Impacts of cover crop, soil steaming, and plastic mulch on field-grown tomato production and virus-induced gene silencing in Antirrhinum, Penstemon, Petunia, Rosa, and Rudbeckia

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Impacts of cover crop, soil steaming, and plastic mulch on field-grown tomato production and virus-induced gene silencing in Antirrhinum, Penstemon, Petunia, Rosa, and Rudbeckia

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A Thesis
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant and Soil Sciences - Horticulture in the Department of Plant and Soil Sciences

Mississippi State, Mississippi

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Weeds and soil-borne diseases can cause large yield losses in field-grown tomato (Solanum lycopersicum) production. Techniques have been developed to reduce soil-based problems. In this study, we evaluated the impacts of cover crops, soil steaming, and plastic mulch to reduce weed and disease pressure in field-grown tomatoes. Four cover crop treatments were grown in the fall and winter before spring planting. Soils were steamed to a target temperature of 71.1 °C for 0, 5, or 20 minutes. Plastic mulch was also used on half of the rows. Yield, weed densities, and disease incidence were recorded.

Reduced flowering time and stringent flowering requirements may reduce the ability to conduct crosses in many plants. Many factors control flowering. Terminal Flowering Locus 1 (TFL1) inhibits flower development. In this study, we attempted to transiently downregulate TFL1 via virus-induced gene silencing (VIGS) in Antirrhinum, Penstemon, Petunia, Rosa, and Rudbeckia.
DEDICATION

I dedicate this thesis to my family. My wife, Jessica, and my son, Wallace, have been the greatest sources of encouragement and motivation throughout this process.
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This work would not have been possible without the support of many people. Dr. Shaun Broderick was a great mentor, supervisor, and friend. The encouragement and support from the rest of my graduate committee (Drs. Richard Harkess, Guihong Bi, Sorina Popescu, and Alen Henn) were appreciated. Dr. Paul Tseng and his graduate students were also instrumental in making the project successful. I want to acknowledge the staff at the Truck Crops Branch Experiment Station: Janie Taylor, Terri Shaw, Clay Cheroni, Devin Phillips, Bill Berch, and Greg West. Their hard work is the reason projects like this are possible. I also think Dr. Michelle Jones and Dr. Ma Nan for providing the VIGS constructs.
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CHAPTER I
WEED AND SOIL-BORNE PRESSURES IN FIELD TOMATO PRODUCTION AND
GENETIC CONTROLS OF FLOWERING

Tomato Production Abstract

A nutrient-rich substrate free of weeds and pathogens is requisite for maximum profitability in field tomato (*Solanum lycopersicum*) production. Many methods can be used to reduce populations of weeds, weed seeds, bacteria, and other pathogens. First discovered in 1888, steam sterilization quickly became a popular method of preparing soils for planting. However, with chemical advancements, methyl bromide became the preferred fumigant for sterilizing soil. Unfortunately, methyl bromide is highly toxic and depletes the ozone layer. While allowed under restricted use, it was phased out in 2005. Additionally, it cannot be used in organic production. Alternatively, soil solarization is a non-chemical sterilization method, but it requires extended periods when the location is out of use. This puts a potentially profitable piece of land out of use for weeks or months. As changes in regulations and demand for organically certified goods have increased, there has been renewed interest in alternative soil-disinfesting methods. Reviewed here are the effects that pests have on vegetable production as well as soil steaming, plastic mulch, and cover crops as tools to reduce weed populations, control soil-borne pathogens, and increase yield in field-grown tomato production.
Pests

Mississippi was once the largest producer of canned tomatoes in the United States. Most (86%) of Mississippi’s tomato production occurs in the field (USDA, 2017). Field tomatoes experience pressure from pests, reducing yield and increasing economic losses. These pests include weeds and diseases.

Common weeds include nutsedge (*Cyperus* spp.), pigweeds (*Amaranthus* spp.), and crabgrasses (*Digitaria* spp.; Webster, 2006). Yellow nutsedge (*Cyperus esculentus*) is a perennial weed that can reduce tomato yields by 20% (Beste et al., 1992). It reproduces from seed and tubers, thus allowing it to establish sizable populations even in unfavorable growing conditions. Pigweed (*Amaranthus palmeri*) is regarded as one of the most destructive agricultural weeds. Some populations have developed herbicide resistance, making it difficult to control (Roberts & Florentine, 2022). Crabgrass (*Digitaria sanguinalis*) is another common weed in tomato production that has evolved resistance to certain herbicides (Zong et al., 2022). Crabgrass has also been shown to decrease soil microbial biomass carbon due to allelopathic root exudates (Zhou et al., 2013). Another weed with destructive allelopathic effects is barnyardgrass (*Echinochloa crus-galli*). Some populations have developed herbicide resistance, affecting crops worldwide (Bajwa et al., 2015).

Southern blight (*Athelia rolfsii*) is a soil-borne disease severely affecting solanaceous crops in warm temperate regions causing more damage in the southern US than any other pathogen (Kator et al., 2015). Southern blight is an aerobic fungus that first attacks host stems. White mycelium and tan sclerotia are visible signs of infection (Kator et al., 2015). Control of the disease is important in field-grown tomato and pepper production.
Soil Steaming

Soil steam sterilization was developed in the late 1800s (Fennimore & Goodhue, 2016). Non-chemical methods to control weeds and soil-borne pests have led to the advancement of steaming technologies (Bond & Grundy, 2001). The efficacy of steaming is influenced by the time and temperature of the steam as well as the physical properties of the soil (Fennimore & Goodhue, 2016).

Steam is an effective method for controlling weeds. Bitarafan et al. (2022) reported steam's efficacy in controlling barnyardgrass in reused soil. The seed viability of 5 weed populations was evaluated after exposing them to 59 °C to 99 °C for 30 to 540 seconds. At 62 °C to 68 °C steam treatment, there was a 50% reduction in weed seed germination, and with 76 °C to 86 °C steam treatment, there was a 90% reduction in weed seed germination. 94 °C was required for total control (Bitarafan et al., 2022).

Steam sterilization efficacy is dependent on the physical properties of the soil. Many methods have been studied to determine how to heat soil with varying physical properties efficiently. One method is to pump steam through pipes buried at least 6 inches in the soil. (Senner, 1934). The steam then rises through the soil profile instead of being forced into the soil. This has proven more effective in sand, loam, and peat soils versus sheet steaming (van Loenen et al., 2003). A steam rake is an early device that utilized rakes constructed out of hollow pipe to deliver steam 6 inches deep (Senner, 1934). Runia (2000) examined the effects of soil type on steam efficacy and indicated sheet steaming as the preferred method to circumvent poor penetration. In sheet steaming, steam is trapped under a tarp and allowed to penetrate the soil. This method most effectively penetrated clay soils and was less successful in penetrating sand, loam, and peat soils (Runia, 2000).
Soil steaming temporarily decreases soil microbiota, which can be measured through microbial biomass carbon. Soils steamed for 150 minutes at 80 °C undergo a 73% reduction in microbial biomass carbon after one steam cycle. After a second steam cycle, it further declines by an additional 49% (Dietrich et al., 2020.) Dietrich et al. (2020) did not report on the recovery of the microbiome, but that has been documented in other research. For example, McSorley et al. (2006) reported on the recovery of Gram-positive bacteria in steam-treated soils. Dietrich et al. (2020) also reported that soil steaming increased soil pH (7.44 to 7.77) and phosphorus availability, indicating that steam treatment influences the physical properties of soil.

Melander & Kristensen (2011) studied the effects of physical soil properties on steam treatment. They evaluated the impact of target soil temperatures, soil type, and moisture content on steam treatment efficacy. Steam treatments of 60 °C, 70 °C, and 80 °C were used on sandy loam and sand soil types. Soil moisture levels, including dry and moist, were evaluated. Sandy soils take longer to reach treatment temperatures than sandy loam soils. Moist soils also require more time to reach target temperatures than dry soils. Moist sand requires the longest time to reach the target temperature. Despite the time requirement, moist sand required the lowest temperature to achieve 90% control of weed seedling emergence, while dry sandy loam required the highest temperature to achieve 99% control (Melander & Kristensen, 2011).

Fennimore & Goodhue (2016) studied the economic viability of steam sterilization. They noted that environmental regulations require a soil buffer if fumigants are used. This reduces the growing area and potential revenue by 9.62%. A combined approach of steam and fumigation was proposed where a portion of the land would be fumigated, and the buffer would be steamed. It was noted that if steam treatment were used on the buffer, revenues would increase by
$56,575/ha for the steamed section. Steam treatment would cost $34,580, leading to a net increase in revenue of 4.2% ($129,745; Fennimore & Goodhue, 2016).

The duration of treatment impacts the cost of soil steaming. Most chemicals have a 5-day post-fumigation re-entry time, while steamed soils can be planted the next day. Fennimore & Goodhue (2016) reported that it took four days for a farmer to steam the field. If enough labor is available, steaming and planting could be completed in less time than the 5-day fumigation re-entry interval, with steamed soils being planted while non-steamed soils are being treated.

The adoption of steaming technology comes at an economic cost. Although the data is regionally significant, the ideas presented can influence the decision to use steam sterilization over other options independent of location. Environmental restrictions, machine and diesel costs, chemical costs, acreage, and farm revenue must all be considered when evaluating the economic viability of a technology like steam sterilization.

**Plastic Mulch**

Plastic mulch has been a common practice for weed control since the 1950s. It effectively reduces weed populations and increases yield in vegetable production (Lamont, 2017). In 2006, an estimated 400,000 acres of plastic mulched cropland were in the United States (Miles et al., 2007).

Plastic mulch has many physical characteristics, such as the polymer type and thickness, but color also impacts plant growth. Bonanno (1996) reported that black plastic mulch provides the best weed management compared to white or clear mulch and is even more effective when herbicides are concurrently used. Clear mulches have soil-warming properties useful for soil solarization; however, in a cropping system, it does not have the same weed suppression ability as a mulch that transmits no light (Bonanno, 1996).
Brown et al. (1989) studied the impact of black plastic mulch on Southern blight control in bell pepper. They reported reduced disease incidence and severity in plots mulched with black plastic. This reduction was even more prevalent in plots with black plastic mulch in combination with spun-bonded polyester row covers. Disease incidence and severity data correlated with yield data. There was an increase in yield in mulched rows. Previous studies (Brown et al., 1984) noted that yield increase was due to balanced soil moisture, weed suppression, and increased temperature; however, they proposed that disease incidence severity was decreased by preventing dead tissues found in the soil from encountering plant material (Brown et al., 1989).

Tarara (2020) evaluated the effect of plastic mulch on microclimate modification. Changes in microclimate include moisture, temperature, and solar radiation. These factors directly affect yield. Tomato yields, biomass, and soil nitrogen increase with black plastic use (Tarara, 2000).

**Cover Crops**

Cover crops have been widely adopted in many crop management plans. Their benefits include increasing microbial activity in the soil, reducing erosion, and preventing leaching of nutrients. Another key function is their role in weed suppression in field production. Cover crops achieve these functions through physical methods as well as allelopathic interactions.

Physical methods are key when competing with weeds. Kunz et al. (2016) highlighted the need for water, space, nutrients, and light as the main source of competition with weed species. They utilized the following weed species: *Chenopodium album, Matricaria chamomilla, Stellaria media* var. Vill., and *Veronica persica* var. Poir. They utilized the following cover crop species: *Sinapis alba, Raphanus sativus* var. niger, *Vicia sativa*, two mixtures, and a fallow control. There was a 59% and 43% reduction in weed densities 5 and 9 weeks after sowing,
respectively (Kunz et al., 2016). Cover crop mixtures were more successful than single cover crops when exposed to a stress factor. They also examined the physical and biochemical/allelopathic effects of cover crops. They utilized cover crop extracts generated using 0.125 g of fresh plant material per mL of H$_2$O, which were then agitated, centrifuged, and filtered. Amine detection was performed to identify allelopathic compounds, including allylamine, benzylamine, 4-hydroxybenzylamine, and glucosinolates (Kunz et al., 2016). The extracts were used in germination tests to determine their effect on weed suppression. Sinapis alba and Raphanus sativus var. niger had the greatest effect (Kunz et al., 2016). This is likely due to the known allelopathic compounds found in Brassicaceae plants.

Summers et al. (2014) studied how mixed species cover crops affect disease prevalence and severity in tomato production in Maryland, New York, and Ohio. They assessed the impact of mixed-species cover crops on tomato production. The following cover crops were used: vetch and rye, mixed species hay, vetch, radish and vetch, turnip and rye, clover and rye, and bare ground. They evaluated the effect cover crops had on disease incidence. Diseases present at all locations included early blight and Septoria blight. Southern blight was noted in Maryland. Cover crop treatment efficacy was variable on disease incidence. Combination plots (clover and rye; vetch and rye) experienced more Septoria blight incidence than plots with vetch alone (Summers et al., 2014). Similar results were seen in early blight. Vetch and rye plots had significantly more early blight than rye plots. Turnip and rye and clover and rye plots had the least early blight across all treatment years compared to bare ground (Summers et al., 2014). The effects of cover crops depend on multiple factors, including weed pressure, physical properties of the soil, and nutrient content.
Virus-Induced Gene Silencing Abstract

Flower induction and initiation are of major interest in plant research. Flowers have genetic, biochemical, and environmental controls that play a part in their induction and initiation. Flower initiation has been well studied; however, there are still knowledge gaps in how to use this information to enhance flowering further. Many genes play a role in determining flower onset, length, type, etc. The function of these controls can vary, with some being inhibitory in nature while others promote flowering. Terminal Flowering Locus 1 (TFL1) is a key regulator of flower initiation. TFL1 is an inhibitory gene that blocks flowering when transcribed (Wickland & Hanzawa, 2015). TFL1’s relationship with Flowering Locus T (FT) is what keeps flower induction regulated. Many different genes control the series of events that must take place for flowering to occur. Understanding these genes and their function can lead to advancements in floral regulation.

Role of TFL1 & FT

About 70 years of research have been conducted to find a mechanism to induce flowering. Flowering Locus T (FT) was eventually identified, but initiating flowering remains a challenge today (Zeevaart, 2008). More work must be done to find methods to induce flowering in various plant materials easily. Understanding the role of genes that interact with the FT, such as Terminal Flowering Locus 1 (TFL1), is critical to further this work. When TFL1 is expressed, it has a role in maintaining indeterminate growth in the meristem (Hanzawa et al., 2005). FT prevents plants from transitioning from the vegetative to the flowering state.

Both TFL1 and FT are thought to be homologs of phosphatidylethanolamine-binding proteins (Hanzawa et al., 2005). This class of proteins plays a role in regulating pathways that dictate growth. Hanazaw et al. (2005) worked to determine whether a single amino acid change
could convert a flower repressor (*TFL1*) to a flowering activator (*FT*). Accelerated flowering occurs when *TFL1* contains non-functional mutations (Hanano & Goto 2011). Because *TFL1* and *FT* share more than 60% sequence homology and 71% amino acid homology, experiments were designed to determine whether a single amino acid change could convert a flowering repressor (*TFL1*) to a flowering activator (*FT*). Through sequence overlap extension PCR, altered versions of *TFL1* and *FT* were generated and transformed into *Arabidopsis* plants containing both *tfl1* and *ft* mutant alleles. Plants transformed with the *ft* (*Y85H*) mutant appeared like *TFL1*. Amino acid substitutions H88Y in *TFL1* and Y85H in *FT* changed *TFL1* into a floral promoter and *FT* into a floral repressor (Hanzawa et al., 2005). This demonstrates the highly conserved nature of specific amino acids within these genes.

The thorough research of flower initiation at the molecular level provides great insight into the identification and role of *TFL1* and *FT* in the induction/initiation of flowers. The experiments reviewed dictate the function of each of these genes and help give an understanding of the methods they use to accomplish their role in floral development. It also demonstrates that *TFL1* and *FT* have been evolutionarily conserved.

**Changes in *TFL1***

To further identify *TFL1* and *FT* functions, Hanano and Goto (2011) fused *TFL1* to a transcriptional activator domain (VP16) and a transcriptional repressor domain (SRDX). The floral development patterns, such as the number of rosette leaves and flower buds, were then observed to see the impact of the changes. They found that *TFL1* does not affect the transcriptional activation domains for flower initiation. *TFL1*-SRDX, the repressor domain, showed similar results to the *tfl1* mutant. Additionally, mutations in *TFL1* or repression/downregulation of *TFL1* lead to accelerated flowering time. Plants with *tfl1* exhibit
inflorescences that shift from indeterminate to determinate growth patterns. They also have fewer flower buds and terminal flowers at the shoot apices. Additionally, alterations in $TFL1$ expression impact multiple aspects of flower development, not just flower initiation. These data suggest $TFL1$ downregulation would lead to faster flower initiation (Hanano & Goto, 2011).

A single amino acid substitution causes a change in $TFL1$ and $FT$’s role in flowering. Two structural differences (a ligand binding residue and a divergent external loop) are responsible for the functional differences between $FT$ and $TFL1$ (Ho & Weigel, 2014). Ho and Weigel (2014) further demonstrated the sequence homology between $FT$ and $TFL1$, noting that mutations in four different residues could yield an $ft$ that mimics $TFL1$. Those four changes could occur at Glu-109, Trp-138, In-140, or Asn-152 and alter the surface charge of the protein (Ho & Weigel, 2014).

Mutations in $TFL1$ or repression/downregulation of $TFL1$ lead to altered flowering patterns (Hanano & Goto, 2011). For example, plants with altered $TFL1$ expression can exhibit inflorescences that shift from indeterminate to determinate. They also can have fewer flower buds and terminal flowers at the shoot apices. Hanano and Goto (2011) demonstrated that alterations in $TFL1$ expression impact multiple areas of flowering, not just flowering time.

**$TFL1$ in Perennials**

At the molecular level, the sequence and function of $TFL1$ and $FT$ are conserved across many different taxa. The examples provide a basis for $TFL1$’s role in annual flowering plants. The same is true for many perennial species. For example, $KSN$, a $TFL1$ homolog, has been implicated in playing a critical role in flowering in *Rosa* and *Fragaria* (Iwata et al., 2012). Rosaceae plants exhibit continual flowering when $TFL1$ is downregulated.
*Koushin* (*KSN*), the *TFL1* homolog in *Rosa*, is a flower inhibitor. Transcription of this gene inhibits floral activator transcripts and prevents flowering (Randoux et al., 2014). Knockouts of this gene continually flower (Randoux et al., 2014). Kurokura et al. (2013) determined that *KSN* is critical in transitioning from the vegetative (i.e., juvenile) to the reproductive (i.e., mature plant) state. However, more work must be done to understand the role all the factors play in controlling flowering (Kurokura et al., 2013).

Iwata et al. (2012) explored the role of *TFL1* homologs in continual flowering in *Rosa* and *Fragaria*. They used primers designed to amplify *RoKSN*, *FvKSN*, and other *TFL1* homologs to isolate and amplify homologs in *Rosa* and *Fragaria*. They identified a 9 Kbp retrotransposon likely belonging to the *Copia* retrotransposon family in the second intron of *RoKSN* in continually flowering roses. Total RNA extraction was used to test the impact of the retrotransposon on mRNA accumulation. No *RoKSN* accumulation was detected in continually flowering roses. This indicates that an absence of functional *KSN* leads to continued flowering. They then used the understood genetic structure to map the genomes. Total RNA extraction was used to test the impact of the retrotransposon on mRNA accumulation. Through PCR, total transcript accumulation was identified. This indicated how altering *TFL1* levels impacts flowering. Flowering is a key component in many different horticulture and agriculture applications with great economic importance.

**Photoperiodic Requirements**

*TFL1* and *FT* do not act alone; they work with environmental and physiological controls such as day length and phytohormones. For example, *Fragaria* has differing photoperiodic requirements for flower initiation (Kosekela et al., 2012). Kosekela et al. (2012) studied the photoperiodic requirements of mutant *tfl1* alleles. Mutations in *tfl1* reversed the photoperiodic
requirement; however, TFL1’s ability to override the photoperiodic requirement is limited to floral development, not vegetative. TFL1’s control is exclusive to reproductive tissue. The photoperiodic repression that TFL1 expression exhibits is shown to enhance floral initiation. In addition to this work, Kosekela et al. (2012) also evaluated the control of TFL1 on the perennial growth cycle. Photoperiodic regulation of TFL1 expression is important in short-day plants such as Fragaria vesca. It regulates the cycle between vegetative and reproductive states (Koskela et al., 2012). Each part of floral induction does not operate independently.

Kosekela et al. (2012) reported photoperiodic requirements as well. Specifically, short-day (SD) varieties exposed to long photoperiods had active TFL1 mRNA expression, while short photoperiods suppressed it, leading to floral induction (Koskela et al., 2012). The authors altered mRNA expression to understand the impact of photoperiods on flower development. They concluded that in SD of F. vesca, FvTFL1 mRNA expression is photoperiodically regulated and necessary for cycling between vegetative and reproductive phases (Koskela et al., 2012). Kosekela et al. (2012) demonstrated that each part of floral induction operates in concert. The highly complex systems used to induce flower development are organized to work together. Genetic, environmental, and biochemical processes are all intertwined in the floral initiation pathway.

**Conclusion**

The highly complex systems plants utilize to induce flower development have evolved to work together. Genetic, environmental, and biochemical processes are all intertwined in the floral initiation pathway. The articles reviewed give great insight into the role TFL1 and FT play in the induction/initiation of flowers. The information presented dictates the function of each of these genes and helps elucidate the methods they use to accomplish their role in floral
development. It also demonstrates the conserved relationship between *TFL1* and *FT* across plant families. It provides a good basis to study downregulating *TFL1* to alter flower initiation and frequency. The field of plant growth and development is full of interesting and intriguing topics; however, few topics have the impact flowering does. Further work in this area will continue to drive research, education, and economics. Overcoming flower inhibition has significant impacts on research and the economy.
CHAPTER II

IMPACTS OF COVER CROPS, SOIL STEAMING, AND PLASTIC MULCH ON FIELD-GROWN TOMATO PRODUCTION

Abstract

Weeds and diseases result in yield loss in field tomato production. Various methods can be utilized to mitigate their impacts, such as herbicides, plastic mulch, cover crops, solarization, and fumigation. We tested the efficacy of steam, mulch, and cover crops in reducing weeds and diseases of field-grown tomatoes. Clover, vetch, and cereal rye were sown the prior fall. Before planting, the cover crops were cut and sprayed with glyphosate. The field was cultivated. Six rows were formed in each of the three complete blocks. Beds were sown with yellow nutsedge, pigweed, and large crabgrass. Each row received a steam treatment of 0, 5, or 20 minutes (time at 71.1 °C) from a steam generator that produces 308.4 kg/h of steam. Half of each plot was covered in plastic mulch, and at least 5 tomato transplants were planted in each subplot. Tomatoes were harvested, graded, and weighed throughout the experiment. The incidence of Southern blight was recorded. Weed density measurements were also taken. Steam and mulch significantly increased the market, cull, and total tomato yields. Steam treatments of 5 and 20 minutes more than doubled the total yield in mulched beds. Southern blight incidence occurred at 4.9% and 8.1% with 5 and 20 minutes of steam, respectively. 37% of plants in non-steamed soils became infected. Mulch and steam decreased weed coverage. Cover crops were not found to be a
significant factor. Soil steaming will enable farmers to reduce herbicide input while increasing tomato yields.

**Introduction**

Tomatoes (*Solanum lycopersicum*) are among the South’s most-grown vegetables. Two of the most common challenges tomato growers face are weeds and diseases (Gay et al., 2010). Weed pressures are higher in field production than in containers, greenhouses, or high tunnels. Some of the most problematic weeds in field tomato production are yellow nutsedge, barnyardgrass, large crabgrass, and Palmer amaranth. These species reduce profitability by competing with crops for nutrients, light, water, and space. Diseases are also of concern when growing tomatoes. A common disease in southern tomato production is *Athelia rolfsii*, which causes southern blight. It is one of the most damaging pathogens to susceptible crops in southern states (Kator et al., 2015). Southern blight disrupts the vasculature of the tomato, causing the plant to wilt and die (Dixit et al., 2016).

Soil steam sterilization was first discovered in 1888 (Gay et al., 2010). The industrial revolution and the popularity of the railroad led to the use of steam. However, with the advancement of modern chemistries, steam sterilization fell out of favor with the introduction of soil fumigation. Methyl bromide dominated the field of soil sterilization for many years. Changes in EPA regulations and a general concern for the environment revitalized interest in alternative methods of sterilization (Bitarafan et al., 2022). One important consideration is the economic feasibility of steam sterilization. Vidotto et al. (2011) noted no relevant differences in price between herbicides, mulching, and soil steaming.

A much older agricultural practice is growing cover crops, which reduce soil erosion, add organic matter to soils, and increase soil nitrogen levels. Legume species are used for the latter
purpose. Many cover crops, including legumes, cereal, and Brassicaceae, affect weed growth by outcompeting weeds for space (Haramoto & Gallandt, 2004). The biomass from the cover crops works by providing shade to reduce weed densities. Cover crops also have allelopathic attributes via naturally produced compounds such as benzoazinoids and glucosinolates (Mennan et al., 2020).

Mulching is another common weed management practice. Organic and plastic mulches are used in vegetable production. Plastic mulch works by inhibiting light and removing factors that promote the germination of weed seeds (Rajabariani et al., 2012).

There are many benefits of non-chemical weed control (Bond & Grundy, 2001); however, the combined effects of cover crops, mulch, and soil steaming in weed and disease management have not been documented. Therefore, we aimed to assess the efficacy of steam, mulch, and cover crops to reduce weed and disease stress in field-grown tomatoes. The goals were to decrease weed and disease populations using soil steaming and plastic mulch, increase yield in field production, and identify appropriate steaming times needed to reduce weeds and diseases.

**Materials and Methods**

*Experimental site.* Work was conducted during the 2021 and 2022 spring cropping seasons at the Truck Crops Branch Experiment Station in Crystal Springs, MS (lat. 31°56’45.8’’N, 90°22’40.4’’ W). A 70 m by 70 m field was sectioned into three blocks. This layout allowed for a split-split plot design. Year served as the whole plot factor, with cover crop as the split-plot factor. The split-split plot factor was a factorial arrangement of steam treatment and plastic mulch. This arrangement was a randomized complete block design. Each treatment had three replications. Figure 2.1 presents the field layout visually.
Figure 2.1 Visual representation of split-split-plot design.

Split-split plot design with three complete blocks is represented above. Dashed boxes denote the four cover crop treatments: 1) crimson clover, 2) hairy vetch, 3) cereal rye, and 4) fallow control. Numbers above each box indicate how treatments were arranged. Values on the left indicate steam time in minutes at 71.1 °C at a depth of 10.16 cm. Gray rows indicated plastic mulch and white rows indicated bare soil.
**Cover crops.** Four cover crop treatments were used in this study. They were *Trifolium incarnatum* (crimson clover), *Vicia villosa* (hairy vetch), *Secale cereale* (cereal rye), and a fallow control. They were sowed in the fall before the spring planting season: November 2020 and 2021. Crimson clover was sown at 69 kg/ha. Hairy vetch was sown at 84 kg/ha. Cereal rye was sown at 84 kg/ha.

Cover crops were mowed to a height of 13 cm in the spring of 2021 and 2022 before soil treatment and planting. They were then sprayed with glyphosate (Roundup Powermax®, 48.7 % active ingredient) at 868 g/ha to prevent their regrowth.

**Site preparation.** Following cover crop spraying, the field was tilled to a depth of 10 cm. 24.4 m long by 0.6 m wide rows were formed – 6 in each block. Before planting, the field was amended based on soil test recommendations from the Mississippi State University Soil Testing Laboratory. Recommended fertilization rates were 3 kg of 0-20-20 (N-P-K; P₂O₅ and K₂O; Bumper Crop, Schulenburg, TX) and 0.9 kg of 33-0-0 (50% CH₄N₂O and 50% (NH₄)₂SO₄; Bumper Crop, Schulenburg, TX) per row.

**Weeds.** Four weed species were used in this study. They were *Cyperus esculentus* (yellow nutsedge), *Digitaria sanguinalis* (large crabgrass), *Amaranthus palmeri* (Palmer amaranth), and *Echinochloa crus-galli* (barnyardgrass). Weed seeds and tubers (yellow nutsedge) were broadcasted onto the prepared rows and then subjected to a roller to ensure good soil contact. Weed coverage (%) was assessed 10 weeks after treatment (WAT) and rated on a 0 to 100% scale. A 0 indicated no weeds present, and 100 indicated complete coverage.

**Steam treatment.** Steam treatments were randomly assigned with durations being 0, 5, or 20 minutes. The steam generator (Steam-Flo model SF-20, Sioux Corporation, Beresford, South Dakota, USA) delivered steam at 308.4 kg/h to raise soil temperatures. Steam is delivered to the
beds through a permeable nylon sleeve (Figure 2.2). The sleeve was placed on the center of each bed and covered with a non-permeable, heat-resistant tarp. The tarp edges were secured with a chain to minimize steam loss (Figure 2.2). Soil temperature was monitored at a depth of 10 cm using a temperature probe connected to a 4-channel temperature monitoring system (Signals 4-Channel, Thermoworks, American Fork, Utah, USA). Treatment time began once the temperature reached 71 °C. Plots assigned 0 minutes were not exposed to steam treatment.

Figure 2.2    Steam delivery to experimental field plots.

A) A permeable sleeve releases steam that is B) captured under a heat-resistant tarp secured with chains to reduce steam loss.
**Plastic mulch.** Following steam treatment, drip tape was placed on each row to provide irrigation during the study. Additionally, plastic mulch was randomly assigned to half of the rows in each block. A 0.0254 mm black plastic mulch was used. A mid-season image highlighting the placement of plastic mulch can be seen in Figure 2.3.

![Weed emergence in steamed and unsteamed, non-mulched plots midseason](image)

The left row was treated with 5 minutes of steam, and the right row was not steamed.

**Plant material.** *Solanum lycopersicum* cv. Roadster was used in this study. Roadster is a determinate tomato cultivar that typically yields 227 to 340 g per season. Transplants were generated by sowing seeds into 72-cell plug trays filled with soilless potting media (ProMix BX, Premier Horticulture, Quebec, Canada) and grown in a greenhouse. Transplants were fertilized at
each irrigation with 200 mg N·L$^{-1}$ of 20N-4.4P-16.6K (Peters Professional General 20-10-20, The Scotts Co., Marysville, OH, USA). Transplants were allowed to develop at least four true leaves before transplanting into the field. At least five transplants were planted 61 cm apart in each treatment plot, as shown in Figure 2.4.

Figure 2.4  Steamed, non-mulched row in block 2.

*Solanum lycopersicum* cv. Roadster transplants planted 61 cm apart in a row treated with 5 minutes steam and not mulched. Taken 4 WAT.
**Disease assessment.** Plants were monitored for *Athelia rolfsii* (southern blight) infection development. Tomato crowns were visually assessed twice a week for signs of infection. The presence of mycelia and sclerotia was documented.

**Statistical analysis.** All data were analyzed using regression analysis via the least-squares means method in JMP Pro 16.1 (SAS Institute Inc., Cary, North Carolina, USA). After determining the assumptions for analysis of variance were met, treatment means were separated using Tukey’s tests at an alpha value of ≤ 0.05. Means sharing a same letter in the figures indicated that the values are not significantly different.

**Results and Discussion**

**Effects of steam.** Steam treatment significantly reduced large crabgrass (*P* < 0.05). Five minutes and 20 minutes of steam reduced large crabgrass by 74% and 95%, respectively. A decline in Barnyardgrass populations was observed but not found to be significant. Palmer amaranth densities were too low throughout the study to see a decline, suggesting they were outcompeted by other weeds or exhibited poor germination. Yellow nutsedge was the most prominent weed species throughout the two-year study. Yellow nutsedge densities increased with 5 and 20 minutes of steam treatment compared to non-steamed plots in 2021 and had no effect in 2022 (Figure 2.5; Figure 2.6). Yellow nutsedge’s method of propagation through tubers is likely the cause. The experimental field contained existing nutsedge populations. While the top 10 cm temperatures reached temperatures that would kill tubers, nutsedge tubers can be found at deeper depths. Germination for nutsedge is favorable under warm soil conditions (Wills, 1975). We hypothesize that the steaming treatment warms soils at deeper depths to temperatures that break tuber dormancy, resulting in an increased population of actively growing nutsedge.
Figure 2.5  Mean weed coverage (%) after soil steam treatments separated by year and weed species.

Mean weed coverage (%) 10 weeks after treatment (WAT) separated by weed species separated by year comparing soil steam treatments of 0, 5, and 20 minutes.

Figure 2.6  Mean cumulative 2021 and 2022 weed coverage (%) after soil steam treatments separated by mulch and weed species.

Values displayed indicated weed coverage (%) 10 weeks after treatment (WAT) separated mulch treatment comparing soil steam treatments of 0, 5, and 20 minutes.
Steam treatment significantly increased tomato yield \((P < 0.05)\). The effect of steam was evident on tomato yield regardless of mulch. In 2021, 5 and 20 minutes of soil steaming yielded 101 and 103 t/ha, respectively, compared to 39 t/ha in mulched, non-steamed rows; 5 and 20 minutes of steam treatment yielded 94 and 108 t/ha, respectively, compared to 4 t/ha in non-mulched, non-steamed rows. Similar results were noted in 2022, with 5 and 20 minutes of steam treatment yielding 59 and 64 t/ha increases over the 0 minutes of steam treatment. In non-mulched rows, 5 and 20 minutes of steam treatment yielded 54 and 60 t/ha, respectively, compared to 7 t/ha with 0 minutes. Steam treatment also improved marketable yield, with 5 and 20 minutes of steam treatment increasing yield to 30 and 37 t/ha, respectively, compared to 10 t/ha with 0 minutes of steam.

Figure 2.7  Total yield (t/ha) after soil steam treatments and separated by mulch.

Values displayed indicate total yield (t/ha) separated by year, comparing soil steam treatments of 0, 5, and 20 minutes based on mulch treatment.
Figure 2.8   Cumulative 2021 and 2022 total yield (t/ha) after soil steam treatments and separated by mulch.

Cumulative market, cull, and total yields (t/ha) separated by mulch treatment comparing soil steam treatments of 0, 5, and 20 minutes.

Over 37% of tomato plants were infected with southern blight in non-steamed rows. In steamed rows, southern blight incidence was below 10%. The greatest reduction in southern blight was seen with 5 minutes of steam treatment. Differences in southern blight infection rates may have resulted from uneven distribution of *A. rolfsii* sclerotia in the soil. Another possibility may be that the germinating nutsedge tubers brought sclerotia to the roots of the tomato as they grew toward the soil surface, causing infection (Kator et al., 2015).

**Effects of mulch.** Mulch provided significant (*P* < 0.0001*) control of the total weed emergence compared to non-mulched rows. Plastic mulch did not reduce Palmer amaranth or barnyardgrass, but yellow nutsedge populations were reduced by 50%, and large crabgrass populations were reduced by 64%. Palmer amaranth and barnyardgrass populations were
minimal in steamed and non-steamed plots, likely due to an inability to compete with the other weed species or poor germination.

Plastic mulch alone improved yield ($P = 0.0008$ Figure 2.11), and mulched rows produced 10 and 8 t/ha more than non-mulched plots, respectively. Despite showing inhibitory effects in previous research (Brown et al., 1989), plastic mulch did not affect the incidence of southern blight in this study.

![Figure 2.9](image)

**Figure 2.9**  Mean cumulative 2021 and 2022 weed coverage (%) after mulch treatment.

The bars represent weed coverage (%) 10 weeks after treatment (WAT) comparing mulched and non-mulched rows.
Figure 2.10  Mean cumulative 2021 and 2022 weed coverage (%) after mulch treatment.

The bars indicate weed coverage (%) 10 weeks after treatment (WAT) separated by weed species comparing mulched and non-mulched rows.

Figure 2.11  Total yield (t/ha) after mulch treatment.

The bars indicate total yield (t/ha) separated by mulch treatment comparing year.
Effects of cover crop. Cover crops were not significant in reducing weeds or disease in either year of this study. They also had no impact on yield. Previous studies noted the physical effect cover crops had by providing competition for weed germination in terms of light, nutrients, and water. Allelopathic methods of weed suppression have also been noted. Compounds most notably associated with allelopathic properties include phenolics, flavonoids, and terpenoids (Kunz et al., 2016). It has been noted that certain flavonoids and phenolics are thermosensitive (Chaves et al., 2020). Steaming could inactivate their efficacy; however, no beneficial effects on yield, disease, or weed suppression were observed in non-steamed plots.

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Figure 2.12  ANOVA table for all study parameters regarding weed coverage (%).
Parameters indicated with an asterisk (*) are significant (P < 0.05).
Figure 2.13  ANOVA table for all study parameters regarding total yield (t/ha).
Parameters indicated with an asterisk (*) are significant ($P < 0.05$).

**Conclusion**

Steam treatment significantly reduced weed stress from large crabgrass. It also significantly reduced disease incidence compared to the non-steamed treatment. Plastic mulch reduced weed stress from all species tested but did not impact disease stress. Cover crops were not significant in this study.

Despite equipment requirements and operating expenses, soil steam sterilization significantly reduces weed and disease stress in field-grown tomato production without adding to total farm operation costs. In addition, this technology provides the benefit of reducing herbicides which benefits the environment and reduces the risk of developing herbicide resistance.
CHAPTER III
VIRUS-INDUCED GENE SILENCING IN ANTIRRHINUM, HELIANTHUS, PENSTEMON, PETUNIA, ROSA, AND RUDBECKIA

Abstract

Traditional plant breeding of angiosperms requires flowers of the parental lines to make crosses. Breeders are limited in conducting crosses when plants do not freely flower. Flowers often need specific environmental and developmental cues to initiate flowering and may only flower for a short time. **Terminal Flowering Locus 1 (TFL1)** inhibits flower development. When it is downregulated in **Arabidopsis**, flower initiation occurs. We used tobacco rattle virus (TRV) based virus-induced gene silencing (VIGS) vectors to transiently downregulate **TFL1** via RNA interference. This work was performed in **Antirrhinum** (snapdragon), **Penstemon** (beardtongue), **Petunia, Rosa cv. Lady Banks**, and **Rudbeckia** (black-eyed Susan). In this study, we cloned a conserved region of **TFL1** into TRV-based VIGS vectors. The plants were inoculated with TRV-**TFL1** vectors through **Agrobacterium** and grown in growth chambers. Inoculation efficacy was visually monitored with Green Fluorescent Protein (GFP) fused to TRV's coat protein. This method may expedite flower breeding and possibly implicate **TFL1** as a universal gene for inhibiting flower development.
Introduction

Plant breeders primarily utilize flowers for generating crosses. Breeding is hindered if plants have a short flowering window or an extended juvenile stage. For example, pecans can take years to flower, resulting in slow breeding efforts (Wood, 1993). Many experimental methods have been attempted to hasten flowering, such as pruning (Hehnen et al., 2012) and exogenous phytohormone application (Iqbal et al., 2017). Other approaches have involved molecular techniques. For example, the University of California developed FasTrack breeding, which uses a transgenic parent transformed with a constitutively expressed Flowering Locus T (FT) gene. This causes transgenic plants to bloom continuously. When crossed with a non-transgenic parental line, approximately 50% of the F1 progeny contain the FT locus transgene, while 50% do not. The transgene-containing seedlings may either be used for additional crosses or discarded. Although this technique successfully reduces the time required for flower initiation, it has a few limitations. For example, breeders are limited to using the FT-transgenic plant as one of the parental lines. Additionally, the transgenic parental lines must be generated before crosses can be made, which can be time-consuming.

Flower initiation has genetic, phytochemical, and environmental controls that regulate flower development (Irish, 2010); this research focused on the genetic controls. Many genes play a role in determining flower onset and duration. Some genes inhibit flowering, while others promote it. Terminal Flowering Locus 1 (TFL1; Hanano & Goto, 2011) is an inhibitory gene that blocks flowering in most plants studied. TFL1 inhibits FT, thus blocking flower initiation (Wickland & Hanzawa, 2015). Additionally, it has been implicated in inhibiting flower development and slowing the transition from a juvenile to a mature plant (Kobayashi et al., 1999). Koushin (KSN) is a TFL1 homolog in Rosa that inhibits flower development (Randoux et
In previous studies, knockouts of KSN exhibited phenotypes that continuously flowered (Randoux et al., 2014).

An effective way to downregulate genes is through RNA interference using virus-induced gene silencing (VIGS). Plants contain enzymes that recognize, bind to, and digest double-stranded messenger RNA (mRNA). This protects plants from mRNA-based viruses and can be used to regulate endogenous gene expression. The digestion process forms small interfering RNA (siRNA) that are typically 21 to 25 bp long. The siRNA then binds to complementary mRNA sequences and propagates mRNA degradation of the virus or endogenous gene (Broderick & Jones, 2014).

Double-stranded mRNA can be generated for target endogenous plant genes using VIGS, which is a useful tool for analyzing specific gene functions. Unlike forming transgenic plants, VIGS offers transient downregulation of target genes by incorporating a fragment (usually 100-800 bp) of the endogenous target plant gene in a viral vector (Macfarlane, 2010). After inoculation of the plants with the VIGS vectors, double-stranded mRNA is generated of the target gene within the plant, and the target gene is downregulated.

An altered genome of tobacco rattle virus (TRV) is often used as the backbone of VIGS vectors because it has a broad host range and was used in this study (Macfarlane, 2010). One VIGS construct in our study carries a portion of Phytoene Desaturase (PDS). PDS plays a role in chlorophyll formation and function. When downregulated, chlorophyll within the leaves does not develop properly, resulting in white leaves. A negative control (empty vector) for each TRV2 construct was also included.
This study aimed to determine if transient downregulation of TFL1 will allow accelerated and continued flower development in *Rosa, Rudbeckia, and Penstemon* and if it could be adapted to enhance breeding programs.

**Materials and Methods**

*Experimental site.* Work was conducted at the Truck Crops Branch Experiment Station research laboratory in Crystal Springs, MS (lat. 31°56′45.8″ N, 90°22′40.4″ W).

*Plant material.* *Rudbeckia hirta, Penstemon barbatus, Petunia x hybrida,* and *Rosa cv.* Lady Banks were used in this study. *Rudbeckia* and *Penstemon* transplants were generated by sowing seeds into 72-cell plug trays filled with a soilless potting meeting (ProMix BX, Premier Horticulture, Quebec, Canada) and grown in a greenhouse. When transplants were four weeks old, they were transplanted into 1 L (15.24 cm diameter) pots and placed in growth chambers (Percival Scientific, Perry, IA, USA) at 22 °C daytime and 18 °C nighttime temperature with 16-h/8-h light/dark photoperiod. Thirty *Rosa cv.* Lady Banks plants were received from Bracy’s Nursery (Amite City, LA). They were cut back and transplanted into 1 L (15.24 cm diameter) pots filled with a soilless potting media (ProMix BX, Premier Horticulture, Quebec, Canada). They were placed next to the *Rudbeckia, Penstemon,* and *Petunia* in the growth chambers. All plants were fertilized at each irrigation with 200 mg N·L⁻¹ of 20N-4.4P-16.6K (Peters Professional General 20-10-20, The Scotts Co., Marysville, OH, USA).

*Sequence identification.* Sequence data for *TFL1* (Genbank 831683) was accessed from the National Center for Biotechnology Information (NCBI). Basic Local Alignment Search Tool (BLAST) searches were performed to locate potential homologs in other species like *Helianthus* and *Antirrhinum.* This allowed us to identify conserved regions of homology that could be used as VIGS targets in our target species. Sequences were aligned to identify regions of *TFL1* that
are conserved across plant families (*Asteraceae* and *Plantaginaceae*). Efforts to identify conserved *PDS* regions for potential VIGS-target sequences have been previously reported. We used sequences 5´ - TGGAGCGCTTTACTTGTCTTC - 3´ and 5´ - TCCATTGAAGCCAAATACTTTTG - 3´ (Yin-Chih et al., 2010).

**Primer design and vector construction.** The data from literature and BLAST searches were used to design degenerate primers specific to *TFL1* (Genbank 831683). Degenerate primers contain universal coding amino acids that accommodate small sequence variations. Primers were designed to flank *TFL1* and its homologs in our target species. Actively growing leaf tissue was harvested from each plant species. Total RNA was isolated using Trizol according to the manufacturer’s instructions (ThermoFisher Scientific, Waltham, MA, USA), and cDNA was generated using an Invitrogen cDNA synthesis kit (ThermoFisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) was used to amplify *TFL1* from each species using the previously designed degenerate primers. Samples were then separated using gel electrophoresis. *TFL1* was then gel purified by cutting the bands corresponding to the size of the TFL1 fragment.

We utilized information gathered from literature and BLAST searches to synthesize gene strands from Eurofins Genomics (Louisville, KY, USA) to be utilized for VIGS vector construction. (See Table 3.1.) *TFL1* sequences were based on the data from the initial BLAST searches. *PDS* sequences were based on literature reviews that proposed a universally conserved *PDS* sequence (Yin-Chih et al., 2010). Gene strands varied from 236 base pairs to 396 base pairs. The gene strands were designed with restriction sites to allow for ligation into TRV2’s multiple cloning site (MCS). Primers were designed to correspond to the gene strands (Table 3.2).
Table 3.1  VIGS Gene Strands (from 5´ to 3´)

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<td>CCTACGTCTAGATTGCTGATGAGGAAGGAACTCTATGATATTTGCAATGCCAAACACAGGCCAGAGGTCTGTACGGTGTGTTTGTTCTTTTCAAGCAGAAACGAAGGCAGTCGGTGAACCCACCTTTCTCAAAG</td>
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<tr>
<td>PDS</td>
<td>GATGCCAAGGCCAAACATAGGAATCCACAGGTTTGTGTTTGTTCTTTTCAAGCAGAAACGAAGGCAGTCGGTGAACCCACCTTTCTCAAAG</td>
</tr>
<tr>
<td></td>
<td>GATGCCAAGGCCAAACATAGGAATCCACAGGTTTGTGTTTGTTCTTTTCAAGCAGAAACGAAGGCAGTCGGTGAACCCACCTTTCTCAAAG</td>
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<tr>
<td></td>
<td>GATGCCAAGGCCAAACATAGGAATCCACAGGTTTGTGTTTGTTCTTTTCAAGCAGAAACGAAGGCAGTCGGTGAACCCACCTTTCTCAAAG</td>
</tr>
<tr>
<td></td>
<td>GATGCCAAGGCCAAACATAGGAATCCACAGGTTTGTGTTTGTTCTTTTCAAGCAGAAACGAAGGCAGTCGGTGAACCCACCTTTCTCAAAG</td>
</tr>
<tr>
<td></td>
<td>GATGCCAAGGCCAAACATAGGAATCCACAGGTTTGTGTTTGTTCTTTTCAAGCAGAAACGAAGGCAGTCGGTGAACCCACCTTTCTCAAAG</td>
</tr>
</tbody>
</table>

35
Table 3.1 (continued)

<table>
<thead>
<tr>
<th>Helianthus CRTISO</th>
<th>CCTACGTCTAGAGTTAGTTACATGGCCTACTTCATCATCTT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CCACCGCTTTCTCTCTGTTTTCCCGCCACCTCTTGCTGCGG</td>
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<tr>
<td></td>
<td>AACTCGTGAGTTCTTTTTCATACTACTACGCTACGTTGTTA</td>
</tr>
<tr>
<td></td>
<td>CGGTGTCGCCGGAGCCGTCAATGTGACTGATTGAAAGGT</td>
</tr>
<tr>
<td></td>
<td>CCGAAGCCGTAATTCGTCAGATTGGACAGAGATGTT</td>
</tr>
<tr>
<td></td>
<td>TCGGATATGAGAATGCTCAGAAAAGATTTTTCCA</td>
</tr>
<tr>
<td></td>
<td>CCGGAACCTGACATTTATCGGGGACCTAATTGAAAGTG</td>
</tr>
<tr>
<td></td>
<td>GCTATCATAGGTGCTGGCTGCGATGCTCAACCCGCGG</td>
</tr>
<tr>
<td></td>
<td>TGGAGCTTTTGAGATCAAGGGCATGAGGTGGATATATACGA</td>
</tr>
<tr>
<td></td>
<td>GTCAAGGACCTTTATCGGTGGAGATCCCCGTCCT</td>
</tr>
</tbody>
</table>

Table 3.2 VIGS Primer Sequences (from 5´ to 3´)

| Universal PDS F | TGGAAGCCYGCTTTACTTGCTTC |
| Universal PDF R | TCCATTGAAGCCAAATCTTTTGTTT |
| Asteraceae TFL1 F | TACAACCTATCAAGGCAAGT |
| Asteraceae TFL1 R | AGCAGAAACACAAACCTTG |
| Rosa PDS F | GGCACGTCTAGATTCAGTGG |
| Rosa PDS R | TATGGATCCTGAGCTTCAACA |
| Plantaginaceae CEN F | TCATCGGACCCTAGTGAT |
| Plantaginaceae CEN R | CTGGCAATTTGAGGAGACA |

TRV2: TFL1 vectors were constructed through digestion, ligation, and transformation reactions. BamHI and XbaI restriction sites were added to the synthesized gene strands to allow for double digestion following Eurofins Genomics’ protocol ("Optimizing Restriction Endonuclease Reactions"). Digestion reactions were precipitated using an overnight ethanol precipitation protocol. Target sequences were then ligated into their TRV2 vector’s MCS using T4 DNA ligase. The vectors were then transformed into Escherichia coli strain DH5α for easier vector replication and long-term storage. They were then re-isolated and transformed into

[36]
*Agrobacterium tumefaciens* strain GV3101. TRV1 was transformed into a separate culture of *A. tumefaciens* strain GV3101 (Broderick & Jones, 2014). VIGS constructs were sequence verified with Eurofins Genomics (Louisville, KY, USA).

Several tobacco rattle virus-based VIGS vectors were utilized in this study. (Table 3.3 and Figure 3.1.) A TRV vector containing a full-length green fluorescent protein (GFP) was received from China Agricultural University for use in this study (Figure 3.2). Three positive controls were included (TRV2, TRV2:GFP, and TRV2:*PDS*) to ensure the efficacy of the inoculation protocol. A VIGS workflow highlights key steps in constructing the TRV2 vectors (Figure 3.3).

Table 3.3  VIGS Vectors and Description.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRV2:GFP:<em>TFL1</em></td>
<td>TRV2 vector containing a conserved region of <em>Terminal Flowering Locus 1</em> to induce gene silencing of <em>TFL1</em> and a full-length green fluorescent protein fused to tobacco rattle virus’ coat protein allow for visualization of the location of the virus within the host plant</td>
</tr>
<tr>
<td>TRV2:GFP:MCS</td>
<td>A negative control to the TRV2:GFP:<em>TFL1</em> vector</td>
</tr>
<tr>
<td>TRV2:<em>PDS</em></td>
<td>This vector serves as a positive control for post-transcriptional gene silencing. When phytoene desaturase is down regulated, chlorophyll formation is prohibited. This is visualized by the leaves turning white.</td>
</tr>
<tr>
<td>TRV1</td>
<td>This vector is required for co-inoculation with all TRV2 vectors.</td>
</tr>
</tbody>
</table>
Figure 3.1 TRV2 vector map (Liu et al., 2002).

Figure 3.2 TRV2:GFP:MCS vector map (Tian et al., 2014).
Figure 3.3  Workflow detailing the steps of VIGS (Broderick et al., 2020).

Virus-induced gene silencing workflow detailing vector construction, *Agrobacterium* transformation, inoculation, and confirmation.
**Inoculation.** Eight *Rudbeckia, Penstemon, Petunia,* and *Rosa* were inoculated with each vector. Inoculum was grown according to Broderick & Jones (2014). 25 mL of each inoculum were grown. The TRV2 vectors were combined in a 1:1 ratio with TRV1 vectors just before inoculation. The plant material was inoculated via an apical meristem application (Broderick & Jones, 2014). We also tested needless syringe infiltration (Singh et al., 2018) and vacuum infiltration (Zhang et al., 2017).

**Analysis.** Inoculation efficacy was visually evaluated. PDS silencing is manifest in the development of white leaves, and GFP fluorescence was visualized using an 100 W ultraviolet lamp (Analytik Jena, Jena, Germany) in a dark room. Photos of the assessments highlight the differences in inoculation methods (See Figures 3.4-3.7).

Figure 3.4  *Rudbeckia hirta* TRV2:MCS negative control (left) and TRV2:GFP:MCS treated (right) under UV light.

Negative control (left) for GFP fluorescence. *Rudbeckia* trichomes reflecting UV light. GFP treated (right) with mild GFP fluorescence on the leaf blades.
Figure 3.5  *Penstemon barbatus* TRV2:MCS negative control (left) and TRV2:GFP:MCS treated (right) under UV light

*Penstemon barbatus* negative control on the left showing no signs of fluorescence. TRV2:GFP:MCS treated on the right shows fluorescence following vacuum infiltration.

**Results & Discussion**

We successfully generated and sequence-confirmed TRV2 VIGS constructs (Table 3.4).

Table 3.4  Sequence confirmed TRV2 VIGS constructs

<table>
<thead>
<tr>
<th>Species</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Antirrhinum</em></td>
<td>TRV2:CEN</td>
</tr>
<tr>
<td><em>Helianthus</em></td>
<td>TRV2:TFL1</td>
</tr>
<tr>
<td><em>Penstemon</em></td>
<td>TRV2:PDS, TRV2:TFL1</td>
</tr>
<tr>
<td><em>Petunia</em></td>
<td>TRV2:PDS</td>
</tr>
<tr>
<td><em>Rosa</em></td>
<td>TRV2:PDS, TRV2:KSN</td>
</tr>
<tr>
<td><em>Rudbeckia</em></td>
<td>TRV2:PDS</td>
</tr>
</tbody>
</table>

VIGS constructs containing TFL1, TFL1 homologs, and PDS.
Apical meristem inoculation was not effective in *Rudbeckia, Penstemon*, or *Rosa*; however, it was effective in *Petunia* evident by photobleaching. Needleless syringe infiltration showed partial transfection in *Rudbeckia*. Vacuum infiltration showed positive signs of transfection in *Penstemon*.

**Conclusion**

The purpose of this study was to successfully inoculate *Rudbeckia, Penstemon*, and *Rosa* to downregulate TFL1 transiently. No alterations in flowering were observed. Inhibition of chlorophyll synthesis was evident in *Petunia* but was not visualized in other species. Fluorescence was noted in *Penstemon* and *Rudbeckia*; however, the presence of viral infection was not confirmed through sequencing and may have been the result of the refracted light from trichomes. Future work may utilize alternative virus vectors for gene silencing.
REFERENCES


