Impatiens Necrotic Spot Virus Resistance in Transgenic *Impatiens walleriana* & *Lycopersicon esculentum*

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**ABSTRACT**

*Impatiens walleriana* is a popular ornamental crop, with a wide variety of flower colors and low light requirements. Impatiens necrotic spot virus (INSV) is a significant pathogen of *I. walleriana* and other ornamental and vegetable crops. Micro-Tom is a model tomato cultivar used for research due to its small size and short time to fruiting. This project evaluated *I. walleriana* and Micro-Tom transformed with *Agrobacterium*. The construct contained GFP (green fluorescent protein) and hygromycin antibiotic-resistant selectable markers, and the antisense sequence of open reading frame of INSV nucleocapsid protein (N). The N gene is expected to confer INSV resistance by RNA interference or gene silencing. The presence of transgenes was confirmed by PCR. Transgenic *Impatiens* was selfed for two generations. Transgenic Micro-Tom was selfed for 4 generations. Spinach was used as an INSV reservoir. *Impatiens*, spinach and Micro-Tom were mechanically inoculated with INSV and evaluated visually, with assay tests, ELISA testing, and PCR. Spinach was successfully infected with INSV six times of seven attempts. *Impatiens* and Micro-Tom had no successful inoculations of three and five attempts, respectively.
**GENERAL AUDIENCE ABSTRACT**

*Impatiens walleriana*, also known as impatiens or ‘Bizzy Lizzy,’ is a popular ornamental plant. It has a wide variety of flower colors and grows well in shade. Impatiens necrotic spot virus (INSV) is an incurable virus that causes disfiguring dead spots on plants. Micro-Tom is a miniature tomato used for research due to its small size and short time to fruiting. This project tested impatiens and Micro-Tom transgenic plants that had been genetically modified using bacteria. The bacteria had been modified to contain ‘markers’ which allow researchers to confirm the modifications were successful. It also contained a small piece of genetic material from the virus, which was expected to make the plants resistant to the virus by interfering with virus movement and reproduction. These transgenic plants were self-pollinated for multiple generations and tested to confirm the transgene was present. “Wild-type” (not genetically modified) spinach was infected with the virus by hand and infected spinach leaves were used to try to infect impatiens and Micro-Tom. The plants were inspected visually and leaves were tested for presence of the virus. Spinach was successfully infected with INSV six times of seven attempts. Impatiens and Micro-Tom had no successful inoculations of three and five attempts, respectively.
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Chapter 1: Introduction and Literature Review

Introduction

Impatiens necrotic spot virus (INSV), a member of genus *Orthospovirus*, family *Tospoviridae*, is an incurable, disfiguring disease of plants (Whitfield et al. 2005), causing more than a billion dollars in damage to hundreds of floriculture and food crops worldwide every year (Goldbach and Peters 1994). INSV is transmitted by the tiny insect *Frankliniella occidentalis*, Western Flower Thrips via a propagative manner. Once acquired, INSV will be present in the infected thrips for the remainder of its life. INSV-infected thrips feed on and infect plants, which become reservoirs of disease and must be removed and destroyed to prevent the virus from spreading (Hausbeck 2006). Western Flower Thrips (WFT, or simply, 'thrips') have a complex and fast life cycle, are difficult to control, and quickly become resistant to insecticides (Guo et al. 2015).

*Impatiens Walleriana* is a popular herbaceous ornamental plant that is susceptible to INSV. Transgenic plants of other species such as tobacco have been developed that are resistant to INSV (Pang et al. 1993). To evaluate resistance in transgenic plants, operators must be able to infect plants with the virus, either by hand ("mechanically") or with the infected vector that normally transmits the virus, such as thrips.

Dr. Yingui Dan developed a transgenic *Impatiens walleriana* and an accompanying transgenic dwarf *Solanum lycopersicum*, "Micro-Tom," both of which include a construct expected to confer resistance to INSV. The transgenic Micro-Toms were developed to be inoculated in parallel and act as verification that mechanical inoculations were successful. There is no current protocol for mechanical infection of *Impatiens walleriana* with the virus.

This research project is to attempt to develop a working protocol for infecting *Impatiens* with INSV. This method would be used to inoculate self-fertile members of the transgenic *Impatiens*
and Micro-Tom developed for INSV resistance, and evaluate their levels of resistance to the virus.
**Impatiens walleriana**

'Deceitful above all plants, and desperately wicked.'
(J. D. Hooker on *Impatiens* (Grey-Wilson 1980))

*Impatiens walleriana* is a popular ornamental herbaceous plant, native to East Africa. It was introduced to J.D. Hooker at the Kew Gardens in England in the 1800s by samples sent by Kirk and Waller, from an expedition up the Zambezi River with Dr. Livingston (Christenhusz 2010). In 1980, C. Grey-Wilson estimated the number of *Impatiens* species to be at least 850, (1980) while in 2007 Raymond Morgan estimated there to be over 1,000 (2007). New *Impatiens* species have been reported in the literature every year from at least 2010 through 2016. Despite the large family size, only a few *Impatiens* species are easily available commercially in the United States, most commonly *Impatiens walleriana* and, since the 1970s, *Impatiens hawkerii* (Morgan 2007).

**Physical structure, ploidy, reproduction**

*Impatiens walleriana* is an herbaceous perennial, growing 25 - 76 cm tall, with extra-floral nectaries on stems and leaves (Grey-Wilson 1980). It is a diploid plant ($2n = 2x = 16$) (Jones and Smith 1966). It has a gametophytic count of 8 and sporophytic count of 16 (Goldblatt 1988). *Impatiens* species all share a trait that gives them their family name – the explosive dehiscence (release of material by splitting) of their seedpods. When ripe, a seed pod capsule’s elastic valves forcefully roll back at the slightest touch, “impatiently” flinging seeds out (Morgan 2007). Figure 1 shows the spring-like valves in unopened and dehisced seedpods.
Impatiens flowers and leaves become brittle and fragile when dried, making preservation and study challenging - thus J.D. Hooker’s characterization of the plant as deceitful and wicked.

Flowers of all Impatiens are zygomorphic – that is, they are symmetrical if split down the middle top to bottom, but not in any other dimension (Grey-Wilson 1980). The blooms generally have five parts ("5-merous") and are resupinate – they twist 180 degrees before opening so that a flower is ‘upside down’ when it opens. Impatiens walleriana has five petals, five sepals, one of which becomes an elongated spur, and five stamens which fuse into an androecium, a cap which initially covers the gynoecium (stigma and ovary) (Caris et al. 2006) (Grey-Wilson 1980). Figure 2 shows the petals and elongated spur sepals.
Grey-Wilson theorizes that the androecium cap prevents the bloom from being self-pollinated (although it does not prevent different blooms on the same plant from providing pollen) (1980).

Figure 3 shows the androecium of a dissected, unopened, flower. The foamy structures on the outside of the anthers dry after opening into a whitish threadlike mass. In Figure 3, the anthers in the dissected flower have not yet spread apart to present their bed of colorful pollen. In Figure 4, the bloom has opened naturally and the anthers have spread apart.
The androcium dries as the ovary below swells. When the adroecium falls off, the stigma is exposed and is ready to be pollinated. Figure 5 shows the ready stigma with the rest of the flower parts removed.

The Value of *Impatiens walleriana* in the United States

*Impatiens* is a popular bedding and potted ornamental plant in the United States. There are many colors and flower forms to choose from and it performs well in shade. According to the USDA National Agricultural Statistics Service’s Census of Agriculture, *Impatiens walleriana* sales were valued at almost $115 million in 2014. Comparing the United States Department of Agriculture
National Agricultural Statistics Service censuses from 2009 and 2014 showed that only Begonia, Geranium, Pansy and Petunia had more sales. *Impatiens*’ value was down from $174 million in 2009. These numbers do not capture the total value of *Impatiens* sales, as the USDA does not track sales by retailers that did not grow the plants themselves. The profits made by big-box stores that buy plants at wholesale and sell them “as-is” are not accounted for, (USDA 2016a) so we can assume the crops tracked in the USDA’s census are even more valuable than the numbers show.

New Guinea Impatiens (*Impatiens hawkerii*) is tracked separately and in 2014, its sales were valued at $10 million. The USDA uses “*Impatiens* – New Guinea” and “*Impatiens* – other” in its online databases but it stresses in its training materials for census workers that there are only two categories of Impatiens to enumerate – New Guinea and *walleriana* (USDA 2016a). There are hundreds of other *Impatiens* species (Grey-Wilson 1980) but it is unusual to see anything but these two sold in a retail store [personal experience and conversation with floriculturists].

**Impatiens major pests & diseases**

*Impatiens walleriana* is susceptible to bacterial spots and rots, fungus, Botrytis, mites, aphids, thrips and other invertebrates, and Impatiens necrotic spot virus (INSV) and Tomato spotted wilt virus (TSWV) (University of California 2016). *Impatiens walleriana*’s 2014 USA value dropped almost $60 million, nearly a third of its value, from two years previous. This is probably due to problems with viruses such as INSV, and the fungal disease downy mildew. Articles such as “Impatiens: Is There Life After Downy Mildew?” (Daughtrey and Palmer 2014) and “The Sky is Falling - Impatiens Downy Mildew” (Schoellhorn 2016) advised growers to use substitute plants due to the outbreak and spread of downy mildew and have dampened the market for *Impatiens*. 
Breeding disease resistance in such a valuable crop as *Impatiens walleriana* could help to recover the demand for this popular plant.
Micro-Tom, a dwarf cultivar of *Solanum lycopersicum*

'...diminutive in every respect.'
(Harbaugh BK 1989)

Micro-Tom is a dwarf tomato bred in the 1980s by J.W. Scott and B.K. Harbaugh at the Gulf Coast Research and Education Center at Bradenton, Florida. About 15 cm tall (NBRP 2012), it was introduced as a novel variety of ornamental vegetable. Micro-Tom's fast growth and small size has helped it become a popular model plant for research (Carvalho et al. 2011). Figure 6 shows fully-grown Micro-Toms fruiting in 10 cm pots.

![Figure 6: Fruiting Micro-Tom in 10 cm pot. Photo: Pris Sears](image)

**Physical structure, ploidy, reproduction**

Micro-Tom is a self-fertile, open pollinated, (Harbaugh BK 1989) determinant phenotype, (Martí et al. 2006) diploid (Menda et al. 2013) dicot (Carvalho et al. 2011). As with other tomatoes, it has a sporophytic count of 24 (Goldblatt 1988) and 12 chromosomes. Flowers are easily self-pollinated by vibrating the mature flower. It takes about 100 days from seeding to fruiting (Harbaugh BK 1989). Individual plants bear 30 - 50 fruit. Fruits generally are 2 - 3 cm (Figure 7), weigh 2 - 8 grams (personal research, (Harbaugh BK 1989) and have 20-30 seeds (NBRP
although individual fruits have been found that have no seeds or as many as 75 seeds (personal research)].

Micro-Tom stands 10 - 20 cm high (Harbaugh BK 1989) and has mutations in SELF-PRUNING (SP) and DWARF (D) genes (Martí et al. 2006). It is resistant to Fusarium wilt, gray leafspot, fruit cracking, blossom-end rot, blotchy ripening and graywall (Harbaugh BK 1989).

The value of *Solanum lycopersicum* in the United States

Micro-Tom itself is not an important food or ornamental crop, but according to the USDA, "Fresh and processed tomatoes account for more than $2 billion in annual farm cash receipts" in 2016. Only China produced more tomatoes than the USA (USDA 2016b). Fresh and processed tomatoes are the second most popular vegetable in the USA, with only potatoes being consumed more often (USDA 2016a).

Tomato major pests & diseases

Tomatoes are susceptible to fungal blights and wilts, bacterial and viral spots and necrosis, mildews and a host of insect pests (UC Master Gardener Program of Contra Costa County 2015). Tomatoes are the namesake of TSWV, the Tomato spotted wilt virus, an *Orthotospovirus* closely related to INSV. TSWV was first reported in the United States in the 1990s and over the next several years became a significant problem in tomatoes and other crops (Margaria and Rosa 2015). The best way to minimize damage from TSWV is by using virus-resistant cultivars
(Csinos and Martinez 2016). INSV resistant cultivars could be similarly effective, and there may be cross-resistance between the closely related TSWV and INSV.
**Impatiens Necrotic Spot Virus (INSV)**

*'INSV has caused devastating plant death...'*

(Hausbeck 2006)

**Plant Viruses**

The first plant virus identified was Tobacco mosaic virus (TMV), *Virgaviridae* family, in the late 1800s, although it was not clear at the time what the infectious agent was (van der Want and Dijkstra 2006). TMV is a model plant virus due to its stability, longevity, and ease of transmission.

**Tospoviridae Family**

INSV is a plant virus in the genus previously known as *Tospovirus*, family *Bunyaviridae*. In 2017 *Bunyaviridae* was abolished and the order *Bunyavirales* was created (Adams et al. 2017). Family *Tospoviridae* was created; the 11 species previously in *Tospovirus* are now in genus *Orthotospovirus*.

INSV is closely related to TSWV, also an *Orthotospovirus*. TSWV was discovered first in the early 1900s in Australia (Whitfield et al. 2005). During 1915-16 in Australia, tomatoes with a devastating wilting virus disease were studied by Samuel et al. (1930). They could not find evidence that it was transmitted via seeds or in the soil. Only with difficulty were they able to transmit it via sap of crushed infected plants. After testing several types of insects as vectors, they discovered it was consistently transmitted by thrips. In 1984, TSWV was proposed to be part of the *Tospoviridae* family, which up until then had only been known to infect mammals (Whitfield et al. 2005).

INSV can be difficult to distinguish from TSWV and the symptoms of either can be mistaken for fungal, bacterial or other infestations. TSWV is more of a problem in the field and INSV is more prevalent in the greenhouse (Daughtrey et al. 1997). Depending on the host plant, symptoms of
both viruses include necrotic lesions, flecks, and rings, stunting, chlorosis, bronzing, white leaf spots, mosaicking, mottling, vein and stem necrosis, and leaf collapse (Daughtrey et al. 1997). The specific virus infecting a plant can be distinguished using assays, enzyme-linked immunosorbent assay (ELISA) kits and Polymerase Chain Reaction (PCR).

The Orthotospovirus genus is named for the first found member (Tomato Spotted Wilt Virus). INSV was first recognized as being distinct from TSWV in 1990, isolated from Impatiens hawkerii (New Guinea Impatiens) (Law and Moyer 1990).

Tospoviridae family viruses are almost exclusively transmitted by arthropods, except for the well-known Hantavirus, which is vectored by rodents (Ullman et al. 2002). Other Bunyvirale order members infect mammals, not plants, and cause infections in humans such as encephalitis, hemorrhagic fever, and Hantavirus (CDC 2013). INSV is transmitted by tiny, approximately 2mm, Western Flower Thrips (Frankliniella occidentalis). The insect and the virus are now found on every continent (Pappu et al. 2009).

**Physical structure of Orthotospovirus**

Tospoviridae family viruses form spherical virons covered in a lipid bilayer membrane that originates from host cells. Two glycoproteins encoded by the virus emerge from the membrane surface. The virus genome has three single-stranded RNA parts that are "closely associated with the nucleocapsid protein" (Whitfield et al. 2005)
The 80-120 nm diameter (Ullman et al. 2002) spherical virion is encased in a host-derived membrane, shown in blue in Figure 8. Viral encoded proteins, shown in green, protrude from the surface. Structure N is the nucleocapsid protein. L is the polymerase enzyme.

**Orthotospovirus susceptible plants, cost to industry**

1,090 species of plants in 85 families are susceptible to TSWV (Morse and Hoddle 2006), while over 300 plant species are vulnerable to INSV (Lebas and Ochoa-Corona 2008). Many vegetable crops are susceptible, as well as most ornamentals - roses and poinsettias are the *only* flower crops not susceptible to TSWV and INSV (Daughtrey et al. 1997). Infected plants develop a variety of symptoms, the best known being necrotic rings on their leaves. The virus is incurable and infected plants become reservoirs for the disease. They must be scouted out and destroyed to avoid infection spreading to nearby plants (Daughtrey et al. 1997). While costs to the industry are difficult to quantify, in Georgia alone reduction in crop value, damage and cost of control of plant viruses including INSV and TSWV accounted for $3.2 million in damage to ornamental crops in 2014, (Little 2016) up from $0.6 million in 2011 (Williams-Woodward 2013). Even plants that are not infected suffer from thrips feeding damage, which causes discolored dead patches and leaves behind frass (droppings).
Transgenic resistance to INSV

Plants have an antiviral defense response - post-transcriptional gene silencing. This response can be leveraged by genetically modifying plants to express viral protein (Badillo-Vargas et al. 2015). In our study, we used pathogen-derived resistance. The genetic code for the viral protein was introduced to the plants via an Agrobacterium plasmid. The plasmid contained the antisense sequence of an open reading frame (ORF) taken from INSV, with instructions for making a nucleocapsid protein (N). Successfully transformed plants express double-stranded RNA which is processed by Dicer, an enzyme, into short-interfering RNAs (siRNA) (Pooggin et al. 2003). The siRNAs guide the RNA-induced silencing complex (RISC) to target matching viral genetic sequences and degrade them, preventing the virus from completing proteins for movement and replication (Galvez et al. 2014).

Frankliniella occidentalis - Western Flower Thrips

'...Thrips suck...'
(Vyavhare and Kerns 2017)

Frankliniella occidentalis species

There are more than 5,000 members of the insect order Thysanoptera, (Figure 9) also known as thrips ('thrips' is both singular and plural). Thrips cause damage to plants when they insert their eggs under plant tissues, when they feed on flowers, buds and leaves, and when they transmit disfiguring, incurable orthotospoviruses. Only ten of the 5,000 thrips species are vectors of orthotospoviruses (Morse and Hoddle 2006). Of those, only three are known to transmit INSV - Frankliniella occidentalis, F. schultzei, and F. intonsa. F. occidentalis (also known as Western Flower Thrips, WFT or 'thrips') is the most significant of these three, due to its presence all over
the world and the ability to vector INSV, TSWV, and multiple other orthotospoviruses (Ullman et al. 2002).

**Frankliniella occidentalis**, Western Flower Thrips

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phlaeothripidae</td>
<td>3,000+ species</td>
</tr>
<tr>
<td>(Suborder)</td>
<td>(Family)</td>
</tr>
<tr>
<td>Thysanoptera</td>
<td>5,000+ species</td>
</tr>
<tr>
<td>(Order)</td>
<td></td>
</tr>
<tr>
<td>Terebrantia</td>
<td>2,000+ species</td>
</tr>
<tr>
<td>(Suborder)</td>
<td></td>
</tr>
<tr>
<td>7 other families</td>
<td></td>
</tr>
<tr>
<td>(Family)</td>
<td></td>
</tr>
<tr>
<td>Thripidae</td>
<td>1,700+ species</td>
</tr>
<tr>
<td>(Family)</td>
<td></td>
</tr>
<tr>
<td>Frankliniella</td>
<td></td>
</tr>
<tr>
<td>(Genus)</td>
<td></td>
</tr>
<tr>
<td>F. occidentalis</td>
<td>(Species)</td>
</tr>
</tbody>
</table>

**Figure 9: Western Flower Thrips (Frankliniella occidentalis) family tree. Figure by Pris Sears, from (Mound 2009)**

Western Flower Thrips (WFT) are native to western North America and stayed there until the 1960s (Cluever et al. 2015). They spread across the USA in the 1970s and 80s and moved on to Europe and the rest of the world as trade barriers fell and transportation improved (CABI 2014). The tiny 2 mm adult lays even tinier 200 µM eggs, using her saw-like ovipositor to insert them in the host plant's tissues (CABI 2014; Cluever et al. 2015). Eggs are resistant to surface pesticides, as they are protected by plant tissue. Thrips are flat, smaller than a grain of rice, and hide in buds and crevices, traveling long distances unseen on host plants. Thrips have wings and due to their light weight can travel for miles on wind currents (Daughtrey et al. 1997). WFT's small size, wide host range and protected eggs all contribute to what Morse and Hoddle term a successful "Invasion Biology" (Morse and Hoddle 2006).
Thrips Life Cycle

Western Flower Thrips have a complex, multi-stage life cycle, illustrated in Figure 10. WFT eggs are laid in leaf tissue and hatch and emerge in 2 to 4 days. During the first and second instars (developmental phases) the 1 mm thrips have legs but not wings. They hide in buds and crevices, feeding on the host plant. This is the only part of the life cycle when thrips are able to acquire Orthotospoviruses. It lasts 3 - 6 days. If a thrips feeds on an infected plant at this point, as the thrips grows the virus spreads from its gut throughout its body and the thrips becomes infective for the rest of its life (Ullman et al. 1992). As the end of the virus-susceptible phases of the cycle nears, the second instar thrips moves down to the soil where it undergoes two pupal instars. These stages last for 2 to 5 days and the thrips is not mobile and does not eat (Lewis 1973; Robb 1988).

If it did not eat infected plant tissues in the first two instars, a thrips will not acquire the virus in the pupal stages or as an adult. The virus is blocked from disseminating by the mid-gut of uninfected adult thrips. The virus is unable to spread to the salivary glands, so the adult thrips does not become infective (Ullman et al. 1992). The final pupa hatches into an approximately 2 mm winged adult, which lives about 30 days at 25°C (Robb 1988).

Thrips are haplodiploid with diploid females and haploid males. WFT are also parthenogenic - a single female thrips can produce unfertilized male eggs and establish a whole new population (Robb 1988).
The mechanism of INSV acquisition by thrips

According to Mound (2005), thrips feed by piercing and sucking, not rasping. A thrips punches a hole in a leaf using a cone-shaped mandible, and then inserts mouthparts that suck up cell contents and inject saliva. A first or second instar WFT acquires INSV by feeding on an infected plant and sucking up infected materials. The virus replicates in the thrips and it can infect plants for the rest of its life. Virus-carrying thrips may survive periods with no plant hosts, such as when in transit or between crops, and start a new outbreak when plants become available again (Ullman et al. 1993).
The mechanism of plant virus infection by thrips

As mentioned earlier, Mound describes thrips feeding behavior as puncturing. The thrips then alternates between suctioning up cell contents and pumping saliva into the plant, both via the same feeding tube (Mound 2005). Thrips make many small feeding punctures, sucking from and salivating into single cells or small areas. They also engage in longer feeding sessions in which they salivate briefly, then feed for long periods, potentially destroying the leaf all the way through. They can transmit viruses during either feeding behavior (Whitfield et al. 2005).

There are several methods researchers use to infect plants with viruses. Some researchers use the actual vector and keep live infectious thrips populations in mesh cages (15 mesh/cm) (Green 1984). Maintaining and controlling thrips is not practical in many settings. "Rub inoculations" or "sap inoculations" are a common method of lab bench virus infection. Infected tissues are ground up with a buffer and applied onto leaves that have been scratched, pricked or dusted with an abrasive such as carborundum to cause small wounds (Hull 2009) (Green 1984) (Samuel 1931). Mandal et al. (2008) had success in mass-inoculating tobacco, tomato and peanut with TSWV using an airbrush.

Agroinfiltration is a method used to infect plants with *Agrobacterium* containing transgenes and may also be appropriate for infection with infected tissues or cloned viral genomes (Hull 2009) (Padmanaban 2005). An agrobacterium culture is injected into leaf tissue with a syringe.

Another method of virus transmission is grafting an infected plant portion to the target. Top cleft, side cleft and approach grafts are among the techniques which may be used (Green 1984). Barker transmitted potato V virus (PVV) by grafting infected tomato shoot tips into removed shoot apices of uninfected potatoes (Barker 1997).
Research problems - avoiding/controlling thrips

Thrips have many advantages that make them a difficult pest to control.

**Thrips physical characteristics**

Thrips are tiny, approximately 1 mm immature and ~2 mm as adults. They can hide and be transported in unopened buds. They are weak fliers, but can be carried on drafts and wind for long distances. They can also be pulled into vents by air currents, entering HVAC systems, and then distributed through entire facilities.

**Thrips Life cycle timing**

Thrips' 30-day life span is short enough that they can become resistant to pesticides quickly but long enough that they may live through greenhouse cleaning, crop changes and other temporary lacks of food.

**Thrips Pesticide Resistance**

The egg placement and five-stage life cycle improve thrips' resistance to pesticides. Eggs are physically protected by being inserted into plant tissues, so surface drenches do not touch eggs. Any infested plant will probably be hosting thrips at all life stages, which requires repeated pesticide treatments to kill adults and then the eggs when they hatch. Susceptible thrips in the exposed life stages die first, and thrips with resistance survive to breed more resistant generations (Gao et al. 2012). At the beginning of 2017, the Michigan State University pesticide resistance database listed 175 cases of Western Flower Thrips resistance around the world (Whalon 2017).
**Thrips feeding habits**

As described earlier, thrips are piercing and sucking feeders. Thrips are "polyphagous" - they will eat many different plants and will survive happily on weeds if preferred host plants are not available (Van Driesche 2013).

**Defense against Thrips**

**Pesticides and thrips**

Pesticides are an important weapon against thrips but must be used carefully. Pesticides that taste bad to thrips may cause them to stop feeding quickly, spreading the virus but not acquiring a lethal dose (Casey and Sutliff-Shipley 2007). As discussed, thrips can quickly acquire resistance to pesticides. One large California nursery which was studied sprayed 35 times in a year for thrips with little effect (Parrella and Costamagna 2007). Gao et al. (2012) listed four mechanisms of insecticide resistance development. Metabolically detoxifying the agent, being physically resistant to agent penetration, target site changes, and behavior changes all contribute to resistance. Thrips are already good at metabolically dealing with toxins due to their polyphagous feeding behavior. Some have been found to have better physical resistance, which gives more time for metabolic defenses. Gao et al. (2012) also noted resistance to initially effective spinosad pesticides has been acquired in thrips within 2 years of introduction, attributed to changes in nicotinic acetylcholine receptors. Pesticides need to be rotated, although there is disagreement on how often and whether to rotate based on mode of action or mechanisms of resistance acquisition. Growers can use spinosads selectively to bring down populations, and then use less effective methods when populations are low. In addition to pesticide rotation, growers should use an Integrated Pest Management (IPM) defense approach that includes exclusion and biological control (Gao et al. 2012).
**Thrips exclusion**

Various types of mesh and screen are used to prevent thrips from coming into greenhouses through vents and doors. Bell and Baker studied more than 20 commercially available meshes and found that some are much more effective than others, with the best excluding 93% of thrips with only moderate airflow reduction (2000).

**Thrips predators**

Another tool that can be used in an Integrated Pest Management IPM program against thrips is the introduction of predatory arthropods that harass and eat thrips. The Veilleux lab used *Amblyseius cucumeris* (aka *Neoseiulus cucumeris*) in reach-in plant incubators containing potatoes and found, similarly to Van Driesche et al.’s research on greenhouse ornamentals, that these predators suppress thrips but do not completely eradicate them (2006). Other commercially available predators include the "minute pirate bugs" (*Orius* spp), and predatory mites *Amblyseius swirskii* (Cluever et al. 2015).

**Thrips monitoring - sticky cards, indicator plants**

These are not control measures but are useful for monitoring presence and populations of thrips. Thrips seem to prefer blue - Muvea et al., caught 1.5 to 1.7 times more Western Flower Thrips with blue sticky cards than with yellow cards (2014). Even with yellow cards, thrips are more attracted to plants with cards in them (Allen and Matteoni 1991).

Indicator plants are grown alongside crops and ideally show damage from feeding and disease symptoms quickly. Allen and Matteoni investigated petunia, gloxinia, globe amaranth, tomato, pepper, and three species of tobacco as indicator plants for WFT (1991). Petunia was superior in many ways - it is very attractive to thrips, damage is easily seen on leaves (flower buds should be removed), and orthotospoviruses quickly cause necrotic damage at the site of infection but do not
become systemic, so the plant does not become a source of new infections. Not surprisingly, thrips may still like the crop of interest more than they like indicator plants (Casey and Sutliff-Shipley 2007).
Chapter 2: Materials and Methods

Plant Transformation

Dr. Yingui Dan’s lab transformed *Impatiens walleriana* using the transformation method described in Dan et al., (2010) and a method developed for cotyledonary node culture (Baxter 2005). These methods were used to transform *Impatiens* with *Agrobacterium* pCAMBIA1304 vectors containing INSV constructs. See Appendix B for pCambia1304 diagram. The T-DNA region of pCambia 1304 includes GFP (green fluorescent protein) and hygromycin antibiotic-resistant selectable markers. The VR1 construct (Dan, unpublished work) includes the antisense nucleocapsid coat protein (N) of INSV and replaced the GUS gene, between positions 731-2569 in pCambia 1304, with Bgl II and BstE II restriction enzymes. The N gene confers resistance by RNA interference gene silencing, binding to gene transcripts that match sequences and prevent translation (Guo et al. 2015). See Appendix C for complete INSV N protein gene sequence. Micro-Tom was also transformed with the same construct, using a protocol described in Dan et al. (Dan et al. 2006).

Dr. Dan screened transformed *Impatiens* using Southern blot and provided two copies each of 18 F0 (the first plants regenerated after transformation) single insertion lines of *Impatiens*, and two copies each of ten F0 multiple-insertion lines. She also provided over 100 F1 seeds per line for two lines of Micro-Toms containing the virus resistant candidate gene, one line of control seeds with a "hollow vector" (pCAMBIA1304 with no INSV genetic material) and wild-type Micro-Tom seeds.
Plant Material Selection: Transgene Screening with GFP and Hygromycin Resistance

Selectable Markers

An Olympus SZX16 with reflected fluorescence system was used to inspect putative transgenic Micro-Toms grown from T1 seed for expression of the GFP Marker. Hygromycin painting was used to test for resistance. A #4 round brush was used to paint one terminal leaflet on four each of two potentially resistant plant lines. The right side of the leaflet was painted with a 100 mg/L hygromycin and two drops of Tween-20 solution according to Carter (2012). Non-resistant plants were expected to show necrosis on treated leaves. Leaf tips were marked with Sharpie® pens to indicate which had been treated.

Plant Material Selection: Transgene Screening with PCR - Micro-Tom

To confirm presence of the transgene in Micro-Toms to be inoculated with INSV, we conducted PCR on DNA extracted from leaves of 36 plants grown on light benches. Individuals included one third generation putatively transgenic line V10-5-C, 14 fourth generation putatively transgenic line V10-5-C-#, 17 second generation putatively transgenic line V21-1-#, and four wild-types.

Fresh leaf tissue was collected, frozen in liquid nitrogen and stored in a -80°F freezer until DNA was extracted according to a modified CTAB protocol as described in Veilleux et al. (2012). DNA quantity was confirmed with a NanoDrop.

PCR primers tested were SL SQEF/R to amplify a segment of the tomato squalene epoxidase gene sequence (800 bp), GFP.INSV - from the GFP gene to the middle of the INSV coat protein (507 bp product) to verify the presence of T-DNA, INSV.Nos, from the middle of the INSV coat protein to the Nos Terminator (583 bp product) also to verify the presence of T-DNA, and INSV,
to amplify a portion of the INSV sequence without adjacent sequences (480 bp product) which should only amplify the viral coat protein (See Appendix A for primer information).

An initial pilot PCR was done on five samples. A 10 min denaturation at 95°C was followed by 35 cycles of 30 sec 95°C denaturing, 30 sec of 55°C annealing, 1.5 min of 72°C extension and a final two min 72°C extension. An additional PCR was done on all 36 samples, using only the SL_SQEF/R wild-type, GFP.INSV and INSV primers. The reaction mix included two µL Micro-Tom DNA from a 50 ng/µL stock, 12.5 µL Immomix™ (Bioline, Taunton, MA), 0.5 µL 50mM MgCl, 0.5 µL of each primer, brought to total volume of 25 µL with water. A 10 min denaturation cycle at 95°C was followed by 35 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min), and a final 5 min extension at 72°C. PCR was run on a 1% agarose / 1x TAE gel with 0.1% EtBr and visualized with a Bio-Rad (Hercules, CA).

**Plant Material Selection: Transgene Screening with PCR - Impatiens**

Ten putatively transgenic *Impatiens walleriana* were selected for greenhouse growth, self-pollination, and seed collection. To confirm presence of the transgene we extracted DNA and conducted PCR. Fresh leaf tissue was collected into 5 ml tubes, frozen in liquid nitrogen and stored in a -80°F freezer until DNA was extracted (Veilleux et al. 2012). PCR was done, amplifying a 318 bp product of the hygromycin resistance gene from pCAMBIA 1304 with HPT5F and HPT5R primers and an *Impatiens* gene using primers from GenBank accession *Impatiens walleriana* FIM gene for FIMBRIATA (lwfimbF and lwfimbR) designed from Genbank accession AB106271.1 to yield a 212 bp product (Veilleux et al. 2012). See Appendix A for primer sequences.
Initially Lazio Spinach was chosen as the spinach variety as Liu reported it to be highly susceptible to INSV (Liu, Sears et al. 2009), and seeds were available from the Osborne Seed Company in 2015. By 2016 they were no longer available, so cvs. 455, Victoria and Giant spinach seeds were requested from the United States Department of Agriculture Germplasm Resources Information Network (USDA GRIN).

Multiple crops of spinach were grown in reach-in and walk-in incubators and in isolation cages in greenhouse & on light benches. See Table 5 for a summary of spinach types used, sources, and germination rates.

Some plants were grown in a BioQuip "Thrips-Proof Mesh" 45 cm x 45 cm x 76 cm cage as shown in Figure 11 to prevent infestation with thrips in a multi-use glass greenhouse and on lab
light-benches. A hole was made in the sheet metal face of the cage, to accommodate a 2.5 cm PVC hose for drip irrigation. The seam was caulked where the pipe entered the cage. Each pot had a drip irrigation stake.

**Population Generation & Maintenance: Micro-Tom**

Dr. Dan provided over 100 F1 seeds per line for Micro-Toms where the T0 lines had been transformed with the virus coat protein gene, V21-1, Vc, V10-2, one F1 line of control seeds with a "hollow vector" (pCAMBIA1304 with no INSV genetic material but still containing hygromycin resistance and GFP), C2-1 and wild-type Micro-Tom seeds. Initial germination tests showed V21-1 and V10-2 to be the most vigorous.

Seeds were started in Magenta boxes with peat pellets and media as described for *Impatiens*, in domed flats on light benches, and in plastic bags on moist brown paper towels.

There were initially some germination issues with Micro-Toms in peat pellets. Five lots of ten seeds from wild-type and two lines of transgenic Micro-Toms and three lots of ten discolored seeds were weighed and compared to see if there was a weight difference that might be contributing to poor germination.

Micro-Toms were grown in cages in greenhouse and on light bench as shown in previous section, and in pots on open light benches. They were also grown from vegetative cuttings. Cuttings were taken from fruiting plants and dipped in Rootone® rooting hormone. Stems were inserted through holes in plastic wrap covered cups of water and placed in a reach-in plant incubator. After 10 days, the cuttings developed callus and began rooting. After 20 days, the roots were examined for GFP expression with an Olympus microscope and EGFP filter. Blooming transgenic and wild-type Micro-Toms were self-pollinated by vibrating the back of blooms with an electric toothbrush with the brush attachment removed.
Fruits were weighed and seeds counted for 11 individuals from the C21 line T2 generation, five from V10 T2 and one from V21 T2. Fruit was processed (see Appendix H for protocol) and seeds were stored in tubes in boxes, in a walk-in Environmental Growth Chambers seed storage unit at 10°C, 15% humidity.

Other tomatoes grown included M82 tomato from Jared Carter's research, Yellow Pear Tomato, Black Krim Tomato, and Brandywine Red Tomato from Burpee.

**Population Generation & Maintenance: Impatiens**

Transgenic *Impatiens* were received as 46 rooted cuttings in peat pellets and media in Magenta GA-7 Plant Culture Boxes sealed with Micropore tape (see Appendix E for protocol). Kept in a reach-in incubator, the plants needed to be subcultured every couple of months. Boxes were opened on a flow hood and the plantlet had more media added or a cutting of 3-4 cm was taken and transferred to a new box with a peat pellet prepared by adding 50 ml of *Impatiens* grafting medium, protocol provided by Dr. Dan (Appendix E). Peat pellets drying out were rejuvenated by adding 5 ml de-ionized water with a needleless syringe and Fisher 0.2 μm nylon syringe filter.

There were problems with mold contamination with some plants. Rescue was attempted (see Appendix F for protocol) but was not very successful. Copies of surviving lines and wild-types were removed from peat pellets, rinsed in water, and planted in Pro-Mix in 10 cm square pots. They were covered with clean Magenta boxes propped open slightly to acclimate and then kept long-term on light benches at 75 μ E m⁻² s⁻¹, 14 h photoperiod.

*Impatiens* clones for virus inoculation were started in peat pellets, in domed trays in a reach-in incubator. The incubator system was infested with thrips, which quickly infested the cuttings. The incubators were treated with *Amblyseius cucumeris* Slow Release predatory mites from Evergreen Growers Supply. The mites reduced thrips levels sufficiently to not interfere with
plant growth but plants with any level of thrips were not appropriate candidates for INSV inoculation, as they are difficult to control and could spread the virus to other plants.

Large Perma-Nest plant trays and domes were purchased from Park Seed. A rectangle was cut out of the dome for ventilation and sealed with thrips-proof mesh and hot glue. A hole was drilled in the side of the tray and it was fitted with an angled 1" plastic tubing for watering. The tubing was sealed with a cork when not in use and the dome was sealed to the tray with Micropore tape. This arrangement, as shown in Figure 12, allowed the plants to be grown in an incubator or open light bench and not be infested with thrips.

![Figure 12: Isolation tray on light bench. Tray 58 x 30.5 x 6 cm, dome 58 x 30.5 x 10 cm.](image)

**Impatiens self-pollination**

Ten *Impatiens* transgenic clones were chosen for generating seed, nine with single insertions of the transgene, one with a double insertion. Cuttings were rooted in peat pellets, and then transferred to the greenhouse in mid-February in 3.8 L pots with ProMix, 14-14-14 and Osmocote top dress. One drip emitter per pot sat on top of media and provided regular water and
fertilizer. At 57 days, all but the double-insertion Impatiens were blooming. They were self-pollinated by using forceps to remove an androecium with loose pollen and rub the pollen on exposed stigma on the same plant. Forceps were cleaned with ethanol between plants. Over six weeks, all but the epinastic plant were self-pollinated at least 100 times each. Seedpods were covered with 4 cm x 7 cm bags to capture seeds at pod dehiscence. Plants became infested with thrips in the greenhouse and were autoclaved six weeks after pollination.

**Impatiens Pollen Measurements**

To see if there were differences in pollen size among the fertile and non-fertile Impatiens, we collected pollen from open flowers. Pollen was stained with acetocarmine and measured using an eyepiece micrometer with a Zeiss Standard Microscope 16, 40x lens. Ten pollen grains per plant were measured.

**Population Generation & Maintenance: Screening for Ploidy Changes**

Both transgenic Impatiens and Micro-Tom had instances of poor germination and some Impatiens were infertile. Some of the transgenic Micro-Tom had phenological differences such as short internodes and folded leaves. Germination problems and phenological changes could be caused by ploidy changes due to regeneration of parents and repeated subcultures of Impatiens cultured in magenta boxes. Ploidy changes could be ruled out with flow cytometry (Owen et al. 1988). Three single-insertion and one wild-type Impatiens and two each of two transgenic Micro-Toms and one wild-type were tested by flow cytometry.

**Virus Proliferation**

With the proper permitting for transportation and storage, we received about 150 mg of INSV infected tissues from USDA. Spinach is susceptible to INSV transmitted by mechanical
inoculation and can be used as a reservoir for fresh and frozen infected tissue to inoculate other plants. Six spinach cultivars (Lazio, Bloomsdale, Viroflay, 455, Victoria, and Giant) were tried as candidates for virus proliferation. See Table 1 for sources. INSV is a fragile virus requiring ice, or liquid nitrogen for transportation and a -80°C freezer for long-term storage. About 75 mg of tissue ground in chilled Fisher Scientific Phosphate Buffer Solution, 0.1 M (Catalog No. S25472) was used to inoculate spinach grown from seed. Agdia INSV ImmunoStrip® Test strips (Catalog #ISK 20501, Elkhart, IN) were used to confirm infection, using directions provided by vendor. Spinach leaves were harvested in 15 mL Falcon™ conical tubes in liquid nitrogen and then transferred to a -80 freezer for storage. After freezing, this tissue was also confirmed to be infected with INSV by PCR. The tissue was used to inoculate Impatiens, tomatoes, and more spinach. Subsequent generations of infected spinach were also used as sources of fresh tissue for inoculations.

**Plant Inoculation Attempts 1 - 11**

1. The first inoculation attempt was done on ten eight week old Lazio spinach plants (two controls) that had been grown in a cage on a light bench in a lab. Infected tissue (2 mg) was ground in 10 ml buffer, pH 7. Inoculum was taken on ice from freezer to the spinach in another building. See Appendix G for details of protocol.

2. The second inoculation attempt was done on 15 eight week old Lazio spinach plants (three controls), using about half the remaining INSV infected tissue from the USDA. Differences from the first attempt: plants were moved in cage to the freezer with the infected tissues in another building and more inoculum was used.

3. The third inoculation attempt was done on 16 wild-type Impatiens four-week-old cuttings rooted in peat pellets in a sealed tray and dome in a reach-in incubator. Infected spinach tissues
from the second inoculation attempt were used for inoculum. Plants were moved in an insulated tray to another building. Eight plants were treated with the carborundum rub method (Appendix G), on the right half of a leaf. Three plants were controls, treated only with buffer and carborundum. Eight plants were treated by infiltration. A slit was cut in the back of a leaf vein with a scalpel, not piercing the front of the leaf. A 1 ml flat-tipped needle-less syringe filled with inoculum solution was pressed against the slit as the plunger was depressed. Three controls were infiltrated with buffer only. Leaf tips were marked with Sharpie pen.

4. Inoculation attempt three was repeated, with five-week-old *Impatiens* rooted cuttings taken from attempt three plants.

5. Inoculation attempt three was repeated except eight six-week-old rooted cuttings of *Impatiens* had INSV inoculum infiltrated into their primary stems.

6. Six wild-type Micro-Toms and six wild-type M82 one-month-old tomatoes started from seed were inoculated with infected tissues from attempt 2.

7. Eight wild-type M82 tomatoes (three controls), ten wild-type Micro-Toms (four controls), and six Victoria spinach (two controls) all one month old started from seed plants were inoculated with infected tissues from attempt 2. Plants were left in the dark for 24 h before inoculation (Hull 2009). 5 ml chilled buffer was ground in mortar and pestle on ice with 1 g inoculum and 500 mg carborundum. Leaves were rubbed gently four times across with swab dipped in inoculum.

8. Six transgenic Micro-Toms (two controls), four wild-type Micro-Toms (two controls) and Victoria spinach were inoculated with INSV infected tissues from attempt 2. All were three weeks old, started from seed. Differences from attempt 7: buffer was not chilled. Gloved hand dipped in inoculum was used rather than swabs. One spinach and one transgenic Micro-Tom
were intentionally treated roughly, pinching the leaf to crack it and rubbing inoculum across leaf multiple times.

9. Three wild-type M82 tomatoes (one control), 11 transgenic Micro-Toms (three controls), 2 Victoria Spinach, and six wild-type Micro-Toms (two controls) were inoculated using fresh INSV infected spinach tissue from attempt 8. All plants were ten weeks old, started from seed, except the M82 plants that were three weeks old, started from seed. Four fresh infected spinach leaves were ground in chilled buffer on ice. Carborundum was powdered directly onto two leaves per plant, which were rubbed three times on top and bottom with a gloved finger dipped in inoculum (Kumar, Ullman et al. 1993).

10. Four Victoria spinach four-week-old seedlings were inoculated with frozen tissues from attempt 2. Ground frozen infected tissues with carborundum and buffer, on ice. Using gloved hand, dipped thumb and first finger in inoculum, held stem with the other hand, pulled leaf through thumb and forefinger four times, two leaves per plant.

11. Four Yellow Pear tomatoes (1 control), six Black Krim tomatoes (two controls), six wild-type Micro-Toms (three controls), 15 transgenic Micro-Toms (four controls), five M82 tomatoes (two controls), and two Victoria spinach were inoculated with fresh INSV infected spinach tissue from attempt 9. All were eight-week-old seedlings. The spinach with the heaviest line on the assay strip test was the donor plant. Changes from attempt 10: leaf pulled through thumb and forefinger three times.

**INSV Screening**

There are several ways to determine if a plant has a virus. Viruses such as INSV may cause leaf necrosis. A first line of screening is visual inspection of the plants. Tests that are more
conclusive are also available. Assay strips, ELISA and PCR are other methods used in this research.

**INSV Infection Screening with Visual Inspection**

INSV can cause necrotic spots and rings on infected plants. Visual inspection is a useful first step in detecting INSV infections. Visual inspection includes examining the top and bottom of the leaf to see if lesions penetrate all the way through the leaf, counting lesions, measuring lesions, and estimating percentage of leaf surface covered with lesions.

**INSV Infection Screening with Assay Strips**

Agdia ImmunoStrip® for Impatiens necrotic spot virus (INSV) were used throughout this research to test for INSV infection. Instructions provided by vendor were followed. Leaf tissue (0.15 g, about 13 square cm) was taken from donor plants with scissors and forceps. Tools were cleaned between samples by dipping in 95% ethanol and passing through flame. Tissue was inserted into an extract bag with SEB1 buffer (proprietary formulation) and plastic mesh. The closed end of a Sharpie® pen was rubbed over the layers of mesh and tissue until tissue and buffer were homogenized (5 - 10 sec). A test strip containing INSV antibodies was lowered into a channel at the side of the bag until it made contact with the solution. A negative test shows one line on the strip. A positive test shows two lines.

**INSV Infection Screening with ELISA**

A Double Antibody Sandwich (DAS) ELISA (Enzyme Linked Immunosorbent Assay) 96 well plate from Agdia was used to test frozen samples of plants from inoculation attempt 11 for INSV. This qualitative serological test also came with polyclonal capture antibody and alkaline phosphatase enzyme conjugate. PNP Substrate Tablets were also bought from Agdia. The protocol provided by the vendor was followed for an initial 8-well strip pilot, then again for the
remaining 88 wells (https://orders.agdia.com/assets/site/docs/m16.pdf). The wells are coated with the capture antibody and incubated. Plant samples were ground in and diluted with general extraction buffer, added to wells, incubated, and the plate was washed. Enzyme conjugate was added, the plate was incubated and washed, PNP solution was added, plate was incubated and washed, then read in a Synergy BioTek 3 reader with Gen5 software settings Lucifer yellow, Greiner 96 flat bottom, absorbance detection at 405 nm.

**INSV Infection Screening with PCR**

Putatively infected *Echinacea* plants were tested with PCR using infected spinach tissues from inoculation attempt two as INSV-infected standards. Primers INSVsF and INSVsR were used with an Applied Bio-Systems high capacity reverse transcription kit. (Thermo Fisher Catalog number: 4368814). A 5 min denaturation at 95°C was followed by 30 cycles of 1 min 95°C denaturing, 30 sec of 42°C annealing, 1 min of 72°C extension and a final 5 min 72°C extension.
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<td>Seed</td>
<td>Burpee Packed for 2017</td>
</tr>
<tr>
<td>Brandywine Red</td>
<td>Seed</td>
<td>Burpee Packed for 2017</td>
</tr>
<tr>
<td>Impatiens Red Accent Transgenic</td>
<td>Rooted F0 plants in culture</td>
<td>Dr. Dan</td>
</tr>
<tr>
<td>Impatiens Red Accent Wild Type</td>
<td>Seed</td>
<td>Park Seed</td>
</tr>
<tr>
<td>Petunia Supercascade Blue</td>
<td>Seed</td>
<td>Burpee</td>
</tr>
</tbody>
</table>
Chapter 3: Results

Plant Material Selection: Transgene Screening with GFP and Hygromycin Resistance

Selectable Markers

GFP screening was inconclusive in Micro-Tom. GFP expression was expected to be weak due to prior experience with a pCambia construct with GFP driven by a single 35S promoter in transgenic tomato (Carter 2012).

Hygromycin screening in the greenhouse presented problems, as thrips seemed especially attracted to the hygromycin and Tween-20 painted leaves, causing extensive damage and occasionally obfuscating the result of the hygromycin reaction (Figure 13).

V10-2 and V21-1 plants proved to have good germination and hygromycin resistance rates, while C21 and Vc plants did not (Table 2).

<table>
<thead>
<tr>
<th>Plant</th>
<th># Seeds</th>
<th># Germinated</th>
<th># Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic Micro-Tom C21-1</td>
<td>24</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Transgenic Micro-Tom V10-2</td>
<td>24</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Transgenic Micro-Tom V21-1</td>
<td>6</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Transgenic Micro-Tom Vc</td>
<td>24</td>
<td>5</td>
<td>Could not distinguish thrips from hygromycin damage</td>
</tr>
<tr>
<td>Wild Type Micro-Tom</td>
<td>18</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Wild Type Tomato M82</td>
<td>12</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 13: Hygromycin-resistance testing on Micro-Tom.
Figure 13 shows Micro-Tom leaves painted on the right side of the mid-vein with a hygromycin-Tween20 solution. Discolored leaf tips on the left and center photographs are due to using Sharpie pens to mark treated leaves. On the left is a 2 cm hygromycin-treated leaf on hygromycin-resistant transgenic Micro-Tom line V21-1 #1 on a light bench. It showed no damage 12 days after painting with hygromycin. In the center is a non-hygromycin-resistant transgenic Micro-Tom line Vc on a light bench, showing yellowing and necrosis 12 days after painting with hygromycin. The treated leaflet is about 1.5 cm across. On the right is a non-hygromycin-resistant wild-type Micro-Tom in a greenhouse, showing extensive light patches and black frass specks indicative of thrips feeding damage on treated side of an approximately 2.5 cm wide leaf.

**Plant Material Selection: Transgene Screening with PCR - Micro-Tom**

Primers designed to amplify the SL_SQE tomato squalene epoxidase gene, a segment spanning GFP and INSV, another segment spanning INSV and Nos, and INSV were used on two putative transgenic tomatoes, T2 plant V10-5-C and its T3 progeny, V10-5-C (Figure 14).

![Figure 14: Multiplex PCR SL_SQE, GFP.INSV, INSV and INSV.Nos on T2 putatively transgenic Micro-Tom (T2 V10-5-C) and its T3 progeny (T3 V10-5-C)](image)

The product was amplified at 55 °C and showed that both samples had the expected 800 bp product from the squalene epoxidase gene (lanes 1 and 2), the 507 bp band spanning the GFP and INSV coat protein genes (lanes 5 and 6) and the 480 bp INSV band (lanes 7 and 8). The
INSV.Nos primer's expected product (583 bp) spanning the GFP and Nos genes did not appear (lanes 3 and 4) (Figure 14).

![Image of multiplex PCR gel](image)

**Figure 15:** Multiplex PCR SL_SQE, GFP.INSV, INSV and INSV.Nos, T1, T2 and T3 generations putatively transgenic Micro-Toms.

A multiplex PCR with all four sets of primers was used on five putative transgenic tomato plants representing generations two, three and four (T1, T2 and T3) after the initial transformation in two independent transformations, V10 and V21 (Figure 15). Subsequent numbers in plant designations represent generations. V21-2 is T2 and is the second progeny of T0 V21.
Figure 16: Simplex PCR GFP.INSV, multiple individuals in two lines of putatively transgenic Micro-Toms (V21 & V10) and wild-types. Two reliable bands were detected, one expected to be an overlap of the two products of GFP.INSV at 507 bp and INSV at 480 bp, and the squalene epoxidase gene at 800 bp. INSV.Nos again did not appear at the expected 583 bp. Nonspecific light bands at approximately 200 and 350 bp were also apparent. As a result of these difficulties, we used simplex reactions for the SQE to verify the presence of tomato DNA and for the GFP.INSV amplicons to verify the presence of the transgene for characterization of additional germplasm.

Once we established that two simplex reactions would provide the necessary information about which tomatoes were transgenic, we characterized 19 additional putative transgenic tomatoes in two different families (nine individuals in family V10 and ten individuals in family V21) to determine if segregation of the transgene was occurring. Additional numbers indicate subsequent generations. V10-2-5-C-6, for example, is the sixth progeny of V10-2-5-C. Only a single plant (lane 23, T3 plant number V10-2-5-3) appeared to be negative for the transgene (Figure 16).
although a faint GFP.INSV product at the expected 480bp band could be seen for this individual, making its characterization inconclusive.

**Plant Material Selection: Transgene Screening with PCR - *Impatiens walleriana***

Of the ten putatively transgenic *I. Walleriana* chosen for self-pollination, all were tested with multiplex PCR and expressed a 318 bp amplicon of Hpt5 within the T-DNA of pCambia1304, whereas the wild-type expressed only the 212 bp amplicon of *I. Walleriana* FIM gene. (Figure 17).

![Figure 17: PCR Impatiens Hpt5, FIM, 10 T₀ individuals and wild-type.](image)

Table 4 shows the number of transgene insertions in the individuals shown in Figure 17 confirmed by Southern blot, the presence of Hpt5 confirmed by PCR, and fertility confirmed by multiple hand pollinations.

**Table 3: Impatiens PCR Key (Figure 16)**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Plant</th>
<th>Transgene Insertions</th>
<th>Hpt5</th>
<th>Fertile or Sterile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68</td>
<td>Not analyzed</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>1</td>
<td>Yes</td>
<td>Fertile</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>1</td>
<td>Yes</td>
<td>Fertile</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>1</td>
<td>Yes</td>
<td>Fertile</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>1</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>1</td>
<td>Yes</td>
<td>Fertile</td>
</tr>
<tr>
<td>8</td>
<td>17</td>
<td>1</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>2</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>1</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>11</td>
<td>Wild Type</td>
<td>Not analyzed</td>
<td>No</td>
<td>Not analyzed</td>
</tr>
<tr>
<td>12</td>
<td>68</td>
<td>Not analyzed</td>
<td>Yes</td>
<td>Sterile</td>
</tr>
<tr>
<td>13</td>
<td>Master Mix</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
## Population Generation & Maintenance: Spinach

### Table 4: Spinach Growing Results

<table>
<thead>
<tr>
<th>Type</th>
<th>Growing conditions</th>
<th>Media</th>
<th>Germination rate</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloomsdale Long-standing</td>
<td>Greenhouse, in domed flats in cage</td>
<td>Miracle-Gro*</td>
<td>75% @ 12 days</td>
<td>Grew well</td>
</tr>
<tr>
<td>Viroflay</td>
<td>Greenhouse, in domed flats in cage</td>
<td>Miracle-Gro*</td>
<td>20% @ 12d</td>
<td>Grew well</td>
</tr>
<tr>
<td>Lazio</td>
<td>Reach-in growth chamber, in domed flat</td>
<td>Fafard superfine germinating mix</td>
<td>100% @ 16d</td>
<td>Grew well, bolted @ 45 days</td>
</tr>
<tr>
<td>Lazio</td>
<td>Walk-in growth chamber, in domed flat</td>
<td>Vigoro all-purpose potting mix</td>
<td>55% @ 15d</td>
<td>Grew poorly, weak, yellowed</td>
</tr>
<tr>
<td>Lazio</td>
<td>Walk-in growth chamber, in domed flat</td>
<td>ProMix BX</td>
<td>47% @ 10d</td>
<td>Grew poorly, weak, yellowed</td>
</tr>
<tr>
<td>Lazio</td>
<td>Reach-in growth chamber in domed flat</td>
<td>ProMix BX</td>
<td>60% @ 20d</td>
<td>Grew well, became infested with thrips</td>
</tr>
<tr>
<td>Bloomsdale current year</td>
<td>Wet towels in plastic bag</td>
<td>No media</td>
<td>40% @ 19d</td>
<td></td>
</tr>
<tr>
<td>Victoria</td>
<td>Wet towels in plastic bag</td>
<td>No media</td>
<td>80% @ 12d</td>
<td>Germinated well, grew well on light bench</td>
</tr>
<tr>
<td>Giant (USDA) 455</td>
<td>Wet towels in plastic bag</td>
<td>No media</td>
<td>60% @ 12d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wet towels in plastic bag</td>
<td>No media</td>
<td>15% @ 12d</td>
<td>Poor germination</td>
</tr>
</tbody>
</table>

Miracle-Gro potting mix F1144 N 0.21%, K 0.14%, P 0.07%, Fe 0.10%
Reach-in growth chamber settings: 20°C, humidity 60%, 16 hr photoperiod, 122 umol light
Vigoro all-purpose potting mix NPK 0.07/0.04/0.03
Wet brown paper towels in plastic bag on light bench

## Population Generation & Maintenance: Micro-Tom

Micro-Tom tomatoes grown in a cage in the greenhouse with drip irrigation did not become infested with thrips, while Micro-Toms outside the cage did. However, neighboring experimental tomatoes in tubs of water developed powdery mildew, which spread to all of the Micro-Toms.
Micro-Toms set fruit readily upon self-pollination. See Appendix D for seed totals. From seed planting to fruit color breaking took about 100 days.

Wild-type Micro-Tom tended to have better germination rates than transgenics. Some seeds looked visually different from others, with a black color to the outer shell. Seeds were weighed; although the mean seed weights of the transgenic and discolored seeds were numerically lower than the wild-type, there were no significant differences due to variation within classifications (Figure 18).

![Figure 18: Seed Weight Comparisons, Micro-Tom](image)

**Micro-Tom Fruit Weight & Seed Counts**

Fruit were harvested, weighed and seeds counted from 11 C2-1-# 2nd generation, five V10-2# 2nd generation and one V21-1-# second-generation lines. Individual fruit weights ranged from 0.8 to 6.1g. Seeds per fruit ranged from 0 to 75. Number of fruits harvested from each plant ranged from three to 13. Line V10-2-5 had the most fruit and seeds and was chosen for subsequent breeding and inoculation (Figure 19).
**Population Generation & Maintenance: Impatiens**

*Impatiens* grew well in a high-dome tray with a thrips-proof mesh and watering tube, sealed with micro-pore tape, as illustrated below in Figure 20.

![Impatiens approximately 8cm in peat pellets growing in sealed tray and dome](image)

The first lab location where research *Impatiens* were grown on open light benches had been home to pet plants in the past and the potted *Impatiens* became infested with mealy bugs. They
were treated with Marathon and moved to another lab space that had not hosted plants in the recent past. Light was kept low at 75 \( \mu \) E m\(^{-2}\) s\(^{-1}\), plants watered twice a week without fertilizer and flowers pinched off. Plants remained small but could be maintained or used for cuttings at 2.5 years.

![Impatiens growth](image)

**Figure 21: Illustration of Impatiens growth after long-term maintenance on light bench**

Figure 21 shows on the left, a representative wild-type *Impatiens* cv. Accent after 2.5 years in a 10 cm pot on a light bench. On the right is another wild-type *Impatiens* cv. Accent after 2.5 years in 10 cm pot on light bench, then transferred outside for two months of growth (June/July) in an approximately 1.5 m pot containing Miracle-Gro potting mix (N .21%, K .14%, P .07%, Fe .10%).

**Impatiens Self-Pollination**

Over six weeks of self-pollination of ten independently derived transgenic *Impatiens*, one double-insertion line and nine single-insertion lines, most pollinated ovaries aborted within days of being pollinated. Only three of the single-insertion plants were self-fertile, with 55, 39 and 183 seeds collected per plant number 19, 31, and 39, respectively (Table 6, Figure 22, Figure 23).
Figure 22: Impatiens pollination and seed counts

Table 5: Successful *I. walleriana* pollinations, days from pollination to harvest, seeds per fruit

<table>
<thead>
<tr>
<th>Plant #</th>
<th>Days Pollination-Harvest</th>
<th>Seeds/fruit</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>19</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>19</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>31</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>31</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>31</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>31</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>39</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>39</td>
<td>22</td>
<td>56</td>
</tr>
<tr>
<td>39</td>
<td>22</td>
<td>45</td>
</tr>
<tr>
<td>39</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>39</td>
<td>unrecorded</td>
<td>13</td>
</tr>
<tr>
<td>39</td>
<td>21</td>
<td>26</td>
</tr>
</tbody>
</table>
**Impatiens Pollen Measurements**

One-way analysis of variance comparing pollen length and width showed significant differences among individuals but not between fertile and infertile self-pollinated *Impatiens* plants.

---

*Figure 23: Impatiens fruit days from pollination to harvest and seeds per fruit.*

*Figure 24: One-Way ANOVA of Impatiens pollen length x width

#19A, 31A, 39B, wt (wild-type) self-fertile. #8B double-insertion*
Visually double-insertion plant number eight was epinastic, with thickened leaves and petals. It eventually grew more normally and its first bloom opened 48 days after the other *Impatiens* began blooming. It only had enough blooms to be pollinated six times, whereas the others ranged from 27 to 50 times each.

Other phenotypic differences noted were flower petals that tore after incomplete release from the androecium upon opening (Figure 25) and double flowers that were fused at the stem (Figure 26).

Figure 25: Impatiens petal tearing from incomplete release. Photo: Pris Sears

Figure 26: Impatiens left - fused buds, center - back of fused blooms, right - side view of open fused blooms. Photos: Pris Sears
Screening for Ploidy Changes

Of the three single-insertion and one wild-type Impatiens tested with flow cytometry, the results were inconclusive. Of the two transgenic Micro-Toms and one wild-type tested with flow cytometry, all were found to be diploid.

Plant Inoculation Attempts 1 -11

1. 30 days after the first inoculation with carborundum rub treatment on eight spinach plants, no symptoms were shown, and plants were assumed to not be infected.

2. 14 days after the second carborundum rub inoculation attempt on 12 spinach plants, necrotic lesions began to form on some plants. Plants were tested with Agdia ImmunoStrip® for INSV. Asymptomatic as well as plants with lesions tested positive. Some infected spinach were still alive 3.5 months after inoculation, at which time they were autoclaved.

3. Carborundum rub treatment Impatiens did not show any symptoms after the third inoculation attempt. Infiltration treatment showed necrotic symptoms on the treated leaf, which abscised on most plants. One infiltration subject developed a lesion on another leaf and several plants had margin browning (Figure 27). The plant with the lesion and two other inoculated subjects were tested with Agdia ImmunoStrips® but were negative.
4. Inoculation attempt three was repeated but no plants were infected.

5. Inoculum was injected into the stems of eight *Impatiens* but no plants were infected.

6. Inoculated Micro-Toms were tested with assay strips. None were positive. There was a lot of damage to treated leaves from carborundum.

7. One treated M82 tomato developed lesions but neither it nor any of the other inoculated tomatoes and spinach tested positive for INSV with assay strips.

8. Roughly treated spinach was successfully infected with INSV, confirmed with assay strip. None of the tomatoes was infected.

9. Both of the inoculated Victoria Spinach tested positive for INSV with assay strips at 13 days. None of the other subjects was infected.

10. Three of the four inoculated Victoria Spinach tested positive for INSV with assay strips at 14 days.

11. Only the inoculated spinach tested positive for INSV.
**INSV Screening**

**INSV Infection Screening with Visual Inspection**

While visual inspection is a valuable tool for identifying INSV infected plants, it is not foolproof. Necrotic spots are not always present or may be subtle, or may be present for non-INSV-related reasons.

**INSV Infection Screening with Assay Strips**

Assay strips work well and are easy to use, especially for plants that may be infected but not showing any symptoms. Figure 28 shows on the left a 6 cm wide INSV infected spinach cv. Bloomsdale Long-Standing leaf with lesion, in the center, a 4 cm wide asymptomatic infected leaf, and at the right, a fragment of the center leaf in an Agdia ImmunoStrip® for INSV assay test showing a top control line and a bottom positive reaction line.

At around $5 each, the assay strip tests may be too expensive for large-scale testing. Tests are good for about a year and then may have erratic results. A few INSV-inoculated transgenic Micro-Toms showed a faint second line but did not have any symptoms. Subsequent testing with ELISA was inconclusive.

![Figure 28: INSV infected spinach with and without symptoms](image)
INSV Infection Screening with ELISA

The pilot and subsequent ELISA tests both had inconclusive results. The vendor recommended re-doing the test.

INSV Infection Screening with PCR

Figure 29 shows the spinach tested with PCR was positive for INSV at the expected 600 bp. The *Echinacea* was negative. The first lane is Hyperladder 50 bp, 1,2,3 are *Echinacea* that were showing necrotic spots, 4 is INSV-inoculated spinach. Primers within the INSV coat protein gene were used to amplify a 600 bp product

![Image of gel with lanes labeled 1 to 4, showing bands at 600 bp](image_url)

Figure 29: PCR Lanes 1-3 INSV negative Echinacea, lane 4 INSV positive spinach
**Chapter 4: Discussion**

**INSV Testing**

The first line of detecting INSV infection in a plant, visual inspection, can fail when plants are infected but asymptomatic. Agdia INSV ImmunoStrip® Test strips are quick and easy to use, costing about $5 per test. We found a few INSV-inoculated transgenic Micro-Toms had no symptoms but showed a faint positive line on assay strip testing. A technician from Agdia was consulted and agreed with the theory that the assay test could have been reacting to the transgene itself, as it contains DNA from the virus's coat protein. ELISA tests are machine-readable and can produce more quantitative results than the presence/absence lines on assay strips. ELISA plates come with 96 wells, at a cost of about $2.50 per well. The ELISA test itself has many steps and requires practice to do well. PCR might be a better method of detecting presence of the virus if primers were designed that matched the virus but not the transgene.

**Spinach**

Spinach was easy to grow from seed, with drip or hand irrigation, in greenhouses or on light benches. It was successfully inoculated with INSV using carborundum rub and has a large leaf that can be freeze-dried or frozen and used for years as inoculum. Specimens were kept alive on light benches for months after infection. It may be a candidate for a model plant transformed with an INSV resistance vector. It has been already successfully transformed with *Agrobacterium tumefaciens* (Zhang and Zeevaart). The main drawback of spinach was its brittle leaves and stems, which are easily cracked or broken when it is moved.
\textbf{Micro-Tom}

Micro-Tom is an ideal model plant - it is small, grows quickly, and fruits prolifically. Despite many attempts, we were unable to infect Micro-Tom plants with INSV using rub inoculations. One contributor to this difficulty may be the $Sw$-5 gene cluster. The $Sw$-5 cluster contains multiple genes, which confer orthotospovirus resistance and have been introgressed into cultivated tomato from \textit{S. peruvianum}. One gene in the cluster, $Sw$-5\textit{b} provides broad resistance to a variety of orthotospoviruses, including TWSV, Tomato chlorotic spot virus (TCSV), Groundnut ringspot virus (GRSV) and INSV (Dianese et al. 2011) (De Oliveira et al. 2016). The sequenced genome of the 'Heinz 1307' tomato cultivar was released in 2012 (The Tomato Genome Consortium 2012). The $Sw$-5 cluster is on Chromosome 9 (Solyc09g098130.1.1) (Andolfo et al. 2013). BLASTing a partial coding sequence of a functional $Sw$-5\textit{b} allele (Spassova et al. 2001) shows 98\% homology to the allele in Heinz 1307. Whole genome sequencing (WGS) reads of Micro-Tom mapped to the Heinz 1307 genome (build 3.0) show complete coverage of this region indicating the cluster is both present in Micro-Tom and homozygous for the allele present in Heinz 1307. $Sw$-5\textit{b} is a dominant gene and its presence should confer broad resistances to orthotospoviruses including INSV. To confirm the presence of the $Sw$-5 resistance gene in tomatoes, PCR could be done as described in Asprelli (Asprelli and Gallardo 2015).

\textbf{Impatiens walleriana}

\textit{Impatiens walleriana} is a popular ornamental plant that blooms in many different colors and grows well in low light. Although \textit{I. walleriana} typically respond well to hand pollination, the ten transgenic R0 \textit{I. walleriana} that were grown for seed only had 30\% fertility, with most pollinations ending in the ovules dropping. Kwong successfully hand pollinated wild-type \textit{I.
walleriana at several stages of stigma development (1991) and Parker achieved 100% success with outdoor hand-pollination in Costa Rica (2005).

The transgenic *I. walleriana* grown for seed displayed some phenotypic differences from wild-types. The double-insertion individual started out much smaller than the others and took longer to bloom. The remaining nine plants looked visually similar, with occasional fused blooms and incompletely opened blooms. Ploidy changes in the plants could have caused infertility but bloom size changes were not noted and measuring pollen did not find significant differences between fertile and infertile individuals. Transgene placement in the genome could account for the observed sterility and phenotypic differences.

Among culturing techniques used, stock *Impatiens* were most successfully kept long-term on an isolated light-bench rather than in tissue culture or in greenhouses or incubators. Thrips and other pests were best avoided by growing in isolated areas that had not held other plants recently. Recent occupation by pet plants was a source of thrips and other pests such as whiteflies and mealy bugs. A thrips-proof isolation cage was protection in an infested greenhouse if the cage was not opened. A sealed tray and dome with watering tube kept plants pest free in infested reach-in incubators but had high humidity that interfered with visibility.

Many plants may readily be mechanically infected with a virus by wounding the plant and introducing the sap of infected plant tissues. Multiple attempts at mechanical INSV inoculations of *Impatiens* with carborundum rub and infiltration were unsuccessful. Other virus transmission methods that could be tried include using agroinfiltration, particle bombardment, the thrips insect vector, grafting, or the parasitic plant Cuscuta ("dodder"). Agroinfiltration requires virulence genes to be subcloned into *Agrobacterium* which is infiltrated into leaves with a syringe (Padmanaban 2005). Franz et al, successfully inoculated *Vicia faba* seedlings with a *Nanovirus*
by bombarding them with gold particles coated with viral DNA (Franz et al. 1999). Using the vector by feeding thrips on infected tissues of a more easily infected crop such as spinach, and then letting them feed on Impatiens might pass the virus on. This would require careful isolation and management of the infectious thrips. Scion and rootstock grafting is typically most successful among plants of the same species, but Li et al. transmitted viruses from the herbaceous Nicotiana occidentalis to the woody Prunus avium via approach grafting (1996). Cuscuta L., a vining parasitic plant known as dodder, can pass viruses between very dissimilar plants (Hull 2014). Dodder is grown attached to an infected plant, then trained around a target plant of another species. The vine transmits the virus to the target. Virus transmission can be increased by shading the healthy plant and pruning the dodder. This technique would require careful management of the dodder, as it is considered a noxious weed. Dodder may also harbor other viruses than the one of interest.

Using grafting to transfer viruses in Impatiens has not been investigated but I. walleriana has been successfully grafted onto I. olivier (I. sodenii) rootstock and lived for years (Kollmann et al. 1985). A combination approach could be designed, using the vector to infect wild-type reservoir plants and then grafting segments onto donor transgenic candidates for resistance.

I. walleriana is subject to multiple pests, viruses, and molds. While INSV is a serious pathogen of I. walleriana and prompted our efforts into deriving transgene based resistance, the water mold "impatiens downy mildew" (Plasmopara obducens) has become a priority due to its devastation of the I. walleriana industry since 2011 (Keach et al.). Transgenic virus resistance has been successfully deployed for several horticultural plants and seemed a logical choice for INSV. The downy mildew crisis has all but wiped out the use of Impatiens as a bedding plant and requires challenging efforts to mount resistance breeding. Research has been done on the
effects of culture temperature and moisture, fungicides, (Harlan et al. 2017) and breeding on Impatiens downy mildew. Harlan, et al. found that *I. walleriana* cultured at 30°C did not develop downy mildew after inoculation. *P. obducens* is known to develop resistance to fungicides, which also have drawbacks of health risks and high costs (2017). Interspecific hybrid breeding with resistant varieties and species of *Impatiens* such as *I. laurenti* and *I. lyallii* has showed some success, with flowers similar to the better known *I. walleriana* (Keach et al. 2016). *I. hawkerii* is resistant to downy mildew but difficult to cross with *I. walleriana* Keach et al. (2016). Most downy mildews only affect one host. *Plasmopara obducens* only affects *Impatiens* (Catlin 2012) and the literature shows no evidence of cross-resistances to other downy mildew pathogens with better known mechanism of resistance. Until more is known about the biology of *P. obducens* and the genetic mechanism of resistance that some *Impatiens* have, growing at high temperatures and breeding resistant varieties are two current approaches for improving *Impatiens*.

Another potential strategy to combat Impatiens downy mildew is, rather than introduce resistance genes, genetically modify the plant to interfere with the processes that make the plant susceptible to the mildew. Identifying S (susceptibility) genes allows loss-of-function gene editing, which has been successful against other pathogens, such as bacterial blight in rice (Liu et al.). Arabidopsis mutant downy mildew S-genes have been identified which are required for susceptibility to downy mildew (van Damme et al. 2008). Huibers reports orthologs of Arabidopsis downy mildew S-genes DMR1 in tomato, although silencing the DMR1 gene caused sterility and reduced height (2013). Meru and Porterfield used bioinformatics to predicate downy mildew S-genes DMR1 and DMR6 in watermelon and squash (2017). A similar method could be used to seek candidate S-genes in *Impatiens*. 
Barriers to marketing genetically modified ornamental plants

When marketing genetically modified plants, regulatory requirements, consumer attitudes, and patent ownership must be considered. Although at least 30 species of ornamental plants have been genetically modified at least once (Deroles et al. 2002), very few have been brought to market. The International Service for the Acquisition of Agri-Biotech Applications (ISAAA) lists two GM Crop Events approval entries for Japanese company Suntory's modified flower color hybrid blue tea roses in the USA in 2011 but no other ornamentals are listed as of November 2017.

In May, 2017, the USDA announced that more than a dozen varieties of orange, red, and purple petunia sold in the USA were unauthorized genetically engineered plants and issued instructions on destruction of seeds and plants. Originally developed in the 1980s using maize genes to modify color, the petunias were unpopular with a public that at the time strongly disliked genetically modified organisms. Although they were approved for trialing in Florida, they were never officially brought to market. It is unclear how they did enter the market (Servick 2017), but due to lack of approvals all are to be destroyed.

A Japanese/Australian company, Florigene, has marketed genetically modified flower color purple carnations in the USA since 2011 as cut flowers. Plants are grown in Ecuador and Columbia; cut flowers can be purchased online. Vendors such as Sam's Club make no specific mention that the flowers are genetically modified. Suntory's blue roses were approved in the USA in 2011 but initial trials apparently did not go well as they were reselected and only announced as being available to florists in October 2017 (Sparks 2017).

The cost to genetically modify an ornamental crop is not insignificant, and as it is a very diverse industry, any given modified plant species is not going to enjoy a large market share. Consumers
do not seem to be bothered by cut flowers with genetically modified flower color and already accept a variety of patented and sterile ornamental plants. The public might also be receptive to ornamental bedding and house plants which have been modified for improved health, color, size or scent.

Many components of the plant genetic modification process are covered by patents. For instance, in 1998 Novartis/Sandoz was assigned patent to many methods of transforming a plant for INSV resistance, although it was allowed to expire in 2010 (Van Grinsven et al. 1998). Researching and licensing can be costly and time-consuming. Dobres and Yoshikazu Tanaka, Chief Operating Officer of Japan's Institute for Plant Science, suggest costs to operate, regulatory obstacles and patent licensing costs may be more of an issue for developers than consumer concerns about genetically modified plants (Dobres 2008) (Yoshikazu 2014).
## Appendices

### Appendix A: Primer Sequences Used, 5' to 3'

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Appendix B: pCAMBIA1304, a legacy plasmid vector from the Cambia organization. 1: Hygromycin resistance, 3: Kanamycin for bacterial selection, 0: puc18 polylinker in Lac Z a, 4: reporter gene mgfp5:gusA. INSV genetic material replaced GUS, between positions 731-25

http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1304/
Appendix C: INSV N protein gene sequence, isolate SV-L1 nucleocapsid protein (N) gene, complete cds, GenBank: KF745140.1

>gi|573033258|gb|KF745140.1| Impatiens necrotic spot virus isolate SV-L1 nucleocapsid protein (N) gene, complete cds

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November 24, 2017.
### Appendix D: Micro-Tom generations, transgene status, seed counts

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<td>3/31/2016</td>
<td>09/30/16</td>
<td>79</td>
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Appendix E: Impatiens grafting medium protocol, developed by Dr. Yingui Dan

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>For 1 liter</th>
<th>For 3 liters</th>
<th>for 6 liters</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS Basal medium with vitamins</td>
<td>2.2 g/l</td>
<td>6.6g</td>
<td>13.2g</td>
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<td>(Prod No: M519, PhytoTechnology Laboratories)</td>
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</tr>
<tr>
<td>IBA</td>
<td>1 mg/L</td>
<td>3mg</td>
<td>6mg</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5 g/l</td>
<td>15 g</td>
<td>30 g</td>
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</table>

Weigh dry ingredients and add to water with stir bar on stir plate. pH to 5.8. Put one peat pellet, hole up, in each Magenta box to be used. Add 50 ml grafting medium per box. Put lids on loosely, autoclave on liquid setting.
Appendix F: Contaminated Impatiens Rescue Protocol developed by Dr. Yingui Dan

Prepare autoclaved Magenta boxes with peat pellets and media (Appendix 5)
Prepare five 50 ml conical tubes with the following contents. Do not fill all the way to the top, there needs to be room for the explant and forceps.
1. Ethanol (70% ethanol/30% sterile DI water) 30 seconds to 1 minute
2. Bleach + tween (30% bleach/70% sterile DI water + 2 drops tween) 30 seconds
3. 100% Sterile DI water 1 minute
4. 100% Sterile DI water 1 minute
5. PPM (20% PPM/80% sterile DI water) 1 minute

Work under flow hood with sterilizer for scalpel and forceps.
Open Magenta box with contaminated plant.
Take a cutting - enough stem to go in peat pellet and two to five leaves or remove entire plant if it comes out easily. Close contaminated box quickly to reduce mold movement.
Put explant into each tube for specified times.
Close tube and shake or roll very gently, then extract explant and put it in the next tube.
This process is very hard on Impatiens.
Without further rinsing, open sterile magenta box, quickly put explant into the peat pellet, close, seal with micropore tape and return to incubator.
Each session needs fresh materials in the first four tubes. The PPM can be re-used.
Appendix G: Modified Protocol of Dr. Hsing-Yeh Liu (United States Department of Agriculture, Agricultural Research Service) to propagate INSV in spinach

Grind freeze-dried infected spinach tissue in 0.1 M phosphate buffer, pH 7.0 (1:50, wt/vol) and use carborundum to wound the leaf and then put on the solution.

Select a middle leaf, not a top one or bottom one. Mark it with a paper tag or Sharpie marker, number the plant and the leaf. Record what leaves are being used. Inoculate two leaves per plant. Use a small brush to put a small amount of carborundum on left half of a leaf.

Use a pipette to place 10 microliters of solution on top of the carborundum on the left half of leaf. Hold back of leaf to keep it steady and use a sterile cotton swab to gently swab twice (left, then right).

Control plants will receive carborundum and be swabbed with buffer with no inoculant in it.

Lesions on the leaves should begin appearing within several days, and when 30 - 70% of the leaf surfaces are covered with lesions, harvest by cutting leaves into small pieces for ease of weighing later, put into freezer-safe tubes and store in -80° freezer until needed.
Appendix H: Micro-Tom Seed Processing Protocol

Cut open fruit horizontally with scalpel, slice outer skin and flesh all the way around
Seeds are in a gelatinous matrix - scoop them out and put into a small glass flask with an inch of
1% HCL, 99% water
Write the plant/fruit # on the flask with sharpie
Label a 3" square of aluminum foil with plant/fruit #
Put stir bar into flask
Put on stir plate, no heat, mid-speed
Stir 3-5 minutes until jelly is dissolved and seeds are separated
When the solution looks foamy, they should be finished
When done, take off stir plate and take to sink.
Pour contents into strainer over beaker to capture HCL solution for re-use
Rinse seeds in strainer under gentle tap water, get them clumped into middle of strainer
Turn strainer upside down over labelled foil and tap sharply, seeds should fall onto the foil
Spread seeds into a single layer
Rinse strainer; make sure there are no seeds stuck in it
Use forceps to remove any tissue clumps that are not seeds from the foil
Put foils in vegetable dehydrator for 14 hours at 95 degrees
One layer of foil per level in the dryer (do not stack foil or let them overlap)
Label storage vials and put one desiccant pellet in each vial
When seeds are dry, scrape seeds off foil store into labeled vials.
Individual fruits from one plant do not need to be stored separately but individual plants must be
labelled and stored separately
References


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Robb KL (1988) Analysis of Frankliniella occidentalis (Pergande) as a pest of floricultural crops in California greenhouses. University of California, Riverside, CA, USA


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