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Satellite Tobacco Necrosis Virus: A New Vector in Plant Genetic Engineering

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Abstract

Cloned full-length copies of genomes of RNA-viruses, when introduced into a permissive host cell (bacterium, animal cell), can lead to a productive infection presumably initiated by a single complete transcript. Satellite Tobacco Necrosis Virus (STNV) requires coinfection by a helper virus such as TNV for replication; it only codes for one protein, the structural coat protein. Introduction of a cloned full-length copy of STNV present on a plasmid into a plant cell and coinfection with TNV leads to productive infection as shown both at the RNA level (Northern) and at the protein level (Western). Therefore, it now becomes possible to manipulate the genetic information of a plant RNA virus. Synthetic DNA linkers were introduced at various positions of the STNV genome and the effect of these insertion mutations on the viral progeny could be established. Insertions which affect the reading frame of the coat protein block not only viral protein synthesis but also viral replication. Restoration of the reading frame may allow to recover viability, depending on the position of the inserted amino acids in the viral three-dimensional structure of the coat protein. Some linker insertions in the region following the coat gene are also detrimental to viability, while other insertions in the 3'-untranslated region are stably transmitted to the progeny. This system for the genetic manipulation of an RNA virus offers many possibilities, not only to study the functional role of various regions of the genome, but perhaps also for expression of heterologous genes in plant cells.

Keywords: heterologous gene expression, insertion mutants, cloned RNA viruses

Abbreviations: BMV Brome Mosaic Virus; STNV Satellite Tobacco Necrosis Virus; TMV Tobacco Mosaic Virus; TNV Tobacco Necrosis Virus

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Upon infection of a cell by a virus, the latter imposes its genetic program on the host. It can do so because the viral genome is either replicated more frequently, or translated more efficiently, or both. The net result is usually an enormous synthesis in the host cell of "viral" genetic information. From the early days of genetic engineering, scientists have tried to make use of this property. For example, DNA bacteriophages have played a very important role in the early development of genetic engineering, e.g. bacteriophage lambda and the single-stranded phages M13 and Fd. Also the double-stranded DNA-containing Cauliflower Mosaic Virus has been extensively studied with the aim of using the system for introducing a foreign replicon in the plant host cell (cf. paper by Lebeurier in this volume). But the very large majority of plant viruses have an RNA molecule as their genome. RNA viruses can also be extremely efficiently replicated and translated, for example, cells infected by TMV may become filled up with the viral progeny.

But how can we genetically engineer RNA viruses? Obviously, it is very difficult to make structural modifications directly at the RNA level, as there are no enzymes known with the same type of specificity as restriction enzymes, and as the chemical and enzymatic fragility of large RNA molecules represents a real barrier. The alternative, however, is to clone the viral information by making a cDNA copy which is then integrated in a bacterial plasmid and in this form further propagated. In this way, full-length copies of bacteriophage Qβ RNA (Taniguchi et al. 1978) and MS_2 RNA (Devos et al., 1978) were obtained. How can we obtain a copy of a viral RNA? Some plant viral RNA genomes contain at their 3'-end a poly-A sequence, and copying by reverse transcriptase can then be primed by oligo-dT as is done for mRNA cloning. Most viral RNA genomes, however, do not end in a poly-A tail, but the poly-A sequence can be added *in vitro* by using a polyadenylation enzyme from *E. coli* (Devos et al., 1976). Another possibility is to determine the sequence at the 3'-end of the viral RNA and then use a chemically synthesized complementary oligonucleotide.

Using one of these techniques, a number of plant viral RNA genomes have now been cloned. This has provided important data on the organization of the viral genetic information and the structure of the virus-coded proteins. However, can they be used for genetic engineering purposes? This requires that the viral genetic information can also be expressed in an appropriate host cell. It was first observed by Weissmann et al. (1978) that cloned bacteriophage Qβ cDNA was infectious. Presumably, occasional transcripts in the bacterial cell which extended over the total viral RNA genome and beyond were trimmed to the correct size and then started the replication cycle. Also cloned poliomyelitis genetic information was expressed in mammalian cells (Racaniello and Baltimore, 1981). A number of systems derived from plant RNA viruses have recently been developed from expression purposes. The first is the viroid system; in this case the genome is a circular RNA of about 316 nucleotides. It was shown both by Tabler and Sänger (1984) and by Visvader, Forster and Symons (1985) that cloned viroid cDNA was infectious, provided either that there was a tandem repeat of more than one copy, or that the viroid cDNA information was flanked by a terminal repetition of at least 11 nucleotides. The infection was obtained either by introducing the bacterial plasmid directly into the plant cells or else by *in vitro* transcription and transfer of the RNA transcripts into plant cells. But viroids are not known to code for protein and therefore the system cannot be used for studying heterologous gene expression. A second system was very recently reported by French et al. (1986) and involves expression in plant cells of cloned genetic information of Brome Mosaic Virus. This virus has a fragmented genome and three components are essential for biological activity. All three viral RNA-molecules have been cloned and replicated in plasmid form. Using a system which was developed in our laboratory (Contreras et al., 1982) *in vitro* transcripts could be obtained which started with a cap structure. Introduction of the capped RNA transcripts corresponding to the viral fragments 1, 2 and 3 into plant cell protoplasts resulted in biological activity. Moreover, the BMV coat gene could be replaced by the chloramphenicol acetyl transferase gene. This experiment proved that a foreign gene could be made in the plant by genetically engineered RNA virus information. However, it should be noted that infection was only possible with protoplasts and so far there is no evidence that transfer from cell to cell of the modified viral RNA-molecules is possible.

We have developed another system, based on cloned full-length Satellite Tobacco Necrosis Virus (STNV) cDNA (Van Emmelo et al., 1980). STNV is strictly dependent for its replication upon co-infection with Tobacco Necrosis virus (TNV). Very often the necrotic lesions caused by TNV are diminished by STNV. In the presence of an appropriate helper, STNV can be replicated and expressed in many plant species, both dicots and monocots. STNV is a very small virus with a genome of only 1229 nucleotides, the entire sequence of which is known (Ysebaert et al., 1980). It codes only for its coat protein and this coat protein gene is located in the first half of the genome, the second half is untranslated. We had originally chosen to work with STNV because

it is one of the smallest RNA viruses known and, moreover, much progress has been made in establishing the three-dimensional structure (Liljas et al., 1982; Jones and Liljas, 1984). Unlike typical spherical plant viruses such as Tomato Bushy Stunt Virus or Southern Bean Mosaic Virus which have a triangulation number of 3 (Harrison, 1980), STNV has a triangulation number of 1 and hence the viral shell is composed of only 60 coat protein monomers. The particle is about 170Å in diameter and the three-dimensional structure is now known at a resolution of 2.5Å (Jones and Liljas, 1984). All 60 subunits are equivalent and each subunit has three calcium ions bound. The coat protein subunit contains two β-sheets, each with 4 antiparallel strands. The coat protein polypeptide is 195 residues in length; the N-termini form bundles of three α-helices extending into the RNA region at the threefold axis.

STNV RNA has the remarkable property of being very well expressed not only in prokaryotic systems but also in eukaryotic systems (although this RNA-molecule does not contain a cap structure!). We have shown that when the viral cDNA information in *E. coli* is placed under control of an inducible promoter, synthesis of STNV coat protein can occur, although the viral monomers do not assemble into virus-like particles (Van Emmelo et al., 1984).

How can we obtain expression of the cloned genetic information in plant cells? The first system we developed was based on the *Salmonella* phage SP6 promoter followed by the complete STNV cDNA information. Using an *in vitro* transcription system it was possible to obtain macromolecular RNA transcripts (much longer than viral unit length). Cowpea plants were infected with TNV together with these *in vitro* transcripts and productive infection was indeed observed.

But a more efficient system was found based on direct transfer of plasmid DNA (together with TNV) into plant cells. This was sufficient to initiate an infection. The test system used to follow these events was to isolate RNA from the leaves, and after separation of the RNA by gel electrophoresis to reveal the STNV specific progeny RNA and replicative RF molecules by hybridization with STNV-specific probes. It turned out that the viral information was exactly excised or copied and the progeny viral RNA was the exact length of the wild type viral genome. The cDNA clone was flanked by two inverted GC segments and we could show that these two segments were indeed essential. Removal or shortening of one resulted in loss of activity. On the other hand bacterial promoters present on the plasmid played no role.

Once we had a system in hand which allowed us to express cloned STNV cDNA information, we could start to manipulate the latter and in this way study the structure-function relationship of the viral genome. We introduced a 14-nucleotide-long synthetic DNA fragment in a number of positions of the cDNA. This linker contained the recognition sites for the restriction enzymes Nco I and Eco RI. Using these synthetic linkers as probes we could show that the plasmids used for infection of plants gave rise to progeny viral RNA, which now contained the replica of the inserted linker in the RNA genome. Many such mutants were analyzed and characterized. Mutants in the coat protein which interrupt the reading frame do not allow the further synthesis of single-stranded progeny RNA but replication still goes on. Possibly a functional coat protein is required for asymmetric synthesis of positive strands. A region immediately following the coat protein on the genome seems to be particularly sensitive as insertion mutants here result in poor viability. But most mutations in the 3'-untranslated sequence of the STNV-RNA do not interfere with the infectivity. Obviously, having now available a large set of such mutants with introduced restriction sites it is possible, by making appropriate combinations, to check also the viability of various deletions. Some deletion mutants, especially when the deletion is rather small, are still viable, and also some longer molecules with partial duplications have been constructed and shown to be viable.

In the next phase we have restored the reading frame of the mutants in the coat protein gene by an additional insertion of 4 nucleotides. Theoretically, these mutants should now code for a coat protein with 6 additional amino acids. Out of 3 such constructs, 2 were viable and 1 was not viable. It was of interest to correlate this result with the three-dimensional structure as established by Strandberg and his colleagues (Jones and Liljas, 1984). It turned out that at positions 162 and 198 (nucleotide number) the additional amino acids were inserted in a region exposed on the outside of the virus particle, while insertion at position 434 would disrupt an internal folding region and this presumably is not viable. Obviously, it will be of interest to determine how these molecular warts on the particle influence the exact 3D-folding of the virus.

Conclusions

We have developed a method to transfect productively plants with cloned viral cDNA. We have shown that this virus cDNA can be genetically engineered and mutations can be introduced both in the coding part and in the non-coding part. We have also shown that this mutant phenotype can be stably transferred to progeny. The system as developed now could already be applied, e.g., for construction of functional but avirulent deletion mutants which would interfere with replication of more pathogenic forms. In the future one may want to use the system for heterologous gene expression in plant cells. Obviously, many problems have still to be overcome, but potentially the cloned viral RNA genomes offer intriguing perspectives. Obviously if the purpose is to obtain synthesis of a heterologous protein in the plant, it is necessary that the latter becomes systemically infected. This may be possible, e.g., by introducing a copy of the TNV-replicase gene under control of the Agrobacterium system into the plant genome. If the TNV replicase is then induced it would lead to a low level of enzyme which would in turn switch on replication of a non-pathogenic satellite RNA which codes for a protein of interest. This then would result in an amplification of the signal and may provide a very efficient approach for heterologous gene expression in plant cells.

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