Increased gene expression with polycistronic expression units to confer resistance to rice tungro bacilliform virus (RTBV)

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Publication Date:
2000

Permanent Link:
https://doi.org/10.3929/ethz-a-003927330

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Increased gene expression with polycistronic expression units to confer resistance to rice tungro bacilliform virus (RTBV)

A dissertation submitted to the
Swiss Federal Institute of Technology Zürich

for the degree of
Doctor of Natural Sciences

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February 2000
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxy acetic acid</td>
</tr>
<tr>
<td>aph4</td>
<td>aminoglycoside phosphotransferase</td>
</tr>
<tr>
<td>b, bp</td>
<td>base, basepair</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxygenin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamminotetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GLH</td>
<td>Green leafhopper</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>IRRI</td>
<td>International Rice Research Institute</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani (medium)</td>
</tr>
<tr>
<td>MES</td>
<td>2-morpholinoethane sulfonic acid x H2O</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige-Skoog (medium)</td>
</tr>
<tr>
<td>NAA</td>
<td>α-naphthyl acetic acid</td>
</tr>
<tr>
<td>nt, nts</td>
<td>nucleotide, nucleotides</td>
</tr>
<tr>
<td>PAT</td>
<td>phosphinothricin acetyltransferase</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylenglycol</td>
</tr>
<tr>
<td>PPT</td>
<td>phosphinothricin</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RTBV</td>
<td>Rice Tungro Bacilliform Virus</td>
</tr>
<tr>
<td>RTSV</td>
<td>Rice Tungro Spherical Virus</td>
</tr>
<tr>
<td>T, U, C, A, G</td>
<td>bases thymine, uracil, cytosine, adenine, guanine</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris/acetic acid/EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/boric acid EDTA buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>X-Gluc</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
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Rice tungro disease (RTD) is one of the most devastating rice diseases in South East Asia. It is caused by a complex of two viruses, rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). There are no suitable natural sources of RTD resistance. Therefore, we have investigated a transgenic approach, following the concept of pathogen-derived resistance (Sanford and Johnston, 1985). Our efforts were concentrated on RTBV, a pararetrovirus containing a double-stranded DNA genome. Despite intensive previous research efforts it was not possible until now to produce any form of RTBV resistance. A possible factor for the lack of protection could have been the low expression level of the transgenes used. Therefore, we developed a new strategy to increase gene expression by translational coupling of the gene of interest to a selectable marker gene. Expression units producing bifunctional, dicistronic mRNAs, translatable by leaky scanning, were constructed. Various RTBV open reading frames (ORF), as well as an ORF of the selectable marker PAT, coding for resistance to phosphinothricin, were tested for their ability to allow expression of a downstream β-glucuronidase (GUS) or an aph4 ORF in transiently or stably transformed rice cells. The sequences of RTBV ORFs I and II are naturally adapted to the leaky scanning mechanism. However, they had negative effects on the expression of the reporter genes, which were not related to translation but probably to RNA stability. With RTBV ORF II the problem could be solved by increasing the total GC content of that sequence. The PAT ORF had to be adapted for leaky scanning by removal of all internal ATG codons. With the adapted upstream ORFs, downstream translation was efficient. With aph4 as downstream ORF, transgenic rice plants expressing high level of dicistronic mRNAs could be generated.

We attempted to achieve RTBV resistance by expression of different RTBV proteins in full-length or truncated form, or by antisense RNA, trans-activation of a cytotoxic protein (a virus induced hypersensitive response) or homology dependent gene silencing.

The approaches of expression of full-length RTBV-proteins and of an induced hypersensitive response could not be tested, because no or only sterile plants could be regenerated. However, 20 independent transgenic lines with modified RTBV proteins, antisense RNA or silenced genes with sequence homology to the RTBV-genome were tested at the International Rice Research Institute (IRRI). After tungro infection all of the lines became infected and displayed disease symptoms. However, some lines containing transcriptionally silenced RTBV homologous genes and one line containing the amino-terminal part of RTBV ORF IV accumulated slightly lower RTBV-titers. The latter line also displayed reduced
disease symptoms with less stunting and increased tillering compared to infected control plants. The potential of genetic approaches to control RTD is discussed.
ZUSAMMENFASSUNG


Wir versuchten durch die Expression verschiedener RTBV Proteine, die in voller Länge oder in verkürzter Form in Reis transformiert wurden, oder durch Antisense RNA Expression eine Resistenz gegen RTBV zu erzeugen. Ausserdem versuchten wir durch einen speziellen vom Virus induzierten Mechanismus, der ein zytotoxisches Protein aktivieren sollte, eine hypersensitive Abwehr zu

Nach Virusinfektion durch den natürlichen Virusüberträger zeigten alle Linien Symptome der Reis Tungro Krankheit. In den Linien jedoch, die Virus homologe stillgelegte Sequenzen enthielten und in einer Linie, die eine carboxy-verkürzte Version von RTBV ORF IV exprimierte, wurden weniger RTBV-Partikeln akkumuliert. Die letztere Linie hatte zudem weniger Symptome, was durch höheren Wuchs und mehr Blätter im Vergleich zu anderen infizierten Pflanzen sichtbar wurde. Die Möglichkeiten zur Kontrolle der Tungro Krankheit mit gentechnologischen Methoden werden diskutiert.
1 INTRODUCTION

1.1 Rice tungro disease

Rice tungro disease is one of the most devastating rice diseases in South and Southeast Asia, causing annual losses in the order of $1.5 \times 10^9$ (Herdt, 1991). Tungro is occurring epidemically throughout whole regions (Thresh, 1991). Major outbreaks occurred in Bangladesh, India, Indonesia, Malaysia, The Philippines and Thailand (Hibino, 1989) and grain yield reduction up to 100% has been reported (Hassanuddin et al., 1988).

Tungro is caused by a complex of two viruses, rice tungro bacilliform virus (RTBV, Fig. 1a) and rice tungro spherical virus (RTSV, Fig. 1b). The symptoms are yellowing and reddening (Fig. 1c) of the leaves, stunted growth and reduced tillering. Plants infected with RTBV alone show similar symptoms. Plants infected with RTSV alone show no obvious symptoms except very mild stunting. However, RTBV on its own is not leafhopper transmitted implicating a so far unknown helper-activity from RTSV (Hibino, 1989).

RTBV and RTSV are transmitted in the semipersistent manner by rice green leafhoppers (Nephotettix spp). Nephotettix virescens D is the most efficient vector of tungro disease (Hibino, 1983) (Fig. 1d). The insects feed on the phloem and xylem of vector-susceptible cultivars (Dahal et al., 1990). They retain tungro infectivity for 2 to 5 days after acquisition.

Fig. 1: Rice tungro disease (RTD). a, b) Electron micrograph of the bacilliform particles of RTBV (a) and the spherical particles of RTSV (b, Dasgupta et al., 1991). Bar is 100 nm. c) RTD infected leaves with orange leaf discoloration. d) The green leafhopper Nephotettix virescens D (c and d from IRRI, 1983b).

Despite integrated tungro management schemes (Sama et al., 1991), it was not possible to control the disease and to avert major outbreaks. Therefore, several research programmes focus on finding resistance to RTD (Herdt, 1991).

Natural resistance to the viruses and the vector has been found in routine screening experiments in different rice varieties, also including modern varieties.
(Hibino et al., 1987; Cabunagan et al., 1993). However, these varieties are not suitable for sustainable rice production. First, vector populations have become adapted after a few years of cultivation of the resistant varieties (IRRI, 1983a; Dahal et al., 1990). Second, resistance traits often cosegregate with traits like low yield or minor grain quality which makes it very difficult to transfer the resistance to new cultivars by breeding. So far, none of the genes conferring resistance to the viruses was identified or even transferred to commercial rice cultivars.

1.1.1 Rice tungro spherical virus (RTSV)
RTSV is a picoma-like virus of the sequiviridae family. Isometric particles with a diameter of 30 nm can be seen by electron microscopy (Fig. 1b). The particles each encapsidate a molecule of positive single-stranded polyadenylated RNA of 12.4 kb (Shen et al., 1993). For a review see Hull (1996).

1.1.2 Rice tungro bacilliform virus (RTBV)
RTBV is named after its bacilliform particles that are elongated icosahedrons of about 130 x 30 nm (Fig. 1a). The size varies with the virus isolate (Hull, 1996). The particles contain a circular double-stranded DNA genome of about 8000 bp (Fig. 2) with two site-specific discontinuities resulting from a replication by reverse transcription. RTBV is a pararetrovirus and belongs to the Caulimoviridae family comprising caulimoviruses, badnaviruses and two other genera (Mayo and Pringle, 1998). It has only recently been proposed that RTBV belongs to the "RTBV-like viruses" genus with RTBV being it's only member. Therefore, in this study, RTBV is still assigned as a member of the badnaviruses. Pararetroviruses share many similarities with animal retroviruses. Similar features are the same distribution of the gag-pol functions, the involvement of reverse transcriptase in the replication cycle, a more-than-genome length transcript with terminal repeats and the usage of the pregenomic RNA as polycistronic mRNA (Rothnie et al., 1994). Unlike the retroviruses, which encapsidate RNA and transcribe their RNA from a genome copy integrated into the host DNA, pararetroviruses encapsidate DNA and transcribe RNA from an episomal form of DNA that is not integrated into the host genome (Hohn and Fütterer, 1991). One promoter controls the synthesis of the pararetroviral pregenomic RNA. This RNA serves as a template for reverse transcription by the virus-encoded reverse transcriptase and also for the expression of the virus encoded proteins (Hull, 1996; Hohn and Fütterer, 1997).
1.1.2.1 Genome organisation

Transcription of the circular, double stranded DNA of RTBV starts at nucleotide 7404 or 7405 and ends at the polyadenylation signal (AATAAA) at nucleotide 7620, yielding a primary transcript of more than genome length with a terminal redundancy of 215 or 216 nucleotides (Fig. 2; Bao and Hull, 1993; Chen et al., 1994). The coding potential of RTBV includes four large open reading frames (ORFs). The first three ORFs are closely packed and have the interface ATGA, the ATG being the start codon of the downstream ORF and the TGA being the stop codon of the upstream ORF (Hay et al., 1991). ORF IV is separated from ORF III by a short noncoding region, and there is a longer intergenic region between ORF IV and ORF I, containing several short ORFs (sORF, Fig. 2). Specialised translation mechanisms allow the expression of all four RTBV ORFs (Fütterer and Hohn, 1991; Fütterer et al., 1994; Fütterer et al., 1996b) despite of the limitations of translation of polycistronic mRNAs by eukaryotic ribosomes (Kozak, 1989b; Kozak, 1999).

1.1.2.2 Eukaryotic translation

Eukaryotic translation mechanisms restrict translation initiation in most cases to the start-codon nearest the 5' end of a mRNA which is the unique site of translation initiation. Translation of a further downstream open reading frame (ORF) will be very inefficient. Translation in eukaryotes begins with association of 40S ribosomal subunits with the 5' cap of mRNAs, followed by a linear scanning towards initiation codons. This phenomenon of linear migration of the ribosomes on the mRNA has been formulated as the scanning model (Kozak, 1989b). Once the 40S ribosomal subunits have recognised a start codon, the translation machinery will assemble and this complex has no capability to restart translation at a downstream ORF. The scanning model postulates that 40S ribosomal subunits stop at the first AUG if that codon occurs in a favourable context defined by a purine in position -3 and G in position +4 (A/GNN AUG G; Kozak, 1989a; Kozak, 1999). Deviating contexts will allow a certain number of ribosomal subunits to bypass the codon and initiate further downstream (leaky scanning). For the translation of a downstream located ORF it is essential that no (or only a few) additional translation start sites (AUGs) are preceding the translation start of the second ORF.
1.1.2.3 **RTBV Translation mechanisms**

*Ribosome shunt*

The ribosome shunt enables translation downstream of the long RTBV leader sequence containing several short ORFs. In the shunt mechanism, ribosomal subunits begin scanning at the 5' end but then are induced by structural motives to bypass RNA regions by jumping from a shunt donor site to an RNA internal shunt acceptor site. For RTBV, the “shunt acceptor” is thought to be located between the first and second AUU codons upstream of ORF I. Translation is initiated at the second AUU codon of ORF I. This mechanism has been reported by Fütterer et al. (1993).

Fig. 2: Organisation of the RTBV genome. The inner circle represents the double-stranded DNA genome with the four large open reading frames (ORF I, II, III, IV) including short ORFs (sORFs) (open boxes) and intergenic regions (spaces between the boxes). The promoter region is located upstream of the transcription start at position 7404/5, the polyadenylation signal was found at 7620, allowing the production of an RNA with a terminal repeat of 215/ 216 nucleotides. Arrows indicate the full-length (dark grey) and the spliced (pale grey) transcripts, respectively. The location of the splice donor (SD) and the splice acceptor (SA) site is shown. The translation start codons of the ORFs are indicated. The outer arcs represent the size of the encoded proteins. The features on p194 are shown including coat protein (CP), aspartate protease (PR), reverse transcriptase (RT) and ribonuclease (RH). The specialised mechanisms that enable the expression of RTBV proteins are indicated.
Leaky scanning

Expression of RTBV ORF I, II and III is enabled by the unusual translation mechanism of leaky scanning. Leaky scanning is possible, because no internal AUG codons are located in the region between the 3' end of the leader and the start of ORF III. In this region only the start codons of ORF I and ORF II could intercept scanning ribosomal subunits. Translation efficiency of the unusual AUU start codon is about 10% that of an AUG codon in the same context (Fütterer et al., 1996b). Thus, many ribosomes are likely to pass the ORF I AUU codon in a still initiation competent state. Mutation of the AUU to a proper AUG start-codon abolished infectivity of the viral DNA in agroinfection (Fütterer et al., 1996b) probably because of abolishing the "leakiness" of the upstream start-codon. The next AUG codon is the start-codon of ORF II, which is in a poor context (Kozak, 1992) and is consequently also bypassed by some of the scanning ribosomes (Fütterer et al., 1997). The third AUG codon in this line is the start-codon of ORF III and the remaining ribosomes will start translation. The potential of this translational mechanism for gene expression on dicistronic mRNAs was evaluated in this study.

Incomplete splicing

Associated with RTBV infection two new RNA species appear in infected plants. The full-length RNA and a spliced product, the mRNA for p46 (Fütterer et al., 1994). Splicing connects a short open reading frame (sORFA) in the RTBV leader sequence in frame with the 5' end of ORF IV and releases an intron of 6.3 kb. In infected rice plants the spliced RNA is only present in very low amounts and usually only detectable by RT-PCR analysis.

1.1.2.4 Coding potential

The following chapter describes the gene products of the four RTBV ORFs. The corresponding proteins are named after the predicted size of the protein (Fig. 2). Although the detailed functions of most of the gene products are not known, the information provided may lead to a better understanding of their potential role.

ORF I; p24

ORF I is the first ORF on the full-length transcript and is located downstream of a leader sequence of 666 nucleotides. Particular features of the ORF are the absence of an AUG start codon and of any AUG codon within the ORF that could inhibit leaky scanning. The protein function is not known. Hay et al. (1994) suggested that p24 might be associated with particle assembly, because the product of ORF I could be located within the virus particles. A comparison to the homologous proteins of other badna-viruses suggests an involvement of p24 in
mealybug transmission (Hohn and Fütterer, 1997), however, so far RTBV has not been reported to be transmissible by any insect on its own.

**ORF II; p12**
For the 12 kDa protein (p12) no definite function has been assigned. However, sequence features also existing in the ORF II of badnaviruses and the ORF III of caulimoviruses suggest a common role of the protein in the viruses' life cycles. p12 of RTBV and the ORF II gene product of the badnavirus commelina yellow mottle virus (CoYMV) were shown to be associated with purified virions (Hull, 1996; Cheng et al., 1996). p12 of RTBV, the ORF II gene product of the badnavirus cacao swollen shoot virus (CSSV) and the ORF III gene product of cauliflower mosaic virus possess a non-specific nucleic acid binding activity (Mougeot et al., 1993; Mesnard and Carrière, 1995; Jacquot et al., 1996; Jacquot et al., 1997). The C-termini of the proteins contain basic, hydrophobic and proline residues, supporting the nucleic acid binding activity. Such residues are also present in the C-termini of bacterial histone-like proteins (Mougeot et al., 1993) suggesting an influence of p12 on genomic DNA condensation. Moreover, mutational analysis of the RTBV ORF II 5' end suggested that a tetramerization site is located in the N-terminal region of the protein (Etienne Herzog, personal communications).

**ORF III; p194**
The amino acid sequence of the RTBV ORF III product contains sequence domains corresponding to the viral coat protein (CP), an aspartate protease (PR), a reverse transcriptase (RT) and a ribonuclease H (RH), ordered from the N- to the C-terminus (Fig. 2; Hay et al., 1991). Consequently, a polyprotein is encoded containing the module for replication and assembly. These modules are the analogues of the gag and pol genes of retroviruses. The viral protease is at least partially responsible for the processing of p194. The cleavage sites at the N- and C-terminal extremities of the RT/RH domain have been characterised. It has been demonstrated that the PR/RT/RH polyprotein can be processed to yield two proteins of 55 kDa and 62 kDa when expressed in insect cells from the 3'part of gene III (Laco and Beachy, 1994).

Two coat proteins of 37 and 33 kDa were found in purified RTBV particles (Qu et al., 1991). Recently, Marmey et al. (1999) showed that RTBV virions contain only a single coat protein species of 37 kDa, the second peptide of 34 kDa in fact being most likely a degradation product of the 37 kDa protein generated during virus purification. It was deduced from mass spectrum analysis that amino acids 477 and 791 of p194 correspond to the N- and C-terminal residues of the 37 kDa coat protein (p37). In the C-terminal region of p37, a Cys/His motif and a basic domain were found, which are highly conserved in plant pararetrovirus coat proteins. This
motif is the equivalent of the "Zinc-finger" motif of retroviral gag proteins and consequently is thought to be involved in specific RNA binding during packaging of the pregenomic RNA into virions (Rothnie et al., 1994).

**ORF IV; p46**

RTBV differs from other badnaviruses in having an ORF downstream of the gag-pol analogue. Like the ORF I and ORF II proteins, the ORF IV protein belongs to the terra incognita. However, a regulatory function of the ORF IV gene product has been proposed (Hohn and Fütterer, 1997). Similar to other regulatory proteins of retro- and pararetroviruses, ORF IV is located at the 3' end of the genome and is expressed from a separate RNA. As described above, p46 is expressed from a spliced mRNA (Fütterer et al., 1994). However, the gene product was reported to be undetectable in western blot analysis (Hay et al., 1994). RTBV ORF IV has a sequence motif that is characteristic for a leucine zipper motif similar to those that are involved in protein-protein interactions (Gruissem, 1990). It is speculated that they interact with host proteins related to cell cycle regulation (T. Hohn, personal communications). Moreover, putative di-/polymerisation domains have been found (J. Fütterer, personal communication), suggesting that the protein might be involved in the production of larger protein-complexes.

### 1.2 Pathogen-derived resistance to viruses in transgenic plants

Viral epidemics can result in a large reduction of the yield of commercial crops. Various methods to control them consist either of so-called conventional methods or non-conventional methods. Conventional methods comprise the use of virus-free germplasm and the eradication of infected plants, timing of planting so as not to coincide with the presence of the vectors, and the use of chemicals to control such vectors. Disadvantages of these methods are that they are expensive, and the chemicals are damaging the environment. Breeding for resistance has the most potential in the long term. Unfortunately, breeding programmes are time consuming, complicated and expensive, especially when virus resistance genes are linked to an agricultural undesirable trait and/or inherited in a complex manner. In addition, there are few natural sources of resistance and virulent strains might quickly overcome the resistance.

The concept of non-conventional protection was developed in the early 1980s, and was published for the first time in 1985, when Sanford and Johnston reported the simple and elegant concept of pathogen-derived resistance (Sanford and Johnston, 1985). Subsequently, there have been numerous attempts to generate virus resistance in transgenic plants based on this concept through the expression
of virus derived genes or genome fragments (Beachy, 1993; Wilson, 1993; Baulcombe, 1994; Lomonossoff, 1995).

This concept predicts that expressing genetic material of a pathogen in a host will disrupt the essential pathogenic processes and hence result in resistance to the pathogen. Pathogen-derived resistance has been obtained by expressing various forms of coat protein (CP) genes, the viral replicase genes (in full-length or deleted form), protease or movement proteins. The proteins or parts thereof are acting as dominant negative competitor during virus infection (Padidam et al., 1999). Other sequences as antisense RNA, satellite RNA, defective interfering RNA and virus-specific "neutralising" antibody genes conferred also resistance in several cases (for reviews see: Hull, 1994; Pappu et al., 1995; Baulcombe, 1996; Palukaitis and Zaitlin, 1997).

1.2.1 RNA-mediated resistance

Initially, it was anticipated that pathogen-derived resistance would operate through expression of the respective protein, and such a mechanism has been suggested by various reports (e.g. Powell-Abel et al., 1986; Brederode et al., 1995). However, it was shown that untranslatable constructs were also able to confer resistance (Lindbo and Dougherty, 1992; van der Vlugt et al., 1992). This type of resistance requires only the synthesis of RNA and became known as RNA-mediated resistance. It characteristically provides a high level of resistance that can not easily be overcome by a high inoculum dose, compared with, e.g., coat-protein-mediated resistance (see Lomonossoff, 1995). Moreover, RNA-mediated resistance avoids the accumulation of any foreign protein and the risks involved with trans-encapsidation are limited. However, this resistance only affects closely related viruses, implying the requirement for a high degree of sequence homology of the virus to the transgene. In addition to the phenotype of extreme resistance, another form of induced RNA-mediated resistance has been referred to as "recovery", because the virus-infected plants undergo a transition from a diseased to a healthy appearance. In such plants, a systemic infection initially occurs; however, each new leaf that subsequently appears displays fewer symptoms. Eventually, virus-free leaves emerge that are completely resistant.

The mechanisms behind RNA-mediated resistance remain largely unknown; however, similarities have recently been shown with homology-dependent gene silencing, a process affecting the expression of transgenes and homologous sequences (Baulcombe, 1996; Wassenegger and Pélassier, 1998).
1.2.2 Homology-dependent gene silencing

The stability of transgene expression for the expression of heterologous sequences is an important aspect in the development of transgenic plants for commercial applications. Homology-dependent gene silencing, a phenomenon whereby the presence of (multiple) transgene(s) can lead to the inactivation of one or all of the transgenes, thus came as an unwelcome surprise (for reviews see: (Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe and English, 1996b). This gene inactivation is a great disadvantage in applied plant biotechnology and a survey held in 1994 among 30 biotechnology companies revealed that almost all of them had encountered problems associated with gene silencing (Finnegan and McElroy, 1994).

Gene silencing can occur either at the transcriptional level (no transcription), called transcriptional gene silencing (TGS) or at the post-transcriptional level (transcription, but increased degradation of the transcript), called post-transcriptional gene silencing (PTGS). Because of their different mode of action, TGS and PTGS processes could be regarded as two separate gene silencing phenomena although both had predominantly been observed in transgenic plants containing multiple homologous copies of either an endogenous gene or a transgene.

1.2.2.1 Transcriptional gene silencing (TGS)

Transcriptional gene silencing (TGS) is defined by the inactivation of (trans)gene-specific nuclear RNA synthesis. The reason for this seems to be a mechanism that can inactivate repeated DNA sequences by methylation and/or heterochromatinisation (Wassenegger and Pélissier, 1998). Once TGS is established, it is meiotically stable and independent of the plant development. Transcriptional inactivation can act in cis (silencing events that affect the expression of transgenes integrated at a single locus, irrespective of the copy number) and in trans (silencing effect of one locus on another, Vaucheret et al., 1998).

Transgenes can undergo TGS in cis when one or multiple copies integrate at a locus located in or next to silent hypermethylated genomic sequences. Heterochromatin can then spread into the gene and affect its expression (Pröls and Meyer, 1992). Transgenes can also undergo TGS in cis when multiple copies become methylated although they integrated at a hypomethylated locus. This case resembles local heterochromatin formation (Ye and Signer, 1996).

Active transgenes can also become silent and methylated in trans when brought (e.g. by crossing) into the presence of an unlinked silenced homologus transgene (Matzke et al., 1989; Vaucheret, 1993). This can affect any transgene that is
expressed under the control of the same promoter, irrespective of the coding sequence being expressed (Vaucheret, 1993; Matzke et al., 1994). This specificity indicates that the promoter of the transgene is the target for this form of transcriptional silencing (Thierry and Vaucheret, 1996). The mechanism for this trans-silencing effect may be the ability of a silencing locus to interact with any other position of the genome by direct DNA-DNA pairing (Vaucheret et al., 1998). Alternatively, the mechanism could involve the production of diffusible RNA by the silencing locus that leads to heritable silencing and methylation of homologous target loci via an RNA-DNA interaction (Wassenegger and Pélissier, 1998). However, it has not yet been shown that such a silencing locus was able to silence the expression of extra-chromosomal copies of a target transgene (Vaucheret, 1994).

1.2.2.2 Post-transcriptional gene silencing (PTGS)

RNA-mediated resistance is associated with post-transcriptional gene silencing (PTGS, Lindbo et al., 1993; Mueller et al., 1995; Sijen et al., 1996). Not related to virus resistance, PTGS was observed in transgenic plants affecting the transgene and the endogenous gene (Napoli et al., 1990), a mechanism also called co-suppression. Unlike TGS, co-suppression has been shown to be developmentally regulated and meiotically reversible (Dehio and Schell, 1994). The induction of the silencing was shown to depend upon environmental conditions but once it was established it was maintained during the subsequent development of the plant.

Models to explain co-suppression and RNA-mediated resistance

Co-suppression and RNA-mediated resistance require sequence homology between the transgene and endogenous gene or the transgene and incoming virus, respectively. There are currently two models, that attempt to explain post-transcriptional silencing (Fig. 3; Vaucheret et al., 1998; van den Boogaart et al., 1998; Baulcombe, 1996). The models are not mutually exclusive, and both are assuming the presence of an RNA degradation mechanism driven by an RNA-dependent RNA polymerase (RdRP).

The first model involves a threshold level of RNA (Fig. 3b). This model proposes that the plant is somehow able to sense the transcription level of the transgene and, if the level is too high, the plant targets the mRNA for degradation. Once the threshold level is reached, the RdRP, present in the cytoplasm of plants, would produce short, antisense RNA molecules with sequence homology to the target RNA. Annealing of the antisense RNA molecules would lead to the formation of a duplex RNA structure that could be a target for degradation by double-strand, specific RNases.
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Fig. 3: Initiation of homology-dependent resistance (modified from Baulcombe, 1996).  

a) Susceptible transgenic plant. In most transgenic lines, a viral transgene is transcribed, the corresponding mRNA (and protein; not shown) accumulates, and the plant is susceptible to the transgene-homologous virus.  
b) The threshold model for homology-dependent resistance. In this model, there is a mechanism that detects high levels of the transgene mRNA (+++). This mechanism suppresses (red symbol) accumulation of nucleus- and virus-derived RNAs that are homologous to the transgene.  
c) The qualitative model for homology-dependent resistance. This model does not postulate a mechanism that monitors the level of transgene expression. Instead, it requires the production of an aberrant RNA (aRNA) that activates the suppression of viral and transgene RNA accumulation.

Because not all PTGS appearances could be explained by the threshold level model, another model involves a qualitative feature of the mRNA, known as aberrancy (Baulcombe and English, 1996). This aberrant mRNA (aRNA) would be distinct from the normal mRNA and would induce a sequence-specific degradation mechanism. In this mechanism, aRNA serves as a template for the RdRP affecting all RNA with sequence homology (Fig 3c). The origin of the aRNA is not clear. English et al. (1996) suggested that transgene methylation could be directly responsible for the production of aRNA, but aRNA could also be caused by transgene features (e.g., untranslatable, no introns, or other unusual characteristics).

Overall, TGS and PTGS phenomena may reflect natural (and poorly understood) mechanisms of plant defence acting at the DNA or RNA level against transposons or invading pathogens like viruses (Kumpatla et al., 1998). TGS may recruit cellular components acting against invading DNA that integrates into the genome. Therefore, TGS is an important point to consider during plant transformation. PTGS may recruit cellular components acting against invading DNA that replicates extra-chromosomally in the nucleus or invading RNA that replicates in the cytoplasm and it is very likely that this mechanism is important for virus resistant transgenic plants.
1.3  The challenge: increased and selectable gene expression

Before this work started, already several attempts had been made to obtain RTD resistant plants by genetic engineering (Klöti, 1996; summarised by Azzam et al., 1999). Despite a large number of independent transgenic plants tested for tungro resistance, none of these plants provided protection. A possible factor for the lack of protection was the low expression level of the transgenes. In almost all lines, expression of the genes of interest was not detectable although very sensitive detection methods were used (Klöti, 1996). Therefore, a novel strategy was designed to select for increased gene expression in transgenic plants by coupling the gene of interest to the selectable marker sequence.

1.3.1  Polycistronic expression units for genetic engineering of plants

In genetic engineering of plants for applied purposes, more than one transgene is normally introduced. One encodes a selectable property, e.g. resistance to an antibiotic or a herbicide, and is required only for the selection of the transformed cells. In addition, one or more protein(s) causing the intended alteration of a plant property have to be expressed. For the latter, no selection can be applied. Transgene expression is notoriously variable between independent transformants, even when the two genes are tightly linked on a single DNA fragment or in one transgene locus. The selectable gene is often expressed at high levels, because the transformation procedure selects for this phenotype. Expression of the payload gene(s), however, can vary over several orders of magnitude. Linking (coupling) the expression of an a priori unselectable gene to that of a selectable gene could significantly facilitate the generation of transgenic plants expression high levels of the gene of interest.

1.3.2  Possible approaches to achieve a transcriptional coupling

A number of approaches have been tested so far.

(i) Transcription from bi-directional promoters might be co-ordinately influenced by position effects. However, an analysis with the divergent mannopine synthase (mas) promoters revealed that this is not the case and that the relative expression of the two transcripts can vary drastically in independent lines (Peach and Velten, 1991).

(ii) Fusion of open reading frames (ORFs) for two proteins that should be coexpressed, e.g. a selectable marker and the protein of interest. This approach requires that either the resulting fusion protein can perform both activities (Datla et al., 1991; Elmayan and Tepfer, 1994; Hosoyama et al., 1995; Khan and Maliga,
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1999) or that it is processed post-translationally to yield two separate active peptides (Marcos and Beachy, 1997).

(iii) Expression of several independent proteins from one polycistronic mRNA. This approach might appear futile since the eukaryotic 5'-cap-dependent translation mechanism usually leads to translation initiation at only 5' proximal start codons, thus precluding initiation at start codons located downstream (Kozak, 1989b; Kozak, 1999). However, polycistronic expression units with independent, non-overlapping ORFs are used as already mentioned by RTBV (Fütterer et al., 1997), and several other plant viruses (Bonneville et al., 1989; Gowda et al., 1989; Scholthof et al., 1992; for a review: Fütterer and Hohn, 1996a). Possibly also the expression of two proteins from the tomPRO1 locus of tomato is regulated by polycistronic expression units (García-Ríos et al., 1997).

1.3.3 Dicistronic mRNAs

Attempts to use dicistronic constructs with a selectable marker gene in the second position for generation of transgenic plants with high levels of expression of the first ORF have failed so far. In one case, the presence of the first ORF reduced expression of the second by a factor of 500-1500, thus precluding sufficient expression of the selectable marker to allow tight selection (Angenon et al., 1989). Plants regenerated with such constructs usually contained rearranged transgene copies, lacking the upstream ORF (Angenon et al., 1989; lida et al., 1992). In another example, the expression of an nptII ORF downstream of the PVY coat protein ORF was reduced several orders of magnitudes compared to the monocistronic control, but its expression was sufficient for selection of transgenic plants (Lough et al., 1997). In yet another case, two different reporters could be expressed from an apparently dicistronic mRNA; however the exact translation pattern is very unclear (Cho et al., 1995). So far, this method of linking two genes has not found wide application, mainly because the strong reduction of expression of the selectable marker by an upstream ORF makes the selection procedure too uncertain.

Plant viruses with polycistronic expression units use specialised mechanisms to efficiently express the downstream ORFs. The caulimoviruses encode a translational transactivator (TAV) that apparently restores initiation capacity to ribosomes that have already performed one translation initiation-elongation-termination cycle (Bonneville et al., 1989; Gowda et al., 1989; Scholthof et al., 1992). TAV has been used to activate polycistronic translation in transgenic plants (Zijlstra and Hohn, 1992). However, TAV causes phenotypes in transgenic plants that make it unsuitable for a general application (reviewed in Hohn and Fütterer,
1997). Furthermore, the requirements \textit{in cis} for efficient transactivation are not completely understood, although it is clear that transactivation can work on model RNAs (Fütterer and Hohn, 1991; Fütterer et al., 1993; Edskes et al., 1996).

For stable transformation of mammalian cells, dicistronic expression constructs have been used with great success. In these constructs, internal ribosome binding sites from animal picornaviruses direct internal initiation of translation to the downstream ORF (Kobayashi et al., 1996), reviewed in Houdebine and Attal (1999). Internal initiation occurs on certain plant viral mRNAs (reviewed in (Fütterer and Hohn, 1996a) and some internal initiation has been detected in dicistronic contexts in plants and \textit{in vitro} (Hefferon et al., 1997; Ivanov et al., 1997; Niepel and Gallie, 1999; Skulachev et al., 1999). It remains to be seen if sequences will prove useful for efficient expression of any gene in transgenic plants.

We have assessed the potential of dicistronic expression constructs, that are translatable by leaky scanning, a translation mechanism used by RTBV and other viruses.

1.3.3.1 \textit{Translation from a polycistronic mRNA in plant cells}

Leaky scanning is a mechanism that allows access to downstream AUG codons as it has already been described (see section "Eukaryotic translation"). Leaky scanning is enabled by the absence of a strong upstream translation start context and no internal ATGs until the downstream ORF start-codon. The model of a dicistronic expression unit is shown in Fig. 4.

![Fig. 4: Dicistronic expression cassette](image)

<table>
<thead>
<tr>
<th>Requirements of the upstream ORF for leaky scanning:</th>
<th>optimal context for translation initiation:</th>
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<tbody>
<tr>
<td>-suboptimal start codon context</td>
<td>+1 ANN ATG G (G)</td>
</tr>
<tr>
<td>-no internal AUG</td>
<td></td>
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**Fig. 4:** Dicistronic expression cassette, predicted to produce an mRNA from which two independent ORFs can be translated by leaky scanning. It consists of a promoter (P), a terminator (T), an upstream ORF with a start codon in a weak sequence context (grid left border of the ORF) and no internal ATG codons, and a downstream ORF, for which no special requirements exist. The optimal context for translation initiation is also shown (for plants not well defined).
1.4     Aim of the work

We attempted to confer to rice resistance to RTBV by applying the concept of pathogen-derived resistance. To achieve this goal, resistance mechanisms depending on protein- or RNA-expression, silencing, or virus-induced cell death were to be tested. For an increased expression of viral gene products, the potential of dicistronic constructs, translatable by leaky scanning, was to be assessed. For this purpose, leaky scanning was to be enabled either by the natural absence of internal start codons in the upstream ORF or by their removal through site-directed mutagenesis. This was to be done with RTBV-proteins for virus resistance and with a selectable marker ORF (PAT) for general application of this translation mechanism. Selection of transgenic rice with such adapted ORFs should be demonstrated. Plants with an increased and selectable expression should be tested for protection in a virus resistance assay.
2 MATERIAL AND METHODS

2.1 Microbiological methods

2.1.1 Bacterial strains

*Escherichia coli* strains XL-1 Blue (Stratagene, La Jolla, Ca) and DH5α (Gibco BRL, Gaithersburg, MD) were used for production of recombinant plasmid molecules. *E. coli* were grown at 37°C either on solid (1.2% agar, Difco, Detroit, MI) or in liquid LB broth (Difco). Liquid cultures were grown on a shaker at 220 rpm in test tubes for preculture or in one litre Erlenmeyer flasks for plasmid isolation. *E. coli* transformed with pBSK- (Stratagene, La Jolla, Ca), pDH51 (Pietrzak et al., 1986), or derivatives thereof were grown in media supplemented with 50 μg/ml ampicillin.

2.1.2 Preparation of competent cells of *E. coli*

*E. coli* cells competent for uptake of foreign plasmid DNA were produced following the protocol of (Inoue et al., 1990) using DMSO.

2.1.3 Transformation of competent *E. coli* cells

1 ng of plasmid DNA or ca. 100 ng ligation products were added to 100 μl competent *E. coli* cells immediately after thawing of the frozen bacteria on ice. The suspension was carefully mixed with a pipette tip and incubated on ice for 30 min. A heat shock of 42°C for 2 min was applied, followed by an incubation on ice for another 5 min. 400 μl of LB were added and the bacterial suspension was incubated at 37°C for 30 min. Aliquots of the suspension were spread evenly on solid LB supplemented with the selective agent. The bacteria were incubated at 37°C overnight.

2.1.4 Storage of bacteria

For short-term storage, bacteria were grown on solid LB supplemented with ampicillin and stored at 4°C. Every six weeks bacteria were restreaked on fresh LB plates and incubated at 37°C overnight, followed by another six week storage period at 4°C. For long-term storage, single colonies of bacteria were picked and inoculated into 2 ml LB supplemented with ampicillin as the selective agent. After overnight growth, 625 μl of the suspension were added to 375 μl 40% glycerol to bring the final glycerol concentration to 15%. The bacteria were quick-frozen in liquid nitrogen and stored at -70°C.
2.2 DNA methods

2.2.1 Single colony plasmid DNA isolation
A rapid and simple method was used to isolate very small amounts of plasmid DNA from single bacterial colonies in order to determine the success of plasmid ligations (Rusconi, pers. comm.). Overnight-grown colonies were picked with plastic inoculation loops, streaked out on a masterplate and incubated in 12 μl lysis buffer (25 mM TrisHCl, pH 7.5 25 mM Na₂EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml RNase, 10% glycerol, v/v, bromophenol blue). The bacteria were lysed for 5 min. 2 μl phenol/chloroform (1 : 1, v/v) were then added. The mix was vortexed for 30 s and centrifuged at 12,000 rpm in a tabletop centrifuge (Hettich, Tuttingen, FRG) for 3 min. The supernatant was examined by gel electrophoresis in the presence of ethidium bromide and compared with the original plasmid. The masterplate was incubated overnight at 37°C.

2.2.2 Small-scale plasmid isolation
Small amounts of DNA suitable for restriction analysis were isolated by a small-scale preparation based on lysis in boiling LiCl of bacterial cells and ethanol precipitation of the plasmid DNA (Wilimzig, 1985). For molecular cloning the Plasmid Miniprep Kit (JET prep miniprep system from GENOMED GmbH, Cat. # 140 050) was used for more purified plasmid DNA.

2.2.3 Large-scale plasmid isolation
To obtain plasmid DNA amounts in the mg range for gene transfer experiments, large volumes of bacterial suspensions were lysed by alkaline treatment, and the nucleic acids purified by column chromatography (Qiagen AG, Switzerland). For lysis and purification the Plasmid Maxi Protocol of Qiagen was followed.

2.2.4 DNA gel electrophoresis
DNA molecules were separated by horizontal gel electrophoresis in TAE buffer (40 mM Tris acetate, 1 mM Na₂EDTA, pH 8). For analytical purposes, agarose (Bioprobe, Montreuil, F), and for DNA fragment isolation, low melting agarose (FMC, Rockland, ME) were used. Ethidium bromide was added to the gel to a final concentration of 0.5 μg/ml.
2.2.5 Enzymatic manipulation of DNA

DNA was cleaved by restriction endonucleases (Boehringer, Mannheim, FRG, New England Biolabs, Beverly, MA, GibcoBRL, Gaithersburg, MD) according to the instructions of the manufacturers. DNA fragments were ligated with T4 DNA ligase (New England Biolabs). Ligation of DNA fragments containing blunt ends was performed at RT overnight, whereas sticky ended fragments were ligated at 16°C overnight. 3' protruding ends of linear DNA were removed using the 3' exonuclease activity of T4 DNA polymerase. 3' recessive ends of linear DNA molecules were filled in with the Klenow enzyme (New England Biolabs) in presence of deoxynucleotides (Ausubel et al., 1994). 5' phosphate residues of linearised vector DNA molecules used for molecular cloning were removed to prevent religation of the vector plasmid. Calf intestine phosphatase (CIP, Boehringer) was used for this purpose. Before CIP incubation restriction enzymes were inactivated by heat treatment or removed by binding them to a synthetic resin (Strataclean, Stratagene, La Jolla, CA) according to the instructions of the manufacturer. CIP was inactivated by heat treatment at 75°C for 10 min (Ausubel et al., 1994) and removed using Strataclean resin.

2.2.6 DNA fragment isolation

Linear DNA fragments used for further enzymatic treatments were isolated from agarose gels after electrophoresis. Agarose slabs containing the desired DNA fragment were excised with a scalpel blade under UV light. DNA fragments were isolated by melting the agarose in a high salt buffer and subsequently binding the DNA to glass particles. DNA fragments of 70 bp to 10 kb were isolated with the Qiaquick Gel Extraction Kit (Qiagen AG, Switzerland).

2.2.7 Plasmid construction and DNA amplification

Expression constructs with a CaMV 35S promoter were derived from PLS1GUS (Fig. 5a), which contains a standard 35S promoter fragment followed by the expression enhancing first 60 nucleotides of the native 35S transcript and a standard CaMV termination/polyadenylation signal. The introduced GUS ORF was flanked by short linkers containing XhoI, BamHI and NcoI sites upstream and PstI and SphI downstream. In some plasmids, the GUS ORF was replaced by an aph4 ORF also containing the upstream XhoI, BamHI sites (Fig. 5b). Modified PAT ORFs were inserted into plasmid UBI-GUS (Fig. 5c), which contains the maize ubiquitin 1 promoter, a GUS ORF flanked by PstI, HindIII and NcoI sites upstream and PstI and SphI downstream, and the 35S terminator cassette. In another series
of PAT constructs the maize ubiquitin promoter was replaced by the enhanced CaMV 35S promoter (Fig. 5f).

Fig. 5: Monocistronic and dicistronic expression cassettes. a: PLS1GUS construct used in this work as cloning intermediate and as monocistronic standard, consist of the CaMV 35S promoter enhanced by the transcriptional or translational enhancer S1 (Futterer et al., 1990). b: Construct derived from PLS1GUS containing the aphlV sequence. c: pUBIGUS contains the maize ubiquitin 1 promoter [region from -730 to +1090; derived from plasmid pUbiCAT (Christensen et al., 1992) after subcloning; numbering with respect to the transcription start]. d, e, f: Dicistronic expression cassettes, consist of the CaMV 35S promoter (d, f) or the maize ubiquitin 1 promoter with its first intron (e), with derivatives of RTBV ORFs I, II or partial (p) IV, or the PAT ORF as upstream ORF, either GUS or aphlV as downstream ORF, and a CaMV 35S terminator. Restriction sites used for molecular cloning and for characterisation of transgenic plants are indicated.

2.2.7.1 Constructs containing RTBV-Sequences

Fragments coding for RTBV ORF I, II and IV from plasmid pRTRB1162 (Dasgupta et al., 1991) were amplified by PCR in a thermal cycler (Techne witec AG, Switzerland).

**PCR conditions:** 2 μg plasmid DNA, 500 nM of each primer, 250 μM of each deoxynucleotide, 3.2 mM MgCl₂, and 1 U high-fidelity Pfu-polymerase (GibcoBRL) were incubated in a reaction mixture of 50 μl total volume, not necessarily covered
with 50 μl paraffin oil. DNA molecules were denatured at 94°C for 1 min followed by 30 amplification cycles of primer annealing at 53°C for 1 min, extension at 72°C for 2 min and denaturation at 94°C for 1 min. The terminal delay was set for 7 min at 72°C.

Primers for PCR were designed to introduce suitable restriction sites and modifications of the start- and stop-codon context of each RTBV ORF (Table 1). The amplified DNA was cloned as XhoI-SalI fragment into the XhoI-site of PLS1 upstream of the GUS- or aph4 sequence (Fig. 5c).

<table>
<thead>
<tr>
<th>plasmid name</th>
<th>Forward primers for start region (5’ - 3’) (XhoI-site underlined)</th>
<th>Reverse primers for stop region (5’ - 3’) (SalI-site underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORFla</td>
<td>GTTCTCGAGCATATGGAGTCACGT TACG</td>
<td>GGGTAAGTGTGACTTATGAGCTTGATGC</td>
</tr>
<tr>
<td>pORFlb</td>
<td>GTTCTCGAGATATGGAGTCACGT TACG</td>
<td></td>
</tr>
<tr>
<td>pORFlc</td>
<td>GTTTCTCGAGATATGGAGTCACGT TACG</td>
<td></td>
</tr>
<tr>
<td>pORFII</td>
<td>CCCCTCGAGACATGAGCGCTGAT TACCC</td>
<td>AGGCGTCGACTTATGAGCTTGATATTTTC</td>
</tr>
<tr>
<td>pORFIIΔC</td>
<td>CCCCTCGAGACATGAGCGCTGAT TACCC</td>
<td>GGTGGTGTGACTTATGACTGACCTTGAG</td>
</tr>
<tr>
<td>pORFIV</td>
<td>GGCATCTCGAGTCCATGATAATAG AGTACCC</td>
<td>GGAGGTCGACTATGCTTTTTTCTTTCGC</td>
</tr>
</tbody>
</table>

Table 1: Primers used for amplification of RTBV ORF I, II and partial IV

2.2.7.2 Modifications of RTBV-Sequences

Mutations of the ORFs were introduced by PCR or insertion of suitably designed oligonucleotides (Table 2). The PCR-conditions were the same as described above. Mutations were designed without any change of the aminoacid sequence. Detailed descriptions of the RTBV sequences in modified and unmodified versions are shown in the appendix. The start-codon context of each RTBV ORF and the linker regions between RTBV ORF I, II or IV and GUS ORF are described in Fig. 7. The start-codon context of each RTBV ORF and the linker regions between RTBV ORF I, II or IV and aph4 ORF are shown in Fig. 11.
Material and Methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>annealing region</th>
<th>Forward and reverse primers (5' – 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>lmut1</td>
<td>177-143</td>
<td>ACAGGAGGTACTTCTGCTTCTTCTTTTCCCCAG</td>
</tr>
<tr>
<td>lmut2</td>
<td>364-422</td>
<td>TCCTCGACCTCAAGCGACGAAGGAGCTGAGAAGCGAGTTTTAG AAAATCAAAACTTA</td>
</tr>
<tr>
<td>linter1</td>
<td>160-194</td>
<td>GCAGAAGTACCTCCTGCTACGATAATATTAGC</td>
</tr>
<tr>
<td>linter2</td>
<td>381-348</td>
<td>CCGCTTGAGGTGAGATCTTGTCTAGGAAGCTG</td>
</tr>
<tr>
<td>I1mut1</td>
<td>763-746</td>
<td>AGTGAACACCCCAATTAA</td>
</tr>
<tr>
<td>I1mut2</td>
<td>791-811</td>
<td>GTCAACTTTGGGGAGCTCTCAAGGGT</td>
</tr>
<tr>
<td>I15'b</td>
<td>725-695</td>
<td>CTGAATCTAGGCTTTGAAACTTCTTGAGGC</td>
</tr>
<tr>
<td>I1inter</td>
<td>751-801</td>
<td>TGGGATCTCTAGGCAATAGCTAGCCGAGGACGACGTCAACT TGCCA</td>
</tr>
<tr>
<td>I1M3 3'</td>
<td>835-796</td>
<td>CTTGGCATCAAAGAGCTTTGGAACACTTTACGCTTGCA</td>
</tr>
<tr>
<td>I1M4 5'</td>
<td>762-745</td>
<td>GTGAACACCCAGTGGAAC</td>
</tr>
<tr>
<td>I13'</td>
<td>869-826</td>
<td>CGCTTTGAGGAGATCGACAGAGAGACTAACACCTTGACAGACCC</td>
</tr>
</tbody>
</table>

**Table 2:** Primers and oligonucleotides used for introduction of mutations in RTBV ORF I and II. Numbers refer to the position on the RTBV-genome. pORF IIΔN was produced by an Xhol/XbaI digestion of pORF II mut2 (Fig. 7), releasing a 96 bp fragment of the 5'end of ORF II. With an Xhol-XbaI-adapter oligonucleotide, containing an ATG in-frame with ORF II, the plasmid was religated.

2.2.7.3 Constructs containing PAT-sequences

The native PAT sequence deriving from pAB1 (Bliffeld et al., 1999) was amplified by PCR and introduced between the HindIII and the Ncol sites upstream of the GUS sequence. Mutations of the PAT ORF were introduced by PCR (Table 3). The PCR-conditions were the same as described above. The translation start context was CATATGAGT, and the sequence linking the PAT-ORF and GUS-ORF was ATCTAAACCATTG (respective start- and stop-codons underlined).
Material and Methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer for start region (5' - 3') (HindIII-site underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar5'a</td>
<td>GGGAAGCTTGAATTATGAGTCCGGAACG</td>
</tr>
<tr>
<td>Reverse primer for stop region (5' - 3') (PstI/-Ncol/-BglII-site underlined)</td>
<td></td>
</tr>
<tr>
<td>Bar3'</td>
<td>GGGCTGCAGAGCTCCATGGTTAGATCTCGGTAGCAGGACGGCAG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>annealing region</th>
<th>Forward and reverse primers (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>bar int3'</td>
<td>472-423 GCG TGC CCC GGG GGG CGT ATC CGA GCG GGC GTA CGC TCG GG</td>
</tr>
<tr>
<td>bar int5'</td>
<td>457-515 CGC CCC CCG GGG CAC GCT GCG GGC GGC CGG CTT CAA GCA GAA CTG CCA CGG CTG GGG</td>
</tr>
<tr>
<td>Bar5'b</td>
<td>91-72 CCG CCG GGA CTG CCT CGG CCG GCA GAC GCT GCG GGC GGC GGC CTT CAA GCA GAA CTG GCA CGA CGG</td>
</tr>
<tr>
<td>Bar5'c</td>
<td>91-71 CCG CCG GGA GAT CTG CCT CGG CCG GCA GAC GCT GCG GGC GGC GGC CTT CAA GCA GAA CTG GCA CGA CGG</td>
</tr>
<tr>
<td>Bar5'd</td>
<td>91-71 CCG CCG GGGTGT CCG CCT CGG CCG GCA GAC GCT GCG GGC GGC GGC CTT CAA GCA GAA CTG GCA CGA CGG</td>
</tr>
</tbody>
</table>

Table 3: Primers used for the introduction of mutations in PAT ORF. Numbers refer to the position on the PAT-sequence (the A of the start codon ATG being position +1).

In another series of constructs the maize ubiquitin promoter was replaced by the enhanced CaMV 35S promoter (Fig. 5f). For variation of the start codon context, oligonucleotides containing an ATG in frame with the modified PAT ORF and respective flanking restriction sites were cloned into the HindIII-BspEI-site on the 5'end of the PAT ORF (Table 4).

**HindIII-BspEI-adapter oligonucleotides**

<table>
<thead>
<tr>
<th>T</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+1</td>
</tr>
<tr>
<td>aAGCTTGATATCGGC</td>
<td>ATAG</td>
</tr>
</tbody>
</table>

Table 4: Oligonucleotides used for the variation of the start codon context. Every possible combination of nucleotides on position −3 and +4 with respect to the +1 position of the start-codon (in bold) was designed. The corresponding reverse primers are not shown.

For stable transformation of rice, the GUS sequence of dicistronic constructs containing the ubiquitin 1-promoter CaMV 35S-terminator cassette was replaced by an aph4 ORF. For that purpose a polylinker was introduced between the BglII and Ncol of the linker region of plasmid BVall (Fig. 9). This polylinker introduced the restriction sites Smal, Xhol, BamHI (Table 5). The aph4 ORF was cloned into the BamHI-site of this polylinker.
Table 5: linker sequences of the PAT-constructs with restriction sites.

2.2.7.4 Constructs for transient expression experiments

For the evaluation of trans-silencing effects in a transient expression assay, different plasmids were used. They contained different combinations of reporter open reading frames and promoters. As reporter the glucuronidase gene (GUS) or the firefly luciferase (LUC) were used. As promoter sequences with sequence homology to an internal transgene, the CaMV 35S promoter and the RTBV promoter were used. As promoter without sequence homology, the maize ubiquitin 1 promoter (UBI) was used. As terminator sequences either the CaMV 35S- (35S) or the nos-terminator were used. A construct containing the maize ubiquitin 1 promoter and the renilla luciferase (renilla) reporter ORF was used as internal standard, because no sequence homology was present.

Table 5: linker sequences of the PAT-constructs with restriction sites.

<table>
<thead>
<tr>
<th>linker region</th>
<th>Sequence (restriction-sites underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAT-ORF and GUS-ORF</td>
<td>AGATCTAACCATGGT (<em>BgII/- Ncol-site</em>)</td>
</tr>
<tr>
<td>PAT ORF and aph4-ORF</td>
<td>GAGATCTAACCCGGGCTCGAGGATCCC CCCTATG (<em>BgII/- SmaI/- Xhol/- BamHI-site</em>)</td>
</tr>
</tbody>
</table>

Fig. 6: Summery of the constructs used in the transient trans-silencing expression assays. a-c: constructs coding for the glucuronidase gene (GUS) with promoter-sequences, the token int indicates the presence of the RTBV-derived intron. d-f: constructs coding for the firefly luciferase gene (LUC) with promoter sequences (Brunner, 1998). g: pUBI-renilla construct coding for the renilla luciferase (renilla). References of the constructs are indicated.
2.2.7.5 **Constructs containing RTBV-sequences in antisense direction**

The construction of the construct containing RTBV-sequences in antisense direction is described in details in Henrich (1996) and Klöti (1996).

2.2.8 **Sequencing of inserted fragments**

Sequencing of the inserted fragments was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.). All synthesised RTBV- and PAT-sequences including modified sequences were confirmed by sequencing in 5' and 3' direction. Primers for sequencing were designed to recognise either the 3'end of the CaMV 35S promoter (TGATATCTCCAGTGACGTAAGGG) or the 3'end of the maize ubiquitin 1 promoter (TTTTTAGCCCTGCCTTCATACGC) or the 5'end of the downstream GUS sequence (TTTTTGTATTTACCGGTTGGG). In a total volume of 20 μl, 0.5 μg of the template DNA, 8 μl of the Terminator ready reaction mix, containing dNTPs and polymerase (Sequencing Ready Reaction Kit; Perkin-Elmer Corp.) and 500 nM of respective 5' or 3' primer were used. DNA molecules were denatured at 96°C for 1 min followed by 45 amplification cycles of primer annealing at 50°C for 15 s, extension at 60°C for 4 min and denaturation at 96°C for 1 min. Samples were separated on a gel (4.75% acrylamide gel, 8.3 M urea in 1x TBE buffer) connected to a DNA Sequencer 373 (Applied Biosystems) coupled with the Genescan software for Macintosh Operating System.

2.2.9 **Isolation of DNA from rice plants**

DNA was isolated either with the Nucléon Phytopure Plant DNA extraction kit (Amersham Life Science, England) or by N-cetyl-N,N,N-trimethylammonium bromide (CTAB) precipitation (Murray and Thompson, 1980). The first 10-20 cm of young rice leaves (five to eight weeks old) without the leaf tip were shock-frozen and ground with a mortar and pestle to a fine powder in the presence of liquid nitrogen. After isolation, DNA was precipitated with 99% ethanol at -70°C for 30 min and washed once with 70% ethanol, briefly dried in the laminar flow hood and dissolved in 50 μl H₂O. An aliquot of 1 μl was loaded on an agarose gel to judge the quantity and quality of the DNA.

2.2.10 **DNA labelling**

Digoxigenin (DIG) labelled DNA probes for Southern hybridisation were produced with a PCR DIG labelling kit (Boehringer) using the same PCR parameters as described above. Labelled fragments were separated from non-incorporated nucleotides using Quiaquick PCR Purification Kit (Qiagen AG, Switzerland).
2.2.11 DNA detection by Southern blot analysis

Specific plant DNA sequences were detected by preparation of Southern blots and hybridisation (Southern, 1975) to DIG-labelled DNA probes at 42°C. For this purpose approximately 5 μg plant DNA were cleaved by an overnight treatment with restriction endonucleases and loaded on an agarose gel. After electrophoretic separation, the 0.8% agarose gel was incubated for 10 min in 0.25 M HCl and subsequently denatured in 0.5 M NaOH, 1.5 M NaCl for 30 min. Finally, the gel was washed three times in neutralisation buffer (0.5 M TrisHCl, pH 7.2, 1.5 M NaCl, 1 mM Na₂EDTA) and blotted on a positively charged nylon membrane (Boehringer) using 20 x SSC, crosslinked by UV-irradiation (UV Stratalinker® 1800, Stratagene). In a rotating oven, pre-hybridisation was performed for a minimum of 30 min in 12ml DIGeasy HYB solution (Boehringer) per membrane (10 x 20cm). Hybridisation was performed overnight at 42°C in 7 ml DIGeasy HYB solution (Boehringer) with 50 μl of the synthesised DNA probe. Following hybridisation, the membrane was washed twice in 2 x SSC, 0.1% SDS for 5 min at RT, once in 0.2 x SSC, 0.2% SDS for 15 min at 67°C and finally in 0.1 x SSC, 0.1% SDS for 15 min at 67 °C. Washing was followed by an immunoreaction of DIG with an anti-DIG-alkaline phosphatase (AP)-conjugate and a chemiluminescent detection reaction with a 1,2 dioxyethane enzyme substrate (CDP-Star, Tropix, Bedford, MA). Hybridisation signals were visualised by exposing the filters to a Kodak X-Omat AR film (Kodak, Rochester, USA) for 5 min to several hours at RT. For rehybridisation wet membranes were rinsed with H₂O and washed in 0.4 M NaOH, 0.1% SDS for 30 min at 62°C. Subsequently, membranes were incubated for 30 min in 0.1 x SSC, 0.1% SDS, 200 mM TrisHCl, pH 7.5 at 62°C and finally rinsed with 2 x SSC.

2.3 RNA methods

2.3.1 Isolation of RNA from rice plants

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) or the peqGOLDRNAPure extraction solution (peqLab, Biotechnologie GmbH) followed by a chloroform extraction and isopropanol precipitation. Total RNA was isolated from the first 10-20 cm of young rice leaves (five to eight weeks old) without the leaf tip or from 8 d old seedlings which have grown on a MS (Murashige and Skoog, 1962) selection medium supplemented with 20 mg/l hygromycin B. Leaf material was shock-frozen and ground with a mortar and pestle to a fine powder in the presence of liquid nitrogen. After isolation, RNA was precipitated with 99% ethanol at -70°C for 30 min and washed once with 70%
ethanol, briefly dried in the laminar flow hood and dissolved in 50 μl of RNase-free, diethylpyrocarbonate (DEPC)-treated H₂O. An aliquot of 1 μl was loaded on an agarose gel to judge the quantity and quality of the RNA.

2.3.2 RNA detection by northern blot analysis
Specific plant RNA sequences were detected by preparation of northern blots and hybridisation to DIG-labelled DNA probes at 42°C. The procedure is very similar to DNA analysis by Southern blotting. Approximately 7 μg total RNA was used for blotting. Total RNA was separated by electrophoresis on a denaturing 2.2M [6.6% (w/v)] formaldehyde gel containing 0.8%-2% agarose. After electrophoretic separation, the gel was washed twice for 20 min in DEPC-treated water to remove the formaldehyde and blotted on a positively charged nylon membrane (Boehringer) using DEPC-treated 20 x SSC. Hybridisation and detection was performed as described above.

2.4 Protein and immunological methods

2.4.1 Isolation and gel electrophoresis of plant proteins
100 mg – 500 mg of leaf material of transgenic plants were homogenised in Tris-buffered saline Tween (TBS-T) buffer (20 mM Tris base, 137 mM NaCl, 4mM HCl, 0.1% Tween 20, pH 7.6) either by preceding powdering in liquid nitrogen or homogenising with an appropriate mill at RT. After centrifugation the supernatant was mixed 3:1 with 4x loading buffer ((Lämmli, 1970); 10% glycerol, 0.25 M Tris pH 6.8, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue), boiled for 5 minutes and clarified by centrifugation for 10 minutes. 15 μl aliquots were fractionated on 12.5% - 15% mini-SDS-polyacrylamid gels following the manufacturer’s instructions (Biorad). Proteins were visualised by gel staining with Coomassie Brilliant Blue dye.

2.4.2 Western blot analysis and immunodetection
Proteins separated by SDS-polyacrylamide gel electrophoresis (Mini-PROTEAN II Cell, Bio-rad, Hercules, California 94547, USA) were blotted onto a nitrocellulose membrane (Schleicher und Schuell, 37582 Dassel, Germany) as described in (Maniatis et al., 1989). Protein was detected with the ECL Chemoluminescence Western Blotting Kit (Amersham, Pharmacia Biotech, UK); according to the manufacturer instructions. The primary antibodies used were raised in rabbits against the recombinant RTBV ORF II and IV (Hay et al., 1994). The antibodies
were affinity purified and kindly provided by R. Hull (John Innes Centre, Norwich, UK). Signals were visualised by exposing the filters to a Fuji RX film (Fuji, Tokyo, Japan) for 5 s to several min at RT.

2.5 Transient expression assays with suspension-cells

2.5.1 Culture of suspension cells
Suspension cells were cultured in MS salts and vitamins (Murashige and Skoog, 1962) provided by Duchefa (Harlem, The Netherlands) plus 2mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 6% sucrose and 2.5 mM 2-morpholinoethanesulfonic acid (MES). For transgene expression analysis, suspensions were cultured on solid R2-medium (Ohira et al., 1973) supplemented with different agents: 30 mg/l hygromycin B; 30 μM 5-azacytidine; 3 or 30 μM Trichostatin A.

2.5.2 Histochemical GUS assay
Expression of GUS was visualised by transferring the plant material (undifferentiated transgenic callus or leaves) to a filter sterilised GUS substrate mixture containing 100mM Na phosphate (pH 7.0), 10 mM Na-EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, Biosynth, Switzerland), and 0.1% Triton X-100 as described by (Mendel et al., 1989). Material was incubated at RT or 37°C for 10 minutes to 24 hours and analysed with a stereomicroscope or a microscope.

2.5.3 Protoplast preparation and transfections
Protoplasts from Oryza sativa (line Oc, TP 309 lines GUS1-4) were isolated from cell suspension cultures and transfected with plasmid DNA by PEG-mediated transformation as described previously (Chen et al., 1994) with modifications. For cell-wall digestion, 3 ml of the very fine suspension culture was incubated in 12 ml of enzyme solution (3% Cellulase R-10, 1% Macroenzyme R-10, 44mM Mannitol, 1mM CaCl₂) for 16-20 h at 27°C and slight shaking. For increased suspension volume the volume of the enzyme solution was adapted.

The protoplasts were transformed by the PEG method with 0.5 to 10μg plasmid DNA, cultured in K3 medium (K3 macro elements, B5 micro elements, 0.4 M sucrose, 0.25 g/l D(+)Xylose, 100 mg/l inositol, 1 mg/l pyridoxine, 10 mg/l thiamine, 1 mg/l nicotinic acid, 0.1 mg/l 2,4-D, 1 mg/l 1-naphthylacetic acid (α), 0.2 mg/l N⁶-benzyladenine, pH 5.6) and harvested 16 h later. The proteins were
extracted using GUS extraction buffer (50mM NaH₂PO₄/Na₂HPO₄, pH 7.0, 10 mM Na₂EDTA, 10 mM β-mercaptoethanol, 0.1% w/v N-lauroyl-sarcosine, 0.1% Triton X-100) or using 1x passive lysis buffer (PLB) provided by the luciferase assay system (Promega Corporation, Madison, WI 5371-53788, USA). Protein concentration was measured by Bradford staining (Bio-rad, Hercules, California 94547, USA)

2.5.4 Enzyme assay for glucuronidase (GUS)
Fluorimetric GUS assays were performed as described (Jefferson et al., 1987). GUS enzyme activity was determined as the production in nmol per minute per microgram of protein of 4-methylumbelliferone (MU) from 4-methylumbelliferyl-β-D-glucuronide (MUG; Duchefa, Haarlem, NL).

2.5.5 Enzyme assay for luciferase
Luciferase activity (firefly luciferase and renilla luciferase) was detected with the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI 5371-53799, USA). The detection was performed according to the supplier's instructions. The light emission was measured with the Lumat LB 9507 luminometer (EG&G Berthold, Switzerland).

2.5.6 Enzyme assay for phosphinothricin acetyltransferase (PAT)
The PAT assay was performed in GUS extraction buffer also used for the GUS assay. To 13μl of extraction buffer 0.8 μl PPT stock (1 mM phosphinothricin in 50 mM Tris-HCl pH 7.5 and 2 mM EDTA) and 1.3 μl ¹⁴C-AcCoA was added. For each sample a control reaction was performed under the same conditions without PPT. After incubation at 37°C for 30 minutes, 6 μl of the reaction mix was spotted on a TLC plate (silica gel/ TLC cards, Fluka) and transferred to a chromatography tank containing 75% isopropanol an 25% ammoniak. After the solvent front had nearly reached the upper edge of the plate, the dried plate was exposed to X-ray film for several days to 3 weeks.

2.6 Growth of rice plants, embryo culture and transformation

2.6.1 Plant material
Rice plants (Oryza sativa), of the Japonica cultivar Taipei 309 were grown in a greenhouse at 28°C (day) and 21°C (night) temperatures, respectively. The photoperiod was 12 h light supplemented with 400 W fluorescent lamps (MT 400
DL/BH, 400 W mercury lamp, Iwasaki, Tokyo, JP) if a minimum of 100,000 lx sunlight measured outside the greenhouse was not reached during the day. The humidity was set to 80% and 60% during the day and night, respectively. Three rice plants were grown together in one pot (12/18, Migros, Zürich, Switzerland) filled with 0.25 l Perlite and 2.5 l sterilised, sieved soil. The soil was supplemented with 1 g/l initial fertiliser (Plantamaag 4 D, NPKMg 20-10-15-3.6, Maag AG, Dielsdorf, Switzerland), 1 g/l long time fertiliser (Nutricote, NPK 16-10-10, Maag AG, Switzerland) and 0.25 g/l NaFeEDTA. The rice plantlets were transferred into these pots when they had reached an age of two weeks. Initially they were germinated and grown under sterile conditions on solid half strength MS medium without hormones in a culture chamber (Weiss, Reiskirchen, FRG) at 30°C.

2.6.2 Isolation of immature zygotic embryos and culture of embryogenic cell suspensions (ECS)

Immature caryopses were collected between 10-12 days after pollination. The caryopses were surface-sterilised by rinsing in 70% ethanol for 2 min and subsequently incubating in 6% Ca(ClO)₂/0.1% (v/v) Tween 80 for 20 min followed by three washes with sterilised water. Embryos were squeezed out of the caryopsis after cutting off the ventral end with a scalpel and placed scutellum side up on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2 mg/l 2,4D. After 1-2 weeks (26°C, in the dark), embryos were either used for microprojectile bombardment or transferred to R₂ liquid medium (Ohira et al., 1973) supplemented with 3% sucrose, 1mg/l 2,4D and 1mg/l thiamine and sub-cultured every 5 to 7 days to establish embryogenic cell suspensions (ECS). After sub-culturing for 6 to 12 weeks the suspension cells were used for microprojectile bombardment. Prior to microprojectile bombardment the tissue was preplasmolysed on liquid R₂ medium containing 12% sucrose for 30 min.

2.6.3 Gene transfer with the particle inflow gun (PIG)

Rice immature zygotic embryos and ECS were transformed by microprojectile bombardment with a particle inflow gun (PIG, Finer et al., 1992). DNA-coated gold particles (gold powder, spherical, 1.5-3 μm diameter, Aldrich, Buchs, CH) were used. Prior to coating particles were washed with H₂O and autoclaved in 50% glycerol at a concentration of 50 mg/ml. 50 μl particle suspension and 5 μg plasmid DNA were then mixed and vortexed at full power. While reducing the power, 50 μl 2.5 M CaCl₂ and 20 μl 0.1 M spermidine free base were added to the suspension. Vortexing on full power was continued for another 3 min. The particles were gently spun down and the supernatant removed and replaced by 300 μl 99%
ethanol. The suspension was again vortexed at full power for 1 min. After a short centrifugation pulse to pellet the particles, the supernatant was removed and replaced by 50 µl H2O.

The preplasmolysed immature zygotic embryos or ECS were transferred on solid plasmolysis medium R2 medium containing 12% sucrose and placed into the vacuum chamber of the PIG at a distance of 14 cm from the filter. A 500 µm nylon mesh was positioned 9 cm from the filter. 8 µl coated particle suspension corresponding to 400 µg gold particles and 0.8 µg DNA were loaded on the filter. The chamber was evacuated to 100 mbar and the particles were accelerated by a helium jet generated by 6 bar pressure for 50 ms. The vacuum was released and tissue was incubated for another 24 h on the plasmolysis medium.

2.6.4 Selection of bombarded tissue for transformation events
One day after bombardment, the tissues were cultivated in R2 liquid medium for 1 week. The liquid cultures were placed in an incubation shaker (Infors, Bottmingen, CH) at 90 rpm and 30°C in the dark. To select for transformation events, hygromycin B (Duchefa, Haarlem, NL) was supplemented as a selective agent after 1 week. The initial concentration was 30 mg/l hygromycin B, but concentration was reduced to 20 mg/l for selection of putative transgenic calli transformed with dicistronic expression constructs with low expression levels. After 2-3 weeks developing calli were placed on R2 solid selection medium for 2-3 weeks containing 6% sucrose, 100 mg/l inositol, 2 mg/l 2,4-D, 20 or 30 mg/l HygB, 2 mg/l 2,4-D and 0.5% agarose to allow the development of embryogenic structures.

2.6.5 Regeneration of transgenic tissue
Resistant calli were transferred to solid R2 regeneration medium supplemented with 2% sucrose, 3% sorbitol, 1 mg/l zeatin (Duchefa), 0.5 mg/l IAA (Sigma), MS vitamins, and 0.65% agarose (Sigma). The regenerating tissue was incubated at 30°C in a light incubator (Weiss, Reiskirchen, FRG) and subcultured in intervals of 2-3 weeks until the first green shoots developed. Shoots that had reached a length of 2-3 cm were transferred to 1/2 MS rooting medium without hormones supplemented with 1.5% sucrose and 0.3% gelrite. After cultivation for 2-4 weeks at 30°C in a Weiss incubator, plantlets were transferred directly to the greenhouse and planted in soil.

2.6.6 Selection of transgenic calli with phosphinothricin (PPT)
Embryogenic cell suspensions (ECS) were cultivated on R2 selective medium supplemented with 3% sucrose, 1mg/l 2,4D and 1mg/l thiamine and with various
amounts of glufosinate ammonium (Riedel-de Haën), the ammonium salt of PPT (0.5 to 100 mg/l). The cells were cultivated on solid or in liquid selective medium for 4 weeks.

2.7 Virus resistance assay
The virus resistance assay was performed at the International Rice Research Institute, Philippines (Fig. 6) in collaboration with the virology group of Ossmat Azzam.

Fig. 6: Facilities at the International Rice Research Institute. a: IRRI breeding varieties, b: administration building, c: greenhouse containment facilities, d: collection of adult green leafhoppers (GLH; *Nephotettix virescens*) from a RTBV and RTSV infected plant, e: transgenic and non-transgenic rice plants individually covered by mylar cages, f: inoculation, 4-6 GLH are placed per plant.

The transgenic seeds were soaked in running water, sown (5 plants per pot) and grown for 11 days. Adult virus-free green leaf hopper (GHL). *Nephotettix virescens* were allowed to feed 4 days on RTBV and RTSV infected Taiching native 1 (TN1) plants to aquire the tungro viruses. They were collected (Fig. 6e) and transferred to the test plants (Fig. 6f). In separate mylar cages 4-6 GHL had access to the plants for 24 hours, then the plants were treated with insecticide and were grown for 27 days.
2.7.1 Assessment of symptom severity (SS)
The seedlings were scored individually using the standard evaluation system for rice (Hassanuddin et al., 1988) 13 days after virus infection based on following scale:

1   no symptoms
3   0-10% plant height reduction, with no distinct leaf discoloration
5   11-30% plant height reduction, with no distinct leaf discoloration
7   31-50% plant height reduction, with yellow to orange leaf discoloration
9   more than 50% height reduction, with yellow to orange leaf discoloration

2.7.2 Enzyme linked immunosorbent assay (ELISA)
ELISA was performed on day 13 and 27, using the method of Clark and Adams (1977), applied to tungro-associated viruses by Hibino et al. (1990) and modified by Cabauatan and Koganezawa (1997). Antisera directed against RTBV and RTSV, respectively were used.
3 RESULTS

3.1 The concept of leaky scanning: translation from a polycistronic mRNA in plant cells

In the dicistronic gene expression constructs produced in this work the gene of interest was linked to a selectable marker gene in order to select for expression of dicistronic mRNAs in transgenic plants. Prior to plant transformation the dicistronic expression units had to be tested for their ability to express two ORFs from the same messenger RNA molecule in transient protoplast assays. The scanning model of translation predicts, that an upstream ORF start-codon located in a strong context and containing numerous internal ATG-codons totally inhibits the capability of scanning ribosomes to start downstream translation by leaky scanning (Kozak, 1989b; Kozak, 1999; see also introduction). The sequences flanking the ATG codon modulate the proportion of scanning ribosomes that recognise this start-codon and start translation of the ensuing ORF (Kozak, 1997). The concept was therefore to produce dicistronic constructs which contain a first ORF with a start codon in an unfavourable (“weak”) sequence context, and no (or only a few) additional translation start sites (ATGs) preceding the second ORF. We expected that the RTBV-ORF I and II were naturally adapted sequences for this expression mechanism (Fütterer et al., 1997): in the natural context, RTBV ORF I starts with an unconventional AUU codon and the ORF II start codon has a weak sequence context and both ORFs lack internal start codons. We also examined a PAT ORF and a part of the RTBV ORF IV with 5 or 2 internal ATG codons, respectively as ORFs a priori not suitable for leaky scanning.

3.2 RTBV ORFs as upstream ORFs in dicistronic expression units

3.2.1 Unmodified sequences

Unmodified RTBV ORFs I and II and a truncated form of ORF IV (IVΔC) were incorporated into an expression cassette, in which the two open reading frames were regulated by a single CaMV 35S promoter and a CaMV terminator (Fig. 7a). In this first series of constructs, an influence of the following parameters of the upstream ORF on gene expression were compared (Fig. 7): the internal ATG content (no internal ATGs in ORF I and II versus 2 internal ATGs in ORF IVΔC), the translation start codon context (optimal translation start versus sub-optimal translation start and no translation start in ORF I constructs; Fig. 7b: pORF I a-c) and the length of the coding sequence (ORF II and ORF IIΔC). A schematic description of the constructs is shown in Fig. 7b (unmodified sequences). A detailed description of the RTBV ORFs I, II and IVΔC is shown in the appendix.
Results

<table>
<thead>
<tr>
<th>a</th>
<th>CaMV 35S</th>
<th>RTBV ORF I, II and IV</th>
<th>GUS</th>
<th>T</th>
</tr>
</thead>
</table>

### b Unmodified sequences

<table>
<thead>
<tr>
<th>pORFIa</th>
<th>CAT ATG GAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORFIb</td>
<td>GAT ATG GAG</td>
</tr>
<tr>
<td>pORFIc</td>
<td>GAT ATG GAG</td>
</tr>
<tr>
<td>pORFII</td>
<td>GAC ATG AGC</td>
</tr>
<tr>
<td>pORFIIΔC</td>
<td>AT-rich</td>
</tr>
<tr>
<td>pORFIVΔC</td>
<td>TCC ATG AAT</td>
</tr>
</tbody>
</table>

### Modified sequences

<table>
<thead>
<tr>
<th>pORFIa mut1</th>
<th>CAT ATG GAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORFIc mut1</td>
<td>GAT AGT GAG</td>
</tr>
<tr>
<td>pORFIΔC mut1</td>
<td>CAT ATG AAG</td>
</tr>
<tr>
<td>pORFII mut1</td>
<td>GAC ATG AGC</td>
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<tr>
<td>pORFII mut2</td>
<td>GAC ATG AGC</td>
</tr>
<tr>
<td>pORFIIΔC mut2</td>
<td>AT-rich</td>
</tr>
</tbody>
</table>

Fig. 7: Schematic overview of the dicistronic expression cassettes used in transient protoplast expression assays. a: Expression cassettes consist of the CaMV 35S promoter (CaMV 35S), enhanced by the transcriptional or translational enhancer S1 (Futterer et al., 1990), the CaMV 35S terminator (T), an upstream RTBV ORF and the downstream GUS ORF (GUS). b: The upstream ORF present in a construct can be deduced from the plasmid name (e.g. pORF Ia: version of RTBV ORF I). The extent of the RTBV ORFs is indicated below the left open box (numbering of the
RTBV genome positions is according to Hay et al., 1991). The internal features of the upstream RTBV ORFs are: +GC: increased GC content; AT/GC-rich: high natural percentage of AT/GC in the sequence; ATTTA: all ATTTA motives mutated; internal ATGs/ *: internal ATGs do exist, within the sequence. The start codon context of each RTBV ORF is indicated, nucleotides complying with the optimal context are in bold, translated codons are underlined. The sequence linking the upstream ORF to the downstream ORF is shown between both boxes, respective stop and start codons are underlined. All variations of ORF I and II contain the same linker sequence.

### 3.2.2 Transient expression assays - unmodified sequences

For transient expression in protoplasts the RTBV ORF was placed upstream of an ORF coding for a β-glucuronidase (GUS; Jefferson et al., 1987). The expression efficiency of the downstream GUS ORF was compared to a monocistronic GUS expression cassette (see Fig. 5a; PLS1 GUS = 100%). All dicistronic constructs were found to have a reduced GUS activity (Fig. 8, constructs in italics). Among all the constructs with unmodified RTBV sequences in the upstream position, the ORF IV construct yielded the highest GUS activity (61%), surprisingly despite the presence of the two internal ATG codons. ORFs I and II yielded almost no GUS expression. For ORF I containing constructs, this lack of expression was quite independent of the translation of the upstream ORF, since the start codon context of the first ORF did not influence downstream expression; a strong reduction of GUS expression was even found with the ORF I construct pORF Ic with an ATG to AGT start codon mutation (Fig. 8). This lead to the assumption that the reduced GUS expression of ORF I and II constructs could be an effect of the RTBV upstream sequences on mRNA levels, as has been observed before in some batches of rice protoplasts (Fütterer et al., 1997). Both ORFs are located within a conditionally removed intron (Fütterer et al., 1994) and therefore are generally AU-rich. They also contain two AUUUA motifs each. Such AU-rich elements (AREs) can be triggers for rapid RNA degradation (Myer et al., 1997). In contrast, pORFIVΔC contains a RTBV-sequence with higher GC-content and exon-like character. These structural features supporting increased mRNA-stability might have been responsible for the high expression obtained with this construct.
### Results

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Description of the Upstream ORF</th>
<th>Transient Downstream GUS Expression in % of the Monocistronic Control (=100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORF Ia</td>
<td>CAT ATG GAG</td>
<td>0.4</td>
</tr>
<tr>
<td>pORF Ib</td>
<td>GAT ATG GAG</td>
<td>0.5</td>
</tr>
<tr>
<td>pORF Ic</td>
<td>GAT ATG GAG</td>
<td>7</td>
</tr>
<tr>
<td>pORF Ia mut 1</td>
<td>CAT ATG GAG</td>
<td>0.2</td>
</tr>
<tr>
<td>pORF Ic mut 1</td>
<td>GAT ATG GAG</td>
<td>3.1</td>
</tr>
<tr>
<td>pORF Id mut 1</td>
<td>CAT ATG AAG</td>
<td>2.4</td>
</tr>
<tr>
<td>pORF II</td>
<td>GAC ATG AGC</td>
<td>3.5</td>
</tr>
<tr>
<td>pORF II mut 1</td>
<td>GAC ATG AGC</td>
<td>2.2</td>
</tr>
<tr>
<td>pORF II mut 2</td>
<td>GAC ATG AGC</td>
<td>2.7</td>
</tr>
<tr>
<td>pORF II,C</td>
<td>GAC ATG AGC</td>
<td>41</td>
</tr>
<tr>
<td>pORF II,C mut 2</td>
<td>GAC ATG CTA</td>
<td>84</td>
</tr>
<tr>
<td>pORF IV,C</td>
<td>TCC ATG ATAT</td>
<td>61</td>
</tr>
</tbody>
</table>

**Fig. 8:** Expression downstream of unmodified (italics) and modified (bold) RTBV ORFs in dicistronic expression constructs. To visualise the localisation of the RTBV and GUS ORFs on the dicistronic expression unit, the structure of the plasmid is shown in the upper part of the figure. Translation start context and RTBV ORF internal sequence features are described as in Fig. 7. Expression of a GUS ORF downstream of the respective RTBV ORF variants in transfected rice protoplasts is shown in the right panel. Values represent the average of between three and more than ten independent transfection experiments and are shown relative to plasmid pLS1GUS (Fig. 5; 100%). Error bars show the standard deviation.

### 3.2.3 Modified sequences

Based on the assumption that problems in expression of ORF I and II constructs were linked to mRNA instability, a second series of expression units was...
Results designed. The ORF I and II coding sequences were modified without alteration of the coding potential or introduction of internal ATG codons (Fig. 7b; modified sequences). For ORF I the AT-content was reduced from 72% (46% A; 26% T) to 67% (42% A; 25% T) and for ORF II from 64% (40% A; 24% T) to 54% (35% A; 19% T). The modified sequences were incorporated into the same gene expression cassettes as the unmodified sequences (Fig. 7a). For ORF I, one modified version was tested that did not contain an AUUUA-motif and with a generally higher GC-content. This version was tested with all variations of the start codon context (Fig. 7b, pORF 1 a, c, d mut1; modified sequences). For ORF II, two modified versions were tested, one only lacking the AUUUA-motifs and another version with an additionally increased GC-content (Fig. 7b, pORF II mut1, mut2). Furthermore, also 5' and 3' end deletion variants were constructed (Fig. 7b, pORF IIΔC mut2, IIΔN mut2). A detailed description of the modified sequences of ORF I and II is shown in the appendix.

3.2.4 Transient expression assays - modified sequences

The modified sequences were tested in transient expression assays (Fig. 8). The expression efficiency of the downstream GUS ORF was compared to the monocistronic GUS expression cassette (Fig. 5a, page 19; PLS1 GUS = 100%). No alteration of expression was observed for any modifications of ORF I (Fig. 8, pORF la, c, d mut1). Also no alteration of expression occurred with ORF II constructs in which only of the AUUUA motifs were modified (pORF II mut1). However, an additional increase of the GC content of ORF II increased GUS expression levels up to 27% of the monocistronic control (pORF II mut 2). The deletions of the 5' region and the 3' region of the modified ORF II further increased expression up to 84% and 41%, respectively (pORF IIΔC mut2 or -ΔN mut2).

3.3 The PAT ORF as upstream ORF

3.3.1 The sequences

The examples of the RTBV ORFs show that a leaky scanning mechanism can indeed function in an artificial context allowing significant expression of a downstream ORF. To test whether this strategy also would work for expression of "normal" ORFs, the phosphinothricin acetyltransferase (PAT) ORF from Streptomyces hygroscopicus (Thompson et al., 1987) was placed upstream of a GUS ORF. PAT confers resistance to the herbicide phosphinothricin (PPT). A successfully adapted PAT ORF could be used as an upstream selectable ORF in combination with any ORF of interest as the downstream ORF. This ORF was chosen among a variety of suitable selectable marker genes because it is
Results

relatively short (183 codons) and contains only five internal ATG codons, three in-frame and two out-of-frame. To be useful as a selectable marker it is critical that the modified PAT ORF still encodes a functional protein. While the mutation of an out-of-frame ATG does not necessarily alter the coding potential, mutations of an in-frame ATG change the amino acid sequence of the encoded protein with a potential impact on protein activity. The chosen PAT ORF contains only one conserved internal methionine, which is the first in-frame ATG. The two downstream methionines are not conserved in different PATs and they could be altered to amino acids found at the corresponding positions of the PAT of Streptomyces viridochromogenes (Wohlleben et al., 1988) without loss of enzyme activity. A schematic description of the constructs with the different PAT ORF mutants is presented in Fig. 9. Detailed descriptions of the modified in comparison to the native PAT sequences are shown in the appendix.

3.3.2 Transient expression assays - impact of the coding sequence

The PAT mutants included changes of the first internal conserved methionine (M) to valine (V), leucine (L) or threonine (T). They were all tested for their activities. The enzyme function was tested by radioactive labelling of PPT by acetylation. The reaction mix was separated by chromatography on a TLC plate (Fig. 9, middle panel) and the plate was exposed to a X-ray film. All mutant constructs resulted in a reduced but still clearly detectable PAT activity. The major reduction of activity was found with the first in-frame mutation of the conserved methionine (BV, BVall, BLall, BTall) as compared to the wild-type sequence (BWT). The other downstream mutations (in-frame-non-conserved and out-of-frame ATGs) did not have an impact on enzyme activity (compare BV to BVall). Replacing the conserved methionine by a leucine may have the least impact on enzyme function (BLall compared to BVall and BTall). A quantitative assay would be required for more precise conclusions on the enzyme activities. The translational leakiness of the different mutants of the PAT ORF was tested with the GUS assay (Fig. 9, right panel). Downstream GUS expression was reduced to almost zero by the native PAT ORF (construct BWT) or by the PAT ORF with only one internal ATG codon mutated (construct BV). However, additional mutations altering the other ATGs (BV, L, T all) by eliminating all internal potential start codons increased downstream expression to 8-16% of the PAT-less monocistronic control (UBI-GUS).
Fig. 9: Schematic representation of the PAT dicistronic expression cassettes and transient expression in protoplast assays. PAT dicistronic expression cassettes consist of the maize ubiquitin 1 promoter with its first intron (Ubiquitin), an upstream PAT derivative (modified), the downstream GUS ORF and the CaMV 35S terminator (T). The start codon context of the PAT ORF and its internal features are indicated. The PAT versions differ by the number of the internal ATG codons (*) and by different replacements of the conserved methionine codon (M) by codons for valin (V), leucine (L) or threonine (T). The sequence linking the PAT ORF to the GUS ORF is shown, respective stop and start codons are underlined. For PAT activity, measured by acetylation of PPT, only the region of the TLC plate with the acetylated PPT is shown. pABI encodes a monocistronic PAT ORF under the control of the rice actin 1 promoter (Bliffeld et. al., 1999). GUS expression is shown relative to the monocistronic pUbiGUS (100%).

3.3.3 Transient expression assays –
impact of the translation start codon context
Translation efficiency is thought to be regulated by the context of the translation start-codon (Kozak, 1989a). The degree to which initiation can be modulated by the sequence context has not yet been studied systematically in plant cells.
Therefore, in a second series of PAT dicistronic expression constructs, the impact for translation efficiency of each nucleotide T, C, A, G in the key positions -3 and +4 of the initiation site was examined (Fig. 10). These positions are numbered relative to position +1, the A of the translation start codon ATG. The translation start codon context of a derivative of BVall (Fig. 9) was mutated systematically. The maize ubiquitin 1 promoter of BVall was replaced by the CaMV 35S promoter and every possible combination of the nucleotides in the respective positions was constructed.

![Diagram](image)

**Fig. 10:** Systematic analysis of the influence of nucleotides located at position +4 and -3 relative to the PAT ORF start codon in dicistronic expression constructs. All constructs tested here are derivatives of BVall shown in Fig. 9. The maize ubiquitin 1 promoter was replaced by the CaMV 35S promoter (35S). **A:** Translation start context, nucleotides at position -3 and +4 are coloured. **B:** Downstream GUS expression is shown in comparison to a monocistronic construct (100%). **C:** Average GUS expression arranged for nucleotides at key positions -3 and +4. The coloured boxes correspond to each nucleotide at position +4 or -3 and represent each the average expression of four expression constructs.

Rather than measuring PAT directly, the downstream GUS ORF was used as reporter for PAT translation efficiency, because changing the sequence context at position +4 necessarily altered the PAT coding sequence, which might have an
impact on PAT activity or stability. With such constructs, a high downstream GUS expression should be measured when the upstream PAT translation was poor. Less GUS expression should be measured with better PAT translation efficiency.

In transient expression assays a strong impact of the presence of particular nucleotides was found. A “strong” translation start was found for nucleotides G or A at position −3 and nucleotide G at position +4. This is the consensus translation start context for eukaryotic genes. An especially “weak” translation start was found for nucleotides T or C at position −3 and C at position +4. The combination of nucleotides on position −3 and +4 behaved in an additive way: a “strong” −3 position in combination with a “weak” +4 position resulted in medium downstream GUS expression of around 11-16%. The same result was found with a “weak” −3 position and a “strong” +4 position. Two “strong” positions resulted in almost no downstream GUS expression (1%). Two “weak” positions resulted in high downstream GUS expression of 30-40%.

The average GUS expression for each nucleotide at key position −3 or +4 is presented in the right panel of Fig. 10. This shows the influence of each nucleotide in the translation start context. For example, the average GUS expression for T at −3 was calculated as the mean GUS expression of the four constructs with a T at −3. When a nucleotide had a strong influence for translation initiation, this average was low (A on −3; G on +4). On the other hand a nucleotide with a weak influence for translation initiation, produced high average GUS expression (T on −3; C on +4).

3.4 Stable transformation of rice

3.4.1 Objectives for stable transformation

The goal for rice transformation with dicistronic constructs was to produce plant lines, which were selected for high expression of the gene of interest. The translational fusion with the selectable marker ORF should lead to increased expression of the linked ORF products, the proteins of interest.

For the specific purpose of producing virus resistant plants, the RTBV ORFs were positioned upstream and the selectable marker ORF, aph4 conferring resistance to the antibiotic hygromycin, was positioned downstream (Fig. 11a). All the sequences used for stable transformation have been tested in transient expression assays (see previous sections). Our objectives were, first, whether the results obtained in transient expression assays are useful to predict expression behaviour in transgenic plants, and second, to examine if the dicistronic expression context would lead to increased levels of recombinant viral gene products. In previous transformation experiments with RTBV derived sequences, only very few of
several hundred transgenic rice lines had detectable levels of RTBV-derived RNAs (Klöti, 1996).

For a general application of this method, PAT dicistronic expression constructs were produced with the leaky PAT ORF located in the upstream position and the aph4 ORF in the downstream position (Fig. 11b).

**Fig. 11:** Dicistronic expression constructs used for stable transformation. a: RTBV-sequences, b: PAT-sequence. Constructs are similar to those used for transient expression (Fig. 7), but the downstream ORF (right box) was exchanged by the aph4 ORF. Translation start context and internal sequence features are described as in Fig. 7. The restriction sites used for Southern analysis are indicated.

### 3.4.2 Rice variety and transformation methods

Japonica rice variety Taipei 309 was chosen as a model plant for rice transformation. It is known for its good tissue culture properties and is easy to transform (Christou and Ford, 1995). A high transformation efficiency was a basic prerequisite for our project because we expected decreased expression levels of aph4 products in the dicistronic context. Two different types of totipotent cells were
used as targets: embryogenic suspension cells and scutellum cells of immature zygotic embryos. Immature embryos were precultured for several days to produce embryogenic callus from scutellum cells. These calli were either used for bombardment or for establishing embryogenic cell suspensions (ECS). Particle bombardment was the method of choice for both cell types, because the transformation efficiency is high and the plasmids containing the dicistronic expression units were ready to use. In addition, with particle bombardment it is possible to transform ECS, which are the most abundant source of totipotent cells (Klöti, 1996).

3.4.3 Selection system of transgenic Taipei 309 calli based on PPT

A PPT based selection system had to be established for rice as a prerequisite for a general use of the dicistronic PAT cassettes. Protocols for PPT selection in rice had been published previously (Datta et al., 1992; Khanna et al., 1996). To test whether this method would work in our hands, non-transgenic embryogenic cell suspensions and cell suspensions transfected with an actin-bar, CaMV 35S GUS expression cassette were subjected to PPT selection (Fig. 12).

Fig. 12: rice suspension calli cultivated on solid selective medium containing phosphinothricin (PPT). a-c: increasing concentration of PPT in the medium. a: 0 mg/l PPT, b: 5 mg/l PPT, c: 10 mg/l PPT. Brownish sections are indicated by an arrow.

In liquid selection medium no difference in cell proliferation of non transformed cell suspensions was observed for various concentrations of PPT (0.5-100 mg/l). On solid medium the cells were growing well up to a concentration of 5 mg/l PPT. Some brownish sections appeared. With a concentration of 10 mg/l PPT cell proliferation was slightly inhibited. However, even a concentration of 100 mg/l PPT did not stop cell proliferation. Cells transiently expressing GUS after particle bombardment could be identified by GUS staining. Under the described conditions, however, we never obtained expressing sectors which would have indicated proliferation of transgenic cells. As a consequence, it was not possible to establish a PPT-based selection system for rice.
3.4.4 Dicistronic constructs used in stable transformation

Transgenic rice was selected with the antibiotic hygromycin. The “leaky” expression cassettes as well as those with no downstream GUS expression in transient expression assays were used for transformation. These latter non-expressing constructs were included since it appeared possible that their properties might be different in stably transformed cells. Furthermore, it was not possible to obtain expressing dicistronic constructs with ORF I upstream elements. For the establishment of a rice transformation protocol with dicistronic expression cassettes, different hygromycin concentrations (20 to 30 mg/l) were used. It turned out that a concentration of 20 mg/l did not allow a stringent selection. Consequently, many regenerated plants did not contain the expected transgene (Table 6). On the other hand a concentration of 30 mg/l appeared to be too high for certain dicistronic constructs. Particularly those with a low downstream expression, as measured in transient GUS assays, did not yield transgenic plants.

<table>
<thead>
<tr>
<th>plasmid</th>
<th>total number of lines</th>
<th>regenerated lines</th>
<th>Southern positive</th>
<th>northern positive</th>
<th>Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORF Ic</td>
<td>39</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
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<td>20</td>
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<td></td>
</tr>
<tr>
<td>pORF IIΔC</td>
<td>36</td>
<td>21</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pORF II mut 2</td>
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<td>2</td>
<td>1</td>
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</tr>
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<td>48</td>
<td>12</td>
<td>12</td>
<td>8</td>
<td>3 (6.25%)</td>
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<tr>
<td>pORF IIΔN mut 2</td>
<td>58</td>
<td>18</td>
<td>18</td>
<td>10</td>
<td>9 (15.5%)</td>
</tr>
<tr>
<td>pORF IVΔC</td>
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<tr>
<td>pBH</td>
<td>45</td>
<td>2</td>
<td>2</td>
<td></td>
<td>1 (2.2%)</td>
</tr>
</tbody>
</table>

Table 6: Summary of rice transformation. Lines in italics were transformed with unmodified dicistronic expression cassettes, the lines in bold with modified cassettes. The total number of lines reflects the amount of lines which were proliferating on selective medium.

With pORF II mut 2 and pORF IVΔC constructs the transformation efficiency reached the level obtained with monocistronic selectable marker gene constructs (Table 6). The failure to regenerate transgenic (Southern positive) plants with pORF Ic, pORF II and pORF IIΔC reflects the problem of expression levels of the resistance gene as well as the decreased concentration of hygromycin used in
these transformation experiments. Lines containing full-length ORF II were not fertile.

3.5 Plant analysis

3.5.1 Analysis of plants transformed with dicistronic expression units
To verify the presence of the gene of interest a probe complementary to nts 234-456 of the aph4-sequence was used. This sequence is present in all constructs. The detection is easy because the same probe is applicable to all different constructs. Another advantage of the analysis of transgenic plants transformed with dicistronic expression units is that the presence of the marker sequence automatically proves the presence of the gene of interest. Transgenic plants obtained from conventional approaches using independent single genes, often only contained the marker sequence but did not contain the gene of interest (Burkhardt, 1996).

3.5.2 Southern analysis of plant material
Regenerated TP309 lines were tested for the presence of the transferred DNA sequences in order to identify independent lines and plants that had integrated complete copies of the transgenes. The transgenes were detected by hybridisation to digoxigenin-labeled probes. Plant DNA was extracted from primary transgenic R0 plants. Integration of the introduced dicistronic sequence was tested by hybridisation to total plant DNA digested with appropriate restriction enzymes that were known to produce a fragment of defined size (see Table 7). With a second digestion, independence of the transformants was tested by using a restriction enzyme cutting only once in the plasmid used for transformation. In this case, fragments of various length corresponding to the 3' end of the transgene and the contiguous plant DNA were expected.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Enzyme(s) used</th>
<th>expected size (bp)</th>
<th>enzyme used</th>
<th>expected size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pORF Ic</td>
<td>EcoRV/ Sphl</td>
<td>2200</td>
<td>EcoRV</td>
<td>variable</td>
</tr>
<tr>
<td>pORF II</td>
<td>EcoRV/ Sphl</td>
<td>1920</td>
<td>EcoRV</td>
<td></td>
</tr>
<tr>
<td>pORF IIΔC</td>
<td>EcoRV/ Sphl</td>
<td>1870</td>
<td>EcoRV</td>
<td></td>
</tr>
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<td>EcoRV</td>
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<td>1870</td>
<td>EcoRV</td>
<td></td>
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<td>EcoRV/ Sphl</td>
<td>1820</td>
<td>EcoRV</td>
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</tr>
<tr>
<td>pORF IVΔC</td>
<td>EcoRV/ Sphl</td>
<td>2000</td>
<td>EcoRV</td>
<td></td>
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<tr>
<td>pBH</td>
<td>MunI</td>
<td>3500</td>
<td>XbaI</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: restriction enzymes used for analysis of the integration of the different plasmid constructs and the size of the expected genomic fragments.

3.5.3 First series of transformation

The first plants analysed were those that had been transformed with unmodified dicistronic sequences (Table 6, lines in italics). Besides the ORF IVΔC sequence none of these cassettes produced a downstream GUS expression higher than 10% of the monocistronic control in transient assays (Fig. 8). In this early stage of the work, the selection regime to produce plant lines was weaker, the hygromycin concentration was reduced from 30 mg/l to 20 mg/l. The majority of the regenerated plant lines were not transgenic (see Fig. 13), probably because of the decreased selection pressure and the low expression of the downstream aph4 sequence. Only two plant lines transformed with pORF IVΔC were transgenic (Fig. 13, ORF IV 5 and 8). One of these lines contained a complete integrated copy of the dicistronic expression unit (Fig. 13, ORF IV 5), whereas the other line did not contain the complete copy (Fig. 13, ORF IV 8). No plants were obtained containing dicistronic pORF I, pORF II or pORF IIΔC cassettes. This was in accordance with the negative results from transient GUS expression assays (Fig. 8). The failure to generate plants with these constructs also indicated that the dicistronic constructs could not easily rearrange to produce a selectable monocistronic gene. The Southern blot analysis included two transgenic lines transformed with plasmid paRNA14 (Fig. 13, AS). These lines are further described in chapter “Strategies
for virus resistance by genetic engineering - antisense RNA". Both lines had integrated the transgene in multiple copies.

**Fig. 13:** Southern blot analysis of 17 putative transgenic plant lines transformed with dicistronic expression units. **A:** Structure of the plasmid used for transformation. The restriction sites, the sizes of the released fragments (2.2 kb for ORF I and 2.0 kb for ORF IV\(\Delta C\)) and the position of the probe in the aph4 sequence are indicated. **B:** Analysis of lines transformed with unmodified sequences: pORF IV\(\Delta C\) (ORF IV) and pORIF \(1c\) (ORF I). Two putative transgenic lines transformed with paRNA14 (AS) are also shown. The position of the expected fragment is marked by an arrow. DNA was digested as described in Table 7. In lane “a” plant DNA was digested with EcoRV and SphI to release the expression cassette, in lane “b” DNA was digested only with EcoRV to verify the independence of the lines. An EcoRV/SphI digestion of plasmid pORF IV\(\Delta C\) is shown in lane P.

### 3.5.4 Second series of transformation

Southern blot analysis of plants transformed with dicistronic expression units containing modified sequences is shown in Fig. 14. The analysis of all regenerated plants revealed that only those constructs with upstream ORFs allowing at least 10\% GUS expression in transient assay (Fig. 8 and 9) produced transgenic plants under the same selection regime (30 mg/l hygromycin) that was employed for conventional monocistronic expression constructs. The number of transgenic plants generated for a particular construct correlated with the efficiency of the corresponding transient GUS expression (compare Fig. 8: transient downstream GUS expression, and Table 6: Southern positive). The DNA integration patterns were similar to those of other transgenic lines obtained under the same conditions with conventional selection cassettes.
**Fig. 14:** Southern analysis of 28 putative transgenic plant lines transformed with constructs from the second series of dicistronic expression units (pORF II\(\Delta\)C mut 2: ORF II\(\Delta\)C, pORF II\(\Delta\)N mut2: ORF II\(\Delta\)N, pORF II mut 2: ORF II, pORF IV\(\Delta\)C: ORF IV) and with the adapted dicistronic PAT construct (pBH:BH). DNA was digested as described in Table 7. Lane a: digestion to release the expression cassette, lane b: single digestion to verify the independence. Lane WT: DNA from an untransformed plant. M: molecular weight marker. The size of the expected fragment is indicated by an arrow. An EcoRV/SphI digestion of plasmid pORF II\(\Delta\)N is shown in lane P.
3.5.5 Northern blot analysis of plant material
mRNA from transgenic plants was analysed by northern blot analysis. Total plant RNA was extracted from young leafs of Southern positive R₀ plants or from T₁ and T₂ seedlings germinated in the presence of hygromycin. Transgene-derived dicistronic mRNA was either detected by hybridisation to a digoxigenin-labeled aph4 probe complementary to nts 234-456 of the aph4-sequence or by hybridization to probes consisting of digoxigenin-labeled fragments of the gene of interest (e.g. the RTBV ORF IV sequence).

3.5.6 Evidence for dicistronic mRNA
To test whether the upstream viral sequence and the downstream aph4 sequence are indeed located on the same mRNA molecule, total RNA was isolated from seedlings of a segregating T₁ progeny of a transgenic line containing the 5' part of RTBV ORF IV. RNA was hybridised with separate probes corresponding either to the ORF IV or the aph4 sequence (Fig. 15). Both probes detected a mRNA of the same size and equal expression level. Thus, it was concluded that the same mRNA molecule was detected. The transcript levels of the segregating plants correlated with their resistance to hygromycin.

Fig. 15: Northern analysis of a segregating transgenic line transformed with pORF IVΔC. In a schematic description the location of the two different probes and the start- and stopcodons of the separate ORFs are shown. Total RNA was isolated from green leaf tissue of a population of T₁ seedlings germinated in the presence of hygromycin (left panel) and hybridised with probes for ORF IV or aph4 (middle panel). RNA levels correlated with hygromycin resistance. 1, 2: seedlings grow well in the presence of hygromycin. 3: seedlings grow poorly in the presence of hygromycin.
3.5.7 Expression of dicistronic mRNA

Plants containing dicistronic ORF II and IV expression cassettes accumulated very high levels of mRNAs of the expected size (Fig. 16). The steady state level of the dicistronic mRNA varied between different plant lines. In most cases, dicistronic mRNA levels were 2 to 5 times higher than those from a monocistronic gene in a line that was regarded previously as a “strong mRNA expresser” (Fig. 16, HYG s). In some cases, the expression was similar to a “weak mRNA expresser” (Fig. 16, HYG w). In two independent plant lines transgenic mRNA was very low (ORF II\&N 6) or not detectable (ORF II\&N 8, 10). Expression levels were very stable in the T₁ and T₂ generation of plants analysed (Fig. 16, ORF II\&N 1 a, b: T₁ generation; c, d: T₂ generation) and did not vary in homozygous T₂ plants (Fig. 16, ORF IV 5 a, b, c).

Experiment 1:Northern analysis of 20 randomly chosen transgenic plants. RNA was detected with a probe complementary to nts 234-456 of the aph4 coding sequence. In a schematic description the location of the probe on the dicistronic and the monocistronic mRNA molecule and the start/- stop codons of the separate ORFs are shown. Total RNA was isolated from green leaf tissue of T₁ seedlings germinated in the presence of hygromycin. ORF II\&C, ORF II\&N, ORF IV\&C indicate the RTBV sequence located on the dicistronic mRNA. Numbers below the denotation refer to the lanes in Fig. 14, where DNA from the same lines was analysed. Letters a, b, c, d indicate the progeny of one line. HYG indicates lines transformed with a monocistronic aph4 expression unit. Expression levels of the monocistronic mRNA are specified with strong (s) or weak (w). For the calculation of the expected size of the dicistronic and monocistronic transcripts, 100 bp for the leader, 200 bp for the CaMV 35S terminator and 200bp for a polyA tail were added to the length of the aph4 sequence and the RTBV sequence.
3.5.8 RNA production in "abnormal" lines

While expression in most lines appeared to be as expected, some lines developed abnormal expression patterns which are shown in Fig. 17. Lines contained a monocistronic (HYG) or dicistronic transgene (ORF IV and ORF II) or an antisense sequence (paRNA 14).

![Fig. 17: Northern analysis of eight transgenic lines. Total RNA was isolated from green leaf tissue of T₁ seedlings (HYG, pORF IV) or of T₀ adult plants (pORF II, paRNA 14). Numbers below the denotation of the plant lines refer to the lanes in Fig 7 and Fig 8, where DNA from the same lines was analysed. In the right column the quality and concentration of the total plant RNA separated on the gel is shown.](image)

The detected mRNA of HYG had the size of the monocistronic aph4 mRNA. As already shown in Fig. 16, expression of the dicistronic transcript was about 5 times higher than the expression of the “strong” monocistronic transcript (ORF IV 5 and HYG s). One ORF IV line, transformed with a dicistronic expression unit, produced a hybridisation signal of the size of the monocistronic mRNA (Fig. 17, ORF IV 8). A smear of aberrant smaller RNAs was also detected. Southern blot analysis indicated that the line did not contain the complete dicistronic expression unit, because no fragment of the expected size was detected (Fig. 13, ORF IV 8). Apparently, only the aph4 sequence was expressed after rearrangement of the dicistronic expression cassette. This line was an exception, because the other lines contained at least one complete copy of the dicistronic expression cassette (Fig. 14). A full-length ORF II line (Fig. 17 ORF II 1) produced also a smear of aberrant smaller RNAs in addition to the hybridisation signal of the dicistronic mRNA. It contained the transgene in multiple (rearranged) copies. The smear of aberrant smaller RNAs in both lines (rearranged ORF IV and full-length ORF II) was not caused by degradation of RNA before blotting (Fig. 17, right column). No transgene expression was detected in another ORF II line and in two paRNA 14 lines (Fig. 17, ORF II 2; paRNA 14 1, 2). Multiple copies of the transgene were found by Southern blot analysis in all three lines. The ORF II lines and the paRNA 14 lines were not fertile (Table 6).
3.5.9 Western analysis of proteins encoded by dicistronic mRNA

Protein expression was detected by western blot analysis (Fig. 18). Soluble proteins were extracted from leafs of northern positive or negative transgenic plants. As a positive control, RTBV infected leaf material was used. The proteins were separated on 15% polyacrylamide gels and were blotted on nitrocellulose membranes. A polyclonal antiserum raised against the ORF II protein and the ORF IV protein (kindly provided by Roger Hull, John Innes Centre, Norwich, UK) was used for detection. In transgenic rice lines containing the carboxyterminal subfragment of ORF II (expected size around 8.7 kDa) a specific protein, smaller than 14.3 kDa and bigger than 6.5 kDa, was detected (Fig. 18). The presence of this signal proved that the RTBV-protein was really translated as separate ORF and not fused to the downstream aph4 gene product. The level of protein expression correlated to the mRNA expression level (Fig. 16). Immunoreactive signals, found in the negative control, are due to unspecific binding of the antibody to plant proteins. The positive control yielded a strong immunoreactive signal of the expected size (12 kDa). In transgenic rice lines containing the aminoterminal subfragment of ORF IV no specific protein of the expected size of around 15 kDa was detected (see Fig. 27). The signal of the expected size was also not detected in positive controls (RTBV infected plant material). This indicated that the ORF IV protein antibody probably was not able to detect the ORF IV protein efficiently. The same observation was made previously (Hay et al., 1994)

![Fig. 18: Western analysis of 7 transgenic ORF II AN lines. The numbers above the membrane refer to the denotation in Figs. 14 and 16. As negative control (WT), total protein of a non-transformed line was loaded on the gel. As positive controls, total protein of RTBV infected plants was used: transgenic line 8 rt and non transformed wild-type (WT rt). Arrows indicate protein size markers in kDa.](image-url)
3.6 Strategies for virus resistance by genetic engineering

The following strategies were pursued to control RTD: (i) virus resistance that does depend on protein expression; the virus-derived transgenic protein serves as dominant negative competitor in order to disturb viral replication. (ii) antisense RNA mediated resistance; expression of specific antisense sequences in order to destabilise viral mRNA. (iii) trans-activation of a cytotoxic protein; virus specific gene expression mechanisms induce cell death in order to restrict the virus to the initially infected cell. (iv) virus resistance dependent on gene silencing; this mechanism is known as RNA-mediated resistance against RNA-viruses, and invading homologous sequences are silenced by post-transcriptional gene silencing. With DNA-viruses, also transcriptional gene silencing may be possible.

3.6.1 (i) Expression of RTBV proteins

Pathogen-derived resistance can be achieved by expression of complete or mutated viral proteins in transgenic plants (Sanford and Johnston, 1985). Our strategy was to increase the level of transgene expression in rice because low expression levels may be a reason that this approach so far was not successful with RTBV (Azzam et al., 1999). The transcriptional fusion with the selectable marker gene sequence on dicistronic mRNAs increased the level of RTBV gene product. The sequence requirements for leaky scanning did not allow the generation of plants expressing the RTBV coat protein or the viral replicase on dicistronic mRNAs. Both sequences are located within RTBV ORF III and have already been tested for virus resistance and the attempts were not successful (Klöti, 1996). With other virus and host plants this strategy was resulting in strong forms of genetically engineered, pathogen-derived resistance (Carr and Zaitin, 1993; Hackland et al., 1994). The strategy to increase transgene expression by leaky scanning was designed for RTBV ORF I, II and IV.

3.6.1.1 RTBV ORF I

The function of RTBV ORF I is unknown. It has the capacity of encoding a 24 kDa protein and contains a motif which is common for Badna viruses. The protein was only detected in degraded but not in intact RTBV particles, suggesting that the product of ORF I could be located within the virus particles, possibly associated with particle assembly (Hay et al., 1994). Overproduction of any viral protein could interfere with replication, infection or movement of the virus. Therefore, the complete ORF I was cloned in a dicistronic construct and tested for expression. Unfortunately, no ORF I-transgenic rice plant could be regenerated and therefore it was not possible to test this approach in a virus resistance assay.
3.6.1.2 **RTBV ORF II**

The exact function of RTBV ORF II is not known. The corresponding protein pII contains a protamin like C-terminal sequence. pII is presumably an analogue of CaMV pIII (Mougeot, 1995) which is a minor capsid protein of 15 kDa. Due to their C-terminal, protamin-like sequence CaMV pIII and RTBV pII strongly bind to DNA (Mesnard and Carrière, 1995). The C-terminal region of RTBV pII interacts with the RTBV coat protein. Mutational analysis of the RTBV ORF II 5' end suggested that a tetramerization site is located in the N-terminal region of the protein (E. Herzog, personal communications).

ORF II in its natural full-length form was cloned into the dicistronic expression context. In addition ORF II was cloned in its 3' end- and 5' end-deleted form. ORF II and both deletion variants might compete as negative dominant competitor with the natural virus-encoded ORF II protein and potentially imbalance or disturb viral replication. It was not possible to produce a fertile transgenic plant expressing full length ORF II (see Table 6). Therefore only plants containing 3' end and 5' end deleted versions of ORF II were tested for virus resistance.

3.6.1.3 **RTBV ORF IV**

The function of RTBV ORF IV is not known. However, it was speculated that RTBV ORF IV codes for a regulatory protein which seems to be present only in very small quantities in RTBV infected plants (Hohn and Fütterer, 1997). Regulatory proteins in retro- and pararetroviruses are expressed from separate RNAs (either spliced or produced from a subgenomic promoter). In addition their genes are located at the 3'end of the genome. Both features are shared by RTBV ORF IV. An interaction with host proteins related to cell cycle regulation is speculated (Thomas Hohn, personal communications). It would not have been possible to express a full length version of ORF IV sequence in a dicistronic context because of its sequence properties. Therefore, a 3'end deletion variant of ORF IV was transformed into plants. The gene product of the 3'end deletion of ORF IV expressed by the dicistronic mRNA might compete with regulatory RTBV pIV. This possibility has been tested in a virus resistance assay.

3.6.2 (ii) Antisense RNA

Expression of a viral antisense RNA (asRNA) sequence in a transgenic plant may lead to inhibition of viral replication when the asRNA is directed to essential viral genes or untranslated regions (Bourque, 1995). In order to disturb RTBV replication, different sequences of the RTBV genome were fused to the 3' end of the aph4 sequence in a single transcriptional unit (Fig. 19). The principle of selecting for high expression was the same as for the dicistronic expression units. However, for an untranslated asRNA, translational mechanisms do not have to be
considered. Therefore, the aph4 sequence was located in the upstream position. Four different constructs were generated (Henrich, 1996; Klöti, 1996). One resulting plasmid, paRNA14, contained 451 nucleotides from nt 7408 to nt 7859 of the RTBV genome in antisense direction. paRNA14 contained regions complementary to the untranslated leader region which includes features like the 5'-splice site of the intron (Fig. 2, SD; nts 7503/4), the polyadenylation signal at nt 7598, translation regulating sequences and a putative packaging signal (Fütterer et ai., 1994; Rothnie et ai., 1994). Two additional asRNA constructs homologous to the 3’ end of RTBV ORF IV, pORF 780 and pORF 90, were constructed. pORF 780 contained a 732 bp antisense sequence from nt 6482 to nt 7214, pORF 90 contained a 92 bp antisense sequence from nt 7122 to nt 7214. Another construct termed pORF L was homologous to the leader region of RTBV and to 18 codons of ORF I. This region included the translation start and the binding site of tRNA\textsuperscript{met} at nt 1. This site initiates the synthesis of the minus-strand DNA by the RNA-dependent DNA polymerase function of the reverse transcriptase (Qu et al., 1991). paRNA14 was transformed into rice several times. Although, transgenic, antisense RNA expressing lines could be obtained, none of these lines was fertile. Plants generated with the other three constructs have already been tested for virus resistance in 1997 (Andreas Klöti, unpublished results). Individual lines have been re-evaluated in the course of this work.

Fig. 19: Schematic representation of the antisense RNA expression units. Different sequences in antisense orientation of the RTBV-genome were fused to the 3’ end of the aph4 sequence in one transcription unit (Antisense; see text). Transcription is regulated by the CaMV 35S promoter and CaMV 35S terminator. Restriction sites used for Southern analysis are indicated.

3.6.3 (iii) Transactivation

In this approach, the rapid induction of cell death in virus infected cells should protect the transgenic plants from infection. A putative transsplice event induced by the invading virus should activate the barnase, a cytotoxic bacterial RNase (Mariani et ai., 1990). In this way, the spread of the virus would be blocked and it would be restricted to the initial infected cell.

This possible RTBV induced RTBV resistance mechanism involving a putative transsplice event was tested in transient expression experiments (Fütterer, unpublished results; see Fig. 20). The RTBV ORF IV is expressed from a spliced RNA (Fütterer et ai., 1994). Some of the data obtained in these experiments
suggested that besides a normal splicing with splice donor and splice acceptor on one mRNA also trans-splicing might occur. The expression of a reporter ORF (CAT) interrupted by an RTBV derived intron (Fig. 20, 1) was compared with similar expression cassettes lacking either the promoter or the splice donor site (Fig. 20b, 2 and 3).

Fig. 20: Evaluation of a transsplice effect in transient protoplast expression assays. a: Genomic organisation of RTBV. ORFs and the splice donor and splice acceptor site are indicated with their positions within the genome relative to the transcription start site. b: Expression units and transient CAT expression. 1: Expression cassette with the RTBV promoter in which the reporter ORF (CAT) is interrupted by an RTBV derived intron. The expression of this construct is 100%. 2: Promoterless expression cassette. 3: Expression cassette with a deleted splice donor site. 4: RTBV-derived expression unit providing the splice donor site. The splice donor site and the splice acceptor site are located on different transcription units. 5: Conventional monocistronic CAT expression cassette. 6: "Dicistronic" expression construct consisting of a transcription unit covering an aph4 ORF, followed by the 3' part of the RTBV intron with a splice acceptor site followed by an ATG-less Barnase ORF. The CAT expression of 3-6 is caused by adding one construct after the other.
No expression was found with expression cassettes lacking either the promoter or the splice donor site. However, it was possible to express the CAT ORF, when the first exon and the first part of the intron was expressed from one transcription unit and the second part of the intron and the second exon from another one (Fig. 20b, 3 4). These results might reflect an experimental artefact, caused by recombination of the high number of DNA copies introduced into cells during transfection. Alternatively, they might indicate a mechanism also active in infected rice plants. Interestingly, the particular organisation of the RTBV expression unit with a polyadenylation signal located within the 5' region of the intron would allow the synthesis of an RNA comprising the first exon and parts of the intron. This RNA could serve as one partner of a trans-splice reaction. This so-called strong-stop RNA has been detected in transgenic plants as the most abundant transcript produced from the RTBV promoter (Klöti et al., 1999). It is most likely also produced by the invading virus. Therefore, we transformed plants with a "dicistronic" expression construct consisting of a transcription unit covering an aph4 ORF, followed by the 3' part of the RTBV intron including the splice acceptor site and an ATG-less Barnase ORF (Fig. 20b, 6). The Barnase ORF was constructed such that it would be in frame with the ATG in the exon part of the RTBV strong-stop RNA after a splice event. In protoplasts, it was possible to express in this way the bacterial RNase barnase, which led to an strong reduction of cellular gene expression activity (Fig. 20b, 4, 5, 6).

Unfortunately, it was not possible to regenerate transgenic plants with these constructs and therefore it was not possible to test this approach in plants.

3.6.4 (iv) Homology induced gene silencing

Gene silencing and virus resistance can be related phenomena (Vaucheret et al., 1998). An active copy of a transgene may be silenced when brought into contact with a silenced homologous copy. Silenced transgenes that are derived from a viral cDNA may also suppress the accumulation of viruses that have a homologous nucleotide sequence by post-transcriptional gene silencing (PTGS) (Baulcombe, 1996). In addition, transcriptional gene silencing (TGS) can affect any transgene that is expressed under the control of the same promoter, irrespective of the coding sequence being expressed (Vaucheret, 1993; Matzke et al., 1994). A transgenic rice plant containing a silenced promoter- or coding sequence homologous to RTBV sequences might therefore have the potential to display resistance to RTBV. Four different transgenic rice lines containing the GUS gene (Jefferson et al., 1987) have been evaluated for their potential to silence invading genes with sequence homology to the transgene (Fig. 21, 22 and Table 8). The GUS gene was regulated by either the CaMV 35S (pHCintG) or the RTBV promoter (pHRintG). Both plasmids are shown in Fig. 21. In both cases, a
shortened version of the intron/leader region of RTBV (RTBVint) was introduced as an expression enhancer (Klöti, 1996). RTBVint contained RTBV sequences from nucleotides 7404 to 7682 and from nucleotides 5924 to 5977 from plasmid pC4CABB (Fütterer et al., 1994). A silencing mechanism directed against the intron could also interfere with RTBV infection. The transgenic plants obtained with these constructs displayed different transgene expression patterns.

It has already frequently been shown, that episomal DNA or RNA can induce the silencing of chromosomal DNA (Covey et al., 1996; Angell and Baulcombe, 1997; Al Kaff et al., 1998). It remained, however, unclear, whether the trans-silencing activity of a chromosomal gene would be strong enough to downregulate multiple extrachromosomal gene copies as would be required for virus resistance based on such a mechanism.

A similar situation as in a virus infection could exist in transient protoplast expression experiments where also a large number of gene copies are introduced into cells. Therefore suspension cultures were established from selected rice lines with silenced and non-silenced transgenes. In such an easily obtainable, reproducible system, a trans-silencing activity could be tested.

3.6.4.1 Pre-evaluation for silencing effects in rice suspension cultures

Suspension cultures from seeds of transgenic lines transformed with pHClntG or pHRIntG (Fig. 21A) were established. These lines were called GUS 1, 3, 4. Prior to protoplast isolation, the expression status of the endogenous transgenes was tested in suspensions (Fig. 21B) and in cross-cuts from the corresponding seedlings 6 days after germination (Fig. 21C). The GUS expression was visualised with a histochemical GUS assay. The aph4 expression was tested by cultivation of the suspensions on selective medium containing hygromycin. GUS 1 was not silenced, blue cells appeared after GUS staining and the suspension was growing well in the presence of hygromycin. GUS 3 was almost completely silenced with only some blue staining cells. GUS 4 was completely silenced showing no blue cells after extensive GUS staining. Both suspensions were sensitive to the selective agent hygromycin. The silencing status of both transgenes, GUS and aph4, was equal in all suspension lines, no matter which promoter sequence was regulating transgene expression (Fig. 21B). The silenced transgenes, GUS and aph4 could be reactivated by adding the methylation inhibitor 5-azacytidine to the culture medium, indicating that the transgenes were silenced by DNA methylation. However, the silenced transgenes could not be reactivated by adding Trichostatin A, a potent inhibitor of the histone deacetylase. Both, DNA methylation and hypoacetylation of core histones are frequently associated with repression of gene expression by transcriptional gene silencing and the application
Results 59

of 5-azacytidine and Trichostatin A reactivated (trans-) genes successfully (Kumptla and Hall, 1998; Selker, 1998). The silencing status of the lines was not altered by protoplast isolation. However, in 6 day old seedlings the silencing status of GUS 3 and 4 was different. Seedlings of line GUS 3 were completely silenced and seedlings of line GUS 4 expressed at very low levels (Fig. 21C).

Fig. 21: A: pHClntG/ pHRintG: schematic description of plasmids that were stably transformed. CaMV 35S and RTBV represent the CaMV 35S promoter and the RTBV promoter respectively. The RTBV intron (RTBVint) is described in the text. △7412-7434 indicates a 22 bp deletion in the RTBV intron region only present in line GUS 4. Boxes in grey represent the sequences homologous to the RTBV genome. B: Phenotype of GUS suspension cultures 1, 3 and 4 cultivated on different media. C: Phenotype of respective seedlings, 6 days after germination. Sections are from the shoot at the base. 5 AC: 5-azacytidine, a DNA methylation inhibitor.
3.6.4.2 Evaluation of trans-silencing effects in transient

To detect possible trans-silencing effects, the expression of various reporter genes in transgenic and non-transgenic protoplasts was compared (Fig. 22). The endogenous transgenes of the protoplasts, their silencing status and the regulating promoter sequence are described in Fig. 21 and are also displayed in Fig. 22. Seven different plasmids containing different combinations of reporter open reading frames and promoters (Fig. 6, 22A) were used for protoplast transfection. Three plasmids per batch were co-transfected: one plasmid was coding for firefly luciferase (LUC), the other with the homologous promoter sequence was coding for glucuronidase (GUS). 3 or 10 μg DNA of both plasmids were used. The third plasmid was coding for renilla luciferase and 0.5 μg plasmid DNA were used for transfection. In protoplast batches transfected with plasmids coding for LUC, a possible trans-silencing effect would suggest transcriptional gene silencing (TGS), because only promoter sequences shared homology with the endogenous silenced transgenes. In batches transfected with plasmids coding for GUS, additionally effects caused by post-transcriptional gene silencing (PTGS) could be detected. Besides the coding sequence also the RTBV-derived intron was homologous to the endogenous transgenes. The plasmid coding for the renilla luciferase did not have any sequence homology to endogenous transgenes, therefore, all data were normalised to the expression of the renilla luciferase as an internal standard. The same protein extracts were used for measuring LUC and GUS activities.

No effect of silenced transgenes on LUC expression was observed in trans. Also with lower plasmid DNA amounts (3μg DNA), no reduction of LUC expression was found. A down-regulation of homologous genes by transcriptional silenced endogenous genes was not seen in protoplast transfection assays.

We expected that the GUS expression would be more difficult to determine, because of the variable GUS expression in the lines containing an endogenous gene coding for the glucuronidase. This problem was very apparent when the set of data obtained from the non-silenced line GUS 1 was analysed. These data could not be evaluated, because the endogenous GUS expression was too high. However, a significant reduction of GUS expression was found in both silenced lines, GUS 3 (CaMV 35S-promoter) and GUS 4 (RTBV-promoter). Their expression levels were reduced by 20%-60% compared to non transgenic WT suspension. This suggested the presence of a trans-silencing activity in lines GUS 3 and GUS 4 which was directed against the transcribed region of the reporter gene. The particular region(s) was not further defined. The extend to which PTGS might be effective in a transient expression assay might be very variable, particularly if a threshold model would apply. This model states that a certain RNA level has to be exceeded, before PTGS sets in (Baulcombe, 1996).
Fig. 22: Evaluation for silencing effects in rice protoplasts isolated from suspension cultures WT and GUS 1, 3, 4. A: Schematic presentation of plasmids that were used for protoplast transfection; pUBI-renilla which was used as internal standard is not shown (see Fig. 6, page 23). The promoter sequences of the plasmids: UBl, CaMV 35S, RTBV and the reporter sequences luciferase (LUC) or β-glucuronidase (GUS) are indicated. B: Three different experiments are represented (I, II, III). In experiment I, 3 μg, in experiments II and III 10 μg of plasmid DNA were used, respectively. Values are shown relative to the expression in non-transgenic suspension WT (=100%) and represent the average of 3 independent batches of transfections. Yellow, grey, and red bars correspond to the expression regulated by the maize ubiquitin 1 (UBl), the CaMV 35S (CaMV 35S) and the RTBV promoter, respectively. The promoter regulating the endogenous GUS expression is indicated by (R) (RTBV promoter) and by (C) (CaMV 35S promoter). Silencing of endogenous GUS is indicated (silenced).
# 3.7 Tests for virus resistance

## 3.7.1 Lines and transgenes

At the International Rice Research Institute (IRRI) 20 independent transgenic lines produced at the ETH Zürich were tested for resistance to RTD (Table 8).

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<td></td>
<td>10A, 10B</td>
<td>++</td>
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<tr>
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<tr>
<td>pORF IVAC</td>
<td>Dicistronic expression construct, expressing the N-terminal part of the ORF II gene product</td>
<td>5, 5 T₂, heterozygous, 5 T₃, homozygous</td>
<td>+++ (all generations)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9A, 9B</td>
<td>+</td>
</tr>
<tr>
<td>pORF 780</td>
<td>Antisense sequence of a large region of ORF 4 (nts 6482-7241 of the RTBV genome)</td>
<td>AS1L</td>
<td>+++</td>
</tr>
<tr>
<td>pORF 780</td>
<td>Antisense sequence of ORF I (nts 7858-123)</td>
<td>AS21</td>
<td>++</td>
</tr>
<tr>
<td>pORF 780</td>
<td>Antisense sequence of the RTBV leader region including translation start of ORF I (nts 7858-123)</td>
<td>AS22</td>
<td>+++</td>
</tr>
<tr>
<td>pORF90</td>
<td>Antisense sequence of a small region of ORF 4 (nts 7122-7214)</td>
<td>AS1S</td>
<td>+++</td>
</tr>
<tr>
<td>pHRintG (Fig. 21)</td>
<td>plant containing the GUS gene under the control of the RTBV promoter</td>
<td>GUS 1</td>
<td>+++</td>
</tr>
<tr>
<td>pHRintG (Fig. 21)</td>
<td></td>
<td>GUS 2</td>
<td>+</td>
</tr>
<tr>
<td>pHCintG (Fig. 21)</td>
<td>plant containing the GUS gene under the control of the CaMV 35S promoter with RTBVint</td>
<td>GUS 3</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 8: Summary of lines tested at the IRRI. First column indicates the plasmid, which was used for transformation of the lines. Column 2 contains a short description of the transgene. The numbers of the third column (plant lines) correspond to the numbers in Southern and northern analyses of Figs. 14 and 16. The lines GUS 1 and GUS 3 are referring to the suspensions tested in transient protoplast expression assays. The transgene expression level is indicated in column 4: (-) no detectable expression. The scale for detectable mRNA levels by northern blot analysis was (+) for weak and (++) for high RNA-levels.
3.7.2 Efficiency of virus infection
In the CL4 containment facility uniform infection of the rice lines was achieved with 4-6 adult viruliferous *Nephotettix virescens* D. From most lines 30 plants were individually infected 11 days after germination. From some lines, less plants were tested due to the limited number of seeds. Virus infection was determined by symptomatology (assessment of symptom severity (SS) and ELISA. Infection of plants with viruliferous *N. virescens* carrying RTBV and RTSV occasionally results in transmission of only one virus. Less than 0.4% of the plant population escaped the infection by RTBV and in general, about 7.5% of the plants were infected with RTBV only but not with RTSV. In ORF II\(\Delta\)N line 8A (T\(_2\)) 40% of the individual plants were infected with RTBV only but not with RTSV.

3.7.3 Assessment of the symptom severity (SS)
The symptom severity of RTD was assessed 13 days after virus infection by visual inspection of the plant phenotype. The height of the infected plants and the leaf discoloration was compared to non-infected plants and they were scored according to an established scale (Hassanuddin et al., 1988). All transgenic lines displayed symptoms of virus infection. The average symptom severity of the lines was around 7 (Fig. 23a; 31-50% plant height reduction, with yellow to orange leaf discoloration, orange section in Fig. 23b). The differences between individuals of one transgenic line are represented by the different colour of the section referring to the respective scores (Fig. 23b). Particular individuals from the lines containing RTBV ORF IV sequences displayed lower SS-scores (Fig. 23a; 23b, green sections), indicating that the plants were less damaged by virus infection. The same observation was made with some other plants, in particular of the T\(_2\) generation, containing the amino-terminally deleted sub-fragment of RTBV ORF II (II\(\Delta\)N).
Fig. 23a: Mean value of all lines sorted by the construct used for transformation. The average SS score is shown on the y axis, the transgene construct and the number of plant lines (between brackets) is shown on the x-axis.

Fig. 23b: Symptom severity scores (SS) for 34 transgenic lines that were tested for virus resistance 13 days after inoculation. Bright green sections, dark green sections, orange sections and brown sections indicate the percentage of plants displaying SS scores of 3, 5, 7 and 9, respectively. This score is ranging from 1 for resistant to 9 for sensitive plants. Lines are sorted for different constructs (IIC, IIIN, IVC, AS, GUS). The numbers of the dicistronic plant lines (IIC, IIIN, IVC) refer to numbers in Southern and northern blot analyses (Figs. 14 and 16). Lines GUS 1 and GUS 3 correspond to suspensions tested in transient protoplast assays. AS indicates lines containing antisense sequences. WT indicates the non-transgenic control line. Dicistronic and antisense lines are the T₁ progeny of primary transformants (not labelled). T₂ indicates the T₂ progeny, specified with he (heterozygous) and ho (homozygous). Lines GUS 1, 2, 3 are homozygous.

The phenotype of line ORF IIΔN 8A (T₂), line ORF IVΔC 5 (T₂/ho) and 5 (T₂/ho) and line GUS 2 was reassessed 24 days after virus infection in comparison to uninfected plants and to the infected WT plants (Fig. 24). The plants of the ORF IIΔN line were reduced in height, compared to the non infected control, but leaf discoloration was less prominent (Fig. 24d). This may be due to an infection with RTBV only. The plants of the ORF IVΔC line 5 (hetero- and homozygous) were also reduced in height. However, the plants still were significantly taller than all infected transgenic and non-transgenic plants (Fig. 24 e, f) and displayed a "slightly" recovered phenotype. A long term observation of 30-40 after virus infection of the plants was not possible because the experiment had to be terminated due to administrative problems.
Results

3.7.4 Detection of rice tungro viruses by ELISA

The enzyme-linked immunosorbent assay (ELISA) was performed 13 days and 27 days after virus infection. No plant line resistant to RTBV was found. ELISA data confirmed the results of the symptomatological assessment, since RTBV coat protein could be detected in each line. Fig. 25 displays the data of RTBV-titers determined by ELISA at d 13 (dark gray box) and d 27 (light grey box). On day 13, virus particles were generally more abundant than on the day 27 as is typical for RTD progression.

The values of lines (IIC) at day 13 could not be evaluated precisely due to technical problems and are not shown, however, values at day 27 were significantly increased, indicating high virus titers.

Most of the lines (IIN) had high RTBV titers at day 13 and day 27. The only exception was line ORF II\ldots N 8A (T\textsubscript{2}). Here the values measured at day 13 were significantly decreased compared to the other lines transformed with the same construct (IIN) and even to the plants of the T\textsubscript{1}-generation of the same line (8A). Note, that 40% of line 8A (T\textsubscript{2}) was infected with RTBV only.
Slightly decreased values were found with the ORF IV 5 lines. Especially, the homozygous T2-line displayed reduced titers at both time-points. Lines containing an antisense sequence of the RTBV genome (AS) did not show any effect on virus titers.

All GUS transgenic lines (GUS) contained sequences homologous to RTBV (compare with Fig. 21). The lines had already been tested in transient expression assays for a trans-silencing effect on transfected DNA. In these assays, silenced endogenous genes reduced GUS expression levels probably due to post-transcriptional gene silencing (see chapter “Homology induced gene silencing”). In the virus resistance assay, the lines GUS 1, 2, 3 displayed reduced virus titers at day 13, irrespective of the initial silencing status of the transgenes. At day 27 reduced titers were found only in initially unsilenced line GUS 1, whereas the other two lines displayed higher titers.

![Fig. 25: ELISA titers of 23 transgenic lines found in a virus resistance assay. Dark grey box: average titer on day 13. Pale grey box: average titer on day 27. The numbers of the plant lines correspond to the numbering in Fig. 23b. y axis displays the absorbance at 405 nm. The absorbance is proportional to the amount of RTBV coat protein.](image)

3.7.5 Analysis of transgenes during virus infection

Transgene expression was analysed in infected plants to screen for possible effects of virus infection on transgene expression levels. It should be possible to correlate the transgene expression to virus proliferation. In particular those lines with special phenotypic appearance and reduced virus titers were tested.

3.7.5.1 RNA level

A northern blot analysis was performed 4 days after virus infection. Samples of uninfected line ORF IIΔN 5 and line ORF IVΔC 5 (T2 ho) were compared to their infected counterpart. No difference in transgene expression was found. The homozygotic line ORF IVΔC 5 (T2 ho) and the line ORF IIΔN 8A (T2) were tested with a another northern blot analysis 24 days after virus infection (Fig. 26).
Surprisingly, in line ORF IV\textDelta C 5 the transgene expression varied among individual plants. Most likely, this variation was due to virus infection, as the uniform expression of the homozygous line was verified previously by northern blot and segregation analysis.

A comparable variation of mRNA expression in individual plants was found in ORF II\textDelta N line 8A (T\textsubscript{2}) although in general the expression levels were lower than those of line ORF IVC 5. However, the homozygous state of this line was not confirmed, and the variation may be due to segregation. It was surprising that the line was expressing transgenic mRNA. In previous segregation and northern blot analyses, transgenes in plants of the same generation and of the T\textsubscript{1} generation were apparently silenced (see Fig. 16).

<table>
<thead>
<tr>
<th>a) IV 5 (T\textsubscript{2} Ho)</th>
<th>b) II\textDelta N 8A (T\textsubscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>day 13</strong></td>
<td><strong>day 27</strong></td>
</tr>
<tr>
<td>SS</td>
<td>RTBV</td>
</tr>
<tr>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td>7</td>
<td>1.9</td>
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<tr>
<td>7</td>
<td>1.9</td>
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<tr>
<td>7</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>1.7</td>
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<td>7</td>
<td>2.2</td>
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<td>5</td>
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<td>2.1</td>
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<td>1.3</td>
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<td>1.0</td>
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<tr>
<td>3</td>
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<tr>
<td>3</td>
<td>0.8</td>
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<td>3</td>
<td>1.9</td>
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<td>7</td>
<td>0.0</td>
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<tr>
<td>3</td>
<td>2.3</td>
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<tr>
<td>c) WT</td>
<td></td>
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<tr>
<td>7</td>
<td>2.4</td>
</tr>
<tr>
<td>7</td>
<td>2.5</td>
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<tr>
<td>7</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**Fig. 26:** Correlation of SS-scores, RTBV-/RTSV-titers, and transgene-derived mRNA levels (24 days after inoculation) in lines (a) ORF IV\textDelta C 5 (T\textsubscript{2} ho), (b) ORF II\textDelta N 8A (T\textsubscript{2}) and (c) WT. The symptom severity-score (SS), the RTBV/RTSV titers as values of the absorbance at 405 nm at day 13 and day 27 and the mRNA-levels ranging from 0 to 6 are shown. RNA was isolated from virus infected leaves, and hybridised with a probe corresponding to the aph\textsubscript{4} part of the bicistronic mRNA.
It was not possible to correlate the SS scores of individual plants with transgenic mRNA levels or with the virus titers, emphasising that the effects found in this experiment are not very strong.

3.7.5.2 Protein level

ORF II protein levels in uninfected lines were compared with infected lines (Fig. 18). ORF II protein resulting from virus infection (8inf and WTinf) was much more abundant than protein encoded by the transgenes. The presence of the carboxyterminal subfragment of ORF II (lane 8) did not have an impact on virus derived protein levels (lane 8inf).

Detection of ORF IV derived proteins was not efficient. No distinct protein could be detected in transgenic or virus infected plants (Fig. 27). Instead, in the infected non-transgenic plants (WT) a signal was found at the top of the protein gel. In callus material of a line containing the aminoterminal subfragment of ORF IV, a similar signal at the top of the gel was observed (Fig. 27b). Interestingly a comparable signal was neither found in the infected ORF IV transgenic lines (5T1, 5ho, 9B) nor in the uninfected WT. It remains unclear whether the signal found at the top of the gel is specific for the ORF IV protein. However, it was speculated that the ORF IV protein was involved in protein-protein interactions. The band on the top of the gel might be a protein-multimer. The absence of the high molecular weight band in transgenic infected plants could be due to a transgene induced down-regulation of ORF IV protein. This might be related to the improved phenotypical appearance of the ORF IV lines.

![Fig. 27: Western blot analysis of 3 ORF IV lines](image)

Proteins analysed in (a) are isolated from non-infected or infected leafs, proteins analysed in (b) are isolated from transformed callus. The numbers on top correspond to the numbers in Figs. 14 and 16. The predicted protein size is indicated by a black arrow, the observed signal by an open arrow. ho: homozygous. WT: non-transgenic plant. x: callus line.
4 DISCUSSION

4.1 RTD resistance with genetically modified plants

Tungro is the most important virus disease of rice in tropical Asia. Currently, there are no conventional sources of resistance. Tungro has become increasingly important since the mid-1960s, when planting of high-yielding cultivars was started and double-cropping of rice became common in irrigated areas (Hibino, 1996). An integrated tungro management to solve the tungro problems to some extent involves the use of chemicals active against the virus vectors, a synchronous planting, allowing a break between successive plantings, or a varietal rotation using rice varieties with different green leaf hopper resistances (Manwan et al., 1985). Despite considerable efforts of many Asian countries, major outbreaks of devastating scale could not be averted. Therefore, intensive research is done to find resistance by genetic approaches in national and international research institutes of tropical countries like Malaysia, Indonesia, The Philippines and also in research institutes of many temperate climate countries like Switzerland, United Kingdom and the United States (The Rockefeller Foundation, 1999). In this study we describe transgenic approaches to obtain resistance against RTBV.

Despite the efforts of several groups to engineer resistance to RTBV it was not possible until now to produce any form of resistance to any plant pararetrovirus. In other cases, the concept of pathogen-derived resistance developed in 1985 by Sanford and Johnston has found a broad application and numerous attempts to generate virus resistance were successful (Wilson, 1993; Lomonossoff, 1995; Beachy, 1993; Baulcombe, 1996; Pinto et al., 1999). Successful examples have been reported almost exclusively for RNA-viruses, and only few cases of resistance to the single-stranded DNA gemini-viruses are known (Hong et al., 1996; Bendahmane and Gronenborn, 1997; Duan et al., 1997). The fact that the DNA of double-stranded DNA pararetroviruses like RTBV or CaMV persists in the nucleus of infected cells and that their replication cycle involves a reverse transcription step might help them to overcome the common concepts of resistance that work for RNA-viruses. The circular double-stranded DNA genome present in many copies in the nucleus of an infected cell is certainly much more stable than the cytoplasmic RNA genomes of most other plant viruses. The pararetroviruses share the reverse transcriptase activity with retroviruses like HIV. This activity might enable them to mutate rapidly during their replication cycle (Pooggin et al., 1998). As a consequence, the virus might quickly overcome single-gene based resistance by mutation. This presumably high mutation rate of pararetroviruses and RNA viruses has to be considered when choosing the
Discussion

strategy to engineer resistance. Mechanisms requiring perfect sequence homology between a transgene and an infecting virus, like e.g. RNA-mediated resistance (reviewed in the introduction), may result in a less durable resistance. Therefore, mechanisms relying on disturbance of functional protein interactions might provide protection against a broader range of virus strains and for a longer period. However, such mechanisms require the stable expression of viral proteins or variants of such proteins.

Various attempts to confer resistance of rice to RTBV failed (Azzam et al., 1999). A possible reason for the lack of protection could have been the low expression level of the transgenes used thus far (Klöti, 1996). In most experiments it was not possible to generate a transgenic plant line highly expressing RTBV-gene products, even though a large number of different genes and gene combinations was transferred into rice. In almost all lines expression of the genes of interest was not detectable although very sensitive detection methods were used. Therefore, in this study a new expression strategy was designed to increase gene expression by translational coupling. This coupling was achieved by the unusual leaky scanning translation mechanism.

4.2 The concept of translational coupling

The coupling of two ORFs on a single, dicistronic mRNA offers a number of advantages in genetic engineering. Most important seems to be the automatic selection of the expression of the non-selectable gene of interest, if the other ORF encodes a selectable function. The increase of expression is due to the selection pressure during the generation of transgenic plants. Coupling will also allow early recognition of problems that may be associated with particular genes of interest, e.g. toxicity of the encoded product or RNA instability. In both cases, regeneration of transgenic plants will be much less efficient. Therefore, transformation with dicistronic expression cassettes will reduce the amount of work that usually is invested in regeneration and analysis of lines that express only the selectable marker but not the gene of interest.

Despite these obvious advantages, the coupling of two ORFs has not found a wide application so far. The problem is that the translation of two independent ORFs located on one mRNA is normally not possible in eukaryotic cells, because eukaryotic ribosomes usually can initiate translation only once and can enter the mRNA only at the 5' cap resulting in translation of only 5' proximal ORFs (Fütterer and Hohn, 1996). Therefore, in polycistronic expression units expression of a downstream ORF is strongly reduced by an upstream ORF.

Due to this translational restriction, strategies were developed to express polyproteins and separate individual polypeptides after translation. Halpin et al.
(1999) engineered two reporter proteins on one mRNA which were linked by a sequence of 16-20 amino acids derived from the foot-and-mouth disease virus (FMDV). This sequence could mediate cleavage at its own C-terminus in a heterologous protein context and the authors showed efficient cleavage of the polyprotein and co-ordinate expression of the two reporter genes in transgenic tobacco. A similar approach was reported by Marcos and Beachy (1997). A cotransfected protease was derived from tobacco etch potyvirus (TEV). Problems associated with this approach are the specificity and extent of processing, potential pleiotropic effects of the included protease, and the possible instability or inactivity of the resulting proteins, which may have improper N- or C-termini. However, plant-viruses provide strategies to express genes in a cap-independent way. Therefore, in various attempts the potential of virus-derived strategies was examined to express separate proteins on a single mRNA in dicistronic expression cassettes: Hefferon et al. (1997), Ivanov et al. (1997) and Skulachev et al. (1999) reported of the potential use of internal ribosome entry sites (IRES) derived from potato virus X or from a crucifer infecting tobamovirus (crTMV), respectively. A similar translation signal which stimulates translation from internal sequences of an mRNA was found in the genome of the barley yellow dwarf virus (BYDV, Wang et al., 1997) and the tobacco etch virus (TEV, Niepel and Gallie, 1999). In transformation systems of mammalian cells, such IRES have been used with great success (reviewed in Houdebine and Attal, 1999). However, in transgenic plants it still has to be shown whether the reported examples will allow reliable and efficient internal ribosome entry. Plant virus-derived IRES sequences did function on the 5' proximal end of the mRNA, but not efficiently as real intercistronic sequences in dicistronic expression units.

4.3 The unusual leaky scanning translation mechanism

With this study we showed for the first time that the leaky scanning mechanism of translation offers an alternative that could be used in any transformation system, provided that the first ORF on the dicistronic mRNA has appropriate properties. The unusual leaky scanning translation mechanism is naturally used by RTBV, but also by related badnaviruses to express several proteins from one polycistronic mRNA (Fütterer et al., 1997). Polycistronic translation by leaky scanning occurs also with a phytoreovirus RNA (Suzuki et al., 1996) and some mRNAs produced by complex mammalian retroviruses (Schwartz et al., 1990; Carroll and Derse, 1993). This mechanism is possible since the upstream ORFs of these viruses lack internal AUG codons that could intercept scanning ribosomal subunits. Ribosomes that have failed to recognise the first start codon can thus migrate to a second or even third ORF. The start codons are probably missed because their sequence
context is not optimal for initiation. The sequences flanking the AUG codon modulate the proportion of scanning ribosomes that recognise it and start translation of the ensuing ORF (Kozak, 1997).

In dicistronic constructs useful for plant transformation, the upstream ORF should therefore have a start codon in an unfavourable sequence context to allow the passage of initiation-competent ribosomal subunits to a downstream ORF. In addition the ORF should contain only very few, or preferably no internal ATG codons that might serve as alternative initiation sites.

4.3.1 Adaptation of ORFs to dicistronic expression

In our model dicistronic expression units RTBV ORF I or II, which are naturally adapted to the leaky scanning mechanism, and other ORFs adapted by mutagenesis were tested. RTBV ORFs I and II do not contain any internal ATG codon. A 5' part of RTBV ORF IV and a "normal" not virus-derived PAT ORF with 2 or 5 internal ATG codons, respectively were used as originally non-adapted sequences.

The naturally adapted sequences RTBV ORF I and II as upstream ORFs did not allow any pronounced downstream GUS reporter expression. However, a GUS ORF positioned downstream of the 5' part of RTBV ORF IV was highly expressed despite the presence of two additional internal ATG codons. This was an unexpected result because with the PAT ORF, such codons were very inhibitory—as would be expected from the scanning model of translation in eukaryotes (Kozak, 1989b; Kozak, 1999). It is not clear, why the internal ATG codons of RTBV ORF IV are not interfering with downstream expression. In transfected protoplasts, the RNA levels were not determined. Therefore, we cannot exclude that the presence of the ORF IV sequence leads to higher steady state RNA levels by somehow increasing mRNA stability and thus allowing a higher expression of the β-glucuronidase. However, the northern data of the transgenic plants expressing dicistronic ORF IV-aph4 mRNA disprove these arguments because mRNA was not more abundant in the ORF IV-lines compared to the ORF II-lines.

An effect of the upstream ORF sequence on mRNA stability is the most likely explanation for the low expression obtained with the unmodified RTBV ORFs I and II. Another reason may be a reduced production of dicistronic ORF I and II mRNA. However, the sequence characteristics of the deduced mRNA sequences favour the hypothesis of mRNA instability. Both ORFs are located in a conditionally removed intron of the RTBV genome (Füterer et al., 1994). It is possible that sequence features that contribute to rapid degradation of excised introns also confer instability to these dicistronic mRNAs. The attempts to increase expression levels by sequence modifications failed with ORF I. Further sequence
modifications would be necessary, e.g. a further increase of the GC content or some deletion analyses in order to identify specific sequences destabilising elements within the ORF I. With ORF II, a general increase of GC content improved expression significantly. Additional increases were obtained with deletion variants lacking either the first 105 nts or the last 42 nts of the ORF. The further increase of GUS expression for the 3' part of ORF II in comparison with the other ORF II versions could be explained by an RNA increase due to the lack of destabilising elements within the deletion variant. Another explanation could be the weaker upstream start codon context of the 3' part of ORF II, causing an increased leakiness and better downstream expression.

The adaptation of the PAT ORF was performed by elimination of all internal ATGs. Only one conserved methionine exists within this sequence, which was replaced by the different amino-acids, valine, leucine or threonine. The mutated PAT proteins retained significant PAT activities.

4.3.2 Modulation of expression levels by variation of the start codon context

In the best case, with the adapted 3' part of ORF II, 84% expression of a downstream GUS ORF was obtained compared to the same GUS ORF on a monocistronic mRNA. More frequently, dicistronic downstream expression levels varied between 10% and 50%. The adapted PAT-GUS dicistronic construct was found to be in the range of about 10% downstream GUS expression. With a modulation of the start-codon context we tried to increase this expression.

With the adapted PAT ORF the translation start-codon context was modulated to quantify the contribution of nucleotides located at flanking key positions –3 and +4. A strong contribution of each nucleotide to translation initiation was found. A strong translation start was found in the presence of G and A at position –3 and G at position +4. Translation start was particularly weak when T and C were present at position –3 and C at position +4. The dicistronic GUS expression compared to a monocistronic control was varying between 1% for a strong translation start context and 30-40% for a weak context. The present study is the first in which the impact of each nucleotide on the key flanking positions of translation start was quantified in plant cells.

The strong contributions of A⁻³ and G⁺⁴ have been verified by mutagenesis in many different experimental situations (Descombes and Schibler, 1991; Dinesh Kumar and Miller, 1993; Cao and Geballe, 1995). The data found in this study coincide with consensus sequences of the respective positions in eukaryotic genes (Chandrashekhar et al., 1997). Furthermore, the optimal context for
initiation of translation in eukaryotic mRNAs is a G residue following the ATG codon (position +4) and a purine, preferably A, three nucleotides upstream position −3, (Kozak, 1987; Kozak, 1991).

The study illustrated also another practical advantage of dicistronic expression constructs. The impact of changes in the protein coding region on expression can be easily monitored by measuring the expression of the linked GUS ORF. Possible effects of the respective changes on protein stability or activity (e.g. by changes of the first codon at position +4 of the PAT ORF) have not to be considered and no special antisera or RNA probes are required.

With different nucleotide combinations within the upstream translation start context, regulated expression of two proteins located on one single mRNA could be achieved. Other possibilities to differentially affect translation of the two ORFs could be the introduction of short upstream ORFs (Fütterer and Hohn, 1992) or variation of the length of the 5′ untranslated leader region (Johnston and Rochon, 1996). For a general application of this system, it remains to be seen how far PAT expression can be reduced to still support effective selection. A translationally down-regulated PAT ORF might be useful for selection of plant lines with particularly high RNA levels.

4.4 Plant transformation

Transformation methods in plant genetic engineering are continuously developed. Using protoplast technology and direct DNA transfer, the first transgenic rice plants were recovered by Toriyama et al. (1988) and Zhang et al. (1988). Since then many reports describing the generation of transgenic rice were published and the range of transformed genotypes, transformation methods and efficiencies expanded and improved significantly. The protoplast technology lost its importance due to the reported problems. Transgenic plants very often developed abnormal phenotypes like albinism, stunted growth and sterility. When this work started, the most common method for rice transformation was the particle bombardment of calli derived from the scutellum of immature rice embryos (Christou et al., 1991) or the bombardment of embryogenic cell suspensions. This rice transformation method made it possible to generate high numbers of transgenic rice plants with desired features and improved breeding qualities (Wünn et al., 1996; Burkhardt et al., 1997; Lucca, 1999; Ye et al., 2000).

However, the production of plants with desirable new phenotypes created by the introduction of foreign DNA did not advance as it was predicted (Finnegan and McElroy, 1994). Often these problems were linked to transgene inactivation or mRNA destabilisation. The plants produced in the present study were selected for increased mRNA levels of the gene of interest.
4.4.1 The use of unconventional expression constructs

Transgenic plants were obtained with dicistronic expression constructs containing 3' end truncated RTBV ORF IV sequences, modified RTBV ORF II sequences in full-length or 5' end and 3' end deleted form, and a modified PAT sequence. In the expression constructs, the selectable marker sequence was located downstream of the "leaky" sequence coding for the protein of interest. The leakiness of the upstream sequence was tested in advance in transient expression assays. For leaky upstream sequences, it was possible to apply the same selection regime as during conventional transformation procedures. The strong signals of RTBV ORF IV and II sequences in northern analysis indicated that the dicistronic expression strategy was an efficient way of expressing transgenes in plants. However, the detection of ORF IV protein was not efficient. The reason for this may be an insufficient recognition of the protein by the ORF IV antibody or instability of the protein. An additional problem was the lack of a positive control since it was not possible to detect RTBV ORF IV protein in infected plants (Hay et al., 1994). Therefore, this detection problem may also be related to specific features of the protein. In contrast, the production of RTBV ORF II-protein from dicistronic expression constructs was demonstrated.

It was a priori unclear to what extent the results obtained in the transient expression assay would allow to predict the properties of the constructs in selection of transgenic plants. Therefore constructs with no or very low expression of the downstream GUS ORF were included in the stable transformation experiments. It turned out that the rapid expression analysis in protoplasts allowed a very reliable prediction on the transformation success of a particular construct. It was impossible to regenerate plants with constructs with poorly expressed downstream ORFs, as e.g. in the case of RTBV ORF I. The protoplast system can therefore serve as a simple test system to evaluate future transgene expression in plants. Similar observation were made in a comparison of a natural and a modified Bt gene (Wünn et al., 1996).

The failure to obtain stable transformants with dicistronic constructs containing RTBV ORF I and unmodified ORF II sequences was probably caused by sequence features located on the RTBV ORF moiety that contributed to rapid degradation of these dicistronic mRNAs. Any such RNA-stability or -production problem would probably also occur, when these sequences were used as monocistronic expression units. This was at least experienced with the expression of RTBV ORF I in monocistronic expression constructs in transgenic plants (Klöti, 1996). In these experiments the transgenic plants had integrated the ORF I sequence as detected by Southern blot analysis. However, the gene of interest, the ORF I product, was not expressed. In the present study we were able to
identify the low expression of RTBV ORF I and unmodified ORF II in an early stage by a low regeneration efficiency of the respective plant material. This experience emphasised an advantage of the dicistronic selection system. However, these examples also showed that the coupling of a gene of interest to the selectable marker does not guarantee regeneration of transgenic plants. A sufficient expression level of the selectable marker sequence was important because it has to be considered that all plants generated with constructs that produced only low (<30%) expression of the downstream ORF in transient protoplast assays were stunted and infertile. It remains to be seen whether this phenotype was accidental or was due to the toxic effects of the strong selection procedure, or in the case of ORF II, might have been linked to a feature of the expressed sequence.

The failure to obtain stable transformants with constructs which poorly expressed downstream ORFs also indicated that activation of expression of the selectable marker by plasmid rearrangements was very rare. Even though Southern blot analysis showed that rearrangements can occur, as is always the case with methods of direct gene transfer. It is likely that these rearrangements did not frequently change the linear order of the different elements in dicistronic expression cassettes.

### 4.4.2 RNA-levels, -quality and gene silencing

With the strategy to express genes in a dicistronic context, we hoped to increase RNA-levels and as a direct consequence to increase the probability of obtaining a desired phenotype. It has been previously reported that plants with very high mRNA expression levels are prone to silencing (Angell and Baulcombe, 1997; Al Kaff et al., 1998). This phenomenon has been explained by the threshold model for homology-dependent gene silencing (Baulcombe, 1996). This model postulates a cellular mechanism that detects high levels of mRNA and leads to subsequent RNA degradation. Since we obtained plants with high expression of dicistronic mRNA, we may expect higher frequencies of silenced plants. However, the frequency of silencing appears at least not to be higher for dicistronic compared to monocistronic constructs. Previously, we have observed gene silencing in transgenic rice at a frequency of 20-30% (Klöti et al., unpublished observations). In general, to evaluate the propensity to be silenced, transgenic lines would have to be observed over more generations; we have analysed expression in one ORF IV line over four generations and the expression was stable.

Another model to explain homology dependent gene silencing suggests that silencing is triggered by the production of aberrant RNAs (aRNAs) from a (trans)gene (Baulcombe, 1996; Wassenegger and Pélissier, 1998). The aRNA
serves as template for a cellular degradation machinery that consequently degrades all RNAs homologous to the aRNA (Wassenegger and Pélissier, 1998). The transformation procedure with dicistronic expression cassettes and the selection pressure may also cause gene rearrangements that lead to aRNA production causing gene silencing. Indeed we found usually many rearranged gene copies integrated into the genome. However in most cases we observed stable mRNA expression. Furthermore, DNA rearrangement changed the linear order of the regulatory elements of the dicistronic expression units only very seldom. In only two plants only marker sequence was expressed. Despite the selection pressure towards high gene expression, we also obtained transgenic lines with no detectable expression in northern blot analysis. It is worthwhile to mention that the expression of these transgenes must have been ceased after the transfer of the calli to regeneration medium, as before this time they were exposed to a selection pressure. In addition, we found two lines, transformed with dicistronic expression units that possibly produced aRNA. One line did not contain the expected fragment of the dicistronic expression cassette and was expressing the selectable marker sequence only. The other line contained multiple copies of the transgene. In both cases, the transgenic sequences might not have been flanked by proper promoter-/terminator-sequences and Southern blot analysis indicated that illegitimate recombination probably occurred within the regulatory elements. As a result of this lack of regulating expression elements, irregular termination of mRNA synthesis is most probable. Baulcombe and English (1996) reported that irregular transcription termination can lead to aRNA. It would also be possible that the production of aRNA was preceding the silencing of the transgenes in dicistronic lines containing multiple copies of the transgene. Notably we observed aRNA production and silencing mainly with lines where we never could produce fertile plants (full-length protein of RTBV ORF II and a particular antisense RNA in construct paRNA 14). Maybe, the products of these ORFs were toxic to some degree and favoured selection for rearrangement in transformed cells.

To reduce the transgene copy number, alternative methods like Agrobacterium-mediated transformation may be useful. Advantages of this method include a low copy number of the integrated transgene, the transfer of relatively large DNA segments, and high quality and fertility of transgenic plants. Efficient Agrobacterium-mediated rice transformation protocols have been published previously (Hiei et al., 1994; Aldemita and Hodges, 1996; Dong et al., 1996; Toki, 1997).

However, the correlation between the frequency of silencing and transgene copy number is disputable (Kohli et al., 1999; Pinto et al., 1999) and was also not evident for our own work.
4.5 The general use of dicistronic expression constructs

Most natural ORFs in their native forms will not be usable in dicistronic expression units, translated by leaky scanning, because they lack the special sequence features required for an upstream ORF. From this work it can be expected that several ORFs could be adapted in a similar way as the PAT ORF. Other selectable ORFs could possibly be adapted for the use in dicistronic expression constructs, e.g. the mouse dihydrofolate reductase ORF, conferring resistance to methotrexate. It is also a short ORF and contains few ATG codons. The internal methionines in different aminoglycoside phosphotransferases are not conserved and could probably also be altered without interference with protein function.

However, for many ORFs adaptation will not be possible or will prove to be too demanding, if the functional consequences of too many amino acid changes have to be evaluated. As an alternative, the adapted PAT ORF could be used as the upstream, selectable ORF. In this case, the problem of loss of the payload ORF through transgene truncation or rearrangement may be slightly more relevant. In a former study, some variation of mRNA length was found in transgene transcripts of plants expressing a 3' end fusion of an RTBV antisense RNA and the aph4 sequence (Henrich, 1996). The situation may be different with translated 3' end mRNA sequences. Therefore the majority of regenerated plants would still be expected to contain the intact, dicistronic cassette. Selection with PPT or related compounds did not work for rice in our hands. However, this selective agent is useful for a large number of plant species and the adapted ORF could thus be used in many transformation systems. Furthermore, the coupling allows an easy detection of the expression of the gene of interest because in this way the expression is linked to a phenotype like resistance to an antibiotic or a herbicide. The PAT ORF as selectable marker would allow an easy selection of the plant progeny still expressing the gene of interest. Silenced plants could be eliminated by herbicide treatment, without laborious analysis of transgene expression. A further advantage is the possibility to use the same probe in Southern or northern blot analyses with all different genes of interest when fused to the same marker sequence.

The use of dicistronic mRNAs also would reduce the number of promoters required. In addition to the RNA mediated gene silencing mechanisms discussed above, silencing can also be provoked by repetitive DNA elements, particularly promoters (Vaucheret, 1993; Vaucheret et al., 1998). It could be beneficial for long term expression stability to avoid repeated use of certain promoters (Finnegan and McElroy, 1994). Applications requiring the transfer of several genes are e.g. metabolic engineering or quantitative disease resistance. Additionally, the relative expression levels of two proteins from a dicistronic mRNA could probably be regulated more reliably on the translational level than by
transcription of independent genes. However, a limiting factor may be, that tissue specific promoters can not be used in a dicistronic system in combination with a selectable marker. Selection on callus level would be impossible.

4.6 Virus resistance tests

For applied purposes of genetically engineered virus resistance, true immunity would be the most desirable trait. However, a confinement of invading viruses to initial infection sites by a hypersensitive response would also be useful. Engineered tolerance might be achieved by an overall reduction of virus titers and could lead to economically useful effects. On a long term this strategy may be less safe since the virus would have a good opportunity to evolve to a more successful pathogen again.

In many of the published cases of engineered virus resistance a form of "true immunity" may have been achieved (Register and Beachy, 1988; Lindbo et al., 1993) However, in other cases increases of tolerance or recovery from infection have been also described (Guo and Garcia, 1997; Duan et al., 1997; Ingelbrecht et al., 1999). "Recovery" is characterised by a delayed form of resistance which is only induced a week or more post inoculation with a virus (Baulcombe, 1996). It leads to a healthy appearance of new plant parts, even though older parts show symptoms of infection.

Our assays with tungro infection of various transgenic lines clearly showed that none of the lines displayed any strong resistance. However, in some lines (GUS 1, 2, 3 and ORF IVÅC) RTBV accumulated to slightly lower levels at the latest time-point analysed. Line ORF IVÅC also showed reduced symptoms with less stunting and increased tillering. However, while in the described "recovery" phenotypes, new plant material tends to be virus-free (Lindbo et al., 1993; Swaney et al., 1995; Goodwin et al., 1996) in our rice lines still considerable virus levels were detected. No particular increase of disease tolerance could be observed with plants transformed with the amino- (IIÅN) or carboxy- (IIÅC) terminal deletion of ORF II. However, one single T₂ progeny of line IIÅN had decreased virus titers and an improved phenotype. The analysis of virus titers showed that this was due to significantly reduced infection by RTSV, the other tungro related virus. With our data we can not determine if this was due to specific transgene features that became apparent only in this particular generation of the line, or due to irregularities of the inoculum used for this line. A repetition of the resistance assay would be necessary to allow final conclusions.
4.6.1 Evaluation of the virus resistance assay

For an evaluation of the data we obtained in the virus resistance assay, three aspects have to be considered in particular: (i) the duration of the experiment, (ii) the complexity of the assay and (iii) the use of segregating lines.

(i) A simulation model of the dynamics of rice tungro disease (RTD) was established to assess the potential of disease management during the first 60 days of crop growth after transplanting (Holt and Chancellor, 1996). According to this model, the experiment had to be terminated too early. At day 27 after virus infection later effects could not be tested. The potential recovery of line ORF IV 5AC could therefore not be further followed and a repetition of the experiment with a monitoring over a longer period will be necessary.

(ii) The assay is very complex. Many parameters are influencing the results, because the two tungro associated viruses RTBV and RTSV, the insect vectors, and the status of the plants are involved. The relative virus titers in the plants from which the inoculum is derived, the age and the sex of the insects, the age of the plant during inoculation and even differences in germination among different batches of seeds may cause variation of infection. A variation was also seen with two different batches of non-transformed, control lines. Strong forms of resistance may easily be detectable in this system. It is, however, questionable if the assay could clearly determine weaker forms of resistance. Therefore, a system with fewer parameters would be more suitable for analysis. At IRRI it is now possible to obtain a uniform RTBV infection mediated by Agrobacterium tumefaciens (Sta Cruz et al., 1999). With this technique, RTBV alone is able to infect the test plants. Thus, the helper component of RTSV and the uncertainties of virus transmission by the insect vector can be omitted. An infection with RTBV alone would allow to analyse more precisely the influence of the transgene. Moreover, such a test system would not be restricted to tropical countries, and could be established in any laboratory.

(iii) Most of the tested lines were T1 lines, i.e. progeny plants of the primary regenerants and therefore hemizygous for the integrated transgene. Only one ORF IV T2 line and the three GUS lines were homozygous. In a segregating T1 population 25% of the progeny are expected be homozygous, 50% hemizygous and another 25% lost the transgene due to Mendelian segregation. This has to be considered in the analysis of the assay, because 25% of the population lacking the transgene would dilute any resistance effect. The remaining 75% differ in the dosage of the transgene. The transgene in homozygous plants is expressed from two gene copies, in hemizygous plants only from one. Expression levels may be either positively or (if silencing occurs) negatively correlated to the copy number. To allow proper interpretation of the data, molecular analyses were performed. However, no correlation between expression and virus titers was observed. In
general, it would be more favourable to test homozygous lines with well defined expression characteristics, if such lines would be available.

4.6.2 Mechanisms for engineered resistance

In this study different approaches for pathogen derived resistance were pursued. Besides the expression of RTBV proteins in transgenic plants, protection of the plants by a virus induced trans-activation of a cytotoxic protein or by antisense RNA was attempted. Furthermore, virus resistance due to homology-dependent gene silencing was examined.

The trans-activation of the cytotoxic barnase, a bacterial RNase (Mariani et al., 1990) would restrict the virus to the initially infected cell. In a similar example such a pathogen derived virus induced resistance worked with the African cassava mosaic geminivirus (ACMV, Hong et al., 1996). In this example a cytotoxic protein was coupled to the ACMV promoter which was activated in trans upon infection by the virus. The trans-activation in our system relied on a trans-splice event which might be specific for RTBV gene expression and was first tested in protoplasts. Unfortunately no transgenic plants could be regenerated and it was therefore impossible to test this approach in plants. The success of such a concept depends on restricting the cytotoxic activity to the virus infection sites. Most likely, the barnase was expressed already in non-infected cells and thus prevented survival of transformed cells. An explanation for this would be that a trans-splicing might also occur in non-infected plants. This will be tested with reporter gene constructs in the future.

Different viral antisense sequences were transformed into rice in order to destabilise viral mRNA. No improved tolerance of plants was observed with our test lines which all expressed the antisense RNA. It is speculated that in reported cases of antisense RNA mediated virus resistance PTGS is involved (Lindbo and Dougherty, 1992; Ravelonandro et al., 1993). This process is less probable to occur in plants produced by selection for expression as is the case with our plants. A particular antisense sequence containing 451 nucleotides from nt 7408 to 7859 of the RTBV genome complementary to the untranslated leader region was transformed into rice several times (Klöti, 1996; Henrich, 1996). Transgenic, RNA-expressing lines were obtained, but at a much lower frequency compared to other constructs and the regenerated lines were not fertile. A similar observation was made with dicistronic constructs containing a full-length sequence of RTBV ORF II. The few lines obtained were not fertile. This abnormality may be due to toxic effects. Therefore, until now it was also not possible to test if these sequences would be able to confer a form of resistance against RTBV.
Down-regulation of genes by homology-dependent gene silencing can provide very strong forms of virus resistance (Baulcombe, 1996). This phenomenon has been called RNA-mediated virus-resistance and is mediated by post-transcriptional gene silencing (PTGS, Mueller et al.; 1995; English et al.; 1996; Angell and Baulcombe, 1997; Guo and Garcia, 1997; Marano and Baulcombe, 1998; Ratcliff et al., 1997; van den Boogaart et al., 1998). However, PTGS-phenomena have not been observed in monocot plants until recently. Pinto et al. (1999) obtained resistance against the rice yellow mottle virus (RYMV), an RNA-virus. Resistance apparently was mediated by a PTGS mechanism. In another monocot, sugarcane, PTGS was also reported recently (Ingelbrecht et al., 1999).

We also tried to evaluate the potential of homology-dependent gene silencing with transgenic rice-lines. We assumed that besides PTGS also transcriptional gene silencing (TGS) could mediate resistance the DNA-virus RTBV. We therefore examined the effect of transcriptionally silenced reporter transgenes (GUS) to silence homologous genes. In transient expression assays with protoplasts, we produced a similar situation as in virus infection where also a large number of gene copies is introduced into cells. We did not find transcriptional gene silencing of genes with homologous promoter sequences. However, a negative effect on transcribed regions was observed, which could be related to PTGS. The transient GUS-expression was significantly reduced in protoplast containing the (transcriptionally) silenced GUS-gene compared to non-transgenic protoplasts. The observation that transcriptionally silenced genes may mediate also trans-silencing by PTGS, could be explained by a model proposed by Wassenegger and Péllissier (1998). In this model a tight connection of TGS and PTGS was postulated, in which both types of silencing are mediated by the same RNA-based mechanism, the production of a small antisense mRNA. However, this signal has yet to be defined (Wassenegger and Péllissier, 1998). On the other hand it is also possible that both, TGS and PTGS may influence a single gene independently.

We possibly found an RNA-mediated reduction of homologous sequences also during virus infection. The lines containing transgenes coding for the β-glucuronidase also contained sequences homologous to RTBV. This may explain the reduced virus titers observed with lines GUS 1, 2 and 3. The homologous sequence present in all tested lines GUS 1, 2, 3 was the RTBV-derived intron. The particular region causing reduced expression during virus infection could be the RTBV-derived intron. This effect, however, was not strong enough to protect the plants from infection.

With the available data of the virus resistance assay we cannot determine whether the improved phenotype in line ORF IV was due to a weak RNA-mediated resistance mechanism or to a completely different protein-based mechanism. Some changes in RNA-levels of a homozygous line (Fig. 22) suggested a partial
gene silencing, however, the more healthy phenotypes did not correlate with low but rather with strong RNA-expression, suggesting a protein mediated mechanism. In the cases of ORF II\textsubscript{AC} and II\textsubscript{AN} probably only a protein mediated mechanism would have been possible. In previous examples of RNA homology-dependent virus resistance, activity was apparent only against strains that were identical or very similar to that of the transgene (Mueller et al., 1995). In those lines containing the RTBV-ORF II variants, the nucleotide sequence was changed to some degree to increase mRNA stability, and therefore as a consequence, the sequence homology could have been insufficient to trigger silencing.

4.7 Perspectives in research for resistance to Rice Tungro Disease

For efficient practical control of RTD high-level, broad spectrum resistance to tungro viruses is required. A delay in resistance development or the development of milder symptoms in transgenic plants may help to reduce the spread of virus disease under field conditions (Thomas et al., 1997). This was recently proposed by authors who focussed on resistance to RTSV, the other tungro related virus (Sivamani et al., 1999). They hope to be able to control the spread of tungro disease, because "the delay in infection by RTSV may be sufficient to escape disease by late infection by the tungro viruses". We have still to prove if a similar delay of virus spread would be possible with our RTBV-derived resistance strategy. In a positive case, it may be favourable to combine both forms of weak resistance by crossing in order to pyramidise the different resistance traits in one single transgenic plant.

However, reliable control of RTD would only be achieved with strong forms of resistance which cannot be overcome by the viruses after some generations of rice cultivation. The presumably high variation of RTBV and RTSV (Druka and Hull, 1998) may overcome resistance mechanisms that are based on perfect sequence homology between a transgene and the infecting virus, like it is the case in PTGS-based resistance. Therefore, such transgenic plants may be protected less durably. In contrast, mechanisms relying on disturbance of functional protein interactions, might provide protection against a broader range of tungro virus variants and for a longer period.

Risks associated with the strategy to express viral sequences in a transgenic plant have been discussed. One is that functional proteins might complement other viruses defective or not adapted for the respective host. Furthermore, recombination of virus genomes with the transgene occurred (Osbourne et al., 1990; Allison et al., 1996; Kiraly et al., 1998) and heteroencapsidation with other viruses in transgenic plants expressing a coat protein was also possible (Greene and Allison, 1994). These processes, however, can also occur in plants naturally
cointected by different viruses. If the resistance is based on expression of
defective viral proteins, these risks are very small.
However, no food products are totally without risk. Preceding a potential
application in pest control of transgenic plants laws and regulations secure that
released plants do not affect consumers health or the ecosystem more than
conventional plants. It is important that the release of a new variety is controlled on
scientific, sociological, ethic and political levels. However, judgement should rely
on the basis of biological characteristics of foods and not on the basis of the
processes employed in their development. A negation of the benefits and
advantages of gene technology relenting just on public fashion and cheap
propaganda may be one of the highest risks of this technology.

4.8 Future aspects of the project
With this study we established the possibility to select for genes of interest on
dicistronic mRNAs. With the PAT ORF on the upstream position it should be
possible to express other RTBV proteins as the RTBV coat protein or the RTBV
replicase from the downstream position. The existing lines which had an improved
phenotype will be tested again in a virus resistance assay. Infection will be done
with agroinoculation in order to minimise the number of parameters and to
precisely determine the extend of protection. In a long-term assay, effects
occurring later in disease development will be monitored. In case of a tolerance
the lines will be eventually crossed to plants carrying a resistance trait against
RTSV, in order to pyramidise both traits.
The potential of the other approaches is still open. Downregulation of RTBV genes
by silencing may work, but probably other transgenic lines would be required.
More work has to be done to transform rice with constructs containing the trans-
splice acceptor site, in order to prove the trans-splice mechanism in plants. New
constructs will just contain an ATG-less reporter gene containing a splice acceptor
site. The reporter gene would be activated in trans by a splice event after crossing
with plants expressing the strong stop RNA including the RTBV-derived splice
donor site. Eventually, the barnase will be transformed again, perhaps with a low
Barstar expression to be able to regenerate transgenic plants that may be tested
for virus resistance.
5 LITERATURE


Wohlleben, W., Arnold, W., Broer, I., Hillemann, D.I., Strauch, E.I. and Pühler, A., 1988. Nucleotide sequence of the phosphinothricin N-


6 PUBLICATIONS


Oral presentations:

Poster presentations:


7 APPENDIX

7.1 Sequence of RTBV ORF I cDNA and modified ORF I

Definition Rice tungro bacilliform virus (infectious clone) ORF's p24, p12, p194, p46
EMBL Name RTBVPHIL, Accession X57924
Organism pararetrovirus
Updated Jun 04, 1991
Created Jun 04, 1991
Coding region 570 nt, annealing region of PCR primers is underlined (reverse primer in dashed).
Mutations are in bold face

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Updated Jun 04, 1991
Created Jun 04, 1991
7.2 Sequence of RTBV ORF II cDNA and modified ORF II (1)

Definition  Rice tungro bacilliform virus (infectious clone) ORF's p24, p12, p194, p46
EMBL Name: RTBVPHIL, Accession: X57924
Organism  pararetrovirus
An analysis of the sequence of an infectious clone of rice tungro bacilliform virus, a plant pararetrovirus. Nucl. Acids Res. 19:2615-2621

Updated  Jun 04, 1991
Created  Jun 04, 1991

Coding region sequence  333 nt. Mutations are in bold face

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<tr>
<td>2</td>
<td>K A P P K K G I K R X Y P A</td>
</tr>
<tr>
<td>1</td>
<td>340 GAG GATCCCCGGAAGCTTCC ATG GTC 366</td>
</tr>
<tr>
<td>1</td>
<td>GAG GATCCCCGGAAGCTTCC ATG GTC 366</td>
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</table>
7.3 Sequence of RTBV ORF II cDNA and modified ORF II (2)

**Definition**
Rice tungro bacilliform virus (infectious clone) ORF’s p24, p12, p194, p46

**EMBL**
Name: RTBVPHIL, Accession: X57924

**Organism**
pararetrovirus

**Citation**

**Updated**
Jun 04, 1991

**Created**
Jun 04, 1991

**Coding region sequence**
333 nt, annealing region of PCR primers is underlined (reverse primer dashed).

**Mutations**
are in bold face

| ORF II | atgaagcgtgattaccaactttcaaggaacccctttgaaagttttaaa 48

| I1S‘b ---------------

| 1 ATGAGCGTCTATTCCCAAGGAGGCCCTGAGAAGTTTGAAGTTTGAAG 48

| ORF II MUT 2 M S A P Y P T F R E A L E R F R

| 49 aac cta gaa tca gat aca gcc gga aaa gat aag ttt aat tgg gta ttt 96

| I1M45'-------------------------

| 49 AAC CTA GAA TCA GAG GCC GAG AAG GAC AAG TCG AAG TCG TCG 96

| N L E S D T A G K D K F N W V F

| IIN:

| ATG CTA G

| 97 act cta gaa aat ata aag tca gcc ggc gac gat gta aat tta gca ttt aag 147

| ___________________________

| 97 ACT CTA GAA ATA AAG TCA GCC GGC GAC GTC AAC TCG ACG TCA MAG 147

| T/M L E N I K S A A D V N L A S K

| 148 ggt tta gta cca ctt tac gct tta cca gaa att gat aaa aag att aat 195

| ___________________________

| 148 GGc TTG GTA CAA CTT TAC GCT TCG CAG GAG ATG AAC TCG ACG 192

| G L V Q L Y A L Q B I D K/-K IN

| 196 aat ctt aca acc ctt gat aag tta cct aca act aat gta gga gtt aag 243

| ___________________________

| 193 AAC CCG ACA ACC CAA GTT AGT AAG TTA CCT ACA ACT AAT GGA AGT AGT 240

| N L/P T T Q V S K L P T T S G S S

| IIC:

| TAA,

| 244 tca gca gga gct ata gta cct gca ggt aat aac cca aag gtt cag tac 291

| ___________________________

| 241 TCA GCA GGA GCT ATA GTA CCT GCA GGT AGT AAC ACC CAA GAT GCG TAC 288

| S A G A I V P A G S N T Q G/ Q

| II

| 292 aac gca cca ctt aag aaa gga att aca aga aaa tat cca gca taa gtc 339

| ___________________________

| 289 AAA GCA CCA CCT AAG AAA GGA ATT AAA AGA AAA TAT CCA GCA TAA GTC 336

| K A P F K K G I K K R K Y P A

| 340 GAGGATCCGCGGAGCGTTCC ATG GTC 365

| ___________________________

| 337 GAGGATCCGCGGAGCGTTCC ATG GTC 362
### 7.4 Sequence of RTBV ORF IV cDNA

**Definition**
Rice tungro bacilliform virus (infectious clone) ORF's p24, p12, p194, p46

**EMBL Name:** RTBVPHIL, Accession: X57924

**Organism**
pararetrovirus

**Citation**

**Updated**
Jun 04, 1991

**Created**
Jun 04, 1991

**Coding region**
414 nt, internal atg-codons are underlined

**Sequence**

ORFIVAC

```
atgaatagtagtacccg
3500 +-----------------------+-------------------+
| taccatgccacattagacagagaacaggtctgcgttctatagcttgatcaagagaattctttc |
| tactcaatccccatataagacagacaacaggtctgcgttctatctgtcaagagaattctttc |
| tactatatcctagtggctgaagcagagatgctgactttttacactgttgagagcagagttgactg |
| atgagttaggtgtatatctgctgatctgtctttcagagcagagttgactgactttttacactgt |
| tactcaatccacatataagacagacaacaggtctgcgttctatctgtcaagagaattctttc |
| tactcaatccccatataagacagacaacaggtctgcgttctatctgtcaagagaattctttc |
| tactcaatccccatataagacagacaacaggtctgcgttctatctgtcaagagaattctttc |
```

**wt**

```
R L R V F F C E E R S M E Y I Y I Y H I K etc...
------ ORFIV stop -----
```

**ORFIV HYG**

```
CGTGGTTCTCTTTTCGCAGAAGAAAAGAGGTCAGTGCAGGCAGCGCGAAATGAAGCGCGTAG
R V F F C E E R S I V G D P T E K A
```

**ORFIV GUS**

```
CGTGGTTCTCTTTTCGCAGAAGAAAAGAGGTCAGTGCAGGCAGCGCGAAATGAAGCGCGTAG
R V F F C E E R S I V G D P G K L P W
```

---

*GTCGAGTCGTATAG*
7.5 Sequence of phosphinothricin acetyl transferase cDNA

Definition: Streptomyces hygroscopicus phosphinothricin resistance gene (pat)

EMBL Name: Shbarpa, Accession: X17220

Organism: Bacteria; Firmicutes; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces


Updated: sep 12, 1993
Created: apr 03, 1990

Coding region sequence: 333 nt, annealing region of PCR primers is underlined (reverse primer dashed). Mutations are in bold face.

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<th>Protein</th>
</tr>
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<tr>
<td>ATGAGTGCCGAACCGCCGGCGGACATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M M M M M M MM</td>
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<td></td>
</tr>
<tr>
<td>BWT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/L/Tall</td>
<td>ATGAGTCCGGAACCGCCGGCGGACATC</td>
<td>MSPERRPADI</td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BVall</td>
<td>---Bar5' b---------</td>
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</tr>
<tr>
<td>240</td>
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<tr>
<td>BLall</td>
<td>---Bar5' c''-</td>
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<tr>
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<tr>
<td>BTall</td>
<td>---Bar5' d--------</td>
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</tr>
</tbody>
</table>

Mutations are in bold face.

bar int5

Annex 104

Appendix
Cécile Alexandra Henrich

19.12.1971

Ulm/ Donau (Germany)

research work for Ph.D. thesis at the Institute of Plant Sciences, ETH Zürich, Switzerland, in the group of Prof. I. Potrykus under the supervision of Dr. Johannes Fütterer on "Increased gene expression in rice with polycistronic expression units to confer resistance to rice tungro bacilliform virus (RTBV)".

Diploma thesis at the Institute of Plant Science under the supervision of Ingo Potrykus, ETH Zürich. Title: "Towards genetically engineered resistance of rice to Rice Tungro Bacilliform Virus (RTBV)".

Studies in Biotechnology at the Ecole Supérieure de Biotechnologie de Strasbourg (E.S.B.S.), Strasbourg (France). The E.S.B.S. is the EUCOR association of the Upper Rhine Universities, Albert-Ludwig-Universität, Freiburg i.Br, Technische Universität, Karlsruhe (Germany), Biozentrum Basel (Switzerland), and Université Louis Pasteur, Strasbourg (France).

Degree: M.Sc. in Biotechnology

Studies in Agrobiology at the Universität Hohenheim, Stuttgart (Germany).

Degree: Vordiplom in Agrobiology

1982-91 Königin-Charlotte-Gymnasium, Stuttgart (Germany)

Degree: Abitur
ACKNOWLEDGEMENTS

I am very grateful to Prof. Dr. Ingo Potrykus for accepting me as PhD student and for his interest during the course of the work. I appreciate all the possibilities he offered me.

I especially thank Dr. Johannes Fütterer for the possibility to perform this project in his laboratory, for his continuos support, scientific guidance and helpful discussions.

My cordially thanks go to my friends and colleagues Stéphane Bieri, Andreas Klöti, Murielle Uzé, Marcela Bliffeld, Daniel Werthmüller and the other past and present members of the group of Prof. Dr. Ingo Potrykus for the inspiring, warm and pleasant working atmosphere and all the support I received. I appreciate the technical support of Alessandro Galli and the help in administrative matters of Brigitte Spaargaren. Katrin Konya and Sabine Klarer are acknowledged for the maintenance of the plants in the greenhouse.

I thank Prof. Dr. Nikolaus Amrhein for accepting to co-examine this PhD thesis.

I am very thankful to Dr. Ossmat Azzam, Ed Coloquio, Filomena Sta Cruz and Esquiron Baguioso for their advice and support in testing the transgenic lines for virus resistance. I would like to thank all members of the virology group at IRRI for the pleasant working atmosphere.

I thank Peter for his support and love.