CANDIDATE GENES FOR DISEASE RESISTANCE IN *PETUNIA HYBRIDA*

by

PENG JIANG

(Under the Direction of Dayton Wilde)

ABSTRACT

Susceptibility genes are plant genes targeted by pathogens to establish infection. To develop genetic resistance to pathogens in *Petunia hybrida,* the genes for *mildew locus O* (*PhMlo1*) and eukaryotic translation initiation factor 4E (*PheIF4E*) were identified and characterized. The genomic sequence of petunia *Mlo* and *eIF4E* was determined using petunia EST sequences with homology to tomato *SlMlo1* and *SleIF4E*. Two approaches were taken to examine whether eliminating *PhMlo1* expression could confer powdery mildew resistance. First, an EMS-mutagenized population of *P. hybrida* 'Mitchell' was developed after optimizing EMS exposure levels. High-resolution melting (HRM) analysis was used to screen DNA from M2 plants for *PhMlo1* variation. Two SNPs were identified that resulted in the missense mutations S130L and G176E. The G176E substitution was predicted by Provean software analysis to be deleterious to *PhMlo1* function. The second approach involved the reduction of *PhMlo1* expression through RNA interference (RNAi). A *PhMlo1* RNAi construct was developed and introduced into petunia by *Agrobacterium*-mediated transformation. We examined whether resistance to powdery mildew (*Podosphaera xanthii*) could be obtained in *P. hybrida* by knocking down the susceptibility gene *PhMlo1*. Ten independently-transformed shoots were rooted and the regenerated T0 plants were propagated vegetatively. Real time-PCR

analysis found that four *PhMlo1*-RNAi lines had reduced levels of *PhMlo1* expression compared to transgenic controls. In two experiments with whole plants, leaves of *PhMlo1*-RNAi lines and controls were inoculated with *P. xanthii* conidia. The time course of infection and the infected leaf area were examined over a 16-day incubation in a growth chamber. RNAi lines showed delayed infection and a reduced area of infection compared to transgenic controls. The transgenic lines were self-pollinated and T1 progeny were produced and examined for powdery mildew resistance and pleiotropic effects on growth. Powdery mildew resistance was observed in the progeny of transgenic petunia, but the knockdown of *Mlo* had a stronger effect on growth than seen in other plant species.

INDEX WORDS: *Petunia hybrida*, ornamental breeding, powdery mildew, HRM, RNAi

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CHAPTER 1

Introduction and Literature Review

1.1 Petunia propagation and disease susceptibility

Many plants are vegetatively propagated in the ornamental horticulture industry. Compared to seed germination, vegetative propagation has several advantages. The offspring are genetically identical and therefore advantageous traits can be preserved. Bypassing sexual reproduction also reduces the time to produce progeny. However, vegetative propagation can expose plants to infection by pathogens such as powdery mildew and potyviruses (Baker, 1987; Daughtrey and Benson, 2005). The vegetatively propagated petunia plants infected with powdery mildew in New Jersey were destroyed, but it is possible that other greenhouse businesses might have unknowingly distributed powdery mildew through regular marketing channels (Kiss *et al.*, 2008). Vegetative propagation of petunia cultivars is conducted in Central and South America (Havrylenko, 2005), where several species of powdery mildew have been documented, and cuttings are exported to the United States and Europe. Meanwhile, eleven viruses have been detected by immunoelectron microscopical examination of naturally infected petunias or of mechanically inoculated test plants (Lesemann, 1996b). The introduction of vegetatively propagated *Petunia* hybrids led to increasing virus infections of propagation material and mother stocks because phytosanitary measures were not undertaken by breeders and propagators (Lesemann, 1996b).

Powdery mildew is an important pathogen during the petunia cutting process. Powdery mildew is a fungal disease that affects a wide range of plants, and is caused by many different species of fungi in the order Erysiphales. Powdery mildew fungi reproduce both sexually and asexually. Asexual spores produce conidia and the sexual reproduction is via ascospaes in chasmothecia, a type of ascocarp. Within each ascocarp are several asci. Over time, ascospores mature and are released and spread to other plants to initiate new infections. Infected plants display white powdery spots on the leaves and stems. As the disease progresses, the spots get larger and denser as large numbers of asexual spores are formed, and the mildew may spread up and down the length of the plant. Powdery mildew fungi are host specific biotrophic parasites; that is, they can grow only on living plant tissue. They attack a variety of plants, including many ornamental plants. Plants in the Solanaceae family such as petunia, tomato, tobacco and eggplant can be seriously damaged by powdery mildew infection. With an increasing number of petunias grown from cuttings, powdery mildew is becoming more common in Petunia (Daughtrey and Benson, 2005).

Thirty-three viruses have been detected in naturally infected petunias by immunoelectron microscopical examination of naturally infected petunias or of mechanically inoculated test plants (Feldhoff *et al.*, 1998; Lesemann, 1996a). Many of these viruses belong to the genus Potyvirus, one of the largest virus groups. Potyviruses are unique in their diversity of inclusion bodies which appear during the infection cycle. The pinwheel- or scroll-shaped inclusion bodies in the cytoplasm of the infected cells are most characteristic, and these cylindrical inclusion (CI) bodies formed by the virus-encoded CI protein form are an important feature in the potyvirus determination. Viruses can also infect petunia in combination with other viruses, such as potato

Y, tobacco mosaic, tomato mosaic, alfalfa mosaic, cucumber mosaic, petunia vein-clearing and broad bean wilt 1 virus (Lesemann, 1996a).

The impact of plant pathogens can be significant because of the size of the petunia market. The value of wholesale garden petunias in 2011 among 15 states in the U.S was over $38.6 million dollars (USDA, 2012). *Petunia* is genus of 35 species of flowering plants of South American origin (Maberly, 1990). Most petunias are diploid with 14 chromosomes and are interfertile with other petunia species. The common garden petunia, *Petunia hybrida*, was first obtained by hybridization of *P. integrifolia* and *P. axillaris* in 1834, and it soon spread to European gardens (Sink, 1984). Today it is cultivated all over the world and is the most important solanaceous species utilized for ornamental purpose.

Over the past two decades petunia has served as an excellent model system for uncovering the molecular, biochemical and physiological studies. The advantages of petunia as a good model system for scientific research are: relatively short life cycle; existence of plants with a range of habits and genetic diversity; hundreds of seeds from a single pollination; abundant genomic resources from related solanaceous species. While the petunia genome sequence remains to be completed, useful genetic and molecular resources are available, including EST and cDNA collections, oligo arrays, and mutant transposon insertion lines (Gerats and Strommer, 2008).

1.2 Plant pathogens and "susceptibility genes"

In crop plants, a strategy for developing disease resistance involves the use of natural or induced mutations in susceptibility (S) genes. All plant genes that facilitate infection and support compatibility can be considered as susceptibility genes (van Schie and Takken, 2014). The physical interaction between host eukaryotic initiation factor *eIF4E* or *eIF(iso)4E* and the viral genome-linked protein (VPg) is critical for viral infection by several members of the genus potyvirus. In solanaceous plants, knockdown of *eIF4E* factors led to resistance in tomato, tobacco and pepper (Combe *et al.*, 2005; Hwang *et al.*, 2009; Piron *et al.*, 2010b).

Powdery mildew can be controlled by chemical control and crop rotation, however, the most effective way to control powdery mildew is through genetic resistance, using susceptibility genes to prevent infection. The *Mildew resistance locus O* gene (*Mlo*) was first identified in barley (Buschges *et al.*, 1997b). The *Mlo* genes encode a plant-specific and sequence-diversified class of seven transmembrane proteins that form a multigene family in both monocot and dicot plants (Buschges *et al.*, 1997b; Devoto *et al.*, 1999). In Arabidopsis, this family is composed of 15 *Mlo* genes (Devoto *et al.*, 2003). So far, *Mlo* genes have been identified and isolated from more than 20 plants. In the Solenaceae, it was found that naturally occurring broad-spectrum resistance to powdery mildew in a Central American tomato accession was caused by loss of *Mlo* function (Bai *et al.*, 2008b; Zheng *et al.*, 2013b; Acevedo-Garcia *et al.*, 2014; Appiano *et al.*, 2015). The role of *Mlo* in powdery mildew susceptibility has also been confirmed in pepper (Zheng *et al.*, 2013b).

1.3 Pathogen resistance through susceptibility gene mutation

Plant breeding is the science of changing the traits of plants in order to produce desired characteristics. Plant improvement can be accomplished through many different techniques ranging from simply selecting plants with desirable characteristics for propagation to more complex molecular techniques. Mutation breeding is the process of exposing seeds to chemicals or radiation in order to generate plants with increased genetic variation. Traditional mutation breeding has been an effective approach to producing horticultural varieties with improved traits (Ahloowalia et al., 2004). However, it requires the phenotypic analysis of a large number of plants. From 1930 to date more than 2600 mutagenic plant varieties have been released, which have been derived either as direct mutants (70%) or from their progenies (30%) (Maluszynski, 2000). Twenty-five percent of released mutagenic species are ornamentals or decorative plants.

Targeted mutation breeding uses genomics to increase the efficiency of mutation breeding. Overall, it requires candidate gene sequence data, mutation screening techniques and screening populations. Candidate gene sequence from the target species is needed for genetic screening. Sequenced genomes of over 100 plant species are currently available, and genomic analysis of more than 100 other plant species is in progress (CoGepedia, https://genomevolution.org/wiki/index.php/Sequenced_plant_genomes). Expressed Sequence Tags (EST) databases have been developed for several ornamental plants, such as petunia. Two current mutation screening techniques are frequently used nowadays. TILLING is a reverse genetics approach that identifies mutations through nuclease cleavage of DNA mismatches (Till *et al.*, 2010). High resolution melting (HRM) detects mutations due to changes in melting

properties of PCR products with DNA mismatches; and it has been used to discover induced variability and natural variability in target genes of several horticultural plants (Chen and Wilde, 2011; Li *et al.*, 2010). High-throughput sequencing of PCR products of candidate genes is an alternative to DNA mismatch detection, particularly useful for outcrossing, polyploid crops like potato (Muth *et al.*, 2008). Genetic diversity in plants can be induced using chemical agents such as ethyl methanesulfonate (EMS) or physical agents such as gamma rays. EMS primarily causes single nucleotide polymorphisms (SNPs), which can alter encoded proteins through premature termination, mis-splicing, and codon changes. The size of the population screened for natural or induced candidate gene polymorphisms depends on genomic characteristics such as ploidy and the allelic diversity. For example, novel alleles of melon *eIF4E* (translation initiation factor 4E) were identified by the TILLING of 2483 EMS-mutagenized M_2 plants (Gonzalez *et al.*, 2011). EMS-induced mutations in tomato eIF4E were identified by sequencing eIF4E genes from 3008 M2 plants (Rigola *et al.*, 2009) and by the TILLING of 4759 M3 plants (Piron *et al.*, 2010b).

An advantage of petunia as a molecular model is the tools developed to knockdown gene expression. Gene silencing in petunia can be accomplished by RNA interference and zinc-finger nuclease (Gerats and Strommer, 2008). Virus-induced gene silencing (VIGS) is a high-throughput analysis of gene knockdown technique. Comparing other gene knockout methods, the constructs of VIGS can be created by cloning gene fragments into a viral vector without inverted repeats. Several plant viruses have been used as VIGS vectors. In petunia, a modified tobacco rattle virus (TRV) is used for VIGS, and the induced phenotype can be observed 10 days after inoculation (Spitzer *et al.*, 2007). Although transposable genetic elements in plants have been studied extensively, their mutagenic capacity has been exploited in only a few plant species such

as maize, antirrhinum and petunia. Representatives of all three major groups of class II elements have been identified in petunia; they are HAT-, CACTA- and Mutator-like elements. In addition, two transposable elements in petunia such as *Act1* and *dTph1* have also been used in both forward and reverse genetics studies (Gerats, 2009).

The role of *Mlo* in powdery mildew susceptibility has been confirmed in plants, such as Arabidopsis, pea, tomato, pepper, wheat, and strawberry (Bai *et al.*, 2008a; Consonni *et al.*, 2006a; Humphry *et al.*, 2011; Jiwan *et al.*, 2013; Pavan *et al.*, 2011; Varallyay *et al.*, 2012a; Zheng *et al.*, 2013a). *Mlo* mutants display loss of susceptibility resembling that described for nonhost resistance (Humphry *et al.*, 2006). In Arabidopsis, the triple mutant *Atmlo2*, *Atmlo6*, and *Atmlo12* is completely resistant to powdery mildew, restricting fungal development at the host cell entry level, while double or single mutants show partial resistance (Consonni *et al.*, 2006a). Conservation of *Mlo*-based resistance across monocot and dicot plant species implicates a common mechanistic basis for this type of plant immunity. It further implies potential application of this trait for plant breeding of many other agriculturally and economically important plant species (van Schie and Takken, 2014).

1.4 Objectives

Based on the research described above, we hypothesized that genetic resistance to plant pathogens of petunia could be obtained by reducing the expression of susceptibility genes. To test this hypothesis, the following objectives were pursued:

1. Identify and characterize the relevant *Mlo* and *eIF4E* genes in petunia.

2. Knockdown *Mlo* and/or *eIF4E* function in petunia by targeted mutation breeding and RNAi.
3. Examine the effect of *Mlo1* knockout/knockdown on powdery mildew resistance and plant growth and development.

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CHAPTER 2

Isolation and Characterization of the *PhMlo1* and *PheIF4E* Family Genes

2.1 Introduction

Powdery mildew is a common fungal disease of many higher plants, including monocots like barley and wheat and dicots like *Arabidopsis,* grape, tomato, and petunia. Powdery mildew genera such as *Erisyphe* (*Golovinomyces*), *Leveillula*, *Microsphaera*, *Podosphaera (Sphaerotheca)* and *Oidium* are found as a white to gray powdery growth on leaves and sometimes stems and flowers (Braun, 1987a; Jones *et al.*, 2001; Bolay, 2005; Kiss *et al.*, 2008). A powdery mildew species *Oidium longipes* has been reported recently a threat to commercial petunia crop in the U.S. (Kiss *et al.*, 2008). To combat against powdery mildew pathogen attack, plant resistance is an important way of controlling powdery mildew disease. Compared with fungicide application, plant resistance has economic and environmentally-friendly features.

Resistance and susceptibility to plant disease are conferred by resistance genes and susceptibility genes respectively. Host plant resistance can be achieved by introducing resistance genes like dominantly inherited resistance (*R*) genes or by disabling susceptibility (*S*) genes in horticultural crop breeding (Pavan *et al.*, 2010). In barley (*Hordeum vulgare*), powdery mildew disease resistance could be conferred by R genes or loss of S genes, such as the recessive alleles of the *Mildew resistance locus o* (*Mlo*) gene (Jorgensen, 1992; Jorgensen, 1994). While R genes usually provide strain-specific resistance, *Mlo*-mediated resistance is broad spectrum and durable

(Jorgensen, 1992; Lyngkjaer *et al.*, 2000). Thus, *Mlo*-mediated resistance has been utilized and proved successful in European barley agriculture (Jorgensen, 1992; Lyngkjaer *et al.*, 2000). Recently, *Mlo* has also been studied in dicot species such as *Arabidopsis* (Consonni *et al.*, 2006a), *Solanum lycopersicum* (tomato) (Bai *et al.*, 2008a) and *Vitis vinifera* L. (grapevine) (Feechan *et al.*, 2008). In *Arabidopsis*, a family of 15 genes highly similar to barley *Mlo* have been isolated. However, only three closely-related members *AtMlo*2, *AtMlo6* and *AtMlo12* play a role in powdery mildew susceptibility (Panstruga, 2005). In addition, *Pen1*, the *Arabidopsis* homologue of *Ror2* (an S gene for powdery mildew resistance in barley), has also been isolated and been found to function in powdery mildew resistance (Sanderfoot *et al.*, 2000; Collins *et al.*, 2003). In tomato (*Solanum lycopersicum* var. *cerasiforme*), a natural allele *ol-2* confers broad-spectrum and recessively inherited resistance to powdery mildew *Oidium neolycopersici*. Bai et al (2008) cloned tomato *Mlo* gene (*SlMlo)* and demonstrated that the *SlMlo* coding region was disrupted by a 19 bp deletion, which accounted for the powdery mildew resistance of the *ol-2* locus. Feechan *et al.* (2008) analyzed the cDNAs of seventeen *VvMlo* genes, and found that the four family members that exhibited upregulated transcription following powdery mildew infection were orthologous to the *Arabidopsis AtMlo2*, *AtMlo6* and *AtMlo12* and tomato *SlMlo* genes.

Since disruption and mutation of *Mlo* genes in barley, Arabidopsis, and tomato led to durable and broad spectrum resistance to powdery mildew, we hypothesized that powdery mildew resistance in petunia could be obtained through mutation of a petunia *Mlo* ortholog. We isolated the genomic and cDNA sequence of an *Mlo* ortholog in petunia and characterized its gene structure and expression in order to facilitate the targeted mutation breeding of powdery mildew resistance.

Another significant breakthrough in natural resistance gene mechanisms was the discovery of the key role of translation initiation factors *eIF4E* and its isoform *eIF*(iso)*4E* in plant resistance to RNA viruses (Robaglia and Caranta, 2006). *eIF4E* binds to the cap structure of mRNA with *eIF4G* to form the *eIF4F* complex (Browning, 1996). Higher plants encode two distinct types of the *eIF4F* translation initiation complex: (1) *eIF4F*, which contains *eIF4E* and *eIF4G*, and (2) *eIF*(iso)*4F*, which contains *eIF*(iso)*4E* and *eIF*(iso)*4G* (Combe *et al.*, 2005; Gallie and Browning, 2001; Rodriguez *et al.*, 1998). These two complexes have the same function for the in vitro translation of some mRNAs, however they differ in *in vivo* expression patterns and show some specificity for different capped cellular mRNAs (Rodriguez *et al.*, 1998). In dicots, several genes code for *eIF4E* and *eIF4G* proteins. For example, in Arabidopsis, three genes code for proteins of the *eIF4E* subfamily and one codes for *eIF*(iso)*4E*. In tomato, two genes code for *eIF4E* proteins, and one codes for *eIF*(iso)*4E* (Piron *et al.*, 2010a). Although *eIF4E* mutations have been implicated in resistance to several viral genera (Stein *et al.*, 2005; Nieto *et al.*, 2006), most often resistance is to potyviruses. The Potyvirus genus is one of the largest population among plant viruses and it causes economic damage to many ornamental crop species. *eIF4E*-mediated resistance to potyviruses results from amino acid changes in the *eIF4E* protein (Le Gall *et al.*, 2011). The exact mechanism of the resistance may due to an altered binding with the potyviral protein VPg (Yeam *et al.*, 2007).

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2.2 Materials and methods

2.2.1 Plant material and sampling

Seeds of *Petunia hybrida* “Mitchell Diploid “(MD), a doubled haploid variety, were grown in pots with Fafard soil 2B in a growth room at 22 ℃ with 16 hours light and 8 hours dark. One-month-old seedling leaves were cut for DNA and RNA extraction for the isolation of *PhMlo1* and *eIF4E* genes. One month old seedling roots and leaves, flower petals and anthers, and mature leaves were sampled for expression of these genes. Root samples were isolated from the young plants with the same growth stage for sampling leaves. At flowering, open flower petals, stigmas, and immature anthers were sampled. The tissues were frozen in liquid nitrogen at sampling and stored in -80 ℃ freezer. All the sampling was repeated twice.

2.2.2 Isolation of gDNA and cDNA sequences of *PhMlo1, PheIF4E,* and *PheIF*(iso)*4E* gene

Leaf tissues ground in liquid nitrogen and DNeasy® Plant Mini Kit (Qiagen, Germany) were used in DNA extraction. The extraction procedures followed the user’s manual supplied by the kit producer. The DNA was quantified employing NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). The information of the primers used was listed in Table 1. PCR was performed using a 2720 Thermocycler (Applied Biosystems). Reactions were conducted in a 25 µl volume with 20 ng genomic DNA, 0.2 µM of each primer (only in the case of TAIL PCR, 50 µM random primer AD1 were used) , 2.5 mM $MgCl_{2,}$ 1 U Taq polymerase (Go Taq, Promega) and 1×PCR buffer. For the amplification of *PhMlo1* internal fragments, PCR reactions were denatured at 94 ˚C for 5 minutes, followed by 45 cycles of thermo shafting of 94 ˚C 20 seconds, 55-65 ˚C (based on the tm of primers) 20 seconds, 72 ˚C for 1 min. For TAIL PCR, the first amplification programs using primer MSW5C and RD2 was, 94˚C for 4 min followed by 3 cycles of thermo shafting: 95˚C for 15 second, 59˚C 1 min, 72˚C 2 min, then 94˚C 15 second, 25˚C 3 min, 26˚C ramp to 44 with rate 0.3˚C/second, and 72˚C 2 min. The program was then

followed by 94˚C 7 second, 42˚C 1 min, 72˚C 1 min for 10 cycles. Finally, 14 cycles of following programs were performed: 94˚C 7 seconds, 59˚C 1 min, 72˚C 1 min, 94˚C 7 second, 42˚C 1 min, 72˚C 1 min. The PCR products were diluted to 1/50 and 1 μL was used in the second amplification using nested primer MSW5B and RD2, and then the product was diluted in the same way and used in the third amplification using the third nested specific primer MSW5A and RD2. The PCR program for second and third amplification using nested primers was, 94˚C 1 min followed by 15 cycles of thermo shafting: 94˚C 12 seconds, 59˚C 25 seconds, 72˚C 45 seconds, 94˚C 12 seconds, 59˚C 25 second, 72˚C 45 second, 94˚C 12 second, 42˚ 1 min, 72˚ 45 second. For each programs, a final extension at 72 ˚C for 5 minutes was performed.

For the isolation of cDNA sequences, one month old petunia seedling leaves were used for RNA extraction with RNeasy®Plant Mini Kit (Qiagen, Germany). The procedure followed the kit producer's instruction. One microgram RNA was treated with RQ1-DNase I (Promega, USA) in a 10 μL volume reaction, and finally used 1 μl stop buffer to terminate the reaction and proceeded to reverse transcription. M-MLV Reverse Transcriptase (Promega, USA) was used in first strand cDNA synthesis, the 25 μL reaction included 1×M-MLV RT buffer, 100 U M-MLV reverse transcriptase, 40 U RNasin®, 0.5 μg Oligo$(dT)_{15}$, and the total RNA treated with RQ1-DNase I. The reverse transcription procedure followed that described by Chen *et al*. (2003). The synthesized cDNA was diluted into 1/50, and used in RT-PCR with primers listed in Table 1. The RT PCR conditions and thermo programs were the same as that used in the amplification of the internal fragment of *PhMlo1* using genomic DNA.

After amplification, PCR products were isolated by agarose gel electrophoresis and purified using a PureLink™ Quick Gel Extraction Kit (Invitrogen, USA) and cloned using pGEM®-T Easy Vector System (Promega, USA). Plasmids were prepared using StrataPrep®Plasmid Miniprep Kit (Stratagene, Cedar Creak, TX). Plasmid DNA samples were sequenced by MWG Operon (Huntsville, AL).

2.3.3 Phylogenetic analysis

Petunia MLO, eIF4E and eIF(iso)E protein sequences were used to search the Genbank protein database for MLO, eIF4E and eIF(iso)E proteins in other plant species. MEGA version 4 (Tamura *et al.*, 2007) was used in phylogenetic analysis. The multiple alignment of protein sequences was performed using ClustalW with the default setting to generate an unrooted phylogenetic tree using the Neighbour Joining method. Branching arrangement was evaluated by bootstrap analysis with 1,000 replicates.

2.3.4 *PhMlo1*, *PheIF4E* and *PheIF*(iso)*4E* gene expression analysis using real time quantitative PCR

Several different tissues of petunia were used in RNA extraction by the CTAB method (Jaakola et al., 2001). The extracted RNA was measured and used in cDNA synthesis using the same procedures as for the isolation of cDNA sequence of *PhMlo1* gene. The cDNA samples were diluted into 1/20, 1/80, 1/200, 1/800. The real time PCR condition, procedures and instrument were the same as above, except for the primers and annealing temperature (Table 1). PCR was conducted in triplicate for each of two biological repeats. ME13F and MJ2R for *PhMlo1* gene (produced a 265 bp band), and EF1αF and EF1αR for *Elongation factor 1α* gene (EF1α) as a

reference were used in expression analysis using real time PCR. Those primers produced single product and negative control produced nothing. EF1α was recommended the most suitable reference gene for petunia line Mitchell (Mallona *et al.*, 2010). The result data analysis adopted similar method as that in copy number estimation. After amplification efficiency was calculated, the data for 1/200 dilution was used for relative quantity estimation.

Petunia leaf tissues are polysaccharide abundant. In order to guarantee the accuracy of the real time quantitative PCR results, we adopted CTAB extraction method to obtain high quality and high concentration of genomic DNA, which was reported by Doyle and Doyle (1987), Sharma (2002). Our extraction was based on the protocol described by Jaakola et al. (2001) for RNA extraction with modification. In each extraction, 200 mg leaf tissue was ground in liquid nitrogen, and mixed with 750 μL extraction buffer (2% CTAB, 2% PVP Mol WT 360,000, 100 mM Tris-HCl (pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, 2% β-mercaptoethanol, and 100 μg RNase A.). After incubated at 65 ℃ for 10 minutes, the mixture was extracted using same volume of phenol-chloroform once and then chloroform twice. The DNA was precipitated using 2 volume of ethanol and then dissolved in 200 μL SSTE (1.0 M NaCl, 0.5%SDS, 10 mMTris-HCl pH 8.0, and 1 mM EDTA pH 8.0). The procedure of phenol-chloroform, chloroform extraction and DNA precipitation using ethanol was repeated. After careful washing with 70% ethanol twice, the DNA was dried then dissolved in ddH_2O. The DNA samples was measured with NanoDrop 8000 spectrophotometer. A serial dilution of the DNA samples started with initiation dilution 60 ng/ μL (1×) was made, 1×, 1/2×, 1/5×, 1/10×, 1/25×, 1/50×, 1/100×, 1/400×, 1/800×, 1/1000×, 1/1600×. Cut tip pipette tips were used to make the dilutions and reduce DNA sheering. For each dilution, even distribution of DNA in water was obtained by allowing

dispersion for 24 hours and using pipette assisted mixing. In the preliminary real time PCR analysis, the data generated from 6 serial dilutions within 1/10× to 1/800× presented nearly perfect linear plot like Figure 4A, and the 6 dilutions were selected in copy number estimation.

Brilliant® II SyBR® Green QPCR low ROX Master Mix (2×) was used in real time relative quantitative PCR on a LightCycler® 480 II Real Time PCR Instrument (Roche) to determine the expression level of *PhMlo1* gene in petunia genome. PCR condition was, in a 20 μL reaction, 1 × buffer (included florescent dye, Mg++,and Taq polymerase), 0.25 μM of each primer, and the 2 μL DNA for each dilution were incorporated. The program was, 95 ℃ 10 minutes, then 40 cycles of 95 ℃ 17 seconds, 60-65 ℃ based on Tm of the primers (Table 1) for 20 seconds, extension at 72 ℃ for 30 seconds and recording florescence data. Primer pair ME14F and MJ1R from *PhMlo1* gene producing a 184 bp product, and EF1αF and EF1αR designed form petunia EF1αgene producing a 120 bp product were used in real time relative quantitative PCR. Those primers were selected previously through examination of the quality of the real time PCR products using agarose gel electrophoresis and Tm Calling based on Melting Curve analysis, during which PCR products were denatured at 95 ℃ for 1 minute, cooled to 40 ℃ for 1 minute, and then heated to 95 ℃ at 0.02 ℃/second, while continuously measuring florescence with 25 data acquisitions/ ℃. LightCycler® 480 SW 1.5 software was used to collect and processing florescence data. In addition, negative control showed no products for those primers. For the real time quantitative PCR, cross point (CP) value was obtained using Fit Points analysis with automatic setting. Triplicate PCRs were performed for each of two biological repeats. The quantity of amplified products was calculated based on the PCR efficiency described by Pfaffl et al. (2001).

2.3 Results

2.3.1 Isolation of *PhMlo1* , *PheIF4E*, and *PheIF(iso)4E* gene sequences

The full-length genomic sequence of *PhMlo1* was isolated previously in our lab by Dr. Yihua Chen (unpublished). There are 15 and 17 *Mlo* gene in Arabidopsis and tomato, respectively (add references). The petunia genome sequence is not publicly available yet. To look for additional petunia *Mlo* genes, I aligned 17 tomato tomato *Mlo* genes in petunia EST and petunia corolla transcriptome database. Four partial cDNA sequences were found that showed weak similarity (E value < e-100) with tomato *Mlo* family genes.

The sequence of *eIF4E* and *eIF(iso)4E* gene in tomato (LOC543653 and EU119958) was used to search the petunia EST database [http://genomics3.biotech.ufl.edu:8080/bq/blastquest.jsp?] and five ESTs for each gene showing high similarity with tomato *eIF4E* and *eIF(iso)4E* cDNA sequence were found. With PCR primers designed from these ESTs, the corresponding genomic sequences were obtained by PCR-based cloning from *Petunia hybrida* DNA.

2.3.2 Feature of petunia *Mlo1*, *eIF4E*, and *eIF(iso)4E* gene structure

Petunia *Mlo1* gene has 15 translated exons, more than that in barley *Mlo1* (12 exons) and *Arabidopsis Mlo2* (14 exons). The 4th and 5th intron in petunia *Mlo1* gene are significantly longer than any introns in barley and *Arabidopsis Mlo* gene, as a result, the size of genomic DNA sequence of *PhMlo1* gene is over 5Kb (Fig.1A). Alignment of its deduced protein sequence with that of several other species showed that in petunia *Mlo1* gene additional introns appeared in the position corresponding to barley *Mlo* gene exon 2, 10 and 11, which caused more number of

exons in this gene in petunia (Fig. 1A, B). The petunia MLO protein showed similar structures to barley, *Arabidopsis*, and tomato orthologs, including seven transmenbrane regions (Devoto *et al.*, 1999), a calmodulin-binding domain (Kim *et al.*, 2002), and two conserved domains identified by Panstruga (2005a) in the highly polymorphic C-terminus (Fig.1B). An unrooted phylogenetic tree was constructed based on the MLO protein family identified from several different plant species including monocot plant barley and dicot plant *Arabidopsis*. Petunia and tomato belong to the *Solanaceae* family. Petunia MLO1 sequence is closely related to tomato MLO1 and formed a group in the tree. They also shared sequence identity with other members in dicot plants within the same large clade including Arabidopsis MLO2, MLO6, pepper *CaMlo1* and *CaMlo2* etc. (Fig. 2), which showed association with powdery mildew susceptibility. Four partial cDNA sequences were also identified that had homology to other tomato *Mlo* family members. The size of the petunia *Mlo* family is unknown; the release of the genome sequence of *P. axillaris* and *P.integrifolia* (Sims et al., 2012) will help resolve this question.

The petunia *eIF4E* and *eIF(iso)4E* genes were found to have five exons (Fig. 3). Exon1 to exon3 are critical for the physical interaction for viral infection which associate with the viral genome-linked protein (VPg).

2.3.3 Expression of *PhMlo1*, *PheIF4E*, and *PheIF(iso)4E* gene transcripts

Expression of *PhMlo1* transcripts in petunia was examined by employing real time quantitative PCR. Transcripts of this gene were expressed in all the tissues examined, but at varying levels. The highest level appeared in seedling root tissues (Fig. 5A). Changes of transcript level were detected in the attached petunia leaves in response to wounding treatment. The transcript level

was very low in the attached leaves wounded for 2 and 4 hours, while it increased in the leaves wounded for 8 hours, and reached even higher level in the leaves wounded for 24 hours. However, we did not detect change of *PhMlo1* transcript level in response to H_2O_2 treatments (Data not shown).

Expression of *PheIF4E and PheIF(iso)4E* gene transcripts in petunia was examined by employing real time quantitative PCR. The transcripts of this gene were detected but in varied levels in all the tissues examined. The highest level appeared in seedling root tissues (Fig. 6).

2.4 Discussion

It has been found that *Mlo* genes are organized in small to medium-sized families in plants (Liu and Zhu, 2008; Devoto *et al.*, 2003; Feechan *et al.*, 2008). In Arabidopsis, 15 genes highly similar to barley *Mlo* have been found. Phylogenetic analysis and bioassay show that there are seven clades in *Mlo* gene family, in which clade V contains genes associated with powdery mildew resistance. In Arabidopsis, only the three members in clade V, *AtMlo*2, *AtMlo6* and *AtMlo12* are involved in powdery mildew susceptibility (Panstruga, 2005; Acevedo-Garcia *et al.*, 2014). Among solanaceous species, three *Mlo* genes found to be involved in powdery mildew resistance, *SlMlo1*, *CaMlo1*, and *CaMlo2*, are in clade V, where *PhMlo1* also clusters. In this study, we isolated the *PhMlo1*, *PheIF4E*, and *PheIF(iso)4E* gene in petunia. Sequence comparisons indicate that these family members are homologous to genes involved in pathogen susceptibility in other plant species.

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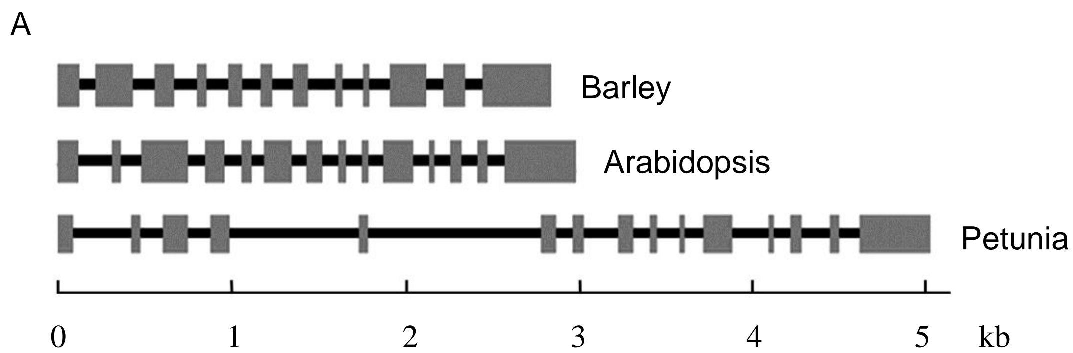
Table 1. PCR primers used in the study

Name	Sequence 5'-3'	Tm (˚C)	Purpose
M836F	CCAGCTCCATATCTTCATCT	58.4	Isolate *Mlo* genomic sequence
M618R	GAATTGGGACGTTGCATAGAG	60.6	Isolate *Mlo* genomic sequence
M5CFA	CTACDTGGGCHRTYGCDGTGG	66.5	Isolate *Mlo* genomic sequence
MWB	GCACGGAGGGGTAACTTG	62.2	Isolate *Mlo* genomic sequence
MSW5C	GCAAATGAAATAAATGAATCCTC	55.6	Tail PCR
MSW5B	CAAGAAAATGCGTGGTCCTGAC	62.7	Tail PCR
MSW5A	TGCATACAAACAAATCCTTCTC	57.1	Tail PCR
AD2	WGTGNAGWANCANAGA	50.3	Tail PCR
M5UF	TCATCTATCAAATTCATCTGTGTATC	59.2	Isolate cDNA sequence
ME7R	TTGCTACTGATGTGATGAATTGC	59.2	Isolate cDNA sequence
MJ2F	GGCGTGGGAGGATGAAAC	62.2	Isolate cDNA sequence
ME14F	CACTTTATGCCTTGGTCACACA	60.8	Copy number assay
MJ1R	AGCAGTTGCCACATTATCACC	60.6	Copy number assay
PS227F	CCGAGGAGGTAAAGACTTCTGAG	64.6	Copy number reference
PS346R	CAGCAGCTTTAGGGGTCTCTTCAAC	66.2	Copy number reference
ME13F	CTTCCACAAAAATGCTGCAGAC	60.8	Expression analysis
M611F	ATCAAGCTCTATGCAACGTC	58.4	Paralog analysis
DMR2	TGACATACAATTGGTAGCCACTTG	61.2	Paralog analysis
MJ2R	GGAGGAACCGTGCAATGG	62.2	Expression analysis

EF1αF	CCTGGTCAAATTGGAAACGG	60.4	Expression reference
EF1αR	CAGATCGCCTGTCAATCTTGG	62.6	Expression reference

Figures and figure legends



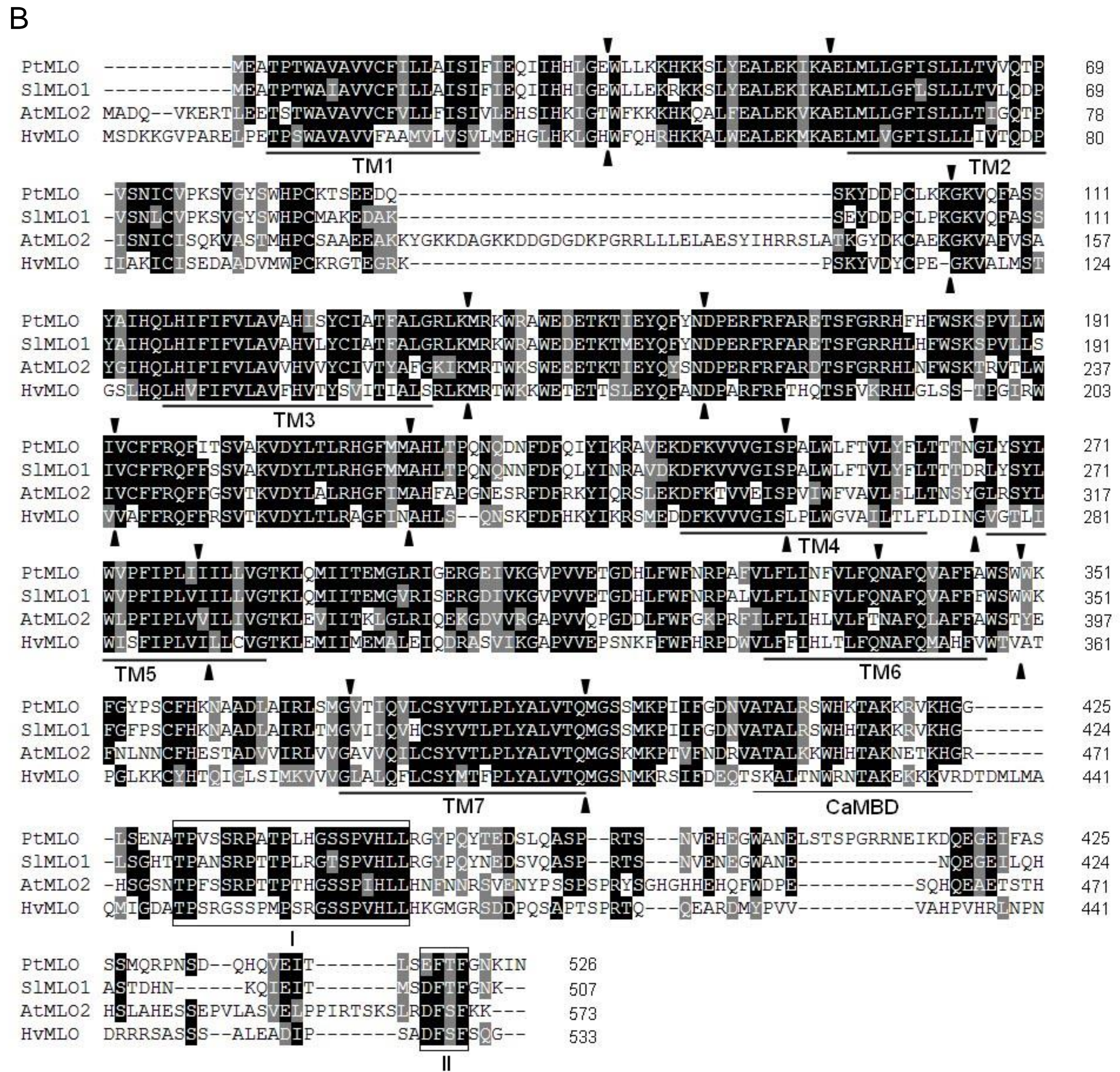


Figure.1. Structure of *PhMlo1* gene and protein. **A**. Genomic DNA structure of petunia *Mlo* gene. The size presented was the length within start and stop codon. Petunia *Mlo1* gene was 5039 bp. Barley *Mlo1* (2839 bp, from GenBank Accession Y14573) and *Arabidopsis Mlo2* (2972 bp, from GenBank accession CP002684) gene are presented for comparing. Horizontal lines represent introns. Vertical grey boxes indicate exons. **B**.Sequence alignment of *PhMlo1* protein with *SlMLO1*, *AtMLO2*, and *HvMLO* priteins. The MLO proteins were from petunia (*PhMlo1*), tomato (*SlMLO1*, Bai et al, 2008), *Arabidopsis* (*AtMLO2*, Consonni et al, 2006), and Barley (Devoto et al, 1999). The alignment was gernerated by CLUSTALW using defalt parameters.

The position of seven transmenbrane regions (TM1-7) inferred from the experiment determined topology of *HvMLO* (Devoto et al, 1999) is indicated individually. The approxmate position of the calmodulin-binding domain designated CaMDB (Kim et al, 2002b) is indicated by a fine line under the sequence. Two conserved domaines identified by Panstruga (2005a) in the highly polyporphic C-termini are indicated in boxes I and II. Downward and upeard triangles indicated the border of exons for *PhMlo1* and *HvMlo* gene respectively.

Figure. 2. Gene tree of different plant MLO like proteins. Neighbor joining method was used in constructing the tree. The lengths of horizontal lines are proportional to the similarity between protein sequences. Numbers beside the branches indicated support from 1000 bootstrap replicates.

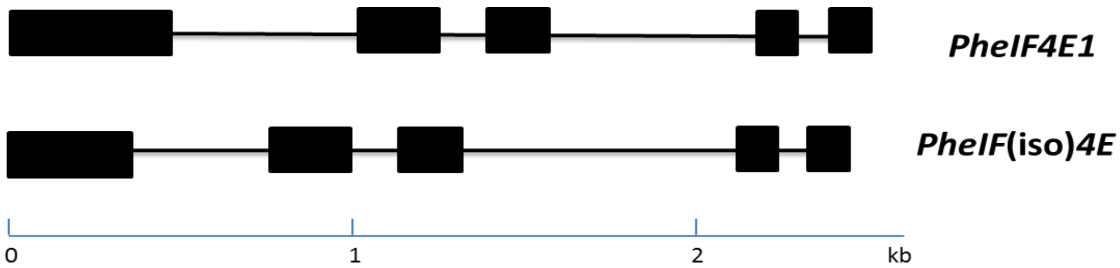


Figure 3. Structure of *PheIF4E* family genes. Genomic DNA structure of petunia *eIF4E1* and *eIF(iso)4E* gene. The size presented was the length within start and stop codon. Horizontal lines represent introns. Vertical black boxes indicate exons.

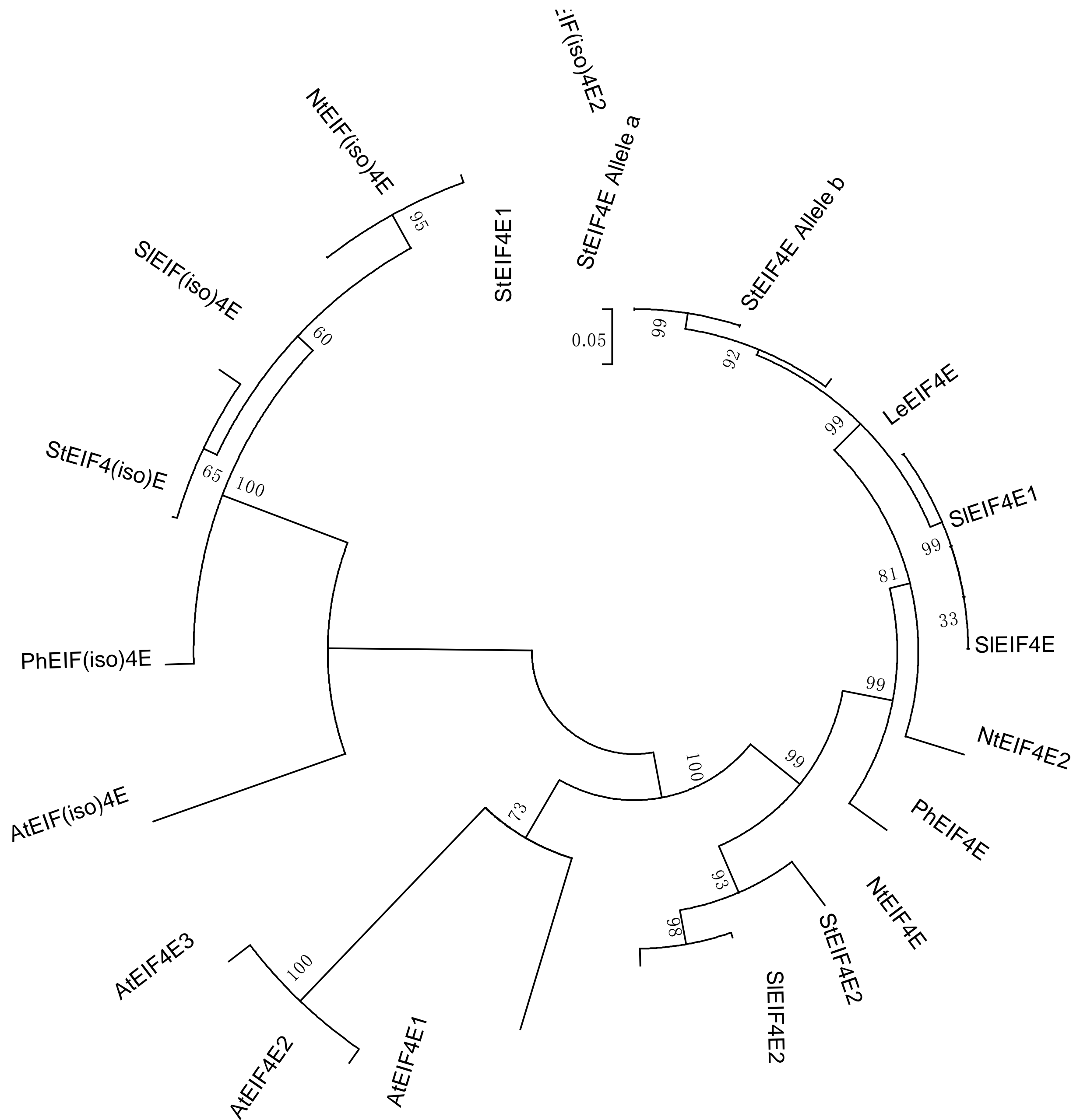


Figure 4. Solanaceae eIF4E mRNA Round Tree. Neighbor joining method was used in constructing the tree. The lengths of horizontal lines are proportional to the similarity between protein sequences. Numbers beside the branches indicated support from 1000 bootstrap replicates.

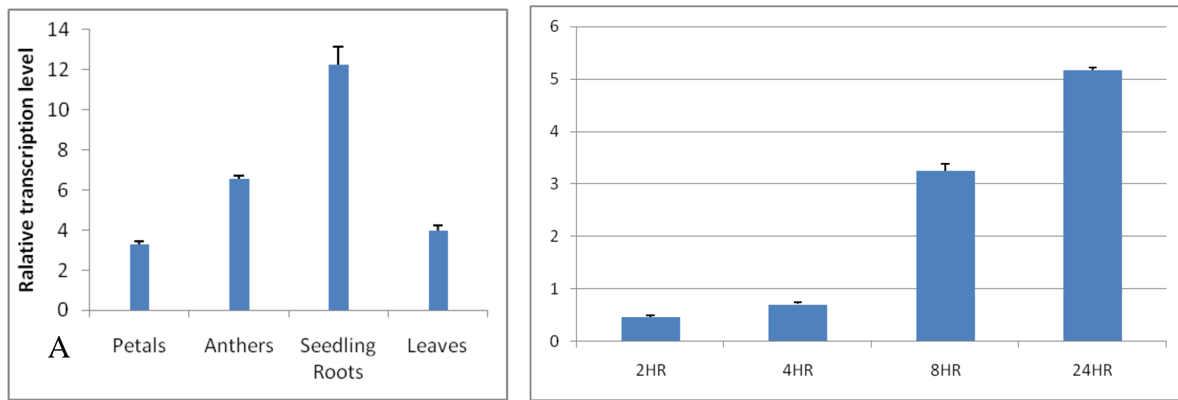


Figure 5. Quantitative Real time PCR analysis of *PhMlo1* gene expression. **A**. Transcript level of *PhMlo1* gene in different petunia tissues. Transcript level of *PhMlo1* gene was normalized against that of the reference gene EFα1 in each DNA sample. Relative normalized transcript levels were shown for different tissues. **B**. Expression of *PhMlo1* gene in petunia leaves in response to wounding treatment. Attached leaves were wounded by rubbing the adaxial surface with sand paper and the leaves were collected at 2, 4, 8 and 24 hours post wounding. Unwounded control leaves were collected at the same time points. Transcript level of *PhMlo1* gene was normalized against reference gene *EFα1* gene in each PCR sample. The mean ratio of normalized level of *PhMlo1* gene transcripts in wounded leaves to that in unwounded leaves was

plotted. For **A** and **B**. The data was calculated from triplicate PCR reactions within two biological repeats. Error bars show standard error.

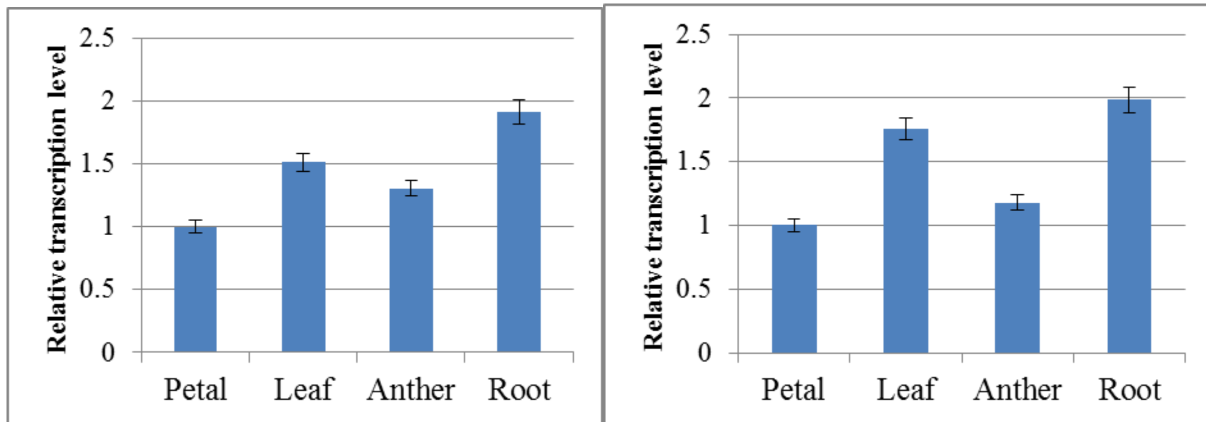


Figure 6. Quantitative RT PCR analysis of *PheIF4E* and *PheIF(iso)4E* gene expression. **A**. Transcript level of *PheIF4E* and *PheIF(iso)4E* gene in different petunia tissues. Transcript level of *PheIF4E* and *PheIF(iso)4E* gene was normalized against that of the reference gene EFα1 in each DNA sample. Relative normalized transcript levels were shown for different tissues.

CHAPTER 3

Knockdown of Susceptibility Gene Function in *Petunia hybrida*

3.1 Introduction

Mutagenesis has been used to introduce genetic variation in ornamental plants for several decades (Schum, 2003; Jain, 2006). More than 560 ornamental varieties from 41 plant species have been officially released from mutation breeding programs (Ahloowalia et al., 2004). Generally, plants with novel traits were identified phenotypically from large, mutagenized populations. More recently, DNA screening techniques such as TILLING (McCallum et al., 2000) have been developed that allow mutagenized populations to be analyzed genetically, before trait expression. The combination of mutagenesis and DNA screening has enabled the identification of novel alleles in model plants (*e.g.* Dalmais et al, 2013) and horticultural species (Wilde et al. 2012).

Petunia hybrida Vilm. is an interspecific hybrid that is both a top-selling ornamental (USDA, 2009) and a genetic model (Gerats and Vandenbussche, 2005). Mutagenesis of this herbaceous ornamental can be a useful tool for crop improvement and basic research. A potential application of mutagenesis is in the development of petunias with resistance to pathogens that can infect plants during commercial propagation. These pathogens include powdery mildew (Kiss et al., 2008) and potyviruses (Lesemann, 1996; Feldhoff et al.,1998). Resistance to powdery mildew and potyviruses can be conferred by mutations in specific genes, which have been targets for

TILLING in other crop species (*e.g.* Gottwald et al., 2009; Piron et al. 2010). The TILLING of petunia for mutant alleles in light signaling and anthocyanin metabolism genes was recently reported (Berenschot and Quecini, 2013). There are published protocols for the mutagenesis of petunia with ethyl methanesulphonate (EMS), but they vary in terms of concentration (0.1% to 0.5%), exposure time (10 to 24 hours), and seed pre-treatment (Kashikar and Kashikar,1981; Napoli and Ruehle, 1996; Berenschot et al., 2008). This chapter describes the optimization of mutation parameters and the development of an EMS-mutagenized population that could facilitate TILLING.

TILLING (Targeting Induced Local Lesions IN Genomes, (McCallum *et al.*, 2000)) technology is a straightforward and cost-effective method to obtain resistance to viruses through loss-of-function mutations. TILLING was successfully applied to screen for potyvirus resistant tomato plants by targeting *eIF4E* genes (Piron *et al.*, 2010b). An *eIF4E1* null mutant was demonstrated to confer immunity to strains of *Potato virus Y* and *Pepper mottle virus*. Compared with previous studies demonstrating broad spectrum resistance to potyviruses in the wild tomato relative *Solanum habrochaites* involving eIF4E1 (Ruffel *et al.*, 2005), the induced *eIF4E1* mutant showed a more narrow resistance spectrum (Pavan *et al.*, 2010). These results suggest that some potyviruses may use more than one eIF4E protein to infect their hosts. Among solanaceous plants, the knockout of eIF4E factors has also conferred potyvirus resistance in tobacco and pepper (Piron *et al.*, 2010b; Combe *et al.*, 2005; Hwang *et al.*, 2009).

RNA interference (RNAi), triggered by double-stranded (ds) RNA molecules, is an important tool for determining or silencing the functions of genes. Dicer enzymes of the plant generate 21-

to 24-nt-long small interfering (si) RNAs from the dsRNA precursor. One strand of these siRNAs is recruited by the RNA-induced silencing complex (RISC) complex, which guides functional impairment, either by breakdown or translational inhibition, of any RNA species showing sequence similarity to the inducing small RNA (Pandey *et al.*, 2015). Transient double-stranded RNA-induced RNAi of the *Mlo* gene has been shown to result in a significant decrease in the germination and penetration efficiency of the fungal structures of powdery mildew at the single-cell level in barley (Varallyay *et al.*, 2012b). Agrobacterium-mediated RNAi transformation has been successfully applied to petunia (Thomas Gubitz and Kuhlemeier, 2009). In this study, I took two approaches to knockdown susceptibility genes in petunia: (1) TILLING for induced mutations in *Mlo* and *eIF4E* genes and (2) RNAi reduction of *Mlo* expression.

3.2 Materials and methods

3.2.1 Plant material and sampling

Seeds of *Petunia hybrida* “Mitchell Diploid” (MD), a doubled haploid variety, were grown in pots with Fafard soil 2B in a growth room at 22 ℃ with 16 hours light and 8 hours dark. One month old seedling leaves were cut for DNA and RNA extraction for the isolation of *PhMlo1* and *eIF4E* genes. One month old seedling roots and leaves, flower petals and anthers, and mature leaves were sampled for expression analysis of this gene. Root samples were isolated from the young plants with the same growth stage for sampling leaves. At flowering, open flower petals, stigmas, and immature anthers were sampled. The tissues were frozen in liquid nitrogen at sampling and stored in -80 ℃ freezer. All the sampling was repeated 2 times.

3.2.2 Mutagenesis treatments

Mutagenesis experiments were conducted with seeds of the doubled haploid cultivar *Petunia hybrida* 'Mitchell Diploid'. Three parameters were examined factorially: pre-treatment imbibition (2 treatments), EMS concentration (4 levels), and EMS exposure time (3 intervals). For each of the 24 treatments, there were 3 replicates of 24 seeds. Prior to exposure to EMS (Fluka, USA), seeds were either not treated or were imbibed in 500 μl of sterile deionized water for 12 h in the dark with mild shaking (45 rpm) at room temperature. EMS concentrations (0, 0.1, 0.2 and 0.3% v/v) were tested by adding an appropriate volume of freshly-prepared 0.5% EMS to the imbibed seeds. The seeds were then incubated in the dark at room temperature for 6h, 12h or 24 h with mild shaking (45 rpm). After EMS treatment, the seeds were rinsed 10 times with 1 ml of sterile deionized water and sown in 72 well trays containing soil (Fafard 3B potting mix). Individual seedling were transplanted to pots ($4 \times 4 \times 6$ metric) and maintained in greenhouse (latitude 33.95N and longitude -83.38W).

3.2.3 Mutagenesis data and statistical analysis

For the EMS treatments, a two-factor/one block experimental design was used with imbibition time as a block. Plants were evaluated 10 days after sowing for seed germination and 30 days after sowing for flower number. Analysis of variance (ANOVA) of the obtained data and regression analysis were conducted using SigmaPlot software.

3.2.4 Evaluation of the genotoxic effect

Genome damage was indirectly evaluated by the effect of the mutagen treatments on developmental and physiological parameters in time course analyses from 1 to 30 days after sowing (DAS) and at 15 and 30 DAS for the following characteristics: seed germination (%),

height (mm), flower number and anther quality. Data were individually scored and statistically analyzed by ANOVA and regression analyses.

3.2.5 High resolution melting analysis and amplicon sequencing

Primers were designed to amplify 200-500 bp amplicons corresponding to exons or pairs of exons from *PhMlo1*, *PheIF4E*, and *PheIF(iso)4E*. On a LightCycler 480 (Roche Diagnostics), PCR products were denatured at 95 ℃ for 1 minute, cooled to 40 ℃ for 1 minute, and then heated to 95 ℃ at 0.02 ℃/second, while continuously measuring florescence with 25 data acquisitions/ ℃. DNA melting data were analyzed by LC480 Gene Scanning software with settings for sensitivity and temperature shifting at 0.3 and 5, respectfully. All PCR/HRM experiments presented were repeated at least three times. For sequencing, PCR products were isolated by agarose gel electrophoresis and purified using a PureLink™ Quick Gel Extraction kit (Invitrogen). DNA samples were sequenced by MWG Operon (Louisville , Kentucky).

3.2.6 Construction of intron-spliced-hairpin constructs

Plasmid constructs containing ihp-RNAs of target genes were cloned using the bacteriophage lambda site-specific recombination. First, genes of interest were amplified by PCR using gene specific primers with flanking *attB1* and *attB2* sites. Purified PCR fragments were cloned into the PHK1001 vector to make an entry clone. Entry clones were confirmed by sequencing. PHK1001 construct plasmids were transformed into *Agrobacterium tumefaciens* strain ABI by electroporation.

3.2.7 Petunia transformation

Young petunia leaves from small (3- to 6-week-old) glasshouse-grown plants were used as the explant source. These were surface-sterilized by immersion with gentle shaking for 10 min in 10% commercial bleach (1.5% sodium hypochlorite) containing a few drops of 1% Tween 20, followed by several washes with sterile water. Leaf discs of 6 mm diameter were cut with a paper punch and immediately immersed for about 2 min in an early log-phase culture of the disarmed *Agrobacterium* strain ABI carrying the appropriate binary vector. The *Agrobacterium* culture was grown at 28◦C with vigorous shaking in YN broth (3 g/l Difco Bacto beef extract, 5 g/l Difco Bacto Peptone, 10 g/l Difco Bacto yeast extract, 8 g/l NaCl, pH 7.3) containing an appropriate bacterial selection agent for the binary vector and 20 µM acetosyringone. When the absorbance of the culture at 550 nm reached about 0.6 (equivalent to early log-phase growth for most *Agrobacterium* strains) the culture was used for co-cultivation.

After co-cultivation the leaf discs were blotted with sterile filter paper and placed onto PS medium, consisting of: MS salts, B5 vitamins, 3% sucrose, 3 mg/l BAP, 0.2 mg/l IAA and 0.7% agar at pH 5.8. After 2 days they were transferred to PS medium containing 500 mg/l cefotaxime, which prevents *Agrobacterium* overgrowth, and a selective agent to allow the preferential growth and regeneration of transformed cells carrying the binary vector T-DNA. The preferred selective agent was kanamycin, for which 300 mg/l is optimal for selection with Petunia Mitchell. After 4 weeks, the regenerating shoots were harvested, cut into nodal segments and placed onto fresh PS medium with cefotaxime and kanamycin, in order to generate up to ten clonal copies of each putatively transformed shoot. This step also provides an additional selection step to eliminate non-transformed "escapes". After 4 more weeks, the cloned shoots were dipped into a sterile solution of 100 mg/l IAA and transferred to PR medium (MS salts, B5 vitamins, 3% sucrose)

containing 500 mg/L cefotaxime and 100 mg/l kanamycin. Shoots initiating roots in the presence of kanamycin were designated putative transgenics and transferred to the glasshouse.

3.2.8 Screening of transgenic plants by PCR and real-time PCR

DNAs isolated from putative transgenic plants were used as templates and the PCR was conducted with Taq DNA polymerase (Genscript) reaction with primers RAF and L1GSF in 20 ml PCR reaction. RAF primer is located at the *Mlo* anti-sense specific and the reverse primers L1GSF are GUS hairpin sequence specific.

Transgenic petunia leaves were used in RNA extraction by the CTAB method (Jaakola et al., 2001). The extracted RNA was measured and used in cDNA synthesis using the same procedures as that for the isolation of cDNA sequence of *PhMlo1* gene described above. The cDNA samples were diluted into 1/20, 1/80, 1/200, 1/800. The real time PCR condition, procedures and instrument were the same as that used in *PhMlo1* gene transcription level estimation except for the primers and annealing temperature. PCR was conducted in triplicate for each of two biological repeats. ME13F and MJ2R for *PhMlo1* gene (produced a 265 bp band), and EF1αF and EF1αR for *Elongation factor 1α* gene (EF1α) as a reference were used in expression analysis using real time PCR. Those primers produced single product and negative control produced nothing. EF1α was recommended the most suitable reference gene for petunia line Mitchell (Mallona *et al.*, 2010). The result data analysis adopted similar method as that in copy number estimation. After amplification efficiency was calculated, the data for 1/200 dilution was used for relative quantity estimation.

3.3 Results

3.3.1 Optimization of EMS mutagenesis

Procedures for EMS mutagenesis of *P. hybrida* were optimized to balance high mutation frequency with plant viability and fertility. Three mutagenesis parameters were investigated: the imbibition of seeds prior to EMS treatment, the EMS concentration, and EMS exposure time. The results of combinations of these factors on germination frequency and flower number are shown in Figures 1 and 2, respectively, with summary statistics in Tables 1 and 2. The effect of seed imbibition prior to mutagenesis was significant for both germination and flower number. EMS concentration and exposure time had significant effects on flower number, but not on germination. After a 12h exposure to EMS, the average number of fruit per plant decreased from 2.7 capsules in controls to 0.6, 0.2, and 0 capsules in treatments with 0.1%, 0.2%, and 0.3% EMS, respectively. Based on these results, the mutagenesis of 2000 petunia seeds was conducted with a 12 hour imbibition followed by exposure to 0.1 % EMS for 12 hours. After flowering, the M1 plants were self-fertilized manually to produce the M2 generation. We have screened 865 M1 families, which included 666 M1 families with four M2 progeny plants, along with 199 M1 lines with three or less progenies for each line (more than 3000 M2 plants in total).

3.3.2 Overall characteristics of *P. hybrida* mutagenized populations

The petunia seed germination and flower number were significantly affected by the mutagen dosage, treatment time and imbibition time of EMS. Fifteen days after germination, plant height was investigated. The plant heights are negatively affected by increased mutagen dosage of EMS (Figure 3). The plant height of 12 hour EMS treatment among different dosages presented linear correlation coefficients, 99.8% for 12 hour EMS treatment at 99% probability. Thirty days after

germination, anther qualities on each plant were investigated. The anther quality was classified into four categories. The anther quality decreased as the mutagen dosage and exposure time increased (Figure 4).

The conditions for EMS mutagenesis of *Petunia hybrida* 'Mitchell Diploid' were optimized in preparation for the development of a mutagenized population. Protocols for petunia mutagenesis have been published previously (Kashikar and Kashikar,1981; Napoli and Ruehle, 1996; Berenschot et al., 2008), but there is little consensus in procedures. This may be related to differences in genotypes. The optimized parameters described in the present work most closely resemble those of Berenschot and coworkers (2008), who used a 14-hour seed imbibition followed by 24 hours of exposure to 0.1% EMS. In their study, EMS concentration had only minor effects on viability after 15 days, but seed capsule production was reduced by 25% with 0.25% EMS (Berenschot et al., 2008). In our hands, treatment of seeds with 0.2% EMS reduced the number of capsules by 79.8%. The application of our EMS mutagenesis protocol to 2000 petunia seeds and manual fertilization of the resulting plants produced an M2 population suitable for TILLING (Jiang et al., 2013).

In order to increase the likelihood of inducing genetic variation in *P. hybrida*, a protocol was developed for mutating petunia with EMS while minimizing deleterious effects on viability and fertility. A mutagenized population was produced that is suitable for screening by genetic techniques such as TILLING.

3.3.3 High resolution melting analysis for the identification of allelic variation in *PhMlo1*, *PheIF4E* and *PheIF(iso)4E*

A population of approximately 865 M1 plants was developed from EMS-mutagenized seeds of *P. hybrida* 'Mitchell Diploid'. Four M2 plants per family were screened for polymorphisms induced in susceptibility genes, starting with *PhMlo1*. Leaves from a family were pooled for DNA extraction and high-resolution melting (HRM) analysis. Six pairs of primers were used to amplify *PhMlo1* exon 4 to exon 9. M2 families were found that exhibited variation in exon 6 (Fig. 5) and exon 4 (Fig. 6). A second HRM screen with individual family members identified the *PhMlo1* variants (Fig 5B), which were confirmed by sequencing.

PROVEAN analysis predicted that the G176E substitution in exon 6 would have a deleterious effect on protein function (Fig 5D). The S130L substitution in exon 4, on the other hand, was predicted to be neutral (Fig 6). For this mutant, we randomly selected 14 petunia lines and sequenced *PhMlo1* exon 4 region. Seven of them showed this substitution in exon 4, so we expected that this is likely a natural mutation in *Petunia hybrid*a (data not shown).

For *eIF4E* family genes, exons 1 through 3 are critical for the physical interaction for viral infection through an association with the viral genome-linked protein (VPg). One pair of primers was designed to amplify *PheIF4E* exon 2 to exon 3 for HRM screen. Two pairs of primers were used to amplify *PheIF(iso)4E* exon 1 to exon 3 for HRM screen. However, no mutations were detected in *PheIF4E* and *PheIF(iso)4E* in M2 petunia lines (data not shown).

3.3.4 Genetic transformation and initial characterization of transgenic petunia plants

To study the involvement of *PhMlo1* in powdery mildew infection, we used a gene silencing strategy. A DNA construct containing a self-complementary hairpin structure of *PhMlo1* gene was developed by Dr. Yihua Chen (unpublished). The construct contained the target gene sequences in forward and reverse orientations separated by a part of GUS gene as an intron (Fig. 7A). Putative transgenic shoots formed on the selection medium after four weeks. The developed shoots were excised from explants and cultivated on the shoot regeneration medium supplemented with selective agent for shoot propagation. Induced shoots showed vigorous and continuous growth on the selection medium (Figure 7B). At least ten clones of transgenic line were kept on the selection medium for propagation and the rest of the induced shoots were transferred to rooting medium. After 4-6 weeks, rooted plantlets were transferred to Magenta boxes and then to the greenhouse for acclimatization (Figure 7B). Leaf tissue from putative transformants was analyzed by PCR using gene specific primer RAF-L1GSF, located in the forward orientation *PhMlo1* and GUS gene region (Figure 7C). DNA samples from nine selected transgenic plants showed RAF-L1GSF PCR amplicon, suggesting that most transgenic lines are the result of *PhMlo1*-RNAi sequence insertion into host plant chromosome successfully (Figure 8). In total, 9 *PhMlo1*-RNAi transgenic lines were obtained.

3.3.5 Reduced expression of target genes in transgenic petunia plants

Expression of *PhMlo1* genes in representative transgenic petunia lines was investigated by Realtime-PCR. In *PhMlo1*-RNAi lines, *PhMlo1* mRNA transcript levels decreased significantly (50%–80% reduction compared to wild-type plants) (Figure 8). The results indicated that the *PhMlo1*-RNAi construct was effectively silencing the expression of the target gene.

3.4 Discussion

Targeted mutation breeding and transgenic breeding are new breeding methods have been applied on many crops. However, they have not been applied to the development of disease resistant ornamental plants. We examined both breeding methods to knockout/knockdown susceptibly genes in petunia in order to generate a disease-resistant cultivar. For target mutation breeding, a level of the chemical mutagen EMS was determined that reduced survival of mutagenized petunia plants by 50%. An HRM screen of more than 3000 M2 petunia mutants found one induced mutation and one natural mutation, corresponding to a deleterious mutation and neutral mutation respectively. However, progeny of the M1 plant with the deleterious mutation was lost due to a lack of vigor. Therefore, we used the RNAi method in order to knockdown the *PhMlo1* gene in petunia. Nine plants with *PhMlo1*-RNAi T-DNA insertions were produced, and four of these transgenic plants had significantly reduced levels of *PhMlo1* expression.

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Tables

Table 1. Analysis of variance of effect of mutagenesis parameters on seed germination (day 10). *P = 0.05; **P = 0.01; ns = not significant.

Source	DF	SS	MS	F	
Imbibition (IMB)	1	0.066544	0.066544	12.73	**
EMS exposure (EXP)	2	0.019351	0.009675	1.85	ns
EMS concentration (CONC)	3	0.003558	0.001186	0.23	ns
IMB x EXP	2	0.012402	0.006201	1.19	ns
IMB x CONC	3	0.064162	0.021387	4.09	*
EXP x CONC	6	0.120158	0.020026	3.83	*
Error	6	0.031369	0.005228		
Total	23	0.317544			

Table 2. Analysis of variance of effect of mutagenesis parameters on flower number (day 30). *P = 0.05; **P = 0.01; ns = not significant.

Source	DF	SS	MS	F	
Imbibition (IMB)	1	18.6345	18.6345	46.38	**

EMS exposure (EXP)	2	4.2732	2.1366	5.32	*
EMS concentration (CONC)	3	8.1507	2.7169	6.76	*
IMB x EXP	2	0.0175	0.0088	0.02	ns
IMB x CONC	3	2.0912	0.3485	0.87	ns
EXP x CONC	6	2.3546	0.7849	1.95	ns
Error	6	2.4107	0.4018		
Total	23	37.9324			

Table 3. *PhMlo1*, *PheIF(iso)4E* and *PheIF4E* primers used in HRM.

Name	Oligonucleotide sequence (5'-3')	Amplicon region	Size (bp)
Mlo-A-For	TGAATGCAGGAAAAGTCCAA	Petunia *Mlo* gene Exon 4	240
Mlo-A-Rev	AAGTTCGACTGTTGTGCGACT		
Mlo-B-For	TTGAGGCATGACCATTTTTAGA	Petunia *Mlo* gene Exon 5	150
Mlo-B-Rev	GGGTGAAGTTTCGAGTTCCA		
Mlo-C-For	TGACTGCAGATCCTGAGAGGT	Petunia *Mlo* gene Exon 6	150
Mlo-C-Rev	TTGTCGAGTCTCTCGGTATCAC		
Mlo-D-For	GAGCAAGTCTCCCGTGCTAC	Petunia *Mlo* gene Exon 7	200
Mlo-D-Rev	ATAACAAGGGGTGCAGTGGA		
Mlo-E-For	CTGCAGGCACATTTAACTCC	Petunia *Mlo* gene Exon 8 and 9	360
Mlo-E-Rev	CCTGTCCAAAAAGACGGAAA		
Mlo-F-For	CTGCAGGCACATTTAACTCC	Petunia *Mlo* gene Exon 10	360
Mlo-F-Rev	CCTGTCCAAAAAGACGGAAA		
eIF4E-A-For	AATATCCACCACCCAAGCAA	Petunia *eIF4E* gene Exon2 and 3	350
eIF4E-A-Rev	TTTCATTGGCAGCATTCTTG		
eIF(iso)4E-A-For	CACCGAAGCACCGGTAGAG	Petunia *eIF*(iso)*4E* gene Exon1	340
eIF(iso)4E-A-Rev	TCGCAAAACATATCCAACAGA		
eIF(iso)4E-B-For	GTTTCAAACCCTGAGCCACT	Petunia *eIF*(iso)*4E* gene Exon2 and 3	390
eIF(iso)4E-B-Rev	CGGCAGTCTTAGTCCACAGG		

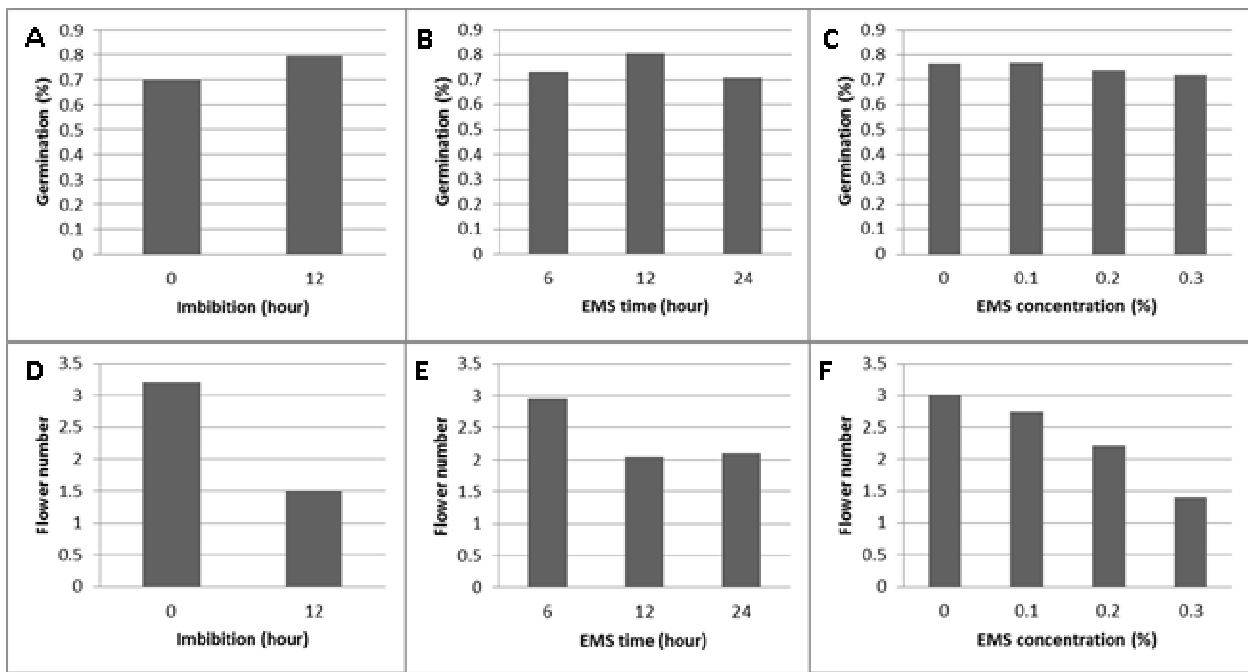
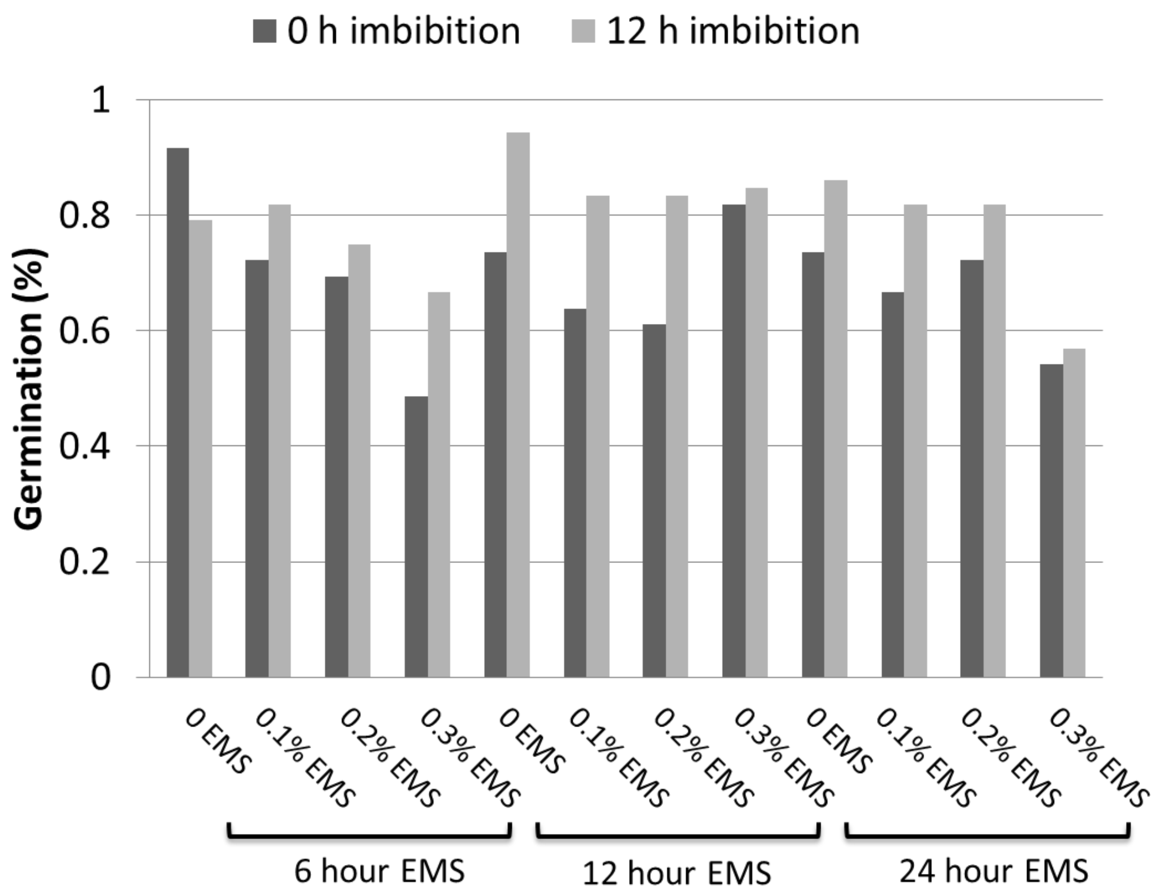


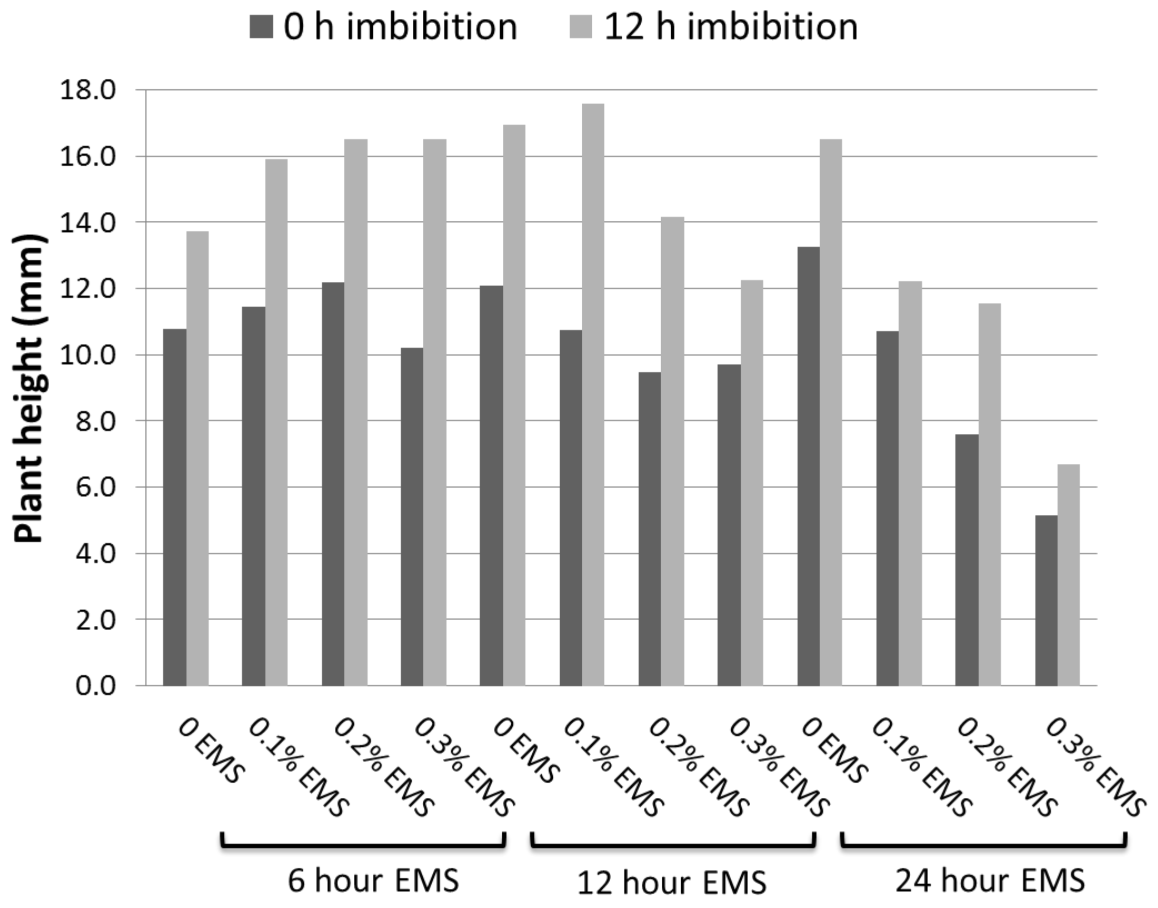
Figure 1. A-C. Germination rates are shown among different imbibition time, EMS treatment time and EMS concentration on 10 days after sowing. D-F. Flower numbers are shown among different imbibition time, EMS treatment time and EMS concentration on 30 days after sowing.



0 h imbibition
12 h imbibition
Germination (%)
1
0.8
0.6
0.4
0.2
0
0 EMS
0.1% EMS
0.2% EMS
0.3% EMS
0 EMS
0.1% EMS
0.2% EMS
0.3% EMS
0 EMS
0.1% EMS
0.2% EMS
0.3% EMS
6 hour EMS
12 hour EMS
24 hour EMS

Figure 2. Germination rates are shown among each treatment on 10 days after sowing.

A.



0 h imbibition
12 h imbibition
Plant height (mm)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0 EMS
0.1% EMS
0.2% EMS
0.3% EMS
0 EMS
0.1% EMS
0.2% EMS
0.3% EMS
0 EMS
0.1% EMS
0.2% EMS
0.3% EMS
6 hour EMS
12 hour EMS
24 hour EMS

B.

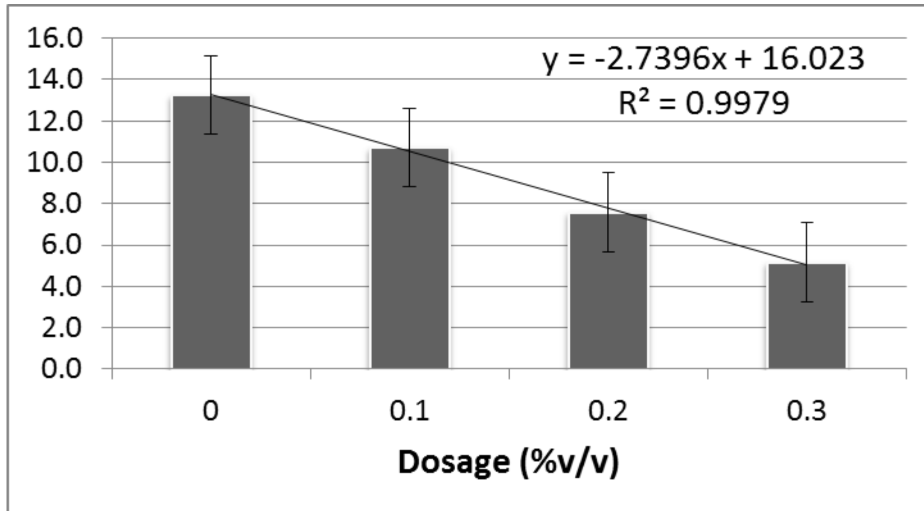


Figure 3. A. Plant heights are shown among each treatment on 15 days after sowing. **B.** The plant height of 12 hour EMS treatment among different dosages presented linear correlation coefficients, 99.8% for 12 hour EMS treatment at 99% probability.

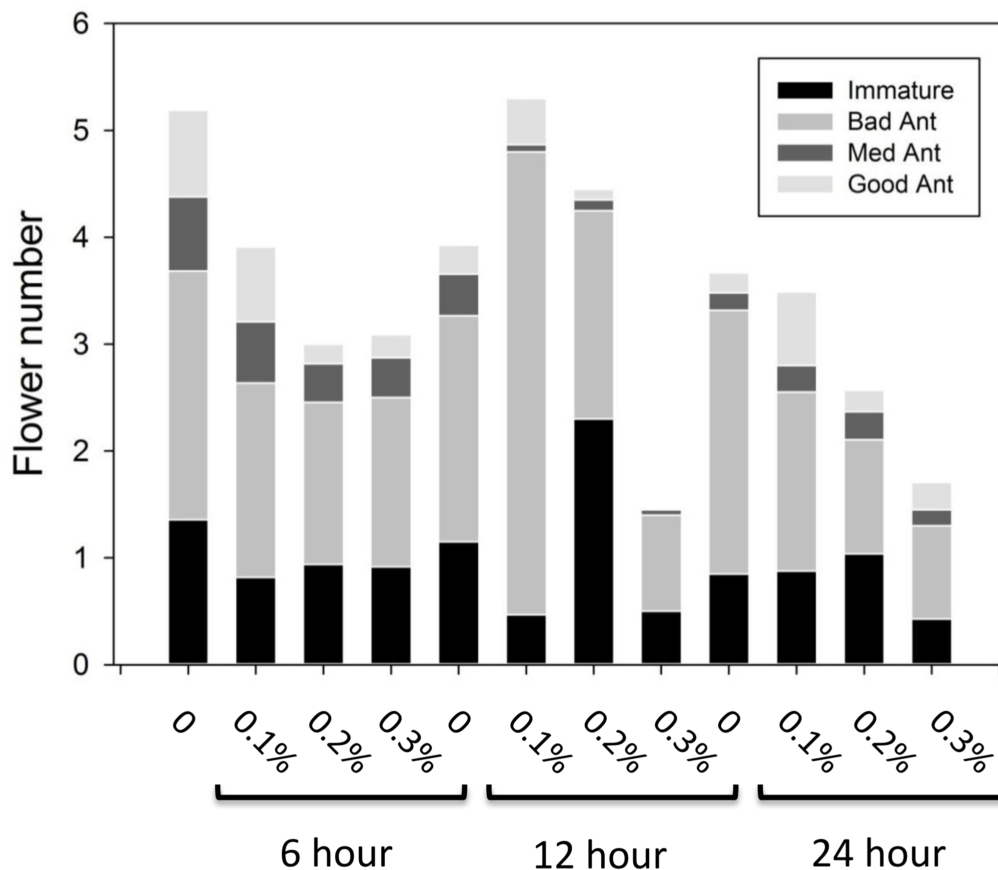


Figure 4. Anther qualities are shown among different imbibition time, EMS treatment time and EMS concentration on 30 days after sowing. Immature: all five anthers are yellow green and not fully developed. Bad Anther (Bad Ant): some of filaments are not straight. Microsporangia are not fully developed. Light yellow pollen looks dry and unhealthy. Medium Anther (Med Ant): most of anthers are fully developed. Yellow pollen covered surface of anthers. Good Anther

(Good Ant): all five anthers are fully developed. Dark yellow round pollen covered surface of all five anthers.

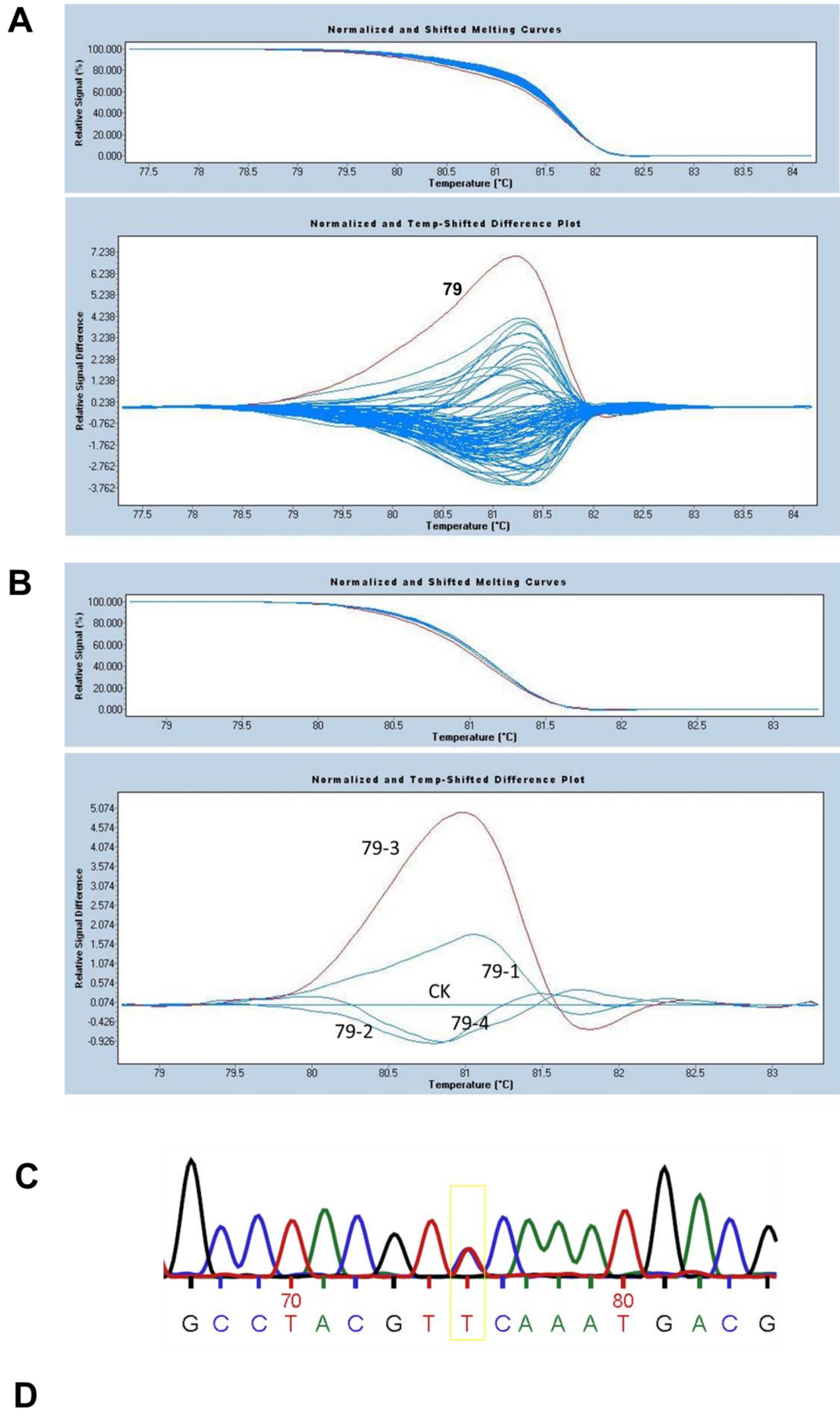
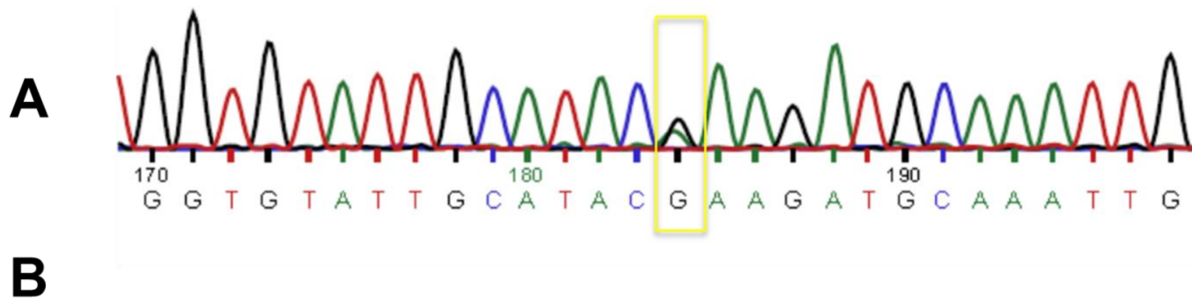


Fig. 4. **A**. Initial HRM screen detected variation in *PhMlo* exon 6 in M2 family #79. **B**. HRM analysis of individual family members.

Variant	PROVEAN score	Prediction (cutoff= -2.5)
G176E	-5.820	Deleterious

Figure 5. **A**. Initial HRM screen detected variation in *PhMlo1* exon 6 in M2 family #79. **B**. HRM analysis of individual family members. **C**. Sequencing of *PhMlo1* exon 6 confirmed a heterozygous mutation (G to A) in family member 79-3. **D**. The exon 6 polymorphism at position 176 was predicted by PROVEAN analysis to be deleterious to MLO function.

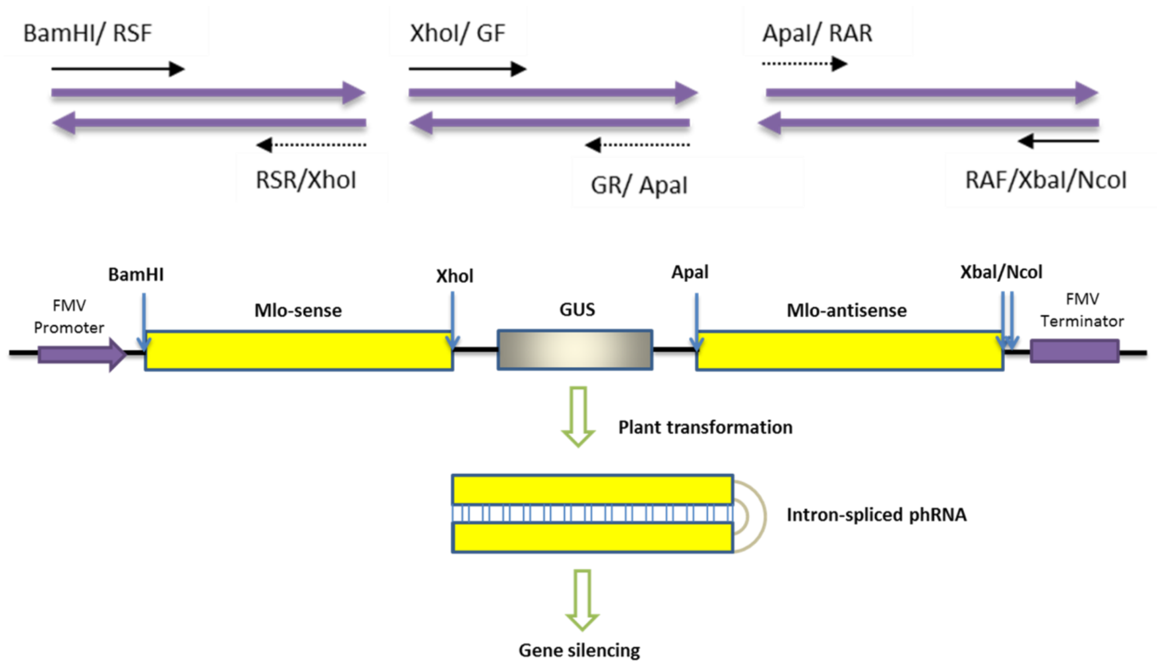


Variant	PROVEAN score	Prediction (cutoff= -2.5)
S130L	4.481	Neutral

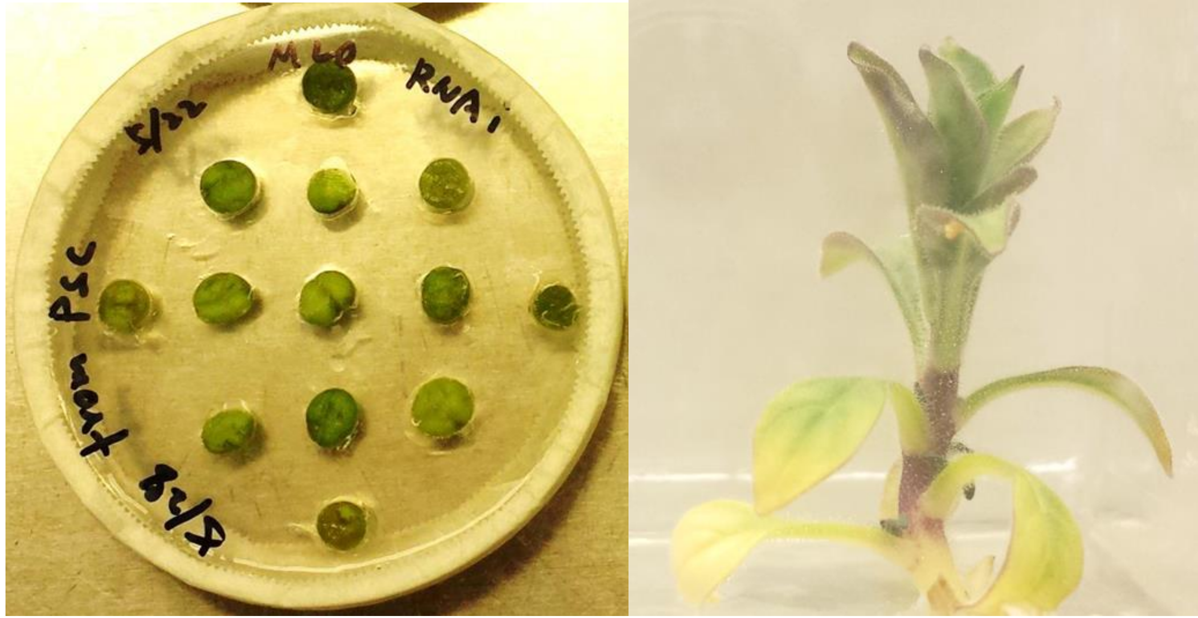
Figure 6. A. Sequencing result of line 633-1 exon4, position 130 show a heterozygous mutation (G to A). **B.** Exon4 polymorphism on MLO position 130 is predicted by PROVEAN, which is neutral (Variants with a score = to or < than -2.5 are considered "deleterious", > than -2.5 are considered "neutral").

Figure 7.

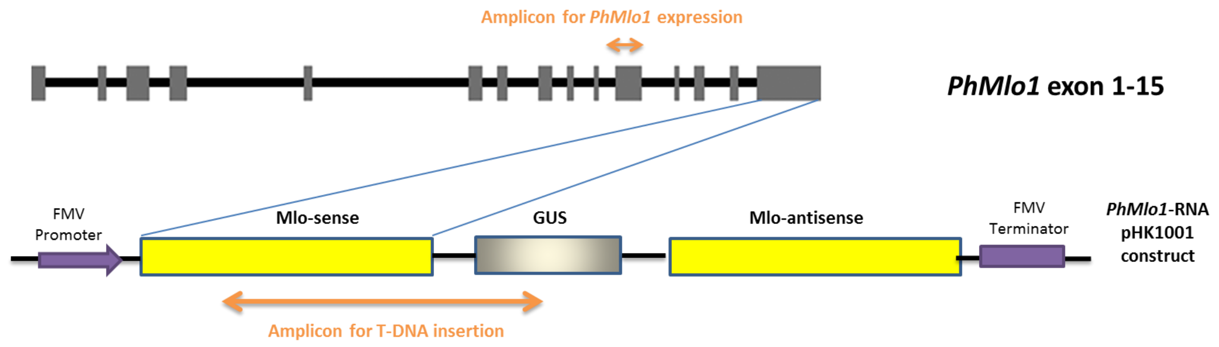
A.



B.



C.



D.

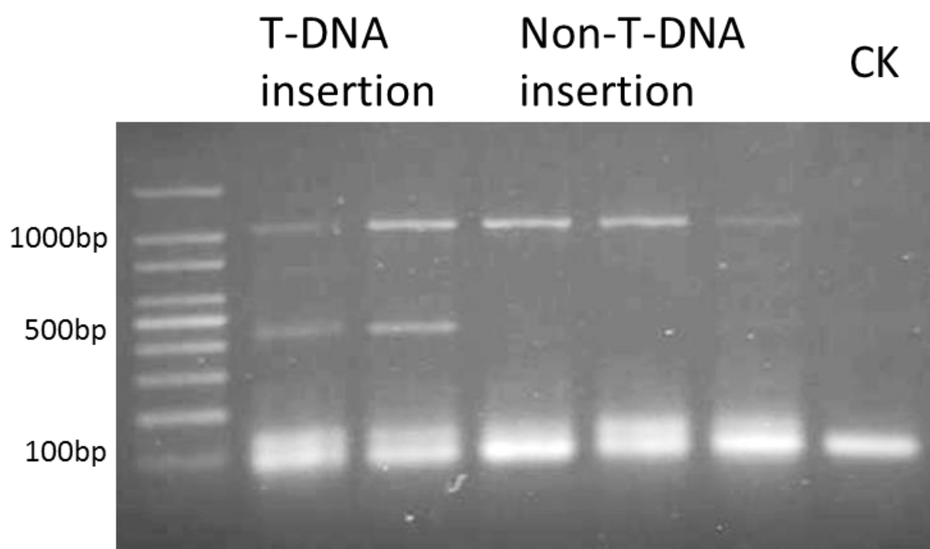


Figure. 7. Production and initial analysis of transgenic petunia plants. **A.** Schematic diagrams for *PhMlo1*-RNA construct. Two PCR fragments of *PhMlo1* are present in opposite orientations. Transgene expression is under the control of a FMV promoter (purple arrow) and an FMV terminator (purple box). The vertical blue arrows indicate restriction sites used to release the inserts. **B.** Regeneration of plum transformants. Left: Petunia leaves infect by *A. tumefaciens* carrying *PhMlo1*-RNAi vector. Middle: Putative transformants rooted in Magenta box. C. Schematic diagrams for amplicon of *PhMlo1*-RNA construct T-DNA insertion and amplicon of *PhMlo1* expression. **D.** PCR test of five transgenic lines, two of them show RNAi construct T-DNA sequence.

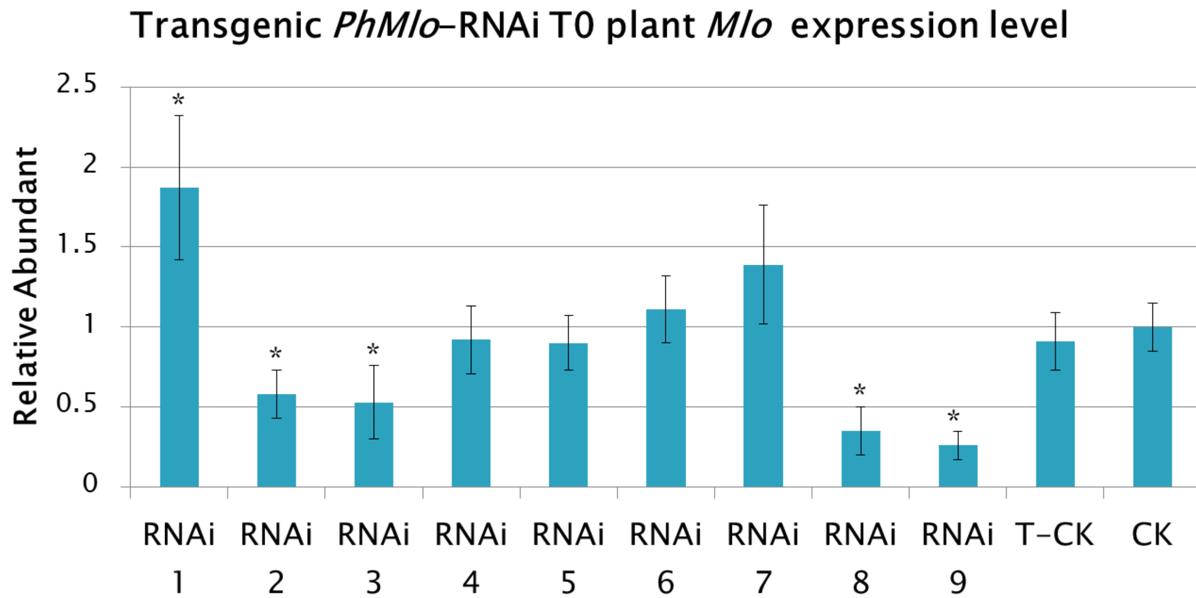


Figure 8. Expression of *PhMlo1* genes in representative transgenic petunia lines was investigated by qRT-PCR. In *PhMlo1*-RNAi lines, *PhMlo1* mRNA transcript levels decreased significantly (50%–80% reduction compared to wild-type plants). The results indicated that the *PhMlo1*-RNAi construct was effectively silencing the expression of the target gene. Bars represent standard errors; * indicates significance at $P<0.05$ according to Student's t test when compared with wild type control.

CHAPTER 4

Effect of *PhMlo1* Knockdown on Powdery Mildew Resistance and Plant Growth

4.1 Introduction

Powdery mildew, caused by the obligate biotrophic fungus, is one of the most important diseases of petunia worldwide (Kiss *et al.*, 2008). While broad-spectrum resistance against this pathogen exists in barley as a result of a natural and induced mutation in the *Mlo* gene (Buschges *et al.*, 1997b), petunia varieties exhibit only gene-for-gene interaction-based, race-specific resistance. As new races of the pathogen can easily break this type of resistance, there is a need for developing broad-spectrum resistance in petunia.

MLO is a transmembrane protein and functions as a negative regulator that suppresses plant defenses in uninfected tissues (Humphry *et al.*, 2006). It is involved in protection against cell death as well as in responses to biotic and abiotic stresses (Buschges *et al.*, 1997b). In the absence of the functional protein, successful entry of powdery mildew hyphae into host epidermal cells is inhibited in both monocot and in dicot plant species (Buschges *et al.*, 1997b; Cadle-Davidson, 2009; Bai *et al.*, 2008b; Micali *et al.*, 2008). In Arabidopsis, 15 *Mlo* genes have been found, and three of them (*AtMlo2*, *AtMlo6*, and *AtMlo12*) are responsible for powdery mildew resistance (Micali *et al.*, 2008). For solanaceous plants, 17 *Mlo* genes have been found in tomato (Zheng *et al.*, 2013a). Three of them (*SlMlo1*, *SlMlo2* and *SlMlo6*) show exceptional similarity to the Arabidopsis *Mlo* orthologs with powdery mildew resistance function.

Mlo was discovered in barley and later mapped and characterized as a membrane-anchored protein (Buschges *et al.*, 1997a). The role of *Mlo* in PM susceptibility has been confirmed in Arabidopsis, pea, tomato, pepper, wheat, and strawberry (Bai *et al.*, 2008a; Consonni *et al.*, 2006a; Humphry *et al.*, 2011; Jiwan *et al.*, 2013; Pavan *et al.*, 2011; Varallyay *et al.*, 2012a; Zheng *et al.*, 2013a). *Mlo* is required for susceptibility to adapted pathogens, and *Mlo* mutants display loss of susceptibility resembling that described for nonhost resistance (Humphry *et al.*, 2006).

In Arabidopsis, the triple mutant *Atmlo2*, *Atmlo6*, and *Atmlo12* is completely resistant to powdery mildew, restricting fungal development at the host cell entry level (Consonni *et al.*, 2006a). These discoveries, together with the knowledge that the *Mlo* family is ubiquitously present in higher plant species (Devoto *et al.*, 2003), led to the identification of powdery mildew resistance on the basis of natural *mlo* loss-of-function alleles in tomato (Bai *et al.*, 2008a; Zheng *et al.*, 2013b) and pea (Humphry *et al.*, 2011; Pavan *et al.*, 2011). Pepper *Mlo2* is also required for susceptibility to the adapted bacterial pathogen *Xanthomonas campestris* but not for avirulent bacteria (Kim and Hwang, 2012). Conservation of *Mlo*-based resistance across monocot and dicot plant species implicates a common mechanistic basis for this type of plant immunity. It further implies potential application of this trait for plant breeding of many other agriculturally and economically important plant species (van Schie and Takken, 2014).

Solanaceous plants are attacked by many powdery mildew fungi worldwide. For example, *Leveillula taurica* and *Golovinomyces orontii* are both known as pathogens of many wild and cultivated species of the Solanaceae, including important crops such as tomato, eggplant, and tobacco (Braun, 1987b). In addition, these two pathogens infect many solanaceous ornamentals as well as *Podosphaera xanthii* (Braun, 1987b). All three of these powdery mildew species are species complexes that consist of a number of phylogenetically distinct lineages with different host ranges (Hirata *et al.*, 2000). Molecular tools, in particular the analysis of the internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA (rDNA), were useful in the precise identification of a large number of powdery mildew anamorphs (Braun *et al.*, 2006; Cunnington *et al.*, 2005). It was found *Mlo* has other functions other than powdery mildew resistance. Since fungal penetration and establishment depend on focal membrane and cytoskeleton rearrangement mediated by *Mlo* (Consonni *et al.*, 2006b), the pleiotropic effects of mutating of *Mlo* gene have been described. It was found that lack of Arabidopsis *Mlo7* affect root hair growth and pollen tube growth and guidance (Kessler *et al.*, 2010). For barley, Arabidopsis and tomato, it was found that reduced cell death suppression affects growth, senescence, and lesions (Jarosch *et al.*, 1999; Kumar *et al.*, 2001; Zheng *et al.*, 2013b). The objectives of this research were to identify a powdery mildew strain and to examine the effect of the knockdown of *PhMlo1* on powdery mildew infection and growth in petunia.

4.2 Materials and methods

4.2.1 Screening of transgenic plants by PCR and real-time PCR

DNA isolated from putative transgenic plants was used as template and the PCR was conducted with Taq DNA polymerase (Genscript) reaction with primers RAF and L1GSF in 20 ml PCR

reaction. The RAF primer is located at the *PhMlo1* anti-sense specific and the reverse primers L1GSF are GUS hairpin sequence specific.

Transgenic petunia leaves were used in RNA extraction by the CTAB method (Jaakola et al., 2001). The extracted RNA was measured and used in cDNA synthesis using the same procedures as that for the isolation of cDNA sequence of *PhMlo1* gene described above. The cDNA samples were diluted into 1/20, 1/80, 1/200, 1/800. The real time PCR condition, procedures and instrument were the same as that used in *PhMlo1* gene transcription level estimation except for the primers and annealing temperature. PCR was conducted in triplicate for each of two biological repeats. ME13F and MJ2R for *PhMlo1* gene (produced a 265 bp band), and EF1αF and EF1αR for *Elongation factor 1α* gene (EF1α) as a reference were used in expression analysis using real time PCR. Those primers produced single product and negative control produced nothing. EF1α was recommended the most suitable reference gene for petunia line Mitchell (Mallona *et al.*, 2010). The result data analysis adopted similar method as that in copy number estimation. After amplification efficiency was calculated, the data for 1/200 dilution was used for relative quantity estimation.

4.2.2 Powdery mildew identification

Two powdery mildew strains were collected from Athens, Georgia and Long island, New York. Powdery mildew can be identified to species by ITS sequencing. Amplification using the PMITS1/2 primer pair works well on fresh tissue and has reportedly worked well on herbarium specimens (Cunnington et al, 2003). 100 mg of fresh or dried plant tissue containing heavy mycelial growth and sporulation were removed and genomic DNA were extracted using the

DNEasy Plant Mini Kit. Primers were prepared to a concentration of 0.8 μM (0.8 pmol/μl) in Nanopure water: PMITS1 5'-TCGGACTGGCCYAGGGAGA-3' PMITS2 5'-TCACTCGCCGTTACTGAGGT-3' The expected amplicon from PMITS primers was 700-800 bp in size (Cunnington et al, 2003).

When a product of the correct size is amplified, gel-purifying is necessary since fragments of other sizes may also amplify. 42 μl of reaction mixture with 8 μl of loading buffer were mixed and dispensed into three lanes joined together in a 1.5% agarose gel. Bands were excised and cleaned using the QiaQuick gel extraction kit or equivalent, paying special attention to instructions in the kit which relate to direct sequencing. After elute in 30-50 μl Buffer EB, amplicon were sequenced using PMITS1 and PMITS2. DNA samples were sequenced by MWG Operon (Louisville, Kentucky). Sequenced powdery mildew ITS regions were aligned in NCBI BLAST database.

4.2.3 Powdery mildew inoculation

Aqueous suspensions of conidia were prepared by vortexing excised mildew colonies in 1.5 ml microcentrifuge tubes with 1 ml of water and 0.05% Tween 20. The inoculated plants were grown at 25 C with 80% RH under natural light supplemented with artificial light to provide a photoperiod of 16 h per day. Six leaves on each transgenic line were inoculated by 10μl of *Podosphaera xanthii* (1×10^{-5} conidia/ml) from two geographic regions in the eastern United States. Latent period (i.e., the time from inoculation until the appearance of the first conidia) was determined by daily inspection for conidia under a dissecting microscope. Inspection took place each morning from day 4 to day 16. Lesion area was calculated from colony diameters measured in two perpendicular directions (a and b) 16 days after inoculation. Because the two diameters

were often unequal, we calculated lesion area as an ellipse (πab/4, in square millimeters) (Huang *et al.*, 1998). Each treatment had six replicates and the experiment was conducted twice.

4.2.4 Analysis of progeny

RNAi plants were self-pollinated. T1 progeny were grown along with parental and control lines. Effectiveness of RNAi in the F1 progeny was confirmed using PCR and real-time PCR. The germination rate, seeds per fruits, and growth rate of the F1 plants were measured.

T1 progeny were grown in pots with Fafard soil 2B in a growth room at 22 ℃ with 16 hours light and 8 hours dark. Individual seedling were transplanted to pots (4×4×6 inch) and maintained in greenhouse (latitude 33.95N and longitude 83.38W).

4.3 Results

4.3.1 Reduced expression of target genes and powdery mildew resistance in T0 transgenic petunia plants

Expression of *PhMlo1* genes in representative transgenic petunia T0 lines was investigated by Realtime-PCR, as shown in Chapter 3. In *PhMlo1*-RNAi T0 lines, *PhMlo1* mRNA transcript levels decreased significantly (50%–80% reduction compared to wild-type plants) (Chapter 3, Figure 8). The results indicated that the *PhMlo1*-RNAi construct was effectively silencing the expression of the target gene.

Two powdery mildew wild strains were obtained from Athens, Georgia and Long island, New York. ITS region sequencing indicated that these two stains are *Podosphaera xanthii*. Eight *PhMlo1*-RNAi transgenic lines along with a null-vector transgenic control and wild type petunia

"Mitchell" were inoculated by *P. xanthii* from two geographic regions in the United States. Both *P. xanthii* strains formed mildew colonies and produced abundant sporulation on the inoculated hosts (Figure 1). For the *P. xanthii* GA strain, two T0 *PhMlo1*-RNAi lines (2 and 3) show significant resistance (Figure 2). For *P. xanthii* NY strains, three T0 *PhMlo1*-RNAi lines (2, 3, and 8) show significant resistance (Figure 2). *PhMlo1*-RNAi T0 lines No. 2, No. 3, and No. 8 showed delayed infection and a reduced area of infection compared to transgenic controls (Figure 3).

4.3.2 Reduced expression of target genes and powdery mildew resistance in T1 transgenic petunia plants

The T0 transgenic lines were self-pollinated and T1 progeny were produced and examined for *PhMlo1* expression level. For T1 plants, *PhMlo1* expression level of ten *PhMlo1*-RNAi petunia No.2 plants, ten *PhMlo1*-RNAi petunia No.3 plants, ten *PhMlo1*-RNAi petunia No.8 plants, and three of each No.1, No.4, No.5, No.6 and No.7 plants were measured (Figure 4-7).

For ten T1 petunia No.2 plants, three showed normal *PhMlo1* expression level, while seven of them showed significantly lower expression level (Figure 4). For ten T1 petunia No.3 plants, two of them showed normal *PhMlo1* expression level, while eight of them showed a significantly lower expression level (Figure 5). For ten T1 petunia No.8 plants, three of them showed normal *PhMlo1* expression level, while seven of them showed significant lower expression level (Figure 6). Three T1 of each No.1, No.4, No.5, No.6 and No.7 plants *Mlo* expression level were measured, all of them show normal expression level (Figure 7).

The T1 progeny were examined for powdery mildew infection. The T1 plants selected for *Mlo* expression measurements were inoculated by powdery mildew *P. xanthii*. Inoculation results were collected in 16 days. For ten T1 petunia No.2 plants, seven of them showed partial powdery mildew resistance, while three of them showed no powdery mildew resistance (Figure 8).

For ten T1 petunia No.3 plants, seven of them showed partial powdery mildew resistance, while ? of them show no powdery mildew resistance (Figure 9). For ten T1 petunia No.8 plants, six of them showed partial powdery mildew resistance, while four of them showed no powdery mildew resistance (Figure 10). Three T1 of each No.1, No.4, No.5, No.6 and No.7 plants with normal *Mlo* expression level, all of them showed no powdery mildew resistance (Figure 11). Overall, *PhMlo1*-RNAi T1 lines with lower *Mlo* expression level showed a reduced area of powdery mildew infection compared to transgenic controls.

4.3.4 Pleiotropic effect in *PhMlo1*-RNAi transgenic plants

To characterize the impact of *PhMlo1* silencing on plant development and fertility, *PhMlo1*-RNAi T0 was self-crossed, and the resulting T1 progeny were grown along with parental and control lines on soil under standard greenhouse conditions. The effectiveness of RNAi of knocking down *Mlo* expression in the T1 progeny was confirmed using real-time PCR (Figure 4-7). Fruits from three plants per transgenic line were collected, and the number of seeds per fruits was counted. The number of seeds per fruits was reduced in the *PhMlo1*-RNAi lines comparing to CK (Table 1). Seed germination for *PhMlo1*-RNAi lines 3 was significantly lower than other transgenic line and CK (Figure 12). The growth rates of the T1 plants were significantly reduced compared to the CK plants. Significant differences in plant terminal height were also observed.

The T1 No.2 and T1 No.3 plants were 70% and 54% of the control, respectively (Figure 14). On the whole, the *PhMlo1*-RNAi lines presented a semi-dwarf phenotype and their fertility was affected, suggesting a cumulative effect of the silencing of *PhMlo1* genes (Figure 13).

4.4 Discussion

Only *PhMlo1*-RNAi T0 and T1 plants with reduced *Mlo* expression showed resistance to powdery mildew. The *PhMlo1*-RNAi lines with reduced *PhMlo1* expression presented a semi-dwarf phenotype and their fertility was affected, suggesting a cumulative effect of the silencing of *PhMlo1* genes. It was found in Arabidopsis that a *AtMlo7* mutation had an effect on plant germination, however, *AtMlo7* is in clade III and has no function of powdery mildew resistance (Acevedo-Garcia *et al.*, 2014). Pleiotropic effects on the *Mlo* mildew resistance gene were found in barley in different genetical backgrounds. It was detected that grain weight was higher in the powdery mildew resistant lines than non-resistant in barley (Bjornstad and Aastveit, 1990). We have aligned *PhMlo1*-RNAi T-DNA with other *Mlo* family members through NCBI BLAST, no homologs were found. It is the first time found that knock down of an *Mlo* gene with powdery mildew function affect plant growth negatively. For thirty T1 progeny of lower *PhMlo1* expression level, eight of them showed normal *PhMlo1* expression level, while twenty-two of them showed significant low expression level. A *PhMlo1*-RNAi line with partial powdery mildew resistance could be further crossed with marker assistant breeding to generate a new powdery mildew resistant petunia cultivar.

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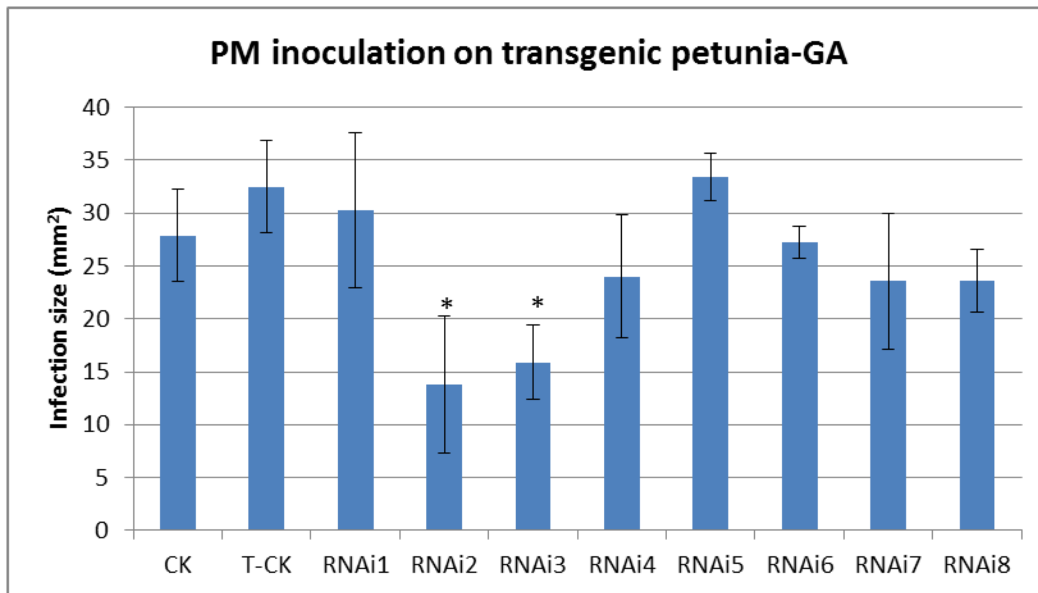
Table1. Seeds number of *PhMlo1*-RNAi T0 fruits.

	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	T-CK	CK
Mean	152	104	193	210	182	134	185	104	214	237
SD	13.45	22.65	23.71	3.51	32.13	6.66	17.79	16.26	8.89	36.80

Figure 1. Transgenic petunia inoculation measurement. Inoculation was conducted by applying ~100 conidia to the center of the leaf with a sterile pipette tip. Fifth youngest leaves inoculated from each of the six host plants. Lesion area calculated as an ellipse ($\pi ab/4$, in mm^2)



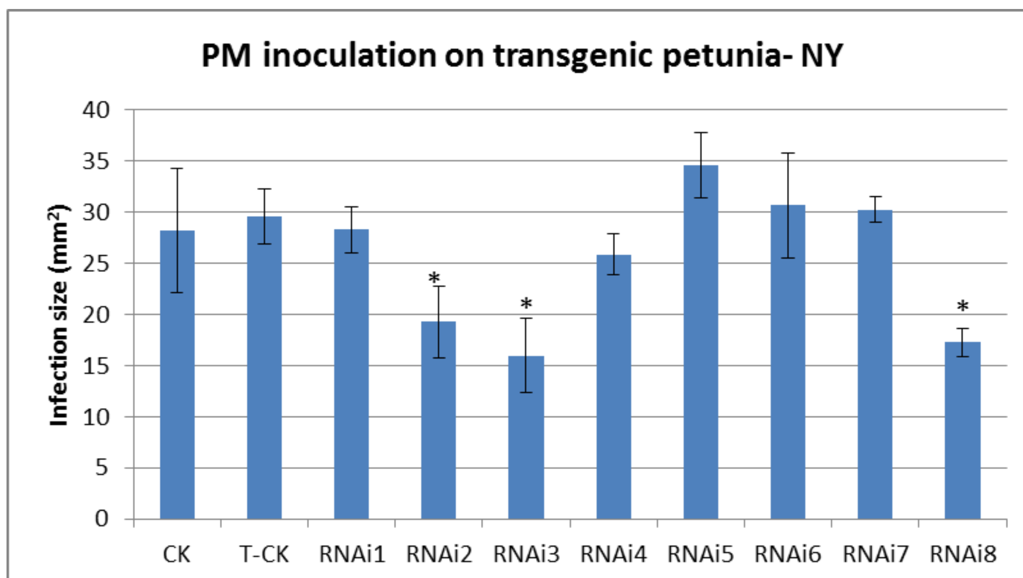


Figure 2. Lesion size for isolates of *Podosphaera xanthii* from two geographic regions in the United States measured on eight *PhMlo1*-RNAi transgenic lines, null-vector transgenic control and wild type petunia "Mitchell". Lesion size was calculated as an ellipse (πab/4

mm^2) and measured 16 days after inoculation. Bars represent standard errors; * indicates significance at P<0.05 according to Student's t test when compared with wild type control.

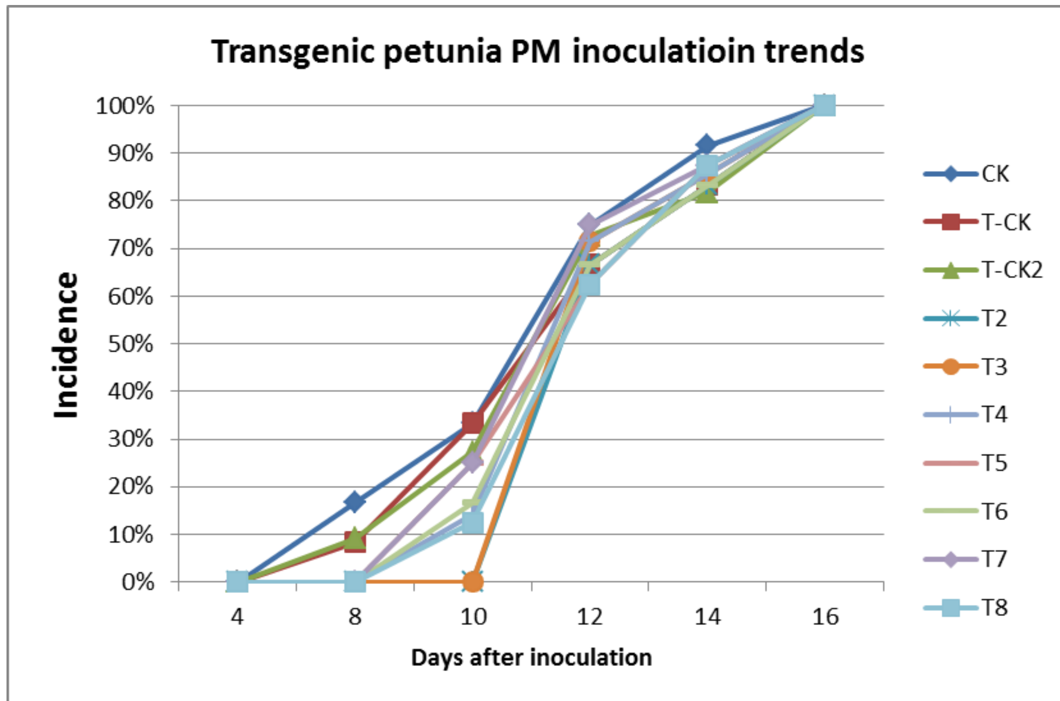


Figure 3. Powdery mildew infection trends of *PhMlo1*-RNAi T0 lines 2, 3, and 8 showed delayed infection and a reduced area of infection compared to transgenic controls.

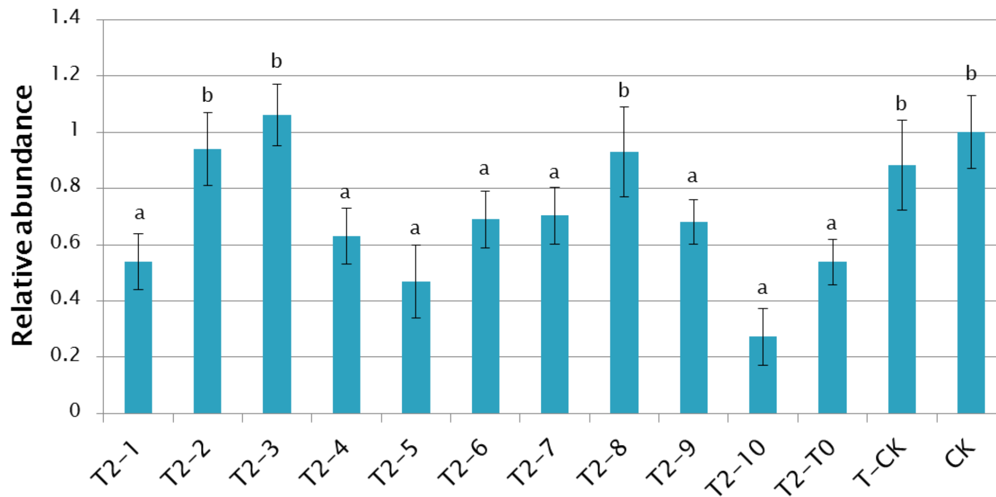


Figure 4. Transgenic *PhMlo1*-RNAi T1 plant No.2 progeny *PhMlo1* expression level.

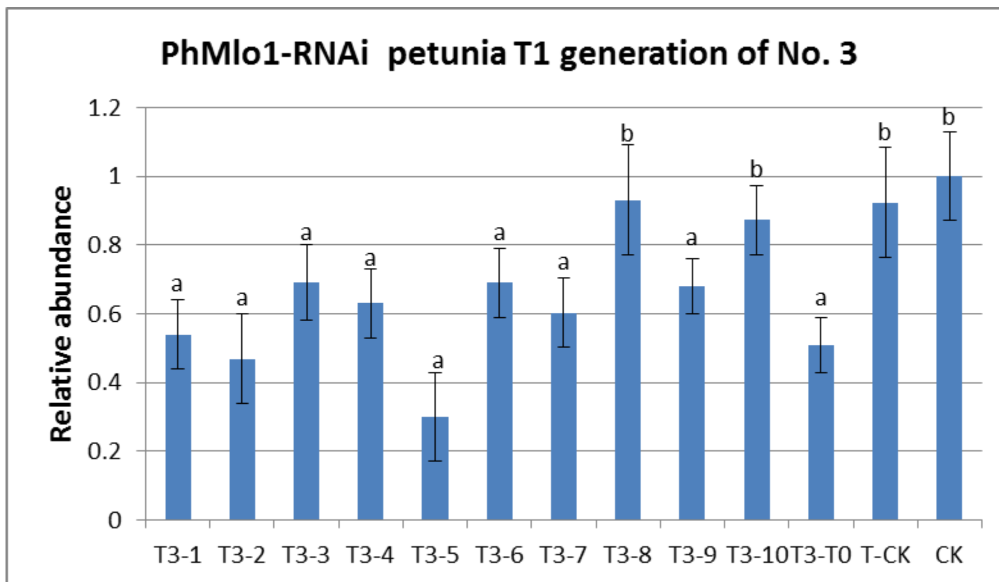


Figure 5. Transgenic *PhMlo1*-RNAi T1 plant No.3 progeny *PhMlo1* expression level.

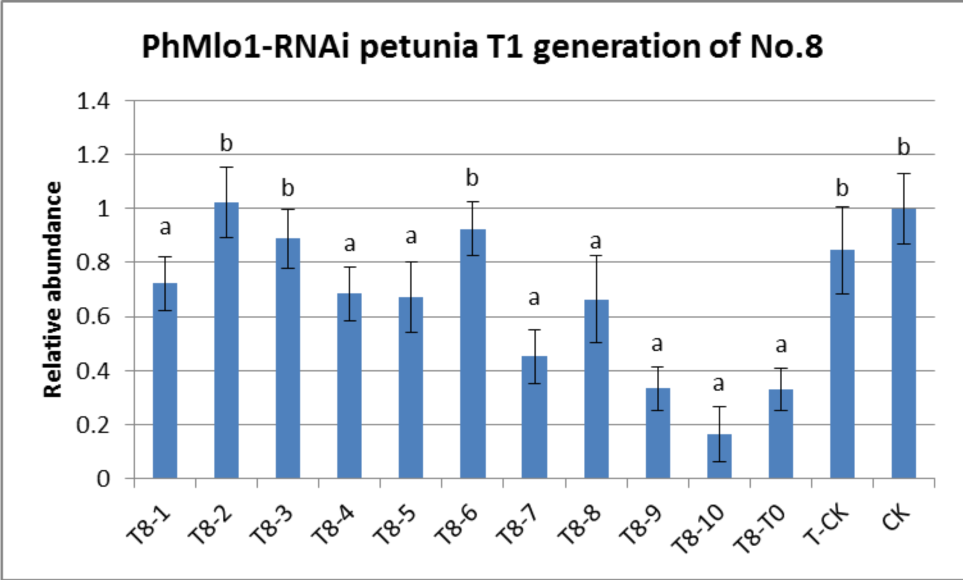


Figure 6. Transgenic *PhMlo1*-RNAi T1 plant No.8 progeny *PhMlo1* expression level.

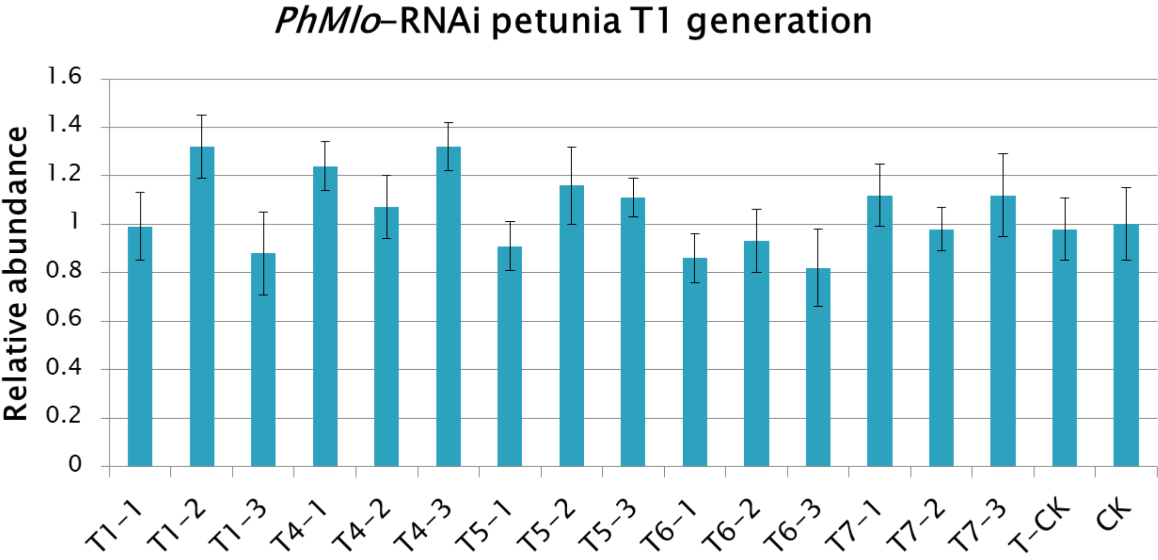


Figure 7. Transgenic *PhMlo1*-RNAi T1 generation progeny *PhMlo1* expression level

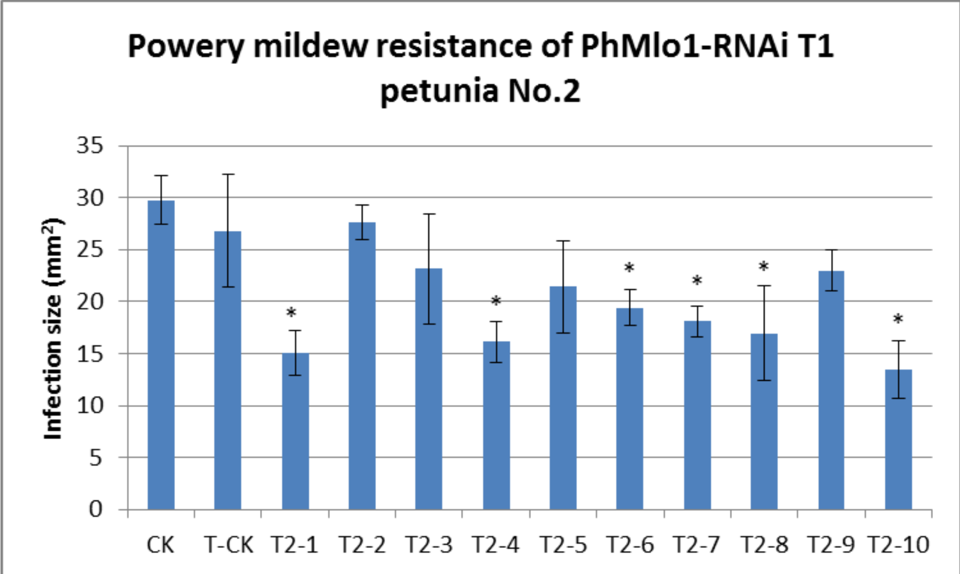


Figure 8. Powdery mildew resistance of 10 Transgenic *PhMlo1*-RNAi T1 No. 2. Bars represent standard errors; * indicates significance at P<0.05 according to Student's t test when compared with wild type control.

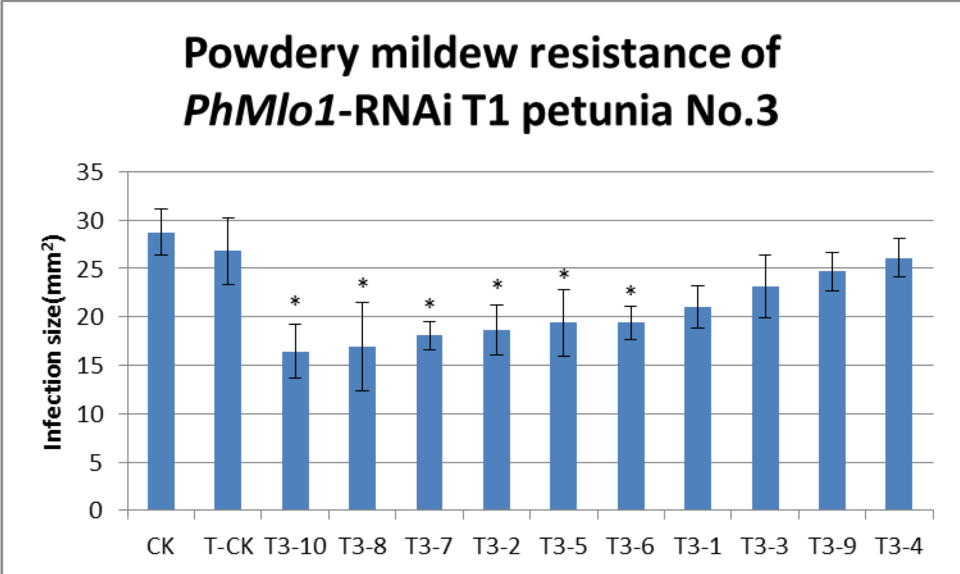


Figure 9. Powdery mildew resistance of 10 Transgenic *PhMlo1*-RNAi T1 No. 3. Bars represent standard errors; * indicates significance at P<0.05 according to Student's t test when compared with wild type control.

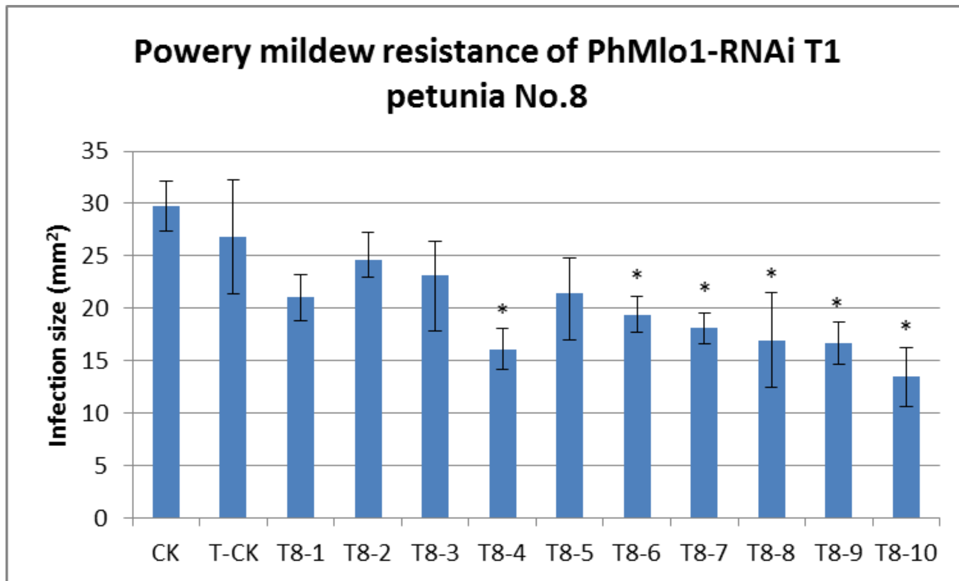


Figure 10. Powdery mildew resistance of 10 Transgenic *PhMlo1*-RNAi T1 No. 8. Bars represent standard errors; * indicates significance at P<0.05 according to Student's t test when compared with wild type control.

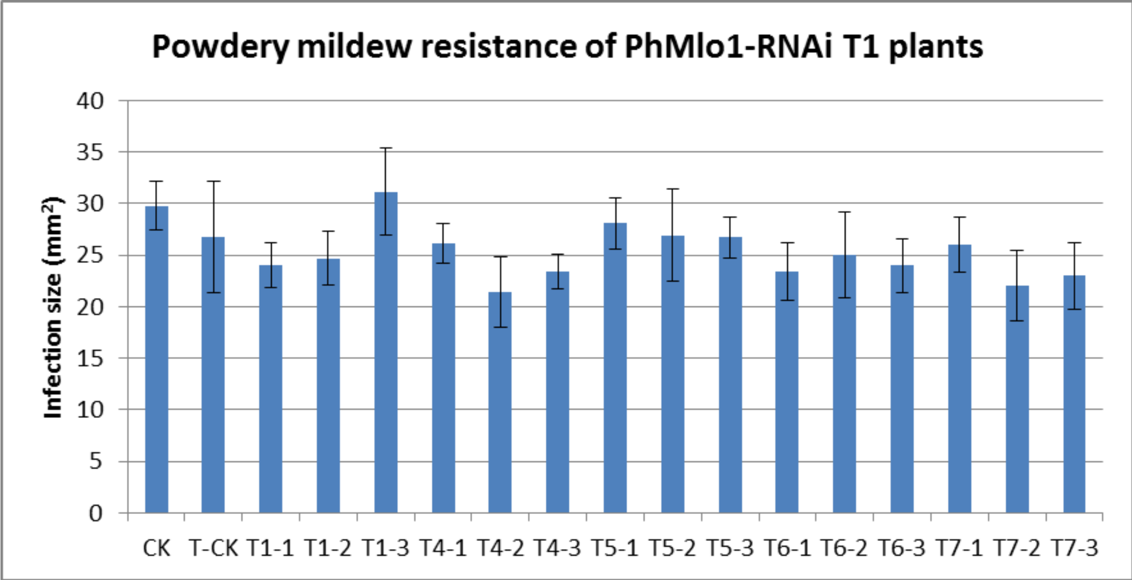


Figure 11. Powdery mildew resistance of transgenic *PhMlo1*-RNAi T1 plants.

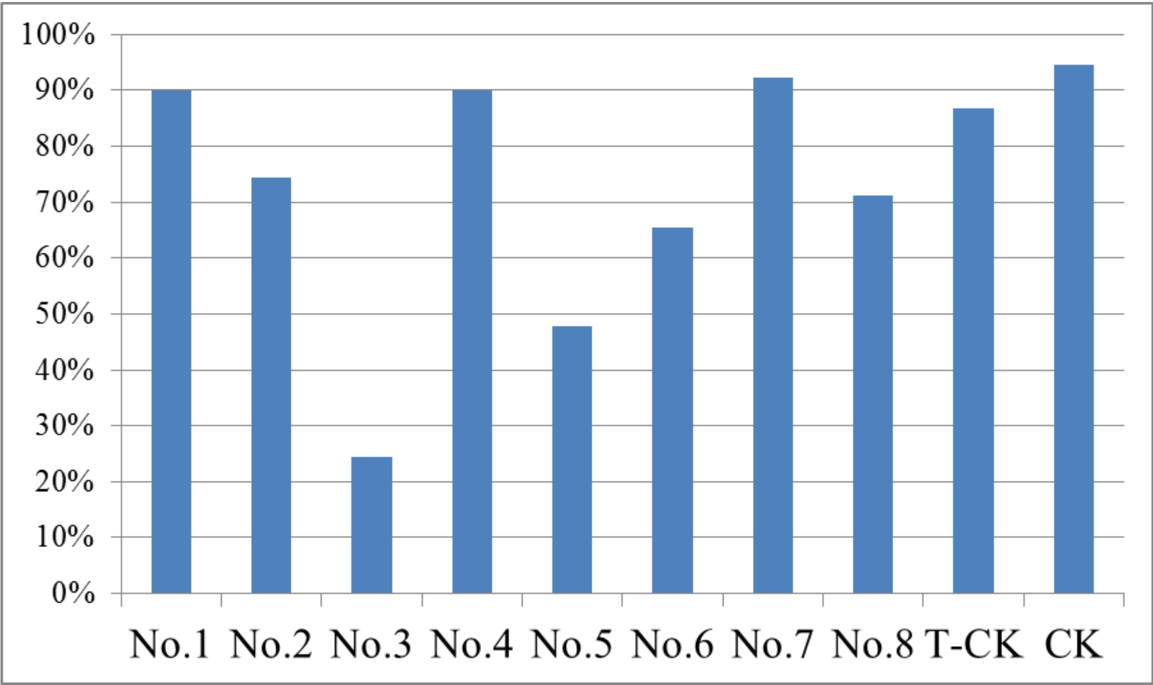
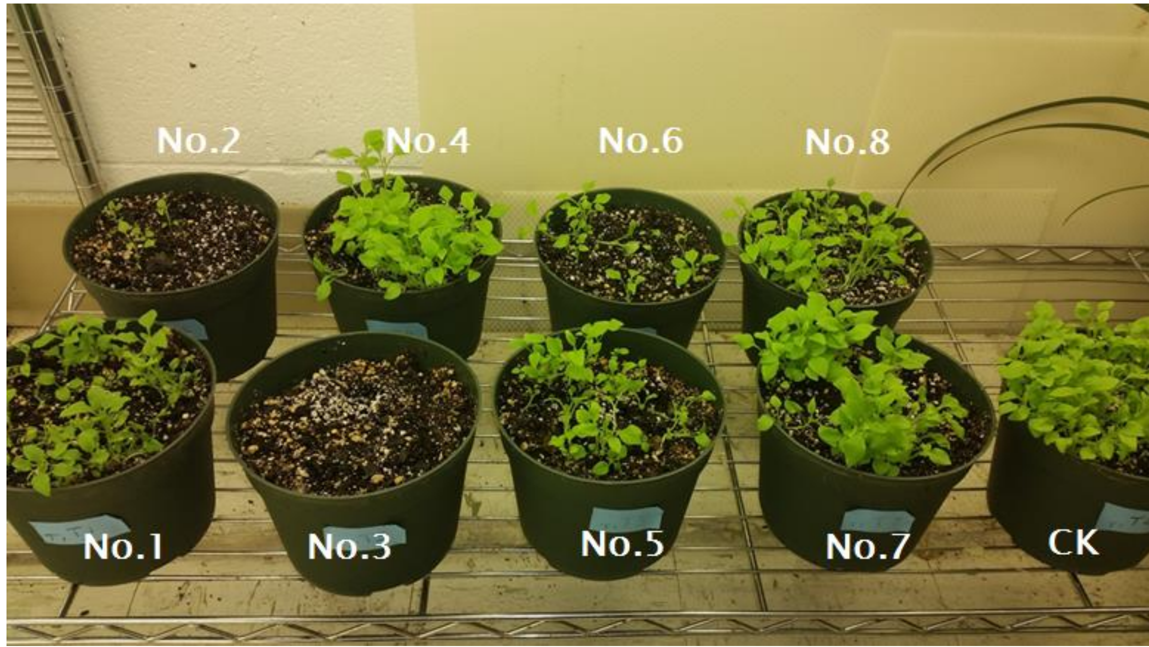


Figure 12. Germination rate of *PhMlo1*-RNAi T1 plants.

A.



B.

No. 1 No. 2 No. 3 No. 8 T-CK CK

Figure 13. Pleiotropic effects of *PhMlo1*-RNAi plants. A. Seed germination and survival of progeny of eight *PhMlo1*-RNAi transgenic lines and CK at twenty days after sowing. B. height were measured for No. 1, No. 2, No. 3, No. 8, T-CK, and CK.

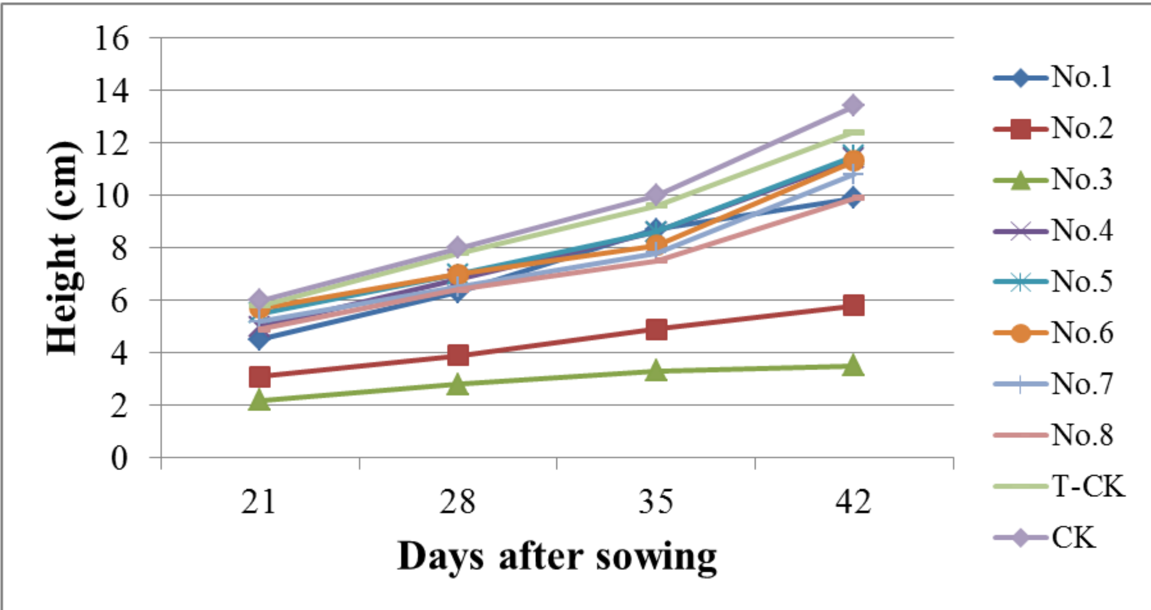


Figure 14. Transgenic lines silenced for *PhMlo1* gene are impaired in their growth.

CHAPTER 5

Conclusions

This research tested the hypothesis that the knockdown of susceptibility genes in *Petunia hybrida* could be used to obtain pathogen resistance. We first isolated petunia orthologs of the susceptibility genes *Mlo1*, *eIF4E*, and *eIF(iso)4E*. In other plant species, these genes are found in small- to medium-size gene families and sequence comparisons demonstrated that the orthologs involved in pathogen susceptibility.

In order to develop induced mutations in petunia susceptibility genes, a large population mutagenized with EMS was generated. A deleterious mutation induced in *PhMlo1* was identified by HRM, but no induced mutations in *PheIF4E* were found. Because the line with the *PhMlo1* mutation did not survive, a second approach involving RNAi knockdown of *PhMlo1* expression was taken. Reduced expression of *PhMlo1* was obtained in some of the petunia lines transformed with a *PhMlo1*-RNAi construct. Reduced *PhMlo1* expression was observed in some, but not all of their progeny.

When challenged with *Podosphaera xanthii,* T0 and T1 lines with reduced *PhMlo1* expression demonstrated reduced infection. However, negative effects on germination and plant growth were also observed in plants with a *PhMlo1* knockdown. In some, but not all, plant species, with *Mlo* mutations, a growth penalty has been observed, but not to the extent that was observed in

petunia. The function of *Mlo* is poorly understood and the petunia RNAi transformants may present an opportunity to understand this gene better. In conclusion, the knockdown of *Mlo* in petunia can confer resistance to powdery mildew, but it also has a negative effect on plant growth.

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