ROLE OF WRKY TRANSCRIPTION FACTORS IN QUANTITATIVE RESISTANCE TO *SCLEROTINIA SCLEROTIORUM*

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Sclerotinia sclerotiorum, a necrotrophic fungal pathogen, infects more than 400 plant species globally, resulting in significant yield loss in the US annually. Challenges in controlling these diseases arise from the quantitative resistance displayed by host plants, with most commercial crop varieties lacking adequate resistance. Previous GWAS identified several WRKY transcription factors associated with *S. sclerotiorum* resistance. Preliminary evaluations of T-DNA insertional mutants indicated that *wrky3* and *wrky4* mutants are hypersusceptible to S. sclerotiorum while *wrky27* mutants exhibited increased resistance. This study aimed to elucidate *WRKY3*, *4*, and *27* roles in *S. sclerotiorum* resistance. Overexpressing *WRKY4* in arabidopsis showed increased resistance against *S. sclerotiorum*. The differential expression analysis revealed *WRKY27* downregulation during infection. Additionally, sunflower orthologs displayed differential expression notably *HaWRKY3-1,3-2,3-3* and *HaWRKY27* upregulation in resistant lines. These findings contribute to understanding the molecular mechanisms underlying host resistance to *S. sclerotiorum*, offering insights for enhancing host resistance against this pathogen.

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DEDICATION

To my parents for their endless support, guidance, and encouragement throughout my life and at every phase of my academic and professional development. To my husband whose patience, love, support, and encouragement enabled me to complete this thesis.

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CHAPTER 1: BACKGROUND AND REVIEW OF LITERATURE

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a polyphagous necrotrophic fungal pathogen that infects over 400 plant species worldwide including economically important crops like sunflower, soybean, rapeseed and alfalfa (Mei et al., 2011). More than sixty names are often used to denote the pathogen such as cottony rot, watery soft rot, stem rot, drop, crown rot, blossom blight and, perhaps most common, white mold (Bolton et al., 2006). White mold is a common name for the *S. sclerotiorum* disease, as when the fungus affects plant cells and causes damage to plant tissue, plants exhibit soft rot or white mold in affected plants parts (Mei et al., 2011; Kamal et al., 2016). The annual losses caused by this disease in the United States have surpassed $200 million (Bolton et al.,2006). During the year 2000, North Dakota and Minnesota lost $24.5 million in canola income due to *Sclerotinia sclerotiorum* (Kamal et al., 2016). There have been reports of considerable yield losses due to sclerotinia stem rot (SSR) of canola in countries all over the world, including Australia and China (Wu et al., 2013; Kamal et al., 2016). Diseases caused by *S. sclerotiorum* have historically been difficult to control due to a lack of high-level resistance in important crops and wide host range making traditional breeding approaches like the use of organic soil amendments, soil sterilization, zero tillage and crop rotation, tillage and irrigation difficult to enhance resistance in the host plant (Kamal et al., 2016; Wang et al.,2019). Host plant shows a complex defense mechanism against necrotrophic *S. sclerotiorum* as plant exhibits quantitative disease resistance. Attempts have been made to find SSR-resistant genotypes, but no entirely resistant commercial crop cultivars have yet been developed (Kamal et al., 2016).

Sclerotinia sclerotiorum taxonomy, nomenclature and biology

Sclerotinia sclerotiorum was formerly named *Peziza sclerotiorum* by Madame M. A. Libert in 1837. This binomial was used until *Peziza sclerotiorum* Lib. and *S. sclerotii* Fuckel were listed as synonyms and the species was moved to the new genus *Sclerotinia* by Fuckel. Hence the name *Sclerotinia libertania* Fuckel was given by Fuckel and used until Wakefield showed that it was against the International Rules of Botanical Nomenclature (Purdy 1979; Bolton et al., 2006). de Bary first used the name *Sclerotinia sclerotiorum* (Lib.) de Bary in 1884 and in 1979, Purdy concluded that the fungus's correct name and authority should be *Sclerotinia sclerotiorum* (Lib.) de Bary (Bolton et al., 2006). Buchwald and Neergaard recommended the conservation of the genus' type species, *Sclerotinia sclerotiorum* (Lib.) de Bary, in 1976. It was approved as a preserved name in 1981 (Bolton et al., 2006).

Sclerotinia sclerotiorum's taxonomic classification, like that of many other organisms, is based on its morphology, anatomy, and genome sequence relatedness.

Kingdom: Fungi

Division: Ascomycota

Class: Leotiomycetes

Order: Heliotales

Family: Sclerotiniaceae

Genus: *Sclerotinia*

Species: *sclerotiorum* (Lib.) de Bary

The Sclerotiniaceae are a family of fungi found in the phylum Ascomycota's order Helotiales. The characteristic shared by all Sclerotiniaceae members is the development of a sclerotial stroma, or a melanized hyphal aggregation. Since Whetzel delineated the

Sclerotiniaceae, other taxonomic criteria have been applied over time, including rRNA gene sequences, the properties of sterile tissues of both apothecia and sclerotia, sclerotial ontogeny, histochemistry, and ultrastructure of sclerotia (Bolton et al., 2006).

As *Sclerotinia sclerotiorum* produces sclerotia, apothecia, and ascospores; the ascomata, and asci are used in its classification. Ascomata is the structure in which asci are borne and asci are the structure that carries the ascospores. Based on this, *Sclerotinia sclerotiorum* has been classified under the subdivision Ascomycota. Apothecia is a disc or cup-shaped structure that is covered with a hymenium. That is why it's under the Discomycetes class. Due to the presence of inoperculate asci on apothecia, the pathogen becomes a member of the Leotiales order. *Sclerotinia sclerotiorum* produces apothecia which are supported by a funnel-like part of the stem called stipe. Because of this character, it's in the family Sclerotiniaceae.

Sclerotinia sclerotiorum **host range**

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating pathogen that causes disease in a wide host range that comprises at least 408 described species of plants from 278 genera in 75 families (Boland and Hall,1994). It infects dicotyledonous plants such as sunflower (*Helianthus annuus*), oilseed rape (*Brassica napus*), edible dry bean (*Phaseolus vulgaris*), soybean (*Glycine max*), dry pea (*Pisum sativum*), chickpea (*Cicer arietinum*), lentils (*Lens culinaris*), peanut (*Arachis hypogaea*), some vegetable crops such as broccoli, cabbage, cauliflower, carrots, celery, etc and in monocotyledonous crops such as onion (*Allium cepa*) and tulip (*Tulipa gesneriana*) (Boland and Hall, 1994; Bolton et al., 2006). *Sclerotinia sclerotiorum* was first detected in 1861 from diseased sunflower (Kamal et al., 2016). In 1915, Shaw and Ajrekar first documented the rapeseed-mustard stem rot disease in 1915 (Kamal et al., 2016). The herbaceous, succulent plants especially flowers and vegetables are a common host for sclerotinia stem rot. High tunnel

vegetable crops like lettuce, tomato, and pepper are all impacted by this disease. The condition is often referred to as timber rot in tomato (Ohio State University, "Sclerotinia White Mold"). sclerotinia stem rot can attack its host plant at any stage of growth that includes mature host plant, and harvested product but most commonly occurs during flowering toward the end of a plant's lifecycle. It is primarily found in tissues that are rich in moisture content and close to the soil. In woody ornamental plants like camelia, it attacks very rarely, typically on juvenile tissue.

Disease cycle and epidemiology

The majority of *Sclerotinia sclerotiorum*'s life cycle is spent as sclerotia (asexual resting propagule) in the soil. Sclerotia are hard-walled, melanized resting structures that survive long-term. Following harvest, the sclerotia from infected plants can be incorporated into the soil, providing a supply of inoculum for future years (Kamal et al., 2016). Sclerotia can germinate myceliogenically or carpogenically during growing season under favorable condition (Sun and Yang, 2000; Kamal et al., 2016).

During carpogenic germination, sclerotia produce apothecial initials (stipes) which later on develop into a cup shape apothecium where the ascospores are formed (Willetes and Wong, 1980). Carpogenic germination also enables sexual recombination which makes carpogenic germination a very important event of this pathogen's life cycle (Nepal and del Río Mendoza, 2012). Apothecia produces spores that can disseminate over several kilometers through air currents (Kamal et al., 2016). *Sclerotinia sclerotiorum* primarily infects host plants via these airborne ascospores (Abawi and Grogan,1979, Schwartz and Steadman,1978). Ascospores take up to five to ten days to get released in field condition (Phillips, 1987). In several crops, ascospores serve as the main inoculum for epidemics. Ascospore generation by apothecia in sclerotinia rot is crucial for epidemics to start (Sun and Yang, 2000).

During myceliogenic germination, sclerotia produce mycelium which consists of fungal thread or hyphae. Mycelia growing from sclerotia or ascospores released into the air by apothecia are what start infections (Kamal er. Al., 2016). Direct mycelial germination allows sclerotia to infect the stem's base. 2–3 weeks after infection, infected and senescing petals lodge on leaves, leaf axils, or stem branches, where they begin infection as water-soaked tan-colored lesions or regions of very light brown discoloration on the leaves, main stems, and branches. Lesions become greyish white, covering most plant sections, and eventually bleached, shredding and breaking. Sclerotia develop when the fungal mycelia aggregates at the end of the growth season. Under ideal environmental conditions, these sclerotia return to the soil on crop wastes or after harvest, overwinter, and the disease cycle is complete (Kamal et Al., 2016).

Environmental factors play a significant role in the development of disease outbreaks. A rainy and cold midsummer is conducive to the disease's growth, according to prior studies. The presence of high moisture content on plant parts favors mycelia growth and facilitates the infection process of the pathogen (Kamal et al., 2016). Conditions like saturated soil and a temperature range of 10 to 20 °C promote the germination of sclerotia (Kamal et al., 2016). *S. sclerotiorum* can grow apothecia in soils with as little as 25% water saturation, which is a relatively low moisture content (Nepal and del Río Mendoza 2012).

Previously, there were no disease predicting models for this illness that take into account environmental factors like moisture, temperature, and light (Sun and Yang 2000). Now, Numerous comprehensive *Sclerotinia spp*. disease forecast model formats have been suggested as tools across diverse crops (Morier-Gxoyiya 2021). For instance, Harikrishnan and del Rio (2008) conducted studies on white mold disease of dry beans under both growth chamber and field conditions, developing logistic regression (LGR) models based on variables such as

temperature, relative humidity, ascospore concentration, and the duration of drying of colonized dry bean flowers which demonstrated the ability to predict disease within an accuracy range of 65% to 91%. In a more recent study, Shahoveisi et al. (2022) investigated white mold disease in canola and dry beans, advancing a machine learning model utilizing the Artificial Neural Network (ANN) algorithm. Remarkably, the predictive accuracy of the ANN model surpassed that of the LGR models by 11% and 4% for canola and dry beans, respectively.

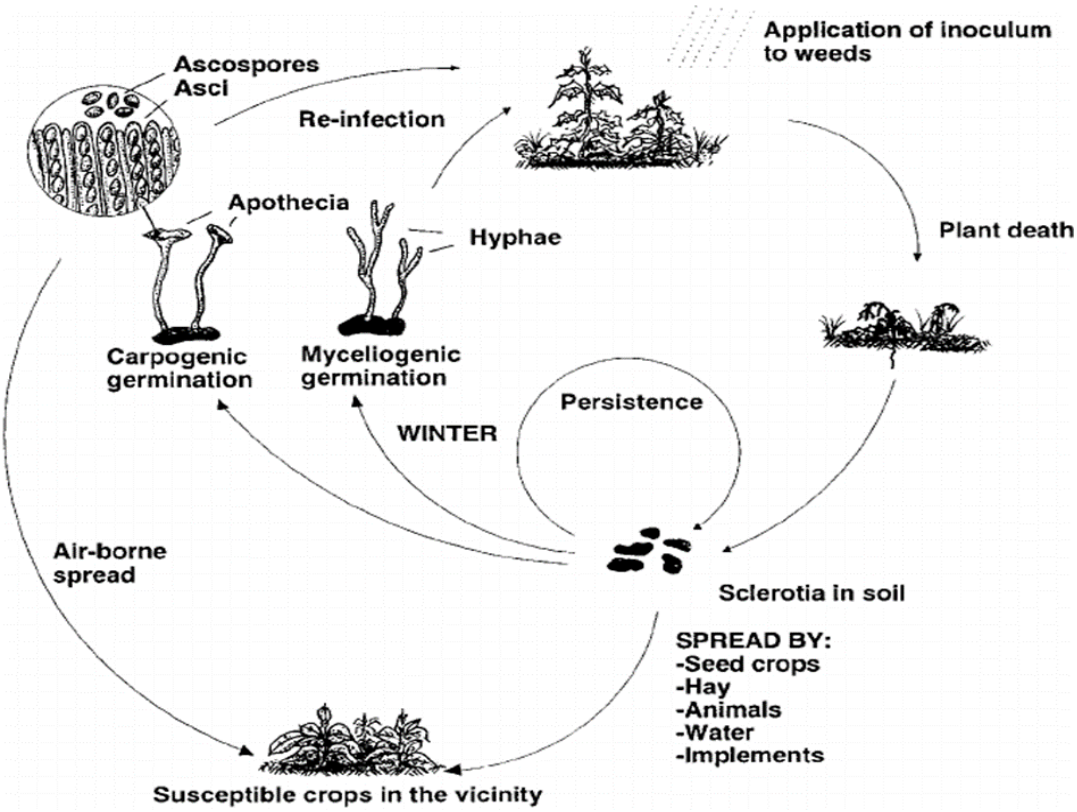

Figure 1. The life history of *Sclerotinia sclerotiorum.*

Drawing courtesy of Dr. Ian Harvey, Plantwise, Lincoln, New Zealand.

Sclerotinia diseases of crop plants

Sunflower, soybean, oilseed rape, edible dry bean, chickpea, peanut, dry pea, lentils, and different vegetables are all threatened by *Sclerotinia sclerotiorum*, as are monocotyledonous species such as onion and tulip (Bolton et al., 2006). Dark lesions arise initially on the stems of

certain infected plants, whereas water-soaked stem lesions appear first on the stems of other hosts. Lesions frequently grow into necrotic tissues, which then form fluffy white mycelium patches, which are the most visible evidence of *Sclerotinia sclerotiorum* infection (Bolton et al., 2006). Sclerotia often develop on or in the flowering and seed-producing parts of the plant and are thus frequently discovered in harvest samples. Massive volumes of sclerotia form in the receptacle of sunflower heads, for example (Bolton et al., 2006). In brassica, the petals provide a food source for *Sclerotinia sclerotiorum* spores to germinate and establish infection (Kamal et al., 2016).

Sclerotinia is found across the world, however they are more frequent in temperate areas. According to certain reports, particularly from North America, the amount of damage caused by them has increased dramatically in recent years (Willets et al.,1980). A $100 million crop loss was estimated in a 1999 sclerotinia head rot epidemic on sunflowers (Anon 2005). Yield losses caused by *S. sclerotiorum*, especially on canola in Sweden, the United Kingdom, and Germany, can range from 50% to 70%. Canola is Canada's third most valuable crop, and its output has been seriously harmed by *Sclerotinia sclerotiorum*. sclerotinia head rot has caused USD $200 million in damage to sunflowers in the United States each year. Sclerotinia white mold on dry beans caused an estimated loss of USD $1.9 million in the state of North Dakota in 2003 (Rothman et al., 2018).

Overview of resistance to necrotrophic pathogens

Sclerotinia sclerotiorum is a cosmopolitan fungal pathogen with a necrotrophic lifestyle (Bolton et al., 2006), which means it kills its host plant and takes up nutrition from the dead tissue of host plant cells to live. *Sclerotinia sclerotiorum* and its close relative *Botrytis cinerea* (which also produces melanized sclerotia) are the quintessential examples of necrotrophic

pathogens (Amselem et al., 2011). Their host-pathogen interaction occurs via the secretion of toxins and degradative enzymes such as oxalic acid often in advance of fungal growth and colonization of the host, also in the field and in the post-harvest period (Kabbage et al., 2015). Oxalic acid affects the host cell and degrades cell wall components (Kabbage et al., 2015). Several extracellular lytic enzymes, such as cellulases, hemi-cellulases, and pectinases, as well as aspartyl protease, endo-polygalacturonases, and acidic protease, display increased activity and breakdown cell organelles in the presence of oxalic acid (Kamal et al., 2016). The fungus colonizes tissues inter- or intracellularly and kills cells before the invading hyphae by enzymatic dissolution. Plant tissue is macerated by pectinolytic enzymes, resulting in necrosis and plant death (Kamal et al., 2016). The dead tissues become the carbon source for the pathogen (Kabbage et al., 2015). Recently, there has been a new observation regarding the fact that, *Sclerotinia sclerotiorum* is a hemi biotrophic nature which means it shows biotrophic characteristics, as well as necrotrophic, necrotrophic phase, seems to be more pronounced (Kabbage et al., 2015).

When a biotrophic pathogen infects the host plant, the host plant detects pathogen elicitors through nucleotide-binding site leucine-rich repeat (NBS-LRR) genes and related complexes, leading to signaling cascades with programmed cell death at the end (Glazebrook 2005; DeYoung and Innes 2006). With this kind of mechanism, gene to gene interaction happens between biotroph and host. In various instances, the host plant can defend itself with just a single resistance gene that gives complete resistance to a specific pathogen strain. In contrast, necrotrophic pathogens are often associated with quantitative trait loci response as there is genetic complexity and a lack of consensus on the genomic locations of disease resistance genes (Pogoda et al., 2021). Due to the quantitative, polygenic nature of resistance to necrotrophs,

relatively little is currently known about the molecular and physiological mechanisms underlying effective defenses.

Inheritance and genetic characteristics of resistance to sclerotinia

The inheritance of sclerotinia resistance is intricate due to several factors. Among these, the differential expression of host resistance specificity against *Sclerotinia sclerotiorum* stands out as a significant challenge (Khan et al.,2020). In addition, differences in pathogen virulence contribute to variable host resistance responses (Khan et al., 2020). Several studies suggest that partial resistance is associated with plant age (Singh et al., 2008). Pathogenicity and/or virulence at seedling versus mature plant stages is different (Khan et al., 2020). There is often variation in aggressiveness of *Sclerotinia sclerotinia* isolates and temperature differences affect the differential resistance expression across genotypes (Uloth et al., 2015).

Several studies showed that, along with sunflower, rapeseed and other brassicas have partial, dominant, or polygenic resistance to sclerotinia resistance owing to epistatic interaction (Disi et al., 2014, Vear and Grezes-Besset 2010). Many genomic regions in various host plants have been identified in conferring partial sclerotinia resistance via QTL mapping. A study suggested, BnaC.IGMT5. is a potential candidate gene that can contribute to resistance to canola via encoding a putative indole glucosinolate methyltransferase enzyme, highlighting the prospective role of Phytoalexins and secondary metabolites (Wu et al., 2013). Although, variation underlying QTL is yet to be explored. There has been another kind of approach, the forward genetic approach using *A. thaliana* which uncovered roles for transcriptional regulation by the Mediator complex, the contribution of pathogen detection by a receptor-like protein, and the importance of cell death modulation during infection (Kabbage et al., 2013; Wang et al., 2013; Wang et al., 2015). The use of a well-developed *A. thaliana* model in genome-wide

association studies offers a promising approach to rapidly identify and evaluate specific genes contributing to quantitative sclerotinia resistance.

Genetic mapping studies of sclerotinia resistance in crop plants and arabidopsis

Genome-wide association mapping of *Sclerotinia sclerotiorum* resistance in various crops such as sunflower, soybean, canola, cabbage, is proving to be a key tool for identifying the underlying quantitative trait loci (QTL). *Arabidopsis thaliana* is a flowering plant weed under the family Cruciferae (Redei 1975) which is often used for studying the genetic architecture of quantitative traits as a model plant right after molecular markers for mapping became available (Weigel 2012). The broad adoption of arabidopsis as a model organism is attributed to its favorable characteristics, including a brief life cycle of approximately 8 weeks and minimal growth needs. Additionally, its amenability to transformation by straightforward methods, such as bacterial-mediated gene delivery via flower spraying, enables efficient transgene incorporation in subsequent generations (Somerville and Koornneef 2002). Arabidopsis is extensively utilized in insertional mutagenesis, a fundamental genetic approach enabling swift identification of genes associated with specific phenotypes through gene tagging (Alonso and Stepanova 2003). Recently, two novel genes contributing to *S. sclerotiorum* resistance were discovered via a genome-wide association study utilizing a diversity panel composed of ninety-eight naturally occurring *A. thaliana* ecotypes inoculated with a single isolate of *S. sclerotiorum* (Badet et al., 2017; Badet et al., 2019). Another study done on 127 soybean accession identified a major gene (Glyma.01 g048000) using Genome-wide association mapping on the whole sequence that can be promising in resistance to sclerotinia (Boudhrioua et al., 2020).

Mapped genes confirmed to influence sclerotinia resistance

Some of the putative resistance genes against *S. sclerotiorum* have been discovered by using several methods, including RNA-sequencing, genome-wide association studies (GWAS), quantitative trait loci mapping, and other methods, (Qasim et al., 2020). In 2013, Mei et al. characterized six QTLs in *B. olerecea* genome in a study for SSR resistance of *B. oleracea*. GWAS has also been useful in finding regions implicated with resistance to SSR. In 2016, Wu et al. described 26 SNPs that are linked to SSR resistance which are found on chromosomes C4, C6, and C8 in a *Brassica napus* genome and predicted 39 genes that are linked to SSR resistance. At the same year, Wei et al. found 17 significant SNP associations with SSR resistance on chromosomes A8 and C6 along with differential gene expression and additionally discovered 24 resistance genes by combining SNP association analysis and transcriptomic studies through GWAS. In 2012 Chen et al. found that overexpression of *AtWRKY28* and *AtWRKY75* in arabidopsis improves host resistance to necrotrophic disease, *S. sclerotiorum.* Sunflower *AtWRKY27* homologue *HaWRKY5* exhibits elevated expression in a *S. sclerotiorum*-resistant line (Giacomelli et al., 2010). Furthermore, the canola *AtWRKY27* homologue *BnaWRKY012* is found within a previously mapped *S. sclerotiorum* resistance QTL area, suggesting that homologues of this transcription factor may play a role in *S. sclerotiorum* resistance in a variety of host plants (He et al., 2016; Wu et al., 2013). The discovery of *BnWRKY33* as a *S. sclerotiorum*-responsive gene highlights its role in positively regulating resistance to this pathogen through the enhancement of gene expression (Liu et al., 2018). Despite all of these efforts to pinpoint potential sources of resistance, further research is required to fully manage the SSR disease genetically.

Previously, we have completed a similar genome-wide association mapping effort in which we phenotyped a considerably larger diversity panel of 325 *A. thaliana* ecotypes at two time points after inoculation for resistance against two *S. sclerotiorum* isolates exhibiting different levels of aggressiveness. We observed considerable variation in resistance to S. sclerotiorum among the 325 ecotypes evaluated (Fig 1). This study yielded a total of 36 loci significantly associated with resistance to one or both isolates of *S. sclerotiorum* (Fig 2). Assessment of candidate genes in linkage disequilibrium with significantly associated markers revealed a group of five WRKY family transcription factors that appear to be associated with *S. sclerotiorum* resistance and among them *WRKY3*, *WRKY4*, and *WRKY27* to be the subject of this proposed research.

Figure 2. Variation among *Arabidopsis thaliana* ecotypes for resistance to *Sclerotinia sclerotiorum*. A. Variation in leaf lesion sizes at 4 days post-inoculation (DPI) with S. sclerotiorum isolate 1980. B. Variation in whole plant resistance at 7 DPI. A highly susceptible ecotype (left) and a partially resistant ecotype (right) are depicted after inoculation with *S. sclerotiorum* isolate 1980 (Underwood, unpublished).

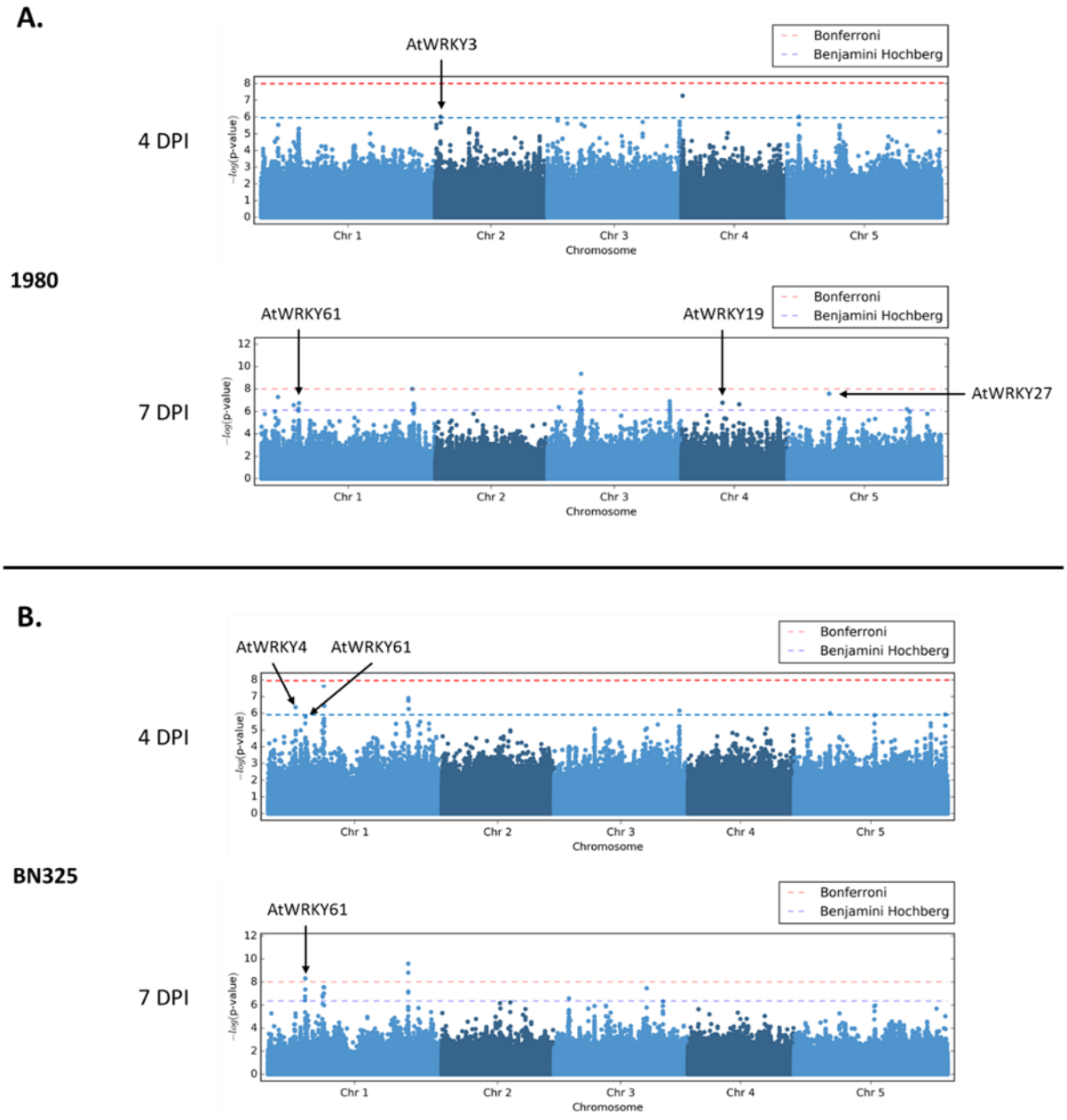

Figure 3. Manhattan plots summarizing genome-wide association mapping using a collection of 325 *Arabidopsis thaliana* ecotypes inoculated on leaves with either aggressive *Sclerotinia sclerotiorum* isolate 1980 (panel A) or less aggressive isolate BN325 (panel B). Disease progression was evaluated at 4 DPI (upper plots) or 7 DPI (lower plots) using a numerical rating scale described above. GWAS was conducted using an accelerated mixed linear model to account for population structure and kinship. Blue and red dashed lines indicate thresholds for significance at a 5% false discovery rate (Benjamini Hochberg procedure) or more stringent Bonferroni correction, respectively. Positions of *AtWRKY3*, *AtWRKY4*, *AtWRKY19*, *AtWRKY27*, and *AtWRKY61* are indicated by arrows (Underwood, unpublished).

WRKY family of transcription factors overview

WRKY transcription factors are known to be one of the largest families of transcriptional regulators in plants and are responsible for the regulation of genes responsive to biotic and abiotic stress. The WRKY TF family member domain consists of two-part, one is a 60 amino acid conserved region that makes up the protein containing the four WRKY amino acids and another is a zinc-finger-like motif (Giacomelli et al., 2010).This DNA binding protein have a number of roles in plant activities, including growth, development, and stress signaling through autonomic and cross-regulation with TF and numerous other genes (Khoso et al., 2022). Ishiguro and Nakamura (1994) identified the first member of the WRKY SPF1 superfamily from the sweet potato (*Ipomoea batatas*). The association of WRKYs in defense responses and development has been reported over time. Plant WRKY genes express quickly in response to pathogen infection or treatment with pathogen elicitors or SA (Lai et al., 2008). For example, a recent study suggested that *AtWRKY38* and *AtWRKY62* (which are also functionally characterized WRKY genes) participate in the basal resistance to bacterial pathogens by interacting with a histone deacetylase (Giacomelli et al., 2010).

In broad terms, it is anticipated that WRKY TF would act as a crucial regulatory protein by precisely interacting with the W-box (TTGAC (C/T)) that controls gene expression (Chi et al., 2013). In recent years, there has been a report of the presence of several WRKY superfamily members in various crops plants including *Arabidopsis thaliana* (75), *Glycine max* (197), *Raphanus sativus* (126), *Oryza sativa* (109), *Sorghum bicolor* (68), *Carica papaya* (52), *Hordeum vulgare* (45) and many more (Wani et al., 2021). In arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), the WRKY TF superfamily has 74 and 109 members, respectively (Pandey and Somssich 2009).

Although WRKY transcription factors were only recently discovered, they are still leading the way in the study of plant defense responses and are quickly becoming one of the most well-characterized groups of transcription factors in plants.

Involvement in plant disease resistance

WRKY family TF can have both positive and negative impact in plant disease resistance. For instance, while responding normally to biotrophic infections, arabidopsis *wrky33* mutants are particularly sensitive to necrotrophic diseases (Zheng et al., 2006). On the other hand, arabidopsis *WRKY7*, *WRKY11,* and *WRK17* mutations increase plant resistance to virulent *P. syringae* strains, and arabidopsis *WRKY25* mutations increase plant tolerance to *P. syringae* (Lai et al.,2008). Plant resistance to *P. syringae* and *E. orontii* is regulated negatively by the structurally similar proteins *WRKY18*, *WRKY40*, and *WRKY60*. Their *HvWRKY1* and *HvWRKY2* barley homologues serve the same purpose as suppressors of basal defense (Lai et al., 2008). In 2012, Birkenbihl et al. did a detailed transcriptome analysis to gain a better understanding of the transcriptional responses mediated by *WRKY33* in response to *B. cinerea* infection. *WRKY33* appears to control the expression of a number of different defense pathway components that are essential for initiating proper host responses to *B. cinerea*. This concludes that *AtWRKY33* is an essential TF for defense against *Botrytis cinerea*. Rice plants overexpressing *OsWRKY53* and *OsWRKY45* have been found to enhance resistance to the rice blast pathogen *M. grisea*. Chen et al. conducted arabidopsis microarray and identified eight WRKY genes upregulated upon exposure to oxalic acid. They proceeded to overexpress *AtWRKY28* and *AtWRKY75* in arabidopsis, revealing an enhanced host resistance against oxalic acid and the necrotrophic pathogen *Sclerotiorum sclerotiorum* (Chen et al., 2013). Differential regulation of genes that encode WRKY TFs in time of infection or the presence of WRKY coding genes inside the

quantitative trait loci have been observed when the plant is attacked by *S. sclerotiorum* (Wang et al., 2013). Overexpression of canola *BnWRKY33* causes enhanced resistance to *S. sclerotiorum* (Wang et al., 2013). Among the WRKY transcription factors identified in our association mapping study, the sunflower *AtWRKY27* homolog *HaWRKY5* exhibits elevated expression in an *S. sclerotiorum*-resistant line (Giacomelli et al.,2010). Furthermore, the canola *AtWRKY27* homolog *BnaWRKY012* is present within a previously mapped *S. sclerotiorum* resistance QTL region, suggesting that homologs of this transcription factor may contribute to *S. sclerotiorum* resistance in multiple host plants (Wang et al., 2014; Wu et al., 2013). *WRKY3* and *WRKY4* are nuclear-localized proteins that detect the TTGACC W-box sequences in in-vitro (Lai et al., 2008). In 2008, Lai et al. created stress conditions by liquid infiltration or spraying causing fast expression of *WRKY3* and *WRKY4*. Pathogen infection and SA therapy increased the stress-induced expression of *WRKY4* (Lai et al., 2008). They identified T-DNA insertion mutants and created transgenic overexpression lines for *WRKY3* and *WRKY4* to assess their function in plant disease resistance directly. *AtWRKY3* and *AtWRKY4* were shown to contribute non-redundantly to resistance against the gray mold pathogen *Botrytis cinerea* (Lai et al., 2008).

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CHAPTER 2: DEVELOPMENT OF ARABIDOPSIS TRANSGENIC LINES OVEREXPRESSING *WRKY4* IN DIFFERENT GENETIC BACKGROUNDS AND EVALUATION OF HOST RESISTANCE TO SCLEROTINIA

Introduction

In the previous year, T-DNA insertion mutants were assessed for their responsiveness to *Sclerotinia sclerotiorum* in relation to five designated WRKY transcription factors (*WRKY3-2*, *WRKY4-2*, *WRKY19*, *WRKY27*, *WRKY61*). Col-0 parent ecotype served as a control. The *WRKY4-2* mutant plants exhibited significantly higher susceptibility to *S. sclerotiorum* at both 4- and 7-days post-inoculation (DPI) when compared to the Col-0 parent ecotype. This result highlighted the role of *WRKY4* in influencing resistance to *S. sclerotiorum*. Subsequently, *WRKY4* expression levels were examined in both resistant and susceptible arabidopsis ecotypes, both in untreated plants and inoculated plants (*S. sclerotiorum* inoculation). Quantitative polymerase chain reaction (qPCR) was employed to evaluate the transcript levels of *WRKY4*. Diverse *WRKY4* transcript levels were observed in untreated plants, with significant variations among different ecotypes. Notably, no clear pattern emerged that could directly associate these transcript levels with resistance or susceptibility. In contrast, in resistant ecotypes, *WRKY4* expression significantly increased by approximately 2-3-fold at 24 hours post-inoculation (hpi) in comparison to control plants whereas the susceptible ecotypes showed unaltered or downregulated *WRKY4* expression levels. These findings consistently support the positive role of *WRKY4* in regulating resistance to *S. sclerotiorum*, prompting further research to explore the temporal dynamics of *WRKY4* expression after *S. sclerotiorum* inoculation and the assessment of additional arabidopsis ecotypes with varying resistance profiles. For that, the objective of this project was to develop transgenic arabidopsis lines overexpressing *WRKY4* from the 35S

promoter in Zdr-6, Col-0, and Lm-2 genetic backgrounds and to evaluate resistance to sclerotinia.

Materials and method

Plant materials and growth condition

Arabidopsis thaliana ecotype Col-0 was used as a control ecotype throughout the study. Three *Arabidopsis thaliana* ecotypes were used, Lm-2 (highly susceptible), Col-0 (Moderately susceptible), and Zdr-6 (Partially resistant) ecotypes. Seeds were directly sown into potting mix (Premier Horticulture Pro-Mix BX) in 4-inch pots and kept in darkness at 4°C for 4 days to induce stratification. Afterward, all plants were grown in a growth chamber under long-day conditions (16h photoperiod at 21°C, 8h darkness at 20°C) with light intensity of approximately 150 uE/m2.

Cloning of *WRKY4*

To generate a binary plasmid carrying *AtWRKY4* under the control of the 35S promoter, *AtWRKY4* genomic DNA fragment from arabidopsis ecotype Col-0 was cloned into the binary vector pMDC32 (Figure 1) (Shin et al., 2012). PCR amplification of *WRKY4* from Col-0 was performed using forward primer (taccgggccccccctcgaggGTTAATTTTGGGGATCGATGTC) and reverse primer (ccgctctagaactagttaatGCAAGAAAATTTGGGTCATAGG) generated by NEBuilder Assembly Tool v2.4.0 to facilitate cloning of *AtWRKY4* genomic DNA fragment into pMDC32 by Gibson Assembly. The *WRKY4* PCR fragment was purified using a GeneJet PCR purification kit (Invitrogen) and subsequently quantified using a Qubit 3.0 fluorometer.

The binary expression plasmid pMDC32 was digested with restriction enzymes PacI and AscI and the digest was electrophoresed on a 1% agarose gel. The vector backbone was cut from the gel under UV transillumination and purified using a GeneJet gel extraction kit. The pMDC32

vector has a Kanamycin resistance marker and hygromycin resistance marker and features two copies of CaMV 35S (2 × 35S) promoter that drives *WRKY4* expression. The vector and insert were assembled using a NeBuilder Hifi DNA Assembly cloning kit according to manufacturer's protocol.

After constructing a recombinant plasmid containing the gene of interest, *WRKY4*, the plasmid was introduced into *E. coli* bacteria via chemical transformation using the HIFI DNA Assembly protocol. Subsequently, the transformed cells were spread onto LB agar plates supplemented with Kanamycin (50 mg/mL) antibiotic and incubated overnight at 37°C. The following day, the plates were examined for bacterial colonies, and selected colonies were streaked onto new plates to ensure purity. A single colony was inoculated into LB broth with the Kanamycin (50 mg/mL) antibiotic, grown in an overnight culture, and utilized for plasmid isolation to confirm the presence of the *WRKY4*/pMDC32 construct. To confirm the successful transformation of *E. coli* with the *WRKY4*/pMDC32 construct, a multi-step screening process was employed. This includes performing colony PCR using WRKY4 specific primer, analyzing PCR product via gel electrophoresis to verify the expected insert size, purification of DNA using Thermo Scientific™ GeneJET Plasmid Miniprep Kit, further confirming the presence of the *WRKY4* insert through restriction digest.

The desired DNA construct was transformed into chemically competent *Agrobacterium tumefaciens* strain GV3101 following the manufacturer's protocol of GoldBio's GV3101 Agrobacterium chemically competent cells transformation protocol. GV3101 Agrobacterium chemically competent cells were used and Kanamycin (GoldBio Catalog # K-120) • Rifampicin (GoldBio Catalog # R-120) served as selectable markers.

Screening of the successful transformants was done using Kanamycin (50 mg/mL) antibiotic. Cells were plated onto LB agar plates containing Kanamycin and the bacterial colony was selected and further expanded.

WRKY4 transformation in arabidopsis, confirmation and selection of transgenic lines

The floral dip method was used to transform the pMDC32 vector containing the *WRKY4* gene into arabidopsis (Zang et al., 2006). In this approach, Seeds of arabidopsis ecotypes Lm-2, Col-0, and Zdr-6 were vapor sterilized, grown in the growth chamber till flowering (3-4 weeks) and developing arabidopsis inflorescences were briefly immersed for a few seconds into a solution consisting of 5% sucrose along with 0.01–0.05% (vol/vol) Silwet L-77 and resuspended Agrobacterium cells carrying the target genes for transfer. Subsequently, the treated plants were permitted to produce seeds, which were then cultured on a selective medium to identify and isolate the desired transformants.

For vapor sterilization of seeds, seeds were aliquoted into 1.5ml tubes. These tubes were then placed in a bell jar, with a rack containing the tubes. A solution comprising 100ml bleach in a 250ml beaker, supplemented with 3ml concentrated HCl, was carefully introduced into the bell jar before sealing. The sealed jar was left in the hood for 4-6 hours to ensure effective sterilization without compromising seed viability, as chlorine gas was released during the process. Once sterilization was complete, the bell jar was opened in the fume hood to avoid exposure to chlorine gas. The tube rack was swiftly removed, and the tubes were capped and labeled for further processing. Seeds underwent vapor sterilization to eliminate any potential contaminants.

For resuspending Agrobacterium cells, a 3ml seed culture of Agrobacterium strains (GV3101 and AGL-1) carrying the pmdc32-*WRKY4* construct was initiated in LB medium

supplemented with antibiotics [Rif (60 mg/ml), Gent (25 mg/ml), and Kan (50 mg/ml)], and incubated for 12 hours at 30°C. Subsequently, a 750ml LB medium in a Fernbach flask was inoculated with 1.5-2ml of the seed culture and incubated for a specific duration (12h for GV3101, 36h for AGL-1) at 30°C. The resulting cells were pelleted by centrifugation, the culture medium was decanted, and the pellet was resuspended in 5% sucrose solution along with 0.01–0.05% (vol/vol) Silwet L-77. This suspension was then applied to the inflorescences of arabidopsis plants.

Plants were covered with a humidity dome for 24 hours post-inoculation, following which the dome was removed, and the plants were allowed to grow to seed. Upon the introduction of *WRKY4* into arabidopsis ecotypes via transformation, the resulting initial generation was denoted as the T0 and it was the hemizygous generation containing both hygromycin-resistant and non-resistant individuals. The T0 generation of *Arabidopsis thaliana* seeds were collected, vapor sterilized, and grown on Petri dishes containing hygromycin and selection medium (0.5x MS, 0.8% Bactoagar, 1% Sucrose, 0.5 g/l MES, 20mg/l Hygromycin) for confirmation of transformation of *WRKY4* into arabidopsis. Only seeds that have successfully integrated the *WRKY4* and expressed the selectable marker developed normal roots and these positive seedlings with 2-4 green leaves constituted the T1 generation which was then grown in pots, subjected to self-pollination, and the resulting seeds were harvested. At this stage, following the selfing, it was anticipated that the population would segregate according to Mendel's classic 3:1 ratio, indicative of a single mutation at the chromosome level. To confirm this genetic segregation, the T1 seedlings were plated on selection media (0.5x MS, 0.8% Bactoagar, 1% Sucrose, 0.5 g/l MES, 20mg/l Hygromycin) and kept in an incubator for 3-5 days.

After the period of 3-5 days, approximately three-quarters exhibited typical root growth, while the remaining quarter displayed a characteristic stunted root phenotype, consistent with the expected ratio. This segregation pattern affirmed the presence of the desired genetic alteration. Subsequently, based on this segregation ratio, the population was advanced to the next generation for further analysis and experimentation. Initially, transgenic lines displaying the desired phenotype were selected from the T1 generation. These chosen lines underwent self-pollination, allowing for the transmission of their genetic material exclusively to the next generation. Following this, the resulting T2 seeds were grown, and the selection process was reiterated to identify individuals exhibiting the desired trait. the selected T2 plants again underwent self-pollination to maintain genetic uniformity within each line. This cycle of selection and self-pollination was iterated through multiple generations, until the T4 generation, to further stabilize the genetic composition of the population. True positive lines were identified (Table 2), and three true positive lines were selected from each ecotype (Lm-2, Col-0, Zdr-6) for further analysis, alongside the wild type.

RNA extraction and quantitative real time PCR

The transcriptional levels of *WRKY4* in the overexpression lines were verified by RT-qPCR. The total RNA was extracted from samples of *arabidopsis* by Genejet RNA extraction kit (Invitrogen, USA), and measured using a Qubit fluorometer (Thermo Scientific, Wilmington, DE, USA). The iScript reverse transcription supermix for RT-qPCR was used to prepare cDNA from RNA samples in order to synthesize first-strand cDNA with oligo-Dt. *WRKY4* primers were designed using Primer3 (http://bioinfo.ut.ee/primer3/) and subsequently validated using the Primer-BLAST tool (www.ncbi.nlm.nih.gov/tools/primer-blast) with a primer size range of 18–23, GC% range of 40–60%. Transcript abundance was measured on the Bio-Rad CFX96

Connect Real-Time system using SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), in 10 µL reactions according to the manufacturer's protocol using the following cycle conditions: 95 °C for 30 s, and 40 cycles of: 95 °C for 15 s and 60 °C for 30 s. Ubiquitin 10 (UBQ10) was used as the housekeeping control for normalization for every reference gene, as described by Underwood et al. (2017). RT-qPCR reactions were carried out and the $2^{-\Delta\Delta CT}$ method was ultimately used to process the data using three biological replicates per treatment and three technical replicates per biological replicate (Schmittgen and Livak 2008).

Arabidopsis inoculation procedures

In adherence to stringent experimental criteria, three distinct arabidopsis transgenic lines were meticulously selected from each ecotype (Zdr-6, Lm-2, and Col-0) as delineated in Table 2. Each line had three replications and each plant was grown on an individual pot along with their parent line (non-transgenic line). One week prior to inoculation, a single sclerotia was plated on potato dextrose agar (PDA), incubated at room temperature for four days and inoculated at 3 weeks old. Three agar plugs were cut from the growing edge of the colony using #3 cork borer and placed into a 50 ml falcon tube containing 25 ml of potato dextrose broth (PDB). The tube was placed in a shaker incubator for 3 days and homogenized using a polytron homogenizer to generate a mycelial suspension. After that sclerotinia inoculum was prepared following several steps. For preparing the standard sclerotinia inoculum, first 1 ml of PDB was transferred to a disposable cuvette to serve as the blank standard, and homogenized mycelium was transferred to another cuvette. Then, the PDB blank cuvette was placed in the Bio photometer for blank reading, and the sample cuvette was placed in the instrument for absorbance readings, which were repeated until 10 readings were obtained. The amount of ground mycelium and PDB required to achieve an optical density (OD) of 0.5 was calculated by averaging the 10

spectrophotometer readings. The calculation involved solving for "y" in the equation 2 ml x 0.5 OD = y ml x (average spectrophotometer reading), with the result multiplied by 1000 to determine the amount of mycelial suspension in microliters. Subsequently, six 2 ml aliquots of inoculum were prepared in microfuge tubes by inverting the tubes to mix the inoculum. The inoculum is now ready for plant inoculation. A sharpie was used to mark two leaves per plant on three plants per pot. Each spot was inoculated with the micropipette which was filled with 10 ul of inoculum mix. After the inoculation the trays were covered with humidity dome and kept in growth chamber at 16h photoperiod at 21°C, 8h darkness at 20°C. The data were collected at 4 days post inoculation (dpi) and 7 days post inoculation (dpi).

Quantitative real-time PCR analysis

Transcript abundance was measured on the Bio-Rad CFX96 Connect Real-Time system (Bio-rad Laboratories, Hercules, CA, US) using Ssoadvanced Supermix (Bio-rad Laboratories, Hercules, CA, US) in 10 µL reactions according to manufacturer's protocol using the following conditions: 95 ℃ for 30 s, and 45 cycles of: 95 °C for 2 s and 60 °C for 5s. There were three biological replicates per treatment and three technical replicates per biological replicates.

The $2^{-\Delta\Delta CT}$ method (Schmittgen, and Livak 2008) was used to calculate the relative expression level of *AtWRKY4*, with *UBQ10* as the internal control.

Evaluation of transgenic lines and statistical analysis

Transformant lines were evaluated visually using the disease severity scale described in table 1. Four biological replicates were used per ecotype, with six technical replicates per biological replicate. Three repetitive trials were conducted. The wild type of each ecotype was employed as a control for comparison with the transgenic lines. As comparison was made to a control group, the Dunnett test, a post hoc non-parametric test, was employed. Dunnett's post-

hoc test was conducted using Excel to determine if statistically significant differences in resistance are observed between T-DNA insertional mutant lines of Col-0, Lm-2 and Zdr-6 compared to their parent line. The threshold for delineating between resistant and susceptible responses was set arbitrarily. Resistant lines were defined as those with a mean rating below 2.5 at 4 dpi and a mean rating below 5 at 7 dpi.

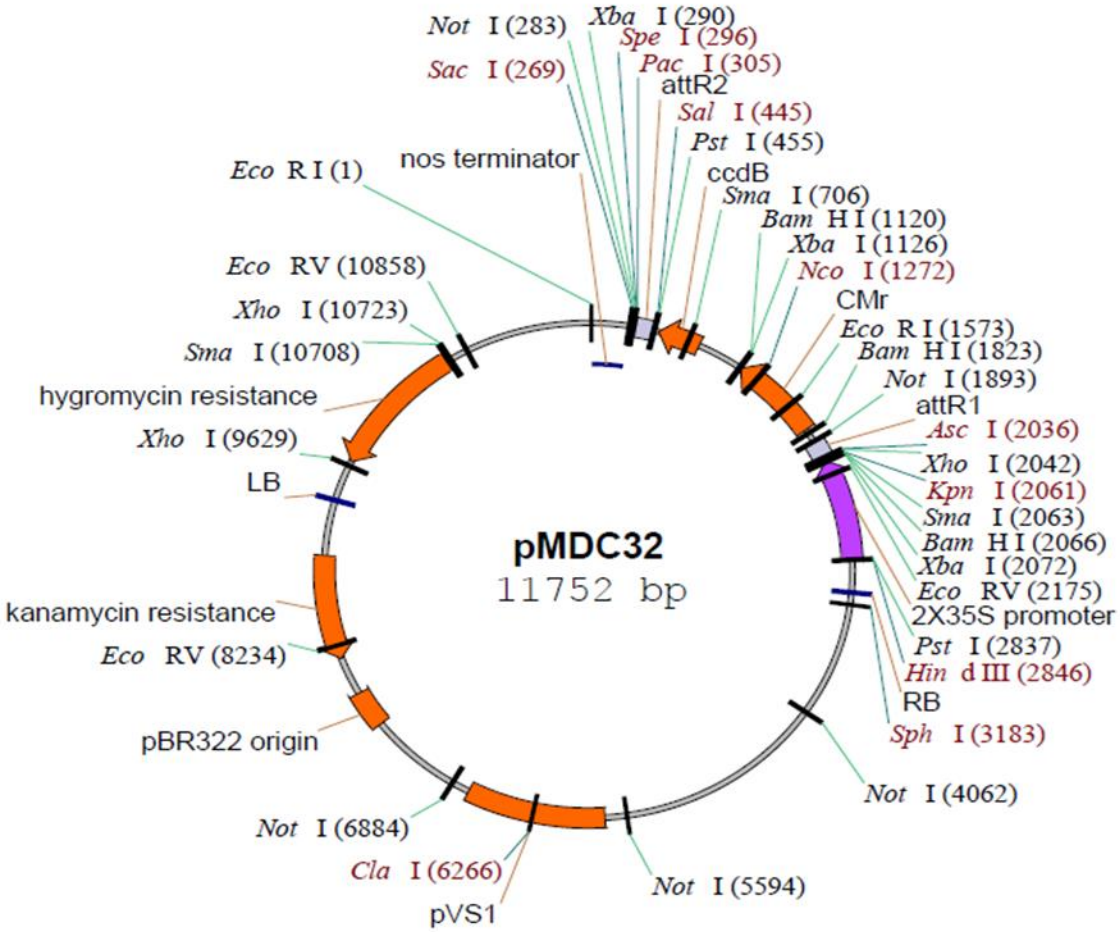

Figure 4. Restriction map of the pMDC32 binary vector. Enzymes are in italics. In red are enzymes that cut this vector only once.

Table 1. Rating scale for visual assessment of *Sclerotinia Sclerotiorum* disease progression after inoculation of arabidopsis leaves.

Rating	Description
0	No visible lesion
1	Lesion confined to inoculation site
2	Lesion <25% leaf area
3	Lesion 25-50% leaf area
4	Lesion 50-75% leaf area
5	Lesion 75-100% leaf area
6	Decay of <25% plant area
7	Decay of 25-50% plant area
8	Decay of 50-75% plant area
9	Decay of 75-100% plant area

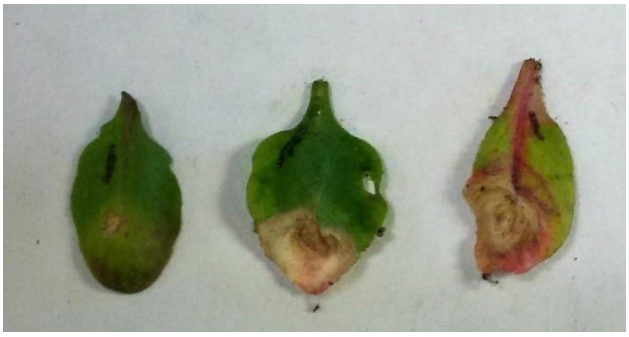

Figure 5. Comparative analysis of *Sclerotinia sclerotiorum* infection severity in arabidopsis leaves utilizing ordinal disease rating scale as outlined in Table 1 of this study. Three distinct leaves, corresponding to disease severity scores 1, 3, and 4 on the established rating scale, are presented. Each leaf represents a distinct level of infection severity, first leaf represents lesion confined to inoculation site, second leaf indicates lesion 25-50% leaf area, last leaf 4 showcases lesion 50-75% leaf area (Underwood, unpublished).

Table 2. List of transgenic lines and their verification results for *WRKY4* overexpression in *Arabidopsis thaliana*

Ecotype	Line Number	Homozygosity	Ecotype	Line Number	Homozygosity	Ecotype	Line Number	Homozygosity
Lm-2	1	Segregating	Col-0	1	Segregating	Zdr-6	1	Segregating
Lm-2	2	Segregating	Col-0	2	False positive	Zdr-6	2	True positive
Lm-2	3	False positive	Col-0	3	True positive	Zdr-6	3	Segregating
Lm-2	4	False positive	Col-0	4	Segregating	Zdr-6	4	True positive
Lm-2	5	Segregating	Col-0	5	True positive	Zdr-6	5	True positive
Lm-2	6	True positive	Col-0	6	True positive	Zdr-6	6	True positive
Lm-2	7	True positive	Col-0	7	True positive			
Lm-2	8	True positive	Col-0	8	Segregating			
Lm-2	9	True positive	Col-0	9	Segregating			
Lm-2	10	True positive	Col-0	10	Segregating			

Results

WRKY4 expression in transgenic lines

The selected transgenic lines of each ecotype were grown along with their wild type as control, and leaves were collected for RNA extraction. Bio-Rad quantitative PCR (qPCR) was performed to analyze the expression of *WRKY4*, providing insights into the genetic modifications and potential impacts on the transgenic lines. In the Lm-2 wild type, following inoculation with sclerotinia inoculum, typical disease ratings observed at 4 days post-inoculation (dpi) range between 4 to 5, escalating to approximately 8 by 7 dpi. Post-inoculation with *Sclerotinia sclerotiorum*, the transgenic *WRKY4* overexpression LM-2 line 6 exhibited approximately 4.5-fold expression, Lm-2 line 9 displayed approximately 43-fold expression, and Lm-2 line 10 showed approximately 87-fold expression greater than wild-type plants that were inoculated with *Sclerotinia sclerotiorum*. In the Col-0 wild-type, upon inoculation with sclerotinia inoculum, the disease rating at 4 days post-inoculation (dpi) typically registered around 3.5, progressively increasing to approximately 6 by 7 dpi. After inoculation with *Sclerotinia sclerotiorum*, both transgenic Col-0 Line 3 and Line 5 displayed approximately 62-63 fold expression, while Col-0 Line 6 exhibited approximately 162-fold expression greater than that observed in wild-type plants inoculated with *Sclerotinia sclerotiorum*. In the Zdr-6 wild-type, subsequent to inoculation with sclerotinia inoculum, the disease rating at 4 days post-inoculation (dpi) is typically observed to be within the range of 1.5 to 2, with a progressive increase noted to approximately 2.5 by 7 dpi. After being infected with *Sclerotinia sclerotiorum*, Zdr-6 Line 2 demonstrated a roughly 60-61 fold increase in expression, Zdr-6 Line 4 exhibited approximately 150-fold expression, and Zdr-6 Line 5 showed around 189-fold expression higher than that observed in wild-type plants exposed to *Sclerotinia sclerotiorum*. From the expression analysis, each transgenic line

consistently demonstrated a prevailing pattern of diminished disease rating when compared to its corresponding wild type (Figure 6).

Sclerotinia sclerotiorum disease evaluation in transgenic lines and statistical analysis

Transgenic lines of each arabidopsis ecotype, along with their respective wild types, were cultivated, and leaves were spot inoculated with ground sclerotinia mycelium in PDB liquid. Visual evaluations at 4 days post-inoculation (DPI) and 7 days post-inoculation (DPI) utilized a categorical 0-9 scale for disease progression (Table 1), providing a standardized and quantifiable approach to symptom severity assessment, promoting consistency, and enabling statistical analysis. Single-factor analysis of variance was employed to discern statistically significant differences among the means of the transgenic lines and their wild-type controls. Subsequently, Dunnett's tests revealed significant differences in disease progression between each ecotype and its respective control while maintaining a standard significance level of 0.05. The standard error of the mean (SEM) measures was calculated to estimate the likely discrepancy in sample means compared with the population mean. In the case of Lm-2, identified as the most susceptible ecotype among the three, a general trend of reduced disease rating compared to the wild type was observed at both 4- and 7 days post-inoculation (dpi). After 4 dpi the disease rating of wild-type Lm-2 was 4.5 whereas at 7 dpi, the disease rating went up to 8 according to the disease progression 0-9 scale. At 7 dpi, transgenic Lm-2 Line 10 exhibited significant differentiation (disease rating 5.6) compared to the wild type. Similarly, the less susceptible Col-0 and partially resistant Zdr-6 demonstrated a consistent pattern of reduced disease rating in their transgenic lines compared to the respective wild types. After 4 dpi the mean disease rating of Col-0 wild type was around 3 which increased to 6 after 7dpi. Notably, at 4 dpi, Col-0 Line 3 and Col-0 Line 5 showed a significant reduction in disease, though, at 7 dpi, none reached significance at

the $p < 0.05$ threshold. Additionally, in Zdr-6, inherently more resistant among the ecotypes (Zdr-6 wild type exhibited disease rating around 2 at 4dpi and disease rating 2.5 at 7dpi), Zdr-6 Line 2 and Line 5 exhibited a significant reduction in disease rating at 7 dpi which was below 1. The graphical representation of sclerotinia's disease response in each ecotype compared to the wild type is presented in Figure 7 and Figure 8.

Discussion

Host plants exhibit quantitative resistance to *S. sclerotiorum*. The genetic determinants of quantitative disease resistance exhibit complexity, with the continuous distribution of heritable phenotypes resulting from combinations of genetic loci. (Wang et al.,2019). Early studies looking for resistance loci in *Arabidopsis thaliana* were conducted using GWAS using a panel of 325 *Arabidopsis thaliana* accession and found 36 loci in the linkage disequilibrium block which were significantly associated with sclerotinia resistance. Subsequent evaluation of candidate genes in linkage disequilibrium and loss of function genomic analysis suggested a potential positive role of *WRKY4* in sclerotinia resistance. The primary objective of this project was to further elucidate the function of *WRKY4* in conferring resistance to *Sclerotinia sclerotiorum*, with the objectives of improving host resistance and enhancing mechanistic understanding of quantitative resistance. The present study was the first to develop transgenic arabidopsis lines overexpressing *WRKY4* using three differentially resistant lines where every transgenic line exhibited a general trend of reduced disease rating compared to the wild type indicating that, *WRKY4* overexpression did make the transgenic lines resistant compared to wild type and also suggesting that, *WRKY4* has a positive role in *S. sclerotiorum* resistance which aligns with our previous loss of function genomic analysis. The possible reason for resistance in transgenic lines is that overexpression may have resulted in the elevated synthesis of proteins involved in plant

defense mechanisms, which ultimately activated the defense signaling pathway in plants. Chen et al. (2013) performed a gene expression profiling study of *AtWRKY28* and *AtWRKY75* in arabidopsis upon *S. sclerotiorum* infection using two salicylic acids (SA)- marker genes (PR1 which encodes a pathogenesis-related protein 1, PAL1 which encodes an enzyme called phenylalanine ammonia-lyase and three jasmonic acid/ethylene (JA/ET) marker genes (PDF1.2 which encodes an antifungal peptide, VSP1, which encodes an acid phosphatase, and LOX2, which encodes a plastidic lipoxygenase) and found that, both *AtWRKY28* and *AtWRKY75* function as transcriptional regulators of SA and JA/ET-dependent defense signaling pathways and activates JA/ET pathway to defend arabidopsis against *S. sclerotiorum*. While our recent investigation suggests that *WRKY4* functions as a positive regulator in plant responses, additional gene expression profiling is needed to identify the induced defense signaling pathways.

In the present study, differentially virulent lines that were transformed with *WRKY4* genes exhibited higher levels of expression in transgenic lines compared to non-transformed lines (wild type) during expression analysis. Possibly, the reason is the position effect where *WRKY4* was highly integrated into the transcriptionally active euchromatin region of the plant genome, which led to strong overexpression, hence resistance. Notably, Zdr-6 line 5 exhibited the highest expression of *WRKY4* during expression analysis. Zdr-6 line 5 also significantly showed the lowest disease rating during disease assessment indicating the key role of *WRKY4* in improving host disease resistance. Subsequently, even though every transformed line showed a high level of expression but not significantly reduced disease rating, lines with higher expression of *WRKY4* showed a trend of reduced disease rating indicating the correlation between overexpression and resistance. The occurrence could be attributed to inconsistencies in the assessment of disease response. Significant results and a more pronounced correlation between

resistance and expression might have been observed with increased replication or a more precise assessment.

In 2008, Lai et al. performed an overexpression study of *WRKY4* in arabidopsis, in the col-0 line to be specific upon *Botrytis cinerea* infection and found no role of *WRKY4* for *Botrytis cinerea* resistance even though *Sclerotinia sclerotiorum* is a close relative of *Botrytis cinerea* (Lai. et al., 2008; Amselem et al., 2011). So, their study was contradictory to our findings. This contradictory effect of *WRKY4* can possibly be attributed to the specific molecular and biochemical interactions between *WRKY4* and the host upon specific host-pathogen interaction. The unique defense responses triggered by *WRKY4* overexpression may be more effective against the defense mechanisms employed by arabidopsis upon *Sclerotinia sclerotiorum* infection. Further investigation is needed to unravel the specific mechanisms underlying these contrasting effects and enhance our understanding of the intricacies of *AtWRKY4* function in plant-pathogen interactions.

While WRKY genes are often associated with plant defense responses, overexpression may impact the plant negatively either by reducing the size of the plant or by reducing yield. In the present study, even though overexpression of *WRKY4* demonstrated a positive impact on plant resistance against *Sclerotinia sclerotiorum*, an assessment of grain yield and evolutionary fitness was not conducted. Overexpressing WRKY transcription factors in plants can result in stunted growth and reduced tolerance to abiotic stress (Wang et al., 2012). Negative impacts on crop yield and resource allocation issues may arise from the altered expression of WRKY genes. Adverse effects can be explained by several possible factors such as gene-specificity varying depending on the specific WRKY gene, plant species, and environmental conditions. As

transgenic Zdr-6 line 5 showed almost immunity upon sclerotinia infection, further study needs to be conducted on Zdr-6 line 5 to assess the impact of *WRKY4* overexpression.

In summary, our results suggest that *WRKY4* improved the resistance in the transgenic lines, especially in Zdr-6 line 5 which showed almost immunity and there is a correlation between overexpression of *WRKY4* and host resistance against *S. sclerotiorum*.

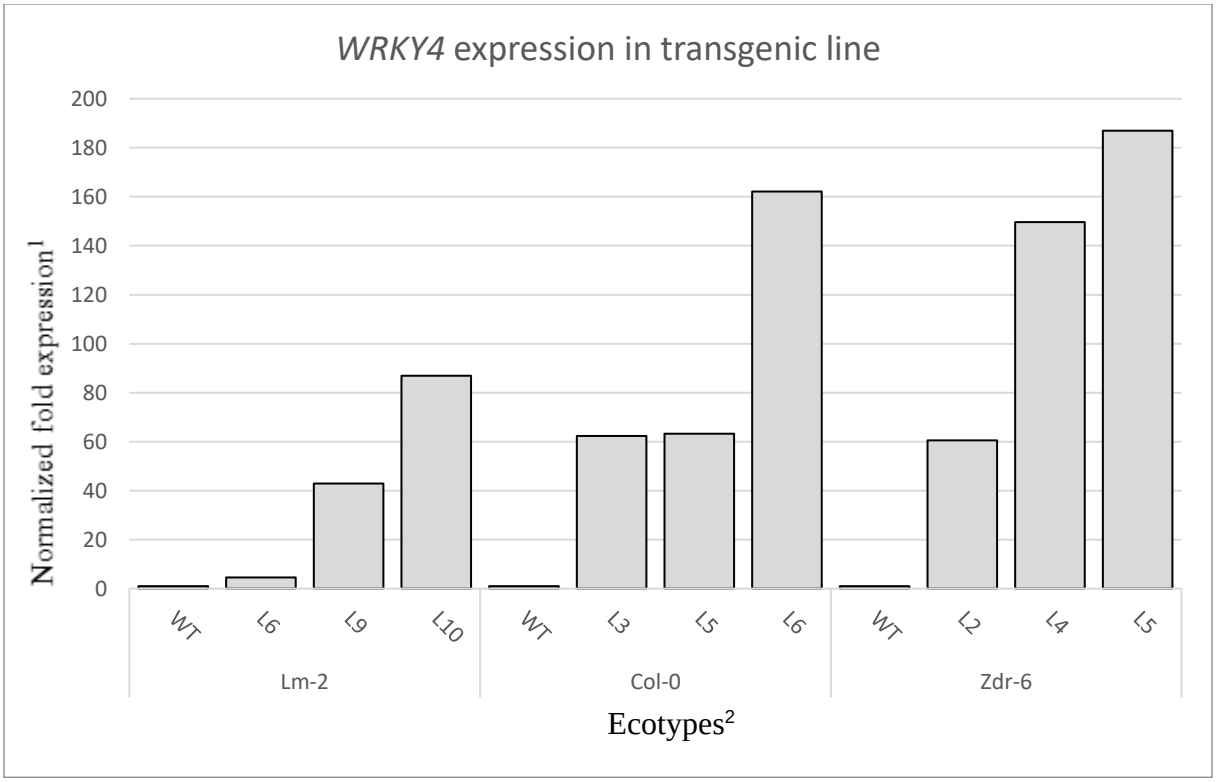

Figure 6. Expression analysis of Lm-2, Col-0, and Zdr-6 transgenic line compared to wild type exhibited reduced disease rating after overexpression.[1,2]

[1] the relative difference in gene expression levels between experimental conditions, calculated by comparing the target gene's expression in treated or transgenic samples to that in control or wild-type samples.

[2] a group of individuals within a plant species that has distinct genetic and phenotypic characteristics, allowing it to thrive in specific environmental conditions, such as a particular habitat, climate, or soil type.

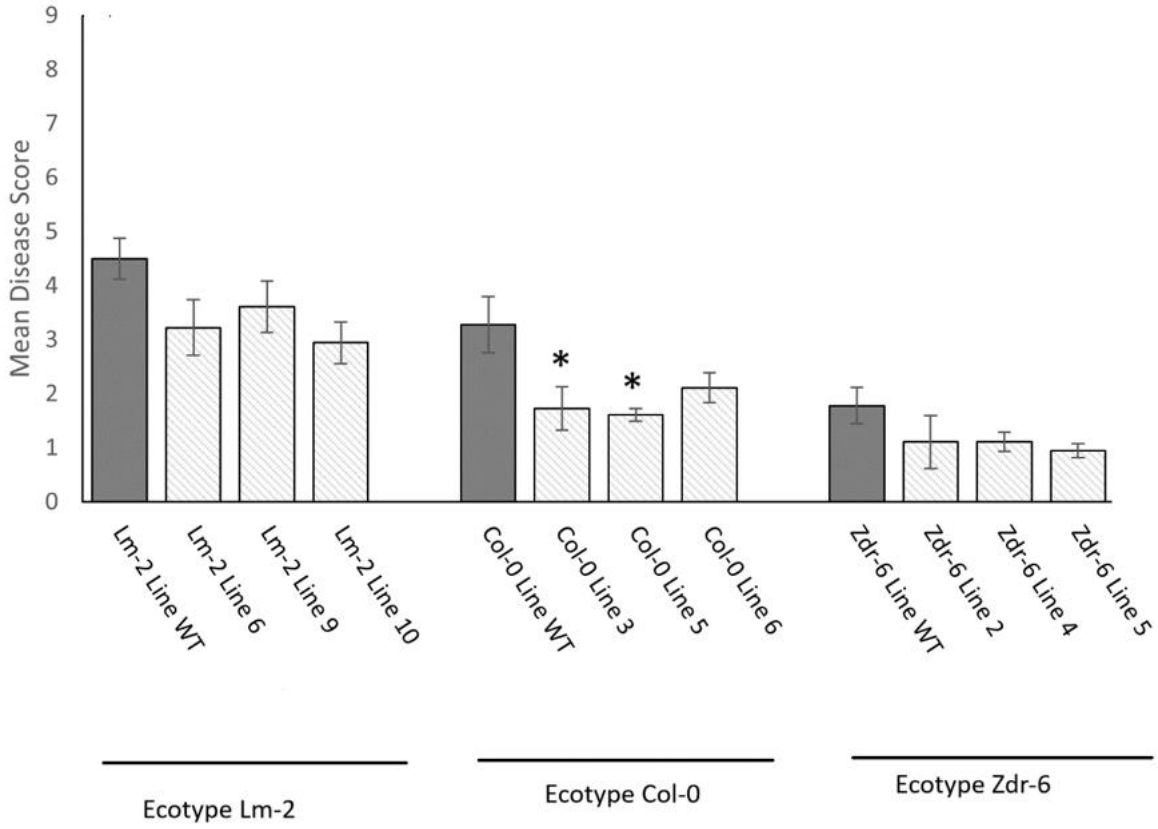

Figure 7. Disease response in Lm-2, Col-0 and Zdr-6 transgenic lines compared to wild type (WT) at 4 days post inoculation (dpi). Reduced disease rating in every transgenic line was observed. At 4 dpi, Col-0 Line 3 and Line 5 showed significantly (*) reduced disease rating ($p < 0.05$)[3].

[3] A quantitative or qualitative assessment used to measure the severity or extent of disease symptoms in plants. It involves assigning a numerical score or qualitative descriptor to indicate the level of infection, damage, or susceptibility observed on the plant. We used a (0-9) disease rating scale which is a continuous scale.

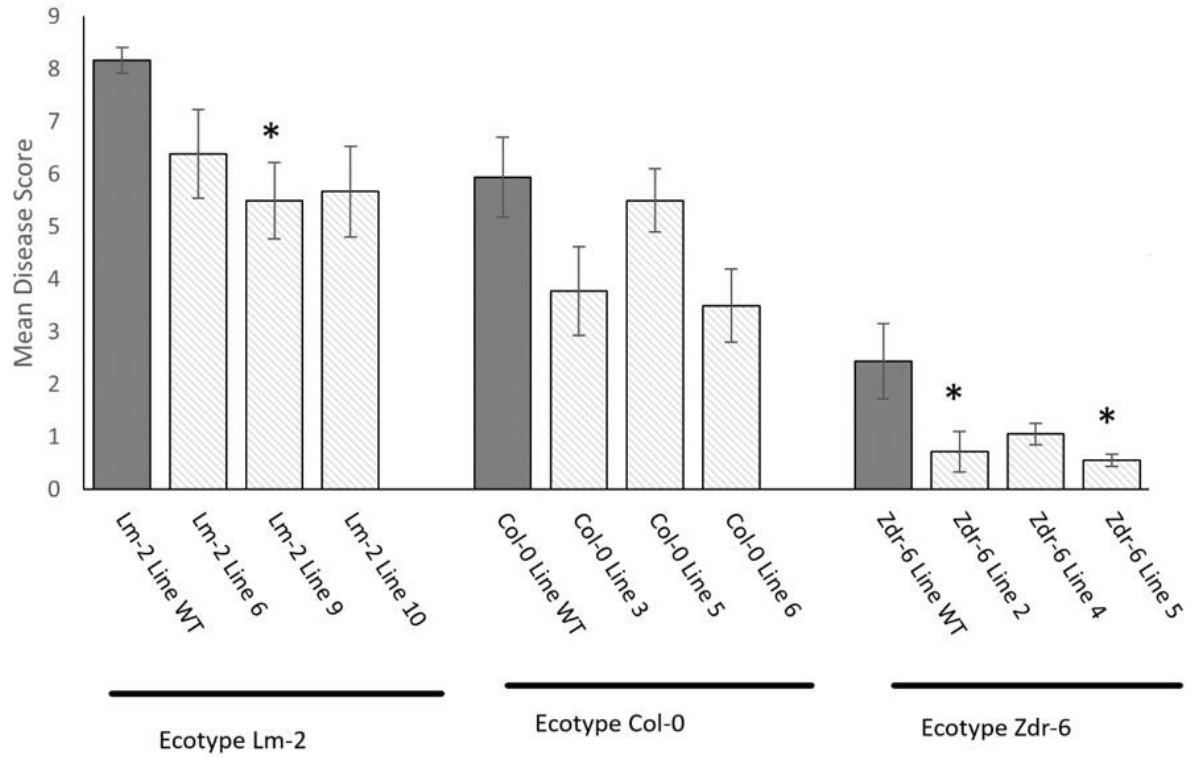

Figure 8. Disease response in Lm-2, Col-0 and Zdr-6 transgenic lines compared to Wild type (WT) at 7 days post inoculation (dpi). Every transgenic line exhibited reduced disease rating. Lm-2 Line 10 showed significantly (*) reduced disease rating at 7 dpi ($p < 0.05$). Zdr-6 Line 2 and Line 4 showed significantly (*) reduced disease rating at 7 dpi ($p < 0.05$).

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CHAPTER 3. EXPRESSION ANALYSIS OF *AtWRKY3, 4,* & *27* IN ARABIDOPSIS AND IT'S ORTHOLOGS IN SUNFLOWER DURING SCLEROTINIA INFECTION

Introduction

To find out genes contributing to sclerotinia resistance and to understand the mechanism of resistance, our lab previously conducted a genome-wide association study using a panel of 325 *Arabidopsis thaliana* accession inoculated with two *S. sclerotiorum* isolates that have different aggressiveness for the identification of loci that provide quantitative resistance against *S. sclerotiorum*. The analysis yielded the identification of 36 loci that exhibited significant linkage to resistance against one or both strains of *S. sclerotiorum*. Among the 36 loci examined, several WRKY transcription factors including *AtWRKY3, AtWRKY4, AtWRKY19, AtWRKY27,* and *AtWRKY61* were identified as candidate resistance genes. Preliminary evaluations of T-DNA insertional mutants indicated that *wrky3* and *wrky4* mutants are hypersusceptible to *S. sclerotiorum* while *WRKY27* mutants exhibited increased resistance. The goal of this project was to further characterize the role of *AtWRKY3, AtWRKY4,* and *AtWRKY27* in resistance to *S. sclerotiorum*. For that, here we conducted expression analysis of *AtWRKY3, 4,* and *27* at 0, 12, 24, & 48 hours post inoculation (hpi) with sclerotinia in 10 ecotypes (5 susceptible,5 resistant) to assess gene expression changes in response to sclerotinia infection and compared the responses of susceptible and partially resistant ecotypes at different time point (0, 12, 24,36,48hpi). To extrapolate the same result in economically important crop, we identified putative sunflower orthologs of *AtWRKY3, AtWRKY4,* and *AtWRKY27* through blast query and conducted the same expression analysis on sunflower orthologs in susceptible and partially resistant lines at different time points (0, 12, 24, & 48 hpi) with sclerotinia to determine if the expression patterns of sunflower orthologs are similar to those observed for the arabidopsis WRKY TFs.

Gene expression analysis, a fundamental molecular biology technique, examines gene activity within cells or organisms. Researchers often do expression analysis to compare the mRNA expression levels of multiple genes quantify messenger RNA levels of specific gene (San Segundo and Sanz-Lozano 2016). This process entails quantifying and characterizing gene expression patterns, whereby genetic information encoded in a gene's DNA is translated into functional products like proteins or non-coding RNAs, such as microRNAs. Methodologies like reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR), facilitate gene expression analysis. These approaches enable scientists to precisely quantify RNA production from specific genes or across the entire genome, offering invaluable information regarding gene regulation and their contributions to a wide array of biological processes and diseases.

Arabidopsis thaliana commonly known as arabidopsis is a widely used model plant in the field of molecular biology and genetics, particularly for gene expression analysis. First, its genetic simplicity, characterized by a small and well-annotated genome, facilitates genetic and genomic investigations (Arabidopsis Genome Initiative, 2000). Second, its short life cycle enables researchers to conduct experiments swiftly, making it ideal for time-course or condition-specific gene expression studies (Koornneef and Meinke 2010). Its genetic homology to other plant species, including important crop plants, means that findings from arabidopsis gene expression studies often hold broader relevance (Provart et al., 2016). Lastly, the availability of diverse arabidopsis mutants enhances the study of specific genes and their effects on gene expression (Alonso et al., 2003).

Sunflower (*Helianthus annuus* var. macrocarpus, Asteraceae: Heliantheae) has emerged as the fourth most significant oilseed crop globally, yielding a remarkable 57 million tons of

seeds in the year 2021 (Filippi et al., 2022). Throughout the annals of sunflower cultivation, diseases have consistently proven to be formidable adversaries, both historically and in the present. Among these challenges, *Sclerotinia sclerotiorum* has emerged as a particularly devastating disease. The USDA National Sclerotinia Research Initiative Strategic Plan, spanning the years 2017 to 2021, revealed the sobering fact that sclerotinia inflicted annual losses reaching as high as $100 million upon the sunflower industry. However, resistance to sclerotinia within the sunflower plant has unveiled a complex genetic landscape, marked by the involvement of numerous genes with subtle effects. Sclerotinia, the fungal antagonist, employs a versatile array of tactics to infiltrate sunflower plants. Its mode of attack is contingent upon the location and mechanism of infection. Root infection stemming from the growth of fungal mycelia precipitates basal stem rot, while the germination of aerial ascospores can incite middle stem rot and head rot eventually (Filippi et al., 2022). The complexity of resistance to sclerotinia within the sunflower population is evident, involving a nuanced interplay of numerous genes with subtle effects (Talukder et al., 2022). Additionally, the pathogen's ability to maintain its inoculum in the soil in the form of resilient, long-lived sclerotia challenges conventional chemical control strategies, a challenge further intensified by the dearth of fully resistant sunflower genotypes (Filippi et al., 2022). Collectively, these factors culminate in a stark reality: sclerotinia stands as a genuine and escalating threat to sunflower cultivation.

Orthologs are genes in different species that evolved from a common ancestral gene via speciation. Using sunflower orthologs of *Arabidopsis thaliana*'s *AtWRKY3*, *AtWRKY4,* and *AtWRKY27* transcription factors is a foundational strategy in plant molecular biology. This approach capitalizes on the evolutionary conservation of these genes across species, allowing researchers to infer potential functions in sunflowers based on their well-characterized

arabidopsis counterparts. The specific objective of the study entailed two primary aims. Firstly, to perform expression analysis of *WRKY3*, *WRKY4*, and *WRKY27* genes in *Arabidopsis thaliana* at 0,12,24, and 48 hours post-inoculation (hpi) with sclerotinia, aiming to compare the responses of resistant arabidopsis ecotypes against susceptible ones. Secondly, to identify sunflower orthologs corresponding to *AtWRKY3*, *AtWRKY4*, and *AtWRKY27*, followed by conducting a similar expression analysis in resistant and susceptible lines of sunflower.

The initial hypothesis posited that *WRKY3* and *WRKY4* would exhibit higher levels of upregulation, while *WRKY27* would demonstrate greater downregulation in resistant arabidopsis ecotypes compared to susceptible ones during sclerotinia infection. Subsequently, it was anticipated that *WRKY3, WRKY4*, and *WRKY27* would manifest a similar pattern of differential regulation in resistant sunflower lines relative to susceptible counterparts upon sclerotinia infection.

Materials and method

Plant materials and growth condition of *Arabidopsis thaliana*

Ten ecotypes of arabidopsis were used as plant material, including five susceptible ecotypes (Wa-1, Lm-2, Bg-2, Shahdara, and Or-0) and five partially resistant ecotypes (Zdr-6, Petergof, Ag-0, Tamm-2, and UKSE06-349). The potting mix (Premier Horticulture Pro-Mix BX) was used to directly sow seeds into a 4-inch pot, and they were kept in a cold dark room for 4 days to induce stratification. It was kept in the cold room for 4 days. Afterward, all plants were grown in a growth chamber under long-day conditions (16h photoperiod at 21°C, 8h darkness at 20°C) with ½ fluorescent light banks and the light intensity of approximately 150 uE/m2 was used. Three replications of each ecotype were done.

Identification of sunflower orthologs of *WRKY3, 4* and *27* and primer design for qPCR

The protein sequence of *WRKY3, 4* and *27* from Arabidopsis.org was downloaded and used as a "BLAST" query against the sunflower genome annotations (Table 1) to find the Sunflower WRKY family ortholog of *AtWRKY3*, *4*, and *27*. The coding sequences of the candidate genes were retrieved from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/, accessed on 24 February 2022). Subsequently, Primer3 v.4.1.0 was used to design the primers following parameters such as annealing preferably on the 3′ of the transcript, primer length between 18–20 bp, GC content between 40–60%, and melting temperature around 60 °C, (Table 2).

Plant material and growth condition of sunflower

Three resistant lines (RHA 801, HA 124, RHA 280) and three susceptible lines (Cabure 1004, RHA 332, HA 277) were obtained from our lab facility. The seeds were sown into potting mix (Premier Horticulture Pro-Mix BX) by us. The samples were stratified for 4-7 days in a cold-room and were then moved to a greenhouse.

Sclerotinia disease inoculations and assessment

Sclerotinia sclerotiorum isolate 1980 was used as the source of inoculation in this study, which is available in the lab facility. First, a few sclerotia were grown from *S. sclerotiorum* isolate 1980 on PDA plates. A 25ml PDB shake culture of sclerotinia isolate 1980 was started in a 50ml conical tube three days before inoculation. This was done by transferring 3 plugs cut with the #3 cork borer from the PDA starter plate into the PDB liquid. The benchtop shaker was used to shake it at 80rpm at room temperature. The inoculum was prepared following the protocol for arabidopsis inoculations.

For arabidopsis, the plants were first thinned out to 3 per pot. Then, the leaves were marked near the petiole on the side of the leaves with a marker, and the marked places of the leaves were inoculated. After that, spot inoculation of 2 leaves per plant was done with 10µl ground mycelium near the leaf tip on the marked side of the leaves. During inoculation, the plants were kept in a growth tray with a transparent cover to maintain high humidity. The day temperature of the growth chamber was adjusted to 18□C to accommodate the humidity dome. The leaf tissues were collected at 5-time points (0,12,24,36 and 48 hpi). The inoculated quartile of the leaves were cut out at the appropriate time-point using a clean razor blade and was transferred to weigh paper on the analytical balance. A sufficient number of inoculated leaf samples was collected for each ecotype in order to obtain 50-100mg tissue. Then the Tissue samples were transferred to a labeled 1.5ml eppendorff tube and the sample weight was recorded. The tissue sample was flash frozen by dropping it into liquid nitrogen to prevent the deterioration of the RNA. After all samples had been collected, the samples were transferred to a freezer box and the sample was stored at -80C freezer.

The protocol for sunflower root inoculations was followed for sunflower orthologs. A quarter teaspoon of *Sclerotinia sclerotiorum*-infested millet was used, and the inoculum was applied in the bottom of the pot beneath the root mass of every 4-5 weeks old sunflower plant. The inoculated plants were arranged in a randomized complete block design. The root tissue samples were collected at different time points (0, 12, 24, 48hpi) by cutting off the bottom of the root mass, and then the soil was rinsed off the tissue before freezing to avoid any contamination. The tissues were collected in Falcon™ 50 mL High Clarity Conical Centrifuge Tubes, were flash frozen by dropping into liquid Nitrogen, and were stored at -80 freezer.

RNA isolation and cDNA synthesis

A total of 50 samples were collected for RNA extraction in arabidopsis, with 5 time points for each ecotype and 10 ecotypes. The Samples were prepared independently by us. Once all samples were collected, RNA isolation was performed using the Genejet RNA purification kit (Invitrogen), and the RNA yield was subsequently checked using the Qubit fluorometer. The iScript reverse transcription supermix for RT-qPCR was used to prepare cDNA from RNA samples in order to synthesize first-strand cDNA with oligo-dT. For sunflower, 24 samples were collected (4 time points for each ecotype) for RNA extraction. The sample was prepared independently, and RNA isolation was performed following CTAB RNA isolation for sunflower roots. After the RNA yield was checked using Qubit fluorometer, the cDNA synthesis of RNA samples was done using iScript reverse transcription supermix for RT-qPCR.

Selection of candidate reference genes and primer

AtWRKY3, *AtWRKY4*, and *AtWRKY27* were selected as the candidate reference genes for gene expression analysis in Biorad qPCR. The existing primers of *AtWRKY3*, *AtWRKY4*, and *AtWRKY27* from our lab facility were used, and Ubiquitin 10 (UBQ10) was used as the housekeeping control for normalization for every reference gene, as described by Underwood et al. (2017). For the sunflower sample, the ACTIN gene was used as the housekeeping gene for normalization (Thomas et al., 2003).

Quantitative real time PCR and gene expression analysis

The qPCR reactions were performed in a 10 µL final volume, which contained 5 µL of 2× SsoAdvancedTM Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 1 µL of Cdna, .4 µL of 10 uM forward primer, .4 µL of 10 uM reverse primer, and 3.2 µL of nuclease-free water. The reactions were run on 386 well plates and a CFX96 Real-Time System (Bio-Rad,

USA) was used with the following cycling conditions: initial denaturation at 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 15 s, and 60 ℃ for 30 s. Three technical replicates were run for all reactions and non-template controls (NTCs) were included in all assays. The PCR efficiency of each primer pair was estimated using CFX Maestro software v. 2.0 (Bio-Rad, USA) by generating a standard curve.

Lastly, the relative gene expression data was calculated using the comparative $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

Table 3. List of sunflower putative orthologs of *AtWRKY3*, *AtWRKY4*, and *AtWRKY27* which were found through blast query.

Transcription Factor	Gene bank accession number	Locus number	% Identity
HaWRKY3	XP_021976320.1	XP_021976320	50.00%
HaWRKY3	XP_022012682.1	XP_022012682	45.79%
HaWRKY3	XP_021983199.1	XP_021983199	44.02%
HaWRKY4	XP_021976320.1	XP_021976320	53.23%
HaWRKY4	XP_022012682.1	XP_022012682	45.70%
HaWRKY4	XP_021983199.1	XP_021983199	44.02%
HaWRKY27	XP_021969654.1	XP_021969654	79.57%
HaWRKY27	XP_022022538.1	XP_022022538	53.12%

Table 4. List of primers of sunflower putative orthologs of *AtWRKY3*, *AtWRKY4* and *AtWRKY27*.

TF	Arbitrary names	Gene bank accession number	Forward Primer	Reverse primer
HaWRKY3/4	*HaWRKY3-1q*	XP_021976320.1	CAAGTGACCCAAAAGCCGTG	ATGATTTGGTGGCGGAAGGT
HaWRKY3/4	*HaWRKY3-2q*	XP_022012682.1	ATTCCGGTAGCAAGGCATCG	CGCTGTGCTGGTCTTGTAGA
HaWRKY3/4	*HaWRKY3-3q*	XP_021983199.1	GACCCGGCGGCTTTATCTAA	GCCCGTATTTTCGCCACTTG
HaWRKY27	*HaWRKY27-1q*	XP_021969654.1	CGGAAGTACGCGAACCAAAC	GTCTTTTCTGTGGCCGGAGA
HaWRKY27	*HaWRKY27-2q*	XP_022022538.1	AGGCTCACAAGTTCATTCTCCA	TGTTTGACTGCTGGAGGACT

Results

Expression analysis of *AtWRKY3, AtWRKY4* and *AtWRKY27*

The expression of *WRKY3, WRKY4*, and *WRKY27* at various time points relative to 0h (control/uninoculated phase) was assessed. Results revealed that in case of *WRKY3*, after inoculation with *Sclerotinia sclerotiorum*, Susceptible arabidopsis ecotypes Bg-2, Oc-0, Shahdara, and Wa-1 showed similar expression at 12h, 24h and 36h compared to 0h or the unioculated phase and eventually showed downregulation at 48h. Even though initially susceptible Lm-2 showed increased upregulation at 12h, but eventually downregulated at later hours. Resistant arabidopsis ecotypes Petergof, Ag-0, Tamm-2, and Zdr-6 showed downregulation at 48h, except for UKSE-06, which demonstrated upregulation at 48h compared to 0h (Fig 9). In case of *WRKY4*, susceptible arabidopsis ecotypes Bg-2 showed around 20 fold increase at 12h ,but over the timecourse showed downregulation compared to 0h or uninoculated phase, other susceptible ecotypes Lm-2, Oc-0, Shah and Wa-1 initially showed upregulation but downregulated over the time course even. While among the five resistant ecotypes, Ag-0, -2, and Zdr-6 displayed upregulation at 48h, and Petergof (Pete) and UKSE-06 (UKSE) showed downregulation (Fig 10). In the case of *WRKY27*, susceptible ecotype Bg-2 initially showed 1.3 fold expression but was downregulated overtime. Other susceptible ecotypes showed gradual downregulation over time. Resistant ecotype Wa-1 showed around 3-fold increase at 12h time point but was downregulated at 48h compared to 0h. Another resistant ecotype Ag-0 showed around 4-5-fold increase at 12h and reduced to below 1.5-2 fold at 24h and 36h timepoint but showed sudden downregulation at 48h. Other resistant ecotype Tamm-2, UKSE and Zdr-6 showed gradual downregulation compared to 0h. (Fig 11).

Overall, expression levels of *WRKY3, 4*, and *27* demonstrated no clear pattern differentiating susceptible lines from resistant lines.

Expression analysis of *HaWRKY3-1q, HaWRKY3-2q, HaWRKY3-3q, HaWRKY27-1q* and *HaWRKY27-2q*

Blast query of *AtWRKY3, AtWRKY4*, and *AtWRKY27* resulted in many putative orthologs. Among them, *WRKY3* and *WRKY4* both showed similar blast hits. Five putative orthologs were selected (*HaWRKY3-1q, HaWRKY3-2q, HaWRKY3-3q, HaWRKY27-1q* and *HaWRKY27-2q*) based on alignment score, alignment details., E- value and % identity. The effects of *HaWRKY3-2q, HaWRKY3-3q, HaWRKY27-1q*, and *HaWRKY27-2q* on susceptible sunflower lines (Cabure 1004, RHA 332, HA277) and resistant sunflower lines (RHA 801, HA124, RHA280) were then at different time intervals in comparison to 0h (the control/uninoculated phase). In *HaWRKY3-1q* susceptible lines, Cabure 1004 exhibited upregulation at 36h but was downregulated at 48h, while HA 277 and RHA 332 showed downregulation. Conversely, *HaWRKY3-1q* resistant lines displayed progressive upregulation (Fig 12A). Similar expression patterns were observed for *HaWRKY3-2q* (Fig 12B), with downregulation in susceptible lines and upregulation in resistant lines, as well as for *HaWRKY3-3q* sunflower lines (Fig 12C). In the case of *HaWRKY27-1q*, susceptible lines demonstrated downregulation, whereas resistant lines, except for RHA280, showed upregulation (Fig 13A). *HaWRKY27-2q* susceptible lines exhibited downregulation, while all *HaWRKY27-2q* resistant lines showed downregulation (Fig 13B).

Overall, the expression analysis of *HaWRKY3-1q, HaWRKY3-2q, HaWRKY3-3q,* and *HaWRKY27-2q* revealed differential regulation in susceptible lines compared to resistant lines, and none exhibited similar regulation to the expression of *AtWRKY3, AtWRKY4*, and *AtWRKY27* in arabidopsis ecotypes.

Discussion

Previous GWAS study and loss of function genomic analysis suggested possible function of *WRKY3, WRKY4,* and *WRKY27* genes in sclerotinia resistance. It was hypothesized that *WRKY3* and *WRKY4* would be more upregulated and *WRKY27* would be more downregulated in resistant ecotypes of arabidopsis compared to susceptible ecotypes during sclerotinia infection. Subsequently, *WRKY3, WRKY4,* and *WRKY27* were expected to show similar kinds of differential regulation in resistant sunflower lines compared to susceptible sunflower lines during sclerotinia infection. As expression analysis of *AtWRKY3, AtWRKY4,* and *AtWRKY27* exhibited no clear pattern to differentiate between resistant ecotypes and susceptible ecotypes of *Arabidopsis thaliana* during the study, so the first hypothesis can be rejected. In contrast, the expression analysis of susceptible and resistant sunflower lines revealed that *HaWRKY3-1, HaWRKY3-2, HaWRKY3-3* and *HaWRKY27-2* were upregulated particularly in resistant lines of sunflowers after sclerotinia infection suggesting their possible function of resistance in resistant ecotypes of sunflower. So, the expression of *WRKY3, WRKY4* and *WRKY27* genes in sunflower were clearly different from the expression of *WRKY3, WRKY4* and *WRKY27* genes in arabidopsis which suggests that, *HaWRKY3-1, HaWRKY3-2, HaWRKY3-3,* and *HaWRKY27-2* may be the functional orthologs of *WRKY3, WRKY4* and *WRKY27* genes in sunflower and can be a key factor contributing to the sclerotinia disease resistance in sunflower. Furthermore, the results suggest that *WRKY3, WRKY4,* and *WRKY27* may not have a significant effect on sclerotinia disease resistance in *Arabidopsis thaliana*. A previous study done by Lai et al. in 2008 suggested somewhat different proposition where *wrky3* and *wrky4* single and double mutant col-0 lines showed comparatively more severe disease symptoms than wild-type col-0 lines after being infected by the necrotrophic fungal pathogen *Botrytis cinerea*. Even though *Botrytis cinerea* and

Sclerotinia Sclerotiorum are very closely related in the phylogenetic tree, the observed discrepancy between the current study's findings and the results reported by Lai et al. in 2008, suggest that *Sclerotinia sclerotiorum* and *Botrytis cinerea*, may trigger distinct defense responses in plants. The function of *WRKY3, WRKY4*, and *WRKY27* might be more pronounced in response to *Botrytis cinerea* and less significant in response to *Sclerotinia sclerotiorum*. Notably this study was the first to evaluate expression analysis of *WRKY3*, *WRKY4*, and *WRKY27* in a wide variety of differentially virulent arabidopsis ecotypes and sunflower lines. Previous studies only considered the Col-0 ecotype when examining the expression of WRKY genes in arabidopsis which suggests a potential limitation in the generalizability of the findings (Lai et al., 2008; Mukhtar et al., 2018; Qin et al., 2022). Specifically, The use of only one ecotype (Col-0) may not capture the full spectrum of responses that could be present in other varying levels of resistant arabidopsis ecotypes as different ecotypes may have different genetic makeup. Furthermore, studying only one ecotype may overlook potential ecotype-specific factors contributing to the regulation of WRKY genes. Similarly, for the genomic expression analysis study of sunflower genes, one to very few lines of sunflower lines are commonly used (Giacomelli et al., 2010; Raineri et al., 2016; Ma et al., 2021). Sunflowers, like arabidopsis, exhibit genetic diversity among different lines or varieties. The selected lines may not be representative of the broader sunflower population, leading to a limited understanding of gene expression patterns. Moreover, different sunflower lines may respond differently to the same environmental factors such as temperature extremes, humidity, soil composition, and pathogens. Overall, a study with limited lines or ecotypes might not adequately represent the variability in gene expression under different conditions. The incorporation of a more extensive and diverse set of arabidopsis

ecotypes and sunflower lines in the present study improves the reliability, generalizability, and applicability of WRKY genes.

Subsequently, the observed differential regulation in resistant ecotypes compared to susceptible ecotypes following sclerotinia infection in sunflower, as opposed to the results in arabidopsis, suggests that the regulatory mechanisms governing the expression of *WRKY3*, *WRKY4*, and *WRKY27* may vary between plant species. The contrasting findings between sunflower and arabidopsis may result from several reasons. One possible reason for the contrasting findings can be due to the species-specific nature of plant-pathogen interactions. For example, a study of comparative whole transcriptome analysis of *S. sclerotiorum* was performed during the infection of *S. sclerotiorum* in two different hosts, *L. angustifolius* and *B. napus* and found that the expression of detoxification-related genes was differentially regulated in *L. angustifolius* and *B. napus* as both species produced different phytotoxin in response to *S. sclerotiorum* infection (Allan et al., 2019). Similarly, arabidopsis and sunflower plants may deploy distinct strategies and utilize specific sets of genes in response to the same pathogen. Further studies like performing RNA-Seq on infected arabidopsis and sunflower samples in the future may provide a comprehensive overview of gene expression changes in response to the pathogen, using tools like KEGG or Reactome may help in understanding the biological pathways that are activated or suppressed in response to the *S. sclerotiorum* infection.

Polymorphism in the genetic sequences of the *WRKY3*, *WRKY4*, and *WRKY27* genes can be another contributing factor to the contrasting differences in responses to pathogens. Polymorphism refers to the presence of genetic variation, such as single nucleotide polymorphisms (SNPs) or insertions/deletions, within a population. Genetic polymorphisms can influence the function of genes and their products, including transcription factors like WRKY

proteins (Lakhneko et al., 2021). Variations in genetic polymorphism exist between species and within genomes where polymorphic variations influence the recognition or signaling pathways involved in response to specific pathogens (Ellegeren and Galtier, 2016). Different ecotypes or lines may carry distinct alleles of the *WRKY3*, *WRKY4*, and *WRKY27* genes and these alleles might have polymorphic sites that affect the proteins' structures or functions, which could result in different outcomes in terms of disease resistance, hence the contrasting finding of the present study. In the present study, DNA sequencing was not conducted to identify polymorphisms in genes. To identify polymorphisms in the *WRKY3*, *WRKY4*, and *WRKY27* genes, DNA sequencing through methods like Sanger or next-generation sequencing can be deployed in the future.

Finally, another possible explanation of the contrasting findings is the differences in the experiment environment, sampling techniques, and inoculation procedure. In the present study, arabidopsis was cultivated in a growth chamber, providing enhanced environmental control. Conversely, sunflower pots were grown in a greenhouse, introducing increased variability compared to growth chamber studies, with challenges in temperature control that also influenced soil moisture and infection dynamics. Differences in the experimental environment may contributed to the different growth conditions for the sclerotinia which potentially influenced the contrasting differential expression of WRKY genes in arabidopsis and sunflower lines. In the case of sunflowers, root inoculation was implemented using sclerotinia-infested millet, and subsequent collection of root samples was carried out. In contrast, for arabidopsis, leaf inoculation was administered using a PDB mycelial suspension, and subsequent collection of leaf samples was conducted. The viability and growth characteristics of sclerotinia-infested

millet may exhibit variations in comparison to the infection mediated by the PDB mycelial suspension.

This study is subject to certain limitations and shortcomings that warrant acknowledgment. Firstly, the selection of representative arabidopsis lines may influence the study outcomes, and the use of different sets of arabidopsis ecotypes could have yielded divergent results. Secondly, variations in the inoculation procedure may have introduced inconsistencies, potentially impeding sufficient growth of sclerotinia in resistant arabidopsis lines and consequently precluding the manifestation of differential expression between susceptible and resistant ecotypes. Thirdly, experimental errors during the Bio-Rad qPCR analysis, wherein the critical cDNA quantity could be compromised during protocol execution, may contribute to the non-observation of differential regulation in *WRKY3*, *WRKY4*, and *WRKY27* genes in arabidopsis. In summary, the differential gene expression analysis of *WRKY3*, *WRKY4*, and *WRKY27* in sunflowers indicates a potential role in sclerotinia resistance, meriting further investigation. The characterization of the roles of these WRKY genes in sunflowers is further recommended for a more comprehensive understanding.

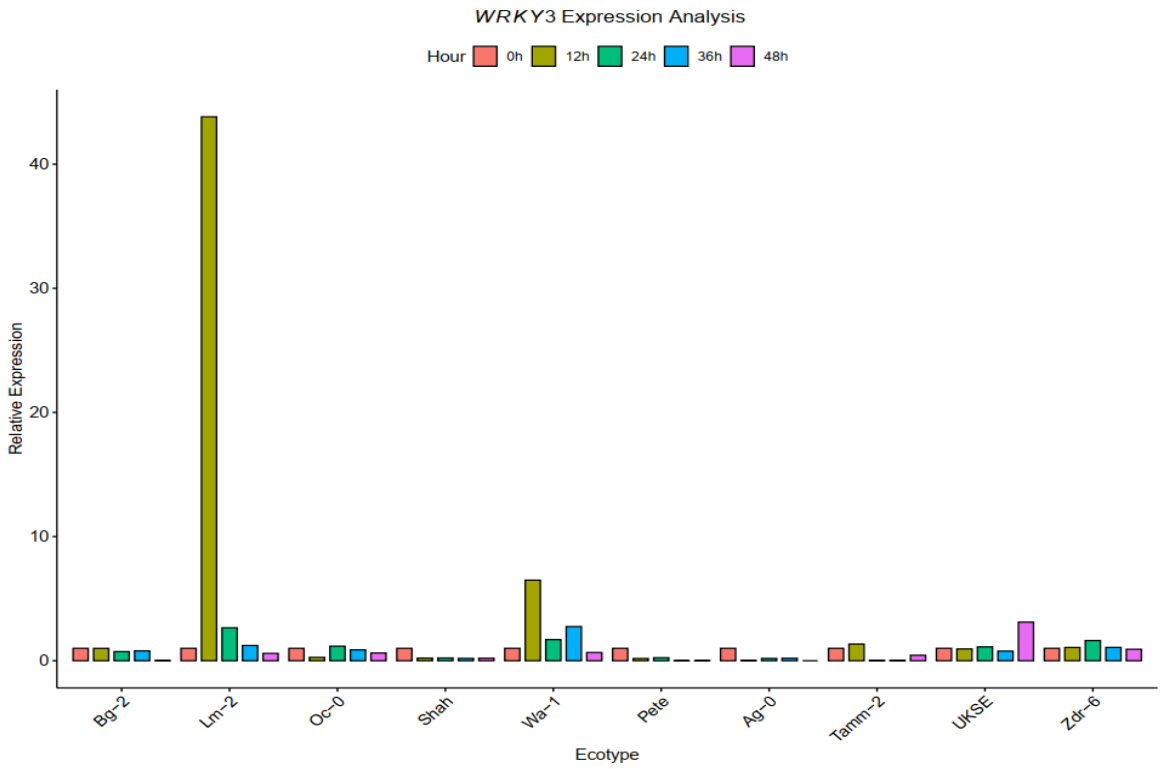


Figure 9. Expression analysis graph depicting the relative expression of *AtWRKY3* at 48h compared to 0h in susceptible (Bg-2, Lm-2, Oc-0, Shahdara, Wa-1) and resistant (Petergof, Ag-0, Tamm-2, Zdr-6) *WRKY3* arabidopsis ecotypes. Here, the X-axis represents the susceptible and resistant lines, while the Y-axis represents the relative expression. The graph depicts the relative expression of *AtWRKY3* at 48h compared to 0h in susceptible (Bg-2, Lm-2, Oc-0, Shahdara, Wa-1) and resistant (Petergof, Ag-0, Tamm-2, Zdr-6) *WRKY3* arabidopsis ecotypes. Susceptible lines exhibited downregulation, with an initial upregulation in *WRKY3* Lm-2 at 12h followed by downregulation at 48h. Resistant lines, except for UKSE-06, showed downregulation at 48h.

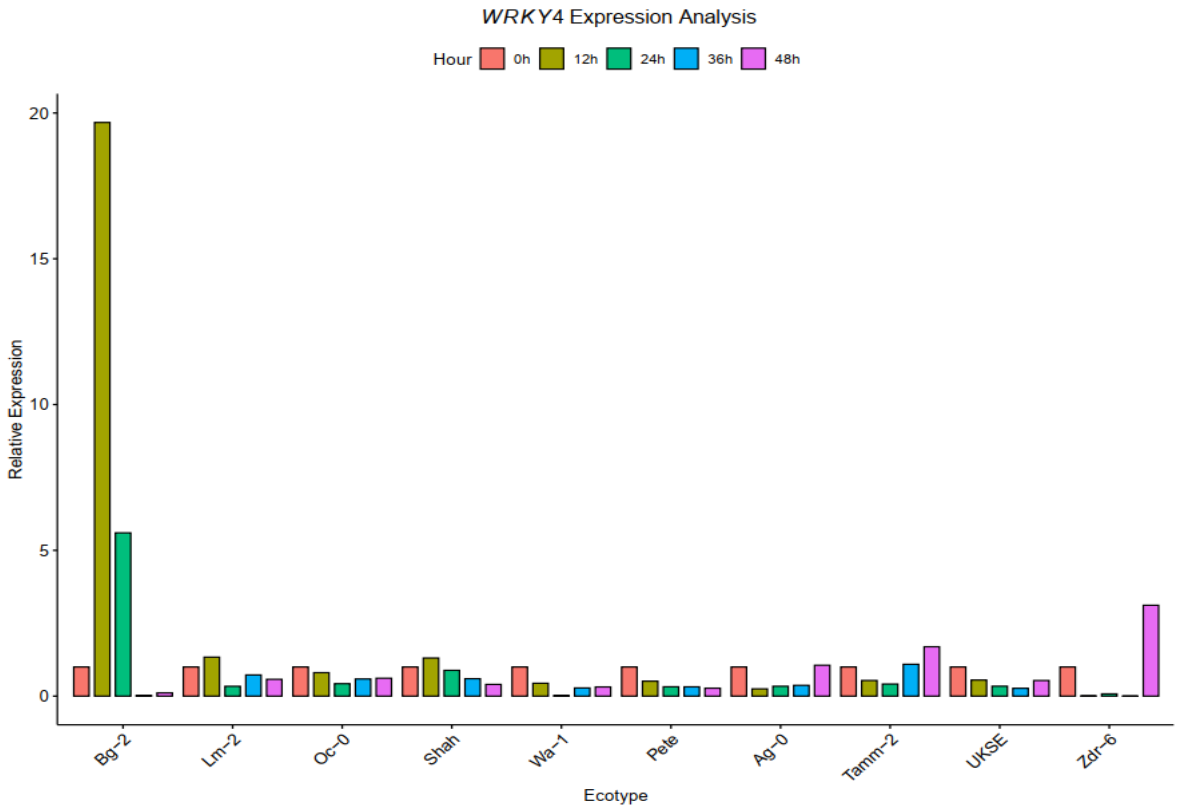


Figure 10. Expression analysis graph depicting the relative expression of *AtWRKY4* at 48h compared to 0h in susceptible (Bg-2, Lm-2, Oc-0, Shahdara, Wa-1) and resistant (Petergof, Ag-0, Tamm-2, Zdr-6) *WRKY4* arabidopsis ecotypes. Here, the X-axis represents the susceptible and resistant lines, while the Y-axis represents the relative expression. Susceptible *WRKY4* arabidopsis ecotypes showed downregulation at 48h, whereas among the five resistant ecotypes, Ag-0, -2, and Zdr-6 exhibited upregulation at 48h, and Petergof (Pete) and UKSE-06 (UKSE) displayed downregulation.

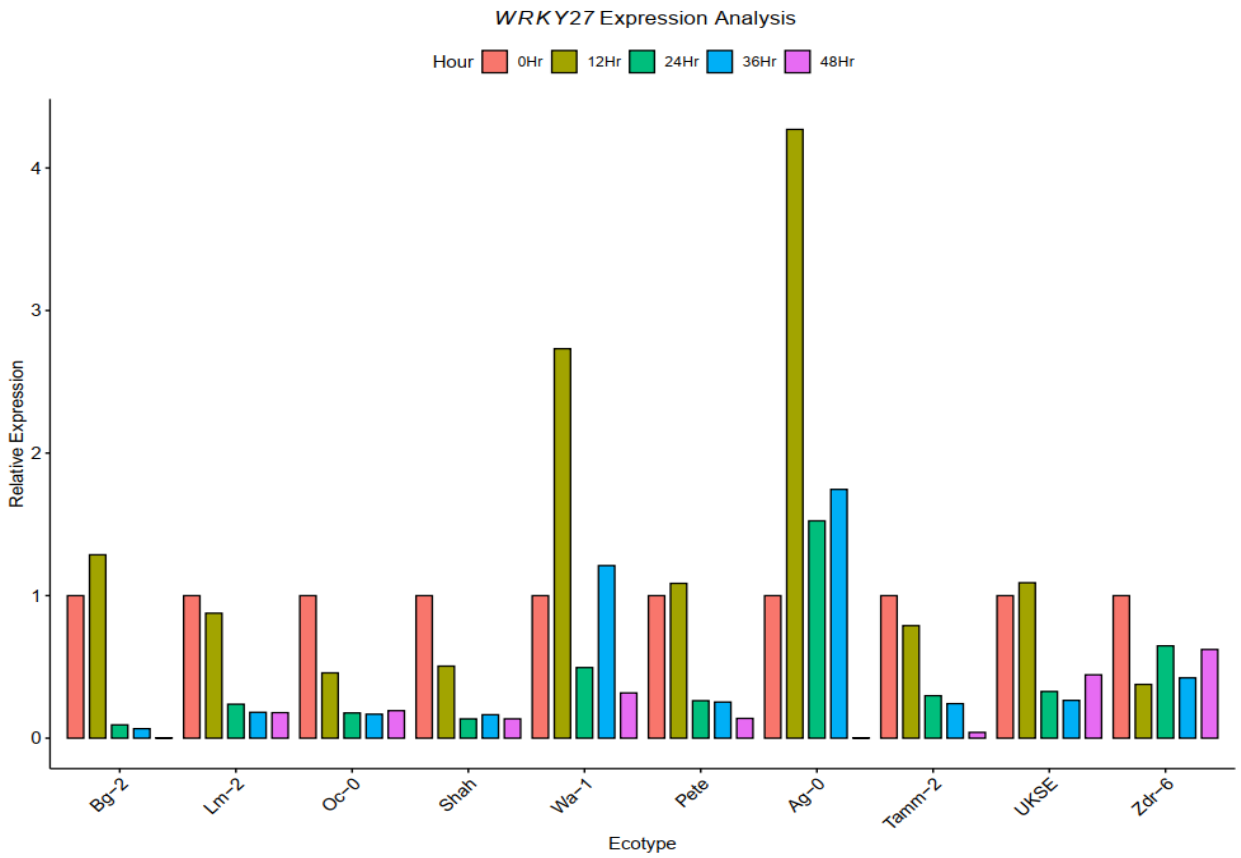
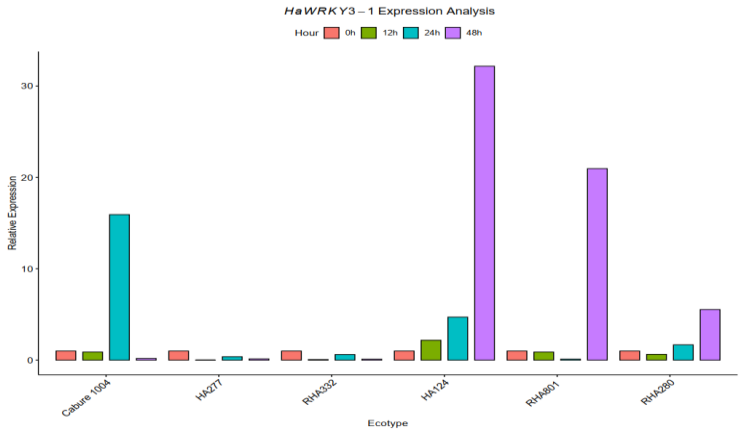
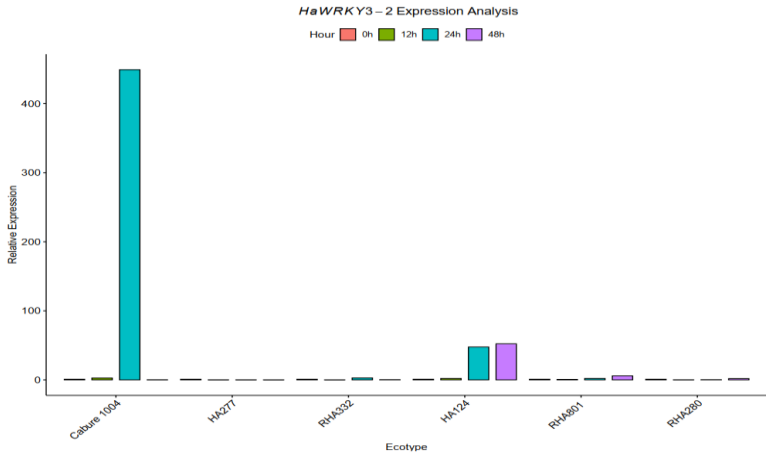


Figure 11. Expression analysis graph depicting the relative expression of *AtWRKY27* at 48h compared to 0h in susceptible (Bg-2, Lm-2, Oc-0, Shahdara, Wa-1) and resistant (Petergof, Ag-0, Tamm-2, Zdr-6) *WRKY4* arabidopsis ecotypes. Here, the X-axis represents the susceptible and resistant lines, while the Y-axis represents the relative expression. Susceptible *WRKY27* arabidopsis ecotypes (Bg-2, Lm-2, Oc-0, Shahdara, Wa-1) displayed downregulation at 48h compared to 0h, while resistant *WRKY27* arabidopsis ecotypes (Petergof, Ag-0, Tamm-2, Zdr-6) exhibited downregulation at 48h.

A.



B.



C.

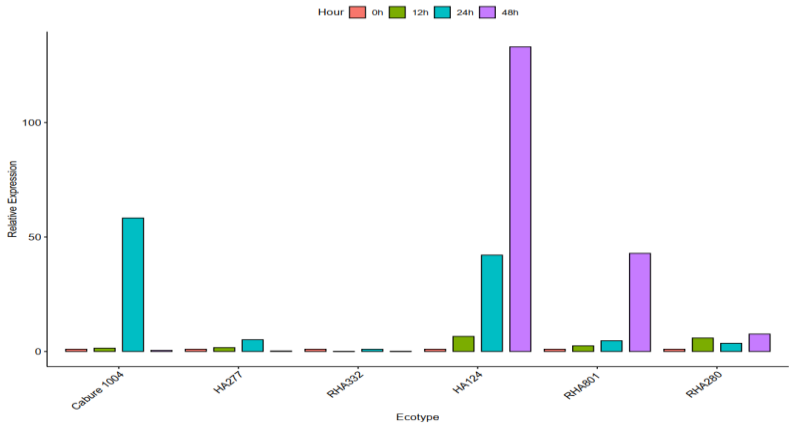
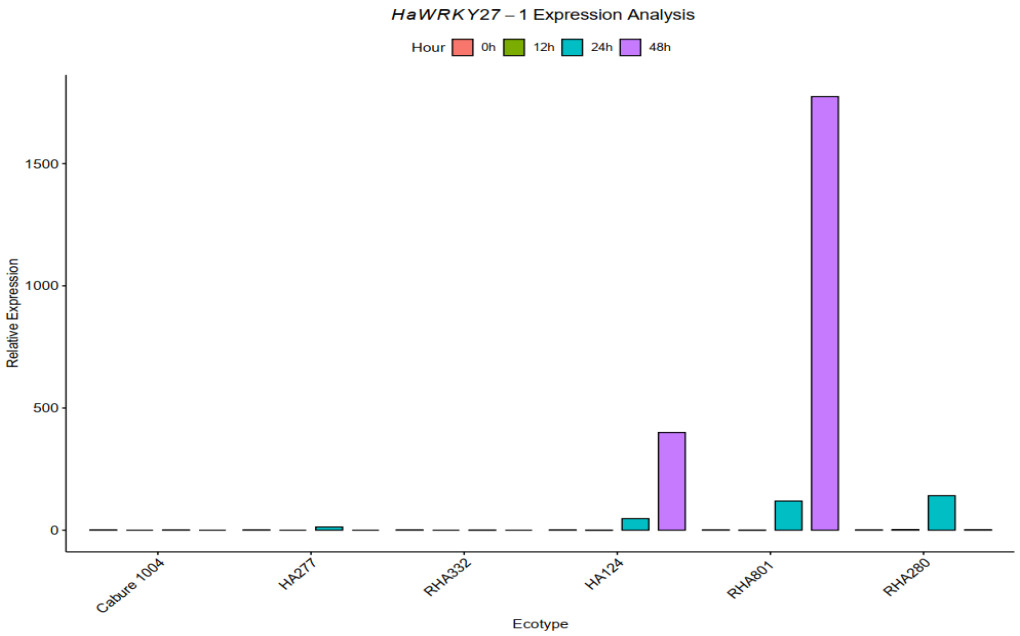


Figure 12. Expression analysis of *HaWRKY3-1q*, *HaWRKY3-2q*, and *HaWRKY3-3q* in susceptible (Cabure 1004, HA 277 and RHA 332) and resistant sunflower lines (HA 124, RHA 801 and RHA 280) to *Sclerotinia sclerotiorum*. Here, the X-axis represents the susceptible and resistant lines, while the Y-axis represents the relative expression compared to 0h. Plants were inoculated with *Sclerotinia sclerotiorum* infested millet seeds at 4-5 weeks old sunflower plants.

A.



B.

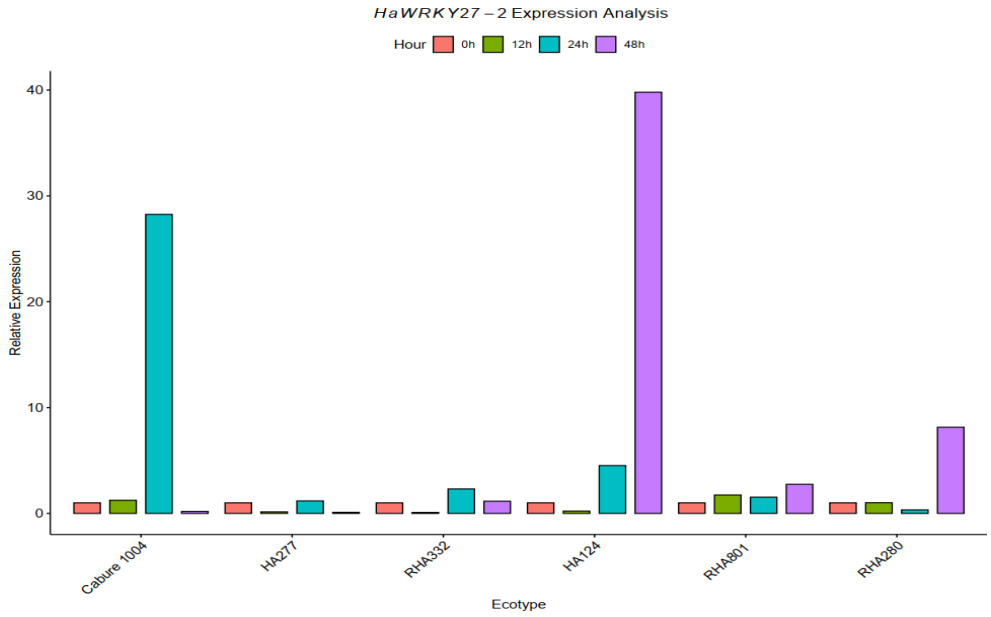


Figure 13. Expression analysis of *HaWRKY27-1q* and *HaWRKY27-2q* in susceptible (Cabure 1004, HA 277 and RHA 332) and resistant sunflower lines (HA 124, RHA 801 and RHA 280). Here, the X-axis represents the susceptible and resistant lines, while the Y-axis represents the relative expression compared to 0h. In *HaWRKY27-1q* sunflower lines, susceptible lines exhibited downregulation, whereas resistant lines, with the exception of RHA280, displayed upregulation (Panel A). Meanwhile, *HaWRKY27-2q* susceptible lines demonstrated downregulation, whereas all *HaWRKY27-2q* resistant lines exhibited downregulation (Panel B).

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