Mechanisms of Herbicide Resistance in Polyploid Monocot Weed Species

by

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Abstract

Herbicide resistance is currently the largest threat facing the field of weed science. With evolved resistance represented by 21 out of 31 herbicide modes of action, and no new active ingredients to alleviate resistance pressure, it is crucial for scientists to approach weed management from a new perspective. Molecular genetics is a growing field that, until recently, has been underutilized by weed scientists. Genomics, functional gene annotation, and differential gene expression analysis are tools now being applied to weed science that allow us to better understand weed species and how herbicide resistance evolves, particularly in polyploid species. This dissertation begins with a literature review to discuss the framework of and provide background on the research, particularly into the chosen species, *Poa annua* (annual bluegrass) and *Digitaria ischaemum* (smooth crabgrass). The second chapter details an extensive survey of herbicide resistant populations of *P. annua* across the United States to identify potential target-site mutations across four common modes of action and six herbicides. Due to the polyploid nature of *P. annua*, new sequencing methods were utilized outside of the standard methodology, as the conflicting subgenomes were introducing noise during Sanger sequencing. Ultimately, 1,349 *P. annua* populations were collected for resistance screening and 389 populations were identified as resistant to at least one mode of action.

In the third chapter a potential quinclorac-resistant biotype of *D. ischaemum* (AL_R1) was identified in the field. The evolution of quinclorac-resistant biotypes of *D. ischaemum* is detrimental to the turfgrass industry, as it removes one of the only postemergence herbicides, as well as the only grass selective herbicide available for controlling crabgrass. A greenhouse dose-response study was conducted in order to confirm the resistance status compared to a known quinclorac-susceptible population of *D. ischaemum* (AL_S1). All replicates of AL_S1 were

controlled at or below the standard rate, while none of the AL_R1 replicates were controlled at more than 55% of the highest rate use, validating its status as a resistant population. AL_S1 was then selected as the reference biotype for assembling the *D. ischaemum* genome, as detailed in chapter 4. Previous research indicated *D. ischaemum* is a polyploid species, and investigations were made into analyzing the subgenomes to confirm this. *D. ischaemum* was successfully assembled into an allotetraploid configuration, with subgenomes C and D to account for the existing genome of *D. exilis*, a tetraploid with subgenomes A and B. The assembled genome showed evidence of segmental allopolyploidy in *D. ischaemum*, given large sections of subgenome C were identified in subgenome D. Comparative analyses were also performed with existing genomes of *D. exilis* and *D. insularis* to determine if either species was a progenitor or shared a common ancestor with *D. ischaemum*, but no similar parentage was established between the species.

The final chapter utilized AL_R1 and AL_S1 to perform a differential gene expression analysis of inherently expressed genes to elucidate potential target-site genes for quinclorac. The mechanism of resistance to quinclorac is currently unknown, and this study sought to uncover genes with potential mutations and genes that were differentially expressed between the resistant and susceptible without being treated with quinclorac. Ultimately, no target-site mutations were identified from known mutations to other synthetic auxins, and while the differential expression study indicated clear differences between AL_R1 and AL_S1, no genes stood out as potential targets. The mechanism of action of quinclorac is a complex process that likely involves proteins still unknown, which highlights the need for further research to fully comprehend the molecular pathways induced.

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Abbreviations

WGD Whole genome duplications

TSR Target site resistance

NTSR Non-target site resistance

ALS Acetolactate synthase

ACCase Acetyl-CoA carboxylase

PSII Photosystem II

EPSPS 5-enolpyruvylshikimate-3-phosphate synthase

psbA Photosystem II protein D

IWGC International Weed Genomics Consortium

SOA Site of action

SNP Single nucleotide polymorphism

MSMA monosodium methanearsonate

ACS 1-aminocyclopropane-1-carboxylate synthase

RCBD randomized complete block design

ARM agricultural research management software

TE transposable element

BUSCO Benchmarking Universal Single-Copy Orthologs

EDTA extensive de-novo TE annotator

LTR long terminal repeats

LAI LTR assembly index

PCA Principal component analysis

HE homoeologous exchange

PPO protoporphyrinogen oxidase

HPPD 4-hydroxyphenylpyruvate dioxygenase

GS glutamine synthetase

TIR1 transport inhibitor response protein

AUX/IAA auxin/indole-acetic acid

β-CAS β-cyanoalanine synthase

CESA cellulose synthesis catalytic subunit A

MITE miniature inverted-repeat transposable elements

MYA million years ago

IR inverted repeats

LSC large single-copy

SSC small single-copy

AFB auxin signaling F-box

SCF SKP1-cullin-F-Box

DEG differentially expressed gene

ACO 1-aminocyclopropane-1 carboxylate oxidase

ACC 1-aminocyclopropane-1 carboxylate

Chapter 1: The Impact of Polyploidization on the Evolution of Weed Species *Poa annua* (annual bluegrass) and *Digitaria ischaemum* (smooth crabgrass)

Abstract: Whole genome duplication via polyploidization is a major driver of diversification within angiosperms and it appears to confer the most benefit during times of rapid environmental change. Polyploidization offers expanded access to novel phenotypes that facilitate invasion of new environments and increased resistance to stress. These new phenotypes can arise almost immediately through the novel interactions among or between transcription factors of the duplicated genomes leading to transgressive traits, and general heterosis, or they can occur more slowly through processes like neofunctionalization, and subfunctionalization. These processes are characterized by the changes within homologs of the duplicated genomes, homoeologs. It has been proposed that redundant homoeologs are released from selective constraints and serve as an additional source of adaptive genetic variation, particularly in neo and meso-polyploids. Current practices in weed management create rapid environmental change through the use of chemicals, practices that are meant to cause the extirpation of the designated weed and represent a strong recurrent selective event—a scenario that should favor polyploidy species. This brings about the discussion of two weed species: *Poa annua* (annual bluegrass), a known tetraploid, and *Digitaria ischaemum*, a suspected polyploid. Both species are native to Eurasia and have spread globally, and have developed resistance to different herbicides, indicating the need to better understand the species. The growing contingent of research in weed genomics, driven by herbicide resistance evolution is rapidly improving our understanding of weed molecular biology and will aid in improving understanding of the impacts of ploidy levels on weed evolution and adaptation in the future.

Introduction: All extant diploid angiosperms have been traced back to polyploid ancestors (Scarpino et al., 2014). Whole genome duplications (WGDs) are major drivers of adaptation and are responsible for the trajectory of flowering plant evolution. Phylogenetic analyses and molecular dating have traced an ancient genome-wide duplication event shared by all extant seed plants (Jiao et al., 2011). Ancient WGD, served as a major force in speciation and diversification in highly plastic angiosperm genomes. Compared to gymnosperms, angiosperms are more likely to endure the impact that polyploidy has on a genome, as less than 5% of gymnosperms are polyploid (A. R. Leitch & Leitch, 2008). While polyploidy is gaining traction as a viable and beneficial means of adaptation, polyploidization has previously been described and is still commonly referred to as an evolutionary "dead end", as ancient WGD were seen scarce (Arrigo & Barker, 2012; Van De Peer et al., 2017). Polyploidy studies are continuing to rise in prevalence, and more cases of ancient and neopolyploid cases are being discovered and suggests that polyploidization via whole genome duplication is more common than previously thought (Barker et al., 2016; Hohmann et al., 2015; Yang et al., 2018). In rare instances polyploids could have had an evolutionary advantage on their non-polyploid competition, especially in times of stress or environmental upheaval, providing means to survive over their counterparts (Van De Peer et al., 2017). Recent studies provided evidence that there is an increased tolerance to genomic changes in polyploids relative to diploid progenitors, including how polyploid lineages were established and the rates at which this occurs, and the mechanisms they used to spread and maintain themselves (Schoenfelder & Fox, 2015; Shimizu-Inatsugi et al., 2017). Within the field of weed science, the selective forces of climate change and the ever-increasing size of highly managed tracts of land around the world may favor polyploids. Here we ask if polyploidy confers an advantage to the weediness of specific species: *Poa annua* (annual bluegrass), which

is a known tetraploid, and *Digitaria ischaemum* (smooth crabgrass), a suspected polyploid. Both species are boons in turfgrass systems, as both present among the most common and most troublesome species in turfgrass (Van Wychen, 2020).

Definitions About Polyploidy: Polyploids are organisms that contain more than two sets of their chromosomes, or simply, a species that has more copies than diploids (Glover et al., 2016). *Polyploidization* itself is defined as whole genome duplication, where it has doubled in the form of either *allopolyploidy* or *autopolyploidy*, or as a combination of both forms (Table 1). *Allopolyploids* are generated through the hybridization of two or more different species each contributing unique subgenomes, while *autopolyploids* arise from the duplication of a single species' genome. On a gene level, the multiple copies of genes or chromosomes in *allopolyploids* are referred to as *homoeologs*. Not to be confused with *homologs*, *homoeologs* are related genes that lie in the different subgenomes of an allopolyploid (Mason & Wendel, 2020). *Homologous genes* share a common ancestor, while *homoeologous genes* have the same parental origin, meaning these genes are duplicates within the context of WGD (Mable, 2003). Within homologous genes, there are *orthologs* and *paralogs*: *orthologs* are genes descended from a common ancestor in different species that share the same function or formed due to a speciation event. *Paralogs* are genes derived from a single gene as the result of a duplication event (Sonnhammer & Koonin, 2002). *Homoeologs* and *orthologs* can be construed as analogous, as homoeologs are orthologous genes within a polyploid species that occur on different subgenomes. *Homoeologs* originated through speciation and were recombined in the same genome through *allopolyploidization* (Glover et al., 2016). The correct usage of "homoeolog" has been debated and the sheer amount of different terms can lead to some confusion.

Paleopolyploidy is defined as polyploidy that occurred millions of years ago (Blanc & Wolfe, 2004; Soltis et al., 2009). Genes associated with *paleopolyploidy* can also be referred to as *paleologs*. Determining whether an organism is a *paleopolyploid* or used to be a difficult task because progenitor species could not be identified through cytological tools or DNA markers (Levy & Feldman, 2002). Advances in genomics has eased the process of identification with whole genome assemblies providing the necessary data for synteny plots, gene trees constructed from gene family analyses, and Ks plots from transcriptome assemblies (Gao et al., 2018; Husemann & Stoye, 2009; Leebens-Mack et al., 2019). More recent polyploids have two different categories: *mesopolyploid*, if formed after *paleopolyploids*, but not as recent as *neopolyploids*, the species that most recently experienced polyploidization (Cheng et al., 2018; Ramsey & Schemske, 2002). *Neopolyploidy* can also be described as a species that has experienced an artificially induced chromosome duplication (Comai, 2005). *Aneuploidy* is another term associated with polyploidy, as it signifies when there is an abnormal number of chromosomes compared to the wild type, which is commonly found in triploid (and sometimes pentaploid) populations (Huettel et al., 2008; Müntzing, 1936).

History of Polyploid Evolution: The earliest concepts of polyploidy came about in the early 1900s. The independent rediscovery of Mendel's work by de Vries, Correns, and Tschermak was the beginning of a golden age of genetics (Corcos & Monaghan, 1990). Geneticists originally associated specific characteristics with morphological characteristics as opposed to genetic characteristics like karyotype (DeVries, 1915; Ramsey & Ramsey, 2014). Using morphological characteristics as a form identification was soon displaced by the acceptance of chromosomes as hereditary units (Roberts, 1929). While certain plants, like maize, had already been determined to be polyploid (Kuwada, 1911), the term polyploidy was coined by (Winkler, 1917), who

created the first artificial polyploid. (Winge, 1917) had some of the most influential thoughts on the subject, proposing hybridization followed by the doubling of chromosomes (Harlan and Dewet, 1975; Soltis et al., 2014). Stebbins (1950) could be considered one of the most important thinkers on the importance of polyploidy, with fourteen chapters in his book *Variation and Evolution in Plants* dedicated to the subject. Scientists were tasked with the painstaking endeavor of manually counting chromosomes under a microscope using the squash method, until the genomics era eventually brought about flow cytometry, a more accurate way to measure cellular contents, including DNA and chromosomes (Kron et al., 2007; Windham et al., 2020).

Much of what is understood about the history of polyploidization has come from studying crops (Beasley, 1940; Mcfadden & Sears, 1946). Thus far, genomic studies on *Triticum* (wheat) and *Gossypium* (cotton) have contributed the most to the current knowledge (Feldman & Levy, 2009; Flagel et al., 2008). Cultivated wheat is a good example of how studying polyploidization can be useful. Cultivated wheat is classified in three different cytogenic categories: diploid, tetraploid, and hexaploid. While the wild type progenitors for the diploid and tetraploid varieties have been determined, studies have shown that the hexaploidy varieties, like bread wheat (*T. aestivum*) have formed as a byproduct of cultivated tetraploid and wild diploid progenitors as a result of polyploidization (Feldman, 2001; Feldman & Levy, 2005). In allohexaploid bread wheat, there are three identifiable subgenomes, A, B and D, which is seen as an AABBDD genome. These subgenomes are known to have derived from diploid progenitors *T. uratu* (AA) and *Aegilops tauschii* (DD). The progenitor of the BB subgenome is extinct but is likely derived from a diploid closely related to *Aegilops speltoides* (Dubcovsky & Dvorak, 2007; Gornicki et al., 2014). The ability to identify these subgenomes provides a history of polyploidization in wheat,

visualizing its progenitors, its center of origin (likely in southwest Asia), and estimating when the polyploidization likely occurred (Feldman, 2001; Savage et al., 1994).

Polyploidization is a seemingly irreversible process, but all polyploid plants eventually undergo the process of diploidization. The process of a polyploid becoming a diploid again is a result of *genomic downsizing*, where genomes have been significantly reduced as a result of loss of DNA fragments, segmental DNA loss, and gene silencing, mainly to stabilize the genome (Adams & Wendel, 2005; Bird et al., 2019). Genomic downsizing most likely occurs immediately following a chromosomal duplication event. Drastic alterations to the genome are referred to as *genome shock*; a plant might not be prepared for such intense changes to its genome and these stabilization events could possibly occur to counteract the shock (McClintock, 1984). There is a case to be made that there are no true extant diploids and should be considered to be paleopolyploids (Levy & Feldman, 2002). Combined with the fact that all diploid angiosperms are descended from polyploid ancestors, genomic downsizing over the course of millions of years could contribute to this claim (Feldman & Levy, 2005; Force et al., 1999). An example of this is present in corn (*Zea mays*); it has paleopolyploid characteristics and has origins as a segmental allopolyploid, but its genome was so drastically altered and silenced that it is a cytogenic diploid (Gaut & Doebley, 1997; Soltis & Soltis, 1999) .

Duplicate genes in polyploids have many different pathways they can take: they can develop a new function (*neofunctionalization*), retain the ancestral function (*subfunctionalization*), or most common, accumulate deleterious mutations and decay (Force et al., 1999). In the process of trying to maintain its status as a diploid, some plants will undergo the process of *instantaneous subfunctionalization*, which occurs immediately following genomic merger in order to retain all duplicate genes (Flagel et al., 2008). Different loss-of-function mutations can develop in both

copies, but both copies must be retained in order to keep its ancestral function (Cheng et al., 2018). Upland cotton (*Gossypium hirsutum*) demonstrates subfunctionalization in the reciprocal silencing of its *adhA* homoeolog; the homoeolog is silenced rather than deleted, retaining all copies present (Adams et al., 2003). Larger populations are more likely to experience neofunctionalization rather than subfunctionalization because the genetic drift in large populations is going to be so slow that parental alleles are likely going to be silenced by deleterious mutations before fixation can occur (Soltis et al., 2010).

Advantages of Polyploidy in Evolution: Polyploidization allows organisms to react and survive; by their very nature, polyploids have a much higher range of genetic diversity than diploids, which certain environmental factors, such as habitat disturbance, nutritional stress, physical stress, and climate changes, can trigger new phenotypes, like increased allelopathic effect (Hegarty & Hiscock, 2007; Omezzine & Haouala, 2017; Ramsey, 2011; Te Beest et al., 2012). New phenotypes may arise through heterosis, gene redundancy, or the formation of transgressive traits (Comai, 2005; McCarthy et al., 2016; Wei et al., 2019). The effects of heterosis were first identified by Darwin, whose experimental crosses in over sixty species resulted in more vigorous hybrids, i.e. heterosis (Darwin, 1892). There are two main models involved in heterosis: the dominance model and overdominance model. The dominance model hypothesizes that the slightly deleterious recessive alleles are complemented by superior dominant ones in hybrids (Hochholdinger & Baldauf, 2018). The overdominance model is used to describe polyploidization, as the progressive heterosis associated with polyploids is more complex due to the increasing vigor with increasing number of genomes (Birchler et al., 2010). While heterosis generally results in polyploids with better phenotypic performance than its parent species, plants with transgressive traits display extreme phenotypes outside of the range of

its progenitors (McCarthy et al., 2016). Heterosis and transgressive traits have been shown to be potential improvements for epigenetic mechanisms in allopolyploids, like histone modification or cytosine methylation (Renny-Byfield & Wendel, 2014). Gene redundancy acts as a protective feature, shielding polyploids from the effects of deleterious mutations with the numerous copies present (Wendel, 2000). Even allelopathy (the ability to suppress growth in another plant), which is present in both diploids and polyploids, has been shown to increase in polyploids compared to diploids. Hexaploid barnyardgrass (*Echinochloa crus-galli*) shows considerable allelopathic tendencies and Omezzine et al. (2017) was able to show that allelopathy increased as ploidy increased in fenugreek (*Trigonella foenum-graecum*) (Khanh et al., 2007).

Allopolyploids provide some evidence of increased fitness over their progenitors. When diploid parents are crossed, typically their offspring have an increase in performance; polyploids produced more viable seed in extreme heat and drought conditions and differences in stomatal pore sizes that improved drought survival over their diploids counterparts (Godfree et al., 2017; Madlung, 2013). For example, cultivated wheat *(T. aestivum*) is an allohexaploid that has managed to survive over its B genome donor (Feldman & Levy, 2009). Allopolyploids also have more potential for ecological adaptation over their diploid counterparts, as shown through diploid and allopolyploid species of *Cardamine*; while different diploid species had a tendency to prefer only one environment, the allopolyploid species was able to grow and survive in all the environments tested (Shimizu-Inatsugi et al., 2017). The ability to alter phenotypes, as in functional trait divergence or generalized trait plasticity is one of the leading hypotheses regarding overall increased fitness in polyploid species (Van De Peer et al., 2017; Wei et al., 2019). Polyploid crops have huge adaptation potential and further studies are necessary to show

the role of genetic variation resulting from polyploidy in this potential (Ramsey & Ramsey, 2014; Schiessl et al., 2017).

The study of neopolyploids furnishes strong insights in the evolution of polyploid species. *Spartina anglica*, (common cordgrass) is an invasive neoallopolyploid weed species that arose in the last 200 years (Baumel et al., 2002). The neo-dodecaploid weed arose at the end of the 19th century as a result of a genome duplication between the already hybrid species *Spartina* x *townsendii*, which is a cross between hexaploids *Spartina alterniflora* and *Spartina maritima* (Ainouche et al., 2004). The duplication of the two unique subgenomes in *Spartina* x *townsendii* cements *S. anglica* as an allopolyploid as opposed to an autopolyploid. Compared to its progenitors, *S. anglica* has been shown to have increased fitness with its prolific seed production, fertility, and extensive lateral clonal growth, which was not seen in its sterile progenitor *Spartina* x *townsendii*. Baumel et al. (2002) was able to demonstrate that rapid, non-Mendelian changes involving preferential sequence elimination or modification of methylation patterns may occur in the earliest stages of polyploid stabilization. Other neopolyploids, like *Senecio* and *Tragopogon* have also been established within the last two hundred years (Abbott & Lowe, 2004; Soltis et al., 2004) . The development of new polyploids aids in understanding gene silencing, cytosine methylation, and parental 'non-additivity' play an active role in polyploidization and improving overall understanding of the process (Adams & Wendel, 2005).

Herbicide Resistance: In this modern era, herbicide resistance is the biggest problem currently faced with weeds. The two types of herbicide resistance typically dealt with are target site resistance (TSR) and non-target site resistance (NTSR). TSR develops directly against a mode of action as a mutation to the target site within a gene (such as single nucleotide polymorphisms (SNPs)), changes to target-site gene expression, or receptor interactions (Gaines et al., 2020).

NTSR relates to metabolism, as there are no direct changes to the genetic code (Sammons & Gaines, 2014). This can be seen as reduced absorption, translocation, or sequestration of the herbicide in the vacuole (Powles & Yu, 2010). Both TSR and NTSR, is more often than not, monogenic: this can be seen as a SNP, a single unregulated metabolic enzyme, or even as a widely duplicated gene (Délye, 2013; Jugulam & Shyam, 2019). Herbicide resistance has evolved to 21 of the 31 major sites of action and 168 herbicides, with both TSR and NTSR mechanisms (Heap, 2024). The modes of action with the most resistance thus far are to inhibitors of acetolactate synthase (ALS), photosystem II (PSII), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), acetyl-coA carboxylase (ACCase), and synthetic auxins (Heap 2024). While glyphosate, the singular EPSPS inhibitor, is the most used herbicide worldwide, there are more herbicides dedicated to the inhibition of ALS than any other mode of action, which makes sense as to why more species have evolved resistance to ALS herbicides (Heap and Duke 2018; Tranel et al. 2002). Regarding target-site resistance, TSR mutations have been identified for almost every mode of action, including eight mutations on the *ALS* gene, five mutations on the *photosystem II protein D* (*psbA*) gene, three mutations on the *EPSPS* gene, and seven mutations on the *ACCase* gene (Murphy and Tranel 2019). Target-site mutations to synthetic auxin herbicides have been identified for herbicides like 2,4-D, dicamba, fluroxypyr, and picloram, but the target site itself is variable based on herbicide use (Walsh et al. 2006; LeClere et al. 2018; de Figueiredo et al. 2022). Despite this, the specific synthetic auxin of interest to this paper, quinclorac, has had no confirmed target-site mutations.

Theoretically, target site resistance should be more common in polyploid weeds, since theoretically polyploids have a more flexible expression profile that allows them to silence adaptive alleles or loci with fitness costs when the allele offers no adaptive advantage (Otto,

2007; Otto & Whitton, 2000). TSR fitness costs, or adverse impacts on a plants reproductivity due to resistance alleles, have been identified, but the level of costs varies among different plant species and modes of action (Cousens & Fournier-Level, 2018; Vila-Aiub et al., 2009). In general, fitness costs have been associated with ALS, ACCase, and PSII herbicides, which is especially evident in PSII herbicides because of the reduced photosynthetic capacity (Jansen & Pfister, 1990). Fitness costs in ACCase inhibitors should have no association with polyploidy in grass species because ACCase inhibitors only affect the plastid isoform of grasses (Murphy & Tranel, 2019). A reduction in fitness has been identified in glyphosate resistant goosegrass (*Eleusine indica*), rigid ryegrass (*Lolium rigidum*) and perennial ryegrass (*Lolium perenne*) (Han et al., 2017; Preston et al., 2009; Yanniccari et al., 2016). However, fitness costs in glyphosate resistant biotypes seem to be present on a case-by-case basis. The TIPS double mutation in the *E. indica* population came at a very high resistance cost: resistant *L. rigidum* populations may or may not have a fitness penalty, depending on the resistance allele present, and the fitness cost in *L. perenne* is not associated with a target-site mutation, but rather high EPSPS activity. The Pro-106-Ser mutation, the most common target-site in glyphosate resistant biotypes, endows a low-level glyphosate resistance and is seemingly negligible in fitness costs compared to mutations endowing high level resistance, like the TIPS mutation (Vila-Aiub et al., 2019). Studies have shown that herbicide resistance alleles do not universally endow some type of fitness cost, but there is more of a cost in diploid species over polyploid (Vila-Aiub et al., 2009; Yanniccari et al., 2016). There has been no investigation comparing the fitness cost of herbicide resistant polyploid species to the cost seen in diploid species, or even delving into the costs of herbicide resistance in any polyploid species. While there have been reviews showing the fitness costs of different herbicides, all data and conclusions are drawn from diploid species (Vila-Aiub et al., 2009).

More studies should be performed in order to ascertain whether polyploidy plays a role in reduced fitness in association with herbicide resistance.

Quinclorac: Evolved resistance to the synthetic auxin quinclorac is concerning from a turfgrass perspective as quinclorac is unique among synthetic auxin herbicides for its grass-in-grass specificity (Grossmann, 1998). However, the mechanism behind the selective nature of quinclorac is currently beyond our understanding: much like how synthetic auxins like 2,4-D or dicamba affect broadleaves and not grasses, quinclorac only targets grasses belonging to the PACMAD clade of Poaceae, which is comprised of all the C_4 grasses (Hodkinson, 2018). The C_3 clade BOP and the Chloridoideae subfamily of PACMAD are tolerant to quinclorac, which eliminates the method of carbon utilization as a possible reason for the selectivity of the herbicide. No taxonomic or phylogenetic study has been performed on the PACMAD and BOP clades to better understand sensitivity or tolerance to quinclorac, and all tolerance levels identified are based on specified usage by the herbicide labels (CDMS Advanced Search Database; Schoch et al., 2020). Beyond the taxonomic peculiarities of quinclorac selectivity, the mechanism of evolved resistance has yet to be resolved. The mode of action for quinclorac is understood on a basic level, where application of quinclorac leads to two separate pathways based on if the affected species is a broadleaf or grass. In broadleaf species, quinclorac acts as a typical synthetic auxin, displaying the expected injury response with epinasty and overgrowth (Grossmann, 1998). In grass species, the injury response is more similar to a systemic herbicide, as the plants will exhibit necrosis rather than the characteristic twisting of stems and cupping of leaves (Grossmann and Kwiatkowski, 1995). The injury caused by quinclorac applications in sensitive grasses has been associated with the overproduction of cyanide as a byproduct of

ethylene biosynthesis, however the differential response between broadleaves and grasses is still not understood.

***Poa annua*:** Annual bluegrass (*P. annua*), is a winter annual monocot weed species that has established itself on every continent, including Antarctica (Molina-Montenegro et al., 2012). Though *P. annua* thrives in mild climates and is a desirable turfgrass species in some parts of the world, its heat sensitivity is limiting to its aesthetics (Yelverton, 2015). Known for its bright green color, *P. annua* has been named the most troublesome weed for turfgrass, as it is prolific, hardy underfoot, and stands out against traditionally darker turf varieties (Mitich, 1998; Van Wychen, 2020). *P. annua's* nature as an exceptional weed species likely lends itself to its polyploid status. *P. annua* is an allotetraploid formed from a WGD following the hybridization of *P. supina* and *P. infirma* (Mao and Huff, 2012). Pandit et al. (2011) showed in an extensive study on rarity and invasiveness that diploid plants were more likely to be rare, while polyploids were more likely to be invasive. It has also been determined that polyploid species are less likely to experience inbreeding depression, due to the balancing effect of the presence of multiple gene copies (Rosche et al., 2017). The combination of higher seedling growth rates and diminished inbreeding depression creates an argument that polyploids are more invasive and therefore more competitive than diploids, which could be argued for *P. annua.*

Along with its hardiness and widespread distribution, *P. annua* is troublesome due to the number of evolved resistance types. To date, *P. annua* has reported resistance to twelve modes of action, more than any other species besides *L. rigidum*, which also has reported resistance to twelve modes of action (Heap, 2022). This includes resistance to the aforementioned herbicide groups, ALS, ACCase, PSII, and EPSPS. As a member of the BOP clade of Poaceae, *P. annua* is naturally tolerant to quinclorac (Hodkinson, 2018). Due to all these characteristics, *P. annua* is

an ideal polyploid species to use to understand the incidence of herbicide resistance across the United States.

***Digitaria ischaemum*:** *Digitaria* is a genus of around 200 species yet there is little to no genetic information on any of them. The only two species with known ploidy levels in the genus, until recently, were *D. sanguinalis* (large crabgrass) and *D. exilis* (fonio millet), a hexaploid and tetraploid species, respectively (Leitch et al., 2019). Despite knowing the ploidy level of these species, the progenitor species are still undetermined. In turfgrass, *Digitaria* spp. are considered one of the most common and troublesome weeds to control, which leads one to assume that understanding the genetic makeup of this genus may help in managing incidences of the weeds (Van Wychen, 2020). The *Digitaria* weed species that have generally been problems for turfgrass managers are *D. sanguinalis*, *D. ciliaris* (Southern crabgrass), and *D. ischaemum* (smooth crabgrass). These three grasses are all visually similar to each other, mainly distinguished by the lack of hairs on leaf blades in *D. ischaemum* (Jones et al., 2021). Due to the fact that the only known ploidy levels for *Digitaria* are that of polyploid species, and the phenotypic similarities to *D. sanguinalis*, it can be hypothesized that *D. ischaemum* is a polyploid. Recent flow cytometry work by the IWGC elucidated that *D. ischaemum* and another *Digitaria* species, *D. insularis* (sourgrass) confirmed the two are tetraploid species, and like the other *Digitaria* spp., their progenitors are currently unknown (Montgomery et al., 2024).

D. ischaemum is a particularly unique case for studying herbicide resistance in polyploids, because unlike *P. annua*, resistance has only been reported in two herbicides belonging to two modes of action: fenoxaprop-ethyl, an ACCase inhibitor, and the synthetic auxin quinclorac (Derr, 2002; Abdallah et al., 2006; Putri et al., 2024). As a member of the PACMAD clade of Poaceae, *D. ischaemum* should be susceptible, but its evolved resistance to quinclorac is one that

should be studied. However, the genomic resources required for analyses involving mechanisms of resistance are currently limited for *D. ischaemum*.

The Genome Issue for Polyploids: The sheer magnitude and complexity of polyploid genomes makes it difficult to perform large-scale genetics studies (Schiessl et al., 2017). While there have been polyploid genomes fully sequenced, the genomes sequenced have been relatively small, genome size wise, outside of the massive undertaking of sequencing the allohexaploid wheat genome (Zimin et al., 2017). Advances in genomics have made whole genome sequencing easier and cheaper as a whole, but it is improving the possibility of sequencing polyploid genomes. Research into weed genomics has room for improvement, and the development of weed genomics provides potential for greater understanding in how weed species evolve and the role polyploidy is playing and has played in weed evolution (Patterson et al., 2019; Ravet et al., 2018). With the increase of weed species developing resistance to herbicides and no new herbicide actives, there was an obvious need for a well-established weed genomics database, which the International Weed Genomics Consortium (IWGC) was founded to achieve (Duke & Dayan, 2022). The first polyploid weed genome sequenced was barnyardgrass in 2017, and since then a major boom in polyploid genome production has occurred (Guo et al., 2017; Kyriakidou et al., 2018). Before the formation of the IWGC, twenty-four weed genomes had been produced since the release of the barnyardgrass genome, seven of which are polyploid species (Montgomery et al., 2024)**.** Comparatively, the IWGC has produced thirty-one weed genomes, eleven of which are polyploid species, that should mostly be publicly available by the end of 2024. (Montgomery et al., 2024). The genome for *P. annua* is among the genomes produced outside of the IWGC, and the *D. ischaemum* is a genome is a goal among the IWGC (Benson et al., 2023). The state of molecular genetics in turfgrass weed science in particular is leagues

behind agricultural weed science because, as a field focused on aesthetics, the economic impact of turfgrass is considerably less than crops (Kassel, 2024; UT Institute of Agriculture, 2023; USDA, 2023)

Objectives: The current state of weed management is severely limited with the increased resistance to herbicides and the lack of new active ingredients. While weed species in general are difficult to manage, there has been a severe lack of trying to understand how herbicide resistance evolves, the extent herbicide resistance is occurring, and the effects polyploidy may have on understanding herbicide resistance. The overall purpose of this study was to focus on target-site resistance mechanisms of herbicide resistance in the species *P. annua* and *D. ischaemum*. Both species are banes to turfgrass systems, and this project aimed to elucidate more information that could be beneficial for practical management and molecular weed genomics. The objectives of this study were to 1) determine the occurrence of target-site resistance in *P. annua* across the United States and improve sequencing techniques for identifying TSR mutations in polyploid species; 2) to confirm the incidence of a quinclorac-resistant population of *D. ischaemum*; 3) produce a reference-level genome of *D. ischaemum* and analyze its subgenome architecture; 4) elucidate potential mechanisms of resistance to quinclorac in *D. ischaemum* through RNA sequencing and transcriptomic analyses. The goal of this study was to provide genetic and genomic techniques and resources into studying herbicide resistance in polyploid species.

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Tables:

TABLE 1-1: Important terms relating to polyploidy and their definitions.

Term	Definition	Source
polyploidy	condition where an organism contains more than two sets of homologous chromosomes, or more than a diploid, as a result of whole genome duplications	Glover et al., 2016
allopolyploidy	polyploidy generated through hybridization between two distinct species followed by genome doubling	Glover et al., 2016
autopolyploidy	polyploidy generated through intraspecific hybridization	Glover et al., 2016
homolog	a gene in two species that are derived from the same ancestor	Mable, 2003
ortholog	a homologous gene within two species that share the same function, formed as a result of a speciation event	Sonnhammer and Koonin, 2002
paralog	a homologous gene within the same species that do not have the same function, formed as a result of a duplication event	Sonnhammer and Koonin, 2002
homoeolog	genes that originated due to a speciation event but were recombined due to allopolyploidization	Glover et al., 2016; Mason and Wendel, 2020
paleopolyploidy	ancient polyploidy, formed millions of years ago	Blanc and Wolfe, 2004; Soltis et al., 2009
neopolyploidy	the most recent cases of polyploidy, can be used to describe artificially created polyploids	Ramsey and Schemske, 2002; Comai, 2005
mesopolyploidy	bridge between paleo and neopolyploidy	Cheng et al., 2018
aneuploidy	situation where there is an abnormal number of chromosomes in a cell	Huettel et al., 2008; Müntzing, 1936
subfunctionalization	process where newly formed genes will retain some subset of the ancestral gene function	Flagel et al., 2008; Force et al., 1999
neofunctionalization	process where newly formed genes will obtain some new function	Force et al., 1999
target site resistance	herbicide resistance mechanism that is the result of a change to the genetic code	Sammons and Gaines, 2014
non-target site resistance	herbicide resistance mechanism that is a result of a change in the metabolism of a plant	Sammons and Gaines, 2014

Chapter 2: Survey of Target Site Resistance Alleles Conferring Resistance in *Poa annua*

Abstract: *Poa annua* L. (annual bluegrass) is a common weed in turfgrass and has been reported resistant to 12 different herbicide sites of action, with various combinations of multiple-herbicide resistance having been identified. To quantify the extent of herbicide-resistant *P. annua*, the ResistPoa Project (resistpoa.org) surveyed 1349 *P. annua* populations for resistance to nine sites of action and one plant growth retardant. Herein, we report results from sequencing of known target site mutations found in 5-enolpyruvylshikimate-3 phosphate synthase (*EPSPS*), acetolactate synthase (*ALS*), photosystem II protein D (*psbA*), and *α-tubulin* genes. Populations were sequenced using either capillary or amplicon sequencing (AmpSeq), depending on the complexity of the gene, and were analyzed for target-site resistance. After additional resistance screening, a total of 389 suspected resistant populations were sequenced—131 for ALS, 83 for EPSPS, 93 for psbA, and 82 for *α*-tubulin. From the resistant populations, 64 displayed resistance to multiple sites of action. After sequencing, it was determined that target-site resistance was the common form of resistance for all sites of action outside of psbA with 65.6% of ALS populations, 73.5% of EPSPS, 39.8% of psbA, and 91.5% of *α*-tubulin having presented a target-site mutation.

Introduction: *Poa annua* L. (annual bluegrass) is listed as one of the top five most common weeds in turfgrass, and within the past two years, has risen to the most troublesome weed in turfgrass and second most troublesome weed overall, behind only *Amaranthus palmeri* (Palmer amaranth) (Van Wychen, 2020). *Poa annua* has been reported resistant to twelve sites of action (SOA), which equals that of *Echinochloa crus-galli* (barnyardgrass) and is only slightly fewer than that of *Lolium rigidum* (rigid ryegrass) (Heap, 2022). Across species, resistance is most

common to 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS), acetolactate synthase (ALS), α-tubulin, and photosystem II protein D (psbA) inhibitors.

Poa annua is a polyploid formed from a whole genome duplication of the hybrid between *P. infirma* and *P. supina* approximately 2.5 million years ago (Mao & Huff, 2012; Tutin, 1957). Ubiquitous in nature, *P. annua* has been found on every continent, including Antarctica (Molina-Montenegro et al., 2012), and is largely a cosmopolitan weed of managed turfgrass. It is prevalent in the winter and spring season in most dormant warm-season turfgrass systems, and is present as a perennial in cool-season turfgrasses, especially if prophylactic management practices protect it from stress, disease, and insect-inducing annual death (Carroll et al., 2022; La Mantia & Huff, 2011). In cool season turfgrass management, the perennial subspecies of *P. annua* (ssp. *reptans*) may be considered a desirable turfgrass for putting surfaces, but the lack of heat tolerance in ssp. *reptans* limits its desirability in warmer climates due to summer decline (Huff, 2003; Yelverton, 2015).

Herbicide resistance is one of the few phenomena in which humans can observe evolution in action (Powles & Yu, 2010). Resistance is a natural, evolved response to human agricultural practices, and with over 500 unique instances of herbicide resistance, the ability to identify resistance mechanisms is important for developing alternative management practices (Heap, 2022; Norsworthy et al., 2012).

Resistance occurs as either target-site resistance (TSR) or non-target-site resistance (NTSR). Target-site resistance presents as direct changes to a gene, like single nucleotide polymorphisms (SNP), multiple nucleotide polymorphisms, codon deletions, changes to target-site gene expression, or receptor interactions, while NTSR presents as a mechanism related to reduced absorption or translocation of a herbicide or changes in metabolic interactions (Délye et al.,

2013; Gaines et al., 2020). Two concerning evolutionary drivers of herbicide resistance are cross-resistance and multiple- resistances. Cross-resistance is associated with both TSR and NTSR, as it deals with resistance for multiple herbicides conferred by a single mechanism, either by altered target-sites for herbicides with the SOA or a number of NTSR mechanisms, like enhanced metabolism or reduced translocation, regardless of herbicide SOA (Beckie & Tardif, 2012; Hall et al., 1994). Multiple-resistance can be described as the expression of more than one resistance mechanism across different herbicide classes and can be a result of both TSR and NTSR mechanisms (Hall et al. 1994). While most mechanisms of NTSR are still not very well understood, TSR is more straightforward and is often elucidated through sequencing alone. Target-site mutations have been found specifically for *ALS, acetyl-CoA carboxylase, α-tubulin,* synthetic auxins, *EPSPS*, *glutamine synthetase, phytoene desaturase, psbA*, and *protoporphyrinogen oxidase* genes (Murphy & Tranel, 2019).

There has been more resistance associated with ALS-inhibiting herbicides than any other SOA, and there are also more known target-site mutations on the *ALS* gene than any other SOA. These include the nucleotide coding positions Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653, and Gly654, with mutations at Pro197 and Trp574 being the most common; however, some mutations endow a level of cross resistance. Mutations at the Ala122, Ala205, Ser653, and Gly654 sites tend to provide some level of resistance to only imidazolinones and Arg377 to only sulfonylureas, while Pro197 provides cross resistance to sulfonylureas and triazolopyrimidines, and Asp376 and Trp574 provides resistance across all classes of ALS-inhibiting herbicides (Beckie & Tardif, 2012; Tranel, P.J., Wright, T.R, and Heap, I.M., 2022). Regarding *P. annua*, the Ala205 and Trp574 mutations have been determined to provide high levels of cross

resistance across the ALS-inhibiting herbicide family (Brosnan et al., 2016; McElroy et al., 2013).

In mitotic-inhibiting herbicides, Thr239 and Met268 mutations on the *α-tubulin* gene both provide an intermediate to high level of resistance to dinitroaniline herbicides but have not been described as providing cross resistance to pyridine herbicides (Yamamoto et al., 1998). The Leu136 mutation however has been shown to provide varying levels of resistance to herbicides in both the dinitroaniline and pyridine chemical families (Russell et al., 2021).

Photosystem II (PSII)-inhibiting herbicides are unique in the fact that there are two distinct herbicide resistance action committee (HRAC) groups associated with inhibition: group 5 D1 Serine 264 binders and group 6 D1 Histidine 215 binders. Thus far there are five known target-sites on the *psbA* gene that endow herbicide resistance to PSII-inhibiting herbicides. These include the nucleotide coding positions Val219, Ala251, Phe255, Ser264, and Asn266 (Dumont et al., 2016; Hirschberg & McIntosh, 1983; Masabni & Zandstra, 1999; Mechant et al., 2008; Park & Mallory-Smith, 2006; Perez-Jones et al., 2009). Because group 5 herbicides encompass more resistance, the Ser264 target site mutation has shown more instances of cross resistance between herbicide families, whereas the Asn266 mutation is the only known mutation endowing resistance to group 6 herbicides. Overall Val219 shows resistance to triazinone and urea herbicides, Ala251 to triazinones, Phe255 to triazinones and ureas, Ser264 to triazines, triazinones, and urea herbicides, and Asn266 to nitrile herbicides (Beckie & Tardif, 2012). The Ser264 mutation was first identified in *P. annua* (Kelly et al., 1999) and is the most commonly identified TSR mutation in the species, with the Val219 mutation being the only other TSR mutation identified thus far (Mengistu et al., 2000).

There are two known target-site mutations to glyphosate, the only EPSPS-inhibitor. These mutations are Thr102 and Pro106 (Baerson et al., 2002; Yu et al., 2015). There have been several target-site mutations at the Pro106 location, including mutations Pro106Ala, Pro106Ser, Pro106Thr, and Pro106Leu; however, Pro106Ala and Pro106Leu are the only documented mutations in *P. annua* (Baerson et al., 2002; Brunharo et al., 2018; Cross et al., 2015; Wakelin & Preston, 2006). Yu et al., 2015 reported a double point mutation, Thr102Ile and Pro106Ser (TIPS) that conferred a high level of glyphosate resistance in *Eleusine indica* (goosegrass), however this mutation has yet to be reported in *P. annua*. Gene amplification has also been associated with *P.* annua as a TSR mechanism, with studies showing a 7-fold increase in *EPSPS* copy number relative to *ALS* (Brunharo et al., 2018). Nontarget-site resistance, specifically reduced translocation of glyphosate throughout the plant, has also been well documented in association with EPSPS resistance; however, the exact mechanism is still unknown (Jugulam & Shyam, 2019).

In response to the widespread proliferation of *P. annua* in turfgrass, the ResistPoa Project was formed with the goal of characterizing the distribution of resistant populations and improving management practices, both chemical and non-chemical strategies (Bagavathiannan et al., 2018). Research was conducted to characterize target-site resistance through various gene-sequencing approaches. Populations suspected of resistance based on site history were collected in field conditions and were screened for susceptibility to as many as ten herbicides representative of nine different SOAs and a single plant growth retardant. Four target sites were selected for sequencing: *ALS*, *EPSPS*, *psbA*, and *α-tubulin*. These four genes were chosen due to the propensity of *P. annua* resistance to these sites of action and because there are known target-site resistance mechanisms (Binkholder et al., 2011; Isgrigg et al., 2002; Kelly et al., 1999; McElroy

et al., 2013). Given the known herbicide use history of most sampled sites combined with reports that many collected populations had escaped standard herbicide treatments, it was hypothesized that most populations would present with TSR mutations.

Materials and Methods: *Herbicide Screening.* *P. annua* populations were collected across the United States from areas representing four major turfgrass sectors including, golf courses, home lawns, athletic fields, and sod farms. Populations were primarily selected based on herbicide use history and reports from turfgrass managers that plants had escaped standard herbicide treatment and were suspected resistant. To achieve representative sampling of warm-season and cool-season turfgrass across climate gradients, sample collection spanned multiple USDA plant hardiness zones, with the majority of samples collected between zones 5a and 9b (Figure 1). Approximately 25-30 plants, or about 400 tillers, were collected for each unique population. Coordinates (latitude and longitude) were recorded for each sampling location to facilitate visualization of spatial trends in suspected resistance and target-site mutations.

Sampled populations were screened for suspected resistance to as many as 11 unique herbicides (preemergence and postemergence) and plant growth retardants based on regional use patterns and collaborator participation in advanced studies. Herein, we report on the screening process for herbicides corresponding to the four target sites presented in this study: glyphosate (EPSPS inhibitor), foramsulfuron/trifloxysulfuron (ALS inhibitor), simazine (PS-II inhibitor), and prodiamine and pronamide (mitotic inhibitor). Postemergence testing was conducted on each collected population by transplanting 20 *P. annua* tillers into flats filled with herbicide free-native soil (indicative to region collection) and established in a greenhouse environment. A total of 10 flats were established for each population and were fertilized and irrigated as needed to

prevent stress. Additional plants were transplanted into separate flats to produce seed for storage and advancement to subsequent studies as appropriate.

Pertinent information for each herbicide treatment is presented in Table 1. Due to regional limitations in herbicide availability, some sites used trifloxysulfuron to screen for suspected ALS-inhibitor resistance while others used foramsulfuron. Treatments were applied one week after transplant using a water carrier volume of 40 gallons per acre and a CO_2-pressurized backpack sprayer equipped with 11004 flat-fan spray nozzles (TeeJet, Spraying Systems Co.). Populations were visually evaluated for injury 21 days after initial treatment (DAT), and populations that survived the standard rate of herbicide treatment were labeled "suspected resistant" and moved forward in the sequencing process.

For preemergence screening, two methods of testing (either seedling emergence or hydroponics) were implemented at the discretion of the cooperators based on local infrastructure and available resources. Because the objective of this screening process was to identify potential herbicide-resistant plants, the method used for initial preemergence screening was not a major concern. For seedling emergence, seeds were harvested from plants not sprayed in the post emergence screening. *Poa annua* panicles were harvested by hand using scissors and were dried at 35-38ºC in forced air ovens for a minimum of seven days. A 2-mm sieve was used to screen the dried seeds to remove debris. Seed was stored at 4ºC for a minimum of seven days prior to preemergence testing. Twenty seeds from each population were placed on the surface of native soil in 10cm pots. Two replicates of each population were used for screening. Seeds were covered with 0.5cm of soil, irrigated and left for two days to allow for imbibement prior to herbicide treatment. Herbicides were mixed and diluted with tap water and applied to pots using the same CO_2-pressurized backpack system described in the post-emergence trial. To avoid

volatilization and/or photodegradation, another 1 cm of untreated soil was placed on the soil surface immediately after spraying and pots were lightly misted with 0.64cm of irrigation to incorporate the herbicide. 21 DAT, the number of seedlings emerged 1 cm above the soil surface were recorded as unaffected and expressed as a percentage of the controls. If both replicates emerged, the population was categorized as suspected resistant. In the hydroponics system, a whole-plant assay was conducted to ascertain plant response to prodiamine and pronamide at 0.1 to 1.0mM (Cutelle, et al. 2009). Populations that did not present with characteristic root clubbing associated with mitotic inhibition were labeled as suspected resistant.

Sequencing. After the initial resistance screening process, populations suspected resistant to HRAC groups 2, 3, 5, and 9 were prepped for sequencing. A single "Auburn" population with no known resistance (susceptible to all herbicide types), was also sequenced as a check for target-site mutations between resistant and susceptible sequences. Ribonucleic acid (RNA) was extracted using the Direct-zol™ RNA Miniprep kit (Zymo Research, Irvine, CA). Extracted RNA was then converted to complementary DNA (cDNA) using the qScript cDNA synthesis kit (Quanta Bioscience, Darmstadt, Germany) and was subsequently amplified in standard polymerase chain reactions (PCR) for each gene of interest. Primers and PCR conditions are presented in Table 2.

Degenerate primers were created to simplify the sequencing process and decrease the number of primers used. The primers and corresponding annealing temperature for each gene region are included in Table 2. A 5µL sample of each PCR product was mixed with 1µL of 6× loading dye and run in a 1.5% agarose gel to visualize if the reaction was successful. The PCR product was cleaned using the E.Z.N.A.® Cycle Pure kit (Omega Bio-Tek, Norcross, GA). Populations resistant to the ALS, psbA, or EPSPS target sites were sequenced by capillary electrophoresis

(Eurofin Genomics, Louisville, KY). Populations resistant to α-tubulin binding site disruptors (prodiamine and pronamide), as well as ALS and EPSPS populations that failed capillary sequencing, were sent for amplicon sequencing (AmpSeq) via Amplicon-EZ (GENEWIZ®, South Plainfield, NJ). AmpSeq is a PCR based method of next generation sequencing (NGS) that works in 500bp amplicons, so the multiple primer sets were needed to scale across *ALS* and *α-tubulin* (Rutland et al., 2022).

Analysis. All sequencing results were visually inspected in CLC Genomics Workbench 21 (QIAGEN Sciences, Germantown, MD). Given the high volume of sequences being analyzed, CLC Genomics Workbench 21 provided a space for bulk analysis to be conducted. The susceptible "Auburn" population was mainly utilized in chromatogram comparisons, while the transcriptome data was used to search for SNPs in the alignments. Chromatograms from capillary sequencing were visually inspected for known SNPs while FASTQ files produced from AmpSeq were analyzed for SNPs via read mapping and variant calling. *Poa infirma* and *P. supina* sequences were extracted from the *P. annua* transcriptome (PRJNA265116, GCZY00000000) and used for subsequent read mapping (Chen et al. 2016). FASTQ files were filtered of low-quality reads before read mapping and each FASTQ file was mapped to both subgenomes for target-site mutation identification. After read mapping the standard variant detection was used to identify potential variant sites at a 20% frequency threshold. Target-site resistance was considered confirmed if the related SNP was present. Previously reported target-site SNPs are presented in Table 3.

Population attributes including sample identification number, georeferenced sampling location (longitude, latitude), site name, corresponding industry sector (golf course, athletic field, sod production, lawn care operator), preliminary sequencing results, and TSR mutation results for all

populations were processed into a standardized format using Excel, and exported as comma delimited (.CSV) files. Outputs were subsequently imported into ArcGIS Online (ESRI, Redlands, CA) and plotted to visualize the spatial distribution of suspected and confirmed target site mutations. Symbols were used to distinguish sample populations based on specific attributes. United States Department of Agriculture (USDA) plant hardiness zones were delineated using freely reproducible data from the PRISM Climate Group at Oregon State University and USDA-ARS.

Results: *Herbicide Screening.* At the end of the collection period, 1349 populations were collected and screened for resistance. Passing the initial screening for resistance traits were 463 populations, and of these, 389 populations were sequenced; the reduction from passing the screen to sequencing was due to plants presenting with a resistance type not screened for target-site mutations, and plants not surviving transport to Auburn.

The USDA Plant Hardiness Zone is the standard for determining growing conditions for plants to thrive. Collected populations ranged from the 5a to 9b zones, with a large majority of populations present in the transition zone, a stretch of land between the northern and southern regions where no complete adaptation has occurred for cool-season and warm-season grasses (Patton, 2012). Figure 1 depicts the spatial distribution of all collected populations across USDA Hardiness Zone and Figure 2 maps populations based on specific resistance type and whether a target-site mutation was present.

Sequencing and Analysis. The tetraploid status of *P. annua* required special consideration during the sequencing process. While the chloroplast-encoded *psbA* gene was easily sequenced with capillary sequencing, we found that the nuclear-encoded *ALS* -and *EPSPS* genes were variable in the results, and it was nearly impossible to correctly sequence *α-tubulin* with

capillary sequencing, presumably due to multiple gene copies. AmpSeq provided us with the ability to align sequences to genes based on the *P. annua* subgenomes rather than relying on a single consensus sequence.

No novel mutations were discovered throughout the sequencing process. Single, known mutations were identified for each gene in *P. annua* conferring resistance to its concomitant SOA. These common mutations were Trp574Leu in ALS, Thr239Ile in α-tubulin, Pro106Ala in EPSPS, and Ser264Gly in psbA populations. Of the four genes, 65.6% of ALS, 73.5% of EPSPS, 39.8% of psbA, and 91.5% of α-tubulin contained these target-site mutations. Chromatogram analysis revealed the majority of psbA populations presented without a mutation. Analysis of read mappings from ALS, EPSPS, and α-tubulin populations revealed that more presented a target-site mutation than not. Table 4 describes the number of populations sequenced and whether or not they contained a target-site mutation.

Discussion: *ALS***.** Detected mutations were exclusively at the Trp574 codon. An issue faced in sequencing the ALS populations was that the 5'end, or the first 500bp of the *ALS* gene would not amplify. Because there are known mutations within this region (Ala122, Pro197, and Ala205), this could lead to missing TSR in the 34.4% of populations labeled with no mutation. Along the remainder of the *ALS* gene, Asp376, Ser653, and Gly654 mutations were not observed in any population. Pro197 and Ala205 mutations have been known to endow resistance to a number of sulfonylurea herbicides in annual bluegrass; however, the diversity of cross resistance endowed by the Trp574 mutation contributes to its evolutionary importance to the weed (Bernasconi et al., 1995; Brosnan et al., 2016; Guttieri et al., 1992; McElroy et al., 2013). It is unknown why there were difficulties in amplifying the 5' end of *ALS* in *P. annua*, as previously published methods were utilized (S. Chen et al., 2015). The only solution now is to conduct transcriptome

sequencing; however, this would be prohibitively expensive for the remaining populations. Regardless, this is unlikely to discount the fact that most populations contained the Trp574 substitution.

***EPSPS*.** The Pro106 mutation associated with target-site resistance in glyphosate-resistant populations was the only mutation discovered in this sequencing survey. Yu et al. (2015) reported a double point mutation that enhanced glyphosate resistance levels of the Pro106 mutation: the Thr102Ile and Pro106Ser mutation, or TIPS. While the Pro106 mutation alone provides a low level of resistance, the TIPS mutation provides resistance up to 180-fold compared to the wild-type (Yu et al., 2015). This double mutation has not been reported in any *P. annua* biotypes and was not found in the 84 sequenced populations. This study did not account for the potential for copy number variation to be a potential cause of resistance in the *P. annua* populations screened, although it has been documented (Brunharo et al., 2018). Gene amplification is a potential mechanism of TSR for the 26.5% of glyphosate-resistant populations that did not present with a target-site mutation.

***psbA*.** Unlike the other genes, approximately 60% of the psbA populations sequenced did not present with a mutation. Target-site resistance is a common mechanism within the *psbA* gene. The first incidences of herbicide resistance occurred due to over reliance on PSII herbicides, and the *psbA* gene was one of the first mutation sites found in all weeds (Hirschberg & McIntosh, 1983; Ryan, 1970). This begs the question as to why the majority of screened psbA populations did not present with a target-site mutation?

Possible reasons for why psbA populations differed from other screened SOA include possible NTSR mechanisms and difficulties within the screening process. Increased metabolism due to cytochrome P450 enzymes and glutathione S-transferase activity is a commonly recognized

cause of herbicide resistance (Burnet et al., 1993; Jugulam & Shyam, 2019; Svyantek et al., 2016).; however, no NTSR studies were performed in this experiment. The screening process in this experiment was limited to spraying populations in flats or trays. With simazine being a root-absorbed herbicide (Cremlyn, 1990), this restricts the amount of herbicide that could be translocated throughout the plant. Without adequate exposure to the roots, it will be more difficult to identify resistant populations. Other herbicide resistance screening processes have previously been described for triazine resistance, mainly through measuring chlorophyll fluorescence (Ali & Machado, 1981; Norsworthy et al., 1998; Oorschot & Leeuwen, 1992). It may be necessary for future studies to pursue alternative methods of herbicide screening to detect simazine resistance.

α-tubulin. Quantifying resistance to the dinitroaniline herbicides was the most difficult to produce given the combination of a polyploid genome in *P. annua* and the presence of multiple copies of the *α-tubulin* gene (J. Chen et al., 2021; Patterson et al., 2019). A majority of resistant populations (91.5%) contained the Thr239-Ile mutation, a known mutation associated with the inhibition of microtubule assembly (Anthony et al., 1998). By using a combination of amplicon sequencing and degenerate primers instead of typical Sanger sequencing with a single primer pair, all of the potential *α-tubulin* gene copies could be captured, which reduced the chance that a mutation was not amplified. This methodology for identifying mutations using AmpSeq was previously reported in Rutland et al. (2022).

Multiple resistance. One of the most concerning aspects of this survey was the level of multiple resistance present. After the initial pre-screen, 16.5% of total populations collected presented with resistance to two or more herbicide families. This included a mix of populations presenting no mutations for either resistance type, a single mutation corresponding to one resistance type,

and a mutation for each resistance type found. The presence of single mutations in populations screened for multiple resistance indicates the possibility of the co-existence of TSR and NTSR within *P. annua* populations (Mithila & Chandrima, 2019). This level of screened populations with multiple target-site mutations could represent a larger issue for *P. annua* management in the future.

NTSR. In this survey, populations were labeled as TSR if a population contained a known target-site mutation; however, populations could not be labeled as NTSR since metabolism, translocation, and nontarget-site experimentation were not performed in this study. Populations with no target-site mutation were labeled as "no mutation" and may be considered for future NTSR testing. Because NTSR could not be confirmed, "no mutation" populations may or may not be resistant, or they may just be a susceptible population misidentified as resistant during initial screening.

Implications for Management. The ability of *P. annua* to thrive and evolve surpasses almost every weed species in turfgrass. With resistance to twelve different modes of action, the need to fully understand resistance mechanisms and how to control *P. annua* seems to present a dire scenario for practitioners reliant upon chemical control strategies alone. Research presented herein was limited to four general target site mechanisms representative of a large, yet admittedly difficult to quantify, proportion of chemical control strategies within the turfgrass industry. Mapping only shows the extent to which resistance is widespread and suggests that regional tactics, albeit important, are not sufficient to circumvent the problem.

Mutations in 66.6% of the 389 populations sequenced across four different SOA were identified by sequencing techniques. Overall, more TSR was confirmed than potential NTSR for all herbicide groups except for PSII inhibitors. This, however, ignores the possibility of co-

occurrence of TSR with NTSR—a known possibility (Bai et al., 2019; Chen et al., 2020). Multiple resistance was observed in 16.5% of the populations sequenced—suggesting an increasingly problematic scenario to *P. annua* management in turfgrass and one that practioners and specialists are already anecdotally familiar with.

The origin of resistance is scientifically well understood but is complicated by a poorly understood and as-of-yet measured movement mechanism within turfgrass systems. Undoubtedly, spread is due to human dispersal mechanisms (Greve & Pertierra, 2022; Wódkiewicz et al., 2017). And while herbicide selection pressure is the main driving force of resistance, it is unclear if gene flow between populations with different resistance alleles or herbicide-induced evolution is driving the potential for multiple resistance (Busi et al., 2013; Beckie et al., 2019). These results further indicate the tenacity of *P. annua* as a weed species, and the need for alternative management practices.

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Tables:

TABLE 2-1: Selected herbicide treatments applied to control *Poa annua*. All rates were standard.

HRAC	Application timing	Active* ingredient	NIS[a] (% v/v)	Active ingredient rate		Trade name	Manufacturer	Formulation	Product rate
				lb a.i. $acre^{-1}$	Kg a.i. ha^{-1}				$acre^{-1}$
2	POST	foramsulfuron	0.25	0.26	0.03	Revolver	Bayer Crop Science	0.19 lb gal^{-1}	17.4 fl oz
2	POST	trifloxysulfuron	0.25	0.03	0.03	Monument 75WG	Syngenta Crop Protection	75%	0.53 oz
3	PRE	prodiamine	0.25	1	1.12	Barricade 4FL	Syngenta Crop Protection	4 lb gal^{-1}	1 lb ai
3	PRE	pronamide	0.25	1.03	1.16	Kerb SC	Corteva Agriscience	3.3 lb gal^{-1}	2.5 pt
5	POST	simazine	0.25	1	1.12	Princep 4L	Syngenta Crop Protection	4 lb gal^{-1}	1 qt
9	POST	glyphosate	0.25	1	1.12	Round-up	Bayer Crop Science	4 lb gal^{-1}	32 fl oz

*Either foramsulfuron or trifloxysulfuron were used for ALS-inhibitor screenings based on regionally availability.

TABLE 2-2: List of primers, degenerate nucleotide sequences, and PCR reaction temperatures used.

Gene	Primer	Sequence 5' to 3'	PCR Temp
psbA	Poa_psbA1F	TGCAGCTGCTACTGCTGTTT	54ºC
	Poa_psbA1R	ACGCGACCTTGACTATCAACT	
EPSPS	SMPoaEPSPS_F	TGTCCGAGGGAACAACTGTG	54ºC
	SMPoaEPSPS_R	ACGAACAGGTGGGCAGTTAG	
***α*-tubulin**	Tua_ampseq_1F	GRCACCARTCSARAACTGGA	57ºC
	Tua_ampseq_1R	GTABGGSACMAGRTTGGTCTG	
	Tua_ampseq_2F	CCWACCTACACCAACCTSAAC	
	Tua_ampseq_2R	GRCACCARTCSACRAACTGGA	
ALS	poa_als_1F	TCACCCGTTCCATCACCAAG	56ºC
	poa_als_2R	ACACCAAATGCAAGCAGCAG	
	poa_als_3F	CCTCATGGGTCTTGGCAACT	
	poa_als_3R	TTCAAGCCCTCCAAAGCGAT	
	poa_als_4F	TCGCTTTGGAGGGCTTGAA	
	poa_als_4R	TGGCCGCTTGTAKGTGTAAT	
	poa_als_5F	ATTACACKTACAAGCGGCCA	
	poa_als_5R	TCTTGATTGCTGCACGGACT	
	poa_als_J8F_1553	AGGAGTTGGCACTGATTCGT	
	poa_als_J8R_1915	TGCCATCACCATCCATGATA	

TABLE 2-3: List of currently known target-site mutations sites in ALS, a-tubulin, EPSPS, and psbA genes.

ALS	**α-tubulin**	**EPSPS**	**psbA**
Ala122	Leu125	Thr102	Val219
Pro197	Leu136	Pro106*	Ala251
Ala205	Val202		Phe255
Asp376	Thr239*		Ser264*
Trp574*	Met268		Asn266
Ser653			
Gly654			

*****Mutations that were identified in sequencing survey

TABLE 2-4: Number of total *Poa annua* populations sequenced by state, percentage of populations found with target-site resistance mutations and without mutations, and percentage of populations with multiple resistance. *Multiple resistance indicates that a population was sequenced multiple times based on pre-screen data.

State	ALS		EPSPS		psbA		a-tubulin		Multiple Resistance*	Mutations	Total Sequenced
	Sequenced	Mutation	Sequenced	Mutation	Sequenced	Mutation	Sequenced	Mutation	# of Populations		
AL	11	8	4	4	10	8	21	18	9	38	46
FL	6	1	0	0	9	1	2	1	3	3	17
GA	13	3	2	0	11	1	0	0	10	4	26
LA	2	2	2	2	0	0	0	0	2	4	4
MS	8	7	0	0	21	16	5	5	5	28	34
NC	59	46	53	43	0	0	31	31	12	120	143
TN	18	8	10	6	15	6	22	19	11	39	65
TX	7	7	3	0	9	4	1	1	4	12	20
VA	7	4	9	6	18	1	0	0	8	11	34
Total	131	86	83	61	93	37	82	75	64	259	389
Percent	65.6%		73.5%		39.8%		91.5%		16.5%	66.6%	

Figures:

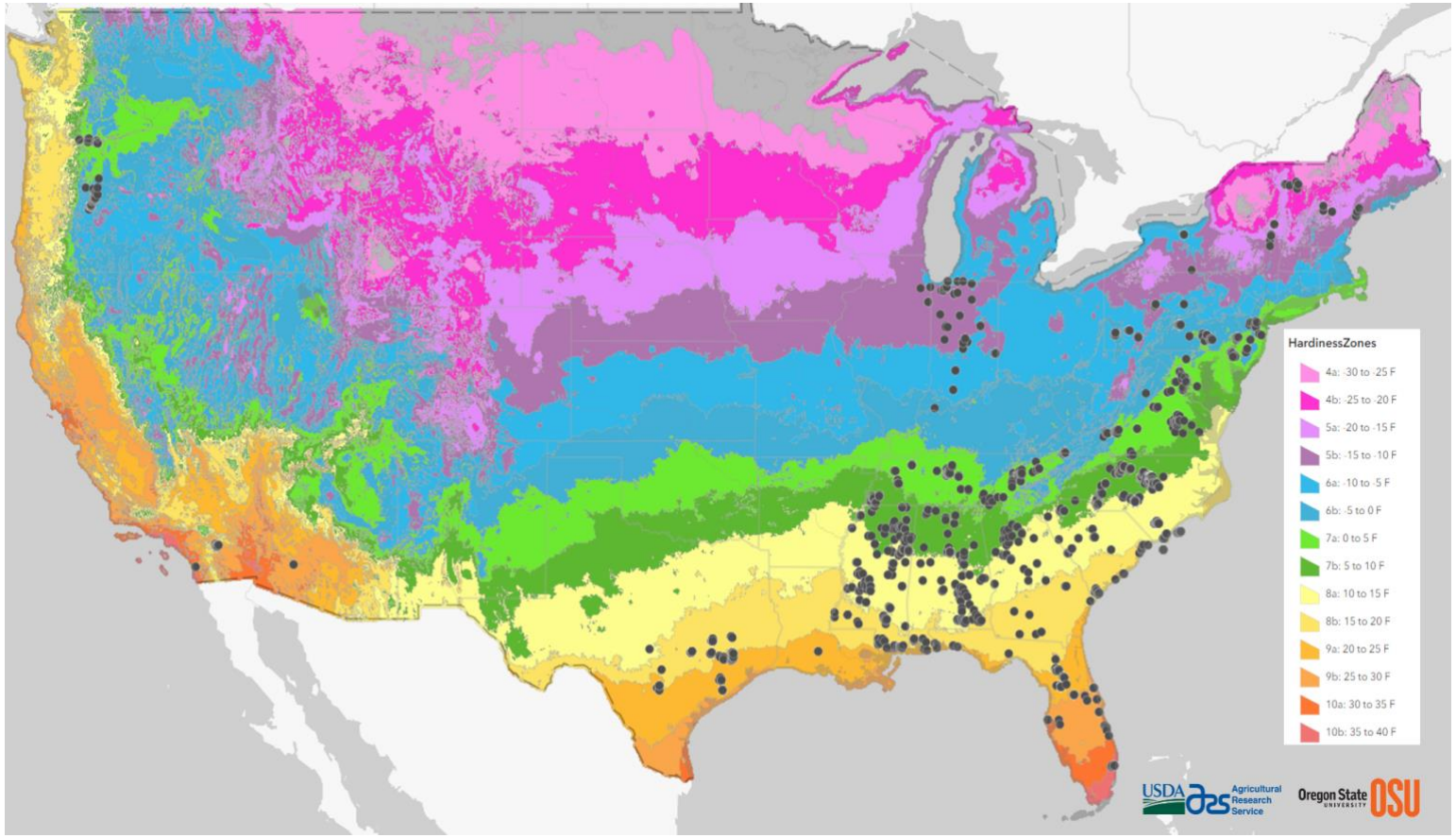

FIGURE 2-1: Map of collection sites for all *Poa annua* populations collected across the United States Department of Agriculture (USDA) plant hardiness zones. Black points indicate a population; however, vicinity of collection sites prevents all 1367 populations from appearing on the map. USDA plant hardiness zones were delineated using freely reproducible data from the PRISM Climate Group at Oregon State University and USDA-ARS

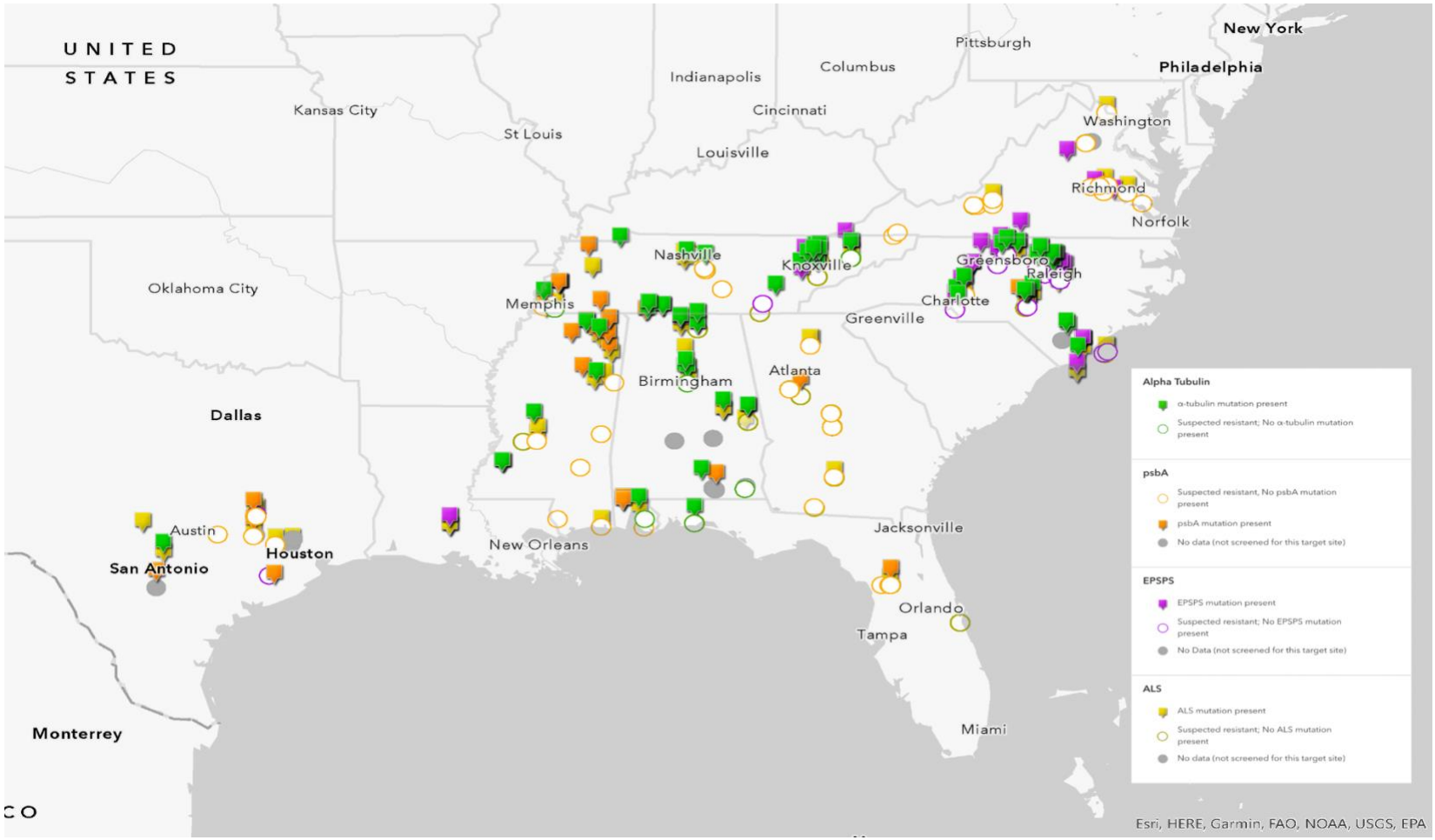

FIGURE 2-2: Map depicting suspected resistant populations and their respective resistance type. Points indicate populations with a target-site mutation, while outlined circles indicate populations that screened resistant, but contained no target-site mutation. Grey points indicate populations that screened resistant but were unable to be sequenced for target-site mutations. Yellow points indicate resistance to ALS inhibitors, green points indicate resistance to mitotic-inhibitors, purple indicates resistance to EPSPS inhibitors, and orange indicates resistance to photosystem II inhibitors.

Chapter 3: Identification of a potential quinclorac-resistant smooth crabgrass (*Digitaria ischaemum*) population in Alabama

Abstract: Quinclorac is a synthetic auxin herbicide that possesses a grass-in-grass selectivity for controlling several troublesome grassy weeds in cool-season and several warm-season species. *Digitaria* is considered one of the most problematic weeds in turf and potential resistance to quinclorac in species like smooth crabgrass (*D. ischaemum*) confounds this issue. A potential resistant smooth crabgrass population, referred to as "AL_R1" was identified at the Auburn University Sports Surface Field Laboratory and a subsequent dose-response experiment was conducted in the greenhouse to confirm its resistance status with comparison to a known susceptible population, "AL_S1." AL_R1 was deemed resistant based on comparison to a known susceptible as no resistant populations were controlled 55% at the highest rate.

Introduction: *Digitaria* spp. (crabgrasses) is consistently surveyed as one of the most common and most troublesome species of weeds to control in turfgrass (Van Wychen, 2020). With the commonality of crabgrasses in turfgrass systems, one might assume that there are numerous methods of controlling crabgrass, however this is not the case. Most control programs emphasize the use of preemergent Group 3 herbicides like pendimethalin (Pendulum; BASF, Ludwigshafen, Germany), prodiamine (Barricade; Syngenta, Basel, Switzerland), and dithiopyr (Dimension; Corteva, Indianapolis, IN, USA), Group 14 herbicides like oxadiazon (Ronstar; Envu, Cary, NC, USA), or Group 29 herbicides like indaziflam (Specticle; Envu, Cary, NC, USA) before crabgrass emergence (Gannon et al., 2015). If a crabgrass escapes a preemergence program or is identified too late for a preemergent program to be implemented, there are limited options to control emerged crabgrasses (Fidanza et al., 1996).

Postemergence control options for crabgrass include dithiopyr, fenoxaprop, monosodium methanearsonate (MSMA), and quinclorac. Dithiopyr has been shown to have early postemergence control, but beyond the three-leaf stage is less effective (Enache & Ilnicki, 1991). Fenoxaprop, an Acetyl-CoA carboxylase inhibitor, (Acclaim; Envu, Cary, NC, USA) has acceptable crabgrass control up to the 5-tiller stage but can injure turfgrasses in higher temperatures (Dernoeden and Fry 1986). MSMA, an organic arsenical, is known to have crabgrass control, but due to environmental concerns is now only available for limited use on golf courses and sod farms (Keigwin, 2013).

Quinclorac (3,7-dichloro-8-quinolinecarboxylic acid; Drive XLR8, BASF, Ludwigshafen, Germany) is a synthetic auxin unique for its grass-in-grass selectivity. It is currently unknown why synthetic auxin herbicides only affect broadleaf species, and why quinclorac can target certain grass species as well as broadleaves (Grossmann & Kwiatkowski, 2000; McSteen, 2010). The ability of quinclorac to selectively target specific grass species makes it a beneficial herbicide for turfgrass systems. The mode of action for quinclorac has been debated for decades: it has been positioned as a cellulose synthesis inhibitor and a synthetic auxin, and quinclorac demonstrates two different mechanisms of control between broadleaf and grass species (Grossmann & Kwiatkowski, 1995; Koo et al., 1997; Tresch & Grossmann, 2003). In broadleaf species, quinclorac acts as a typical synthetic auxin, displaying typical responses like epinasty and overgrowth when applied, while grass species exhibit a necrotic response to quinclorac applications. The response to quinclorac in grasses has been associated with the accumulation of cyanide and ethylene levels due to a stimulation in 1-aminocyclopropane-1-carboxylate synthase (ACCase) enzyme (Grossmann & Kwiatkowski, 1995).

Quinclorac has labeled usage in warm-season turfgrass fairways and roughs for species like bermudagrass (*Cynodon dactylon*), Kentucky bluegrass (*Poa pratensis*), buffalograss (*Bouteloua dactyloides*), and zoysia (*Zoysia* spp.). Roughs and fairways can often be contaminated by warm-season weed species like crabgrasses, barnyard grasses (*Echinochloa* spp.), and paspalum (*Paspalum* spp.), which quinclorac can control with little to no injury to the desired turfgrass species. Quinclorac is known specifically for its ability to control crabgrass, especially with small-tillered populations and mature crabgrass populations beyond the four-tiller stage. Crabgrass in the growing stages from two-tillered to four-tillered are known to escape quinclorac applications, but the reasoning behind this escape is unknown (Frank, 2022).

Smooth crabgrass (*Digitaria ischaemum)*, in particular, is a warm-season annual grass weed species that has been identified as one of the most common and troublesome weeds in turf (Van Wychen, 2020). Even though smooth crabgrass is an extremely common weed in turfgrass, only two different types of resistance has been reported: fenoxaprop-ethyl (Group 1, inhibition of acetyl-CoA carboxylase) and quinclorac (Group 4, auxin mimics) (Heap, 2024).

Despite the decades-long use of quinclorac, there have been limited reports of resistance to the herbicide, and no reports of target-site mutations. Quinclorac-resistant populations of smooth crabgrass have been confirmed in California and Mississippi, and both displayed nontarget-site resistance mechanisms (NTSR) (Abdallah et al., 2006; Putri et al., 2024). Neither population was associated with a target-site mutation, however this does not eliminate the possibility a mutation could be endowing resistance. Target-site resistance (TSR) mutations have been identified in other synthetic auxin herbicides, like 2,4-D, dicamba, and fluroxypyr, but no mutations have been seen with quinclorac-resistant smooth crabgrass, or other quinclorac-resistant species (de Figueiredo et al., 2022; LeClere et al., 2018).

A smooth crabgrass population (AL-R1) in Auburn, AL was suspected of resistance due to escaping standard field treatments of quinclorac, formulated as Drive XLR8, as seen in Figure 1. This population was discovered in a research plot with no long-term use of quinclorac, so it is unknown how the resistance trait spread to the field. A dose response assay was conducted against a known susceptible smooth crabgrass population (AL_S1) to determine the level of resistance to quinclorac.

Materials & Methods: AL_R1 was originally collected from the field in August 2022 and AL_S1 was collected in July 2023. Both populations were transplanted to the greenhouse where each was grown to seed and mature seed was collected, cleaned, and placed in cold storage (5ºC). In January 2023 AL_R1 and AL_S1 were seeded into trays with Miracle Gro® potting mix (Scotts Miracle-Gro Products Inc., Marysville, OH) and then in March 2023 seeds began to germinate. Individual seedlings were then transplanted 4x4 pots filled with Marvyn sandy loam. Transplanted seedlings were allowed to grow to the 4-leaf stage before proceeding with the dose response assay.

A dose response screening was conducted as a randomized complete block design (RCBD). The RCBD protocol was designed using Agricultural Research Management software (ARM, GD Solutions, Brookings, SD) with three replicates and repeated. The initial screening for AL_R1 and AL_S1 was March 30th, 2023, and the second run began April 11th, 2023. Each run was kept on separate tables in the greenhouse. Foliar applications were made using a hand-held CO_2-pressurized sprayer calibrated to 280 L ha^{-1}at 206 kPa. Treatments were 9 rates of quinclorac: 105, 210, 420, 841, 1681, 3363, 6726, 13452 g ai ha^{-1} and compared to a nontreated check. These rates correspond to 0.125X to 16X the standard rate of quinclorac. The rates were log transformed into log rates to maintain equal spacing at 0 for the nontreated check, and the

remaining rates at 0.301, 0.602, 0.903. 1.204, 1.505, 1.806, 2.107, and 2.408, respectively. Visual injury ratings from 0% (no visible injury) to 100% (complete plant death) were collected at 7, 14, 21, 28, and 35 days after treatment (DAT). At 35 days, fresh weight was collected from each population with above ground tissue to determine biomass reduction.

Fresh weight was transformed to a percentage based on the nontreated control for graphing purposes. Data was subjected to Analysis of Variance (ANOVA) and Fisher's LSD ratings were calculated in R v4.3.2 using packages *tidyverse* v2.0.0 and *agricolae* v 1.3-7, while biomass reduction and dose response curves were modeled in GraphPad Prism 10 using the log (inhibitor) vs response (variable) slopes, shown in Equation 1. Model equations were selected based on best fit of commonly applied dose-response curves.

Results & Discussion: The population AL_R1 was hypothesized to be resistant to quinclorac, and based on the lack of field control and visual injury, as shown in Figure 2, was confirmed resistant. At 35 DAT, all susceptible replicates were controlled 100% at the standard rate, while no resistant populations were controlled 55% at the highest rate (Figure 3). The ANOVA indicated that there was a significant difference between each run at for injury response, so each run was plotted separately (Table 1). Non-linear regressions were modeled for other time points but considered redundant and thus not shown. No linear regression could be calculated for the resistant population, as 100% control was not achieved; therefore, no I_{50} values could be determined for the resistant population. No level of resistance could be determined as IC_{90} values were calculated separately, as they were not included in the model (Table 2). Fresh weights were also taken at 35 DAT. Biomass reduction was calculated relative to the non-treated population for each run. The ANOVA indicated there was a significant difference between runs for AL_R1, but not AL_S1, so biomass reduction was pooled for AL_S1. A significant interaction was

present across treatment rates ($P < 0.01$) when calculating biomass reduction for AL_S1, but no significant treatment interaction was seen for AL_R1, as shown in Figure 4. Fisher's LSD test groupings are also indicated in Figure 4.

The actual level of resistance to quinclorac for AL_R1 could not be calculated within the dose rate utilized. Identifying this population as resistant is interesting because the research plot where AL_R1 was found had no history of repeated quinclorac use. A similar case occurred where a smooth crabgrass population was discovered in Indiana that was determined to be 80X resistant, but with no extensive use of quinclorac over ten years (Patton, 2023; Reicher et al., 2009). This substantial level of resistance brings up more questions beyond just showing quinclorac resistance is present in these situations: why are these populations so resistant given the little amount of quinclorac that has been applied and how might this resistance be determined.

Resistance to quinclorac is not fully understood, but there are multiple proposed mechanisms, mostly focused on non-target site resistance pathways. NTSR is a polygenic mechanism of herbicide resistance, involving the interaction of numerous metabolic or systems. It has been shown that there is generally an increased accumulation of ethylene and cyanide in quinclorac-susceptible species compared to inherently tolerant ones due to the induction of ACCase (Abdallah et al., 2006; Grossmann & Kwiatkowski, 1995). This situation has been reported in quinclorac-resistant populations of smooth crabgrass, where there was no increase in ethylene or cyanide accumulation compared to susceptible populations (Abdallah et al., 2006; Putri et al., 2024). NTSR mechanisms have been associated with quinclorac resistance: Some method of accumulation or distribution of quinclorac, but not metabolism, has also been suggested as a means of selectivity (Chism et al., 1991). There are some unrealized detoxification mechanisms

that affect quinclorac resistant species, but the genetic basis of NTSR is still not fully understood (Délye, Jasieniuk, et al., 2013).

Given the high level of quinclorac resistance seen in AL_R1, it stands to reason that this resistance is likely due to a target-site mutation, or another mechanism of TSR. Target-site resistance has been shown to have higher levels of resistance compared to NTSR. Certain mutations to the *ALS* (acetolactate synthase) gene are known to have higher levels of resistance, and resistance to glyphosate has been shown to stem from the number of *EPSPS* (5-enolpyruvate-shikimate-3 synthase) gene copies are present (Patterson et al. 2018; Murphy and Tranel 2019). Higher levels of herbicide resistance have also been identified in a combinatorial manner, where NTSR mechanisms, namely enhanced metabolism, work in tandem with TSR (Gaines et al. 2020).

There have still been no mutations known to endow resistance to other synthetic auxins identified in quinclorac resistant populations or novel mutations. Generally, the auxin pathway works by auxin binding to transport inhibitor response (TIR1) proteins, a member of the auxin F-Box (AFB) family, to promote the transcription of auxin responsive genes by recruiting auxin/indole-acetic acid (AUX/IAA) proteins (Abel & Theologis, 1996; Dharmasiri et al., 2005). In a low auxin scenario, TIR1 and auxin are not bound, which inhibits the transcription of auxin responsive genes, while in a high auxin (or synthetic auxin application) scenario, auxin activates the SCF^{TIR1} complex, which ubiquinates AUX/IAA proteins and tags them for degradation, stimulating the transcription of auxin responsive genes like transcription factors, signaling, or stress proteins (Taiz & Zeiger, 2006). TSR mechanisms in synthetic auxins are becoming more understood as more target-site mutations are being identified: deletion of the degron tail in IAA2 endowing resistance to 2, 4-D (*IAA2*$_{\Delta 27}$), a glycine to asparagine mutation within the degron

endowing resistance to dicamba, 2,4-D, and fluroxypyr, multiple mutations across AFB5 that endowed resistance to picloram, as well as searching for potential novel mutations in the genes *IAA, TIR1,* and *AFB*s (de Figueiredo et al., 2022; LeClere et al., 2018; Walsh et al., 2006). However, quinclorac's structure is different from the other synthetic auxins, and has been shown to not bind to known TIR1 and AFBs, it is likely there may be a different target site for quinclorac altogether (Prusinska et al., 2023). The inhibition of cellulose biosynthesis has also been proposed and argued as an alternative mode of action for quinclorac (Koo et al., 1997; Tresch & Grossmann, 2003). Indaziflam is currently one of more successful means of controlling smooth crabgrass, however there has been no reported resistance associated with the species and quinclorac is vastly different from indaziflam and other cellulose biosynthesis inhibitors: indaziflam works as a preemergent herbicide while quinclorac has postemergence control and the two chemical structures are different (HRAC, 2024).

It is currently unknown why quinclorac can selectively control smooth crabgrass, and there are some interesting taxonomic implications behind the list of species quinclorac can and cannot control. Smooth crabgrass and other similarly affected grass species are all grasses in the PACMAD clade of Poaceae, (specifically the Panicoideae subfamily), while most tolerant species are grasses in the BOP clade, as shown in Tables 3 and 4. The BOP clade is composed solely of C_3 grasses, while PACMAD is composed of C_3 and C_4 grasses (Hodkinson, 2018). The reason behind the different species presenting with susceptibility or tolerance is currently unknown. One might assume it could be associated with the differential anatomy (meaning the presence of the Kranz anatomy in C_4 species) between the two subclades, as all susceptible species are C_4 grasses. However, there are C_4 grasses (like bermudagrass, zoysia, and seashore

paspalum (*P. vaginatum*)) tolerant to quinclorac and some C_3 grasses (colonial (*Agrostis capillaris)* and seaside bentgrass (*A. pallens)*) that are susceptible.

Beyond taxonomy and anatomy, smooth crabgrass is also likely a polyploid species, which introduces more potential pathways for target site mutations to evolve (Rutland et al., 2021). However removing potential polyploidy from the equation doesn't dismiss the possibility of target-site resistance either as standing genetic variation has been identified as the cause for herbicide resistance mutations in areas where no herbicide applications have been made (Barrett & Schluter, 2007; Délye, Deulvot, et al., 2013; Kersten et al., 2023). Inherent resistance alleles hidden within a polyploid genome not yet available could be the reason for these highly resistant populations of smooth crabgrass to emerge suddenly after no known use of quinclorac.

Research Implications: More work is needed to determine if this population is resistant via a target-site or nontarget-site mechanism, but it is clear that AL_R1 is resistant to quinclorac. Identification of this population further expands the growing issue of quinclorac resistant smooth crabgrass. With quinclorac resistance becoming more prevalent, it is imperative to use resistant populations to our advantage to study how the resistance mechanism functions. Combination preemergence/postemergence programs have shown good control with other herbicides, but quinclorac is one of the few postemergence control options for crabgrass (Chism & Wayne Bingham, 1991; Johnson, 1997). Given the losses already incurred for crabgrass control with MSMA and the lack of effective postemergence options, ensuring the use of quinclorac for turfgrass as a control option is vital.

Finding target site mutations for quinclorac has been particularly difficult because the target-site itself is not known. However, the combination of known target-site mutations in other synthetic auxins, NTSR mechanisms for quinclorac resistance, and Poaceae phylogeny can give us an idea

of where we need to look next. Comparative genetic analyses between species inherently tolerant and susceptible to quinclorac may elucidate target genes responsible for increased detoxification or single nucleotide morphisms in genes previously ignored. There may be some effect that polyploidy and standing genetic variation has endowed in relation to tolerance, susceptibility, or resistance to quinclorac that has yet to be uncovered.

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Tables:

TABLE 3-1: ANOVA for dose-response study for % injury and biomass reduction. Treatment and runs for both the resistant population AL_R1 and susceptible population AL_S1 were both significantly different for % injury, thus runs could not be pooled for this analysis. For biomass reduction, run was only significantly different for AL_R1, thus AL_S1 was pooled. AL_S1 also displayed significant difference among treatments, so Fisher's LSD values were calculated. Df: degrees of freedom; Sum Sq: sum of squares; Mean Sq: mean square; Pr(>F): p-value.

% Injury							
Population		**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
AL_R1	**Treatment**	8	18310	2289	18.48	<2.00E-16	***
	Run	1	3816	3816	30.81	7.01E-08	***
	Residuals	260	32199	124		7.01E-08	
		Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
AL_S1	**Treatment**	8	289380	36173	122.765	<2.00E-16	***
	Run	1	1378	1378	4.677	0.0315	*
	Residuals	260	76609	295			
Biomass Reduction							
Population		**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
AL_R1	**Treatment**	8	38.7	4.8	0.192	0.991	
	Run	1	1143.4	1143.4	45.336	2.82E-08	***
	Residuals	44	1109.7	25.2			
AL_S1		**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	
	Treatment	8	232.79	29.098	6.795	8.90E-06	***
	Run	1	0.05	0.054	0.013	0.911	
	Residuals	44	188.41	4.282			

significance codes: **0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '**

TABLE 3-2: Model parameters for susceptible population AL_S1: I_{50}, I_{90} values, log equivalents, R^2, Top, Bottom, and Hillslope from dose-response screening for quinclorac. I_{50}: effective concentration that gives a response halfway between Top and Bottom; log(I_{50}): log value of I_{50}; I_{90}: effective concentration that gives a response at 90% between Top and Bottom; log(I_{90}): log value of I_{90}; R^2: goodness of fit of curve; Top: the high plateau on the Y-axis; Bottom: the low plateau on the Y-axis; Hillslope: the steepness of the curve.

	Visible Injury (%)							
	I_{50}	**log(I_{50})**	**I_{90}**	**log(I_{90})**	**R^2**	**Top**	**Bottom**	**Hillslope**
Run 1	1.769	0.2478	16.0479	1.21	0.8577	104.3	-51.69	0.9964
Run 2	2.026	0.3067	3.0639	0.49	0.8979	99.20	-2.332	5.312

TABLE 3-3: Taxonomic classification of grasses with known tolerance to quinclorac. Species are organized by Poaceae clades, BOP or PACMAD, subfamilies, and status as a C_3 or C_4 species. Eleven of the sixteen species are C_3 grasses and five are C_4 grasses.

Tolerant Grasses				
BOP	**Subfamily**	**Scientific Name**	**Common Name**	**C_3 or C_4**
Pooideae	Poodinae	*Poa annua*	annual bluegrass	C_3
		Poa pratensis	Kentucky bluegrass	C_3
		Poa trivialis	rough bluegrass	C_3
	Agrostidodinae	*Agrostis stolonifera**	creeping bentgrass	C_3
	Loliodinae	*Festuca arundinacea*	tall fescue	C_3
		Festuca rubra spp. *commutata* Gaudin*	Chewings fescue	C_3
		*Festuca brevipila**	hard fescue	C_3
		Festuca rubra L. spp.*	red fescue	C_3
		Lolium multiflorum	annual ryegrass	C_3
		Lolium perenne	perennial ryegrass	C_3
Oryzoideae	Oryzinae	*Oryza sativa*	rice	C_3
PACMAD				
Chloridoideae	Cynodonteae	*Cynodon dactylon*	bermudagrass	C_4
		Eleusine indica	goosegrass	C_4
		Bouteloua dactyloides	buffalograss	C_4
	Zoysiinae	*Zoysia*	zoysiagrass	C_4
Panicoideae	Paspaleae	*Paspalum vaginatum**	seashore paspalum	C_4

*moderately tolerant

TABLE 3-4: Taxonomic classification of grasses with known tolerance to quinclorac. Species are organized by Poaceae clades, BOP or PACMAD, subfamilies, and status as a C_3 or C_4 species. Two of the sixteen species are C_3 grasses and fourteen are C_4 grasses.

Susceptible Grasses				
BOP	**Subfamily**	**Scientific Name**	**Common Name**	**C_3 or C_4**
Pooideae	Agrostidodinae	*Agrostis capillaris*	colonial bentgrass	C_3
		Agrostis pallens	seaside bentgrass	C_3
PACMAD				
Panicoideae	Anthephorinae	*Digitaria ischaemum*	smooth crabgrass	C_4
		Digitaria sanguinalis	large crabgrass	C_4
	Andropogonodae	*Axonopus*	carpetgrass	C_4
		Eremochloa ophiuroides	centipedegrass	C_4
	Paspaleae	*Paspalum notatum*	bahiagrass	C_4
	Boivinellinae	*Echinochloa crus-galli*	barnyardgrass	C_4
		Echinochloa colona	junglerice	C_4
	Cenchrinae	*Cenchrus clandestinus*	kikuyugrass	C_4
		Setaria pumila	yellow foxtail	C_4
		Setaria viridis	green foxtail	C_4
		Setaria faberi	giant foxtail	C_4
		Stenotaphrum secundatum	St. Augustinegrass	C_4
	Panicinae	*Brachiaria platyphylla*	broadleaf signalgrass	C_4
		Panicum repens	torpedograss	C_4

Figures:

FIGURE 3-1: Mixed smooth crabgrass with populations controlled by and escaping control with a standard rate of quinclorac (Drive XLR8). This figure demonstrates the classic segregating populations.

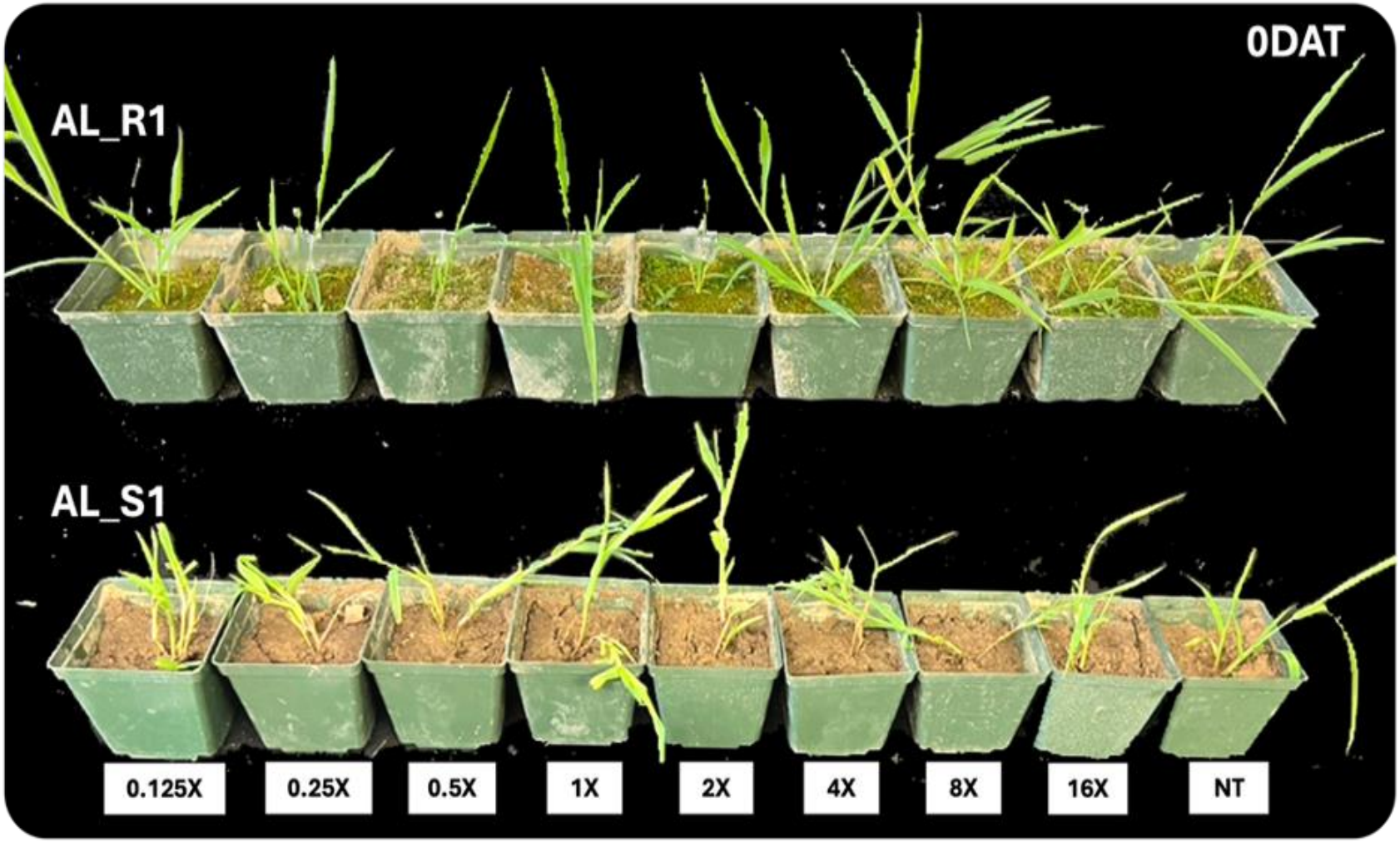

FIGURE 3-2A: Comparison of plant injury from quinclorac application along increasing doses between AL_R1 and AL_S1 0 d after treatment.

FIGURE 3-2B: Comparison of plant injury from quinclorac application along increasing doses between AL_R1 and AL_S1 35 d after treatment.

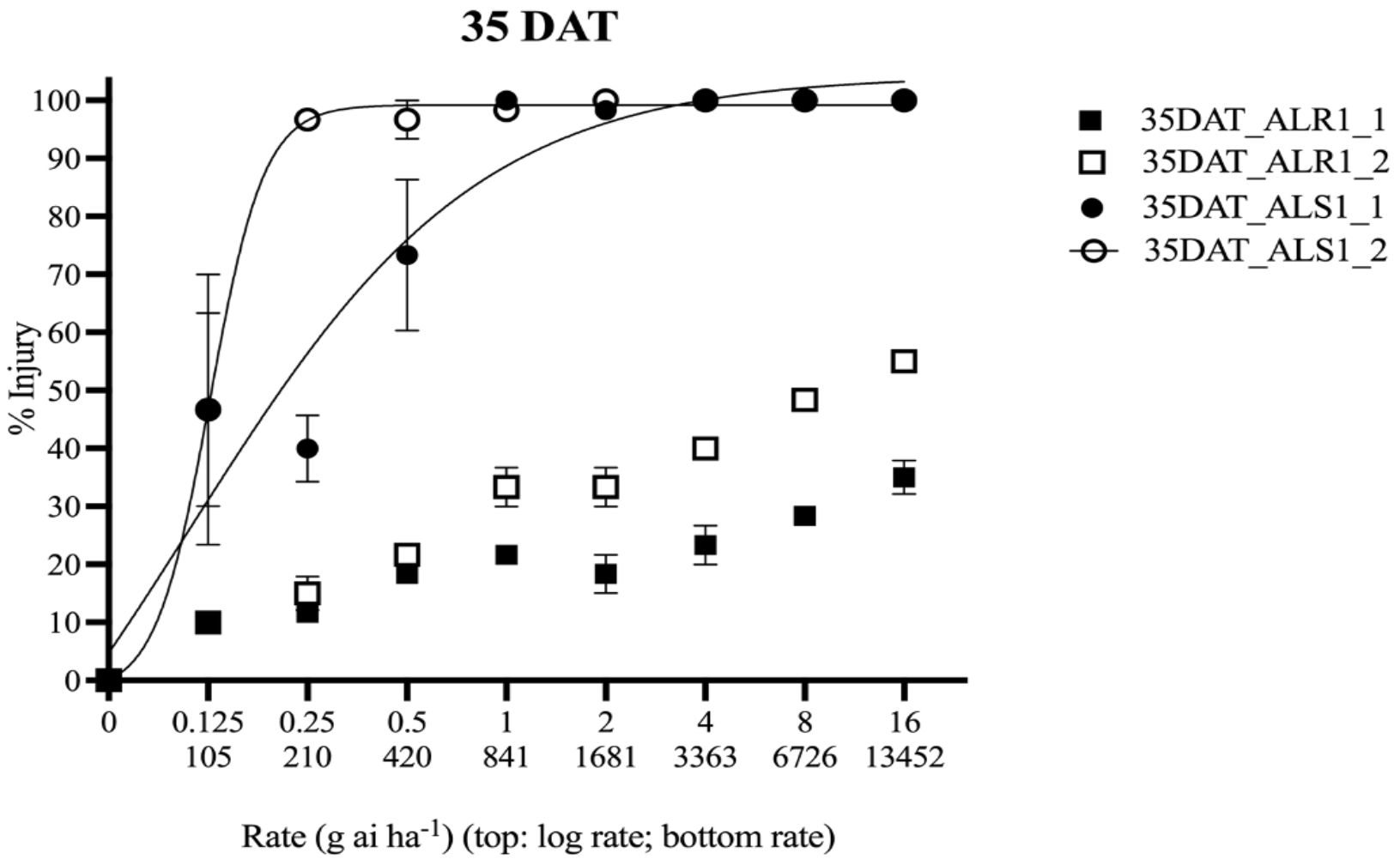

FIGURE 3-3: Percent visible injury response relative to nontreated control of smooth crabgrass populations AL_R1_1, AL_R1_2, AL_S1_1, and AL_S1_2 at 35 d after treatment. Non-log transformed rates presented for reference. Nonlinear regressions could only be modeled for AL_S1. Vertical bars represent standard error (P=0.05)

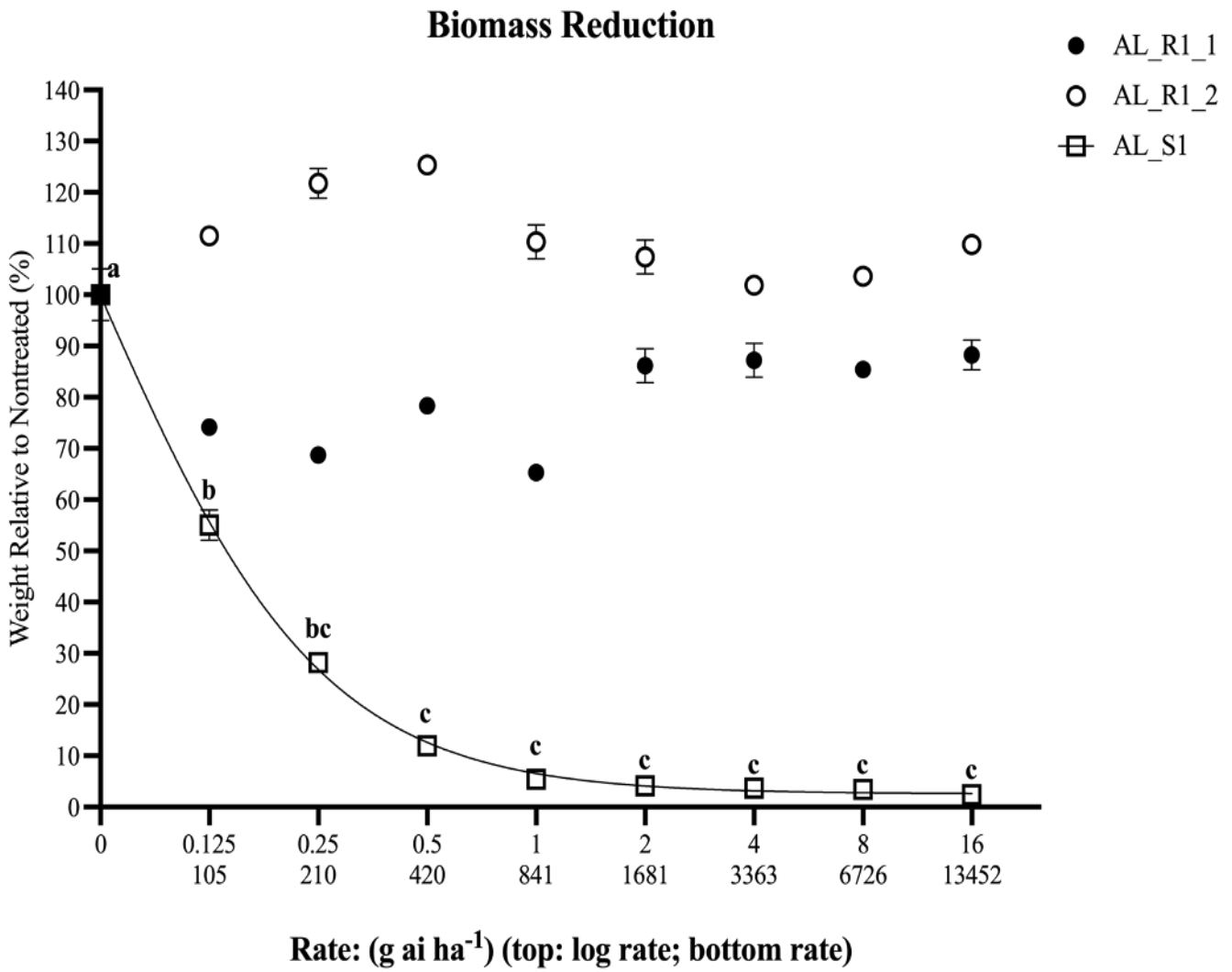

FIGURE 3-4: Biomass reduction 35 d after treatment presented as a percentage relative to the nontreated. No curve could be calculated for AL_R1_1 or AL_R1_2 as there were no differences among treatments at (α=0.05). Vertical bars represent standard error (P=0.05). Letters above AL_S1 curve indicate Fisher's LSD groupings.

Equations

EQUATION 3-1: Where Y is equal to injury (%), Top and Bottom are plateaus, I_{50} is the rate of the herbicide that gives a response halfway between the Top and the Bottom, X is the log rate of the herbicide, and Hillslope is the steepness of the curve.

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{[(\log I_{50} - X) * Hillslope]}}$$

Chapter 4: The Reference Genome and Subgenome Analysis of Allotetraploid *Digitaria ischaemum* (Smooth Crabgrass)

Abstract: *Digitaria ischaemum* is a common weed species in turfgrass that consistently causes issues for turfgrass management due to its prolific nature. The evolution of herbicide resistant biotypes of *D. ischaemum* hinders any ability to control the weed species, and the lack of genomic resources hinders investigation into the mechanisms of resistance to herbicides like quinclorac. The evolved resistance to quinclorac in *D. ischaemum* is an issue for managing turfgrass as it removes one of the only postemergence methods of control for *D. ischaemum* in turfgrass. Herein a reference-level genome was assembled, annotated, and analyzed for *D. ischaemum.* A known quinclorac susceptible biotype, AL_S1, was selected then sequenced and assembled using PacBio HiFi long read sequencing, Hi-C sequencing, and Bionano optical genomics mapping. *D. ischaemum* was previously determined to be a tetraploid through flow cytometry, and the assembly was successfully resolved into two subgenomes, labeled C and D, with 9 chromosomes on each subgenome, at 4n=36. The assembly was 644 Mbp in size with a scaffold N50 of 39.09 and a BUSCO score of 98.9%. Subsequent subgenome analyses indicated that *D. ischaemum* possesses traits of a segmental allopolyploid, and is likely a neoallopolyploid, although further investigation needs to be done to confirm this claim. The *D. ischaemum* genome is subject to a confidentiality period with the International Weed Genomics Consortium and will be publically available once the period has passed.

Introduction: As the need arises, the field of weed science is slowly being introduced to genomics. Typically weed science is focused on eliminating undesirable species, but the lack of new herbicide modes of action is driving the weed science industry to become more sustainable,

which relies on understanding weed species at a molecular level (Duke & Dayan, 2022; Patterson et al., 2019). This is especially important in the turfgrass industry, as there are less available selective herbicides for grass systems to control grass weeds. Turfgrass managers are often trying to control weed species in turfgrass that belong to the same family or even subfamily, like *Cynodon dactylon* (bermudagrass) and *Eleusine indica* (goosegrass) (Johnson, 1980; Schoch et al., 2020). Approaching genomics for weeds species (particularly weeds in the Poaceae family) is more difficult than crops as there is a definitive lack of resources compared to model species like *Arabidopsis thaliana* and crops. Currently the majority of publicly available Poaceae genomes (across NCBI, Phytozome, and the International Weed Genomics Consortium (IWGC)) are related to cereal crops like *Oryza* (rice), *Zea* (maize), *Hordeum* (barley), and *Triticum* (wheat), which is expected due to the economic impact produced by these crops ((U.S. Agricultural Baseline Projections, 2024)). The IWGC was formed in response to the lack of genomic information regarding weed species and has already produced 17 Poaceae weed genomes, compared to the 18 available on NCBI and 5 on Phytozome, as described in Table 1 (Montgomery et al., 2024). The production of weed genomes can aid in determining the genetic foundation of the evolution of weedy traits like herbicide resistance, while also providing a framework for understanding weed biology. Weed species can be as diverse intraspecies as it is interspecies, which creates difficulties when assembling genomes and performing comparative genomics analyses (Martin et al., 2019). One weed genus in particular that requires much more investigation is *Digitaria*.

Digitaria spp. (crabgrasses), named for their finger-like inflorescences (i.e. digitus), are a genus in Poaceae consisting of about 200 species (Döring, 2022). First cultivated for grains—and still cultivated with some species like *D. exilis* (fonio millet)—*Digitaria* is now more commonly

identified as a weedy genus (Mitich, 1988). *Digitaria* as a genus in North America has been frequently recognized as one of the most common weeds in grass crops, and the most common in turf, specifically *D. ischaeumum* (smooth crabgrass) and *D. sanguinalis* (large crabgrass) (Van Wychen, 2020). The weediness of *Digitaria* is associated with its hardiness and prolific nature: a summer annual C4 species, *Digitaria* spp. thrive in tropical environments and can germinate past expected periods when temperatures remain around 20-30ºC (Jones et al., 2021). *Digitaria* spp. are known for their seed production, as single plants with limited competition can put off around 150,000 seeds and 700 tillers in temperate areas like the United States (Mitich, 1988). *Digitaria* spp. are monoecious and self-pollination is its typical mechanism of reproduction, however cross-pollination can occur by wind and hybridization between species can occur (Ebinger, 1962). Despite the prolific nature of *Digitaria* spp., there is still much to uncover regarding the species.

D. ischaemum is a summer annual weed that presents with similar physical characteristics to *D. sanguinalis* and *D. ciliaris* (Southern crabgrass), but is distinguished by the lack of hair on leaf blades and sheaths, the pubescence on its collar region, and its membranous ligule (Bryson & DeFelice, 2009). It is a prolific weed that is difficult to control in turfgrass due to limited management options. Consistent mowing is the most utilized method of weed control in turfgrass, however the decumbent growing pattern of *D. ischaemum* and other *Digitaria* spp. actually increases weed density (Busey, 2003). In situations where herbicides are utilized, preemergence applications are preferable, however it is reasonable to assume that escapes can occur (Fidanza et al., 1996). In postemergence situations, *D. ischaemum* has been shown to be difficult to manage due to the disparity of control between growth stages with different herbicides (Dernoeden & Fry, 1986; Enache & Ilnicki, 1991). The evolution of herbicide

resistance traits creates even more issues when trying to manage the weed species. There are known resistances to fenoxaprop-ethyl, a Group 1 (acetyl-CoA carboxylase (ACCase) inhibitors) herbicide and quinclorac, a Group 4 (synthetic auxin) herbicide (Heap, 2024). As a common weed species, *D. ischaemum* populations resistant to these herbicides can cause major issues in turfgrass systems. Quinclorac, a synthetic auxin, is one of the only herbicides that possesses grass-in-grass activity, specifically the ability to control *Digitaria*, which is especially useful for postemergence control (Grossmann & Kwiatkowski, 2000). However, the mechanism of action for quinclorac and the mechanism of resistance are still not fully understood (Abdallah et al., 2006). The production of a reference genome for a quinclorac-susceptible species like *D. ischaemum* provides more resources for the determination of quinclorac's mode of action, as well as a potential foundation for understanding *Digitaria* biology.

There is currently no genomic data publicly available for *D. ischaemum*, which causes issues when trying to perform genetic analyses like differential gene expression or phylogenic studies to determine ancestry. Within the *Digitaria* genus, only one species, *D. exilis* has a full chromosome-level assembly publicly available (available on NCBI at GCA_902859565.1). Within the IWGC, the genome for *D. insularis* (sourgrass) has been completed, but is subject to a year-long confidentiality period. *D. exilis* and *D. sanguinalis* are also the only *Digitaria* species to have known ploidy levels, with *D. exilis* ranging from diploid to tetraploid, and *D. sanguinalis* ranging from tetraploid to hexaploid (Adoukonou-Sagbadja et al., 2007; Bennett, 1998; Gould, 1963). *D. insularis* was determined by the IWGC to be a tetraploid species through flow cytometry (Montgomery et al., 2024). Known *Digitaria* C-values are relatively small compared to other monocots within the Poaceae family: ranging from the smallest, *D. coenicola* at 602.70 Mbp, to largest, *D. setigera* (East Indian crabgrass) at 2239.30 Mbp (2019).

The goal of this study was to produce a reference-level genome for *D. ischaemum*. A novel genome would provide a foundation for future analyses involving herbicide resistant biotypes. Comparative genomics were also performed between *D. ischaemum*, *D. insularis*, and *D. exilis* to better understand *Digitaria* spp. on a taxonomic level.

Materials and Methods: Plant Selection: A population of *D. ischaemum* (AL_S1) was selected as the reference genome in this project for its known susceptibility to quinclorac in the field. For confirmation of susceptibility, AL_S1 was grown to seed, collected, and tillered into individual pots at the 3-leaf stage. AL_S1 was subjected to a dose-response assay in a randomized complete block design (RCBD) repeated in time from 0.125-16X the standard rate of quinclorac (841 g ai ha^{-1}) and deemed susceptible, as the population did not survive the 0.5X rate.

DNA and RNA Extraction: AL_S1 was grown to full maturity in the greenhouse and 6g of fresh growing, new leaf samples were sent for DNA extraction, PacBio HiFi sequencing, and Hi-C sequencing through Corteva Agriscience (Indianapolis, IN, USA). RNA was extracted from the same AL_S1 population that DNA was extracted from. Leaf tissue (old and young), stems, roots and seeds were combined into a single tube for three replicates to ensure at least one sample was of sufficient quality. Tissue was crushed with a mortar and pestle after flash freezing with liquid nitrogen, then transferred to a 5mL tube. 1000µL of Trizol reagent was added after grinding tissue and vortexed for 5 minutes, then centrifuged for 5 minutes at 15,000RPM. The supernatant was then transferred to a 1.5mL tube and centrifuged again for 1 minute to ensure no debris remained. In a new tube, 700µL of supernatant and 700µL of 100% ethanol were added to the tube and vortexed briefly. 650µL of the supernatant/ethanol mixture were then transferred to a column tube and centrifuged for 30 seconds in a centrifuge at 4ºC. The flow through was then discarded and this step was repeated until all of the supernatant/ethanol mixture was transferred

to the column tube. At this point, the RNA extraction proceeded with instructions based on the Zymo Direct-zol Miniprep Kit (Zymo Research, Irvine, CA, USA). At the elution step, 50µL of DNase/RNase free water was added instead of 100µL to increase RNA concentration. RNA quantity and quality was then checked on the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was then sent to Corteva Agriscience for RNA Isoform sequencing (Iso-Seq).

Genome Sequencing, Assembly, and Annotation: Genome sequencing, assembly, and annotation was conducted in accordance with the IWGC, utilizing a genomics pipeline outlined by (Raiyemo et al., 2024)). Estimated genome size and ploidy were determined using flow cytometry (Montgomery et al., 2024). The genome was constructed using Bionano Optical genome mapping, PacBio HiFi long-read sequencing, and Hi-C sequencing.

The genome was then configured into contigs and scaffolds, using *Hifiasm* v0.16.1(Cheng et al., 2021) to assemble the PacBio HiFi reads and the resulting contig set was aligned to the Bionano map set using the Bionano Genomics Solve software v3.7. Sequence reads were mapped against each other to determine best orientation and polished against the PacBio HiFi data using *minimap2* v2.2.4 (Li, 2018)**.** Errors were identified and corrected using samtools-mpileup (Danecek et al., 2021). Reads that were not scaffolded into contigs were assigned to Chr00. *Juicer* v0.7.0 (Durand, Shamim, et al., 2016) was used to validate the hybrid scaffold into pseudomolecules, which was then visualized using *Juicebox* (Durand, Robinson, et al., 2016). Once the genome was fully assembled, the draft genome was corrected using *Pilon* (Walker et al., 2014). The pipeline then annotated the genome assembly by first identifying transposable element (TE) families using *RepeatModeler* v2.0.2 (Flynn et al., 2020), annotating the TEs with *RepeatMasker* (http://www.repeatmasker.org/RepeatMasker/), and finally softmasking the

genome with *BEDTools* v2.30.0 (Quinlan & Hall, 2010) against the TE annotations. The annotation of the softmasked genome then proceeded by using *Multiloc2* (Blum et al., 2009), *InterProScan5* v82 (P. Jones et al., 2014), and *MMSeqs2* (Steinegger & Söding, 2017) to identify subcellular protein localizations, predict protein domains, and functional annotation by whole-protein homology, respectively.

Genome Analysis: Statistical analysis was performed on the assembled genome using a variety of programs: *Assemblathon2* was used to determine the assembly size, N50 and L50 scaffold lengths, and GC content (Bradnam et al., 2013). The genome was then processed through *BUSCO* (Benchmarking Universal Single-Copy Orthologs) (Manni et al., 2021) in genome mode utilizing the embryophyta_odb10 lineage dataset to determine completeness of the genome. Another Gtf/Gff Analysis Toolkit (*AGAT*) was utilized for statistics regarding number of genes, gene, exon, and CDS length, the average exon per gene, and number of tRNAs present. *Orthofinder* v2.5.4 was used to determine the number of genes in orthogroups and the percentage of those genes in orthogroups.

A number of standard reports were also produced from the genome pipeline including: stats on the telomeric regions, karyotyping and gene density via R package *RIdeogram* ((Hao et al., 2020; Krzywinski et al., 2009), and comparative analyses of the subgenomes using *SubPhaser* (Jia et al., 2022). *EDTA* v2.2 (extensive de-novo TE annotator) was utilized outside the genome pipeline to identify and annotate the repetitive regions (Ou et al., 2019). The *EDTA* package included *RepeatMasker, TIR-Learner (Su et al., 2019), Generic Repeat Finder (Shi & Liang, 2019), HelitronScanner (Xiong et al., 2014), TEsorter (Zhang et al., 2022), LTRharvest (Ellinghaus et al., 2008), LTR_Finder (Xu & Wang, 2007), LTR_Finder_parallel (Ou & Jiang, 2019) and LTR_retriever (Ou & Jiang, 2018)* for a robust analysis of the repeat landscape. The

LTR assembly index (LAI) was calculated using *LTR_retriever* v3.0.1 separately from *EDTA.* Subgenome analysis was also conducted via *SubPhaser* to phase homoeologous subgenomes and estimate the LTR insertion age by identifying subgenome specific repetitive DNA sequences, more specifically 15bp long k-mers, or 15-mers. *SubPhaser* was also used to compare other *Digitaria* species available against *D. ischaemum* to identify potential progenitor subgenomes. Chloroplast Genome Assembly: Raw PacBio HiFI *D. ischaemum* data was aligned to the *D. sanguinalis* plastid genome (NCBI accession OZ156447) using *minimap2* V2.26 (Li, 2018) with the -map-hifi option and *Samtools* V1.19 (Danecek et al., 2021) was used to filter out reads shorter than 8000bp in with the minimum length option -m and and a query value less than 60 with the option -q. The filtered reads were then de novo assembled into the plastid genome with *HiCanu* V2.0 (Nurk et al., 2020) using the -pacbio-hifi option and setting the plastid genome size to 0.14 Mbp as an estimate to match that of *D. exilis*. The chloroplast genome was then annotated with the MPI-MP reference set built into the Chlorobox *GeSeq* V2.03 tool, and then visualized using the *OGDraw* (Greiner et al., 2019; Tillich et al., 2017).

Results and Discussion: Genome Sequencing, Assembly, and Annotation: The PacBio HiFi sequencing resulted in ~6.3 Gbp of raw data and was constructed into a chromosome-level assembly as a single haplotype, with a genome size of 644 Mbp. Flow cytometry estimated the genome size for *D. ischaemum* to be 625 Mbp, so the actual genome size is only slightly larger (Montgomery et al., 2024). The final assembly consisted of 19 scaffolds, representing 9 chromosomes across two subgenomes determined by *SubPhaser*, indicating that *D. ischaemum* is an allotetraploid species (4n=36). The subgenomes were designated as C (Chr01C-09C) and D (Chr01D-09D), and Chr00, which contained all unscaffolded contigs. The *D. ischaemum* subgenomes were labeled C and D to accommodate the existing subgenome names of the *D.*

exilis genome (A and B), and the *D. insularis* subgenomes were labeled E and F, as it was assembled after *D. ischaemum (Wang et al., 2021)*.

Statistical analysis of the *D. ischaemum* genome assembly indicates the successful construction of a high quality, reference-level genome. The BUSCO score for the *D. ischaemum* genome was found to be 98.9%, with 11.65% genes being complete and single copy, 87.24% genes being complete and duplicated, and only 1.12% genes being fragmented or missing. The scaffold N50 was 39.09 Mbp, scaffold L50 was 8, and LAI was determined to be 19.59, indicating reference-level genome (Table 2) (Ou et al., 2018). The genome annotation identified 62,161 protein coding genes, with a mean gene length of 2703 bp, mean CDS length of 1057 bp, and mean exon length of 264 bp (Table 2). The GC content of the assembly was 45.41%, following the general trend of monocot genomes to positively correlate GC content with a small genome size (Šmarda et al., 2014).

Genome Analysis: Subgenomes, Karyotype, and Herbicide Resistance Genes: *D. ischaemum* was hypothesized to be a polyploid species, as its close relatives *D. sanguinalis* and *D. exilis* are hexaploid and tetraploid species, respectively. A differential analysis of 15-mers was used to produce a principal component analysis and heat map of the *D. ischaemum* chromosomes, indicating that *D. ischaemum* is an allotetraploid with two distinct subgenomes (C and D) (Figure 1). The PCA1 described of 83.9% of the variation between the two subgenomes, indicating high variation, PCA2 described 2.6% of the variation, showing clear clustering between C and D chromosomes. The C and D subgenomes and k-mers specific to each subgenome were formatted in a Circos plot along with the enriched subgenomes, normalized proportions of the subgenomes, the density of k-mers specific to each each subgenome, long terminal repeat (LTR) density, and homology between the subgenomes (Figure 2). The Circos

plot indicates that *D. ischaemum* is fairly homologous, with the D subgenome carrying homoeologous exchanges (HEs) from the C subgenome. This could be indicative of *D. ischaemum* being a segmental allopolyploid, meaning *D. ischaemum* displays traits of both allo- and autopolyploids (Mason & Wendel, 2020). In the segmental allopolyploid model, HEs can be balanced or duplication-deletion, where sections of one subgenome are deleted and replaced with the same sections from the other subgenome (Stebbins, 1971). With the D genome containing all of the HEs, it is likely that the C genome is the preferential or "dominant" subgenome, where evolution has been biased towards the C subgenome and HEs have occurred to retain this progenitor.

The Circos plot reveals more evolutionary information beyond HEs, as we can see with the innermost circle displaying homologous blocks between the subgenomes. With Chr02C and Chr02D and Chr08C and Chr08D there are clear inversions of these chromosomes between subgenomes, indicated by the mirrored syntenic blocks, although these are not true "inversions" as the subgenomes represent two distinct species (Figure 2). These chromosomal differences, and ones not easily identified are more evident in the karyotype plot produced by *RIdeogram* (Figure 2). The heat map of the karyotype is filled with gene density across each chromosome, and common genes associated with herbicide resistance have been used as markers in each chromosome. The differences between Chr02C and Chr02D can be seen due to the "flip" in gene density and is even more evident between Chr08C and Chr08D due to the location of the EPSPS gene. Other evident chromosomal differences based on the karyotype are present in Chr03, Chr05, Chr07, and Chr09. The ACCase marker present on Chr03D is missing on Chr03C, there is an extra HPPD maker present on Chr05D not present on Chr05C, and Chr07D and Chr09D are both ~14% larger than Chr07C and Chr09C (Table 3).

These small differences between the subgenomes and HEs indicate that the two progenitor species are likely highly related species, but the makeup of the repeat landscape indicates that these are different species and not the result of autopolyploidization.

The genome assembly and annotation pipeline plotted the most common genes related to herbicide resistance to the karyotype, *protophyrinogen oxidase* (*PPO*) *1*, *PPO2*, *acetyl-CoA carboxylase* (*ACCase*), *4-hydroxyphenylpyruvate dioxygenase* (*HPPD*), *glutamine synthetase* (*GS*), *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*), and the *acetolactate synthase* (*ALS*) small and large subunits (Figure 3). The target sites for quinclorac are not currently known, so they were not included in the karyotype, however the locations of potential target sites (i.e. genes with known mutations for other synthetic auxin herbicides) were identified. Mutations on *transport inhibitor response 1* (*TIR1*) gene have been associated with resistance to picolinate herbicides, and the genes were identified on both subgenome C and D of Chr01, Chr03, Chr09, and subgenome C of Chr06 of *D. ischaemum* (Walsh et al., 2006). Resistance to dicamba, fluroxypyr, and 2,4-D have been associated with mutations to the *auxin/indole-3-acetic acid* (*AUX/IAA*) gene family, and these were identified on every chromosome in *D. ischaemum* except Chr07D (de Figueiredo et al., 2022; LeClere et al., 2018). Another potential target site for quinclorac is the *β-cyanoalanine synthase* (*β-CAS*) gene, which was shown to endow resistance to quinclorac specifically in a resistant biotype of *Echinochloa crus-galli* (barnyardgrass), and these genes are located on both subgenomes of Chr03 and Chr06 (Gao et al., 2017). The last potential target site gene for quinclorac is the *cellulose synthesis catalytic subunit A (CESA)* gene, as it has been suggested that quinclorac may have some activity in inhibiting cellulose biosynthesis, and mutations to CESA6 have resulted in resistance to isoxaben (Desprez et al., 2002). The CESA gene was identified on both subgenomes of Chr01, Chro02, Chr03, Chr04, and

Chr08. Knowing the location of these potential target sites may be beneficial for understanding potential gene duplications or gene expression.

Repeat Landscape: The transposable element (TE) content of *D. ischaemum* was determined to be about 42.56% of the genome. LTRs comprised the majority of the repetitive elements identified at 29.74%, DNA transposons consisted of 11.74%, and miniature inverted-repeat transposable elements (MITEs) consisting of 1.09% of the repetitive elements (Table 4). *D. ischaemum* interestingly contains a much higher percentage of Gypsy-LTRs (17.35% count of all repeat elements and 52.68% of the length of repeat elements) than Copia-LTRs (5.05% count of all repeat elements and 8.54% of the length of repeat elements). This trait has been previously reported in *D. exilis:* a 6.7:1 Gypsy/Copia ratio compared to the 6.2:1 presented in *D. ischaemum (Wang et al., 2021)*. This supports Wang et al. (2021)'s proposal that the ratio of Gypsy/Copias is indicative of some common ancestral property, although it is unknown what property produces this ratio.

While *EDTA* was used to identify and produce an annotation of the repeat elements across the *D. ischaemum* genome, *SubPhaser* was used to estimate the LTR-insertion age (Figure 3). It is estimated that all LTR-insertions occurred less than 3 million years ago (MYA), and both subgenomes substantiate this estimation as Subgenome C had a LTR-insertion at around 1.29 MYA and Subgenome D had a LTR-insertion around 1.97 MYA (Jedlicka et al., 2020). So based not only on karyotype and the present HEs, LTR-insertion age indicates that there are two progenitor species of *D. ischaemum* and is indicative of segmental allopolyploidy.

Comparative Genomics: Differential K-mer Analysis and Plastid Genome Assembly: Despite the *Digitaria* genus consisting of around 200 species, there are no confirmed diploids to trace lineage back from *D. ischaemum*. The species with known ploidy, *D. insularis, D. exilis* and *D.*

sanguinalis, are tetraploid species and hexaploid species, respectively. None of the species with known ploidy levels have had progenitor species identified, and only *D. exilis* has a known timeline for its allopolyploidization event, at ~3.1 MYA (Wang et al., 2021). The differential k-mer analysis produced by *SubPhaser* compared each subgenome of *D. ischaemum, D. insularis*, and *D. exilis* utilized 15 k-mers from each species. *D. sanguinalis* was excluded from this analysis as at the time the study was conducted, no genome had yet been produced.

As plastid genomes are maternally inherited, the maternal progenitor can be identified by assembling the chloroplast (Greiner et al., 2015). *SubPhaser* confirmed that *D. insularis*, *D. exilis*, nor *D. sanguinalis* share a progenitor with *D. ischaemum* by the differential k-mer analysis of the subgenomes. However, future taxonomic research could benefit from the production of the plastid genome, as it is an easy confirmation test for the maternal progenitor. The *D. ischaemum* plastid was constructed from the raw PacBio HiFi reads and assembled with *HiCanu* into a plastid genome that was 158,824 bp long, which is larger than the average monocot plastid (Figure 6) (Mohanta et al., 2020). The plastid assembly has not experienced any loss of major features, as it has retained both inverted repeats (IR) A and B, the large single-copy region (LSC) and the small single-copy region (SSC). *D. ischaemum* also follows the trend that plastid is AT rich, with a GC-content of 38.24% (Table 5).

Research Implications: A reference-level genome for *D. ischaemum* was produced and analyzed for future use in studying the molecular foundation of herbicide resistance and other traits related to weediness. *D. ischaemum* was determined to be a tetraploid and is likely a neoallopolyploid with two phylogenetically similar progenitors, as *SubPhaser* was able to phase the subgenomes. *SubPhaser* was designed with neoallopolyploids in mind, specifically for species with closely related subgenomes and potentially extinct diploid progenitors (Jia et al.,

2022). *D. ischaemum* also exhibits qualities of a segmental allopolyploid, as subgenome D presented with evidence of homoeologous exchanges from subgenome C. The production of this genome will be beneficial for studying how weedy polyploid species evolve and for understanding how herbicide resistance can evolve. Future phylogenetic research for the *D. ischaemum* should include determining its progenitor species, when its whole genome duplication event occurred, and potentially using RNASeq to determine subgenome dominance. For *D. ischaemum*, using this genome for more studying resistance to quinclorac is especially important, as the appearance of quinclorac-resistant biotypes of susceptible species like *D. ischaemum* is detrimental for turfgrass systems, as it removes one of the only available selective herbicides for grass-weed control in grasses. The availability of the *D. ischaemum* genome provides an essential resource for studying quinclorac-resistant biotypes and comparing to susceptible biotypes, particularly for differential gene expression studies or functional gene discovery.

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Tables:

TABLE 4-1. Poaceae genomes available on different genomics platforms, the number of unique species available on each platform, and the number of grass weed genomes available on each platform.

	NCBI	**Phytozome**	**IWGC***
genomes available	841	156	17
unique species	151	25	8
weed genomes	18	5	17

***4 genomes are subject to a year-long confidentiality period

TABLE 4-2: Summary of assembly statistics for *Digitaria ischaemum* genome.

Assembly size (Mbp)	644.33
Number of Chromosomes	4n=36
Scaffold N50 (Mbp)	39.09
Scaffold L50	8
GC content (%)	45.41
Complete BUSCO (%)	98.9
LTR assembly index (LAI)	19.59
Protein-coding genes	62161
Mean gene length (bp)	2703
Mean CDS length (bp)	1057
Mean exon length (bp)	264
Mean exon per gene	4.9
Number of tRNA	1295
Number of genes in orthogroups	47992
Percentage (%) of genes in orthogroups	74.03

TABLE 4-3: Length of chromosomes across both subgenomes and the difference in length between each chromosome. The longer subgenome is highlighted in the color corresponding to the subgenome (yellow, subgenome C; blue, subgenome D).

Subgenome	Length (bp)	Subgenome	Length (bp)	Difference (bp)	Percent Change (%)
Chr01C	44880976	Chr01D	45768067	887091	1.97654124
Chr02C	42847379	Chr02D	41477545	1369834	3.3025918
Chr03C	39863487	Chr03D	40812455	948968	2.38054438
Chr04C	39633005	Chr04D	39088769	544236	1.39230785
Chr05C	33262940	Chr05D	37189739	3926799	11.8053275
Chr06C	31425685	Chr06D	30729326	696359	2.26610567
Chr07C	28081100	Chr07D	31989442	3908342	13.9180516
Chr08C	27767727	Chr08D	29370719	1602992	5.77285998
Chr09C	26925677	Chr09D	30649582	3723905	13.8303115

TABLE 4-4: Summary of repetitive element annotations.

	Total	Sequences:	19	
	Total	Length:	644483057	bp

	Class	Count	bpMasked	%masked
DNA Transposon	DTA (hAT)	22300	5542009	0.86
	DTC (CACTA)	50845	13802622	2.14
	DTH (PIF/Harbinger)	14456	2707296	0.42
	DTM (Mutator)	50235	12318571	1.91
	DTT (Tcl/Mariner)	32611	6108948	0.95
	Helitron	142326	35158838	5.46
LTR	Copia	25731	23437521	3.64
	Gypsy	88472	144486721	22.42
	unknown	38088	23731711	3.68
MITE	DTA (hAT)	8244	1387754	0.22
	DTC (CACTA)	3771	524444	0.08
	DTH (PIF/Harbinger)	11083	1815571	0.28
	DTM (Mutator)	10119	1798320	0.28
	DTT (Tcl/Mariner)	11676	1477937	0.23
	Total Repeats	509957	274298263	42.56%

TABLE 4-5: Summary of plastid genome statistics

Assembly size (bp)	158824
LSC length (bp)	100830
SSC length (bp)	12556
IR length (bp)	45438
Overall GC content (%)	38.24
Protein-coding genes	184
Number of rRNA	8

Figures

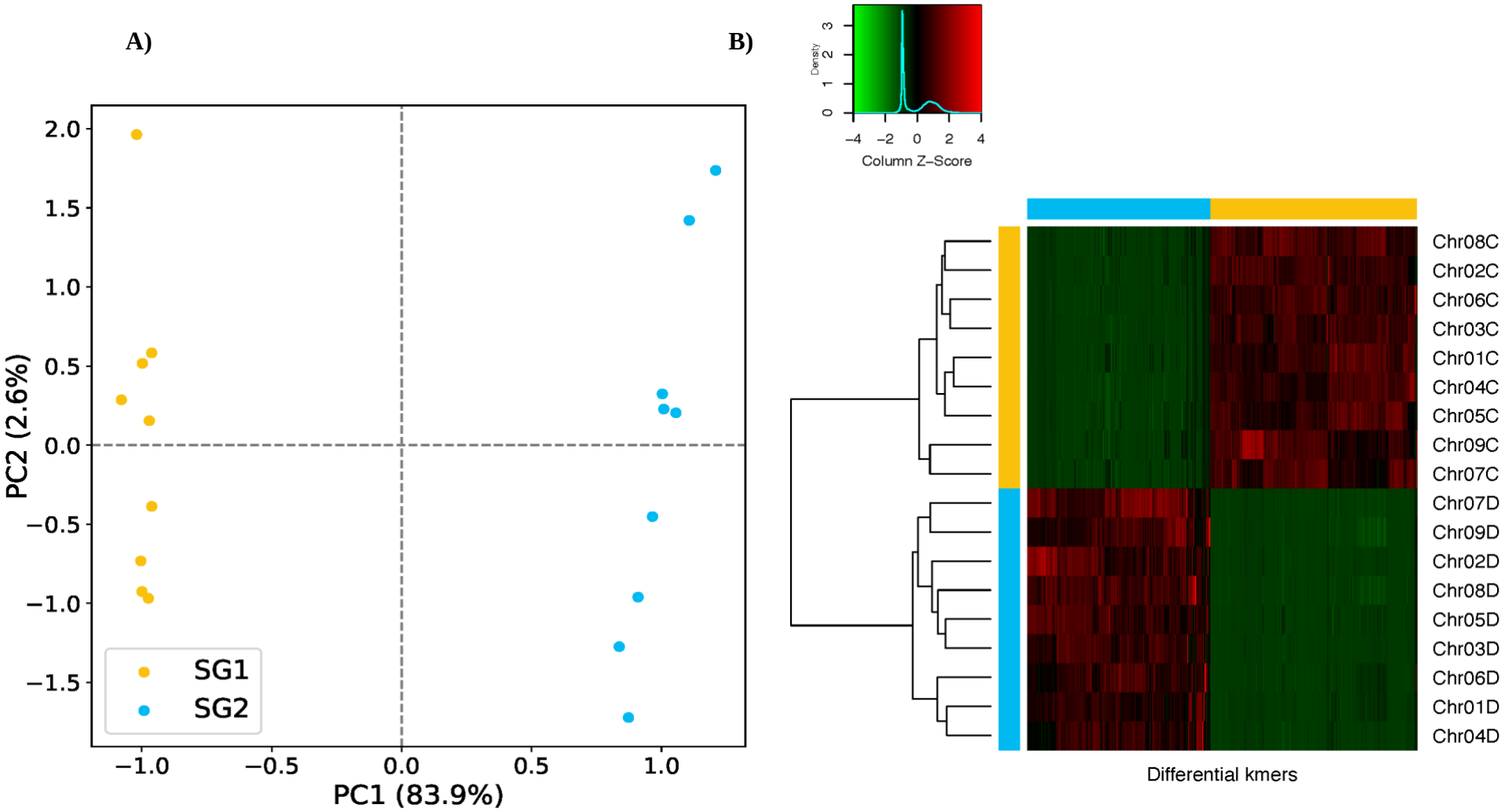

FIGURE 4-1: A) Principal component analysis of *Digitaria ischaemum* chromosomes between subgenomes B) heatmap of 15-mers differential k-mer analysis of the subgenomes.

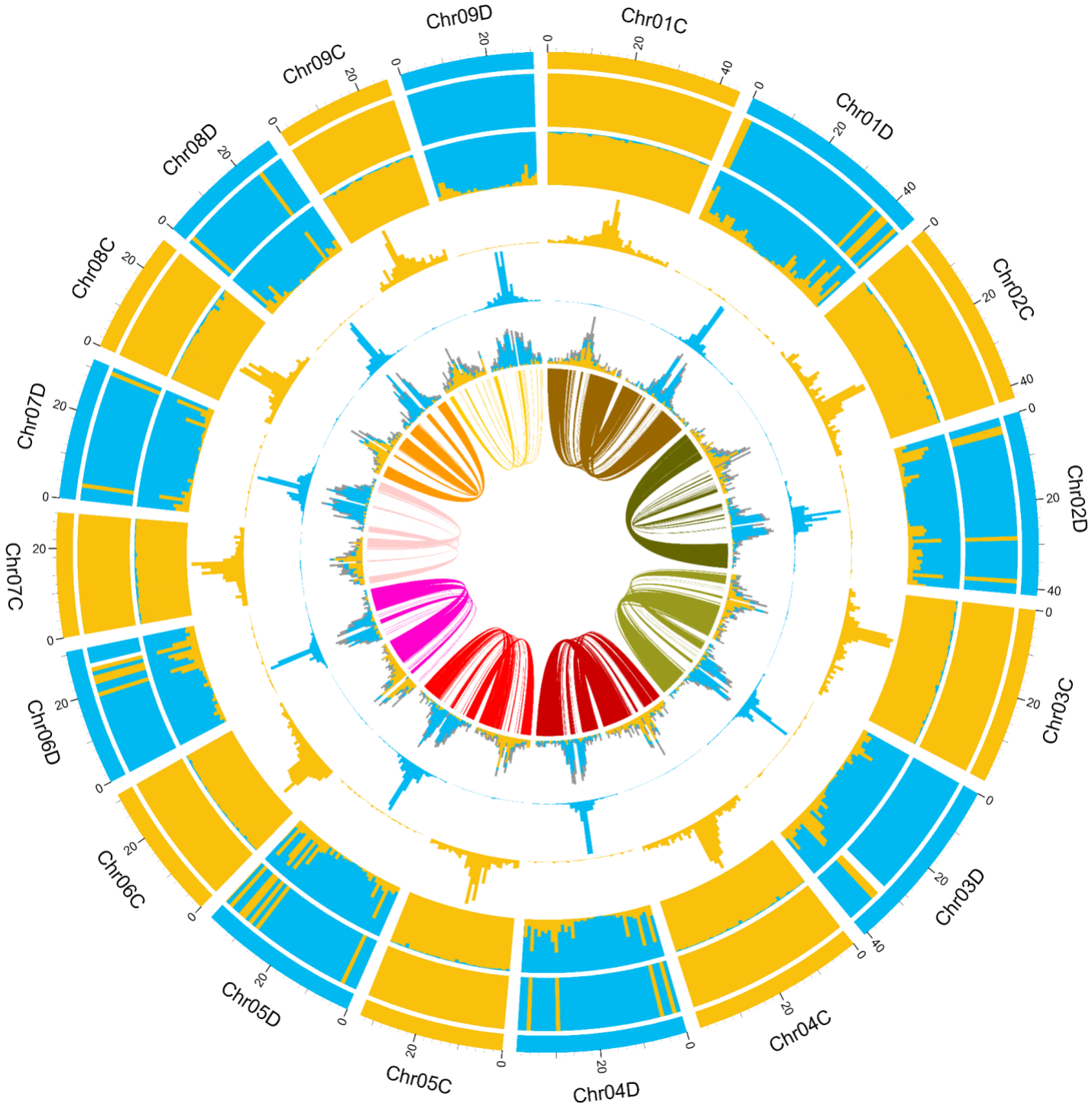

FIGURE 4-2: Circos plot depicting two subgenomes of *Digitaria ischaemum*. Window size was set to 1000000 bp. Subgenome C is represented by yellow and subgenome D is represented by blue. Outer to inner rings indicate: 1) karyotype 2) enriched subgenome 3) normalized proportion of each subgenome 4) density of SG1-specific k-mers 5) density of SG2-specific k-mers 6) density of LTR-RTs, where grey indicates non-specific LTRs 7) homologous blocks between subgenomes.

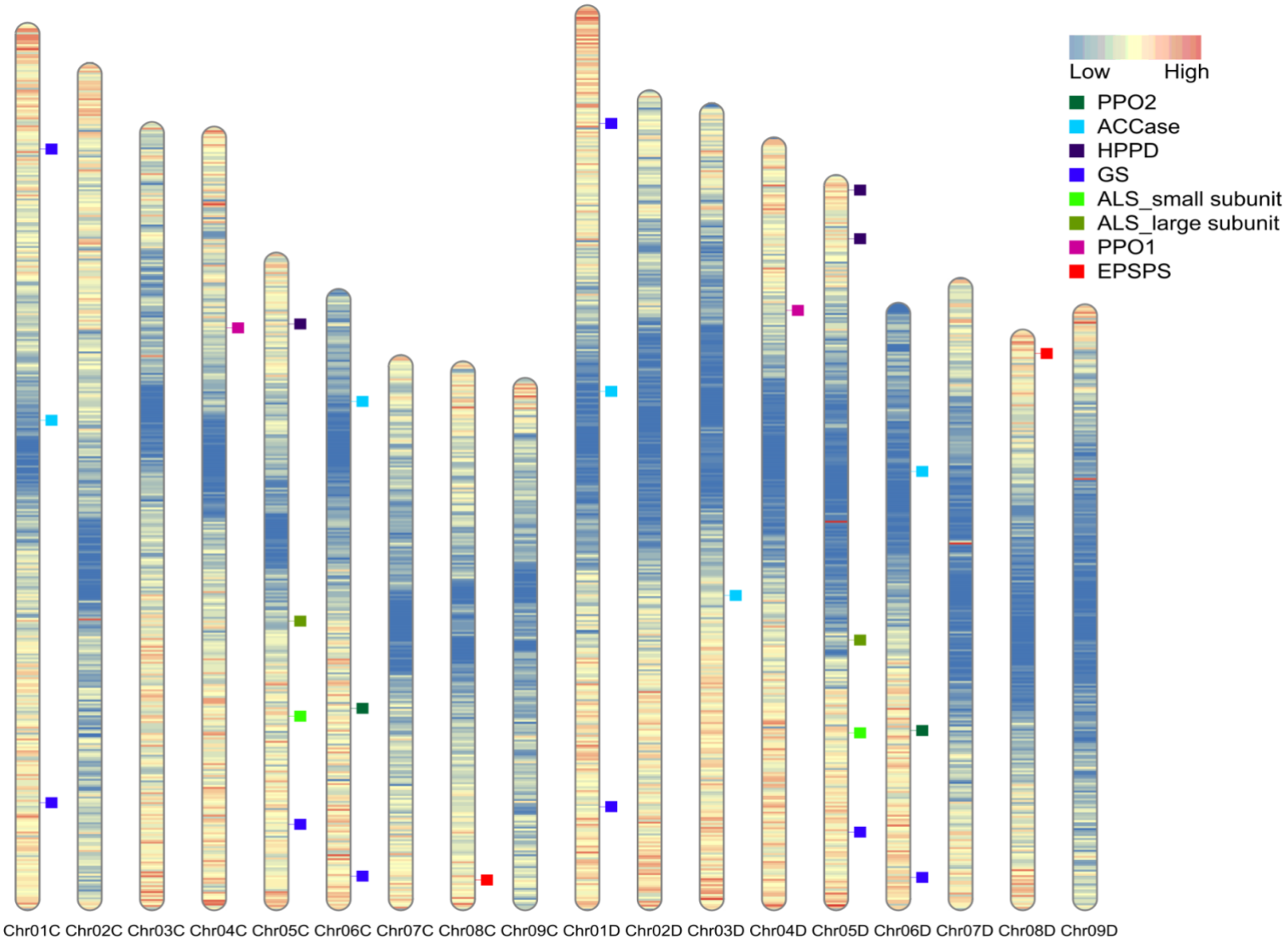

FIGURE 4-3: Karyotype plot of Chr01C-09C and Chr01D-09D. The heat map indicates areas of low to high gene density across each chromosome, and the legend indicates common genes related to herbicide resistance traits. Genes include *protoporphyrinogen oxidase* (*PPO*) *1*, *PPO2*, *acetyl-CoA carboxylase* (*ACCase*), *4-hydroxyphenylpyruvate dioxygenase* (*HPPD*), *glutamine synthetase* (*GS*), *acetolactate synthase* (*ALS*), *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*).

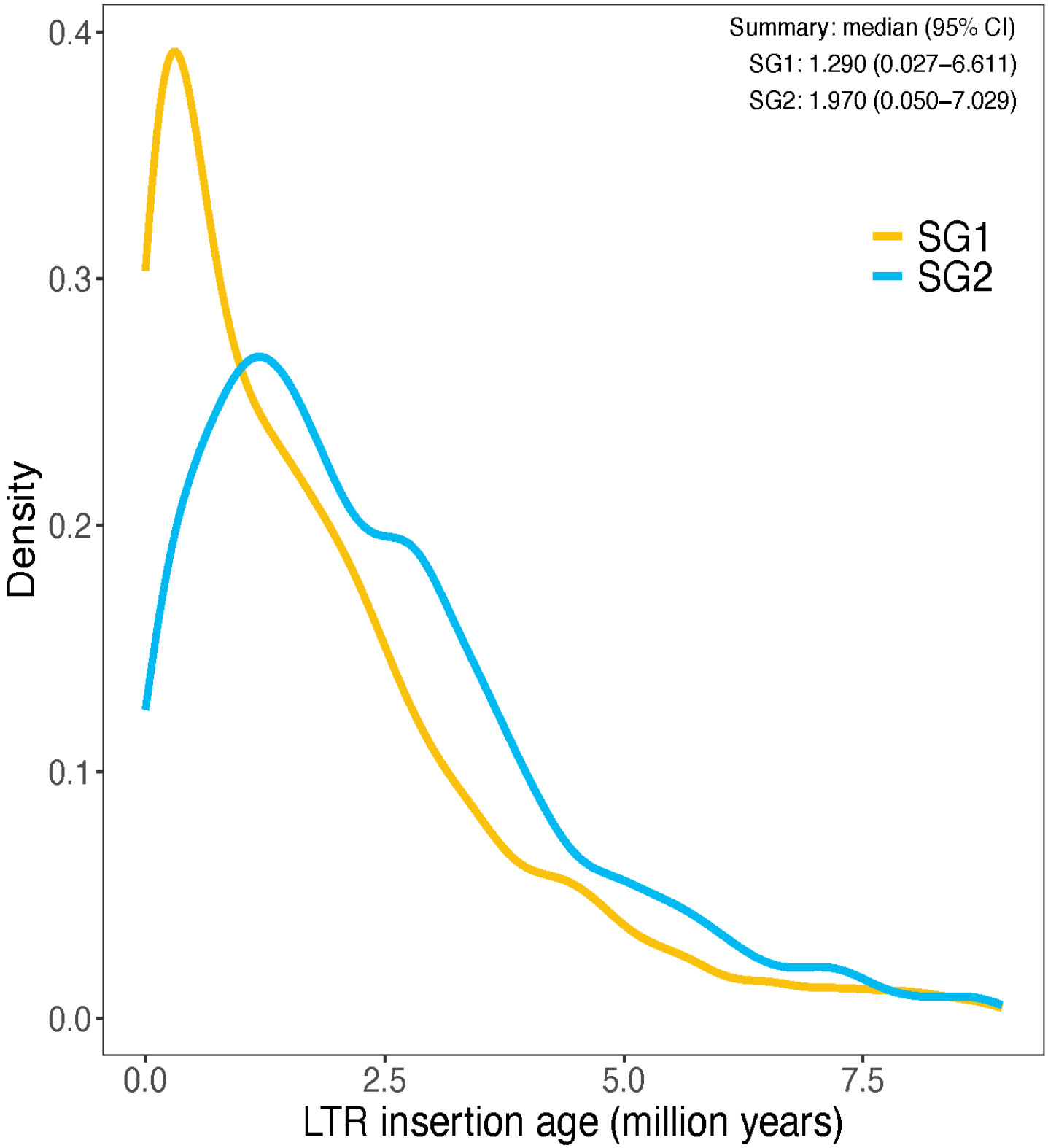

FIGURE 4-4: LTR insertion age of Subgenome 1 (C) and Subgenome 2 (D), based on median values.

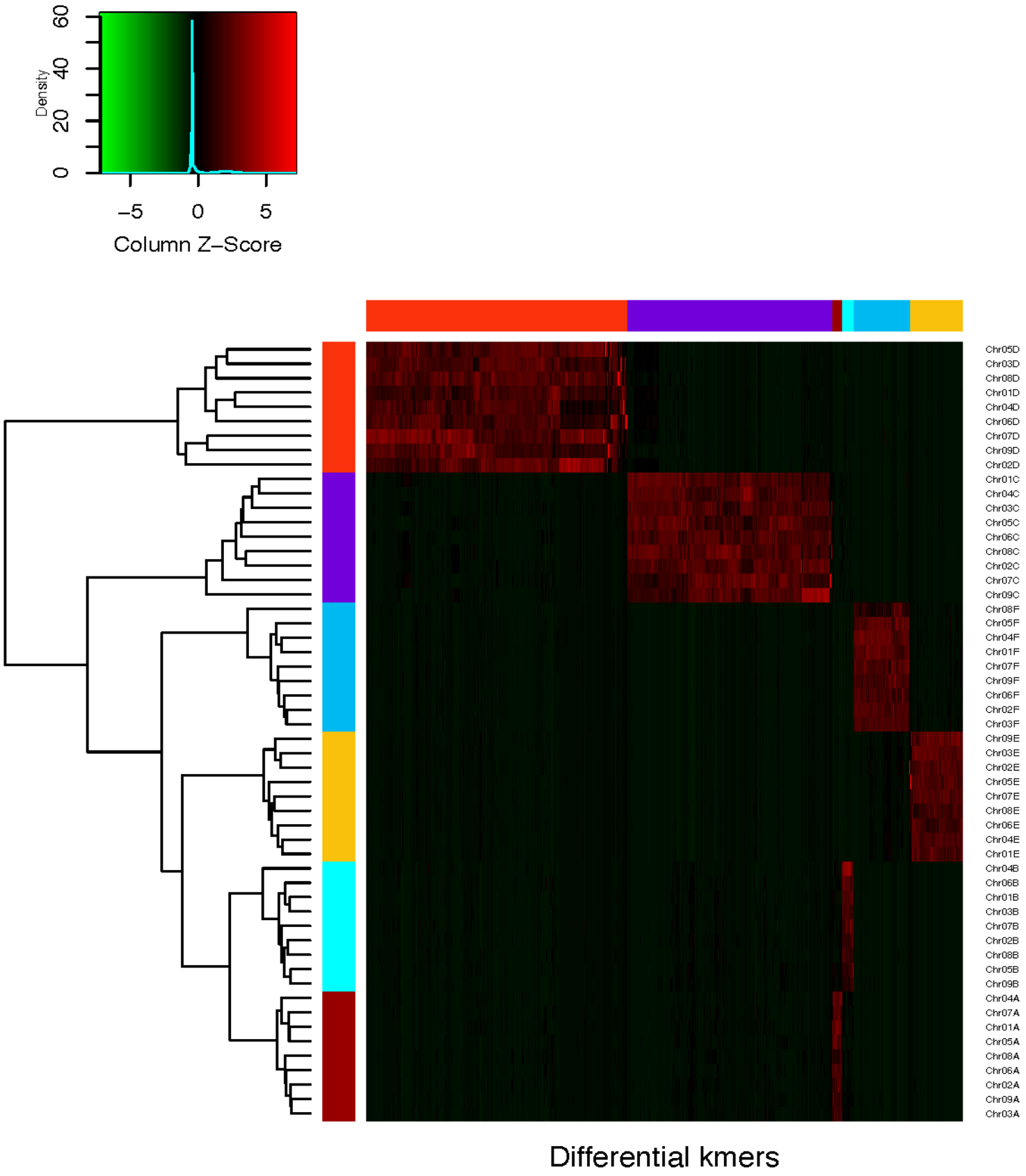

FIGURE 4-5: Differential k-mer tree of 15-mers from *Digitaria ischaemum* (subgenomes C and D), *Digitaria insularis* (subgenomes E and F) and *Digitaria exilis* (subgenomes A and B).

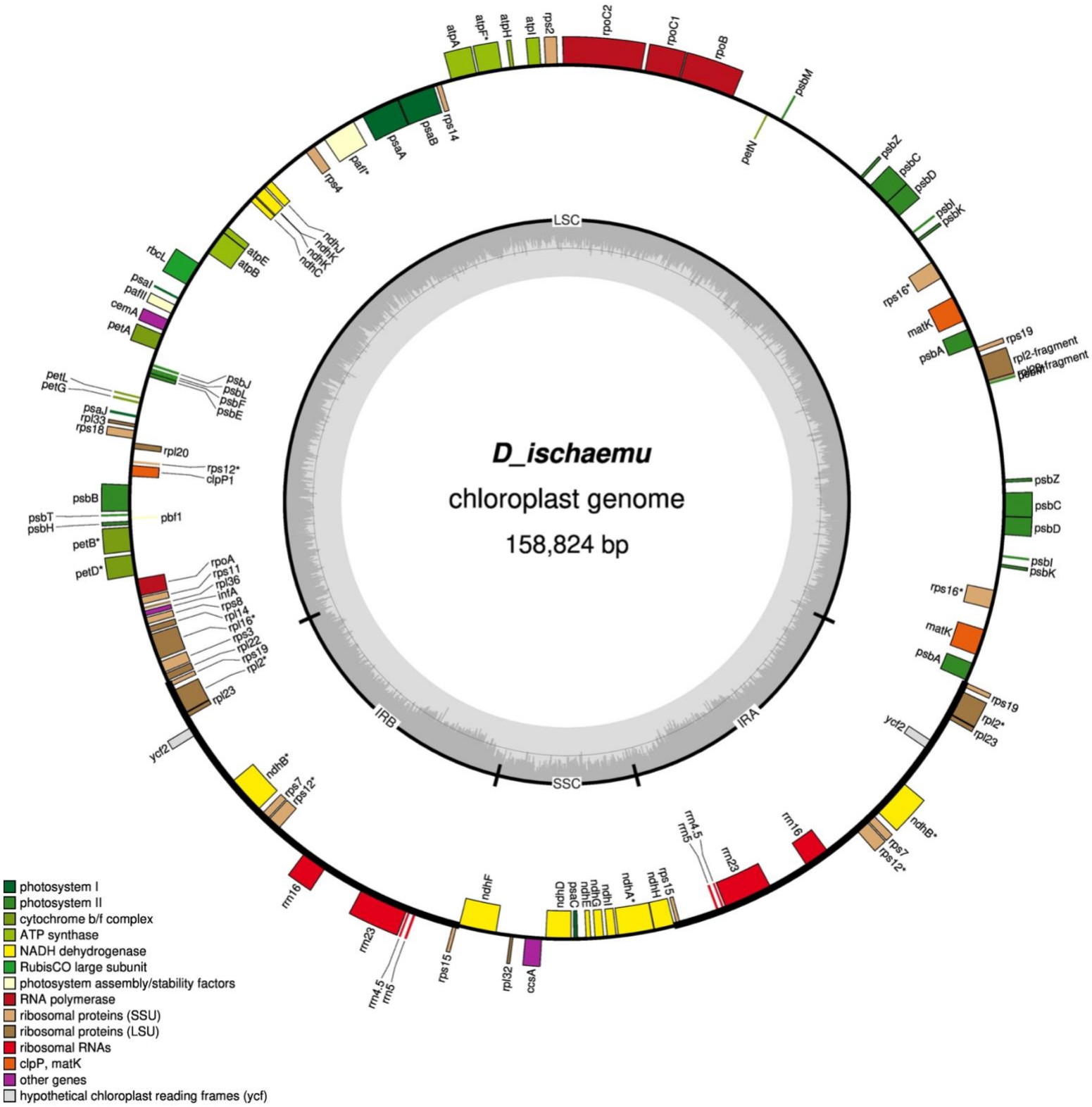

Figure 6. Plastid genome assembly of *Digitaria ischaemum.* Assembled plastid was 158,824 bp long, with retained both inverted-repeat (IR), long single-copy (LSC), and short single-copy (SSC) regions at 45,438 bp, 100,830 bp , and 12,556 bp long, respectively.

Chapter 5: Analysis of inherent gene expression and the potential target-sites in quinclorac-resistant smooth crabgrass

Abstract: The plant hormone auxin governs various aspects of plant growth and development through its interaction with the Aux/IAA gene family and the SCF ubiquitin ligase complex. Synthetic auxin herbicides, specifically quinclorac, are thought to exploit this hormonal pathway but exhibit selective efficacy between specific grass families. This study aimed to elucidate the mechanisms underlying quinclorac resistance in a population of *Digitaria ischaemum* by investigating potential target-sites, focusing only on inherently expressed genes. Two populations, quinclorac-resistant (AL_R1) and susceptible (AL_S1), were subject to six replicates of RNA sequencing. Each replicate of AL_R1 and AL_S1 was utilized for a differential gene expression study, then subjected to individual transcriptome assemblies to compare specific gene sequences. Several differentially expressed genes and gene families were identified, however no specific target-site mutations with auxin resistance were found in the selected genes related to auxin signaling, cellulose biosynthesis, or cyanide detoxification. Differential gene expression analysis revealed substantial differences between resistant and susceptible populations, underscoring the complexity of the mechanisms of resistance to quinclorac and highlights the need for further research into the molecular basis of quinclorac resistance and potential unknown proteins implicated in this process.

Introduction: Auxin is an important plant hormone associated with plant growth and development, particularly in regards to vascular tissue development, root formation, phototropism, and apical dominance (Went & Thimann, 1937). There are numerous natural auxin analogs, the most common being indole acetic acid (IAA), which will simply be referred to as auxin at this point forward. In addition to auxin, the auxin/indole-3-acetic acid (Aux/IAAs) is

an important gene family of repressor proteins (Abel & Theologis, 1996). The Aux/IAAs are composed of four subunits: domains I, II, III and IV. Domain II is essential for binding to the SCF ubiquitin ligase complex, as it contains the degron motif where binding to the complex occurs, while domains III and IV work in tandem by dimerizing and binding to auxin response factors to repress the expression of auxin responsive genes (Luo et al., 2018). When auxin levels are high, auxin binds to transport inhibitor response protein (TIR1) (a subunit of the SCF^{TIR1} complex), acting as a molecular glue between TIR1 and Aux/IAA at the degron, inducing a large variety of auxin responsive genes by ubiquinating Aux/IAA, thus tagging it for degradation and leaving the auxin response factors unbound and available for expression (Taiz & Zeiger, 2006). Auxin and the AUX/IAA gene family have been instrumental in the development of synthetic auxin herbicides, like 2-4,D and dicamba. Synthetic auxins act as natural auxins and enhance the binding activity between TIR1 and Aux/IAAs (Tan et al., 2007). Analogs of TIR1, the auxin signaling F-box protein (AFB1-5), have been shown to show selective binding between particular synthetic auxins, like TIR1 to 2,4-D and picloram to AFB5 (Calderón Villalobos et al., 2012). The role of auxin is massive in all plant development and there are still major gaps in understanding the phytohormone, particularly in the differential responses seen between monocot and dicot species with synthetic auxin herbicides. It is unclear why synthetic auxins do not affect monocots in the same manner as dicot species, considering auxin's molecular foundation is conserved between monocots and dicots (McSteen, 2010). Only one synthetic auxin, quinclorac, is known to have activity in monocot species.

Quinclorac (3,7-dichloroquinoline-8-carboxylic acid) is a synthetic auxin herbicide unique for its grass-in-grass selectivity. While quinclorac acts in the typical synthetic auxin manner when applied to broadleaf species (i.e. epinasty, unsustainable growth), certain grass species exhibit a

vastly different response with chlorosis and tissue necrosis due to overproduction of cyanide with the stimulation of the 1-aminocyclopropane-1-carboxylate synthase (ACS) enzyme (Grossmann & Kwiatkowski, 1995). It has also been suggested that quinclorac acts as a cellulose biosynthesis inhibitor, but results have varied in proving this is the case or not (Koo et al., 1997; Tresch & Grossmann, 2003). While the outcome of quinclorac application is understood to be the result of accumulation of reactive oxygen species leading to cell death, the mechanism of evolved resistance to quinclorac is not (Fipke & Vidal, 2016). Previous literature has indicated the likelihood for a target-site mechanism to be the cause of resistance, but limitations to discovering this target site are due to the fact that a target gene for quinclorac binding is not known (Abdallah et al., 2006; Grossmann & Kwiatkowski, 2000).

Other known target-site mutations across synthetic auxin herbicides provide genes to investigate for potential target-site for quinclorac. Target-site mutations have been identified in broadleaf and grass species; mutations in the degron of Aux/IAA in *Kochia scoparia* endowed cross resistance to 2,4-D, dicamba, and fluoroxypyr, and a deletion of the degron tail in *Sysbrium orientale* endowed resistance to 2,4-D (de Figueiredo et al., 2022; LeClere et al., 2018). Mutations to TIR1 and AFB5 in *Arabidopsis* provided resistance to picolinate herbicides (Walsh et al., 2006). Regarding cellulose biosynthesis, mutations in the cellulose synthesis catalytic subunit A (CESA) have endowed resistance to isoxaben (Desprez et al., 2002). Target-site mutations associated with quinclorac resistance, specifically with the ability to detoxify cyanide via β-cyanoalanine synthase (β-CAS), have been identified in *Echinochloa crus-galli (Gao et al., 2017)*. Quinclorac resistance in *E. crus-galli* has also been attributed to the suppression of ACS and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (Gao et al., 2018). Thus, analyzing the auxin receptors, *TIR1* and *AFB1-5*, the *Aux/IAA* gene family, *ACS*, *β-CAS*, and *CESA* may reveal

mutations that could be associated with quinclorac resistance. Despite the idea that a target-site mutation is the likely cause of resistance to quinclorac, non-target site resistance (NTSR), has also been commonly associated with resistance to synthetic auxin, and quinclorac-resistance in *D. ischaemum* in particular. Reduced translocation and increased metabolism of synthetic auxins mediated by cytochrome P450 (CP450s) has been commonly associated with resistance to 2,4-D and dicamba (Gaines et al., 2020; Jugulam & Shyam, 2019). Resistance to quinclorac has been attributed mainly to mechanisms of increased detoxification of cyanide via ß-CAS and herbicide metabolism in *E. crus-galli* and *D. ischaemum* (Gao et al., 2017; Putri et al., 2024). The suppression of ethylene biosynthesis via the ACS pathway has also been suggested as a potential mechanism of resistance to quinclorac in *E. crus-galli (Gao et al., 2018).*

There are numerous hypotheses on what the root cause of resistance to quinclorac. The purpose of this study was to identify potential target-site mutations in a quinclorac-resistant population of *D. ischaeumum*, and to determine if there are any inherently differentially expressed genes that could elucidate potential resistance mechanisms.

Materials & Methods: *Population Collection and Identification.* Previous research identified both a smooth crabgrass population resistant to quinclorac (AL_R1) and a population susceptible to quinclorac (AL_S1) in the Auburn area. These populations were subjected to a dose-response study to determine the resistance status to quinclorac, and their respective resistance statuses were confirmed.

Each population was vegetatively cloned in flat trays with Miracle-Gro Potting mix in the greenhouse until the three-leaf stage, then tillers were transplanted into four-inch square pots to maturity for 4 weeks. Populations were irrigated daily and fertilized once a week.

RNA Extraction and Next Generation Sequencing. RNA extraction was performed for both AL_R1 and AL_S1 populations; 6 replicates were performed for each population. Approximately 150mg of young leaf tissue was collected from single plants grown in greenhouse conditions and flash frozen with liquid nitrogen before being ground with mortar and pestle. Powdered tissue was then transferred to 5mL centrifuge tubes and 1000 µL of TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added then vortexed for 5 minutes. Tubes were transferred to ice for 5 minutes then centrifuged for 5 minutes. Supernatant was then transferred to a new 1.5mL tube and centrifuged again to ensure no debris remained. An equivalent amount of 100% ethanol was added to the supernatant, vortexed and then transferred to a spin column tube. The RNA extraction then proceeded with the reagents and protocol from the Zymo Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA). RNA quality and quantity was measured using a NanoDrop™ One spectrophotometer. All RNA samples were then sent to Novogene (Beijing, China) for RNA sequencing. Samples were evaluated for RNA quality using the Agilent 2100 bioanalyzer and then sent for library preparation. Libraries were quality checked again and then pooled into a single tube and ran on Illumina NovaSeq 6000 to produce 150bp paired-end reads. Data was released after a read quality check for a percentage of reads containing N >10% (N represents the base that cannot be determined) and low-quality reads (Q score ≤ 5)

Transcriptome Assembly and Identification of Potential Mutations. Each replicate (AL_R1_1-6 and AL_S1_1-6) were assembled into individual transcriptomes using TRINITY v2.15.0 *(Grabherr et al., 2011)* and functionally annotated using TRINOTATE v3.2.0 (https://github.com/Trinotate). Reads were automatically trimmed during TRINITY run using the -Trimmomatic option. HMMER v3.3.2, BLASTP v2.13.0, and BLASTX v2.13.0 were used to

load the Sqlite database for annotation. Specific genes (*TIR1, AFBs, ACS, Aux/IAA, ß-CAS, CESA*) related to resistance in synthetic auxins were then extracted from each replicate using a custom trinotateExtractor python script and analyzed for potential target site mutations using CLC Genomics Workbench 21.0 (Qiagen, Hilden, Germany).

Differential Gene Expression. Each replicate was processed for differential gene expression analysis. Transcripts were first processed for quality control with FASTP v20 (Chen et al., 2018); https://github.com/OpenGene/fastp) using the default parameters to remove adapters, Poly A sequences, low-quality reads ($Q < 30$), and reads < 15bp in length after trimming. Filtered reads were then aligned to the *D. ischaemum* genome (International Weed Genomics Consortium, preliminary data currently in confidentiality period) using HISAT2 v2.2.1 (Pertea et al., 2016); http://daehwankimlab.github.io/hisat2/). A gene count matrix was produced using STRINGTIE v2.1.6 (Pertea et al., 2016); https://github.com/gpertea/stringtie), which was then analyzed using the *DESeq2* package (Love et al., 2014); https://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html) in RStudio. The genes with an absolute value of log2 fold change ≥ 2 (upregulated genes) or ≤ -2 (downregulated genes) and a false discovery rate (FDR) < 0.01 were considered significant differentially expressed genes (DEGs).

Results & Discussion: AL_R1 and AL_S1 were both previously subjected to a dose-response study to determine respective resistance statuses. Both AL_R1 and AL_S1 were collected in Auburn, AL, in different areas with no continued use of quinclorac; AL_R1 was identified after a failure to control the present *D. ischaemum* population with quinclorac, and AL_S1 was collected in an area with no known herbicide applications. AL_R1 was determined to be resistant, however a true resistance level could not be calculated as the population never reached

100% injury at the highest rate of 16X the standard application rate (841g ai ha^{-1}), compared to AL_S1 which was deemed susceptible as it reached 100% injury at the standard application rate.

Transcriptome Assembly and Identification of Potential Mutations. Following RNA extraction and Illumina sequencing, each sample produced at least 47M raw pair-reads (7G raw data/sample), as shown in Table 1. Each replicate (AL_R1_1-6 and AL_S1_1-6) was assembled into individual transcriptomes for identification of potential target-site mutations. AL_S1 was used as the basis for the *D. ischaemum* genome used for subsequent RNASeq analysis.

Despite the likelihood that this is a target-site mutation causing resistance to quinclorac, no target-site mutations have yet been identified in association with quinclorac resistance in *D. ischaemum*. For this study *TIR1, AFBs, Aux/IAAs, ACS, ß-CAS, and CESA* gene sequences from AL_R1 and AL_S1 were compared to first identify the mutation sites and motifs. Alignments of TIR1 and AFBs revealed no known mutations (as seen with picolinate herbicides at G147, G441, and W574 loci, and W134, W220, C451, and R609 loci, respectively (Walsh et al., 2006). With regard to the Aux/IAAs, no mutations were found within the degron region or the deletion of the degron tail previously seen in relation to 2,4-D and fluroxypyr resistance (de Figueiredo et al., 2022; LeClere et al., 2018). For ß-cas, unlike the *E. crus-galli* populations presented by Gao et al. (2018), the AL_R1 and AL_S1 replicates both presented with the same K295 mutation that only appeared in the resistant *E. crus-galli* population, as seen in Figure 1. The R1064 mutation associated with isoxaben resistance was also not present in the AL_R1 and AL_S1 *CESA* gene replicates, as seen in Figure 2 (Desprez et al., 2002).

Beyond these initial comparisons, no firm claims could be made for potential novel target-site mutations. Since synthetic auxin herbicides generally do not affect monocots, there are few other monocot peptide or nucleotide sequences available to compare the *D. ischaemum* sequences

against. The unknown parentage of the *D. ischaemum* progenitor species also adds another blockade in distinguishing potential target-site mutations from simple subgenome differences. Because no target-site mutations were identified, the next step is to identify potential mechanisms for NTSR.

Differential Gene Expression. Unlike typical gene expression studies, this experiment aimed to understand if there was an inherent difference in gene expression between the resistant and susceptible populations. Giacomini et al. (2018) presented research that indicated resistant transcripts were constitutively differentially expressed between herbicide resistant and susceptible populations, whether the populations were treated or untreated, and that NTSR genes can be overexpressed in both resistant and susceptible populations, regardless of treatment, and are still detectable in untreated populations . Differential gene expression studies take into account all up and downregulated genes, and this can be variable for treated resistant and susceptible populations: susceptible populations generally experience overexpression of stress-related genes that may have no role in the actual mechanism of resistance to the treatment (Gardin et al., 2015; Giacomini et al., 2018). Thus, all replicates were left untreated for this experiment. The tetraploid status of *D. ischaemum* also creates issues when performing a standard RNASeq study, as a allopolyploid, there may be homoeolog expression dominance, but no progenitor species are yet known to compare expression levels (Grover et al., 2012; Hu et al., 2021). Principal component analysis (PCA) indicates a clear difference in basic gene expression between the susceptible and resistant replicates (Figure 3). The PCA distinctly grouped the resistant and susceptible replicates with only a 5% difference between replicates, and a 65% difference in inherent gene expression between the AL_R1 and AL_S1. There were 4340 DEGs between AL_R1 and AL_S1, 2343 of those being downregulated and 1997 being upregulated

(Figure 4). The total number of genes that were differentially expressed among the genes of interest in this study, along with the total number of transcripts extracted from the individual transcriptomes are presented in Table 2. The comparison of genes was condensed to the 794 most differentially expressed (based on log-fold change and FDR values) and then specifically within those 749 genes those relevant to NTSR were selected, revealing 26 DEGs (Figure 5). More genes were found to be upregulated in the resistant replicates (415) compared to being upregulated in the susceptible replicates (379). The most downregulated and upregulated genes overall were also selected for comparison based on their log-fold changes and FDR values (Table 3a and 3b). None of the 26 DEGs selected for their expression level in regard to NTSR were present in the top 40 most differentially expressed genes. Interestingly, there were genes within the ABC, CP450, and GST that were both up and downregulated in the 26 selected NTSR genes, as well as genes related to auxin transport and stress (heat shock proteins). The differential expression of heat shock proteins is intriguing because these samples were not treated with quinclorac. The upregulation of a stress-response gene family in the absence of the herbicide stressor could be an avenue for future research. However, there were no unique occurrences of specific gene among these gene families that would indicate the mechanism of resistance to quinclorac is associated with NTSR without an additional metabolism study.

Another gene present among the most differentially expressed genes that is of any interest to this study would be 1-aminocyclopropane-1-carboxylate oxidase (ACO). ACO is directly related to ethylene biosynthesis by catalyzing 1-aminocyclopropane-1-carboxylate (ACC) to produce ethylene, and its byproducts cyanide and carbon dioxide, (Figure 6) (S. F. Yang & Hoffman, 1984). There were 7 differentially expressed ACO transcripts: 6 transcripts were upregulated, all associated with ACO homolog 1 or homolog 1-like, and a single transcript downregulated,

associated with ACO homolog 4-like. Other studies have indicated how the expression of ACS and ACO plays a role in quinclorac resistance, where reduced expression of these genes was present in resistant biotypes of *E. crus-galli* (Gao et al., 2018). RNASeq studies also showed that untreated resistant controls showed a much lower basal expression of ACO1 compared to the untreated susceptible control (X. Yang et al., 2021). Regarding the full ethylene biosynthesis pathway, no other genes besides ACO along the ethylene biosynthesis pathway, which includes L-methionine, S-adenosylmethionine (SAM) synthetase, SAM, ACS, and ACC, presented with a differential response in expression. However, the expression of ACO1 is a response to the application of quinclorac, not a potential target for quinclorac binding. So, the differential expression of ACO1 is a good indication that the population is indeed resistant, however it does not provide more information into how the gene in particular was expressed.

Research Implications: There were difficulties in determining specific up and downregulated genes in this study, as there were 183 differentially expressed genes in this study that were solely identified as "unknown proteins," 13 of which were among the top 100 most differentially expressed genes. The lack of species available for comparison is still a glaring issue for molecular research into weed science; the unknown proteins present in this study could play a role in quinclorac resistance; however this is a moot point until the function of these unknown proteins can be identified.

The lack of known target-site mutations brings into question the target-site itself: if not TIR1, AFBs, Aux/IAAs, then where is quinclorac binding? Recent studies indicate that TIR1 or AFBs may not be a target-site at all for quinclorac, as quinclorac showed a low binding affinity for any TIR1 or AFB receptors in *Arabidopsis* (Prusinska et al., 2023). Going on the assumption that quinclorac does indeed bind to TIR1 or an AFB, what in the ethylene stimulus pathway in grass

species is triggering the accumulation of HCN rather than the typical epinastic response seen in broadleaf species?

While transcriptome analysis alone might elucidate a known mutation, it can be difficult to parse through every transcript. It could be useful for future research to perform a qPCR analysis to look more specifically at the genes of interest. Additional metabolism studies would also be useful to see if detoxification of cyanide or herbicide metabolism is playing a role in resistance; the resistance status of a species typically tolerant to quinclorac to begs the question if detoxification and metabolism is a byproduct of quinclorac not binding to its target gene (whatever it may be) rather than the root cause of resistance. Given the large amount of differentially expressed genes between the resistant and susceptible replicates, we know for certain there is a significant difference between AL_R1 and AL_S1, which may likely be attributed to a target-site mutation, the issue just lies with determining where the mutation is occurring.

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Tables:

TABLE 5-1. Sequence statistics for AL_R1 and AL_S1 replicates

Sample	Raw reads	Raw data (G)	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
AL_R1_1	52325532	7.8	99.18	0.03	97.94	94.05	56.14
AL_R1_2	48915848	7.3	99.26	0.03	97.95	94.09	56.19
AL_R1_3	48945130	7.3	99.14	0.03	97.93	94.1	56.37
AL_R1_4	47202118	7.1	98.99	0.03	97.89	94.03	56.4
AL_R1_5	55763884	8.4	99.15	0.03	97.92	94.05	56.18
AL_R1_6	58429644	8.8	99.06	0.03	97.65	93.61	55.91
AL_S1_1	69128594	10.4	98.94	0.03	97.92	94.02	57.3
AL_S1_2	57145038	8.6	99.15	0.03	97.95	94.12	57.01
AL_S1_3	56962284	8.5	98.37	0.02	98.03	94.4	57.17
AL_S1_4	59220922	8.9	98.74	0.03	97.58	93.42	57.01
AL_S1_5	63960724	9.6	99.03	0.02	98.08	94.46	56.87
AL_S1_6	54113002	8.1	99.18	0.02	98.09	94.42	56.85

TABLE 5-2. Table of genes of interest, with the total transcripts extracted from each replicate and the number of differentially expressed genes. The differentially expressed genes for IAA also included various auxin-responsive genes

	# of transcripts	Downregulated	Upregulated	Total DEGS
unknown proteins	NA	506	390	896
P450	5310	52	32	54
ABC	5645	4	26	30
GST	2080	11	15	26
IAA/auxin-related	1977	10	3	13
TIR1	171	0	0	0
AFBs	10864	0	0	0
ACS	90	0	0	0
ACO	572	1	6	7
ß-CAS	48	0	0	0
Cellulose synthase	45	5	0	5
Heat shock protein	1282	5	22	27

TABLE5-3A. Top 20 downregulated genes in AL_R1 replicates with log-fold changes and FDR values against AL_S1 replicates

	Gene_Location	Fold	FDR	Gene
Down	DIGISCHR04DG324780	-24.673871	1.92E-15	probable L-type lectin-domain containing receptor kinase S.5 [Panicum virgatum]:(InterPro|IPR011009|Protein kinase-like domain superfamily)
	DIGISCHR06CG443720	-16.828533	1.12E-88	uncharacterized protein LOC101767640 [Setaria italica]:(InterPro|IPR011990|Tetratricopeptide-like helical domain superfamily)
	DIGISCHR09DG632490	-14.512518	6.82E-66	uncharacterized protein LOC101777413 [Setaria italica]:(InterPro|IPR007658|Protein of unknown function DUF594);
	DIGISCHR04DG333350	-13.996357	4.94E-61	uncharacterized protein LOC120695147 [Panicum virgatum]:(InterPro|IPR036397|Ribonuclease H superfamily)
	DIGISCHR03DG236030	-13.922665	1.70E-60	zinc finger BED domain-containing protein RICESLEEPER 2 [Setaria italica]:(InterPro|IPR036236|Zinc finger C2H2 superfamily)
	DIGISCHR02DG153910	-13.874825	5.02E-60	187-kDa microtubule-associated protein AIR9 [Panicum hallii]:(PANTHER|PTHR31149|EXPRESSED PROTEIN);
	DIGISCHR03DG262600	-13.512458	3.45E-57	probable GTP diphosphokinase CRSH1%2C chloroplastic [Panicum hallii]:(InterPro|IPR043519|Nucleotidyltransferase superfamily)
	DIGISCHR05DG431830	-13.489422	8.48E-57	quinone oxidoreductase PIG3-like [Setaria italica]:(InterPro|IPR014189|Quinone oxidoreductase PIG3)
	DIGISCHR02CG109570	-13.346769	1.05E-55	peroxidase 2 [Oryza sativa Japonica Group]:(InterPro|IPR000823|Plant peroxidase)
	DIGISCHR01DG103910	-13.268622	4.00E-55	uncharacterized protein LOC101756762 [Setaria italica]:(InterPro|IPR006502|Protein of unknown function PDDEXK-like);
	DIGISCHR04CG279190	-13.099129	1.23E-53	60S ribosomal protein L17 [Panicum virgatum]:(InterPro|IPR005721|Ribosomal protein L22/L17%2C eukaryotic/archaeal)

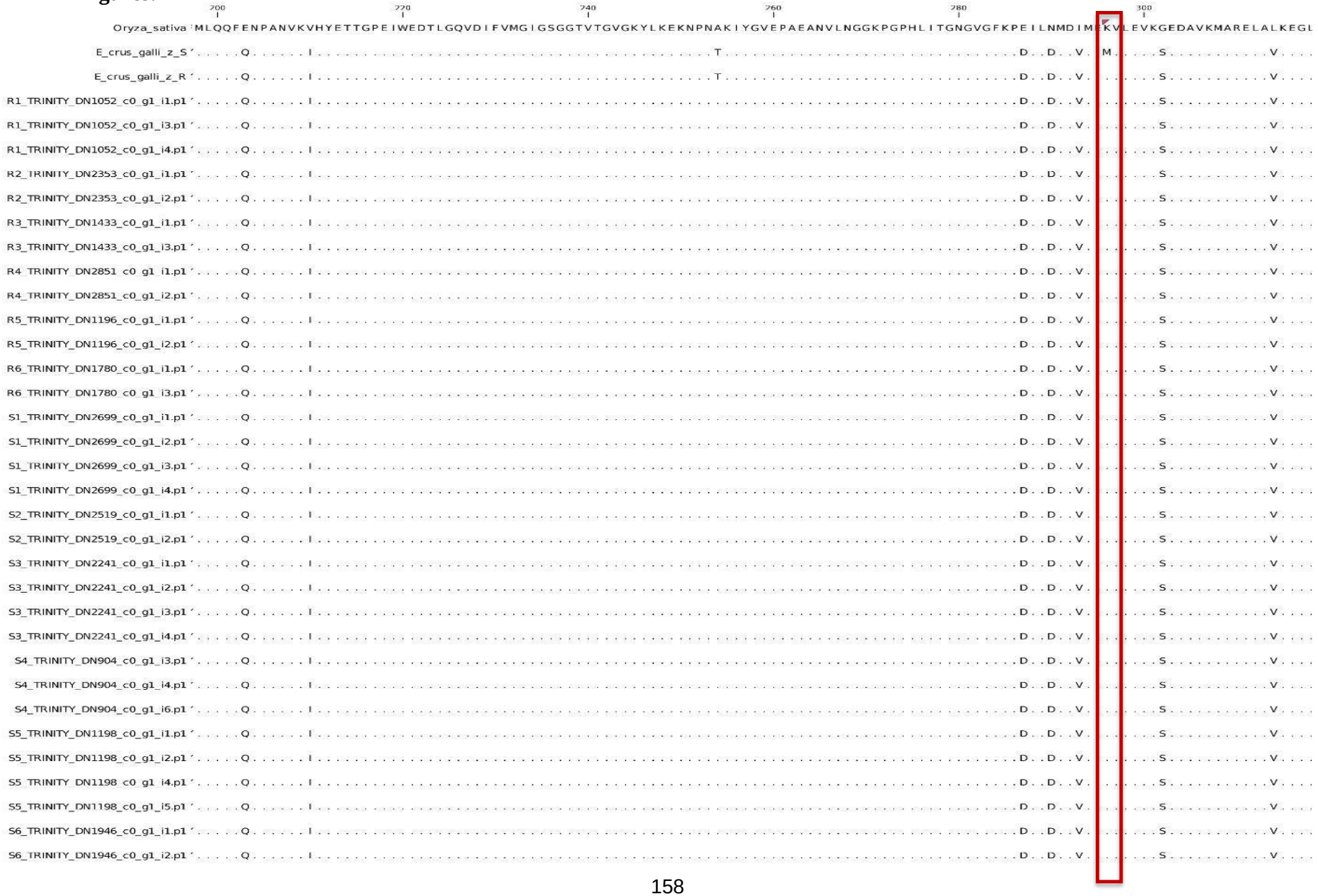
DIGISCHR08DG576920	-12.728486	1.70E-50	1-aminocyclopropane-1-carboxylate oxidase homolog 4-like [Panicum virgatum]:(InterPro|IPR027443|Isopenicillin N synthase-like)
DIGISCHR09CG606960	-12.561296	3.29E-49	receptor kinase-like protein Xa21 [Setaria italica]:(InterPro|IPR032675|Leucine-rich repeat domain superfamily)
DIGISCHR02CG127940	-12.527779	8.74E-49	uncharacterized protein LOC101778712 [Setaria italica]:(MobiDBLite|mobidb-lite|consensus disorder prediction);
DIGISCHR08DG580220	-12.489592	1.14E-48	phosphatidylinositol 4-kinase gamma 4-like [Panicum virgatum]:(InterPro|IPR029071|Ubiquitin-like domain superfamily)
DIGISCHR00G632970	-12.486152	1.04E-48	phosphatidylinositol 4-kinase gamma 4-like [Panicum virgatum]:(InterPro|IPR029071|Ubiquitin-like domain superfamily)
DIGISCHR09DG621730	-12.351299	2.16E-47	mannose/glucose-specific lectin isoform X3 [Setaria italica]:(InterPro|IPR036404|Jacalin-like lectin domain superfamily)
DIGISCHR05CG366770	-12.212615	2.57E-46	40S ribosomal protein S14-2 [Panicum virgatum]:(InterPro|IPR001971|Ribosomal protein S11)
DIGISCHR01DG079460	-12.176055	5.11E-46	uncharacterized protein LOC101757026 [Setaria italica]:(InterPro|IPR003615|HNH nuclease)

TABLE 5-3B. Top 20 upregulated genes in AL_R1 replicates with log-fold changes and FDR values against AL_S1 replicates

	Gene_Location	Fold	FDR	Gene
Up	DIGISCHR06DG475470	23.1602809	1.01E-13	ATP-dependent RNA helicase SUV3L%2C mitochondrial-like [Panicum virgatum]:(InterPro|IPR027417|P-loop containing nucleoside triphosphate hydrolase);
	DIGISCHR02CG123310	22.311697	8.44E-13	probable 26S proteasome non-ATPase regulatory subunit 3 [Panicum virgatum]:(PANTHER|PTHR10758|26S PROTEASOME NON-ATPASE REGULATORY SUBUNIT 3/COP9 SIGNALOSOME COMPLEX SUBUNIT 3);
	DIGISCHR05CG384960	15.4403581	1.31E-74	receptor kinase-like protein Xa21 [Triticum dicoccoides]:(InterPro|IPR032675|Leucine-rich repeat domain superfamily)
	DIGISCHR03CG197180	14.811905	1.13E-68	LOW QUALITY PROTEIN: agglutinin-like [Panicum virgatum]:(InterPro|IPR004265|Dirigent protein)
	DIGISCHR05CG384930	14.7567761	2.69E-68	probable LRR receptor-like serine/threonine-protein kinase At3g47570 [Triticum dicoccoides]:(InterPro|IPR011009|Protein kinase-like domain superfamily)
	DIGISCHR04CG317540	14.0689895	7.03E-62	uncharacterized protein LOC112893400 isoform X1 [Panicum hallii]:(InterPro|IPR023933|Glycoside hydrolase%2C family 2%2C beta-galactosidase)
	DIGISCHR09CG610020	13.6932555	1.06E-58	0
	DIGISCHR05CG385000	13.64835	2.33E-58	probable leucine-rich repeat receptor-like protein kinase At1g35710 [Panicum virgatum]:(InterPro|IPR032675|Leucine-rich repeat domain superfamily);
	DIGISCHR01CG023960	13.3792643	5.45E-56	uncharacterized protein LOC101753379 [Setaria italica]:(InterPro|IPR006502|Protein of unknown function PDDEXK-like);
	DIGISCHR09CG610010	13.1574568	4.16E-54	disease resistance protein Pik-2-like [Panicum virgatum]:(InterPro|IPR027417|P-loop containing nucleoside triphosphate hydrolase)

DIGISCHR04DG341530	12.0152897	9.21E-45	GDSL esterase/lipase At1g28600 [Zea mays]:(InterPro|IPR036514|SGNH hydrolase superfamily)
DIGISCHR05CG375940	11.9661373	1.98E-44	F-box/FBD/LRR-repeat protein At1g13570-like [Setaria italica]:(InterPro|IPR006566|FBD domain);
DIGISCHR02DG173400	11.9129273	4.55E-44	uncharacterized protein LOC120661032 [Panicum virgatum]:(InterPro|IPR034904|Fe-S cluster assembly domain superfamily);
DIGISCHR07CG499420	11.8351792	2.79E-43	extensin-like [Setaria italica]:(MobiDBLite|mobidb-lite|consensus disorder prediction);
DIGISCHR03DG242980	11.8230356	3.34E-43	L-gulonolactone oxidase 2 [Brachypodium distachyon]:(InterPro|IPR036318|FAD-binding%2C type PCMH-like superfamily)
DIGISCHR02CG130270	11.7894965	3.09E-43	0
DIGISCHR03CG199880	11.7667381	7.10E-43	uncharacterized protein LOC101761729 [Setaria italica]:(InterPro|IPR039306|Myosin-binding protein)
DIGISCHR09DG629280	11.7203069	1.19E-42	uncharacterized protein LOC120686840 [Panicum virgatum]:(PANTHER|PTHR35166:SF6|OS05G0193700 PROTEIN);
DIGISCHR09CG597500	11.6416033	6.93E-42	disease resistance protein RGA2-like isoform X3 [Panicum virgatum]:(InterPro|IPR027417|P-loop containing nucleoside triphosphate hydrolase)
DIGISCHR09CG612580	11.6394664	6.85E-42	dextranase isoform X3 [Panicum virgatum]:(InterPro|IPR039218|REM family);

Figures: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220
240
260
280
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E_crus_galli_z_S
E_crus_galli_z_R
R1_TRINITY_DN1052_c0_g1_i1.p1
R1_TRINITY_DN1052_c0_g1_i3.p1
R1_TRINITY_DN1052_c0_g1_i4.p1
R2_TRINITY_DN2353_c0_g1_i1.p1
R2_TRINITY_DN2353_c0_g1_i2.p1
R3_TRINITY_DN1433_c0_g1_i1.p1
R3_TRINITY_DN1433_c0_g1_i3.p1
R4_TRINITY_DN2851_c0_g1_i1.p1
R4_TRINITY_DN2851_c0_g1_i2.p1
R5_TRINITY_DN1196_c0_g1_i1.p1
R5_TRINITY_DN1196_c0_g1_i2.p1
R6_TRINITY_DN1780_c0_g1_i1.p1
R6_TRINITY_DN1780_c0_g1_i3.p1
S1_TRINITY_DN2699_c0_g1_i1.p1
S1_TRINITY_DN2699_c0_g1_i2.p1
S1_TRINITY_DN2699_c0_g1_i3.p1
S1_TRINITY_DN2699_c0_g1_i4.p1
S2_TRINITY_DN2519_c0_g1_i1.p1
S2_TRINITY_DN2519_c0_g1_i2.p1
S3_TRINITY_DN2241_c0_g1_i1.p1
S3_TRINITY_DN2241_c0_g1_i2.p1
S3_TRINITY_DN2241_c0_g1_i3.p1
S3_TRINITY_DN2241_c0_g1_i4.p1
S4_TRINITY_DN904_c0_g1_i3.p1
S4_TRINITY_DN904_c0_g1_i4.p1
S4_TRINITY_DN904_c0_g1_i6.p1
S5_TRINITY_DN1198_c0_g1_i1.p1
S5_TRINITY_DN1198_c0_g1_i2.p1
S5_TRINITY_DN1198_c0_g1_i4.p1
S5_TRINITY_DN1198_c0_g1_i5.p1
S6_TRINITY_DN1946_c0_g1_i1.p1
S6_TRINITY_DN1946_c0_g1_i2.p1

FIGURE 5-1. Alignment of AL_R1_1-6 and AL_S1_1-6 ß-CAS amino acid sequences against *Oryza sativa* (AAV48542.1), susceptible *Echinochloa crus-galli* (ATY36228.1), and resistant *Echinochloa crus-galli* (ATY36229.1). Red box indicates the mutation site, which shows no Met295Lys mutation between AL_R1 and AL_S1 replicates

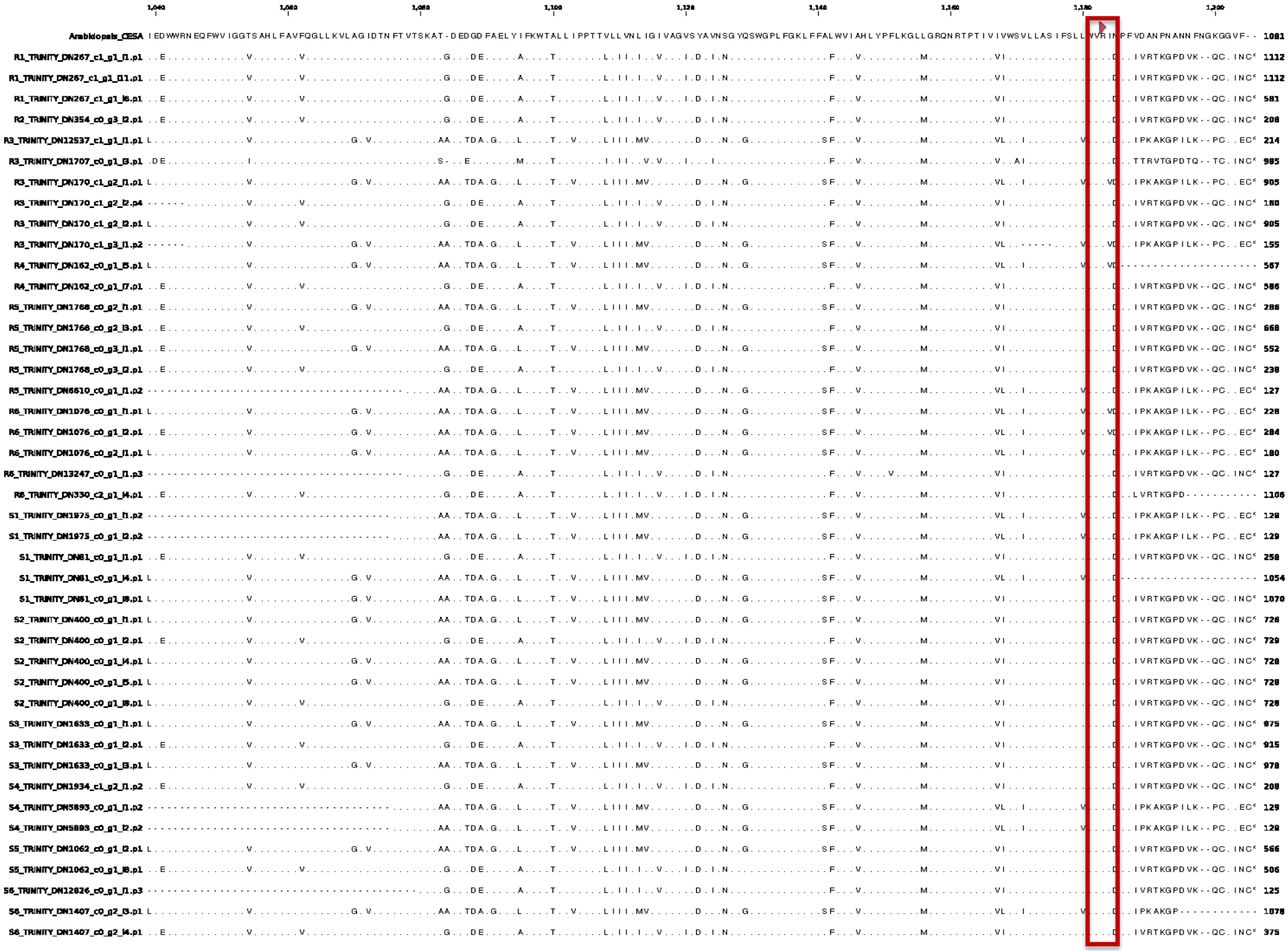

FIGURE 5-2. Alignment of AL_R1_1-6 and AL_S1_1-6 CESA amino acid sequences against *Arabidopsis thaliana* (NP_194967.1)**.** Red box indicates the mutation site, which shows no Arg1064Trp mutation in AL_R1 or AL_S1 replicates

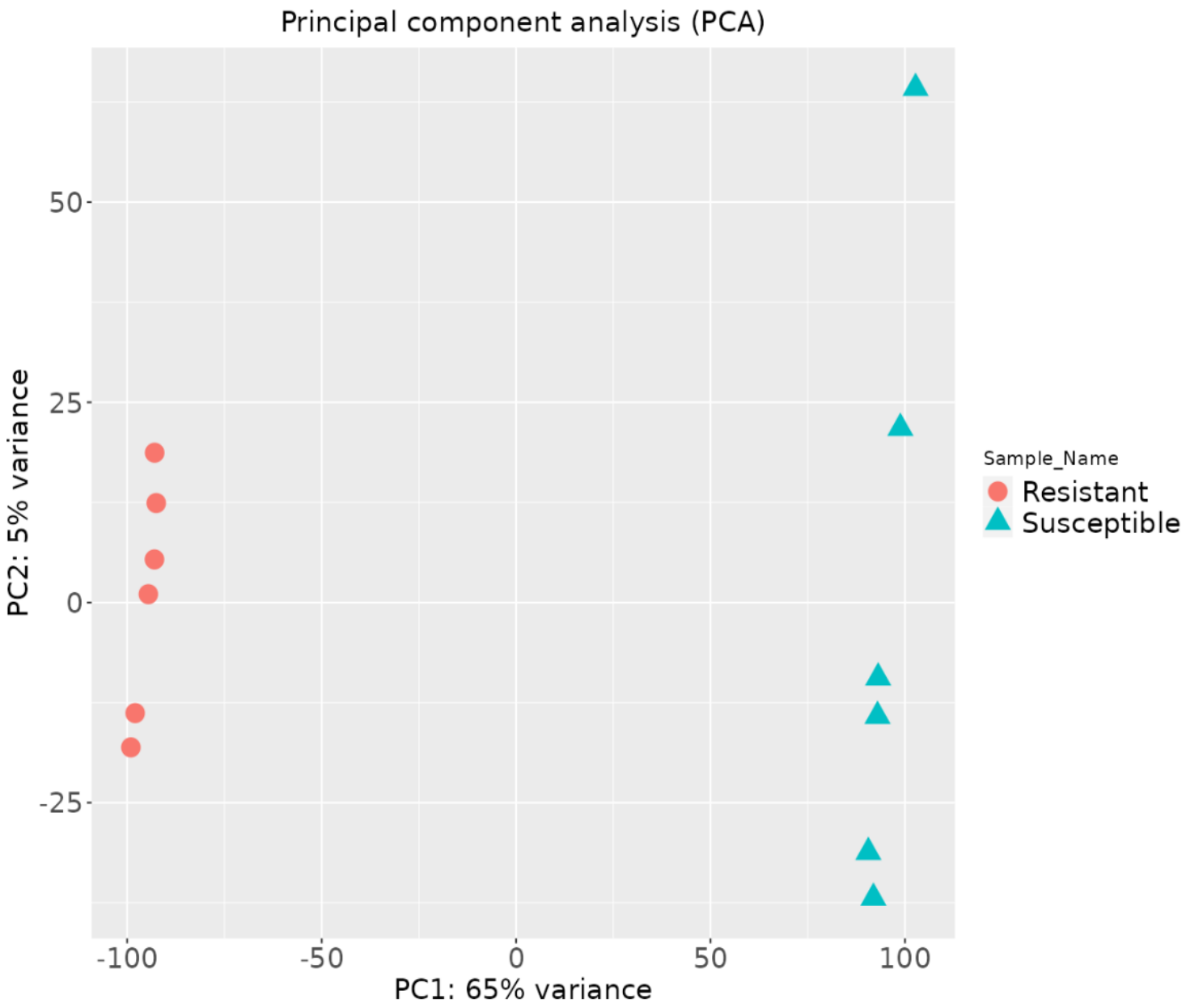

FIGURE 5-3. Principal component analysis for untreated AL_R1 and AL_S1

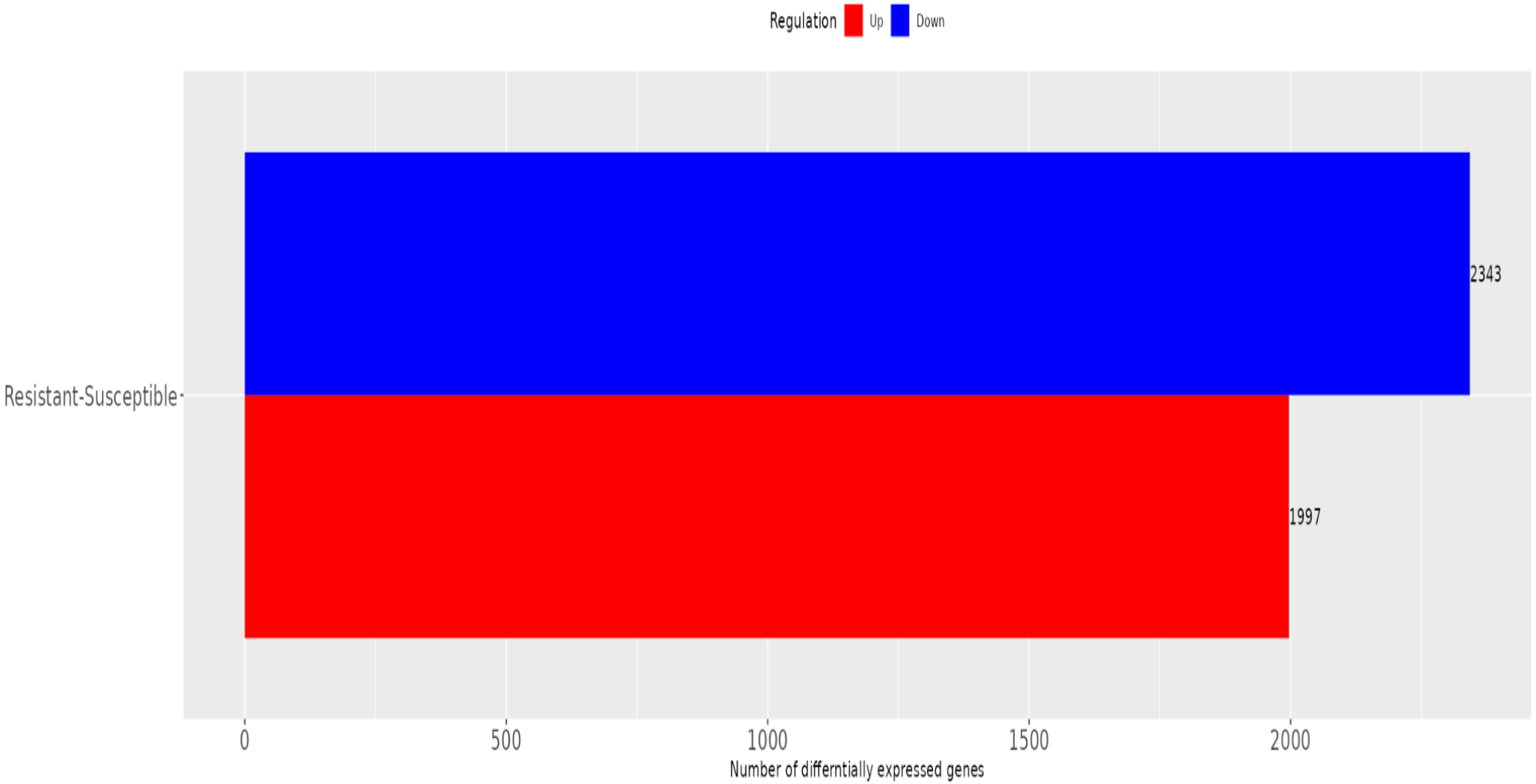

FIGURE 5-4. Comparison of the number of upregulated and downregulated genes present across all replicates of AL_R1 and AL_S1.

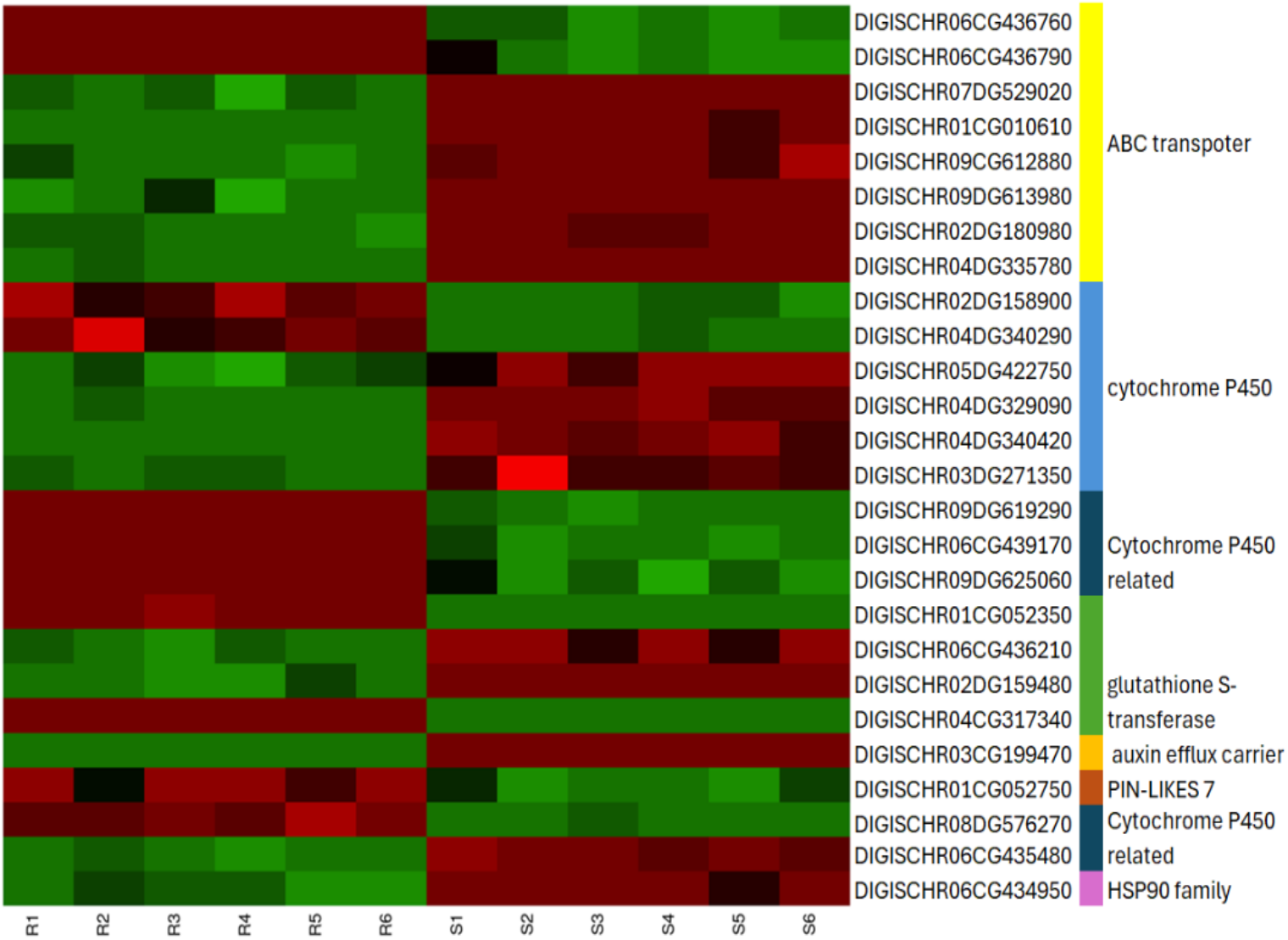

FIGURE 5-5. Heat map of common NTSR related genes from within the top 100 most differentially expressed genes.

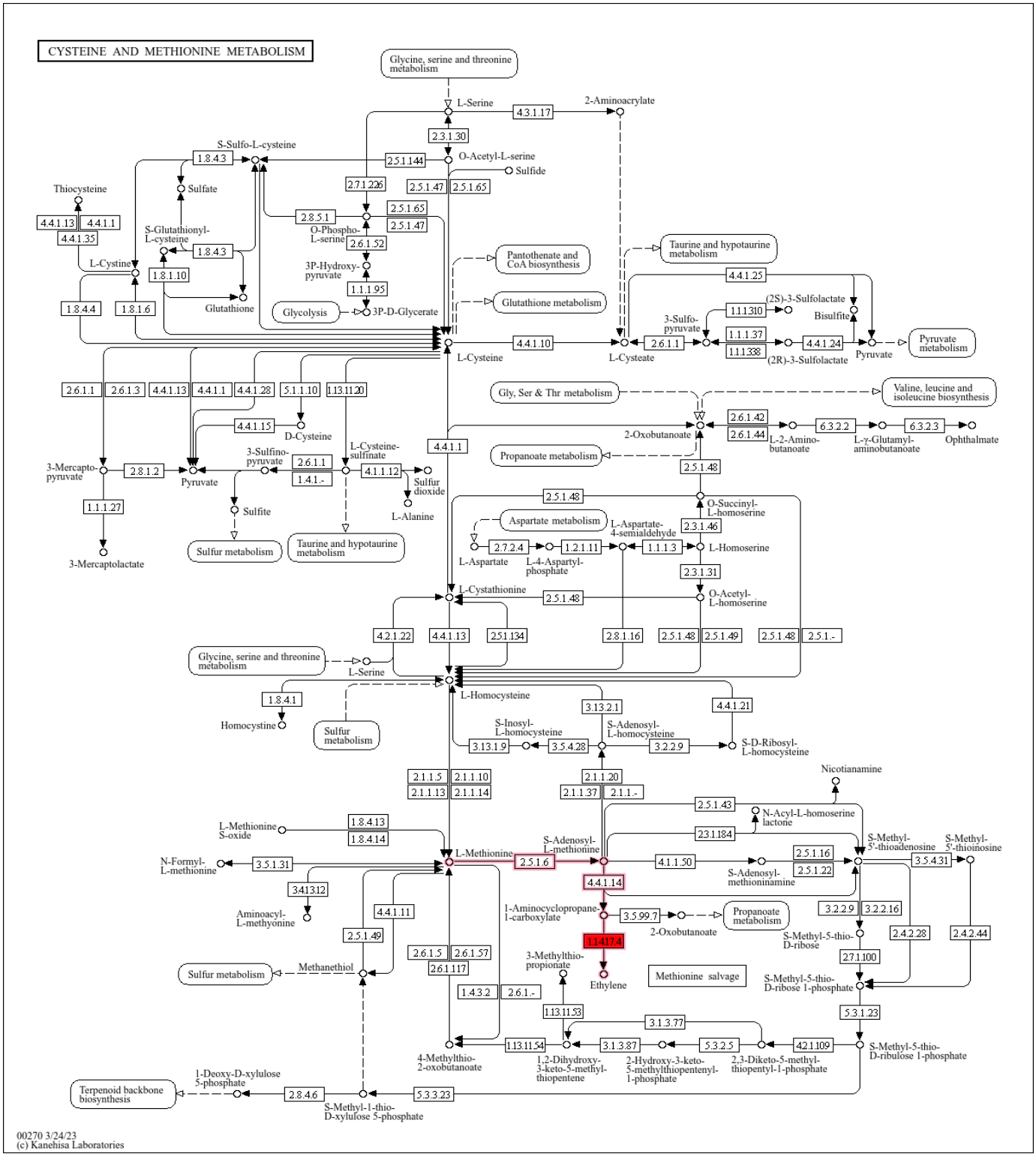

FIGURE 5-6. KEGG pathway for cysteine and methionine metabolism; highlighted pathway indicates ethylene biosynthesis pathway. The red marked cell indicates the downregulated 1-aminocyclopropane-1-carboxylate oxidase gene.