Genetic transformation of rice (Oryza sativa L.) to confer resistance to rice tungro bacilliform virus (RTBV)

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Genetic Transformation of Rice (*Oryza sativa* L.) 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

presented by
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March 1996
Rice terraces in Banaue, Philippines
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>b, bp</td>
<td>base, basepair</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorphenoxy acetic acid</td>
</tr>
<tr>
<td>(d)NTP</td>
<td>(deoxy) nucleoside triphosphate</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
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<tr>
<td>DIG</td>
<td>digoxigenin</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECS</td>
<td>embryogenic cell suspension</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>Hyg</td>
<td>hygromycin</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>MS</td>
<td>Murashige-Skoog (medium)</td>
</tr>
<tr>
<td>NAA</td>
<td>α-naphtalene acetic acid</td>
</tr>
<tr>
<td>NPK</td>
<td>nitrogen/phosphorous/potassium (Kalium)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PIG</td>
<td>particle inflow gun</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</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>RTD</td>
<td>rice tungro disease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SS</td>
<td>symptom severity</td>
</tr>
<tr>
<td>SSC</td>
<td>salt/sodium citrate</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>X-Gluc</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
</tr>
</tbody>
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Summary

Rice tungro disease (RTD) is caused by the combination of two different viruses, rice tungro bacilliform virus (RTBV); [a doublestranded (ds)DNA virus] and rice tungro spherical virus (RTSV); [a singlestranded (ss)RNA virus], which are transmitted by insects. RTD is the most important viral disease of rice and causes average annual losses estimated in the range of 1.5 billion US$ in South and South-East Asia. It is mainly RTBV that is responsible for the severe disease symptoms, causing yield reductions up to 100%. No natural resistance to the viruses could be transferred to commercial rice varieties by classical breeding, and resistances to the vector have been overcome by the insects.

The aim of this work was to induce resistance to RTBV in rice, using the newly developed methods of plant genetic engineering. To evaluate an efficient method, suitable for the transfer of many constructs to rice, the methods of tissue electroporation and particle bombardment were compared. By tissue electroporation, plasmids were transferred to the scutella of wheat embryos and to rice suspension cells. By particle bombardment, plasmids were transferred to rice scutella and rice suspension cells. With both methods, fertile transgenic rice plants could be produced. In terms of transformation efficiency, particle bombardment was superior to tissue electroporation and was chosen for the transfer of 32 different plasmids to rice.

The strategies for conferring RTBV resistance included expression or production of different RTBV components within the rice plant: expression of the putative viral coat protein, expression of the viral replicase including (mutated) subfragments and production of antisense RNA against an important part of the viral transcript. The expression of the transgenes, controlled by the Cauliflower mosaic virus 35S or the RTBV promoter, enhanced by an RTBV-derived intron, was localized in sections of leaf sheaths and blades with the help of a visual marker gene.

A total of 514 transgenic plants of the japonica cultivars Taipei 309 and Kinuhikari were regenerated and most were analysed in Southern analysis. Selected lines were analysed for the expression of the transgenes. In different lines, mRNA or protein from the transgenes could be detected. A total of 52 independent, transgenic lines were tested for virus resistance by inoculation by the natural vector in greenhouse tests at the International Rice Research Institute (IRRI). So far, no resistant line could be identified.
Zusammenfassung


Um Resistenz gegen RTBV zu erzeugen, wurden die Reispflanzen dazu veranlasst, verschiedene Teile des Virus selbst zu synthetisieren. Dazu gehören das Hüllprotein, (mutierte) Teile oder die gesamte Replikase und Antisense-RNS-Moleküle, welche gegen eine wichtige Region im Virustranskript gerichtet sind. Um die Expression der Transgene in der Reispflanze zu lokalisieren, wurden Genkonstrukte, in welchen die Expression eines visuellen Markergens entweder vom Cauliflower Mosaik Virus 35S- oder vom RTBV Promoter gesteuert und von einer Kurzform des RTBV Introns verstärkt wird, in Reis übertragen.

1. INTRODUCTION

1.1 Rice

Rice (*Oryza sativa*), besides wheat and maize, is one of the three major food crops in the world. The rice varieties grown world-wide can be classified into the indica, japonica and javanica subspecies. While indica rice varieties are grown in tropical climate, japonica varieties are more temperate varieties and javanica varieties are grown in Africa mainly. Bangladesh, China, India, Indonesia, Thailand and Vietnam are the world's largest producers of indica rice, accounting for about 79% of the global rice production in 1991. By 1991, Asia in total accounted for 92% of the world rice production of more than 500 million tons, grown on nearly 150 million hectares of land. Most of the production is grown for domestic consumption. Only 3-4% of the world rice production was traded from 1980 to 1991 (IRRI 1993).

By 1990, the world population was 5.3 billion. With an expected growth of nearly 100 million persons per year, the world population will reach 8.4 billion by 2025. Low developed countries will experience population increases of 200-500%, compared to only 10-30% increase of industrialized countries. To keep up with this enormous growth, the world's rice production will have to increase by almost 70% over the next 30 years (IRRI 1993). This increase of production will have to go in parallel with a significant loss of land for rice production, due to salinity, urbanization and changes in the use of the arable land from growing rice to growing cash crops or grazing animals. Therefore, more rice will have to be produced on less land. Around 50% of the potential rice harvest are lost every year due to insects, viral and fungal diseases, and competing weeds (Grayson *et al.* 1990). Preventing these losses could contribute significantly to an increased rice production.

1.2 Rice tungro disease

A total of 15 rice viruses have been described worldwide (Hibino 1989). Since the late 1960s, coinciding with the release of modern rice varieties (Thresh 1988; Hibino 1989), several viral diseases, including rice tungro disease (RTD) have become increasingly important in the tropical rice growing countries. RTD was known before as "penyakit merah" in Malaysia and as "mentek" in
Indonesia (Ou 1984). By now, RTD is widespread in the main rice-growing areas of South-East Asia, occurring epidemically throughout whole regions (Thresh 1991). Major outbreaks occurred in Bangladesh, India, Indonesia, Malaysia, The Philippines and Thailand (Hibino 1989).

RTD is a composite disease (Hibino et al. 1978) associated with rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). Both viruses are transmitted from plant to plant by an insect vector, mainly the Green Leafhopper (GLH), *Nephotettix virescens* Distant (Fig. 1 a; Hibino 1983). Probably, RTBV and RTSV are adsorbed on the surface of the cuticular walls of leafhopper mouths and are ejected when the leafhoppers feed on plants (Hibino 1989). Transmission is semipersistent, GLH retain tungro infectivity for 2 to 5 days after acquisition only and lose infectivity after molting (Ling 1966). The vector acquires RTBV only if it has been exposed to RTSV-infected plants before (Hibino et al. 1978), implicating a so far unknown helper-activity from RTSV for acquisition of RTBV. The insects feed on the phloem and xylem of vector-susceptible cultivars (Dahal et al. 1990). The disease can not be transmitted mechanically (Hibino 1989).

RTSV infection alone causes no or only mild symptoms, whereas combined infection with RTBV and RTSV leads to severe symptoms like stunting and yellow or orange discolouration of the leaves (Fig. 1 b; Rivera and Ou 1965). Grain yield reduction as high as 100% has been reported (Hasanuddin et al. 1988). RTD is the most important viral disease of rice and has been considered by Herdt (1996) as the major biotic stress for rice, causing average annual losses in South and South-East Asia estimated at 1.5 billion US$ (Herdt 1988; Herdt 1991).

![Figure 1: Rice tungro disease (RTD). a) The vector, green leafhopper (GLH), *Nephotettix virescens* D. b) RTD infected leaves with orange leaf discolouration (a and b from IRRI 1983b) c) The bacilliform particles of RTBV (from Dasgupta et al. 1991). Bar is 100 nm.](image-url)
1.2.1 Natural resistance to the vector

Several resistances to the vectors have been found in routine screening experiments in different lines, including modern varieties (Hibino et al. 1987). Such varieties tend to escape infection. Unfortunately, vector populations have become adapted to these varieties after a few years of cultivation (IRRI 1983a, Manwan et al. 1985, Dahal et al. 1988, Dahal et al. 1990).

1.2.2 Natural resistance to the viruses

Several authors have reported varieties resistant to the viruses, Balimau Putih, Utri Merah and ARC11554 (Cabunagan et al. 1993; Daquioag et al. 1986). However, it is often difficult to distinguish between resistance to the viruses and resistance to the vector. Further, resistance traits are often linked to traits like yield or grain quality which makes it very difficult to transfer the resistances to new cultivars by breeding. So far, no genes conferring resistance to the viruses were identified and transferred to commercial rice cultivars.

1.2.3 Tungro viruses

1.2.3.1 Rice tungro spherical virus (RTSV)

RTSV is a picorna-like virus. The particles are isometric, have a diameter of 30 nm and contain a positive single-stranded (ss) RNA genome of 12.4 kb (Shen et al. 1993).

1.2.3.2 Rice tungro bacilliform virus (RTBV)

1.2.3.2.1 Classification

RTBV is classified as a member of the badnaviruses, a new group of non-enveloped bacilliform plant viruses containing circular double-stranded (ds) DNA, which infect mainly monocotyledonous plants (Lockhart 1990). The bacilliform particles of RTBV are approximately 35 nm in diameter with a length of 150 to 350 nm (Fig. 1 c; Hibino et al. 1978). Members of the same group are Commelina yellow mottle virus (CoYMV, Medberry et al. 1990), cocoa swollen shoot virus (CSSV, Hagen et al. 1993) and sugarcane bacilliform virus (SCBV, Bouhida et al. 1993).

Together with the hepadna- and the caulimoviruses, the badnaviruses are members of the newly proposed pararetroviruses (Qu et al. 1991). Unlike the retroviruses, which encapsidate RNA and transcribe their RNA from a genome
copy integrated into the host DNA, the pararetroviruses encapsidate DNA and transcribe RNA from an episomal form of DNA that is not integrated into the host genome (Hohn and Fütterer 1991).

Pararetroviruses have various features in common with animal retroviruses, like the involvement of reverse transcriptase in the replication cycle, the functional organization of the gag-pol polyprotein, a more-than-genome length transcript with terminal repeats, usage of the pregenomic RNA as polycistronic mRNA for open reading frames (ORFs) up to the gag-pol region and, with exception of the hepadnaviruses, tRNA-priming of the minus-strand DNA synthesis (Hohn and Fütterer 1991).

Pararetroviruses start the replication cycle by the synthesis of a terminally redundant genome-length transcript under the control of the viral promoter. When primed by a host cell tRNA, this transcript is used as a template by the viral-encoded reverse transcriptase (RT) for synthesis of the minus-strand DNA. The RNA in the RNA:DNA hybrid is degraded by the ribonuclease H (RNaseH), leaving one or more specific RNA fragments, which are used by the RT, now acting as a DNA-dependent DNA polymerase, to prime synthesis of the plus-strand viral DNA, completing the circular double-stranded DNA genome (reviewed by Hohn and Fütterer 1991).

1.2.3.2.2 Primary transcript / promoter

The circular genome of RTBV is 8.0 kb in length (Hay et al. 1991, Qu et al. 1991) and one major transcript has been found (Qu et al. 1991). This transcript starts at nucleotide 7404 or 7405 and ends at the polyadenylation signal (AATAAA) at nucleotide 7598, yielding a primary transcript of more than genome length with a terminal repeat of 193 or 194 nucleotides (Fig. 2; Bao and Hull 1993). Sequences upstream of the genomic position 7390 were reported to direct phloem-specific expression in young transgenic plants and in bombarded tissue (Bhattacharyya-Pakrasi et al. 1993). Chen et al. (1994) defined the active TATA box and showed that considerable promoter activity in transformed protoplasts was observed still with only 50 bp of upstream sequence from the transcription initiation site. Furthermore, downstream elements in the RTBV leader sequences are involved in RNA production (G. Chen, FMI, Basel; pers. comm.). Yin and Beachy (1995) reported that a fragment 164 to 43 nucleotides upstream of the transcription initiation site was essential for promoter activity and that a fragment 164 nucleotides upstream to 45 nucleotides downstream the transcription initiation site contained sufficient information for phloem-specific gene expression.
Figure 2: The double-stranded DNA-genome of RTBV. Four open reading frames (ORFs) are encoded in the plus strand. ORF1 starts with an unusual AUU codon. Located in ORF3 are the coat protein (cp), an aspartic protease (prot), a reverse transcriptase (RT) and a RNaseH. Transcription starts at 7404/5 and the polyadenylation signal (polyA) was found at 7598. The primary transcript is indicated in blue. The large intron and the 5’- and 3’-splice sites are indicated in dark blue. The promoter region is indicated in yellow colour. Several short ORF (sORF) are located between ORF4 and ORF1 within the long leader of the pregenomic RNA. By removal of the intron, the first sORF with a start-codon (AUG) is spliced to the beginning of ORF4.

1.2.3.2.3 Open reading frames

Four open reading frames (ORFs) greater than 300 nucleotides are located in the plus strand of the RTBV genome (Fig. 2, Qu et al. 1991). ORFs 1/2 and 2/3 share overlapping stop/start codons (ATGA). Whereas in CoYMV and in SCBV no genes have been found downstream of the gag-pol core (a common arrangement in retroviruses of the structural (gag) and enzymatic (pol) functions), in RTBV there is a fourth ORF encoding a potential protein of 46 kDa (Hay et al. 1991, Qu et al. 1991). Several short ORFs (sORF) are located in the intergenic region between ORF4 and ORF1.

Unknown functions of ORF1 and ORF2

ORF1, with the capacity of encoding a 24 kDa protein, does not contain an AUG start codon (Hay et al. 1991). Protein synthesis could be initiated at the
AUU codon (Füßerer et al. 1996), leading to a protein of 198 amino acids. Mutation of the AUU to a proper AUG start-codon abolished infectivity of the viral DNA in agroinfection (Füßerer et al. 1996).

In ORF1, no significant sequence homologies to related viruses have been found. Antiserum raised against a recombinant protein product of ORF1, detected proteins in degraded RTBV particles but not in intact ones, suggesting that the product of ORF1 could be within the virus particles, possibly associated with particle assembly. However, the detected proteins were much larger than expected (Hay et al. 1994).

ORF2 is capable of encoding a protein of 12 kDa (p12). The function of p12 is still unknown. Yambao et al. (1994) speculated that the product of p12 could be a constituent of the viral capsid in addition to the major coat protein, similar to the gene III product of caulimoviruses.

Nucleocapsid, protease and replicase in ORF3

ORF3 encodes a polyprotein of 194 kDa (1675 amino acids). The polyprotein is probably cleaved post-translationally, yielding several viral proteins (Laco et al. 1995).

Nucleocapsid. At amino acids 776 to 789 of RTBV ORF3, a cysteine-histidine (Cys-His) box was found: Cys-X2-Cys-X4-His-X4-Cys, with X standing for nonconserved amino acids. This motif is highly conserved among retroviruses and is the most conserved region among CoYMV, CaMV and RTBV. The Cys-His element is supposed to bind RNA while packaging the RNA of retroelements in order to separate it as template for reverse transcription from the pool of cellular RNAs (Covey 1986, Füßerer and Hohn 1987; Bowles et al. 1993). Two coat proteins of 37 kDa and 33 kDa, found in purified RTBV particles, were reported by Jones et al. (1991) and Qu et al. (1991). As only the 37 kDa protein could be detected in infected rice tissue, Qu et al. (1991) concluded that the 37 kDa protein might be the major coat protein of RTBV, while the 33 kDa protein is a processed product of the 37 kDa protein which may arise during virus purification or storage. The amino terminus of the 33 kDa coat protein was located by Qu et al. (1991) at amino acid 502 (nucleotide 2497) of ORF 3. This protein probably contains the Cys-His motif. However, Kano et al. (1992) partially sequenced tryptic fragments of a 32kDa putative coat protein and found a peptide sequence that is located around 100 amino acids further downstream of the calculated carboxy-terminal end of the putative
33kDa coat protein. Thus, the location of the RTBV coat protein encoding sequence within the ORF3 is still not clear.

**Protease and replicase.** Enzymatic domains were identified by homologies to highly conserved sequences in other retroid elements (Argos 1988; Füttgerer and Hohn 1987; Qu et al. 1991). Homologies were found to an aspartic protease (LIDSGS; ORF3 amino acids 986-992), a reverse transcriptase (YIDDILI; ORF3 amino acids 1339-1345) and to an RNaseH (dispersed sequence homologies between ORF3 amino acids 1486 and 1607). Laco and Beachy (1994) indeed found in a heterologous baculovirus system that a 87 kDa protein, including the protease and the reverse transcriptase domains, was cleaved by the protease (Laco et al. 1995) into two smaller proteins of 62 or 55 kDa, respectively. Both the proteins, having the same amino terminus, showed protease and DNA polymerase activities. Only the 55 kDa protein also showed RNaseH activity.

**Unknown function of ORF4**

In ORF4, with the capacity of encoding a protein of 46 kDa, a leucine zipper similar to those that are involved in protein-protein interactions (Gruissem 1990) and a putative dimerisation domain have been found (J. Füttgerer, pers. comm.). The 5' end of ORF4 is spliced in frame to a short ORF in the RTBV leader sequence, removing an intron of about 6.3 kb (Füttgerer et al. 1994). The function of the putative protein is unknown.

### 1.3 Strategies for a control of viral diseases

#### 1.3.1 Conventional methods for controlling RTD

To some extent damage caused by tungro disease can be reduced by measures like appropriate planting time, varietal rotation according to GLH-resistance genes, use of insecticides and by synchronous planting, allowing a break between successive plantings (Manwan et al. 1985). Despite considerable efforts to control RTD in Indonesia, major outbreaks occurred in central Java, Indonesia, and in Mindanao, Philippines, in 1995. Tungro-resistant varieties could therefore substantially contribute to control this devastating disease.
1.3.2 Genetically engineered resistance to viruses

A plant systemically infected by one strain of a particular virus may become resistant to a second infection by a related strain of the same virus. This phenomenon is known as "cross protection" (Fraser 1992). Sanford and Johnston (1985) postulated the concept of pathogen-derived resistance. They stated that certain gene products of a virus present in the plant either in a dysfunctional form, in excess, or at an inappropriate time during viral replication could disrupt infection by an invading virus. Since then, many authors reported successful conferring of virus resistance to plants by transferring certain viral sequences to the plant genome (for reviews see Fitchen and Beachy 1993; Wilson 1993).

Resistance to a virus is defined as "a property of a plant that reduces or prevents virus multiplication, spread within the plant, or symptom expression", and protection is "a property conferred to a plant that interferes with the virus infection cycle" (Hull and Davies 1992).

1.3.2.1 Coat protein-mediated resistance or protection

Powell-Abel et al. (1986) reported that transgenic plants, expressing the coat protein of tobacco mosaic virus (TMV) showed a delay in disease development when challenged with the virus. Since then, coat protein-mediated resistance or a delay of symptom development has been achieved in a range of crops like tobacco, tomato, alfalfa, cucumber, potato, papaya, sweet corn, muskmelon, rice and sugarbeet with viruses of different families, including tobamoviruses and potyviruses (reviewed in Beachy et al. 1990, Gonsalves and Slightom 1993, Hackland et al. 1994; Stark and Beachy 1989). In cereals, Murry et al. (1993) reported resistance to the potyvirus maize dwarf mosaic virus (MDMV) in maize, and Hayakawa et al. (1992) reported resistance to the whitefly-transmitted tenuivirus rice stripe virus (RSV) in rice.

No unifying mechanism that leads to this coat protein-mediated resistance could be found; rather, a number of different mechanisms are probably functional with different host-virus systems. Several authors reported transgenic plants in which the transgenic coat protein could not be detected but that were protected against the virus (Stark and Beachy 1989; Farinelli and Malnoë 1993). However, the present consensus in most cases is that the endogenous coat protein prevents co-translational disassembly, an early event in the establishment of viral infection (reviewed by Reimann-Philipp and Beachy 1993, Hackland et al. 1994). This mechanism is supported by the fact that often, but
not always, coat protein-mediated resistance is overcome by direct inoculation of the viral RNA (Powell-Abel et al. 1986). So far, coat protein-mediated protection has been reported for more than 20 RNA viruses. For DNA viruses, one single report has been published up to now. Kunik et al. (1994) reported delayed disease symptoms and recovery from the disease in tomato plants, transformed with the capsid protein gene from tomato yellow leaf curl virus (TYLCV), a geminivirus (ssDNA virus).

1.3.2.2 Replicase-mediated resistance

Transferred sequences coding for functional or defective components of the viral replicase complex can induce a high level of resistance to the virus and to closely related strains. Golemboski et al. (1990) transformed tobacco with an ORF from the tobacco mosaic virus (TMV) genome, coding for a 54 kDa protein with sequence homologies to viral RNA-dependent RNA polymerases and found the plants highly resistant to TMV infection. Only mRNA, but no protein could be detected in the transgenic plants (Carr and Zaitlin 1991). Expression of replicase-derived sequences is considered to interfere with normal function and assembly of viral replicase enzyme complexes, leading to a general, marked inhibition of viral replication in the cells of these transgenic plants (reviewed in Carr and Zaitlin 1993). The resistance conferred by the 54 kDa-replicase differed to the coat protein-mediated resistances in so far as the resistance was effective against both intact virus and viral RNA, that it was not overcome by high inoculum concentrations and that it did not break down over time or when the incubation temperature was increased (Carr and Zaitlin 1993). Transgenic tobacco, transformed with a truncated version of the cucumber mosaic virus (CMV) replicase, was resistant to CMV infection (Anderson et al. 1992). The truncated replicase lacked the motif Gly-Asp-Asp (GDD; Rezaian et al. 1984), common among RNA viruses. Resistance to the bacteriophage Qβ could be conferred by transforming E. coli with a subunit of the RNA-dependent RNA polymerase of Qβ, with mutation of the Gly residue (Inokuchi and Hirashima 1990).

1.3.2.3 RNA-mediated resistance

De Haan et al. (1992) reported that the phenotype of the resistance to tomato spotted wilt virus (TSWV) was identical in plants carrying translationally competent or defective coat protein genes. This suggested that in this case the "coat protein-mediated resistance" was actually mediated by the RNA.
Protection was observed against various strains of TSWV but not against two related strains with only about 80% nucleotide homology to TSWV. In several experiments, plants transformed with translationally defective or antisense coat protein sequences, exhibited levels of resistance similar to those with translationally competent gene constructs (Kawchuk et al. 1990; Van der Vlugt et al. 1992). In these examples, the coat protein produced from the translationally competent gene constructs was either not detectable or its expression level was not correlated with the degree of protection.

Resistance to virus infection was also observed after transformation of antisense constructs from viral sequences other than the coat protein of TMV (Powell-Abel et al. 1989) and tomato golden mosaic virus (TGMV, a geminivirus; Day et al. 1991). In these examples, a direct correlation between RNA levels and resistance was observed.

It is not understood why transgenic plants, producing sense RNA, exhibit resistance to the virus. This resistance may share features with a phenomenon called "sense suppression" (Matzke and Matzke 1993; Smith et al. 1994). Sense suppression often results when exogenous copies of a plant gene, under the control of a constitutive promoter, are transferred to the plant. Instead of an expected increase in gene expression, expression of both the endogenous and exogenous genes is often reduced. Different mechanisms have been proposed to explain this phenomenon, including methylation of DNA (Matzke and Matzke 1991) and cytoplasmic activities, targeting specific RNA sequences for inactivation (Lindbo et al. 1993; Müller et al. 1995).

1.3.2.4 Other strategies

Cooper et al. (1995) showed that a defective movement protein of TMV in transgenic plants conferred resistance to multiple viruses, whereas the functional analogue increased susceptibility.

Ribozymes are small RNA molecules derived from certain viroids that catalyze the cleavage of RNA with high sequence specificity (Bruening 1989). Theoretically by combining a ribozyme sequence with an antisense RNA, target RNAs could be specifically cleaved (for a description see Wilson 1993).

Satellite RNAs are infrequently found in some plant RNA virus isolates. They are RNA molecules without any homology to viral RNA that replicate with the help of and are encapsidated within the virus. Transgenic plants expressing a cucumber mosaic virus (CMV) satellite RNA gene were tolerant to CMV (Harrison et al. 1987). In such plants, the satellite RNA was replicated to high
levels on infection, while the virus itself was replicated to much lower levels than usual, with nearly no symptoms.

1.3.2.5 A prerequisite: Rice transformation

1.3.2.5.1 Agrobacterium-mediated transformation

Since the first reports about successful Agrobacterium tumefaciens-mediated transformation in higher plants in 1984 (Horsch et al. 1984, De Block et al. 1984), a lot of efforts have been invested by many laboratories to establish different transformation techniques for a broad range of plant species. Only recently, Agrobacterium-mediated transformation has been applied to rice (Chan et al. 1992; Hiei et al. 1994).

1.3.2.5.2 Direct gene transfer to protoplasts

The first transformation technique using direct gene transfer to plants was the delivery of the DNA to protoplasts mediated by the presence of polyethylenglycol (PEG) or by electroporation (Potrykus et al. 1985, Lörz et al. 1985, Uchimiya et al. 1986). This transformation technique is applicable for both dicots and monocots. As differentiated monocot cells have lost their totipotency, protoplasts for monocot transformation are usually prepared from suspension culture cells that are derived from a regenerable tissue like the scutellum of the embryo. However, the establishment of embryogenic suspension cultures often takes a long time during which the culture cells undergo somaclonal variations (Phillips et al. 1994). These variations often lead to regenerated plants that show abnormal traits like albinism or sterility (Fromm et al. 1990, Datta et al. 1992).

1.3.2.5.3 Tissue electroporation

Direct transformation of regenerable cells without prior establishing suspension cultures and removing the cell wall avoids unnecessary long tissue culture periods. Although it is not likely that a large macromolecule like a plasmid can penetrate an intact cell wall (Potrykus 1990), some authors reported electroporation-mediated delivery of plasmid DNA to intact plant cells (Morikawa et al. 1986, Lindsey and Jones 1987, Dekeyser et al. 1990). D'Halluin et al. (1992) regenerated transgenic plants after electroporating both maize immature embryos that had partially digested cell walls and embryogenic maize callus wounded mechanically by cutting. Songstad et al. (1993) and Klöti et al. (1993) showed that plasmid DNA can be delivered to certain recipient
cells, including regenerable cells from maize or wheat embryo scutella, without prior wounding. Xu and Li (1994) regenerated fertile transgenic rice plants of the indica variety IR36 after electroporation of mature rice embryos that were cut in two halves beforehand.

1.3.2.5.4 Particle bombardment

Particle bombardment was conceived by Sanford et al. (1987) and allows the delivery of DNA into any plant cell, even through several cell layers (McCabe and Christou 1993). It involves the acceleration of DNA-coated microparticles. To penetrate the plant cell wall, acceleration is achieved by means of an explosion of gun powder (Sanford and Klein 1987), instantaneous water vaporisation (McCabe and Christou 1993) or a burst of gas like helium (Finer et al. 1992). The biolistic approach proved to be applicable for a large number of plants, including the monocots rice, maize, wheat, barley, sorghum, sugarcane, rye, Lolium multiflorum, red and tall fescue and banana (for a review see Vain et al., in press). At present, particle bombardment is the most widely used method for transformation of monocots.

1.4 Aim of this work

With the recently developed tools of genetic engineering of plants, resistance to rice tungro bacilliform virus should be conferred to tropical (Indica) rice. To evaluate an efficient rice transformation system, particle bombardment and tissue electroporation were compared. The use of different target materials like the scutella of immature embryos or embryogenic suspension cells, was tested. Possible strategies for conferring RTBV resistance included expression or production of different RTBV components within the rice plant like the viral coat protein, the viral replicase or antisense RNA against an important part of the viral transcript. Suitable promoters for the expression of the transgenes in the vascular tissue and the use of an intron for an increase of the expression levels were tested.
2. MATERIAL AND METHODS

2.1 Plasmid DNA methods

2.1.1 Bacterial strains

For amplification of recombinant plasmid DNA, competent *Escherichia coli* strains XI-1 blue (Stratagene, La Jolla, CA) and DH5α (Life Technologies, Basel) were transformed as described in Inoue *et al.* (1990). Bacteria were grown at 37°C in LB growth medium (1% (w/v) NaCl, 1% (w/v) Bacto tryptone and 0.5% (w/v) Bacto yeast extract [both Difco, Detroit, MI]) either on solid medium, containing 1.5% (w/v) agar (Difco) or in liquid on a shaker at 150-220 rpm. Recombinant bacteria, containing pUC19-derivatives, were selected and amplified by addition of 100 mg/l ampicillin to the growth medium.

2.1.2 Single colony plasmid DNA preparation from bacteria

A simple and rapid method was performed to visualize the size of transformed ligation products on agarose gels (Rusconi, pers. comm.). The plasmids were released from the bacteria by lysing overnight grown colonies for 5 minutes in 12 μl lysis buffer (25 mM TrisHCl, pH 7.5, 25 mM Na₂EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml Rnase A, 10% v/v glycerol, 0.001% (w/v) bromophenol blue). Proteins and debris were extracted with 2 μl of phenol-chloroform (1:1) by vortexing and centrifuging for 3 minutes at 12'000 rpm in a table top centrifuge (Hettich, Tuttingen, FRG). The size of the released plasmid DNA was compared with the original plasmid and with DNA molecular size markers (1 kb ladder; Life Technologies, Basel) on 0.8% agarose gels.

2.1.3 Small-scale preparation of plasmid DNA

Small amounts of plasmid DNA were isolated from 1 ml of bacterial overnight cultures. Single colonies were grown in 2 ml of growth medium. 1 ml of the bacteria culture was transferred to a 1.5 ml tube and was centrifuged for 15 seconds in a table top centrifuge. The cell pellet was resuspended in 200 μl of TELT-buffer (Wilimzig 1985), containing 2.5 M LiCl, 50 mM Tris (pH 7.5), 60 mM Na₂-EDTA, 0.4% Triton X-100 and 1 mg/ml lysozyme. After boiling the suspensions for 1 minute, the tubes were centrifuged for 15 minutes at 4°C.
The slimy pellet was removed and the DNA was precipitated with 2 volumes of ethanol. After an incubation of 5 minutes at RT, the tubes were centrifuged for 5 minutes, the supernatant was removed, the pellet was dried briefly and dissolved in 20 µl of water. The yield of plasmid DNA was generally around 20 µg.

2.1.4 Large-scale preparation of plasmid DNA

Large amounts of plasmid DNA were isolated from 250 ml of bacterial overnight cultures. The isolations were performed using purification columns from Qiagen (Hilden, FRG), following the suppliers instructions. The yield of plasmid DNA was generally around 0.5 mg. The quality of the isolated plasmid DNA was sufficient to be used in plant transformation experiments using particle bombardment and tissue electroporation.

2.2 Constructions

The various DNA fragments were restricted, modified and ligated and PCR was performed using general cloning protocols (Maniatis et al. 1982) and according to the supplier's recommendations.

2.2.1 Plasmids

Plasmid pBC17 (kindly provided by T. Klein, DuPont, Delaware, USA) carries two anthocyanin regulatory genes, C1 (Cone et al. 1986) and B-peru (Goff et al. 1990), both under the control of CaMV 35S promoters and interrupted by the maize Adh intron.


2.2.2 Construction of pHCX

The plasmids pHCX (Fig. 3) with the RTBV genes, controlled by the CaMV 35S promoter are derivatives of plasmid pCintG, containing the CaMV 35S promoter-terminator cassette from plasmid pTZDH (Füterer et al. 1989), a GUS gene (Jefferson 1987) and a shortened version of the intron from rice tungro bacilliform virus (RTBV).
2.2.2.1 Intron

The shortened version of the RTBV intron contains the first exon and parts of the intron of the RTBV ORF4 mRNA from nucleotides 7404 to 7682 and from nucleotides 5924 to 5977 (nucleotides from the circular RTBV genome of 8002 bp) from plasmid pC4CABB (Fütterer et al. 1994). In the following, this sequence is termed "RTBV intron" (Fig. 4). After splicing, nucleotide 7503 is linked to nucleotide 5973, fusing three codons, including the start-codon from ORF4, to the 5'-end of the coding region. These codons, together with the following HindIII-site that was used to clone-in the different sequences, lead to a common N-terminus of the RTBV-proteins: MAQGQA. These six amino acids are followed by two to six additional amino acids, encoded by the linker used for cloning. These linkers contain an additional start-codon (exceptions:
plasmids pH3, pH2ZPol/pHRZPol) possibly leading to another, smaller protein without additional amino acids at the N-terminus (see table 1).

### Figure 4: RTBV intron
Nucleotides 7404 to 7682 and nucleotides 5924 to 5977 from the RTBV genome were linked by a BstBI site. Numbers indicate the nucleotides in the RTBV genome. Yellow lines indicate where the RNA will be spliced. Amino acids from the exons are given in green colour.

#### 2.2.2.2 Tag-peptide

The coding sequence for the 10 amino acid tag-peptide from the influenza virus hemagglutinin protein (Fig. 5), derived from plasmid pHATO was cloned into pCintG. The BamHI-KpnI (partial digest) fragment from plasmid pCintG containing the CaMV 35S terminator was replaced by the BamHI-KpnI fragment from pHATO, containing the tag-sequence and the CaMV 35S terminator, yielding pCintG(tag).

### Figure 5: DNA- and protein sequence of the tag-epitope
The 10-amino-acids epitope from the influenza virus hemagglutinin protein is detected by commercially available antibodies. The protein sequence that represents the carboxy-terminus of all the proteins is given in green colour. Capitals represent the tag peptide.

#### 2.2.2.3 RTBV sequences

All RTBV sequences were derived from plasmid pRTRB1162 (Dasgupta et al. 1991), containing a more than full-length copy of the RTBV sequence. The RTBV genes were either amplified by PCR with forward primers including
5' nonviral extensions with a *HindIII* site and with reverse primers including 3' nonviral extensions with a *BamHI* site, or were derived directly from the RTBV sequence (details see in table 1).

<table>
<thead>
<tr>
<th>PCR primers (forward / reverse primer)</th>
<th>Description of the constructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-gggaagctctaatATgGAGTCACGTTACG-3'</td>
<td><strong>pHC1</strong>: Fragment was derived by PCR.</td>
</tr>
<tr>
<td>5'-gggggatccGTAGCTTGATGCTTAAGG-3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HindIII</th>
<th>102</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttcataag</td>
<td>GAG TCA CGT ... CAT CAA CTT ACggatccaaca tag</td>
</tr>
<tr>
<td>MAQGQA S N M</td>
<td>ORF1 aa 12 -199</td>
</tr>
</tbody>
</table>

| pHC3 | was cloned using two linker-oligonucleotides: 5'-agcttcgctgatcggcggGAGCCTATAGCATTATTA-3' and 5'-ATGGTCTAAGCAGCTccgggctcgagctcgga-3' that, hybridized together, show compatible ends for *XhoI* resp. *PflMI*-sites. This linker was ligated to a *XhoI*-opened fragment, showing *HindIII*-Sacl-*XhoI*-sites and a *PflMI*-Ndel fragment cleaved from pRTBV1162. The product was cleaved by *HindIII* and *Ndel* and was cloned into plasmid pHCPol in exchange to the respective *HindIII*-Ndel fragment. |

<table>
<thead>
<tr>
<th>HindIII</th>
<th>SacI</th>
<th>XhoI</th>
<th>997</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttcataag</td>
<td>GTC CATCAGAAGAGCCGCTAGGTTAAGAC</td>
<td>GAT GAC TCGgaatccactag</td>
<td></td>
</tr>
<tr>
<td>MAQGQA S E L E P G</td>
<td>ORF3 aa 2-1655</td>
<td>D P T</td>
<td>MYPYDVPDYA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pHCPol</th>
<th>was derived by PCR.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-gggaagctcctagATGAAATGCTAGAAATAC-3'</td>
<td></td>
</tr>
<tr>
<td>5'-gtggagctcAGTCACGACAGCT-3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HindIII</th>
<th>NcoI</th>
<th>3747</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttcattG GTA GAA ATG CAG ... GAT GAC TCGgaatccactag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAQGQA S M</td>
<td>ORF3 aa 919 - 1655</td>
<td>D P T</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pHCPol(Xh)</th>
<th>was cloned in two fragments that were linked together by the newly created <em>XhoI</em> sites. The first fragment, derived by PCR, was cleaved by <em>HindIII</em> and <em>XhoI</em> and the second PCR-derived fragment was cleaved by <em>XhoI</em> and <em>BamHI</em>.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-gggaagctcctagATGAAATGCTAGAAATAC-3'</td>
<td></td>
</tr>
<tr>
<td>5'-agtgcctcGATGAAATGCTAGAAATAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HindIII</th>
<th>NcoI</th>
<th>3747</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttcattG GTA GAA ... TAT cTc GaAG AATGGAATGAC</td>
<td>GAT GAC TCGgaatccactag</td>
<td></td>
</tr>
<tr>
<td>MAQGQA S M</td>
<td>aa 919 - 1339 L E E aa 1339-1655</td>
<td>D P T</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pHCZPol</th>
<th>Fragment was derived by PCR.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-aagcttcctaggATGGGATTAGACGAGACAGATAG-3'</td>
<td></td>
</tr>
<tr>
<td>5'-aagcttcctaggATGGGTTAGACGAGACAGATAG-3'</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HindIII</th>
<th>NcoI</th>
<th>3394</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttcattG GTA TTA GAC ... GAT GAC TCGgaatccactag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAQGQA S M</td>
<td>ORF3 aa 801 - 1655</td>
<td>D P T</td>
</tr>
</tbody>
</table>
Material and methods

**pHCPol(HiXh)** was derived from pHCPol(Xh) by deleting the HindIII-XhoI fragment. After digestion, the restriction sites of the remaining vector were filled-in by the Klenow enzyme and the blunt ends were ligated together, again resulting in a HindIII site. To create the right frame for the ORF/tag fusion, the HindIII site was opened again, filled-in and religated.

<table>
<thead>
<tr>
<th>HindIII NcoI</th>
<th>2375</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttagcttgcaagga</td>
<td>ATA CTT ... GAT GAC TCggatccaaca tag</td>
</tr>
<tr>
<td>MAQGQA</td>
<td>S</td>
</tr>
</tbody>
</table>

**pHCPol(XhBa)** was derived from pHCPol(Xh) by deleting the XhoI-BamHI fragment. After digestion, the ends of the opened vector were filled-in by the Klenow enzyme and were ligated together, again resulting in an XhoI site. To create the right frame for the ORF/tag fusion, the XhoI site was opened again, filled-in and religated.

<table>
<thead>
<tr>
<th>HindIII NcoI</th>
<th>3747</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttagcttgcaagga</td>
<td>GAA ATG CAG ... TTA TAT ctcgatccagatccaaca tag</td>
</tr>
<tr>
<td>MAQGQA</td>
<td>S</td>
</tr>
</tbody>
</table>

**pHCC23**: Fragment was derived by PCR.

<table>
<thead>
<tr>
<th>HindIII NcoI</th>
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</tr>
</thead>
<tbody>
<tr>
<td>aagcttagcttgcaagga</td>
<td>TCT ATA GAA ... GAA AAT TTggatccaaca tag</td>
</tr>
<tr>
<td>MAQGQA</td>
<td>L</td>
</tr>
</tbody>
</table>

**pHCC24**: Fragment was derived by PCR.

<table>
<thead>
<tr>
<th>HindIII NcoI</th>
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</tr>
</thead>
<tbody>
<tr>
<td>aagcttagcttgcaagga</td>
<td>TCT ATA GAA ... GAG ATT Acggatccaaca tag</td>
</tr>
<tr>
<td>MAQGQA</td>
<td>L</td>
</tr>
</tbody>
</table>

**pHCR** was cloned in three fragments that were linked together by the newly created XhoI- and ApaI sites. The first fragment was cleaved by HindIII and ApaI, the second fragment was cleaved by ApaI and XhoI and the third fragment [same fragment as the second fragment of pHCPol(Xh)] was cleaved by XhoI and BamHI.

<table>
<thead>
<tr>
<th>HindIII NcoI</th>
<th>2375</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttagcttgcaagga</td>
<td>TCT ATA GAA ... TGT AGA gggggg CCT AGA ...</td>
</tr>
<tr>
<td>MAQGQA</td>
<td>L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HindIII NcoI</th>
<th>2375</th>
</tr>
</thead>
<tbody>
<tr>
<td>aagcttagcttgcaagga</td>
<td>TCT ATA GAA ...</td>
</tr>
<tr>
<td>MAQGQA</td>
<td>L</td>
</tr>
</tbody>
</table>
pHC4: The sequence for construct pHC4 was derived directly from the RTBV sequence as HindIII-BamHI fragment.

Table 1: RTBV sequences in the different plasmids. PCR (forward/reverse) primers (Capital letters indicate viral sequences, others are non-viral extensions.) and descriptions of the constructions are listed. DNA- and amino acid (aa; single letter code in green colour) sequences, as present in the constructs are given. In the DNA sequences, capital letters indicate nucleotides from the viral genome. Numbers indicate the nucleotides within the RTBV genome, restriction sites are indicated. Protein sequences generally end with the amino acids DPT, encoded by the linker, and the tag peptide MYPYDVPDYA. Further, the constructs pHCC23, pHCC24 and pHCPol(XhBa) have 1, 1 and 3 additional amino acids after the specific RTBV sequences.

The RTBV sequences were ligated to the large HindIII-BamHI fragment of pCintG(tag), replacing the uidA gene and yielding a translational in-frame fusion of the RTBV sequence with the tag sequence (Fig. 3).

Where possible, internal portions of the cloned PCR fragments were replaced by restriction fragments from pRTRB1162 to minimize the PCR derived parts. The remaining PCR parts were analysed by sequencing.

2.2.2.4 Hygromycin-resistance cassette

For selection of stably transformed cells, a hygromycin-resistance cassette (CaMV 35S promoter::aph4::CaMV 35S terminator) from plasmid pCIB900 (Ciba, Basel) was included in all the constructs into the Nael site from pCintXtag as filled-in Asp718I fragment. By restriction analysis with SmaI, a clone was selected where the hygromycin-resistance cassette had integrated in the same direction as the GUS cassette (Fig. 3).
2.2.3 Construction of pHRX

The plasmids pHRX, containing the different RTBV genes, the promoter, part of the leader and the RTBV intron (nucleotides 6724 to 7682 and 5924 to 5977 linked at a BstBI site, Fig. 4), are derivatives of the plasmid pHrintG (Fig. 6). Cla-SphI or BstBI-SphI fragments from plasmid pCintXtag, containing the RTBV genes fused to the tag sequence, were cloned into pHrintG, replacing the respective fragments.

![Figure 6: Construction of plasmids pHRX. Cla-SphI or BstBI-SphI fragments from pCintXtag, containing the RTBV genes fused to the tag sequence, were cloned into pHrintG, replacing the respective fragments.](image)

2.2.4 Construction of paRNA14

A Cla-HindIII fragment from plasmid pRTRB1162 (Dasgupta et al. 1991), comprising 451 nucleotides (nts 7408 to 7859 from the RTBV genome), was cloned in reverse direction to the 3'-end of aph4, before the CaMV 35S terminator. By fusing the two sequences, transcription of aph4 assures the production of the antisense RNA, not disturbing the translation of the aph4 transcript. The resulting plasmid was termed paRNA14 (Fig. 7; J. Fütterer, pers. comm.).

![Figure 7: Plasmid paRNA14. 451 bp from the untranslated leader of RTBV are linked in antisense orientation to the 3'-end of aph4. Numbers indicate RTBV sequences.](image)
2.3 Tissue culture and transformation

2.3.1 Cultures

2.3.1.1 Plants

Rice plants (Oryza sativa L.) of the indica cultivars Tetep, IR24, IR43, IR 58 and IR72 and the japonica cultivars Taipei 309 and Kinuhikari were grown in the greenhouse at 28°C day and 21°C night temperatures (Fig. 8 a,b). The photoperiod was 12 h light supplemented with 400 W fluorescent lamps (MT 400 DL/BH, 400 W mercury lamp, Iwasaki, Tokyo) if a minimum of 100,000 lx, measured outside the greenhouse, was not reached during the day. Humidity was set to 80% during the day and 60% during the night.

Seedlings were transplanted to pots 20 days after germination. The soil was paddled and fertilized with 1 g/l Nutricote (NPK 16-10-10; Maag, Dielsdorf, CH), 1 g/l Plantomaag 4D (NPKMg 20-10-15-3.6; Maag) and 0.25 g/l FeEDTA (Fluka, Buchs, CH). Three plants were grown in one pot, containing 2 l soil (Fig. 8 c-f).

Wheat plants (Triticum aestivum L., cv. Sonora) were grown in the greenhouse at 18°C day (18 h) and 15°C night temperatures.

2.3.1.2 Germination of R1-rice seedlings

Rice seeds from primary transformants were dehusked and surface sterilized by rinsing for 1 min in 70% ethanol and subsequent incubation in 6% Ca(ClO)₂/0.01% TritonX-100 for 1 hour, followed by three washes in sterile water. The seeds were germinated on MS-medium (Murashige and Skoog 1962), supplemented with 30 g/l sucrose and solidified with 4 g/l agarose. Six days after germination they were used for histochemical GUS assays.

To exclude those F1 plants from further cultivation that have lost the transgene due to mendelian segregation, seedlings were transferred 4 days after germination for 5 days onto a metal grid within a glass jar, containing water, supplemented with 10 mg/l Hygromycin B up to the metal grid. Growing exposed to the selective agent, transgenic plantlets developed healthy roots, whereas non-transgenic plantlets died.
Figure 8: Rice cultures a) Greenhouse in Eschikon, Zürich b) Growth chamber for rice c) Nursery pot with germinated rice seedlings d) Four weeks old rice plants, 7 d after transplanting e) Four months old TP309 plants at flowering stage f) Flowering rice panicle g) Intact and dehusked caryopses at around 10, 13, 15 and 25 d after pollination (top to bottom) h) Immature zygotic rice embryos, isolated at different times after pollination.
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2.3.1.3 Isolation and culture of rice immature zygotic embryos

Immature caryopses, 10 to 15 days after pollination, with still liquid endosperm were collected from the plants in the greenhouse (Fig. 8 g). Intact or dehusked caryopses were surface sterilized by incubation for 1 minute in 70% ethanol and subsequent incubation in 6% \( \text{Ca(ClO)}_2 \) 0.01% TritonX-100 for 10 minutes, followed by three washes in sterile water. Immature embryos (Fig. 8 h; only the three oldest stages were used) were excised aseptically and were placed scutellum upside on callus induction medium (MSS3; MS salts and vitamins, supplemented with 2 mg/l 2,4-D, 30 g/l sucrose, 50 mg/l cefotaxime [Duchefa, Haarlem, NL] and solidified with 3.5 g/l agarose Type I [Sigma, Buchs, CH]). The embryos were cultured at 26°C (indica varieties) or 30°C (japonica varieties) respectively in the dark for 3 days, before they were screened for contaminations. 3 to 4 days after isolation, the coleoptiles emerging from the embryos were removed and the embryos were further cultured for 2 to 3 days.

2.3.1.4 Isolation of wheat immature zygotic embryos

Wheat spikelets were surface sterilized in 70% ethanol for 5 minutes and the embryos (8-12 days after pollination; Fig. 12 e), were aseptically excised and plated on MS medium supplemented with 500 mg/l glutamine, 100 mg/l enzymatic casein hydrolysate, 2.0 mg/l 2,4-D (Murashige and Skoog 1962, modified by Vasil et al. 1990), 6% sucrose and solidified with 8 g/l agarose.

2.3.1.5 Establishment of rice embryogenic cell suspensions

Immature zygotic embryos of the rice cultivar TP309 were precultured for 2 weeks on MSS3 medium lacking cefotaxime. Five to ten of the large embryo-derived calli were transferred to 25 ml of liquid R2 culture medium, supplemented with 1 mg/l 2,4-D, 1 mg/l thiamin and 30 g/l sucrose. Cultures were maintained at 30°C in the dark on a rotary shaker at 90 rpm and the medium was changed weekly. Small cell clusters, derived from the large calli, separated and started to proliferate independently. After 2 weeks, the large embryo-derived calli were removed. As soon as the suspension had increased sufficiently in callus mass, it was subcultured weekly and was used for bombardments (Fig. 10 d-g). Suspensions of the japonica cultivar Kinuhikari (a gift from R. Terada; derived from mature embryo-derived calli) were cultured in medium, containing R2 salts and MS vitamins, 100 mg/l inositol, 30 g/l sucrose and 2 mg/l 2,4-D.
2.3.1.6 Production of rice callus material from seeds

Seeds were surface sterilized as described before and were plated on callus growth medium (N6 macro elements, B5 micro elements and vitamins, 500 mg/l proline, 300 mg/l enzymatic casein hydrolysate, 2 mg/l 2,4-D, 30 g/l sucrose; Li et al. 1993). After 3 days, the coleoptile was removed, the mature embryo was separated from the attached endosperm and was placed scutellum side-up on fresh callus growth medium, supplemented with 30 mg/l Hygromycin B. The calli were subcultured weekly. Only the most embryogenic structures were selected and were transferred to fresh medium.

2.3.2 Particle bombardment

For all bombardments, a modified (Iglesias 1994) particle inflow gun (PIG), constructed according to Finer et al. (1992), was used (Fig. 9). Bombardments were carried out following the protocol of Iglesias (1994). Gold particles (spherical gold powder, 1.5-3 μm diameter, Aldrich, Buchs, CH) were suspended in 50% glycerol at a concentration of 50 mg/ml and were autoclaved. Coating the particles with plasmid DNA was performed following a modified protocol of Klein et al. (1988). For 5 shots, 5 μl of plasmid DNA (1 μg/μl) were added into a tube containing 50 μl of particle suspension. While vortexing vigorously, 50 μl of 2.5 M CaCl₂ and 20 μl of 100 mM spermidine were added dropwise. Samples were vortexed for 2 minutes and coated particles were sedimented by a brief centrifugation for 30 seconds at maximum speed in a table top centrifuge. After resuspending the particles in 200 μl of ethanol, samples were again briefly centrifuged and coated particles were resuspended in 35 μl of sterile, distilled water. Before removing 7μl- aliquots for bombardment, the suspension was mixed well by vortexing. The particle suspension was placed in the center of the screen of a metallic syringe filter unit (Sartorius, Nr. SM 16214), serving as particle holder. The target plates were placed on the adjustable shelves at 11.5 or 14 cm distance from the particle holder. A nylon baffle grid (500 μm nylon mesh) was placed between the filter and the target at a distance from the filter of 9 cm in order to reduce gas impact on the tissue. The chamber was evacuated to 100 mbar and the particles were accelerated by a helium jet generated by 6-7 bar pressure for 50 milliseconds (Fig. 9).
2.3.2.1 Bombardment of precultured rice embryos

30 to 60 minutes before bombardment, 12 precultured embryos were placed in the center of a 5 cm petri dish (Fig. 10 b,c) containing plasmolysing medium (MSS10; MS salts and vitamins, supplemented with 100 g/l sucrose and solidified with 5 g/l agarose Type I. The precultured embryos were bombarded with the culture plates at a distance of 14 cm from the particle holder. After bombardment, the embryos were incubated overnight on the plasmolysing medium.

2.3.2.2 Bombardment of rice embryogenic cell suspensions

Two to four days after subcultururing, the suspension calli were used for bombardment. One hour before bombardment, the suspension calli were incubated in liquid plasmolysing medium, containing 100 g/l sucrose. After 30 minutes, this medium was removed and the suspension calli were spread in the center of a culture plate with plasmolysing medium, solidified with 5 g/l agarose type I (Fig. 10 g). With one container of suspension, 2 to 4 plates could be prepared, depending on the age of the culture. Before bombardment, as much as possible of the remaining liquid was removed from the cell clusters by pipetting and by drying the plates for 5 to 10 minutes in the air flow. The suspension calli were bombarded with the culture plates at a distance of 11.5 cm from the particle holder. After bombardment, the suspension calli were incubated over night on the plasmolysing medium.
2.3.2.3 Bombardment of etiolated rice seedlings

IR72 seeds were sterilized as described before and germinated at 26°C in the dark on MS medium solidified with 5 g/l agarose. Five days after germination the shoots of the seedlings were placed side by side on MS medium, containing MS salts and vitamins, 100 g/l sucrose, solidified with 5 g/l agarose type I for plasmolysis. After one hour, the coleoptiles were bombarded on this medium in batches of eight to ten with the target plates at a distance of 11.5 cm from the particle holder.

2.3.3 Tissue electroporation

For all electroporation experiments, a Biorad Gene pulser™ with capacitance extender was used (Fig. 11 e).
2.3.3.1 Electroporation of embryos

**Electroporation chamber.** A special electroporation device was constructed in which the embryos could be fixed (Fig. 11). The reaction chamber has a diameter of 7 mm, the distance between the electrodes is 4 mm. The bottom part is made from one piece of acrylic glass. The electrode-holders and the electrodes are made of brass, the electrodes are gold-plated (Nickelflash plus 2 µm gold-plated; W. Flühmann AG, Dübendorf, Switzerland). The bottom electrode is tightened in the reaction chamber by a rubber grummet. The electric pulses, discharged from the capacitor, have an exponential decay wave form.

**Fixation of the embryos on agarose discs.** A 22 x 60 mm Thermanox™ coverslip (Nunc Inc., Naperville, IL) was placed on a ceramic plate. Above this slide a tunnel with three microscope slides was formed. The medium (MS medium solidified with 30 g/l agarose (Sea Plaque; FMC Bioproducts, Rockland, USA) containing 15% sucrose and 6 mM CaCl₂) was boiled and 1 ml was added into the tunnel to form a 1 mm thick agar layer (Fig. 12 a). A moistened filter paper was placed into the cover of a 9 cm petri dish and the thermanox coverslip with the polymerized agarose was transferred into the petri dish. With the top of a 1.5 ml centrifuge tube, discs, 9 mm in diameter, were pricked out (Fig. 12 b).

For fixation, ten embryos were placed on a droplet of 4% alginate on an agarose disc (Fig 12 c,d). The discs with the embryos were placed upside down on the upper electrode (Fig. 11 d).

**Electroporation of fixed embryos.** The electroporation chamber was sterilized by rinsing with 70% ethanol, was fixed and filled with chilled electroporation buffer (35 mM aspartic acid monopotassium salt, 35 mM glutamic acid monopotassium salt, 5 mM calcium gluconate, 5 mM MES and 0.4 M mannitol, pH 5.8; Tada *et al.* 1990) containing the plasmid DNA. The agarose disc with the fixed embryos was carefully placed in the chamber and air bubbles in the chamber were replaced with buffer. A single electric pulse was discharged from a 960-µF capacitor. Immediately after the delivery of the pulse, the embryos were removed and were washed for 1 minute in MS medium containing 3% sucrose. The discs with the embryos were placed on MS medium containing 3% sucrose and 8 g/l agarose and were incubated at 26°C.
Figure 11: Electroporation chamber device allowing fixation of tissues. a) Top view of the bottom part of the chamber setup. b) Top part of the setup. c) Transverse section of the reaction chamber with added top and bottom electrodes and rubber grummet. d) Transverse section of the reaction chamber. e) Gene pulser with capacitance extender from Biorad. f) Electroporation device in working position. g) Gold-plated electrodes, brass electrode holders and rubber grummet.
Optimization of parameters. Ten embryos were used per treatment. Electroporations were carried out with a plasmid DNA concentration of 50 µg/ml, a single electric pulse of 275 V/cm, sucrose concentrations in the culture medium of 6% (w/v) before and 3% (w/v) after electroporation, varying the respective parameter in the individual experiments.

2.3.3.2 Electroporation of embryogenic cell suspensions

Electroporation chamber. Suspension calli were electroporated in a Teflon® chamber with two gold-plated electrodes inserted from both sides, with a distance of 4 mm between the electrodes. The cylindric chamber could be placed inside a 2 ml centrifuge tube. During centrifugation, the suspension calli were retained by a nylon mesh (Fig. 13).
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**Electroporation of rice TP309 suspension calli.** 0.5 to 1 g of TP309 suspension calli were transferred to a sterile petri dish. 2 ml of 0.3% (w/v) Macerozyme (Yakult, J; in CPW salts; 5 mM MES, 10% mannitol; D'Halluin et al. 1992) solution were added for 3 minutes, followed by washing five times with washing solution (CPW salts; 5 mM MES, 10% mannitol). The calli were transferred to a 2 ml centrifuge tube and were preincubated three hours in electroporation buffer at RT, the buffer was changed three times. After this preincubation, the buffer was removed and 1.2 ml of fresh buffer and 100 μg of plasmid DNA were added. The anode was inserted into the chamber and the nylon mesh was added on top of it. 130 μl of the buffer containing the plasmid DNA and 50 to 100 mg of the preincubated calli were added. The cathode was fixed in place, avoiding air bubbles in the chamber. After delivery of the electric pulse the electrodes were removed and the chamber was placed in a 2 ml centrifuge tube lacking the lid. After addition of 0.8 ml of suspension culture medium, the chamber was closed with the lid of the centrifuge tube and the tube was centrifuged for a few seconds at 2000 rpm in a table top centrifuge. With a sterile glass stick, the nylon mesh with the suspension calli was transferred into a compartment of a 6-well dish, containing 5 ml of suspension culture medium.

**2.3.4 Selection of resistant rice calli after bombardments**

*Precultured TP309 embryos.* After over-night incubation on plasmolysing medium, the embryos were transferred to semi-solid culture medium (MSS3-H30), containing 30 mg/l Hygromycin B. After 5 to 7 days, the embryos were transferred into a beaker containing 25 ml of liquid R2-selection medium (Ohira et al. 1973), supplemented with 1 mg/l 2,4-D, 1 mg/l Thiamin, 30 g/l sucrose and 30 mg/l Hygromycin B. The liquid medium was changed weekly. Resistant colonies (Fig. 14 a,b) were transferred to R2-callus increasing medium (R2I),
containing R2 salts, MS vitamins, 100 mg/l inositol, 2 mg/l 2,4-D, 60 g/l sucrose, 30 mg/l Hygromycin B, solidified with 5 g/l agarose Type I.

**Precultured indica embryos.** After over-night incubation on plasmolysing medium, the embryos were transferred to culture medium MSM3, containing 30 g/l maltose (Ghosh Biswas and Zapata 1993). The embryos were transferred weekly to fresh medium supplemented with 50 mg/l Hygromycin B (MSM3-H50). Resistant colonies, growing on the surface of the embryos were separated after having reached a seize of 2-3 mm in diameter and were further cultured on fresh medium.

**Suspension calli.** After over-night incubation on plasmolysing medium, the bombarded cell clusters were transferred into beakers, containing 25 ml of culture medium and were incubated on a rotary shaker at 90 rpm at 30°C in the dark. After one week, the medium was replaced by medium, supplemented with 30 mg/l Hygromycin B and was changed weekly. Resistant colonies were picked from the liquid and were transferred to R2I-medium.

### 2.3.5 Selection of resistant rice calli after electroporation

After two days in liquid culture medium, the suspension calli were transferred to semi-solid callus increasing medium (R2I), containing 30 mg/l Hygromycin B. Resistant colonies were isolated after 4 weeks and were transferred to fresh medium.

### 2.3.6 Regeneration of rice plants from resistant calli

Resistant calli growing on callus increasing medium (Fig. 14 c), were transferred weekly to fresh medium. Only the most embryogenic parts of the calli (Fig. 14 d) were isolated and further cultured.

Embryogenic calli of appropriate size (at least 5 mm in diameter) were transferred to R2-regeneration medium (R2R), containing R2 salts, MS vitamins, 100 mg/l Inositol, 1 mg/l zeatin (Duchefa, Haarlem, NL), 0.5 mg/l IAA (Sigma, Buchs, CH), 20 g/l sucrose, 30 g/l sorbitol, 30 mg/l Hygromycin B, solidified with 8 g/l agarose Type I. After placing the calli on R2R-plates, the plates were air dried for 15 minutes to enhance desiccation of the calli (Rancé et al. 1994). The calli were subcultured every 2 to 4 weeks. Calli with shoots of 2 cm in length (Fig. 14 e) were transferred to rooting medium (Fig. 14 f), containing MS salts and vitamins, 30 g/l sucrose, solidified with 3 g/l gelrite (Scott, Fiskeville). Plantlets were transferred to soil (Fig. 14 g) after 1 to 2 weeks and were grown to maturity (Fig. 14 h,i) within 3 to 4 months (cv. TP309) or 2 to 3 months (cv. Kinuhikari).
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Figure 14: Selection of resistant colonies and regeneration of transgenic rice plants. a,b) Resistant colonies (arrows), emerging from bombarded precultured embryos in liquid selection c) Resistant colonies growing on semi-solid callus-increasing medium d) Embryogenic callus after two weeks on callus increasing medium e) Shoots and roots regenerating from embryogenic callus f) Plantlets on rooting medium g) Plants, four weeks after transplanting to soil h,i) Fertile transgenic TP309 plants.
2.4 Analysis of the transformed plants

2.4.1 Molecular analysis

2.4.1.1 Generation of digoxigenin-labeled specific probes

Southern and Northern analyses were performed using the Digoxigenin-system from Boehringer Mannheim (Boehringer 1993). A 350 bp probe from the hygromycin resistance gene (aph4) and a 275 bp-probe from the RTBV leader region were synthesized. Digoxigenin-linked dUTP nucleotides were incorporated into the specific probes by PCR using specific primers. In the PCR, 45 cycles (1 min denaturation at 94°C, 2 min annealing at 53°C, 3 min extension at 72°C) were run with 100 pg of the plasmid pHCC23 as the template and with 4 pM each of the forward primer GATCAAGCAAGCGAGAG (RTBV nts 7409-7425) and the reverse primer AACACTTAATCTTAGAAGG (RTBV nts 7670-7652). The PCR product was purified by passing through a PCR product purification column (Boehringer). Incorporation of DIG-nucleotides into the PCR product was determined following the suppliers instructions. For hybridization, 20 ng of denatured probe per ml hybridization solution were used.

2.4.1.2 Isolation of plant genomic DNA and Southern analysis

Genomic plant DNA was isolated from leaves using a CTAB extraction method, modified after Murray and Thompson (1980). Plant material was powdered in liquid nitrogen and thawed in 0.8 ml of extraction buffer (50 mM Tris-HCl, pH 8.0; 700 mM NaCl; 10 mM EDTA; 1% [w/v] CTAB; 1% [v/v] β-mercaptoethanol). After heating the samples for 15 minutes at 56°C, debris and proteins were extracted with 0.8 ml CO (chloroform/octanol 24:1 [v/v]). 80 μl of CTAB (10% [w/v] CTAB in 700 mM NaCl) were added and nucleic acids were precipitated with 1 ml of precipitation buffer (extraction buffer without NaCl and β-mercaptoethanol). Nucleic acids were redissolved in 0.4 ml 1 M NaCl, treated with RNaseA (1μl of RNase A stock, 2 mg/ml in 10 mM Tris-HCl, pH 8.0; 15 mM NaCl). The DNA was precipitated with 2.5 volumes of ethanol. Finally the DNA was washed and resuspended in sterile water.

Approximately 10 μg of total genomic DNA was digested with appropriate endonucleases and fragments were electrophoretically separated on 0.9% agarose (Sigma, Type I) gel. The gels were incubated for 10 minutes in 0.25 M HCl, then the DNA was denatured for 30 minutes in 0.5 M NaOH, 1.5 M NaCl. After washing the gel in neutralization buffer (0.5 M Tris-HCl pH 7.4;
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1.5 M NaCl; 1mM EDTA) two times for 20 minutes, the DNA was transferred to a positively charged nylon membrane (Boehringer) to which DNA was covalently bound by baking the membrane for 30 minutes at 120°C. Membranes were hybridized with digoxigenin-labeled probes and were washed and detected following the suppliers instructions (Boehringer). Signals were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY).

2.4.1.3 Isolation of total RNA and Northern analysis

Total RNA was extracted from either leaves of young seedlings, leaves from primary transformants or from seed derived callus using a modified method of Chomczynski and Sacchi (1987). Leaves were powdered in liquid nitrogen and thawed in 1.8 ml of extraction buffer (RNA-Clean; Axonlab, Switzerland). After adding 200 µl of chloroform, the suspension was mixed, incubated for 15 minutes at 4°C and centrifuged for 15 minutes in a table top centrifuge. To precipitate the RNA, the aqueous phase was transferred to a fresh tube, mixed with one volume of isopropanol, mixed well and incubated for at least 15 minutes at 4°C. The RNA was collected by centrifugation for 15 minutes at 4°C. The pellet was washed with cold 70% ethanol and resuspended in 80 µl of 1 mM EDTA. The RNA concentration was determined photometrically at 260 nm and the quality was checked on a 1% agarose gel.

5-30 µg of total plant RNA were lyophilised and dissolved in 8µl of 1 mM EDTA. 8 µl of denaturing buffer (50% deionized formamide, 12% formaldehyde, 5 mM EDTA in 2x MAE buffer) and 1 µl of 0.4 mg/ml ethidium bromide were added, samples were denatured for 10 minutes at 65°C and placed on ice. Samples were electrophoresed under denaturing conditions (1% agarose gel containing 6% formaldehyde) and were transferred overnight with 20xSSC to Hybond N membrane (Amersham). Filters were hybridized with a digoxigenin-labeled DNA probe and were washed and detected following the suppliers instructions. Signals were visualized on Kodak X-Omat AR film.

2.4.1.4 Isolation of plant proteins and Western analysis

100 mg of mature embryo-derived callus were powdered in liquid nitrogen and thawed in 300 µl of buffer (Lämmli 1970; 10% glycerol, 0.25 M Tris pH 6.8, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue). The suspension was boiled for 5 minutes and was clarified by centrifugation for 10 minutes at 4°C. 40 µl aliquots were fractionated on 12.5% mini-SDS-polyacrylamide gels following the manufacturer's instructions (Biorad). Proteins were transferred to nitrocellulose membrane (Schleicher und Schuell), using a mini transfer cell (Biorad),
following the manufacturer's instructions. The membranes were blocked in TBS containing 0.1% Tween-20 (TBST) and 5% dried milk, washed and incubated in TBST with a rabbit anti-tag antibody, diluted 1:1000. After washing, antigen-antibody complexes were detected with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins, using the enhanced chemiluminescence procedure (ECL Western blotting, Amersham, UK).

2.4.2 Histochemical GUS assay

2.4.2.1 Assay with bombarded material

Expression of GUS was visualised by transferring the plant material to a filter-sterilized GUS substrate mixture containing 100 mM 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). After incubation at RT or 37°C for 10 minutes to 24 hours, the plant material was analysed with a microscope.

2.4.2.2 Assay with transformed plants

Six day-old seedlings were sliced with a thin razor blade. Slices were immediately transferred to GUS-staining solution (50 μl in compartments of a 96-well-plate). After 16 hours at 37°C, 250 μl of 96% ethanol were added to extract the chlorophyll from the tissue. After 30 minutes, the turbid liquid was removed and 300 μl of sterile water were added. Finally, the sliced tissues were examined in glycerol with an inverted light microscope.

2.4.3 Virus resistance tests

Seeds were soaked in running tap water for 2 days, sown in clay pots (4 plants per pot) and grown for 14 days. Adult green leafhopper (GLH), *Nephotettix virescens* that had had access to Taichung Native 1 (TN1) plants infected with RTBV and RTSV for 4 days were allowed an inoculation access of 24 hours (3 insects per seedling) in mylar cages. Seedlings were sprayed with insecticide and were grown for 40 days for symptom development. ELISA was performed after 20 and 40 days, using the method of Clark and Adams (1977), applied to tungro-associated viruses by Hibino et al. (1990). The symptom severity (SS) was scored visually by experienced personnel on the basis of plant height and leaf colour (Hasanuddin et al. 1988).
3. RESULTS

3.1 Transformation methods

A basic prerequisite for this project is a reliable and efficient method for rice transformation, yielding fertile transgenic plants in a time as short as possible. Two transformation methods were evaluated, particle bombardment and tissue electroporation.

Two different types of totipotent cells were used as target plant material: Scutellum cells of immature zygotic embryos and embryogenic suspension cells. Embryos were used either freshly isolated or were precultured for several days, producing embryogenic callus from scutellum cells. Embryogenic cell suspensions (ECS) were established from precultured mature or immature embryos.

3.1.1 Transformation by tissue electroporation

3.1.1.1 Electroporation of immature zygotic embryos

A special electroporation chamber was designed for efficient transformation of embryo scutellum cells (described in "Material and methods").

3.1.1.1.1 Transient expression in immature embryos

First, different plant materials like immature embryos and suspension cells of rice and wheat were electroporated using parameters adapted from Tada et al. (1990; electroporation buffer without chloride ions) and Dekeyser et al. (1990; voltage and capacity). Plasmid pBC17, containing C1 and Bperu, two regulatory genes from the anthocyanin biosynthesis pathway, was used as visual marker. These genes induce production of anthocyanin in transformed cells within a few hours after transformation (Cone et al. 1986; Goff et al. 1990). Without any pretreatment, different cells, including the regenerable scutellum cells of immature zygotic wheat embryos could be transformed under the applied conditions. Since wheat embryo scutellum cells responded well, different steps in the electroporation protocol were optimized with wheat embryos. 24 hours after transformation, anthocyanin producing cells could be detected on the embryos (Fig. 15 a-b). The anthocyanin accumulated in the vacuoles of the
cells (Fig. 15 c). Sectors of red cells were observed a few days or weeks after transformation in experiments in which only single stained cells were found after one day (Fig. 15 d,g). This cell division activity indicates that transformed cells survived electroporation.

Figure 15: Tissue electroporation of immature embryos. a-c) Wheat immature embryos transiently expressing anthocyanin 24 h after electroporation. Anthocyanin is accumulated in the vacuoles. Bars are 1 mm. d) Sectors of anthocyanin expressing cells 96 h after electroporation in the scutellum of a wheat embryo. e,f) Colocalization (arrows) of anthocyanin accumulation (e) and of transient GUS expression (f) within individual cells of a wheat immature embryo 24 h, resp. 48 h after electroporation. g) Anthocyanin accumulating tissue in young regenerating shoot in wheat, 5 weeks after electroporation. h) Rice immature embryo, 24 h after electroporation. Anthocyanin accumulating cells (arrow) on the coleoptile side of the embryo.
After co-transformation with pBC17 and pAct1-D containing the *uidA* gene under control of the rice actin promoter, both anthocyanin accumulation and GUS staining could be detected in individual cells of the scutellum (Fig. 15 e,f; arrows).

### 3.1.1.1.2 Optimization of electroporation conditions

Several experiments were performed to optimize different parameters of the electroporation procedure (Fig. 16). Best transformation (highest number of transformed cells) was obtained with electric pulses of 225-325 V/cm (Fig.-16 a). With pulses higher than 325 V/cm, the tissues were damaged and only few transformed cells could be detected. The number of transformed cells increased linearly with the DNA concentration (Fig. 16 b). Subsequent experiments were carried out with single electric pulses of 275 V/cm discharged from the 960 μF capacitor and with a plasmid DNA concentration of 50 μg/ml. The osmotic strength of the electroporation buffer in the range of 0.2 to 0.8 M was found to have no influence on the number of transformed cells (data not shown). Plating the embryos for a short time on MS medium containing 6% sucrose before electroporation (Fig. 16 d) and culturing them on MS medium containing 3% sucrose after transformation (Fig. 16 c) proved to be optimal for maximal transient expression.

Under optimal electroporation conditions (summary in table 2), transformation of wheat scutellum cells with plasmid pBC17 resulted in more than one hundred anthocyanin accumulating cells per embryo. As many cells strongly accumulating anthocyanin were surrounded by non-accumulating cells (Fig.-15 c), diffusion of anthocyanin to neighbouring cells can be excluded. Therefore all the accumulating cells were considered as transformed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>optimal value as determined</th>
</tr>
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<tbody>
<tr>
<td>voltage, discharged from a 960 μF capacitor</td>
<td>275 V/cm</td>
</tr>
<tr>
<td>plasmid DNA concentration in the buffer</td>
<td>linear increase (100 μg DNA/ml)</td>
</tr>
<tr>
<td>sucrose concentration in the medium before/after electroporation</td>
<td>6%/ 3%</td>
</tr>
<tr>
<td>incubation time on medium before electroporation</td>
<td>short (30 min)</td>
</tr>
</tbody>
</table>

**Table 2:** Optimized parameters for tissue electroporation (of wheat immature embryos).
Figure 16: Transient expression in wheat immature embryos after tissue electroporation. Values on the y-axis represent numbers of anthocyanin-expressing cells per embryo 24 h after transformation. **a)** Optimization of the voltage discharged from a 960-μF-capacitor. **b)** Influence of plasmid DNA concentration on transformation efficiency. **c)** Influence of the sucrose concentration in the culture medium before and after electroporation. **d)** Influence of the preincubation time of isolated immature wheat embryos on medium containing 6% sucrose before electroporation.

3.1.1.3.3 Arrangement of the embryos in the electroporation chamber

Since it was observed that embryos always had transformed cells only on one side, it was speculated, that the negatively charged DNA molecules move towards the anode during the delivery of the electric pulse. This influence of the relative orientation of the embryos in the electroporation chamber was demonstrated by the experiment, shown in table 3. By arranging the embryos in the reaction chamber, it was clearly demonstrated that only cells, facing the cathode get transformed during electroporation (Table 3 b-d).
Results

<table>
<thead>
<tr>
<th>orientation</th>
<th>transformed cells in scutellum</th>
<th>transformed cells on coleoptile side</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wheat</td>
<td>rice</td>
</tr>
<tr>
<td>a randomly</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>b embryos fixed at the anode; scutellum facing the cathode</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>c embryos fixed at the anode; coleoptile facing the cathode</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d embryos fixed at the cathode; scutellum facing the anode</td>
<td>0</td>
<td>-</td>
</tr>
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</table>

Table 3: Orientation of embryos in the electroporation chamber. Values represent red spots (anthocyanin-expressing cells) 24 h after electroporation. 

To transform the scutellum cells rather than the cells on the coleoptile side of the embryo, the embryos were fixed on small discs of agarose (see figure 12 in "Material and methods"). These discs with the fixed embryos were inserted into the chamber and the electrodes were appropriately connected to the electroporation machine (see figure 11 d in "Material and methods").

3.1.1.1.4 Different susceptibility of tissues to gene transfer by electroporation

With the experiment in table 3, it could also be demonstrated, that the different parts of the embryos differently respond to electroporation-mediated DNA transfer:

- Embryos randomly placed in the electroporation chamber were found to have more transformed cells in the scutellum than in other tissues (Table 3 a).
- Fixed embryos with the scutellum facing the cathode had an average of 21 cells per embryo showing anthocyanin accumulation (Table 3 b).
- Fixed embryos with the coleoptile side facing the cathode had a sevenfold decrease in the number of transformed cells (Table 3 c).

This indicated that the scutellum cells of wheat embryos are far more responsive to DNA-uptake or expression than any other cells on the surface of
the embryo. Further it was found, that cells of wheat embryos, isolated 8-12 days after pollination (see figure 12 e in "Material and methods") were much more responsive to electroporation-mediated gene transfer than cells of embryos that were either younger or older (data not shown).

In contrast to these findings with wheat embryos, a different tissue specificity was found with rice embryos. While the non-regenerable cells on the coleoptile side of the embryo could easily be transformed under the applied conditions (Fig. 15 h), no transformed cells at all could be detected in the scutellum (Table 3 b,c). It was not investigated, whether wounding of scutellum cells could increase the transformation rate. Instead, experiments were continued using rice embryogenic suspension cells as targets.

3.1.1.2 Electroporation of TP309 embryogenic cell suspensions

For electroporation of embryogenic suspension cells, a reaction chamber with removable electrodes was designed that fits into a 2 ml centrifuge tube. This allowed removal of buffer and washing the calli by centrifugation, retaining the plant material with a nylon mesh (described in "Material and methods").

3.1.1.2.1 Transient expression in suspension calli

The first experiment was performed with the conditions optimized for immature embryos. A few transiently transformed cells could be detected one day after transformation, using plasmid pHClintG (Fig. 17). After enzymatic pretreatment (incubation for 3 min in 0.3% (w/v) macerozyme solution), a tenfold increase of the number of transiently expressing cells could be observed. The optimal voltage was determined at 500 V/cm. Subculturing the suspension two days before electroporation proved to be important for maximal transformation efficiency (data not shown).

Under optimized conditions, nearly 400 transiently GUS expressing cells per gram fresh weight of suspension calli were found (Table 8, Fig. 22). These results of successful DNA transfer to regenerable rice cells encouraged selection for stably transformed rice calli.
3.1.1.2.2 Stable transformation of suspension calli

For stable transformation, rice suspension calli were electroporated under the described optimized conditions using plasmid pHCntG. After several weeks, more than 20 hygromycin resistant colonies had evolved from the experiments. In a histochemical GUS assay (Jefferson 1987), callus material from only one of the resistant clones stained blue (Fig. 17 a). Southern analysis confirmed that this callus was transgenic (Fig. 17 c). Several plantlets, regenerated from this clone, were transferred to the greenhouse and developed to fertile plants (Fig. 17 b).

3.1.2 Transformation by particle bombardment

3.1.2.1 Bombardment of precultured immature zygotic embryos

For bombardment of the embryos with a gold particle/DNA suspension, a particle inflow gun (PIG) was used.
3.1.2.1.1 **Optimization of bombardment conditions**

Several steps and parameters of the bombardment procedure were optimized for transformation of rice embryos. For the optimization experiments, the GUS-reporter system (Jefferson 1987) with plasmid pHClintG was used. In the following experiments, blue cells or clusters of blue cells are termed "blue spots" (Fig. 18).

The following parameters were tested:

*Particle amount per shot*: In bombardment experiments, an insufficient amount of particles leads to low transformation efficiency, whereas an excess of particles causes unnecessary destruction on the target tissue. Embryos were bombarded with variable amounts of particles, ranging from 100 to 1000 μg per shot. An optimum of 500 μg particles per shot was found, as the number of blue spots increased up to this concentration but did not further increase with higher concentrations (Fig. 19 a).

*Coating of particles*: Three different protocols for preparing the particle/plasmid suspensions were compared: Coating of particles with plasmids and resuspending the coated particles in water, coating of particles with plasmids and resuspending the coated particles in plasmid solution and suspending uncoated particles in plasmid solution. The protocols including coating of the particles yielded better results than the protocol with uncoated particles. Resuspending the coated particles in water gave better results than resuspending them in plasmid solution (Fig. 19 b).

*Plasmolysis before bombardment*: Vain et al. (1993) demonstrated that preculture of tissue on high osmotic pressure improved transformation efficiency. Plasmolysis of the cells prevents the cells from bursting when hit by the particles. Therefore, embryos were plasmolysed on MSS10, containing 10% sucrose, for 5 to 120 min. A clear increase in transformation frequency was found with plasmolysed embryos compared to non-plasmolysed embryos. No apparent variations could be found for incubation times between 5 and 60 minutes, indicating that plasmolysis of embryo scutellum cells is a fast process. However, embryos plasmolysed for as long as two hours before bombardment showed clearly less blue spots (Fig. 19 c). In all the following
experiments the embryos were plasmolysed for about 30 to 60 minutes before bombardment.

**Choice of cultivar.** For a comparison of transformation efficiencies using different rice cultivars, embryos of the indica varieties IR43 and IR58 and the japonica variety TP309 were bombarded. Average numbers of blue spots per embryo and therefore efficiency of DNA transfer did not significantly vary among the tested cultivars (Fig. 19 d).

![Graphs showing optimization of the bombardment parameters for immature embryos.](image)

**Figure 19:** Optimization of the bombardment parameters for immature embryos. Values on the y-axis represent blue spots (transiently GUS-expressing cells) per embryo 24 h after bombardment. **a)** Influence of the particle amount per shot. **b)** Influence of the coating procedure; coating versus non-coating and resuspending the particles in water or DNA-solution. **c)** Influence of the time on culture medium with high osmotic pressure before bombardment. **d)** Influence of the cultivar.

### 3.1.2.1.2 Stable transformation of immature embryos

**Transformation of indica cultivars.** With the optimized bombardment protocol, more than 2400 precultured immature embryos of the indica cultivars IR24, IR43, IR58, IR72 and Tetep were bombarded. After two to five weeks on
selection, resistant colonies started to grow on the surface of the calli (Fig. 20). Among these bombarded embryos, ten embryos of the cultivar IR58 yielded resistant colonies that were proliferating for two to three weeks on selection. However, the resistant calli died, before Southern analysis could be performed.

![Figure 20: Bombarded indica embryos on hygromycin selection. Resistant colonies (arrows) are growing on bombarded precultured immature embryos of the cultivar IR58.](image)

Even if all the resistant colonies, derived from the 2400 embryos had been transgenic, transformation frequency was still too low for the purpose of transforming many different constructs. Therefore, a more efficient transformation system for rice was needed. An efficient system was found by using japonica rice cultivars instead of the recalcitrant indica varieties.

*Transformation of japonica cultivar TP309.* Precultured immature TP309 embryos were bombarded with plasmid pHClntG. Resistant colonies were selected in liquid culture medium. From the liquid selection medium, they were transferred to semi-solid callus-increasing medium and further to regeneration medium. Several plants were transferred to the greenhouse and developed to fertile plants. Transformed plant material, tested at different stages during the transformation, selection and regeneration procedures, stained deep blue in histochemical GUS assays (Fig 21 a,b).

![Figure 21: Stable transformation of TP309 by particle bombardment. a) Hygromycin-resistant colony, transformed with pHClntG, growing on bombarded TP309 precultured embryo after selection of 3 weeks in liquid medium. b) Plantlet, regenerating from transformed callus. c) Fertile, transgenic plant.](image)
3.1.2.2 Bombardment of embryogenic suspension calli

As an alternative target material for particle bombardment, embryogenic cell suspensions (ECS), established from embryogenic callus from the scutellum cells of immature embryos, were used. For the bombardment of ECS the suspension calli were spread on semi-solid medium plates. After bombardment, the calli were transferred back to liquid culture medium. Plants were transferred to the greenhouse and developed to fertile plants.

3.1.3 Particle bombardment versus tissue electroporation

To compare the transformation frequencies, an embryogenic TP309 cell suspension was transformed by particle bombardment and by tissue electroporation, using the optimized protocols.

In the bombarded suspension, the number of transiently expressing cells was twice as high as in the electroporated suspension (Table 4). With respect to the amount of DNA used, particle bombardment was around 140 times more efficient than tissue electroporation (Table 4).

<table>
<thead>
<tr>
<th>method</th>
<th>blue cells per µg fresh weight</th>
<th>blue cells per µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>particle bombardment</td>
<td>740</td>
<td>372</td>
</tr>
<tr>
<td>tissue electroporation</td>
<td>391</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 4: Number of transiently GUS-expressing cells (per gram freshweight suspension culture or per µg DNA) after transformation by particle bombardment and tissue electroporation, using plasmid pHClntG.

Transient GUS-expression in cells transformed by electroporation was clearly weaker than in cells transformed by particle bombardment, where expression usually was high, resulting in transformed cells that stained deep blue (Fig.22).

**Figure 22:** GUS expression in suspension calli. a,b) After particle bombardment c,d) After tissue electroporation. Bars are 500 µm (a,c; stereo microscope) or 50 µm (b,d; inverted light microscope).
With both methods, tissue electroporation and particle bombardment, fertile, transgenic rice plants were obtained. Particle bombardment was chosen as routine transformation system as more transgenic plants can be produced considering working time and required amount of DNA. Unfortunately, nothing is known so far about the influence of the transformation method on the quality and quantity of plasmid DNA integration into the plant genome.

### 3.2 Strategies for conferring resistance to RTBV

To confer resistance to RTBV several strategies were followed: Expression of the viral coat protein, expression of the viral replicase, expression of viral proteins with functions unknown so far and production of antisense RNA against the leader of the pregenomic mRNA.

In the strategies where the expression of proteins was intended two different approaches were followed:

- Expression of natural viral proteins with the intention to disturb protein balances during viral replication
- Expression of mutated viral proteins that might compete with the virus-encoded proteins for substrates.

In the following the different strategies are described. For plasmid construction details refer to the chapter "Material and methods". A summary with all the constructs is given in table 5.

#### 3.2.1 Expression of the coat protein

The incorporation and expression of viral coat protein genes has provided one of the strongest forms of genetically engineered, pathogen-derived resistance yet developed (Hackland et al. 1994).

In RTBV preparations, two coat proteins with apparent molecular weight of 37 and 33 kDa were found (Qu et al. 1991). The amino terminus of the 33 kDa coat protein of RTBV was determined at amino acid 502 (nucleotide 2497). The amino terminus of the 37 kDa coat protein could not be determined because of an unknown modification. The carboxy terminal ends of both proteins were not localized either. Since in Western analysis of infected rice leaves only the 37 kDa protein could be detected, Qu et al. (1991) concluded that the smaller protein might be a processed product of the 37 kDa protein, arising during virus purification or storage.
It is likely that the N-terminus of the 37 kDa protein is located about 40 amino acids upstream of that of the 33 kDa protein. In order to express this putative 37 kDa coat protein, the RTBV sequence encoding amino acids 462-821 was cloned in plasmids pHCC23 and pHRC23. This region contains the cystein-histidin motif \(\text{CYICQDENHLANRC;}\) close to the carboxy-terminal end. This element is conserved among retro-elements and is supposed to bind RNA during packaging of the pregenomic RNA in order to separate it from the pool of cellular RNAs as template for reverse transcription (Bowles et al. 1993). The RTBV sequence in plasmids pHCC24 and pHRC24 starts at the same position as the sequence in plasmid pHCC23, but is lacking the RNA binding motif at the 3'-end. If this RNA binding element has a function in the replication of RTBV, the non-functional mutant could interfere with the virus-encoded coat protein by competing for the presence in the capsid structure.

### 3.2.2 Expression of the replicase

Viral enzymes involved in the replication of the viral genome, like reverse transcriptase (RT), RNA-dependent RNA polymerase or RNaseH, are summarized as “replicases”. In several cases, plants transformed with sequences from a viral replicase, became resistant to infection with these viruses (Carr and Zaitlin 1993). Enzymatic domains from RTBV were identified by homologies to highly conserved sequences in other retroid elements (Argos 1988; Füttener and Hohn 1987; Qu et al. 1991; Hay et al. 1991): an aspartic protease (\(\text{LIDSGS;}\) ORF3 amino acids 986-992), a reverse transcriptase (\(\text{YIDDILI;}\) ORF3 amino acids 1339-1345) and a RNaseH (dispersed sequences between ORF3 amino acids 1486 and 1607). Laco et al. (1995) indeed proved protease, DNA polymerase and RNaseH activities for proteins containing these domains. The sequence in plasmid pHCPol contains the viral protease, the RT and the RNaseH domains. As the sequence contains the viral aspartic protease, the resulting protein might be cleaved to smaller fragments.

Anderson et al. (1992) and Longstaff et al. (1993) mutated the GDD box present in all RNA-dependent RNA polymerases and found that plants transformed with these mutated replicase sequences were highly resistant to the respective viruses.
In order to produce a similarly inactivated RTBV-RT, in plasmids pHCPol(Xh) and pHRPol(Xh) the sequence coding for amino acids IDD was mutated to encode LEE by PCR-mediated mutation, at the same time creating an Xhol restriction site.

Subfragments of the polyfunctional RT were cloned to follow a strategy that was successful with some RNA viruses, where subfragments of the polymerase gene produced high levels of protection (Wilson 1993). Sequences in the plasmids pHCPol(XhBa) and pHCPol(HiXh) were both derived from the sequence in plasmid pHCPol(Xh) and contain the viral protease or the RNaseH domains, respectively.

The RTBV sequence in plasmids pHZPol and pHZPol is identical with the sequence in pHCPol, additionally comprising a zinc-finger-like structure at the amino-terminus, that might have nucleic acid-binding activity (Berg 1990; De Rocquigny et al. 1992).

The RTBV sequence in plasmids pHCR and pHRR includes the coat protein, the replicase and the aspartic protease. Two sites were mutated. First, 14 amino acids from the cys-his motif in the coat protein region were mutated to two glycins. This mutation destroys the RNA binding activity of the protein, but probably preserves the structure of the remaining part (De Rocquigny et al. 1992). Second, as in plasmid pHCPol(Xh), the conserved sequence IDD of the reverse transcriptase was mutated to LEE.

The RTBV sequence in plasmid pHC3 represents the complete ORF3. The viral protease, present in the polyprotein, may cleave all the proteins contained within the large 192 kDa protein to natural lengths.

3.2.3 Expression of proteins with unknown functions

The functions of the products of ORFs 1 and 4 are unknown. As an over-production of any viral protein could interfere with replication, infection or movement of the virus, complete ORFs 1 and 4 were cloned to plasmids pHC1 and pHR1 and pHC4 and pHR4 respectively. Additionally, two subfragments from ORF 4 were cloned. The sequences in plasmids pHC4(HiNh) and pHR4(HiNh) or pHC4(BgBa) and pHR4(BgBa)
include a putative dimerisation domain or a putative leucine zipper, respectively.

<table>
<thead>
<tr>
<th>ORF</th>
<th>construct</th>
<th>nr.</th>
<th>description of the RTBV sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pHc1</td>
<td>5</td>
<td>complete ORF1; amino acids 12-199</td>
</tr>
<tr>
<td></td>
<td>pHR1</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>pHc3</td>
<td>6</td>
<td>complete ORF3; amino acids 2-1655</td>
</tr>
<tr>
<td></td>
<td>pHCPol</td>
<td>8</td>
<td>polymerase; including protease, RT, RNaseH</td>
</tr>
<tr>
<td></td>
<td>pHCPol(Xh)</td>
<td>9</td>
<td>polymerase; including protease, mutated RT (IDD mutated to LEE), RNaseH</td>
</tr>
<tr>
<td></td>
<td>pHRPol(Xh)</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHCPol</td>
<td>7</td>
<td>polymerase; including protease, RT, RNaseH, additional zinc-finger motif at amino terminal end</td>
</tr>
<tr>
<td></td>
<td>pHCPol(XhBa)</td>
<td>10</td>
<td>“polymerase”; including protease</td>
</tr>
<tr>
<td></td>
<td>pHCPol(HiXh)</td>
<td>11</td>
<td>“polymerase”; including RNaseH</td>
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<tr>
<td></td>
<td>pHCC23</td>
<td>1</td>
<td>coat protein; including Cys-His motif</td>
</tr>
<tr>
<td></td>
<td>pHRCC23</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHCC24</td>
<td>2</td>
<td>coat protein; without Cys-His motif</td>
</tr>
<tr>
<td></td>
<td>pHRCC24</td>
<td>27</td>
<td></td>
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<tr>
<td></td>
<td>pHCR</td>
<td>3</td>
<td>ORF3; including coat protein (Cys-His motif replaced with Gly-Gly), protease, mutated RT (IDD mutated to LEE), RNaseH</td>
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<tr>
<td></td>
<td>pHRR</td>
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<td></td>
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<td>pHc4</td>
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<td></td>
<td>pHR4</td>
<td>43</td>
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<tr>
<td></td>
<td>pHc4(BgBa)</td>
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<td>amino terminal half of ORF4; amino acids 1-237</td>
</tr>
<tr>
<td></td>
<td>pHR4(BgBa)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>pHc4(HiNh)</td>
<td>14</td>
<td>carboxy terminal half of ORF4; amino acids 174-409</td>
</tr>
<tr>
<td></td>
<td>pHR4(HiNh)</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

| Intergenic region | paRNA14 | 39 | antisense RNA from leader of pregenomic RNA; nucleotides 7408-7859, linked in antisense orientation to 3'-end of aph4 |

Table 5: Plasmid constructs with construct number and description of the expressed RTBV sequences. In plasmids pHcX, the RTBV genes are controlled by the CaMV 35S promoter, in plasmids pHRX, the RTBV genes are controlled by the RTBV promoter.

### 3.2.4 Antisense RNA to the untranslated leader

The 5'-end of the primary transcript of RTBV was determined by Bao and Hull (1993) at nucleotides 7404/05, resulting in an untranslated leader of the primary transcript of nearly 700 bp. Located within this untranslated leader are functions like the 5'-splice site of the intron (nts 7503/04), the polyadenylation signal at nt 7598 and the binding site of tRNA\textsuperscript{met} at nt 1 (Qu et al. 1991), that initiates the synthesis of the minus-strand DNA by the RNA-dependent DNA polymerase-function of the RT.
With the aim to disturb the functions within the untranslated leader by production of antisense RNA, a Cla-HindIII fragment from plasmid pRTRB1162 (Dasgupta et al. 1991), containing 451 nucleotides from 7408 to 7859, was cloned in reverse direction to the 3'-end of aph4. By fusing the two sequences, transcription of aph4 assures the production of the antisense RNA, not disturbing the translation of the aph4-product. The resulting plasmid was termed paRNA14.

Table 5 summarizes all the plasmids.

3.3 Plasmid constructions

3.3.1 Promoter analysis

For controlling expression of the RTBV sequences, two different promoters were selected: The CaMV 35S promoter that has been reported to be constitutively active in rice tissues (Battraw and Hall 1990) and the homologous RTBV promoter that should assure expression of the transgene in the tissue where the virus accumulates. Bhattacharyya-Pakrasi et al. (1993) reported RTBV promoter-dependent phloem-specific GUS expression in transgenic rice plants and Saito et al. (1986) showed with electron microscopic studies that RTBV virus particles accumulate primarily in the cytoplasm of the companion cells in phloem tissues.

In plasmids pHCintG and pHRintG the GUS gene was either controlled by the CaMV 35S or the RTBV promoter, respectively. In order to analyse precisely the locations of expression of the transgenes both plasmids were transformed to the rice cultivar TP309 by particle bombardment of precultured embryos. Two independent lines, transformed with pHCintG and three independent lines, transformed with pHRintG were used for analysis.

GUS expression was uniformly high in callus material, transformed with either construct (Fig. 23 a,b).
Expression studies were performed with R1 progeny plants. Plants were analysed from 3 to 60 days after germination. Leaf tissue was sectioned at variable thickness and examined for GUS expression. After extracting chlorophyll by immersing the tissue in ethanol, the slices were observed in glycerol using an inverted light microscope.

Not all the R1-progeny plants from the same primary transformant expressed GUS at the same level. By staining several plants of a R1-population six days after germination, typical expression patterns in sections from the shoot and from a leaf blade (see figure 24) for the two different promoters could be observed:

In sections through the shoot close to the base, both promoters expressed GUS in the blade of the 4\textsuperscript{th} leaf (still enclosed within the older leaves). While in the blade of the 4\textsuperscript{th} leaf the RTBV promoter was mainly active in the vascular bundles and in the epidermis cells, the CaMV 35S promoter was nearly uniformly active in all the cells. Both promoters similarly expressed strongly in the vascular bundles of the 3\textsuperscript{rd} leaf sheath (Fig. 25 a-f). In the blade of the 2\textsuperscript{nd} leaf, expression was nearly uniform for the CaMV 35S promoter (Fig. 25 g), while the RTBV promoter driven expression was restricted to the vascular bundles (Fig. 25 i). Both promoters were active in the guard cells of the stomata. In the sheath of the 1\textsuperscript{st} leaf, expression was equally uniform with both promoters (Fig. 25 h,k).
Figure 25: GUS expression in six day-old seedlings.  

- a-f) Sections from the shoot at the base of the 2nd leaf blade (ph: phloem; xy: xylem; gc: guard cell) 
- g,i) Vascular bundle of the 2nd leaf blade 
- h,k) Midrib of the 1st leaf sheath.
In older plants, the activity of the CaMV 35S promoter was usually uniformly high in all the tissues of the leaf sheath and blade, whereas the activity of the RTBV promoter was restricted to the phloem cells, if expression still could be detected at all (Fig. 26).

Figure 26: GUS expression in 60 day-old R1 plants. Expression in midribs of old leaves, controlled by a) the CaMV 35S promoter or b) the RTBV promoter. Weak promoter activity can be observed only in the phloem (arrow).

However, a certain variability of promoter activity in different R1 plants was observed. Often, GUS expression controlled by the RTBV promoter in leaf blades was similar to the expression controlled by the CaMV 35S promoter (Fig. 27). The reason and the frequency of this observation still have to be determined. Promoter activity of the CaMV 35S promoter in old plants was variable. Whereas in some leaves expression was extremely high, in other leaves expression was only weak or could not be detected at all.

Figure 27: GUS expression in the blades of the 3rd leaf of 10 day-old R1 rice seedlings. a) Expression controlled by the CaMV 35S promoter. b) Expression controlled by the RTBV promoter.

3.3.1.1 RTBV promoter-deletions

Seven constructs with deletions within the RTBV promoter and leader (Fig. 28; constructs kindly provided by G. Chen, FMI, Basel) were transformed to the rice cultivar TP309 by particle bombardment of precultured embryos. Between 15 and 22 plants were regenerated per construct (Table 6).
Figure 28: Promoter and untranslated leader of RTBV. Plasmid p33 contains the full-length promoter and leader, plasmids p34 to p37 have 5'-deletions in the promoter, plasmids p30 to p32 have deletions within the leader. All the constructs contain the RTBV intron, the GUS gene (uidA) and the hygromycin-resistance cassette. Black numbers represent nucleotides within the RTBV genome. Red numbers indicate nucleotide positions relative to the transcription start at nucleotide 7404/5.

<table>
<thead>
<tr>
<th>construct</th>
<th>regenerated plants</th>
<th>independent lines</th>
<th>GUS positive lines (fertile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p33</td>
<td>21</td>
<td>3</td>
<td>3 (3)</td>
</tr>
<tr>
<td>p37</td>
<td>22</td>
<td>2</td>
<td>2 (2)</td>
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<tr>
<td>p34</td>
<td>18</td>
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<td>p30</td>
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<td>4</td>
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<td>15</td>
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</tr>
<tr>
<td>p32</td>
<td>21</td>
<td>4</td>
<td>4 (3)</td>
</tr>
</tbody>
</table>

Table 6: Transformation of RTBV promoter/leader deletion constructs.

In histochemical assays (Fig. 29), GUS expression was visualized in segregating R1-populations (exceptions: For p35 and p36, primary transformants were analysed). Normally, 75% of the tested plants expressed GUS, as expected from mendelian segregation of selfed, hemizygous primary transformants, with the transgenes inserted at one single locus.
Figure 29: GUS expression in 6 day-old R1-seedlings. GUS gene expression is controlled by the RTBV promoter. Sections from the shoot at the base (left) and from the blade of the 2nd leaf (right). Plants from constructs p36 and p35 are R0-plants. p33: Full-length construct; p37: deletion in the promoter up to -280; p34: deletion in the promoter up to -220; p36: deletion in the promoter up to -100 (b: bulliform cells); p35: deletion in the promoter up to -50; p32: deletion in the leader from +52 to +83; p31: deletion in the leader from +8 to +30; p30: deletion in the leader from +8 to +83.
The deletions to -280 and -220 (relative to the transcription start; constructs p37 and p34) resulted in expression patterns similar to the full-length promoter (construct p33). However, only around 40% of the plants expressed GUS at all, less than was expected from mendelian segregation. In addition, plants transformed with construct p37 often expressed GUS only relatively weak in the vascular bundles, compared to the strong GUS expression in the vascular bundles from construct p33 (Fig. 29).

Further deletion of the RTBV promoter to -100 or -50 (relative to the transcription start; constructs p36 and p35) resulted in expression restricted to the epidermis cells. Strong GUS expression was detected in the bulliform cells of the upper epidermis (Fig. 29).

Deletions from nt +8 to +30 or to +83 (relative to the transcription start; constructs p31 and p30) in the RTBV leader resulted in a drastic increase of GUS expression levels compared to the full-length construct (Fig. 29). Deletion from nucleotides +52 to +83 (relative to the transcription start; construct p32) in the RTBV leader resulted in a decrease of expression levels, restricting expression to vascular bundles and some epidermis cells. GUS expression in the blade of the youngest leaf, still enclosed within the sheaths of the older leaves, was decreased.

As the RTBV particles accumulate in the phloem of infected plants, the transgenes should be expressed in this tissue. Both promoters, the CaMV 35S and the full-length RTBV promoter have been shown to be active in this tissue in young plants. Therefore, both promoters are suitable for controlling expression of the transgenes.

3.3.2 RTBV Intron

Introns have been shown to enhance expression of genes in eukaryotic systems (e.g. McElroy et al. 1990). All the constructs share a shortened version of the RTBV intron ("RTBV-intron"). The influence of the RTBV intron on expression in rice tissue was tested using four different constructs containing the GUS gene, with or without the RTBV intron: pCG and pCintG, controlled by the CaMV 35S promoter and pRG and pRintG, controlled by the RTBV promoter (kindly provided by J. Füetterer).
Results

Germination and GUS expression was visualised 24 hours after bombardment. With both promoters, the RTBV intron increased the number of transformed cells by a factor of 2.7 (Fig. 30).

![Figure 30: Influence of the RTBV intron. The coleoptiles of etiolated, bombarded rice seedlings, bombarded with different constructs, after GUS staining. The constructs contain the GUS gene, driven by the CaMV 35S (pCmtG, pCG) or the RTBV promoter (pRmtG, pRG) respectively, either containing or lacking the RTBV intron. Average numbers of blue spots per coleoptile are given.]

### 3.3.3 Construction of the plasmids pHCX

The constructs containing RTBV genes, controlled by the CaMV 35S promoter and containing the RTBV intron (plasmids pHCX), are derivatives of plasmid pCintG (see figure 3 in "Material and methods"). For detection of the gene products in transgenic plants, the RTBV sequences were fused to a sequence, coding for a 10 amino acid-tag-peptide from the influenza virus hemagglutinin protein. This small epitope allows detection of all the products with one single antibody (Kolodziej and Young 1990).

For selection of stably transformed cells, a hygromycin-resistance cassette was included in all the constructs. All RTBV sequences were derived from plasmid pRTRB1162 (Dasgupta et al. 1991), encoding the complete sequence of RTBV. The RTBV sequences were either amplified by PCR or were derived directly from the RTBV sequence. The RTBV sequences were ligated into plasmid pCintG(tag), replacing the uidA gene and fusing the RTBV sequence in frame to the tag sequence (details see in "Material and methods").
The resulting constructs were named pHCX, with H standing for the hygromycin-resistance cassette, C for the CaMV 35S promoter and RTBV intron and X representing the respective RTBV sequence (Fig. 3, Table 5).

3.3.4 Construction of the plasmids pHRX

The constructs containing the RTBV genes, driven by the RTBV promoter (plasmids pHRX), are derivatives of plasmid pHRintG (see figure 6 in "Material and methods"). The specific RTBV sequences, derived from the plasmids pHCX were cloned into pHRintG by replacing the BstBI-SphI fragment of pHRintG with the respective fragment of pHCX, containing the variable RTBV sequences with the fused tag sequence. The resulting constructs were named pHRX, with H standing for the hygromycin-resistance cassette, R for the RTBV promoter and the RTBV intron and X representing the respective RTBV sequence (Fig. 6, Table 5).

3.4 Analysis of the regenerated plants

A total of 23 different plasmid constructs with RTBV sequences was transferred by particle bombardment to precultured TP309 embryos or suspension calli of the varieties TP309 or Kinuhikari. Between 24 and 70 embryos per construct were bombarded, from which up to 29 plants were regenerated. In average, one plant was regenerated from every four bombarded embryos (24%) and two plants from every plate with suspension calli. Totally 257 TP309 and 106 Kinuhikari plants were regenerated from hygromycin-resistant calli. The primary transformants were assigned a two-part identifier to indicate the transgene and the individual plant: K 12.4, for example, describes plant 4 carrying transgene 12. In addition, the letter K marks plants of the cultivar Kinuhikari.

3.4.1 Fertility

Fertility varied among the regenerated plants. While some of the plants were fully fertile, some yielded only a few seeds. However, as from a few seeds enough seeds can be obtained in the next generation, the plants were divided into fertile or sterile, independent of the number of seeds. Around 50% of the plants derived from bombarded TP309 embryos and from bombarded TP309 suspension calli were fertile. Only 30% of the plants derived from bombarded Kinuhikari suspension calli were fertile (Table 7).
<table>
<thead>
<tr>
<th>target material</th>
<th>fertile plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precultured immature TP309 embryos</td>
<td>47 %</td>
</tr>
<tr>
<td>TP309 ECS</td>
<td>50 %</td>
</tr>
<tr>
<td>Kinuhikari ECS</td>
<td>30 %</td>
</tr>
</tbody>
</table>

Table 7: Percentage of fertile plants, regenerated from different target material.

### 3.4.2 Test of transgene integration by Southern analysis

In order to identify independent lines among the primary transformants and to identify plants that had integrated complete copies of the transgenes, Southern analysis was performed with all the plants.

Genomic plant DNA was isolated from leaves of the primary transformants and was digested with either EcoRI or SacII to cleave the plasmid-derived sequences at one single site within the hygromycin resistance gene. Differently sized fragments of transgenic DNA were released, depending on the site of integration.

The size of the integrated RTBV sequences was tested in a second restriction digest. The RTBV gene with flanking sequences was cut out by digesting the plant genomic DNA with KpnI or BamHI. Direct comparison of the size of the fragment derived from the plasmid used in transformation with the fragment derived from the genomic plant DNA allowed identification of plants that had integrated complete transgene copies.

Figure 31 shows an example of a Southern analysis of 13 plants, regenerated from a bombardment experiment, using construct pHCPol(HiXh). Plants showing the same pattern of hybridizing fragments in the a-lanes were considered as being derived from the same transformation event (examples in Figure 31; plants 8 and 10). Plants showing variable patterns were considered as independent lines. Plants showing a fragment of the expected size in the b-lanes (examples in Figure 31: 1.5 kb; plants 8, 10, 11, 14, 15) were considered as having integrated at least one complete copy of the RTBV transgene. All larger or smaller fragments hybridizing to the labeled probe in the b-lanes were considered as rearranged copies of the transgene (extreme examples in Figure 31; b-lanes of plants 5 and 11). Many of the primary transformants had multiple, complete or rearranged, transgene insertions.
Figure 31: Southern of 13 plants, transformed with plasmid pHCPol(HiXh) (construct number 11). The plant genomic DNA was cut either with EcoRI (a; single cut) or with KpnI (b; releases a 1.5 kb fragment). As negative control, plant genomic DNA from a non-transformed plant was loaded (lane n) and as positive control, plasmid pHCPol(HiXh) was digested with KpnI, yielding a 1.5 kbp fragment (lane p).

Several plants did not contain any fragments hybridizing to the leader probe (see examples in Figure 31; plants 4 and 6). Hybridization of the same membrane with the hygromycin probe revealed, that these plants had integrated incomplete copies of the transgene, probably with a deletion in the RTBV leader, but with a functional copy of the hygromycin-resistance gene (see example in figure 32; plant 5.7).

In all the analyses done, not a single plant was identified that had escaped from the applied selection; all the plants were transgenic.

The results of the Southern analysis of the regenerated plants are summarized in table 8.

Among all the primary transformants, containing RTBV sequences and tested in Southern analysis, 119 independent transgenic lines were identified. From these 119 lines, only 37 (31%) had integrated at least one complete copy of the respective RTBV gene. From these 37 lines, 25 (68%) were fertile. From the different constructs transformed, between zero and five fertile lines with at least one complete copy of the transgene integrated in the genome, were obtained.
Figure 32: Hybridization with different probes. Southern blot with plants, transformed with pHCl (construct number 5) was hybridized with two different probes, leader (A) and hyg (B). Plant genomic DNA was loaded either uncut (lane a), digested with EcoRI (lane b) or with KpnI (releases a 1.2 kb fragment; lane c).

<table>
<thead>
<tr>
<th>construct number</th>
<th>regenerated plants</th>
<th>independent lines</th>
<th>fertile lines</th>
<th>lines with complete copy</th>
<th>fertile lines with complete copy</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) pHCC23</td>
<td>20</td>
<td>12</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>(2) pHCC24</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>(3) pHCR</td>
<td>21</td>
<td>5</td>
<td>3</td>
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<td>0</td>
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<td>(5) pHCI</td>
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<td>1</td>
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<tr>
<td>(6) pHC3</td>
<td>34</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>(7) pHCZPol</td>
<td>16</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(8) pHCPol</td>
<td>17</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>(9) pHCPol(Xh)</td>
<td>17</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>(10) pHCPol(XbBa)</td>
<td>18</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>(11) pHCPol(HxXh)</td>
<td>16</td>
<td>12</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>(12) pHC4(BaBa)</td>
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<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(13) pH4</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(14) pHC4(HxNh)</td>
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<td>nt</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>2</td>
<td>0</td>
</tr>
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<td>(27) PHRC24</td>
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<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
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<td>(28) PHRR</td>
<td>24</td>
<td>7</td>
<td>7</td>
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<td>0</td>
</tr>
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<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
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<tr>
<td>(39) paRNA14</td>
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<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<td>(40) PHRZPol</td>
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<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(41) PHRZPol(Xh)</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>9</td>
<td>9</td>
<td>5</td>
<td>5</td>
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<tr>
<td>(43) PHR4</td>
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<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(44) PHR4(HxNh)</td>
<td>23</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>summary</td>
<td>357</td>
<td>119</td>
<td>84</td>
<td>37</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 8: Results of the Southern analysis of all regenerated plants. From 119 independent lines, 25 had integrated a complete copy of the RTBV transgene and were fertile. (nt: not determined)
3.4.3 Analysis of transgene transcription

Northern analysis was performed with the lines that had integrated complete copies of the RTBV transgene and that had produced enough R1 seeds to test the lines for resistance in the R1 generation.

The transcripts of the hygromycin-resistance gene were usually easy to detect. However, expression levels varied among the individual lines. Figure 33 shows the analysis from two transgenic primary transformants where equal amounts of total plant RNA from leaves were compared. Both plants had only one or two copies of the *aph4* gene integrated in their genomes, but the expression level in plant 5.6 was much higher than in plant 2.30.

![Figure 33: Northern analysis with leaf material of two transgenic lines. Both lines were transformed with plasmids, containing the *aph4* gene, controlled by the CaMV 35S promoter. Variable amounts of transcripts were detected from the two lines. As negative control, total RNA from non-transformed plants was loaded. The transcript is indicated in blue, the probe in red colour.](image)

Compared to *aph4*, the RTBV transgenes were expressed at much lower levels. Two blots with equal amounts of total plant RNA were hybridized with either the probe from *aph4* or from the RTBV leader. After parallel exposure of both blots to X-ray film, the transcript from *aph4* could easily be detected, while the transcripts from the RTBV genes could not be detected (Fig. 34).
Results

**Figure 34:** Northern analysis with leaf material of three transgenic lines. The lines contain RTBV sequences from the coat protein (line 1.18; CaMV 35S promoter), the polymerase (line 7.8; CaMV 35S promoter) or the mutated ORF3 (line 28.8; RTBV promoter). As negative control (neg), total RNA from non-transformed plants was loaded, as positive control, total RNA from plants, transformed with plasmid pHRintG (line 33.1; RTBV promoter/GUS). M: RNA marker. The (spliced) transcripts are indicated in blue, the probes in red colour. The blots were hybridized with probes from either the RTBV leader or the aph4 gene (hygromycin-resistance) and were exposed to x-ray film for equal time. With the leader probe, weak signals of around 300 bp were detected in lines 1.18 and 33.1 (arrows). With the hyg probe, signals of around 1200 bp were detected with all transgenic lines.

Transcripts that hybridized to the leader probe were found in several lines (lines 5.6 and 6.13 in figure 35 a and several lines in figure 35 b/c). As the expression level was generally low, many signals could be detected only after over-exposure of the X-ray film (Fig. 35 c). In several lines, more than one transcript was detected and often the detected transcripts did not have the expected size. Additionally, many of the lines produced an RNA of uniform size of around 300 basepairs (Fig. 34, 35).

As this small RNA was detected by the leader probe, it can be expected that this RNA is produced due to the RTBV-polyA-signal in the RTBV intron (see figure 2). This transcription termination signal naturally stops transcription of the RTBV genome, producing the more than full-length primary transcript with redundant ends.
Figure 35: Northern analysis of transgenic lines. 

**a)** Total RNA from leaf material of two transgenic lines. The plants contain complete sequences from RTBV ORF1 (line 5.6; CaMV 35S promoter) and ORF3 (line 6.13; CaMV 35S promoter). As negative control, total RNA from non-transformed plants was loaded. From line 5.6, RNAs of 1400, 5200, 5800 and 6200 basepairs were detected (arrows). From line 6.13, an RNA of 5500 basepairs was detected. From both lines, weak signals of around 300 bp were found (arrows). Stronger signals were detected on an over-exposed film (not shown).

**b,c)** Total RNA from callus material of 14 transgenic lines. The membrane was hybridized with the probe from the RTBV leader. In most of the lines, strong signals were obtained from small, 300 base RNAs. Signals from larger RNAs were only obtained after over-exposure of the x-ray film (b: 2h, c: 16 h exposure). Total RNA from callus material of two transgenic lines containing the GUS gene, controlled either by the CaMV 35S promoter (line 4.1) or the RTBV promoter (line 33.1) serve as positive controls.
The expected and the detected sizes of the transgene mRNAs in the presented Northern blots (Fig. 35) are listed in table 9.

<table>
<thead>
<tr>
<th>lines</th>
<th>construct</th>
<th>expected lengths of spliced mRNA [bases]</th>
<th>detected mRNA size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>300 b</td>
</tr>
<tr>
<td>5.6</td>
<td>pHCl</td>
<td>1060</td>
<td>+</td>
</tr>
<tr>
<td>6.13</td>
<td>pHC3</td>
<td>5450</td>
<td>+</td>
</tr>
<tr>
<td>1.3</td>
<td>pHCC23</td>
<td>1570</td>
<td>+</td>
</tr>
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<td>1.18</td>
<td>pHCC23</td>
<td>1570</td>
<td>+</td>
</tr>
<tr>
<td>1.27</td>
<td>pHCC23</td>
<td>1570</td>
<td>+</td>
</tr>
<tr>
<td>2.31</td>
<td>pHCC24</td>
<td>1400</td>
<td>+</td>
</tr>
<tr>
<td>27.10</td>
<td>pHRC24</td>
<td>1400</td>
<td>+</td>
</tr>
<tr>
<td>38.2</td>
<td>pHR1</td>
<td>1060</td>
<td>+</td>
</tr>
<tr>
<td>38.7</td>
<td>pHR1</td>
<td>1060</td>
<td>+</td>
</tr>
<tr>
<td>10.4</td>
<td>pHCPol(XhBa)</td>
<td>1750</td>
<td>+</td>
</tr>
<tr>
<td>11.8</td>
<td>pHCPol(HiXh)</td>
<td>1440</td>
<td>+</td>
</tr>
<tr>
<td>11.11</td>
<td>pHCPol(HiXh)</td>
<td>1440</td>
<td>-</td>
</tr>
<tr>
<td>7.6</td>
<td>pHCPol</td>
<td>3060</td>
<td>+</td>
</tr>
<tr>
<td>40.5</td>
<td>pHRCpol</td>
<td>3060</td>
<td>+</td>
</tr>
<tr>
<td>K42.1</td>
<td>pH4(BgBa)</td>
<td>1200</td>
<td>+</td>
</tr>
<tr>
<td>K42.10</td>
<td>pH4(BgBa)</td>
<td>1200</td>
<td>+</td>
</tr>
<tr>
<td>neg. contr.</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>33.1</td>
<td>pHrinG</td>
<td>2300</td>
<td>+</td>
</tr>
<tr>
<td>4.1</td>
<td>pHCintG</td>
<td>2300</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 9: Expected and detected sizes of the transgene mRNAs in the presented Northern blots (Fig. 35). For the calculation of the expected size of the (spliced) transcripts, 100 bp for the leader, 200 bp for the CaMV 35S terminator and 200 bp for a polyA tail were added to the length of the RTBV gene. The "detected sizes" are all estimated from the presented Northern blots.

Also with the plants, transformed with the GUS genes (positive-control lines 33.1 and 4.1) the detected transcripts were larger than expected and the small 300 bases-RNA was produced. These plants express the functional GUS gene strongly in leaves, as well as in callus material (see figures 23 and 25).

In leaves from primary transformants, transformed with the antisense-construct paRNA14, high levels of RNA could be detected (Fig. 36). As the RTBV sequence was added in antisense orientation at the 3'-end of aph4, the RTBV-antisense sequence was transcribed at high levels and was obviously not disturbing translation of the APH4 protein.
Figure 36: Northern analysis of plants, transformed with paRNA14. 451 basepairs from the leader of the pregenomic RNA of RTBV were linked in antisense orientation to the 3'-end of aph4. aph4 and the leader sequence are transcribed in one mRNA (indicated as blue arrow). Three plants from three independent lines (indica variety IR43; transformed by PEG-mediated transformation to protoplasts by G.C. Ghosh Biswas, data not shown) produced mRNAs of 1.6 kb. As positive control, total RNA from a plant, transformed with a CaMV 35S promoter::aph4::CaMV 35S terminator construct was loaded on the gel. This control plant produced mRNA, that was only 1.2 kb in size. As negative control, total RNA from a non-transformed plant was loaded.

3.4.4 Analysis of protein synthesis

As stable mRNA and protein accumulation are controlled by different factors, it is important to examine both expression levels (Hanley-Bowdoin and Hemenway 1992). The same lines that were tested for mRNA accumulation in figure 35 b,c were tested for protein accumulation. Western analysis was performed with proteins isolated from mature embryo derived callus. Soluble proteins were separated on 12.5% polyacrylamide gels and were blotted to nitrocellulose membrane. A polyclonal antiserum raised against the tag-peptide was used to detect the proteins.

In most of the lines, no specific protein was detected. Signals, as found in the negative control, are due to unspecific binding of the antibody to plant proteins. In line K42.10, containing the amino-terminal subfragment of the RTBV ORF4 (expected protein around 26 kDa), a specific protein smaller than 30 kDa was detected (Fig. 37).

Figure 37: Western analysis of 8 transgenic lines. As negative control, total protein of a non-transformed line was loaded on the gel. A protein smaller than 30 kDa is detected by the tag-antibody in the line K42.10. In all the lines, including the negative control, unspecific detection of plant proteins occurred.
3.4.5 Virus resistance tests

In a first experiment, 20 transgenic lines were tested for resistance to RTD in the greenhouse at the International Rice Research Institute (IRRI) on the Philippines (Table 10).

<table>
<thead>
<tr>
<th>plants</th>
<th>construct</th>
<th>promoter/ description of RTBV sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3/1.6/1.18/1.27</td>
<td>pHCC23</td>
<td>35S/ coat protein; including Cys-His motif</td>
</tr>
<tr>
<td>2.31</td>
<td>pHCC24</td>
<td>35S/ coat protein; without Cys-His motif</td>
</tr>
<tr>
<td>27.1</td>
<td>pHRC24</td>
<td>RTBV/ coat protein; without Cys-His motif</td>
</tr>
<tr>
<td>5.3</td>
<td>pHC1</td>
<td>35S/ ORF1</td>
</tr>
<tr>
<td>38.2/38.7</td>
<td>pHR1</td>
<td>RTBV/ ORF1</td>
</tr>
<tr>
<td>8.2</td>
<td>pHCPol</td>
<td>35S/ polymerase</td>
</tr>
<tr>
<td>7.6</td>
<td>pHCZPol</td>
<td>35S/ polymerase with zinc-finger</td>
</tr>
<tr>
<td>40.5</td>
<td>pHRRZPol</td>
<td>RTBV/ polymerase with zinc-finger</td>
</tr>
<tr>
<td>10.4</td>
<td>pHCPol(XhBa)</td>
<td>35S/ protease</td>
</tr>
<tr>
<td>11.8/11.11</td>
<td>pHCPol(HiXh)</td>
<td>35S/ RNaseH</td>
</tr>
<tr>
<td>42.1/K42.1/K42.10</td>
<td>pHHR4(BgBa)</td>
<td>RTBV/ 5'-half of ORF4</td>
</tr>
<tr>
<td>K44.10</td>
<td>pHHR4(HiNh)</td>
<td>RTBV/ 3'-half of ORF4</td>
</tr>
<tr>
<td>33.1</td>
<td>pHRintG</td>
<td>RTBV/ GUS gene</td>
</tr>
</tbody>
</table>

**Table 10:** Lines, carrying complete copies of the transgenes, tested for virus resistance.

Fourteen days after germination, 56 plants per line were exposed individually to three adult viruliferous *Nephotettix virescens*, carrying RTBV and RTSV, each. The plants were split into two groups, using two different virus isolates. One of the tungro virus isolates was originally collected in Laguna, Philippines and has been maintained in the greenhouse for nearly 20 years ("greenhouse population"). RTBV viruses from this population had been used for sequencing of the RTBV genome (Hay et al. 1991). The other isolate was collected in Famy, Laguna, Philippines, one month before the beginning of the experiment. Twenty days after infection, the state of health of the plants was assessed on the basis of height reduction and leaf discolouration, the characteristic symptoms. The average symptom severity score (SS), ranging from 1 for resistant to 9 for sensitive plants (Hasanuddin et al. 1988) was determined. Further, the plants were tested serologically by ELISA for RTSV- and RTBV-titers 20 and 40 days after infection.

Unfortunately, none of the tested lines showed any form of resistance to RTBV. Nearly all the treated plants had developed severe symptoms (height reduction and leaf discolouration) 20 days after infection (Fig. 38 f) and had virus-titers
Results comparable to the infected control plants. Several individual plants from different lines had low RTSV- but high RTBV-titers, showing a reduction in plant height but without leaf discolouration (Fig. 38 g). These plants got infected by RTBV only, as it frequently occurs when plant populations are infected with viruliferous *N. virescens*. Three individual plants, one each from the lines 1.27, 7.6 and from the non-transformed negative control, had low titers of RTBV and RTSV and did not show any characteristic disease symptoms (Fig. 38 h). As both titers, from RTBV and from RTSV were low, these symptom-free plants must be considered as escapes from infection (Dahal et al. 1988).

*Figure 38: Test of F1-generation plants for virus resistance*. a) Containment facilities at IRRI. b) Plants, covered individually by mylar cages. c) Adult green leafhoppers (*GLH; Nephotettix virescens*). d) 3 GLH are placed per plant. e) After infection, plants are screened for symptoms. f) Non-infected control plant (left) and plant infected with RTBV and RTSV. g) Control plant (left) and plant infected with RTBV only. h) Control plant (left) and plant that probably escaped from infection.
None of the lines had recovered after 40 days, as determined by ELISA, using the youngest leaves (data not presented).

The average symptom severity (SS) of the lines was between 6 and 9 (Fig. 39 a) and the average RTBV- and RTSV-titers, as determined by ELISA, were uniformly high (Fig. 39 b).

![Figure 39: Resistance tests of transgenic lines. The data are mean values of 28 plants, infected with the greenhouse population. a) Symptom severity of the tested lines. Symptom severity score (SS), ranging from 1 for resistant to 9 for sensitive plants b) Titers of RTBV and RTSV in the youngest leaf of the plants, 20 days after infection. The titers of the non-infected control plants are zero.]

In a second experiment, another 32 transgenic lines were tested for resistance by exposing 28 R1-progeny plants per line to viruliferous vectors infected with the greenhouse-virus population. These transgenic lines had integrated rearranged copies of the transgenes in their genome, as determined by Southern analysis.

Similarly to the first experiment, none of the lines showed resistance to RTBV 20 days after infection, nor had recovered after 40 days (data not presented).
4. DISCUSSION

4.1 Evaluation of transformation methods

The first transformation system available for rice was PEG- or electroporation-mediated direct gene transfer to protoplasts (Toriyama et al. 1988; Zhang et al. 1988). Direct, PEG-mediated gene transfer to protoplasts of indica varieties, followed by regeneration of transgenic plants was a well established system in the laboratory when this work was started (Datta et al. 1990; Datta et al. 1992; Ghosh Biswas et al. 1994a). Unfortunately, transgenic rice plants derived from protoplast transformation very often developed abnormal phenotypes like albinism, stunted growth and sterility. These abnormalities are most likely due to the relatively long tissue culture periods involved in establishment of embryogenic cell suspensions (ECS) of indica varieties and the protoplastation of the cells. Tissue culture cells are not under a complete functional selection and mutations or epigenetic changes can accumulate (Lynch et al. 1994; Fütt erer and Potrykus 1995). Alternative sources for totipotent cells, allowing regeneration of plants, would probably avoid these problems. Despite the reports of successful transformation of rice mesophyll protoplasts (Gupta and Pattanayak 1993) or protoplasts isolated from the scutellum of rice immature embryos (Ghosh Biswas et al. 1994b) and following regeneration of rice plants, protoplast transformation was not followed up for this work, as more promising methods had become feasible. Recent reports from several authors about successful transformation of different monocot species by alternative transformation methods encouraged us to evaluate novel techniques. Christou et al. (1991) reported production of transgenic indica and japonica rice varieties by particle bombardment of the scutellum cell layer of immature rice embryos. D'Halluin et al. (1992) reported transgenic maize plants derived from tissue electroporation of maize suspension calli.

Therefore, the two methods of tissue electroporation and of particle bombardment were evaluated for production of fertile, transgenic rice plants.

4.1.1 Tissue electroporation

When this work on tissue electroporation was started, several reports on electroporation-mediated transfer of plasmid DNA to intact plant cells had been published (Morikawa et al. 1986, Lindsey and Jones 1987, Dekeyser et al. 1989).
However, it was generally doubted that macromolecules like DNA could penetrate the cell wall of intact cells after delivery of electrical pulses (Potrykus 1990). Therefore, some of the published work involved steps for wounding intact cells before electroporation, either mechanically or enzymatically (D'Halluin et al. 1992).

Despite initial concerns, tissue electroporation was tried with non-pretreated, intact tissue. Surprisingly, gene transfer to intact cells from immature embryos of wheat and rice (Klöti et al. 1993), as well as from rice suspension calli could be demonstrated. Not all the cells that were present in the electroporation chamber responded similarly to the electrical pulses. Cells from the scutellum of wheat immature embryos for example were more responsive to electroporation-mediated gene transfer than the cells on the opposite side of the embryo. With immature embryos of rice, a different pattern was observed. While no transformed cells could be detected in the scutellum, cells from the coleoptilar side responded much better. These findings suggest that electroporation-mediated penetration of the cell wall by macromolecules like DNA indeed is difficult. However, the structure of the cell wall of certain cells at certain developmental stages seems to allow the transport of macromolecules across the cell walls. It was further demonstrated, that during the delivery of the electric pulses the negatively charged DNA molecules move towards the anode, supporting observations of Xie et al. (1992).

The regenerable rice scutellum cells could not be transformed by electroporation under the applied conditions. Since successful electroporation-mediated transformation of enzymatically pretreated maize suspension cells had been demonstrated by D'Halluin et al. (1992), experiments were continued using rice embryogenic suspension cells of the variety Taipei 309 (TP309) as targets. A short enzymatic pretreatment, like the one described by D'Halluin et al. (1992) for maize suspension cells, also increased the number of transiently transformed cells in electroporated rice suspensions. Several parameters for the electroporation-mediated transfer of plasmid DNA to rice suspension cells could be optimized and finally, hygromycin resistant calli could be obtained after electroporation with a plasmid containing both a hygromycin-resistance and a GUS (Jefferson 1987) gene. Fertile, transgenic, GUS expressing plants could be regenerated from these calli.

For electroporation of embryos or suspension calli, special reaction chambers were designed, that had several advantages, compared to the commercially
available electroporation cuvettes, which were designed for electroporation of bacteria suspensions:
1. The chambers were convenient for adding and removing tissues and suspension calli.
2. The relatively small chamber volume minimized the required amount of plasmid DNA.
3. The chambers were made from inert materials like acrylic glass or Teflon®, that could be washed and sterilized. The electrodes were gold-plated to reduce corrosion during electroporation. Therefore, the chambers could be reused many times.
4. The reaction chamber for electroporation of embryos allowed fixation of the embryos in the chamber; transformation of the scutella could be favoured over transformation of the cells on the opposite side of the embryo.
5. The reaction chamber for electroporation of suspension calli offered the possibility of washing the calli after electroporation easily by adding washing buffer, followed by a sterile centrifugation of the chamber in a tabletop centrifuge.

4.1.2 Particle bombardment
In the first experiments transformation of the indica rice varieties IR24, IR43, IR58, IR72 and Tetep was tried, as the trait of tungro virus resistance should be conferred to indica rice. It is well known that indica varieties are much more recalcitrant to transformation than japonica varieties (Ayres and Park 1994) and no transgenic indica plant could be obtained in this work. Therefore, transformation of the japonica cultivar TP309 was started. As the evaluation of different strategies which could lead to RTBV resistant rice was one of the aims of this work, rice varieties which are easy to transform had to be used. Later, genes which are identified to confer RTBV resistance can be transferred to indica varieties, either by transformation of these varieties or by classical breeding. By bombardment of precultured immature embryos with plasmids, containing both a hygromycin-resistance and a GUS gene, followed by selection in liquid culture medium for several weeks, fertile transgenic GUS expressing plants could be regenerated.

Also embryogenic cell suspensions were used as target material. The plants regenerated from these experiments were as fertile as the plants from the experiments with bombarded embryos. This could be due to the short time that is needed for the establishment of ECS from TP309. Compared to the use of
immature embryos, ECS as target material offered several additional advantages:

1. Subculture of ECS is less laborious than isolation of immature embryos and no continuous supply of fresh material is required.
2. Production of ECS as target material can be planned precisely, whereas isolated immature embryos often are contaminated, spoiling the planned experiments.

Particle bombardment and tissue electroporation were compared directly by transformation of ECS. Particle bombardment was superior to tissue electroporation in terms of efficiency of gene transfer into the cells. Particle bombardment yielded more transformed cells per freshweight ECS and per microgram of plasmid DNA. Additionally, the protocol for particle bombardment was less laborious than the one for tissue electroporation. Unfortunately, nothing is known so far about the influence of the transformation method on the quality and quantity of plasmid DNA integration into the plant genome. Transformed, transiently GUS expressing cells derived by particle bombardment expressed GUS at much higher levels than cells, transformed by tissue electroporation. This indicates that much more plasmid copies get transferred into the cells mediated by particles than by voltage. The influence of probably thousands of plasmid copies within the cells on plasmid integration is not known. Problems like abnormal traits of the primary transformants or silencing of the transgenes in subsequent generations may be due to the circumstances of plasmid integration. Despite these concerns, the method of particle bombardment was chosen for the transfer of the constructs to rice.

4.2 Genetic engineering for virus resistance

An overview about the different strategies of genetically engineered virus resistance has been given in the chapter "Introduction“. In most cases, resistance was obtained by expressing a normal or mutated viral gene product in transgenic plants (Beachy 1993; Wilson 1993). In these transgenic plants, expression of functional viral proteins at the onset of virus infection could interfere with an ordered progression through the viral replication cycle, and expression of mutated viral proteins might interfere with the function of normal viral proteins by competition. A similar approach might be feasible for engineering resistance to RTBV. So far, no success has been achieved with
double-stranded DNA viruses. With the little knowledge available so far about the function of many of the RTBV gene products, replication and transmission, it is difficult to predict which approach, if any, will lead to resistant rice plants. In this work, plants were transformed with plasmids, carrying the genes for different viral proteins or RNA:
- The putative RTBV coat protein and a subfragment of the coat protein
- The RTBV replicase, mutated replicase and subfragments of the replicase
- RTBV proteins with unknown functions and subfragments of these proteins
- Antisense RNA to the leader of the pregenomic RNA.

4.2.1 Transgene expression

The transgenes in the plants can be functional only if they are expressed at the right time and in the right amount at the right place. On the other hand, expressed transgenes can be a constraint for the plant if they are expressed at too high levels at the wrong place. Therefore, it is important that the transgenes are controlled by appropriate promoters.

Right time of expression: Rice plants are most susceptible to tungro infection when they are still young, before and right after transplanting the seedlings from the nursery to the field in the age of a few weeks. The yield of a plant that becomes infected late will not be seriously affected, but the ratoon crop will be lost (IRRI 1983b). Therefore the transgenes should be expressed when the plant is still young.

Right amount of expression: Predictions about the required amount of expressed transgene for an effective protection against the virus are nearly impossible. Many strategies like the expression of functional or dysfunctional coat protein count on an interaction with the protein, encoded by the transgene, with the invading virus. However, several authors reported transgenic plants in which the transgenic coat protein could not be detected but that were protected from the virus (Stark and Beachy 1989; Farinelli and Malnoë 1993). As long as the mechanism of protection for a certain transgene-virus interaction is not known, predictions of required transgene expression levels are impossible. Screening of a range of different expression levels is obtained:

1. By testing different, independent lines, that express the transgene at variable levels due to the "position effect" (Dependence on the integration of the transgene into transcriptionally more or less active parts of the plant genome).
2. By testing segregating populations, where the plants are either homozygous or hemizygous for the transgene.

**Right place of expression:** Transgenes that protect against a virus have to be expressed either where the virus enters the plant, disassembles, replicates and assembles, or moves. Sta. Cruz *et al.* (1993) found RTBV particles in the cytoplasm of both xylem and phloem cells (sieve tubes, phloem parenchyma cells, companion cells and xylem parenchyma cells in leaf tissue), but never in mesophyll cells. Saito *et al.* (1986) showed with electron microscopic studies that the virus accumulates primarily in the cytoplasm of the companion cells in phloem tissues. As RTBV is insect transmitted, the virus particles are probably brought directly to the cells of the phloem or xylem, when the insect is sucking from the sap in the vascular bundle. The transgenes should therefore be expressed in the cells of the vascular bundle.

### 4.2.1.1 Promoter analysis

For the control of expression of the transgenes, two promoters were selected: The CaMV 35S promoter, that has been reported to be active constitutively in almost all the rice tissues (Battraw and Hall 1990), but mainly in the vascular bundles (Terada and Shimamoto 1990), and the homologous RTBV promoter, that should assure expression of the transgene in the tissue where the virus accumulates. Bhattacharyya-Pakrasi *et al.* (1993) reported RTBV promoter-dependent phloem-specific GUS expression in transgenic rice plants.

In plasmids pHCintG and pHRintG the GUS gene was either controlled by the CaMV 35S or the RTBV promoter, respectively. To visualize expression of the transgenes, these two constructs were transferred to rice. Both promoters were highly active in callus material, transformed with either construct. Strong GUS expression was found with both promoters in the vascular bundles of young plants. In very young tissue, the RTBV promoter was also active in many cells of the epidermis. Both promoters were active in the guard cells of the stomata. In contrast to the RTBV promoter, the CaMV 35S promoter was also active in mesophyll cells. In older plants, RTBV promoter activity was restricted to the phloem cells or could not be detected at all. It still has to be determined when exactly in the development of the plant the activity of the RTBV promoter is reduced.

These results encouraged us to use both promoters for the control of the transgenes, as both were active in the vascular bundles of young plants.
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For an enhancement of the expression levels, an intron derived from RTBV was included in all the constructs. Expression levels were found to be enhanced by this intron by a factor 2.7 with either promoter.

4.2.1.2 Detailed analysis of the RTBV promoter

Since little is known about the RTBV promoter and because only a few promoters have been analysed in monocot plants, a deletion analysis of the RTBV promoter in transgenic rice was included in this work. Our particular interest was directed to promoter elements located downstream of the transcription start site which were required for promoter activity in protoplasts (Chen et al. 1994) but were not included in the promoter constructs tested in R. Beachys group in transgenic rice (Bhattacharyya-Pakrasi et al. 1993). Seven plasmids containing the GUS gene, controlled by the RTBV promoter with different mutations in the promoter or the leader were transferred to rice. The full-length promoter contained the RTBV sequence from 680 nucleotides (nt) upstream (-) to 278 nt downstream (+) the transcription start (Fig. 28). Deletions in the promoter up to -280 or up to -220 nt resulted in similar expression patterns at slightly lower levels. Deletion up to -100 nt completely abolished GUS expression in the vascular bundles. These results suggest, that an element, responsible for vascular bundle-specific expression is located between -220 and -100 nt.

Deletions up to -100 or -50 nt respectively resulted in a completely different expression pattern. Promoter activity was restricted to the epidermal cells and was enhanced therein compared to the full-length promoter. It remains to speculate, whether or not this epidermis-specific expression is just the remaining expression after removal of the elements, responsible for vascular bundle-specific expression. Since this promoter activity in the epidermis cells was much higher than with any longer promoter, an element that influences (partial suppression) the expression in the epidermis cells, located between -220 and -100 nt, is suggested.

While this work was in progress, a study by Yin and Beachy (1995) was published, describing a similar analysis. Deletion of the promoter up to -571 nt reduced promoter activity to 25% of the activity of the full-length promoter (from -731 nt). Deletions up to -164 nt did not further reduce expression levels. However, deletion up to -43 nt completely abolished promoter activity. These results are partially in agreement with our results, as promoter activity was also slightly reduced in our experiment, after deletion of the promoter up to
-280. Therefore, it is suggested that more than one, functionally redundant element within the RTBV promoter is responsible for expression in the vascular bundles. One of these elements is proposed to be located around -571 nt. This would explain both the weak expression from the deletion up to -280 in our experiments and the observed decrease of GUS expression level after deletion of -731 to -571 nt, or -571 to -414 nt, reported by Yin and Beachy (1995) (Fig. 40). A second element is proposed to be located between -164 and -100 nt. This would explain both the loss of expression in the vascular bundles from the deletions up to -100 and -50 nt and the observation of Yin and Beachy (1995), that deletion of -164 to -43 nt does not destroy expression in the vascular bundles, but reduces expression levels.

However, Yin and Beachy (1995) did not report about promoter activity in the epidermal cells after the deletion up to -43 nt. What is the reason for this difference? Besides the additional seven nucleotides from -50 to -43 nt, our constructs contained more of the sequence downstream the transcription start than the constructs of Yin and Beachy (1995). These downstream elements of RTBV were required for promoter activity in protoplasts in a report published by Chen et al. (1994). Whereas our constructs contained RTBV leader sequences up to +278 nt, the constructs of Yin and Beachy (1995) contained only the part of the leader up to +43 nt.

The influence of these downstream elements in transgenic plants was demonstrated by deletions within the leader: Deletions from +8 to +30 or to +83 nt increased GUS expression compared to the full-length leader. Deletion from +52 to +83 nt reduced expression.

However, these results disagree with results from Chen (1995), who found that transient GUS expression levels in rice protoplasts were reduced with the deletions from +8 to +30 or to +83 nt. While a deletion from +8 to +35 reduced expression levels to 34%, the deletion from +8 to +83 completely abolished expression. The reason for this difference could be the different assay systems used (transgenic plants compared to transient expression in protoplasts). This explanation is supported by the observation of Chen (1995) who found that in bombarded rice seedlings the same deletion constructs (Δ +8 to +35 or to +83) reduced expression only to 36 or 22%, respectively, compared to the construct with a full-length leader.

Our results from the construct with the deletion from +52 to +83 are in agreement with the results from Chen (1995). In all the experiments deletion of this part of the leader lead to a reduction of expression. Chen (1995) found a position- and orientation-independent DNA element within this region which
enhanced expression from the RTBV promoter. Interestingly, the expression pattern from the histochemical GUS-assay of the transgenic plants of the deletion +52 to +83 was alike the expression pattern of the full-length promoter, reported by Yin and Beachy (1995), whose constructs contained only the leader up to +45.

It is suggested that secondary structures of the RTBV leader, as calculated by G. Chen (FMI, Basel; pers. comm.), influence expression on a post-transcriptional level (indicated as stem-loop structures in figure 40). Deletions of +8 to +30 or to +83 nt may destroy the ability of the leader to build stem-loop structures and deletion of +52 to +83 nt may allow the leader to form different secondary structures. Alternatively, elements further downstream may be involved, that are brought into the right distance by the different deletions. This subject will have to be further investigated.

![Figure 40: Proposed elements within the RTBV promoter and leader. Numbers represent nucleotides upstream (-) or downstream (+) the transcription start (+1). +vb: Proposed elements, positively influencing expression in the vascular bundles. -epid: Proposed element, negatively influencing expression in the epidermal cells. Within the leader, secondary structures are proposed, that influence expression post-transcriptionally.](image)

**4.2.2 Transformation of 32 different plasmids**

By bombardment of precultured immature TP309 embryos and ECS of TP309 and Kinuhikari, 32 different plasmids (with RTBV sequences or GUS genes) were transferred to rice.

Totally 514 TP309 and Kinuhikari plants were regenerated from hygromycin-resistant calli. Around 50% of the plants derived from bombarded TP309 embryos or suspension calli and 30% of the plants, derived from bombarded Kinuhikari suspension calli were fertile. The Kinuhikari ECS was established from embryogenic callus, derived from the scutellum of mature embryos, while the TP309 ECS was established from immature embryos. This could be the reason for the difference in fertility.

Among all the primary transformants, containing RTBV sequences and tested in Southern analysis, 119 independent transgenic lines were identified. From these 119 lines, only 37 (31%) had integrated at least one complete copy of the respective RTBV gene. From these 37 lines, 25 (68%) were fertile. Between
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zero and five fertile lines with at least one complete copy of the transgene were obtained from the different plasmids. Not a single plant that was regenerated from hygromycin-resistant calli was not transgenic, indicating that the applied selection with Hgromycin B was very tight.

Two reasons are responsible that from the large number of regenerated plants only relatively low numbers of independent, fertile lines with complete copies of the transgenes integrated in the genome were obtained:

1. Liquid selection of hygromycin-resistant colonies was done in relatively large beakers with many bombarded embryos or ECS cells cultured in one aliquot of medium. After isolation of two hygromycin-resistant colonies from the same beaker, it was not clear, whether these two colonies belonged to the same or to different lines. Only after testing plant genomic DNA, isolated from the regenerated plants in Southern analysis, independent lines could be identified. Therefore, from some lines, many plants were regenerated.

2. The plasmid DNA used for particle bombardment was used in natural, circular form. For integration, linearization anywhere within the plasmid sequence had to occur. In case this happened within the sequence of the gene of interest, rearranged or incomplete copies of the transgene were integrated into the genome.

In future experiments, selection of resistant colonies should be done on semi-solid medium or the bombarded material should be aliquoted into smaller volumes for selection in liquid medium. The plasmids, used for transformation, should be linearized by appropriate restriction endonucleases to minimize integration of rearranged or incomplete transgene copies.

For all the strategies like expression of the coat protein, the replicase and proteins of unknown functions, fertile plants with complete transgene integration were obtained. However, not for all the individual constructs that were transferred this aim could be reached (see table 8). For above mentioned reasons, especially the plasmids with very long RTBV sequences seldomly integrated in full-length into the plant genome. Furthermore, with the constructs, containing sequences from RTBV ORF4, it was difficult to obtain resistant calli and fertile plants. Whether this was due to the specific sequences from RTBV ORF4, which might code for a regulatory function that may be deleterious to plant cells when expressed, can only be speculated.
Unfortunately, no fertile plants, transformed with the antisense RNA construct, were obtained.

4.2.3 Expression analysis

In many of the transgenic lines transcripts could be detected that hybridized to the specific probe from the leader region, which is common to all the RTBV transgene transcripts. Several of the detected transcripts had the size, which could be expected from the transgene sequence. However, in several lines, the transcripts were larger than expected, several expressed more than one transcript and some showed transcripts, that seemed to appear in more than one line. The nature of these different, unexpected RNAs, that hybridized to the specific probe, remains to be investigated.

Generally, the levels of accumulated RTBV transcripts that were detected in Northern analysis were very low, compared to the levels of accumulated mRNA from the hygromycin-resistance gene (aph4). This is not surprising, as during cell culture, cells were selected for expression of aph4.

One reason for these low expression levels could be the presence of the natural RTBV poly(A) site within the RTBV intron. This transcription termination signal around 200 nucleotides after the transcription start is probably responsible for the small RNA that is detected uniformly in most of the tested lines, mainly in RNA isolations from callus material (Fig. 35 b,c), but also from leaf material (Fig. 35 a). To find large amounts of this small RNA in the tissues was surprising, as it can not be found naturally in infected rice leaves (H. Rothnie, FMI, Basel, pers. comm.). It is unknown whether this RNA serves a function in the virus replication cycle. Furth et al. (1994) reported poor poly(A) site recognition when located in an intron and Sanfaçon and Hohn (1990) reported reduced recognition efficiency of promoter proximal poly(A) sites. In CaMV, an equivalent small RNA, that was produced at low level, could be found in infected plants and was probably very stable (Sanfaçon and Hohn 1990). Since with the plants transformed with the GUS gene (expressing GUS at high levels in callus as well as in leaves) the same situation with large amounts of the small RNA, but only small amounts of the longer transcripts is found, it is suggested, that the longer transcripts are produced but are not stable enough to be detected in large amounts in Northern analysis. The short RNA in contrast, probably accumulated because it was more stable.
For detection of the proteins in the transgenic plants, the RTBV sequences had been fused to a sequence, coding for a 10 amino acid-tag-peptide from the influenza virus hemagglutinin protein. This small epitope allows detection of all the products with one single antibody. From all the tested lines, only in one line a protein of the expected size could be detected (Fig. 37). This line, K42.10, produces a subfragment of the gene product of RTBV ORF4 with unknown function.

Small amounts of protein, below the level of detection in Western analysis, could still be produced by the other plants, although no proteins could be detected. The proteins, which in many cases are only small subfragments of the respective viral proteins, might not be stable since these protein fragments may not get properly folded.

High levels of transgene transcripts were detected in the primary transformants, that were transformed with the plasmid, producing antisense RNA. In this plasmid, part of the DNA-sequence from the leader of the RTBV pregenomic RNA was linked in antisense orientation to the 3' end of aph4. That translation of APH4 was not disturbed by the 3' extension of aph4 was obvious, as transgenic plants could be selected with Hygromycin B. Northern analysis revealed, that aph4 with the 3' extension was transcribed efficiently. Unfortunately, the plants were not fertile. Not enough independent lines were produced to conclude whether this was due to the construct or whether it was bad luck.

In order to make sure, that non-selectable transgenes are transcribed efficiently, linkage of the sequence of interest to the selectable gene could be generally advantageous. Constructs should be designed, in which not only sequences for the production of antisense RNA, but also sense sequences are linked with the selective gene. This could be done by expression of the two genes polycistronically, as it is done by RTBV, where the ORF1 is translated from a weak AUU-start codon, whereas ORF2 and ORF3 have proper start codons. Placing aph4 with an AUU-start codon in front of any gene of interest, silently mutating all the AUG triplets within the aph4 sequence, would probably assure production of significant transgene levels in hygromycin resistant cells.
4.2.4 Virus resistance tests

Since it is not known which levels of RNA or protein are required for protection or resistance to the virus, 52 lines that had produced enough seeds for testing the R1 generation were tested at the International Rice Research Institute (IRRI) for resistance to rice tungro disease. The plants that were tested included lines from all the strategies, except antisense RNA.

The plants were inoculated by the natural, viruliferous vector, *Nephotettix virescens*, carrying RTBV and RTSV. The plants were splitted into two groups, using two different sources for the viruses. One of the tungro virus isolates used in these tests was collected in Laguna, Philippines and has been maintained in the greenhouse for nearly 20 years. RTBV viruses from this population had been used originally for sequencing of the RTBV genome (Hay et al. 1991). The other isolate was collected in Famy, Laguna, Philippines, one month before the beginning of the experiment. Twenty days after infection, the plants were assessed on the basis of characteristic symptoms, like height reduction and leaf discolouration. The average symptom severity score (SS), ranging from 1 for resistant to 9 for sensitive plants (Hasanuddin et al. 1988) was determined. Further, the plants were tested serologically by ELISA for RTSV- and RTBV- titers 20 and 40 days after infection.

The tested plants were R1 progeny plants of the primary regenerants. The transgene, integrated hemizygously in the genome of the primary regenerants, is segregating in the R1 population. Selfed plants that have the transgenes inserted at one single locus in the genome are expected to yield 25% of homozygous and 50% of hemizygous progenies. In another 25% of the progenies, the transgene was lost due to Mendelian segregation. Therefore, in the best case 75% of the plants from a tested line could be expected to exhibit resistance to the disease.

Unfortunately, none of the tested lines showed any form of resistance to RTBV. Nearly all the treated plants had developed severe symptoms 20 days after infection and had virus-titers comparable to the infected control plants. None of the lines had recovered after 40 days. Three individual plants, one each from the lines 1.27, 7.6 and from the non-transformed negative control, had low titers of RTBV and RTSV 20 days after infection and did not show any characteristic disease symptoms. As both titers, from RTBV and from RTSV were low, these symptom-free plants must be considered as escapes from infection, as it frequently can be observed infecting plant populations (Dahal et al. 1988).
4.3 Perspectives

No RTBV-resistant line could be identified so far. Low expression of the transgenes was observed with all the lines. By a different architecture of the construct, maybe polycistronic expression of the gene of interest with the selectable marker as described above, could yield high transgene expression levels.

With the optimized protocol for rice transformation by particle bombardment, additional constructs can easily be transferred to rice. Some of these constructs will contain different sequences for the production of antisense RNA.

4.4 Why genetically engineered tungro resistance?

4.4.1 Risks of genetically engineered, virus resistant plants

It has been demonstrated by several groups that the RNA molecules of RNA viruses, infecting transgenic plants that express the coat protein of another RNA virus, can recombine with the expressed transgene (Osbourne et al. 1990; Greene and Allison 1994). Further it was demonstrated by Lecoq et al. (1993) that heteroencapsidation can occur in transgenic plants expressing a coat protein gene, and that this can indeed affect the vector transmission properties of the infecting virus.

However, it has to be evaluated what impact such plants, expressing viral genes really have on recombination and heteroencapsidation with other viruses. Plants expressing viral sequences, and which are then infected with a virus, are in a situation similar to plants coinfected with both viruses naturally. The products of the transgenes are generally synthesized from the plant genome at levels much lower than during viral infection. Therefore, the transgenic plants do not provide better chances for recombination or heteroencapsidation than that can be found naturally within a doubly infected plant. For DNA viruses however, probably replicating in the nucleus of the host cell, the situation for recombinations might be slightly different, as the transgenes are present in all the cells, even where they are not expressed.

Before the release of transgenic plants, containing viral sequences, specific risk/benefit analyses have to be done. The matter is beyond where only scientific evaluation is appropriate and public perception of such a release will be required.
In the case of tungro disease resistant rice, perception from the side of the rice farmers can certainly be expected, as the farmers, whose fields are affected by tungro disease, loose nearly the whole harvest and so the income for supporting their family. For preventing these losses with (genetically engineered) tungro resistant varieties, the resistant trait has to be transferred to the local varieties that are adapted to the specific locations and that are accepted by the consumers in terms of quality. This transfer of the resistance trait to local varieties can be done by the local breeding centers or even by skilled farmers themselves. To prevent a fast break-down of the virus resistance due to a large scale planting of varieties that all contain the same resistance gene, several resistance genes, that protect the plant by different mechanisms, are required. These genes should either be incorporated in the same plant, in order to minimize the possibility for the virus to overcome the multiple resistance, or they should be used in different varieties which should be cultivated in rotation with each other.

4.4.2 Perspectives for rice production

By 1990, the world population was 5.3 billions. With an expected growth of nearly 100 millions per year, world population will reach 8.4 billions by 2025 (IRRI 1993). Today, enough food to feed the world is produced theoretically with large industrialized over-production in high developed countries and dramatic food shortages in low developed countries. Distribution of the harvests over long distances causes immense problems and creates additional dependencies. The high population growth rates of 200-500% in low developed countries will soon lead to a deficit in worldwide food production. To keep up with this enormous growth, the world’s rice production will have to increase by almost 70% over the next 30 years (IRRI 1993). Today, many countries like the Philippines depend on rice imports. Even the highly industrialized Japan had to import rice in 1994. As soon as China has to start large scale rice imports due to decrease in domestic harvests, no rice will be available on the relatively small world market of only 3-4% of the world rice production. Corruption at all levels of the political systems accounts as one main reason for the insufficient rice production in many countries. It is worthwhile to note that a poor country like Vietnam, that suffered for a long time under the US trade embargo, is not only producing enough rice for its domestic demand, but also is one of the leading rice exporters. Further reasons for the frequent rice shortages in many countries are wars, lacking infrastructure (as a consequence of the reasons mentioned before), rapidly growing population and various calamities. Food
shortages increase the risk for wars and solving the problems that lead to food shortages is not easy and takes time. Solving these problems while the population suffers from hunger is impossible. Therefore, food-, but especially rice production has to be increased in the countries that do not produce enough rice for domestic use.

Genetic engineering of plants alone certainly cannot solve any of these problems and by itself, will not increase food production in a sustainable way. However, as part of agronomical programs that respect the individual circumstances of a country or a region, genetically engineered crops can contribute to an increased food production. It is not possible to increase the yield indefinitely without immense fertilizer inputs. Preventing yield losses of up to 50% due to insect pests, viral and fungal diseases and weeds, would increase rice harvests significantly. Together with various other measures, transgenic, virus resistant rice plants could contribute to an increased rice production.
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Curriculum vitae

May 20 1967  Born in Richterswil, Switzerland
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Publications


