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Reduction of *MLO1* expression in petunia increases resistance to powdery mildew

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ABSTRACT

Cultivars of *Petunia hybrida* have been found to be susceptible to *Podosphaera xanthii* and other powdery mildew species. We examined whether powdery mildew resistance could be obtained through the knockdown of the petunia gene *MLO1* by RNAi. Transformation of petunia with an *MLO* RNAi construct resulted in transgenic plants in which *PhMLO1* expression was significantly reduced, but not completely silenced. The growth of *Podosphaera xanthii* on plants with reduced *PhMLO1* expression was delayed and limited in extent compared to control plants. Powdery mildew resistance was found to be heritable in T_1 progeny. The reduction in *PhMLO1* expression was also associated with lower seed production, reduced germination frequency, and reduced plant height in T_1 progeny. The knockdown of *PhMLO1* increased resistance to powdery mildew in petunia, but pleiotropic effects on plant growth and development were also observed.

1. Introduction

Powdery mildew has been reported to infect petunia cultivars in Europe and the US, most likely during commercial propagation (Kiss et al., 2008; Kiss and Bereczky, 2011). Species of powdery mildew that infect petunia include *Podosphaera xanthii*, *Golovinomyces orontii*, and *Oidium longipes*, and co-infection by these pathogens has been observed. While phytosanitary practices can reduce the incidence of disease, vegetatively-propagated cultivars of petunia would benefit from wide-spectrum resistance to powdery mildew. This research examined the potential of developing petunia cultivars with genetic resistance to powder mildew through the knockdown of a susceptibility gene *MLO* (*mildew resistance locus O*).

Powdery mildew species require the expression of the plant gene *MLO* to penetrate the epidermal cells of the host plant. *MLO* encodes a calmodulin-binding, seven-transmembrane protein that plays a role in plant defense, although its biochemical function has not been established (Acevedo-Garcia et al., 2014). Naturally occurring, loss-of-function mutations in *MLO* have conferred powdery mildew resistance to crop species such as barley (*Hordeum vulgare*; Jørgensen, 1992), tomato (*Solanum lycopersicum*; Bai et al., 2008), and pea (*Pisum sativum*; Humphry et al., 2011).

Resistance to powdery mildew has also been developed by approaches that induce the mutation of *MLO* or reduce its expression. Functional mutations in *MLO* have been induced randomly in barley by exposure of seeds to chemical or physical mutagens, followed by phenotypic analysis for resistance to the powdery mildew species *Blumeria graminis* (Büschges et al., 1997). Ethylnitrosourea (ENU) mutagenesis of pea conferred resistance to *Erysiphe pisi* through the randomly induced mutation of *MLO* (Santo et al., 2013). *MLO* homeologs of hexaploid wheat were inactivated specifically by TALENs (transcription activator-like effector nucleases), resulting in resistance to *B. graminis* (Wang et al., 2014).

The knockdown of *MLO* expression by antisense or virus-induced gene silencing resulted in powdery mildew resistance in pepper (*Capisicum annuum*; Zheng et al., 2013), strawberry (*Fragaria x ananassa*; Jiwan et al., 2013), and rose (*Rosa hybrida*; Qiu et al., 2015). The genomic sequence of petunia *MLO1* was identified (Jiang et al., 2015) based on its close homology to the *MLO* family member responsible for powdery mildew susceptibility in tomato, *SlMLO1* (Bai et al., 2008). We investigated whether the knockdown of *PhMLO1* by RNAi could be a strategy for developing powdery mildew resistance in petunia.

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2. Materials and methods

2.1. Plant and fungal material

Petunia hybrida "Mitchell Diploid", a doubled haploid variety, was grown in pots with Fafard soil 2B in a growth room at 22 °C with a 16 h photoperiod. Plants for transformation were transferred to a greenhouse as seedlings. The powdery mildew species *Podosphaera xanthii* was obtained from Dr. Margery Daughtrey at the Long Island Horticultural Research & Extension Center. *P. xanthii* was maintained on infected petunia plants in a growth chamber at 25 °C with 80% relative humidity.

2.2. Generation of PhMLO1 RNAi transgenic petunia

An RNAi construct was developed from a PCR-amplified sequence of exon 15 of *PhMLO1* (GenBank accession KT833172). Sense and antisense copies of the *PhMLO1* fragment, which flanked a spacer region derived from the GUS gene, were ligated between figwort mosaic virus (FMV) promoter and terminator sequences. This construct was subcloned into the binary vector pHK1001. pHK1001 with and without the RNAi construct were introduced into *Agrobacterium tumefaciens* strain ABI by electroporation. Petunia leaves from 3–6 week-old greenhouse-grown plants were surface-sterilized and transformed with *Agrobacterium* using the method of Jorgensen et al. (1996). Kanamycin-resistant transformants were regenerated, transferred to soil, and then propagated by rooted cuttings. The T_0 plants were manually self-fertilized to generate T_1 progeny.

2.3. Nucleic acid isolation and analysis

To obtain DNA from petunia leaves, tissue was ground in liquid nitrogen and DNA was isolated using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). DNA was quantified with a NanoDrop 8000 spectrophotometer. The presence of the RNAi construct in putatively transformed plants was determined by PCR with specific for the *PhMLO1* sense fragment and the GUS fragment spacer. RNA was isolated from frozen petunia leaf tissue by the CTAB method of Jaakola et al. (2001) and quantified spectrophotometrically. To synthesize cDNA, RNA was treated with RQ1-DNase I (Promega, USA) and reverse-transcribed using M-MLV reverse transcriptase with oligo$(dT)_{15}$ as a primer. Semi-quantitative RT-PCR was conducted with primers for *PhMlo1* (forward: CTTCCACAAAAATGCTGCAGAC; reverse: GGAGGAACCGTGCAATGG) and *EF1α* (forward: CCTGGTCAAATTGGAAACGG; reverse: CAGATCGCCTGTCAATCTTGG), which served as the reference gene (Mallona et al., 2010). PCR was conducted in triplicate for each of two biological repeats. Relative quantification of gene expression was done by the ΔΔCt method (Pfaffl, 2001). Student's *t*-test was used to evaluate the differences between the wild-type control and transgenic plants, with $p < 0.05$ considered statistically significant.

2.4. Powdery mildew assay

Aqueous suspensions of *P. xanthii* 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 suspensions were adjusted to 1×10^5 conidia/ml and 10 μl was applied to each of six leaves per plant. The inoculated plants were maintained in a growth chamber at 25 °C with 80% RH and a 16 h photoperiod. Leaves were inspected each morning from day 4 to day 16 after inoculation. The lesion area was determined after 16 days by the method of Frenkel et al. (2010). Briefly, the colony diameter was measured in two perpendicular directions (a and b) and the area was calculated as an ellipse (πab/4). Each treatment had six replicates and the experiment was conducted twice. Student's *t*-test was used to evaluate the differences between the wild-type control and transgenic plants, with $p < 0.05$ considered statistically significant.

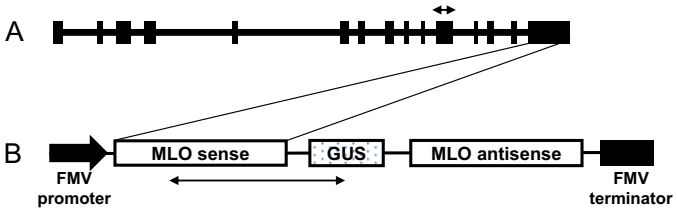

Fig. 1. RNAi construct development. (A) PCR amplified region of exon 15 of *PhMLO1* and (B) ligation of the PCR amplicon in sense and antisense orientation between the figwort mosaic virus (FMV) promoter and terminator sequences. A spacer derived from the *E. coli* β-glucuronidase gene (GUS) separated the *PhMLO1* amplicons. The double arrows in (A) and (B) indicate the regions amplified in the PCR analysis, respectively, of *PhMLO1* expression and the presence of the RNAi construct.

2.5. Analysis of plant growth and development

After self-fertilization of RNAi transgenic plants, transgenic controls, and nontransgenic controls, the average number of seeds per pod was calculated from three pods per plant. The seeds were treated with 100 μM gibberellic acid (GA_3), sown in Fafard soil 2B, and the frequency of germination in a 22 °C growth room (16 h photoperiod) was determined. Three seedlings per line were transplanted individually into 4″ pots, transferred to a greenhouse, and their height was measured weekly between three and six weeks after sowing.

3. Results

3.1. Knockdown of PhMLO expression by RNAi

An RNAi construct was developed based on gene sequence from the last exon of *PhMLO1* (Fig. 1). The construct was introduced into leaf discs of *P. hybrida* 'Mitchell Diploid' and nine independent *PhMLO1*-RNAi transformants were regenerated. *PhMLO1* expression in leaves was examined by semi-quantitative RT-PCR and four transgenic lines (lines 2, 3, 8, 9) were found to have significantly lower transcript levels than untransformed and transformed control lines (Fig. 2). Five transgenic lines (lines 1, 4, 5, 6, 7) were PCR-positive for the *MLO* RNAi transgene, but exhibited no reduction in *PhMLO1* expression. Transgenic line 9, which had the lowest level of *PhMLO1* expression, died before it could be analyzed further.

3.2. Powdery mildew resistance in transgenic petunia

The susceptibility of transgenic petunia lines and controls to the powdery mildew *Podosphaera xanthii* was examined by treating leaves with a 10 μl drop containing approximately 1000 conidia. After 16 days, there was no difference in size between the lesions on the non-transgenic control, the transgenic control, and the transgenic lines without *MLO* silencing (Fig. 3). In contrast, transgenic lines with reduced *PhMLO1* expression (lines 2, 3, 8) had lesions that were significantly smaller. In addition, infection by *P. xanthii* was delayed on transgenic lines 2, 3, and 8 compared to controls (Fig. 4). Powdery mildew was first recorded on control plants 8 days after inoculation, compared to 10 days for line 2, and 12 days for lines 3 and 8.

3.3. Stability of powdery mildew resistance in transgenic progeny

Transgenic petunia lines and controls were self-fertilized in order to examine the stability of RNAi knockdown of *PhMLO1*