# Effect of Artificial dsRNA on Infection of Pea Plants by *Pea seed-borne mosaic virus*

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**Abstract**

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The effect of direct application of artificial dsRNA molecules derived from the viral coat protein gene and the possibility of RNA interference induction leading to protection against *Pea seed borne mosaic virus* (PSbMV) have been investigated. Effectivity of virus transmission was not afected by the dsRNA treatment. Simultaneous application of dsRNA and virus inoculation as well as dsRNA application one day before PSbMV inoculation led to a significant short-term decrease in the PSbMV concentration in experimental pea plants. Either the curative effect of the artificial dsRNA biolistic application was not noticed.

**Keywords**: *Pisum sativum*; PSbMV; RNA vaccine; qRT-PCR

Epidemics of many plant viruses often have negative impacts on agricultural production, leading to very significant economic losses. Generally, the primary strategy to control the spread of plant diseases is prevention. Host resistance is considered to be the most durable and relatively cheap approach. The traditional method of introducing resistance genes into varieties through classical breeding is time consuming, and in many cases suitable progenitors of resistance are either not available or unknown. With recent progress on GMO (transgenic) plants, it has been shown that plant resistance to viral infection can be created in this manner. The molecular breeding process is faster and more genetically controllable (Prins *et al*. 2008). The success of this approach was first demonstrated in tobacco plants, with an inserted gene encoding the TMV coat protein, which showed resistance (Beachy 1999). In many cases, in resistant transgenic genotypes it was not possible to detect the presence of transgenic protein, e.g. the viral coat protein. Subsequent analyses showed that the induction of resistance is connected with the presence of small interfering RNA (Baulcombe 1996; Smith *et al.* 2000). Currently, the principle of RNA-mediated resistance has been successfully used for the construction of transgenic plants bearing viral genomic segments which serve as inducers of the gene silencing mechanism and/or viral RNA degradation, and inducing resistance against viruses (Chen *et al.* 2004; Niu *et al.* 2006). Although the mechanism of RNA interference is considered to be a plant defence system, it is necessary to bear in mind that this is a complex dynamic system which can be affected by other cellular factors and/or viral suppressors (Smith *et al.* 2000; Waterhouse & Helliwell 2003). Little is currently known about the possibility of activating a specific RNA interference by direct application of artificial dsRNA, and thereby to induce resistance or curing via a non-transgenic approach in plants. The goal of the present study was the preparation of artificial *Pea seed borne mosaic virus* (PSbMV) dsRNA molecules, their use as an RNA-vaccine, and the evaluation of their effect on PSbMV infection of pea plants.

## MATERIAL AND METHODS

**Plant material and virus isolation**. Pea plants (cv. Raman) were cultivated in pots under greenhouse conditions. They were grown in Klasmann #4 substrate in a growth chamber at temperatures of 22/18°C (day/night) under a periodic 16/8 h (day/night) cycle. Experimental pea plants were mechanically inoculated with PSbMV isolate PSB117CZ (Šafářová *et al.* 2008).

**Preparation of synthetic PSbMV dsRNA**. Artificial dsRNA was prepared using the Replicator RNAi Kit (Finnzymes) according to the manufacturer's instructions. For the synthesis of the target molecule, the pGEM-T plasmid carrying the complete gene sequence for coat protein (PSbMV isolate PSB117CZ) was used. The suitable primers (PSB117cp0_F: 5'-TAATACGACTCACTATAGGGAGCAACGCAGAGCCAGTTCG-3', PSB117cp0_R: 5'-GGAAAAAAATGCTGTGTGCCTCTCCGTGT-3') for the synthesis of dsRNA were designed in a conservative area of the coat protein (CP) gene (Šafářová *et al.* 2008) using PrimerSelect software (Lasergene, DNAStar Inc., Madison, USA). Their characteristics were determined by the IDT OligoAnalyzer (ver. 1.3). The concentration of the synthesized dsRNA was determined spectrophotometrically.

**dsRNA application**. The dsRNA was applied using an airgun bombardment device, as described by Predajňa *et al.* (2010). Either 10 µl of dsRNA (2 µg) or 10 µl of $dH_2O$ as a negative control supplemented with 5% carborundum (400 mesh) was applied onto the second upper leaf of the experimental pea plants.

**Experiment I**. Five pea plants (10 days old) for each experimental group were dsRNA treated –1, 0, 1 and 2 days before or post- mechanical inoculation with PSbMV. 8 mm diameter leaf discs were collected from the treated leaf and from one leaf above, one and two weeks after inoculation for virus quantification.

**Experiment II**. Five PSbMV infected pea plants for each experimental group were dsRNA treated 21 days post inoculation (31 days old). Samples (8 m diameter leaf discs) were collected from the treated leaf and from one leaf above at 0, 1, 2, 3, 7, and 10 days after treatment for virus quantification.

In both experiments, triplicate bulk samples were prepared. Experiments were repeated three times, independently. The symptoms were observed at one-week intervals.

**Virus quantification**. Isolation of total RNA from the leaf samples was performed using the NucleoSpin® Plant RNA Kit (Macherey-Nagel, Düren, Germany). For quantitative analysis, all RNA samples were diluted 10×. The newly designed primers PSbMVCIF (5'-AGAAACATGGAGAAATCCGGT-3') and PSBMV6K1R (5'-CCATCTGATTTTGCCACCATTCA-3') for the virus quantification, and reference β-tubulin primers (Die *et al.* 2010) were used as control. A quantitative RT-PCR reaction was performed in a 20 µl reaction volume using the Verso SYBR 1-Step QRT Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. The thermal profile used was: reverse transcription (42°C for 30 min), polymerase activation (95°C for 15 min), followed by 40 amplification cycles (95, 55 and 72°C – each for 20 s). The specificity of amplicons was checked in the subsequent melting analysis (70–96°C). For determination of the relative virus concentration, a comparative analysis between the Ct virus and the β-tubulin genes was carried out using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Data significance analyses were carried out using one-way ANOVA statistical analysis and Tukey-Kramer test, $P = 0.05$ (NCSS 2001, Statistical Solution Ltd.).

## RESULTS AND DISCUSSION

The designed PSB117cp0_F/ R primer pair proved to be suitable for the synthesis of artificial PSbMV dsRNA, yielding molecules of about 500 bp in length. When using 1 µg of template DNA, about 11.1 µg dsRNA (with a concentration of 222 ng/µl) were obtained. This amount of dsRNA obtained was used in virus silencing experiments to study its suitability as an RNA vaccine.

In the first experiment, the influence of dsRNA treatment on effective virus inoculation and replication was studied. Pre- and post-inoculation treatment with artificial dsRNAs did not impact viral transmission. All of the pea plants were successfully PSbMV infected, which was confirmed by quantitative RT-PCR. A significant decrease in the concentration of PSbMV in comparison with the control pea plants treated with $dH_2O$ was noticed only in leaves treated with dsRNA one day before PSbMV inoculation (–1 day post inoculation (DPI)), evaluated after one (33%) and two (8%) weeks (Figure 1). One week after dsRNA application, a significant decrease in PSbMV concentration (55%) was also detected in leaves simultaneously mechanically inoculated and dsRNA treated (0 DPI); however, two weeks after application the viral concentration in these leaves was comparable with the PSbMV-infected plants non-treated with dsRNA. Later, due to viral infection, as well as damage during mechanical inoculation and dsRNA application, the leaves yellowed and dried up; rendering them unsuitable for virus quantification. Thus, it was not possible to evaluate the treated and/ or inoculated leaves longer than for two weeks. The detected virus concentration correlated with the observed intensity of symptoms; plants treated with dsRNA before and on the day of viral inoculation developed bigger leaves after one week and showed a weaker mosaic compared to the control plants. However, after two weeks the symptoms of plant infection became similar, which is typical for PSbMV infection.

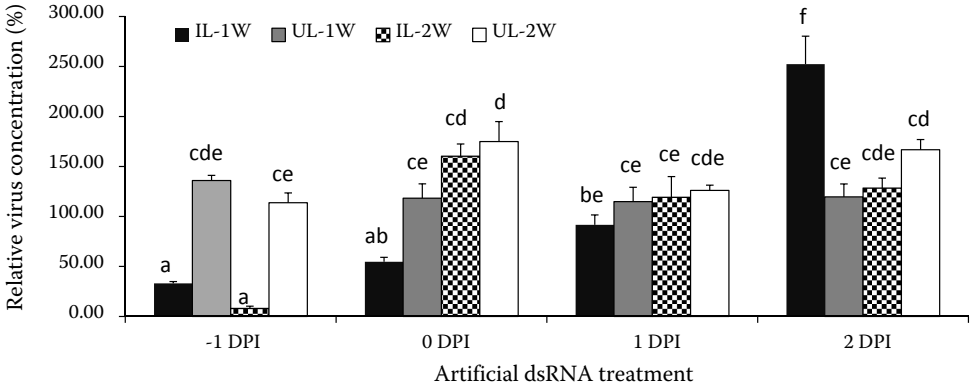


Figure 1. Effect of artificial *Pea seed borne mosaic virus* (PSbMV) dsRNA treatment on PSbMV concentration
DPI – day(s) post inoculation, IL – inoculated leaf, UL – upper non-inoculated leaf, 1W – evaluated one week after inoculation, 2W – evaluated two weeks after inoculation; the bars represent standard deviation; the same letter indicates values not significantly different at $P = 0.05$

Until now, artificial dsRNA molecules were produced in *E. coli*, and the bacterial crude extract was used for the induction of plant resistance against the viruses (Tenlado *et al.* 2003). Even though quite expensive, it seems that the *in-vitro* dsRNA synthesis can produce sufficient amounts of artificial dsRNA molecules for laboratory studies. Here, we have demonstrated that the *in-vitro* synthesized PSbMV dsRNA can affect the infection of pea plants by PSbMV.

In the case of the protective effect of the co- and post-inoculation of *Pepper mild mottle virus* with its homologous dsRNA in *Nicotiana bethamiana*, all of the plants inoculated within 5 days after treatment were resistant (Tenlado *et al.* 2003), an effect which is not often found. Artificial application of different dsRNA molecules induced an obvious resistance only in a portion of the tested plants, e.g. against *Sugarcane mosaic virus* (SCMV) infecting sugarcane (Gan *et al.* 2010) and *Plum pox virus* (PPV) infecting tobacco (Tenlado *et al.* 2003). For the different hairpin molecules derived from *Tobacco mosaic virus* (TMV) and *Potato Y virus* (PVY) genomes, Sun *et al.* (2010) showed that their antiviral activity can vary, and can induce only local resistance at the site of application. The temporal decrease in virus concentrations after pre-, co- and post-application of dsRNA molecules observed in our experiment is consistent with their finding. The treatment with artificial dsRNA can imbalance the system dynamics, leading to a delay in virus spread and reducing the symptom severity (Niehl *et al.* 2012); however, the prolongation of protection is more problematic and should not be expected.

The second experiment was aimed at verifying the curative effect of the biolistic application of artificial dsRNA onto PSbMV infected pea plants. As the treatment was delayed, being applied three weeks after viral inoculation, no significant decrease in the virus concentration in the treated plants was observed, either in treated or in upper (untreated) leaves. The virus concentration varied between 100 to 331% in the treated and control plants (Figure 2). This observation indicates that in the case of systemically

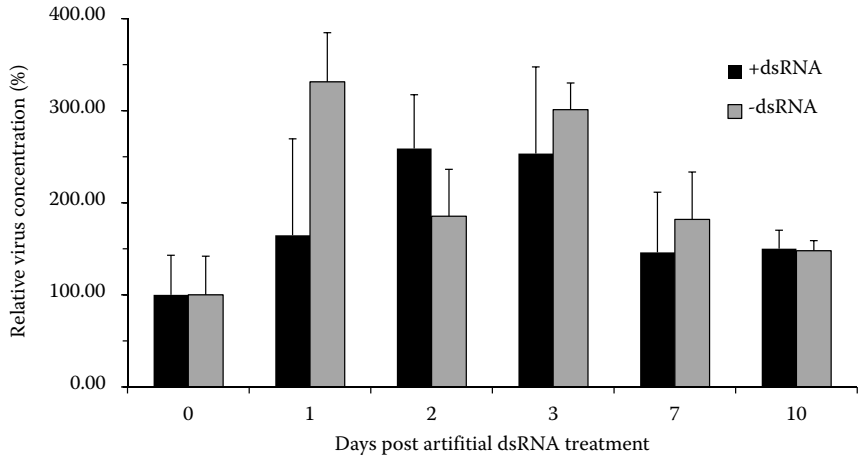


Figure 2. Effect of artificial PSbMV dsRNA treatment on PSbMV concentration in fully PSbMV infected pea plants
+dsRNA – treated leaf, –dsRNA – upper untreated leaf; the bars represent standard deviation

infected plants the local application of dsRNA has not led either to a reduction of virus concentrations in the plants nor to the recovery of the plants from PSbMV infection.

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