Functional analysis of the cellular RNA-directed RNA polymerase (RdRP) in higher plants

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Dedicated to
my paternal and maternal
grandparents

"*Everything soft and tender reminds me of you*"

Although I never saw my grandparents, I feel their presence would have made a difference to my life...

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TABLE OF CONTENTS

I Introduction

"Golden age" in biology is the period when persuasive discoveries demonstrated that deoxyribonucleic acid (DNA) was the genetic material. The first evidence came from the work of Avery, MacLeod and McCarty (Pneumococcus transformation) and subsequently from Hershey's experiment [infection of *Escherichia coli* (*E. coli*) with T2 phage]. The most remarkable event was the discovery by Watson and Crick (1953) on the structure and replication of DNA. These discoveries and other important ones that followed led to the realisation that DNA has two fundamental functions: 1) serve as a template for its own replication, 2) to carry the genetic information that brings about the specific phenotype of the cell. DNA is transcribed into ribonucleic acid (RNA), and the RNA is then translated into amino acids to produce proteins. However, it was not for long that DNA was exclusively entitled as genetic material. Experiments with Tobacco Mosaic Virus (TMV) which has RNA as genome demonstrated that RNA is equally competent for the title. It was difficult to reconcile with the notion that RNA also has properties of genetic material, but elaborate studies on retroviruses enlightened the vision. Since then several studies were made to understand the structure and composition of genetic materials and one of them was the discovery of polymerases.

Discovery of the first DNA-directed enzyme in *E. coli* by Kornberg (1956) led to a better understanding of DNA replication. This enzyme was designated as DNA polymerase I (pol I), and was shown to possess three basic and important properties: 5'→3' polymerase activity, 3'→5' exonuclease activity, and 5'→3' exonuclease activity. Subsequently, two additional polymerases were discovered in *E. coli*, pol II and pol III, both of which lacked 5'→3' exonuclease activity. This marked the beginning of identification of additional polymerases. The eukaryotic polymerases exhibited fascinating variations on the basic themes of structure and function characteristics of prokaryotic polymerases. Eukaryotes were found to have at least four nuclear DNA polymerases: α, β, δ, and ε. Polymerase α and δ are involved in DNA replication while polymerase ε and β are involved in DNA repair. An additional polymerase (γ) is located in mitochondria (mt) and is presumably responsible for mtDNA replication. Polymerase α is analogous to *E. coli* pol I, ε to pol II, and δ to pol III. Beside these, the RNA dependent DNA polymerases that are directed by single-stranded RNA, perform transcription in reverse. These enzymes were termed

reverse transcriptase and were first found in RNA tumour viruses (retroviruses), where they convert RNA genomes into duplex DNA.

Discovery of RNA polymerases led to a better understanding of the process of RNA synthesis from DNA. It was found that this process occurs in three steps: initiation, elongation and termination. Interestingly, RNA polymerases were found to exist in two active forms in *E. coli*. The core enzyme is composed of α, β and β′ subunits. Association of an additional subunit σ with the core enzyme results in transition into the holoenzyme state, which is involved in initiation of transcription. In contrast to prokaryotes, eukaryotes have three nuclear RNA polymerases, specialised for transcription of different classes of genes. Based on their functions, they have been categorised in three classes: class I (or A) synthesises ribosomal RNA (rRNA) and is located in nucleolus; class II (or B), pre-mature messenger RNA (mRNA) (mRNA with introns); and class III (or C), transfer RNA (tRNAs) and 5S RNA. Class II and III are located in the nucleoplasm.

Over the period of time, research related to RNA viruses also progressed with the aim to understand how RNA replicates and how RNA transfers genetic information. RNA viruses are categorised into three major classes, differentiated by whether infectious virion particles contain the genome as dsRNA, positive-strand (messenger-sense) RNA, or negative-strand RNA. It was found that they replicate their RNA genome without DNA intermediates. Instead of using DNA they copy RNA from RNA. Copy of RNA from RNA templates was performed by a class of virus-encoded enzymes called RNA-dependent RNA polymerases (RdRps). This process usually takes place in concert with other viral and/or host factors. RdRps are found in all three classes of RNA viruses and they share multiple sequence motifs that are conserved. Additionally, they were found to exhibit similarity to DNA-dependent RNA and DNA polymerases and to reverse transcriptases (reviewed by Ahlquist, 2002).

Surprisingly, it was found that not only in viruses but also in higher biological systems copy of RNA from RNA exists. Interest on the enzymes that carry this process expanded with the discovery of the cellular RNA-directed RNA polymerase (RdRP) in plants. However, this enzyme class does not share obvious similarities with virus-encoded RdRps except the fact that both polymerase families synthesise complementary RNA from RNA template.

I.1 Recognition of RdRPs

RdRP activities have been detected in a number of plant species (Astier-Manifacier and Cornuet, 1971; Duda, *et al*., 1973; Astier-Manifacier and Cornuet, 1978; Duda, 1979; Boege and Sänger, 1980; Dorssers *et al.*, 1982; Takanami and Fraenkal-Conrat, 1982; Khan *et al*., 1986) but, so far, only the RdRP from tomato leaf tissue had been purified and its corresponding gene cloned (Schiebel *et al*., 1993a, b and 1998). The purified tomato RdRP *in vitro* catalysed transcription of single-stranded RNA and DNA molecules into short complementary RNAs (cRNAs) (Schiebel *et al*., 1993b). The tomato enzyme was found to be inducible upon viroid infection (Schiebel *et al*., 1998). In addition, Xie and co-workers (2001) found increased virus accumulation in RdRP-deficient tobacco plants. These results strongly suggested that RdRP activity plays an important role in a plant defence mechanism against viruses either restricting virus proliferation or virus spreading. Although similar RdRP activities have not been reported for organisms others than plants, sequences homologous to plant RdRPs have been found in further eukaryotes as for example in *Dictyostelium discoideum* (Martens *et al*., 2002), *Neurospora crassa* (Cogoni *et al*., 1999), and *Caenorhabditis elegans* (Smardon *et al*., 2000; Sijen *et al*., 2001).

Studies on plants carrying a transgene encoding the Tobacco Etch Virus coat protein showed loss of transgene and viral mRNA upon infection with Tobacco Etch Virus. Hence, resulting in induction of a highly specific antiviral state. This observation led to the hypothesis that the RdRP would copy a small segment(s) of RNA. These small RNAs would then hybridise with the target RNA, rendering the RNA non-functional, and RNases would target the partially double-stranded RNA or viral RNA complex for degradation. Hence, RdRP could play an essential role in posttranscriptional gene silencing (PTGS) (Lindbo *et al*., 1993). Support for this hypothesis came from characterisation of *Arabidopsis thaliana* knock-out mutants that were impaired in PTGS. In parallel, two independent groups reported that one of their PTGS-deficient mutants (sde1 and sgs2) carried an inactivated RdRP gene (Dalmay *et al*., 2000b; Mourrain *et al*., 2000). Because transgene expression was shown to be affected also in virus-free plants, the SDE1/SGS2 was not assumed to play a major role in virus defence but would be a key enzyme of PTGS. This posed the possibility that the tomato RdRP differed from the *A. thaliana* SDE1/SGS2. Despite the protein similarity between the tomato RdRP and the SDE1/SGS2 (Dalmay *et al*., 2000b, and this work), certain members of a RdRP gene family may exist. Thus, it was important to examine how many different RdRPs are expressed in plants.

Moreover, it would be interesting to find out whether these RdRPs have different biological functions and whether they also have a function in normal gene regulation.

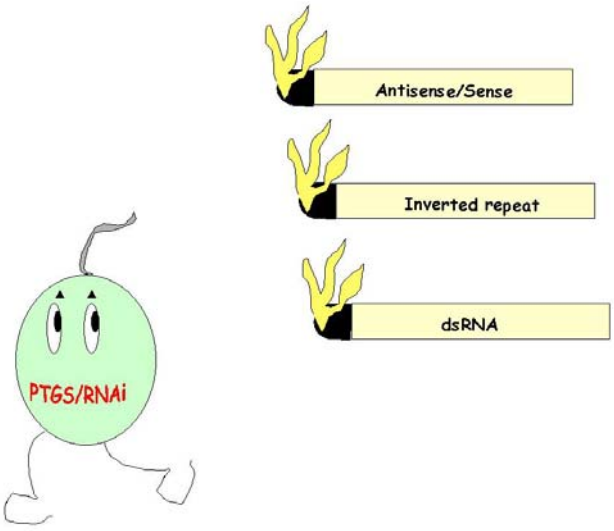
I.2 The principle of PTGS

First examples of PTGS described as the co-suppression phenomenon, were uncovered serendipitously in plants (Napoli *et al*., 1990; Van der Krol *et al*., 1990). It was observed that expression of transgenes being introduced into plants became occasionally suppressed. Moreover, it was found that endogenous genes sharing sequence homology with the silenced transgenes became co-suppressed. These findings indicated that silencing was based on a sequence-specific RNA-targeting and -degradation process preventing the accumulation of cytoplasmic transcripts (reviewed in Kooter *et al*., 1999). The fact, that since 1990 an enormous number of reports on silencing were published indicated that PTGS was not exceptional. A general conclusion was that increased transgene dosage resulted in silencing and not in enhanced expression as one would have expected.

I.2.1 PTGS and its counterpart

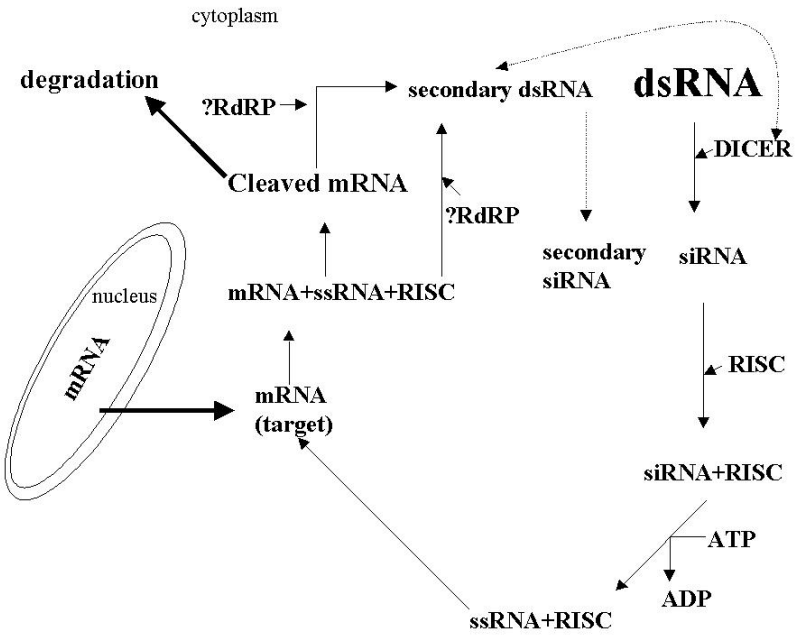
The main factors that promote PTGS were found to be high transcription rates of transgenes (Elmayan and Vaucheret, 1996; Que *et al*., 1997) or arrangement of transgenic loci as inverted repeats (IRs) (Van Blokland *et al*., 1994; Stam *et al*., 1997). Based on these findings, the hypothesis was set forth that formation of transgene-specific duplex RNA would induce silencing. This hypothesis was supported by the observation that simultaneous expression of a sense and an antisense transgene can act as a potent inducer of silencing (Waterhouse *et al*., 1998). It was speculated that the sense and antisense transcripts can hybridise to produce double-stranded RNA (dsRNA). However, it was not until 1997 that English and co-workers presented first experimental evidence demonstrating that IRs must be transcribed to initiate PTGS. In 1998, work of Hamilton and co-workers shed light on the potency of IR to trigger PTGS, where a transgene containing two copies of a 5' untranslated region (5'UTR) as an IR could silence the expression of homologous genes in much higher percentage (96%) than a transgene without the 5'UTR IR structure. Previously, it was assumed that based on their ability to form cruciforms or hairpins, IRs become *de novo* methylated (reviewed in Sijen and Kooter, 2000). Upon ectopic pairing with homologous sequences the methylated IRs would induce *de novo* methylation of paired DNA. Transcription of the methylated homologous DNA was then suggested to result in production of aberrant RNA (abRNA)

that would finally induce PTGS (Baulcombe and English, 1996). More recent studies on this subject revealed that transcription of IRs and back-folding of the transcripts into dsRNA are essential for PTGS induction (Smith *et al*., 2000; Wang and Waterhouse, 2000; De Buck *et al*., 2001). In summary, the current models favour the view that dsRNA promotes PTGS in plants. This was reminiscent of RNA interference (RNAi) that was discovered in *C. elegans* (Fire *et al*., 1998) and other animals as for example *Drosophila melanogaster* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), fish (Wargelius *et* al., 1999; Yx et al., 2000) and mouse (Wianny and Zernicka-Goetz, 2000). RNAi was initiated upon injection of dsRNA and RNA molecules sharing sequence homology with the dsRNA became efficiently degraded. Due to the phenomenological similarities between RNAi in animals and PTGS in plants, these processes were proposed to be related (Fire *et al*., 1998). Further support for this assumption came from studies on virus-induced gene silencing (VIGS) (Ruiz *et al*., 1998) and from RNA-mediated virus resistance (Ratcliff *et al*., 1997). It is believed that plants defend themselves against virus infection by exploiting the requirement of most plant viruses to replicate via the RNA/RNA pathway that involves production of dsRNA intermediates. VIGS in plants takes place if there is sequence similarity between the virus and either a transgene or an endogenous nuclear gene (Lindbo *et al*., 1993; Kumagai *et al*., 1995). Similar to PTGS, the mechanism is post-transcriptional and can be targeted in a sequence-specific manner, against the transgene mRNA as well as the RNA genome of the virus (Lindbo *et al*., 1993; Smith *et al*., 1994; Goodwin *et al*., 1996; Guo and Garcia, 1997).



I.2.2 The mechanism of PTGS/RNAi

Enhanced understanding of silencing phenomena revealed that PTGS and RNAi seemed to require several closely related gene products (Catalanotto *et al*., 2000; Ketting and Plasterk, 2000). Introduction of dsRNA sharing homology with endogenous genes or introduction of transgenes transcribing dsRNA trigger a process wherein dsRNA is processed into short (21-25 nucleotides) interfering RNAs (siRNAs) (Zamore *et al*., 2000; Tuschl, 2001). Fragmentation of dsRNA was shown to require DICER, the RNaseIII family member (Bernstein *et al*., 2001). Many studies in diverse biological systems, led to a better understanding of the siRNA sequence context, the composition of their 3' and 5' ends and the polarity of the RNA strands with respect to initiation of silencing. siRNAs, the hallmark of silencing, associate with a ~250 to ~500 kilo Dalton (kDa) nuclease complex denoted RNA-induced silencing complex (RISC) (Hammond *et al*., 2000). By an ATP-dependent step RISC-associated siRNA unwind and render single-stranded RNA (ssRNA) bound to RISC (Nykänen *et al*., 2001). In a sequence-specific manner, this complex targets complementary RNA molecules leading to RNA cleavage and finally to degradation of the target (Elbashir *et al*., 2001). However, recently it was observed, that, instead of RNA degradation, the target RNA can become a template for a RdRP (as shown in Scheme/1). During this process, the ssRNA serves as primer that is extended by a RdRP by copying the target RNA from 3' to 5' direction (Lipardi *et al.*, 2001; Sijen *et al.*, 2001). At present, it is not clear whether un-primed target RNA could also serve as template for a RdRP. It is conceivable that cleavage of the target might produce molecules having 3'end structures that stimulate complementary RNA strand synthesis by a RdRP (Han and Grierson, 2002; Tang *et al*., 2003). Both, the primed and un-primed RdRP synthesis steps would result in production of secondary dsRNA, which subsequently could be diced to produce secondary siRNAs (Lipardi *et al.*, 2001; Sijen *et al.*, 2001; Vaistij *et al*., 2002; Van Houdt *et al*., 2003). These secondary siRNAs would undergo the same pathway as primary siRNAs. Based on this process silencing can spread from the primary target region into upstream RNA sequences. This process is termed 'transitive RNAi' (Sijen *et al.*, 2001).

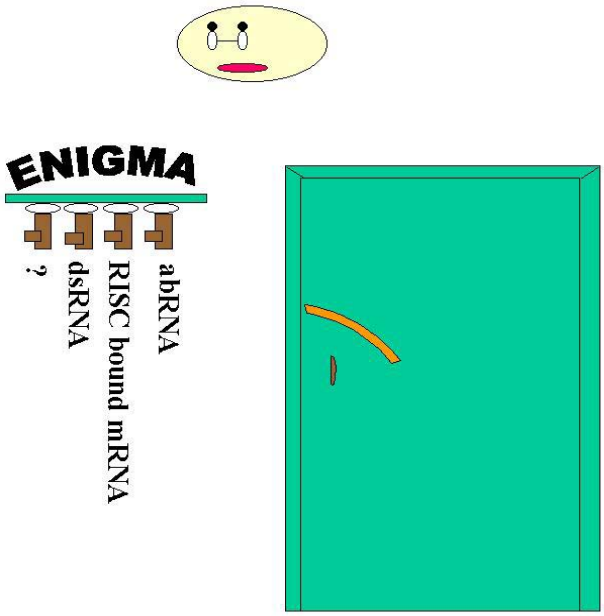


PTGS/RNAi mechanism

Scheme/1: Process triggered upon production or introduction of double-stranded RNA (dsRNA). Details are described in the text.

In addition to the primed RdRP transcription of target RNA, RdRP is postulated to use abRNA as template for the synthesis of complementary RNA (Vaucheret *et al*., 2001). Although not yet defined, abRNA is presumed to represent improper mRNA molecules that may derive from highly expressed transgenes or from low expressed repetitive sequences (Dougherty and Parks, 1995; English *et al*., 1996; Wassenegger and Pélissier, 1998; Grant, 1999). Experimental evidence for generation of abRNA in silenced plants comes from Metzlaff and co-workers (1997) and Han and Grierson (2002). Alternatively, abRNA may be produced by endogenous genes which are epigenetically modified, resulting in premature termination of transcription. Although, evidence for direct correlation between the two is lacking, it is postulated that *de novo* methylation or condensed chromatin structure impedes with the processivity of the RNA polymerase. Additionally, inefficient polyadenylation could also be a factor enhancing abRNA production (Fagard *et al*., 2000; Cogoni *et al*., 2000; Dalmay *et al*., 2000a). Recently, biochemical analysis showed that a wheat RdRP could convert exogenous single-stranded RNA into dsRNA, *in vitro* (Tang *et al*., 2003). However, collective observation indicates that irrespectively of whether primed or un-primed, RdRPs require single-stranded RNA

for synthesis of complementary RNA, and that RdRPs are definitively involved in PTGS and RNAi. Nevertheless, the nature of RdRP substrates needs to be elucidated.



I.2.3 Silencing a powerful tool for functional genomics

"*Every problem has a gift for you in its hands*."

Richard Bach

Silencing, once perceived as an unpredictable and unprecedented side effect that affected transgene expression in plants, now serves as a tool for functional genomics. To determine the function of a gene of interest and to create plants with novel traits, technologies that enable gene inactivation are useful tools. For a long time insertional mutagenesis, were the only reliable method to destroy gene functions. Today, constructs expressing dsRNA, usually in the form of self-complementary hairpin RNA (hpRNA) were found to specifically and efficiently down-regulate gene activities. Therefore, use of gene silencing instead of traditional approaches is advantageous. This technique has been found to silence a wide range of genes in a sequence-specific manner. Reduction in expression of genes can be achieved from mild to complete depending on the construct used. This feature enables the investigation of genes required for basic cell function or development, hence partial block in expression of such genes may give viable plants with phenotypes indicative of the

role of the target gene. Silencing of gene expression could also be achieved by direct introduction of trigger molecules e.g., dsRNA or siRNA into the cells. This technique has been found to be effective in the diverse biological systems. All these features collectively make RNA-mediated gene silencing a favourable tool for gene functional genomics.

In plants, numerous examples of IR gene construct-mediated silencing have been described (Hobbs *et al*., 1993; Dehio and Schell, 1994; Van Blokland *et al*., 1994; Cluster *et al*., 1996; Depicker *et al*., 1996; Jorgensen *et al*., 1996; Stam *et al*., 1998). However, it turned out that production of transgene constructs containing IRs was troubleshooting. Most of the commonly used *E. coli* strains do not propagate plasmids bearing direct IRs. This problem could be solved by introduction of spacer sequences. Upon spacing of the two reversely oriented DNA regions plasmids could be stably maintained in *E. coli* (Das Gupta *et al*., 1987; Lobachev *et al*., 1998) and it is one of the most efficient ways to produce stable IR transgene constructs in *E. coli* (Waterhouse *et al*., 1998). Importantly, *in planta*, IR constructs with a spacer sequence were observed to be even more efficient in inducing RNAi than direct inverted repeats lacking the spacer (Smith *et al*., 2000; Stoutjesdijk *et al*., 2002). Spacer sequences varying from 500 bp up to 1022 bp in length were demonstrated to result in efficient silencing (De Buck *et al*., 2001). Moreover, spacers representing a functional intron were found to be the most potent inducers (Wesley *et al.*, 2001). Based on these findings improved plant RNAi vectors were produced.

Other factors that influence the efficiency of silencing are the size and the sequence context of the dsRNA. Though, precise minimum sequence length needs to be unravelled, dsRNAs longer than 500 bp have been found to be efficient inducers of silencing (Wesley *et al*., 2001). With respect to the sequence context or location of targets within mRNAs, regions specific to 3' ends (e.g., untranslated regions) have been reported to elicit the degree and frequency of silencing (English *et al*., 1996; Sijen *et al*., 1996; Stoutjesdijk *et al*., 2002). Besides IR constructs, experimental analysis with antisense nuclei acids were also successful in achieving selective gene inactivation (Rothstein *et al*., 1987; Takayama and Inouye, 1990; Hamilton *et al*., 1990; Stam *et al*., 2000). It is believed that efficient production of antisense transcripts can be achieved by using strong promoters as for example the Cauliflower Mosaic Virus (35S) promoter, makes them accessible to hybridise with corresponding sense RNA. The resulting dsRNA would then in turn, trigger the dsRNA-induced degradation pathway (Stam *et al*., 2000; Van Houdt *et al*., 2000). Alternatively, it can not be excluded, that strong expression of antisense transgene

constructs initiate the "normal" transgene-induced PTGS process involving production of transgene-specific abRNA followed by RdRP-mediated synthesis of dsRNA.

It has been observed that virus-induced gene silencing is independent of cellular RdRPs. RNA viruses replicate via dsRNA intermediates, and this step is carried out by viral encoded RdRps. This was supported by experiments using the *A. thaliana* RdRP (SDE1) mutants which were anticipated to be impaired in PTGS. To investigate SDE1 involvement in virus-induced PTGS, viruses containing a part of a cDNA sequence sharing homology with an endogenous gene showed silencing of the endogenous gene in wild-type as well as in the SDE1-deficient plants (Dalmay *et al*., 2000b). This demonstrated that induction of VIGS was independent of the SDE1 activity. It may further give rise to the hypothesis that dsRNA-mediated silencing is in general independent of RdRPs. Hence, it was speculated that silencing induced by genome-integrated IR and antisense constructs would not require RdRP activity as both of these constructs have the potential to form dsRNA.

I.3 Aim of the thesis

Many of the details and ramifications of silencing still need to be explored. However, what we already see is an elegant system that not only recognises genome-invading nucleic acids but also marshals a defence against them. The current findings reflected the importance of RdRP activities and it appeared that different homologues of RdRPs exist in nature. These homologues can be grouped into a gene family. Insights into the protein sequence of all family members could reveal regions that are conserved among all RdRPs. These observations prompted us to initiate further investigations that aimed at the identification, isolation and characterisation of tomato RdRP genes. For plants with small genomes (e.g., *Arabidopsis* and rice), it is possible to sequence the entire genome and to carry out mutagenesis of all genes. Thus, with these approaches it is feasible to gain information about the genetic make-up of these biological systems. However, it is not suitable to follow such strategies for biological systems with more complex and larger genomes. Therefore, the current work involved alternative experimental approaches, namely RNA-mediated gene silencing strategies, to gain information about the number, sequence, and function of different tomato RdRP genes. In order to achieve these goals the following experimental approaches were chosen:

- At first, all known putative RdRP genes in plants will be identified by carrying out a database homology search using the amino acid sequence of the tomato RdRP (Schiebel *et al*., 1998) as the family member. All retrieved sequences exhibiting similarity with the tomato RdRP will be manually aligned and compared. Sequences will then be screened for highly conserved regions.

Subsequently, all sequences of each plant species will be aligned to search for sequence homology and diversity within one species. This analysis will be carried out to find different RdRP homologues. Identification of homologues may then allow to design primers that would enable specific amplification of each homologue.

- Isolation of RdRP cDNA clones from tomato will involve commonly used molecular biology techniques. Designed primers will be used to amplify the cDNA reverse transcribed from total RNA of tomato. Amplification with polymerase chain reaction (PCR) will be performed using various primer combinations under different conditions.
- Characterisation of putative RdRP homologues will mainly focus on the determination of their biological function. This will be achieved by following an experimental approach based on RNA-mediated gene silencing and on virus-induced gene silencing (VIGS). As stated earlier, these mechanisms serve as powerful tools to analyse gene functions by blocking gene expression. Loss of gene function may result in the establishment of an altered phenotype. Conclusions drawn from the phenotype may finally give insights into the possible function of the RdRPs.

Blocking of gene expression by RNA-mediated gene silencing will mainly involve generation of constructs that have been reported to be efficient inducers of RNAi. Introduction of these constructs into plant, analysis of the resulting transgenic plants, monitoring of phenotypes, production of homozygous lines, analysis of homozygous lines for the inheritance of the constructs and monitoring of phenotypes in these lines will follow. Generation of the “silencing constructs” will include double-stranded and antisense transcript-producing transgenes. For construct design basic features will be considered that render the transcripts to be an efficient inducer of silencing (size of the targeting sequence, introduction of functional introns into IR constructs, etc.). Importantly, highly conserved regions are present among all members of the RdRP gene family. In view of this, constructs will be produced that either contain RdRP gene-specific or conserved

sequences. The former transgenes will enable inactivation of only one RdRP homologue whereas the latter may allow silencing of the RdRP gene family. Previous experiments showed that, plants containing the full-length cDNA of the tomato RdRP in antisense orientation exhibited floral and leaf anomalies (Wassenegger, unpublished results). These phenotypic alterations ("TK-phenotype") comprised robust flower development, male sterility and leaves developing thick mid-ribs in combination with peculiar venation (see on page 13). As the antisense cDNA included the conserved regions no assignment to one of the putative tomato RdRP could be made. Thus, it was assumed that specific down-regulation of only one of the RdRPs would render possible to determine which of the homologues is associated with the TK-phenotype.

IR constructs that aim at RdRP gene-specific inactivation should i) exclude conserved sequence motifs, ii) exhibit minimal homology with the previously isolated tomato RdRP, iii) contain a 3'end-specific gene fragment, and iv) contain a targeting sequence with a size of $\geq$ 500 base pairs. Constructs will contain two copies of the cDNA fragments as inverted repeats with an intron as spacer and will be placed under the control of a strong viral promoter. Such IR constructs will produce a transcript capable of back-folding into dsRNA ("panhandle" structure) thereby triggering RNAi. Control construct will contain direct repeats of the same fragments also spaced by an intron. These constructs will produce linear transcripts lacking double stranded regions. In addition, direct repeat containing cDNA fragments from the previously isolated tomato RdRP having exactly the same features will be designed. These controls will be used to analyse transgene stability, transgene expression, and efficiency of transcript splicing.

Antisense constructs will be designed with fragments of new and also the previously isolated homologues to block expression of RdRP homologues. It was known that induction of gene silencing by antisense constructs is not as dominant as exhibited by IR. This feature makes them favourable for functional analysis of essential genes as it is assumed that gene expression will be only blocked partially. Constructs will be used that include conserved regions and 3'end-specific cDNA fragments. The antisense transgenes will also be placed under the control of a strong viral promoter. Such constructs will produce antisense transcript which are anticipated to anneal with sense mRNA. Whether RNAi will be induced by these constructs or whether another RNA-mediated process triggers gene silencing is not clear. Annealing of the antisense RNA with sense RNA would produce dsRNA that may initiate RNAi. However, it is conceivable that a low concentration of dsRNA is not capable to start the RNAi process. Nevertheless,

degradation of the dsRNA may take place that would result in partial gene suppression. Control constructs will contain the same fragments in sense orientation. In addition to these constructs, sense construct containing full-length cDNA of the RdRP homologue will be designed to over-express the endogenous gene. All these constructs will be introduced into tobacco plants by *Agrobacterium*-mediated transformation to obtain stable transgenic plant lines.

Tobacco SR1Wild-type

Tobacco SR1TK plant line

Tobacco SR1Wild-type

Tobacco SR1TK plant line

Photographs: Flower and Leaf morphology of the SR1TK plant lines.

Virus-induced RdRP gene silencing will be achieved by introducing gene-specific fragments of the RdRP homologue into Potato Virus X (PVX) and subsequent infection of tobacco plants using *in vitro* transcripts of the recombinant PVX strains as inoculum. Inoculated plants will be analysed for replication of the viruses and monitored for development of phenotypes.

II Materials and Methods

II.1 Materials

II.1.1 Chemicals and consumables

All chemicals used throughout the work were purchased from the following companies: Roche (Mannheim), Roth (Karlsruhe), Merck (Darmstadt), Amersham Pharmacia Biotech (Freiburg), Gibco BRL (Eggenstein) and Sigma (München). The consumables were from: Roth (Karlsruhe), Amersham Pharmacia Biotech (Freiburg), Whatman (Schleicher and Schüll, Düren) and Sigma (München).

II.1.2 Enzymes and reaction Kits

Restriction enzymes either from New England Biolabs (Schwalbach), Promega (Mannheim), Roche (Basel, Switzerland) or Fermentas (St. Leon-Rot) were used for DNA manipulation. The following Kits were used:

5'/3' RACE Kit	Roche
SP6/T7 Transcription Kit	Boehringer
cDNA Cycle Kit	Invitrogen
mCAP™ RNA capping Kit	Stratagene
Ladderman™ labeling Kit	BioWhittaker
TaKaRa Ex Taq™	BioWhittaker
pGEM®-T easy Vector Systems	Promega
T4 DNA ligase Kit	Promega
T4 DNA polymerase	Promega
Plasmid (Midi or Maxi) Kits	Qiagen
QIAprep spin Miniprep Kit	Qiagen
QIAquick gel extraction Kit	Qiagen
RNeasy Plant Total RNA Kit	Qiagen

II.1.3 Host strains

All recombinant plasmids were propagated in the *Escherichia coli* strain INVα F´. Before conjugation, recombinant pPCV702SM plasmids were propagated in the *E. coli* SM-10 strain. Growth conditions were incubation on plates as well as in liquid medium [Luria-Bertani (LB) + antibiotics] at 37°C overnight.

Name	genotype	source
INVα F'	F'*end*A1*rec*A1 *hsd*R17(r_k-, m_k +) *sup*E44*thi*-1 *gyr*A96 *rel*A1Φ80*lac*ZΔ(*lac*ZYA-*arg*F)U169	Invitrogen
SM-10	thi thr leu suIII (Simon *et al.*, 1983)	Kind gift from C. Koncz

Agrobacterium strain:

Agrobacterium tumefaciens GV3101 (pMP90RK Gm^r, Km^r, Rif^r) (Koncz and Schell, 1986) was used for *Agrobacterium*-mediated gene transfer. Growth conditions were YEB + antibiotics (Gm + Rif + Sm + Sp) at 28°C for 2-3 days.

II.1.4 Plants

Lycopersicon esculentum cultivar Rentita, *Nicotiana tabacum* cultivar Petita Havana SR1, *Nicotiana glutinosa* and *Nicotiana benthamiana*.

II.1.5 Vectors

The pGEM-T easy (~3.0 kb) from Promega was used to clone polymerase chain reaction (PCR) products having 3' A-overhangs. Cloning was done according to manufacturers instructions. Selection medium was LB + ampicillin (100µg/mL).

The pT3T7SM (~3.2 kb) (Wassenegger, unpublished) is a modified vector derived from the pT3T7 (Boehringer). The vector was used for sub-cloning of gene fragments. Selection medium was LB + spectinomycin (75µg/mL) + streptomycin (25µg/mL).

The pPCV702SM (~10.8 kb) (Wassenegger *et al*., 1994) is a modified pPCV702 plasmid (Koncz and Schell, 1986) that contained the streptomycin/spectinomycin resistance gene instead of the carbenicillin resistance gene. In addition, a polylinker sequence was introduced between the 35S promoter and the pAnos signal sequence. Selection medium was LB + spectinomycin (75µg/mL) + streptomycin (25µg/mL).

The pP2C2S (9.713 kb) is a Potato Virus X (PVX) expression vector (Chapman *et al*., 1992) that was used to *in vitro* transcribe infectious PVX RNA. Selection medium was LB + ampicillin (100µg/mL).

II.1.6 Oligonucleotides

All primers that were used for sequencing were either synthesised by Metabion GmbH (Martinsried, Germany) or by MWG (Ebersberg, Germany). Below is a list of gene- and vector-specific primers that were used to sequence recombinant clones. Primer sequences are presented in the 5' to 3' direction.

Primers used for sequencing:

T7	TAA TAC GAC TCA CTA TAG GG
T3	AAT TAA CCC TCA CTA AAG GG
sp6	ATT TAG GTG ACA CTA TAG AA
35S-18A	GGA TGA CGC ACA ATC CCA CTA TC
PolyA-blue	ATC GCA AGA CCG GCA ACA GGA TTC
RdRP7500Int4fr	GCT GAG ATT ACC TTC TCG AAG
RdRP7620Int4rev	GAT AGA TCC TAG AGA AGG AG
2440forward	GAA GGG AAA TGT GGT TGT TG
2440reverse	CAA CAA CCA CAT TTC CCT TC
3070forward	GCT GAT ATG GAA GTT GAT GG
3070reverse	CCA TCA ACT TCC ATA TCA GC
RevCfrRdRP²	GCC GGA AAG GAA GTG TCT TG
RdRP²fr1010	GCA GTC TAT CTG TTT CGA CCC
3'HomfrBamHI	CTC CAA GGA TCC GCA CCC AAT C
XbaIRdRP²rev	GCG ATA CTC TAG GTG CAA TCT C
TomRes specific R2	CTG AAA TTT GAT TCT CAA AAT G
TomRes specific R6	GCA AGG CAT GGC ATC ACT CCA CTT
RdRP²rev1010	GGG TCG AAA CAG ATA GAC TGC
5'RdRP²revC	CAA GAC ACT TCC TTT CCG GC
5'RdRP² internal sp	GAG GTG CTA AAC AGT TGA CC
5' RdRP² internal t7	CAT GTT GCA ACC AGG ATG TG
RdRP²revXbaI	CTC TGA ATT TCC TCT AGA GTG AC
PVX5582forward	ATA AGG GCC ATT GCC GAT CTC
PVX5740reverse	TGT GTT GTG CTA GCT GGT GC

II.1.7 Buffers, media and solutions

All standard solutions, buffers, and media were prepared according to Sambrook *et al*., 1989. Media for cultivating bacteria were sterilised by autoclaving (20min, 121°C, 1-2bar). Thermo labile components such as antibiotics were sterile filtered (0.2µm) and added to

the media after autoclaving and cooling to 55°C. Compositions of non-standard solutions or buffers are listed at the end of the method section.

II.1.8 Matrices and membranes

Radioactive labelled probes, used for Southern and Northern hybridisation, were purified by chromatography using Sephadex G50 (Amersham Pharmacia Biotech) columns. Nylon Hybond transfer membranes (positively charged and uncharged) were from Qiagen. Whatman paper 3M was from Whatman. Miracloth was from CalBiochem.

II.1.9 Equipments

Centrifuges: Eppendorf table centrifuge, Heraeus Christ Minifuge-2, Sorvall Superspeed RC2-B, Beckman J2-21M;

UV-Transilluminator: Cromato-Vue with 306 nm or 366 nm (San Gabriel, USA);

Gel electrophoresis apparatus: 19cm x 13cm and 13cm x 13cm, mini gel apparatus and power supplies (Bio-Rad);

PCR Thermocycler: Crocodile I and II (Appligene, France);

Photo camera systems: Nikon CoolPix 990, standard Polaroid Camera systems;

Incubators: Hybridisation Ovens (Hybaid, MWG, Germany); Dry bath (Heraeus, Hanau); bacterial cultures incubator (HAT, Infors, Basel); phyto-chambers VT PH500 (Heraeus Vötsch, Balingen);

Autoradiography: Kodak films (size 18cm x 24cm) BioMax MR, single emulsion; developing machine (AGFA, CURIX 60);

Computer software: Windows 98 operating system; Chromas 15 (data analysis and technical graphics); DNASIS 2.1 (sequence comparison and translation); MS Powerpoint; Macromedia FreeHand 8.0, Adobe Photoshop 5.0 and Nikon view version 3 (plants photographs).

II.2 Methods

All experiments related to genetic engineering were performed according to the regulations of the “S1-Richtlinien” and were officially approved by the “Regierung von Oberbayern, Aktenzeichen 250-7321-12/92, 05.Oct.92". Transfer of the permission from Max-Planck Gesellschaft to the Fraunhofer Gesellschaft was given by the "Regierung von Oberbayern, Aktenzeichen 821-8763.11.311" on 27th of March 2000.

Throughout the work, the guide to molecular biology techniques (Sambrook *et al*., 1989) was used as a reference manual.

II.2.1 Standard molecular cloning

II.2.1.1 DNA modifications

II.2.1.1.1 Plasmid DNA restriction

Plasmids were digested with restriction endonucleases according to the manufactures instructions using the buffers supplied with the enzyme. Double digestions with different enzymes were carried out in one reaction when a common buffer was recommended. If no common buffer was recommended then plasmid was stepwise digested with enzymes. First plasmid was digested with one enzyme in supplied buffer, gel-purified (II.2.1.3) and subsequently, it was digested with the second enzyme in recommended buffer. Three units of the respective enzyme were used per microgram of DNA. Reactions were stopped by phenol extraction and precipitated according to standard protocols (Sambrook *et al.*, 1989).

II.2.1.1.2 T4 DNA Polymerase fill-in reactions

In case of incompatible cohesive ends of inserts and vectors, the corresponding cloning sites were filled-in by the T4 DNA polymerase (Promega) to produce blunt ends that can be joint upon ligation.

Reaction mix:

Digestion mix	30µL
dNTPs mix(2.5mM)	2µL
Enzyme(8u/µL)	2µL
5x buffer	10µL
Water	18µL

	60µL

The reaction was incubated at 37°C for 1h and subsequently phenolised and precipitated according to standard protocols (Sambrook *et al.*, 1989).

II.2.1.2 Agarose gel electrophoresis

Analytical as well as preparative gel electrophoresis of plasmid DNA and PCR fragments was performed as described by Sambrook *et al*., 1989. The percentage of the agarose gels

used ranged from 0.8%-1.5% (w/v) made in 1x TAE, supplemented with the fluorescent intercalating dye ethidiumbromide (0.1µg/mL). To determine the fragment size and estimate the concentration, DNA markers [λ DNA (Promega) cut by PstI (λ-PstI marker) or a 1 kb ladder (New England Biolabs)] with known sizes of fragments were co-electrophoresed. Bands were visualised using an ultraviolet (UV) transilluminator at 306nm and photographs were taken using Polaroid films and standard Polaroid camera systems.

Fragment sizes of DNA markers were:

1 kb ladder (bp): 10002, 8001, 5001, 4001, 3001, 2000, 1500, 1000, 500-517.

λ-PstI (bp): 11501, 5077, 4749, 4507, 2838, 2459, 2443,2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 200, 216, 211, 164, 150, 94, 87, 72, 15.

50x TAE

Tris base	2M
Glacial acetic acid	5.71%(v/v)
EDTA pH 8.0	50mM

II.2.1.3 Gel extraction

For purification of DNA fragments, preparative gel electrophoresis was carried out. Onto a UV transmittable plastic sheet, gels were placed and fragments of interest were excised with a sterile scalpel. DNA was extracted from the gel pieces using the QIAquick gel extraction Kit.

II.2.1.4 Ligation

Ligation reactions were performed using T4 DNA ligase (Promega). The molar ratio between insert and vector was 3:1 for blunt end cloning, and 1:3 for sticky end cloning. Ligations were incubated in total volumes of 10µL or 15µL, incubated at room temperature for 1h, and then at 12-14°C for 12 to 16 hours.

II.2.1.5 Preparation and transformation of competent *Escherichia coli* cells

Competent INVα F' and SM-10 cells

Competent cells were prepared using TSB medium. A 100mL culture of INVα F´ or SM-10 cells was grown overnight at 37°C with vigorous shaking (225rpm). From the overnight culture 200µL was added to 50mL SOB in a 100mL flask. The fresh culture was allowed

to grow under the above conditions for 3-4 hours until the OD_{600nm} reached 0.4-0.5. The culture was then aseptically transferred into sterile, disposable, ice-cold 50mL polypropylene tubes (Falcon). Cells were recovered by centrifugation in a Heraeus Christ Minifuge-2 (3000rpm/10min/4°C). After decantation of the medium, tubes were inverted on a sterile tissues for 1min to drain last traces of medium. The pellet was re-suspended in 3.5mL TSB and stored on ice for 30min. Finally, 100µL aliquots were dispensed into 1.5mL sterile microfuge tubes and after shock-freezing in liquid nitrogen the tubes were stored at -80°C. Transformation efficiencies of the cells was tested as described (Sambrook *et* al., 1989).

SOB:

Tryptone/peptone	2% (w/v)
Yeast extract	0.5%(w/v)
NaCl	10mM
KCl	2mM

After autoclaving 10mL of 2M Mg^{2+} was added.

TSB:

LB (pH 6.1)	
PEG (3.300)	10%(w/v)
DMSO	5% (v/v)
Mg^{2+} (Mg^{2+}: 1:1 of 1M $MgCl_2$ and $MgSO_4$)	20mM

Transformation mix:

Ligation mix	10µL
Sterile water	70µL
5x KCM	20µL
Competent cells (thawed)	100µL

5x KCM:

KCl	0.50M
$CaCl_2$	0.15M
$MgCl_2$	0.25M

The 5x KCM medium was filter sterilised and stored at 4°C.

The transformation mix was incubated for 30min on ice and then transferred to 42°C for 90sec. Subsequently, the tubes were incubated on ice for 1-2min before 800µL of SOC was added. The mix was incubated at 37°C for 1-3 hours (preferably with shaking). Finally, the transformation mix was plated on LB agar plates (400µL-600µL/plate) containing the suitable antibiotic(s). Plates were incubated at 37°C overnight. Single colonies from these plates were selected for recombinant plasmid analysis.

SOC:

SOB + 20mM glucose (stock: 2M)

Luria-Bertani (LB) medium (1L):

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	5g

adjust pH 7.0 with NaOH (1M)

LB plates with antibiotic(s)

For LB plates 15g of Bacto-agar (Difco) was added to 1L of liquid LB medium. After autoclaving, the medium was cooled to 50°-55°C before antibiotic(s) were added. Approximately 30mL medium was poured into a 85mm petridish and the agar was solidified by storing at RT for 2h. Plates that were not immediately used were stored at 4°C.

Antibiotics	**Stock**	**Final concentration**
Ampicillin (Amp)	50mg/mL	100µg/mL
Streptomycin (Sm)	150mg/mL	25µg/mL
Spectinomycin (Sp)	100mg/mL	75µg/mL
Kanamycin (Km)	50mg/mL	25µg/mL

II.2.1.6 Identification and analysis of recombinant plasmids

Single bacterial colonies were picked and inoculated in liquid media. The culture was incubated at 37°C overnight under shaking. If vector- or gene-specific primers were available "pooled PCR" of recombinant clones was carried out.

II.2.1.6.1 Pooled polymerase chain reaction (pooled PCR) of *E. coli* transformants

Most of the transformants were initially screened for the presence of inserts by performing a PCR using crude total DNA as template. Crude DNA was prepared as follows:

With a sterile 20µL Gilson tip single colonies were picked from LB agar plates and struck out on a new LB agar plate (master plate). The master plate was provided with a grid as described for "recombinant plasmid screening by hybridisation" (Sambrook *et al*., 1989). Each square of the grid was numbered. Subsequently, the bacteria that were left at the tip were washed off into a sterile 1.5mL Eppendorf tube containing 25µL of sterile water. Into each numbered tube five independent colonies were transferred. Once pooling was finished, the tubes were incubated for 5min at 95°C to lyse the cells. The heated samples were spun in an Eppendorf centrifuge (14000rpm) for 2min at RT. A 2µL aliquot of the supernatant was taken for PCR amplification under standard conditions using specific primers. The master plate was incubated overnight at 37°C. Cultures showing amplification were selected and each colony of a "positive pool" that was picked from the master plate was separately incubated. Cultures of the single colonies were proceeded for DNA isolation.

II.2.1.6.2 Plasmid DNA mini-preparation

The overnight bacterial culture was submitted for plasmid DNA mini-preparation using the QIAprep spin Miniprep Kit. Isolation was performed according to the instructions of the manufacturer.

II.2.1.6.3 Mini-preparation of *Agrobacterium* DNA

Cultures of 1.5mL were centrifuged in an Eppendorf centrifuge (14000rmp, 2min) to pellet the cells. After centrifugation the supernatant was discarded and the pellet was re-suspended in 400µL TE buffer (10mM Tris buffer, 1mM EDTA, pH 8.0). Volume of 66µL of 10% (w/v) Sarkosyl and 134µL of Proteinase K (2mg/mL stock made in sterile water) were added before the cells were incubated, first at 50°C for 10min and then at 37°C for 1h. The cell suspension was phenolised twice and the aqueous layer was precipitated by adding 1mL isopropanol. To pellet the DNA, the sample was centrifuged at room temperature for 2min using an Eppendorf centrifuge (14000rpm). The DNA pellet was washed with 70% (v/v) ethanol and re-suspended in 200-300µL TE (10mM Tris buffer, 1mM EDTA, pH 8.0).

II.2.1.7 Polymerase Chain Reaction -*TaKaRa Ex Taq*

Polymerase chain reaction was used through out the work for various purposes mainly, to isolate gene-specific fragments and to screen recombinant plasmids for the presence of inserts. In addition the PCR technology was applied to introduce restriction enzyme sites at the ends of DNA fragments. Standard reaction mixes and standard amplification conditions were applied for most reactions. Deviations from the standard protocol are mentioned in the text.

II.2.1.7.1 Standard PCR Reaction Mix

Template	< 1µg
Primer 1	20pmol (0.2-1.0µM final concentration)
Primer 2	20pmol
10x buffer (+ Mg^{2+})	5µL
dNTPs mix (2.5mM each)	4µL
Ex-taq enzyme (5units/µL)	0.3µL
Add sterile water to a final volume of 50µL.	

II.2.1.7.2 Standard PCR Conditions

All the PCR amplifications were performed using *TaKaRa Ex-taq* PCR Kit and the Crocodile II Thermocycler according to the following conditions:

Initial denaturation	2min	94°C	
Denaturation Annealing Polymerisation	40sec 2min 3min	94°C 58°-64°C 72°C	30 cycles
Final extension	10min	72°C	

Annealing temperature was variable according to the T_m value of the primers

II.2.1.7.3 5'/3' Rapid amplification of cDNA ends (RACE)

The first cDNA strand as well as dA-tailed cDNA was synthesised from total RNA according to the manufacturers instructions using the 5'/3'RACE Kit (Roche). In case of the $RdRP^2$ 3'end PCR amplification, the first cDNA strand used for amplification was synthesised with the oligo dT-anchor primer (RACE Kit). For the $RdRP^2$ 5'end amplification, the first cDNA strand was synthesised using a gene-specific primer. The cDNA produced was proceeded for addition of a homopolymeric tail (A-tail) according to manufacturers instructions. This dA-tailed cDNA was thereafter directly used to amplify the 5'end. PCR reaction mixes were prepared according to manufacturers instructions under standard PCR conditions.

II.2.1.7.4 cDNA synthesis

Two µg of total RNA (isolated using the RNeasy Plant Total RNA Kit from Qiagen), was reverse transcribed to synthesise single strand cDNA using the Invitrogen cDNA Cycle Kit. The single strand cDNA was directly used for the standard PCR reaction mix and proceeded for amplification under standard PCR conditions

II.2.1.7.5 Re-amplification of PCR products

In most cases, PCR products obtained from cDNA or dA-tailed cDNA failed to show visible bands on agarose gels. Hence, re-amplification of these PCR products was performed using a 1µL aliquot. Re-amplification was performed using the oligo dT-anchor primer in combination with gene-specific nested primers. The reaction mixes were prepared according to the 5'/3' RACE instruction manual and amplification was performed under standard PCR conditions. The amplified products were run on 1% (w/v) agarose gels using the TAE buffer systems.

Primers used for PCR amplifications:

Name	**Sequence** (5'→ 3')
TomRes specific 1	CTC AAA TTT GAA TCA AAG AAC AGG
TomRes specific R2	CTG AAA TTT GAT TCT CAA AAT G
TomRes specific 2	AAG TGG AGT GAT GCC ATG CCT TGC
TomRes specific 3	ATG TTG CTC CAA GGA TAC GCA CCC
TomRes specific R3	ACC ACC CAT GCT CTC CAA AAC
TomRes specific R6	GCA AGG CAT GGC ATC ACT CCA CTT
RdRP14-5 fr	CTG ACG ATT TAC ATC CAT TT
RDRP 6-3	CCC CTG ATT GTA GCA ACC CC
5' RdRP^{2}revB	CGC CAA ACT GTA CCT CCA ACC
5' RdRP^{2}revC	CAA GAC ACT TCC TTT CCG GC
3'HomfrBamHI*	CTC CAA GGA TCC GCA CCC AAT C
3'HomRevBamHI*	GGA TAA GGC AGG ATC CAA CAA C
RdRP2790SalI* fr	GTA TTT GTC GAC AGA GAA CCT GATATG GCC
RdRP3450SalI* rev	GGA TTA GTC GAC CAT AAA CAC ACC AGG GAA AG
RdRP2800Not* fr	GAG AAC CTG ATG CGG CCG CGA GTG ACC
RdRP3440Not* rev	ATC TGG ATT AGG CGG CCG CAA ACA CAC C
RdRP^{2}fr SmaI*	GAA GTC CCG GGT ATA GAC ATG
RdRP^{2}rev XbaI*	CTC TGA ATT TCC TCT AGA GTG AC
RdRP7500 Int4fr	GCT GAG ATT ACC TTC TCG AAG
RdRP7620 Int4rev	GAT AGA TCC TAG AGA AGG AG
pNOS1020BiUSrev	CTC TAA TCA TAA AAA CCC ATC TC
Oligo dT-anchor	GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV
Anchor primer	GAC CAC GCG TAT CGA TGT CGA C
710BiUS-35S	AAG CAA GTG GAT TGA TGT G
RdRP1-intron4-XbaI-fr*	TGG ATC TAG ATG TCA CAA TTG AG
RdRP1-intron4-XbaI-rev*	ACA ATC TAG ATG GTG AAG TAC TCT T

R = rev = reverse.
fr = forward.
V = A, C or G.
TRs = TomRes specific.
RACE Kit primers = Oligo dT-anchor and Anchor primer.
*Primer sequences are gene-specific but have some base pair substitutions. These substitutions were made to create a suitable restriction enzyme site. The primer name includes the name of restriction enzyme whose site was created by these substitutions.
#All primer stocks had 1µg /µL concentration.

II.2.1.8 Cryopreservation of bacterial cells

Validated recombinant bacterial clones were maintained as glycerol stocks [360µL glycerol (87%) + 640µL bacterial culture]. Stocks were frozen at -80°C.

II.2.2 Generation and characterisation of transgenic tobacco plants

II.2.2.1 Conjugation

The pP702SM derivatives were transformed into *E. coli* SM-10 by heat shock (II.2.1.5). Transformation mixes were plated on LB agar plates containing Km (25µg/mL), Sm (25µg/mL), and Sp (75µg/mL). To obtain an overnight culture for conjugation with *Agrobacterium*-GV3101, a single colony of the transformed SM-10 was inoculated in 2mL of LB + $MgCl_2$ (10mM) liquid media. For an overnight culture of the *Agrobacterium* GV3101 (pMP9ORK) a 10µL aliquot of a glycerol stock was inoculated in 2mL LB + $MgCl_2$ (10mM) liquid media. The transformed SM-10 culture was allowed to grow at 37°C overnight and that of *Agrobacterium* GV3101 (pMP9ORK) was grown at 28°C overnight. From the overnight *Agrobacterium* GV3101 culture, an aliquot (50-100µL) was transferred on LB (10mM $MgCl_2$) plate and dried for 10-15min. This spot was marked at the bottom of the plate, then a drop of the transformed SM-10 overnight culture was laid over the *Agrobacterium* drop. After drying for 10-15min, the plate was incubated at 28°C for two days. After two days incubation, a single colony streak was made on YEB plates containing Sm (300µg/L), Sp (100µg/L), and Rif (100µg/L). These plates were incubated at 28°C for two days. Single colonies were picked, inoculated in 3mL YEB media containing Sm (300µg/L), Sp (100µg/L), and Rif (100µg/L) and the culture was allowed to grow at 28°C for two days (shaking). A 650µL aliquot of the culture was used to make a glycerol stock and 1.5mL was proceeded for mini-preparation of *Agrobacterium* DNA (II.2.1.6.3).

II.2.2.2 Re-transformation into INVα F´

After mobilisation of the pPCV702SM derivatives into *Agrobacterium*, transconjugants were analysed by re-transformation into INVα F´ cells. One microgram Agrobacterium DNA isolated from a single colony culture of a transconjugant was directly transformed into INVα F´ cells (II.2.1.5). For each construct three independent colonies of the INVα F´ transformants were picked and cultured at 37°C overnight. From these overnight cultures, plasmid DNA was isolated (II.2.1.6.2). The plasmid DNA was cut with suitable restriction

enzymes and the restriction patterns were compared with those of the original construct. In case of identical patterns, the corresponding *Agrobacterium* strains were used for tobacco leaf disc transformation.

II.2.2.3 *Agrobacterium*-mediated "leaf disc transformation" of tobacco plants

For most of the pPCV702SM derivatives stable plant transformation was performed by the "Zentrale Einheit für Pflanzen-Transformationen" (ZEPT) of our institute in Schmallenberg with the assistance of Frau Barbara Henke and Frau Christiane Fischer. Introduction of the T-DNA into the *Nicotiana tabacum* cv Petita Havana SR1 genome was carried out following the "leaf disc transformation" procedure (Horsch *et al.*, 1985). Wild-type tobacco plants were grown on MS medium (Murashige and Skoog, 1962) under sterile conditions in glass containers and young leaves with a length of about 4cm were used for transformation. The transconjugated *Agrobacteria* were grown in 20mL Minimal A (MinA) media at 28°C for 1-2 days. Cultures that became slightly cloudy due to growth of the cells (OD_{600nm} <0.1) were taken for transformation. The *Agrobacterium* suspension was poured into sterile glass containers and leaves of wild-type tobacco plants that had been cut into 8-10 pieces of about ~1cm² were transferred to the suspension. Leaf pieces were totally moistened by gentle shaking before the "leaf discs" were transferred onto MS plates (6-8 pieces/plate). Plates were incubated in a phytochamber under moderate light for 3-4 days. Subsequently, the "leaf discs" were washed three times in liquid half-MS (½MS) medium. For the third washing the medium was complemented with carbencillin (500mg/L) to kill the *Agrobacteria*. The washed "leaf discs" were placed onto "shoot plates" and maintained under sterile conditions in a phytochamber under standard light conditions. Every two weeks the leaf discs were put onto fresh shoot medium until calli developed. Calli were separated and shoots from each callus were cut when they had ~1cm of height and were placed onto root medium. Transgenic plants were maintained under sterile conditions until roots developed. Two to three weeks after the rooting the small plants were transferred into soil.

II.2.2.4 Self-pollination, genetic crosses and sterilisation of seeds

Tobacco is a self-fertile plant. Therefore, at the flowering stage young buds could be covered with plastic bags to allow self-pollination and to prevent cross-pollination. Three to four months after transfer of transgenic plants into soil, seeds were obtained. Seeds were collected when the green capsules turned to brown and were stored at 4°C. Primary

transformants carrying a transgene were expected to segregate according to Mendel's rules. Siblings developed that either maintained or that lost the transgene. To select progeny of the T_1 generation that carried the transgene, seeds were sown and genomic DNA was isolated from progeny plants for PCR amplification. PCR amplification was carried out using transgene-specific primers. T_1 progeny plants showing PCR products of the expected size were considered to carry the transgene and were chosen for further analysis. Genomic DNA of these plants was used for Southern analysis to confirm the presence of the transgene and to determine as explained in the "Results" part whether the transgene was in a homozygous or heterozygous state. To get additional evidence for transgene homozygosity, genetic crosses between progeny plants of selfed primary transformants and SR1 wild-type plants were performed. Young buds were emasculated and pollen from wild-type tobacco was dusted onto the stigma. Seeds were collected and stored in Eppendorf tubes at 4°C. Two weeks after storage, about 100 seeds were sterilised and plated onto kanamycin-containing MS medium. Sterilisation was done by incubation of the seeds in 70% (v/v) ethanol for 2-3min followed by a 7% (v/v) sodium-hypocholride treatment (10min). Before plating the sterilised seeds onto the selection medium, they were washed with sterile water for at least 6 times. Subsequently, 10-20 seeds per plate were dispensed. The plates were incubated in a phytochamber under standard light conditions until seedlings were grown. By counting the number of germinating seeds *versus* non-germinating seeds, the germination percentage was calculated. The growth of germinating seeds that developed into seedlings was monitored. Due to the presence of the kanamycin, non-transgenic seedlings developed white cotyledons and died within few weeks. By contrast, seedlings from transgenic plants had green cotyledons and developed further. The ratio between developing and dying seedlings was counted. If all of the germinated seeds developed green cotyledons the parental T_1 transgenic plant was considered to carry the transgene in a homozygous state. It should be noted that this statement can only apply for primary transformants that contained a single transgene copy. Plants bearing multiple transgene copies at independent loci can not be analysed according to the above procedure.

YEB-Rif-Gm medium

Nutrient Broth	0.5% (w/v)
Yeast Extract	0.1% (w/v)
Peptone	0.5% (w/v)
Sucrose	0.5% (w/v)

$MgSO_4$ (2mM), Rifampicin (100µg/mL), and Gentamycin (40µg/L) were added after autoclaving and cooling of the medium.

MinA

K_2HPO_4	1.0% (w/v)
KH_2PO_4	0.45% (w/v)
$(NH_4)_2SO_4$	0.1% (w/v)
Na.citrate x $2H_2O$	0.05% (w/v)

Medium was autoclaved and after cooling it to 55°C, 1mL of a 20% (w/v) filter-sterilised $MgSO_4$ x $7H_2O$ solution and 4mL of a filter-sterilised 3M glucose solution was added.

MS medium

MS-salt mix (Sigma)	0.44% (w/v)
Sucrose	3.0% (w/v)

The pH was adjusted to 5.7 with a 1M NaOH stock solution.

MS Plates

MS plates were made by adding 0.9% (w/v) of Agar agar (Merck) to 1L of liquid MS media before autoclaving.

Shoot medium

MS	
NAA (Sigma)	0.2mg/L
BAP (Sigma)	1.0mg/L
Carbencillin (Sigma)	500.0mg/L
Kanamycin (Sigma)	100.0mg/L

Root medium

½MS	
Carbencillin (Sigma)	500.0mg/L
Kanamycin (Sigma)	100.0mg/L

II.2.3 Growth of transgenic plants

All primary transformants were grown in soil under standard conditions (25-30°C, 70-90% humidity, and 12 hours light/day) to obtain seeds and to monitor appearance of phenotypic alterations.

II.2.4 Characterisation of transgenic plants

Transgenic plants were analysed by Southern and Northern analyses. Southern analysis was performed to analyse the arrangement and the copy number of the integrated T-DNAs. Northern analysis was performed to detect transgene expression.

II.2.4.1 Extraction of genomic DNA from plants

Genomic DNA was extracted from plants according to the method of Dellaporta and co-workers (1983). One gram of fresh leaf tissue or of stored samples (-80°C) were quick frozen in liquid nitrogen and ground to fine powder in a mortar and pestle. The powder was transferred into 30mL Oak Ridge tubes (Nalgene) containing 15mL extraction buffer. One millilitre of 20% (w/v) SDS was added to the tubes and mixed by vortexing. Tubes were then incubated at 65°C for 10min. After incubation 5mL of 5M Potassium acetate was added and the sample was mixed by vortexing. Tubes were then incubated on ice for 20min prior to centrifugation in a Sorvall Superspeed RC2-B (17000rpm/4°C/20min). After the centrifugation, the supernatant was filtered through Miracloth into 30mL Oak Ridge containing 10mL isopropanol. The tubes were gently inverted several times and incubated at –20°C for 30min. After centrifugation (20000rpm/4°C/15min) the supernatant was discarded and the DNA pellet was dried by inverting the tubes on paper towel for 10min. The DNA was re-suspended in 0.7mL high salt TE buffer (pH 8.0) and the solution was transferred into 1.5mL Eppendorf tubes. Tubes were spun in an Eppendorf centrifuge (14000rpm) at RT for 10min. The supernatant was transferred into 1.5mL Eppendorf tubes and 75µL of 3M sodium acetate (pH 4.6), and 500µL isopropanol were added. Samples were gently mixed to precipitate the DNA. The precipitate was centrifuged in an Eppendorf centrifuge (14000rpm/RT/30sec) to pellet the DNA. The DNA pellet was washed with 70% (v/v) ethanol and the tubes were re-centrifuged as before (14000rpm/RT/1min). The supernatant was discarded and the pellet was air-dried. Finally, the pellet was dissolved in 400µL-500µL TE buffer (pH 8.0) or water. Samples were treated with DNase-free RNase (10-20µL of a 500µg/mL stock) and a 5µL aliquot of the sample was electrophoresed on a 0.8% (w/v) agarose gel to determine the quality and quantity of the DNA.

Extraction buffer

Tris (pH 8.0)	100mM
EDTA (pH 8.0)	50mM
NaCl	500mM

10mM β-mercaptoethanol were freshly added to inactivate DNase activities.

High salt TE		**TE**	
Tris (pH 8.0)	50mM	Tris (pH 8.0)	10mM
EDTA (pH 8.0)	10mM	EDTA (pH 8.0)	1mM

Loading buffer (1 BP: 1XC)

Bromo-phenol blue sodium salt (Sigma)	0.2%(w/v)
Xylene cyanol FF (Sigma)	0.2%(w/v)
Sucrose in water	40.0%(w/v)

II.2.4.2 Southern blot analysis

Genomic DNA (10-15µg) was digested with suitable restriction enzymes overnight. Digested samples were phenolised by mixing the samples with equal volumes of phenol:chloroform. Samples were centrifuged in an Eppendorf centrifuge (13000rpm/RT/30sec) to separate the aqueous and organic phases. The aqueous layers were transferred into new 1.5mL Eppendorf tubes and mixed with loading dye (1/10 volume). Digested DNAs were electrophoresed [0.8 to 1.5% (w/v) agarose gels] at 130mA for 7-8 hours. In case of Southern hybridisation of genomic DNA as well as of PCR products, after the run, gels were photographed under UV-light and processed for DNA transfer to positively charged nylon membranes according the following procedure:

1x **Depurination** (0.2N HCl) for 10min.
Rinse with water.
1x **Denaturation** (0.5M NaOH, 1.5M NaCl) for 45min.
Rinse with water.
2x **Neutralisation** (0.5M Tris, 1.5M NaCl, 0.001M EDTA; pH 7.2) for 15min.

The treated agarose gel was proceeded for capillary transfer as described by Sambrook *et al.*, 1989. The DNA was UV-cross-linked (0.3J/cm² at 254nm) and then was submitted to overnight hybridisation according to Amasino (1986). The DNA immobilised on the membrane was hybridised to a random primed α^{32}P dCTP labelled DNA probe which was made according to manufacturers instructions (Ladderman™ labeling Kit). Purification of the probes was performed by chromatography through Sephadex G50 columns. 200µL fractions were collected in 1.5mL Eppendorf tubes. The first 700µL fraction is expected to not contain any radioactivity and was discarded. Fractions were obtained by placing the labelled probe and subsequently 200µL water aliquots onto the column. After the first 700µL fraction that was discarded, the next three fraction usually contained the leading peak of radioactivity (2-3K impulse per sec-IPS) comprising the labelled DNA fragment.

Fractions were collected until a second peak was eluted from the column. This peak consisted of unincorporated nucleotides. After hybridisation in a Hybaid Hybridisation Oven (MWG, Germany) at 42°C for 12-16 hours, the blot was washed with Wash buffer I (42°C, 2 hours) and then with Wash buffer II for 15-30min at 65°C. After washing, the filter was dried for 15-20min and covered with Saran Wrap. Identification of hybridising DNA fragments was done by autoradiography. Depending on the signal strength the exposure time varied from one hour to several days. Exposure was performed at -80°C using signal amplification screens [Perlux Extra Rapid Screens 200; Harkness Hall (UK)]. In case of re-hybridisation with another probe, the blot was stored for 2-3 weeks to let the radioactivity of previous hybridisation cease off.

Wash buffer I		**Wash buffer II**	
NaPi (pH 7.2)	0.25M	NaPi (pH 7.2)	0.05M
EDTA (pH 8.0)	1.00mM	EDTA (pH 8.0)	1.00mM
SDS	1%(w/v)	SDS	1%(w/v)

NaPi = 1M Na_2HPO_4 + 1M NaH_2PO_4 (desirable pH attained by mixing the two buffers as described by Sambrook *et al.*, 1989).

II.2.4.3 RNA isolation from plants

Total RNA was extracted from liquid nitrogen frozen young leaves in a 8M Guanidine-HCl buffer (pH 7.0) according to the method of Logemann *et al*., 1987.

II.2.4.4 Northern blot analysis

RNA samples were denatured according to the following procedure:

RNA (5µg-10µg)	20.0µL
Formaldehyde 37% (w/v)	20.0µL
MEN (10x)	4.4µL
Ethidiumbromide (10mg/mL stock)	0.1µL

Samples were incubated at 55°C for 15min before cooling to 0°C on ice. After cooling the samples were centrifuged in an Eppendorf centrifuge at 14000rpm for 15sec.

Subsequently, samples were mixed with loading buffer (1BP:1XC) and electrophoresed on a RNA-denaturating agarose gel [~1.2% (w/v); see below] for 3-4 hours.

The gel was proceeded for Northern analysis as follows:

1x **Denaturation** (0.05M NaOH, 1.5M NaCl) for 20min.
Rinse with water.
2x **Neutralisation** (0.5M Tris pH 7.4, 1.5M NaCl) for 15min.
1x **DEPC** Water for 5min.

The denatured agarose gel was proceeded for capillary transfer of RNA onto uncharged nylon membranes as described in Sambrook *et al.*, 1989. The RNA was UV-cross-linked (0.3J/cm² at 254nm). The membrane was proceeded for pre-hybridisation at 42°C for 6h and hybridised in a Hybaid Hybridisation Oven (MWG, Germany) at 42°C for 12-16 hours. The RNA immobilised on the membrane was hybridised to a random primed α^{32}P dCTP labelled DNA probe made according to manufacturers instructions (Ladderman™ labeling Kit). Purification of the probes was done by chromatography through Sephadex G50 columns as described before (II.2.4.2). After hybridisation the blot was washed with Wash buffer I at 22°C for 30min and with Wash buffer II at 65°C for 15min. After washing, the filter was dried for 15-20min and covered with Saran Wrap. Visualisation of hybridising RNA was done by autoradiography. Depending on the signal strength the exposure time varied from several hours to several days. To increase signal strength an intensifying screen was placed behind the X-ray film. Whenever intensifying screens were used, exposure was performed at -80°C. Size determination of hybridising RNA was done by comparing the hybridisation fragments with the size of the ribosomal RNA (rRNA). Below are the sizes of the rRNAs that are commonly found in plants.

rRNA	Length in nucleotides
25S	3400
18S+23S	1800
16S	1500

S= Svedberg units (unit for sedimentation coefficient)

8M Guanidine-HCl buffer (1L)

Guanidium hydrochloride	8M
2-Morpholinoethane sulfonic acid (MES)	20mM
EDTA	20mM

The pH was adjusted with NaOH to 7.0 and 50mM β-mercaptoethanol was freshly added to inactivate RNase activities.

10x MEN

MOPS	200mM
Sodium acetate	50mM
EDTA (pH 7.0)	10mM

Northern pre-hybridisation/hybridisation buffer

20xSSC	125mL
De-ionised formamide	250mL
50x Denhardt's	10mL
SDS [10%(w/v)]	50mL
t-RNA (12.5mg/mL)	5mL
Dextran-sulphate (Sigma)	10%(w/v)

50x Denhardt's reagent

Ficoll (type 400, Pharmacia)	1%(w/v)
Polyvinylpyrrolidone	1%(w/v)
Bovine serum albumin	1%(w/v)

Wash I		**Wash II**	
SSC	1.0x(v/v)	SSC	0.1x(v/v)
SDS	0.1%(v/v)	SDS	0.1%(v/v)

20x SSC pH 7.0

NaCl	3M
Tri-Sodiumcitrate	0.3M

RNA-Denaturating gel

Water	100.0mL
MEN(10x)	13.5mL
Agarose	1.4g = 1.23%(w/v)

The solution was mixed, boiled, then cooled to 60-70°C, and finally 21mL of formaldehyde 37% (w/v) was added.

DEPC water contained 0.1% (v/v) DEPC and was autoclaved.

II.2.4.5 Radiolabelled DNA probes

The different gene fragments used as probes in Southern and Northern hybridisations were i) the RdRP²-specific fragment cTomRes$_{8X/S}$ (III.2.1.1.1), ii) the spacer-specific fragment I$_{X510}$ (Intron4) (III.2.1.1.1), iii) the RdRP¹-specific (Rd¹$_{5Sac}$) fragment with a size of ~560 bp was isolated from the RdRP24 clone (Schiebel *et al*., 1998) by SacI digestion, iv) the nptII-specific (npt$_8$) probe that was isolated by SphI digestion of the pPCV702SM was ~820 bp long fragment, v) the PVX-specific fragment (PVX$_p$) with a size of ~1.3 kb was isolated by HindIII digestion of the PVX vector (pP2C2S), and vi) the GFP-specific probe (G$_{FP}$) that was isolated from the GFP-pT3T7 (Wassenegger, unpublished results) by double digestion with BamHI/XbaI was ~850 bp long fragment.

II.2.4.6 Inoculation of plants with the *in vitro* transcripts

Plants were manually inoculated with infectious PVX-RNA. Infectious PVX-RNA was synthesised from the pP2C2S derivatives by *in vitro* transcription using the T7 polymerase. All plasmids were linearised with suitable restriction enzymes and proceeded for T7 transcription according to previously described procedures (Chapman *et al*., 1992; Baulcombe *et al*., 1995) using the SP6/T7 Transcription Kit. All the *in vitro* transcripts were capped using the mCAP^{TM} RNA capping Kit to protect the 5'end of the transcripts from degradation as well as to enable their *in vivo* translation. Transcripts were analysed on formaldehyde gels to examine transcript quality and quantity. Samples were directly used for inoculations according to the standard methods of Chapman and co-workers (1992) and Baulcombe and co-workers (1995), respectively.

III Results

III.1 Amino acid alignment of the RNA-directed RNA polymerase (RdRP) homologues within and across plant species

III.1.1 Data base search and alignment

The RNA-directed RNA polymerase (RdRP) with a size of 127 kilo dalton (kDa) was previously purified from the *Lycopersicon esculentum* cultivar Rentita (tomato) and characterised by Schiebel *et al.*, 1993. Isolation of the first full-length ~3.6 kilo base pairs (kb) cDNA clone and identification of RdRP homologues in four additional higher plant species [*Petunia hybrida* cv V26, *Triticum aestivum* (wheat), *Nicotiana tabacum* cv Petit Havana SR1, and *Arabidopsis thaliana* ecotype Columbia] came from Schiebel *et al*. (1998). They aligned the amino acid sequences obtained from these four homologues with the tomato RdRP and found that there were regions that are almost identical among all putative RdRPs. To update this information, a new homology search (Table A) was carried out using the 1114 amino acid (AA) long sequence of the tomato RdRP as a family member. The amino acid sequences that matched the tomato RdRP were aligned and compared. The alignment revealed the presence of homologues from *A. thaliana* (accession numbers AC006917, AC012329, AF080120), *Pinus taeda* (BE431396), *Solanum tuberosum* (BF460205) (potato) and two Expressed Sequence Tags (ESTs) from tomato (AI896465, AI774429). Alignment of these sequences showed that *A. thaliana* has, at least, three different types of RdRPs. This was recognised by the presence of three highly conserved regions. Moreover, the same conserved regions were found in all plant homologues (Table A). Regions flanking these motifs were highly variable. The three types of RdRPs identified by this alignment were numbered as follows:

A. thaliana RdRP AC006917 similar to the tomato RdRP = **RdRP^{1}**
A. thaliana RdRP AF080120 = **RdRP^{2}**
A. thaliana RdRP AC012329 identical with the SDE1 = SGS2 gene = **RdRP^{3}**

Based on the fact that, at least, three different RdRPs exist in *A. thaliana*, it was speculated that also in tomato three RdRPs might be present.

Table A: Alignment of highly conserved regions between the RdRP homologues.
Bold amino acid residues represent the conserved sequences. Full-length RdRP sequences were not available from all plants. Therefore, the sequences that are presented in the three blocks are not derived from identical plant species. Alignment was done manually by aligning the best fitting amino acid residues. Location of the three conserved regions within the cDNA of tomato RdRP is indicated in Table A/1 (page 43).

Conserved region 1 (19 amino acids)

PSAFQIRYGGYKGVVG**VDP**	RdRP[1], tomato
PSS**FQIRYGGYKGVVAVDP**	RdRP[1], tobacco
PSAFQIRYGGYKGVVAVDP	RdRP[1], *Petunia*
PSAFQIRYGGYKGVVAVDP	RdRP[1], *A.thaliana*
PSAFQIRYGGYKGVIAVDR	RdRP[2], *A.thaliana*
PSAFQIRYGGYKGVIAVDR	EST, putative RdRP[2], tomato
PC**A**Y**QIRY**A**GF**K**GVV**A**RW**P**	RdRP[3] (SDE1/SGS2), *A.thaliana*

Conserved region 2 (19 amino acids)

VVVA**KNPCLHPGDIRVLK**A	RdRP[1], tomato
VVVA**KNPCLHPGDIRVLR**A	RdRP[1], tobacco
VVVAKNPCLHS**GDVRVLQ**A	RdRP[1], *A.thaliana*
A**VVAKNPCLHPGDIRILEA**	putative RdRP[1], *Pinus*
VVVT**KNPCLHPGDIRVLD**A	RdRP[2], *A.thaliana*
VVVT**KNPCLHPGDVRVLE**A	putative RdRP[2], potato
VAIA**KNPCLHPGDVRILEA**	RdRP[3] (SDE1/SGS2), *A.thaliana*

Conserved region 3 (22 amino acids)

RPHPNECSGSDLDGDIYFVCW**D**	RdRP[1], tomato
RPHPNECSGSDLDGDIYFVCW**D**	RdRP[1], tobacco
RPHPNECSGSDLDGDIYFVCW**D**	RdRP[1] *A.thaliana*
-**PHPNECSGSDLDGDIYFVS**W**D**	putative RdRP[1], wheat
RPHPNECSGG**DLDGDLYFIC**W**D**	putative RdRP[2], potato
RPHPNECSGG**DLDGD**Q**FFVS**W**D**	RdRP[2] *A.thaliana*
RPHTN**EA**S**GSDLDGDLYFVA**W**D**	RdRP[3](SDE1/SGS2), *A.thaliana*

III.1.2 A tomato EST comprising sequence homology with the *Arabidopsis thaliana* RdRP[2]

The amino acid sequence of an auto-translated tomato EST (AI774429) with a size of 433 bp displayed strong homology to the *A. thaliana* RdRP[2]. The 5'-3' orientation of the sequence as it was presented in the data base represented a fragment of an antisense strand of a putative RdRP mRNA. Therefore, the complementary strand of this sequence was translated using the DNASIS® standard codon usage programme. The amino acid sequence of the continuous open reading frame (ORF) revealed the presence of the conserved region

1, PSAFQIRY (Table A), that is present in all RdRP homologues. On alignment with the three members of the *A. thaliana* RdRP family, it was inferred that the EST resembles more the $RdRP^2$ than the $RdRP^1$ or $RdRP^3$ sequence (data not shown).

III.1.3 Isolation of a $RdRP^2$-specific cDNA fragment from tomato

The tomato EST (AI774429) was used to attain the restriction map of the sequence and to design the gene specific primers, TRs1 (forward, II.2.1.7.5) and TRsR2 (reverse, II.2.1.7.5). Using this primer-pair and the *L. esculentum* cultivar Rentita genomic and cDNA [genomic DNA (II.2.4.1) cDNA synthesis (II.2.1.7.4)] a fragment (TomRes) corresponding to the EST was amplified by the polymerase chain reaction (II.2.1.7). Both amplified products (genomic and cDNA) were expected to be ~320 bp in size (Fig. 1).

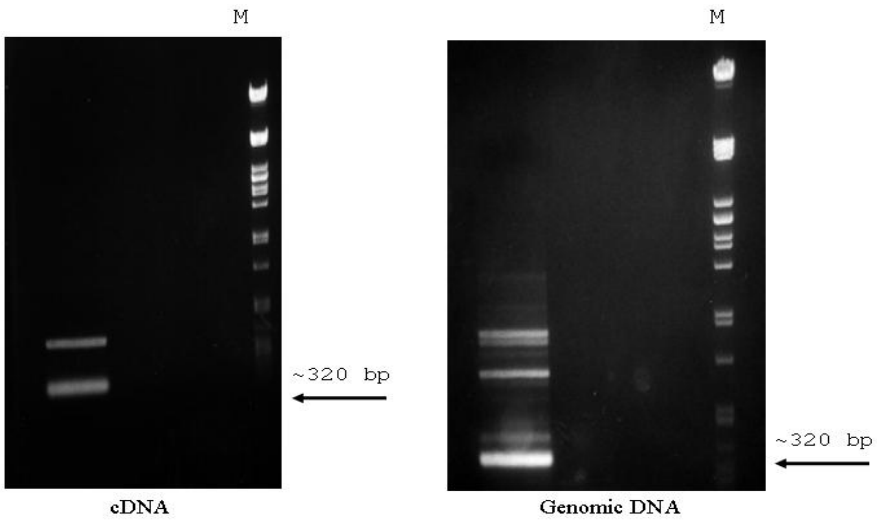


Figure 1: Isolation of a TomRes-specific EST fragment from tomato.
PCR amplification using the TRs1/TRsR2 primer pair (II.2.1.7, annealing temperature 50°C) of the EST TomRes-specific fragment from tomato cDNA and genomic DNA.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2).

PCR products with the corresponding size were gel-purified and cloned into the pGEM-T easy vector (II.1.5). Because no internal EcoRI restriction endonuclease sites were found within the EST but were flanking the vector cloning site at both ends, the entire insert of positive recombinant pGEM-T easy plasmids could be released by this enzyme. Two positive clones, the $gTomRes_{320}$ (from genomic DNA) and the $cTomRes_{320}$ (from cDNA) were sequenced with sp6/T7 primers (II.1.6). Sequence comparison of the two clones with the EST showed a single A→C transversion leading to an amino acid substitution from

isoleucine (I) to leucine (L). Leucine was one of the conserved amino acid among all the RdRPs. This may indicate that the published EST contained an error, while the gTomRes$_{320}$ and the cTomRes$_{320}$ clones comprised the authentic sequence (see also III.1.4).

III.1.4 Isolation of a 3'end-specific fragment of the RdRP^2 gene from tomato and tobacco

Using 5'/3' rapid amplification of cDNA ends (RACE; II.2.1.7.3), the first cDNA strand was synthesised from total RNA (II.2.4.3) of the *L. esculentum* cultivar Rentita. This strand was used for PCR amplification with the 3'end-specific primer, TRs2 (forward, II.2.1.7.5) and the oligo dT-anchor (RACE, II.2.1.7.5) according to the standard PCR amplification method (II.2.1.7). The PCR-amplified product was separated on a 1% (w/v) agarose gel but after electrophoresis no band was visible on the ethidiumbromide-stained gel under UV light. Therefore, re-amplification (II.2.1.7.5) was performed with the nested primer, TRs3 (forward, II.2.1.7.5) that bound ~170 bp downstream to the TRs2 primer binding site. As a reverse primer the anchor primer of the RACE Kit was used (II.2.1.7.5). To identify the RdRP^2 3'end-specific fragment, Southern hybridisation of the PCR products was carried out (II.2.4.2). The re-amplified PCR products were hybridised with the cTomRes$_{320}$ (III.1.3) as probe. The probe had an overlap of ~260 bp with the expected 3'end fragment. After hybridisation, a positive signal was detected at a position that corresponded to a fragment with a size of ~1.5 kb (data not shown). This gene-specific fragment was gel-purified (II.2.1.3) and cloned into the pGEM-T easy vector (II.1.5). Recombinant clones were screened for the insert, by EcoRI digestion of the plasmid (II.2.1.1.1). As mentioned above (III.1.3), EcoRI sites were located at both ends of the pGEM-T easy cloning site enabling the release of the entire insert by this enzyme. However, insert-specific fragments with sizes of ~840 bp, ~520 bp and ~120 bp were detectable on the gel (data not shown) indicating that two additional EcoRI sites were present within the ~1.5 kb fragment. This assumption was confirmed by sequencing three of the clones (cTomRes$_{1.5}$/1-3) with sp6/T7 primers (II.1.6). Base substitution during PCR or sequencing are inevitable. To deduce the authentic gene sequence of the RdRP^2 cDNA 3'end, the sequences of the three independent cTomRes$_{1.5}$ clones were compared. Sequence deviations that were present in the minority of the clones were regarded as PCR or sequencing errors. Following this strategy a clone could be selected that contained the

authentic tomato RdRP^2 cDNA 3'end. Moreover, with respect to the tomato EST, sequence analysis confirmed the A→C transversion that was already found in the gTomRes$_{320}$ and the cTomRes$_{320}$ clones (III.1.3).

The 3'end of the *N. tabacum* cv Petit Havana SR1 RdRP^2 cDNA was isolated by using the same primer combinations and applying the same procedure as described for tomato (see above). It should be noted that sequence comparison of the Rentita RdRP^1 (full-length) with the tobacco RdRP^1 (full-length) showed 90% identity at the DNA and 86% identity at the amino acid level. In view of this extensive homology, the PCR product amplified from the tobacco cDNA was hybridised against a tomato RdRP^2-specific cDNA fragment as probe. The fragment with a size of ~310 bp was obtained by HindIII digestion of the cTomRes$_{1.5}$ clone. After hybridisation, the strongest positive signal was detected at a position that corresponded to a fragment with a size of ~1.5 kb (Fig. 2A and B).

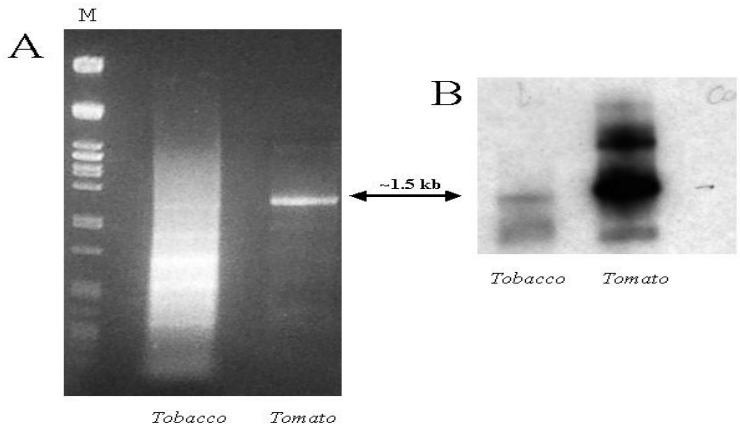


Figure 2: Isolation of the RdRP^2 3'end from tobacco.
Re-amplification (II.2.1.7.5) was carried out using the nested primer (TRs3) and the anchor primer (RACE Kit) (annealing temperature 64°C).
A) The 1% (w/v) agarose gel (II.2.1.2, 50mA, Two hours) of re-amplified PCR product obtained from tobacco and tomato cDNA.
B) Southern blot [II.2.4.2, 40000 Impulse per sec (IPS), 12 hours hybridisation, 45min exposure time] of the RdRP^2 3'end re-amplified PCR product from tomato and tobacco hybridised with a ^{32}P-labelled ~310 bp fragment that was obtained by HindIII digestion of cTomRes1.5 clone. The tomato re-amplified product was taken as control.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2).

This fragment was gel-purified (II.2.1.3) and cloned into the pGEM-T easy vector (II.1.5). The clones were EcoRI-digested and those containing an insert with a size of ~1.5 kb were sequenced with sp6/T7 primers (II.1.6). Sequence data revealed that the inserts had two EcoRI sites. However, these sites were located close to the ends of the insert. Thus, an insert with a size of ~1.5 kb was released upon EcoRI digestion. Sequence comparison of

three tobacco RdRP^2 cDNA clones (cTob$_{1.5}$) with the sequence of the cTomRes$_{1.5}$ clone demonstrated that all sequenced cTob$_{1.5}$ clones contained the RdRP^2 homologue. In order to examine whether sequence substitutions occurred during PCR amplification or sequencing, the sequence data of the three independent positive clones were compared. Sequence deviations that were present in the minority of clones were regarded as PCR or sequencing errors. Following this strategy a clone could be selected that contained the authentic tobacco RdRP^2 cDNA 3'end. However, because the TRs2/TRs3 primers that were used to amplify the tobacco RdRP^2-specific 3'end were deduced from the tomato sequence it could not be excluded that mismatches within the primer binding sites were present in the cTob$_{1.5}$ clone.

As stated earlier, the entire RdRP^1 sequence from tomato and tobacco exhibited high identity at the DNA level. To verify that the same applies for the RdRP^2 sequences of these species, the sequence of the cTomRes$_{1.5}$ was compared with the cTob$_{1.5}$ sequence. Similarity between the two was 90.9% at the DNA and 89% at the amino acid level. Based on the high degree of homology between the two plant species, it should be possible to utilise tomato-specific RdRP^2 sequences for the initiation of RNAi in tobacco plants. Although functional analysis of RdRP homologues was planned in the tobacco system, characterisation of RdRP gene sequences was concentrated on tomato homologues. This was done for two reasons: i) production of transgenic tobacco plants is feasible and faster than tomato transformation and ii) procedures for RdRP protein isolation were established for tomato (Schiebel *et al*., 1993a, b and 1998). Moreover, one of the goals of this work was to identify which RdRP was involved in the establishment of the "TK-phenotype" in *N. tabacum* SR1 (I.3, Photographs). This phenotype was found in tobacco plants carrying a full-length tomato RdRP^1 cDNA antisense construct (Wassenegger, unpublished results).

III.1.5 Isolation of the RdRP^2 cDNA 5'end from tomato

Using the 5'RACE technology, first strand cDNA synthesis was performed with the RdRP^2 gene-specific primer TRsR3. To the 3'end of the first cDNA strand a homopolymeric tail was added (II.2.1.7.3). The dA-tailed cDNA was used for PCR amplification with the nested RdRP^2 gene-specific primer TRsR6 (II.2.1.7.5) in combination with the oligo dT-anchor (RACE, II.2.1.7.5). The TRsR6 primer bound 127 bp downstream to the TRsR3 primer binding site. The PCR products were analysed on a 1% (w/v) agarose gel (II.2.1.2) and then processed for Southern hybridisation (II.2.4.2). To identify the gene-specific

fragment, the blot was hybridised with the cTomRes$_{320}$ (III.1.3) as probe. The probe had an overlap of ~60 bp with the expected 5'end fragment. After hybridisation, a positive signal was observed at a position that corresponded to a fragment with a size of ~1.5 kb (Fig. 3A and B).

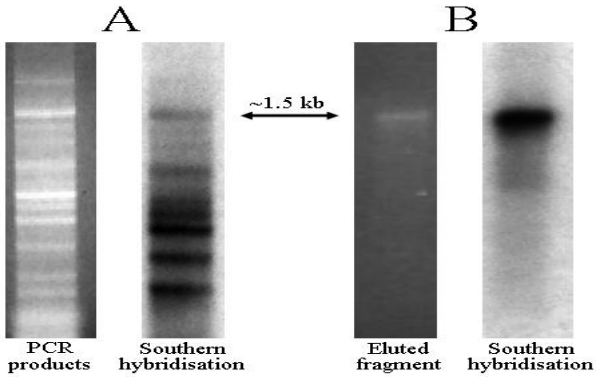


Figure 3: Isolation of the tomato RdRP^2 5'end.
PCR products obtained with the nested TRsR6 (II.2.1.7.5) and oligo dT-anchor (RACE, II.2.1.7.5) primers (annealing temperature 64°C).
A) The 1% (w/v) agarose gel (II.2.1.2, 50mA, two hours) of the PCR products and the corresponding Southern blot [II.2.4.2, 40000 Impulse per sec (IPS), 12 hours hybridisation, two hours exposure time] hybridised with the ^{32}P-labelled cTomRes$_{320}$ (III.1.3,) probe is presented.
B) RdRP^2 5'end-specific positive PCR fragments with a size of ~1.5 kb were gel-purified (II.2.1.3) and run on a 1% (w/v) agarose gel to confirm the previous Southern hybridisation (A). The gel-purified RdRP^2 5'end-specific fragments were probed with the ^{32}P-labelled cTomRes$_{320}$ [III.1.3, 40000 Impulse per sec (IPS), 12 hours hybridisation, 48 hours exposure time].

The gene-specific 5'end fragment was gel-purified (II.2.1.3) and cloned into the pGEM-T easy vector (II.1.5). The transformants were verified for the insert by EcoRI digestion as described previously (III.1.3). Upon EcoRI cleavage inserts with a size of about 1.5 kb but with variable lengths were released. Three clones carrying inserts with different lengths were sequenced with the sp6, T7, 5'RdRP^2 internal sp, and the 5'RdRP^2 internal t7 primers (II.1.6). Sequences that overlapped with cTomRes$_{320}$ (III.1.3) were considered as positive. To, again, overrule sequence substitutions that might have occurred during PCR amplification or sequencing, the sequences of three independent positive clones (c5TomRes$_{1.5}$/1-3) were compared. Sequence comparison revealed that the length of different inserts varied from about 1.45-1.6 kb (including the dA-tail). Because, the 3'end-

specific TRsR3 primer was used for first strand cDNA synthesis (II.2.1.7.3) different insert sizes must be due to variable 5'ends. These variations could be the result of inefficient reverse transcription or of assaying mRNA templates with heterogeneous 5'ends. The RdRP^2 5'end sequence was deduced from the c5TomRes$_{1.5}$/3 clone having the longest insert. In order to obtain the entire RdRP^2 cDNA sequence, this 5'end (c5TomRes$_{1.5}$), cTomRes$_{320}$, and the 3'end (cTomRes$_{1.5}$) sequences were assembled revealing a continuous RdRP^2 cDNA sequence of ~3.1 kb. The cDNA sequence was translated using the DNASIS® programme (standard codon usage). The amino acid sequence of the continuous open reading frame (ORF) was manually aligned (best fit) with the amino acid sequences of all other RdRPs (data not shown). In comparison with other homologues, the tomato RdRP^2 ORF appeared to lack ~120 AA at the N-terminus.

The missing RdRP^2 5'end was isolated again using the 5'RACE technology. The first cDNA strand was synthesised with the 5'-specific 5'RdRP^{2}revB primer (II.2.1.7.5). The homopolymeric tail was added to the first cDNA strand in order to obtain a dA-tailed cDNA (II.2.1.7.3). The dA-tailed cDNA was used as template for PCR amplification with the nested 5'RdRP^{2}revC primer. The nested primer bound 33 bp downstream to 5'RdRP^{2}revB primer binding site (II.2.1.7.5). The PCR products were electrophoresed on a 1% (w/v) agarose gel. The products were further processed for Southern hybridisation (II.2.4.2). A 5'end-specific fragment with a size of ~480 bp was used as probe. This probe was obtained by digestion of the c5TomRes$_{1.5}$ clone (see above) with EcoRI/BamHI (II.2.1.1.1). The overlap of this probe with the expected RdRP^2 5'end was ~60 bp. A hybridisation signal was detected at a position that corresponded to a fragment with a size of ~530 bp (Fig. 4A and B). The 5'end-specific fragment was eluted from the gel (II.2.1.3) and cloned into the pGEM-T easy vector (II.1.5). The ~530 bp gel-purified PCR product was analysed for the presence of the insert by EcoRI cleavage (II.2.1.1.1). As no internal sites were detectable, the recombinant clones were expected to release the entire ~530 bp insert upon EcoRI digestion. Similar to c5TomRes$_{1.5}$ clones (see above), these clones also showed variable 5'ends giving inserts ranging from about 400-500 bp. Clones containing the largest inserts were sequenced with sp6/T7 primers (II.1.6). Sequences that comprised a part of the c5TomRes$_{1.5}$ sequence were considered as positive. Sequence comparison of three independent clones (cTomRes$_{500}$) helped to determine base substitutions that might

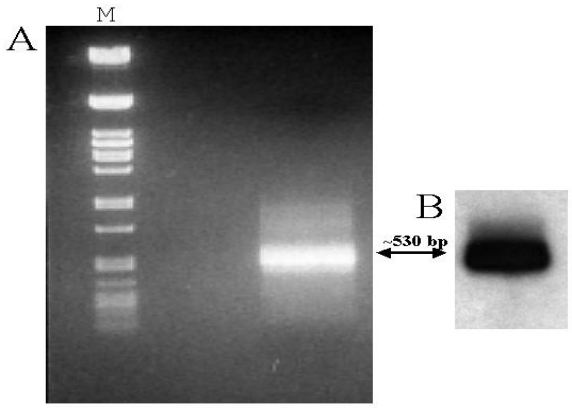


Figure 4: Isolation of the missing tomato RdRP[2] 5'end.
PCR products obtained with the nested 5'RdRP[2] RevC (II.2.1.7.5) and the oligo dT-anchor primers (RACE, II.2.1.7.5) (annealing temperature 63°).
A) The 1% (w/v) agarose gel (II.2.1.2, 50mA, two hours) of the PCR-amplified products.
B) Southern blot [II.2.4.2, 40000 Impulse per sec (IPS), 12 hours hybridisation, Five hours exposure time] of the PCR products hybridised with the ^{32}P-labelled ~480 bp fragment (see text). Positive hybridisation signal corresponded to a fragment size of ~530 bp.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2).

have occurred during PCR amplification or sequencing. Computer-supported assembly of the cTomRes$_{500}$ sequence with the previously assembled ~3.1 kb sequence revealed a continuous RdRP[2] cDNA full-length sequence of ~3.5 kb.

Table A/1: Amino acid sequence (1119 AA) of the RdRP[2] cDNA. The ~3.5 kb long RdRP[2] cDNA sequence was translated using the DNASIS® programme (standard codon usage). Bold amino acids represent the three conserved regions (1, 2 and 3) found on alignment of different RdRP homologues.

MGVEKRATAT VRVSNIPQSA IAKDLSNFFD SLIGKGSVFA CDIHSEHKNW KSRGHGRVQF
ETLQDKLHCL SLAEQGNLLF KGHQLSLVSS FDDIITRPVE PKCRFQAGIL HTGLLVEKDV
MQVLETWEDV KTLIMPERKC LEFWVSHAEE CYRLEVQFGD VTEGTLCSVE NQKSALLLKL
KHAPKLYQRV SGPAVASKFS ADRYHICKED CEFLWIRTTD FSNIKSIGCS SSLCWETEDG
WLSSDLFSSL PCCNQDVIDL DLDKVGDIYS GSEFVPLVRI PSDLKLPYEI LFQLNSLVQT
QKISLGAINP NLIEVLSKLE LDTAMMILQK MHKLQSICFD PLLFIKTRLH VLGKNNKNRP
SSSYSRLVNH SMMSVHRVLV TPSKIYCLGP ELETSNYIVK NFASHASDFL RVTFVEEDWG
KLFPNAVSMS VEQGIFAKPY RTKIYHRILS ILREGIVIGT KRFFFLAFSA SQLRSNSVWM
FASNEYVKAE DIREWMGCFN KIRSISKCAA RMGQLFSTSV QTMEVKLQHV EILPDIEVTS
DGVSYCFSDG IGKISQAFAR QVAQKCGLNH **TPSAFQIRYG GYKGVIAVDR** NSFRKLSLRG
SMLKFESKNR MLNITKWSDA MPCYLNREIV ILLSTLGVED KAFEDLLDNH LCLLGKMLTT
NEAALDVLES MGGGEVKKIL MRMLLQGYAP NQEPYLSMML QSHFENQISD LRSRCRIFIP
KGRILVGCLD ETGILKYGQV YVRITMTKAE LQNGQQNFFQ KVDETTAVVR GK**VVVTKNPC**
LHPGDVRVLE AVYEVTLEEK TWVDCIIFPQ KGE**RPHPNEC SGGDLDGDLY FICWD**ESLIP
CQTVTPMDYT GRRPRIMDHE VTLEEIQRFF VDYMISDTLG AISTAHLVHA DREPDKALNS
KCLQLATLHS MAVDFAKTGA AAEMPRFLKP REFPDFMERW DKPMYISEGV LGKLYRGVMK
SYIRRNSDDL SADRAIQDAY DHDLLVEGYE AFTETAKTHK AMYLDSMNSL LNYYGAEKEV
EILTGNLRQK SVYLQRDNRR YFELKDRILV SAKSLHKEVK GWFTSCCQED DHQKLASAWY
HVTYHPSYCH ESANCLGFPW VVGDILLNMK SHNTRKTIP

This sequence was translated using the DNASIS® programme (standard codon usage). The amino acid sequence was aligned with all known RdRP homologues. The alignment showed that the RdRP^2 cDNA sequence comprised the entire ORF (Table A/1).

III.2 Designing of RdRP^1 and RdRP^2 transgene constructs for stable plant transformation

No developmental abnormalities were reported for mutated *A. thaliana* plant lines carrying a non-functional RdRP^3 gene (SGS2, SDE1) (Dalmay *et al*., 2000b; Mourrain *et al*., 2000). This observation suggested the unlikelihood of RdRP^3 to be involved in normal gene regulation. The fact that some of the tobacco plants expressing a full-length RdRP^1 antisense construct established an aberrant TK-phenotype (Wassenegger, unpublished results) led to the speculation that RdRP^1 might be involved in normal gene regulation. However, it was reported that RdRP^1 activity increased upon virus or viroid infection (Astier-Manifacier and Cornuet, 1971; Van der Meer *et al*., 1984; Schiebel *et al*., 1993a). In addition, Xie and co-workers (2001) found increased virus accumulation in RdRP^1-deficient tobacco plants pointing to an involvement of this enzyme in control of virus replication rather than in normal gene regulation. If the RdRP^1 contributes to combat viruses and the RdRP^3 is essential for transgene-mediated PTGS what is the biological function of the RdRP^2 in plants? To gain information about this function, strategies were developed that aimed at the suppression/over-expression of the RdRP^2 gene. For this purpose RdRP^2 sense, antisense, inverted repeat, and direct repeat constructs were designed and introduced into tobacco plants. RdRP homologues contain highly conserved motifs (Table A). Therefore, gene constructs that are specific to only one of the RdRP sequences must lack such common motifs. Moreover, to avoid any cross-hybridisation between two nucleic acid sequences, regions of minimal homology should be selected. Minimal homology between the tomato RdRP^1 and RdRP^2 genes were found by comparison of their full-length cDNA sequences. The two full-length sequences showed an overall identity of 51.7% whereas the 3'ends of the genes displayed with 53.5% a slightly higher identity. However, 3'ends have been shown to more efficiently induce PTGS when compared to 5'end-specific gene fragments (English *et al*., 1996; Stoutjesdijk *et al*., 2002). In addition, it was shown that sequence identity of more than 78% was required to initiate RNAi (Parrish *et al*., 2000). Thus, to generate RdRP constructs that would be capable to

efficiently but specifically induce RNA-mediated gene silencing, RdRP^1 and RdRP^2 3'end fragments with sizes between 600 and 800 bp were screened for.

III.2.1 Designing of repeat constructs for endogenous RdRP suppression

III.2.1.1 Isolation and cloning of RdRP-specific 3'end fragments

III.2.1.1.1 Isolation and cloning of RdRP^2-specific 3'end fragments

As it was known from Hamilton and Baulcombe (1999), both transgene-induced and virus-induced PTGS in plants, involves processing of double-stranded RNA (dsRNA) into 21-25 nucleotide (nt), short interfering RNA (siRNA). In a sequence-specific manner, the siRNA mediates suppression of genes through targeted RNA degradation. In 2001, this observation was supported by the work of Thomas and co-workers. They found that complete complementarity between the inducer RNA and the target was required in that a 27 nt long RNA containing a single mismatch failed to initiate silencing of a reporter gene. In view of this, not only the overall identity of two fragments needs to be considered but also the homology of small regions with size of 23 nucleotides. Therefore, the tomato and tobacco RdRP^1 3'end-specific cDNA regions were at first compared with the RdRP^2 sequences of these plant species. The comparison revealed that sequence identities between them was less than 54%. Subsequently, the RdRP^1 sequence stretch displaying the longest continuous homology with the RdRP^2 gene (tobacco and tomato) was determined. In case of the RdRP^1 (tomato) and the RdRP^2 (tomato) this region comprised 11 nt. In case of the RdRP^1 (tomato) and the RdRP^2 (tobacco) this region comprised 8 nt. In none of these sequence comparisons, a continuous 23 nt long region was found. In addition, comparison of the RdRP^2 (tomato) with the RdRP^1 (tobacco) exhibited a stretch of only 8 continuous nucleotides. Based on these observations the tomato RdRP^2 3'end fragment with a size of ~800 bp was considered as a suitable sequence to suppress expression of the endogenous tobacco RdRP^2 gene. In summary, this fragment exhibited 53.5% homology with the tomato RdRP^1 and 52.8% homology with the tobacco RdRP^1. No common region of continuous 23 nt was present.

Inverted repeat (IR) constructs were used to enable *in vivo* synthesis of transcripts that can fold back into a hairpin structure forming dsRNA (panhandle structure). Production of such molecules was anticipated to initiate RNAi in plants (Wang and Waterhouse, 2000). RdRP^2 direct repeat (DR) constructs were expected to synthesise

linear transcripts that can not form dsRNA by intra-molecular hybridisation. Because these transcripts lack a dsRNA portion they would represent inefficient PTGS-inducing RNA molecules. To produce the IR and DR constructs a ~800 bp tomato $RdRP^2$ gene-specific fragment was isolated from the $cTomRes_{1.35Bam}$ clone. This $cTomRes_{1.35Bam}$ clone resulted from a PCR amplification of the $cTomRes_{1.5}$ clone (III.1.4) using the 3'HomforwardBamHI and the 3'HomReverseBamHI primers (II.2.1.7.5). It contained a fragment with a size of ~1.35 kb. By using the 3'HomforwardBamHI and the 3'HomReverseBamHI primers, BamHI sites were introduced at both ends of the ~1.35 kb fragment as shown below.

```
3'HomforwardBamHI
CTCCAAGGaTCCGCACCCAATC
GAGGTTCCTAGGCGTGGGTTAG........ cTomRes1.5....GTTGTTG ATCCTGCCTTATCC
                                              CAACAACCTAGGACGGAATAGG
                                                   3'HomReverseBamHI
```

The PCR product was run on a 1% (w/v) agarose gel and the expected ~1.35 kb fragment ($Rd^2_{1.3}$) was gel-purified (II.2.1.3) before cloning into the pGEM-T easy vector (II.1.5). The recombinant plasmids ($cTomRes_{1.35Bam}$) were analysed by BamHI digestion (II.2.1.1.1). Plasmids releasing a ~1.35 kb fragment were regarded as positive. The gene-specific fragment for the repeat constructs was isolated by digestion of this $cTomRes_{1.35Bam}$ clone with BamHI and XbaI. The corresponding 810 bp long fragment ($cTomRes_{810}$) was gel-purified and cloned into the BamHI/XbaI sites of the binary vector pPCV702SM (II.1.5; II.2.1.4; II.2.1.5). In a first screen, recombinants were analysed by "pooled PCR" (II.2.1.6.1). The amplification was done under standard PCR conditions (II.2.1.7.2) using the 710BiUS-35S as forward and the pNOS1020BiUS as reverse primers (II.2.1.7.5). The PCR products were electrophoresed on a 1% (w/v) agarose gel and samples showing the amplification of a fragment with the expected size of ~1.1 kb (Fig. B/I) were selected for further examinations. In order to find the colonies carrying the recombinant plasmid, DNA of each colony of a "positive pool" was isolated (II.2.1.6.2) and analysed by digestion with BamHI/XbaI (II.2.1.1.1). To determine the orientation of the insert, plasmids releasing a ~810 bp long BamHI/XbaI fragment were further analysed with HindIII and EcoRI, respectively. Based on the directed BamHI/XbaI cloning step the $cTomRes_{810}$ fragment should be integrated in antisense orientation with respect to the 35S promoter (Fig. B/I). Release of ~300 bp and ~270 bp fragments from the HindIII-cut plasmid verified that the recombinant pPCV702SM plasmid ($702cTR^-_8$) contained the $cTomRes_{810}$ in antisense

orientation (data not shown). This result could be confirmed by the EcoRI digestion. As calculated (Fig. B/I) a fragment with a size of ~700 bp was released (data not shown).

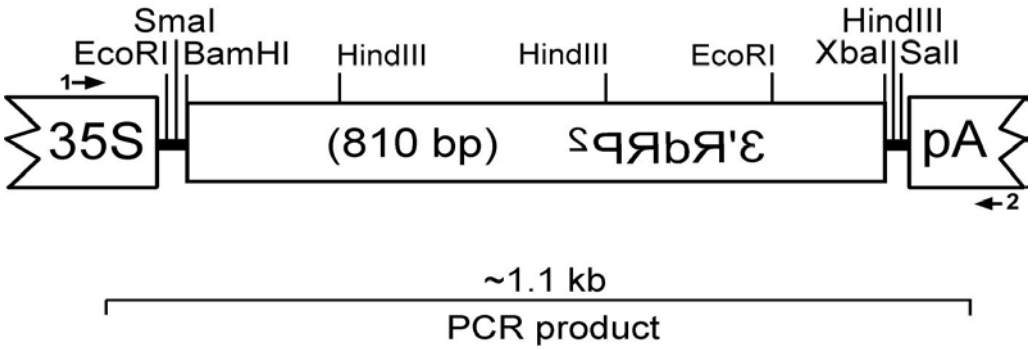


Figure B/I: Physical map of the 702cTR$^{-}_{8}$ construct obtained after the introduction of the 3'RdRP^{2} fragment (cTomRes$_{810}$) in antisense orientation with respect to the 35S promoter. In addition to the polylinker restriction sites the internal EcoRI and HindIII sites are presented. Arrows indicate the binding sites of the primers that were used for "pooled PCR" amplifications (II.2.1.6.1; II.2.1.7.5). The size of the PCR product that was amplified with the 710BiUS-35S (1) and pNOS1020BiUS (2) primers is given.

Construction of all inverted and direct repeats included introduction of an intron spacer between the two gene-specific fragments for two reasons. Firstly, direct inverted repeats lacking a spacer are unstable in most of the commonly used *E. coli* strains. Secondly, inverted repeat constructs with an intron were found to be more efficient in inducing RNAi than direct inverted repeats (Smith *et al*., 2000; Wesley *et al*., 2001). From the characterisation of the entire RdRP^{1} gene, it was known that this gene has four introns. The RdRP^{1} intron4 was chosen as a spacer for all repeat constructs due to its small size. Intron4 was PCR-amplified from the pGEM$_{I-3/4}$ clone that contained a RdRP^{1} gene-specific fragment with a size of ~2.1 kb. This ~2.1 kb fragment was PCR-amplified from EcoRI-digested tomato genomic DNA (*L. esculentum* cv. Rentita) using the exon-specific RDRP14-5 and RDRP 6-3 primers (II.2.1.7.5). The PCR product should include intron3 and intron4. To utilise intron4 as a spacer, a PCR amplification was carried out with the pGEM$_{I-3/4}$ clone using the exon-specific primers RdRP1-intron4-XbaI-fr and RdRP1-intron4-XbaI-rev (II.2.1.7.5). These primers introduced XbaI sites at both ends as shown below:

```
RdRP1-intron4-XbaI-fr
TGGATcTAGATGTCACAATTGAG
ACCTGGTACTACAGTGTTAACTC....Intron4....AAGAGTACTTCACCAACTATATTGT
                                      TTCTCATGAAGTGGTaGATcTAACA
                                         RdRP1-intron4-XbaI-rev
```

pGEM$_{I-3/4}$ clone

The PCR-amplified products were seperated on a 1% (w/v) agarose gel (II.2.1.2). A fragment with a size of ~540 bp was gel-purified and digested with XbaI. This fragment (I_{X510}) now having a size of ~510 bp was cloned into the unique XbaI site of the $702cTR^{-}_{8}$ plasmid. In a first screen, total DNA isolated from pooled colonies was PCR analysed using the 710BiUS-35S as forward and the pNOS1020 BiUS as reverse primers (II.2.1.6.1). PCR products were electrophoresed on a 1% (w/v) agarose gel and samples containing a fragment with the expected size of ~1.6 kb (Fig. B/II) were selected for further examination. In order to find the colonies carrying the recombinant plasmid containing intron4 (spacer), DNA of each colony of a "positive pool" was isolated (II.2.1.6.2) and analysed by digestion with XbaI (II.2.1.1.1). Plasmids releasing a ~510 bp fragment were further analysed with PstI to determine the orientation of the intron. To be functional in the repeat RdRP constructs, introduction of intron4 into the $702cTR^{-}_{8}$ required (+)-orientation.

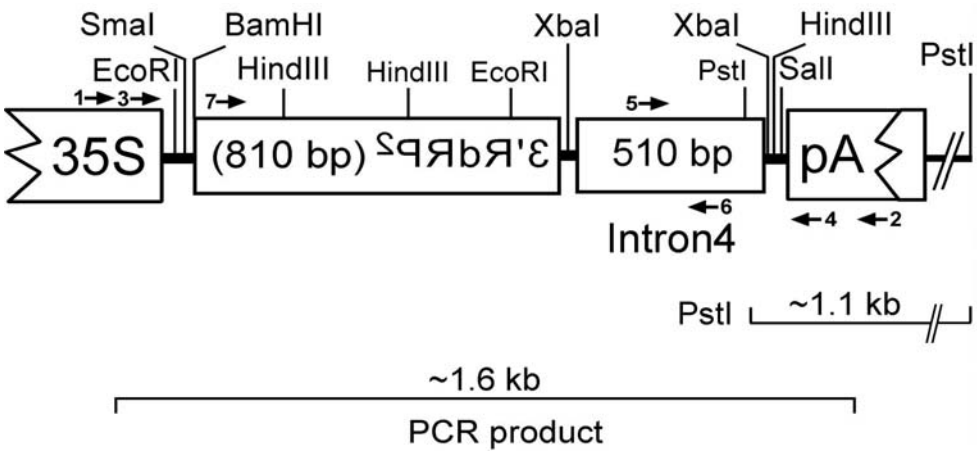
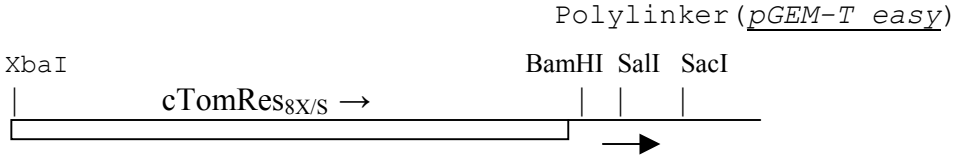


Figure B/II: Physical map of the $702cTR^{-}_{8\text{-}S}$ construct obtained after the introduction of the I_{X510} fragment (intron4) in sense orientation. Only the most relevant restriction sites are presented. Arrows indicate the binding sites of the primers that were used for PCR amplifications or sequencing. The size of the PCR product that was amplified with the 710BiUS-35S (1) and pNOS1020BiUS (2) primers is given. Double diagonal lines correspond to the most downstream PstI fragment that is not presented in full-length.
The following pimers were used for different analyses:
1 = 710BiUS-35S; 2 = pNOS1020BiUS; 3 = 35S-18A; 4 = PolyA-blue;
5 = RdRP7500Int4forward; 6 = RdRP7620Int4reverse; 7 = 3'HomRevBamHI (II.1.6; II.2.1.7.5).

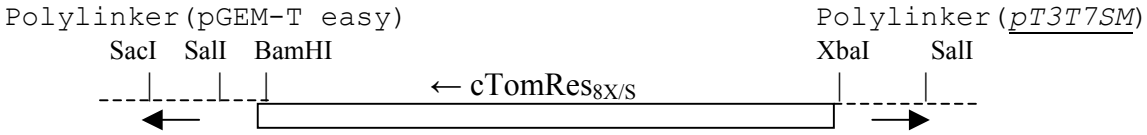
Thus, recombinant clones ($702cTR_{8\text{-}S}$) were screened for the presence of intron4 in a 35S promoter-related orientation being identical to the orientation of this sequence within the endogenous $RdRP^{1}$ gene. For (+)-orientation of intron4, digestion of recombinant clones

with PstI should release a ~1.1 kb long fragment. The 702cTR$^{-}_{8\text{-}S}$ clone containing the expected fragment was selected for the introduction of the second gene copy.

In order to introduce the second copy in either sense or antisense orientation with respect to the orientation of first copy, the 702cTR$_{8\text{-}S}$ was linearised with SalI. Because the cTomRes$_{810}$ fragment did not contain suitable cloning sites, a strategy was designed to introduce SalI sites to both of its ends. A ~855 bp long XbaI/SacI gene-specific fragment (cTomRes$_{8X/S}$) was isolated from the cTomRes$_{1.35Bam}$ clone (see above). At the 3'end of this fragment one SalI site was located upstream to the SacI site within the poly-linker sequence of the pGEM-T easy vector:



To introduce a SalI site at the 5'end, the fragment was sub-cloned into the XbaI/SacI linearised pT3T7SM plasmid (II.1.5). Recombinant plasmids (PT$_{Rd²8/sal}$) were analysed by SalI digestion. Plasmids releasing a ~850 bp long fragment were regarded as positive. From one of the positive clones this fragment (cTomRes$_{8s}$) was gel-purified and cloned into the linearised 702cTR$_{8\text{-}S}$. Recombinant plasmids releasing a ~850 bp SalI fragment were analysed in more detail.



To determine the orientation of the cTomRes$_{8s}$, recombinant plasmid were digested with BamHI, EcoRI, BamHI/XbaI and XbaI, respectively, and separated on a 1% (w/v) agarose gel (Fig. 5, see also Figures B/III and C). As depicted in Figure B/III, the BamHI-digested IR construct should bear a ~2.1 kb fragment whereas DR constructs should give a ~1.3 kb fragment (Fig. C). Upon EcoRI digestion IR construct should release two ~700 bp and one ~730 bp fragment. By contrast, DR constructs should produce two ~700 bp and one ~620 bp fragment. With BamHI/XbaI both repeat constructs should release one ~510 bp and two ~810 bp fragments. Finally, IR constructs would release a ~510 bp and DR constructs should produce one ~510 and one ~850 bp XbaI fragment.

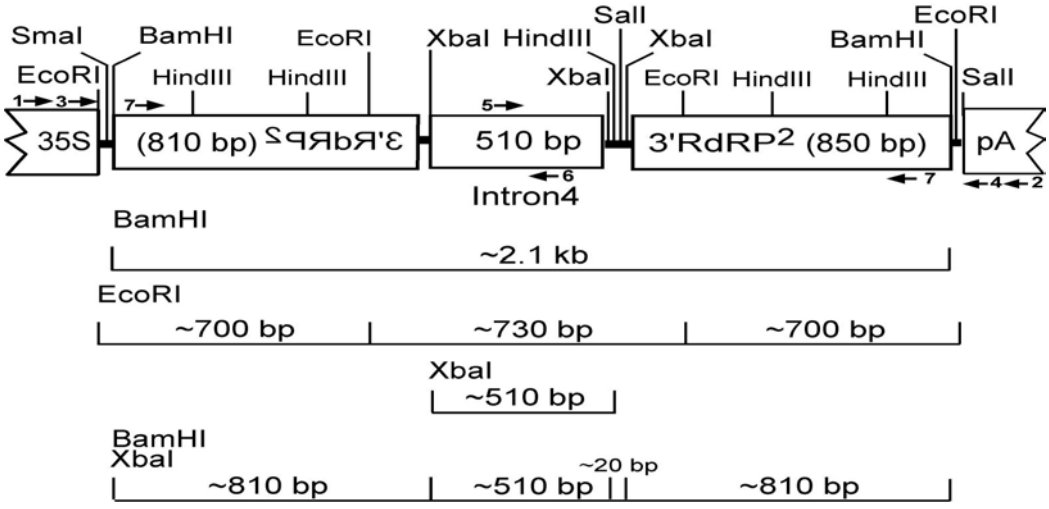


Figure B/III: Physical map of the 702cTR$_{IR}$ construct obtained after introduction of the 3'RdRP^2 fragment (cTomRes$_{8s}$) in sense orientation. This construct was used to generate the transgenic SR1IR/Inv' plant lines. In addition to the polylinker restrictions sites, the internal BamHI, EcoRI and XbaI sites of the 702cTR$_{IR}$ as well as the calculated lengths of the corresponding fragments are shown. Arrows indicate the binding sites of the primers that were used for PCR amplification.
1 = 710BiUS-35S; 2 = pNOS1020BiUS; 3 = 35S-18A; 4 = PolyA-blue;
5 = RdRP7500Int4forward; 6 = RdRP7620Int4reverse; 7 = 3'HomRevBamHI (II.1.6; II.2.1.7.5).

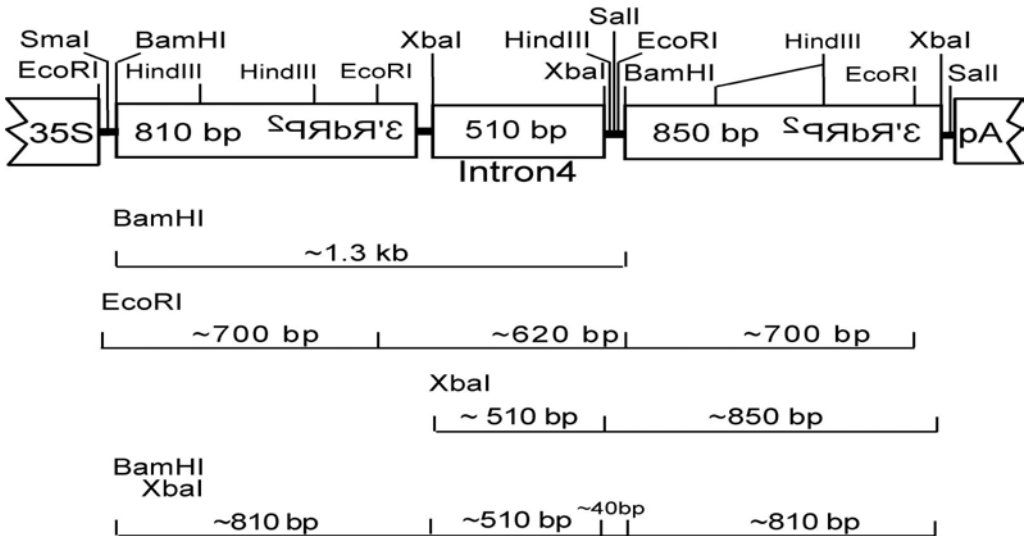


Figure C: Physical map of the 702cTR$_{DR}$ construct obtained after introduction of the 3'RdRP^2 fragment (cTomRes$_{8s}$) in antisense orientation. This construct was used to generate the transgenic SR1tan plant lines. In addition to the polylinker restrictions sites, the internal BamHI, EcoRI and XbaI sites of the 702cTR$_{DR}$ as well as the calculated lengths of the corresponding fragments are shown.

The agarose gel photograph of the restriction analysis (Fig. 5) showed that the recombinants, 702cTR$_{IR}$ and 702cTR$_{DR}$, displayed the expected BamHI, EcoRI, BamHI/XbaI and XbaI restriction pattern. In addition, single digestion with either BamHI or XbaI confirmed that the orientation of the second copy corresponded to the restriction patterns that were expected for the 702cTR$_{IR}$ and 702cTR$_{DR}$.

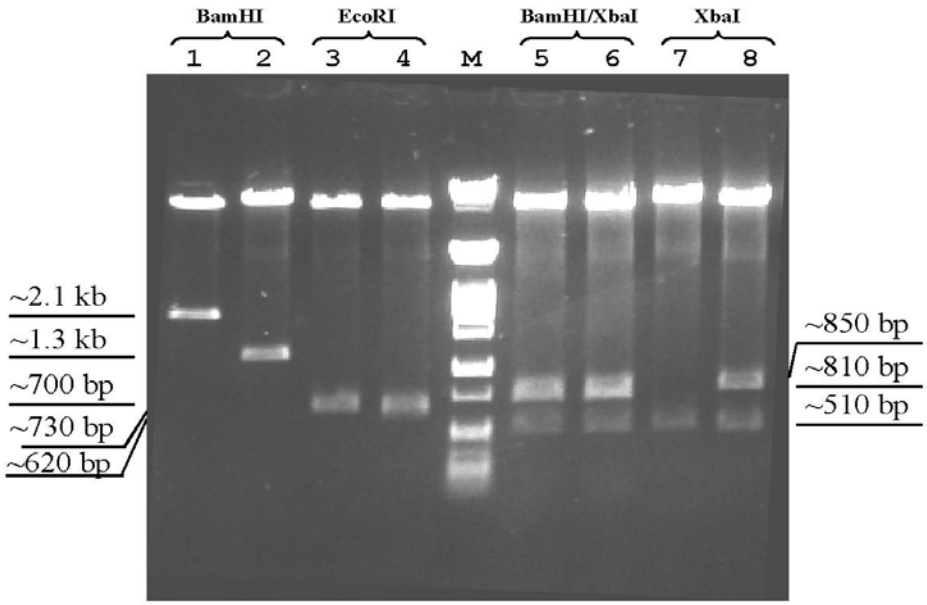
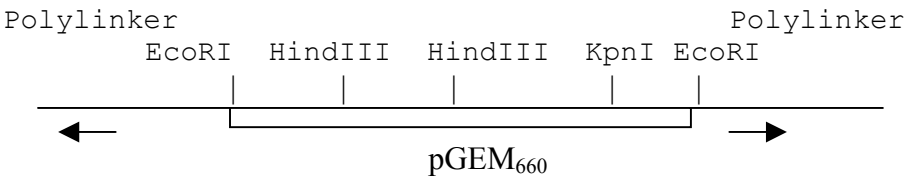


Figure 5: Restriction analysis of the 702cTR$_{IR}$ and the 702cTR$_{DR}$ constructs.
Lanes 1, 3, 5, and 7: 702cTR$_{IR,}$ plasmid DNA; Lanes 2, 4, 6, and 8: 702cTR$_{DR}$ plasmid DNA.
In lanes 3 and 4 two fragments with sizes of ~700 bp are detectable.
In lanes 5 and 6, two fragments with sizes of ~810 bp are detectable.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2); 1% (w/v) agarose gel.

III.2.1.1.2 Isolation and cloning of RdRP^1-specific 3'end fragments

A RdRP^1 DR construct was generated to serve as an internal control. A ~660 bp long RdRP^1 3'end fragment displaying sequence homology with the RdRP^2 cDNA of 53.5% was chosen to generate the RdRP^1 DR construct. The RdRP24 plasmid (Schiebel *et al.*, 1998) served as template to amplify the RdRP^1-specific fragment using the RdRP2800Not as forward and the RdRP3440Not as reverse primers (II.2.1.7.5). A PCR product with a size of ~660 bp was gel-purified (II.2.1.3) and cloned into the pGEM-T easy vector (II.1.5). The recombinant clones (pGEM$_{660}$) were verified for the presence of the insert with EcoRI, KpnI, and HindIII.

Location of the restriction sites is indicated below:



The $pGEM_{660}$ clones should release a fragment with a size of ~660 bp upon EcoRI digestion. With KpnI the recombinant plasmid (~3.6 kb) will be linearised and a ~130 bp fragment will be released by HindIII. The picture of the ethidiumbromide-stained agarose gel showed that all recombinant $pGEM_{660}$ clones displayed the expected EcoRI, KpnI and HindIII restriction pattern (data not shown). The ~660 bp long gene-specific fragment (Rd^1_{660}) was cut out from one of the $pGEM_{660}$ clones by EcoRI, was gel-purified and cloned into an EcoRI-linearised pPCV702SM (II.1.5; II.2.1.1.1). The initial screening of recombinants was done by pooled PCR as previously described (III.2.1.1.1) using the same primer pair. PCR products were electrophoresed on a 1% (w/v) agarose gel and samples showing amplification of a fragment with the expected size of ~930 bp (Fig. D/I) were selected for further analysis. In order to find the colonies carrying the recombinant plasmid, DNA of each colony of a "positive pool" was isolated (II.2.1.6.2) and analysed with SacI and HindIII, respectively, to determine the orientation.

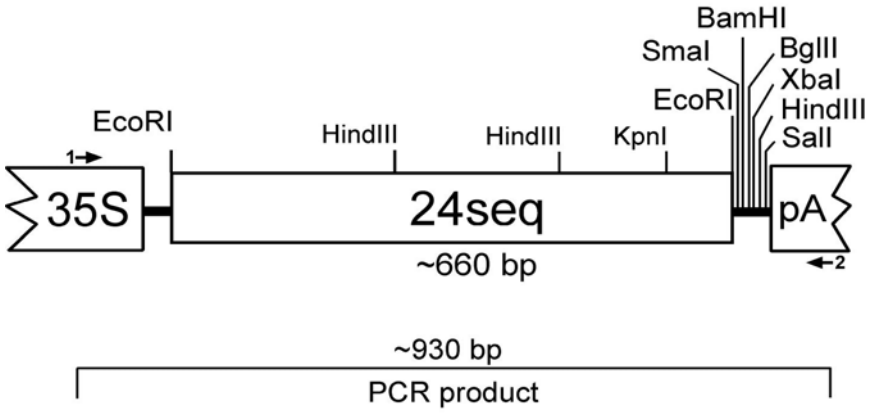


Figure D/I: Physical map of the $702Rd^1_{DR}$ construct obtained after the introduction of the 24seq fragment (Rd^1_{6S}) in sense orientation. In addition to the polylinker restrictions sites, the internal HindIII and KpnI sites of the $702Rd^1_{DR}$ as well as the calculated lengths of the corresponding fragments are shown. Arrows indicate the positions of the primers that were used for PCR amplifications. The size of the PCR product that was amplified with the 710BiUS-35S (1) and pNOS1020BiUS (2) primers is given.

Digested samples were run on a 1% (w/v) agarose gel. Release of a ~560 bp long fragment from the SacI-cleaved plasmid verified that the recombinant pPCV702SM plasmid ($702Rd^{1}_{6+}$) contained the Rd^{1}_{660} fragment in sense orientation (data not shown). The HindIII digestion confirmed this result. With HindIII (Fig. D/I), ~300 bp and ~130 bp long fragments were released (data not shown).

The $702Rd^{1}_{6+}$ plasmid was linearised with XbaI to introduce the I_{X510} spacer fragment. Isolation and cloning of the I_{X510} fragment into the$702Rd^{1}_{6+}$ was done as described for the $702cTR^{-}_{8}$ clone (III.2.1.1.1). In a first screen, total DNA was isolated from "pooled colonies" and PCR-analysed using the 710BiUS-35S as forward and the pNOS1020BiUS as reverse primers (II.2.1.6.1). The PCR products were electrophoresed on a 1% (w/v) agarose gel and samples showing a fragment with the expected size of ~1.44 kb (see Fig. D/II) were selected for further examinations. In order to find the colonies containing the spacer, DNA of each colony of a "positive pool" was isolated (II.2.1.6.2) and analysed with XbaI. Plasmids releasing a ~510 bp long fragment were further analysed with PstI to determine the orientation of the spacer. For a functional intron4, recombinant clones ($702Rd^{1}_{6+S}$) were expected to release a ~1.1 kb PstI fragment (Fig. D/II). One of the $702Rd^{1}_{6+S}$ plasmids carrying the ~1.1 kb fragment was selected for introduction of the second gene copy.

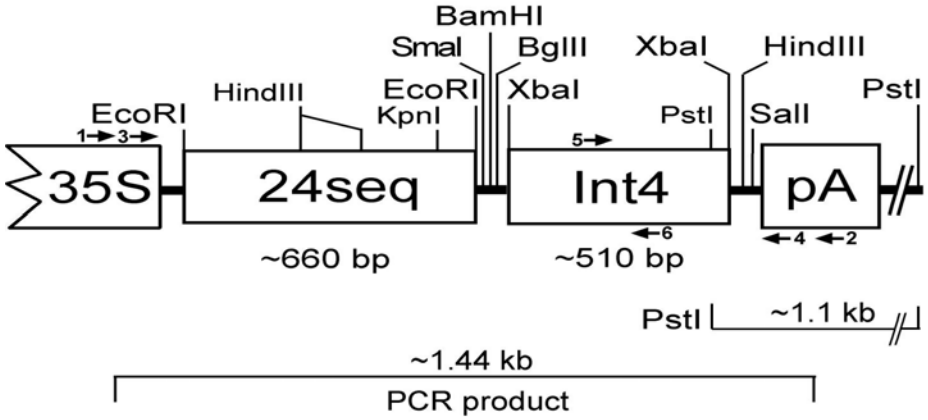
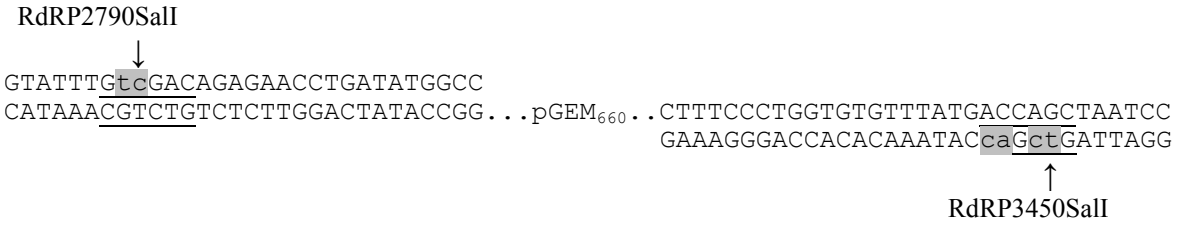
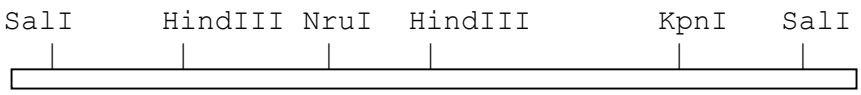


Figure D/II: Physical map of the $702Rd^{1}_{6+S}$ construct obtained after the introduction of the Intron4 fragment (I_{X510}) in sense orientation. Only the most relevant restriction sites are indicated. Arrows indicate the positions of the primers that were used for PCR amplifications or sequencing. The sizes of the PstI fragment and the PCR product that was amplified with the 710BiUS-35S (1) and pNOS1020BiUS (2) primers are given. Double diagonal lines correspond to the most downstream PstI fragment that is not presented in full-length. The following primers were used for different analyses:
1 = 710BiUS-35S; 2 = pNOS1020BiUS; 3 = 35S-18A; 4 = PolyA-blue;
5 = RdRP7500Int4forward; 6 = RdRP7620Int4reverse (II.1.6; II.2.1.7.5).

The second copy of the RdRP[1]-specific fragment was introduced into the 702Rd$^{1}_{6+S}$ in sense orientation with respect to the 35S promoter. To enable cloning of the second copy with SalI, a RdRP[1]-specific fragment was PCR-amplified from the pGEM$_{660}$ clone using the RdRP2790SalI as forward and the RdRP3450SalI as reverse primers (II.2.1.7.5). These primers introduced SalI sites at both ends as shown below:



The PCR products were electrophoresed and a fragment with an expected size of ~670 bp was detectable on a 1% (w/v) agarose gel. The fragment was gel-purified and digested with either HindIII, NruI or KpnI to verify whether it represented the RdRP[1] sequence. Location of restriction sites within the PCR fragment are indicated below:



PCR fragment (~670 bp)

Upon HindIII cleavage fragments with sizes of ~130 bp, ~200 bp and ~340 bp were released. NruI cuts the PCR fragment into ~250 bp and ~420 bp long fragments and KpnI into ~100 bp and ~570 bp long fragments. Because all three restriction patterns corresponded to the patterns that were deduced from the pGEM$_{660}$ sequence the ~670 bp long PCR-amplified fragment was digested with SalI to release the ~660 bp SalI fragment (Rd$^{1}_{6S}$). The Rd$^{1}_{6S}$ fragment was gel-purified and cloned into the SalI-linearised 702Rd$^{1}_{6+S}$ vector. To examine the orientation of the Rd$^{1}_{6S}$ sequence, recombinant plasmids were digested with SalI, KpnI and HindIII, respectively (Fig. 6, also see Figure D/III). As depicted in Figure D/III, the appearance of a ~660 bp SalI fragment indicated the presence of the Rd$^{1}_{6S}$ sequence. The KpnI-digested DR construct should bear fragments with sizes of ~130 bp and ~1.0 kb. Upon HindIII digestion, the DR construct should release two ~130 bp, one ~200 bp and one ~860 bp long fragment. The agarose gel photograph of the restriction analysis (Fig. 6) showed that the recombinant, 702Rd$^{1}_{DR}$, displayed the expected SalI, KpnI, and HindIII restriction patterns.

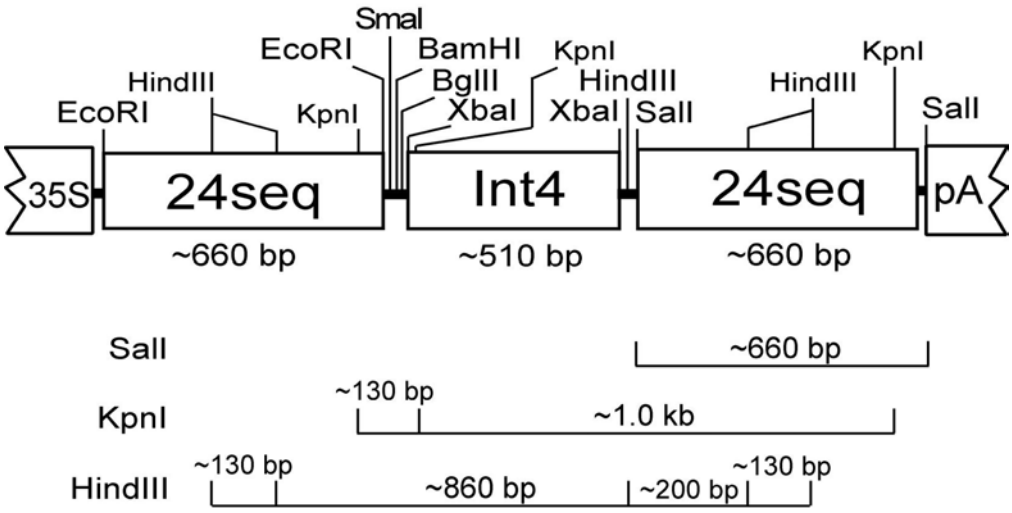


Figure D/III: Physical map of the 702Rd$^{1}_{DR}$ construct obtained after the introduction of the 24seq fragment (Rd$^{1}_{6S}$) in sense orientation. This construct was used to generate the transgenic SR1dem plant lines. In addition to the polylinker restriction sites, the internal HindIII, KpnI and SalI sites of the RdRP^{1} and the spacer sequences are shown. The calculated sizes of the corresponding fragments are presented.

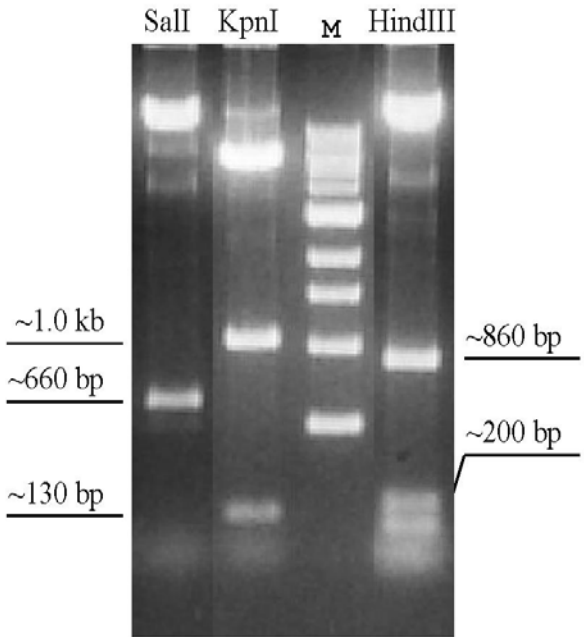


Figure 6: Restriction analysis of the702Rd$^{1}_{DR}$ construct.
Two fragments with sizes of ~130 bp are released upon HindIII digestion.
M = DNA length standard (1kb ladder; II.2.1.2); 1% (w/v) agarose gel.

All positive repeat constructs were sequenced. Due to the large size of the constructs, clones were sequenced with the 35S-18A, RdRP7500Int4fr, PolyA-blue, and RdRP7620Int4rev primers (II.1.6). The binding sites and orientations of the primers are shown in Figures B/III, and D/II. The 35S-18A and PolyA-blue primers were specific for the binary vector whereas the RdRP7500Int4fr and RdRP7620Int4rev primers were specific for the spacer (intron4). From the sequence data of the 702Rd$^{1}_{DR}$ clone, the orientation of the repeated copies and the spacer could be determined. Furthermore, no alteration of the sequences within and around the cloning sites was found. Hence this construct was chosen for further experiments. Sequence data of the 702cTR$_{IR}$ and the 702cTR$_{DR}$ clones revealed the orientation of the first copy (cTomRes$_{810}$ fragment), the sequence of the spacer, and the sequences flanking the cloning sites. Sequencing of the702cTR$_{DR}$ clone with the RdRP7500Int4fr primer showed the junction between the spacer and the second copy thereby, indicating that the orientation of the first copy was in accordance with previous restriction analysis. However, sequencing of the702cTR$_{DR}$ clone with PolyA-blue was not successful. Thus, no sequence data of the junction between the second copy and polyadenylation (pA) signal was available. On the other hand, for unknown reasons sequencing of the second copy (cTomRes$_{8s}$) fragment of the 702cTR$_{IR}$ plasmid was neither achieved with the RdRP7500Int4fr nor with the PolyA-blue primers. Hence, sequence with these primers failed to provide any information about the orientation of second copy in the 702cTR$_{IR}$ plasmid. It was presumed that secondary structure formation of the repeated copies could be the cause of failure of sequencing reactions. Hence, strategies were designed to re-isolate and sub-clone the internal fragments of the 702cTR$_{DR}$ plasmid. Analysis of the sub-clones should then provide information about sequence of the second copy and the pA signal junction.

A ~1.3 kb long PstI fragment was cut out and sub-cloned into the PstI site of the pT3T7SM vector (II.1.5). This PstI fragment comprised the 3'end of the second copy and the vector sequence that included pA signal sequence. Clones were verified for the presence of the insert by restriction analysis with PstI and those which released a ~1.3 kb fragment (pTRd$^{2}_{1.3}$) were sequenced with the T7/T3 primers (II.1.6). Whereas in case of the 702cTR$_{IR}$ plasmid, sequence information about the orientation of second copy was lacking. Therefore, it was important to sub-clone a fragment that included either a part of intron and second copy or second copy and pA signal sequence. In view of this, PstI-mediated sub-cloning was unsuitable as in the702cTR$_{IR}$ plasmid, PstI restriction sites were

located within the junction of the intron and the second copy as well as within the junction of second copy and pA signal sequence. Due to the presence of PstI restriction sites within both junctions, digestion with PstI would have released fragments only corresponding to second copy. Also, no other suitable restriction site was available to isolated fragments containing junction sequences. Therefore, PCR amplification was carried out. The $702cTR_{IR}$ was linearised with the KpnI and used as a template for PCR amplification. The PCR amplification was carried out under standard conditions using the RdRP7500Int4forward and the pNOS1020BiUS as reverse primers (see Fig. B/III, for primer binding site). The PCR fragment with a size of ~1.2 kb was gel-purified and cloned into the pGEM-T easy vector. Recombinant clones ($pGRd^2_{1.2}$) that released EcoRI fragments with sizes of ~160 bp, ~430 bp and ~700 bp were sequenced with the sp6/T7 primers (II.1.6). Sequencing of the $pTRd^2_{1.3}$ and $pGRd^2_{1.2}$ clones revealed that the orientation of the second copy within the $702cTR_{DR}$ and the $702cTR_{IR}$ clones was in accordance with previous restriction analysis (III.2.1.1.1; Fig. 5). In addition to this, sequencing data showed no alterations within or around the cloning sites.

III.2.2 Designing of the sense and antisense constructs to suppress expression of $RdRP^1$ and $RdRP^2$

III.2.2.1 Isolation and cloning of RdRP-specific 3'end fragments into the pPCV702SM vector

III.2.2.1.1 Cloning of $RdRP^2$-specific 3'end fragments

The ~1.35 kb fragment ($Rd^2_{1.3B}$) was cut out from the $cTomRes_{1.35Bam}$ clone (III.2.1.1.1) by digestion with BamHI, gel-purified, and cloned into the BamHI-linearised pPCV702SM (II.1.5). As cloning was not directional, introduction of the $Rd^2_{1.3B}$ fragment in either antisense or sense orientation was possible. Initial screening of recombinants for the presence of insert was done as described (III.2.1.1.1) by pooled PCR using the same primer pair. The PCR products were electrophoresed on a 1% (w/v) agarose gel and samples showing amplification of a fragment with the expected size of ~1.6 kb (Fig. F) were selected for further analysis. In order to find the colonies carrying the recombinant plasmid, DNA of each colony of a "positive pool" was isolated (II.2.1.6.2) and analysed with HindIII and EcoRI (Fig. 7, also see Figures E and F). As depicted in Figure E, the

HindIII-digested plasmid having the $Rd^2_{1.3B}$ fragment in sense orientation with respect to the 35S promoter should bear ~300 bp and ~230 bp long fragments. Upon EcoRI digestion the same plasmid should release ~520 bp and ~130 bp long fragments.

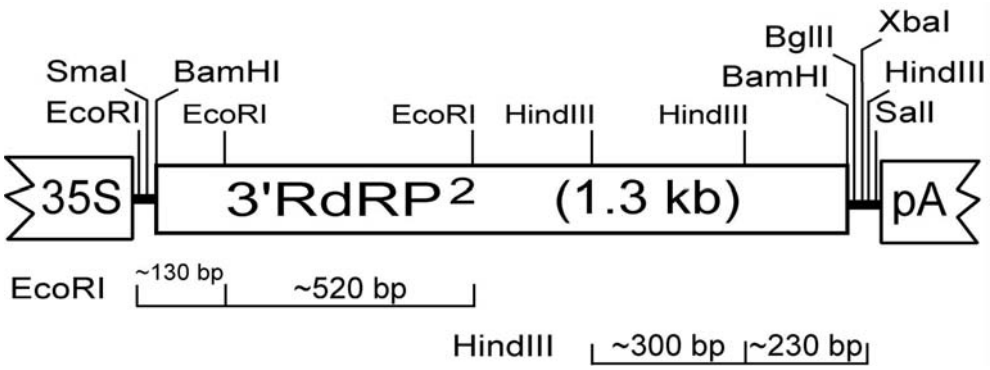


Figure E: Physical map of the $702Rd^2_{B+}$ construct obtained after introduction of the 3'RdRP^2 fragment ($Rd^2_{1.3B}$) in sense orientation. This construct was used to generate the transgenic SR1B$^+$ plant lines. In addition to the polylinker restriction sites, the EcoRI and HindIII sites of the $702Rd^2_{B+}$ as well as the calculated lengths of the corresponding fragments are shown.

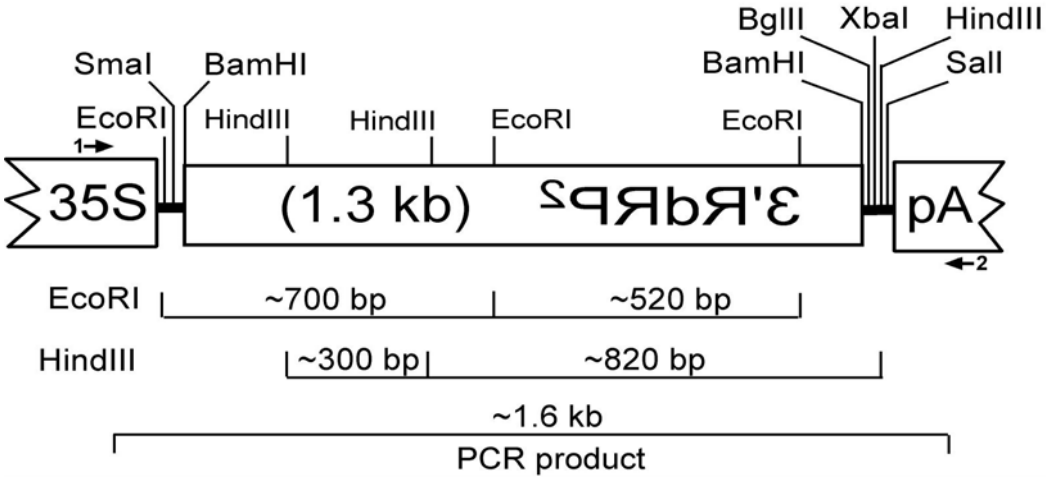


Figure F: Physical map of the $702Rd^2_{B-}$ construct obtained after introduction of the 3'RdRP^2 fragment ($Rd^2_{1.3B}$) in antisense orientation. This construct was used to generate the transgenic SR1Bam$^-$ plant lines. In addition to the polylinker restriction sites, the internal EcoRI and HindIII sites of the $702Rd^2_{B-}$ as well as the calculated lengths of the corresponding fragments are shown. Arrows indicate the positions of the primers that were used for PCR amplifications. The size of the PCR product that was amplified with the 710BiUS-35S (1) and pNOS1020BiUS (2) primers is given.

By contrast, recombinant plasmid having the $Rd^2_{1.3B}$ fragment in antisense orientation were expected to release ~300 bp and ~820 bp HindIII fragments (Fig. F). Upon EcoRI digestion the same plasmid should bear ~520 bp and ~700 bp long fragments (Fig. F). The agarose gel photograph of the restriction analysis (Fig. 7) showed that the recombinants, $702Rd^2_{B+}$ (lanes 1 and 3) and $702Rd^2_{B-}$ (lanes 2 and 4) displayed the expected HindIII and EcoRI restriction pattern, respectively. To confirm these results, both the clones were sequenced with the 35S-18A and the PolyA-blue primers (II.1.6). Sequence data revealed that all clones contained the $Rd^2_{1.3B}$ fragment. Moreover, its orientation in the $702Rd^2_{B+}$ and $702Rd^2_{B-}$ clones was in accordance with the restriction analysis.

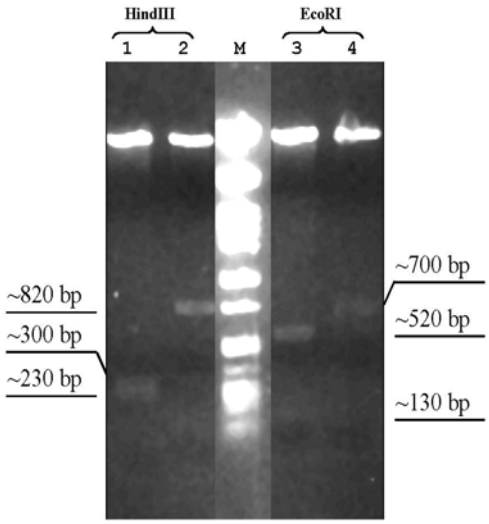


Figure 7: Restriction analysis of the $702Rd^2_{B+}$ and the $702Rd^2_{B-}$ constructs.
Lanes 1 and 3: DNA of the $702Rd^2_{B+}$ plasmid; Lanes 2 and 4: DNA of the $702Rd^2_{B-}$ plasmid.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2); 1% (w/v) agarose gel.

III.2.2.1.2 Cloning of $RdRP^1$-specific 3'end fragments

The ~2.2 kb long $RdRP^1$-specific 3'end fragment ($Rd^1_{2.2SX}$) was cut out from the RdRP24 clone (III.2.1.1.2) by SmaI/XbaI (II.2.1.1.1). To enable cloning of the $Rd^1_{2.2SX}$ fragment into the pPCV702SM (II.1.5) in sense orientation with respect to the 35S promoter, generation of compatible cloning ends was required. For this purpose the binary vector was linearised with HindIII, at first. The HindIII site was filled-in with T4 polymerase (II.2.1.1.2) to convert this site into a SmaI-compatible blunt-end site. Subsequently, the "blunt-end vector" was digested with XbaI and gel-purified (II.2.1.3). The $Rd^1_{2.2SX}$ fragment was introduced and transformants were analysed for the presence of the insert by

"pooled PCR" using the same primer pair as described (II.2.1.6.1; III.2.1.1.1). The PCR products were electrophoresed on a 1% (w/v) agarose gel and samples showing the amplification of a fragment with the expected size of ~2.5 kb (Fig. G) were further analysed. DNA of each colony of a "positive pool" was isolated and analysed with EcoRI, HindIII and BamHI, respectively.

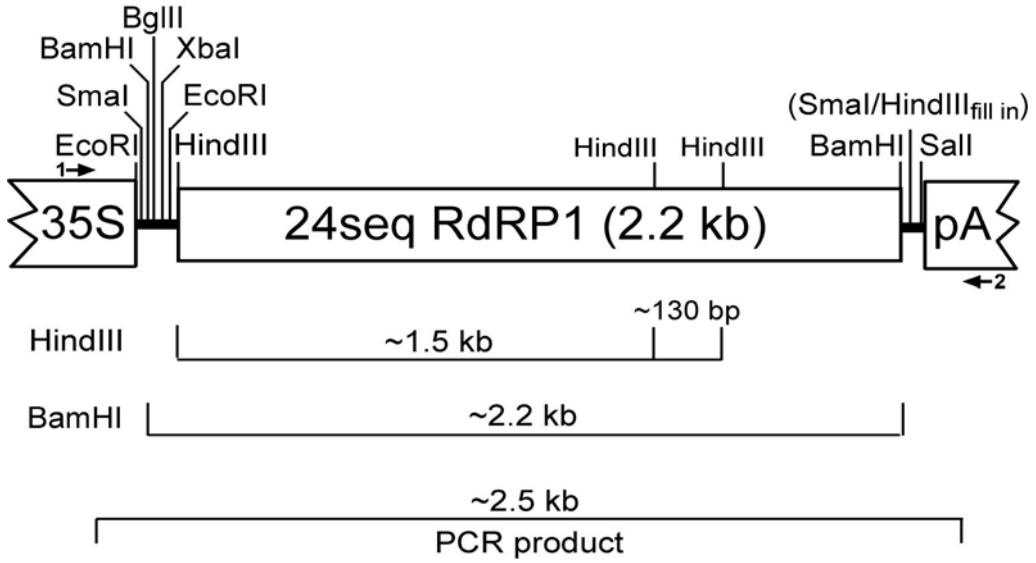


Figure G: Physical map of the 702Rd$^{1}_{S+}$ construct obtained after introduction of the 3'RdRP^{1} (Rd$^{1}_{2.2SX}$) fragment in sense orientation. This construct was used to generate the transgenic SR1S^{+} plant lines. In addition to the polylinker restriction sites, the HindIII sites of the 702Rd$^{1}_{S+}$ plasmid and the calculated sizes of the corresponding fragments are shown. Arrows indicate the positions of the primers that were used for PCR amplifications. The size of the PCR product that was amplified with the 710BiUS-35S (1) and pNOS1020BiUS (2) primers is given.

Based on the directed HindIII$_{fill-in}$/XbaI cloning step the Rd$^{1}_{2.2SX}$ fragment should be only integrated in sense orientation. As depicted in Figure G, the two EcoRI sites within the 702Rd$^{1}_{S+}$ were separated by only ~30 bp. Therefore the plasmid is virtually linearised by EcoRI. Upon HindIII digestion the 702Rd$^{1}_{S+}$ plasmid should bear fragments with sizes of ~130 bp and ~1.5 kb. With BamHI, the entire Rd$^{1}_{2.2SX}$ fragment with a size of ~2.2 kb should be released. In Figure 8 restriction analysis of one 702Rd$^{1}_{S+}$ clone is presented. The fragment sizes corresponded to the expected EcoRI, HindIII and BamHI patterns. Based on this analysis, the 702Rd$^{1}_{S+}$ clone was considered positive and sequenced with the 35S-18A, PolyA-blue, 2440forward, 2440reverse, 3070forward and 3070reverse primers (II.1.6). Sequence data revealed that it contained the Rd$^{1}_{2.2SX}$ in sense orientation.

To produce an antisense RdRP[1]-specific pPCV702SM derivative the ~2.2 kb long RdRP[1]-specific 3'end fragment ($Rd^1_{2.2BX}$) was cut out from the RdRP24 (III.2.1.1.2) clone by BamHI/XbaI. The $Rd^1_{2.2BX}$ fragment was gel-purified (II.2.1.3) and cloned into the BamHI/XbaI-linearised binary vector. Recombinant plasmids were analysed for the presence of the insert by "pooled PCR" as described previously (II.2.1.6.1; III.2.1.1.1) using the same primer pair. The PCR products were electrophoresed on a 1% (w/v) agarose gel (II.2.1.2), and samples showing amplification of a fragment with the expected size of ~2.5 kb (Fig. G) were selected for further analysis. DNA from each colony of a "positive pool" was isolated (II.2.1.6.2) and analysed with EcoRI, HindIII and BamHI (II.2.1.1.1).

Figure H: Physical map of the $702Rd^1_{A-}$ construct obtained after introduction of the 3'RdRP[1] fragment ($Rd^1_{2.2SX}$) in antisense orientation. This construct was used to generate the transgenic $SR1A^-$ plant lines. In addition to the polylinker sites, the internal HindIII sites of the $702Rd^1_{A-}$ are presented. Furthermore, the calculated lengths of the EcoRI and HindIII fragments are shown. Due to the unique BamHI site the $702Rd^1_{A-}$ construct could be linearised by this enzyme.

As depicted in Figure H, recombinant plasmids were expected to release a ~2.2 kb fragment upon EcoRI cleavage. With HindIII ~130 bp and ~1.5 kb fragments should be visible and with BamHI, the plasmid was expected to become linearised. In Figure 8, restriction analysis of one of the $702Rd^1_{A-}$ clones is presented. It shows the expected EcoRI, HindIII and BamHI restriction pattern. The clone was sequenced with the 35S-18A, PolyA-blue, 2440forward, 2440reverse, 3070forward and 3070reverse primers (II.1.6) and the sequence data revealed that the $Rd^1_{2.2BX}$ fragment was cloned in antisense orientation.

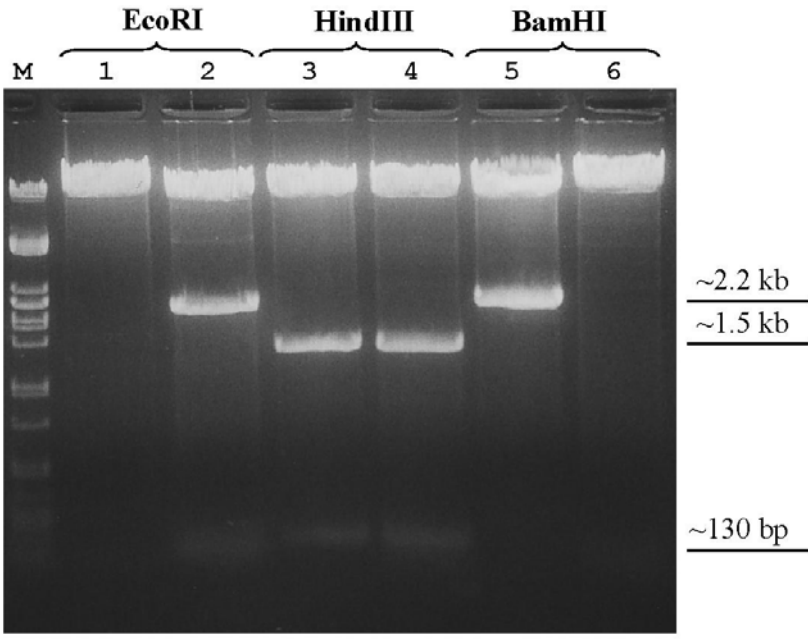


Figure 8: Restriction analysis of the 702Rd$^{1}_{S+}$ and the 702Rd$^{1}_{A-}$ constructs.
Lanes 1, 3 and 5: DNA of the 702Rd$^{1}_{S+}$ plasmid; Lanes 2, 4 and 6: DNA of the 702Rd$^{1}_{A-}$ plasmid.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2); 1% (w/v) agarose gel.

III.2.3 Designing of the sense constructs to over-express the RdRP^{2}

III.2.3.1 Cloning of the full-length RdRP^{2} cDNA into the pPCV702SM vector.

III.2.3.1.1 Assembly of the tomato full-length RdRP^{2} cDNA

Independent clones containing the RdRP^{2} 5'end (III.1.5), the cTomRes$_{320}$ (III.1.3) and the RdRP^{2} 3'end fragments (III.1.4) were available. However, no unique restriction site was present in the overlapping regions of the cTomRes$_{500}$, c5TomRes$_{1.5}$, cTomRes$_{320}$ and the cTomRes$_{1.5}$ fragments that would have allowed to assemble the entire RdRP^{2} cDNA sequence by simple cloning steps. Nevertheless, to obtain a full-length cDNA clone, a PCR amplification-mediated strategy was utilised. For this purpose, the RdRP^{2}forwardSmaI and RdRP^{2}reverseXbaI primers (II.2.1.7.5) were designed to amplify a ~2.7 kb long RdRP^{2} 5'end fragment from Rentita cDNA. The RdRP^{2}forwardSmaI primer was specific for the 5' untranslated region and was designed to introduce a SmaI site at the 5'end of the fragment. The RdRP^{2}reverseXbaI primer binding site was in an overlapping region of the

5'end fragment and the 3'end cTomRes$_{1.5}$ fragment. This overlapping region had an unique XbaI site, which was later used to join these 5' and 3'end fragments.

RdRP^{2}forwardSmaI
↓
GAAGTCCCGGgTATAGACATG
CTTCAGGGCCTATATCTGTAC....cDNA....

The Rd²$_{2.7}$ fragment was gel-purified (II.2.1.3) and cloned into the pGEM-T easy vector (II.1.5). Recombinant plasmids (pGEM$_{2.7}$) were digested with EcoRI (II.2.1.1.1) and analysed on a 1% (w/v) agarose gel (II.2.1.2). Plasmids releasing fragments with sizes of ~410 bp and ~2.3 kb were considered to be positive for the Rd²$_{2.7}$ fragment (data not shown). One of the positive clones (pGEM$_{2.7}$) was selected for the assembly of the tomato full-length RdRP^2 cDNA. The full-length RdRP^2 cDNA clone was assembled by joining the pGEM$_{2.7}$ with a sub-fragment of the PT$_{Rd²8/sal}$ plasmid (III.2.1.1.1). To isolate this ~855 bp long RdRP^2 3'end sub-fragment (cTomRes$_{8X/S}$), the PT$_{Rd²8/sal}$ clone was double digested with XbaI and SacI. The cTomRes$_{8X/S}$ fragment was gel-purified and then introduced into the XbaI/ SacI sites of the pGEM$_{2.7}$ clone. Recombinant plasmids (pGEM$_{3.5}$) were verified by double digestion with XbaI and SacI (II.2.1.1.1). Clones releasing a fragment with a size of ~855 bp were considered as positive (data not shown). The pGEM$_{3.5}$ were sequenced with sp6/T7 primers (II.1.6) to gain information about the junction between the RdRP^2 and the flanking vector sequences. Sequencing of the pGEM$_{3.5}$ clone revealed that the 5'end fragment had been assembled to the 3'end fragment resulting in the full-length RdRP^2 cDNA.

III.2.3.1.2 Introduction of the full-length RdRP^2 cDNA into the pPCV702SM vector

The pGEM$_{3.5}$ clone was double-digested with SmaI and SalI (II.2.1.1.1). The resulting fragment with a size of ~3.5 kb (Rd²$_{3.5}$) was gel-purified and cloned into the SmaI/SalI sites of the pPCV702SM (II.1.5). Based on the directed SmaI/SalI cloning step the Rd²$_{3.5}$ fragment was expected to integrate in sense orientation with respect to the 35S promoter (Fig. I). To verify the orientation, recombinant plasmids were cut with EcoRI, HindIII and PstI (Fig. I), respectively, and the digested samples were electrophoresed on a 1% (w/v) agarose gel (Fig. 9).

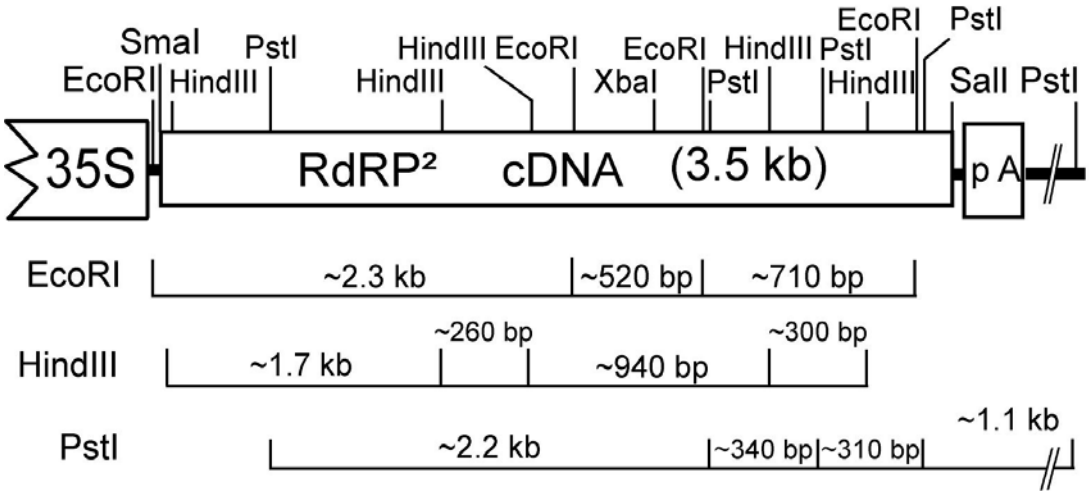


Figure I: Physical map of the 702Rd²$_{3.5+}$ construct obtained after introduction of the RdRP^2 cDNA fragment (Rd²$_{3.5}$) in sense orientation. This construct was used to generate the transgenic SR1Rd^{2+} plant lines containing the full-length RdRP^2 cDNA. In addition to the polylinker restriction sites, the internal EcoRI, HindIII and PstI sites of the 702Rd²$_{3.5+}$ as well as the calculated length of the corresponding fragments are shown. Double diagonal lines indicate that the most downstream PstI fragments are not presented in full length.

As depicted in Figure I, the EcoRI-digested 702Rd²$_{3.5}$ plasmids containing the Rd²$_{3.5}$ fragment in sense orientation should bear fragments with sizes of ~520 bp, ~710 bp and ~2.3 kb. Upon Hind III digestion the expected fragments were ~260 bp, ~300 bp, ~940 bp and ~1.7 kb in size. Finally, with PstI ~310 bp, ~340 bp, ~1.1 kb and ~2.2 kb fragments should be released. The agarose gel photograph (Fig. 9) shows one example of a positive clone (702Rd²$_{3.5}$). The restriction pattern exhibited by positive clone for all three enzymes was, as deduced from the map presented in Figure I.

To verify and compare the entire sequence of the RdRP^2 cDNA with previous sequence data (III.1.6), the 702Rd²$_{3.5+}$ clones were sequenced with 11 different primers (II.1.6, sequencing primers presented in Italics). Sequence comparison revealed five base pair substitutions of which three resulted in amino acid changes. The changes comprised a methionine (M) to valine (V), an aspartic acid (D) to glycine (G) and a tyrosine (Y) to histidine (H) substitution. According to their biochemical properties amino acids can be grouped. Because M and V are members of the same group the M to V substitution was not expected to affect the RdRP^2 function. However, as D and G as well as Y and H are not in the same group, these substitutions might have an affect on the activity of RdRP^2. Thus, it could not be excluded that expression of the RdRP^2 full-length would not code for a functional protein. The three independent clones 702Rd²$_{3.5+}$ that were sequenced all

showed the above changes indicating that these sequence alterations occurred before assembly of the 5'end and 3'ends.

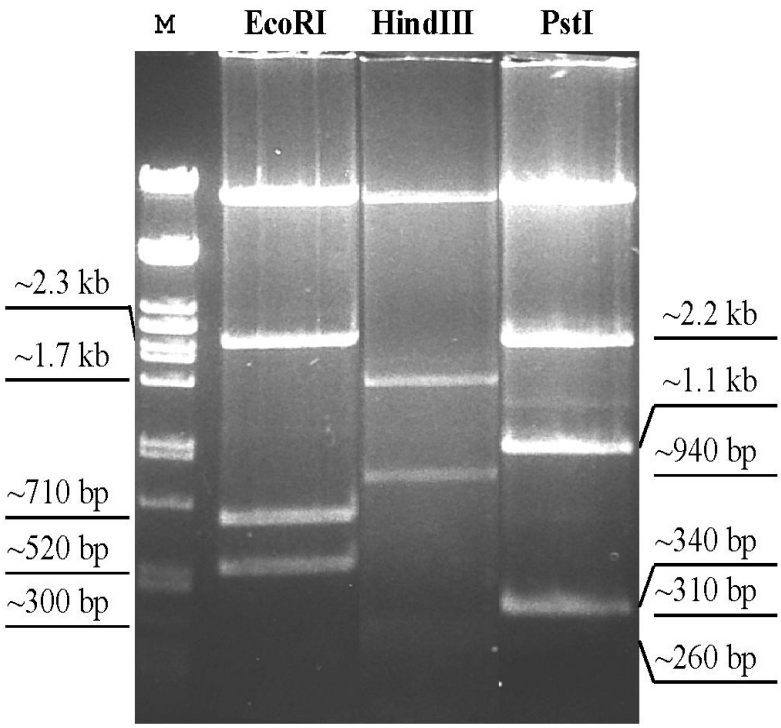


Figure 9: Restriction analysis of the 702Rd²$_{3.5+}$ construct.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2); 1% (w/v) agarose gel.

III.3 Generation and characterisation of transgenic *Nicotiana tabacum* plants

All transgenic plants were generated by *Agrobacterium tumefaciens*-mediated "leaf disc transformation" (II.2.2.3). For this purpose the pPCV702SM derivatives were mobilised into the *A. tumefaciens* strain GV3101 (pMP90RK) (II.1.3; Koncz and Schell, 1986) using the *E. coli* SM-10 (II.1.3; Simon *et al*., 1983) as helper strain for conjugation (II.2.2.1). The *A. tumefaciens* strains that were used for transformation are summarised in table B. Successful introduction of pPCV702SM derivatives was verified by re-transformation of the constructs into the *E. coli* INVα F´ strain (II.1.3) and subsequent characterisation of the transformants (II.2.2.2). Characterisation comprised restriction analysis of isolated plasmid DNA as described (Fig. 5, 6,7, 8 and 9).

Table B: *A. tumefaciens* strains that were used for "leaf disc transformation" (II.2.2.3).

A. tumefaciens	**Re-transformed into *E. coli***	**Figure No.**	**Restriction enzyme**
GV702cTR$_{IR}$	702cTR$_{IRinv}$	5	BamHI, EcoRI, XbaI, BamHI/XbaI
GV702cTR$_{DR}$	702cTR$_{DRinv}$	5	BamHI, EcoRI, XbaI, BamHI/XbaI
GV702Rd$^{1}_{DR}$	702Rd$^{1}_{DRinv}$	6	HindIII, KpnI, SalI
GV702Rd$^{2}_{B+}$	702Rd$^{2}_{B+inv}$	7	EcoRI, HindIII
GV702Rd$^{2}_{B-}$	702Rd$^{2}_{B-inv}$	7	EcoRI, HindIII
GV702Rd$^{1}_{S+}$	702Rd$^{1}_{S+inv}$	8	BamHI, EcoRI, HindIII
GV702Rd$^{1}_{A-}$	702Rd$^{1}_{A-inv}$	8	BamHI, EcoRI, HindIII
GV702Rd$^{2}_{3.5+}$	702Rd$^{2}_{3.5+inv}$	9	EcoRI, HindIII, PstI

Table C: Primary transformants generated by *Agrobacterium*-mediated transformation.

Single copy transgenic plants	***A. tumefaciens* used for plant transformation**	**Integrated construct**	**Number of plants Regenerated**
SR1IR(1a2), SR1IR(14), SR1Inv'(4), SR1Inv'(22)	GV702cTR$_{IR}$	RdRP^{2} inverted repeat (Fig.B/III)	20
SR1tan(10)	GV702cTR$_{DR}$	RdRP^{2} direct repeat (Fig.C)	10
SR1dem(18)	GV702Rd$^{1}_{DR}$	RdRP^{1} direct repeat (Fig.D/III)	10
SR1Bam^{+}(2)	GV702Rd$^{2}_{B+}$	RdRP^{2} 3'end sense (Fig.E)	10
SR1Bam^{-}(5)	GV702Rd$^{2}_{B-}$	RdRP^{2} 3'end antisense (Fig.F)	10
SR1S^{+}(40)	GV702Rd$^{1}_{S+}$	RdRP^{1} 3'end sense (Fig.G)	10
SR1A^{-}(6 and 8)	GV702Rd$^{1}_{A-}$	RdRP^{1} 3'end antisense (Fig.H)	10
SR1Rd^{2+} (2 and 17)	GV702Rd$^{2}_{3.5+}$	RdRP^{2} cDNA sense (Fig.I)	10

III.3.1 Characterisation of transgenic plants

Genomic DNA of all primary transformants was analysed by Southern hybridisation (II.2.4. 2). Southern analysis allowed to determine the number of genome-integrated T-DNA copies and provided a brief overview about the arrangement of the T-DNA. Plants that were selected for further characterisation were self-pollinated to attain homozygous T_1 generations (II.2.2.4). In the frame of this work, homozygous T_1 plants containing the $702cTR_{DR}$, $702Rd^2_{B+}$, and $702Rd^2_{B-}$ constructs were produced. The homozygous state of T_1 generations was verified by genetic crosses with tobacco wild-type plants (II.2.2.4) and by molecular characterisation of individual T_1 plants. Transgenic plants that exhibited the TK- phenotype were also analysed for copy number and were crossed with wild-type tobacco plants.

III.3.1.1 Southern analysis of primary transformants displaying no obvious phenotypic alterations

For each construct (Table C), 10-20 independent transformants were analysed for copy number and complete integration of the T-DNA.

III.3.1.1.1 Southern analysis of plant lines transformed with the $RdRP^2$ repeat T-DNA constructs

All primary transformants were initially screened for the $702cTR_{IR}$ T-DNA integration with DraI-restricted genomic DNA by Southern analysis. Due to the presence of unique DraI sites within the right (RB) and left (LB) T-DNA border sequences of the pPCV702SM (II.1.5) the entire ~7.2 kb long T-DNA of the empty vector can be released. In Figure J, the physical map of the $702cTR_{IR}$ transgene depicts the location of the DraI sites at the two borders and the two additional DraI sites located within the $RdRP^2$ fragments. The sizes of the fragments that were expected upon hybridisation with the ^{32}P-labelled $RdRP^2$-specific $cTomRes_{8X/S}$ (III.2.1.1.1) and the spacer-specific I_{X510} probes (III.2.1.1.1) are also shown.

The DraI-restricted genomic DNA from inverted repeat plants was expected to show one positive hybridisation signal with the I_{X510} probe at a position corresponding to a fragment with a size of ~960 bp and additional hybridisation signal(s) corresponding to the endogenous $RdRP^1$-intron4. Whereas with the $cTomRes_{8X/S}$ probe, three positive hybridisation signals at positions corresponding to fragments with sizes of ~960 bp, ~2.5 kb (RB) and ~5.9 kb (LB) should light up. The number of band(s) that correspond to the

endogenous RdRP^2 gene that were expected to appear are not known. From 20 independent primary transformants, only one plant (SR1Inv'4) showed a DraI pattern that corresponded to a full-length-integrated T-DNA construct when probed with cTomRes$_{8X/S}$. Four plants (SR1IR1a2, SR1IR14, SR1Inv'4 and SR1Inv'22) showed a positive signal corresponding to fragments with sizes of ~960 bp and ~2.5 kb. Only the SR1Inv'4 plant out of these four plants showed the ~5.9 kb band, whereas the other three showed larger hybridising fragments.

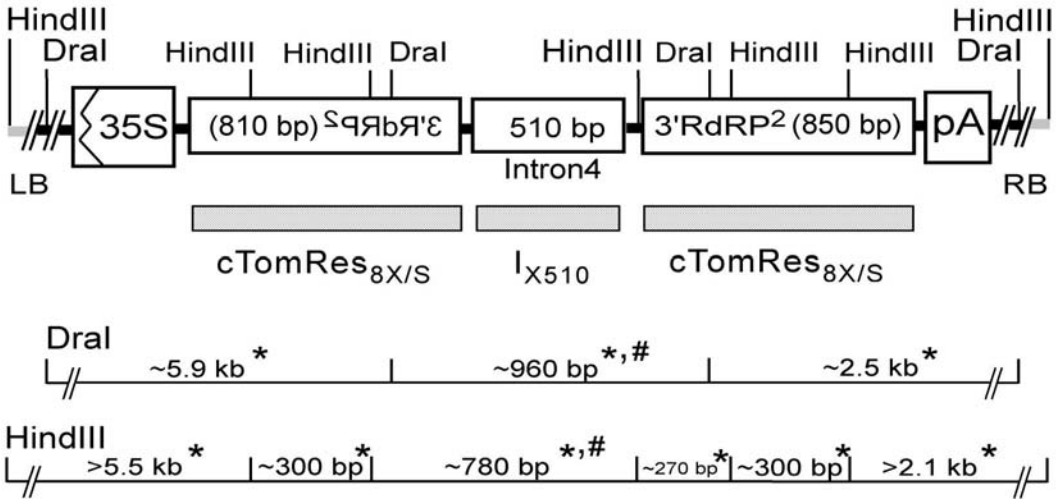


Figure J: Physical map of the 702cTR$_{IR}$ T-DNA. The DraI and HindIII sites as well as the sizes of the corresponding fragments are shown. Fragments that are expected to hybridise with the cTomRes$_{8X/S}$ (*) and I$_{X510}$ (#) probes are depicted. Double diagonal lines indicate that the most up- and downstream DraI fragments as well as the T-DNA/plant DNA border fragments (grey lines) are not presented in full length.
Grey boxes represent the fragments that were used as probes.
LB = left border; RB = right border.

With the cTomRes$_{8X/S}$ probe, DraI-restricted genomic DNA from a wild-type tobacco plant exhibited a hybridisation signal at a size of ~5.9 kb, which corresponded to the endogenous RdRP^2 gene (Fig. 21/IIC, lane 2). For the I$_{X510}$ probe a hybridising fragment with a size of ~10 kb, specific for the endogenous RdRP^1-intron4, was detected (data not shown).

A Southern blot of DraI-restricted genomic DNA of the inverted repeat plants SR1IR1a2, SR1IR14 probed with the I$_{X510}$ fragment is shown in Figure 10A (lanes 1 and 3). The appearance of a positive signal at a position corresponding to fragment with a size of ~960 bp, indicated no major deletions or rearrangements within this fragment. The blot was re-hybridised with the cTomRes$_{8X/S}$ fragment. Two hybridising fragments with sizes

of ~960 bp and ~2.5 kb were detected (Fig. 10B; lanes 1 and 3). Similarly, Southern analysis with DraI-restricted genomic DNA of the SR1Inv'4 and SR1Inv'22 plant lines showed two hybridising fragments with sizes of ~960 bp and ~2.5 kb (Fig. 12; lanes 1 and 2, respectively). As stated earlier, only one of these plants displayed the expected ~5.9 kb long hybridising fragment. However, all of three plants (SR1IR14, SR1Inv'22, SR1IR1a2) showed a third hybridisation signal corresponding to fragments with sizes of ~7.0 kb (Fig. 10B; lane 3), ~9.5 kb (Fig. 12; lane 2), and ~6.3 kb (Fig. 10B; lane 1), respectively. The ~5.9 kb fragment detected with the "DraI Southern analysis" of the SR1Inv'4 plant coincided with the endogenous RdRP^2 fragment, but the intensity of this band corresponded to the intensity that is expected for two fragments. Further restriction analysis revealed that indeed in this plant a LB fragment was present (see below-HindIII pattern).

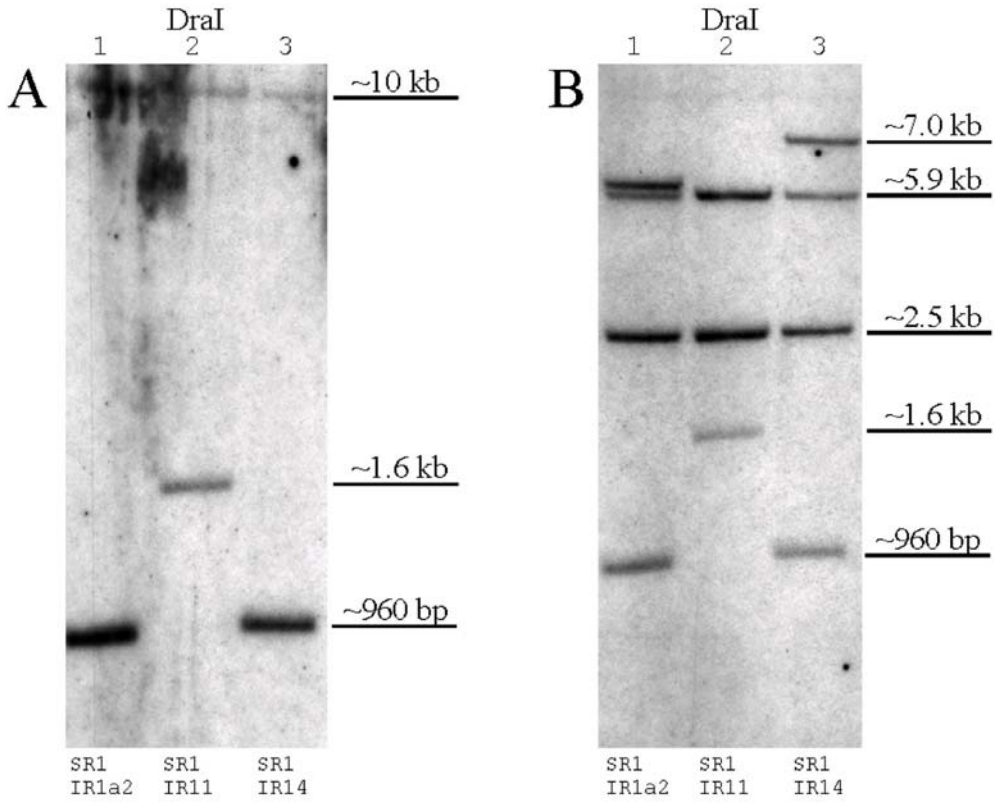


Figure 10: Southern blot of genomic DNA isolated from primary transformants.
A) The blot was hybridised against the ^{32} P-labelled I_{X510} spacer-specific probe (III.2.1.1.1). The ~10 kb long hybridising fragment corresponds to the endogenous RdRP^1 gene.
B) Re-hybridisation of the blot with the ^{32}P-labelled cTomRes$_{8X/S}$ RdRP^2-specific probe. (III.2.1.1.1). The hybridising fragment with a size of ~5.9 kb corresponded to the endogenous RdRP^2 gene.
Sizes of hybridising DNA fragments are indicated.

In summary, these data showed that deletions and/or rearrangements within the LB fragment are present in the three plants analysed. The fact that fragments with sizes of ~960 bp and ~2.5 kb lighted up demonstrated that no major deletions or rearrangements occurred within the internal and the RB fragments. Despite the deletions or rearrangements within the LB fragment, all of the three SR1IR1a2, SR1IR14 and SR1Inv'22 lines were proceeded for determination of copy number along with the SR1Inv'4.

In principle, the number of genome-integrated T-DNA copies can be determined by using an enzyme cutting only once within the T-DNA and using a probe that overlaps the unique restriction site. Provided that the T-DNA integrated without rearrangements, the next restriction sites for this enzyme will be in the flanking plant DNA. Counting the hybridising fragments will then reveal the copy number of T-DNA insertions. Because, the probe overlaps with the internal restriction site, a single copy insertion will always produce two hybridising fragments. Multiples of two shows that more than one copy integrated. The usage of an overlapping probe has the advantage that apart from determination of the copy number, information about the LB and RB fragment can be obtained. Appearance of odd number bands will indicate insertions of rearranged T-DNA copies.

Unfortunately, there was no suitable enzyme cutting only once within the $702cTR_{IR}$ and $702cTR_{DR}$ T-DNA constructs. However, HindIII appeared to be suitable for copy number analysis. Within the $702cTR_{IR}$ and $702cTR_{DR}$ T-DNA constructs all the HindIII sites were localised in the region between the 35S promoter and the pA signal sequences (Fig. J and Fig. K, respectively,). In addition to copy number determination, HindIII allowed to verify the results obtained previously, when genomic DNA of these constructs was Southern-analysed with DraI. The physical map of the $702cTR_{IR}$ transgene indicates the HindIII sites and the sizes of hybridising DNA fragments that are expected for probing with the $cTomRes_{8X/S}$ and the I_{X510} fragment (Fig. J). When probed against the $cTomRes_{8X/S}$ fragment, HindIII-restricted genomic DNA from plants containing IR constructs was expected to show internal fragments with sizes of ~270 bp, ~300 bp (twice) and ~780 bp. Appearance of HindIII fragments with sizes >5.5 kb (LB) and >2.1 kb (RB) would allow to determine the copy number. Apart from this banding pattern, hybridisation signal(s) that correspond to the endogenous $RdRP^2$ gene fragments should be detectable. However, these signals can be easily recognised as they will also appear in the lane containing tobacco wild-type DNA. When probed against the I_{X510} fragment, HindIII-restricted genomic DNA from the plants containing IR constructs was expected to give a

positive hybridising signal at a position that corresponds to a fragment with a size of ~780 bp. Also an endogenous RdRP[1]-intron4-specific band should become visible. It should be noted that Southern analysis does not allow precise determination of fragment sizes. Depending on the size, deviations between 10-300 bp can be assumed (the larger the fragment, the greater the possible deviation). Thus, minor rearrangements within T-DNA fragments can not be excluded by Southern analysis (Wassenegger, 2001)

A Southern blot of HindIII-restricted genomic DNA of the SR1IR1a2, SR1IR14, SR1Inv'4 and the SR1Inv'22 primary transformants that was probed with the cTomRes$_{8X/S}$ fragment is shown in Figures 11 and 12. For the SR1IR1a2 plant line only one T-DNA border fragment with a size of ~7.5 kb lighted up (Fig. 11A). Lack of a fragment with a size of >5.5 kb or >2.1 kb indicated that a second border fragment was missing, thus, implying rearrangements within one of the border sequences.

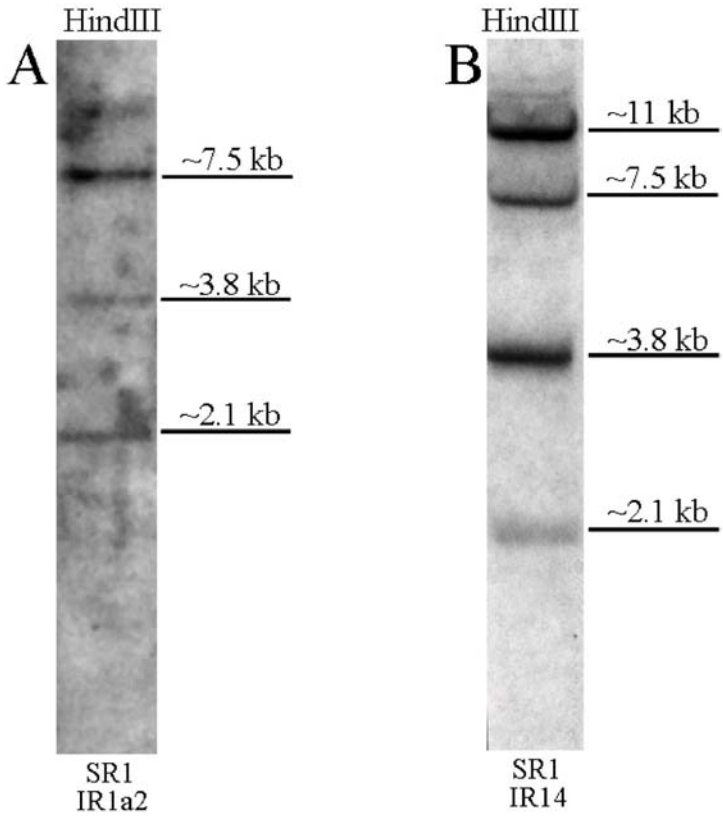


Figure 11: Southern analysis of genomic DNA isolated from primary transformants probed with the ^{32}P-labelled cTomRes$_{8X/S}$ fragment.
Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous RdRP[2] gene. Sizes of hybridised DNA fragments are indicated.

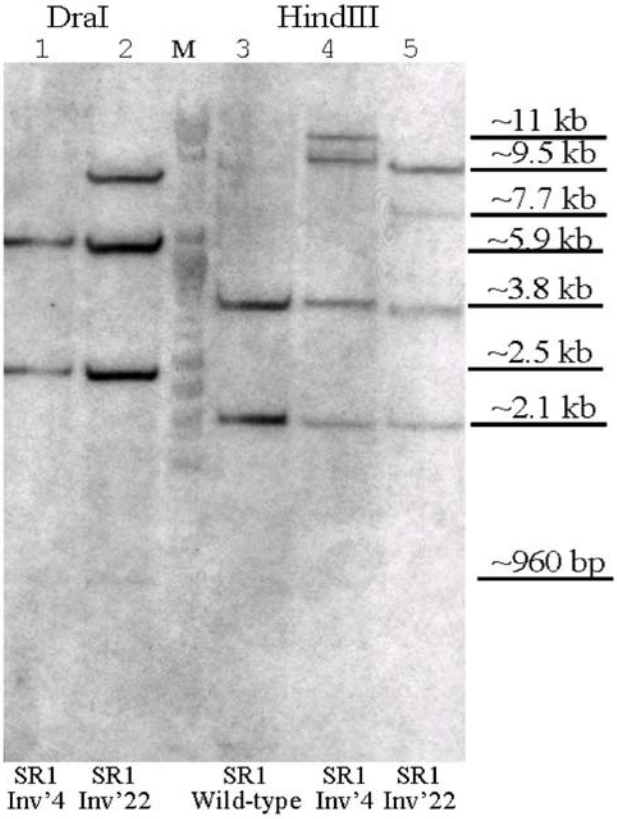
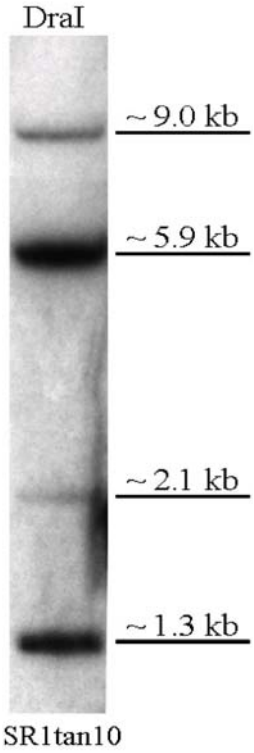


Figure 12: Southern analysis of genomic DNA isolated from primary transformants probed with the ^{32}P-labelled cTomRes$_{8X/S}$ fragment.
Lanes 1 and 2: the ~5.9 kb long hybridising fragment corresponded to the endogenous RdRP^2 gene. Lanes 3, 4 and 5: the hybridising fragments with sizes of ~2.1 kb and ~3.8 kb corresponded to the endogenous RdRP^2 gene.
Sizes of the hybridising DNA fragments are given.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2).

The two large size fragments ranging from ~7 kb to ~11 kb for each of the SR1IR14 (Fig. 11B), SR1Inv'4 and SR1Inv'22 plant lines (Fig. 12, lanes 4 and 5) indicated single copy T-DNA insertions. The additional fragments with sizes of ~2.1 kb and ~3.8 kb corresponded to the endogenous RdRP^2 gene as they were also hybridising with tobacco wild-type DNA (Fig. 12, lane 3). None of the inverted repeat plants showed positive signals corresponding to HindIII fragments with sizes of ~270 bp, ~300 bp and ~780 bp. This finding argued for rearrangements of the RdRP^2 and/or the intron sequences. To confirm this inference, Southern analyses of plant lines containing the control construct (702cTR$_{DR}$) was required. Examination of the control plants would show whether these small fragments are detectable under the hybridisation conditions applied.

As stated earlier, DraI was one of the most suitable enzymes to analyse T-DNA integration patterns. Therefore, all the SR1702cTR$_{DR}$ primary transformants were initially screened for T-DNA integration with DraI using the RdRP^2-specific cTomRes$_{8X/S}$ fragment as probe (III.2.1.1.1). DraI-restricted genomic DNA from RdRP^2 direct repeat plants was expected to give positive signals at positions that correspond to fragments with sizes of ~1.3 kb, ~2.1 kb (RB) and ~5.9 kb (LB). Apart from this, hybridisation signal(s) corresponding to the endogenous RdRP^2 gene should be detectable, as well. When probed against the cTomRes$_{8X/S}$ fragment, out of 10 independent primary transformants three plant lines displayed a DraI pattern that was expected for full-length T-DNA integration (Fig. 13, only the data of one plant is presented).



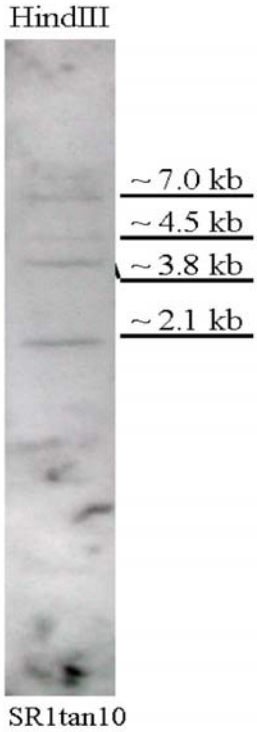


Figure 13: **Figure 14:**

Figures 13/14: Southern analysis of genomic DNA isolated from primary transformants. The blots were hybridised against the ^{32}P-labelled cTomRes$_{8X/S}$ probe. In Figure 13, the hybridising fragment with a size of ~5.9 kb corresponded to the endogenous RdRP^2 gene. In Figure 14, the hybridising fragments with sizes of ~2.1 kb and ~3.8 kb corresponded to the endogenous RdRP^2 gene.
Sizes of the hybridising DNA fragments are given.

The hybridisation patterns of all three DNAs showed the ~1.3 kb and the ~2.1 kb fragments indicating no major rearrangements within the internal and the RB fragments. By contrast, the LB fragment appeared either smaller or larger than calculated. This demonstrated rearrangements in the LB fragment.

Despite the LB fragment rearrangements, the three plants containing the 702cTR$_{DR}$ construct were analysed for determination of the copy number. Similar to the analysis of plant lines containing IR constructs, determination of the copy number of the 702cTR$_{DR}$ T-DNA was examined with HindIII. The physical map of the 702cTR$_{DR}$ transgene depicts the HindIII sites and the sizes of DNA fragments that are expected to light up upon hybridisation with the cTomRes$_{8X/S}$ as probe (Fig. K). In case of single copy insertions, positive signals were expected to correspond to fragments with sizes of ~240 bp, ~300 bp (twice), ~780 bp and two border fragments with sizes ranging from >5.5 kb and >2.1 kb. Out of the three independent transformants, the SR1tan10 line appeared to bear a single copy of the T-DNA. As presented in Figure 14 HindIII fragments with sizes of ~4.5 kb and ~7.0 kb lighted up demonstrating that both of the border fragments were present.

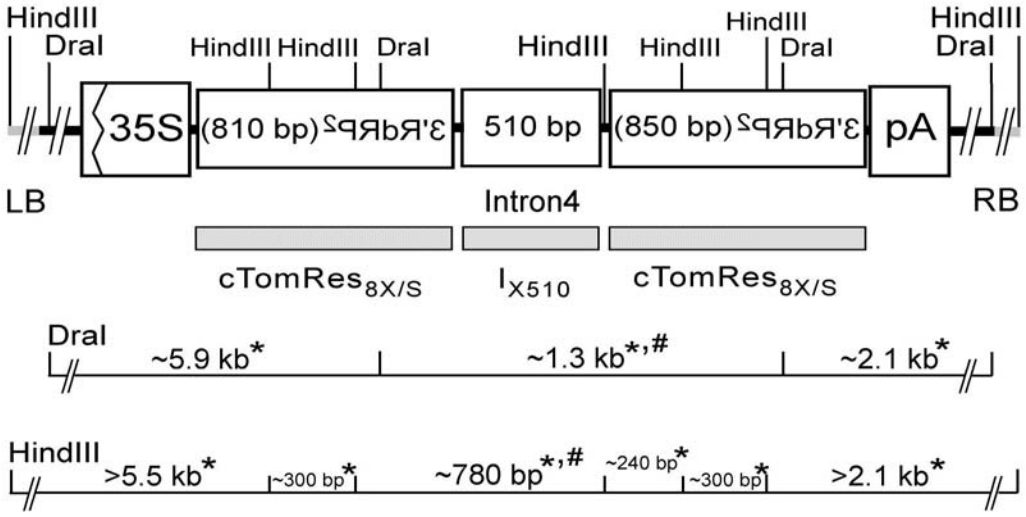


Figure K: Physical map of the 702cTR$_{DR}$ T-DNA. The DraI and HindIII sites of the T-DNA as well as the calculated lengths of the corresponding fragments are shown. The sizes of the fragments that are expected upon hybridisation with the cTomRes$_{8X/S}$ (*) and the I$_{X510}$ (#) probes are depicted. In addition to the cTomRes$_{8X/S}$ and the I$_{X510}$ probes (III.2.1.1.1), the npt$_8$ probe (II.2.4.5) was used (Fig. 21/IIA) which is not indicated in this Figure. The npt$_8$ hybridised to sequences between the pA signal and the right border sequences. Double diagonal lines indicate that the most up- and downstream DraI fragments as well as the T-DNA/plant DNA border fragments (grey lines) are not presented in full length.
Grey boxes represent the fragments that were used as probes.
LB = left border; RB = right border.

In summary, the Southern data of the primary transformants indicated that the SR1IR14, SR1Inv'4, SR1Inv'22 and the control SR1tan10 plant lines contained a single copy of the corresponding T-DNA. The absence of internal HindIII fragments in both, the IR and DR constructs contradicted the detection of internal DraI fragments upon hybridisation with $cTomRes_{8X/S}$ as probe (compare Figure 12, lanes 1 and 2 with Figure 12, lanes 4 and 5). Therefore, the DNA of the single copy primary transformants SR1IR14 and SR1tan10 was digested with HindIII and probed against the I_{X510} fragment (III.2.1.1.1).

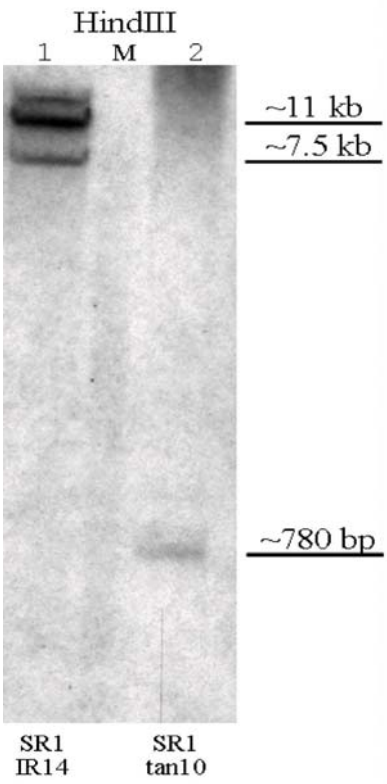


Figure 15: Southern blot of genomic DNA isolated from primary transformants.
The blot was hybridised against the ^{32}P-labelled I_{X510} spacer-specific probe (III.2.1.1.1). Sizes of the hybridising DNA fragments are given.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2).

In Figure J and K, the physical maps of the $702cTR_{IR}$ and $702cTR_{DR}$ transgene depict the HindIII sites and the expected sizes of the I_{X510} probe-specific fragments. A corresponding Southern blot is shown in Figure 15. The hybridisation pattern of the SR1IR14 genomic DNA showed appearance of two large fragments with sizes of ~7.5 kb and ~11 kb instead of the expected ~780 bp arguing for rearrangements in the genome-integrated T-DNA. Detection of a ~780 bp fragment in the HindIII-digested genomic DNA of the SR1tan10 led to the conclusion that the lack of an internal hybridising fragment on the previous blot (Fig. 14) was due to the poor quality of the blot. This assumption was later supported by Southern analysis of the SR1tan10 and the $SR1IR14T_1$ generations

[Fig. 26/I and 29/I, respectively]. From this analysis, fragment corresponding to the endogenous RdRP^{1}-intron4 could not be determined.

III.3.1.1.2 Southern analysis of plant lines transformed with the RdRP^{1} direct repeat T-DNA construct

All GV702Rd$^{1}_{DR}$-transformed plants (Table B) were screened for single copy integration of the T-DNA by Southern analysis using EcoRI. In Figure L, the physical map of the 702Rd$^{1}_{DR}$ transgene construct shows the location of the EcoRI sites within the T-DNA. The sizes of the fragments that were expected upon hybridisation with the ^{32}P-labelled Rd$^{1}_{5Sac}$ (II.2.4.5) and the I_{X510} probes are presented. From 10 independent primary transformants, the SR1dem18 showed the expected EcoRI pattern when probed against the Rd$^{1}_{5Sac}$. The appearance of the internal ~660 bp long fragment and the positive signal that corresponded to the RB fragment (~6.0 kb) indicate single copy insertion and no major rearrangements (Fig. 16A). The minimal length calculated for RB fragments was about 3.1 kb. The additional ~2.5 kb and ~4.5 kb long fragments derived from hybridisation with the endogenous RdRP^{1} gene (Fig. 18/a, lane 2).

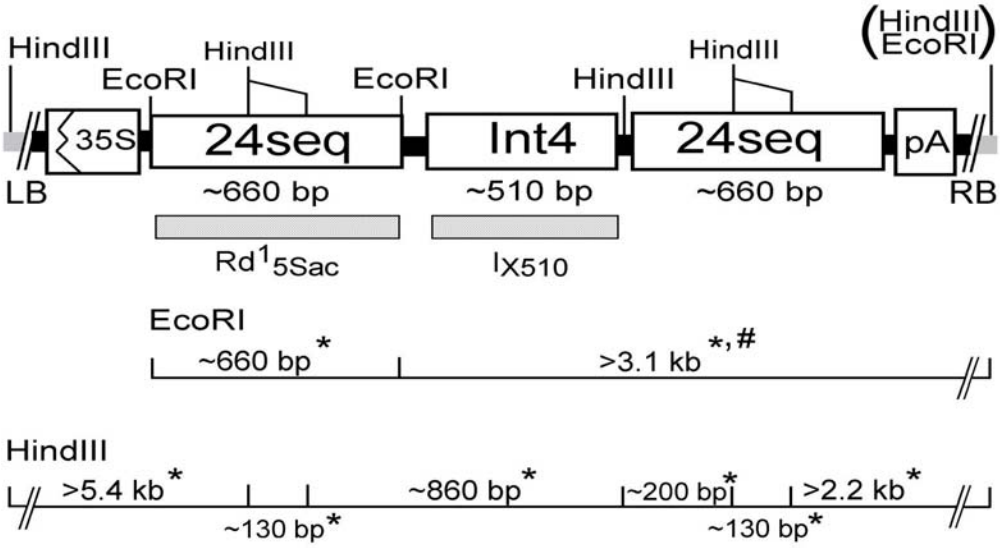


Figure L: Physical map of the 702Rd$^{1}_{DR}$ T-DNA. In addition to the polylinker restriction sites, the internal HindIII and KpnI sites as well as the sizes of the corresponding fragments are shown. Fragment sizes that were expected upon hybridisation with the Rd$^{1}_{5Sac}$ (*) and I_{X510} (#) probes are shown. Double diagonal lines indicate the undetermined length of the T-DNA/plant DNA (grey lines) border fragments.
Grey boxes represent the fragments that were used as probes.
LB = left border; RB = right border.

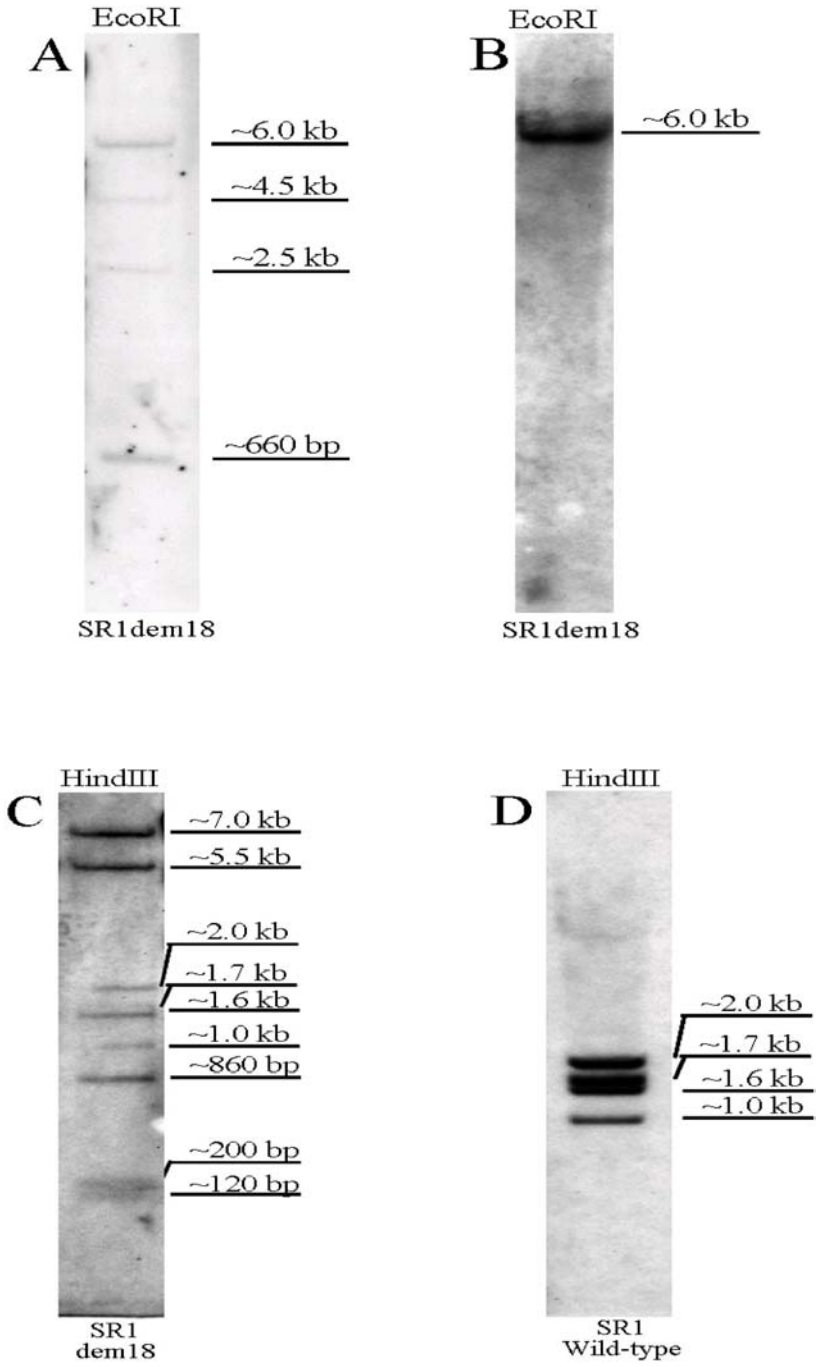


Figure 16: Southern blot of genomic DNA isolated from primary transformant and tobacco wild-type plants.
Sizes of hybridising DNA fragments are indicated.
A) The blot was hybridised against the ^{32}P-labelled $Rd1_{5Sac}$ DNA probe (II.2.4.5). Hybridising fragments with sizes of ~2.5 kb and ~4.5 kb corresponded to the endogenous RdRP[1] gene.
B) The blot was hybridised against the ^{32}P-labelled I_{X510} probe.
C), D) The blots were hybridised against the ^{32}P-labelled $Rd1_{5Sac}$ probe. Hybridising fragments with sizes ~1.0 kb, ~1.6 kb, ~1.7 kb and ~2.0 kb corresponded to the endogenous RdRP[1] gene. In C, the intensity of the ~120 bp long fragment corresponded to the intensity of two fragments.

The ~6.0 kb long RB fragment should include the entire spacer and the second copy of the RdRP[1] fragment. However, because the 3' EcoR1 site of the ~6.0 kb fragment was located at an unknown position in the flanking genomic DNA the fragment size could not give any information about the presence of the spacer fragment. The fact, that the border fragment lights up with the Rd^{1}_{5Sac} probe, provided evidence that, at least, a part of the RdRP[1] fragment had integrated. To confirm the presence of the spacer Southern analysis using EcoRI and the I_{X510} fragment as probe was carried out (Fig. 16B). The observation that an identical ~6.0 kb fragment lighted up under these conditions verified that the SR1dem18 plant contained, at least, a part of the spacer sequence.

To further examine the SR1dem18 plant line Southern analysis was performed using HindIII-digested genomic DNA. The physical map of the corresponding T-DNA is shown in Figure L and the blot hybridised with the Rd^{1}_{5Sac} fragment is presented in Figure 16C. The additional fragments with sizes of ~1.0 kb, ~1.6 kb, ~1.7 kb and ~2.0 kb derived from hybridisation with the endogenous RdRP[1] gene (Fig. 16D). The HindIII hybridisation pattern comprising the internal ~120 bp (twice), ~200 bp and ~860 bp long fragments was identical to the previous HindIII pattern of the $702Rd^{1}_{DR}$ plasmid DNA (Fig. 6, HindIII-lane). This indicated that no major rearrangements within the internal fragments including the spacer and a part of the two RdRP[1] copies were present in the SR1dem18 plant line. The fragments corresponding to sizes of ~5.5 kb and ~7.0 kb verified single copy insertion. Thus, based on these Southern analysis the SR1dem18 primary transformant carried a non-rearranged single copy T-DNA insertion.

III.3.1.1.3 Southern analysis of plant lines transformed with the RdRP[2] 3'end-containing T-DNA constructs

All primary transformants were screened for the $702Rd^{2}_{B+}$ T-DNA integration and copy number by Southern analysis using HindIII-restricted genomic DNA. In Figure M, the physical map of the construct illustrates the location of the HindIII sites and the sizes of the fragments that were expected upon hybridisation with the ^{32}P-labelled RdRP[2]-specific $cTomRes_{8X/S}$ fragment (III.2.1.1.1). The DNA from plants containing the 3'end sense construct [$SR1Bam^{+}$ (Table C)] were expected to show positive signals corresponding to fragments with sizes of ~230 bp and ~300 bp. The minimal length of large size fragments that could give insights about the T-DNA copy number was estimated to be about 6.0 kb for the LB.

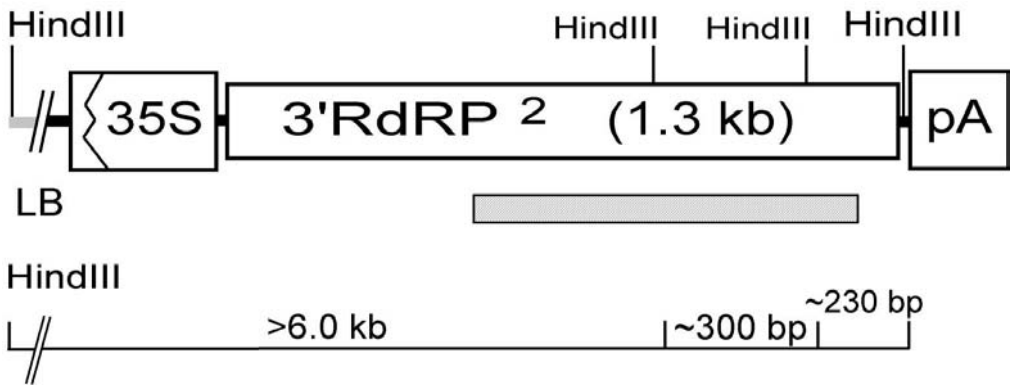
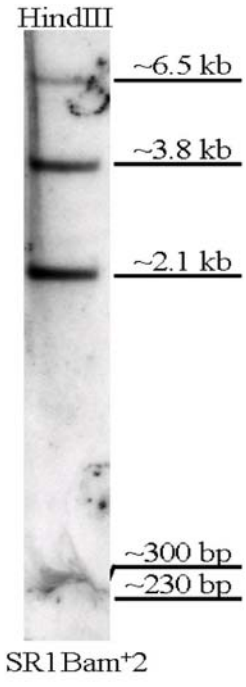


Figure M: Physical map of the 702Rd²$_{B+}$ T-DNA. HindIII sites as well as the sizes of the fragments that were expected upon hybridisation with the cTomRes$_{8X/S}$ probe (III.2.1.1.1) are indicated. Double diagonal lines correspond to the undetermined length of the T-DNA/plant DNA (grey line) border fragments.
The grey box represents the fragment that was used as probe.
LB = left border.



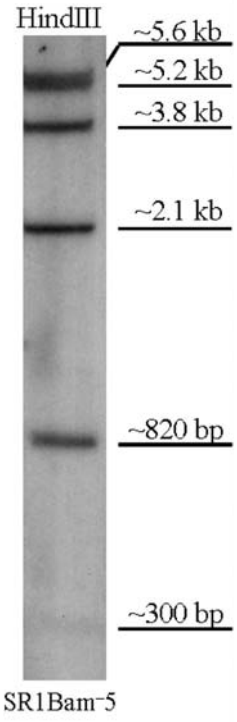


Figure 17/A: **Figure 17/B:**

Figures 17/A and **17/B:** Southern blots of genomic DNA isolated from primary transformants probed with the cTomRes$_{8X/S}$ DNA fragment. Sizes of the hybridising DNA fragments are indicated. The ~2.1 kb and ~3.8 kb long hybridising fragments correspond to the endogenous RdRP^2 gene. Figure 17/A: 0.8% (w/v) agarose gel; Figure 17/B: 1.5% (w/v) agarose gel.

From 10 independent primary transformants, six plants were initially analysed for the presence of the 702Rd²$_{B+}$ T-DNA. A Southern blot of HindIII-restricted genomic DNA revealed that one plant, the SR1Bam^{+}2 appeared to carry a single copy insertion with no detectable rearrangements within the internal fragments. The hybridisation pattern showed positive signals corresponding to fragments with sizes of ~230 and ~300 bp (Fig. 17/A). The restriction pattern was identical to the pattern obtained with the 702Rd²$_{B+}$ plasmid DNA (Fig. 7, lane 1). Appearance of a single ~6.5 kb fragment verified single copy insertion. Because the SR1Bam^{+}2 primary transformant seemed to contain the 702Rd²$_{B+}$ T-DNA as a single copy insertion without any obvious rearrangements. Therefore, it was proceeded for self-pollination to establish homozygous line.

All primary transformants containing the 702Rd²$_{B-}$ T-DNA were examined by Southern analysis using HindIII-digested genomic DNA. In Figure N, the physical map of the construct is presented. The HindIII-restricted genomic DNA from plants containing the 3'end antisense construct [SR1Bam^{-} (Table C)] should reveal hybridising fragments with sizes of ~300 bp and ~820 bp. This restriction pattern for internal fragments was known from the previous analysis of the 702Rd²$_{B-}$ plasmid DNA (Fig. 7; lane 2).

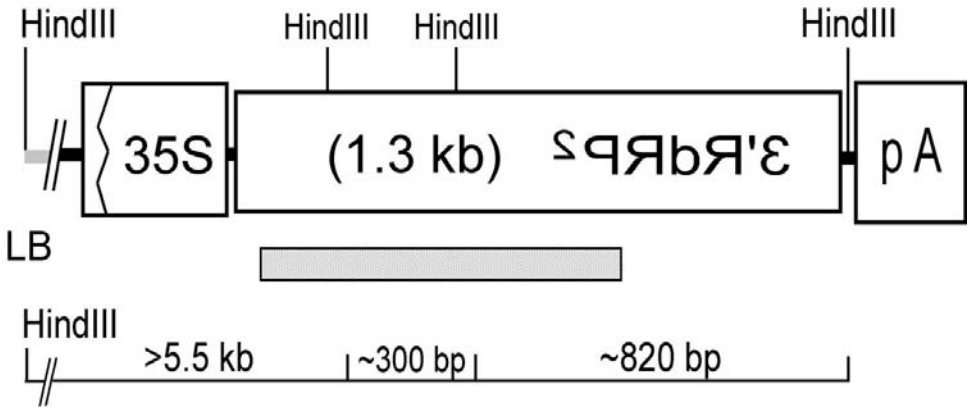


Figure N: Physical map of the 702Rd$^{2}_{B-}$ T-DNA. HindIII sites as well as the sizes of the fragments that were expected upon hybridisation with the cTomRes$_{8X/S}$ probe are indicated. Double diagonal lines correspond to the undetermined length of the T-DNA/plant DNA (grey line) border fragments.
The grey box represents the fragment that was used as probe.
LB = left border.

Appearance of a single fragment with a minimal length of about 5.5 kb would indicate single copy insertion. However, none of the 10 independent primary transformants showed

only a single hybridising fragment of this size. Two plant lines were found to carry two T-DNA insertions. One plant the SR1Bam^{-}5 was further examined and a Southern blot of this plant is shown in Figure 17/B. The presence of the ~300 and ~820 bp long fragments indicated no apparent rearrangements within the internal HindIII fragments. In addition to a ~5.6 kb, a ~5.2 kb fragment became visible demonstrating that one of the two LB fragments was rearranged. Nevertheless, this primary transformant was selected for self-pollination to examine whether the two copies were unlinked and would therefore segregate in progeny.

III.3.1.1.4 Southern analysis of plant lines transformed with the RdRP^{1} 3'end-containing T-DNA constructs

All primary transformants obtained by introduction of the 702Rd$^{1}{}_{S+}$ T-DNA were screened for the copy number by Southern analysis using EcoRI. The physical map of the 702Rd$^{1}{}_{S+}$ construct illustrates the location of the two closely adjacent EcoRI sites, the HindIII sites, and the sizes of the fragments that were expected upon hybridisation with the ^{32}P-labelled Rd$^{1}{}_{5Sac}$ probe (Fig. O).

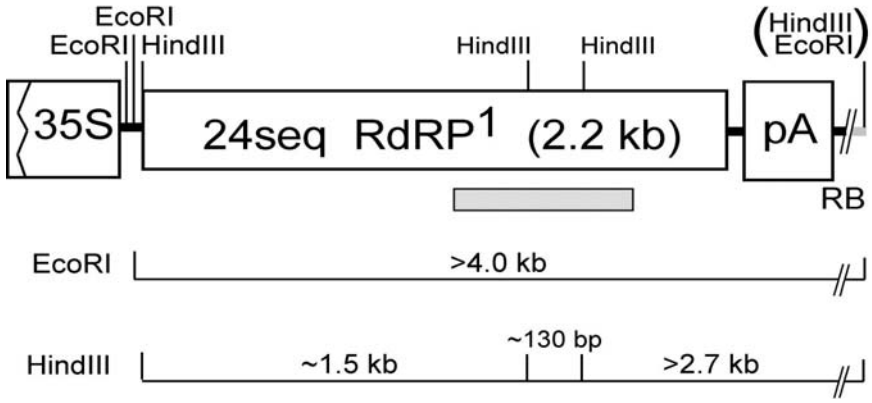


Figure O: Physical map of the 702Rd$^{1}{}_{S+}$ T-DNA. The HindIII sites of the 24seq sequence are shown. The calculated length of the HindIII and EcoRI fragments that were expected upon hybridisation with the Rd$^{1}{}_{5Sac}$ probe are depicted. Double diagonal lines indicate that the T-DNA/plant DNA border fragment (grey line) is not shown in full length. Restriction sites in parenthesis indicate that their next recognition sequences are located in the flanking plant DNA.
The grey box represents the fragment that was used as probe.
RB = right border.

The primary transformants containing a single copy of the 702Rd$^{1}{}_{S+}$ T-DNA were expected to show one EcoRI fragment (RB) with a length that, depending on the next EcoRI site

located within plant genome, would be larger than ~4.0 kb. Hybridisation of HindIII fragments with sizes of ~130 bp and ~1.5 kb would demonstrate that no major rearrangements within the internal fragments are present. Similar to a single EcoRI fragment with a size of >4.0 kb, an unique HindIII RB fragment with a size of >2.7 kb would indicate single copy integration.

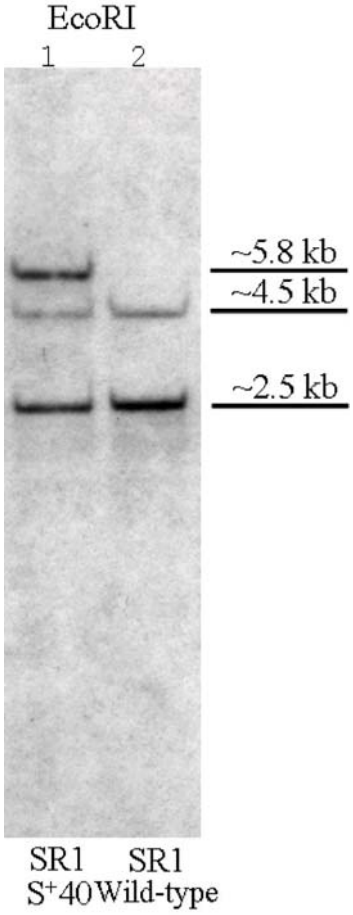


Figure 18/a: Southern blot of genomic DNA isolated from primary transformant and tobacco wild-type plants. The blot was hybridised against the ^{32}P-labelled Rd^{1}_{5Sac} fragment. Sizes of the hybridising DNA fragments are indicated. Hybridising fragments with sizes of ~2.5 kb and ~4.5 kb correspond to the endogenous RdRP[1] gene.

Ten independent primary transformants were examined and two of them seemed to have a single copy T-DNA insertions. Southern analysis of one plant ($SR1S^{+}40$) using EcoRI as well as HindIII is presented in Figure 18. By probing against the Rd^{1}_{5Sac} fragment, hybridisation of a single ~5.8 kb RB EcoRI and a single ~8.0 kb HindIII fragment (Fig. 18/a and 18/bB-I, respectively) was detected. Appearance of these unique fragments indicated single copy integration. In addition to the ~8.0 kb fragment, HindIII fragments with sizes of ~130 bp and ~1.5 kb lighted up (Fig. 18/bB-I and B-II). The sizes of these fragments were in agreement with the length of the HindIII fragments that were detectable when the $702Rd^{1}_{S+}$ plasmid was analysed (Fig. 8; lane 3). This finding inferred that no

major rearrangements within the internal fragments had occurred. The additional fragments with sizes of ~1.0 kb, ~1.6 kb, ~1.7 kb and ~2.0 kb derived from hybridisation with the endogenous RdRP[1] gene (Fig. 18/bA).

All primary transformants of the RdRP[1] antisense construct were screened for the presence of the 702Rd$^{1}_{A-}$ T-DNA by Southern analysis using EcoRI and HindIII.

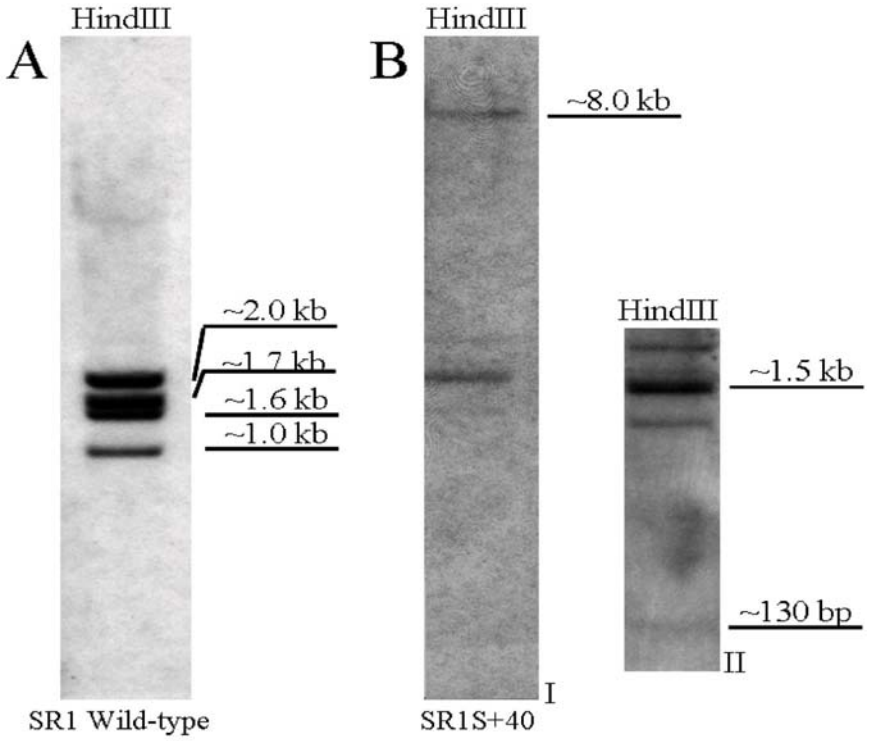


Figure 18/b: Southern blot of genomic DNA isolated from primary transformant and tobacco wild-type plants. The blot was hybridised against the ^{32}P-labelled Rd$^{1}_{5Sac}$ fragment (II.2.4.5). Sizes of the hybridising DNA fragments are given. Hybridising fragments with sizes of ~1.0 kb, ~1.6 kb, ~1.7 kb and ~2.0 kb corresponded to the endogenous RdRP[1] gene.
B-II = overexposed autoradiograph presented in B-I.

In Figure P, the physical map of the 702Rd$^{1}_{A-}$ construct illustrates the location of the EcoRI and HindIII sites as well as the corresponding fragment sizes that are expected upon hybridisation with the Rd$^{1}_{5Sac}$ fragment. EcoRI digestion of genomic DNA from the SR1A^{-} (6 and 8) resulted in the predicted ~2.2 kb long internal fragment (Fig. 19) indicating no major rearrangements. The size of this fragment was in agreement with the length of the EcoRI fragment that was detectable when the 702Rd$^{1}_{A-}$ plasmid was analysed (Fig. 8; lane 2). In order to determine the copy number genomic DNA was digested with HindIII and

probed against the Rd^{1}_{5Sac} fragment (data not shown). Due to the poor quality of the blot internal fragments were not clearly visible. However, the hybridising ~8.0 kb and ~6.5 kb fragments demonstrated that the SR1A^{-}6 as well as the SR1A^{-}8 plant lines contained single copy T-DNA insertions.

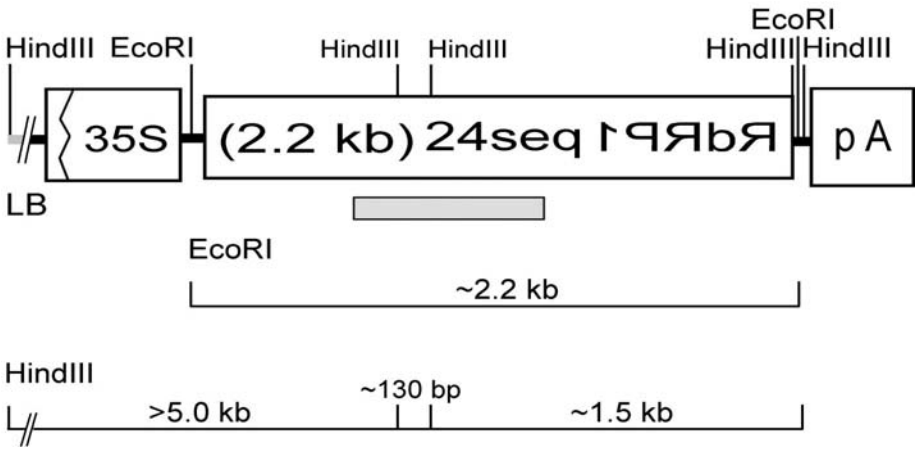


Figure P: Physical map of the $702Rd^{1}_{A}$- T-DNA. The HindIII sites of the 24seq sequence are shown. The calculated lengths of the HindIII and EcoRI fragments that were expected upon hybridisation with the Rd^{1}_{5Sac} probe are depicted. Double diagonal lines indicate that the T-DNA/plant DNA border fragment (grey line) is not presented in full length.
The grey box represents the fragment that was used as probe.
LB = left border.

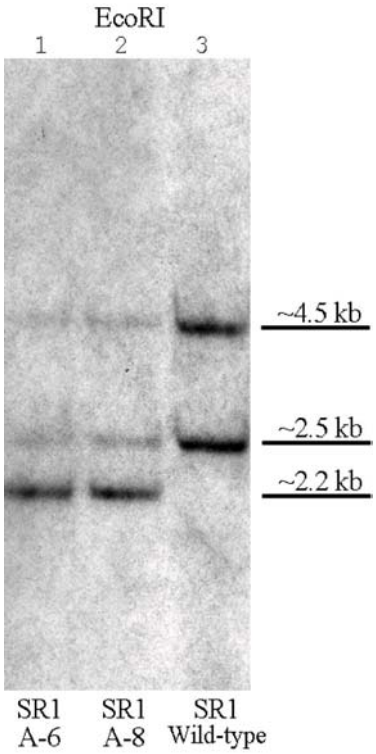


Figure 19: Southern blot of genomic DNA isolated from primary transformants tobacco wild-type plants. The blot was hybridised against the ^{32}P-labelled Rd^{1}_{5Sac} DNA fragment (II.2.4.5). Sizes of the hybridising fragments are given. Hybridising fragments with sizes of ~2.5 kb and ~4.5 kb corresponded to the endogenous RdRP^{1} gene.

III.3.1.1.5 Plant lines transformed with the full-length RdRP^2 cDNA T-DNA construct

All primary transformants were screened for the presence of the 702Rd²$_{3.5+}$ T-DNA construct with HindIII. In Figure Q, the physical map of the 702Rd²$_{3.5+}$ transgene depicts the location of the HindIII sites and the corresponding fragment sizes that are expected upon hybridisation with the cTomRes$_{8X/S}$ fragment. Out of 10 independent primary transformants, two plants (SR1Rd^{2+}, 2 and 17) showed hybridising HindIII fragments with sizes of ~300 bp and ~940 bp (Fig. 20) as would be predicted for non-rearranged internal fragments (Fig. Q, see also Fig. 9; HindIII). Single hybridisation signals corresponding to HindIII fragments (RB) with sizes of ~6.0 kb and ~7.5 kb indicated that the SR1Rd^{2+}17 and the SR1Rd^{2+}2 plant lines, were single copy transformants. The minimal size for a fragment corresponding to the RB was predicted to be about 2.2 kb.

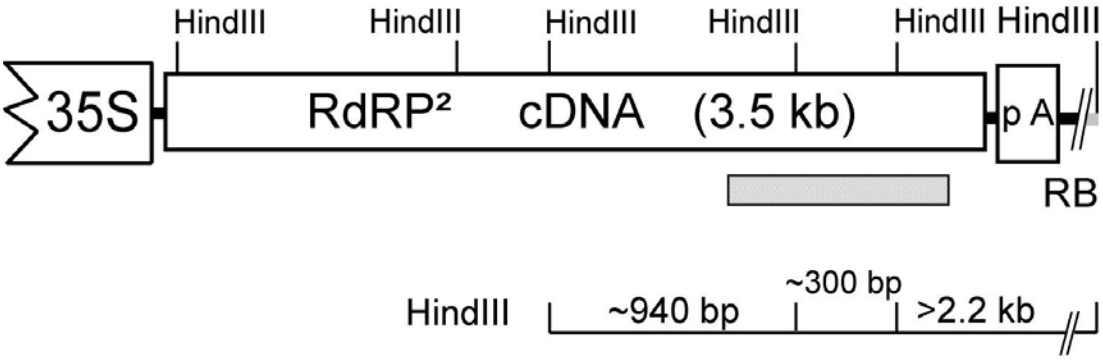


Figure Q: Physical map of the 702Rd²$_{3.5+}$ T-DNA. HindIII sites of the RdRP^2 full-length cDNA are shown. The calculated lengths of the HindIII fragments that were expected upon hybridisation with the cTomRes$_{8X/S}$ probe are depicted. Double diagonal lines indicate that the T-DNA/plant DNA border fragment (grey line) is not present in full length.
The grey box indicates the fragment that was used as probe.
RB = right border.

Due to the large size (~3.5 kb), the cTomRes$_{8X/S}$ probe only overlapped with the 3'half of the HindIII-cleaved RdRP^2 cDNA. For detection of the 5'half of the cDNA a ~700 bp long EcoRV/EcoRI fragment that was isolated from the pGEM$_{2.7}$ clone (III.2.3.1.1) was used as probe. Hybridisation of HindIII-digested genomic DNA from the SR1Rd^{2+}17 and SR1Rd^{2+}2 plant lines lighted up a ~260 bp, ~940 bp and ~1.7 kb fragments (data not shown). Appearance of fragments with these sizes demonstrated that no rearrangements within the 5'half of the cDNA were present. Nevertheless, the presented Southern analysis

indicated that the SR1Rd^{2+}17 and SR1Rd^{2+}2 plant lines both contained non-rearranged single T-DNA insertions.

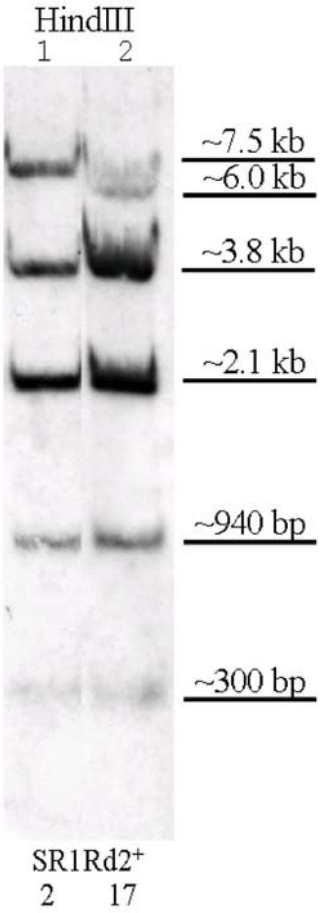


Figure 20: Southern blot of genomic DNA isolated from primary transformants.
The blot was probed with the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment. Sizes of the hybridising DNA fragments are given. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb corresponded to the endogenous RdRP^2 gene. Numbers above the blot represent the lanes and numbers below the blot indicate the number of the plant line.

III.3.1.2 Southern and Northern analyses of primary transformants displaying the TK-phenotype

Out of the primary transformants examined, four plant lines showed the TK-phenotype. These four plants derived from independent transformants either containing RdRP^2-specific (three plants) or a RdRP^1-specific T-DNA construct (one plant).

The primary transformant SR1Bam$^-$32 carrying the 702Rd^2_B. T-DNA exhibited a flower and leaf morphology that was reminiscent of the previously produced SR1TK-plant lines (I.3, Photographs). As was described for the SR1Bam$^-$5 plants (III.3.1.1.3), copy number and arrangement of the T-DNA of the SR1Bam$^-$32 line were Southern-analysed with HindIII using the cTomRes$_{8X/S}$ as probe (Fig. N). In contrast to the SR1Bam$^-$ plants that displayed no TK-phenotype, the SR1Bam$^-$32 plant line seemed to bear a single copy insertion of the T-DNA. On the Southern blot, only a single fragment with a size of ~9.0

kb lighted up (Fig. 21/IA). In addition, appearance of the ~300 and ~820 bp long fragments indicated no major rearrangements within the internal transgene fragments (Fig. 21/IA).

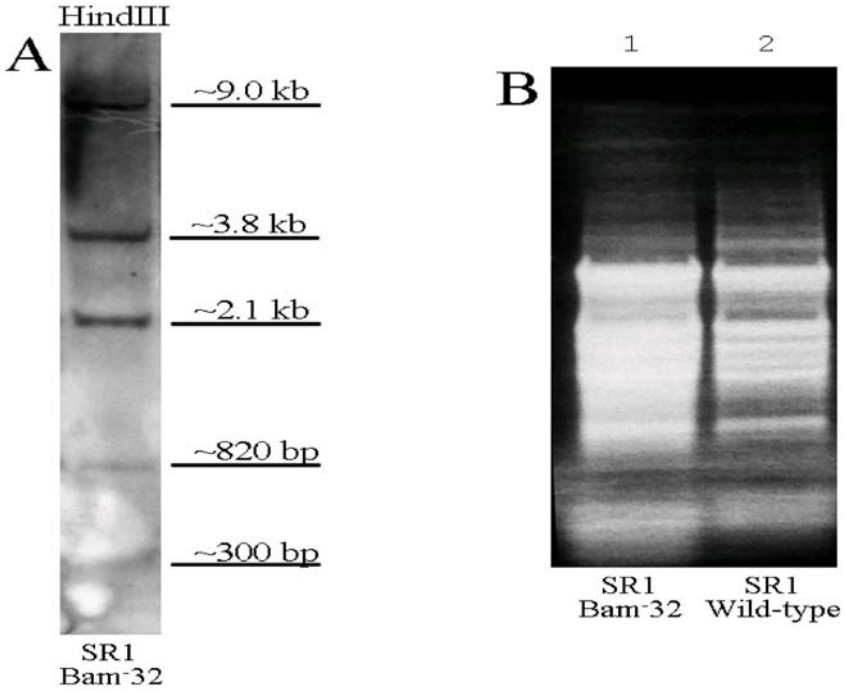


Figure 21/I: Southern and Northern analysis of the primary SR1Bam^{-}32 transformant.
A) Southern blot (II.2.4.2) of genomic DNA (II.2.4.1) probed with the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment (III.2.1.1.1). Sizes of the hybridising DNA fragments are given. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous RdRP^{2} gene
B) Membrane showing the amount of total RNA that was transferred. Membrane was hybridised against the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment. Because no hybridising signal was detectable, the corresponding autoradiograph is not presented.

Because of the appearance of the TK-phenotype it was important to examine whether the RdRP^{2}-specific antisense construct of the SR1Bam^{-}32 line suppressed the expression of the endogenous RdRP^{2} gene. Northern hybridisation was performed with total RNA using the cTomRes$_{8X/S}$ fragment as probe. As a control for the RdRP^{2} steady state mRNA accumulation, tobacco SR1 wild-type RNA was co-analysed. However, no hybridisation signal was visible in either lane (Fig. 21/IB). The fact that no signal was detectable for the non-transgenic plant demonstrated that natural expression of the endogenous RdRP^{2} gene was below the detection limit, at least, under the applied Northern hybridisation conditions. Additionally, absence of positive hybridisation signal for the transgenic plant indicated that either expression was below the detection limit or that the

transgene was not expressed. To further gain information about the expression level, PCR was carried with cDNA that was synthesised from total RNA (II.2.1.7.4) of the SR1Bam^{-}32 plant. PCR was performed under standard conditions (II.2.1.7.2) using the 3'HomRevBamHI primer in combination with pNOS1020BiUS primer (II.2.1.7.5, primer sequences) to ensure that no endogene-specific fragment was amplified. Upon PCR amplification a fragment with a size of ~1.4 kb was expected. PCR products were electrophoresed on a 1% (w/v) agarose gel (II.2.1.2) and processed for Southern hybridisation (II.2.4.2) with the cTomRes$_{8X/S}$ fragment as probe. Detection of the positive hybridisation signal corresponding to a fragment size of ~1.4 kb (data not shown), indicated that the transgene was transcribed but expression was either low or the transcripts were unstable. In order to follow the inheritance of this phenotype in progeny plants, the SR1Bam^{-}32 male sterile plant was proceeded for genetic cross with the tobacco wild-type plant. Progeny plants obtained from this cross were analysed for the presence of the transgene by Southern analysis and were monitored for phenotypic alterations. Out of six progeny plants, two plants had lost the transgene (data not shown). Interestingly, as the four transgene-containing progeny these plants also displayed the phenotypic alterations. In other words, the SR1Bam^{-}32 progeny plants established the TK-phenotype in absence of the transgene.

The primary transformant SR1tan7 bearing the 702cTR$_{DR}$ T-DNA, was Southern-analysed for copy number with HindIII using the npt$_8$ fragment (II.2.4.5) as probe (for the physical map see Figure K). A Southern blot showed three hybridising fragments with sizes of ~8.0 kb, ~10 kb and ~11 kb indicating integration of, at least, three T-DNA copies (Fig. 21/IIA). Southern analysis with DraI-digested genomic DNA and probing against the I$_{X510}$ fragment was performed to examine possible rearrangements within internal fragments. The presence of a hybridising fragment with a size of ~1.3 kb and the high intensity of it as compare to the single copy endogenous RdRP^1-intron4 revealed that the three T-DNA copies carried the internal fragment with no major rearrangements (Fig. 21/IIB). Re-probing of this "DraI blot" with the cTomRes$_{8X/S}$ fragment (III.2.1.1.1) was carried out to see the integration pattern of copies (Fig. 21/IIC). As discussed for the RdRP^2 DR constructs, a non-rearranged T-DNA was expected to show positive signals corresponding to fragments with sizes of ~1.3 kb (internal), ~2.1 kb (RB), and ~5.9 kb (LB). If all three copies were non-rearranged, these fragments should show a three fold higher intensity when compared to a single copy T-DNA insertion. To gain information

about the relative hybridisation signal intensities the blot presented Figure 21/IIC was compared with the autoradiograph of the single copy SR1tan10 plant line (Fig. 13). It turned out that the intensities of the ~2.1 kb long reference fragments being present in both, the SR1tan10 and the SRtan7 lines, were identical (compare Figure 21/IIC, lane 1 with Figure 13). This finding indicated that the SRtan7 carried only one non-rearranged T-DNA copy. Moreover, appearance of only one additional DraI fragment (~8.5 kb) (Fig. 21II/C, lane 1) demonstrated that the two remaining T-DNAs were heavily rearranged. If the ~1.3 kb, ~2.1 kb, and ~5.9 kb long DraI fragments represented the banding pattern of the non-rearranged T-DNA insertion, one would have expected two additional internal and, in total, four additional border fragments. To get clear idea about the transgene arrangement and the T-DNA copy number further analysis are required.

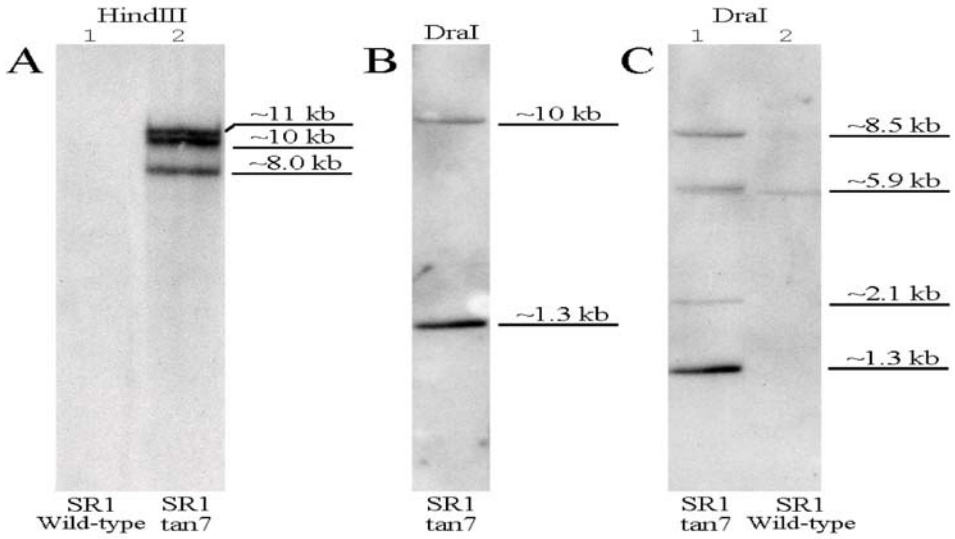


Figure 21/II: Southern blots of genomic DNA isolated from a primary transformant exhibiting TK-phenotype and from tobacco wild-type plants.
Sizes of the hybridising DNA fragments are indicated.
A) The blot was probed with the npt_8 fragment (II.2.4.5).
B) The blot was probed with the I_{X510} fragment (III.2.1.1.1). The hybridising fragment with a size of ~10 kb correspond to the endogenous $RdRP^1$-intron4.
C) The blot was probed with the $cTomRes_{8X/S}$ fragment (III.2.1.1.1). The hybridising fragment with a size of ~5.9 kb correspond to the endogenous $RdRP^2$ gene.

The transgene expression in this plant was analysed by PCR using cDNA as template. PCR amplification of the 5' $RdRP^2$-specific fragment was performed using the 3'HomRevBamHI as forward and the RdRP7620Int4reverse primers while the 3' copy was amplified using the pNOS1020BiUS primer as reverse and the RdRP7500Int4forward

primers (II.2.1.7.5, primer sequences). In Figure B/III, the primer binding sites are indicated. PCR products were run on a 1% (w/v) agarose gel (II.2.1.2) and proceeded for Southern hybridisation (II.2.4.2) with the $cTomRes_{8X/S}$ fragment as probe. The size of the hybridising fragments corresponded to ~1.1 kb for the 5' fragment and ~1.2 kb for the 3' copy (data not shown). These sizes were expected for non-spliced transcripts.

The primary transformant $SR1S^{+}26$ carrying the $702Rd^{1}_{S+}$ was Southern-analysed for copy number with HindIII. The fact that probing against the Rd^{1}_{5Sac} fragment resulted in two hybridising fragments with sizes of ~8.0 kb and ~6.0 kb indicated that two transgene copies were present. Due to poor quality of the blot, internal ~130 bp and ~1.5 kb long HindIII fragments (for physical map see Figure O) were not visible (data not shown). In frame of this work, the main objective was to investigate whether the transgenic plants exhibiting the TK-phenotype carried a transgene copy and if so, whether multiple or single copies were present. To gain more information about the genotype, the $SR1S^{+}26$ plant line needs to be further analysed.

As described for the $SR1Bam^{-}32$ line, Northern analysis was performed to examine whether expression of the endogenous $RdRP^{1}$ gene was affected by the presence of the $702Rd^{1}_{S+}$ transgene. However, when probed against the Rd^{1}_{5Sac} fragment (II.2.4.5) no hybridisation signals were detectable, neither for the $SR1S^{+}26$ nor for the tobacco SR1 wild-type plant. This showed that similar to the $RdRP^{2}$ gene, the natural expression level of the $RdRP^{1}$ gene was below the detection limit. The expression level of the $SR1S^{+}26$ transgene needs to be examined by PCR amplification using cDNA.

In addition to the aforementioned plant lines, one primary transformant containing the $RdRP^{2}$ 3'end-specific fragment that was also present in the TK-phenotype-expressing $SR1Bam^{-}32$ line, was found to develop the phenotype. Importantly and in contrast to the $SR1Bam^{-}32$ line, this $SR1Bam^{+}7$ line carried the $RdRP^{2}$ 3'end-specific sequence in sense orientation. Unfortunately, it was not possible to characterise this plant line within the scope of this work.

III.3.1.3 Northern analysis of primary transformants displaying no obvious phenotypic alterations

T_1 progeny plants of the primary transformants carrying $RdRP^{2}$-specific fragments (SR1IR14, SR1Inv'4, SR1Inv'22, SR1tan10, $SR1Bam^{+}2$, and SR1 $Bam^{-}5$) were analysed for transgene expression by Northern analysis (II.2.4.4). The objective behind analysis of

T_1 generations was to determine the level of transgene expression in homozygous plants. Moreover, it was of interest to follow if any phenotypic alterations would develop in T_1 progeny plants. In the frame of this work, Northern analysis of only single copy primary transformants carrying $RdRP^1$-specific transgenes (SR1dem18, $SR1S^+40$ and $SR1A^-6/8$) and the full-length $RdRP^2$ cDNA transgene ($SR1Rd^{2+}17$) were performed. As a control for endogenous RdRP expression, Northern analysis was performed with total RNA isolated from tobacco SR1 wild-type plants using the $RdRP^1$-specific $Rd^1{}_{5Sac}$ and the $RdRP^2$-specific $cTomRes_{8X/S}$ fragments as probes (II.2.4.4, II.2.4.5). The Northern data confirmed previously obtained results in that expression of both of the endogenous $RdRP^1$ and $RdRP^2$ genes were below the detection limit. On none of the autoradiographs a specific hybridisation signal for a full-length RdRP mRNA was detectable (for $RdRP^2$ see Figure 26/IIB and for $RdRP^1$data not shown).

Total RNA from the SR1dem18 containing the $702Rd^1{}_{DR}$ transgene was probed against the $Rd^1{}_{5Sac}$ fragment. Size estimation of the transgene transcript was done by comparing the hybridising RNA with the ribosomal RNAs (II.2.4.4). Precise size determination are problematic as the transcript (mRNA) sizes were dependent on the length of poly (A) tail, which was expected to range from 50 to 200 nucleotides (nt). All sizes mentioned correspond to transcripts having a poly (A) tail of 100 nt. The appearance of two hybridisation signals with an estimated size of ~1900 nt and ~1400 nt led to the hypothesis that non-spliced and spliced transgene transcripts were present (Fig. 22A). The spliced transcript was expected to be ~510 nt smaller in size than the non-spliced transcript. To obtain support for the above assumption total RNA from the SR1dem18 (carrying single copy) and the SR1dem11 plant line carrying multiple copies of the $702Rd^1{}_{DR}$ construct (III.3.1.1.2) was probed against the I_{X510} fragment. This fragment was intron4-specific and would thus allow to distinguish between non-spliced and spliced transcripts. Both plant lines showed a positive hybridisation signal corresponding to the large-sized RNA (~1900 nt) (for SR1dem11, see Figure 22B). This substantiated the notion that the $RdRP^1$-transgene was expressed and that the primary transcript underwent partial splicing. This finding demonstrated that the intron that spaced the repeated sequences was functional *in planta* and that intron-containing tandem transgene constructs were, in general, moderately expressed *in vivo*.

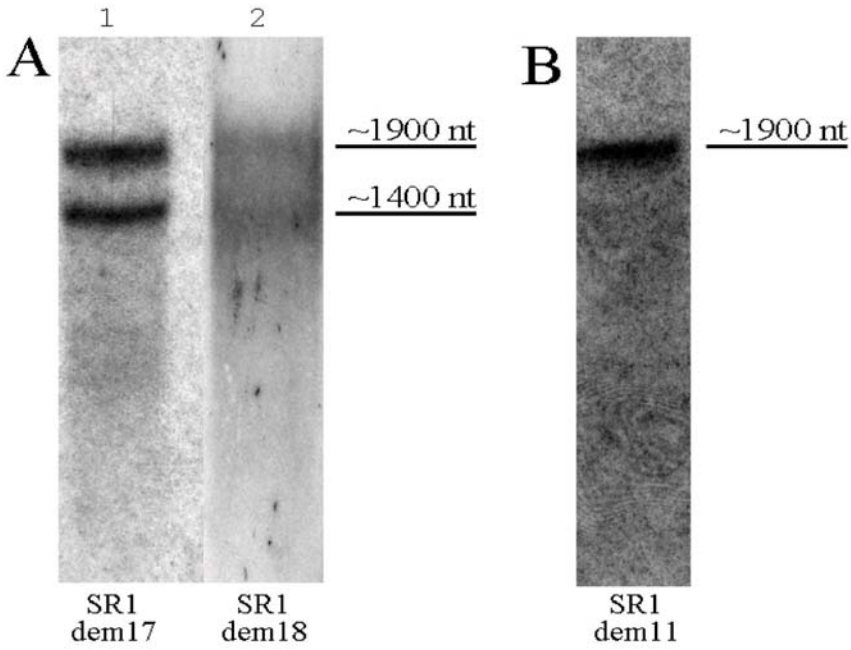


Figure 22: Northern hybridisation of total RNA isolated from primary transformants.
The indicated sizes of hybridising RNAs included a 100 nt long poly (A) tail.
A) The blot was probed against the ^{32}P-labelled Rd^{1}_{5Sac} DNA fragment (II.2.4.5).
B) The blot was probed against the ^{32}P-labelled I_{X510} DNA fragment (III.2.1.1.1). Due to the poor resolution of the SR1dem18 blot after scanning, Northern hybridisation was repeated but using total RNA from the multiple copy plant, SR1dem11. The hybridisation pattern of total RNA isolated from the SR1dem11 plant was similar to that of total RNA isolated from the SR1dem18 line but the signal strength of this RNA was clearly stronger.

Northern analysis of the $SR1S^{+}40$ primary transformant containing the $702Rd^{1}_{S+}$ T-DNA using the Rd^{1}_{5Sac} probe is shown in Figure 23. The hybridisation RNA showed an apparent size of ~2300 nt long RNA as expected for an expressed transgene. Similarly, the $SR1A^{-}6$ and 8 primary transformants containing the $702Rd^{1}_{A-}$ T-DNA were analysed for transgene expression by Northern hybridisation using the Rd^{1}_{5Sac} DNA fragment as probe (Fig. 24). A positive hybridisation signal corresponding to a RNA with a size of ~2300 nt demonstrated that the transgene was expressed in the two plants. Finally, Northern analysis of the $SR1Rd^{2+}2$ and 17 using the $cTomRes_{8X/S}$ fragment as probe revealed transgene expression. Example of a typical Northern blot with total RNA isolated from the $SR1Rd^{2+}17$ line is presented in Figure 25. A positive hybridisation signal that corresponded to a RNA with a size of ~3600 nt was detectable.

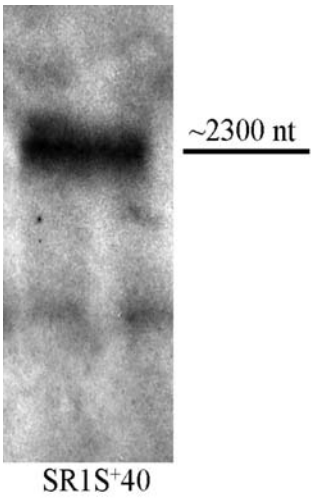


Figure 23:

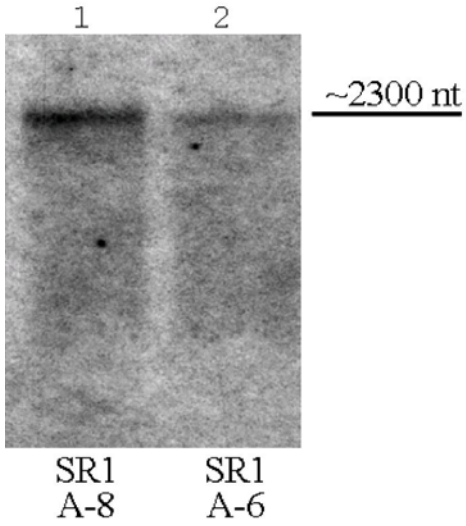


Figure 24:

Figures 23/24: Northern hybridisation of total RNA isolated from primary transformants. Blots were probed against the ^{32}P-labelled Rd^{1}_{5Sac} DNA fragment (II.2.4.5). The indicated sizes of hybridising RNAs included a 100 nt long poly (A) tail.

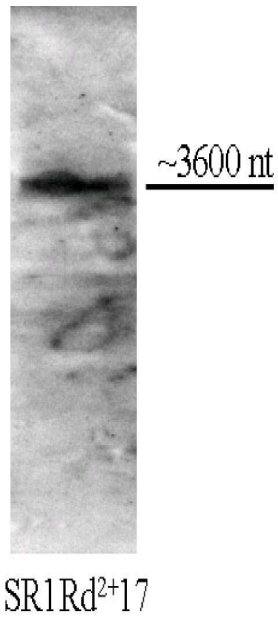


Figure 25: Northern hybridisation of total RNA from the primary transformant.
The blot was probed with the ^{32}P-labelled $cTomRes_{8X/S}$ DNA fragment. The indicated size of the hybridising RNA included a 100 nt long poly (A) tail.

III.3.1.4 Southern and Northern analysis of the RdRP^{2} T_1 generation

Initial screening of the SR1Bam^{+}2 and SR1Bam^{-}5 T_1 progeny plants for the inheritance of the transgene was performed by PCR. Genomic DNA was digested with SphI and PCR-amplified using the 710BiUS-35S as forward and pNOS1020BiUS as reverse primers. PCR-amplified fragments were expected to correspond to a size of ~1.6 kb (Fig. F). Plant

lines that exhibited the expected PCR product were selected for Southern analysis. In T_1 progeny plants, screening for the presence of $RdRP^2$ inverted and tandem repeats was done in the same way but using different primer pairs. Another set of primers was required because any attempt to amplify the entire IR construct failed. Thus, it was decided to separately amplify the 5' and 3' parts of IRs. For detection of the 5' $RdRP^2$ fragment the 710BiUS-35S (forward) and the intron4-specific RdRP7620Int4 (reverse) were used. The 3' $RdRP^2$ was amplified with the forward/reverse primer combination RdRP7500Int4/pNOS1020BiUS. In Figure B/III, the binding positions of the four primers are indicated. For both of the amplifications the expected fragment size that would indicate the presence of the $RdRP^2$-specific sequences was ~1.2 kb. Plant lines that showed PCR products with this size were selected for Southern analysis (data not shown).

Positive plants were screened for the homozygous state of the transgene by Southern analysis (II.2.4.2). Based on the intensity of hybridising fragments it was concluded whether the transgene was present on one or on both chromosomes. As an internal control the intensity of the endogenous $RdRP^2$ gene was utilised. If the intensity of the endogene-specific signals was identical for the DNA of two different plants, it was assumed that equal amounts of DNA were loaded on the gel. A comparison of the transgene-specific signal strength would then allow to directly determine the genotype. The intensity of the transgene-specific signal was expected to be twice as strong for a homozygous plant when compared to heterozygous plants. Southern analysis of the T_1 generation was performed as described for the parental plants.

Genomic DNA from plants of the SR1tan10 T_1 generation was digested with HindIII and probed against the $cTomRes_{8X/S}$ fragment. A Southern blot of three plants is shown in Figure 26/I. It should be noted that, in contrast to the Southern analysis of the parental plant SR1tan10 (Fig. 14), the internal fragments with sizes of ~240 bp, ~300 bp (twice) and ~780 bp were now detectable. This showed that the quality of the previous Southern blot was indeed poor. Moreover, these results demonstrated that no major rearrangements were present within the internal fragments of the transgene. Out of the three progeny plants, the SR1tan10T_1/3 and 5 appeared to be homozygous. The hybridisation signals of these plants were comparatively stronger than that of the plant 6 (Fig. 26/I, compare lanes 1 and 2 with lane 3). Out of the two homozygous plant lines, the SR1tan10T_1/3 was selected to further substantiate its homozygous state by a genetic cross with a tobacco SR1 wild-type plant. Seeds produced were plated onto selection media and

the number of growing versus dying seedlings was counted. All of the germinating seeds survived on kanamycin-containing medium. Because the SR1tan10 plant line was shown to carry a single copy T-DNA insertion (III.3.1.1.1; Fig. 14), development of all seedlings demonstrated that the SR1tan10T_1/3 was homozygous.

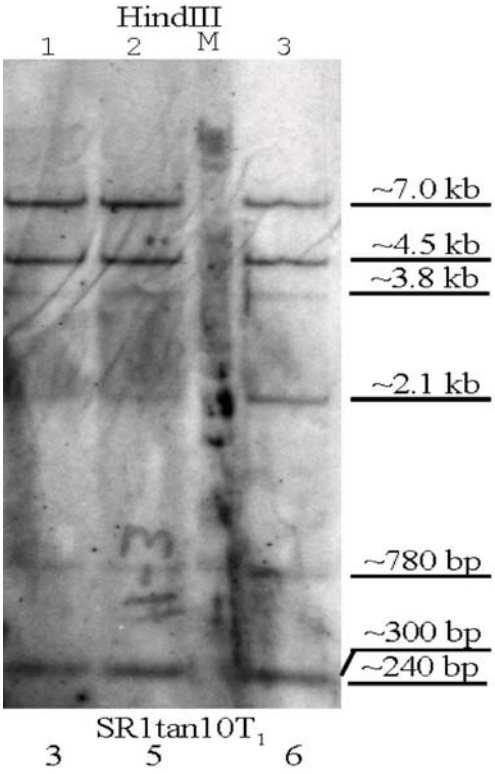


Figure 26/I: Southern blot of genomic DNA isolated from T_1 progeny plants.
The blot was probed with the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment. Sizes of the hybridising DNA fragments are indicated. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous RdRP^2 gene.
Numbers above the blot represent the lanes and numbers below the blot indicate the number of the T_1 plant line.
M = DNA length standard (λ DNA cut by PstI; II.2.1.2).

To examine whether the transgene was expressed in the homozygous SR1tan10T_1/3 plant, Northern analysis was performed with total RNA (II.2.4.4). One blot was probed with the I_{X510} (Fig. 26/IIA) and the second was probed with the cTomRes$_{8X/S}$ fragment (Fig. 26/IIB). Both hybridisation analyses gave one signal which corresponded to the ~2200 nt. The I_{X510} probing was done to distinguish non-spliced from spliced transcripts. Detection of an identical hybridisation pattern with both of the two different probes indicated that the transgene was actively expressed but more importantly this data demonstrated that only non-spliced transcripts were present in this plant. These results

were not in accordance with the finding that transcripts of the SR1dem plant lines were, at least, partially spliced. In summary these data show that the function of intron4 might depend on the context of flanking sequences.

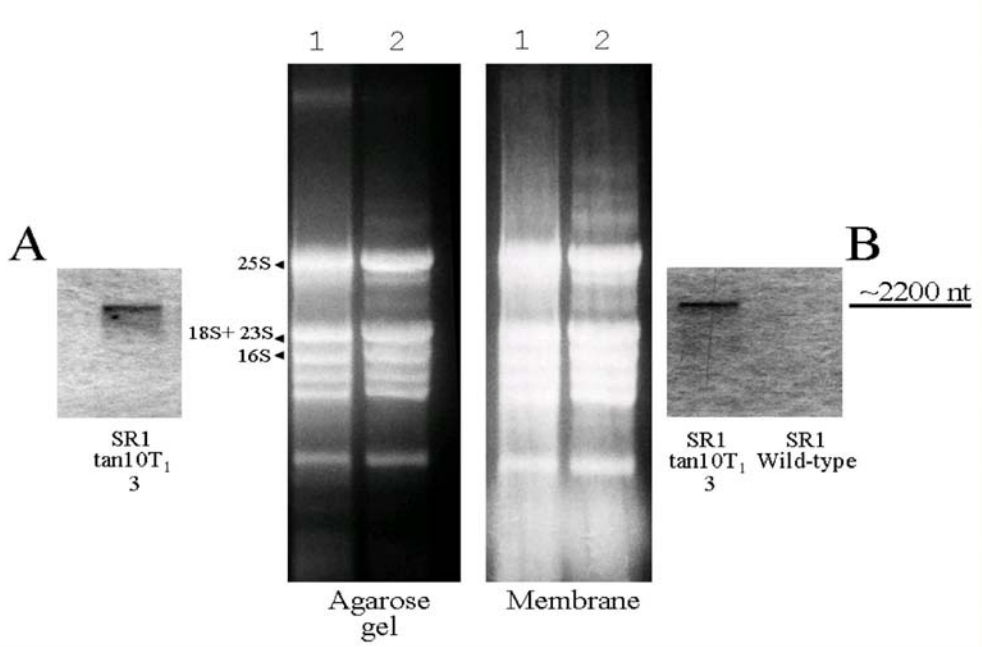


Figure 26/II: Northern analysis of total RNA isolated from T_1 progeny plants.
The indicated sizes of hybridising RNAs included a 100 nt long poly (A) tail.
A) The blot was probed with the ^{32}P-labelled I_{X510} DNA fragment (III.2.1.1.1).
B) The blot was probed with the ^{32}P-labelled $cTomRes_{8X/S}$ DNA fragment (III.2.1.1.1). No positive signals were detectable for the endogenous $RdRP^2$ expression.

The SR1Bam$^-$5 T_1 generation was analysed by Southern analysis with HindIII using the $cTomRes_{8X/S}$ fragment as probe. The hybridisation patterns revealed independent segregation of the two transgene copies that were found to be integrated in the parental plant (Fig. 17/B). Out of five progeny plants analysed, the SR1Bam$^-$5T_1/1 line only carried the ~5.6 kb long parental plant-specific fragment (Fig. 27A, compare SR1Bam$^-$5T_1/1 with SR1Bam$^-$5T_1/2 and see also Figure 17/B). Hence, the SR1Bam$^-$5T_1/1 was selected for further analysis. To determine whether the single copy transgene was in a homozygous state, the SR1Bam$^-$5T_1/1 line was genetically crossed with a tobacco SR1 wild-type plant and the seeds obtained were plated onto selection medium. All of the germinating seeds developed in the presence of kanamycin indicated that SR1Bam$^-$5T_1/1 line was homozygous for the T-DNA. The appearance of a ~1400 nt long hybridising RNA on a

cTomRes$_{8X/S}$ fragment-probed Northern blot (Fig. 27B) demonstrated that the transgene is actively expressed in the homozygous SR1Bam^{-}5T$_1$/1 line.

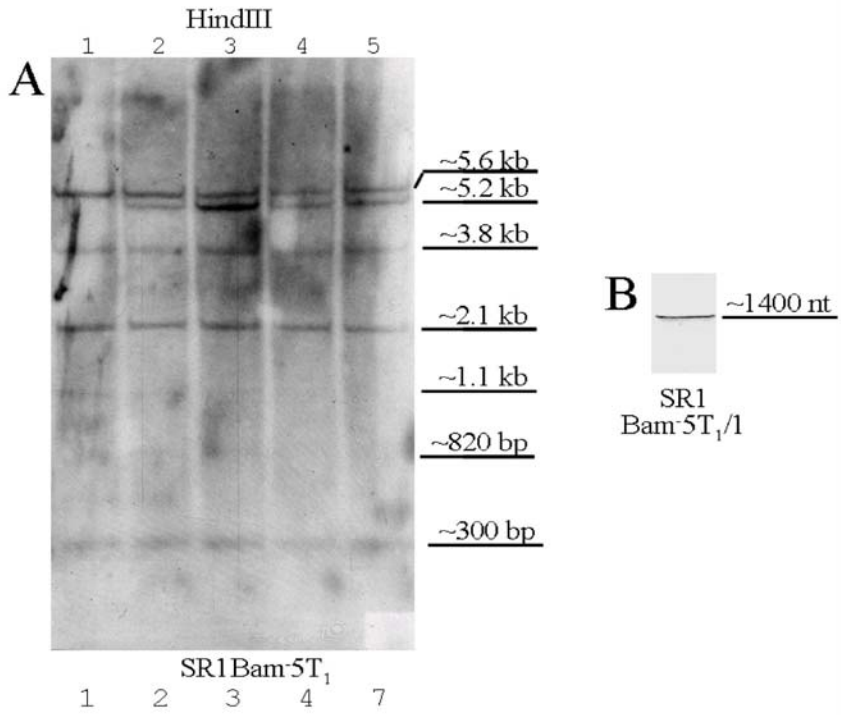


Figure 27: Southern and Northern analysis of T$_1$ progeny plants.
A) Southern blot of genomic DNA isolated from T$_1$ progeny plants using the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment as probe (III.2.1.1.1). Sizes of the hybridising fragments are indicated. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous RdRP^2 gene.
Numbers above the blot represent the lanes and numbers below the blot indicate the number of the T$_1$ plant line.
B) Northern hybridisation of total RNA isolated from the homozygous SR1Bam^{-}5T$_1$/1 plant line. The blot was probed with the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment. The indicated size of the hybridising RNA included a 100 nt long poly (A) tail.

Progeny plants of the SR1Bam^{+}2 parental line were analysed for the inheritance of the transgene by Southern analysis with HindIII and the cTomRes$_{8X/S}$ probe. The corresponding hybridisation patterns of the T$_1$ generation (Fig. 28A) were identical to the pattern of the parental plant (Fig. 17/B). A higher intensity of the band was visible for the SR1Bam^{+}2T$_1$/4 (Fig. 28A, lane 2) when compared to the SR1Bam^{+}2T$_1$/5 line (Fig. 28A, lane 1). Based on this finding, it was assumed that in the SR1Bam^{+}2T$_1$/4 line, the T-DNA was present in a homozygous state. Thus, this plant line was chosen for a genetic cross with a tobacco SR1 wild-type plant. Seeds obtained were plated onto selection media and development of 100% of the germinating seeds confirmed homozygosity. Northern

hybridisation was carried out to examine the expression of the transgene (Fig. 28B). Detection of a Northern signal inferred that the RdRP^2-specific transgene was transcribed into a ~1400 nt long RNA.

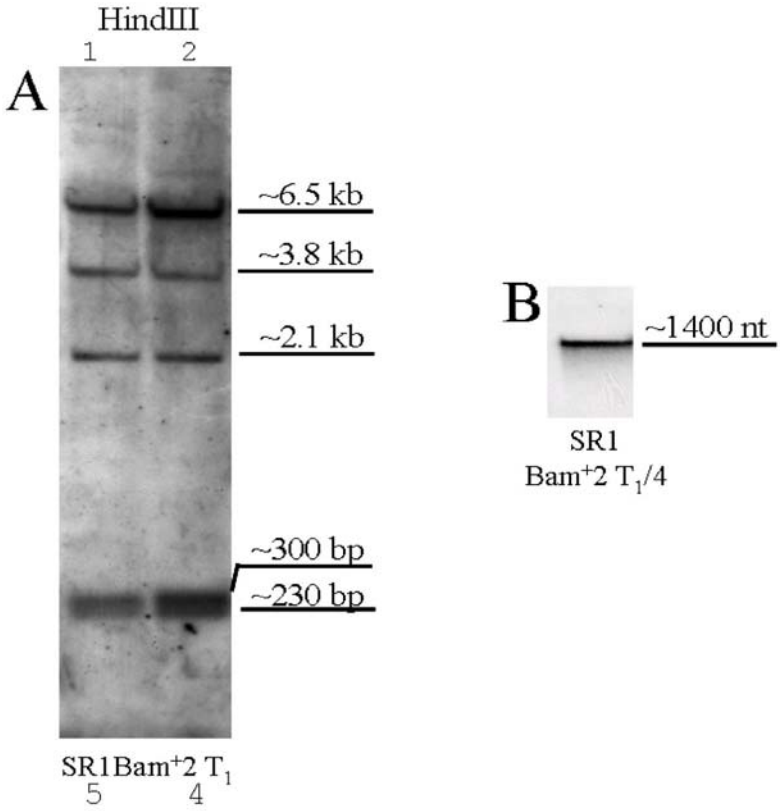


Figure 28: Southern and Northern analysis of T_1 progeny plants.
A) Southern blot of genomic DNA isolated from T_1 progeny plants using the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment as probe. Sizes of the hybridising fragments are indicated. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous RdRP^2 gene.
Numbers above the blot represent the lanes and numbers below the blot indicate the number of the T_1 plant line.
B) Northern hybridisation of total RNA isolated from the homozygous SR1Bam$^+$2T$_1$/4 plant line. The blot was probed with the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment. The indicated size of the hybridising RNA included a 100 nt long poly (A) tail.

Progeny of the SR1IR14 parental plant line was analysed for the inheritance of the transgene by Southern analysis with HindIII and the cTomRes$_{8X/S}$ probe. The corresponding hybridisation patterns (Fig. 29/I) were identical to the pattern of the parental plant (Fig. 11B). Hybridising HindIII fragments with sizes of ~270 bp, ~300 bp (twice), and ~780 bp were absent when the DNA was probed against the cTomRes$_{8X/S}$ fragment (Fig. 29/I). In contrast, internal HindIII fragments with similar sizes were detectable when

the progeny plants of the SR1tan10 T_1 generation were analysed (Fig. 26/I). The fact that this samples were analysed on the same membrane (the membrane was cut in the middle to obtain the autoradiographs presented in Figures 26/I and 29/I) demonstrated that the quality of Southern analysis was proper. This substantiated that the internal SR1IR14 HindIII fragments were indeed absent, thus, confirming T-DNA rearrangements in the SR1IR14 parental plant.

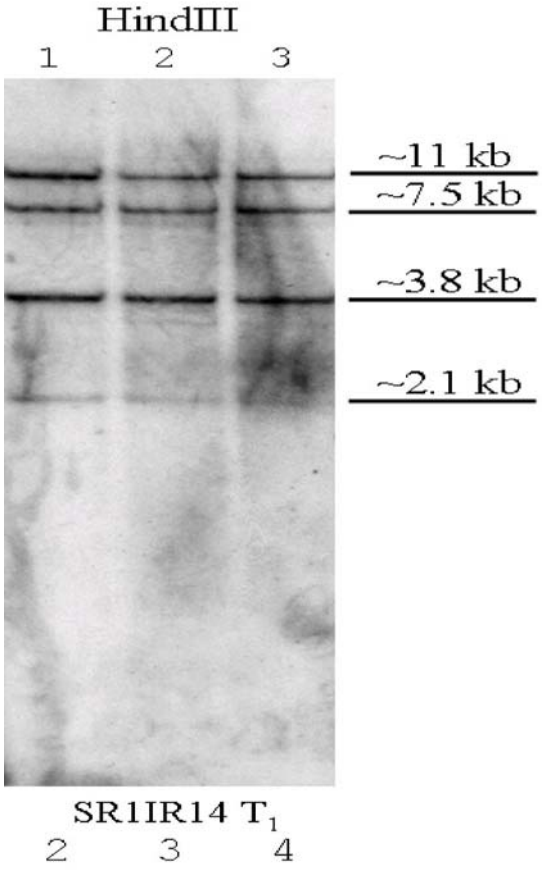


Figure 29/I: Southern blot of genomic DNA isolated from T_1 progeny plants.
The blot was probed with the ^{32}P-labelled cTomRes$_{8X/S}$ DNA fragment (III.2.1.1.1). Sizes of the hybridising DNA fragments are indicated. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous RdRP^2 gene.
Numbers above the blot represent the lanes and numbers below the blot indicate the number of the T_1 plant line.

Northern analysis of one of the SR1IR14T_1/2 progeny plant failed to give positive hybridisation signal with the cTomRes$_{8X/S}$ probe as well as with the intron4-specific I_{X510} fragment as probe.

Genomic DNA from plants of the SR1Inv'4 T_1 generation was analysed for the inheritance of the transgene by Southern analysis with DraI or HindIII. The hybridisation

patterns of DraI-digested genomic DNA from three individuals of the SR1Inv'4 T_1 generation were similar to the pattern of the parental line (Fig. 12, lane 1) when probed against the $cTomRes_{8X/S}$ fragment (Fig. 29/IIA, lanes 4-6). Moreover, as was found for the parental line, the inverted repeat progeny plants also failed to show the expected internal HindIII fragments (Fig. 29/IIB). Genomic DNA from three individual plants of the SR1Inv'22 T_1 generation was digested with DraI and probed with the $cTomRes_{8X/S}$ fragment (Fig. 29/IIA, lanes 1-3). Similar to the T_1 progeny of the SR1Inv'4 plants, the hybridisation patterns were identical to the pattern of the parental SR1Inv'22 plant (Fig. 12; lane 2).

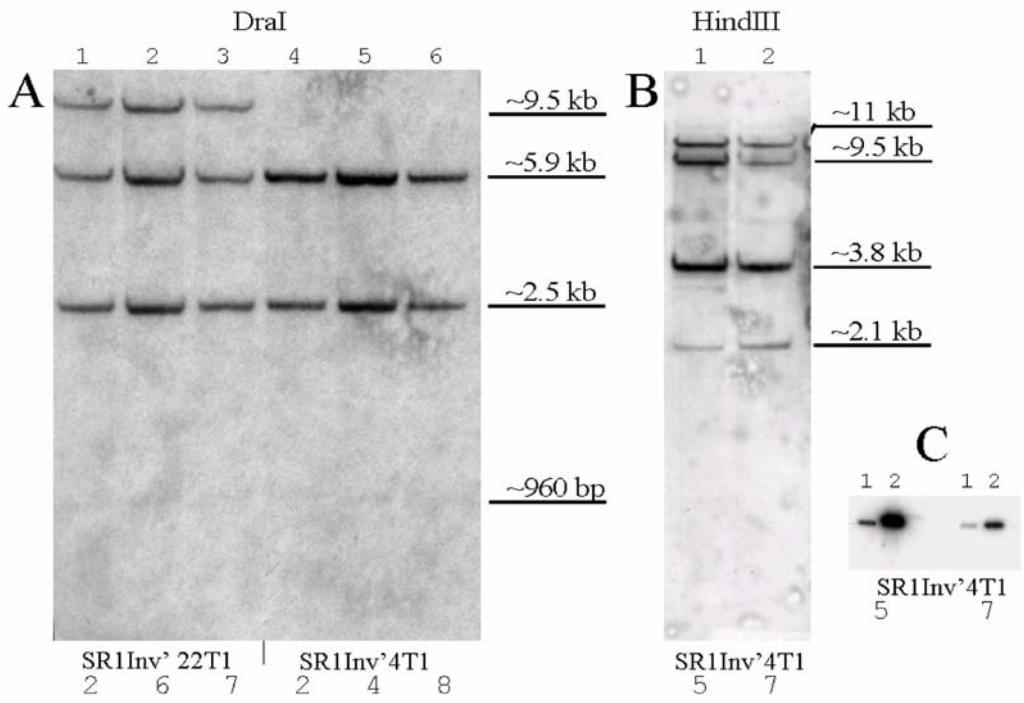


Figure 29/II: Southern and PCR analysis of T_1 progeny plants. DNA fragment sizes of the hybridised DNA fragments are indicated.
A) Southern analysis of DraI-digested genomic DNA using the ^{32}P-labelled $cTomRes_{8X/S}$ DNA fragment as probe (III.2.1.1.1). Sizes of the hybridising DNA fragments are indicated. The hybridising fragment with a size of ~5.9 kb correspond to the endogenous $RdRP^2$ gene.
B) Southern analysis of HindIII-digested genomic DNA using the ^{32}P-labelled $cTomRes_{8X/S}$ DNA fragment as probe. Hybridising fragments with sizes of ~2.1 kb and ~3.8 kb correspond to the endogenous $RdRP^2$ gene.
C) PCR-amplified products, probed with the ^{32}P-labelled $cTomRes_{8X/S}$. Lane 1 = first copy, Lane 2 = second copy.
Numbers above the blots represent the lanes and numbers below the blots indicate the number of the T_1 plant line.

Identical DraI hybridisation patterns of the SR1Inv'22 parental and the progeny plant lines rendered it likely that the hybridisation pattern of HindIII-digested DNA will be also identical. Therefore, genomic DNA of the SR1Inv'22 progeny plants was not analysed with HindIII. In summary, Southern data of the SR1IR14 and the SR1Inv'4 T_1 generation obtained by HindIII restriction confirmed the previous findings pointing to rearrangements of the genome-integrated 702cTR$_{IR}$ T-DNA in all single copy primary transformants.

None of the IR construct-containing plants gave a hybridisation signals upon Northern analysis with total RNA. To examine whether the expression level was below the detection limits or whether transgenes were not expressed, PCR was performed using cDNA as template. The cDNA was reverse transcribed from total RNA of the SR1Inv'4T_1 progeny plants 5 and 7 (II.2.1.7.4). The primers used for PCR amplification were transgene-specific, thereby ensuring no amplification of the endogenous gene fragment. PCR amplification of the 5' RdRP^2-specific fragment was performed using the 3'HomRevBamHI as forward and the RdRP7620Int4reverse primers and the 3' copy was amplified with the 3'HomRevBamHI as reverse and the RdRP7500Int4forward primers (see Fig. B/III for primer binding sites). PCR products were run on a 1% (w/v) agarose gel and proceeded for Southern hybridisation with the cTomRes$_{8X/S}$ fragment as probe (Fig. 29/IIC). The size of the hybridising fragments corresponded to the expected sizes of non-spliced transcripts (~1.1 kb for the 5' fragment and ~1.14 kb for the 3' copy, see Figure B/III). From these data, it was concluded that the transgene was transcribed but that expression was either low or that the transcripts were rather unstable.

III.4 Designing and introduction of RdRP^1 and RdRP^2 transgene constructs for the transient expression in plants

In parallel to projects aiming at suppression of endogenous RdRP gene expression by stable introduction of transgene constructs, investigation with virus-induced gene silencing (VIGS) strategy was carried out. The principle of this technology is based on virus replication-mediated production of RdRP gene-specific double stranded RNA (dsRNA). In other words, plant viruses that contain sequences homologous to nuclear-expressed genes act to induce silencing of the targeted genes (Lindbo *et al*., 1993; Kumagai *et al*., 1995; Baulcombe, 1999; Ruiz *et al*., 1998). As studies dealt with cytoplasmic RNA viruses, it was inferred that the mechanism of VIGS involves destabilisation of target mRNA in the cytoplasm (Smith *et al*., 1994). To obtain RdRP-specific dsRNA, the pP2C2S vector

containing the entire ~9.7 kb long Potato Virus X (PVX) genome was modified (II.1.5). For insertions of foreign DNA into the PVX genome unique ClaI and SalI sites were available. Introduction of DNA fragments (<2.0 kb) into these cloning sites was shown to not affect the infectivity of the virus (Baulcombe, personnel communication). Infectious PVX RNA could be produced by *in vitro* transcription of the pP2C2S derivatives using the T7 polymerase (II.2.4.6). Two derivatives carrying a $RdRP^1$-specific cDNA fragment (PVX/$RdRP^1$) and a full-length green fluorescent protein (GFP) cDNA (PVX/GFP), respectively, were previously generated (Wassenegger, unpublished results) and were used as controls in these studies.

The $RdRP^2$-specific PVX construct was produced by introducing the $cTomRes_{8s}$ fragment into the SalI site of the pP2C2S. The orientation of the insert with respect to the T7 promoter was determined with EcoRI. Recombinants showing ~700 bp, ~2.4 kb and ~2.6 kb long fragments were considered to be in sense orientation. One of the positive clones (PVX/$RdRP^2$) was sequenced with the PVX5582 (forward) and PVX5740 (reverse) primers (II.1.6). Sequencing revealed the presence of $cTomRes_{8s}$ fragment and confirmed the sense orientation of the fragment.

The pP2C2S and the three pP2C2S derivatives were first linearised with unique restriction endonucleases. Both the pP2C2S and PVX/$RdRP^2$ plasmids were linearised with SacI while the PVX/$RdRP^1$ and the PVX/GFP were linearised with SpeI. The linearised plasmids were then used for T7 transcription. The transcripts were electrophoresed on 1.2 % (w/v) formaldehyde gels (II.2.4.4) to examine their quality and quantity. For each assay two bands appeared after the run. Thus, the gels were proceeded for Northern hybridisation using specific probes for each recombinant PVX vector. Appearance of two hybridisation signals at positions corresponding to nucleic acid fragments with sizes of about 7000-7300 nt and about 10.2-10.5 kb pointed to the presence of transcript and residual amounts of the linearised vectors. To verify the synthesis of transcripts, one of the samples was treated with RNase-free DNase. After electrophoresis the fragment in the range of 10 kb was missing on the gel and the fragment in the range of 7000 nt was present indicating that the lower fragment was indeed the transcript. For the pP2C2S, hybridisation signals at positions corresponding to fragments with sizes of ~6500 nt and ~9.7 kb indicated the presence of the transcript and the linearised vector. Nevertheless, without DNase treatment the *in vitro* transcripts were directly used for plant inoculation according to the standard inoculation procedure (II.2.4.6). *Nicotiana glutinosa*,

Nicotiana benthamiana and *Nicotiana tabacum* were chosen for inoculation. It was known that the PVX-GFP successfully replicates in the *N. benthamiana* (Baulcombe *et al*., 1995; Ruiz *et al*., 1998). Therefore, *N. benthamiana* was used as control and as an additional control *N. glutinosa* was selected. As for the stable introduction of transgene *N. tabacum* was used, therefore also for VIGS study this species was chosen as experimental plant. The inoculated plants were monitored for symptom expression until flowering. To prove for virus replication, samples were taken 20 days post inoculation (dpi), and total RNA isolated from the samples was applied to Northern analysis (II.2.4.4).

All of the three *Nicotiana* species exhibited typical PVX symptoms upon infection with the pP2C2S. Figure 30 shows typical examples of symptom expression of PVX-infected *N. benthamiana* and *N. tabacum* plants. Northern analysis of the *N. tabacum* plant using the PVX_p probe (II.2.4.5) revealed a strong hybridising RNA with a size of ~6500 nt confirming PVX infection (data not shown). As a size marker, an aliquot of the *in vitro* transcript was used.

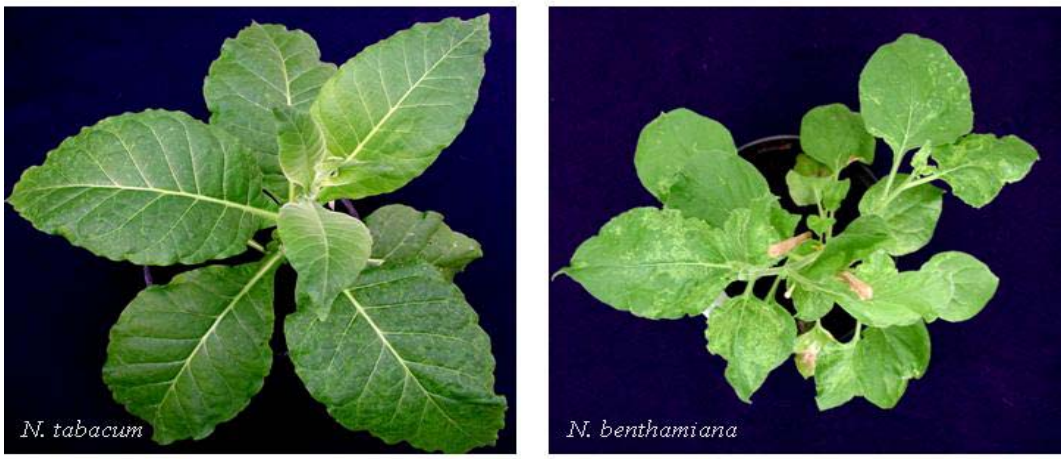


Figure 30: Symptom expression of PVX-infected *N. tabacum* and *N. benthamiana* plants. The photograph was taken 20 dpi with *in vitro* transcripts of the pP2C2S (II.2.4.6).

The PVX-GFP *in vitro* transcripts were used to inoculate *N. glutinosa*, *N. benthemiana* and *N. tabacum* plants. In Figure 31, *N. glutinosa* and *N. benthemiana* plants are shown that transiently expressed the GFP. These results demonstrated that the PVX-

GFP *in vitro* transcripts were infectious, that the PVX-GFP was stable and that it was capable to systemically infect the plant. Northern analysis of a PVX-GFP-infected *N. tabacum* plant that exhibited typical PVX symptoms (Fig. 32) was performed (II.2.4.4). Total RNA from this infected plant was hybridised against the PVX-specific PVX_p and the GFP-specific G_{FP} probes (II.2.4.6) to detect the recombinant virus RNA (Fig. 32). Single positive signals lighted up at identical positions indicating successful replication of the PVX-GFP also in *N. tabacum*. In addition to biochemical characterisation of the PVX-GFP-infected plants, a biological assay was included to examine the stability of the replicating virus. Total RNA from a PVX-GFP-infected *N. glutinosa* was used to inoculate young *N. glutinosa* and tobacco plants. The observation that after 3-4weeks PVX symptom expression was normal on both plant species demonstrated that virus transmission from one plant to the other and from one species to the other was successful.

Figure 31: Symptom and transient GFP expression in PVX-GFP-infected *N. benthamiana* and *N. glutinosa* plants. The photograph was taken 20 dpi using *in vitro* transcripts of the PVX-GFP. The photograph was taken under UV-light. Green sectors resulted from GFP fluorescence whereas red sectors represent GFP-deficient tissue. Appearance of the green areas demonstrated that the PVX-GFP stably replicated and was systemic.

The original PVX-GFP-infected *N. glutinosa* plants were also monitored under UV-light to show that virus replication was accompanied by GFP expression (data not shown). It

turned out that all bleached areas (indicating PVX infection) also showed GFP fluorescence. By contrast, inspection under UV-light of the plants that were infected with total RNA from the *in vitro* transcript-infected plants were found to only have few regions displaying GFP expression (data not shown). The fact, that the majority of the bleached spots did not show GFP fluorescence indicated that the PVX-GFP was unstable.

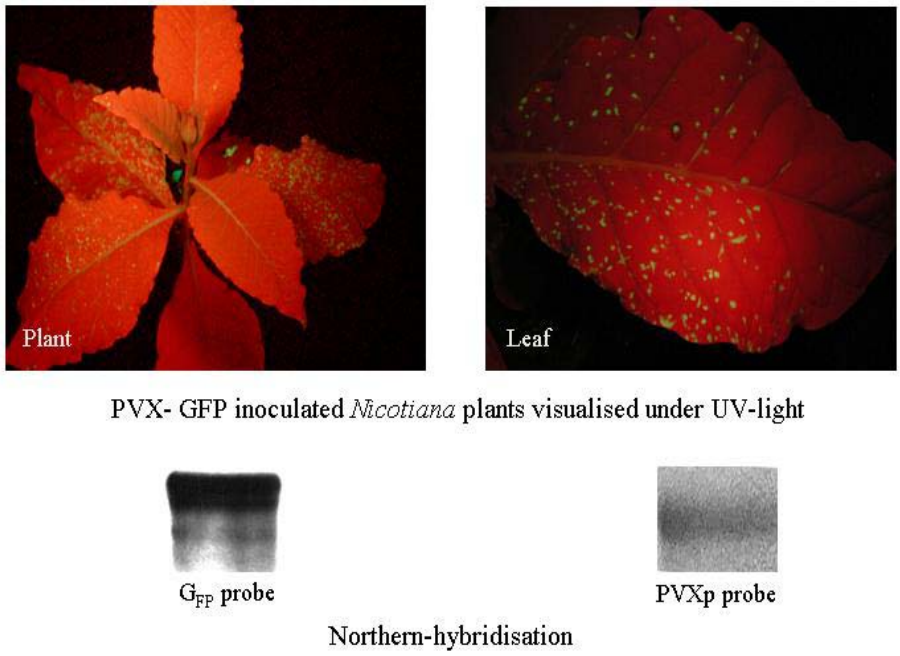


Figure 32: Symptom and transient GFP expression in PVX-GFP-infected tobacco plants. The photograph was taken under UV-light 20dpi using *in vitro* transcripts of the PVX-GFP. Green sectors resulted from GFP fluorescence whereas red sectors represent GFP-deficient tissue. Total RNA from one of the infected plants was used for Northern analysis using the G_{FP} and the PVX_P probes (II.2.4.4; II.2.4.5).

PVX-RdRP[1] *in vitro* transcripts were used to inoculate all three *Nicotiana* species and all infected plants exhibited PVX symptoms. The PVX-RdRP[1]-infected *N. tabacum* plants (Fig. 33) being of main interest for RdRP suppression studies, were Northern-analysed to investigate virus replication. Northern hybridisation against the $Rd^1{}_{5Sac}$ and the PVX_p fragment as probes showed single positive hybridisation signals at identical positions (Fig. 33) indicating successful infection. Northern hybridisation of the same PVX-RdRP[1] positive plant at later stages of growth was found to only accumulate the wild-type PVX as no hybridisation signal was detectable with the RdRP[1]-specific probe

(II.2.4.5). This finding confirmed previous results obtained with the PVX-GFP demonstrating the instability of the recombinant PVX genomes.

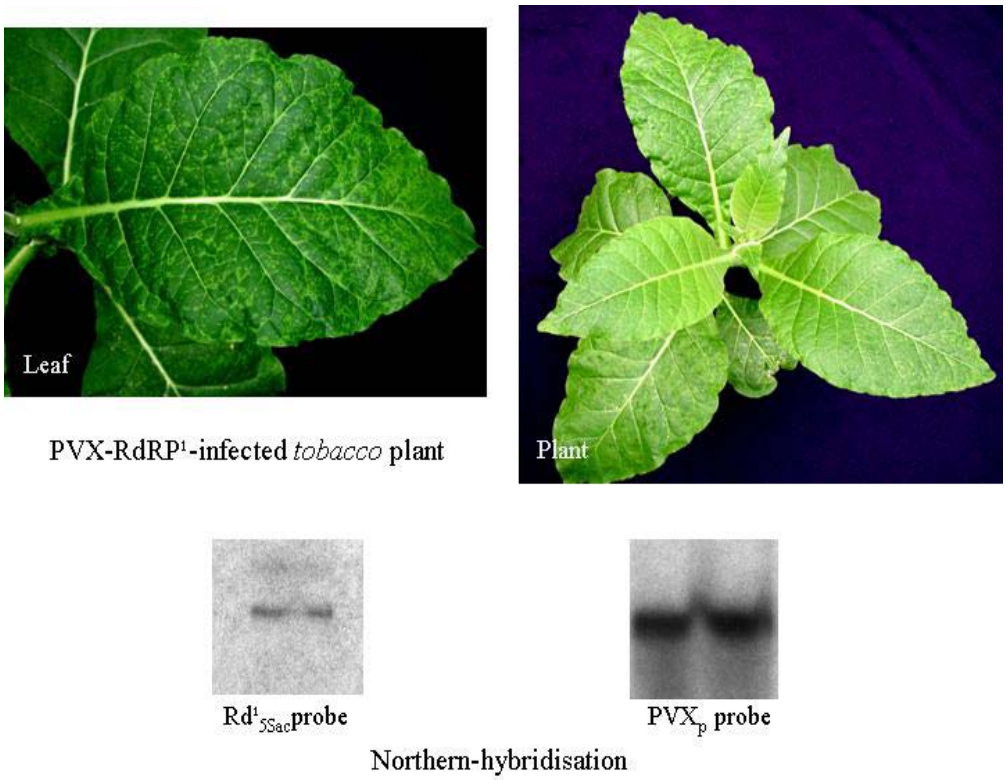


Figure 33: Symptoms exhibited by the PVX-RdRP[1] infected tobacco plant. The photograph was taken 20dpi with *in vitro* transcripts of the PVX-RdRP[1]. Total RNA from one of the infected plants was used for Northern analysis using the Rd^{1}_{5Sac} and the PVX_p probes (II.2.4.4; II.2.4.5).

For unknown reasons, infection upon inoculation with PVX-RdRP[2] *in vitro* transcripts was never successful. Neither symptoms nor Northern hybridisation signals were detectable. It should be noted that in one experiment one of the PVX-RdRP[2] inoculated plants developed severe symptoms, a leave phenotype that was never seen before (Fig. 34). However, the experiment could not be reproduced and detection of PVX RNA in this symptom-expressing plant failed. Northern hybridisation did not give a signal. Inoculation with total RNA extract from leaves exhibiting the altered leaf morphology resulted in appearance of same phenotype in the control plants. One may assume that this

phenotype was caused by another pathogen that was co-isolated with the RNA. However, similar phenotypes were never found on a tobacco plant that was cultivated in our greenhouse.

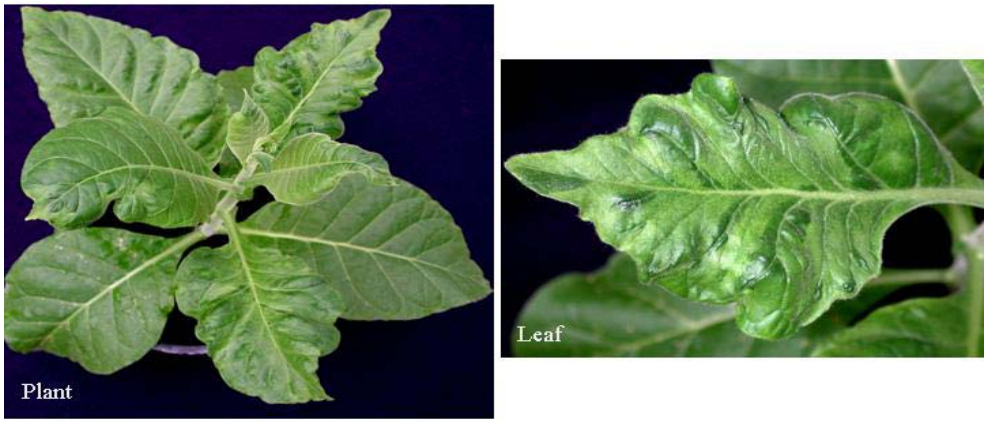


Figure 34: Phenotype of a tobacco plant that was inoculated with *in vitro* transcripts of the PVX-RdRP^2.

In summary, several attempts were made to inoculate plants with *in vitro* transcripts of recombinant PVX cDNA genomes. Although virus replication was detectable in most cases all recombinant PVX were found to be unstable. Moreover, plants frequently recovered from infection but recovery was not identical for all constructs. Therefore, due to the heterogeneity of the results the VIGS strategy failed to give reliable information about the biological function of the RdRP^2 in plants.

IV Discussion

Tremendous amount of information about the genetic make-up of an organism has been obtained from genome-sequencing projects. In view of this work, the genome-sequencing project of *Arabidopsis thaliana* was of great interest. Sequence alignment and comparison provided evidence that, at least, three RdRP homologues exist in *A. thaliana*. Based on the sequence homology with known RdRPs, these three homologues were categorised as $RdRP^1$, $RdRP^2$ and $RdRP^3$. This prompted the speculation that three RdRP homologues are also expressed in tomato. A first line of evidence for this hypothesis came from an auto-translated tomato Expressed Sequence Tag (EST; AI774429) that was obtained from a database search. Alignment with other RdRP homologues showed that this 433 bp long sequence displayed high amino acid similarity with the *A. thaliana* $RdRP^2$. Gene suppression experiments in tobacco and characterisation of *A. thaliana* PTGS mutants gave first insights into the biological functions of the $RdRP^1$ and $RdRP^3$. However, no information about the role of the $RdRP^2$ was available. This lack of knowledge initiated our interest to analyse the $RdRP^2$. The present work comprises isolation and cloning of the full-length $RdRP^2$ cDNA sequences from tobacco and tomato, introduction of PTGS-inducing RdRP constructs into tobacco, and characterisation of the transgenic plants produced.

IV.1 Isolation of the $RdRP^2$ cDNA clones

Deduced from the tomato EST (AI774429) PCR primers were designed. Two EST-primers were used to amplify a ~320 bp long fragment (TomRes) using genomic and cDNA of the *Lycopersicon esculentum* cultivar Rentita, respectively (III.1.3). PCR products from each amplification were cloned and sequenced. Sequence comparison of the two positive clones, $gTomRes_{320}$ and $cTomRes_{320}$ with the EST showed a single A→C transversion resulting in an amino acid substitution from isoleucine (I) to leucine (L). Isolation and characterisation of the tomato $RdRP^2$ 3' fragment ($cTomRes_{1.5}$) that contained the EST sequence (III.1.4) exhibited the same A→C transversion. This finding demonstrated that the tomato $RdRP^2$ clones contained the authentic sequence and that the published EST most likely carried a mutation. Assembly of the $cTomRes_{500}$, $c5TomRes_{1.5}$, $cTomRes_{320}$ and $cTomRes_{1.5}$ sequences revealed the ~3.5 kb long full-length $RdRP^2$ cDNA sequence.

IV.2 Determination of the possible biological role of the RdRP^2 in higher plants

IV.2.1 Analysis of stable transformed plant lines

Since the development of the 'RNA interference' (RNAi) technique, analysis of gene functions in biological systems got revolutionised. RNAi is triggered by double-stranded RNA (dsRNA) which undergoes an endonucleolytic cleavage resulting in formation of short interfering RNAs (siRNAs) with a size of 21-25 nucleotides (nt). In a sequence-specific manner, siRNAs target complementary RNA for degradation. The RNAi technology as well as related RNA-mediated gene silencing techniques were utilised to suppress expression of the endogenous tobacco RdRP^2.

To investigate the function of the RdRP^2, different constructs containing tomato RdRP^2-specific fragments were designed. The fragments were cloned into the pPCV702SM binary vector that contained the Cauliflower Mosaic Virus (35S) promoter, a promoter that is known to be highly active in plants. Inverted repeat constructs (IR) have been reported as potent triggers of RNAi (English *et al*., 1997; Hamilton *et al*., 1998). Consequently, main focus was put on the production of RdRP^2-specific IR constructs. IR constructs were designed under consideration of factors that render them potent RNAi inducers including the introduction of an intron as a spacer and the utilisation of a 3'end fragment that was specific for the RdRP^2 gene. This 3'end-specific fragment was analysed for the absence of conserved regions and was searched for the presence of the longest continuous sequence stretch that shared 100% homology with the other two RdRP homologues. The overall homology of the full-length tomato RdRP^1 and RdRP^2 cDNA sequences was 51.7% and homology of the 3'end-specific fragments was 53.5%. Despite this slightly higher homology, it was not expected that the RdRP^1-specific transgene construct would induce co-suppression of the endogenous RdRP^2 and *vice versa*. This assumption was based on experiments where it was shown that sequence identity of more than 78% was required to initiate RNAi (Parrish *et al*., 2000). Hence, for the RdRP^1 a ~660 bp long and for the RdRP^2 a ~800 bp long fragment was selected. At the beginning of this work, the sequence of the RdRP^3 gene from tomato and tobacco was not available. Therefore, homology between the RdRP^2 and RdRP^3 sequences could not be determined at that time. However, the mutated *A. thaliana* plant lines carrying a non-functional RdRP^3 gene (SGS2, SDE1) (Dalmay *et al*., 2000b; Mourrain *et al*., 2000) were known to lack

developmental abnormalities. Hence, it was assumed that even if cross-interference with the endogenous RdRP^3 will occur, no phenotypic alterations would be displayed in plants carrying RdRP^2-specific transgene constructs. In this context, it is important to note that induction of silencing by IR constructs does most likely not involve RdRP^3 activity. This notion stems from the observation that VIGS is independent of cellular RdRP^3. As was demonstrated with the help of the *A. thaliana* RdRP^3 (SDE1) mutants in which transgene-mediated initiation of PTGS but not VIGS was shown to be impaired (Dalmay *et al*., 2000b). In view of these observations and considering the fact that viruses replicate via dsRNA intermediates it was hypothesised that dsRNA-mediated silencing is in general independent of RdRP^3 (Béclin *et al*., 2002). Therefore, determination of sequence homology with RdRP^3 was of lower significance. At the later part of this work, the entire RdRP^3 cDNA sequence from tobacco became available (Wassenegger, unpublished results) and sequence comparison of the full-length tobacco RdRP^3 cDNA sequence (~3.6 kb) with the RdRP^2 ~800 bp fragment revealed an overall homology of 50%. No 21-25 nt long sequence stretch that shared 100% homology was detectable. Hence, the probability of cross-interference between the tomato RdRP^2-specific sequence and the tobacco RdRP^3 gene could be almost excluded. Based on these data it was assumed that the IR construct (702cTR$_{IR}$) containing the tomato RdRP^2 fragment would specifically suppress the tobacco RdRP^2.

The 702cTR$_{IR}$ plasmid was originally propagated in *E. coli* and then conjugated to *Agrobacterium.* The *Agrobacterium* strain carrying the 702cTR$_{IR}$ plasmid was denoted as GV702cTR$_{IR}$. From this strain the 702cTR$_{IR}$ was isolated and re-transformed into *E. coli*. The plasmid (702cTR$_{IRinv}$) isolated from these transformants was analysed with several restriction enzymes. The corresponding restriction pattern was identical to that of the original 702cTR$_{IR}$ plasmid. Hence, it was shown that the RdRP^2 IR transgene construct (702cTR$_{IR}$) containing an intron as spacer was stable in *E. coli* as well as in *Agrobacterium*. However, none of the primary plant transformants carried a non-rearranged copy of this IR construct. Southern analysis of the single copy primary transformants, SR1IR14, SR1Inv'4, and SR1Inv'22, using a RdRP^2-specific probe failed to detect the expected internal HindIII fragments [~270 bp, ~300 bp (twice) and ~780 bp] indicating rearrangements within the IR construct. These rearrangements were confirmed by hybridisation against the intron-specific I$_{X510}$ probe. This probe was used to detect the ~780 bp long internal HindIII fragment. The SR1IR14 plant showed appearance of two

large hybridising fragments with sizes of ~7.5 kb and ~11.0 kb instead of the expected ~780 bp. In contrast to this, the SR1tan10 primary transformant carrying the 702cTR$_{DR}$ control construct showed a positive hybridising fragment corresponding to this size. Moreover, by Southern analysis the T_1 generation plants derived from the SR1tan10 plant line showed the presence of all expected internal fragments whereas the T_1 generation of the SR1IR14, SR1Inv'4 and the SR1Inv'22 plant lines again showed the rearrangement of the parental plants. These results demonstrated that the failure to detect the internal fragments was not due to the Southern procedure but supported the assumption that the genome-integrated IR construct was rearranged, at least, in plants containing a single copy transgene. These results were in accordance with previous studies where deletions and truncations of IRs have been reported in eukaryotes (Gordenin *et al*., 1993; Collick *et al*., 1996; Akgün *et al*., 1997). However, data obtained by Southern analysis carried out with DraI-restricted genomic DNA that was probed against the I_{X510} fragment contradicted the rearrangement hypothesis. The hybridisation pattern of the internal and the right border (RB) fragments corresponded to the expected pattern. Only the left border (LB) was found to be greater in size than expected. This indicated some rearrangements within the left border fragment. Such rearrangements are known to frequently occur during T-DNA integration and mostly comprise one of the border sequences (Matzke and Matzke, 1998). In order to get an idea why the internal HindIII fragments could not be detected by Southern analysis, PCR of genomic DNA isolated from the SR1IR14 was performed using transgene-specific primers. PCR-amplified products showed fragments with the expected sizes. However, these PCR products were not cloned and sequenced. Thus, it could not be excluded that minor deletions or nucleic acid substitutions were present within the PCR-amplified fragments. The PCR products were digested with HindIII and the cleavage products were proceeded for Southern analysis using a RdRP^2-specific probe. Surprisingly, all internal fragments could be detected irrespectively of whether the template DNA was isolated from IR- or DR construct-containing plants (data not shown). This clearly demonstrated that HindIII sites were present in the PCR products and strongly argued for the presence of HindIII sites in the genome-integrated IR construct. One may speculated that the transgene-specific HindIII sites became *de novo* methylated and were therefore not cut. It is worth to note that IR sequences were reported to be prone for *de novo* DNA methylation in plants (reviewed in Sijen and Kooter, 2000).

Northern hybridisation that was carried out with total RNA isolated from progeny plants carrying the IR construct never gave a positive signal. Hybridising RNA was neither detectable with the RdRP^2-specific probe nor with the intron-specific probe. However, Southern hybridisation of PCR products amplified from cDNA of a T_1 SR1Inv'4 plant showed that non-spliced transcripts were produced by the IR transgene. In view of the aforementioned characterisation of IR rearrangements it is important to mention that these PCR products gave a HindIII restriction pattern that was identical to the one that was obtained with the PCR products amplified from the genomic DNA of the SR1IR14 plant line. This may further support the speculation that the HindIII sites were methylated.

Northern analysis failed to detect any IR-specific RNA. Thus, no evidence for the production of the spliced IR-specific transcripts could be provided. Importantly, no PCR products corresponding to spliced RNA was amplified from the cDNA of T_1 SR1Inv'4 progeny plants. One would have expected such products from IR transcripts by using a single RdRP^2-specific primer. However, any attempt to PCR-amplify an entire IR sequence was problematic and failed to give a product. For non-spliced RNA, intron- and gene-specific primers were used allowing the amplification of only a part of the sequence which lacked an IR structure. Thus, it could not be determined whether spliced RNA was not present in the plant or whether a cDNA derived from spliced transcripts could not be amplified by PCR.

If the spliced transcript was produced, the failure to detect it by Northern analysis could be due to initiation of RNAi. In silenced plants, not only the target mRNA is degraded but also the RNAi-inducing transgene RNA. Thus, it was not possible to state whether dsRNA was transcribed from the IR transgene. Moreover, if dsRNA was produced it is not clear whether this RNA was capable to efficiently initiate RNAi. The homozygous SR1tan10T_1/3 control plant line that carried the RdRP^2 direct repeat construct (702cTR$_{DR}$) showed positive signals upon Northern hybridisation. Transcripts that were detected with the RdRP^2-specific (cTomRes$_{8X/S}$) and the intron-specific (I_{X510}) probes only represented non-spliced transgene RNA. This may indicate that, at least in this plant line, the intron was not functional.

Northern hybridisation of the SR1dem18 primary transformant carrying the RdRP^1 direct repeat construct (702Rd^1$_{DR}$) showed two positive hybridisation signals with the RdRP^1-specific probe (Rd^1$_{5Sac}$). This demonstrated that spliced as well as non-spliced transcripts are synthesised from the transgene. To specifically detect the non-spliced form,

total RNA from the SR1dem18 primary transformant was hybridised against the intron-specific I_{X510} probe. The positive signal obtained corresponded to the upper fragment that was visible on the previous blot using the $Rd1_{5Sac}$ probe. This finding confirmed that non-spliced transcripts were indeed present supporting the hypothesis that the intron was inefficiently spliced out.

Results of this work showed that most likely all single copy transgenic plants had rearranged IRs and none of these plant lines exhibited phenotypic alterations. This could be attributed to dominant silencing properties of IRs resulting in a "negative selection", favouring the development of transformants carrying rearranged IR constructs. It is conceivable that $RdRP^2$ is involved at early stages of development and plants that are deficient in $RdRP^2$ are not viable. This may cause an early death of transformed cells. Consequently, the viable plants obtained may only contain rearranged IR constructs. Transcripts of these constructs would represent inefficient inducers of RNAi explaining the absence of any phenotypic alterations. If so, these findings may support the speculation that the $RdRP^2$ plays a significant role in normal gene regulation. This speculation was supported by the observation that the SR1tan7 carrying the $RdRP^2$ direct repeat construct ($702cTR_{DR}$) exhibited phenotypic alterations identical to that of the SR1TK plants. Southern analysis of the SR1tan7 with HindIII pointed to multiple copy integration of the T-DNA (Fig. 21/IIA). The precise integration pattern could not be clearly determined but Southern analysis using DraI demonstrated that, at least, one T-DNA copy was integrated without major rearrangements (Fig. 21/IIC). Expression of the transgene copies was analysed by PCR using cDNA as template and primers that were expected to amplify the non-spliced transcript. From PCR analysis it became clear that full-length, non-spliced transcripts were produced. This result indicated that a non-rearranged $RdRP^2$ direct repeat transgene copy was indeed expressed.

Primary transcripts produced from the $702cTR_{DR}$ control construct were anticipated to not induce RNAi. Nevertheless, one of the SR1tan7 plant lines established the TK-phenotype indicating that RNA-mediated $RdRP^2$ gene silencing was initiated. It should be noted that, with respect to 35S promoter, the $702cTR_{DR}$ construct contained the $RdRP^2$-specific fragments as a direct repeat in antisense orientation. Although IRs were shown to be the most potent inducers of RNAi, it is well known that highly expressed antisense constructs are also capable to induce gene silencing (Takayama and Inouye, 1990; Stam *et al.*, 2000). However, the efficiency of antisense constructs to induce gene silencing is far

below the potential of IR constructs to induce RNAi. Moreover, not only RNAi initiation but also the efficiency of silencing shows a higher degree of variation when antisense constructs are used. IR construct-induced RNAi leads to almost complete target RNA degradation in most cases whereas antisense RNA-induced silencing frequently results in about 15-50% reduction of target RNA accumulation. In view of functional analysis of essential genes a low rate of RNA degradation can be advantageous in that it allows gradual down-regulation of gene expression instead of complete suppression. In other words, total inactivation of an essential gene may represent a lethal factor. By contrast, partial suppression could result in viable plants with phenotypes indicative of the role of the target gene (Wesley *et al*., 2001). In such a scenario, establishment of the TK phenotype in the SR1tan7 plant can be explained. One of the transgene copies was shown to be expressed. Thus, a moderate amount of antisense transcripts was produced. Assuming that $RdRP^2$ gene knock-outs are lethal, the antisense RNA would initiate only partial $RdRP^2$ inactivation. Hence, the SR1tan7 plant could develop but due to reduced $RdRP^2$ activity the TK phenotype established. As an alternative to antisense RNA-mediated silencing, one may also consider that in the SR1tan7 plant line, $RdRP^2$ suppression was caused by transgene-induced PTGS. A high transcription rate could lead to transcript accumulation exceeding a critical threshold. This in turn may result in production of abRNA. The abRNA would serve as template for the $RdRP^3$ and the resulting dsRNA would finally induce the RNAi mechanism.

Why is the $RdRP^2$ assumed to be involved in establishment of the TK phenotype? The most important reason for this hypothesis is the fact, that the SR1tan7 plant contained a $RdRP^2$-specific fragment. This ~800 bp sequence lacked the highly conserved motifs of the RdRPs and also lacked a 23 nt long region sharing 100% homology with one of the other RdRP homologues. Comparison of the tomato $RdRP^2$-specific fragment (~800 bp) with the tobacco $RdRP^1$ cDNA revealed that the best matching 23 nt region comprised three mismatches. At present, the potential to trigger silencing of sequence stretches containing mismatches has not been studied in detail. Previous studies in plants demonstrated that VIGS could be induced by a virus RNA containing a 23 nt long sequence displaying complete homology with a target RNA. However, a 27 bp long RNA region carrying a single mismatch that divided the 27 nt into 12 and 14 nt of identity with the target failed to initiate VIGS (Thomas *et al*., 2001). Thus, it can not be expected that the $RdRP^2$-specific sequence would interfered with expression of the tobacco $RdRP^1$.

Another argument for involvement of the RdRP^2 results from the finding that the TK-phenotype was never observed in transformants carrying a direct repeat construct of the tomato RdRP^1-specific fragment. Comparison of this ~660 bp fragment with the tobacco RdRP^2 sequence revealed 8 nt as the longest stretch of continuous sequence identity. Thus, it was concluded that the tomato RdRP^1-specific fragment was specific for the RdRP^1 gene of tobacco and cross-interference with the tobacco RdRP^2 gene was unlikely to occur. Consequently, if the RdRP^1 is essential, one would have expected that one of the SR1dem lines established the TK-phenotype. In summary, the results obtained from the characterisation of the primary transformants carrying RdRP^2-IR and -DR transgenes indicated that the plant RdRP^2 could have an essential function in normal regulation of genes being involved in pollen, flower and leaf development.

Apart from the plant line carrying the RdRP^2-DR transgene, the TK-phenotype was also observed in the SR1Bam$^-$32, SR1S$^+$26, and SR1Bam$^+$7 primary transformants. All of these plants carried RdRP transgene constructs that comprised, at least, two of the most conserved regions that are present in all RdRP homologues. The SR1Bam$^-$32 and the SR1Bam$^+$7 plant lines carried (SR1Bam$^-$32) or should carry (SR1Bam$^+$7, not analysed) the RdRP^2 3'end fragment. Under the assumption that the RdRP^2 gene was down-regulated, display of the TK-phenotype by these primary transformants as well as by the RdRP^2-DR transgene-containing plant line, was not unexpected. Based on the transgene polarity, the single copy SR1Bam$^-$32 plant line was anticipated to transcribe RdRP^2 3'end antisense RNA. As stated earlier, this antisense RNA could hybridise with complementary endogenous RdRP^2 mRNA to produce dsRNA. This may have resulted in RNAi-mediated down-regulation of the RdRP^2. To investigate transgene expression of the SR1Bam$^-$32 primary transformant, Northern hybridisation was carried out. Unlike the single copy SR1Bam$^-$5T$_1$/1 plant line, Northern analysis failed to give a positive hybridisation signal. This indicated that in this plant, expression of the transgene was affected. However, PCR amplification using the SR1Bam$^-$32 cDNA as template gave a positive hybridisation signal demonstrating that the transgene was actively expressed. From these findings one may conclude that, under the applied conditions, transgene expression was below the detection level of the Northern hybridisation. Alternatively, one may speculate that in the SR1Bam$^-$32 plant, the transgene transcripts were unstable. An antisense/sense RNA duplex originating from hybridisation of the RdRP^2 antisense RNA and the endogenous RdRP^2 mRNA might be degraded either by the RNAi pathway or by an alternative mechanism

that does not necessarily involve DICER. From Northern analysis it was known that the endogenous RdRP genes are expressed at hardly detectable levels (data not shown). Thus, even low transgene expression could produce sufficient amounts of antisense RNA to bind all RdRP^2 mRNA copies. It is conceivable that such a low concentration of RNA duplex molecules are degraded before RNAi is initiated. Based on the Southern hybridisation data of the PCR products amplified from the SR1Bam$^-$32 and the SR1tan7, it was assumed that the SR1Bam$^+$7 primary transformant displaying the TK-phenotype also carried an actively expressed transgene(s). In frame of this work, this plant line has not been genotypically characterised. Nevertheless, this plant line was expected to carry the RdRP^2 3'end fragment in sense orientation. Without knowledge about the T-DNA integration pattern and transgene expression, it can only be speculated that expression of the endogenous RdRP^2 gene was affected in this plant line. In this context it is worth to mention, that progeny of a genetic cross between the SR1Bam$^-$32 and tobacco wild-type plants displayed the TK-phenotype despite the fact that in these plants, the transgene segregated away as was demonstrated by Southern analysis (data not shown). This observation was striking but it may indicate that the TK-phenotype is based on an epigenetic effect. However, additional experiments and extensive investigations are needed to support this rather speculative hypothesis. In addition to Southern analysis, expression of the endogenous RdRP^2 gene was analysed by PCR using cDNA that was derived from floral buds of progeny as well as from wild-type tobacco plants. PCR amplification was carried out using gene-specific primers. Southern hybridisation of the PCR products revealed that the RdRP^2 was expressed in both plants. However, precise determination of RdRP^2 mRNA concentrations (for example by "Real Time PCR") was not performed and needs to be done in future experiments. In view of the presence of, at least, three RdRP homologues, it would be of great interest to study their expression in different tissues. Because in the present study, the three RdRP homologues derived from leaf-specific cDNAs it can not be ruled out that additional members of the RdRP gene family are present in plants. If these homologues differentially expressed in certain tissues or during development they may have escaped detection. In order to investigate the temporal and spatial expression of RdRPs, *in situ* hybridisation experiments have been initiated in collaboration with the Tabler group from Greece.

Appearance of the TK-phenotype in the primary transformants carrying the RdRP^1 3'end (SR1S$^+$26) and the full-length RdRP^1 cDNA (SR1TK) seemed to contradict the

RdRP^2 hypothesis. However, the TK-phenotype was only manifested in those plant lines carrying RdRP^1 transgene constructs comprising the highly conserved RdRP motifs. Therefore, one may speculate that sequences covering the conserved regions have the potential to impair expression of all three RdRP homologues. In other words, if silencing was induced in some of the SR1S$^+$ and SR1TK plants cross-interference might have led to down-regulation of the RdRP^2 and RdRP^3. The SR1TK plant lines are expected to carry the RdRP^1 cDNA as an antisense construct but the plants have not been genotypically characterised for T-DNA integration and copy number. In addition, no RdRP^1 expression analysis was carried out, so far. Southern analysis of the SR1S$^+$26 plant showed the presence of multiple copies of a RdRP^1 3'end sense fragment. The precise integration pattern of all T-DNA copies could not be clearly determined but irrespective of these short-comings it is noteworthy that PTGS was reported to be often associated with multiple copy T-DNA insertions (Hobbs *et al*., 1993; Dehio and Schell, 1994; English *et al*., 1996). Additionally, it has been observed that plants carrying transgene constructs encoding sense mRNA homologous to endogenous genes could also suppress the expression of the cognate gene (Napoli *et al*., 1990; Van der Krol *et al*., 1990). To examine the transgene expression of the SR1S$^+$26 primary transformants Northern hybridisation was carried out. Unlike the single copy SR1S$^+$40 plant, Northern analysis of this plant line failed to give a positive hybridisation signal. Thus, as was found in the SR1Bam$^-$32 plant line, also in the SR1S$^+$26 plant, transgene expression was affected. Assuming that primary transcription was not impaired one may hypothesise that a silencing process hampered accumulation of the transgene-specific steady state RNA. If so, three possible scenarios must be discussed as candidate mechanism for the induction of RNA-mediated silencing in this plant line. Firstly, production of antisense transcripts by at least one of the copies may result in formation of dsRNA upon hybridisation with the endogenous RdRP^1 mRNA. Antisense RNA could be transcribed by an endogenous promoter that is located adjacent to one of the integrated copies. Secondly, if two copies of the transgene have integrated into the genome as an inverted repeat, dsRNA would be directly transcribed. Thirdly, a high transcription rate of the transgene copies may have led to accumulation of RdRP^1-specific RNA exceeding a critical threshold level.

These observations led to an intriguing question: How could the RdRP^1 transgenes down-regulate the RdRP^2 gene? In order to find an explanation and to verify the speculation that was set forth, the DNA sequences of the regions that are highly conserved

among all RdRP homologues were compared. The tomato RdRP[1] 3'end fragment and the full-length tomato RdRP[1] cDNA both, comprised all three highly conserved regions (Table A). However, comparison of the tomato RdRP[1] conserved region 3 with the tobacco RdRP[2] conserved region 3 revealed no 23 nt region with 100 % homology. The best fitting sequence stretches of 23 nt contained three mismatches and a maximum length of 11 uninterrupted nucleotide identity was found. Comparison of the conserved region 2 exhibited more variability. Unfortunately, comparison of the conserved region 1 was not possible because the tobacco full-length RdRP[2] cDNA was not available. The summarised data of the sequence comparison between the tomato RdRP[1] and the tobacco RdRP[2] were hardly in agreement with the assumption that cross-interference between the highly conserved regions was responsible for the establishment of the TK-phenotype. It should be noted that the full-length tobacco RdRP[2] cDNA sequence was not available and comparison with the full-length tomato RdRP[1] could not be carried out. Thus, it could not be excluded that a 23 nt long region with 100% homology was located in the upstream sequence of the tobacco RdRP[2] mRNA.

Inspite of the above arguments against cross-inference, two hypotheses are set forth to nevertheless explain down-regulation of the endogenous tobacco RdRP[2] in plant lines containing the tomato RdRP[1] gene constructs. As a consequence of high level transgene transcription, the probability of transcription errors may increase. In case that such transcription errors comprise the positions of mismatches within the best fitting sequence stretches, a 23 nt long tomato RdRP[1] transgene transcript displaying 100% identity with the tobacco RdRP[2] mRNA could be generated. Only a few RdRP[1] transgene-carrying primary transformants exhibited the TK-phenotype. This may indicate that transcription error-mediated initiation of cross-interference would be a rare event.

According to a second hypothesis, the percentage of overall homology between two RNA sequence stretches may have an impact on initiation of gene silencing. In order to get some support for this hypothesis, the potential of heteroduplex formation between the tomato RdRP[1] transgene RNA (TOM1C.SEQ) and the tobacco RdRP[2] mRNA (TOB2C.SEQ) was investigated. Comparison of the best-fitting 80 nt sequence stretch exhibited an overall sequence homology of 77,5% (Fig. 35) and covered the conserved region 3. This sequence stretch also included the seven best-fitting 23 nt regions comprising three mismatches.

```
TOM1C.SEQ  GGUAGAUUGUGUUGUAUUCCCUCAGAAAGGAAAAAGACCUCAUCCGAAUGAAUGUUCUGGGAGUGAUUUGGAUGGGGAUA
           ::: :::::  :: : :: :::::::::::  :::: :::::::: :::::::: :: ::  ::::: : ::::: :::
TOB2C.SEQ  GGUGGAUUGCAUUAUUUUUCCUCAGAAAGGUGAAAGGCCUCAUCCAAAUGAAUGCUCCGGUGGUGAUCUUGAUGGAGAUC
                            ----------..-----.------
                             ----------..----.------
                              ---------..----.-------
                               --------..----.--------
                                        ----.--------.--------.
                                         ---.--------.--------.-
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Figure 35: Best-fitting sequence stretch between the tomato RdRP[1] transgene RNA and the tobacco RdRP[2] mRNA. The lines below the sequences show the best-fitting 23 nt regions. Dots within these lines indicate the mismatches.

In contrast to this, the 80 nt sequence stretch of the tomato RdRP[1] gene-specific (~660 nt) transcript (6TOM1) that best-fitted with the tobacco RdRP[2] mRNA (8TOB2) exhibited an overall homology of 64% (Fig. 36). The best-fitting 23 nt sequence stretch of this region contained four mismatches. Significantly, there was only one of such a 23 nt stretches whereas within the conserved region 3 there were seven and these only contained 3 mismatches.

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6TOM1.SEQ    GCAGUGGACUUUCCAAAGACUGGUGUUCCCGCUGAAAUACCAUCUCAGUUGCGCCCUAAAGAAUACCCAGACUUCAUGGA
             :: ::::::::  :::: ::::: : : : ::::: :: :::       :    :: : ::: :  :: :: ::: ::::
8TOB2.SEQ    GCUGUGGACUUCGCAAAAACUGGAGCUGCAGCUGAGAUGCCAAGGUUUCUAAAACCAAGAGAGUUUCCUGAUUUCUUGGA
             --.--------..----.-----
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Figure 36: Best-fitting sequence stretch between the tomato RdRP[1] 3' end-specific transgene transcript and the tobacco RdRP[2] mRNA. The lines below the sequences show the best-fitting 23 nt region. Dots within these lines indicate the mismatches.

In view of the potential to initiate cross-interference, the significant higher percentage of overall homology within the conserved region 3 in combination with the presence of the best-fitting 23 nt sequence stretches could represent a critical difference between the transgenes containing the conserved region and the RdRP-specific transgene constructs. It is reasonable to assume that, *in vivo*, the probability of RNA heteroduplex formation increases proportional to the percentage of the complementarity between two RNA molecules. However, even if heteroduplex formation takes place, evidence that transgene constructs containing the conserved regions of the RdRPs are capable to affect the expression of all three homologues is missing. Moreover, if a heteroduplex-mediated mechanism exists further investigations are needed to find out whether the postulated cross-interference is based on PTGS/RNAi-like processes or whether an alternative mechanism results in RdRP inactivation. Support for this hypothesis came from Sanders

and co-workers (2002). They found that the mRNA of an endogenous gene was silenced by 65 nt long inducer RNAs that only shared homology with the target of 58.5 to 89.2%. Importantly, they reported that the efficiency of silencing increased proportional to the degree of homology between the inducer and the target RNA.

In plants and animal systems, endogenous short RNA species called microRNAs were recently discovered (miRNAs; Park *et al*., 2002; Reinhart *et al*., 2002; Rhoades *et al*., 2002). Functional analysis of miRNAs in animals revealed that they regulate expression of endogenous gene by partially base-pairing with 3' untranslated regions (3'UTR) of mRNAs (Reinhart *et al*., 2000). Expression was found to be regulated translationally rather than post-transcriptionally (Olsen and Ambros, 1999). However, data from Tang and co-workers (2003) supported the idea that in plants, miRNA can function in much the same way as siRNAs do. In view of the above cross-interference hypothesis it is most important to note that Tang and co-workers predicted that despite the mismatches between the miRNAs and the mRNA molecules, target are cleaved. Additionally, Doench and co-workers (2003), demonstrated that a siRNA that was only partially complementary to the 3'UTR of the target RNA was capable to induce translational gene repression. In view of the current work, translational repression of the endogenous tobacco $RdRP^2$ gene may represent an alternative to the cross-interference hypothesis.

IV.2.2 Analysis of plants inoculated with *in vitro* transcripts

In addition to a transgenic approach, a virus-induced gene silencing (VIGS) strategy was applied to determine the biological function of the $RdRP^2$ gene in plants. Recombinant plasmids containing a Potato Virus X (PVX) cDNA into which fragments of the $RdRP^1$, the $RdRP^2$, or the full-length GFP cDNA have been integrated were produced and stably propagated in *E. coli*. From these plasmids, infectious PVX/$RdRP^1$, PVX/GFP, PVX/$RdRP^2$ and PVX RNA molecules were *in vitro* transcribed using the T7 polymerase. Plants inoculated with PVX, PVX/$RdRP^1$ and PVX/GFP RNA showed symptom expression. Northern analysis demonstrated that the viruses had replicated and that the recombinant forms were stable in the inoculated plants. However, when re-analysed at later stages of development, PVX/$RdRP^1$-infected plants lost the recombinant virus and were found to only accumulate PVX. Similarly, inoculation using total RNA isolated from plants that were successfully infected with PVX/GFP *in vitro* transcripts, revealed that the majority of the bleached spots (PVX symptoms) that had developed on infected plants did

not show GFP fluorescence under UV-light. This showed that the recombinant forms of the PVX were unstable in plants. Nevertheless, at early developmental stages, at least, up to 20 dpi, infection and accumulation of recombinant PVX/RdDP^1 and PVX/GFP could be demonstrated for primary plants being inoculated with *in vitro* transcripts. This contrasted the results obtained with the PVX/RdRP^2. PVX/RdRP^2 *in vitro* transcripts were never found to be infectious. Although one of the inoculated plants developed a severe phenotype (Fig. 34), Northern analysis of this plant did not give a positive signal when total RNA was hybridised against a PVX- or PVX/RdRP^2-specific probe. The failure to infect plants with PVX/RdRP^2 was reminiscent of the failure to isolate a transgenic plant containing a non-rearranged RdRP^2 IR construct. The fact that viruses and IR-construct are potent inducers of gene silencing supported the speculation that successful infection with PVX/RdRP^2 would have caused complete down-regulation of the endogenous RdRP^2 gene and that total RdRP^2 gene suppression would have resulted in non-viable plants. Hence, the finding that PVX/RdRP^2 was non-infectious substantiated the hypothesis that the RdRP^2 represents a housekeeping gene being essential for normal plant development. In this context, development of a severe phenotype in one of the PVX/RdRP^2 *in vitro* transcript-inoculated plant was an interesting aspect. One may speculate that in a few inoculated cells, the endogenous RdRP^2 gene was drastically inactivated. RdRP^2-deficient cells successfully combated virus replication but, simultaneously may have produced a signal that was able to spread into newly developing leaves. The signal hypothesis is based on the observation that inoculation using total RNA isolated from the phenotype-bearing plant established the same severe leaf malformations (data not shown). However, a more simple and plausible explanation would be that another, unknown pathogen, most likely another virus, was present in the plant and that this pathogen was transmitted during the inoculation procedure. An argument against this assumption is the fact, that we never observed similar symptoms in our greenhouse where more than hundred tobacco plants were cultivated and numerous mechanical inoculations were made.

IV.2.3 Analysis of plants over-expressing the RdRP^2

In order to over-express the RdRP^2 in tobacco, constructs containing the full-length tomato RdRP^2 cDNA were designed (702Rd$^2{}_{3.5+}$). Full-length cDNA was attained by stepwise assembly of the 5'end PCR-amplified fragments with the 3'end fragment. Positive clones containing full-length cDNA were sequenced to confirm the previous sequencing results

(III.1.6). Sequencing of the full-length clones showed five base pair substitutions of which three resulted in amino acid changes. The changes comprised a methionine (M) to valine (V), an aspartic acid (D) to glycine (G) and a tyrosine (Y) to histidine (H) substitution. Based on the biochemical properties amino acid can be grouped. Because D and G as well as Y and H are not in the same group, it was speculated that these two changes might have an affect on the activity of $RdRP^2$. Moreover, the amino acid changes were located within the highly conserved regions present in all RdRP homologues. Thus, it seemed quite probable that the construct containing the $RdRP^2$ full-length cDNA would not code for a functional protein *in planta*. Primary transformants carrying the $702Rd^2_{3.5+}$ construct were demonstrated to moderately transcribe the transgene (Fig. 25) but none of the transgenic plants displayed any obvious phenotypic alterations. This observation may support the idea that the recombinant $RdRP^2$ was not functional. Less likely but also conceivable is, that the tomato $RdRP^2$ was not functional in tobacco or that over-expression of a functional $RdRP^2$ does not have detectable effects. In the frame of this work, it was not possible to substantiate the results by determining the $RdRP^2$ protein levels. In addition, there was no time left to isolate and introduce into tobacco plants a $RdRP^2$ cDNA clone (tobacco or tomato) that contained the full-length authentic sequence.

IV.3 Future prospects

One major objective of future experiments would be a detailed characterisation of the transgenic tobacco plants displaying the TK-phenotype. These plants can be analysed for the presence of $RdRP^2$-specific siRNAs to obtain clear evidence of whether $RdRP^2$ gene silencing is taking place in TK-phenotype-bearing tissue. siRNAs are a hallmark of silencing. It has been observed that in plants where silencing occurs siRNAs are detected (Hamilton and Baulcombe, 1999). Genetic crosses between homozygous $SR1Bam^{-}5T_1/1$ and $SR1Bam^{+}2T_1/4$ plant lines can be made to monitor progeny plants for the establishment of the TK-phenotype. As was reported, simultaneous expression of sense and antisense transgene constructs enhanced the probability of silencing induction (Waterhouse *et al*., 1998). Thus, provided that $RdRP^2$ suppression correlates with the TK-phenotype, it can be expected that several of the progeny plants will develop this phenotype. Additionally, it was shown that the $RdRP^2$ transgene construct was highly expressed in the $SR1Bam^{-}5T_1/1$ and the $SR1Bam^{+}2T_1/4$ plant lines. It would be interesting to follow if the expression levels will decrease when these transgenes are brought into the

SR1Bam^{-}32 plant. A decrease of expression would indicate that the transgene transcripts of the SR1Bam^{-}5T$_1$/1 and SR1Bam^{+}2T$_1$/4 lines became a target of the silencing machinery that was suggested to be active in the SR1Bam^{-}32 plant.

It would be interesting to get experimental evidences for the hypothesis that the percentage of overall homology between two RNA sequence stretches might have a potential to initiate cross-inference. In view of this, sequence stretches exhibiting different overall homology (for example, 80 bp fragments shown in Figure 35 and 36) can be selected to design similar constructs as used in the present work. Constructs containing these fragments in sense and antisense orientation can be introduced in plants and transgenic plants carrying these constructs can be monitored for the phenotype. In case of transgenic plants carrying fragments that exhibit high percentage of overall homology, establishment of TK-phenotype would be indicative of cross-interference. Additionally, this experiment would help to dissect the percentage and sequence determinants of cross-interference with homologous sequences.

Furthermore, it was assumed that the RdRP^2-specific fragment (~800 bp) and the RdRP^1-specific fragment (~660 bp) are incapable of initiating cross-interference. This assumption can be evaluated by crossing the SR1tan7 plant with the homozygous SR1dem18 plant and analysing the progeny for transgene expression level. It was shown that the RdRP^1 DR transgene construct was highly expressed in the SR1dem18 primary transformant. Therefore, no change in the expression level of transgene when brought in the SR1tan7 would provide an experimental evidence for absence of cross-interference.

V Summary

The cellular RNA-directed RNA polymerase 2 ($RdRP^2$) full-length cDNA was successfully cloned and its authentic sequence has been deduced from the *Lycopersicon esculentum* cultivar Rentita (tomato). Insights into the possible biological function of the $RdRP^2$ was achieved by down-regulating expression of the endogenous tobacco $RdRP^2$ using RNA-mediated gene silencing technologies. Eight different RdRP sequence-containing constructs were produced and introduced into the *Nicotiana tabacum* cv. Petita Havana SR1. Results of the transgenic plants carrying $RdRP^2$-specific IR and DR transgene constructs indicated that the $RdRP^2$ could be involved in normal gene regulation. Appearance of phenotypic alterations in a plant line carrying a $RdRP^2$ DR transgene was indicative of partial down-regulation of the $RdRP^2$. Absence of any phenotypic alterations in plants carrying a $RdRP^1$-specific DR transgene construct supported the observations and conclusions that were drawn from plant lines carrying the $RdRP^2$ IR and DR transgenes. Expression of identical phenotypic alterations in plant lines containing $RdRP^2$ gene fragments, in either sense or antisense orientation, further supported the essential function of the $RdRP^2$. The same applies for the observation, that *Nicotiana* plants could not be infected with the recombinant PVX/$RdRP^2$. The presented data provided indirect experimental evidence that the $RdRP^2$ gene was indeed down-regulated by a gene silencing mechanism. These experiments included the generation of transgenic plants that carried RdRP gene constructs comprising highly conserved RdRP regions.

Transgenic plants were genotypically characterised by Southern and PCR analysis and for most of them, T-DNA copy numbers as well as arrangements of the integrated DNA could be determined. These plants are now available for further experiments. In the frame of this work, homozygous T_1 plant lines containing non-rearranged $RdRP^2$ constructs as a single copy insert were established. In addition to genotypical examinations, transgene expression levels were investigated by Northern analysis and by PCR amplification with cDNA.

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VII Abbreviations

α	Alpha
AA	Amino acid
abRNA	aberrant RNA
Amp	Ampicillin
BAP	6-Benzylaminopurine (Cytokinin)
bp	Base pair
cDNA	complementary DNA
cm	centimetre
cv	cultivar
dCTP	2'-Deoxycytidine 5'-triphosphate
DEPC	Diethyl-pyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
dpi	days postinoculation
DR/tan/dem	direct repeat
dsRNA	double-stranded RNA
EDTA	Ethylenediamine tetraacetic acid
EST	Expressed Sequence Tag
Fig	Figure
Fr/rev	Forward/reverse
g	Gram
GFP	Green Fluorescent Protein
Gm	Gentamycin
h	Hour
hpRNA	hairpin RNA
IR/Inv'	Inverted repeat
kb	Kilo base pairs
KCl	Potassium chloride
kD	kilo dalton
Km	Kanamycin
λ	Lambda

LB	Luria-Bertani
M	Molar
µg	Microgram
µL	microlitre
mA	milli Ampere
mg	milligram
min	Minute(s)
mL	millilitre
mm	millimetre
mM	millimolar
MOPS	3-(morpholino)propanesulfonic acid)
mRNA	messenger RNA
MS	Murashige and Skoog
N	Normal
NAA	1-Naphthaleneacetic acid (Auxin)
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	nanometre
nptII	NeomycinphosphotransferaseII
nt	Nucleotide
OD	Optical density
O/N	overnight
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
pmol	picomole(s)
PTGS	Posttranscriptional gene silencing
RACE	Rapid amplification of cDNA ends
RdRP	RNA-directed RNA polymerase
Rif	Rifampicin
RNA	Ribonucleic acid
RNAi	RNA-mediated interference
RNase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
Sarkosyl	N-Lauroylsarcosine, Sodium Salt

SDS	Sodium dodecyl sulfate
sec	Second(s)
siRNA	small interfering RNA
Sm	Streptomycin
Sp	Spectinomycin
SSC	Saline sodiumcitrate
TAE	Tris-acetate EDTA
T-DNA	Transfer DNA
Tm	Melting temperature
Tris	Tris (hydroxymethyl) aminomethane
t-RNA	Transfer RNA
TRs	TomRes specific
UV	Ultra violet
VIGS	virus-induced gene silencing
v/v	volume per volume
w/v	weight per volume

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