G-10                   GACATAGTATATATTTAAATGTATAATAAAAATAATGTAAACCTAAATTTATAATTCGAA        1980
S-1                    GACATAGTATATATTTAAATGTATAATAAAAATAATGTAAACCTAAATTTATAATTCGAA        1980
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Sobic.002G104200       ATGGAAGTAATGTCTTTTTTATTTTTCCTTGAAAAAAAGGTTACAATAAAACGTGTTATA        2040
G-1                    ATGGAAGTAATGTCTTTTTTATTTTTCCTTGAAAAAAAGGTTACAATAAAACGTGTTATA        2040
G-10                   ATGGAAGTAATGTCTTTTTTATTTTTCCTTGAAAAAAAGGTTACAATAAAACGTGTTATA        2040
S-1                    ATGGAAGTAATGTCTTTTTTATTTTTCCTTGAAAAAAAGGTTACAATAAAACGTGTTATA        2040
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Sobic.002G104200       TATGCAGGCCAACCTTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATG        2100
G-1                    TATGCAGGCCAACCTTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATG        2100
G-10                   TATGCAGGCCAACCTTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATG        2100
S-1                    TATGCAGGCCAACCTTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATG        2100
                       ************************************************************


Sobic.002G104200       AGAACGGGAAGGAATACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTC        2160
G-1                    AGAACGGGAAGGAATACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTC        2160
G-10                   AGAACGGGAAGGAATACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTC        2160
S-1                    AGAACGGGAAGGAATACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTC        2160
                       ************************************************************


Sobic.002G104200       ATTTGGTGAAATCCATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTT        2220
G-1                    ATTTGGTGAAATCCATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTT        2220
G-10                   ATTTGGTGAAATCCATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTT        2220
S-1                    ATTTGGTGAAATCCATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTT        2220
                       ************************************************************


Sobic.002G104200       CCTAAACAACACTGTACTGTCATCTCTCTGCTTAATAAGGAGTTACTATA 2270
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G-1              CCTAAACAACACTGTACTGTCATCTCTCTGCTTAATAAGGAGTTACTATA 2270
G-10             CCTAAACAACACTGTACTGTCATCTCTCTGCTTAATAAGGAGTTACTATA 2270
S-1              CCTAAACAACACTGTACTGTCATCTCTCTGCTTAATAAGGAGTTACTATA 2270
                 **************************************************
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Figure S3.1. Sequence alignment of 4-Hydroxyphenylpyruvate dioxygenase (*HPPD*) gene from resistant genotypes G-200, G-350, and susceptible genotype S-1

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Sobic.002G104200.1    ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA    60
G-200                 ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA    60
G-350                 ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA    60
S-1                   ATGAATGCCCATGCCCACCCAGTTGTGGCCTCCACGGCGTTCGCCCATGCCCTCCTGCCA    60
                      ************************************************************

Sobic.002G104200.1    AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG    120
G-200                 AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG    120
G-350                 AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG    120
S-1                   AACTCCAGCAGCATTCGATCACGACGCACCCCACTCCGCCCAATTTTTGCAGTGCCGCCG    120
                      ************************************************************

Sobic.002G104200.1    CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA    180
G-200                 CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA    180
G-350                 CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA    180
S-1                   CCAGCCCTTGGACAACTCCCGACGACTCTCCGAGCAACAACCAAGACGCTTCTGCACCCA    180
                      ************************************************************

Sobic.002G104200.1    ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC    240
G-200                 ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC    240
G-350                 ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC    240
S-1                   ACGACGACGCCGCCCGTCGTCACCACCACCGCTGACCGTGCTGAAAGCGTTTTCGCCGGC    240
                      ************************************************************

Sobic.002G104200.1    ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC    300
G-200                 ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC    300
G-350                 ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC    300
S-1                   ACCGGCGACCGCTTCCACGTGATGGACTTCCACCACGTCGAGTTCTGGTGCGCCGACGCC    300
                      ************************************************************

Sobic.002G104200.1    GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC    360
G-200                 GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC    360
G-350                 GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC    360
S-1                   GCCTCCGCTGCCGGCCGCTTCTCCTTCGCGCTCGGCGTGCCACTCGCCGCGCAGTCCGAC    360
                      ************************************************************

Sobic.002G104200.1    CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC    420
G-200                 CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC    420
G-350                 CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC    420
S-1                   CTCACCACGGGGAACACCGCGCACGCTTCCCGCCTGCTGCGGTCGCGCTCTGGACCTCTC    420
                      ************************************************************

Sobic.002G104200.1    GCGCTCCTCTTCACCGCCCCGTACGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA    480
G-200                 GCGCTCCTCTTCACCGCCCCGTACGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA    480
G-350                 GCGCTCCTCTTCACCGCCCCGTACGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA    480
S-1                   GCGCTCCTCTTCACCGCCCCGTACGCGCGGCACATCGGCGCCCGCCCCGACGCCGACGCA    480
                      ************************************************************

Sobic.002G104200.1    ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC    540
G-200                 ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC    540
G-350                 ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC    540
S-1                   ACGTCCGCCTCCTCCCCCGTGGTGCCCTCCTTCTCGGCCGACGCCGCGCGCCGCTTCGCC    540
                      ************************************************************

Sobic.002G104200.1    GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG    600
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G-200               GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      600
G-350               GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      600
S-1                 GCCGACTACGGCGGCCTCGCGGTGCGCGCCGTCGCGGTCCGTGTCTCCGACGCCGCCGAG      600
                    ************************************************************


Sobic.002G104200.1  GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      660
G-200               GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      660
G-350                GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      660
S-1                 GCGTTCCGCGCCAGCGTCGCCGCGGGTGCGCGCCCGGCCTTCGCTCCCGCTGAGCTCGGC      660
                    ************************************************************


Sobic.002G104200.1  CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      720
G-200               CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      720
G-350               CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      720
S-1                 CACGGCTTCGTGTTTGCCGAAGTCGAGCTCTACGGAGACGCCGTCCTCCGTTTCGTGAGC      720
                    ************************************************************


Sobic.002G104200.1  TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      780
G-200               TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      780
G-350               TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      780
S-1                 TACCCGGACGACACGGGCGGCGTGGCCTTCCTCCCCGGGTTCGAGAACGTCGCAAACTCA      780
                    ************************************************************


Sobic.002G104200.1  TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      840
G-200               TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      840
G-350               TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      840
S-1                 TCAGCGTGCCCGGCGCCGGACTACGGACTCAACCGGTTCGACCACATCGTCGGCGGCGTG      840
                    ************************************************************


Sobic.002G104200.1  CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      900
G-200               CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      900
G-350               CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      900
S-1                 CCGGACCTGGCTCCGGTCGCCGCGTACATCGCCGGCTTCACGGGCTTCCACGAATTCGAC      900
                    ************************************************************


Sobic.002G104200.1  AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG      960
G-200               AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG      960
G-350               AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG      960
S-1                 AGGGTCAACGGCGACGAAATAGGCACGGCCGAGAGCTCGCTCAACGGCCTGGTGCTGGCG       960
                    ************************************************************


Sobic.002G104200.1  GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG      1020
G-200              GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG      1020
G-350               GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG      1020
S-1                 GACAGCTCGGAGAAGGTGCTCCTCACGCTGCTGGAGCCGGTGCAGGGCACCAAGCGCCGG      1020
                    ************************************************************


Sobic.002G104200.1  AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG      1080
G-200               AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG      1080
G-350               AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG      1080
S-1                 AGCCAGATACAGACGTTCCTGGACCACCATGGCGGGCCAGGAGTGCAGCACCTGGCCATG      1080
                    ************************************************************


Sobic.002G104200.1  ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC      1140
G-200               ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC      1140
G-350               ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC      1140
S-1                 ACCAGTGACGACCTTCTCGGCACGCTGAGGGAGATACGTGCGCGGTCCTCCATGGGCGGC      1140
                    ************************************************************
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Sobic.002G104200.1    TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG          1200
G-200                 TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG          1200
G-350                 TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG          1200
S-1                   TTCGAGCTCCTGCCACCGCCGCCGCCCAGCTACTATGACGGCGTAAAGCGGCTCGCCGGG          1200
                      ************************************************************

Sobic.002G104200.1     GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG         1260
G-200                  GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG         1260
G-350                 GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG          1260
S-1                   GATGTGCTGTCGGAGGCGCAGATTAACGAGTGCCAAGAGCTCGGCGTGCGGGTGGACAGG          1260
                      ************************************************************

Sobic.002G104200.1    GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGCCAACC          1320
G-200                 GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGCCAACC          1320
G-350                 GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGCCAACC          1320
S-1                   GCTGACAATGGCGGAGTTGTGCTCCAAACCTTCACCAAGGCTGCTGGAGACAGGCCAACC          1320
                      ************************************************************

Sobic.002G104200.1    TTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATGAGAACGGGAAGGAA          1380
G-200                 TTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATGAGAACGGGAAGGAA          1380
G-350                 TTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATGAGAACGGGAAGGAA          1380
S-1                   TTGCTCTTGGAGTTTATCCAGAGGATCGGCTGCGTGGAGATAGATGAGAACGGGAAGGAA          1380
                      ************************************************************

Sobic.002G104200.1    TACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTCATTTGGTGAAATCC          1440
G-200                 TACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTCATTTGGTGAAATCC          1440
G-350                 TACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTCATTTGGTGAAATCC          1440
S-1                   TACCAGAGGGGTGGCTGCGGCGGTTTTGCCAAGGATAACGTCATTCATTTGGTGAAATCC          1440
                      ************************************************************

Sobic.002G104200.1    ATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTTCCTAA          1491
G-200                 ATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTTCCTAA          1491
G-350                 ATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTTCCTAA          1491
S-1                   ATTGAGGACTATGACAAAACTCTTGACGCTCCTGCCCATGTGGCTTCCTAA          1491
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Figure S3.2. Linkage map obtained from 606 markers spread across sorghum genome, Genetic distances are indicated on the left side of linkage group in centiMorgans (cM), and the marker names are shown on the right side.
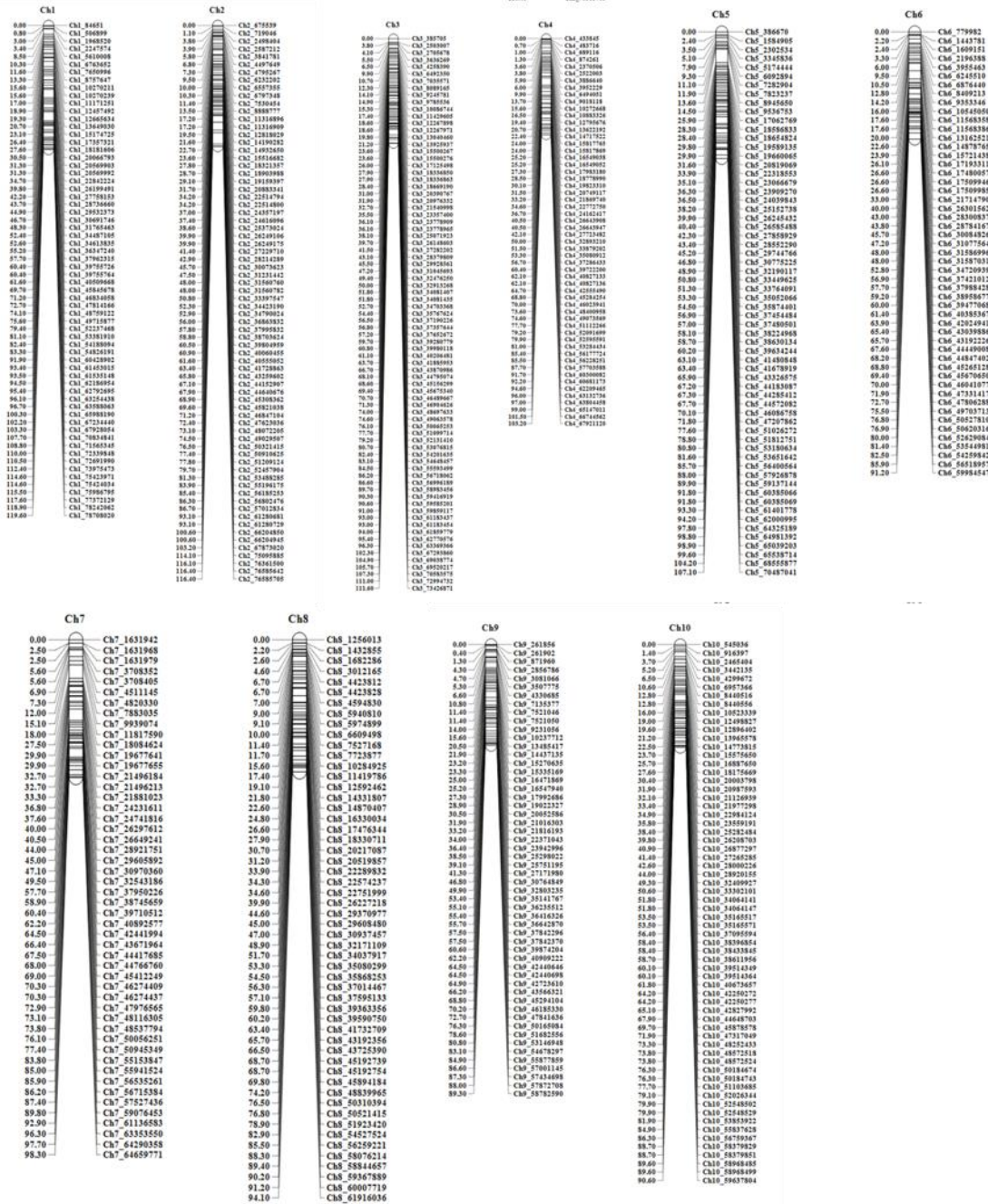
Figure S3.3. Phenotypic distribution of A) S-1 x G-350 F$_2$ progeny and B) parents for Recovery (RE); and Visual injury at four weeks after treatment (VI).
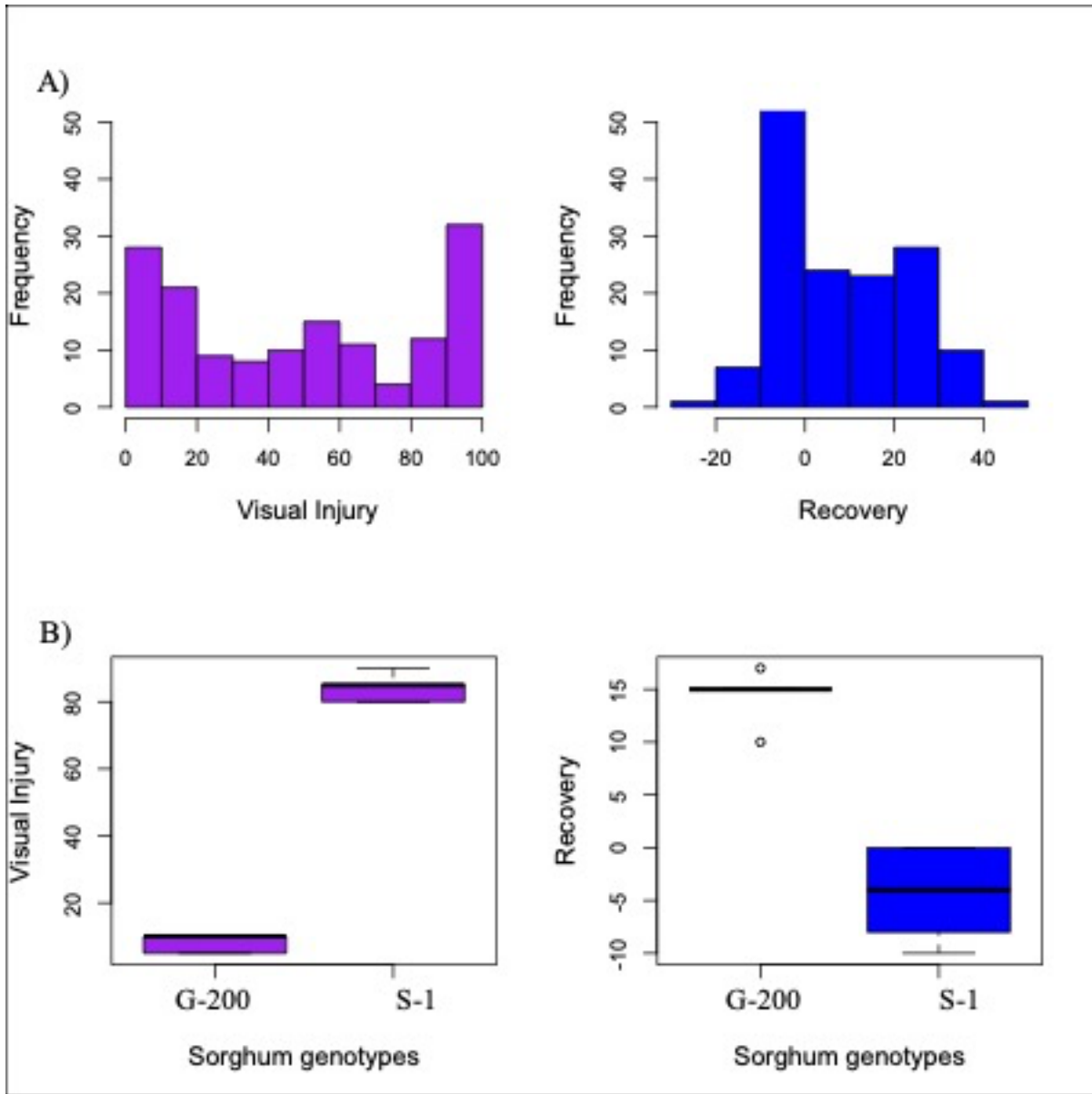
Figure S4.1 Sequence alignment of BTx623 (susceptible), S-1(resistant) and coding sequence
(CDS) of sorghum *ALS*-gene (Sobic.004G155800.2)

```
Sobic.004G155800.2    ATGGCCACCACCGCCGCCGCCGCTGCCGCCGCGCTAGCCGGCGCCACTACCGCTGCGCCC    60
BTx623                ATGGCCACCACCGCCGCCGCCGCTGCCGCCGCGCTAGCCGGCGCCACTACCGCTGCGCCC    60
S-1                   ATGGCCACCACCGCCGCCGCCGCTGCCGCCGCGCTAGCCGGCGCCACTACCGCTGCGCCC    60
                      ************************************************************

Sobic.004G155800.2    AAGGCGAGGCGCCGGGCGCACCTCCTGGCCGCACGGCGCGCCCTCGCCGCGCCCATCAGG    120
BTx623                AAGGCGAGGCGCCGGGCGCACCTCCTGGCCGCACGGCGCGCCCTCGCCGCGCCCATCAGG    120
S-1                   AAGGCGAGGCGCCGGGCGCACCTCCTGGCCGCACGGCGCGCCCTCGCCGCGCCCATCAGG    120
                      ************************************************************

Sobic.004G155800.2    TGCTCAGCGGCGCCACCCGCCACGCTGACGGTGACGGCTCCCCCGGCCACCCCGCTCCGG    180
BTx623                TGCTCAGCGGCGCCACCCGCCACGCTGACGGTGACGGCTCCCCCGGCCACCCCGCTCCGG    180
S-1                   TGCTCAGCGGCGCCACCCGCCACGCTGACGGTGACGGCTCCCCCGGCCACCCCGCTCCGG    180
                      ************************************************************

Sobic.004G155800.2    CCGTGGGGCCCCACCGATCCCCGCAAGGGCGCCGACATCCTCGTCGAGGCTCTTGAGCGC    240
BTx623                CCGTGGGGCCCCACCGATCCCCGCAAGGGCGCCGACATCCTCGTCGAGGCTCTTGAGCGC    240
S-1                   CCGTGGGGCCCCACCGATCCCCGCAAGGGCGCCGACATCCTCGTCGAGGCTCTTGAGCGC    240
                      ************************************************************

Sobic.004G155800.2    TGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGCGTCCATGGAGATCCACCAGGCA    300
BTx623                TGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGCGTCCATGGAGATCCACCAGGCA    300
S-1                   TGCGGCGTCCGCGACGTCTTCGCCTACCCCGGCGGCGCGTCCATGGAGATCCACCAGGCA    300
                      ************************************************************

Sobic.004G155800.2    CTCACCCGTTCCCCCGTCATCGCCAACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTC    360
BTx623                CTCACCCGTTCCCCCGTCATCGCCAACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTC    360
S-1                   CYCMCCCGTTCCCCCGTCATCGCCAACCACCTCTTCCGCCACGAGCAAGGGGAGGCCTTC    356
                      * * ********************************************************

Sobic.004G155800.2    GCCGCCTCTGGCTTCGCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGC    420
BTx623                GCCGCCTCTGGCTTCGCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGC    420
S-1                   GCCGCCTCTGGCTTCGCGCGCTCCTCGGGCCGCGTCGGCGTCTGCGTCGCCACCTCCGGC    416
                      ************************************************************

Sobic.004G155800.2    CCCGGCGCCACCAACCTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATG    480
BTx623                CCCGGCGCCACCAACCTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATG    480
S-1                   CCCGGCGCCACCAACCTAGTCTCCGCGCTCGCCGACGCGCTGCTCGACTCCGTCCCCATG    476
                      ************************************************************
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Sobic.004G155800.2    GTCGCCATCACGGGACAGGTTCCGCGGCGCATGATTGGCACCGACGCCTTCCAGGAGACG    540
BTx623                GTCGCCATCACGGGACAGGTTCCGCGGCGCATGATTGGCACCGACGCCTTCCAGGAGACG    540
S-1                   GTCGCCATCACGGGACAGGTTCCGCGGCGCATGATTGGCACCGACGCCTTCCAGGAGACG    536
                      ************************************************************


Sobic.004G155800.2    CCCATCGTCGAGGTCACCCGCTCCATCACCAAACATAACTACCTGGTCCTCGACGTCGAC    600
BTx623                CCCATCGTCGAGGTCACCCGCTCCATCACCAAACATAACTACCTGGTCCTCGACGTCGAC    600
S-1                   CCCATCGTCGAGGTCACCCGCTCCATCACCAAACATAACTACCTGGTCCTCGACGTCGAC    596
                      ************************************************************


Sobic.004G155800.2    GACATCCCCCGCGTCGTGCAGGAGGCTTTCTTCCTCGCCTCCTCCGGTCGCCCGGGACCG    660
BTx623                GACATCCCCCGCGTCGTGCAGGAGGCTTTCTTCCTCGCCTCCTCCGGTCGCCCGGGACCG    660
S-1                   GACATCCCCCGCGTCGTGCAGGAGGCTTTCTTCCTCGCCTCCTCCGGTCGCCCGGGACCG    656
                      ************************************************************


Sobic.004G155800.2    GTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCCGTGCCGGTCTGGGACACG    720
BTx623                GTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCCGTGCCGGTCTGGGACACG    720
S-1                   GTGCTTGTCGACATCCCCAAGGACATCCAGCAGCAGATGGCCGTGCCGGTCTGGGACACG    716
                      ************************************************************


Sobic.004G155800.2    CCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTT    780
BTx623                CCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTT    780
S-1                   CCCATGAGTCTGCCTGGGTACATTGCGCGCCTTCCCAAGCCTCCTGCGACTGAATTGCTT    776
                      ************************************************************


Sobic.004G155800.2    GAGCAGGTGCTGCGTCTTGTTGGTGAATCAAGGCGCCCTGTTCTTTATGTTGGTGGTGGC    840
BTx623                GAGCAGGTGCTGCGTCTTGTTGGTGAATCAAGGCGCCCTGTTCTTTATGTTGGTGGTGGC    840
S-1                   GAGCAGGTGCTGCGTCTTGTTGGTGAATCAAGGCGCCCTGTTCTTTATGTTGGTGGTGGC    836
                      ************************************************************


Sobic.004G155800.2    TGCGCAGCATCTGGCGAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACA    900
BTx623                TGCGCAGCATCTGGCGAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACA    900
S-1                   TGCGCAGCATCTGGCGAGGAGTTGCGCCGCTTTGTGGAGATGACTGGAATCCCAGTCACA    896
                      ************************************************************


Sobic.004G155800.2    ACTACTCTTATGGGCCTTGGCAATTTCCCTGGCGACGACCCACTGTCTCTGCGCATGCTT    960
BTx623                ACTACTCTTATGGGCCTTGGCAATTTCCCTGGCGACGACCCACTGTCTCTGCGCATGCTT    960
S-1                   ACTACTCTTATGGGCCTTGGCAATTTCCCTGGCGACGACCCACTGTCTCTGCGCATGCTT    956
                      ************************************************************


Sobic.004G155800.2    GGTATGCATGGCACGGTGTATGCAAATTATGCAGTGGATAAGGCGGATCTGTTGCTTGCA    1020
BTx623                GGTATGCATGGCACGGTGTATGCAAATTATGCAGTGGATAAGGCGGATCTGTTGCTTGCA    1020
S-1                   GGTATGCATGGCACGGTGTATGCAAATTATGCAGTGGATAAGGCGGATCTGTTGCTTGCA    1016
                      ************************************************************


Sobic.004G155800.2    TTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCT    1080
BTx623                TTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCT    1080
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109

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S-1                    TTTGGTGTGCGGTTTGATGATCGTGTGACAGGGAAGATTGAGGCTTTTGCAAGCAGGGCT      1076
                       ************************************************************


Sobic.004G155800.2     AAGATTGTGCACATTGATATTGATCCCGCTGAGATTGGCAAGAACAAGCAGCCACATGTG      1140
BTx623                 AAGATTGTGCACATTGATATTGATCCCGCTGAGATTGGCAAGAACAAGCAGCCACATGTG      1140
S-1                    AAGATTGTGCACATTGATATTGATCCCGCTGAGATTGGCAAGAACAAGCAGCCACATGTG      1136
                       ************************************************************


Sobic.004G155800.2     TCCATCTGTGCAGACGTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGC      1200
BTx623                 TCCATCTGTGCAGACGTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGC      1200
S-1                    TCCATCTGTGCAGACGTTAAGCTTGCTTTGCAGGGCATGAATGCTCTTCTGGAAGGAAGC      1196
                       ************************************************************


Sobic.004G155800.2     ACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCAAGCTGAGTTGGATCAGCAGAAGAGA      1260
BTx623                 ACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCAAGCTGAGTTGGATCAGCAGAAGAGA      1260
S-1                    ACATCAAAGAAGAGCTTTGACTTTGGCTCATGGCAAGCTGAGTTGGATCAGCAGAAGAGA      1256
                       ************************************************************


Sobic.004G155800.2     GAGTTCCCCCTTGGGTATAAAACTTTTGATGACGAGATCCAGCCACAATATGCTATTCAG      1320
BTx623                 GAGTTCCCCCTTGGGTATAAAACTTTTGATGACGAGATCCAGCCACAATATGCTATTCAG      1320
S-1                    GAGTTCCCCCTTGGGTATAAAACTTTTGATGACGAGATCCAGCCACAATATGCTATTCAG      1316
                       ************************************************************


Sobic.004G155800.2     GTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAG      1380
BTx623                 GTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAG      1380
S-1                    GTTCTTGATGAGCTGACAAAAGGGGAGGCCATCATTGCCACAGGTGTTGGGCAGCACCAG      1376
                       ************************************************************


Sobic.004G155800.2     ATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCAGCTGGT      1440
BTx623                 ATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCAGCTGGT      1440
S-1                    ATGTGGGCGGCACAGTACTACACTTACAAGCGGCCAAGGCAGTGGTTGTCTTCAGCTGGT      1436
                       ************************************************************


Sobic.004G155800.2     CTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGT      1500
BTx623                 CTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGT      1500
S-1                    CTTGGGGCTATGGGATTTGGTTTGCCGGCTGCTGCTGGCGCTGCTGTGGCCAACCCAGGT      1496
                       ************************************************************


Sobic.004G155800.2     ATCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCT      1560
BTx623                 ATCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCT      1560
S-1                    ATCACTGTTGTTGACATCGACGGAGATGGTAGCTTCCTCATGAACATTCAGGAGCTAGCT      1556
                       ************************************************************


Sobic.004G155800.2     ATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGG      1620
BTx623                 ATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGG      1620
S-1                    ATGATCCGAATTGAGAACCTCCCAGTGAAGGTCTTTGTGCTAAACAACCAGCACCTGGGG      1616
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110

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Sobic.004G155800.2    ATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAATAGAGCACACACATACTTGGGA    1680
BTx623                ATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAATAGAGCACACACATACTTGGGA    1680
S-1                   ATGGTGGTGCAGTGGGAGGACAGGTTCTATAAGGCCAATAGAGCACACACATACTTGGGA    1676
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Sobic.004G155800.2    AACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAAC    1740
BTx623                AACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAAC    1740
S-1                   AACCCAGAGAATGAAAGTGAGATATATCCAGATTTCGTGACAATTGCCAAAGGGTTCAAC    1736
                      ************************************************************


Sobic.004G155800.2    ATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTCCATGCAGCAATCAAGAAGATGCTT    1800
BTx623                ATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTCCATGCAGCAATCAAGAAGATGCTT    1800
S-1                   ATTCCAGCAGTCCGTGTGACAAAGAAGAGCGAAGTCCATGCAGCAATCAAGAAGATGCTT    1796
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Sobic.004G155800.2    GAGACTCCAGGGCCATACCTCTTGGATATAATCGTCCCGCACCAGGAGCATGTGTTGCCT    1860
BTx623                GAGACTCCAGGGCCATACCTCTTGGATATAATCGTCCCGCACCAGGAGCATGTGTTGCCT    1860
S-1                   GAGACTCCAGGGCCATACCTCTTGGATATAATCGTCCCGCACCAGGAGCATGTGTTGCCT    1856
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Sobic.004G155800.2    ATGATCCCTAGTGGTGGGGCTTTCAAGGATATGATCCTGGATGGTGATGGCAGGACTGTG    1920
BTx623                ATGATCCCTAGTGGTGGGGCTTTCAAGGATATGATCCTGGATGGTGATGGCAGGACTGTG    1920
S-1                   ATGATCCCTAGTGGTGGGGCTTTCAAGGATATGATCCTGGATGGTGATGGCAGGACTGTG    1916
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Sobic.004G155800.2    TATTGA
BTx623                TATTGA
S-1                   TATTGA
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111

Appendix B - Publications

Publications from my Ph.D. research not included as a part of this dissertation.

Role of Cytochrome P450 Enzymes in Plant Stress Response

Balaji Aravindhan Pandian [1] [iD], **Rajendran Sathishraj** [1], **Maduraimuthu Djanaguiraman** [1,2] [iD], **P.V. Vara Prasad** [1] [iD] and **Mithila Jugulam** [1,*]

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Abstract: Cytochrome P450s (CYPs) are the largest enzyme family involved in NADPH- and/or O_2-dependent hydroxylation reactions across all the domains of life. In plants and animals, CYPs play a central role in the detoxification of xenobiotics. In addition to this function, CYPs act as versatile catalysts and play a crucial role in the biosynthesis of secondary metabolites, antioxidants, and phytohormones in higher plants. The molecular and biochemical processes catalyzed by CYPs have been well characterized, however, the relationship between the biochemical process catalyzed by CYPs and its effect on several plant functions was not well established. The advent of next-generation sequencing opened new avenues to unravel the involvement of CYPs in several plant functions such as plant stress response. The expression of several CYP genes are regulated in response to environmental stresses, and they also play a prominent role in the crosstalk between abiotic and biotic stress responses. CYPs have an enormous potential to be used as a candidate for engineering crop species resilient to biotic and abiotic stresses. The objective of this review is to summarize the latest research on the role of CYPs in plant stress response.

Keywords: cytochrome P450; plant metabolism; antioxidants; plant stress response

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1. Introduction

Cytochrome P450s (hereafter referred to as CYPs) belong to the oxidoreductases class of enzyme, which represents one of the largest enzyme families containing heme-thiolate as a cofactor. CYPs catalyze NADPH- and/or O_2-dependent hydroxylation reactions in primary and secondary metabolism in many organisms. CYPs are considered as one of the main contributors to the diversity of metabolites formed through oxidation, reduction, hydroxylation, epoxidation, dealkylation, C–C cleavage, desaturation, decarboxylation, dimerization, isomerization, and ring extension reactions [1]. CYPs are mainly anchored to the endoplasmic reticulum to play an essential role in the biosynthesis of different metabolites [2]. The involvement of CYPs in xenobiotic metabolism is well characterized across microorganisms, insects, plants, and humans, imparting resistance to antibiotics, insecticide, herbicide, and drugs, respectively. Further, the CYPs play diverse roles in plants beyond xenobiotic metabolism, including the biosynthesis of hormones, fatty acids, sterols, cell wall components, biopolymers, and several defense compounds (terpenoids, alkaloids, flavonoids, furanocoumarins, glucosinolates, allelochemicals) (Figure 1). CYPs are also implicated in protecting plants from harsh environmental conditions [3], by enhancing the activity of compounds (e.g., flavonoids) with an increased antioxidant activity [4].
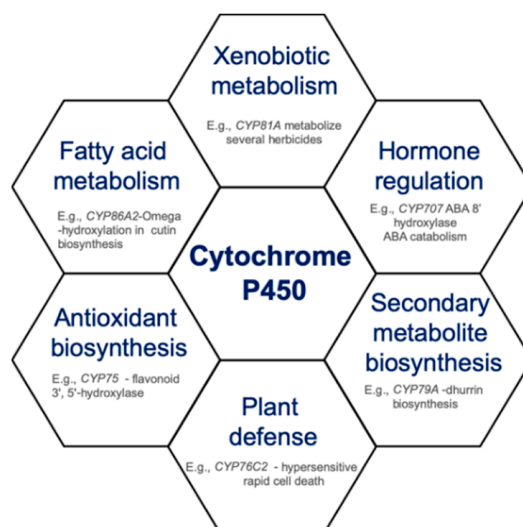
Figure 1. Diverse roles of cytochrome P450s in plants.

The identification and characterization of CYPs can be divided into pre- and post-genomic eras. In the pre-genomic era, the involvement of CYPs was demonstrated by biochemical techniques such as the isolation of CYPs from microsomal fractions, and the inhibition of CYP activity. Being a large gene family with diverse isoforms, the characterization of CYPs with these methods can be challenging because, in most cases, the substrate of the enzyme cannot be easily predicted [5]. However, in the pre-genomics era, the biochemical and molecular processes of CYPs were characterized; however, the relationship between the biochemical process and its direct and indirect effects on several plant functions was not established. Later, with the advent of next-generation sequencing technology (NGS) followed by its rapid advancement and affordability, new avenues to unravel the involvement of CYPs in several plant functions including stress responses were created. Abiotic/biotic stress can be defined as any non-living/living factor(s) that negatively impact the growth and development of plants. The major abiotic stresses affecting, specifically the crop plants include their response to drought, salinity, high/low temperature, heavy metal toxicity, and herbicide application. On the other hand, the major biotic stresses that affect plants include the infestation of insects, pathogens, or weeds. As plants are sessile, they are forced to respond to dynamic environmental changes to sustain their growth and development. Plants can function normally under optimal environmental conditions; however, they are often exposed to a variety of abiotic/biotic stresses or combinations of both [6]. Such exposure can overwhelm their natural defense systems and may result in a substantial yield loss in crops [7]. CYPs have been found to play a major role in hormone signaling, thereby regulating plant response under stress conditions [8–10]. CYPs have been reported to protect plants from drought [8], heat [11], salt [12], heavy metal stress [13], and insects [14] and diseases [15] infestations. In addition, CYPs are also directly involved in the secondary metabolism of plants, facilitating detoxification of external compounds or those that are produced as a byproduct of metabolism in response to stress [16].

The reference genomes and sequence information of many species has been made available as a result of the advancement of sequencing technology and computational capacity in the last decade. Over 300,000 CYP gene sequences have been identified in different organisms, which include ~16,000 plant CYPs [17]. NGS-based technologies such as RNA-Seq, exome seq, and genotyping by sequencing (GBS) combined with several fine mapping methods helped to deduce the function of CYPs. Specific CYP genes involved in plant stress response have also been identified. Such CYP genes have a great potential to be used as candidates for engineering crop species resilient to biotic and abiotic stress. The objective of this review is to summarize the research on the involvement of CYPs in the plant stress response.

2. Classification & Catalysis of CYPs

2.1. Nomenclature and Basic Classification

The structural classification of plant CYPs are based on the similarity of amino acid sequences. The rules for nomenclature and systematic classification of CYPs were set by the "CYP Nomenclature Committee", which assigns names to new CYP genes and also updates the CYP database [18]. CYPs are hierarchically divided into clans, families, and subfamilies. All the cytochrome enzymes will have a code "CYP" followed by a family number, followed by an alphabet that denotes the subfamily of the enzyme. Those enzymes with a 40% amino acid sequence similarity are considered as members of the same family, and those with a >55% identity are grouped into the same sub-family, and those with a >97% similarity are considered as an allelic variant of the same gene [19]. The primary amino acid sequence similarity between different CYPs may be very low, but their secondary structure is relatively conserved [20]. CYPs can be broadly grouped into four types based on origin viz., animal, fungal, microbial, and plant CYPs.

CYPs from humans, vertebrates, and insects are classified as animal CYPs and have 196 families grouped into 11 clans. Fungal CYPs constitute the largest group among other CYPs, with 276 families grouped into 115 clans. Microbial CYPs are not yet classified completely [21]. The plant CYPs have 47 families grouped into 11 clans. In plants, the CYP genes cover ~1% of their genome, implying the abundance and importance for CYPs in plant function [22]. Clan membership parameters have not yet been clearly defined [23,24]. CYPs have also been classified based on their function using the enzyme commission number (EC). The classification of CYPs by the EC number is determined by the type of reaction they catalyze and the type of electron donor with which they interact [25]. The usual electron donor for microsomal enzymes is NADPH-hemoprotein reductase (EC 1.6.2.4). The reactions involving monooxygenation and formation of a single molecule of water were classified under the class oxidoreductases in the sub-subclass of EC 1.14.14 and EC 1.14.15. These sub-subclasses of enzymes act on paired donors, with an incorporation or reduction of molecular oxygen. The differences within these sub-subclasses of enzymes have been primarily because of the involvement of donor proteins. For example, EC 1.14.14 uses reduced flavin or flavoprotein as a donor (e.g., EC 1.14.14.16, steroid 21-monooxygenase, or CYP45021A2), whereas EC 1.14.15 uses a reduced iron-sulfur protein as a donor (e.g., EC 1.14.15.8, steroid 15β-monooxygenase, or CYP106A2). The mitochondrial CYPs utilize a specialized ferredoxin, known as adrenodoxin—an iron-sulfur protein—as their electron donor, and are thus classified under EC 1.14.15 (e.g., EC 1.14.15.15, cholestanetriol 26-monooxygenase, or CYP27A/CYP27A1/CYP27A1'). Those oxidoreductase enzymes involved in the oxidation of a pair of donors, resulting in the reduction of molecular oxygen to two molecules of water, are classified under EC 1.14.19 (e.g., EC 1.14.19.52, camalexin synthase, or CYP71B15). The exceptions to these classifications are CYPs from the CYP74 family that catalyze dehydration reactions that do not require oxygen or an electron donor and are classified under EC 4.2.1 (e.g., EC 4.2.1.121, colneleate synthase, or CYP74D/CYP74D1/CYP74). CYPs that catalyze isomerization reactions have been classified under other intramolecular oxidoreductases (EC 5.3.99). For example, prostaglandin-I synthase (EC 5.3.99.4), or CYP8A1, is an enzyme involved in prostanoid biosynthesis that belongs to the CYP isomerase [25].

2.2. Catalysis of CYP Enzymes

The mechanisms of action of CYPs are extensively studied and documented. CYPs were first reported in rats as a carbon monoxide binding pigment, which absorbs light at 450 nm and later named P-450, where P denotes pigment [26]. The CYP enzymes have heme-thiolate as a cofactor centering porphyrin ring, which makes it one of the metalloenzymes involved in the reduction of molecular oxygen. The most conserved region of CYPs is the heme cofactor, which is also the site of catalysis. The conserved regions and the amino acid sequences of many CYPs have been well-characterized [27–30]. However, the reactions involved in the substrate binding of CYPs are complex and not completely understood [31].

The catalysis of CYPs involves five major steps, step 1: The organic substrate (R) will bind to the heme group of the enzyme; step 2: the substrate binding induces the transfer of an electron from NADPH through cytochrome P450 reductase (CPR) or any other associated reductase to the CYPs that will reduce the iron (Fe) from the ferric state (Fe^{3+}) to the ferrous state (Fe^{2+}); step 3: molecular oxygen binds to ferrous CYPs to form a ferrous CYP–dioxygen complex; step 4: a second electron is transferred from CPR or any other associated reductase to the ferrous CYP–dioxygen complex to form a short-lived peroxo complex and this complex rapidly protonated twice forming one molecule of water and an iron–oxo complex; step 5: the oxygen atom in the iron–oxo complex binds to the organic substrate (R) and forms the oxidized reaction product (RO) (Figure 2) [32].

$$R + O_2 + NADPH \rightarrow RO + H_2O + NADP^+$$
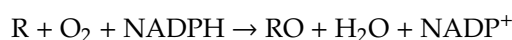
Apart from oxidation, CYPs are also known to be involved in reactions such as dehydrogenation, carbon–carbon bond cleavage, and dealkylation [33–35].
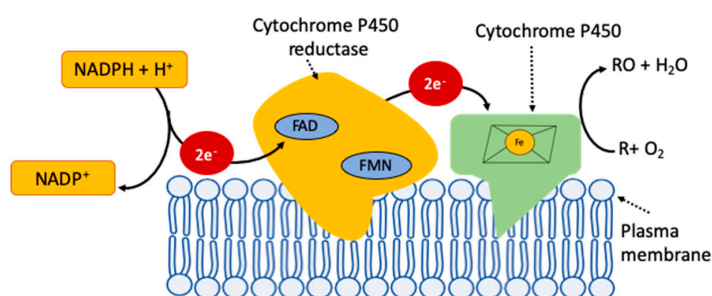
Figure 2. Simplified scheme of catalysis of cytochrome P450 (CYP) system; the CYPs receive two electrons derived from nicotinamide adenine dinucleotide phosphate (NADPH) through cytochrome P450 reductase (CPR) to catalyze the oxidation reaction $R + O_2 + NADPH \rightarrow RO + H_2O + NADP^+$; where R is the substrate and RO is the product of the oxidation reaction.

3. Role of CYPs in Abiotic and Biotic Stress

3.1. Drought Stress

In response to drought (water-deficit) stress, plants trigger multiple enzymatic and hormonal activities to maintain the intracellular ion homeostasis, osmolyte accumulation, and scavenging of ROS such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) [36]. The plant hormone ABA plays a critical role in abiotic stress and activates multiple stress-responsive genes [37]. Enhanced ABA levels have also shown to be correlated with drought stress in plants [38]. ABA is synthesized in plants from a carotenoid precursor (C_{40} carotene) and xanthoxin [39]. ABA levels considerably fluctuate during dehydration and rehydration. However, under such instances, the balance of ABA levels will be maintained by the catabolism of ABA. The high level of ABA accumulation can be catabolized by oxidation or conjugation reactions [40]. Under drought stress, the ABA is converted to 8'-hydroxy ABA and then isomerized to phaseic acid (PA). This process is catalyzed by ABA 8'-hydroxylase (ABA8Ox), an enzyme that belongs to the CYP707 family [41]. The physiological processes controlled by ABA in plants are achieved by the synergistic relationship between the biosynthesis and catabolism of ABA, which is mediated by ABA 8'-hydroxylases. In maize, the *CYP707A* (*ABA8Ox*) gene was found to upregulate when exposed to water deficit conditions [42]. Similarly, *CYP707A1* and *CYP707A2* genes were found to be significantly upregulated under osmotic stress in *Arachis hypogaea* [43] and *Populus simonii* (a highly drought-tolerant tree species found in China) [44]. The same genes (*CYP707A1* and *CYP707A2*) have also been reported to be upregulated under drought stress in *Arabidopsis* [40].

The CYPs have also been found to play a role in the synthesis of leaf lignin and grain formation when exposed to drought stress in plants. For example, CYP96A8 was speculated to be involved

in lignin biosynthesis and other drought response-related functions [45]. Further, CYP86A2 plays a major role in the biosynthesis of epicuticular lipids such as cutin. Mutants of the *CYP86A2* gene in *Arabidopsis* exhibit a reduced cuticle membrane thickness and increased water permeability to help in drought tolerance [46]. The *LEAF CURLING RESPONSIVENESS (LCR)* gene, which encodes CYP86A8 in *Arabidopsis*, was found to be involved in the omega-hydroxylation of fatty acids in the biosynthesis of cutin [47]. Likewise, CYPs have also been reported to be involved in the biosynthesis of cuticular wax along with the *WXP1* gene in transgenic plants of *Medicago sativa* [48]. In response to drought stress, CYPs can directly or indirectly involve in the biosynthesis of several antioxidants, which can reduce the oxidative damage. A citrus CYP gene, *CsCYT75B1* was found to be upregulated during drought stress in *Citrus sinensis*; further, the transformation and overexpression of this gene in *Arabidopsis* significantly enhanced the total flavonoid content and antioxidant activity under drought stress [4]. The *CYP75* family was also found to be involved in flavonoid regulation in grapevine and ferns [49]. The heterologous expression of the *Carthamus tinctorius CYP82G24* gene in *Arabidopsis* induces the expression of several other genes involved in flavonoid biosynthesis [50]. Transcriptome analyses of sorghum plants found upregulation of the *CYP71A25* and *CYP71B2* genes under drought stress along with other drought-responsive genes [51]. Further, two CYPs have been identified to be upregulated in the rice variety Nagina 22 under drought stress, but the specific function of these CYPs have not been characterized [52]. Five uncharacterized CYP genes were found differentially expressed between drought-tolerant and -susceptible genotypes of rice when exposed to long-term drought stress [53].

3.2. Temperature Stress

Temperature fluctuations during plant growth and development are common. If plants are exposed to temperature variations, i.e., 10–15 °C above optimum (heat stress), <20 °C (chilling), or below 0 °C (freezing), for a prolonged period, it can result in irreversible damage to plant growth and development [54–57]. Both heat and cold stress generally affect respiration and photosynthesis, leading to oxidative damage caused by the production of ROS. The role of CYPs in the regulation of non-enzymatic antioxidants such as carotenoids, flavonoids, and hormones (e.g., abscisic acid) and the activation of antioxidant enzymes have been investigated. The expression of CYP genes involved in flavonoid production was found to be differentially regulated under heat and/or cold stress in *Lolium perenne* and *Festuca arundinacea*. The *CYP73A* (trans-cinnamate 4-monooxygenase), *CYP75A* (flavonoid 3',5'-hydroxylase), and *CYP75B* (flavonoid 3'-monooxygenase) genes were significantly upregulated under heat and cold stress in both the species [58]. Prolonged cold stress in *Arabidopsis* induced a 2–4-fold expression of the CYP83A1 gene involved in flavonoid (phenylpropanoids) metabolism [59]. ABA also can play a critical role in cold and heat stress response by increasing the expression of ABA 8'-hydroxylases (*CYP707A* genes) in *Arabidopsis* [60,61]. A *Panicum virgatum* population exposed to long-term heat stress (38/30 °C, day/night, for 50 days) showed a differential expression of 11 CYPs compared with those grown under ambient temperature. Specifically, two *CYP71A1* genes responsible for the biosynthesis of indole alkaloid secologanin were upregulated under heat stress [62]. In contrast, *CYP71* was highly downregulated in the leaves of *Rhazya stricta* exposed to a high-temperature range (40–42.4 °C) [63]. Secologanin is a monoterpene glycoside involved in the biosynthesis of alkaloids. A genome-wide association study of heat-tolerant *Brassica napus* identified that the *CYP71A23* gene is involved in pollen sterility [64]. The same gene was also upregulated in *Panicum maximum* exposed to elevated heat and CO_2 [65]. A transcriptome analysis of a cold-tolerant sorghum genotype revealed the upregulation of two CYPs, the *CYP99A1* and *CYP709C1* genes [66].

3.3. Salinity Stress

Soils with a pH above 8.5 are considered saline, which can affect the crop yields significantly. The undesirable effects of salinity stress in plants include toxicity induced by the accumulation of sugars, amino acids, and various inorganic compounds, and osmotic stress by the reduced uptake of water [67]. Two major mechanisms involved in salinity tolerance in crops include leaf Na^+ exclusion

mediated by high-affinity K$^+$ transporters (HKTs) and ROS homeostasis [68]. The manipulation of a CYP expression can impart tolerance to salt stress. Constitutively expressing the *TaCYP81D5* gene enhances the salinity tolerance in wheat both at the seedling and reproductive stages via accelerating the ROS scavenging activity [69]. The activation of the *AtCYP81D8* gene has been used as a marker for ROS activity [70]. CYPs also play a major role in maintaining ROS homeostasis to provide salinity tolerance. The induction of ABA is highly correlated with the salinity stress response. Low ABA levels are more adaptive for salinity stress [37]. CYP707A was found to be involved in ABA biosynthesis, and thus can indirectly protect plants from salt stress. Salicylic acid signaling is also found to enhance ABA synthesis in plants during salinity stress [71]. The *AtCYP709B3* gene was found highly expressed under salt stress in *Arabidopsis* providing tolerance to salinity [72]. An increased expression of the *CYP709* family was also found in salt-stressed *Robinia pseudoacacia* [73]. The proteome of NaCl-exposed *Physcomitrella patens*, naturally tolerant to high salinity, revealed the expression of 49 CYPs. Under salinity stress, the CYPs were speculated to reduce damage to the cell wall and scavenge the ROS [74].

3.4. Heavy Metal Stress

Metals such as aluminum (Al), iron (Fe), cobalt (Co), zinc (Zn), silver (Ag), cadmium (Cd), nickel (Ni), and mercury (Hg) with relatively high densities (>5 g/cm^3) and toxic at a low concentration are referred to as heavy metals. The disposal of sewage sludge and other anthropogenic activities can increase the concentration of heavy metals in soil [75]. Not all the heavy metals are toxic to plants, and many of them—Zn, Fe, Co, etc.—at low concentrations are essential for plant growth and development. A transcriptome analysis of wheat varieties tolerant to aluminum toxicity resulted in the identification of an increased expression of the *CYP88A* gene (also known as *KAO1*), which is involved in gibberellin biosynthesis [76]. In a different study, *Pisum sativum* with the *KAO1* gene showed stunted growth [77]. The upregulation of multiple CYP genes has been reported in wheat genotypes susceptible to Al toxicity. *CYP81D8*, which is hypothesized to be involved in the metabolism of Al, showed a 4-fold expression in response to Al toxicity in *Arabidopsis* [78]. Two CYP genes (an uncharacterized CYP and *CYP99A1*) have been found to show a 32- and 21-fold increased expression, respectively, to Cd toxicity in rice. In some plants, CYPs are also believed to be involved in the metabolism of heavy metals along with other genes such as glutathione-*S*-transferase (GST) [79]. *Medicago sativa* plants transformed with the human *CYP2E1* and *GST* genes showed potential for phytoremediation in Hg-contaminated soils. Transgenic *M. sativa* expressing both these genes also had a synergistic effect that helped plants to withstand mercury contamination via enhanced metabolism [80]. In roots of *Panax ginseng* treated with Ni and Cd, the upregulation of the gene *CYP71*, involved in the biosynthesis of flavonoids, alkaloids, and other secondary metabolites has been reported [81].

3.5. Herbicide Stress

Plants metabolize xenobiotics such as pesticides that enter into their system; however, the ability to metabolize xenobiotics differs between and within different species. Herbicides are used extensively to control weeds in both crop and non-crop areas. The ability of crop plants to withstand herbicide applications targeted to control weeds is referred to as herbicide selectivity. CYPs are one of the key enzymes involved in conferring selectivity in crop plants via the metabolism of herbicides [82]. A novel mapping method, bulk segregant analysis combined with RNA-Seq (BSR-Seq), was used to map the gene *CYP81A9*, responsible for the metabolism of the acetolactate synthase (ALS) inhibitor herbicide nicosulfuron in maize [83]. Several rice varieties cultivated in Asia are naturally tolerant to the ALS inhibitor herbicide bentazon, and the genetic transformation of the rice *CYP81A6* gene to *Arabidopsis* and *Nicotiana tabacum* proved that CYP81A6 is involved in the metabolism of betazon [84]. The metabolism of herbicides such as chlorsulfuron, triasulfuron, metsulfuron-methyl, bensulfuron-methyl, and tribenuron-methyl by CYP71C6v1 from wheat was demonstrated by a heterologous expression in yeast [85]. Maize is naturally tolerant to 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitor herbicides [86]. A CYP-mediated metabolism of the HPPD inhibitors

mesotrione [87] and tembotrione [88] was reported in maize. Several uncharacterized CYP genes have been found to confer resistance to herbicides with different modes of action such as nicosulfuron (ALS inhibitor), mesotrione (HPPD inhibitor), dicamba (synthetic auxin), diflufenzopyr (auxin transport inhibitor), and carfentrazone (protoporphyrinogen oxidase (PPO) inhibitor) in maize [89,90]. CYPs are also involved in the metabolism of several herbicides in weeds, making them resistant to herbicides. The role of CYPs in the metabolism of herbicides in weeds has been reviewed extensively [82,91,92] and beyond the scope of this review.

3.6. Biotic Stress: Diseases, Insects, and Weeds

The metabolism of polyunsaturated fatty acids synthesizes oxylipins. The two major oxylipins in plants are jasmonic acid and methyl jasmonate. The expressions of genes responsible for the biosynthesis of these oxylipins and levels of these molecules in plants play an essential role in multiple stress signaling pathways, especially during physical injury and disease defense [93,94]. In plants, lipoxygenase is metabolized by several enzymes such as allene oxide synthase (AOS) and hydroperoxide lyase (HPL), which are members of the *CYP74* family [95,96]. Apart from the biosynthesis of oxylipins, CYPs are involved in the jasmonic acid and methyl jasmonate signaling pathways, for instance, in soybean, the *CYP82A3* gene expression is induced by methyl jasmonate which was also found to be induced by several fungal infections. Transgenic *Nicotiana benthamiana* plants overexpressing the *GmCYP82A3* gene were found to be highly resistant to black shank (*Phytophthora parasitica*) and gray mold (*Botrytis cinereal*) [3]. Hypersensitive response (HR) is a common defense mechanism for microbial infection in various crop species. The CYP gene *CaCYP1* from *Capsicum annuum* was found to be involved in the HR, following the infection by *Xanthomonas axonopodis* [97]. The CYP gene *AtCYP76C2* from *Arabidopsis* was found to be associated with hypersensitive rapid cell death, which is a defense mechanism for bacterial canker (*Pseudomonas syringae*) infection [98]. In the head blight-resistant wheat genotype "Ning 7840," *CYP709C3v2* was upregulated along with the chitinase (*Chi1*) gene, which confers tolerance to the fusarium head blight (*Fusarium graminearum*) [99]. A pathogen-induced *CYP82C2* gene and other possible CYPs are involved in the biosynthesis of 4-hydroxyindole-3-carbonyl nitrile with cyanogenic functionality against bacterial canker (*Pseudomonas syringae*) [100]. Camalexin is a secondary plant metabolite which is involved in fungal and bacterial tolerance, where CYP71B15 catalyzes the reaction of dihydrocamalexic acid to form camalexin [101]. The CYP enzyme CYP96A15 (referred to as mid-chain alkane hydroxylase (MAH1)) is involved in epicuticular wax biosynthesis, which is a common structural plant defense mechanism. The upregulation of CYP transcripts during the wounding process has been recorded in *Helianthus tuberosus* [102], *Pisum sativum* [103], and maize [104].

Resistance to green peach aphid (*Myzus persicae*) in *Arabidopsis* was found to be controlled by the CYP family *PAD3* gene, which is involved in camalexin, known as a toxic phytoalexin [105]. The CYPs of a well-characterized *CYP79D* gene family were found to be involved in the herbivore-induced biosynthesis of aldomixes in *Populus trichocarpa* [106]. Cembratriene-ol (CBT-ol) in the trichome glands of *Nicotiana tabacum* plants was found to be converted into cembratriene-diol (CBT-diol) by an uncharacterized CYP hydroxylase, suppressing this CYP increased the CBT-ol content and exhibited resistance to aphids (*Myzus nicotianae*) [107]. CYPs' involvement in the biosynthesis of cutin, lignin, and cyanogenic glucosides can directly or indirectly be associated with plant defense mechanisms against various biological threats [9].

Crop production is challenged by weed infestation resulting in an enormous crop yield loss due to crop–weed competition. The weeds compete with the crops for nutrients, water, and light. Weed control measures are focused directly or indirectly towards improving the competitive ability of the crop plants [108]. Allelopathy is defined as the effects (stimulatory or inhibitory) of a plant on the development of neighboring plants through the release of secondary compounds. Some crops express their allelopathic potential by releasing allelochemicals that suppress the weeds. Allelopathic effects have been documented for crops such as rice, wheat, sorghum, sunflower, rapeseed, and rye [109]. Sorgoleone (2-hydroxy-5-methoxy-3-[(Z, Z)-8',11',14'-pentadecatriene]-pbenzoquinone

which is produced in root hairs of sorghum [110] can suppress some of the major problematic weeds of the United Stated such as *Amaranthus palmeri* [111], *Abutilon theophrasti* [112], *Echinochloa crus-galli* [113], and *Lolium rigidum* [114]. The CYP enzyme CYP71AM1 is involved in the biosynthetic pathway of the allelochemical sorgoleone in sorghum. CYP71AM1 catalyzes the formation of dihydrosorgoleone using 5-pentadecatrienyl resorcinol-3-methyl as a substrate in sorgoleone pathway [115]. Benzoxazinoids are specialized metabolites that are predominantly present in monocot species. Naturally occurring benzoxazinoids DIBOA [4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one] and DIMBOA [4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one] are known to play a role in allelopathic plant–plant interactions and also serve as defense compounds against microbes, insects, and weeds [116]. DIBOA and DIMBOA have proven to inhibit the growth and development of weeds in crops such as wheat [117], maize [118], and rye [119]. Four CYPs belonging to the *CYP71* family play a key role in the biosynthesis of these compounds in wheat [120] and maize [121].

4. Conclusions

CYPs are versatile enzymes involved in multiple processes of plant growth and development and also play an essential role in stress response. CYPs protect plants from abiotic and biotic stresses by the biosynthesis and regulation of hormones, fatty acids, sterols, cell wall components, biopolymers, and several other defense compounds (terpenoids, alkaloids, flavonoids, furanocoumarins, glucosinolates, allelochemicals) [122]. Even though the involvement of several CYPs in different plant stress responses has been identified, the precise function of most of the CYPs is still elusive. The CYP genes involved in desirable plant functions can potentially be used to improve crop varieties, especially for stress tolerance [123]. A complete understanding of the biochemical processes catalyzed by a CYP along with the availability of sequence information are valuable for crop improvement by deploying marker-assisted selection, genetic transformation, or gene-editing techniques. In recent years, crop improvement programs have been focused on developing climate-smart crop varieties that can withstand abiotic stresses, although not by engineering the CYP genes that are known to provide resistance to abiotic stresses. The development of crop varieties by integrating abiotic and biotic stress resistance traits can significantly improve crop productivity [124]. Recently, several CYPs have been characterized and have the potential to be exploited in crop improvement to develop stress-tolerant crops (Table 1).

Table 1. List of cytochrome P450 genes that can be used as candidates in crop improvement; all the list items given here are characterized for their biochemical process and involvement in plant function and traits desirable for crop improvement.

CYP	Identified Species	Biochemical Process	Function	Desirable Trait	Reference
CYP97C1	*Arabidopsis*	Carotenoid ε-ring hydroxylation	Lutein biosynthesis	Abiotic stress resistance	[125]
CYP703A2	*Arabidopsis*	Hydroxylation of lauric acid	Pollen development	Abiotic stress resistance	[126]
CYP83A1 and CYP83B1	*Arabidopsis*	Biosynthesis of glucosinolates	Pungency	Insect resistance	[127]
CYP79A1 and CYP71E1	Sorghum	Tyrosine into *p*-Hydroxymandelonitril	Cyanogenic glucoside (dhurrin) biosynthesis	Insect resistance	[128]
CYP72A1	*Catharanthus roseus*	Secologanin synthase	Indole alkaloid biosynthesis	Disease resistance	[129]
CYP707A	*Arabidopsis*	ABA 8′-hydroxylases	ABA regulation	Abiotic stress resistance	[38]
CYP86A2, A8	*Arabidopsis*	Omega-hydroxylation	Cutin biosynthesis	Insect resistance	[46]
CYP714A3	Rice	Gibberellin regulation	Shoot development	Heavy metal stress	[76]
CYP88A	Wheat	Gibberellin biosynthesis			
CYP86A1	*Arabidopsis*	Omega-hydroxylase	Suberin biosynthesis	Insect resistance	[130]
CYP71C	Maize	DIBOA biosynthesis	Allelopathy	Biotic stress resistance	[118]

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Confirmation and Characterization of the First Case of Acetolactate Synthase (ALS)-Inhibitor—Resistant Wild Buckwheat (*Polygonum convolvulus* L.) in the United States

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Abstract: Wild buckwheat (*Polygonum convolvulus* L.) is a problem weed and ALS-inhibitors (e.g., chlorsulfuron) are commonly used for its management. Recently, a population of wild buckwheat (KSW-R) uncontrolled with ALS-inhibitors was found in a wheat field in Kansas, USA. The objectives of this research were to determine the level and mechanism of resistance to chlorsulfuron and cross resistance to other ALS-inhibitors in the KSW-R population. In response to chlorsulfuron rates ranging from 0 to 16x (x = 18 g ai/ha), the KSW-R wild buckwheat was found >100-fold more resistant compared to a known ALS-inhibitor susceptible (KSW-S) wild buckwheat. Also, >90% of KSW-R plants survived field recommended rates of sulfonylurea but not imidazolinone family of ALS-inhibitors. A portion of the *ALS* gene covering all previously reported mutations known to bestow resistance to ALS-inhibitors was sequenced from both KSW-R and KSW-S plants. The Pro-197-Ser substitution that confers resistance to the sulfonylurea herbicides was found in KSW-R plants. Our results support the evolution of high level of chlorsulfuron resistance as a result of a mutation in the *ALS*-gene in KSW-R buckwheat. This is the first case of resistance to any herbicides in wild buckwheat in the US.

Keywords: chlorsulfuron-resistant buckwheat; herbicide resistance; target-site mutation

1. Introduction

Wild buckwheat (*Polygonum convolvulus* L.) is a summer annual weed, commonly found in small grain crops such as wheat (*Triticum aestivum*) and oats (*Avena sativa*). This weed was introduced into the US from Europe via grain transport and became a problem weed throughout the Great Plains, Northern Plains, Canada's Prairie provinces, and US Midwest. Wild buckwheat is a competitive weed that can cause yield losses of up to 66% in wheat. Apart from competing for nutrients, the vines of wild buckwheat can tangle and climb on the shoots of host plants which interferes in harvesting operations leading to lower yields and poor quality [1,2]. Furthermore, chemical control of wild buckwheat is a challenge in small grain crops, because some auxinic herbicides (e.g., 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenomyacetic acid (MCPA)), widely used for control of broadleaf weeds in cereal crops are not effective in controlling wild buckwheat [3]. Hence, control of wild buckwheat in these crops is largely dependent on the use of acetolactate synthase (ALS)-inhibiting herbicides.

ALS is one of the key enzymes involved in the biosynthesis of branched-chain amino acids (BCAAs) such as valine, leucine, and isoleucine [4]. Inhibition of the ALS enzyme leads to depletion of BCAAs and several secondary effects resulting in plant death [5]. ALS-inhibiting herbicides include five chemical families, viz., sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-thio benzoates (PTB) and sulfonyl-aminocarbonyl-triazolinone (SCT). Since the commercialization of ALS-inhibitors in 1982, they are widely used for effective control of a broad spectrum of weeds compared to other commercial herbicides [6]. Because of extensive use, 165 weed species have evolved resistance to ALS-inhibitor herbicides [7].

Reduced sensitivity to ALS-inhibitors as a result of one or several mutations in the *ALS* gene, the target site of these herbicides is the most common resistance mechanism found in weeds [8]. A total of 29 amino acid substitutions at eight positions on the ALS protein (Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654, positions based on the *Arabidopsis thaliana* sequence Genbank accession NP_190425) that confer resistance to ALS-inhibitors have been reported in many weed species [7]. Enhanced metabolism of ALS-inhibitors by cytochrome P450 monooxygenases (CYPs) activity has also been reported in several ALS-inhibitor-resistant weeds [6], such as waterhemp (*Amaranthus tuberculatus*) [9], Palmer amaranth (*Amaranthus palmeri*) [10], rigid ryegrass (*Lolium rigidum*) [11], late watergrass (*Echinochloa oryzicola*) [12], and blackgrass (*Alopecurus myosuroides*) [13].

Small grain crops such as wheat, oat, and barley (*Hordeum vulgare*) are naturally tolerant to the SU family of ALS-inhibitors [14]. Specifically, SU herbicides chlorsulfuron, triasulfuron, and thifensulfuron are widely used for postemergence control of wild buckwheat in these crops [2]. As a result of repeated selection, the evolution of resistance to ALS-inhibitors was reported in wild buckwheat biotypes in Canada and Australia [7]. ALS-inhibitor-resistant wild buckwheat was first reported in Queensland, Australia for chlorsulfuron in 1993 [7] and later in 2007 in Alberta (Canada), for SU and TP herbicide families [15]. An amino acid substitution, Trp-574-Leu in the *ALS* gene, was found to bestow resistance in the wild buckwheat population from Alberta [15]. Herbicide-resistant wild buckwheat has not been documented in the US; however, recently a population of wild buckwheat (KSW-R) survived chlorsulfuron applications in a wheat field in Marion County in Kansas. The focus of this research was to confirm and characterize the chlorsulfuron resistance in KSW-R wild buckwheat. This research was based on the hypothesis that similar to ALS-inhibitor-resistant wild buckwheat from Canada, one or more mutations in the ALS gene may contribute to chlorsulfuron resistance in KSW-R wild buckwheat. The objectives of this research were to determine the level and mechanism of resistance to chlorsulfuron and cross resistance to other ALS-inhibitors in KSW-R wild buckwheat.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

A wild buckwheat biotype suspected to have evolved resistance to chlorsulfuron was collected from a winter wheat field in Marion County, Kansas in summer 2017. This population was designated as KSW-R and a biotype known to be susceptible to ALS-inhibitors, collected in Kansas (KSW-S) was also used in this study for comparison. The seeds of KSW-R and KSW-S wild buckwheat were planted in plastic trays (25 x 15 x 2.5 cm) with the commercial potting mixture (ProMix Ultimate, Premier Tech Horticulture, Mississauga, ON, Canada) and kept in 4 °C for 3–4 weeks for a cold treatment to break the dormancy and enhance germination. Later, the trays were moved to a greenhouse. Upon germination, at the 2–3 leaf stage, seedlings were transplanted in square pots (6 x 6 x 6 cm) and grown in a greenhouse maintained at 25/20 °C, 15/9 h a day/night photoperiod with a photosynthetic photon flux density of 750 μmol m^{-2} s^{-1} and relative humidity of 60 $\pm$ 10 percent. The plants were fertilized (Miracle GRO®All-purpose plant food, Scotts Miracle-Gro, Marysville, OH, USA) as needed. At the 4–5 leaf stage, KSW-R and KSW-S buckwheat plants were treated with chlorsulfuron (Glean® XP, Wilmington, DE, USA) at 36 g ai ha^{-1} (field recommended rate 1x = 18 g ai ha^{-1}) along with 0.25% nonionic surfactant (NIS) using a bench-type sprayer (Research Track Sprayer, Generation

III, De Vries Manufacturing, Hollandale, MN, USA) equipped with a flat-fan nozzle tip (80015LP TeeJet®tip, TeeJet Spraying Systems, Wheaton, IL, USA) delivering 168 L ha^{-1} at 222 kPa in a single pass at 4.8 km h^{-1}. KSW-R plants that survived the treatment with 36 g ai ha^{-1} of chlorsulfuron were transferred to individual pots and allowed to self-pollinate. Upon maturity, seeds were collected from the self-pollinated plants and used in the dose-response and *ALS* gene sequencing experiments.

2.2. Chlorsulfuron Dose-Response Assay

The KSW-R and KSW-S plants at the 3 to 4 leaf stage grown in square pots (6 x 6 x 6 cm) under greenhouse conditions as described above were used for the whole-plant dose-response assay. Six to ten plants of each KSW-S wild buckwheat treated with 0, 4.5, 9, 18 (field recommended rate), 36 g ai ha^{-1} and the KSW-R wild buckwheat were treated with 0, 9, 18, 36, 72, 144, and 288 g ai ha^{-1} of chlorsulfuron along with 0.25% NIS. The herbicide treatment was applied as described above. The above-ground dry biomass was collected at 3 weeks after treatment (WAT) and oven-dried at 60 °C for 72 h, and then the dry weight of biomass was recorded. The dose-response experiment was repeated following the same experimental procedure, herbicide treatments, and growth conditions.

2.3. Response to Different ALS-Inhibitors

In order to determine the response of KSW-R and KSW-S biotypes to different ALS-inhibitors (SU or IMI), twelve plants of KSW-R and KSW-S wild buckwheat at the 4–5 leaf stage were treated separately with field recommended rates of thifensulfuron, halosulfuron, nicosulfuron, and imazethapyr along with chlorsulfuron (Table 1). Plant survival was assessed at 3 WAT as per [10]. The experiment was repeated following the same growth conditions and methods as above.

Table 1. List of herbicides used in the study.

Herbicide	Trade Name	Chemical Family	Manufacturer	Field Rate
Glean	Chlorsulfuron	Sulfonylurea	DuPont Wilmington, DE http://cropprotection.dupont.com	18 g ai ha^{-1}
Permit	Halosulfuron	Sulfonylurea	Gowan Company, Yuma, AZ www.gowanco.com	36 g ai ha^{-1}
Harmony	Thifensulfuron	Sulfonylurea	DuPont Wilmington, DE http://cropprotection.dupont.com	36 g ai ha^{-1}
Accent	Nicosulfuron	Sulfonylurea	DuPont Wilmington, DE http://cropprotection.dupont.com	36 g ai ha^{-1}
Pursuit	Imazethapyr	Imidazolinone	BASF Corporation, Research Triangle Park, NC, USA	72 g ai ha^{-1}

2.4. ALS Gene Sequencing

To assess if any known mutations in the *ALS* gene of KSW-R wild buckwheat confer resistance to ALS-inhibitors, leaf tissue was collected from 15 KSW-R wild buckwheat plants that survived 288 g ai ha^{-1} of chlorsulfuron along with five non-treated individuals of KSW-S buckwheat. The genomic DNA (gDNA) was extracted using GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific™, Waltham, MA, USA) following the manufacturer's instructions. A wild buckwheat *ALS* gene sequence from a transcriptome assembly deposited at National Center for Biotechnology Information (NCBI) GenBank under the accession GIUI00000000 was used to design primers (*Pc*ALS_F: AGGGAGTCACCAACGTGTTC *Pc*ALS_R: TGGTAAAACCATACCCCCAGT; primer used for sequencing *Pc*ALS_F1: CATGCTGTTGAATAACCAGC) to amplify a portion of the *ALS* gene (~1.8 kb in length) that covers all the previously reported mutation sites. Polymerase Chain reaction (PCR) was performed using T100™ Thermal Cycler (Bio-Rad Inc., Hercules, CA, USA); a mixture containing 50–80 ng of gDNA, 0.5 μM of forward primer, reverse primer and Promega™ ready-to-use

PCR master mix with the following PCR conditions were used, initial denaturation 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s and a final extension at 72 °C 7 min. The PCR products were purified using GeneJET™ PCR Purification Kit (Thermo Scientific™, Waltham, MA, USA) and sequenced by Sanger sequencing platform (GENEWIZ Inc., South Plainfield, NJ, USA), and the sequences were aligned using MultAlin multiple sequence alignment tool (INRA, Paris, France). Further, the Sanger sequencing reads were assembled using the one-click assembly option of EGassembler with default parameters [16]; the assembled sequence contig was translated using the Translate tool—ExPASy [17]. The assembled sequences of wild buckwheat populations KSW-R and KSW-S were deposited at the NCBI GenBank.

2.5. Statistical Analysis

The plant biomass accumulation (in grams per plant) data was converted to percent dry aboveground biomass relative to the non-treated control of the respective, wild buckwheat biotype, i.e., KSW-S and KSW-R. The relationship between herbicide rate and biomass accumulation was estimated by non-linear regression analysis using a three-parameter log-logistic model (1). All the analyses were performed [18] using the 'drc' [19] package in R [20]. To assess the fit of data to various regression models, a "Lack-of-fit" test was performed using the "model fit" function of 'drc':

$$Y = \left\{ \frac{d}{1} + \exp\left[b(logx - loge)\right] \right\} \tag{1}$$

In the three-parameter log-logistic model Equation (1), Y is the response variable, d is the upper limit, b is the slope of the curve, and e is the GR_{50} which is the rate required for 50% reduction of plant biomass [21]. Analysis of variance (ANOVA) was performed following Fisher's LSD test used to separate means at $p \leq 0.05$ using the 'agricolae' package in R [22] to estimate the significant differences in percent dry biomass in response to different rates of chlorsulfuron.

3. Results and Discussion

3.1. Chlorsulfuron Dose-Response

The results of the dose-response assay confirmed a very high level of resistance to chlorsulfuron in KSW-R wild buckwheat. No significant differences were found between two runs of dose-response ($p < 0.05$). The KSW-R plants survived up to 16x (288 g ai ha^{-1}) of the field rate of chlorsulfuron. However, the KSW-S plants were heavily injured at 4.5, and 9 g ai ha^{-1} and completely killed at 18 g ai ha^{-1} of chlorsulfuron (Figure 1). The percent reduction in the above-ground biomass relative to non-treated control did not show a significant difference up to 72 g ai ha^{-1} ($p < 0.05$) rate of chlorsulfuron in the KSW-R; however, 16 and 25% reduction in biomass was found in 144 and 288 g ai ha^{-1} treated plants, respectively (Figure 2). The GR_{50} of KSW-R (703.74) was exponentially higher than KSW-S (3.96), indicating that the KSW-R population is >100-fold resistant to chlorsulfuron than the KSW-S wild buckwheat (Table 2). The GR_{50} of the KSW-R buckwheat was higher than the highest rate used (288 g ai ha^{-1}) in this study; and the biomass reduction of KSW-R buckwheat was <50% even at the highest rate (Figure 2). Previously, GR_{50} values that are more than the highest rate used in the experiments have been reported in several ALS-inhibitor-resistant weeds such as Palmer amaranth [10], henbit (*Lamium amplexicaule*) [23], and mouse barley (*Hordeum murinum)* [24]. Control of wild buckwheat in small grain crops, especially wheat, is primarily dependent upon ALS-inhibitors [2], and therefore, considerable selection pressure is expected in wheat producing regions. Resistance to ALS-inhibitor herbicides has been previously reported in a wild buckwheat population collected from wheat fields in Alberta (Canada) with 10–20-fold resistance to thifensulfuron/tribenuron and florasulam, respectively [15]. Similarly, a biotype of wild buckwheat from Queensland, Australia, also was found to have evolved resistance to chlorsulfuron [7], although the level of resistance or the mechanism of resistance to chlorsulfuron in the Australian biotype is not yet available.
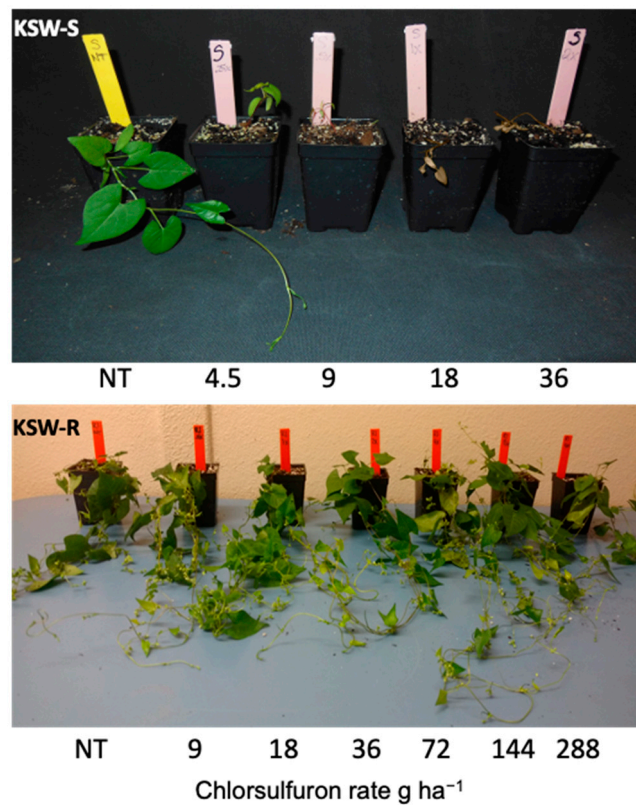
Figure 1. The whole-plant response of KSW-R (resistant) and KSW-S (susceptible) wild buckwheat populations to different rates of chlorsulfuron at 3 weeks after treatment.
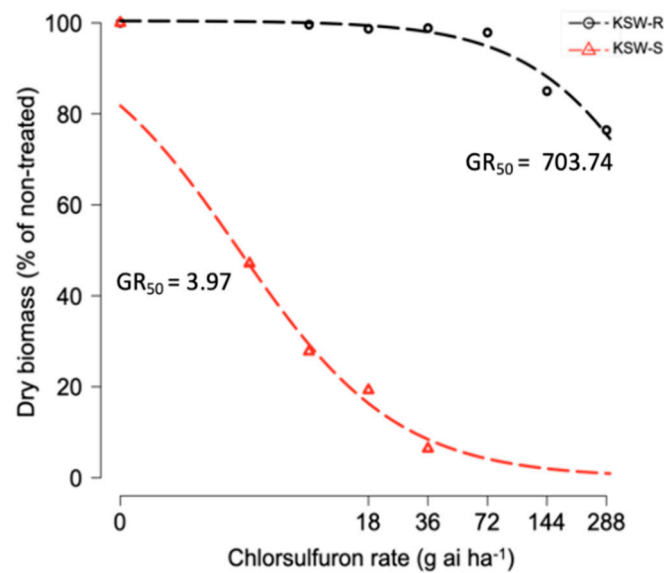

Figure 2. Chlorsulfuron dose-response curves obtained by three parameter log-logistic regression. $Y = \left\{ \frac{d}{1} + \exp[b(log x - log e)] \right\}$ analysis of above-ground dry biomass of KSW-R (resistant) and KSW-S (susceptible) wild buckwheat at 3 weeks after treatment; GR_{50} herbicide rate required for 50% growth reduction.

Table 2. Regression parameters describing the response of wild buckwheat populations KSW-R (resistant) and KSW-S (susceptible) to chlorsulfuron; herbicide rate required for 50% growth reduction (GR_{50}), slope of the curve (b), upper limit (d) and, standard error (SE).

Genotype	GR_{50} (SE)	b (SE)	d (SE)
KSW-R	703.74 (333.11)	1.24 (0.55)	100.46 (3.0)
KSW-S	3.97 (0.79)	1.06 (0.21)	100.01 (4.80)

3.2. Response to Different ALS-Inhibitors

In addition to chlorsulfuron, KSW-R buckwheat survived field recommended rates of other SU herbicides, e.g., thifensulfuron, halosulfuron, and nicosulfuron, but did not survive the application of imazethapyr (IMI herbicide). The KSW-S plant did not survive the applications of the above herbicides (Table 3). Cross-resistance to different ALS-inhibitors has been previously reported to be associated with altered *ALS* gene or due to CYP based metabolism of ALS-inhibitors in weed species [25,26]. Cross-resistance endowed by altered *ALS* gene depends on type of amino acid substitutions and weed species. The single nucleotide polymorphisms in the *ALS* gene resulting in common amino acid substitution at the Pro-197 position confers a high level of resistance primarily to SU herbicides in many weed species [27,28]. Nonetheless, other amino acid substitution at the same position, for example, Pro-197-Ser bestows cross-resistance to SCTs in addition to SUs [29], while Pro-197-Leu substitution provides high or moderate level of cross-resistance to herbicides in different families of ALS-inhibitors [27]. Amino acid substitutions at Ala-122 or Ser-653 confer resistance to both IMI and SU herbicides, while Asp-376-Glu substitution provides resistance to all five chemical families and substitutions at Trp-574 confer resistance to IMIs, SUs, and TPs [27,30].

Table 3. Response of the KSW-R (resistant) and KSW-S (susceptible) wild buckwheat treated with different ALS inhibitors at their field rates at 3 weeks after treatment.

Trade Name	Field Rate	% Survival [a]	
		KSW-R	KSW-S
Chlorsulfuron	18 g ai ha^{-1}	100%	0%
Halosulfuron	36 g ai ha^{-1}	100%	0%
Thifensulfuron	36 g ai ha^{-1}	90%	0%
Nicosulfuron	36 g ai ha^{-1}	90%	0%
Imazethapyr	72 g ai ha^{-1}	0%	0%

[a] A total of 12 plants were treated and the number of plants that survived was expressed as the percent (%) survival.

3.3. Molecular Basis of ALS-Inhibitor Resistance in KSW-R Wild Buckwheat

A high level of resistance to chlorsulfuron in KSW-R wild buckwheat (Table 2) indicates the possible presence of one or more previously reported mutations in the *ALS* gene. A portion of the *ALS* gene from 15 KSW-R and 5 KSW-S wild buckwheat individuals was sequenced. The amplified region includes all eight previously reported amino acid positions, i.e., Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 [27]. A single nucleotide polymorphism (C/T)CC resulting in amino acid substitution at position 197 from proline (CCC) to serine (TCC) was found in all the KSW-R but not in KSW-S wild buckwheat plants (Figure 3). These results suggest that alteration in the *ALS* contributes to chlorsulfuron resistance in KSW-R wild buckwheat. Mutations in the *ALS* gene resulting in amino acid substitution is the most common mechanism of resistance to ALS-inhibitors in weed species [6]. All the 15 KSW-R buckwheat plants sequenced were homozygous for the Pro-197-Ser mutation. A high level of resistance to chlorsulfuron conferred by Pro-197-Ser substitution was previously reported in Palmer amaranth [10] and wild radish (*Raphanus raphanistrum)* [31]. However, in ALS-inhibitor-resistant wild buckwheat from Canada, an amino acid substitution, $Trp_{574}Leu$ in the *ALS* gene was found and this mutation also was shown to confer resistance to SU and TP herbicides [15].

Although a wild buckwheat population resistant to chlorsulfuron was reported from Queensland (Australia), the mechanism of resistance has not been identified.

Figure 3. Nucleotide sequence alignment and analysis of a portion of the *ALS* gene sequence from KSW-R (15 resistant plants) and KSW-S (5 susceptible plants) wild buckwheat. Nucleotide/amino acid numbering refers to the *Arabidopsis thaliana ALS* gene sequence.

Since the Pro-197-Ser substitution is specific for resistance to SU and SCT herbicides in most weed species [27], the KSW-R wild buckwheat was found resistant to only SUs but not the IMI herbicides (Table 3). The evolution of target-site resistance leading to cross-resistance within the single group of herbicides essentially depends on the ALS protein structure and the amino acid domains on the protein. The ALS protein in higher plants generally will have five highly conserved domains, namely, A, B, C, D, and E [31]. The domain A located in the N-terminal end consists of 13 amino acids including the Pro-197 [32]. Any mutations in domain A largely confer resistance to SU and/or TP, but moderate or no resistance to IMIs [27]. The SU herbicides interact with more amino acid residues compared to IMIs due to the difference in binding pockets making, SU resistance most common to several substitutions [29].

The coexistence of both target and non-target site resistance mechanisms such as altered *ALS* gene and enhanced metabolism of ALS-inhibitors in the same weed species has been reported [10,33,34]. Although altered *ALS* gene is known to confer a high level of resistance [28], enhanced metabolism also can bestow a high level of resistance in some weed species [10,35,36]. The presence of enhanced metabolism or other non-target site mechanisms such as reduced absorption or translocation also need to be tested in the KSW-R wild buckwheat to rule out contribution of non-target resistance mechanism to ALS-inhibitor resistance in this weed.

4. Conclusions

In conclusion, weed control in small grain crops such as wheat, oats, and barley are highly dependent on use of ALS-inhibitors. However, due to selection, several summer annual weeds, including wild buckwheat evolved resistance to these herbicides, which is a challenge for effective and efficient weed management. The evolution of resistance to SU herbicides in KSW-R is the first case of herbicide resistance in wild buckwheat in the US. Weed management strategies such as reducing the selection pressure by rotating herbicides with different modes of action, developing effective pre-emergence programs, use of weed-free crop seeds s and other integrated management techniques along with improved stewardship need to be followed to reduce further spread and evolution of weed resistance.

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