PRODUCTION OF HIFH QUALITY MOLECULES FOR ACTIVATION OF PTGS OF THE HOST AGAINST PVY USING BACTERIOPHAGE ϕ6 POLYMERASE COMPLEX

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ABSTRACT

PVY is one of the most damaging plant pathogens listed in the world top-five viruses affecting field-grown vegetables. The developed control strategies do not seem capable to take into account PVY evolution and to suppress risks of new epidemics. The virus is aphid transmitted in nonpersistent manner in which HC-Pro and CP viral proteins act a key role. HC-Pro protein is the viral silencing suppressor of the host gene silencing defense. We produced dsRNA targeting HC-Pro region to counter-attack the viral suppressor proteins and to stop viral replication and propagation trough the plant.

Key words: *PVY, HC-PRO, dsRNA, ϕ6 bacteriophage*

INTRODUCTION

Potato Virus Y is the type species of the *Potyvirus* genus (Brunt, 1992). They have monopartite genomes composed of single-stranded positive-sense RNA, 9,500 to 10,000 nucleotides long (Hollings and Brunt, 1981). RNA of potyviruses is translated into a large precursor polyprotein that is cleaved co- and posttranslationally into mature proteins (Dougherty and Carrington, 1988; Riechmann et al., 1992). The HC-Pro protein is multifunctional; it is involved in aphid transmission of the viral particle and in movement of the virus within the plant, and it is a suppressor of the RNA-dependent gene silencing that recently emerged as a defense mechanism against viruses (Brigneti et al., 1998; Kasschau and Carrington, 1998; Revers et al., 1999).

A conserved PTK domain in the central portion of HC-Pro participate in binding to the DAG motif of the N-terminal region of CP, while a KITC domain at the N-terminal region of HC-Pro is involved in binding to unknown structures of the aphid stylet (Fig. 1)(Mahy et al., 2008).

To counter-attack the viral suppressor protein HC-Pro of PVY we use its sequence to design siRNAs.

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 nucleotides in length, that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. In addition to their role in the RNAi pathway, siRNAs also act in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome; the complexity of these pathways is only now being elucidated. siRNAs were first discovered by David Baulcombe's group at the Sainsbury Laboratory in Norwich, England, as part of post-transcriptional gene silencing (PTGS) in plants (Hamilton et al., 1999). Shortly thereafter, in 2001, synthetic siRNAs were shown to be able to induce RNAi in mammalian cells by Thomas Tuschl, and colleagues (Elbashir et al., 2001). This discovery led to a surge in interest in harnessing RNAi for biomedical research and drug development.

MATERIALS AND METHODS

In vitro dsRNA production system

The dsRNA is synthesized by combined in vitro transcription and replication from a DNA template (According to Replicator RNAi Kit instructions, Finnzymes, Finland). The DNA template for the dsRNA synthesis is produced by PCR using Phusion High-Fidelity DNA polimerase. The PCR primers are designed so that the resulting PCR product contains the target sequence (HC-Pro of PVY) flanked by T7 promoter sequence at the 5' end and ϕ6 qRdRP promoter sequence at the 3'

end. The PCR product is purified and transcribed into single-stranded RNA (ssRNA) by T7 RNA polymerase. This ssRNA is replicated into double stranded RNA (dsRNA) by ϕ6 qRdRP in the same incubation step. (Fig. 2) Primers: HC-Pro dsRNA 1 (5'-TAA TAC GAC TCA CTA TAG GG TAG GAT TCT GTC GAA TGC CGA CAA TTT T -3'),

HC-Pro dsRNA 2 (5'-GGA AAA AAA TAC TGC AGA CCA ACT CTA TAA TGT TT -3').

***In vivo* dsRNA production system**

The system utilizes the T7 RNA polymerase and the polymerase complex of bacteriophage ϕ6 (Fig. 3) to produce high amounts of target-specific dsRNA. The *in vivo* system is set up in *Pseudomonas syringae* cells that constitutively express the T7 RNA polymerase (from plasmid pLM1086). Plasmid pLM991 carries a cDNA copy of the ϕ6 Lkan – segment, which contains all the components required to form the polymerase complex (PC), as well as a kanamycin resistance gene. The other plasmid contains the required ϕ6 S-segment 5'packaging (*) and 3'-replication (†) signals, between which the gene of interest (HC-Pro). Upon transformation into the host cells, the T7 RNA polymerase transiently synthesizes ssRNA from the cDNA plasmids (Fig. 4) (Aalto et al, 2007). The (+)ssRNA of the Lkan – segment directs the translation of viral proteins, which yield empty PCs. Packaging begins with the S-segment specific ssRNA, followed by L-segment specific ssRNA. Upon packaging, an exact complementary strand is synthesized inside the PC particle by the viral RdRP.

RESULTS AND DISCUSSION

We produce dsRNA targeting HC-Pro region to counter-attack the viral suppressor proteins and to stop viral replication and propagation trough the plant.

In vitro dsRNA production method combines the use of T7 RNA polymerase to synthesize ssRNA templates of PVY cDNA. ϕ6 RdRP performs de novo initiation and produces full-length dsRNAs from given template ssRNAs.

In vivo system ϕ6 -specific ssRNA and procapsid proteins are produced simultaneously *in vivo* by transfection with ColEI-based suicide plasmids containing cDNA copies of viral genome segments under control of the T7 promoter. The system is stable at the RNA level. Selection of stable carrier state cells is achieved by inserting a kanamycin resistance (kan) gene in the 39 untranslated region of the L segment. Plasmids containing a T7 promoter and any heterologous cDNA, flanked by the viral packaging and replication signals, are transcribed and packaged into viral PC particles, followed by synthesis of the complementary RNA strand (Aalto et al, 2007).

Medium and large quantities of dsRNA of a given sequence may be generated by *in vitro* or *in vivo* recombinant f6 RdRP-based dsRNA production systems. The *in vitro* system offers an efficient and flexible method to produce high-quality dsRNA from a ssRNA molecule of practically unlimited length such as Potato virus Y HC-Pro gene, around 1445 nt, 456 AA (Fig. 5).

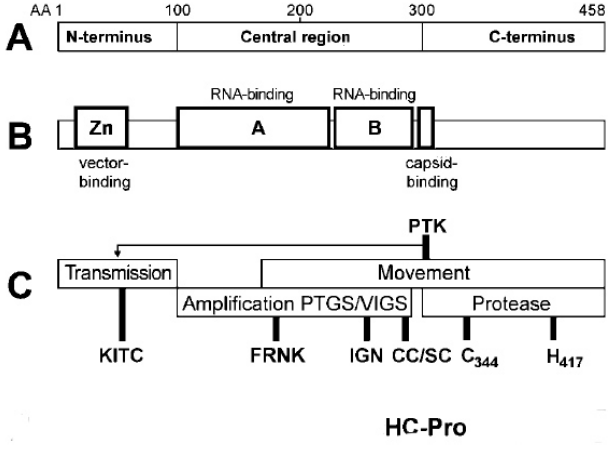

Fig.1 HC-Pro region of PVY (Plisson et al, 2003)

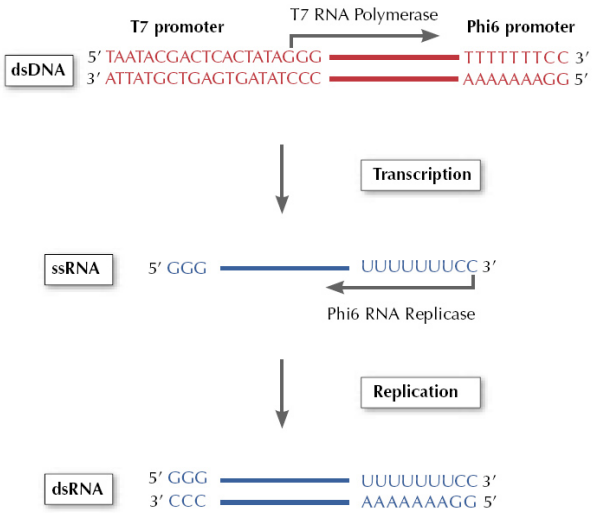

Fig.2 *In vitro* dsRNA production system (Finnzymes, Finland)

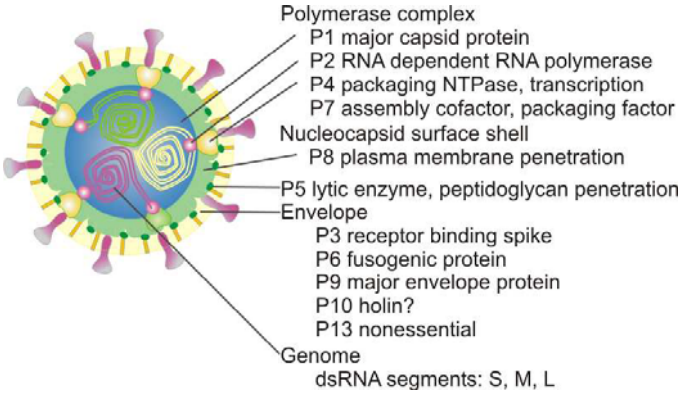

Fig. 3 Structure of bacteriophage ϕ6 (Poranen et al, 2001)

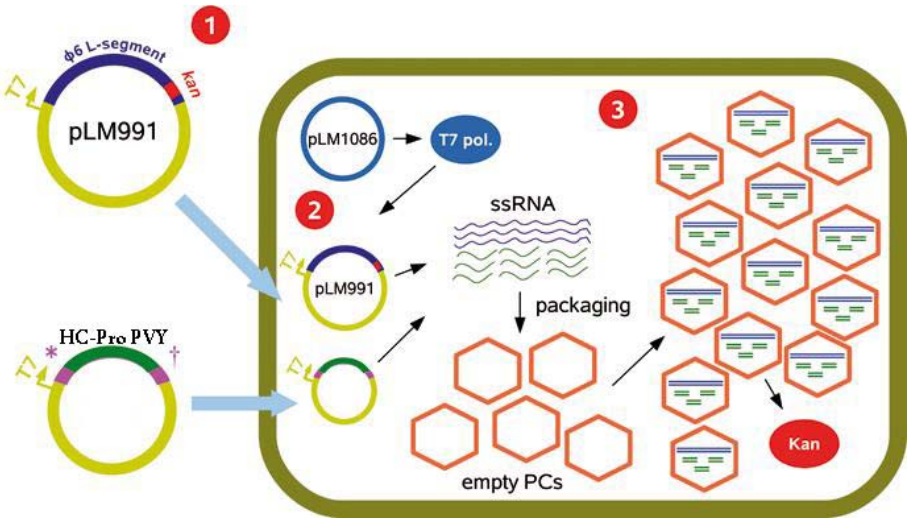

Fig. 4 *In vivo* dsRNA production system (Aalto et al., 2007)

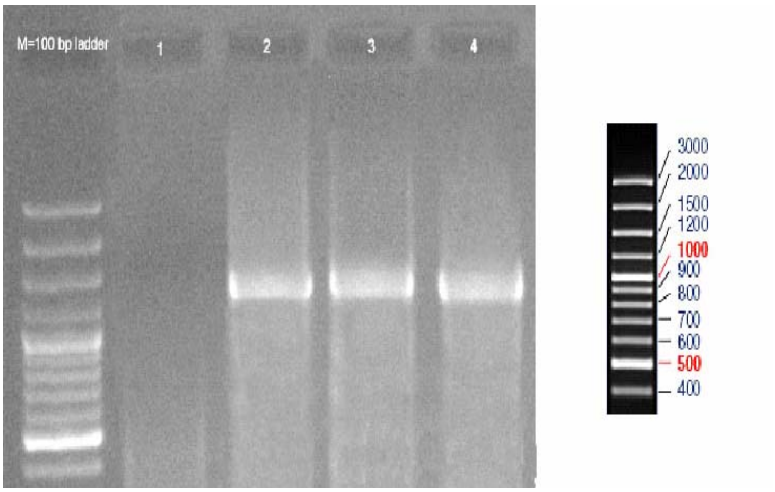

Fig. 5 dsRNA of HC-Pro gene region of PVY (1445 bp)

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