The 22-nucleotides siRNA injection in leaves triggers *Endo-gene* (*Eg4*) silencing in *Prunus persica* fruit pulps

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ABSTRACT

The widespread use of gene silencing against specific endo-genes in plants and animals is in practice. Different methods are used for triggering gene suppression in plants and animals. Peach *Eg4* was targeted through small interfering RNA (siRNA) for down-regulation which belongs to the EGase family and encodes *1-4-beta-glucanase* enzyme in fruit mesocarp, responsible for early softening of mesocarp. A 22-nucleotides (22-nts) siRNA was designed and synthesized *in vitro* within the *Eg4* gene with 2-nts overhangs at 3′ and injected into the peach leaves. Two months later total RNA was isolated from leaves and fruit pulps and analyzed through northern blotting where the 22-nts siRNA was detected. The mRNA of *Eg4* was also probed in the extracts and the results were compared with control samples of leaves and fruits. The presence of *Eg4* mRNA was confirmed in untreated samples while the 22-nts siRNA were not detected. The samples isolated from the treated plants lack *Eg4* mRNA and contained the 22-nts siRNAs. The contemporary presence of *Eg4* mRNA and 22-nts siRNAs were not observed which proves that the presence of siRNA was responsible for the triggering of homology-based degradation of the target mRNA. The experiments were repeated the next year and the same results were obtained. It was concluded from the results that siRNA injected in the leaves is amplified through RNA dependent RNA polymerase (RdRp) and spread systemically in the plant tissues, therefore siRNA injection can be used as a sustainable means for endo-gene silencing.

Keywords: dsRNA, gene silencing, *Prunus persica*, siRNA injection.
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INTRODUCTION

There is a general dislike in public against genetically modified organisms (Wunderlich and Gatto, 2015), therefore alternative strategies are needed which are not protein based and also to obtain the desirable traits. Desirable characters in plants can be created through transforming desirable genes into plants or their suppression of target genes which produce an unwanted trait. Different methods are used for gene silencing like RNA interference (RNAi) also called post transcriptional gene silencing (PTGS) in plants (Vaucheret *et al*., 2001, small interfering RNA (siRNA) (Baulcombe, 2004), micro RNA (miRNA) (Bartel, 2004), transcriptional gene silencing (TGS) Vaucheret & Fagard, 2001), and Virus-induced gene silencing (VIGS) (Burch-Smith *et al*., 2004). To achieve sustainable gene silencing, Baulcombe (2007); Dunoyer and Voinnet (2008) have confirmed the amplification and systemically spreading of silencing signals in plant tissues in the form of double stranded RNA (dsRNA). The role of RNA dependent RNA polymerase (RdRp) in dsRNA amplification in plant tissues was studied by Cuperus *et al*., (2010) which shows the

sustainable strategy for targeting Endo-genes. The primary objective of the project was to develop a methodology to achieve the target of gene silencing without cloning and transformation of desirable DNA sequences in a plant through genetic engineering and tissue culture. Dalakouras *et al.,* (2016; 2018) have shown that endo-genes can be suppressed in *Nicotiana* plants through high-pressure spray containing 22-nts dsRNAs. The method of Dalakouras *et al.,* (2016; 2018) was further modified and *in vitro* synthesized siRNAs were injected into the leaves of peach trees for the downregulation of the *Eg4* gene of the EGase family. The gene showed expression after 90 days after full bloom and was responsible for early softening before the onset of the final softening or melting process (Trainotti, *et al.,* 2006).

A dsRNA of 22-nts was designed and synthesized *in vitro* within the sequence of *Eg4* gene from nucleotide number 958 to 981 with 2 nucleotides overhang at both 3/ ends, which efficiently suppress the target mRNA (Hamilton & Baulcombe, 1999; Bernstein *et al.,* 2001; Elbashir *et al.,* 2001; Meister & Tuschl, 2004; McHale *et al.,* 2013). The 22-nts dsRNA was injected into the leaves of peach tree at various points (Dalakouras, *et al.,* 2016). Two months later, after the injection of dsRNA, the RNA was isolated from fruits and leaves of other branches where siRNA was not injected and resolved on the agarose gel. The presence of siRNA was confirmed in the extracts through northern blotting which proves its systemic spread in the plant tissues and its amplification (Manavella *et al.*, 2012; McHale *et al*., 2013). RNA samples were also evaluated for the presence of mRNAs of *the Eg4* gene. The mRNA of *Eg4* gene was not detected in the fruit pulps of plants injected with siRNA which might have been degraded by RNAi machinery activated by siRNAs on homology bases (Eamens *et al.,* 2008). The results were compared with control plants where siRNA was not observed and *Eg4* mRNA was detected in the fruit pulps. The results prove the systemic spreading of mRNAs, their amplification in plant tissues and downregulation of homology-based endo-genes (Dunoyer and Voinnet 2008; McHale *et al*., 2013), therefore it is suggested that siRNA injection can be used as a time and resources saving means for silencing of endo-genes to achieve desirable characters in the existing plants without going in the complexities of gene cloning and development of transgenic plants through a lengthy process of tissue culture. The process of endo-gene silencing through siRNA injection in leaves is a new approach and it is recommended for endo-gene silencing in existing plants, efficient control of viral and bacterial infections in plants.

Materials and methods

SiRNA designing

The sequence of *Eg4* gene mRNA (1863nts) was downloaded from the NCBI site (Trainotti *et al.,* 2006) and a 59% CG rich sequence of 24-nts was selected from 958-981 nts within the *EG4* gene mRNA. The original sequence was selected as a sense strand and its complementary sequence was designed as an antisense strand. Two last nucleotides overhangs at both ends at *3/* ends of the construct were introduced. The overall length of the dsRNA was 24nts while the pairing sequence of dsRNA was 22ntss and the last 2-nts were overhang at *3/* ends (Chen *et al.,* 2010). The siRNAs constructs were designed commercially by Macrogen Korea. To develop working solutions of siRNAs, 1-ml of each sense and antisense solutions were taken and mixed in 100mM potassium acetate (1ml), 30mM HEPES–KOH, pH 7.4 (1ml) and 2mM magnesium acetate (1ml) and diluted to 100ml with deionized distilled water (ddH_2O). The solution was incubated at 85°C for 1 minute and followed by 37°C for 1 hour (Dalakouras *et al.,* 2016)

Selected *Eg4* mRNA sequence: 958- 981nts

Selected RNA sequence: $U^{\cdot}A^{\cdot}\ U^{1}CGGAAAGGGCAGCCGAAAUGC^{22}$

Sense RNA strand: $U^{*}A^{*}\ U^{1}CGGAAAGGGCAGCCGAAAUGC^{22}$
Antisense RNA strand: $A^{1}GCCUUUCCCGUCGGCUUUACG^{22}A^{*}U^{*}$
SiRNA

$U^{*}A^{*}\ U^{1}CGGAAAGGGCAGCCGAAAUGC^{22}$
$A^{1}GCCUUUCCCGUCGGCUUUACG^{22}A^{*}U^{*}$

Probe designing

For radiolabeled probe designing the *in vitro* synthesized 22nts antisense sequence was used and labelled with P^{32} Ambion® KinaseMax™ 5' End-Labeling Kit, Thermofisher Scientific according to the provided protocol.

***Prunus persica* trees selection and siRNA injection**

Mariadeliza variety of *Prunus persica* (L.) Batsch) was selected for experiments. Two plants were selected and tagged as control plants and two plants as the experimental group. The selected plants were six years old and healthy. The upper epidermis of target leaves was carefully punctured with a needle while the lower epidermis was kept intact. The siRNA was taken in a 5ml syringe. The needle was removed from the syringe and the opening of the syringe was kept on the punctured epidermal point of the leaf. The palm of the left hand was kept firmly below the syringe for support and the solution was injected with pressure. The flow of solution was obvious in the tissues visually. The process of injection was carried out at four different points.

RNA analysis

Total RNA was isolated from leaves and fruits through Maxwell@ RSC 48 RNA extraction kit according to the protocol and run on 1.7% agarose gel (figure #1). The resolved gel was used for northern blotting to confirm the presence of siRNA and *Eg4* mRNA sequences (Alwine, *et al.*, 1977) figure# 2. The 5' P^{32} labelled 22ntss antisense RNA with P^{32} Ambion® KinaseMax™ 5' End-Labeling Kit, Thermofisher Scientific was used to probe the *Eg4* mRNA and the 22ntss siRNA. The process of RNA isolation from leaves and fruits was repeated in the following year (2018) to confirm the sustainability of siRNA in the plant tissues. The results have been shown in Figures # 3 and # 4.

Results and Discussion

RNA samples were extracted from leaves and ripened fruits when ready for collection in July. RNA samples were run on agarose gel and analyzed through northern blotting for confirmation of the presence of 22nts siRNAs in the extracts of leaves and fruit pulps isolated from siRNA injected plants only, which proves its amplification and its systemic spreading in plant tissues (Dunoyer and Voinnet 2008). The fruit pulps of siRNA treated trees lack 1863nts *Eg4* mRNA while the extracts of fruits from control plants show the presence of 1863nts *Eg4* mRNA. These results confirmed that the injected siRNAs have entered the cells, amplified and moved cell to cell in the plant tissues systemically and triggered the silencing machinery (RNAi) which has triggered the degradation of *Eg4* mRNA on homology bases (Baulcombe, 2004; Schwab *et al.*, 2006; Baulcombe, 2007; Dunoyer and Voinnet 2008; Eamens *et al*., 2008;Voinnet 2008;). The results prove that the contemporary presence of 22nts siRNA and its homologous mRNA are not coexisting.

The same process of RNA extraction was repeated next year (2018) from already siRNA treated trees in 2017. The results obtained in the following year also proved the presence of siRNAs in the extracts of leaves and fruit pulps from siRNA injected plants. The availability of 1863ntss *Eg4* gene mRNA was detected in the fruit pulps of non-injected or control plants while it was absent in

the fruit pulps of injected tree samples. The results of the following year proved that the siRNAs are sustainable and amplified in plant tissues (Baulcombe, 2007) and the process of silencing remains active even after the trigger has been discontinued (Baulcombe, 2004).

Conclusion

The injection of 22ntss siRNA with 2ntss overhang in the leaves spreads in plant tissues systemically and suppresses the expression of endo-gene *EG4* on homology base. The suppression of genes by siRNA injection was continuous and sustainable. The simple technique used in the study is recommended for achieving resistance in plants against pathogens and suppression of other genes to increase shelf life in peach fruits.

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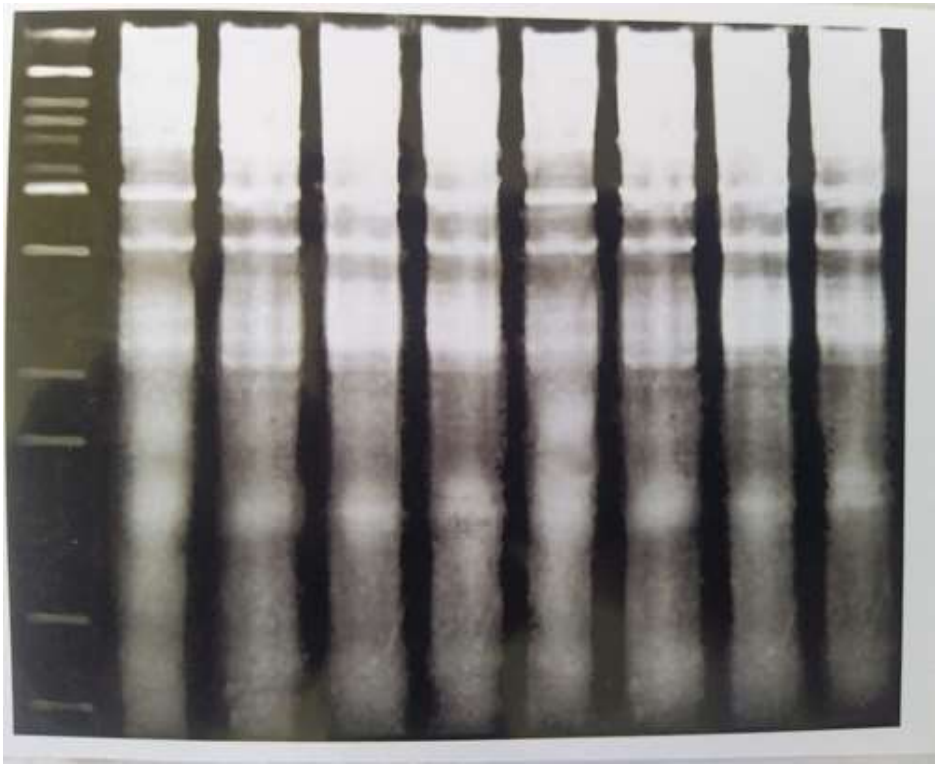

Figure 01: Agarose gel of total genomic RNA extracted from leaf and fruit samples of Mariadeliza peach variety in 2017. Lane 1 in the agarose gel (from the left side) contains the ladder run in parallel to the RNA samples. Lane 2 and 3 contain genomic RNA extracted from the leaves of the Mariadeliza peach variety injected with 22-nts siRNA targeting mRNA of *EG4* gene or Mariadeliza experimental trees 1 and 2 as the experimental group. Lane 4 and 5 contain genomic RNA extracted from the leaves of Mariadeliza variety which are not injected with siRNA or control trees 1 and 2 as a control group. Lane 6 and 7 contain genomic RNA extracted from the fruits of the Mariadeliza variety injected with siRNA or Mariadeliza experimental trees 1 and 2. Lane 8 and 9 contain genomic RNA extracts from the fruit pulps of Mariadeliza control trees 1 and 2. In the RNA samples extracted from control fruits.

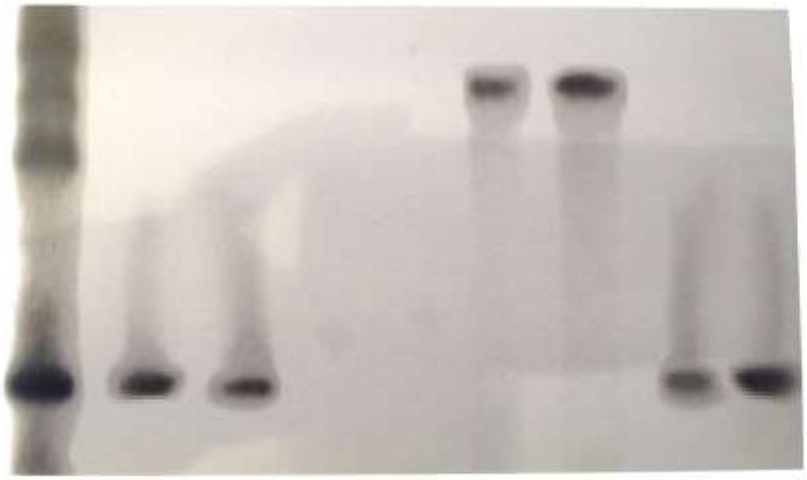

Figure 02: Northern blot analysis of total genomic RNA extracted from leaf and fruit samples of Mariadeliza peach variety in 2017. Lane 1 (from the left side) contains the ladder run in parallel to the RNA samples. Lane 2 and 3 in the blot contain genomic RNA extracted from the leaves of the Mariadeliza peach variety injected with 22-nts siRNA targeting mRNA of *EG4* gene or the Mariadeliza experimental trees 1 and 2 as the experimental group. The hybridization signal of 22nts siRNA is evident in the northern blot. Lane 4 and 5 contain genomic RNA extracted from the leaves of the Mariadeliza variety which are not injected with siRNA or control trees 1 and 2 as a control group where the hybridization signal for 22-nts siRNA is not detected. Lane 6 and 7 contain genomic RNA extracted from the fruits of the Mariadeliza variety not injected with siRNA or the Mariadeliza control trees 1 and 2. In the RNA samples extracted from the control fruits, the hybridization signal of 22-nts siRNA is absent but the hybridization signal for the target mRNA of the *Eg4* mRNA consisting of 1863 nts is detected. Lane 8 and 9 contain genomic RNA extracted from the fruit pulps of Mariadeliza experimental group trees 1 and 2. In the RNA samples extracted from fruit pulps of the experimental group the hybridization signal of 22-nts is detected but the hybridization signal for 1863-nts mRNA is not detected.

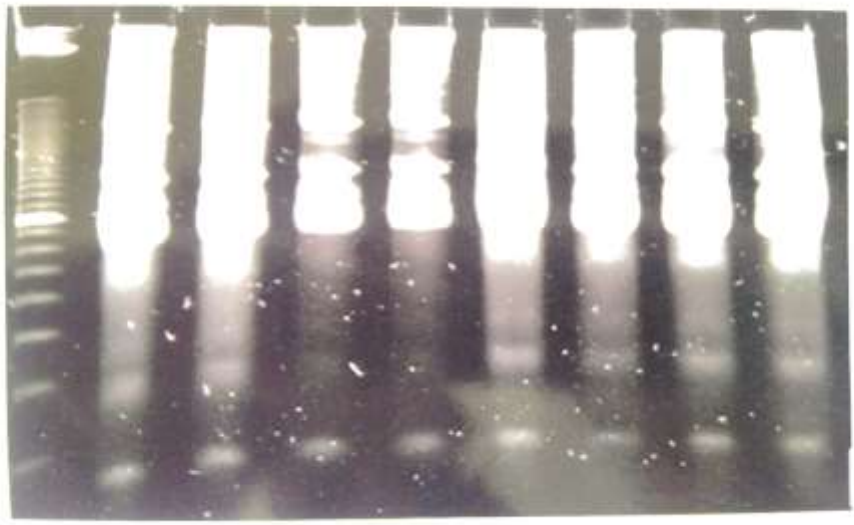

Figure 03: Agarose gel of total genomic RNA extracted from leaf and fruit samples of Mariadeliza peach variety in 2018. Lane 1 in the agarose gel (from left side) contains the ladder run in parallel of the RNA samples. Lane 2 and 3 contain genomic RNA extracted from the leaves of Mariadeliza peach variety injected with 22-nts siRNA targeting mRNA of *EG4* gene or Mariadeliza experimental trees 1 and 2 as experimental group. Lane 4 and 5 contain genomic RNA extracted from the leaves of Mariadeliza variety which are not injected with siRNA or control trees 1 and 2 as a control group. Lane 6 and 7 contain genomic RNA extracted from the fruits of the Mariadeliza variety injected with siRNA or Mariadeliza experimental trees 1 and 2. Lane 8 and 9 contain genomic RNA extracts from the fruit pulps of Mariadeliza control trees 1 and 2.

Figure 03: Agarose gel of total genomic RNA extracted from leaf and fruit samples of Mariadeliza peach variety in 2018. Lane 1 in the agarose gel (from the left side) contains the ladder run in parallel to the RNA samples. Lane 2 and 3 contain genomic RNA extracted from the leaves of the Mariadeliza peach variety injected with 22-nts siRNA targeting mRNA of the *EG4* gene or Mariadeliza experimental trees 1 and 2 as experimental group. Lane 4 and 5 contain genomic RNA extracted from the leaves of the Mariadeliza variety which are not injected with siRNA or control trees 1 and 2 as a control group. Lane 6 and 7 contain genomic RNA extracted from the fruits of the Mariadeliza variety injected with siRNA or Mariadeliza experimental trees 1 and 2. Lane 8 and 9 contain genomic RNA extracts from the fruit pulps of Mariadeliza control trees 1 and 2.

Figure 04: Northern blot analysis of total genomic RNA extracted from leaf and fruit samples of Mariadeliza peach variety in 2018. Lane 1 (from left side) contains the ladder run in parallel to the RNA samples. Lane 2 and 3 contain genomic RNA extracted from the leaves of the Mariadeliza peach variety injected with 22-nts siRNA targeting mRNA of the *EG4* gene or Mariadeliza experimental trees 1 and 2 as the experimental group where the hybridization signal of 22nts siRNA is evident in the northern blot. Lane 4 and 5 contain genomic RNA extracted from the leaves of the Mariadeliza variety which are not injected with siRNA or control trees 1 and 2 as a control group where the hybridization signal for 22-nts siRNA is not detected. Lane 6 and 7 contain genomic RNA extracted from the fruits of the Mariadeliza variety injected with siRNA or Mariadeliza experimental trees 1 and 2 where the Hybridization signal of 22-nts siRNA is confirmed. Lane 8 and 9 contain genomic RNA extracted from the fruit pulps of Mariadeliza control trees 1 and 2. In the RNA samples extracted from control fruits, hybridization signal of 22-nts siRNA is absent but the hybridization signal for the target mRNA of the *Eg4* gene consisting of 1863-nts is detected.