DEVELOPING 2,4-D RESISTANT WHITE CLOVER THROUGH MUTATION BREEDING AND RECURRENT SELECTION

by

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(Under the Direction of Ali Missaoui)

ABSTRACT

White clover (*Trifolium repens* L.) is a key pasture legume in temperate regions worldwide. The high protein content of white clover herbage makes it an ideal component of pastures to complement grasses. In addition, its ability to fix atmospheric nitrogen improves soil fertility. The breeding efforts for white clover have been focused on improving yield, persistence, disease resistance, and abiotic stress tolerance. There isn't much research on improving herbicide resistance in white clover. Therefore, the choice of herbicides as chemical weed control in white clover is very limited. Selective post-emergence herbicides are needed as emerging clover seedlings development is slow and not competitive with faster-growing weeds. In this study, we developed 2,4-D tolerant white clover through mutation breeding and recurrent phenotypic selection. We also performed transcriptomic analysis to find candidate genes responsible for 2,4-D tolerance in white clover. A local white clover ecotype "Durana" was used as recipient of EMS mutation. The concentration of 0.4% EMS resulted in a 72.3% germination rate, indicating it was the optimal dose. An effective dose (ED_{90}) of 2241.7 g ae ha^{-1} 2,4-D was determined and used to screen mutagenized seedlings in the greenhouse. Surviving plants were

crossed back with “Durana” plants to create a base population for recurrent selection. After 6 cycles, the survival rate following a 2,4-D rate (4483.4 g ae ha^{-1}) that was two times the ED_{90} reached 80%. Field and greenhouse experiments comparing resistance levels of the mutant population and three commercial cultivars (“Durana”, Renovation, and Regalgraze) showed greater resistance in the experimental population, with GR_{50} values of 1785.03 g ae ha^{-1} and 3240.61 g ae ha^{-1} in the greenhouse and field trials, respectively. A genetic gain of 9% per cycle was observed in cycles 6 and 9. This study demonstrates that EMS mutagenesis and recurrent selection are effective methods for inducing 2,4-D tolerance in white clover. Transcriptome analysis of 2,4-D tolerant (Experimental cycle 6) white clover and susceptible (“Durana”) revealed stress response, metabolism, and photosynthesis-related genes are responsible for 2,4-D resistance in white clover.

INDEX WORDS: EMS, white clover, recurrent phenotypic selection, mutation breeding, Transcriptome, DEGs.

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DEDICATION

I dedicate this work to my mother, Rokeya Begum, and my wife, Nushrat Jahan Niva, for whole-heartedly supporting and encouraging me throughout my life pursuits. I could never have done anything without you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

White clover (*Trifolium repens*) is a cool-season perennial legume that originates from the Mediterranean region. Also known as Dutch clover, white trefoil, creeping Trifolium, ladino clover, or honeysuckle clover, white clover is one of the most important crops out of the 250-300 species in the genus *Trifolium*. Its origin in the Mediterranean region suggests its adaptability to cool-season climates (Shaw, 1906). White clover has several important characteristics that contribute to its significance as a pasture legume. First is the high genetic variability, which contributes to its adaptability in different environments (Frame, 2003; Seker et al., 2003). This adaptability is crucial for the white clover's success as a forage legume in mixed species pastures in temperate regions. White clover's adaptability allows it to thrive in various soil conditions and climates, making it a valuable resource for farmers and livestock producers. White clover's ability to fix atmospheric nitrogen is another key characteristic that contributes to its importance (Zeven, 1991). This ability to fix nitrogen reduces reliance on costly synthetic nitrogen fertilizers, which can have negative environmental impacts. Furthermore, white clover has a prostrate growth habit and stoloniferous nature, allowing it to spread and form dense ground cover. This characteristic improves the soil structure by protecting the soil from erosion and promoting water infiltration, suppresses weed growth, and enhances overall pasture productivity (J. R. Caradus et al., 1995, 2023b). White clover also has a high nutritive value for grazing livestock. Its high nutritive value is attributed to its relatively high crude protein content, digestibility, and energy value. This makes white clover an excellent option for increasing the

quality of forage available to grazing animals, leading to improved animal performance and productivity. This makes white clover an excellent option for increasing the quality of forage available to grazing animals, leading to improved animal performance and productivity (Fothergill et al., 2006).

Establishing white clover in pasture is an important aspect of sustainable agricultural practices. However, clover establishment is often slow and weed competition can significantly impact the successful establishment and growth of white clover (Gibson & Hollowel, 1966). Weeds not only compete with white clover for nutrients, sunlight, and water but also reduce the overall productivity of the pasture. This can result in decreased germination and growth of white clover seedlings, ultimately impacting their establishment in the pasture. In addition, certain weeds may produce allelochemicals that negatively affect the biological nitrogen fixation of white clover (Scheffer-Basso et al., 2019). Furthermore, white clover's relatively slow growth rate makes it particularly vulnerable to competition from winter weeds such as wild radish (*Raphanus raphanistrum*), cutleaf evening primrose (*Oenothera laciniata*), and common vetch (*Vicia sativa*) (Basinger & Hill, 2021). These winter weeds have a higher growth rate than white clover, giving them an advantage in resource acquisition. Therefore, controlling these winter weeds is critical during white clover establishment to prevent them from dominating the pasture and hindering the growth of white clover. Successful weed control during white clover establishment requires careful consideration and implementation of various management practices. These practices may include herbicide application such as 2,4-D, dicamba, clopyralid, fluroxypyr, and quinclorac mowing, cultivation, and inter-seeding. There are several auxinic herbicides available to control broadleaf weeds in a grass pasture mixture such as WeedMaster (2,4-D + dicamba), Grazon P+D (picloram + 2,4-D), Milestone (aminopyralid), PastureGard

(triclopyr + fluroxypyr), and Surmount (picloram + fluroxypyr) (Hancock & Hermon, 2016). However, these herbicides also kill pasture legumes. Therefore, controlling broadleaf weeds in legume pasture mixture is a concern due to the limited herbicide choice. A white clover cultivar resistant to auxinic herbicides can be a sustainable solution for establishing clover-grass pasture at a low cost.

Auxinic herbicides are a widely used group of herbicides with a mode of action that targets the plant hormone auxin (McCauley et al., 2020a). These herbicides disrupt the normal functions of auxin in plants, leading to growth abnormalities and, ultimately, the death of the sensitive weeds (Magnus Eklund et al., 2010). Resistance to auxinic herbicides can arise through several mechanisms. One mechanism of resistance to auxinic herbicides is the alteration or mutation of genes responsible for the target site of the herbicide (Torra & Alcántara-de la Cruz, 2022). For example, in some cases, resistance to auxinic herbicides has been attributed to modifying genes encoding the first common enzyme in the plant auxin biosynthesis pathway known as AUX1. This modification prevents the herbicide from effectively binding to its target, rendering it ineffective in inhibiting auxin biosynthesis (Teale et al., 2006). Some common auxin mimic herbicides are 2,4-D, dicamba, clopyralid, triclopyr, fluroxypyr, aminopyralid, halauxifen-methyl, Florpyrauxifen-benzyland, and mecoprop. To develop white clover resistant to auxin mimic herbicides, we must understand these herbicides' inheritance and mechanism of resistance. Several studies have been conducted in this regard. For example, Preston et al., 2009 reported that a single allele with a high degree of dominance determined dicamba resistance in kochia. Similar results were also found in other studies. A single dominant gene in wild mustard conferred resistance to dicamba, 2,4-D, and picloram (Jasieniuk & Maxwell, 2005; Jugulam et al., 2005). On the other hand, clopyralid and picloram resistance in yellow starthistle (*Centaurea*

solstitialis) and quinclorac resistance in false cleavers (*Galium spurium*) are both regulated by a single recessive gene (Van Eerd et al., 2005a). Considering the simple inheritance of auxinic herbicide resistance, artificial mutagenesis might be helpful to induce resistance of these herbicides in crop species like white clover. Due to the possibility that a change in only one gene could impact the metabolism and the herbicide's ability to bind to the site of action, resistance to herbicides that target specific sites of action is more likely to develop through artificial mutation.

Mutation breeding is a technique in plant breeding involving induced mutations to generate genetic variation in plants (Raymond et al., 2015). These induced mutations can improve the plants' various traits, such as environmental adaptability, nutritional quality, yield, disease resistance, herbicide resistance, and aesthetic characteristics. Mutation breeding plays a crucial role in improving the genetic potential of plants and enhancing their overall growth and productivity (Nurcholis et al., 2023). The mutation breeding process involves exposing plants to external agents known as mutagens, which can induce changes in the DNA of the plants (Basu et al., 2008). This leads to the formation of new genetic variations that can be beneficial for plant breeders. Mutation breeding has been widely used in the agricultural and horticultural sectors to develop new cultivars with improved traits (Pinthus et al., 1972). Mutation breeding has been an integral part of developing herbicide-resistant crops. The first herbicide-tolerant plant was developed in the laboratory through mutation in tobacco plants (Chaleff & Ray, 1984). In recent years, research efforts to mutate seeds to create crops resistant to imidazolinones and sulfonylureas. Numerous crops, such as soybean (*Glycine max*), sunflower (*Helianthus annus*), wheat (*Triticum aestivum*), corn (Zea mays), lentil (*Lens culinaris*), and canola (*Brassica nepus*), have developed tolerance to or resistance to these herbicides through mutation breeding (Beckie et al., 2011; S. Tan et al., 2005). Most herbicide-tolerant mutants were created through chemical

mutagenesis. Among the chemical mutagens, ethyl methanesulfonate (EMS) is the most effective and widely used for inducing point mutation (Gillmor & Lukowitz, 2020; Muhammad Shah et al., 2014; Sabetta et al., 2011). For example, using EMS, sulfonylurea-resistant Arabidopsis thaliana mutants were created by Haughn & Somerville (1986). As opposed to other chemical and physical mutagens that generate mutations like the deletion of a significant portion of the genome, which results in considerable alterations and also disturbs the features of the variety, EMS causes point mutations or minor nucleotide changes within the genome (Weil & Monde, 2007). The general properties of the treated plant material are unaffected by EMS, and it is easily available and easy to use (Rizwan et al., 2015).

After inducing herbicide resistance through chemical mutation, the next step is to increase the favorable allele frequencies. By repeatedly selecting plants with desired phenotypes, breeders can gradually increase the frequency of favorable alleles in a population. This method is also known as recurrent phenotypic selection. Hull (1945) used the term "recurrent selection" for the first time in 1945. The genetic variability can be maintained through recurrent selection while increasing the favorable allele frequencies of quantitative traits (Singh et al., 2021a). In many species, recurrent selection has been used to improve quantitative traits (Hallauer, 1987; Hallauer et al., 2010). For example, 2,4-D resistant red clover (*Trifolium pretense*) was developed through six cycles of recurrent phenotypic selection (Munoz et al., 2015). Recurrent selection includes three phases, conducted in a repetitive manner: 1) development of selection units, 2) evaluation of the units, and 3) intermating the superior units or the parents of superior selection units to form an improved population for the next cycle of selection (Hallauer & Darrah, 1985; Hallauer & Eberhart, 1970).

In recent years, RNA sequencing has emerged as a powerful tool for crop improvement. This technique allows researchers to analyze the transcriptome of a crop, providing valuable information about gene expression levels and identifying differentially expressed genes under specific conditions such as stress or disease (Stark et al., 2019; Wang et al., 2009). By understanding the expression patterns of genes in crops, researchers can gain insights into the molecular mechanisms underlying important agronomic traits and develop strategies for improving crop yield and resistance to stresses. Furthermore, RNA sequencing can also enable the identification and quantification of RNA sequence variants and isoforms, providing a more comprehensive view of gene expression diversity within crop populations (Tyagi et al., 2022). RNA sequencing, also known as RNA-seq, has revolutionized the field of biology and has become an attractive alternative to other gene expression profiling techniques, such as RT-qPCR and gene expression microarrays (Valdés et al., 2022). This technology allows for the sequencing of the transcriptome, providing a more comprehensive understanding of gene expression in crops compared to other methods. In addition, RNA-seq technology has rapidly evolved in recent years, leading to improved quality and yield of sequencing platforms and reduced costs (Hong et al., 2020). Moreover, the application of RNA-seq technology in crop improvement has allowed researchers to discover new genes, molecular markers, single nucleotide polymorphisms, metabolic pathways, and alternative splicing events (Deshpande et al., 2023; Luan et al., 2017). RNA-seq is becoming very popular to investigate herbicide resistance mechanisms. RNA-seq can reveal Non-Target Site Resistant (NTSR) genes by providing whole-transcriptome comparisons between herbicide-resistant (HR) and herbicide-sensitive (HS) plants (Giacomini et al., 2018). RNA-seq studies had been conducted in short awn foxtail (*Alopecurus aequalis*), black grass (*Alopecurus myosuroides*), alligator weed (*Alternanthera philoxeroides*), flixweed

(*Descurainia Sophia*), barnyard grass (*Echinochloa crus-galli*), and goosegrass (*Eleusine indica*) to investigate different types of herbicide resistance (An et al., 2014; J. Chen et al., 2017a; Gardin et al., 2015; Q. Yang et al., 2016; X. Yang et al., 2013a).

Importance, morphology, and domestication of white clover

White clover is a perennial legume in the Fabaceae family. It is native to Europe and Asia but has been introduced to various parts of the world, including North America (Lattimore, 2021). It is one of the most important forage legumes in temperate areas of the world (Gibson & Hollowell, 1966). Since colonial times, white clover has been used as a forage in North America (Hancock & Hermon, 2016). White clover is grown in various regions across the USA in a wide range of soils and climates. It is grown for forage from Florida to the northeastern United states, but it thrives particularly well in the temperate climate of the Northeast, Midwest, and Pacific Northwest regions. It is among the most important forage legumes in mixed species grazed pastures due to its high nutritive value, nitrogen-fixing ability, and seasonal dry matter distribution (J. R. Caradus et al., 2023a). A meta-analysis by Dineen et al. (2018)showed that including white clover with perennial ryegrass sward can increase milk production, weight gain, and lower nitrogen application rates.

There are three morphological classes that white clovers often fall into small, intermediate, and large. Small white clovers are found in lawns or regions that are densely grazed and rarely grow taller than 0.025 meter (m). These clovers produce little and are not very productive for grazing animals. Compared to small or intermediate white clovers, large or ladino white clovers grow more uprightly, have larger leaves, and bloom later. Ladino white clovers are more productive than other white clover types when managed and fertilized properly. Ladino clovers, on the other hand, have fewer stolons and leaves that are close to the ground, are

unreliable reseeders, and have lower persistence under grazing. Intermediate white clover flowering period and leaf size falls between small and large-type white clovers. Intermediate types typically reseed more dependably than ladinos, possess many stolons and leaves at ground level, and produce more forage than small types. Because of these traits, intermediate types of white clover persist well in grazing situations (Hancock & Hermon, 2016).

White clover is an allotetraploid (2n = 4x = 32) developed through hybridization of two diploid *Trifolium* species (Ansari et al., 1999). A diploid alpine species, *T. pallesecens,* and a diploid coastal species, *T. occidentale,* are the probable ancestral progenitors to white clover. DNA sequence analyses and molecular cytogenetics suggest that the interspecific hybridization occurred during the last major glaciation 13,000-130,000 years ago (Badr et al., 2002; Ellison et al., 2006; Williams et al., 2008). Using embryo rescue, *T. pallescens* and *T. occidentale* were crossed to produce F1 hybrids. They produced a significant number of unreduced gametes and were inter-fertile with white clover. This suggests that the polyploidization event might have created white clover (Williams et al., 2012). Due to allopolyploidization, white clover has increased genetic diversity, which helped them to survive in stressful environments (Griffiths et al., 2019). White clover was domesticated at least 400 years ago in the Netherlands, and with European colonization, it spread worldwide (Kjærgaard, 2003). White clover's success in temperate, Mediterranean, and some subtropical regions has been made possible by its broad habitat tolerance (Davies & Young, 1967; Zeven, 1991). White clover shows disomic inheritance, which means during meiosis, the chromosome pairing is similar to that of nonhomologous pairs of chromosomes in diploids (Williams et al., 1998). It is mostly self-incompatible, with a small number of plants in a population that are self-compatible. Therefore,

a white clover population is a heterogeneous mixture of heterozygous individuals (Williams, 1987).

White clover breeding

White clover seed cultivation can be traced back at least to the 1500s in the Netherlands (Leffel & Gibson, 1973). Breeding of white clover has been focused on improving on-farm productivity, feed quality, persistence, or a combination of these. Moreover, breeders have also worked to improve resistance to pests and diseases, tolerance of drought, production in aluminum toxic soils, herbicide tolerance, and improved yield at low soil phosphorus levels (J. Caradus et al., 2021). The selection strategy used in white clover breeding is primarily determined by the fact that it is a cross-pollinating species. The fact that white clover is consistently grown alongside grass further complicates this evaluating issue.
Additionally, for several features, including yield and persistency, individual genotype performance (measured as spaced plants) does not predict field results in competitive sward circumstances (Marcia et al., 2017). The heterozygous allotetraploid DNA of white clover makes it challenging to choose good breeding lines for breeding operations. Except in a very small number of lines, it is hard for the species to self-pollinate enough generations to establish favorable features because of the gametophytic self-incompatibility mechanism. Due to worries about inbreeding depression, self-pollination is hardly ever used, even in genotypes that are self-fertile. To overcome these challenges, white clover breeders use recurrent selection over several generations to retain genetic variation for all other traits in the population while applying selection pressure on only one feature (Cope & Taylor, 1985). Creating new cultivars using recurrent selection takes longer than other breeding techniques because a large base population must be crossed and maintained to avoid inbreeding depression (Fehr, 1987).

History and uses of auxin herbicides

Natural auxins are important phytohormones consisting of indole-3-acetic-acid (IAA) and its related endogenous molecules, including 4-chloroindole-3-acetic acid, phenylacetic acid, and indole-3-butyric acid, all of which have similar responses in plants and play a crucial role in various aspects of plant growth and development. Synthetic auxin is a selective herbicide and is one of the most important herbicides to control weed growth in agriculture worldwide. As early as the 1940s, academic and commercial laboratories produced many synthetic auxins, including 1NAA, 2,4D, and MCPA (Song, 2014a). Moreover, the first true herbicide ever commercialized was 2,4-D (M. A. Peterson et al., 2016)

Due to their selective action and preferential suppression of dicot weeds in cereal crops, synthetic auxin herbicides heralded a new era in weed management in contemporary crop production (Grossmann, 2003). Auxinic herbicides have been widely used to control dicot weeds in domestic lawns, commercial golf courses, and crops. There are four major chemical groups in auxinic herbicide family, which are quinoline-carboxylates (quinmerac and quinclorac), pyrimidine-carboxylates (fluroxypyr, triclopyr, clopyralid, and picloram), benzoates (dicamba), and phenoxy-carboxylates (2,4-D, 2,4-DP, 2,4-DB, 2,4,5-T, MCPA, MCPB, and mecoprop). One of the first synthetic auxin herbicides is 2,4-D. It is widely and commonly used to control annual and perennial weeds (Peterson, 1967). It was developed during World War II as one of many so-called phenoxy herbicides to increase crop yields for a nation at war (Quastel, 1950). It was commercially released in 1946 and became the first successful selective herbicide. It was allowed for weed control in wheat, maize, rice, and other similar cereal crops because it specifically targets dicots. This herbicide family is credited for starting an agricultural revolution and establishing the foundation for modern weed science when it was first marketed in the

1940s. The low cost of 2,4-D has led to continued usage today, and it remains one of the most commonly used herbicides in the world (Peterson et al., 2016). It has also proven to be a useful chemical probe of auxin action because of the potent and stable xenobiotic compound that is not subject to the many endogenous homeostatic and metabolic mechanisms that can affect IAA (Ljung et al., 2002). Further herbicide research has led to the discovery and development of more commercial auxinic herbicides and considerably more experimental compounds that act via the auxin mode of action (Walsh et al., 2006a).

Different formulations of 2,4-D

2,4-D is a widely used post-emergence systemic herbicide to control broadleaf weeds in a variety of sectors and is found in many commonly used products. Currently, over fifty products containing 2,4-D are labeled in the United States, and a similar number of products contain 2,4-D in combination with other herbicides (Peterson et al., 2016).

In its pure form, 2,4-D acid is a dry crystalline solid and must be formulated to disperse and form a suitable mixture with water readily. To accomplish this, 2,4-D is produced in different forms, including acids, salts, amines, and esters. The two most common formulation types are amine and ester salts, which have gained widespread acceptance in the marketplace. Of these, the dimethylamine salt (DMA) and ethylhexyl ester (EHE) forms account for approximately 90-95% of the total global use (Charles et al., 2001). Amines and esters differ in many respects offering advantages and disadvantages for specific uses. Some general differences in amine and ester formulations include the following:

• Esters are absorbed more quickly than amines on broadleaf weeds and are more efficient under certain environmental conditions and for controlling certain plant species.

• Amine formulations of 2,4-D are essentially non-volatile and pose less potential for vapor movement following application.

• Esters are absorbed more quickly by plants and, therefore, are less likely to be washed away by rain.

Metabolic and physiological process of 2,4-D

Auxinic herbicides in plants induce similar responses to those induced by high exogenous doses of the natural auxin, IAA. At low doses, it promotes plant growth, while at high doses, it drives plant overgrowth, including cupping and stunting of leaves, brittleness, stunting and twisting of stems, and general abnormal growth (Grossmann, 2009a). 2,4-D mainly kills plants in three ways: altering the plasticity of the cell walls, influencing the amount of protein production, and increasing ethylene production (Song, 2014b). When 2,4-D is applied to plants, it is absorbed through roots, stems, and leaves and is translocated to the meristems of the plant (Munro et al., 1992). Roots become thickened and stunted, phloem and xylem tissue in the stem disintegrates or blocks, and leaf growth ceases. Uncontrolled, unsustainable growth ensues, causing stem curl-over, leaf withering, and eventual plant death. Even plant species resistant to 2,4-D may become injured if applied during rapid cell division (tillering or flowering) or rapid growth conditions (Song, 2014b).

In conclusion, the application of auxinic herbicides interferes with plant physiological processes in three stages: first, stimulation of abnormal growth and the initiation of gene expression with characteristics such as stem curling, tissue swelling, and the upregulation of 9-cis-epoxycarotenoid dioxygenase genes (NCED), which encode key regulatory enzymes in ethylene and abscisic acid biosynthesis and 1-aminocyclopropane-1-carboxylate synthase (ACS) genes which encode the rate-limiting enzyme of ethylene biosynthesis; second, inhibition of

abnormal growth and physiological responses such as stomatal closure and ROS production; and ending with senescence and cell death, including disruption of chloroplasts, and tissue necrosis (Grossmann 2009).

2,4-D at the molecular level

Natural auxins at optimum concentration promote plant growth, whereas auxinic herbicides, like 2,4-D, kill plants. 2,4-D is structurally and functionally analogous to the natural auxin IAA. That is, 2,4-D is not only structurally similar to IAA but is also biologically active as an auxin in plants. The main difference between natural auxin (IAA) and 2,4-D is that plants cannot metabolize this phenoxy herbicide as they can with IAA. Although 2,4-D has been used in agriculture for over three-quarters of a century, the molecular mode of 2,4-D action still needs to be thoroughly characterized. As 2,4-D acts and looks like auxin, it was thought that 2,4-D acts like auxin at the molecular level, too. Identifying important elements in the auxin-signaling pathway provided basic clues about the molecular mode action of 2,4-D (Song, 2014a).

Auxin-signaling pathway

Auxin is transported from cell to cell through auxin transporters, which reside in the plasma membrane (PM). There are three types of auxin transporters which are the amino acid permease-like AUXIN RESISTANTS/LIKE AUX (AUX/LAX) proteins, which mediate auxin influx (Swarup et al., 2008; Y. Yang et al., n.d.); the PIN-FORMED (PIN) efflux carriers, which mediate auxin efflux, and the MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (PGP) class of ATP binding cassette (ABC) auxin transporters (Luschnig et al., 1998; Petrášek et al., 2006). After entering the cell through auxin-influx carriers, natural auxin IAA rapidly controls auxin-responsive gene expression by regulating the degradation of Aux/IAA repressor proteins. Aux/IAA proteins are negative regulators of auxin-responsive genes (Mockaitis & Estelle, 2008).

At low concentrations of auxin, Aux/IAA binds to ARF, thus repressing the expression of auxin-inducible gene; at high auxin concentration, auxin serves as "molecular glue" that brings Aux/IAA protein to F-box protein TIR_1 and mediates the degradation of Aux/IAA proteins. Thereby, ARF is alleviated from Aux/IAA, allowing the homo-dimerization of ARFs, and binding to AuxREs, and the subsequent activation of auxin response genes (X. Tan et al., 2007). Research on Arabidopsis revealed that it can encode five TIR_1-like F-box proteins (AFB_1 to AFB_5), with AFB_1, AFB_2, and AFB_3 showing the closet homology to TIR_1 (Dharmasiri et al., 2006; Parry et al., 2009). The AFB_1, AFB_2, and AFB_3 proteins also act as auxin receptors and bind to IAA at different affinity (Dharmasiri et al., 2006; Greenham et al., 2015; Parry et al., 2009). Different AFBs bind to different types of auxinic herbicides. This selective affinity might be due to their auxinic binding pockets controlled by the aromatic ring size in auxinic herbicides (Calderon-Villalobos et al., 2010). For example, AFB_5 mutant is resistant to the auxinic herbicide picloram, suggesting SCF^{AFB5} may be the main receptor for picloram (Walsh et al., 2006b). TIR_1 is required for 2,4-D perception. TIR_1 contains a binding pocket that can bind 2,4-D (Calderon-Villalobos et al. 2010). Moreover, 2,4-D is also recognized by AUXIN BINDING PROTEIN1 (ABP_1), which acts as a PM auxin receptor, suggesting there are multiple pathways to sensor 2,4-D (Petrášek & Simon, 2010; Sauer & Kleine-Vehn, 2011). However, unlike IAA, 2,4-D is not a good substrate for the auxin-binding protein ABP_1 (Lobler & Klambt, 1985) and is poorly transported by auxin efflux carriers (Delbarre et al., 1996).

Interaction of 2,4-D with other hormones

Natural auxins are usually inactivated very quickly by conjugation and degradation (Ljung et al., 2002), while 2,4-D is retained for long periods of time and therefore works as a herbicide. Auxin and auxinic herbicides induce growth by cell elongation instead of cell division.

Hormone interplay is important in regulating plant growth and development (Avilés-Arnaut & Délano-Frier, 2012; Song & Xu, 2013). Several possible mechanisms are involved in 2,4-D controlled plant death mediated by multiple hormones such as ethylene and abscisic acid (ABA). First, one of the most well-known effects of excess auxinic herbicides on dicots is the overproduction of the plant hormone ethylene (Grossmann 2003, 2009). Unlike natural auxins, which are rapidly degraded by plants, 2,4-D lasts for a long time, resulting in the overproduction of ethylene, which may result in several herbicide-related responses, including epinasty and senescence (Iqbal et al., 2017). Ethylene synthesis is induced by various environmental stimuli and is involved in plant response to drought, wounds, defense against pathogens, and auxinic herbicides (Wang et al. 2002). Several enzymes work in ethylene biosynthesis, such as SAM synthase, ACC synthase (ACS), and ACC oxidase (ACO). Both ACS and ACO are encoded by multigene families in plants, and their expression patterns are regulated by several environmental stimuli, including 2,4-D (Lin et al. 2009).

Although the role of ethylene in the molecular mode of action of 2,4-D is still not fully understood, it is clearly a secondary response in plants exposed to 2,4-D. Another effect of excess ethylene production in response to 2,4-D is the stimulation of ABA production. The rate-limiting factor of ABA biosynthesis is the conversion of 9-cis-neoxanthin to cis-xanthoxin by 9-cis-epoxycarotenoid dioxigenases (NCED), and the plastid enzyme NCED catalysis is encoded by a family of NCED genes (Nambara & Marion-Poll, 2005). Several experiments show that 2,4-D induces the expression of NCED genes, consequently increasing the production of ABA (Hansen & Grossmann, 2000). 2,4-D may generate ethylene to induce the production of ABA, and ABA directly mediates plant death via stomatal closure. In a recent study by McCauley et al. (2020b), the primary mechanism of plant death induced by synthetic auxin herbicides involves a

swift increase in the expression of 9-cis-epoxycarotenoid deoxygenase (NCED). This rapid up-regulation leads to the quick synthesis of abscisic acid (ABA) and a sustained accumulation of ABA, subsequently triggering a broad suppression of transcription related to photosynthesis. This sequence of events ultimately culminates in leaf senescence, marking the predominant pathway for plant mortality caused by synthetic auxin herbicides (Gaines, 2020).

Role of reactive oxygen species

One of the main effects caused by 2,4-D application is the production of reactive oxygen species (ROS). It is involved in 2,4-D-induced epinasty by promoting cell expansion and vascular tissue proliferation and signals molecules to induce cellular response against stress conditions (Mittler et al., 2004; Pazmiño et al., 2011). The increased production of ROS has direct consequences on the activation of specific enzymes such as xanthine oxidoreductase (XOD) involved in ureide metabolism, acyl-CoA oxidase (ACX) involved in fatty acid b-oxidation and jasmonic acid biosynthesis, and lipoxygenase (LOX) (Pazmino et al. 2011). Another resource of ROS is the NADPH oxidases from the plasma membrane. It appears that nitric oxide (NO) also plays an important role in 2,4-D response. Ortega-Galisteo et al. 2012 found that NO production is reduced, and S-nitrosylation of peroxisomal proteins increases in pea plants treated with 2,4-D. However, the precise molecular mechanism of reduced NO production in 2,4-D application is not fully understood.

Resistance to auxin herbicides

There are several reasons auxinic herbicide resistance is relatively rare. Auxin governs many complex signaling networks in plants. Any genetic mutation in auxin signaling pathways may decrease the survival capacity of plants in harsh environmental conditions (Jasieniuk & Maxwell, 2005; Mortensen et al., 2012). Auxins also affect the dynamics of cellular metabolism

at various levels of the organization, including specific processes in each cell, such as endocytosis, cell polarity, and cell cycle control. It also plays a role in regulatory processes at the level of a dynamic biological system, directing macroscopic processes such as embryogenesis, organizational and spatial patterning of tissues, and the formation of new organs (Woodward and Bartel, 2005). Due to these crucial and extensive roles of auxins, it is difficult for plants to develop auxinic herbicide resistance.

However, more than 43 cases of resistance to auxinic herbicides have been reported worldwide (Heap, 2023). The molecular basis of most of the current cases of auxin resistance mechanisms is still unknown. In dicamba-resistant *Kochia scoparia* (Preston et al., 2009), auxinic-herbicide-resistant *Sinapis arvensis* (Zheng & Hall, 2001), and 2,4- D resistant *Sisymbrium orientale* (Preston & Malone, 2015), resistance is conferred by single dominant alleles, indicating that resistance could theoretically evolve and become established more quickly than resistance due to complex genetic inheritance (Jasieniuk and Maxwell, 1994). However, recent findings indicate more complex genetic inheritance of 2,4-D resistance. Dicamba-resistant kochia (*Kochia scoparia*) populations, collected from fields in various states across the western United States, have been identified. In contrast to previous studies on prickly lettuce, preliminary research suggests the presence of multiple resistance mechanisms among these biotypes (Goss & Dyer, 2003; Harwood et al., 2001; Preston et al., 2009). Fitness cost analysis indicates a significant penalty associated with a single gene responsible for resistance in a Nebraska population, potentially contributing to the relatively limited dispersal of the resistance mechanism (Wu C, 2017). An examination of a Colorado population confirmed that reduced herbicide translocation was involved in the resistance mechanism. However, there was no discernible difference in the absorption, translocation, or metabolism of the herbicide in a

population from Kansas (Wu C, 2017). Subsequent research revealed that the resistance response in these populations is associated with increased production of chalcone synthase, an enzyme involved in the flavonol biosynthetic pathway (Pettinga et al., 2018). Ongoing research efforts are being conducted to further characterize and understand the intricacies of this resistance mechanism.

In 2012, the first report of a 2,4-D-resistant waterhemp population from Nebraska surfaced (Bernards et al., 2012). Similar resistant populations in other Midwestern states have since been identified, displaying resistance not only to 2,4-D but also exhibiting multiple resistances to ALS- and photosystem II-inhibiting herbicides (Crespo et al., 2017). The Nebraska biotype showed a remarkable 30-fold resistance to 2,4-D compared to the susceptible biotype, a resistance magnitude not previously observed in species resistant to auxin herbicides (Crespo et al., 2017) moderate cross-resistance within the auxin herbicide mode of action was noted, with decreased sensitivity to aminopyralid and picloram.

Preliminary data from a 2,4-D-resistant waterhemp population in Missouri suggested that the resistance mechanism is not attributed to differences in absorption or translocation. Instead, it may involve differential metabolism through cytochrome P450 activity (Shergill LS, 2017). Research on the genetic basis of 2,4-D resistance in a Nebraska population concluded that a single gene confers resistance, while in an Illinois population, it is conceivable that multiple genes contribute to 2,4-D resistance (Sabate S, 2017). It's important to note that not all instances of decreased sensitivity are confirmed resistance; there are also reports of increased tolerance in weed species to 2,4-D (Kruger et al., 2009; Spaunhorst & Johnson, 2017).

Termed "creeping resistance," the gradual and nearly imperceptible shift in herbicide sensitivity has been hypothesized to result from reduced application rates or the use of

ineffective herbicides. This phenomenon may accurately describe resistance to auxin herbicides, given their distinctive mode of action (Gressel J, 1995). Given the recently discovered concepts for auxin pathways, new perspectives can be considered for how weeds may evolve resistance to auxinic herbicides. There are now multiple known mechanisms through which auxinic herbicides may act.

Mutation breeding

Genetic diversity is continuously reduced as breeders increasingly focus on so-called "elite" cultivars. This genetic erosion eventually became a bottleneck, and various techniques to induce mutations and artificially increase variation emerged in the middle of the last century. The goal of mutagenesis breeding is to cause genomic variation with a minimum decrease in viability.

Through the years, mutagenesis has generated vast genetic variability and played a significant role in plant breeding programs worldwide. Records maintained by the joint FAO/IAEA Division in Vienna show that 2965 crop cultivars, with one or more useful traits obtained from induced mutations, were released worldwide during the last 40 years (*Welcome to the Joint FAO/IAEA Mutant Variety Database*, n.d.). Notable examples are several wheat varieties (e.g., durum wheat used in pasta), barley including malting barley, rice, cotton, sunflower, and grapefruit, resulting in an enormous positive economic impact.

Chemical mutagenesis

Chemical mutagens have gained popularity since they are easy to use, do not require any specialized equipment, and can provide a very high mutation frequency. Compared to radiological methods, chemical mutagens tend to cause single base-pair (bp) changes or single-nucleotide polymorphisms (SNPs), as they are more commonly referred to, rather than deletions

and translocations. Of the chemical mutagens, EMS (ethyl methane sulfonate) is today the most widely used. EMS selectively alkylates guanine bases, causing the DNA-polymerase to favor placing a thymine residue over a cytosine residue opposite to the O-6-ethyl guanine during DNA replication, which results in a random point mutation. Most of the changes (70–99%) in EMS mutated populations are GC to AT base pair transitions (Till et al., 2004, 2007).

Recurrent phenotypic selection

The term "recurrent selection" was first used by Hull in 1945. It is defined as the systematic application of repeated cycles of selection and recombination to improve a random-mating population. The objective of recurrent selection is to gradually increase the frequency of favorable alleles that influence quantitative trait(s) in the population while maintaining genetic variability so that further improvement of the trait(s) can be made. Summaries of a large number of experiments have indicated that recurrent selection has been effective in improving quantitative traits in many species (Sprague and Eberhart, 1977; Frey, 1983, 1984; Hallauer, 1986; Hallauer and Miranda, 1988; Hallauer et al., 1988). Recurrent selection includes three phases, conducted in a repetitive manner: 1) development of selection units, 2) evaluation of the units, and 3) intermating the superior units or the parents of superior selection units to form an improved population for the next cycle of selection (Hallauer, 1986, 1987; Hallauer and Miranda, 1988).

RNA-seq analysis

RNA-Seq uses recently developed deep-sequencing technologies. In general, a population of RNA (total or fractionated) is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end

sequencing) or both ends (pair-end sequencing). The reads are typically 30–400 bp, depending on the DNA-sequencing technology used. In principle, any high throughput sequencing technology can be used for RNA-Seq, and the Illumina IG, Applied Biosystems SOLiD, and Roche 454 Life Science systems have already been applied for this purpose. Following sequencing, the resulting reads are either aligned to a reference genome or reference transcripts or assembled *de novo* without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene (Wang et al., 2009).

Researchers have gradually adopted RNA-seq due to its cost drop. RNA-seq can reveal target site resistance and non-target site resistance (NTSR) genes by providing whole-transcriptome comparisons between herbicide-resistant (HR) and herbicide-sensitive (HS) plants (Wang et al., 2009). Following are some examples of research where RNA-seq was used to find herbicide-resistant and susceptible genes in various species. Kohlhase et al. (2019) conducted RNA-sequencing (RNA-seq) analyses on two waterhemp (*Amaranthus tuberculatus*) populations (HPPD-herbicide resistant and sensitive). They collected RNA samples from herbicide-treated and mock-treated leaf samples at three, six, twelve, and twenty-four hours after treatment (HAT). Their results indicated that the response of HPPD herbicide-resistant and susceptible water hemp genotypes to HPPD-inhibiting herbicide is rapidly established as early as 3 hours after herbicide treatment. They identified 89, 62, 61, and 1983 differentially expressed transcripts (DETs) in the resistant water hemp genotype at 3, 6, 12, and 24 HAT, respectively, and 500, 77, 61, and 565 DETs were identified in the susceptible water hemp genotype at 3, 6, 12, and 24 HAT, respectively. The authors identified 11 and 12 gene ontology (GO) terms significantly overrepresented in the resistant water hemp genotype at 3 and 24 HAT. No significant GO terms

were identified at 6 and 12 HAT. Combining all DETs from the resistant water hemp genotype,18 significantly overrepresented GO terms were identified. Combining all DETs from the susceptible water hemp genotype, they identified 39 significantly overrepresented GO terms. They conclude that the resistance observed in this population did not stem from mutation, amplification, or elevated expression of the herbicide target gene HPPD. Instead, biochemical analyses indicate that increased herbicide metabolism is attributed to the involvement of cytochrome P450s. Noteworthy variations in herbicide metabolism were not discernible until 48 hours post-treatment. This implies that the responses to herbicides are inducible, and genes situated upstream of the cytochrome P450s may directly or indirectly react to herbicide exposure.

The susceptible and resistant biotypes of barnyard grass (*Echinochola crus-galli*), with and without treatment with three herbicides (quinclorac, penoxsulam, bispyribac-sodium), were used to generate the first large-scale transcriptome sequencing data using the 454 GS-FLX platform by (X. Yang et al., 2013b). They obtained about 244,653 GO terms related to the biological process, cellular component, and molecular function categories. The authors also identified eight herbicide target-site gene groups and four nontarget-site gene groups were identified in the resistant biotype of *E. crus-galli*.

In another study (Duhoux et al., 2015) performed an RNA-seq analysis of ryegrass (*Lolium rigidum*) to decipher the transcriptomic response to an herbicide (pyroxsulam) inhibiting acetolactate-synthase and found that the transcripts were linked to non-target-site-based resistance. They used a ryegrass biotype from a field where ryegrass control using ALS inhibitors failed. Comparing the resistant and sensitive plants, the authors identified 30 candidate NTSR contigs. Further validation using 212 plants resistant or sensitive to pyroxsulam and/or to the ALS inhibitors iodosulfuron + mesosulfuron confirmed four contigs. The four candidate

contigs identified potentially encoded peptides with functions consistent with herbicide degradation: two cytochromes P450 (CYP72A and CYP81B1), one glycosyltransferase (GTA) and one glutathione-S-transferase (GSTA). However, their expression levels varied among plants: some resistant plants showed a low expression level for all four transcripts, and no resistant plant displayed a high expression level for all contigs.

Chen et al. (2017) performed transcriptome analysis to assess the potential mechanisms of glyphosate resistance in *E. indica*. De novo assembly of the transcriptomic data produced 48852 UniGenes with an average length of 847 bp. They annotated the UniGenes using seven databases. From digital gene expression analysis, they found sixteen candidate differentially expressed genes. These candidate genes were validated by quantitative real-time PCR (qRT-PCR). Among these UniGenes, the EPSPS and PFK genes were constitutively up-regulated in resistant (R) individuals and showed a higher copy number than that in susceptible (S) individuals. The expressions of four UniGenes relevant to photosynthesis were inhibited by glyphosate in S individuals, and this toxic response was confirmed by gas exchange analysis. Two UniGenes annotated as glutathione transferase (GST) were constitutively up-regulated in R individuals and induced by glyphosate in both R and S. In addition, the GST activities in R individuals were higher than in S. From the above discussion, it is safe to say that RNA-seq is a strong tool to investigate the mechanism of herbicide resistance in plants.

Considering the above discussions, the study presented here aims to:

1. Test and select the optimal mutagen dosage for EMS treatment.
2. Induce the tolerance of white clover to 2, 4-D herbicide.
3. Increase the resistance allele frequency through recurrent phenotypic selection.

4. Evaluate the resistance level of the mutant genotype to 2,4-D herbicide in field and greenhouse trials.

5. Perform RNAseq analysis to find the differentially expressed genes and eQTLs between the mutant and parent genotypes.

CHAPTER 2

IMPROVING 2,4-D TOLERANCE IN WHITE CLOVER THROUGH EMS MUTAGENESIS AND RECURRENT PHENOTYPIC SELECTION

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ABSTRACT

White clover is an important cool-season perennial forage legume commonly planted in grass mixtures. One of the obstacles to establishing white clover in grass mixtures or following a grass pasture is sensitivity to herbicides that control broadleaf weeds, such as commonly used auxin herbicides. The objective of this study was to increase 2,4-D herbicide tolerance in white clover through mutation breeding. Seeds of the cultivar "Durana" were incubated in different concentrations of EMS solutions (0.2% to 0.8%) for 15h and planted in Petri dishes for 1 to 2 weeks to test for germination. The EMS concentration of 0.4% resulted in a germination rate of 72.3% and was considered the optimal dose. The effective dose (ED_{90}) was determined to be 2241.7 g ae ha^{-1} 2,4-D and was used to screen mutagenized seedlings in the greenhouse. The surviving plants were crossed back to "Durana" plants to develop a base population for recurrent selection. After 6 cycles of recurrent selection, the survival rate following spraying with 2,4-D rate of 4483.4 g ae ha^{-1} which is twice the rate of ED_{90} reached 80%. A dose-response experiment was conducted in the field and greenhouse to compare the resistance level of the mutant population to three commercial cultivars ("Durana", Renovation, and Regalgraze). The whole plant dose-response trials confirmed the significantly higher resistance to 2,4D herbicide in the experimental population compared to the other three commercial cultivars with GR_{50} values of 1785.03 g ae ha^{-1} and 3240.61 g ae ha^{-1} in the greenhouse and field trial, respectively. A genetic gain (9%) per cycle was observed in cycle 6 and cycle 9, with an average realized genetic of 4.4% percycle. This study shows that EMS mutagenesis and recurrent selection are viable methods to induce 2,4-D tolerance in white clover.

Abbreviations: ANOVA, analysis of variance; CI, confidence interval; EMS, Ethyl methanesulfonate**;** RCBD, randomized complete block design; WAT, weeks after treatment.

INTRODUCTION

White clover (*Trifolium repens* L.) is a cool-season perennial legume of Mediterranean origin. Other common names of white clover are Dutch, white trefoil, creeping Trifolium, ladino, or honeysuckle clover. White clover is one of the most commonly planted of 250-300 species of the genus Trifolium. It is a natural tetraploid with a chromosome number of 2n=4x=32. Being an allotetraploid, its inheritance is disomic. Additionally, white clover is self-incompatible, with only a small proportion of plants in a population being quite self-compatible (Voisey et al., 1994) Usually, white clover is grown in a mixed sward with grasses. It is used for grazing, pasture hay, and ground cover in horticultural situations. It has a high impact on dairy, meat, and wool industries, as it significantly improves the productivity of pastures (Lane et al., 1997). Due to being rich in proteins and minerals, white clover is considered one of the most nutritious forage legumes. It also has high voluntary intake by grazing animals (Harris et al., 1998). Another highly desirable attribute of white clover in pasture systems is its ability to fix atmospheric nitrogen (Griffith et al., 2000). This attribute improves the nitrogen content of the soil, reducing the need for fertilizers. Moreover, it is well adapted to various soil and environmental conditions and combines well with many perennial grasses (Elgersma & Hassink, 1997).

The focus on "elite" cultivars has led to a reduction in genetic diversity during crop evolution. This has caused a bottleneck, which prompted the development of various techniques to induce mutations and increase variation (Rauf et al., 2010). Mutagenesis breeding aims to drive the maximum amount of genomic variation with the least decrease in viability. Over the years, mutagenesis has generated vast genetic variability and played a significant role in plant breeding programs worldwide (Sikora et al., 2011). According to records maintained by the joint

FAO/IAEA Division in Vienna, 2965 crop cultivars with one or more useful traits obtained from induced mutations were released worldwide during the last four decades. Examples of crops that have benefited from mutagenesis include maize (Till et al., 2004), rice (Suzuki et al., 1997), barley (Caldwell et al., 2004), and sorghum (Xin et al., 2008). Chemical mutagens have become popular due to their ease of use and high mutation frequency. EMS is currently the most widely used chemical mutagen. It selectively alkylates guanine bases, causing the DNA-polymerase to favor placing a thymine residue over a cytosine residue opposite to the O-6-ethyl guanine during DNA replication, resulting in a random point mutation. Most changes (70–99%) in EMS mutated populations are GC to AT base pair transitions (Till et al., 2004, 2007). Treatment of rice (*Oryza sativa* L.) suspension-cultured cell clusters with 0.4% EMS for 18-22 h produced as high as 29.4% independent mutant lines with visible phenotypic variations, including a new allelic mutant of elongated uppermost internode caused by two-point mutations in the first exon of the EUI gene (Y. L. Chen et al., 2013).

Several studies have shown that the inheritance of many of the auxin mimic herbicides is controlled by a single gene or, in fewer cases, by two major genes (Preston et al., 2009). Inheritance of wild mustard (*Sinapis arvensis L.*) resistance to dicamba, 2,4-D, and picloram is conferred by a single dominant gene (Jasieniuk & Maxwell, 2005; Jugulam et al., 2005). On the other hand, a single recessive gene controls clopyralid and picloram resistance in yellow starthistle (*Centaurea solstitialis* L.) (Sabba et al., 2003) and quinclorac resistance in false cleavers (*Galium spurium* L.) (Van Eerd et al., 2005b). Study of the inheritance of resistance to some auxinic herbicides, picloram (pyridine group), dicamba, and 2,4-D showed that herbicide resistance in the wild mustard biotype (*Brassica kaber*) is conferred by a single dominant gene and that resistance to all three auxinic herbicides is determined by closely linked genetic loci

based on analysis of backcross progenies (Jugulam et al. 2005). The simple inheritance may offer prospects for inducing herbicide tolerance in crop species through artificial mutagenesis. It is more probable to generate resistance to herbicides that interfere with single sites of action because a change in only one gene may be enough to affect the metabolism and the herbicide's binding potential to the site of action.

The term "recurrent selection" was first used by Hull in 1945 (Cress, 1966). It is defined as the systematic application of repeated cycles of selection and recombination to improve a random-mating population. The objective of recurrent selection is to gradually increase the frequency of favorable alleles that influence quantitative trait(s) in the population while maintaining genetic variability so that further improvement of the trait(s) can be made (Singh et al., 2021b). Summaries of a large number of experiments have indicated that recurrent selection has been effective in improving quantitative traits in many species (Hallauer, 1987b; Hallauer et al., 2010b). Recurrent selection includes three phases, conducted in a repetitive manner: 1) development of selection units, 2) evaluation of the units, and 3) intermating the superior units or the parents of superior selection units to form an improved population for the next cycle of selection (Hallauer & Darrah, 1985b; Hallauer & Eberhart, 1970b).

One of the obstacles to the establishment of white clover, especially in grass mixtures or following a grass pasture, is the application of herbicides that control broadleaf weeds. Among the herbicides frequently used in the Southeastern US pastures are WeedMaster (2,4-D + dicamba), Grazon P+D (picloram + 2,4-D), Milestone (aminopyralid), PastureGard (triclopyr + fluroxypyr), and Surmount (picloram + fluroxypyr). These herbicides mimic auxin activity in the plant and cause uncontrolled disorganized growth in susceptible plants. These herbicides control of a wide range of weeds but kill all pasture legumes. Developing non-GM white clover cultivars

with tolerance to these herbicides can be valuable for incorporating white clover into grass pasture systems while providing significant production cost savings to farmers.

MATERIALS AND METHOD

EMS treatment

Seeds of the cultivar "Durana" were scarified by immersion in 98% sulfuric acid for 8 minutes and washed with distilled water five times. Half of the scarified seeds were transferred to a media bottle (100 mL) containing EMS solution of different concentrations (0.2% ~ 0.8%, 0.1 mL~0.4 mL/50 mL) and shaken for 15h on a rotary shaker set at 30 r.p.m followed by a distilled water wash for 10 minutes. The remaining half of the scarified seeds were treated with only distilled water (50 ml), which acted as a control treatment, followed by shaking for 15h on the rotary shaker. The EMS-treated and control treatment seeds were then incubated separately in Petri dishes for 7 to 14 days for germination testing. The germination percentage was calculated, and the optimal dose of EMS treatment was selected based on the germination percentage.

Screening mutagen plantlets for herbicide resistance has been conducted. 6000 "Durana" seeds were subject to 0.4% EMS treatment. The control "Durana" seeds were treated with distilled water only. The EMS solution was removed and put into 1M NaOH overnight to remove any EMS solution from seeds. The seeds were then rinsed under running water for 5-10 minutes. Rinsed seeds were placed in wet germination paper that was imbibed with diluted solutions containing the appropriate dosage of the 2,4-D amine herbicides (200 μg a.i./L, 400 μg a.i./L, 500 μg a.i./L). After 7 days, 2,4-D amine herbicide was applied again into the Petri dishes at different concentrations (1.5 mg a.i./L, 2 mg a.i./L, 2.5 mg a.i./L, 5 mg a.i./L, and 8 mg a.i./L) with sprayers. Survival rates were estimated, and surviving plantlets from seeds germinating in

the herbicide solution were transferred to the growth medium for the second screening and field experiment. Plants surviving the individual herbicide tests were transferred into big square pots (8.2 cm X 8.2 cm) and maintained until flowering.

Recurrent phenotypic selection

The soil was prepared using a commercial potting mixture (Miracle-Gro® Moisture Control® Potting Mix, Miracle-Gro Lawn Products, Inc., Marysville, OH, USA). Greenhouse conditions were set up at 26/24±3 ∘C day/night temperatures and 15:9 h day/night photoperiods, and the supplemental photoperiod was provided with metal halide lamps (560 μmol m^{-2} s^{-1}). The surviving four plants and 25 "Durana" were transferred into the bee cage and crossed among themselves to generate the base population. The seeds of the base population were planted into 12 trays. Two weeks after planting, six trays were treated with 2,4-D amine at 2802.13 g ae ha^{-1} rate using a CO2-pressurized backpack sprayer with a 1-m-wide handheld boom equipped with two flat fan XR8002 nozzles (Teejet Spraying Systems, P.O. Box 7900, Wheaton, IL 60189) calibrated to deliver 140 L ha-1 at 276 kPa. The plants were clipped at 0.5-1.0 cm above soil level two weeks after herbicide treatment. Two weeks after clipping, the surviving plants were consolidated in empty flats, allowed to grow for 2-3 weeks, and then sprayed again with the same aforementioned herbicide and rate. In total, 60 trays were treated with 2,4-D amine herbicide. After two herbicide treatments and consolidation of the surviving plants, we ended up with 20 2,4-D treated trays, each tray having 128 plants. They were sprayed for the third time with the same rate of 2,4-D herbicide. The number of surviving plants was counted three weeks after spraying. Out of 450 surviving plants, 100 plants were selected, grown in pots until flowering, and then intermated in bee cages. The cycle one seed was harvested in bulk from all 100 plants.

The cycle 1 seeds harvested from the polycross of the surviving base population were planted in twelve 128-cell flats (later mentioned as "flats" only). Two flats of non-treated "Durana" seeds were also planted for comparison. The seedlings were thinned after germination, keeping only 1 plant per cell. The seedlings (10 flats of cycle one seeds, and one flat of non-treated "Durana") were sprayed with 4483.4 g ae ha^{-1} 2,4-D amine at the 3-5 trifoliate stage. Two flats of cycle 1 seedlings and 1 flat of non-treated "Durana" were treated with water and considered as control. At four weeks after herbicide treatment, 250 surviving plants were transplanted into square pots (8.2 cm X 8.2 cm) and maintained until flowering. The plants were crossed into the bee cage at flowering to produce cycle 2 seeds. Cycle 2 seeds were planted in the greenhouse following the same procedure described above and treated with 2,4-D amine herbicide. The surviving plants from cycle 2 selection under 4483.4 g ae ha^{-1} were advanced to selection for agronomic traits under field conditions. One thousand surviving plants were transplanted in the fall of 2018 in a field selection nursery at the University of Georgia Iron Horse Farm (IHF) in Greene County (33°43′37″N 83°18′03″W). About 300 selections were flagged based on vigor, number of stolons, and flower heads. The seeds were harvested from the selections in bulk at the end of June 2018. The cycle 3 seeds harvested from the field nursery were planted in 12 flats in the greenhouse. Ten flats were sprayed with 4483.4 g ae ha^{-1} 2,4-D amine at the 3-5 trifoliate stage, and two flats were used as control. Two hundred and fifty surviving plants at 4483.4 g ae ha^{-1} 2,4-D rate from cycle 3 plants were transferred to large pots to cross in the bee cage and produce cycle 4 seeds. Cycle 5 seeds were also produced using the same methodology as previously described. Cycle 5 seeds were planted in 22 flats in the greenhouse. Twenty flats were sprayed with 4483.4 g ae ha^{-1} 2,4-D amine at the 3-5 trifoliate stage, and two flats were used as control. From the surviving plants, 1300 2,4-D resistant white

clover plants were transplanted in the field nursery to produce large amounts of seeds which were used in field plot trials. At the same time, 250 surviving cycle 5 plants were transferred to large pots for crossing in the bee cage, producing cycle 6 seeds in the greenhouse.

Evaluation of resistance level

Dose-response testing in the field trial

A dose-response trial under field conditions was conducted at the University of Georgia J. Phil Campbell Sr. Research and Education Center ((33.887487◦ N, 83.420966◦ W), Watkinsville, Georgia, in the spring of 2022. The study area has a humid subtropical climate with an average minimum and maximum annual temperature of 11.1 ◦C and 25.6 ◦C, respectively. The mean annual rainfall for Watkinsville was 1230 mm, and the soil type was sandy loam. The field was prepared by disking twice, followed by cultipacking. The trial was set up in a randomized complete block design (RCBD) with four entries, five replications, and seven herbicide rates. Selected entries included the 2,4-D selection (experimental cycle 6) and three commercially available checks: "Durana", RegalGraze, and Renovation for comparison. The seed was planted in 3 rows at 3362 g ha^{-1} in a 1.5 m strip in mid-February. To evaluate the resistance level of the white clover entries, 2,4-D amine (Alligare®)(0.455 g ae ml^{-1}) was applied at 1120.85 (T1), 2241.7 (T2), 4483.4 (T3), 8966.81 (T4), 17933.62 (T5), and 35867.24 (T6) g ae ha^{-1} perpendicular to the planting direction. An untreated check was also included for comparison. Herbicide application was made at the 5-7 trifoliate growth stage. The plots were kept grass-free by hand weeding once a week throughout the duration of the experiment. Irrigation was applied on an as needed basis. Biomass was harvested at eight weeks after treatment (WAT) from the entire plot. The samples were dried at 55° C for 72 hours and weighed

to determine dry biomass. The dry biomass of treated plants was expressed as a percent reduction relative to that of the non-treated control for each entry using the equation:

$$Y = \left[\frac{DWc - DWt}{DWc}\right] X\ 100$$

Where *Y* is the percent dry biomass reduction, *DWc* is the dry biomass of the non-treated control, and *DWt* is the dry biomass of treated plants at 8 WAT.

Greenhouse trial

We conducted another dose-response trial in the greenhouse in the Fall of 2022 to investigate the response of the experimental white clover population in different environments. This experiment included four entries: the 2,4-D tolerant selection (Experimental cycle 6) and three commercially available varieties: "Durana", RegalGraze, and Renovation. All entries were planted into 128 cell trays and thinned to one plant per cell. The study design was a randomized complete block design with five replications. Each replication consisted of 64 plants of each entry and 2,4-D amine applied at 1120.85, 2241.7, 4483.4, 8966.81, 17933.62, and 35867.24 g ae ha^{-1} in a spray chamber (Research TrackSprayer, De Vries Manufacturing, Hollandale, MN, USA) located at the UGA Turfgrass Facility at 187 liters ha^{-1} using a 8002VS nozzle. Plants were allowed to dry and moved to the Riverbend Rd greenhouse (33.9316° N, 83.3619° W). Solid trays, without holes, were placed beneath each tray to catch water and ensure complete foliar and root absorption of the applied herbicide. On the eighth week after herbicide application, clover biomass was harvested, and samples from each replication were dried at 55° C for 72 hours and weighed to determine dry biomass.

Realized genetic gain

To estimate the realized genetic gain, two greenhouse trials were conducted. In the first trial, cycle 3 to cycle 7, seeds were planted in the greenhouse in 128 flat cells. After one week of

planting, the flats were thinned to 1 plant per cell. The study was set as RCBD with three replicates. The plants were sprayed with 2,4-D amine herbicide at 3-5 trifoliate stage at 4483.4 g ae ha^{-1} in a spray chamber located at the UGA Turfgrass Facility at 187 litters ha^{-1} using an 8002VS nozzle. One flat of each cycle was sprayed with only water as control. Plants were allowed to dry under the sun for 1 hr. before moving to the greenhouse. Plants were watered and fertilized regularly. After four weeks of herbicide treatment, the number of white clover survivors in each cycle was counted. Clover biomass was harvested, dried at 65° C for 72 hours, and weighed to determine dry biomass.

For the second realized gain trial, we redeveloped cycle 2 seeds by crossing the remaining seed from cycle 1 plants in the bee cage. Moreover, we continued our recurrent phenotypic selection to increase the favorable allele frequencies and developed cycle 8 and 9 seeds. Therefore, in the second trial, we used cycle 2 to cycle 9 seeds to estimate the realized genetic gain. The experiment followed the same procedure as the first trial described in this section.

Data analysis

The data was analyzed for homogeneity of variance (Bartlett) and normality (Lilliefors) tests. Data were then subjected to Analysis of Variance (ANOVA) and means separations were performed using the Tukey test at 5% probability with the JMP program (JMP®, Version 17. SAS Institute Inc., Cary, NC, 1989–2023). No data transformations were required since assumptions for ANOVA were met (data not shown). Herbicide dose-response data for biomass reduction were analyzed using a four-parameter log-logistic model (equation 2) to determine the dose of 2,4-D that causes 50% biomass reduction (GR_{50}).

$$y = c + \{d - \frac{c}{1} + \exp\left[b(\log x - \log e)\right]\}$$

Where *y* is the response variable (dry biomass as a percentage of non-treated), *d* is the upper limit, *c* is the lower limit, *b* is the slope of each curve, and *e* is the herbicide dose required to cause a 50% reduction in dry biomass referred as GR_{50}, and *x* is the herbicide dose. All nonlinear regression parameter estimates, their standard errors, and 95% confidence intervals (CI) for each population were estimated using the JMP software.

RESULTS

Optimum EMS concentration

Optimizing the mutagen dose before embarking on a mutation breeding program is essential. This is because the dose and exposure time to the mutagen are important in determining the frequency of mutations. Therefore, we conducted experiments to determine the optimum EMS concentration. The highest percentage of germination was obtained with 0.2% (v/v) EMS concentration, which is 87%. A higher concentration of EMS treatment resulted in a lower germination rate. However, 0.3% and 0.35% EMS treatment had 86% and 68% germination rates (Table 2.1). If the concentration of mutagen applied is too low, the survival rate of treated plants is higher with lower mutation frequency (Porch et al., 2009). Therefore, 0.4% was decided to be used as the optimal mutagen dosage for EMS treatment, with an average germination rate of 72% after one week, while the average germination rate for control-treated seeds is near 100% (Table 2.2). The average germination rate of 72% indicates that the embryos have been influenced or mutated by the EMS treatment but still have a relatively acceptable survival rate, making the EMS treatment applicable.

Dose-response

Results from the dose-response experiments in the greenhouse and field indicate that EMS mutation and recurrent selection have increased 2,4-D tolerance in white clover. In the greenhouse trial, treatments and genotypes had a significant effect ($p<0.05$) on survivor rate and biomass reduction. The experimental cycle 6 mutant population had significantly higher survivor (% of non-treated) rates of 97.6%, 69.4%, 60.6%, 36.8%, and 14.6% in treatment T2, T3, T4, T5, and T6, respectively (Figure 1A). We did not see significant treatment differences among the other three cultivars ("Durana", Renovation, and Regalgraze) for treatments T2, T3, T4, T5, and T6. In the case of T1, only Regalgraze had a significantly lower survivor rate of 95% compared to other cultivars. Even at the highest dose (35867.24 g ae ha^{-1}), the mutant population had around 15% survivors, whereas the commercial cultivars were mostly dead (Figure 2.1A). The data from biomass reduction also had a similar trend of survivor rate. The experimental cycle six population had significantly lower biomass reduction at the 2,4-D application of 4483.4 g ae ha^{-1} (T1) and all the higher doses applied (T4, T5, and T6). Even at lower 2,4-D doses, the experimental population had lower biomass reduction, but not significant compared to other cultivars (Figure 2.1B). From the dose-response analysis of the dry biomass reduction, we found that the GR_{50} for experimental was 1785.03 g ae ha^{-1}, the highest among all genotypes (Table 2.3, Figure 2.4).

In field conditions, the experimental cycle 6 mutation population was more tolerant to 2,4-D herbicide than greenhouse application. However, the field trial results followed a similar greenhouse trial trend. The experimental cycle 6 population had significantly lower biomass reduction in all treatments except T1. There wasn't any significant difference among the other three cultivars across all treatments (Figure 2.2). Estimates of the 2,4-D dose required to reduce

dry weight biomass by 50% (GR_{50}) were 3240.61 g ae ha^{-1} for the experimental cycle 6 population (Figure 2.4). The GR_{50} in the field trial is much higher than in the greenhouse trial. This might be due to the fact that in a greenhouse, plants do not develop thick cuticles (H. M. Hull et al., 1975). Therefore, plants likely uptake more herbicides compared to plants in the field. Moreover, we placed plastic trays underneath the flats, which hold water. The plastic trays also hold the herbicide, and plants might uptake more herbicide through their roots.

Realized Genetic Gain

The genetic gain trial was done two times in a greenhouse. In the first trial, cycle 3 to cycle 7 seeds were used. During the second trial, we included cycle 2, cycle 8, and cycle 9 seeds. The average realized genetic gain per cycle ranged from -5.5% to 9%. The highest gain was in cycle 6 and cycle 9, which is 9%. In cycle 3, the realized genetic gain was decreased by 5.5%.

DISCUSSION

The dose-response experiments in both greenhouse and field conditions have provided valuable insights into the effectiveness of EMS mutation and recurrent selection in increasing 2,4-D tolerance in white clover. In the greenhouse trial, the results indicated that treatments and genotypes significantly affected survivor rate and biomass reduction. The greenhouse trial's experimental cycle 6 mutant population showed significantly higher survivor rates than other cultivars, even at higher doses of 2,4-D herbicide. This suggests that EMS mutation and recurrent selection have successfully increased 2,4-D tolerance in the white clover population. The experimental cycle 6 mutant population consistently had higher survivor rates across different treatments of 2,4-D herbicide in the greenhouse trial. The results from the greenhouse trial are supported by previous studies that have used EMS mutation and recurrent selection to improve herbicide tolerance in crops. For example, a study by (Fernanda et al., 2017) reported

that white clover treated with 2,4-D herbicide at 600 g ai ha^{-1} resulted in a 40% reduction in dry matter compared to the non-treated plants. Furthermore, the dose-response analysis in the greenhouse trial revealed that the experimental cycle 6 mutant population had the highest GR_{50} value among all genotypes. In the field trial, the experimental cycle 6 mutant population also exhibited increased tolerance to 2,4-D herbicide compared to the greenhouse conditions. This suggests that the increased tolerance observed in the greenhouse trial is transferable to field conditions. The higher GR_{50} value in the field trial compared to the greenhouse trial indicates that the experimental cycle 6 mutant population showed even greater tolerance to 2,4-D herbicide in the field. The findings of this study support the potential use of EMS mutation and recurrent selection as effective strategies for increasing herbicide tolerance in white clover. These results align with previous studies that have used EMS mutation and recurrent selection to improve herbicide tolerance in other crops. For example, a study by (Taylor et al., 1989) conducted a selection program to increase levels of 2,4-D tolerance in red clover. They found that after four selection cycles, the levels of 2,4-D tolerance in red clover were significantly increased. Furthermore, studies on other weed species, such as wild mustard, prickly lettuce, oriental mustard, and wild radish, have also shown monogenic and polygenic tolerance to 2,4-D herbicide through either EMS mutation and/or recurrent selection (Dang, 2018; Goggin et al., 2016; Jugulam et al., 2005). The use of EMS mutation and recurrent selection is also effective in increasing herbicide tolerance in other plant species. For example, EMS mutant lines of *Arabidopsis thaliana* showed resistance to approximately 50-fold higher concentrations of 2,4-D than wild-type (Hayashi et al., 1998). Moreover, recurrent selection has been used to select for auxinic herbicide tolerance in red clover, wild radish, and Palmer amaranth (Ashworth et al., 2016; Taylor et al., 1989; Tehranchian et al., 2017). These studies collectively highlight the

effectiveness of EMS mutation and recurrent selection in enhancing herbicide tolerance in various crops and weed species. The findings of our study support the idea that recurrent use of 2,4-D herbicides on large plant populations acts as a strong selection pressure for the enrichment of herbicide-tolerant individuals.

One possible explanation for the decrease in genetic gain in cycle 3 could be related to the selection and intermating of 250 more vigorous cycle 2 plants from the field nursery. By specifically selecting cycle 2 plants for agronomic traits, the favorable allele frequency for herbicide tolerance was likely reduced in the cycle 3 population. This reduction in favorable allele frequency may have hindered the potential for genetic gain in cycle 3, leading to a decrease in realized genetic gain. These findings highlight the complex nature of genetic gain trials and the importance of carefully selecting parents in breeding programs to ensure that desired traits are effectively passed on to the next generation. The obtained results from the genetic gain trial indicate that there was variation in the success of genetic gain across different cycles. Factors such as heritability, selection intensity, and phenotypic variation can all influence the efficacy of genetic gain (Hernández-Leal et al., 2019).

CONCLUSION

In this study, we developed 2,4-D tolerant white clover through mutation breeding. Treating white clover seeds with EMS solution (0.4% v/v) for 15h created the necessary mutation to increase 2,4-D tolerance in white clover. We have also found that recurrent phenotypic selection increases the favorable allele frequencies in the white clover mutant population, which we confirmed through field and greenhouse trials using cycle 6 seeds. After 9 cycles of recurrent phenotypic selection, the survivor rate of EMS-treated white clover has

increased by over 80% at 4483.4 g ae ha^{-1} rate. The genetic gain in each cycle wasn't the same but showed an upward trend with each cycle. However, we have not investigated the mechanisms of 2,4-D resistance in our mutant population. Other studies are ongoing to understand the 2,4-D resistance mechanism in this white clover population to identify target genes that can be potentially modified in other crops to induce 2,4-D and possibly other auxin herbicide resistance.

Table 2.1. Germination rates of ""“Durana”" seeds treated with different concentrations (v/v) of EMS solutions.

	0.2% EMS	0.3% EMS	0.35% EMS	0.5% EMS	0.6% EMS	0.7% EMS	0.8% EMS
Germ No.	268	308	179	188	91	26	23
Non germ No.	41	50	84	662	194	304	599
Germ Rate	87%	86%	68%	28%	32%	8%	4%

Table 2.2. Germination rates of ""“Durana”" seeds treated with 0.4% (v/v) of EMS solution.

	0.4% EMS						Average
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	
Germ No.	226	258	156	231	132	171	72.30%
Non germ No.	76	120	64	77	35	88	
Germ Rate	74.80%	68%	71%	75%	79%	66%	

Table 2.3: Estimates of the 2,4-D dose resulting in a 50% reduction in dry biomass (GR_{50}) for "Durana", Experimental, Regalgraze, and Renovation in greenhouse trial and field trial.

Genotype	**Predicted rate (g ae ha^{-1}) (GR_{50})**	**Std Error**	**Lower 95%**	**Upper 95%**	R/S
	Greenhouse				
"Durana"	1339.51	109.22	1125.44	1553.58	1.2
Experimental	1785.04	134.76	1520.91	2049.17	1.7
RegalGraze	1038.79	68.14	905.25	1172.34	1
Renovation	1044.11	86.61	874.35	1213.87	1
	Field				
"Durana"	2100.79	167.33	1640.84	2296.78	1.1
Experimental	3240.61	360.84	2533.38	3947.84	1.6
RegalGraze	1968.81	198.31	1712.1	2489.48	1
Renovation	2027.1	229.13	1578.02	2476.18	1

Table 2.4: Mean survivor (% of non-treated) and realized genetic gain of recurrent phenotypic selection cycles in greenhouse trials.

Cycle	Survivor	Genetic gain
	%	
Cycle 2	53	
Cycle 3	47.5	-5.5
Cycle 4	53.5	6
Cycle 5	55.5	2
Cycle 6	64.5	9
Cycle 7	69	4.5
Cycle 8	75	6
Cycle 9	84	9

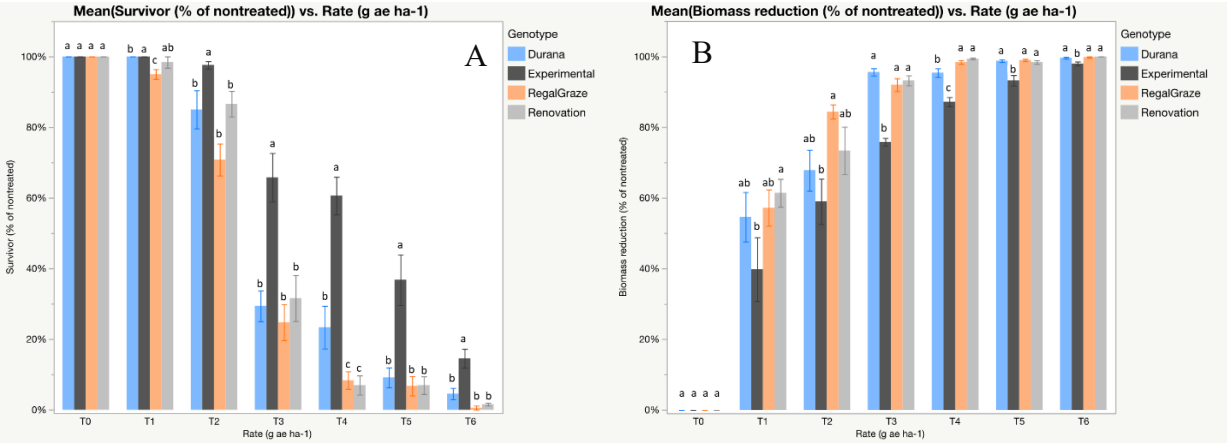


Figure 2.1. (A and B). Survival (A) and biomass reduction (B) of "Durana", Experimental, Regalgraze, and Renovation from greenhouse trial at increasing doses of 2,4-D herbicide. Vertical bars represent the (+/-) standard error of the mean. Values with different letters within each treatment are significantly different.

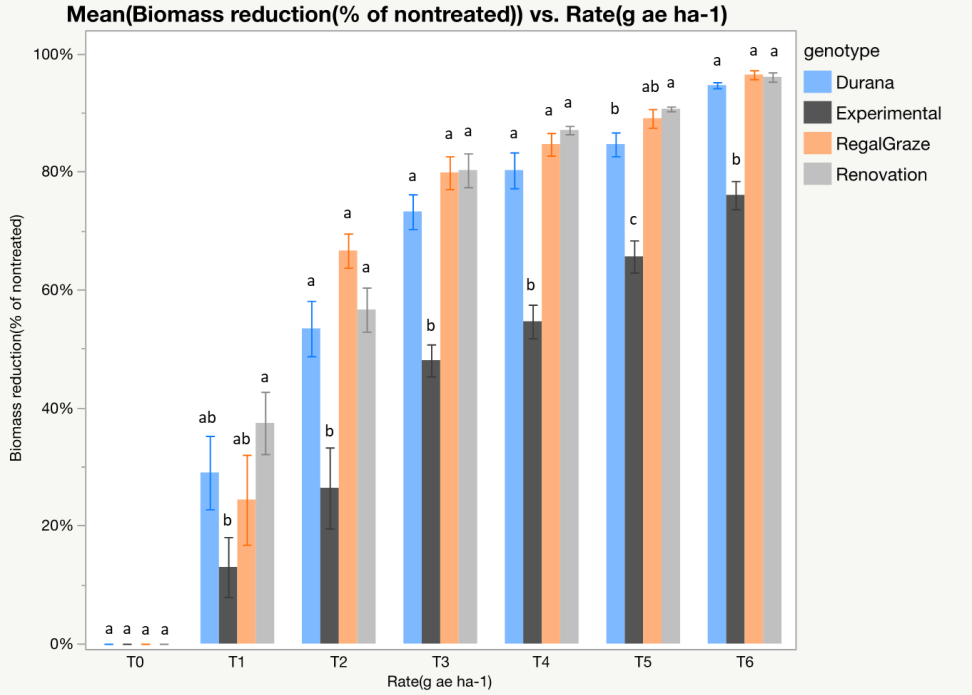


Figure 2.2. Biomass reduction of "Durana", Experimental, Regalgraze, and Renovation from field trial at increasing dose of 2,4-D herbicide. Vertical bars represent the (+/-) standard error of the mean. Values with different letters within each treatment are significantly different.

Figure 2.3. Condition of Experimental, "Durana", Renovation, and Regalgraze (left to right) at 8 weeks after treatment (4483.4 g ae ha^{-1}).

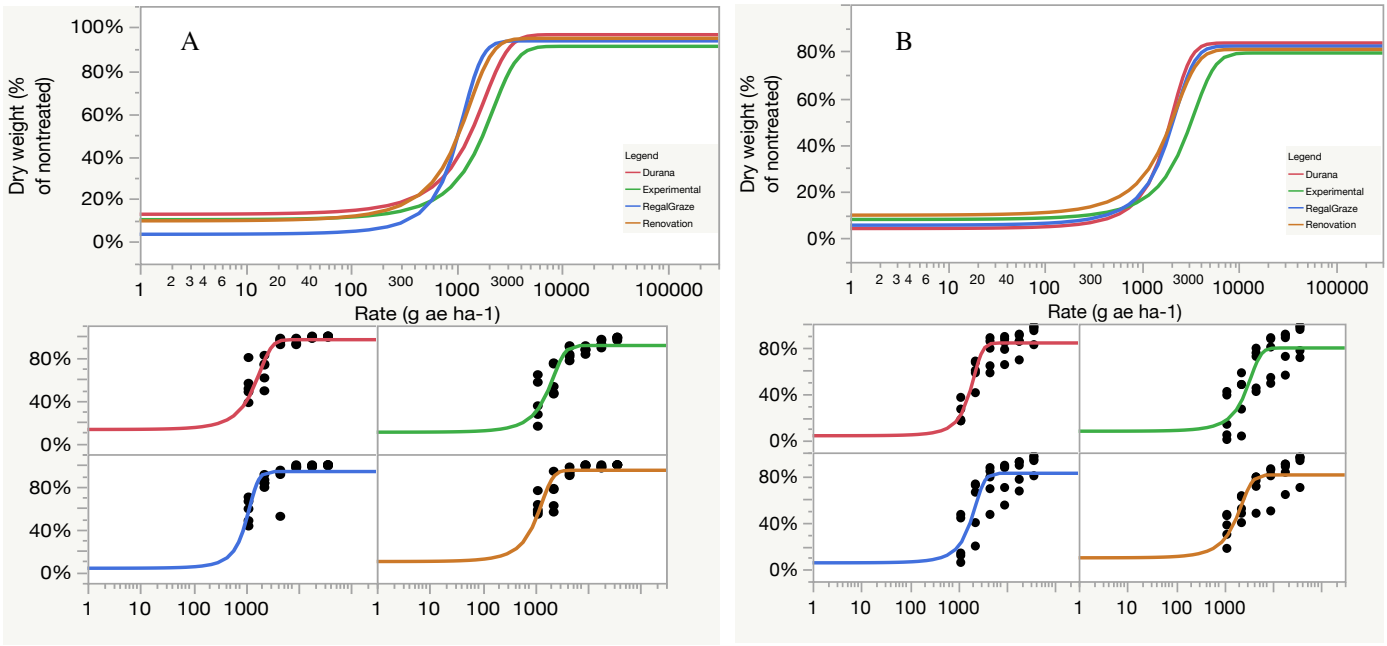


Figure 2.4. Dry biomass response of the "Durana", Experimental, Regalgraze, and Renovation to increasing rates of 2,4-D in greenhouse trial (A) and field trial (B). Symbols and lines represent actual and predicted responses, respectively.

CHAPTER 3

TRANSCRIPTOMIC ANALYSIS OF 2,4-D TOLERANT WHITE CLOVER (*TRIFOLIUM REPENS*)

Ibrahim, R., Kabir, A., Hsieh, C., Vencill, W., Missaoui, A., 2023, will be submitted to *Frontiers in Plant Science*

ABSTRACT

White clover plays a significant role in grass-legume pastures due to its numerous benefits and contributions to overall pasture productivity and animal nutrition. White clover is a valuable component of grass-legume pastures because it fixes nitrogen from the atmosphere, reducing the reliance on synthetic fertilizers and improving the nutrient content of the soil. One of the obstacles of establishing white clover in mixed pasture is the control of broadleaf weeds. Auxinic herbicides such as 2,4-D are an excellent choice to control broadleaf weeds. However, they also kill white clover. Therefore, 2,4-D tolerant white clover was developed at the University of Georgia through mutation breeding and recurrent phenotypic selection. The current study aimed to identify candidate genes involved in 2,4-D tolerance in white clover. Two genotypes, 'Experimental cycle six' as 2,4-D tolerant and "Durana" as 2,4-D susceptible, were selected for transcriptome analysis. De novo and reference-based analyses of transcriptome assemblies were conducted to study the global transcriptome changes due to 2,4-D treatment at 6, 12, and 24 hours after treatment of the two contrasting white clover genotypes. Transcript profiles and gene ontology enrichment analysis indicate that genes related to stress response, metabolism, and photosynthesis are differentially expressed between these two genotypes. A significant number of metabolism-related genes were overrepresented in 'Experimental cycle six', which might be a reason for its 2,4-D resistance ability. The candidate genes detected in this study might be used to develop molecular tools for breeding white clover varieties with improved synthetic auxin resistance potentials.

INTRODUCTION

White clover (*Trifolium repens*) is a perennial legume found in the Fabaceae family, originally native to Europe and Asia but now cultivated globally, including in North America (Lattimore, 2021). It holds significant importance in temperate regions as a forage legume due to its high nutritional value, nitrogen-fixing capability, and seasonal dry matter distribution, especially in mixed species grazed pastures (J. R. Caradus et al., 1996; Gibson & Hollowell, 1966). Studies, such as the meta-analysis by Dineen et al. (2018), have demonstrated that incorporating white clover with perennial ryegrass can enhance milk production, weight gain, and reduce nitrogen application rates. Breeding efforts have focused on improving on-farm productivity, feed quality, and persistence, along with enhancing resistance to pests, diseases, drought, aluminum-toxic soils, herbicides, and low soil phosphorus conditions (Caradus et al., 2023).

In the context of livestock farming in the US, weeds pose a significant challenge, being the primary pest for producers and causing an annual loss of around $2.0 billion (Ditomaso, 2000). To maximize yields of mixed grass-legume pastures, herbicide applications, particularly auxinic herbicides, are necessary. These herbicides target broadleaf weeds while minimizing the impact on desirable legumes in pastures (Ditomaso, 2000). This practice is especially crucial during the seedling establishment phase (Ellis & Young, 1967).

Auxin-type herbicides are categorized into four main chemical groups: quinoline-carboxylates (quinmerac and quinclorac), pyrimidine-carboxylates (fluroxypyr, triclopyr, clopyralid, and picloram), benzoates (dicamba), and phenoxy-carboxylates (2,4-D, 2,4-DP, 2,4-DB, 2,4,5-T, MCPA, MCPB, and mecoprop) (Song, 2014b). Among these, 2,4-dichlorophenoxyacetic acid (2,4-D) is a widely used synthetic auxin herbicide effective against

unwanted dicot plants like weeds (Quastel, 1950). 2,4-D mimics natural auxin at low concentrations, promoting cell division and elongation, while it exhibits herbicidal properties (Grossmann, 2009b; Song, 2014b). High doses of 2,4-D cause abnormal growth, premature senescence, and tissue decay in sensitive dicots (Grossmann, 2000). The specific target site of 2,4-D remains unknown, and it is believed to act at multiple sites within the plant once absorbed (M. A. Peterson et al., 2016). Studies suggest that 2,4-D's mechanism of action involves activating the auxin receptor system, leading to increased auxin responses, elevated ethylene production, and up-regulation of ABA biosynthesis in plants (Schulz & Segobye, 2016; Song, 2014b). High auxin levels have been linked to chloroplast damage, progressive chlorosis, membrane leakage, overproduction of reactive oxygen species (ROS), localized necrosis, and cell death (Grossmann, 2009b; Schulz & Segobye, 2016).

For over 70 years, synthetic auxin herbicides have been employed, yet the precise mechanism leading to plant death remains elusive. The fundamental process of auxin recognition and its pivotal role in plant growth and development have been extensively documented (Hagen & Guilfoyle, 2002a; Kepinski & Leyser, 2005a, 2005b; X. Tan et al., 2007). According to the accepted model, indole-3-acetic acid (IAA), the natural auxin, binds to a receptor from the transport inhibitor response 1 (TIR1)/auxin signaling F-Box (AFB) receptor family. This binding event promotes the recruitment of the Aux/IAA transcriptional repressor, resulting in the formation of a complex involving auxin, TIR1/AFB, and Aux/IAA (Calderón Villalobos et al., 2012). The stability of this complex, where auxin acts as the "molecular glue," enables further interaction with a Skp1-cullin-F-box (SCF) complex, initiating the ubiquitination process of the Aux/IAA protein (Kepinski & Leyser, 2005a). Subsequently, the ubiquitin tag marks the protein for degradation by the 26S proteasome.

Various mechanisms have been identified for herbicide tolerance in weeds and cultivated crops. These mechanisms include regulating enzyme families like cytochrome P450s, glutathione S-transferases (GSTs), and glycosyltransferases. These enzymes play a role in degrading and converting herbicides into non-toxic forms (Busi & Powles, 2017; Ohkawa et al., n.d.; Werck-Reichhart et al., 2000). As outlined by Grossmann (2010), the upregulation of 9-cis-epoxycarotenoid dioxygenase (NCED) and 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, resulting in increased abscisic acid (ABA) and ethylene biosynthesis, represents crucial elements of the auxin herbicide resistance. It has been suggested that auxin herbicide-induced ACC synthase is a primary target process in this mode of action, and upregulation of genes encoding ACC synthase has been observed following IAA treatment (Abel et al., 1995).

The rapid and efficient analysis of complex traits, including resistance mechanisms and metabolic pathways responding to auxinic herbicides such as 2,4-D, has been made possible by high-throughput DNA sequencing. Through this method, specific resistant allele variants of a cytochrome P450 associated with non-target site resistance to 2,4-D in *Amaranthus tuberculatus* have been identified (Giacomini et al., 2020). In wild radish (*Raphanus raphanistrum*) plants resistant to 2,4-D amine treatment at a concentration of 500 g ae ha^{-1}, transcriptome sequencing revealed complex and population-specific changes in auxin signaling, along with heightened plant defense responses, as mechanisms for 2,4-D resistance (Goggin et al., 2018). Whole transcriptome analysis has also been employed to unravel glufosinate resistance in *Amaranthus palmeri* and explore global transcriptional changes linked to herbicide resistance (Rey-Caballero et al., 2016). The transcriptome influenced by IAA has been extensively studied in Arabidopsis, forming the basis for exploring plant responses to synthetic auxin compounds (Goda et al., 2004; Hagen & Guilfoyle, 2002a; Teale et al., 2006). Further analysis of auxin-induced transcriptomes

in etiolated Arabidopsis seedlings revealed that treatment with six distinct auxin compounds led to similar gene expression patterns. However, differences between natural and synthetic compounds were noted (Pufky et al., 2003). While these studies delved into the fundamental aspects of auxin activity governing plant growth and development, understanding genetic regulation and hormonal interactions after synthetic auxin herbicide treatment remains incomplete.

The complex network of plant hormone interactions perturbed by exogenous synthetic auxin herbicide applications provides a unique opportunity to employ transcriptomics to study 2,4-D herbicide response in white clover. This study aims to pinpoint the genes that exhibit differential expression (DEGs) linked to 2,4-D tolerance in white clover. By analyzing specific DEGs that were either up-regulated or down-regulated in the 2,4-D-tolerant plant, experimental cycle six, compared to the 2,4-D-sensitive plant, "Durana," we hope to enhance our understanding of the genetics and molecular mechanisms behind 2,4-D tolerance. We anticipate that this knowledge will guide future breeding initiatives to enhance the tolerance of non-genetically modified white clover to control broad-leaf weeds in grass-legume mixture.

MATERIALS AND METHODS

Plant materials and experimental design

The study utilized the commercial white clover cultivar "Durana" as a susceptible control for 2,4-D herbicide, while experimental cycle 6 genotype were chosen as the tolerant cultivar. The tolerant plant was specifically selected because it survived a 4483.4 g ae ha^{-1} dose of 2,4-D amine. Clones were generated from stem cuttings for both the susceptible and tolerant genotypes to create biological replicates. Forty clones of each genotype type (susceptible and resistant)

were individually potted in plastic containers within a controlled glasshouse environment, maintaining a temperature range of 22°C during the day and 18°C at night, with a 12-hour photoperiod. Plants were watered as needed and fertilized weekly. Twenty-eight days after planting, the 2,4-D herbicide was applied at a rate of 4483.4 g ae ha^{-1} in a controlled spray chamber (Research TrackSprayer, De Vries Manufacturing, Hollandale, MN, USA) located at the UGA Turfgrass Facility using a 8002VS nozzle calibrated to deliver 187 liters ha^{-1} at 275 kpa. After herbicide application, the treated plants were allowed to dry for one to two hours before transferring back to the greenhouse to minimize herbicide volatilization and contamination to non-treated cotton plants. Irrigation was resumed on the treated seedlings after 24 hours. The experiment was conducted following a completely randomized design with two treatments (2,4-D treated and non-treated plants) and five replications per treatment. Leaf tissues were collected from the treated and non-treated white clover plants before herbicide treatment, 6,12, and 24 hours after treatment (HAT). The leaf tissues were immediately frozen on liquid nitrogen and stored at -80°C before total RNA extraction.

RNA isolation

RNA extraction was performed as recommended by the manufacturer using the RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA). The leaves from each treatment group's plants were combined and ground in RNeasy Lysis Buffer (RLT buffer, Qiagen) using a mortar and pestle. The homogenized tissue was then transferred to a QiaShredder column. Subsequently, the Qiagen RNEasy plant mini kit protocol was followed. After the extraction process, an off-column DNase treatment was carried out. Specifically, 60 µL of RNA, 1X RDD buffer (Qiagen), and 2.7 units of DNase were mixed, and H_2O was added to make the total volume 70 µL. The DNAse digestion occurred at room temperature for 30 minutes, after which RNA purification

was performed again on the column using the same Qiagen kit. The RNA was added to 700-µL RW1 buffer, and the Qiagen kit protocol was continued. Post-extraction, the RNA's quantity and quality were assessed through A_{260} and $A_{260/280}$ readings using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis. All samples had RNA concentrations exceeding 200 ng μL^{-1} and $A_{260/280}$ ratios between 2.1 and 2.2. Gel electrophoresis results showed no signs of RNA degradation.

cDNA library preparation

Total RNA samples isolated from white clover leaf tissues treated with 2,4-D (4483.4 g ae ha^{-1}) and untreated control leaves were used to construct cDNA libraries using KAPA Stranded mRNA-Seq Kits (07962193001 and 07962207001) and KAPA mRNA Capture Kits (07962231001 and 07962240001) following the manufacturer's instructions. We performed the library preparation in-house to reduce the cost and train myself. The procedure of cDNA library preparation is as follows.

mRNA Capture

According to the protocol, 100 ng – 4 μg of intact, total RNA in 50 μL of RNase-free water was used. Before starting, the mRNA Capture Beads, mRNA Bead Binding Buffer, mRNA Bead Wash Buffer, and Fragment, Prime, and Elute Buffer were equilibrated to room temperature. The beads were washed with mRNA Bead Binding Buffer before use. The resuspended mRNA Capture Beads were thoroughly mixed by pipetting up and down gently. For each library to be prepared, 52.5 μL (50 μL + 5% excess) of the resuspended mRNA Capture Beads were transferred into an appropriate tube. The tube was placed on a magnet and incubated at room temperature until the solution became clear. The supernatant was carefully removed and discarded, and it was replaced with an equal volume of mRNA Bead Binding Buffer (52.5 μL

per library). The tube was removed from the magnet, and the beads were resuspended by pipetting up and down. The tube was placed on the magnet again and incubated at room temperature until the solution was clear. The supernatant was carefully removed and discarded, and it was replaced with an equal volume of mRNA Bead Binding Buffer (52.5 μL per library). The tube was removed from the magnet, and the beads were resuspended again by pipetting up and down.

For each RNA sample to be captured, 50 μL of resuspended mRNA Capture Beads were transferred into individual tubes. To each tube, 50 μL of the appropriate RNA sample (in RNase-free water) was added. The tubes were placed in a thermocycler, and the 1st mRNA capture was performed at 65°C for 2 min, followed by cooling at 20°C for 5 min. The tubes containing the mixture of mRNA Capture Beads and RNA were then placed on a magnet and incubated at room temperature until the solution became clear. The supernatant was removed and discarded. The tubes were removed from the magnet, and the mixture was thoroughly resuspended in 200 μL of mRNA Bead Wash Buffer by pipetting up and down several times. The tubes were placed on the magnet and incubated at room temperature until the solution was clear. The supernatant was removed and discarded, and the beads were resuspended in 50 μL of RNase-free water. The tubes were placed in a thermocycler, and the 2nd mRNA capture was performed at 70°C for 2 min, followed by cooling at 20°C for 5 min.

Then, 50 μL of Bead Binding Buffer was added to the mixture of mRNA Capture Beads and RNA, and the solution was mixed thoroughly by gently pipetting up and down several times. The tubes were incubated at 20°C for 5 min. The tubes were placed on the magnet and incubated at room temperature until the solution became clear. The supernatant was removed and discarded. The beads were removed from the magnet and resuspended in 200 μL of mRNA Bead

Wash Buffer by pipetting up and down several times. The tubes were placed on the magnet and incubated at room temperature until the solution became clear. The entire volume of supernatant was removed and discarded.

mRNA Elution, Fragmentation, and Priming

The mRNA Capture Beads with captured mRNA, prepared in the previous step, were thoroughly resuspended in 22 μL of Fragment, Prime, and Elute Buffer. The tubes were placed in a thermocycler, fragmented (201-300 bp), and primed at 94°C for 6 minutes. After the fragmentation and priming program, the tubes were immediately placed on a magnet and incubated until the liquid became clear. Twenty microliters of the supernatant containing the eluted, fragmented, and primed RNA were carefully removed into a separate tube. The tubes were then placed on ice, and the process proceeded immediately to 1st strand synthesis.

1st and 2nd Strand Synthesis

On ice, the first strand synthesis reaction was assembled, consisting of 20 μL of fragmented, primed RNA eluted from the beads and 10 μL of 1st strand synthesis master mix. The reaction was mixed thoroughly by gently pipetting up and down several times. The tubes were then incubated following the protocol: primer extension at 25°C for 10 min, 1st strand synthesis at 42°C for 15 min, enzyme inactivation at 70°C for 15 min, and held at 4°C.

On ice, the second strand synthesis and marking reaction were assembled, comprising 30 μL of 1st strand cDNA and 30 μL of 2nd strand synthesis and marking master mix. The reaction was mixed thoroughly by gently pipetting up and down several times. The tubes were incubated at 16°C for 60 min and held at 4°C. The process then proceeded immediately to the 2nd Strand Synthesis and Marking Cleanup. A 1.8X bead-based cleanup was performed by combining 60 μL of the 2nd strand synthesis reaction product with 108 μL of KAPA Pure Beads. The mixture

was thoroughly mixed by pipetting up and down multiple times. The tubes were incubated at room temperature for 5 – 15 min to bind DNA to the beads. The tubes were placed on a magnet to capture the beads and incubated until the liquid became clear. Carefully, 160 μL of supernatant was removed and discarded. While the tubes remained on the magnet, 200 μL of 80% ethanol was added, incubated at room temperature for ≥30 sec, and then carefully removed and discarded. This ethanol-washing step was repeated once more. Residual ethanol was removed without disturbing the beads. The beads were dried at room temperature until all of the ethanol had evaporated, taking caution not to over-dry, which could result in reduced yield. The process then proceeded immediately to A-tailing.

A-tailing

The A-tailing reaction was assembled by combining beads with dscDNA obtained from the previous step and 30 μL of the A-tailing master mix. The mixture was thoroughly mixed by pipetting up and down several times. The tubes were incubated following the protocol: A-tailing at 30°C for 30 min, enzyme inactivation at 60°C for 30 min, and held at 4°C. The process then proceeded immediately to adapter ligation.

Adapter Ligation

The adapter ligation reactions were set up by combining 30 μL of beads with A-tailed DNA, 35 μL of Adapter ligation master mix, and 5 μL of diluted adapter stock. The mixture was thoroughly mixed by pipetting up and down several times to resuspend the beads. The tubes were then incubated at 20°C for 15 min. The process proceeded immediately to post-ligation Cleanup.

1st and 2nd Post-ligation Cleanup

A 1X bead-based cleanup was performed by combining 70 μL of beads with adapter-ligated DNA and 70 μL of PEG/NaCl Solution. The mixture was thoroughly mixed by pipetting

up and down multiple times. The tubes were incubated at room temperature for 5 – 15 min to bind DNA to the beads. Then, they were placed on a magnet to capture the beads and incubated until the liquid became clear. Carefully, 135 μL of supernatant was removed and discarded. While the tubes remained on the magnet, 200 μL of 80% ethanol was added, incubated at room temperature for ≥30 sec, and then carefully removed and discarded. This ethanol-washing step was repeated once more. Residual ethanol was removed without disturbing the beads. The beads were dried at room temperature for 3 – 5 min, or until all the ethanol evaporated. Caution was taken not to over-dry the beads, which could result in reduced yield. The tubes were removed from the magnet. The beads were resuspended in 50 μL of 10 mM Tris-HCl (pH 8.0 – 8.5). The tubes were incubated at room temperature for 2 min to elute DNA off the beads. The same steps were followed for the 2nd post-ligation cleanup. After that, the tubes were placed on a magnet to capture the beads and incubated until the liquid became clear. Twenty microliters of the clear supernatant were transferred to a new tube and the process proceeded to library amplification.

Library Amplification and Cleanup

Each library amplification reaction was assembled with 20 μL of purified, adapter-ligated DNA and 30 μL of Library amplification master mix. The mixture was well mixed by pipetting up and down several times. The library was amplified using the following thermocycling profile: initial denaturation at 98°C for 45 sec, denaturation at 98°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 min, and held at 4°C. The process immediately proceeded to library amplification cleanup.

A 1X bead-based cleanup was performed by combining 50 μL of amplified library DNA with 50 μL of KAPA Pure Beads. The mixture was thoroughly mixed by pipetting up and down multiple times. The tubes were incubated at room temperature for 5 – 15 min to bind DNA to the beads.

The tubes were placed on a magnet to capture the beads and incubated until the liquid became clear. Carefully, 95 μL of supernatant was removed and discarded. While the tubes remained on the magnet, 200 μL of 80% ethanol was added, incubated at room temperature for ≥30 sec, and then carefully removed and discarded. This ethanol-washing step was repeated once more. Residual ethanol was removed without disturbing the beads. The beads were dried at room temperature for 3 – 5 min, or until all the ethanol evaporated. The tubes were removed from the magnet. The dried beads were thoroughly resuspended in 22 μL of 10 mM Tris-HCl (pH 8.0 – 8.5). The tubes were incubated at room temperature for 2 min to elute DNA off the beads. The tubes were placed on a magnet to capture the beads and incubated until the liquid became clear. Twenty microliters of the clear supernatant were transferred to new tubes, and the purified, amplified libraries were stored at 2°C to 8°C for ≤ 1 week before being sent for sequencing.

Sequencing technology, transcriptome assembly, and Differential Gene Expression analysis

The Georgia Genomics and Bioinformatics core sequenced the RNA using the Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA, USA) platform. Before sequencing, the quality of all samples was confirmed using an Agilent® 2100 Bioanalyzer™ (Agilent®, Santa Clara, CA). RNA was acceptable if the RNA integrity number (RIN) exceeded seven. Sequences were generated in High Output Mode with 100 base pair read length and paired-end sequencing. Sequencing the RNA from both strand directions, the paired-end protocol enables better transcriptome coverage. The initial step involved assembling clean reads into contigs using Trinity with default settings (Haas et al., 2013). The de novo assembled transcriptome was then used as a reference to map the individual reads using the Bowtie program. Transcript abundance was measured for each genotype and time point combination as the expected number of fragments per kilobase (kb) of transcript sequence per million mapped reads (FPKM) using

RSEM version 1.1.11. Subsequently, these contigs were further organized into unigenes using CORSET with default parameters, as outlined in previous literature (Davidson & Oshlack, 2014). These unigenes from white clover were then subjected to BLAST searches against a comprehensive database comprising protein sequences from *Arabidopsis thaliana*, rice (*Oryza sativa*), soybean (*Glycine max*), and *Medicago truncatula* using the BLAST2GO program for functional annotation, employing an E-value cutoff of 1e-5 (Altschul et al., 1997). Following this, functional annotations were assigned to the white clover unigenes based on their corresponding homologs in the combined database, including Gene Ontology (GO) and KOG (EuKaryotic Orthologous Groups) annotations (Ashburner et al., 2000; Tatusov et al., 2003). Differentially expressed genes were detected using the DESeq2 package with the adjustment of p-value lower than 0.05 and the absolute value of fold change larger than 2 for each herbicide treatment compared to the water-only treatment.

RESULTS

Transcriptome analysis

There are no reports in the literature of whole transcriptome analysis of the response of white clover to 2,4-D. The selection of specific time points (12 HAT) for the study of the herbicide response was based on studies of gene expression responses to abiotic stress (i.e., cold stress) and synthetic auxin herbicide treatment (i.e., upland cotton) in other plants, including Arabidopsis, upland cotton, and *Brassica napus* (L. Chen et al., 2011; Ishitani et al., 1998; Perez et al., 2022). Analysis of the cDNA fragment libraries before sequencing using capillary-zone electrophoresis (Bioanalyzer) showed the average fragment size in the desired 250-300 bp size range (Figure 3.1). Sequencing of the short-read Illumina libraries generated approximately 120

million reads (paired-end) from 60 million fragments (average) per library. To establish a white clover reference transcriptome database, we performed de novo assembly of all reads using Trinity (default parameters used for software tools) (Grabherr et al., 2011). Finally, we obtained 348,086 transcripts (unigenes) from the de novo assembly with the mean length 814.83 bp and the N50 value 1255 bp. To annotate these unique transcripts, they were used as queries in a BLAST search of a database established by combining the protein sequences of the well-studied plant organisms: *Arabidopsis thaliana*, rice (*Oryza sativa*), soybean (*Glycine max*), and *Medicago truncatula*. The transcripts were also BLAST searched against the Uniport database. The results showed that about 75.8% of transcripts (55,078 ones) have homologs with significant hits (E-value cutoff: 1e-05) in the database.

Differential Gene Expression (DEGs) analysis overview

Using FDR adjusted p-value <.01 and -2<log2FC>2 as cutoff values for differentially expressed genes (DEGs), a total of 3709, 5247, and 5597 DEGs were up-regulated in experimental cycle six genotypes at 6, 12, and 24 HAT, respectively (Figure 3.2 A). A total number of 2900, 5069, and 4925 DEGs were downregulated in the same genotype at 6, 12, and 24 HAT, respectively (Figure 3.2 B). Among these DEGs 2495 and 1859 DEGs were upregulated and downregulated, respectively, in all the time points (Figure 3.2 A & B). On the other hand, in the 2,4-D susceptible genotype "Durana", 3203, 5271, and 4510 DEGs were up-regulated at 6, 12, and 24 HAT, respectively (Figure 3.3 A). DEGs downregulated in "Durana" were 2650, 5892, and 3569 at 6, 12, and 24 HAT, respectively (Figure 3.3 B). 2182 and 1478 DEGs were upregulated and downregulated across all time points in "Durana", respectively (Figure 3.3 A &B). When comparing the treated resistant genotype with the susceptible genotype, 18 and 13 DEGs were upregulated and downregulated at all time points (Figure 3.4 A

& B). The top 20 upregulated and downregulated DEGs at 6, 12, and 24 HAT in experimental cycle six genotypes are shown in Table 3.2, Table 3.3, and Table 3.4. In "Durana", the top 20 upregulated and downregulated DEGs are shown in Table 3.5. Table 3.6 and 3.7 at 6, 12, and 24 HAT, respectively.

Metabolic pathways (GO) responsive to 2,4-D herbicide in white clover

Functional annotation of transcripts in the 2,4-D treated experimental cycle 6 genotypes at 6 HAT revealed the top 20 most enriched (or up-regulated) gene sets, including DNA metabolic process, catalytic activity, response to stimulus, response to chemical, glutathione transferase activity, and hormone-mediated signaling pathway (Supplementary Table S1). On the other hand, chloroplast, photosystem, photosynthetic membrane, photosystem I and II, and plastid thylakoid are among the most significantly down-regulated metabolic pathways (Supplementary Table S1). At 12 HAT, cellular nitrogen compound metabolic process, nucleic acid metabolic process, DNA metabolic process, and protein-containing complex are among the top enriched metabolic pathways in Experimental cycle 6 genotypes (Supplementary Table S2). Chloroplast, plastid membrane, plastid stoma, and chloroplast stroma are among the top downregulated metabolic processes in Experimental cycle six genotypes at 12 HAT (Supplementary Table S2). Cellular nitrogen compound metabolic process, nucleobase-containing compound metabolic process, response to chemical and protein-containing complex and chloroplast, plastid membrane, and photosynthetic membrane were some of the topmost upregulated and downregulated metabolic pathways in Experimental cycle 6 genotypes at 24 HAT, respectively (Supplementary Table S3).

In case of susceptible genotype ("Durana"), protein-containing complex, nucleobase-containing compound metabolic process, nucleic acid metabolic process, and cellular nitrogen

compound metabolic process were upregulated and chloroplast, endonuclease activity, ADP binding, and nuclease activity were downregulated at 6 HAT and belonged to the top 20 metabolic processes (Supplementary Table S4). At 12 HAT, top upregulated metabolic processes were nucleic acid metabolic process and cellular nitrogen compound metabolic process, whereas chloroplast, plastid, and plastid membrane were the top-downregulated metabolic processes. Protein-containing complex, nucleic acid metabolic process, and nucleobase-containing compound metabolic process were the topmost enriched GOs at 24 HAT in "Durana" genotypes. On the other hand, chloroplast, plastid, plastid membrane, organic substance biosynthetic process, and photosynthetic membrane were the topmost depleted GOs at 24 HAT in "Durana" genotypes.

With the comparison of GO term results of the upregulated DEGs in the Experimental cycle 6 genotypes, DNA metabolic process, response to stimulus, response to chemical, glutathione metabolic process was determined to be significantly enriched in biological processes (Supplementary Table S1, Supplementary Table S3, Supplementary Table S3) at all time points. Common molecular functions upregulated at all time points in resistant genotypes were catalytic activity, DNA polymerase activity, and glutathione transferase activity. Most of the top-downregulated metabolic processes belonged to the cellular component. At all-time points, photosystem, photosystem membrane, plastid stroma, and thylakoid membrane were common cellular components in Experimental cycle 6 genotypes (Supplementary Table S1, Supplementary Table S3, Supplementary Table S3).

Analysis of Differentially Expressed Genes

As expected, many auxin-responsive genes were upregulated in experimental cycle six and "Durana" genotypes following 2,4-D treatment at 6 HAT, 12 HAT, and 24 HAT. In

experimental cycle six genotypes NADPH:quinone oxidoreductase, Auxin-responsive GH3 family protein, Tetratricopeptide repeat (TPR)-like superfamily protein, and UDP-glycosyltransferase genes were consistently upregulated at 6 HAT, 12 HAT, and 24 HAT (Table 3.2, Table 3.3, Table 3.4). On the other hand, Inositol-3-phosphate synthase, Chlorophyll a-b binding protein, and Cytochrome P450 CYP736A12 genes were consistently downregulated in the resistant genotype (Table 3.2, Table 3.3, Table 3.4) at all time points. In case of "Durana", common genes that were upregulated at all time points were Receptor-like protein kinase FERONIA, Ferroptosis suppressor protein 1, Auxin-responsive GH3 family protein, and FAD/NAD(P)-binding oxidoreductase family protein (Table 3.5, Table 3.6, Table 3.7). Downregulated genes common at 6 HAT, 12 HAT, and 24 HAT in "Durana" were Inositol-3-phosphate synthase and Chlorophyll a-b binding protein. As 2,4-D resistant experimental cycle 6 genotype was developed through mutation breeding from "Durana", many genes were common in both genotypes. However, unique genes were significantly upregulated or downregulated either in the experimental Cycle six or "Durana" genotype. For example, NADPH:quinone oxidoreductase, Tetratricopeptide repeat (TPR)-like superfamily protein, and Auxin-responsive protein IAA30 genes were uniquely upregulated in Experimental cycle 6 genotype and belonged to the top 20 upregulated DEGs. Genes that were uniquely downregulated in the experimental cycle six genotype were 1-deoxy-D-xylulose 5-phosphate reductoisomerase, Dimethylnonatriene synthase, small heat shock protein, chloroplastic, and Cytochrome P450 CYP736A12.

DISCUSSION

The findings presented in the study shed light on the molecular mechanisms underlying the resistance of experimental cycle six plants to the 2,4-D herbicide. The upregulation of several

auxin-responsive genes in both experimental cycle six and "Durana" plants following 2,4-D treatment suggests a common response mechanism to the herbicide. However, the unique expression patterns observed in experimental cycle six plants provide valuable insights into their resistance.

The molecular pathway involved in plant response to 2,4-D has been outlined in previous reports (Grossman et al. 2010, Song 2014, Gaines et al. 2020). Auxin-responsive gene expression has been extensively characterized and includes three major groups or gene families: Aux/IAAs, SAURs (small auxin-responsive RNAs), and GH3s (Hagen & Guilfoyle, 2002b). In experimental cycle six and "Durana" plants, key genes involved in auxin signaling and metabolism, such as Indole-3-acetic acid-amido synthetase (GH3.3) and Auxin-responsive protein IAA30, were significantly upregulated. Auxin plays a crucial role in plant growth and development, including regulating processes like cell elongation and division (Huang et al., 2019). IAA30 protein is involved in regulating auxin synthesis, transport, and perception, as well as mediating auxin-responsive gene expression. IAA30 functions as a transcriptional regulator, binding to auxin response elements in the promoter regions of target genes and modulating their expression (Cakr et al., 2015). The GH3 genes are involved in auxin herbicide resistance by being upregulated in response to auxin herbicide treatment, potentially leading to the detoxification of the herbicide (Busi et al., 2018). This upregulation of GH3 gene expression may result in the conjugation and inactivation of the herbicide molecule, preventing its adverse effects on plant growth and development. The upregulation of these genes suggests that experimental cycle six plants might have an enhanced capacity to modulate auxin levels, possibly as a defense mechanism against herbicidal stress. Even though both genotypes up-regulate GH3.3 genes, experimental cycle six plants up-regulate additional four GH3 genes which might help them be more resistant to 2,4-D

herbicide than “Durana”. Additionally, the upregulation of genes encoding enzymes like UDP-glycosyltransferase and glutathione S-transferase indicates potential detoxification processes, where the herbicide molecules might be conjugated and rendered less harmful (Zhu et al., 2023).

Furthermore, the unique upregulation of genes such as NADPH: quinone oxidoreductase, Tetratricopeptide repeat (TPR)-like superfamily protein, and Auxin-responsive protein IAA30 in experimental cycle six plants implies the activation of specific pathways involved in herbicide resistance. NADPH: quinone oxidoreductase is associated with oxidative stress response, and its upregulation suggests that experimental cycle six plants might have a more efficient antioxidative defense system against herbicide-induced oxidative damage (Yamauchi et al., 2011). TPR proteins are involved in gibberellin, cytokinin, and auxin responses as well as ethylene biosynthesis (Greenboim-Wainberg et al., 2005). On the other hand, “Durana” plants, while also exhibiting upregulation of certain auxin-responsive genes, showed unique upregulation of genes related to different stress responses, including Ferroptosis suppressor protein 1 and Dormancy-associated protein 1. These genes might be part of “Durana”'s defense strategy against herbicidal stress, but they do not seem to confer resistance comparable to the mechanisms activated in experimental cycle six plants.

The analysis of enriched metabolic pathways further highlighted significant differences between the genotypes. In the experimental cycle 6 genotype, several GO terms related to DNA metabolic processes, response to chemical stimuli, and glutathione metabolic processes were consistently enriched across all time points. Glutathione transferase activity, in particular, was upregulated, indicating an enhanced capacity for herbicide detoxification (Takahashi & Nagata, 1992). Catalytic activity and DNA polymerase activity were also upregulated, suggesting an active involvement in cellular repair and maintenance processes following herbicide exposure

(Stepanova & Alonso, 2016). In contrast, the "Durana" genotype exhibited a different pattern of response. While it also showed upregulation of certain metabolic pathways, including nucleic acid metabolic processes and cellular nitrogen compound metabolic processes, the downregulation of genes related to chloroplasts, plastids, and photosynthetic membranes indicated a compromised photosynthetic capacity, possibly contributing to herbicidal damage.

The upregulation of genes involved in redox homeostasis, detoxification processes, and DNA repair mechanisms, coupled with the downregulation of genes associated with herbicide metabolism and photosynthesis, likely confer a higher level of resistance in the experimental cycle 6 genotype.

CONCLUSION

This study reports the first whole transcriptome response to 2,4-D in white clover. The upregulation of NADPH: quinone oxidoreductase suggests that the resistance of 2,4-D in white clover might be due to rapid metabolism. Upregulation of TPR proteins indicates effects of auxin synthesis in Experimental cycle 6 genotypes. Most genes consistently upregulated among all herbicide treatments were involved in auxin response, metabolism, and hormone signaling. The identification of genes involved in the general auxin herbicide response will enable future progress to be made to understand the physiological processes involved in herbicide-induced plant death and has the potential to expedite research in biotypes that evolve resistance to these herbicide active ingredients. Future work will be needed to validate the genes mentioned above through RT-qPCR or metabolism test.

Table 3.1: Summary of de novo assembled white clover transcriptome.

Data type	Number
Total transcripts	348,086
Total genes	161,853
Percent GC	37.6
N50 (bp)	1,255
Average length (bp)	814.83

Table 3.2: The top 20 up-regulated and down-regulated differentially expressed genes (DEGs) at 6 HAT relative to control in the experimental cycle six white clover plants treated with 4483.4 g ae h^{-1} of 2,4-D herbicide.

Gene ID	logFC	FDR	Function
Up-regulated			
AT3G27890.1	21.9875616	4.45E-14	NADPH:quinone oxidoreductase
AT4G28080.1	17.1991547	1.32E-13	Tetratricopeptide repeat (TPR)-like superfamily protein
AT4G25580.1	16.2075598	2.09E-12	Dormancy/auxin associated family protein
AT2G33830.2	11.2967448	2.49E-39	Dormancy-associated protein 1
AT2G23170.1	10.4447441	2.67E-22	Auxin-responsive GH3 family protein GH3.3
AT2G23170.1	10.2107854	1.27E-19	Auxin-responsive GH3 family protein GH3.3
AT3G47080.1	10.0846336	1.21E-18	Tetratricopeptide repeat (TPR)-like superfamily protein
AT5G38200.1	9.78350274	1.32E-13	Putative glutamine amidotransferase GAT1_2.1

AT1G59500.1	9.77891849	2.37E-17	Auxin-responsive GH3 family protein GH3.4
AT4G37770.1	9.77069715	4.33E-16	1-aminocyclopropane-1-carboxylate synthase 3
AT1G03390.1	9.40595602	1.26E-14	Hydroxycinnamoyl-CoA
AT2G23170.1	9.40254478	7.36E-32	Auxin-responsive GH3 family protein GH3.3
AT3G17600.1	9.32872746	1.97E-15	Auxin-responsive protein IAA30
AT1G10070.1	9.18154852	5.58E-13	Branched-chain-amino-acid aminotransferase 2
AT1G59500.1	9.03213953	2.32E-14	Auxin-responsive GH3 family protein GH3.4
AT2G14960.1	8.96257309	9.05E-17	Auxin-responsive GH3 family protein GH3.1
AT2G15890.1	8.93648021	9.82E-23	CCG-binding protein 1
AT3G44190.1	8.74623884	3.49E-23	Ferroptosis suppressor protein 1
AT5G22140.1	8.55093649	1.86E-14	FAD/NAD(P)-binding oxidoreductase family protein
AT2G14960.1	8.47932306	4.03E-13	Auxin-responsive GH3 family protein GH3.1
Down-regulated			
INO1_HORVU	-10.767713	3.26E-27	Inositol-3-phosphate synthase
INO1_NICPA	-10.294257	1.06E-22	Inositol-3-phosphate synthase
AT4G13510.1	-9.9549943	1.79E-23	Ammonium transporter 1 member 2
AT3G55580.1	-9.5585832	1.81E-35	Ultraviolet-B receptor UVR8
INO1_ARATH	-9.412023	1.21E-17	Inositol-3-phosphate synthase isozyme 1
INO1_POPEU	-9.3271463	9.91E-17	Inositol-3-phosphate synthase 1
AT1G48100.1	-8.9668128	4.06E-19	Polygalacturonase At1g48100
AT5G10170.1	-8.9584541	1.41E-19	Inositol-3-phosphate synthase
AT3G25180.1	-8.2815201	4.54E-15	Dimethylnonatriene synthase

AT1G64780.1	-8.2571151	1.74E-32	Ammonium transporter 1 member 2
AT1G01060.7	-8.0861277	1.15E-07	Protein LATE ELONGATED HYPOCOTYL
AT2G15490.1	-8.0494797	8.16E-34	UDP-glucose flavonoid 3-O-glucosyltransferase 7
AT3G54500.3	-7.8395718	4.78E-131	Protein LNK2
AT5G17050.1	-7.7525562	1.45E-11	Flavonoid 3-O-glucosyltransferase
AT1G61930.1	-7.736793	2.46E-24	Protein S40-6
AT2G05070.1	-7.5548652	1.04E-84	Chlorophyll a-b binding protein 215, chloroplastic
AT2G05100.2	-7.4162181	1.87E-17	Chlorophyll a-b binding protein 215, chloroplastic
AT2G05070.1	-7.3849455	2.14E-125	Chlorophyll a-b binding protein 215, chloroplastic
AT1G30700.1	-7.3789873	4.02E-21	Berberine bridge enzyme-like 8
AT4G14690.1	-7.3661598	1.13E-29	Early light-induced protein, chloroplastic

logFC= log 2 fold change, FDR= false discovery rate p-value.

Table 3.3: The top 20 up-regulated and down-regulated differentially expressed genes (DEGs) at 12 HAT relative to control in the experimental cycle six white clover plants treated with 4483.4 g ae h^{-1} of 2,4-D herbicide.

Gene ID	logFC	FDR	Function
Up-regulated			
NQR_ARATH	22.47360378	6.25E-15	NADPH:quinone oxidoreductase
AT2G23170.1	11.40528673	7.53E-27	Auxin-responsive GH3 family protein
GH33_ARATH	11.19192384	8.49E-24	Indole-3-acetic acid-amido synthetase GH3.3
AT1G10070.1	10.90219246	2.02E-18	Branched-chain-amino-acid aminotransferase 2

AT3G17600.1	10.25201733	7.56E-19	Auxin-responsive protein IAA30
GH34_ARATH	9.991182747	9.35E-18	Indole-3-acetic acid-amido synthetase GH3.4
AT3G22250.1	9.684936798	2.88E-14	UDP-glycosyltransferase 82A1
AT2G23170.1	9.682481653	3.71E-34	Indole-3-acetic acid-amido synthetase GH3.3
AT5G38200.1	9.588809875	2.77E-13	Putative glutamine amidotransferase GAT1_2.1
AT3G01420.1	9.514785979	4.10E-07	Alpha-dioxygenase
GH31_ARATH	9.281127775	3.13E-18	Probable indole-3-acetic acid-amido synthetase GH3.1
AT1G17180.1	8.785521722	2.71E-10	Probable glutathione S-transferase
AT4G13430.1	8.77630481	1.56E-08	3-isopropylmalate dehydratase large subunit
GH31_ARATH	8.765611882	1.30E-31	Probable indole-3-acetic acid-amido synthetase GH3.1
AT3G53140.1	8.660020341	2.30E-20	Isoflavone 4'-O-methyltransferase
U83A1_ARATH	8.582580849	2.51E-10	UDP-glycosyltransferase 83A1
GH32_ARATH	8.525904432	1.26E-11	Indole-3-acetic acid-amido synthetase GH3.2
GH38_ORYSI	8.524918136	5.64E-13	Indole-3-acetic acid-amido synthetase GH3.8
C81E1_GLYEC	8.43204138	7.80E-12	Isoflavone 2'-hydroxylase
AT2G37430.1	8.353994728	5.33E-11	Zinc finger protein ZAT11
Down-regulated			
CB21_RAPSA	-11.99520539	5.26E-37	Chlorophyll a-b binding of LHCII type 1 protein
AT3G54500.3	-10.84837094	5.43E-155	Protein LNK2
AT5G54270.1	-10.78088014	3.49E-19	Chlorophyll a-b binding protein 13, chloroplastic

INO1_NICPA	-10.76599977	1.09E-24	Inositol-3-phosphate synthase
AT5G10170.1	-10.51024548	3.22E-21	Inositol-3-phosphate synthase
INO1_POPEU	-10.3697613	6.15E-20	Inositol-3-phosphate synthase 1
AT1G22360.1	-10.13858122	1.40E-21	Linamarin synthase 1
AT3G55580.1	-9.98267089	2.62E-48	Ultraviolet-B receptor UVR8
INO1_HORVU	-9.530379837	2.29E-42	Inositol-3-phosphate synthase
AT1G22380.1	-9.183844528	3.49E-22	UDP-glycosyltransferase 85A8
AT1G64780.1	-9.179918057	2.96E-45	Ammonium transporter 1 member 2
AT5G62790.2	-9.164910993	1.20E-39	1-deoxy-D-xylulose 5-phosphate reductoisomerase
AT3G25180.1	-9.164668216	3.20E-17	Dimethylnonatriene synthase
AT4G27670.1	-8.897959491	8.02E-32	Small heat shock protein, chloroplastic
AT3G22840.1	-8.893694618	4.17E-14	Early light-induced protein, chloroplastic
AT3G54500.3	-8.777026596	1.55E-07	Protein LNK2
AT5G57260.1	-8.705416713	3.71E-05	Cytochrome P450 CYP736A12
AT1G61520.1	-8.248283665	4.66E-17	Chlorophyll a-b binding protein 3, chloroplastic
AT1G78950.2	-8.163003391	5.96E-07	Beta-amyrin synthase
AT2G20260.1	-8.090473073	7.31E-91	Photosystem I reaction center subunit IV A

logFC= log 2 fold change, FDR= false discovery rate p-value.

Table 3.4: The top 20 up-regulated and down-regulated differentially expressed genes (DEGs) at 24 HAT relative to control in the experimental cycle six white clover plants treated with 4483.4 g ae h^{-1} of 2,4-D herbicide.

Gene ID	logFC	FDR	Function
Up-regulated			
VIT1_PERAM	24.94964134	0.001871344	Vitellogenin-1
NQR_ARATH	23.76442896	1.81E-16	NADPH:quinone oxidoreductase
AT4G25580.1	19.11340382	1.24E-18	CAP160 protein
AT4G28080.1	16.55785875	2.69E-13	Tetratricopeptide repeat (TPR)-like superfamily protein
GH33_ARATH	11.90866908	1.59E-26	Indole-3-acetic acid-amido synthetase GH3.3
AT2G23170.1	11.89557355	9.46E-29	Indole-3-acetic acid-amido synthetase GH3.3
AT1G17180.1	11.14166842	4.41E-16	Probable glutathione S-transferase parA
AT3G03080.1	11.12464827	7.40E-21	2-alkenal reductase (NADP(+)-dependent)
AT3G01420.1	10.97976916	3.19E-09	Alpha-dioxygenase
AT3G44190.1	10.84216154	2.13E-35	Ferroptosis suppressor protein 1
AT1G26320.1	10.8242727	6.87E-16	NADP-dependent alkenal double bond reductase P2
GH34_ARATH	10.72612641	3.58E-20	Indole-3-acetic acid-amido synthetase GH3.4
AT3G22250.1	10.60898683	7.49E-17	UDP-glycosyltransferase 82A1
AT5G22140.1	10.57168918	6.00E-22	FAD/NAD(P)-binding oxidoreductase family protein
GH34_ARATH	10.37236267	1.53E-19	Indole-3-acetic acid-amido synthetase GH3.4
BGIA_MOMCH	10.27095008	8.32E-13	Glu S.griseus protease inhibitor
AT1G53920.1	10.22530628	3.92E-15	GDSL esterase/lipase 5

AT3G17600.1	10.20426561	1.76E-18	Auxin-responsive protein IAA30
AT1G08080.1	10.07562207	3.39E-14	Alpha carbonic anhydrase 7
AT3G09270.1	10.05132539	1.97E-11	Probable glutathione S-transferase
Down-regulated			
AT2G45550.1	-10.91132807	7.79E-11	Geraniol 8-hydroxylase
AT5G57260.1	-8.607831312	4.66E-05	Cytochrome P450 CYP736A12
AT4G39950.2	-8.33880655	2.92E-08	Belongs to cytochrome P450
AT5G64570.1	-8.296773164	6.54E-07	Beta-xylosidase/alpha-L-arabinofuranosidase 2
AT1G64780.1	-7.844377758	8.99E-09	Ammonium transporter 1 member 3
AT1G10330.1	-7.761449744	8.99E-09	Putative pentatricopeptide repeat-containing protein
AT1G74040.1	-7.729550612	0.018909562	Probable 2-isopropylmalate synthase
CB4A_ARATH	-7.525980257	0.035551234	Chlorophyll a-b binding protein CP29.1
AT2G34420.1	-7.499837076	0.008760127	Chlorophyll a-b binding protein
AT1G22380.1	-7.301121872	6.74E-19	UDP-glycosyltransferase 85A8
AT1G22360.1	-7.298548036	2.59E-13	Linamarin synthase 1
RCA_SPIOL	-7.173046248	3.44E-06	Ribulose bisphosphate carboxylase/oxygenase activase
AT1G29930.1	-7.119624547	0.001153122	Chlorophyll a-b binding protein 40
AT4G28660.2	-7.110136179	2.61E-05	Photosystem II reaction center PSB28 protein
AT3G51280.1	-7.021905345	6.71E-09	Protein POLLENLESS 3-LIKE 2
AT5G42260.1	-7.005981275	0.007520786	Cyanogenic beta-glucosidase
AT3G63190.1	-6.999892654	0.000416663	Ribosome-recycling factor
AT1G76160.1	-6.970463791	0.002714123	L-ascorbate oxidase homolog

AT2G29630.1	-6.968342122	9.22E-05	Phosphomethylpyrimidine synthase
AT5G20300.4	-6.929607573	0.014677166	Translocase of chloroplast 90

logFC= log 2 fold change, FDR= false discovery rate p-value.

Table 3.5: The top 20 up-regulated and down-regulated differentially expressed genes (DEGs) at 6 HAT relative to control in the "Durana" white clover plants treated with 4483.4 g ae h^{-1} of 2,4-D herbicide.

Gene ID	logFC	FDR	Function
Up-regulated			
AT3G51550.1	11.6548792	3.65E-05	Receptor-like protein kinase FERONIA
AT2G33830.2	10.9896738	5.69E-51	Dormancy-associated protein 1
AT3G44190.1	10.7737699	9.92E-19	Ferroptosis suppressor protein 1
GH33_ARATH	10.6531547	3.00E-21	Indole-3-acetic acid-amido synthetase GH3.3
GH34_ARATH	10.6400181	2.03E-20	Indole-3-acetic acid-amido synthetase GH3.4
AT2G23170.1	10.0948343	1.49E-25	Indole-3-acetic acid-amido synthetase GH3.3
AT1G10070.1	9.65443792	3.09E-14	Branched-chain-amino-acid aminotransferase2
AT2G23170.1	9.64672249	5.10E-40	Indole-3-acetic acid-amido synthetase GH3.3
AT1G03390.1	9.48654191	8.75E-15	Hydroxycinnamoyl-CoA
GSTX3_TOBAC	9.39849488	6.69E-18	Glutathione S-transferase
AT4G37300.1	9.32083524	1.50E-40	Maternal effect embryo arrest 59
AT3G47080.1	9.20941911	1.43E-20	Tetratricopeptide repeat (TPR)-like superfamily protein
GH31_ARATH	9.16296489	1.56E-17	Indole-3-acetic acid-amido synthetase GH3.1

AT4G37770.1	9.15034975	9.38E-17	1-aminocyclopropane-1-carboxylate synthase3
AT5G22140.1	9.0076251	4.43E-14	FAD/NAD(P)-binding oxidoreductase family protein
GH31_ARATH	8.97844969	8.21E-29	Indole-3-acetic acid-amido synthetase GH3.1
AT4G37390.1	8.8364522	5.94E-11	Indole-3-acetic acid-amido synthetase GH3.1
GH38_ORYSI	8.70724731	3.31E-13	Indole-3-acetic acid-amido synthetase GH3.8
AT2G39980.1	8.45324075	2.19E-13	Uncharacterized acetyltransferase
GH31_ARATH	8.42863226	7.01E-13	indole-3-acetic acid-amido synthetase GH3.1
Down-regulated			
AT3G55580.1	-8.608456	2.94E-33	Ultraviolet-B receptor UVR8
AT3G12320.1	-8.4534037	1.07E-39	night light-inducible and clock-regulated 3
INO1_NICPA	-8.0406266	4.18E-22	Inositol-3-phosphate synthase
INO1_HORVU	-7.9019996	8.39E-30	Inositol-3-phosphate synthase
INO1_ARATH	-7.8719067	2.26E-16	Inositol-3-phosphate synthase isozyme 1
INO1_POPEU	-7.8679685	1.44E-16	Inositol-3-phosphate synthase 1
AT1G48100.1	-7.77044	2.31E-15	Polygalacturonase At1g48100
AT4G13510.1	-7.4330205	2.24E-19	Ammonium transporter 1 member 2
AT1G30700.1	-6.8150584	3.40E-21	Berberine bridge enzyme-like 8
AT3G54500.3	-6.7556934	1.79E-104	Protein LNK2
AT1G01060.7	-6.6380456	2.90E-05	Protein LATE ELONGATED HYPOCOTYL
AT1G64780.1	-6.5536938	3.67E-24	Ammonium transporter 1 member 2
AT4G08570.1	-6.5313559	7.14E-05	Heavy metal-associated isoprenylated plant protein

AT1G61930.1	-6.4956181	4.95E-21	Protein S40-6
AT4G39950.2	-6.4724456	4.69E-05	CYP79B2, CYTOCHROME P450,
CB2G_SOLLC	-6.219449	2.63E-16	Chlorophyll a-b binding protein 3C
AT1G20850.1	-6.2138546	3.49E-05	Cysteine proteinase mucunain
AT5G10170.1	-6.1917418	1.47E-13	Inositol-3-phosphate synthase
AT5G14570.1	-6.1122289	8.93E-11	High affinity nitrate transporter 2.7
AT2G05070.1	-6.0818779	4.58E-86	Chlorophyll a-b binding protein 215

logFC= log 2 fold change, FDR= false discovery rate p-value.

Table 3.6: The top 20 up-regulated and down-regulated differentially expressed genes (DEGs) at 12 HAT relative to control in the "Durana" white clover plants treated with 4483.4 g ae h^{-1} of 2,4-D herbicide.

Gene ID	logFC	FDR	Function
Up-regulated			
AT3G51550.1	18.50664042	4.80E-14	Receptor-like protein kinase FERONIA
AT3G44190.1	12.79366403	1.21E-26	Ferroptosis suppressor protein 1
AT1G10070.1	11.64967966	6.52E-21	Branched-chain-amino-acid aminotransferase 2
GH33_ARATH	11.3472305	2.18E-24	Indole-3-acetic acid-amido synthetase GH3.3
AT2G23170.1	10.84789599	8.30E-30	Indole-3-acetic acid-amido synthetase GH3.3
CORA_MEDSA	10.78837468	1.03E-22	Cold and drought-regulated protein CORA

AT5G22140.1	10.74737125	3.25E-20	FAD/NAD(P)-binding oxidoreductase family protein
GH34_ARATH	10.72792987	4.88E-21	Indole-3-acetic acid-amido synthetase GH3.4
AT2G33830.2	10.47586152	6.46E-47	Dormancy-associated protein 1
AT3G22250.1	10.26413064	6.78E-15	UDP-glycosyltransferase 82A1
AT1G03390.1	10.1165397	4.83E-17	Hydroxycinnamoyl-CoA
AT5G22140.1	10.06960495	7.73E-14	Ferroptosis suppressor protein 1
AT2G23170.1	10.00567813	2.18E-43	Indole-3-acetic acid-amido synthetase GH3.3
AT1G17180.1	9.839362201	9.45E-13	Probable glutathione S-transferase parA
AT1G52340.1	9.834543701	1.78E-17	Borneol dehydrogenase, mitochondrial
AT1G59950.1	9.782181741	3.85E-15	NAD(P)H-dependent 6'-deoxychalcone synthase
E13B_PEA	9.521847061	7.56E-23	Glucan endo-1,3-beta-glucosidase
GH31_ARATH	9.471877623	2.38E-32	Probable indole-3-acetic acid-amido synthetase GH3.1
AT3G51000.1	9.383915107	5.33E-18	alpha/beta-Hydrolases superfamily protein
GSTX3_TOBAC	9.366004465	4.85E-18	Probable glutathione S-transferase
Down-regulated			
CB21_RAPSA	-11.38515356	2.57E-25	Chlorophyll a-b binding of LHCII type 1 protein
INO1_HORVU	-10.88410614	7.77E-25	Inositol-3-phosphate synthase
AT1G29920.1	-10.81601421	1.03E-61	Chlorophyll a-b binding protein 2
AT2G45550.1	-10.80321652	1.96E-10	Geraniol 8-hydroxylase
AT2G05070.1	-10.35586094	1.41E-208	Chlorophyll a-b binding protein 215

AT3G54500.3	-10.35060761	1.21E-80	Protein LNK2
AT2G05070.1	-9.973967664	9.45E-102	Chlorophyll a-b binding protein 215
INO1_POPEU	-9.61029084	3.69E-17	Inositol-3-phosphate synthase 1
INO1_NICPA	-9.490005713	1.84E-19	Inositol-3-phosphate synthase
CB23_SOLLC	-9.355364041	9.20E-85	Chlorophyll a-b binding protein 13
INO1_ARATH	-9.321656333	1.08E-16	Inositol-3-phosphate synthase isozyme 1
AT5G10170.1	-9.257311842	5.97E-16	Inositol-3-phosphate synthase
AT5G54270.1	-9.24327083	9.77E-99	Chlorophyll a-b binding protein 3
AT5G54270.1	-8.855291633	9.46E-93	Chlorophyll a-b binding protein 13
AT1G22360.1	-8.843300819	1.95E-13	Linamarin synthase 1
CB2G_SOLLC	-8.840081909	1.98E-16	Chlorophyll a-b binding protein 3C
AT1G22380.1	-8.646113724	1.99E-13	UDP-glycosyltransferase 85A8
AT2G15490.1	-8.608686525	7.14E-29	UDP-glucose flavonoid 3-O-glucosyltransferase 7
AT1G64780.1	-8.608432613	7.44E-27	Ammonium transporter 1 member 2
AT2G47460.1	-8.576072859	3.28E-23	Transcription factor MYB12

logFC= log 2 fold change, FDR= false discovery rate p-value.

Table 3.7: The top 20 up-regulated and down-regulated differentially expressed genes (DEGs) at 24 HAT relative to control in the "Durana" white clover plants treated with 4483.4 g ae h^{-1} of 2,4-D herbicide.

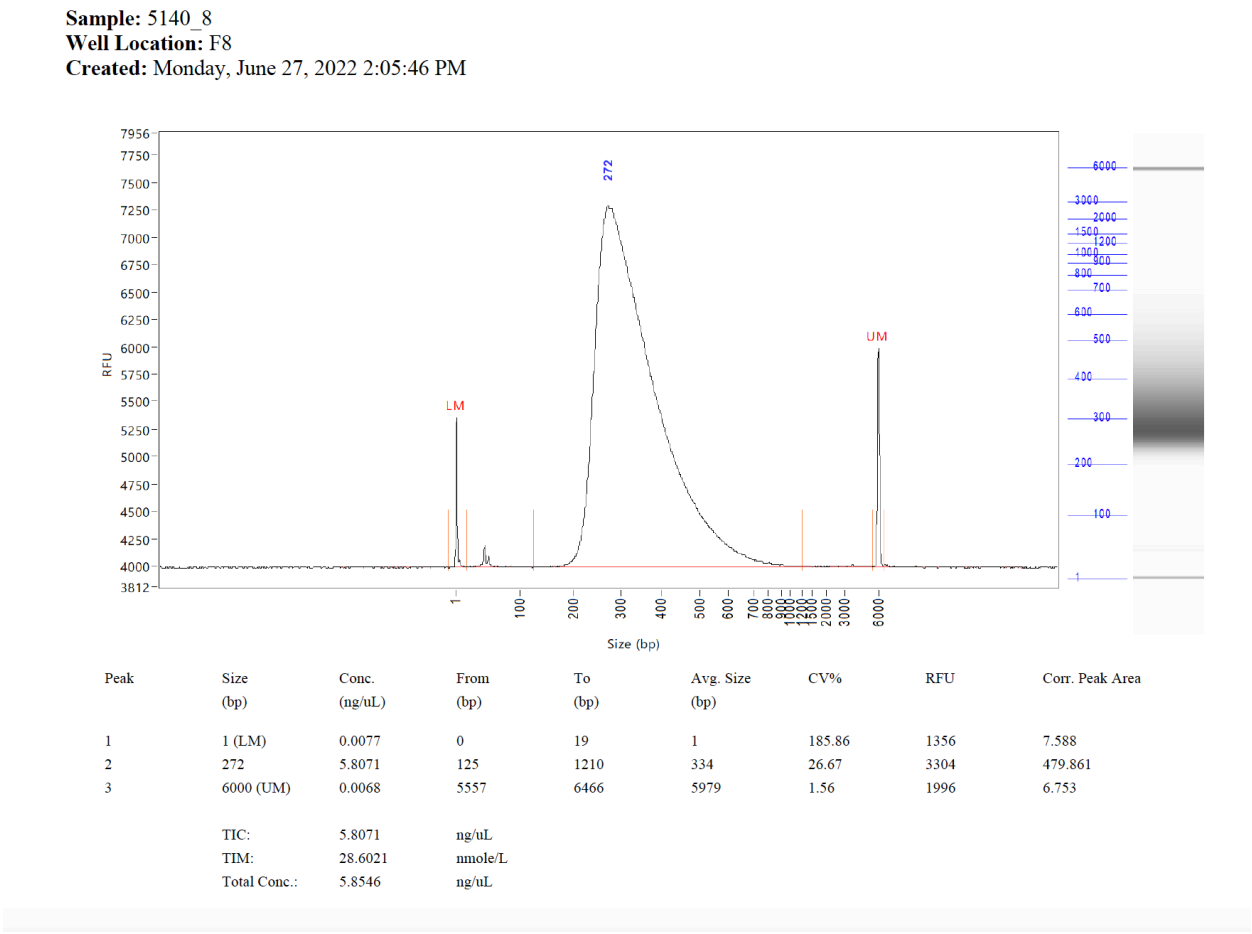
Gene ID	logFC	FDR	Function
Up-regulated			
AT3G51550.1	17.35575606	3.64E-12	Receptor-like protein kinase FERONIA

AT3G44190.1	12.71670809	7.64E-26	Ferroptosis suppressor protein 1
GH33_ARATH	11.86258611	3.97E-26	Indole-3-acetic acid-amido synthetase GH3.3
AT2G23170.1	11.37531543	3.17E-32	Indole-3-acetic acid-amido synthetase GH3.3
GH34_ARATH	10.64688543	2.44E-20	Indole-3-acetic acid-amido synthetase GH3.4
AT5G22140.1	10.60242706	2.60E-19	FAD/NAD(P)-binding oxidoreductase family protein
AT1G58170.1	10.57314363	4.26E-10	Dirigent protein 19
AT1G17180.1	10.48522953	4.07E-14	Probable glutathione S-transferase parA
AT3G22250.1	10.27138269	1.26E-14	UDP-glycosyltransferase 82A1
CORA_MEDSA	10.08007079	1.70E-19	Cold and drought-regulated protein CORA
GSTX3_TOBAC	10.00361381	3.74E-20	Probable glutathione S-transferase
DF230_PEA	9.971839367	2.77E-11	Defensin-like protein 230
AT2G23170.1	9.838064382	3.13E-41	Indole-3-acetic acid-amido synthetase GH3.3
AT1G10070.1	9.801111599	1.24E-14	Branched-chain-amino-acid aminotransferase 2
AT5G22140.1	9.754513551	9.14E-13	Ferroptosis suppressor protein 1
GH34_ARATH	9.696278214	4.93E-18	Indole-3-acetic acid-amido synthetase GH3.4
AT1G03390.1	9.576427428	5.04E-15	Hydroxycinnamoyl-CoA
AT4G20840.1	9.533254486	8.31E-10	Berberine bridge enzyme-like 21
AT3G09270.1	9.52366763	1.18E-09	Probable glutathione S-transferase

GH31_ARATH	9.508893038	8.50E-19	Probable indole-3-acetic acid-amido synthetase GH3.1
Down-regulated			
AT2G45550.1	-10.94361622	3.02E-11	Geraniol 8-hydroxylase
AT1G05260.1	-7.851068753	7.24E-09	Peroxidase 3
AT1G10330.1	-7.694499408	2.21E-09	Putative pentatricopeptide repeat-containing protein
AT3G13610.1	-7.549507585	1.81E-10	Feruloyl CoA ortho-hydroxylase F6H1-3
AT1G18370.1	-7.407092436	1.04E-12	Kinesin-like protein NACK1
AT5G66230.2	-6.840947254	2.75E-07	Chalcone-flavanone isomerase family protein
AT5G64570.1	-6.806789018	4.86E-05	Beta-xylosidase/alpha-L-arabinofuranosidase 2
AT3G27330.1	-6.730163144	1.83E-07	zinc finger (C3HC4-type RING finger) family protein
AT5G37660.2	-6.578142646	1.19E-06	Plasmodesmata-located protein 7
AT2G05790.1	-6.555670603	1.70E-17	Glucan endo-1,3-beta-glucosidase 14
AT5G25080.1	-6.524770814	3.89E-12	Nuclear nucleic acid-binding protein C1D
AT2G20870.1	-6.441076115	6.13E-06	cell wall protein precursor, putative
AT1G22360.1	-6.351885409	0.006735041	7-deoxyloganetin glucosyltransferase
AT1G63100.2	-6.334575198	1.64E-11	Scarecrow-like protein 28
AT1G80740.1	-6.323751473	1.14E-08	Putative DNA (cytosine-5)-methyltransferase CMT1
AT1G22360.1	-6.254317412	2.83E-09	Linamarin synthase 1
AT4G17560.1	-6.130656518	2.14E-05	Large ribosomal subunit protein bL19c

AT1G22380.1	-6.063140531	1.18E-11	UDP-glycosyltransferase 85A8
AT3G27970.1	-6.05918851	0.001094572	RNA exonuclease 4
AT3G15550.1	-5.942781274	2.16E-10	Trichohyalin

logFC= log 2 fold change, FDR= false discovery rate p-value.

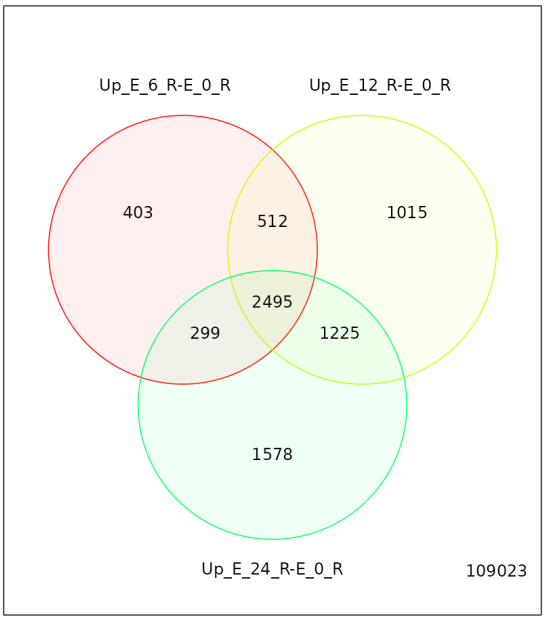


Peak	Size (bp)	Conc. (ng/uL)	From (bp)	To (bp)	Avg. Size (bp)	CV%	RFU	Corr. Peak Area
1	1 (LM)	0.0077	0	19	1	185.86	1356	7.588
2	272	5.8071	125	1210	334	26.67	3304	479.861
3	6000 (UM)	0.0068	5557	6466	5979	1.56	1996	6.753

TIC:	5.8071	ng/uL
TIM:	28.6021	nmole/L
Total Conc.:	5.8546	ng/uL

Figure 3.1: Example of Bioanalyzer electropherogram of cDNA library of white clover (*Trifolium repens*).

A



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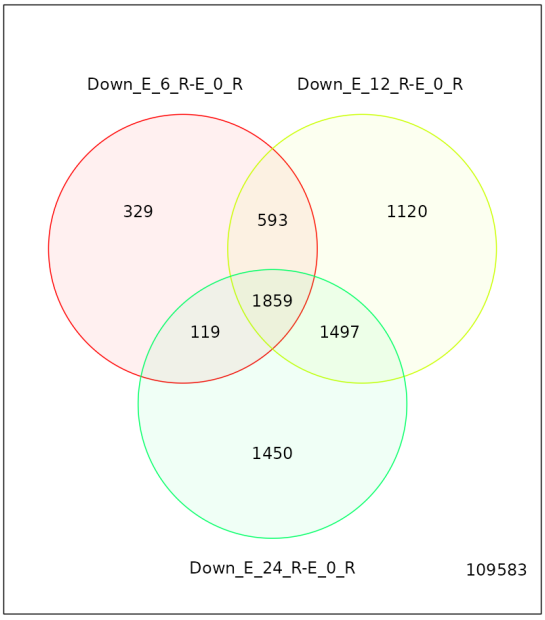
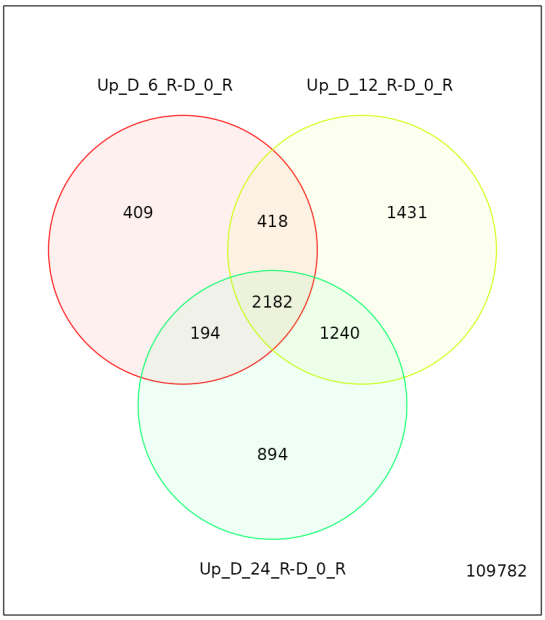


Figure 3.2: Venn diagrams showing transcripts that were either up or down regulated between the untreated condition and 6, 12, 24 hours after treatment (HAT) with 2,4-D in 2,4-D tolerant genotype Experimental cycle 6.

A. Venn diagram depicting shared and uniquely upregulated transcripts at 6 (Up_E_6_R-E_0_R), 12 (Up_E_12_R-E_0_R), and 24 (Up_E_24_R-E_0_R) HAT compared to control.

B. Venn diagram depicting shared and uniquely downregulated transcripts at 6 (Down_E_6_R-E_0_R), 12 (Down_E_12_R-E_0_R), and 24 (Down_E_24_R-E_0_R) HAT compared to control.

A



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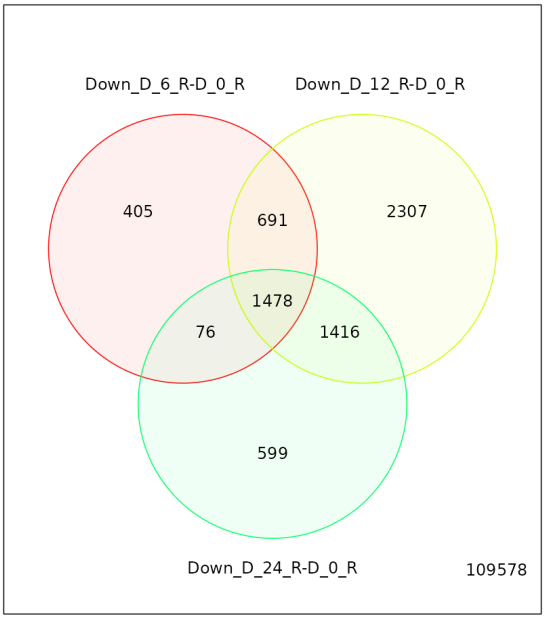
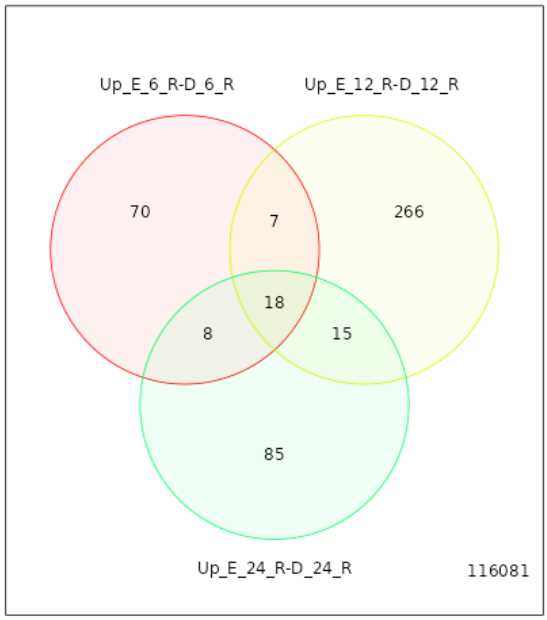


Figure 3.3: Venn diagrams showing transcripts that were either up or down regulated between the untreated condition and 6, 12, 24 hours after treatment (HAT) with 2,4-D in 2,4-D susceptible genotype "Durana".

A. Venn diagram depicting shared and uniquely upregulated transcripts at 6 (Up_D_6_R-D_0_R), 12 (Up_D_12_R-D_0_R), and 24 (Up_D_24_R-D_0_R) HAT compared to control.

B. Venn diagram depicting shared and uniquely downregulated transcripts at 6 (Down_D_6_R-D_0_R), 12 (Down_D_12_R-D_0_R), and 24 (Down_D_24_R-D_0_R) HAT compared to control.

A



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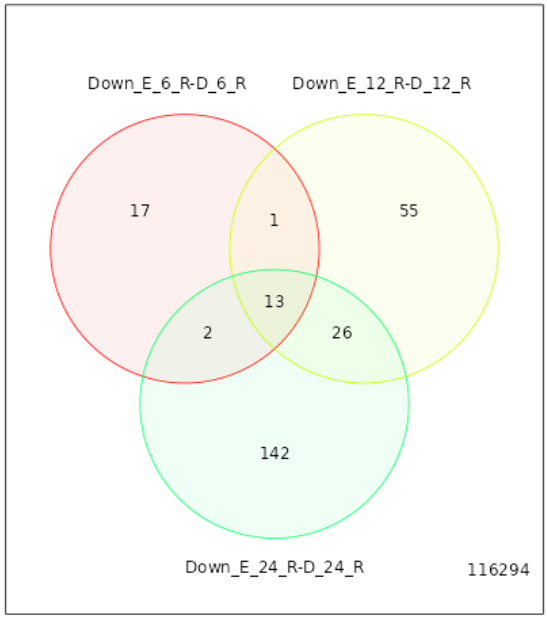
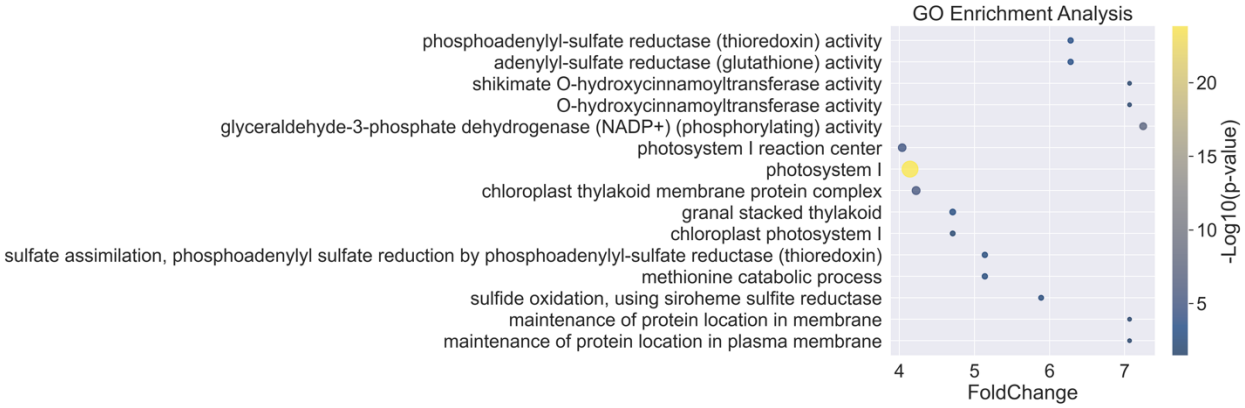
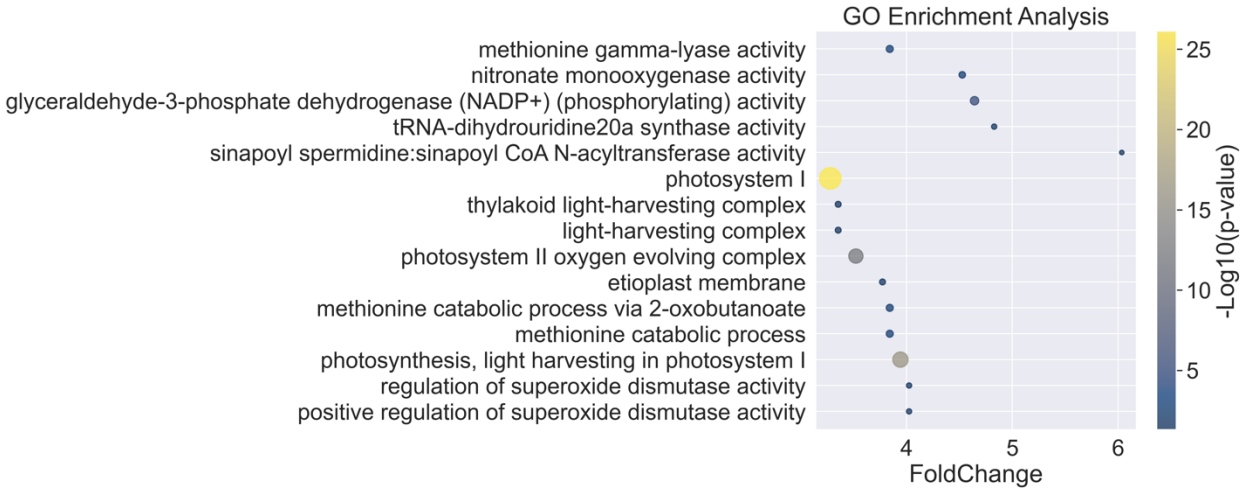


Figure 3.4: Venn diagrams showing transcripts that were either up or down regulated between the 2,4-D resusceptible genotype "Durana" and tolerant genotype Experimental cycle 6 at 6, 12, 24 hours after treatment (HAT) with 2,4-D.

A. Venn diagram depicting shared and uniquely upregulated transcripts at 6 HAT (Up_E_6_R-D_6_R), 12 HAT (Up_E_12_R-D_12_R), and 24 HAT (Up_E_24_R-D_24_R)

B. Venn diagram depicting shared and uniquely downregulated transcripts at 6 HAT (Down_E_6_R-D_6_R), 12 HAT (Down_E_12_R-D_12_R), and 24 HAT (Down_E_24_R-D_24_R).


GO Enrichment Analysis
phosphoadenylyl-sulfate reductase (thioredoxin) activity
adenylyl-sulfate reductase (glutathione) activity
shikimate O-hydroxycinnamoyltransferase activity
O-hydroxycinnamoyltransferase activity
glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) activity
photosystem I reaction center
photosystem I
chloroplast thylakoid membrane protein complex
granal stacked thylakoid
chloroplast photosystem I
sulfate assimilation, phosphoadenylyl sulfate reduction by phosphoadenylyl-sulfate reductase (thioredoxin)
methionine catabolic process
sulfide oxidation, using siroheme sulfite reductase
maintenance of protein location in membrane
maintenance of protein location in plasma membrane
4
5
6
7
FoldChange
20
15
10
5
-Log10(p-value)


GO Enrichment Analysis
methionine gamma-lyase activity
nitronate monooxygenase activity
glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) activity
tRNA-dihydrouridine20a synthase activity
sinapoyl spermidine:sinapoyl CoA N-acyltransferase activity
photosystem I
thylakoid light-harvesting complex
light-harvesting complex
photosystem II oxygen evolving complex
etioplast membrane
methionine catabolic process via 2-oxobutanoate
methionine catabolic process
photosynthesis, light harvesting in photosystem I
regulation of superoxide dismutase activity
positive regulation of superoxide dismutase activity
4
5
6
FoldChange
25
20
15
10
5
-Log10(p-value)

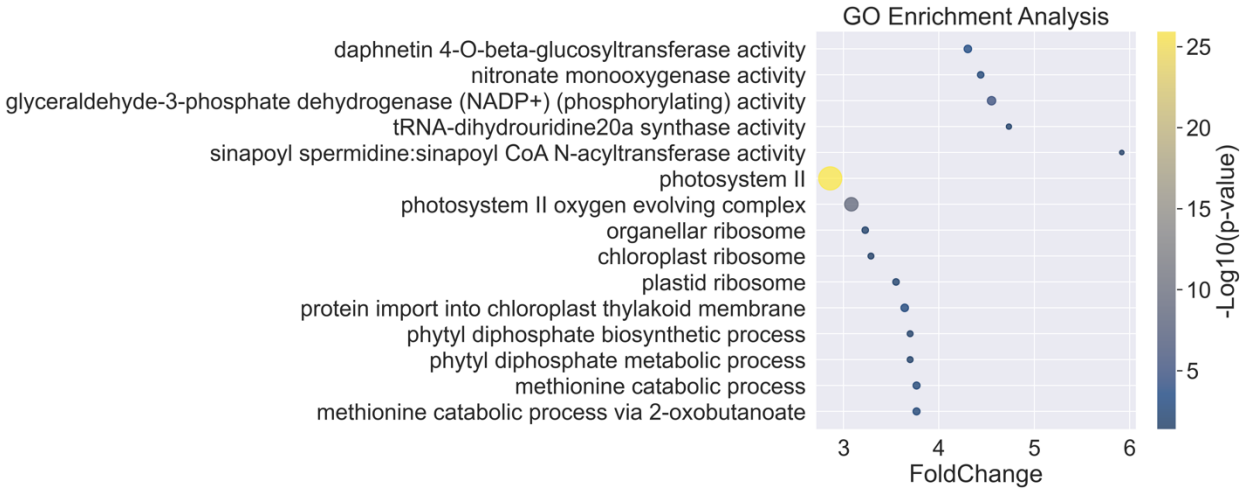
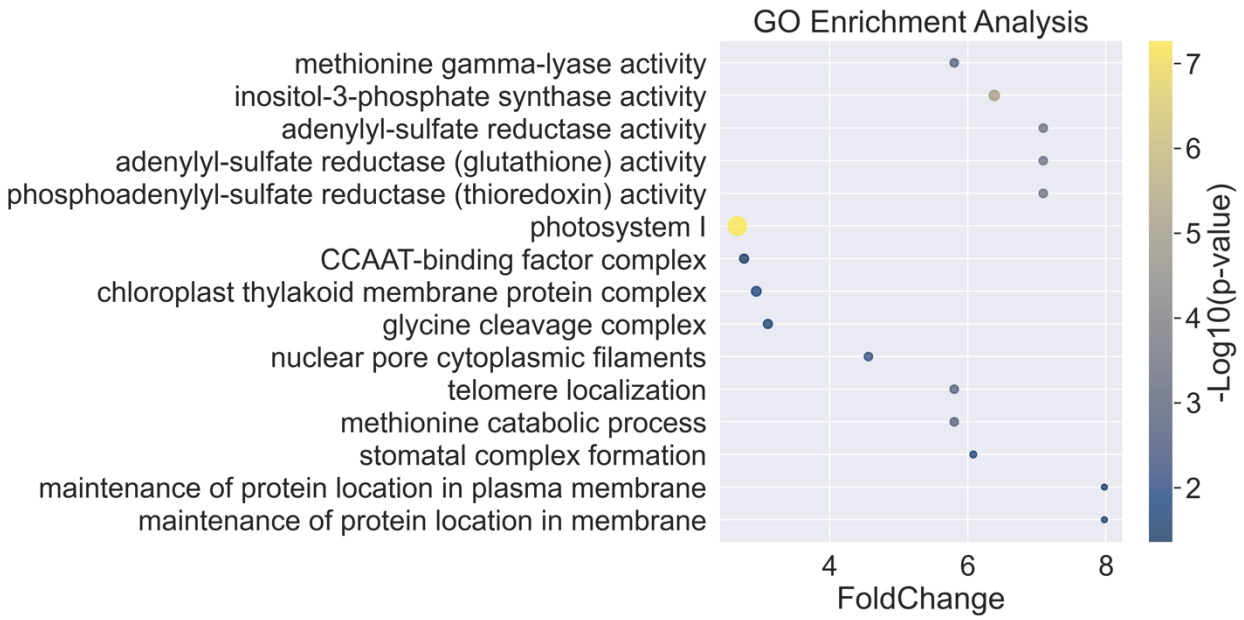
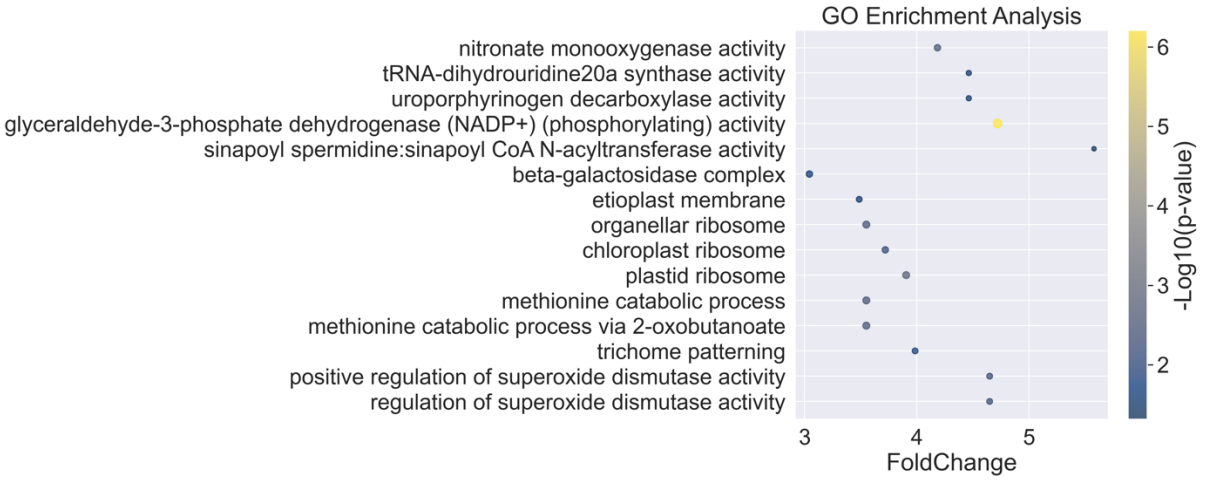

GO Enrichment Analysis
daphnetin 4-O-beta-glucosyltransferase activity
nitronate monooxygenase activity
glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) activity
tRNA-dihydrouridine20a synthase activity
sinapoyl spermidine:sinapoyl CoA N-acyltransferase activity
photosystem II
photosystem II oxygen evolving complex
organellar ribosome
chloroplast ribosome
plastid ribosome
protein import into chloroplast thylakoid membrane
phytyl diphosphate biosynthetic process
phytyl diphosphate metabolic process
methionine catabolic process
methionine catabolic process via 2-oxobutanoate
3
4
5
6
FoldChange
25
20
15
10
5
-Log10(p-value)

Figure 3.5: GO enrichment analysis of DEGs in Experimental cycle 6 genotype treated with 4483.4 g ae ha^{-1} rate of 2,4-D at different time points compared to control. A. GO at 6 HAT, B. GO at 12 HAT, C. GO at 24 HAT.





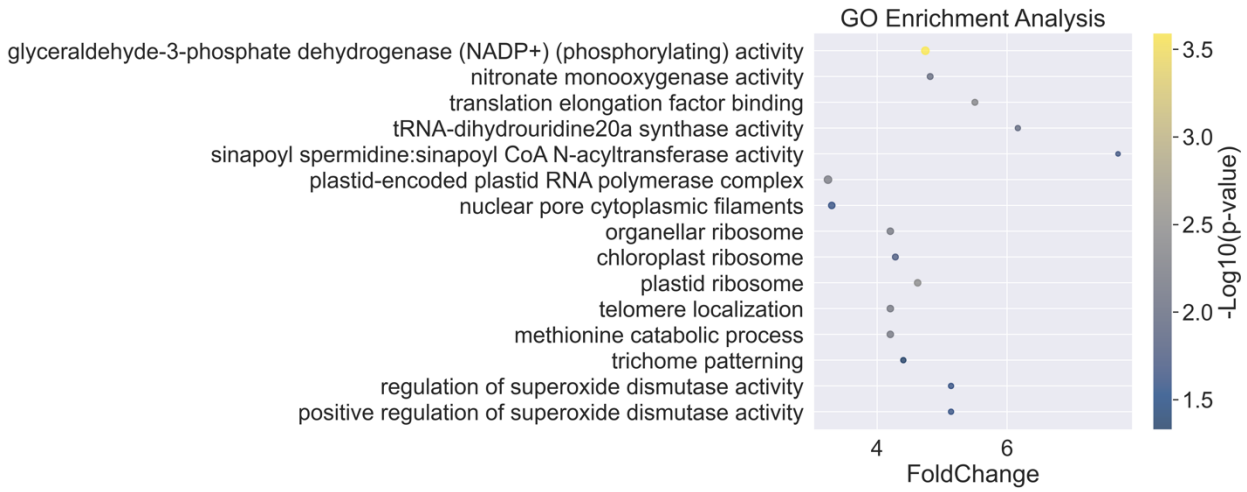


Figure 3.6: GO enrichment analysis of DEGs in Experimental cycle 6 genotype treated with 4483.4 g ae ha^{-1} rate of 2,4-D at different time points compared to control. A. GO at 6 HAT, B. GO at 12 HAT, C. GO at 24 HAT.

Supplemental Materials

Table S1: The top 20 functionally most enriched (UP) and depleted (Down) gene ontology (GO) terms at 6 HAT based on functional annotation of gene sets detected in experimental cycle six white clover genotypes treated with 4483.4 g ae ha^{-1} of herbicide 2,4-D.

GO Term	Adjusted p-value	Gene Count	-log(p-value)	Description	Category
Up-regulated					
GO:0006259	5.03E-34	24	33.2981278	DNA metabolic process	B
GO:0140640	3.35E-33	64	32.4755229	catalytic activity, acting on a nucleic acid	M
GO:0032991	3.84E-33	184	32.41523	protein-containing complex	C
GO:0140097	3.17E-32	16	31.4989544	catalytic activity, acting on DNA	M
GO:0034641	8.57E-30	238	29.0670462	cellular nitrogen compound metabolic process	B
GO:0090304	7.24E-29	137	28.1400625	nucleic acid metabolic process	B
GO:0006139	8.57E-29	184	28.0669582	nucleobase-containing compound metabolic process	B
GO:0050896	4.34E-27	850	26.3620935	response to stimulus	B
GO:0034061	5.66E-27	8	26.2472058	DNA polymerase activity	M
GO:0016779	3.67E-26	17	25.4357915	nucleotidyltransferase activity	M
GO:0004519	1.18E-25	24	24.926374	endonuclease activity	M
GO:0042221	2.59E-25	395	24.5870021	response to chemical	B
GO:0006749	1.17E-24	52	23.9299932	glutathione metabolic process	B

GO:0003964	4.85E-24	8	23.3143223	RNA-directed DNA polymerase activity	M
GO:0046483	6.08E-24	233	23.2161665	heterocycle metabolic process	B
GO:0015074	1.60E-22	9	21.7966961	DNA integration	B
GO:0008233	1.50E-20	53	19.8226984	peptidase activity	M
GO:0004364	1.50E-20	45	19.8226984	glutathione transferase activity	M
GO:1901360	2.18E-20	275	19.6622274	organic cyclic compound metabolic process	B
GO:0009755	1.44E-19	209	18.8402368	hormone-mediated signaling pathway	B
Down-regulated					
GO:0009507	2.86E-91	466	90.5439533	chloroplast	C
GO:0009536	1.34E-75	491	74.87379	plastid	C
GO:0042170	1.07E-72	248	71.9711618	plastid membrane	C
GO:0009521	3.64E-58	79	57.4385863	photosystem	C
GO:0034357	3.49E-57	156	56.4574714	photosynthetic membrane	C
GO:0042651	3.49E-57	156	56.4574714	thylakoid membrane	C
GO:0009535	3.49E-57	154	56.4574714	chloroplast thylakoid membrane	C
GO:0055035	3.49E-57	154	56.4574714	plastid thylakoid membrane	C
GO:0031968	1.03E-48	172	47.9865837	organelle outer membrane	C
GO:0019867	1.62E-48	172	47.7911584	outer membrane	C
GO:0009523	1.31E-43	60	42.8842802	photosystem II	C
GO:0009522	1.54E-39	51	38.8134899	photosystem I	C

GO:0009579	3.89E-36	97	35.4103276	thylakoid	C
GO:0009532	4.92E-36	169	35.307727	plastid stroma	C
GO:0009570	5.65E-36	168	35.2480052	chloroplast stroma	C
GO:0009765	1.32E-32	41	31.8790811	photosynthesis, light harvesting	B
GO:0004518	1.39E-31	17	30.8580116	nuclease activity	M
GO:0031976	1.37E-30	75	29.8635227	plastid thylakoid	C
GO:0009534	1.37E-30	75	29.8635227	chloroplast thylakoid	C
GO:0004519	7.45E-30	14	29.127726	endonuclease activity	M

Table S2: The top 20 functionally most enriched (UP) and depleted (Down) gene ontology (GO) terms at 12 HAT based on functional annotation of gene sets detected in experimental cycle six white clover genotypes treated with 4483.4 g ae ha^{-1} of herbicide 2,4-D.

GO Term	Adjusted p-value	Gene Count	-log(p-value)	Description	Category
Up-regulated					
GO:0034641	2.33E-63	267	62.6329293	cellular nitrogen compound metabolic process	B
GO:0090304	9.48E-61	143	60.0230079	nucleic acid metabolic process	B
GO:0006139	9.48E-61	201	60.0230079	nucleobase-containing compound metabolic process	B
GO:0046483	6.25E-54	259	53.2037978	heterocycle metabolic process	B
GO:1901360	2.78E-47	314	46.5551889	organic cyclic compound metabolic process	B

GO:0006725	1.71E-44	301	43.7679594	cellular aromatic compound metabolic process	B
GO:0140640	1.26E-43	94	42.9006165	catalytic activity, acting on a nucleic acid	M
GO:0006259	1.54E-42	41	41.81224	DNA metabolic process	B
GO:0050896	5.90E-42	1177	41.2290447	response to stimulus	B
GO:0032991	1.06E-41	266	40.9762328	protein-containing complex	C
GO:0140097	2.69E-41	27	40.5695554	catalytic activity, acting on DNA	M
GO:0006950	1.53E-36	913	35.8165292	response to stress	B
GO:0042221	5.18E-36	540	35.2857392	response to chemical	B
GO:0034061	5.32E-36	13	35.2738479	DNA polymerase activity	M
GO:0016779	6.01E-35	26	34.2210283	nucleotidyltransferase activity	M
GO:0004519	1.49E-34	35	33.8265548	endonuclease activity	M
GO:0003964	6.47E-33	12	32.1892242	RNA-directed DNA polymerase activity	M
GO:0015074	5.22E-31	13	30.2820006	DNA integration	B
GO:0004518	1.31E-29	52	28.8825564	nuclease activity	M
GO:0003676	2.44E-29	456	28.6131746	nucleic acid binding	M
Down-regulated					
GO:0009507	2.62E-228	909	227.581865	chloroplast	C
GO:0009536	1.37E-200	967	199.861973	plastid	C
GO:0042170	3.15E-132	428	131.501026	plastid membrane	C
GO:0009532	1.59E-107	358	106.79852	plastid stroma	C

GO:0009570	2.22E-107	356	106.653869	chloroplast stroma	C
GO:0042651	1.14E-104	269	103.944828	thylakoid membrane	C
GO:0034357	1.14E-104	269	103.944828	photosynthetic membrane	C
GO:0009535	1.29E-103	264	102.888497	chloroplast thylakoid membrane	C
GO:0055035	1.29E-103	264	102.888497	plastid thylakoid membrane	C
GO:0019867	3.04E-91	301	90.5168105	outer membrane	C
GO:0031968	4.92E-91	300	90.3076776	organelle outer membrane	C
GO:0009579	3.27E-73	175	72.4858979	thylakoid	C
GO:0009521	1.19E-65	101	64.9253277	photosystem	C
GO:0009941	1.34E-58	228	57.8725408	chloroplast envelope	C
GO:0009526	5.59E-57	228	56.2529599	plastid envelope	C
GO:0031976	8.41E-53	125	52.0749718	plastid thylakoid	C
GO:0009534	8.41E-53	125	52.0749718	chloroplast thylakoid	C
GO:0009523	9.01E-52	79	51.0451697	photosystem II	C
GO:0031967	3.26E-42	235	41.4873572	organelle envelope	C
GO:0031975	3.26E-42	235	41.4873572	envelope	C

Table S3: The top 20 functionally most enriched (UP) and depleted (Down) gene ontology (GO) terms at 24 HAT based on functional annotation of gene sets detected in experimental cycle six white clover genotypes treated with 4483.4 g ae ha^{-1} of herbicide 2,4-D.

GO Term	Adjusted p-value	Gene Count	-log(p-value)	Description	Category
Up-regulated					
GO:0034641	1.32E-44	334	43.8794195	cellular nitrogen compound metabolic process	B
GO:0006139	2.05E-43	257	42.6879552	nucleobase-containing compound metabolic process	B
GO:0006259	7.45E-41	47	40.1277536	DNA metabolic process	B
GO:0090304	1.07E-36	209	35.9725117	nucleic acid metabolic process	B
GO:0140097	1.07E-36	36	35.9725117	catalytic activity, acting on DNA	M
GO:0046483	2.38E-36	326	35.6225882	heterocycle metabolic process	B
GO:0042221	1.52E-30	538	29.8183718	response to chemical	B
GO:1901360	1.94E-30	388	29.7116836	organic cyclic compound metabolic process	B
GO:0032991	3.04E-30	318	29.5167237	protein-containing complex	C
GO:0004519	1.56E-29	46	28.8082643	endonuclease activity	M
GO:0006725	3.74E-29	370	28.4265533	cellular aromatic compound metabolic process	B
GO:0050896	8.47E-29	1155	28.0720592	response to stimulus	B
GO:0003676	4.07E-28	484	27.3903416	nucleic acid binding	M

GO:0140640	4.55E-28	136	27.3420636	catalytic activity, acting on a nucleic acid	M
GO:0034061	8.05E-28	25	27.0941994	DNA polymerase activity	M
GO:0003964	1.15E-27	20	26.9401276	RNA-directed DNA polymerase activity	M
GO:0015074	5.76E-26	21	25.2393972	DNA integration	B
GO:0006950	5.27E-24	885	23.278518	response to stress	B
GO:0008233	6.27E-24	89	23.2024126	peptidase activity	M
GO:0016779	6.98E-24	46	23.1561233	nucleotidyltransferase activity	M
Down-regulated					
GO:0009507	1.67E-169	804	168.776105	chloroplast	C
GO:0009536	1.29E-150	866	149.889431	plastid	C
GO:0042170	9.36E-105	384	104.028556	plastid membrane	C
GO:0034357	8.97E-85	242	84.0470024	photosynthetic membrane	C
GO:0042651	8.97E-85	242	84.0470024	thylakoid membrane	C
GO:0055035	1.27E-83	237	82.8966871	plastid thylakoid membrane	C
GO:0009535	1.27E-83	237	82.8966871	chloroplast thylakoid membrane	C
GO:0009532	1.77E-74	304	73.7521513	plastid stroma	C
GO:0009570	3.26E-74	302	73.4862016	chloroplast stroma	C
GO:0031968	5.87E-74	272	73.2313581	organelle outer membrane	C
GO:0019867	1.32E-73	272	72.8793654	outer membrane	C
GO:0009579	1.70E-58	156	57.7686842	thylakoid	C
GO:0009521	9.83E-56	92	55.0075413	photosystem	C

GO:0009523	4.80E-45	73	44.3189247	photosystem II	C
GO:0009941	8.72E-44	201	43.0596982	chloroplast envelope	C
GO:0009526	1.90E-42	201	41.7211699	plastid envelope	C
GO:0031976	1.86E-39	108	38.7302754	plastid thylakoid	C
GO:0009534	1.86E-39	108	38.7302754	chloroplast thylakoid	C
GO:0009058	3.14E-35	831	34.5029792	biosynthetic process	B
GO:1901576	3.65E-35	807	34.4375891	organic substance biosynthetic process	B

Table S4: The top 20 functionally most enriched (UP) and depleted (Down) gene ontology (GO) terms at 6 HAT based on functional annotation of gene sets detected in "Durana" white clover genotypes treated with 4483.4 g ae ha^{-1} of herbicide 2,4-D.

GO Term	Adjusted p-value	Gene Count	-log(p-value)	Description	Category
Up-regulated					
GO:0032991	1.23E-48	136	47.91073194	protein-containing complex	C
GO:0006139	1.85E-48	126	47.73361542	nucleobase-containing compound metabolic process	B
GO:0090304	4.77E-48	88	47.32128981	nucleic acid metabolic process	B
GO:0034641	5.64E-45	184	44.24875833	cellular nitrogen compound metabolic process	B
GO:0046483	1.64E-36	182	35.78495823	heterocycle metabolic process	B

GO:0140640	2.88E-36	54	35.54029519	catalytic activity, acting on a nucleic acid	M
GO:0042221	7.72E-36	415	35.11246162	response to chemical	B
GO:0043170	7.17E-35	487	34.14430414	macromolecule metabolic process	B
GO:0006259	4.67E-34	22	33.33089871	DNA metabolic process	B
GO:0009755	9.26E-32	234	31.03330893	hormone-mediated signaling pathway	B
GO:0050896	4.60E-30	831	29.33703559	response to stimulus	B
GO:0140097	8.63E-30	17	29.06379027	catalytic activity, acting on DNA	M
GO:0006749	1.74E-29	56	28.76043893	glutathione metabolic process	B
GO:1901360	2.23E-29	229	28.65202486	organic cyclic compound metabolic process	B
GO:0006725	9.18E-29	216	28.03722803	cellular aromatic compound metabolic process	B
GO:0016779	5.44E-28	13	27.26429224	nucleotidyltransferase activity	M
GO:0010033	2.51E-26	322	25.60082277	response to organic substance	B
GO:0006807	3.72E-26	613	25.42954185	nitrogen compound metabolic process	B
GO:0004519	3.74E-26	21	25.4275375	endonuclease activity	M
GO:0034061	1.13E-25	8	24.94694245	DNA polymerase activity	M
Down-regulated					
GO:0009507	2.53E-25	281	24.59760295	chloroplast	C
GO:0004519	2.53E-25	10	24.59760295	endonuclease activity	M

GO:0043531	3.04E-23	2	22.51716922	ADP binding	M
GO:0004518	3.13E-23	17	22.5051129	nuclease activity	M
GO:0042170	1.32E-22	143	21.87833713	plastid membrane	C
GO:0009536	2.69E-22	310	21.56986221	plastid	C
GO:0015074	6.91E-22	2	21.16034664	DNA integration	B
GO:0003964	3.99E-21	3	20.3986391	RNA-directed DNA polymerase activity	M
GO:1901576	2.54E-20	417	19.59455475	organic substance biosynthetic process	B
GO:0009058	1.14E-19	426	18.94433602	biosynthetic process	B
GO:0034061	2.77E-18	8	17.55742142	DNA polymerase activity	M
GO:0140640	7.57E-18	64	17.12116891	catalytic activity, acting on a nucleic acid	M
GO:0006457	1.12E-16	78	15.95238231	protein folding	B
GO:0044249	4.35E-16	376	15.36112423	cellular biosynthetic process	B
GO:0098542	5.49E-16	46	15.26017876	defense response to other organism	B
GO:0009522	5.49E-16	29	15.26017876	photosystem I	C
GO:0009521	1.76E-15	36	14.75486335	photosystem	C
GO:0006952	3.56E-14	84	13.44904576	defense response	B
GO:0070001	3.56E-14	12	13.44904576	aspartic-type peptidase activity	M
GO:0004190	3.56E-14	12	13.44904576	aspartic-type endopeptidase activity	M

Table S5: The top 20 functionally most enriched (UP) and depleted (Down) gene ontology (GO) terms at 12 HAT based on functional annotation of gene sets detected in "Durana" white clover genotypes treated with 4483.4 g ae ha^{-1} of herbicide 2,4-D.

GO Term	Adjusted p-value	Gene Count	-log(p-value)	Description	Category
Up-regulated					
GO:0090304	2.94E-70	136	69.53101785	nucleic acid metabolic process	B
GO:0034641	8.51E-70	271	69.07011052	cellular nitrogen compound metabolic process	B
GO:0006139	3.79E-69	198	68.42178119	nucleobase-containing compound metabolic process	B
GO:0046483	6.34E-61	259	60.19824715	heterocycle metabolic process	B
GO:0032991	1.55E-60	234	59.8104166	protein-containing complex	C
GO:1901360	6.26E-51	323	50.20333867	organic cyclic compound metabolic process	B
GO:0140640	1.85E-49	91	48.73170653	catalytic activity, acting on a nucleic acid	M
GO:0006725	2.56E-49	306	48.592141	cellular aromatic compound metabolic process	B
GO:0050896	2.04E-42	1220	41.68979869	response to stimulus	B
GO:0006259	7.84E-41	48	40.10563912	DNA metabolic process	B
GO:0006950	4.92E-35	938	34.30814213	response to stress	B
GO:0140097	2.07E-34	40	33.68362224	catalytic activity, acting on DNA	M

GO:0003676	2.07E-34	460	33.68362224	nucleic acid binding	M
GO:0003723	6.34E-34	121	33.19771208	RNA binding	M
GO:0043228	6.35E-33	83	32.19707811	non-membrane-bounded organelle	C
GO:0043232	6.35E-33	83	32.19707811	intracellular non-membrane-bounded organelle	C
GO:0006952	4.64E-32	561	31.33354591	defense response	B
GO:0016779	1.13E-30	35	29.94677386	nucleotidyltransferase activity	M
GO:0042221	2.73E-30	536	29.56307486	response to chemical	B
GO:0016070	4.32E-29	88	28.36483956	RNA metabolic process	B
Down-regulated					
GO:0009507	7.10E-197	903	196.1486644	chloroplast	C
GO:0009536	1.72E-172	967	171.7648886	plastid	C
GO:0042170	2.42E-105	407	104.6161782	plastid membrane	C
GO:0009570	3.71E-88	343	87.43065405	chloroplast stroma	C
GO:0009532	7.99E-88	344	87.09756459	plastid stroma	C
GO:0009535	8.83E-86	252	85.05428078	chloroplast thylakoid membrane	C
GO:0055035	8.83E-86	252	85.05428078	plastid thylakoid membrane	C
GO:0034357	1.94E-85	255	84.71327838	photosynthetic membrane	C
GO:0042651	1.94E-85	255	84.71327838	thylakoid membrane	C
GO:0019867	6.11E-72	285	71.21430546	outer membrane	C
GO:0031968	9.30E-72	284	71.03135725	organelle outer membrane	C

GO:0005840	2.48E-64	214	63.60532165	ribosome	C
GO:1901576	2.36E-59	969	58.62729169	organic substance biosynthetic process	B
GO:0009058	2.69E-57	989	56.57063845	biosynthetic process	B
GO:0009579	3.09E-57	162	56.5101514	thylakoid	C
GO:0043232	9.80E-57	585	56.00893002	intracellular non-membrane-bounded organelle	C
GO:0043228	9.80E-57	585	56.00893002	non-membrane-bounded organelle	C
GO:0015074	2.25E-53	5	52.64877823	DNA integration	B
GO:0003964	4.96E-53	6	52.30489536	RNA-directed DNA polymerase activity	M
GO:0006412	1.17E-52	234	51.93215477	translation	B

Table S6: The top 20 functionally most enriched (UP) and depleted (Down) gene ontology (GO) terms at 24 HAT based on functional annotation of gene sets detected in "Durana" white clover genotypes treated with 4483.4 g ae ha^{-1} of herbicide 2,4-D.

GO Term	Adjusted p-value	Gene Count	-log(p-value)	Description	Category
Up-regulated					
GO:0032991	2.23E-70	162	69.65101903	protein-containing complex	C
GO:0090304	4.65E-70	101	69.33285192	nucleic acid metabolic process	B
GO:0006139	3.43E-69	151	68.4645164	nucleobase-containing compound metabolic process	B

GO:0034641	2.18E-67	217	66.66126696	cellular nitrogen compound metabolic process	B
GO:0046483	8.20E-52	224	51.08601173	heterocycle metabolic process	B
GO:1901360	7.17E-43	280	42.14441912	organic cyclic compound metabolic process	B
GO:0006725	4.97E-42	264	41.30337484	cellular aromatic compound metabolic process	B
GO:0003676	5.16E-40	359	39.28709952	nucleic acid binding	M
GO:0140640	1.00E-39	83	38.99960352	catalytic activity, acting on a nucleic acid	M
GO:0050896	2.26E-38	1062	37.64596655	response to stimulus	B
GO:0006259	6.81E-38	37	37.16707476	DNA metabolic process	B
GO:0042221	8.43E-37	501	36.07440446	response to chemical	B
GO:0003723	8.99E-33	96	32.0463166	RNA binding	M
GO:0140097	1.76E-32	30	31.75542782	catalytic activity, acting on DNA	M
GO:0071840	5.06E-31	193	30.29584263	cellular component organization or biogenesis	B
GO:0016070	1.12E-30	64	29.95056322	RNA metabolic process	B
GO:0016043	1.41E-28	187	27.85130647	cellular component organization	B
GO:1902494	6.46E-28	60	27.19004467	catalytic complex	C
GO:0006950	2.71E-27	797	26.56650378	response to stress	B
GO:0043228	2.71E-27	73	26.56650378	non-membrane-bounded organelle	C
Down-regulated					

GO:0009507	5.89E-126	601	125.2295584	chloroplast	C
GO:0009536	2.34E-110	643	109.630885	plastid	C
GO:0042170	3.64E-68	273	67.43849647	plastid membrane	C
GO:1901576	3.06E-53	695	52.51364528	organic substance biosynthetic process	B
GO:0034357	3.06E-53	169	52.51364528	photosynthetic membrane	C
GO:0042651	3.06E-53	169	52.51364528	thylakoid membrane	C
GO:0009535	6.68E-53	166	52.17526553	chloroplast thylakoid membrane	C
GO:0055035	6.68E-53	166	52.17526553	plastid thylakoid membrane	C
GO:0009058	1.27E-52	711	51.89602972	biosynthetic process	B
GO:0005840	2.29E-51	158	50.63955928	ribosome	C
GO:0031968	1.03E-46	191	45.98758919	organelle outer membrane	C
GO:0019867	1.71E-46	191	45.76639263	outer membrane	C
GO:0009570	1.65E-44	208	43.78152351	chloroplast stroma	C
GO:0044249	2.99E-44	629	43.52457346	cellular biosynthetic process	B
GO:0009532	5.48E-44	208	43.26082617	plastid stroma	C
GO:0006412	3.07E-42	171	41.51311253	translation	B
GO:0043043	1.50E-40	173	39.82505371	peptide biosynthetic process	B
GO:0003735	2.76E-40	155	39.55944509	structural constituent of ribosome	M
GO:0005198	5.07E-40	205	39.29495708	structural molecule activity	M
GO:0043604	3.23E-39	199	38.49126392	amide biosynthetic process	B

CHAPTER 4

CONCLUSION

The dose-response experiments conducted in greenhouse and field conditions have provided valuable insights into the effectiveness of EMS mutatagenesis and recurrent selection in increasing 2,4-D tolerance in white clover. The results from the greenhouse trial demonstrated that the experimental cycle 6 mutant population exhibited significantly higher survivor rates and biomass reduction compared to other cultivars, even at higher doses of 2,4-D herbicide. This indicates that EMS mutation and recurrent selection have successfully enhanced 2,4-D tolerance in the white clover population, consistent with findings in previous studies on other crops and weed species. Furthermore, the field trial results reinforced the increased tolerance observed in the greenhouse, demonstrating that the enhanced tolerance observed in controlled conditions translates effectively to real-world field scenarios. The higher GR_{50} value in the field trial compared to the greenhouse trial underscores the robustness of the mutant population's tolerance to 2,4-D herbicide in diverse environments. However, the study also highlighted the complexities involved in genetic gain trials. The decrease in genetic gain observed in cycle 3 was attributed to specific selections made during the breeding process, emphasizing the importance of meticulous parent selection in breeding programs. Factors such as heritability, selection intensity, and phenotypic variation were identified as crucial variables influencing the efficacy of genetic gain. Overall, this research provides compelling evidence supporting the utility of EMS mutation and recurrent selection as potent strategies for enhancing herbicide tolerance in white clover. The findings underscore the adaptability of these methods across different environmental conditions and emphasize the need

for careful consideration of various factors in breeding programs to optimize the selection of desired traits for future generations.

We also investigated the genes responsible for 2,4-D resistance in Experimental cycle 6 genotypes. We performed a time course transcriptome analysis experiment to determine the differentially expressed genes at 6 HAT, 12 HAT, and 24 HAT compared to the non-treated control. To our knowledge, this is the first attempt to investigate the transcriptome in 2,4-D resistant white clover. Many genes involved in auxin response were upregulated at all time points, even in both resistant and susceptible genotypes. However, 70, 266, and 85 genes were upregulated in the resistant genotype compared to the treated susceptible genotype at 6 HAT, 12 HAT, and 24 HAT, respectively, many of which are involved in metabolism, hormone signaling, and gene expression. Additionally, 17, 55, and 142 genes were downregulated at 6 HAT, 12 HAT, and 24 HAT, respectively, many of which are involved in photosynthesis or response to hormones. The vast data set generated from the transcriptomic effort will continue to be a resource for future work on the gene expression profiles uniquely induced by 2,4-D herbicide and other synthetic auxin herbicides. Finally, this knowledge will help plant breeders to develop synthetic auxin herbicide-resistant crop varieties.

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