MOLECULAR ANALYSIS OF GENOME SEGMENT 5 OF RICE RAGGED STUNT VIRUS AND TRANSFORMATION OF INDICA RICE WITH SEGMENT 5 BASED GENES

By

ZHONGYI LI

A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

February, 1996
DECLARATION

I hereby declare that the research described in this thesis is my own work unless acknowledgment is made in the text. There is no material involved in this thesis which has been submitted for any other degree.

Zhongyi Li
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<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
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<tr>
<td>Act1</td>
<td>rice actin 1 gene</td>
</tr>
<tr>
<td>Adh 1</td>
<td>maize alcohol dehydrolase gene</td>
</tr>
<tr>
<td>AOA</td>
<td>(aminooxy) acetic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>bar</td>
<td>phosphinothricin acetyltransferase gene of <em>Streptomyces hygroscopicus</em></td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate, disodium salt</td>
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<tr>
<td>BEU</td>
<td>blue expression unit</td>
</tr>
<tr>
<td>bp</td>
<td>basepairs</td>
</tr>
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<td>BTV</td>
<td>blue tongue virus</td>
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<td>CaMV</td>
<td>cauliflower mosaic virus</td>
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<td>CaMV 35S</td>
<td>the promoter of the cauliflower mosaic virus 35S RNA transcript</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>CIP</td>
<td>calf intestinal alkaline phosphatase</td>
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<td>CRIP</td>
<td>core replication intermediate particle</td>
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<td>csr-1</td>
<td>a mutant acetolactate synthase gene of <em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl-trimethylammonium bromide</td>
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<td>CTP</td>
<td>cytidine triphosphate</td>
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<tr>
<td>ddNTP</td>
<td>dideoxynucleotide triphosphates</td>
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<td>dhfr</td>
<td>mouse dihydrofolate reductase gene</td>
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<tr>
<td>DMSO</td>
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<td>dNTP</td>
<td>deoxynucleotide triphosphates</td>
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<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothretol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetate, tetrasodium salt</td>
</tr>
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<td>Emu</td>
<td>the promoter derived from a modified maize alcohol dehydrogenase 1 promoter and first intron</td>
</tr>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>ERSV</td>
<td><em>Echinocloa</em> ragged stunt virus</td>
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<tr>
<td>FDV</td>
<td>Fiji disease virus</td>
</tr>
<tr>
<td>GCG</td>
<td>a computer software package developed by University of Wisconsin Genetics Computer Group</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<td>guanosine triphosphate</td>
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<td>GUS</td>
<td>β-glucuronidase</td>
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<td>hph</td>
<td>hygromycin phosphotransferase gene of <em>E. coli</em></td>
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<td>presence of <em>hph</em> gene</td>
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<td>Hph'</td>
<td><em>hph</em> gene negative</td>
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<td>IAA</td>
<td>indole-3-acetic acid</td>
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<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
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<td>IR</td>
<td>involving replication</td>
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<td>ISL</td>
<td>inner shell large protein</td>
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<td>inner shell small protein</td>
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<td>kilodalton</td>
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<td>potassium acetate</td>
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<td>LB</td>
<td>Luria broth</td>
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<td>MRDV</td>
<td>maize rough dwarf virus</td>
</tr>
<tr>
<td>NAA</td>
<td>α-naphthalene acetic acid</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium chloride</td>
</tr>
<tr>
<td>npt II</td>
<td>neomycin phosphotransferase II gene of <em>E. coli</em></td>
</tr>
<tr>
<td>NSP</td>
<td>nonstructural protein</td>
</tr>
<tr>
<td>NSRB</td>
<td>nonstructural proteins with RNA binding activity</td>
</tr>
<tr>
<td>NSRP</td>
<td>nonstructural proteins associated with replication</td>
</tr>
<tr>
<td>NSTC</td>
<td>nonstructural proteins forming tubule or associating with cytoskeleton</td>
</tr>
<tr>
<td>NSVR</td>
<td>nonstructural proteins associated with viral release</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide triphosphates</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>OSDV</td>
<td>oat sterile dwarf virus</td>
</tr>
<tr>
<td>OSL</td>
<td>outer shell large protein</td>
</tr>
<tr>
<td>OSS</td>
<td>outer shell small protein</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDR</td>
<td>pathogen-derived resistance</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethyleneglycol</td>
</tr>
<tr>
<td>PIRV</td>
<td>plant-infecting reoviruses</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PRIP</td>
<td>precore replication intermediate particle</td>
</tr>
<tr>
<td>PSV</td>
<td>Pangola stunt virus</td>
</tr>
<tr>
<td>PU</td>
<td>the function of the protein remains unknown</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinyl-pyrrolidine</td>
</tr>
<tr>
<td>PVY</td>
<td>potato virus Y</td>
</tr>
<tr>
<td>RB</td>
<td>RNA binding protein</td>
</tr>
<tr>
<td>RBDSDV</td>
<td>rice black-streaked dwarf virus</td>
</tr>
<tr>
<td>RDRP</td>
<td>RNA dependent RNA polymerase</td>
</tr>
<tr>
<td>RDV</td>
<td>rice dwarf virus</td>
</tr>
<tr>
<td>REO 3</td>
<td>reovirus type 3</td>
</tr>
<tr>
<td>RIP</td>
<td>replication intermediate particle</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RolC</td>
<td>Ri plasmid ORF12 gene of <em>Agrobacterium rhizogenes</em></td>
</tr>
<tr>
<td>ROTA-A</td>
<td>simian serotype A rotavirus SA11</td>
</tr>
<tr>
<td>RRSV</td>
<td>rice ragged stunt virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S (before number)</td>
<td>segment</td>
</tr>
<tr>
<td>SC</td>
<td>spike cap protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SP</td>
<td>spike protein</td>
</tr>
<tr>
<td>SRIP</td>
<td>single-shelled replication intermediate particle</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>SVP</td>
<td>subviral particle</td>
</tr>
<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>seed or plants produced from T&lt;sub&gt;0&lt;/sub&gt; transgenic plant</td>
</tr>
</tbody>
</table>
TEMED  N, N, N', N'-tetramethylethylenediamine

tRNA  transfer RNA

TTP  thymidine triphosphate

TY  tryptone yeast extract

Ubi1  the promoter of maize ubiquitin 1 gene

UidA (gus)  β-glucuronidase gene of E. coli

v/v  volume to volume ratio

WTV  wound tumour virus

X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

The complete nucleotide sequence of the genomic segment 5 of a
Tilovirus was determined. The 2822 nucleotide sequence
contains a single long open reading frame capable of encoding a
polypeptide with a Mr of 44941 Da. Polypeptides encoded by several
truncated cDNAs of 55 were expressed using the pUCEx fusion protein
vector and the highest level of fusion protein was obtained from a construct
encoding a hydrophilic region of the 55 protein. Antibodies raised against
this fusion protein recognized a minor 9kDa polypeptide present in purified
preparations of RRSV particles, infected inset vectors, and in infected
plants. This indicates that RRSV 55 encoded a minor structural protein.

Comparing the RRSV 55 sequence with sequences of other reassortants, did
not reveal any significant sequence similarity. This suggests the
classification of RRSV into a fourth reassortant group, Cryptovirus.
ABSTRACT

Rice ragged stunt virus (RRSV) is a major virus pathogen of rice, second only in importance to rice tungro virus. There is no easily accessible natural resistance gene against RRSV that can be introduced into elite cultivars by conventional breeding. The aim of this study is to investigate genomic segment 5 (S5) of RRSV, develop synthetic resistance gene constructs from this segment and transform Indica rice with these constructs.

The complete nucleotide sequence of the genomic segment 5 of a Thai isolate of RRSV was determined. The 2682 nucleotide sequence contains a single long open reading frame capable of encoding a polypeptide with a Mr of ~ 91kDa. Polypeptides encoded by various truncated cDNAs of S5 were expressed using the pGEX fusion protein vector and the highest level of fusion protein was obtained from a construct encoding a hydrophilic region of the S5 protein. Antibodies raised against this fusion protein recognized a minor 91kDa polypeptide present in purified preparations of RRSV particles, infected insect vectors, and in infected rice plants. This indicates that RRSV S5 encodes a minor structural protein. Comparing the RRSV S5 sequence with sequences of other reoviruses, did not reveal any significant sequence similarities. This supports the classification of RRSV into a separate reovirus genus, Oryzavirus.
The effectiveness of different promoters for use in Indica rice transformation was compared. Plasmids encoding the *uidA* gene under the control of *RolC*, CaMV35S, Emu, *Act1* or *Ubi1* promoters were electroporated into Indica rice protoplasts or delivered into cell suspension cultures by particle bombardment. Transient gene expression in both protoplasts and suspension culture was greatest from plasmids utilising the constitutive promoters, *Act1* and *Ubi1*. Gene expression in stably transformed tissue was examined by bombarding embryogenic Indica rice calli with a *pUbi1-gus* plasmid and a plasmid containing either *hph* or *bar* under the control of the CaMV35S, Emu, *Act1*, or the *Ubi1* promoters. The bombarded calli were placed on the appropriate selection media and stained for GUS activity at 1 day, 3 weeks, and 5 weeks after shooting. Callus bombarded with the *pUbi1-hph* or the *pEmu-hph* constructs gave a dramatic increase in the size of the GUS staining areas with time. No such increase in the size of the GUS staining areas was observed in calli co-bombarded with *pUbi1-gus* and any of the *bar* containing constructs.

Many fertile transgenic Indica rice plants containing one to eight copies of both selectable and non-selectable genes (*uidA* or RRSV S5) were obtained by co-bombardment of calli with either the *pEmu-hph* or *pUbi1-hph* constructs and the non-selectable gene constructs.

Preliminary results from PCR assay of T₁ plants and germination of T₁ seeds showed that both the RRSV S5 gene and *hph* gene were transmitted
to T₁ progeny plants. The results from RT-PCR assays confirmed that the RRSV S5 transgene was also presented in T₁ transgenic plants.

The T₁ seeds produced from these transgenic plants (containing RRSV S5 transgenes) have been sent to our collaborators in China and India where they will be challenged with RRSV.
INTRODUCTION

Rice ragged stunt virus (RRSV) is a major virus pathogen of rice, second only in importance to rice tungro virus. Sporadically it causes widespread epidemics of rice crops in South East Asia, for example, in Thailand in 1990 there was an outbreak that covered over 170,000 hectares (W. Kositratana, personal communication). It can cause yield losses of 20 to 80%. There is no easily accessible natural resistance gene against RRSV that can be introduced into elite cultivars by conventional breeding (Herdt, 1991). However, resistance has been achieved for a number of other viruses by pathogen-derived resistance (PDR), namely transforming plants with viral transgenes (reviewed by Hull and Davies, 1992). Furthermore, a number of groups have reported success in genetic transformation of rice (reviewed by McElroy and Brettell, 1994; Vasil, 1994). Therefore, it may be possible to protect rice from RRSV using the PDR approach. At the commencement of this study there was no molecular information about RRSV. Therefore the strategy I adopted was to investigate a genomic segment of RRSV, develop an Indica rice transformation system, and combine the two aspects to generate rice plants with viral resistance genes against RRSV.
CHAPTER ONE: REVIEW OF LITERATURE --- THE REOVIRIDAE

1.1 Introduction

The Reoviridae is a remarkable virus family. It has a host range that encompasses almost all types of multicellular eukaryotic organisms, from shellfish to plants, insects, fish, birds and mammals (Holmes, 1990). Some members can cause mortality in humans (Kapikian and Chanock, 1990), others can be fatal in domesticated animals such as sheep, cattle and horses (Knudson and Monath, 1990), and some plant-infecting reoviruses, eg. rice ragged stunt virus (RRSV), can have devastating effects on staple crops (Herdt, 1991). The name Reoviridae was proposed in 1959 (Sabin, 1959), and this family currently contains over 50 distinct viruses, classified into nine genera (Table 1.1). Classification as a member of this family is based on the presence of a characteristic multilayered virion, a dsRNA genome with 10-12 segments, a common replication strategy, and a RNA polymerase activity within the virion.

While there has been some research on plant infecting reoviruses, notably wound tumour virus (WTV) and rice dwarf virus (RDV), much more effort has been focused on reoviruses that infect animals. In particular, blue tongue virus (BTV), reovirus serotype 3 (REO 3), and simian serogroup A rotavirus SA11 (ROTA-A) have been intensively studied. Therefore, in this
Table 1.1 Prototype, host range and number of genome segments of the genera of the Family Reoviridae

<table>
<thead>
<tr>
<th>Genus</th>
<th>Prototype</th>
<th>Host range</th>
<th>Segments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoreovirus</td>
<td>wound tumor virus</td>
<td>plants, insects</td>
<td>12</td>
</tr>
<tr>
<td>Fijivirus</td>
<td>fijivirus</td>
<td>plants, insects</td>
<td>10</td>
</tr>
<tr>
<td>Oryzavirus</td>
<td>rice ragged stunt virus</td>
<td>plants, insects</td>
<td>10</td>
</tr>
<tr>
<td>Orthoreovirus</td>
<td>reovirus serotype 3</td>
<td>vertebrates</td>
<td>10</td>
</tr>
<tr>
<td>Aquareovirus</td>
<td>Golden shiner virus</td>
<td>aquatic organisms</td>
<td>11</td>
</tr>
<tr>
<td>Coltivirus</td>
<td>Colorado tick fever virus</td>
<td>mammals, tick</td>
<td>12</td>
</tr>
<tr>
<td>Orbivirus</td>
<td>bluetongue virus</td>
<td>vertebrates, insects</td>
<td>10</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Simian serogroup A rotavirus</td>
<td>vertebrates</td>
<td>11</td>
</tr>
<tr>
<td>Cypovirus</td>
<td>Bombyx mori cytoplasmic polyhedrosis virus type 1</td>
<td>insects</td>
<td>10</td>
</tr>
</tbody>
</table>
review I will not only discuss plant infecting reoviruses, but will also present much of the detailed knowledge gained from study of the above animal reoviruses.

1.2 Biology of plant infecting reoviruses

All of the 10 currently known plant-infecting reoviruses (PIRV) are transmitted to their plant hosts by arthropod insects (Table 1.2), in which they also replicate, thus they have a two part biological cycle; one part in the plant host, the other in the insect host. Some WTV and RDV virus isolates, after prolonged maintenance in infected plants by vegetative propagation, lose their ability to be transmitted by their insect vectors (Black, 1969; Kimura, 1976). None of the PIRVs is spread by mechanical transmission.

All but one of the PIRVs have narrow host ranges restricted to the plant Gramineae. WTV is the clear exception, possessing a wide experimental host range of dicotyledonous plants. Interestingly, WTV was originally isolated from the leafhopper, *Agalliopsis novella*, and has only recently been found in a naturally infected plant (Hillman et al., 1991).

PIRVs typically cause stunting, the formation of galls derived from phloem tissue, excess tillering, abnormal flowering and abnormal seed development (Boccardo and Milne, 1984).

The galls are formed from a proliferation of modified phloem cells and appear to be the exclusive sites of PIRV replication (Hatta and Francki, 1976). These cells contain viroplasms that consist mainly of proteins and
Table 1.2 The host ranges and vectors of definitive plant infecting reoviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Insect vector</th>
<th>Host plant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phytoreoviruses</strong></td>
<td><strong>Cicadellid leafhoppers</strong></td>
<td><strong>Mainly Gramineae</strong></td>
</tr>
<tr>
<td>Rice dwarf virus (RDV)</td>
<td><em>Nephotettix cincticeps</em></td>
<td>Gramineae</td>
</tr>
<tr>
<td>(RDV)</td>
<td><em>N. apicalis, N. virescens,</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Recilia (=Inazuma) dorsalis</em></td>
<td></td>
</tr>
<tr>
<td>Wound tumor virus (WTV)</td>
<td><em>Agallia constricta</em></td>
<td>Dicotyledons</td>
</tr>
<tr>
<td>(WTV)</td>
<td><em>A. quadripunctata</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Agalliopsis novella</em></td>
<td></td>
</tr>
<tr>
<td>Rice gall dwarf virus (RGDV)</td>
<td><em>Nephotettix nigropictus</em></td>
<td>Gramineae</td>
</tr>
<tr>
<td>(RGDV)</td>
<td><em>N. cincticeps, N. malayanus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>N. virescens, Recilia</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(=Inazuma) dorsalis</em></td>
<td></td>
</tr>
<tr>
<td><strong>Fijiviruses</strong></td>
<td><strong>Delphacid hopper</strong></td>
<td><strong>Gramineae</strong></td>
</tr>
<tr>
<td>Fiji disease virus (FDV)</td>
<td><em>Perkinsiella saccharicida</em></td>
<td>Sugarcane</td>
</tr>
<tr>
<td>(FDV)</td>
<td><em>P. vastatrix, P. vitiensis</em></td>
<td></td>
</tr>
<tr>
<td>Maize rough dwarf virus (MRDV)</td>
<td><em>Laodelphax striatellus,</em></td>
<td>Gramineae</td>
</tr>
<tr>
<td></td>
<td><em>Delphacodes propinqua</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Sogatella vibrix</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Javesella pellucida</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Dicranotropis hamata</em></td>
<td></td>
</tr>
</tbody>
</table>
Rice black-streaked dwarf virus (RBSDV) Laodelphax striatellus Gramineae

Pangola stunt virus (PSV) Unkanodes albifascia Pangola

Oat sterile dwarf virus (OSDV) Javesella pellucida Gramineae

J. discolor, J. dubia

J. obscorsa, Dicranotropis hamata

Oryzavirus

Rice ragged stunt virus (RRSV) Nilaparvata lugens Rice

Echinochloa ragged stunt virus (ERSV) Sogetella longifurcifera Echinochloa longifurcifera

S. vibix

Notes:


ERSV: Chen et al., 1989a.

The reproduction of PIRVs within the insect vector allows the virus to be transmitted to host plants during the entire life span of the insect. This can facilitate long distance spread of the virus, for example RRSV has often been transmitting in rice crops in Japan by wind-borne brown planthoppers from southern China (Milne et al., 1984; Oya, 1988). Transovarian transmission of some PIRVs occurs in their insect vectors, but no such transmission has been observed for Oryzium and Oryzina viruses (Finzi and Boccardo, 1983). The attack on rice by the carrier insect may be a key factor in the spread of the virus.

1.3 Virus particle structure and genome organization

1.3.1 Virus particle structure

The Phytovirinae have dsRNA genomes divided into one to nine segments whereas the Fiji- and Oryza-viruses each segment in a segmented genome. All necroviruses appear to package a single copy of each of the different genome segments into every particle. Both attack and spread infecting necroviruses have complex transmission vectors. With few exceptions, the oryza and oryza viruses, which will be discussed in Chapter 3, the virions are double stranded and structures assembled from at least six or seven different polypeptide species. However, most of the necrovirus genomes
RNA (Milne and Lovisolo, 1977; Giannotti and Monsarrat, 1968), and also tubules bearing ordered arrays of virus particles (Boccardo and Milne, 1980).

The replication of PIRVs within the insect vector allows the virus to be transmitted to host plants during the entire life span of the insect. This can facilitate long distance spread of the virus, for example RRSV has often been transmitted to rice crops in Japan by wind-borne brown planthoppers from southern China (Hirao et al., 1984; Oya, 1986). Transovarial transmission of some PIRVs occurs in their insect vectors, but no such transmission has been observed for fijiviruses and oryzaviruses (Francki and Boccardo, 1983).

1.3 Virus particle structure and genome organisation

1.3.1 Virus particle structure

The Phytoreoviruses have dsRNA genomes divided into twelve segments whereas the Fiji- and Oryza- viruses each possess ten segmented genomes. All reoviruses appear to package a single copy of each of the different genome segments into every particle. Both animal and plant infecting reoviruses have complex isometric virions. With the exception of the oryzaviruses and the cypoviruses, which will be discussed in Chapter 4, the virions are double shelled structures assembled from at least six or seven different polypeptide species. Indeed, most of the reovirus genome is...
devoted to encoding particle proteins and almost all stages of the viral life cycle involve the particle.

There are a number of common features among all the double shelled reovirus particles but there are two basic models. These are represented in Fig. 1.1. In this figure I have simplified the structures and allocated a standard nomenclature to the different proteins. As can be seen from Table 1.3 there is variation in the size of proteins within a category, variation in the genomic segments encoding the protein and differences in the names used to define the proteins. For the sake of clarity, I have used the nomenclature described in Fig. 1.1 and Table 1.3 throughout this thesis. In both particle types each of the two shells is composed of two types of subunit (one large; one small). Hence both types of particle contain four structural homologs which I have termed outer shell large (OSL) protein, outer shell small (OSS) protein, inner shell large (ISL) protein and inner shell small (ISS) protein. The ISL, ISS, OSL and OSS proteins have average sizes of approximately 110, 45, 100 and 60 kDa respectively. These four types of proteins are the major proteins in the type 1 particles of orbi-, colti- and rota- viruses and phytoreoviruses, but there are two additional proteins in the type 2 particles of orthoreoviruses, aquareoviruses and fijiviruses. These are the spike (SP) and the spike cap (SC) proteins.

A proportion of particles in purified preparations of PIRVs usually have some of the outer shell protein subunits missing. Such particles are termed cores and not only are they found in the different states of
Fig. 1.1 Diagram of particle structure of two types of reovirus particles. A. Type 1 reovirus particle. B. Type 2 reovirus particle. ISL: inner shell large protein; ISS: inner shell small protein; OSL: outer shell large protein; OSS: outer shell small protein; SP: spike protein; SC: spike cap protein.
Reovirus Particles

Type 1 particle:
- orbiviruses eg blue tongue virus
- coltivirus
- rotavirus
- phytoreovirus

Type 2 particle:
- orthoreovirus eg Reovirus serotype 3
- aquareovirus
- fijivirus
Table 1.3 Coding assignment of double shell proteins of Reoviridae

<table>
<thead>
<tr>
<th>Protein</th>
<th>ISL</th>
<th>ISS</th>
<th>OSL</th>
<th>OSS</th>
<th>SP</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>REO 3</td>
<td>λ1</td>
<td>σ2</td>
<td>μ1C</td>
<td>σ3</td>
<td>λ2</td>
<td>σ1</td>
</tr>
<tr>
<td>size kDa</td>
<td>137</td>
<td>47</td>
<td>72</td>
<td>41</td>
<td>144</td>
<td>49</td>
</tr>
<tr>
<td>segment</td>
<td>L3</td>
<td>S2</td>
<td>M2</td>
<td>S4</td>
<td>L2</td>
<td>S1</td>
</tr>
<tr>
<td>BTV</td>
<td>VP3</td>
<td>VP7</td>
<td>VP2</td>
<td>VP5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>size kDa</td>
<td>103</td>
<td>39</td>
<td>111</td>
<td>59</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>segment</td>
<td>L3</td>
<td>S7</td>
<td>L2</td>
<td>M5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ROTA-A</td>
<td>VP2</td>
<td>VP6</td>
<td>VP4</td>
<td>VP7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>size kDa</td>
<td>102</td>
<td>45</td>
<td>87</td>
<td>37</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>segment</td>
<td>S2</td>
<td>S6</td>
<td>S4</td>
<td>S9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WTV</td>
<td>P3</td>
<td>P8/P9</td>
<td>P2</td>
<td>P5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>size kDa</td>
<td>(108)</td>
<td>48/36</td>
<td>(130)</td>
<td>91</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>segment</td>
<td>S3</td>
<td>S8/S11</td>
<td>S2</td>
<td>S5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RDV</td>
<td>P3</td>
<td>P8</td>
<td>P2</td>
<td>P5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>size kDa</td>
<td>114</td>
<td>46/45</td>
<td>116</td>
<td>90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>segment</td>
<td>S3</td>
<td>S8</td>
<td>S2</td>
<td>S5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: The protein size was determined by the calculation of deduced proteins from the nucleotide sequences of the viruses, but number in bracket represents the protein size estimated in SDS-PAGE gel.
degradation during the purification process but also in the different states of
the virus particle during its replication cycle.

1.3.1.1 Type 1 reovirus particles

Roy and her colleagues (Roy, 1993) have used a baculovirus system
to express and assemble the proteins from different combinations of BTV
genome segments. They have then analysed the resulting structures by
electron microscopy. This work gives exquisite detail of the BTV particle
structure and provides the clearest picture of a reovirus type 1 particle (Fig
1.2A).

The inner protein shell of BTV consists of a smooth scaffold, made
from sixty ISL subunits (as a T=1 lattice), covered with 260 ISS subunit
trimers in a T=13 lattice (Hewat et al., 1992; Roy, 1993). The BTV outer
shell is composed of two major proteins, OSL and OSS (Verwoerd et al.,
1972; Martin and Zweerink, 1972; French et al., 1990). The OSS subunits
appear to be globular and are located (120 per particle) in the channels
formed by each of the six-membered rings of ISS trimers. The OSL subunits
(60 per particle) are sail-shaped spikes, which project 4 nm beyond the OSS
proteins and are located above 180 of the ISS subunit trimers (Roy, 1993).
These two outer shell proteins form a diffuse protein layer which obscures
the structural arrangement.

The structure of a rotavirus particle is not as clearly elucidated as that
of BTV. However, the ROTA-A particle has been studied by electron
Fig. 1.2 Scale model of particle structure of two types of reovirus particles. 
Top: Type 1 reovirus particle: BTV. Bottom: Type 2 reovirus particle: REO 3. 
ISL: inner shell large protein; ISS: inner shell small protein; OSL: outer shell large protein; OSS: outer shell small protein; SP: spike protein; SC: spike cap protein; RDRP: RNA dependent RNA polymerase; RB: RNA binding protein; IR: involving replication; PU: the function of these proteins remains unknown; dsRNA: double-stranded RNA.
microscopy and cryoelectron microscopy (Prasad et al., 1988). The inner shell of ROTA-A is composed of 102 kDa ISL and 45 kDa ISS subunits which assemble into 132 units arranged in a $T=13$ lattice with a skew symmetry. The outer shell is composed of 87 kDa OSL and 37 kDa OSS subunits which form a layer containing 132 small holes that correspond one-to-one with holes in the inner shell (Roseto et al., 1979). These coordinated holes form channels linking the outer surface to the inner core. There are 120 channels along the 6-coordinated centres and 12 along the 5-coordinated centres. These channels may be involved in importing metabolites required for RNA transcription and / or exporting the nascent RNA transcripts for subsequent viral replication processes. The outer shell of rotaviruses is clearly visible under the electron microscope and confers on the particle the appearance of a wheel, hence the name (from Latin "rota" = wheel).

The particle structures of two phytoreoviruses, WTV and RDV have been examined in some detail by electron microscopy and PAGE (Streissle and Granados, 1968; Uyeda and Shikata, 1982). The inner shells of RDV and WTV particles contain ISL subunits, and two different sized ISS subunits which are encoded either by one or two genomic segment(s) (Table 1.3). The outer shells are made from OSL and OSS subunits. In RDV it has been shown that the OSL and OSS subunits interact to form 180 morphological subunits which are arranged as hexamers and pentamers to give icosahedral shells (Uyeda and Shikata, 1982).
1.3.1.2 Type 2 reovirus particles

The double shells of type 2 reovirus particles contain six types of subunit arranged slightly differently from those in type 1 particles. The order of the proteins from the inside surface to the outside surface of the double shells is ISL, ISS, OSL and OSS. The particles contain 12 hollow spikes, made of SP subunits, capped by trimers of SC subunits (Fig 1.1 & 1.2).

The particle structure of Orthoreovirus REO 3 (reovirus serotype 3) has been intensively studied (reviewed by Joklik, 1983). Its inner shell is composed of ISL and ISS subunits interacting in a one-to-two ratio (Joklik, 1983) to form morphological subunits 4 nm in diameter (Schiff and Fields, 1990). The icosahedral inner shell possesses 12 spikes situated at its vertices. The spikes are composed of five subunits of the SP protein (Ralph et al., 1980) and are about 10 nm in diameter. They each possess a central channel about 5 nm wide and project about 5.5 nm beyond the inner shell surface; this is about half the distance through the outer protein shell (Palmer and Martin, 1977). Each spike is capped with a trimer of SC subunits (Banerjea et al., 1988; Bassel-Duby et al., 1987). The structural arrangement of the outer surface of intact REO 3 particles has been difficult to identify, as there appears to be extensive intermeshing of protein subunits. The particle is a nearly spherical triangulated icosahedron with 12 vertices, which, when viewed along five-fold axes of symmetry exhibit 20 peripheral morphological subunits, while particles viewed along two- or
three-fold axes of symmetry exhibit 36 peripheral morphological subunits (Palmer and Martin, 1977). The outer shell has a skewed T=13 symmetry composed of 132 intermeshing morphological subunits (Khaustov et al., 1987) made from OSL and OSS subunits in a 1:1 ratio.

The particle structures of two fijiviruses, Fiji disease virus (FDV) and maize rough dwarf virus (MRDV), have been studied in some detail (Francki and Boccardo, 1983; Milne et al., 1973; Boccardo and Milne, 1975; Hatta and Francki, 1977) and they appear to be similar to that of REO 3. The arrangement of the OSL, OSS, SC and SP subunits are similar, however, the inner shell appears to be comprised of ISL alone (Boccardo and Milne, 1975). Based on these observations, two similar structural models of fijivirus particles have been proposed by Milne and Lovisolo (1977), and Hatta and Francki (1977).

1.3.2 Proteins within the inner shell of reovirus particles

PIRV particles, like animal reovirus particles, generally enclose two to three minor proteins within their inner shell. One of these is always a RNA dependent RNA polymerase (RDRP). Others have functions involved with replication (IR), RNA binding (RB) or functions yet to be determined. A summary of these proteins is shown in Table 1.4.

1.3.2.1 RNA dependent RNA polymerase
RNA dependent RNA polymerase activity has been detected in particles of various plant reovirus: WTV (Black and Knight, 1970; Reddy et al., 1977), RDV (Uyeda and Shikata, 1984), RGDV (Yokoyama et al., 1984), FDV (Ikegami and Francki, 1976), RRSV (Uyeda et al., 1987a; Lee et al., 1987; Lu et al., 1988) and RBSDV (Uyeda et al., 1987a). It has also been measured in particles of the animal reoviruses, REO 3 (Borsa and Graham, 1968; Shatkin and Sipe, 1968), ROTA-A (Cohen, 1977; Mason et al., 1980), and BTV (Van Dijk and Huismans, 1980; Verwoerd and Huismans, 1972).

The enzyme is a minor protein component of the reovirus particle and has a Mr of 120-164 kDa. In WTV, RDV, REO 3, BTV and ROTA-A it is encoded by genomic segment 1 (Suzuki et al., 1992b; Wiener and Joklik, 1989; Roy, 1991; Mitchell and Both, 1990). Nucleotide sequence analysis shows that reovirus RDRPs have the GDD motif found in all known viral RDRPs (Koonin, 1991).

In addition, Loudon and Roy (1991) have suggested that the BTV RDRP protein has both enzymatic and structural roles.

### 1.3.2.2 Other minor packaged proteins

WTV and RDV each possess a ~ 55 kDa protein that is enclosed within the inner shell. While the function of these proteins is unknown, similar proteins of animal reoviruses (Table 1.4) are involved with viral RNA replication and stability (IR proteins), and some have a RNA binding capacity (RB proteins). The BTV IR protein is a viral capping and
Table 1.4 Coding assignment of minor proteins packaged within the inner particle shells of the Reoviridae

<table>
<thead>
<tr>
<th>Reovirus</th>
<th>Protein</th>
<th>VP</th>
<th>VP</th>
<th>VP</th>
<th>RDRP</th>
<th>IR</th>
<th>RB</th>
<th>PU</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV</td>
<td></td>
<td>VP1</td>
<td>VP4</td>
<td>VP6</td>
<td>L1</td>
<td>M4</td>
<td>S9</td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>150</td>
<td>76</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>L1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REO 3</td>
<td>protein</td>
<td>VP1</td>
<td>VP3</td>
<td></td>
<td>L1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>142</td>
<td>83</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>L1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROTA-A</td>
<td>protein</td>
<td>VP1</td>
<td>VP3</td>
<td></td>
<td>S1</td>
<td>S3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>125</td>
<td>98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>S1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WTV</td>
<td>protein</td>
<td>P1</td>
<td></td>
<td>P6</td>
<td>S1</td>
<td>S7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>(155)</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>S1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDV</td>
<td>protein</td>
<td>P1</td>
<td></td>
<td>P7</td>
<td>S1</td>
<td>S7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>164</td>
<td>55</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>S1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes: RDRP: RNA dependent RNA polymerase. IR: involving replication. RB: RNA binding protein. PU: the function of these proteins remains unknown. Number in bracket: protein size was estimated in SDS-PAGE gel.
methylation enzyme (Roy et al., 1990; Roy, 1992), whilst ROTA-A IR protein may be involved in RNA replication (Estes and Cohen, 1989). The BTV RB protein is a component of the mRNA polymerase complex (it may be a helicase and involved in separating template dsRNA molecules for the viral RDRP to transcribe), and is capable of binding virion double-strand RNA, single-strand RNA transcripts, and other nucleic acid species (Roy et al., 1990; Roy, 1992).

**1.3.3 Nonstructural proteins**

Plant reoviruses, such as WTV and RDV, encode 5 or 6 different nonstructural proteins (NSP), whereas animal reoviruses encode 3 or 4 nonstructural proteins. These proteins are present in the cytoplasm of infected cells but not in the viral particles (Cross and Fields, 1976). The functions of most of the plant reovirus nonstructural proteins (NSP) are unknown, however, some of them may have similar functions to those of animal reoviruses. NSP from the latter have been classified into four types: those that have a RNA binding capacity, those that encode microtubules, those associated with viral replication and those associated with the release of virus particles from cells (Table 1.5).

**1.3.3.1 Nonstructural proteins with RNA-binding activity**

Nonstructural proteins with RNA-binding activity (NSRB proteins) of REO 3 have a strong affinity for ssRNA (Huimans and Joklik, 1976), and
may interact with specific regions of reovirus mRNA (Stamatos and Gomatos, 1982). These regions appear to be unique for members of each size class of viral ssRNA. Similarly, one of the two NSRB proteins of ROTA-A has been shown to specifically bind to the conserved terminal sequences (---UGUA---) of the positive sense strand ssRNA of all the ROTA-A genomic segments. This suggests that the function of NSRB proteins is to condense the full complement of viral ssRNAs into precursor subviral particles in preparation for dsRNA synthesis (Gomatos et al., 1981; Huismans and Joklik, 1976). They may also play several other different roles, such as protecting viral mRNA from degradation, regulating translation, recognising and transporting viral mRNAs from the transcribing particles to the site of replication; and discriminating between the rotavirus mRNAs and cellular RNAs (Poncet et al., 1993).

RNA-binding activity has been predicted for a NSP (Pns4) of RDV and WTV by protein sequence analysis (Uyeda et al., 1990a). A ‘zinc-finger’ motif is present in both the WTV and RDV proteins, and there is a purine NTP-binding motif in the RDV NSRB.

1.3.3.2 Nonstructural proteins forming tubules or associating with cytoskeletons

Proteins in this class (NSTC; see Table 1.5) are either tubule subunits or associated in some way with the cytoskeleton of the cell. The REO 3 NSTC protein is associated with the cytoskeleton of infected cells in
Table 1.5 Coding assignment of nonstructural proteins of Reoviridae

<table>
<thead>
<tr>
<th>Protein</th>
<th>NSRB</th>
<th>NSTC</th>
<th>NSRP</th>
<th>NSVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>REO 3</td>
<td>σNS</td>
<td>μNS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>size kDa</td>
<td>41</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>segment</td>
<td>S3</td>
<td>M3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BTV</td>
<td>NS2</td>
<td>NS1</td>
<td>-</td>
<td>NS3/NS3A</td>
</tr>
<tr>
<td>size kDa</td>
<td>41</td>
<td>64</td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td>segment</td>
<td>S8</td>
<td>M6</td>
<td>-</td>
<td>S10</td>
</tr>
<tr>
<td>ROTA</td>
<td>NSP1/</td>
<td>-</td>
<td>NSP2/NSP3</td>
<td>NSP4</td>
</tr>
<tr>
<td>-A</td>
<td>NSP3</td>
<td>/NSP5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>59/36</td>
<td>-</td>
<td>36 / 36 / 21</td>
<td>20</td>
</tr>
<tr>
<td>segment</td>
<td>S5 /S7</td>
<td>-</td>
<td>S8/S7/S11</td>
<td>S10</td>
</tr>
<tr>
<td>WTV</td>
<td>Pns4</td>
<td>Pns7/ Pns10/Pns11/Pns12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>81</td>
<td>59 /39</td>
<td>/39 /39 /19</td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>S4</td>
<td>S6 /S9</td>
<td>/S10 /S12</td>
<td></td>
</tr>
<tr>
<td>RDV</td>
<td>Pns4</td>
<td>Pns6/ Pns9/Pns10/Pns11/Pns12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>size kDa</td>
<td>80</td>
<td>58 /39</td>
<td>/39 /33 /21</td>
<td></td>
</tr>
<tr>
<td>segment</td>
<td>S4</td>
<td>S6 /S9</td>
<td>/S10 /S12 /S11</td>
<td></td>
</tr>
</tbody>
</table>

Notes: NSRB: nonstructural proteins with RNA binding activity. NSRB1 for ROTA-A NSP1 and NSRB2 for NSP3. NSTC: nonstructural proteins forming tubule or associating with cytoskeleton. NSRP: nonstructural proteins associated with replication. NSRP1 for ROTA-A NSP2, NSRP2 for NSP3 and NSRP3 for NSP5. NSVR: nonstructural proteins associated with viral release. The functions remains unknown of the proteins in italics.
particular spatial and temporal patterns, and may serve to anchor to the cell
matrix the viral structures involved in genome synthesis and assembly (Mora
et al., 1987).

The BTV NSTC is a major nonstructural protein and assembles to
form tubules in multimeric forms (Urakawa and Roy, 1988). It has been
postulated these tubules may be involved in the movement of virus particles
through plasmodesmata (Monastyryskaya et al., 1994).

1.3.3.3 Nonstructural proteins associated with replication

ROTA-A encodes three NSRP proteins which I have termed NSRP1
(NSP2), NSRP2 (NSP3; note dual function) and NSRP3 (NSP5). ROTA-A
NSRP2 has consistently been found in complexes isolated from infected
cells that contain RDRP activity and in complexes formed in vitro in
association with NSRB1 (Estes and Cohen, 1989). These data suggested
that NSRP2 may be a component of the viral RDRP (Helmberger-Jones and

ROTA-A NSRP1 is a basic protein and associates with viroplasms in
infected cells (Petrie et al., 1984). It may be involved in the replication of
RNA or packaging of ssRNA into subviral particles.

ROTA-A NSRP3 is a minor neutralizing antigen (Matsuno et al.,
1980) and may be also involved in the replication of RNA.

1.3.3.4 Nonstructural proteins associated with viral release
The ROTA-A nonstructural protein associated with viral release (NSVR) is synthesized as a primary translation product (M, 20 kDa) which is co-translationally glycosylated (Ericson et al., 1982; Kabcenell and Atkinson, 1985). The mature glycoprotein is an integral membrane protein of the endoplasmic reticulum (ER). The cytoplasmic domain of NSP4 has been proposed to be involved in the morphogenesis of virus particles that mature by budding through the membrane of the ER (Ericson et al., 1983; Kabcenell et al., 1988).

BTV NSVRs are associated with intracellular, smooth-surfaced vesicles and the plasma membrane. These proteins may be involved in the final stages of BTV morphogenesis, i.e. the release of BTV from infected cells. BTV NSVR may share some functional similarities with the rotavirus glycoprotein NSVR (Roy, 1992).

**1.3.3.5 Minor nonstructural protein**

The REO 3 σ1s protein is encoded by the second open reading frame in the S1 gene. This basic protein accumulates in the cytoplasm of infected cells, but its function remains unknown.

**1.3.4 Genome structure**

Both plant- and animal-infecting reoviruses have genomes comprised of 10 to 12 segments of dsRNA (Table 1.1). Each virus particle is believed to contain one copy of each dsRNA segment. The dsRNA is tightly
packed together in the centre of the core (Lufitig et al., 1972; Harvey et al., 1981) with adjacent stretches of helix running locally parallel to one another and ordered in a hexagonal arrangement (Harvey et al., 1981). Each segment usually has a single open reading frame (ORF) and encodes a single protein, but there are exceptions, e.g. the segment 6 of MRDV and the segment 7 of RBSVD encode 2 proteins respectively (Marzachi et al., 1991; Azuhata et al., 1993). The nucleotide sequences of the genomes of two plant reovirus, WTV (S4-S12) and RDV (S1-S12) as well as three animal reoviruses REO 3 (S1-S10), BTV (S1-S10) and ROTA-A (S1-S11) have been determined (Nuss and Dall, 1990; Uyeda et al., 1995; Schiff and Fields, 1990; Roy, 1991; Bellamy and Both, 1990; Mitchell and Both, 1990).

The dsRNA segments have several shared features:

1. The 5' and 3' terminal sequences are conserved within each genus (Table 1.6).

2. There are segment-specific inverted repeat sequences adjacent to the terminally conserved nucleotide sequences (Uyeda et al., 1995; Schiff and Fields, 1990; Roy, 1991; Bellamy and Both, 1990; Mitchell and Both, 1990). These strongly conserved terminal sequences and segment-specific inverted repeat sequences in genome segments contain signals important for transcription, replication, or assembly of the viral genome segments (Nuss and Dall, 1990; Dall et al., 1990).
Table 1.6 Terminally conserved nucleotide sequences of Reoviridae

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phytoreovirus</strong></td>
<td></td>
</tr>
<tr>
<td>WTV (S1-S12)</td>
<td>5′-GG U A U U-----U GAU-3′</td>
</tr>
<tr>
<td>RDV (S1-S12)</td>
<td>5′-G/GC/UA A A-----U/C/GAU-3′</td>
</tr>
<tr>
<td>RGDV (S1-S12)</td>
<td>5′-G/GC/UAU/AU-----U GAU-3′</td>
</tr>
<tr>
<td><strong>Fijivirus</strong></td>
<td></td>
</tr>
<tr>
<td>RBSDV (S1-S10)</td>
<td>5′-AAGUUUUU---------GUC-3′</td>
</tr>
<tr>
<td>MRDV (S6, S7, S8)</td>
<td>5′-AAGUUUUU---------UGUC-3′</td>
</tr>
<tr>
<td><strong>Oryzavirus</strong></td>
<td></td>
</tr>
<tr>
<td>RRSV (S1-S10)</td>
<td>5′-G/GAUA---GUGC-3′</td>
</tr>
<tr>
<td>ERSV (S1-S10)</td>
<td>5′-GGAUA---GUGC-3′</td>
</tr>
<tr>
<td><strong>Orthoreovirus</strong></td>
<td></td>
</tr>
<tr>
<td>REO 3 (S1-S10)</td>
<td>5′-GCUA---------UCAUC-3′</td>
</tr>
<tr>
<td><strong>Orbivirus</strong></td>
<td></td>
</tr>
<tr>
<td>BTV serotype 10 (S1-10)</td>
<td>5′-G/GUAUA---ACUUAC-3′</td>
</tr>
<tr>
<td><strong>Rotavirus</strong></td>
<td></td>
</tr>
<tr>
<td>ROTA-A (S1 - S11)</td>
<td>5′-G/GC/GUA ---G/AA/GCC-3′</td>
</tr>
<tr>
<td>ROTA-B (S5, S6, S9)</td>
<td>5′-G/GU/CU/A -----A CC CC-3′</td>
</tr>
</tbody>
</table>

Notes: RGDV: rice gall dwarf virus; RBSDV: rice black-streaked dwarf virus; MRDV: maize rough dwarf virus; ERSV: Echinocloa ragged stunt virus.

Antczak et al., 1982; Asamizu et al., 1985; Azuhata et al., 1992; Estes and Cohen, 1989; Kudo et al., 1991; Marzachi et al., 1991; Roy, 1991; Yan et al., 1992, 1994;
3. The segments contain a short 5' non-coding region and a long 3' non-coding region.

4. The positive sense strands of dsRNA segments of REO 3, ROTA-A, and cypoviruses contain 5' capped termini (Sciff and Fields, 1990). This is probably a general feature of reoviruses but it has not been demonstrated that such capping occurs in any plant reovirus (Nuss and Dall, 1990).

In addition the genes carried by these segments also have certain conserved features:

5. The first initiation codon is usually a strong initiation codon based on Kozak's rules (Kozak, 1981, 1986a, 1986b, 1987).

6. Polyadenylation sequences or signals are not found at the 3' end of reovirus genes.

1.4 Replication

PIRVs replicate in both their plant and insect vector hosts. The replication of PIRVs, particularly WTV and RDV, has been studied in both insects and insect-cell monolayers (Sinha, 1968; Black, 1979). The multiplication of WTV in its vector was first demonstrated by injecting leafhoppers with virus particles and observing an increase in virus concentration with time (Whitcomb and Black, 1961). After feeding leafhoppers on infected plants, the virus takes about 17 days to spread from the intestinal filter chamber to the salivary gland, and several other organs, such as the fat bodies and the brain (Sinha, 1965). Fijiviruses replicate in
most tissues of their planthopper vectors and FDV particles form crystalline aggregates in insect vector cells (Francki and Grivell, 1972).

The adsorption of RDV on the surface of insect vector cell monolayers requires an optimal virus concentration and optimal inoculation period (Kimura, 1986). The viruses probably enter the cells by phagocytosis (Kimura, 1986). WTV can multiply in cell lines derived from vectors and non-vectors (Chiu and Black, 1967; Hirumi and Maramorosch, 1967). Twelve WTV-specific polypeptides have been detected in vector-cell monolayer culture, corresponding to those encoded by the genomic RNA (Nuss and Peterson, 1980). Using this system seven polypeptides were identified as virus particle proteins; the roles of 5 or 6 non-particle proteins remain to be elucidated. Although viroplasm and virus particles are clearly evident in the cytoplasm of WTV infected vector cells, the infection appears not to affect the synthesis of normal cellular proteins in vector-cell monolayers (Black, 1979).

Plant reoviruses also replicate in plant cells frequently causing galls in the infected plant. All the plant-infecting reoviruses are transmitted by their insect vectors into the phloem tissue where virus replication occurs. As with infection of insect cells, viroplasms consist mainly of proteins, RNA and subviral particles (Milne and Lovisolo, 1977; Shikata, 1977, 1981; Giannotti and Monsarrat, 1968; Hatta and Francki, 1981). These viroplasms are considered to play a central role in virus synthesis and assembly (Favali et al., 1974; Favali and Lotti, 1981; Hatta and Francki, 1981). Intact virus
particles are only found in viroplasms and in the cytoplasm, often associated with tubules and helical filaments.

Although considerable effort has been made to understand the replication of plant reoviruses, many details of their replication in both insect and plant cells remain unsolved. Therefore, it may be helpful to review the replication of some animal reoviruses to clarify the possible nature of the plant reovirus replication.

Most information about the animal reovirus replication cycle is based on studies of REO 3, BTV and ROTA-A (Estes, 1990; Gorman et al., 1983; Schiff and Fields, 1990).

The replication cycle of a reovirus is depicted in Fig. 1.3 and is based on the replication cycles of REO 3 and ROTA-A. This replication cycle can be divided into several stages: adsorption, penetration, uncoating, transcription, replication, assembly and virus release.

1.4.1 Adsorption

Only double-shelled particles of ROTA-A attach to cells (Petrie et al., 1981; Elias, 1977). Virus attachment occurs via SC (REO 3) or OSS (ROTA-A) proteins (Fukuhara et al., 1988; Matsuno and Inouye, 1983; Sabara et al., 1985) and does not require cleavage of the outer capsid proteins (Petrie et al., 1983). Glycophorin A on the surface of the cell is the reovirus receptor (Paul and Lee, 1987), however, the rotavirus receptor is not known.
Fig. 1.3 Diagram of the reovirus replication cycle. It is based on two animal reoviruses, REO 3 and ROTA-A replication cycles. The replication cycle is composed of adsorption; penetration; uncoating; transcription of parental genome; capping of transcripts; assembly of the RIPs (replication intermediate particle) including precore RIP, core RIP, single shell RIP; replication; translation; morphogenesis and release. Two penetration pathways were shown: penetration by intact particle and by core particle. Core particles: viruses have lost the outer shell proteins.
Reovirus Replication Cycle

- Direct penetration by core particle
- Cell
  - Adsorption and penetration by intact virus particle
  - Release
  - Morphogenesis
    - Assembly of RIP
    - Replication and translation
    - Capping of transcripts
  - Uncoating
  - Transcription of parental genome
  - Lysosome
  - Vacuole
1.4.2 Penetration

The penetration of reovirus and rotavirus particles into the host cell has been studied primarily by electron microscopy (Silverstein and Dales, 1968; Sturzenbecker et al., 1987). Some of the double shelled particles adsorbing to the outer surface of the cell membrane penetrate as intact particles while others disassemble into core particles which have lost the outer shell proteins (Borsa et al., 1979). The intact virus particles are taken up by phagocytosis, in some cases by receptor-mediated endocytosis (Ludert et al., 1987) and the phagocytic vacuoles migrate toward the centre of the cell where they fuse with lysosomes (Silverstein and Dales, 1968; Sturzenbecker et al., 1987; Suzuki et al., 1985). The core particles appear to be aided in their penetration of the cell membrane by a protein fragment cleaved from the OSL during disassembly (Nibert and Fields, 1992).

1.4.3 Uncoating

Uncoating of reovirus double shelled particles occurs within lysosomes (Silverstein and Dales, 1968; Sturzenbecker et al., 1987). It involves proteolytic digestion of OSL protein (Borsa et al., 1981, Choi and Lee, 1988; Silverstein and Dales, 1968; Crowell and Lonberg-Holm, 1986) and requires a low pH. This represents an essential step in the reovirus infection cycle (Canning and Fields, 1983; Maratos-Flier et al., 1986; Sturzenbecker et al., 1987).
1.4.4 Transcription of the parental genome

Viral cores escape from lysosomes possibly via a non-receptor-mediated pathway (Borsa et al., 1979; Silverstein et al., 1976). Removal of the outer shell activates the viral RDRP which then transcribes the viral genome within the core particles (Banerjee and Shatkin, 1970; Borsa et al., 1974a, 1974b; Chang and Zweerink, 1971; Silverstein et al., 1968, 1972; Skehell and Joklik, 1969).

Reovirus transcription is conservative. Only the negative sense strand of each genome dsRNA segment acts as a template, so that all the transcripts are positive sense strand (Banerjee and Shatkin, 1970; Levin et al., 1970; Skehell and Joklik, 1969); they are derived from the full length of the genomic RNA segments (Skehell and Joklik, 1969). The catalytic sites of the viral RDRP lie within the viral core (Joklik, 1970). After transcription, the genomic dsRNA remains within the viral core, however, the completed transcripts are extruded from the core through a channel in the SP protein (Bartlett et al., 1974; Gillies et al., 1971). These transcripts are then capped (Faust et al., 1975; Furuichi et al., 1975a, 1975b, 1976, 1977a, 1977b; Shatkin and Kozak, 1983; Yamakawa et al., 1982) and serve as mRNAs for protein synthesis and / or as templates for negative sense strand synthesis (Furuichi et al., 1975b).

The mRNAs encoding the RDRP, NSTC, NSRB and OSS are produced first (Lau et al., 1975; Nonoyama et al., 1974; Watanabe et al., 1968). A host-cell repressor protein induced by one of these four “early”
proteins may regulate the transcription of the six “late genes” (see section 1.4.6; Lau et al., 1975; Nonoyama et al., 1974; Zarbl and Millward, 1983). The number of copies of each transcript from the 10 dsRNA varies, and transcription frequency is generally inversely related to gene size (Joklik, 1981; Zweerink and Joklik, 1970).

Very soon after the positive sense strand reovirus RNA molecules are transcribed they become associated with viral proteins. These are the NSRBs, NSTC, and OSS proteins (Antczak and Joklik, 1992). The conserved 3' ssRNA terminal sequences are bound to the NSRBs (Poncet et al., 1993).

1.4.5 Replication of viral dsRNA

Characterization of the replicase particles has shown that a series of replication intermediate particles exist. This morphogenetic pathway starts with a precore replication intermediate particle (PRIP) which becomes a core replication intermediate particle (CRIP) and then a single-shelled replication intermediate particle (SRIP) (Gallegos and Patton, 1989; Patton and Gallegos, 1988, 1990).

The rotavirus PRIP contains the viral positive sense ssRNA associated with the structural proteins, RDRP and IR (Table 1.4) together with four nonstructural proteins (NSRB and NSRP; see Table 1.5) and is transformed into a CRIP particle by addition of ISL protein and loss of NSRB1 protein. The CRIP particle subsequently becomes a SRIP particle by
further addition of ISS and removal of the remaining non-structural proteins (Gallegos and Patton, 1989; Patton and Gallegos, 1988, 1990). The RDRP within the SRIP then synthesizes the negative sense strand RNA using the packaged positive sense strand RNA as template (Acs et al., 1971; Sakuma and Watanabe, 1972; Schonberg et al., 1971). Such particles are now capable of synthesising mRNA and are termed transcriptase particles (Morgan & Zweerink, 1977).

Segment assortment, the process by which each PRIP (and ultimately the mature particle) packages a single copy of each dsRNA segment, may occur at the level of positive sense strand ssRNA, because free negative sense strand ssRNA and free dsRNA are never found in infected cells. It has been suggested, and there is now some supporting evidence (Mattion et al., 1992; Poncet et al., 1993), that nonstructural protein NSRB, an ssRNA-binding protein, may play a role in this process (Gomato et al., 1964; Huismans and Joklik, 1976). Conserved terminal sequences and segment-specific inverted repeats located adjacent to the terminally conserved oligonucleotides have been observed in all reported reovirus segment sequences (Table 1.6, Antczak et al., 1982; Gaillard et al., 1982; Anzola et al., 1987, Nuss and Dall, 1990; Uyeda et al., 1995). The conserved terminal sequences may be used by the virus to distinguish viral RNA from host RNA, and the segment-specific terminal inverted repeat sequences used for the recognition of individual segments (Anzola et al., 1987).
The signals necessary for the synthesis of the negative sense strand viral RNA appear to be located within the 200 bp regions adjacent to the 5' and 3' termini of the positive sense strand RNA (Anzola et al., 1987; Zou and Brown, 1992; Xu et al., 1989; Dall et al., 1990; Gorziglia and Collins, 1992; Chen et al., 1994). Synthesis of the negative sense strand proceeds in the 5' to 3' direction from a single initiation point (Sakuma and Watanabe, 1972). Once the complementary negative sense strand is synthesized, it remains associated with the positive sense strand (Sakuma and Watanabe, 1972). Within the progeny particle the RDRP thus catalyzes only a single round of negative sense strand synthesis (Zweerink, 1974).

1.4.6 Transcription from the progeny dsRNA genome

The “late” viral mRNAs which encode proteins including the OSL, OSS and NSVR appear to be produced by the progeny particles (also termed transcriptase particles - see section 1.4.5; Kudo and Graham, 1966; Watanabe et al., 1967, 1968). There are two possible fates for these particles. They may either be coated with OSL and OSS to form intact particles or they remain uncoated and act as parental particles for PRIP / CRIP / SRIP cycle (Morgan and Zweerink, 1977).

1.4.7 Translation of Reovirus mRNA

Shortly after the viral infection, there is a gradual decrease in host-cell protein synthesis and a gradual increase in viral protein synthesis.
(Ensminger and Tamm, 1969; Gomatos and Tamm, 1963). Most of the proteins synthesized in infected cells are viral proteins (Zweerink and Joklik, 1970). A number of factors regulate the levels of the different viral proteins synthesized. These include the variability in mRNA levels resulting from different rates of transcription of the dsRNA segments (Joklik, 1981; Detjen et al., 1982; Lau et al., 1975; Zweerink and Joklik, 1970) and the variability in the translation frequency of the different mRNAs (Joklik, 1981). As with all genes, the efficiency of translation of reovirus genes is regulated by the nucleotide sequences of the mRNAs at the -3 and +4 positions flanking the initiator AUG codon (Kozak, 1981; Roner et al., 1989). They are also regulated by other nucleotide sequences (Munemitsu and Samuel, 1988; Roner et al., 1989; Belli and Samuel, 1993). For example, it has been found for S1 RNA of REO 3 that the region between two initiation codons, AUG14 and AUG75, plays a major role in determining the relative efficiency of synthesis of two proteins from the reovirus bicistronic S1 mRNA (Belli and Samuel, 1993).

Most rotavirus structural proteins, and all nonstructural proteins, are synthesized on the free ribosomes. The glycoproteins OSS and NSVR are synthesized on ribosomes associated with the membrane of the ER, and they are cotranslationally inserted into ER membrane, a process that is directed by signal sequences at their amino termini (Petrie et al., 1982; Richardson et al., 1986; Soler et al., 1982).
1.4.8 Assembly and release

Details of reovirus assembly and subsequent release from host cells remain essentially unknown. The structural polypeptides can self-assemble to form the viral core and outer capsid. This assembly can be independent of the three minor core proteins, the dsRNA genome and the four nonstructural proteins (Roy, 1992, 1993; Tanaka and Roy, 1994). Subviral rotavirus particles form within viroplasms, and mature by budding through the ER membrane. In this process, particles acquire their outer capsid proteins (Estes, 1990). The release of viral particles from infected cells may result from virus-induced cytolysis and drastic alterations in the permeability of the plasma membrane (Estes, 1990).

1.5 Conclusion

Plant- and animal-infecting reoviruses share many attributes. They have complex particles, complex genome structures and complex life cycles. Reoviruses replicate within sub-particles and seem to require little from their hosts except for nucleotides, amino acids and protein synthesis machinery. It is probably this independence that allows a PIRV to replicate in both plants and insects.

The reovirus sub-particle is like a cell in which the virus resides separate from the rest of the cell. The virus transfers copies of its genome into pre-prepared sub-particles and dons another shell before spreading outside the cell. This minimal interaction of the virus with host proteins
makes the prospect of engineering synthetic resistance to the virus more challenging than achieving the same outcome for other plant-infecting viruses. However, the complexity of the reovirus life cycle may have fortuitously produced stages at which the virus is vulnerable. The wealth of knowledge gained from the study of animal-infecting reoviruses may help me to identify and exploit such chinks in the virus’ armour.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains and media

Bacterial strains used in this study are described and listed in Table 2.1. *Escherichia coli* strains were grown on LB agar medium with selection at 37°C and stored at 4°C. For long term storage, liquid cultures were grown in 2x YT liquid medium with selection to stationary phase, mixed with an equal volume of 50% (v/v) glycerol and stored at -70°C.

The compositions of bacterial growth media used are listed below. Reagents are for 1 litre and solid media contained 1.5% agar (Difco). Antibiotics were filter sterilised (0.22 μM, Millipore) whenever required. Ampicillin or kanamycin were used in selective media at 50 mg / L.

Bacterial culture media

**LB (Luria-Bertain) medium (Miller, 1972)**

- bacto-tryptone: 10 g
- bacto-yeast extract: 5 g
- NaCl: 5 g
- pH: 7.0
Table 2.1 *Escherichia coli* strains used in this study

<table>
<thead>
<tr>
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<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>AD202</td>
<td>F-, <em>ompT hsdSB</em> (rB- mB-; an <em>E. coli</em> B strain)</td>
<td>Nakano <em>et al.</em>, 1993</td>
</tr>
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<td>BL21</td>
<td>F-, <em>ompT: Tn5 hsdSB</em> (rB- mB-; an <em>E. coli</em> B strain)</td>
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<tr>
<td>JM109</td>
<td><em>F' traΔ36 lacIq Δ(lacZ)M15 proAB / recA1 endA1 gyrA96</em> (Nalr) <em>thi</em></td>
<td>Yanisch-Perron <em>et al.</em>, 1985</td>
</tr>
<tr>
<td></td>
<td><em>hsdR17</em> (rK-mK+) <em>supE44 relA1</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Δ(lac-proAB)</td>
<td></td>
</tr>
<tr>
<td>JPA 101</td>
<td><em>F' traΔ36 lacIq Δ(lacZ)M15 proAB</em></td>
<td>J. P. Adelman, unpublished</td>
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<td></td>
<td>Δ(lac, proAB) <em>thi supE44 recA</em></td>
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<td></td>
<td><em>TcR(:Tn10)</em></td>
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</tbody>
</table>
2 x YT medium (Sambrook et al., 1989)

- bacto-tryptone: 16 g
- bacto-yeast extract: 10 g
- NaCl: 5 g
- pH: 7.0

SOB medium (Hanahan, 1985)

- bacto-tryptone: 20 g
- bacto-yeast extract: 5 g
- 5 M NaCl stock: 2 mL
- 1 M KCl stock: 2.5 mL
- 2 M MgCl₂ stock: 5 mL
- 1 M MgSO₄ stock: 10 mL
- pH: 7.0

SOBG medium (Hanahan, 1985)

- SOB medium: 1 L
- 1 M Glucose stock: 10 mL

2.1.2 Virus and insects

Rice plants infected with a Thai isolate of rice ragged stunt virus were kindly provided by Dr. Wichai Kosiritatana (Kasetsart University, Thailand). This isolate was maintained and propagated in rice plants.
(cultivar TN1) in the glasshouse at Kasetsart University, Thailand, by transmitting the virus using viruliferous brown planthoppers *Nilaparvata lugens* as reported by Hibino and Kimura (1982). Freeze-dried infected and healthy brown planthoppers (*Nilaparvata lugens*) were gifts from Dr. Zuxun Gong (Shanghai Institute of Biochemistry, Academica Sinica, China).

### 2.1.3 Plasmids

Plasmid and recombinant plasmids, including those containing viral fragments of RRSV S5, constructed or used in the course of this study, are listed in Table 2.2 to 2.7.

### 2.1.4 Oligodeoxynucleotides

Two M13 sequencing primers were used for sequencing of cDNAs of RRSV S5 cloned in pUC119. The 17-mer Sequencing Primer (-20) #1211 (5' GTAAAACGACGGCCAGT 3') and 24-mer Reverse Sequencing Primer (-48) #1233 (5' AGCGGATAACAATTTCACAGGA 3') were purchased from New England Biolabs.

The oligodeoxynucleotide primers shown below were synthesized in our laboratory by Ms. Lynda Graf and Dr. Paul Keese using the phosphoramidite method (Beaucage and Caruthers, 1981) using a 394 DNA / RNA Synthesizer (Applied Biosystems). Four oligodeoxynucleotide primers were used for cloning the end sequences of RRSV S5. A 20 mer primer S55R (5' CGTGGAGGCTTG ACTCGTCG 3') and a 15 mer primer S55S (5'
Table 2.2 Plasmid vectors used in this study

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<th>Plasmids</th>
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<th>Features</th>
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<td>pUC119</td>
<td>JPA101,</td>
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<td>JM109</td>
<td>having sites for EcoRI, Sacl, Kpnl,</td>
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<td>Smal, Xmal, BamHI, Xbal, SalI,</td>
<td>1987</td>
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<td>Acel, HindII, PstI, SphI, HindIII</td>
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<td>having sites for EcoRI, Sacl, Kpnl,</td>
<td>1988</td>
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<td>Aval, Smal, BamHI, Xbal, SalI,</td>
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<td></td>
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<td>Acel, HindII, PstI, SphI, HindIII</td>
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<td>Promega</td>
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<td>SalI, Xhol, BamHI, Apal, Xbal, NotI,</td>
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<td>NsiI, HindIII, SphI, HindIII</td>
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<td>Short et al.,</td>
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<td>having sites for Kpnl, Apal, DralI,</td>
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<td>Xhol, SalI, AclI, HindII, ClaI, HindIII,</td>
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<td>EcoRV, EcoRI, PstI, Smal, BamHI,</td>
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<td>SpeI, Xbal, NotI, EagI, BstXI,</td>
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Table 2.3 *uidA (gus)* gene constructs used in this study

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<td>pRolC-gus</td>
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<td><em>E. coli uidA</em> gene</td>
<td>M. Graham, unpublished</td>
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<tr>
<td>Acl-gus</td>
<td>DD54 pUC118</td>
<td><em>E. coli uidA</em> gene, nopaline synthase terminator</td>
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Table 2.4 *hph* gene constructs used in this study

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<th>Reference</th>
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<td>p35S-&lt;i&gt;hph&lt;/i&gt;</td>
<td>pTra151</td>
<td>pUC12</td>
<td>CaMV 35S promoter, coding region of the hygromycin phosphotransferase gene (<em>hph</em> or <em>hpt</em> = &lt;i&gt;aphlV&lt;/i&gt;) from <em>E. coli</em>, nopaline synthase terminator</td>
<td>Zhang &lt;i&gt;et al.&lt;/i&gt;, 1991</td>
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<tr>
<td>pEmu-&lt;i&gt;hph&lt;/i&gt;</td>
<td>pEmuHPT</td>
<td>pUC118</td>
<td>Emu promoter, coding region of <em>hph</em> gene, nopaline synthase terminator</td>
<td>Chamberlain &lt;i&gt;et al.&lt;/i&gt;, 1994</td>
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<td>pUbi1-&lt;i&gt;hph&lt;/i&gt;</td>
<td>pLZUHN</td>
<td>pGEM-11Zf(-)</td>
<td>Ubi1 promoter, coding region of <em>hph</em> gene, nopaline synthase terminator</td>
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Table 2.5 *bar* gene constructs used in this study

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<td>pGEM-11Zf(-)</td>
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<td>pAct1-bar</td>
<td>pDM302</td>
<td>pSP72</td>
<td>Act1 promoter, coding region of <em>bar</em> gene, nopaline synthase terminator</td>
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Table 2.6 Recombinant plasmids constructed during this study

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<td>RR292</td>
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<td>RR299</td>
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<td>RR322</td>
<td>1828 (667-2495)</td>
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<td>RR433</td>
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<td>RR474</td>
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Note: Numbers in brackets show the position in RRSV S5 sequence.
Table 2.7 Fusion protein gene constructs based on RRSV S5

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<thead>
<tr>
<th>Constructs</th>
<th>Vector</th>
<th>Source of insert DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS5FL</td>
<td>pGEX-3X</td>
<td>sense sequence of RRSV S5 containing ORF</td>
</tr>
<tr>
<td>pS5FLa</td>
<td>pGEX-3X</td>
<td>antisense sequence of RRSV S5 containing ORF</td>
</tr>
<tr>
<td>pS5H1</td>
<td>pGEX-3X</td>
<td>sense sequence of RRSV S5 containing ORF with internal deletion of SnaBI and NruI fragment</td>
</tr>
<tr>
<td>pS5H2</td>
<td>pGEX-2T</td>
<td>antisense sequence of RRSV S5 containing partial ORF from clone RR541</td>
</tr>
<tr>
<td>pS5H1+2</td>
<td>pGEX-3X</td>
<td>sense sequence of RRSV S5 containing ORF with internal deletion of XbaI and SpeI fragment</td>
</tr>
<tr>
<td>pS5H1+3</td>
<td>pGEX-3X</td>
<td>sense sequence of RRSV S5 containing ORF deleted SnaBI and XbaI fragment</td>
</tr>
</tbody>
</table>
Table 2.8 Synthetic resistance gene constructs based on RRSV S5

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Vector</th>
<th>Source of insert DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>p35S-S5</td>
<td>pJ35SN</td>
<td>sense sequence of RRSV S5 containing ORF</td>
</tr>
<tr>
<td>p35S-S5a</td>
<td>pJ35SN</td>
<td>antisense sequence of RRSV S5 containing ORF</td>
</tr>
<tr>
<td>pRolC-S5</td>
<td>pRolCcas</td>
<td>sense sequence of RRSV S5 containing ORF</td>
</tr>
<tr>
<td>pRolC-S5a</td>
<td>pRolCcas</td>
<td>antisense sequence of RRSV S5 containing ORF</td>
</tr>
<tr>
<td>pUbi1-S5</td>
<td>pUbi1cas</td>
<td>sense sequence of RRSV S5 containing ORF</td>
</tr>
</tbody>
</table>
TTTTTTTTTGATAAA 3') were used for the 5' end sequence, and a 18 mer primer S53S (5' GTACGCTTTTG AACGCGC 3') and a 14 mer primer S53R (5' TTTTTTTTTGCACC 3') were used for synthesis of the 3' end sequence.

Three oligodeoxynucleotide primers were used to confirm the structure of constructs. The 17 mer pGEX primer (5' GCATGGCCTTTTGCGAG GG 3') was used to confirm that the fusion protein expression constructs based on pGEX contained RRSV S5 ORF. Two other oligodeoxynucleotide primers were used to confirm the structure of plant expression constructs. The 18 mer 35S primer (5' GGGATGACGCACAATCCC 3') was used for p35S-S5 and p35S-S5a constructs. The 22 mer Ubi1 primer (5' GGATGATGGCATATGCAGCAGC 3') was used for pUbi1-S5 clone.

Five oligodeoxynucleotide primers were used in PCR assays to confirm the transgenes in transgenic rice plants. The S55R primer and 35S primer, Ubi1 primer (see above) or 18 mer RolC primer (5' GATGCTTTCGAGTTATGGG 3') were used to conform p35S-S5, pUbi1-S5 or pRolC-S5 transgenes. The S53S primer and 35S primer or RolC primer were used to confirm p35S-S5a or pRolC-S5a transgenes. Two other oligodeoxynucleotide primers, 20 mer S5RTPF primer (5' TGGACAAGTTTCGCTGAG ATC 3') and 20 mer S5RTPR primer (5' CACATACTCTGGCAGCTCA 3'), were used in reverse transcription polymerase chain reaction (RT-PCR) to confirm RRSV S5 transgene in transgenic plants.

2.1.5 Plant material
The seeds of Indica rice cultivar *Chinsurah Boro II* used for rice tissue culture and transformation in this study were kindly provided by Dr F. J. Zapata, International Rice Research Institute (IRRI), Philippines.

### 2.1.6 Chemicals and reagents

- (amino-oxy) acetic acid (AOA): Sigma
- Acrylamide: Bio-Rad
- Adenosine triphosphate (ATP): Sigma
- Agarose and SeaPlaque (low melting point): Progen
- Ampicillin: Sigma
- BCIP (5-bromo-4-chloro-3-indolyl phosphate disodium salt): Sigma
- Bialophos: Meiji Seika Kaisha, Ltd., Japan
- Bis-acrylamide (N,N'-methylene-bis-acrylamide): Bio-Rad
- Cellulose phosphate: Sigma
- Cesium chloride: Boehringer Mannheim
- CTAB (cetyl-trimethylammonium bromide): B.D.H, Poole England
- Deoxynucleotide triphosphates: Boehringer Mannheim
- 2,4-dichlorophenoxyacetic acid (2,4-D): Sigma
- Dideoxynucleotide triphosphates: Boehringer Mannheim
- Dimethyl sulphate: Eastman-Kodak
- Dithiothreitol: Sigma
- DNA Tailing Kit: Boehringer Mannheim
DNA-molecular weight standard pUC19 DNA restricted with \textit{Hpall}:

- Bresatec
- dsDNA cycle sequencing system: BRL
- ethidium bromide: Sigma
- Ficoll 70000: Sigma
- formamide: BDH Chemicals
- GENECLEAN II Kit: BIO 101 Inc
- gold particles: Aldrich
- hygromycin: Boehringer Mannheim
- IPTG (Isopropyl-\textbeta-D-thiogalactopyranoside): Sigma
- kanamycin: Sigma
- kinetin (6-furfurylaminopurine): Sigma
- maltose: Sigma
- \textbeta-mercaptoethanol: Sigma
- MOPS (3-[N-morpholino]propanesulfonic acid): Sigma
- NAA (\textalpha-naphthalene acetic acid): Sigma
- NBT (nitro blue tetrazolium chloride): Sigma
- nitrocellulose (0.45 mm, BA85): Schleicher & Schull
- nylon filter (Hybond-N): Amersham
- oligo(dT)-cellulose: Pharmacia
- \([\alpha-^{32}\text{P}]\text{dATP}, [\gamma-^{32}\text{P}]\text{dATP}\) and \([\alpha-^{32}\text{P}]\text{dCTP}\) (3000 Ci / mM): New England Nuclear, Westwood, MA
- pGEM-T Vector System I: Promega
phenol: Wako
Phytogel: Sigma
Polaroid film (Type-667): Polaroid
polyvinyl-pyrrolidone 40,000 (PVP-40): Sigma
QIAGEN-tip 500: QIAGEN
Rainbow coloured molecular weight markers: Amersham
Rapid multiprimer DNA probe labelling kit: Amersham
Sarkosyl (N-lauroylsarcosine): Sigma
Sephadex (G-50 medium) (DEAE G-25): Pharmacia
SPP-1 Bacteriophage DNA restricted with EcoRI: Bresatec
Taq Dye Primer Cycle Sequencing Kit: Applied Biosystem
TEMED (N,N,N′,N′-tetramethylethylenediamine): Sigma
Tetracycline: Sigma
Triton X-100: Sigma
tRNA (from Escherichia coli): Sigma
tungsten particles: Rhone-Poulenc, France
urea: Rhone-Poulenc
Wizard™ PCR preps DNA purification system for rapid purification of DNA fragments: Promega
X-gal (5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside): Sigma
X-ray film (AR, or RP-5): Kodak
All other chemicals were of analytical grade.
2.1.7 Enzymes

All enzymes used in this study were of high purity, obtained from commercial sources, and used according to manufacturer’s instructions. Sources for enzymes are as follows:

*Ampli*Taq DNA polymerase: Perkin Elmer Cetus
calf intestinal alkaline phosphatase (CIP): New England Biolabs
cellulase RS: Yakult Honsha Co., Japan
DNA polymerase I (Klenow fragment): BRESA
Driselase: Sigma
Lysozyme: Sigma
M-MLV H- RT (Superscript): BRL
Pectolyase Y-23: Seishin Pharmaceutical Co., Tokyo, Japan
T4 Polynucleotide Kinase (PNK): New England Biolabs

restriction enzymes: Amersham, New England Biolabs, Boehringer Mannheim, BRL, Promega

ribonuclease A (RNase A): Sigma
ribonuclease H (RNase H): Sigma
T4 DNA ligase: New England Biolabs, Boehringer Mannheim

2.1.8 Buffers and dyes

The final concentrations of the ingredients of various reaction mixtures are listed below. Most of these buffers were prepared as stock
solutions at 10x their final concentrations. Restriction enzyme buffers and ligation buffers were stored in small aliquots at -20°C.

2.1.8.1 DNA buffers

AL-buffer

- 50 mM glucose
- 25 mM Tris-HCl, pH 8.0
- 10 mM EDTA, pH 8.0

Alkaline lysis buffer

- 0.2 N NaOH
- 1% SDS

3M / 5M KOAc buffer

- 5M potassium acetate
- glacial acetic acid
- H$_2$O

1% CTAB 100 mM

- 5% CTAB
- 5 M NaCl
- H$_2$O
CTAB isolation buffer

- Tris-HCl, pH 8.0: 100 mM
- NaCl: 1.4 M
- EDTA, pH 8.0: 20 mM
- CTAB: 2%
- β-mercaptoethanol: 0.2% (v/v)

1x CTAB extraction buffer

- Tris-HCl, pH 8.0: 50 mM
- NaCl: 0.7 M
- EDTA: 10 mM
- CTAB: 1%
- β-mercaptoethanol: 20 mM

1x CTAB precipitation buffer

- Tris-HCl, pH 8.0: 50 mM
- EDTA: 10 mM
- CTAB: 1%

20x SSC buffer

- NaCl: 175.3 g
- Tri-sodium citrate: 88.2 g
- H2O to 1 L
pH 7.0 (adjusted with 10 N NaOH)

Southern hybridization buffer

- Ficoll: 2 g
- BSA: 2 g
- Polyvinylpyrrolidone: 2 g
- 20% SDS: 5 mL
- *E. coli* tRNA (10 mg/mL): 2 mL
- herring sperm DNA (3 mg/mL): 6 mL
- 1 M HEPES, pH 7.0: 50 mL
- 20x SSC buffer: 150 mL

Volume made up to one litre

Khandjian’s hybridization solution (Khandjian, 1987)

- 1 M Tris-HCl, pH 7.5: 10 mL
- NaCl: 11.7 g
- formamide (de-ionized): 100 mL
- 100x Denhardt’s solution: 20 mL
- dextran sulfate: 20 g
- 20% SDS: 10 mL
- 10% sodium pyrophosphate (NaPPI): 2 mL
- 10 mg/mL herring sperm DNA: 2 mL
- H₂O to 200 mL
100x Denhardt’s solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll</td>
<td>2 g</td>
</tr>
<tr>
<td>PVP-40</td>
<td>2 g</td>
</tr>
<tr>
<td>BSA (Pentax Fraction V)</td>
<td>2 g</td>
</tr>
<tr>
<td>H₂O to</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

Preparation of Khandjian’s hybridization solution (200 mL)

Twenty g of Dextran sulfate, 10 mL of 1 M Tris-HCl, pH7.5 and 10 mL of water were put in a 50 mL beaker, mixed with a stirring rod until forming a homogeneous slurry, then heated slightly and carefully in a microwave to form the solution. The mixture was slowly added into 30 mL of boiling water in a beaker (boiled in microwave) while stirring. Overheating can cause dextran sulfate to caramelize. After slight cooling, de-ionized formamide was added. On further cooling NaCl, SDS and NaP Pi were added into the solution. Denhardt’s solution was then added. Herring sperm DNA was heated to 95°C for 10 minutes before adding to the solution. The solution was made up to a volume of 200 mL and stored at room temperature.

Formamide hybridization buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Phosphate buffer, pH 7.2</td>
<td>5 mL</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>1 mL</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>40 μL</td>
</tr>
</tbody>
</table>
herring sperm DNA (10 mg / mL) 200 µL
water 2.4 mL
SDS 1.4 g
formamide (de-ionized) 10 mL
H2O to 20 mL

TAE buffer
Tris 40 mM
sodium acetate (hydrated) 6 mM
EDTA 1 mM
pH (with acetic acid) 7.8

TBE buffer
Tris 50 mM
boric acid 42 mM
EDTA 1 mM
pH 8.3

TE (Tris-EDTA) buffer
Tris HCl, pH 8.0 10 mM
EDTA 1 mM

1/10 TE (Tris-EDTA) buffer
2.1.8.2 Buffers used for enzymatic reactions

Calf intestinal alkaline phosphate (CIP) buffer, T4 Polynucleotide Kinase buffer (PNK buffer) and restriction endonuclease buffers (NEBuffer) were purchased from New England Biolabs.

10x Ligation buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.8</td>
<td>500 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>100 mM</td>
</tr>
<tr>
<td>BSA</td>
<td>500 mg / mL</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>200 mM</td>
</tr>
<tr>
<td>ATP</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

Chemicals except ATP were made up in 90% volume. Ten % volume of 100 mM ATP was added into ligation buffer just before ligation reactions were assembled.
10x PCR reaction buffer

Tris-HCl, pH 8.3 500 mM
KCl 500 mM
MgCl₂ 40 mM
Dithiothreitol (DTT) 100 mM

Phosphate buffer

Na₂HPO₄ 100 mM, 500 mM or 1M
NaH₂PO₄ 100 mM, 500 mM or 1M

Mixed in required proportions (disodium : monosodium) to give a specified pH.

2.1.8.3 Dyes

SDS dye (5x)

Sucrose 25%
SDS 1%
EDTA 5 mM
Bromophenol blue 0.2%

TE buffer was added until SDS dye turns blue. Chemicals were dissolved by heating and stirring.

Sequencing stop dye

Formamide 95%
2.1.8.4 Buffers and dyes used in protein preparation and immunoblots

Coomassie Brilliant Blue

- Coomassie Brilliant Blue R250: 0.25 g
- Methanol: 45 mL
- \( \text{H}_2\text{O} \): 45 mL
- Glacial acetic acid: 10 mL

Solution was filtered through a Whatman No. 1 filter to remove any particulate matter.

Destaining solution

- Methanol: 45 mL
- \( \text{H}_2\text{O} \): 45 mL
- Glacial acetic acid: 10 mL

5x Tris-glycine electrophoresis buffer

- Tris base: 125 mM
- glycine: 1.25 M
- pH 8.3
Just before use, 200 mL of 5x buffer and 10 mL of 10% SDS stock were diluted to 1 L with water to obtain 1x buffer (25 mM Tris base, 250 mM glycine and 0.1% SDS).

2x SDS gel-loading buffer

- 1 M Tris-HCl, pH 6.8: 1 mL
- 10% SDS (electrophoresis grade): 4 mL
- bromophenol blue: 20 mg
- glycerol: 2 mL
- H$_2$O to: 8 mL

Dithiothreitol (200 mM) was added to buffer just before use, by adding 20% (v/v) of 1 M DTT. Two X SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature.

30% Acrylamide / Bisacrylamide

- Acrylamide: 29.2 g
- β'β'-Bis-methylene-acrylamide: 0.8 g
- H$_2$O to: 100 mL

Filter and store at 4°C in the dark bottle.

Electrophoresis transfer buffer

- Tris: 25 mM
- glycine: 192 mM
methanol 20%

One time buffer is made in 80% volume, by dissolving 15.1 g Tris base and 72.0 g glycine in 4 L distilled water.

* Do not adjust pH with acid or base. 1 L methanol is added just before use.

TBS-Tween
Tris-HCl, pH 8.0 10 mM
NaCl 150 mM
Tween-20 0.05%

BCIP buffer
Tris-HCl, pH 9.5 100 mM
NaCl 100 mM
MgCl₂ 5 mM

2.1.8.5 RNA buffers
Solution TE3D (Tris-EDTA, 3 detergents)
water 5.0 mL
Tris 2.42 g
Na₂EDTA 0.74 g
Nonadet P-40 1.0 g
lithium dodecyl sulfate 1.5 g
sodium deoxycholate 1.0 g

phenol-EDTA

phenol 500 g
water 45 mL
0.2 M Na₂EDTA, 0.18 M NaOH 5 mL
8-hydroxyquinoline 0.5 g

3 M ammonium acetate-EDTA

water 807 mL
ammonium acetate 231 g
0.2 M Na₂EDTA, 0.18 M NaOH 2 mL

3.6 M LiCl-EDTA

water 918 mL
0.2 M Na₂EDTA, 0.18 M NaOH 5 mL
LiCl 153 g

formamide-EDTA

formamide 5 mL
0.2 M Na₂EDTA, 0.18 M NaOH 25 μL

10x MOPS buffer
Water, gassed with high purity nitrogen gas 190 mL
NaOH (fine pearl form best) 1 g
Na\textsubscript{2}EDTA 0.74 g
MOPS 10.45 g

RNA isolation buffer
phenol-EDTA 2.5 mL
TE3D 1.25 mL
mercaptoethanol 50 μL

2.1.9 Media used for Indica rice tissue culture

Media used for Indica rice tissue culture and transformation are listed in table 2.9. & 2.10. The medium stocks used in this study are listed below.

2.1.9.1 Stocks solutions for media used for Indica rice tissue culture

MS macroelement stock (20x) (Murashige & Skoog, 1962)

\begin{align*}
\text{NH}_4\text{NO}_3 &\quad 33.0 \text{ g} \\
\text{CaCl}_2\cdot2\text{H}_2\text{O} &\quad 8.8 \text{ g} \\
\text{KNO}_3 &\quad 38.0 \text{ g} \\
\text{MgSO}_4\cdot7\text{H}_2\text{O} &\quad 7.4 \text{ g} \\
\text{KH}_2\text{PO}_4 &\quad 3.4 \text{ g} \\
\text{H}_2\text{O to} &\quad 1 \text{ L}
\end{align*}
MS microelement stock (1000x) (Murashige & Skoog, 1962)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>620 mg</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>2230 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>860 mg</td>
</tr>
<tr>
<td>KI</td>
<td>83 mg</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>25 mg</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>H₂O to</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

MS iron stock (250x) (Murashige & Skoog, 1962)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄·7H₂O</td>
<td>3.48 g</td>
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<tr>
<td>H₂O to</td>
<td>500 mL</td>
</tr>
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</table>

MS Na₂EDTA stock (250x) (Murashige & Skoog, 1962)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA</td>
<td>4.66 g</td>
</tr>
<tr>
<td>H₂O to</td>
<td>500 mL</td>
</tr>
</tbody>
</table>

MS vitamin stock (100x) (Murashige & Skoog, 1962)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Nicotinic acid</td>
<td>25 mg</td>
</tr>
<tr>
<td>Thiamin.HCl</td>
<td>5 mg</td>
</tr>
<tr>
<td>Pyridoxine.HCl</td>
<td>25 mg</td>
</tr>
</tbody>
</table>
myo-inositol 5 g
H₂O to 500 mL

R2 macroelement stock (20x) (Ohira et al., 1973)

KNO₃ 40.0 g
(NH₄)₂SO₄ 3.4 g
CaCl₂.2H₂O 1.5 g
MgSO₄.7H₂O 2.5 g
NaH₂PO₄.H₂O 2.73 g
H₂O to 1 L

R2 microelement stock (1000x) (Ohira et al., 1973)

H₃BO₃ 300 mg
MnSO₄.4H₂O 160 mg
ZnSO₄.7H₂O 220 mg
Na₂MoO₄.2H₂O 12.5 mg
CuSO₄.5H₂O 1.25 mg
H₂O to 100 mL

Kao vitamins (100x) (Kao and Michayluk, 1975)

Inositol 1 g
Nicotinamide 10 mg
Thiamin.HCl 100 mg
2,4-D Stock (1 mg / mL or 4.525 mM) (MW=221.0)

Dissolve 100 mg of 2,4-D in 1 mL of absolute ethanol,
add 3 mL of 1 N KOH,
adjust the volume to 80 mL with distilled H₂O,
adjust to pH 6.0 with 1 N HCl (about 4 mL),
adjust final volume to 100 mL,
Store in fridge.
Table 2.9 Media used for tissue culture of Indica rice, cv. *Chinsurah Boro II*

<table>
<thead>
<tr>
<th>Medium stocks or Chemicals</th>
<th>MSC</th>
<th>R2C</th>
<th>R2S</th>
<th>R2P</th>
<th>MSR</th>
<th>MSG</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>macronutrients</td>
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<td>R2</td>
<td>R2</td>
<td>R2</td>
<td>MS</td>
<td>MS</td>
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<tr>
<td>microelements</td>
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<td>R2</td>
<td>R2</td>
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<td>MS</td>
<td>1/3MS</td>
<td>1/3MS</td>
<td>1/3MS</td>
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<td>MS</td>
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<td>Na₂EDTA</td>
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<td>1/3MS</td>
<td>1/3MS</td>
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<td>MS</td>
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<td>MS</td>
<td>B5</td>
<td>MS</td>
<td>MS</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>Kao</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>organic acids</td>
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<td>-</td>
<td>-</td>
<td>Kao</td>
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<tr>
<td>AOA</td>
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<td>30 g</td>
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<td>30 g</td>
</tr>
<tr>
<td>glucose</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>109 g</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>NAA</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1 mg</td>
<td>0.5 mg</td>
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<td>kinetin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5 mg</td>
<td>-</td>
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<tr>
<td>Phytogel²</td>
<td>2.5 g</td>
<td>2.5 g</td>
<td>-</td>
<td>-</td>
<td>2.5 g</td>
<td>2.5 g</td>
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**Notes:**
- MS: Murashige & Skoog, 1962;
- R2: Ohira *et al.*, 1987;
- B5: Gamborg *et al.*, 1968;
Table 2.10 Media used for transformation of Indica rice, cv. *Chinsurah Boro II*

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NAA Stock (1 mg / mL or 5.37 mM) (MW=186.2)

Dissolve 100 mg of NAA in 1 mL of absolute ethanol,
add 3 mL of 1 N KOH,
adjust the volume to 80 mL with distilled H$_2$O,
adjust to pH 6.0 with 1 N HCl (about 2 mL),
adjust final volume to 100 mL,
Store in fridge.

Kinetin Stock (0.1 mg / mL or 465 μM) (MW=215.21)

Dissolve 50 mg of kinetin in 10 mL of 0.2 N HCl,
bring up to 500 mL slowly with distilled H$_2$O,
store in fridge.

AOA Stock (1 mg / mL) [(aminooxy) acetic Acid]

Dissolve 100 mg AOA in 1-2 mL 0.1 N HCl,
bring up to 100 mL with H$_2$O.
store in fridge.

2.2 Purification of rice ragged stunt virus (Omura et al., 1983)

2.2.1 Virus purification

RRSV was purified from infected rice plants as described by Omura et al. (1983). Four hundred g of infected rice seedlings were cut and homogenised in extraction buffer (3 mL of buffer / g seedling material)
which contains 0.1 M phosphate buffer, pH 7.0 with freshly added 10 mM 
MgCl₂. The homogenate was filtered through 2 layers of muslin cloth, 
centrifuged at 10,000 x g for 15 minutes and filtered through a layer of 
cotton wool and muslin cloth again. The supernatant was clarified by adding 
20% (v/v) CCl₄ and blended in Waring blender for 2 minutes and centrifuged 
at 10,000 x g for 15 minutes. The supernatant was mixed with PEG 6000 to 
a final concentration of 6%, NaCl to a final concentration of 0.3 M and Triton 
X-100 to a final concentration of 1%, and stirred in the cold room for 1 hour. 
The pellets were collected by centrifugation at 10,000 x g for 20 minutes, 
resuspended in 40 mL histidine-MgCl₂ buffer (0.1 M histidine and 10 mM 
MgCl₂, pH 7.0, 10% of the volume of original material by weight). The 
samples were stirred in the cold room for 1 to 2 hour(s) and centrifuged at 
10,000 x g for 10 minutes. The pellet was collected by loading the 
supernatant on to the top of 3 mL of 30% sucrose and centrifuging at 
100,000 x g (70.1Ti) for 2 hours, and then resuspended in 10 mL of 
histidine-MgCl₂ buffer and stirred in the cold room at low speed for 10 
minutes to 2 hours. The pellet was collected by centrifuging at 45,000 x g 
(70.1Ti) for 1.5 hours, and resuspended in 1 to 2 mL of histidine-MgCl₂ buffer. 
The sample was then centrifuged at 10,000 x g for 2 minutes. Partially 
purified RRSV can be obtained in the supernatant.

2.2.2 Observation of RRSV particles using an electron microscope
The purified virus preparations were trapped on a carbonformvar coated grids, which were treated by glow discharge prior to use. The grids were washed with several drops of distilled water and negatively stained by 2% uranyl acetate and drained with a filter paper. The preparations were examined in a JEM-100B electron microscope and virus particles were measured by comparing with a 500 nm long bar.

2.3 Nucleic acid isolation and purification

2.3.1 Isolation and purification of RRSV genomic dsRNA

RRSV dsRNAs were directly extracted and purified from fresh or frozen infected rice plants using a method modified from that for TMV dsRNA isolation (Uyeda et al., 1990b). Ten grams of the infected rice roots or leaves were cut and homogenised in 30 mL of STE (100 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.0), 10 mL of 4% SDS and 40 mL of phenol. An equal volume of phenol was added to the homogenate, shaken, then filtered through a muslin cloth. The emulsion was centrifuged at 4500 x g for 10 minutes and the aqueous phase recovered. This was extracted twice with an equal volume of phenol : chloroform (1:1), and the nucleic acids precipitated by ethanol and 0.3 M sodium acetate. The precipitate was resuspended in 48 mL of STE and 12 mL of absolute ethanol. After centrifugation at 6000 x g for 10 minutes the supernatant was recovered and passed twice through a CF-11 cellulose column (Whatman BioSystems Ltd, England). The CF-11 cellulose column was then washed with 100 mL of
20% ethanol in STE and the dsRNA eluted with 30 mL of STE. The dsRNA was precipitated by mixing with an equal volume of isopropanol and then resuspended in 200 μL of STE.

2.3.2 Isolation and purification of plasmid DNA

2.3.2.1 Minipreparations of plasmid DNA for enzyme analysis

Small-scale preparations of plasmid DNA were obtained by the alkaline lysis method described by Sambrook et al. (1989). A single bacterial colony was cultured overnight in 5 mL 2x YT at 37°C. Cells (1.5 mL) were pelleted by centrifugation at 13,000 rpm for 5 minutes, resuspended in 200 μL of AL-solution (Section 2.1.8.1), mixed with 2 mg/mL lysozyme and kept on ice for 5 minutes. Cells were lysed by mixing with 400 μL of freshly prepared alkaline lysis buffer (Section 2.1.8.1) and kept on ice for 5 minutes, and neutralised by mixing with 300 μL of 3M / 5M KOAc buffer (Section 2.1.8.1) and kept on ice for 5 minutes. The supernatant was collected by centrifuging the lysed cells at 13,000 rpm for 5 minutes, and extracted with an equal volume of phenol: chloroform: IAA (25:24:1). Plasmid DNA was precipitated with 2 volumes of ethanol, washed with 70% ethanol, vacuum dried and dissolved in 30 μL of 1/10 TE.

2.3.2.2 Minipreparations of plasmid DNA for sequence analysis

Minipreparations of plasmid DNA from 5 mL of overnight E. coli cultures containing appropriate plasmids were essentially the same as
described in Section 2.3.2.1, except phenol: chloroform extraction was omitted and DNA was precipitated with 1 volume of isopropanol. The nucleic acids were resuspended in 150 μL STE buffer and treated with RNase A at 100 μg / mL at 65°C for 10 minutes. Plasmid DNAs were then precipitated by adding 150 μL of 1% CTAB, 100 mM NaCl (Section 2.1.8.1) at room temperature for 5 minutes and centrifuged at 13,000 rpm for 5 minutes. DNA was redissolved in 300 μL of 1.2 M NaCl, precipitated again with 2 volumes of ethanol and 0.3 M sodium acetate, then washed with 70% ethanol, vacuum dried and redissolved in 50 μL of 0.1x TE. Purified DNA can be used for cycle sequencing using either ³²P-end labelled primer or dye-labelled primers.

2.3.2.3 Maxipreparations of plasmid DNA

Large-scale plasmid preparation was essentially as described by Sambrook et al. (1989). A single colony of the E. coli strain containing the plasmid was grown overnight in 5 mL of 2x YT medium, inoculated into 500 mL of 2x YT and grown at 37°C overnight with shaking.

Cells were harvested by centrifugation at 4,500 x g for 5 minutes, resuspended in 2.5 mL of AL-buffer (Section 2.1.8.1) containing 2 mg / mL of lysozyme, lysed with 5 mL of alkaline lysis buffer (Section 2.1.8.1), and neutralised with 3.75 mL of 3M / 5M KOAc buffer (Section 2.1.8.1). After removing debris from the lysed cells by centrifugation at 8,500 x g for 10 minutes, the supernatant was treated with RNase A at 100 μg / mL at 37°C
for 30 minutes and extracted with 5 mL of phenol: chloroform: isoamyl alcohol (25:24:1). Plasmid DNA was precipitated with 5 mL of cold isopropanol at -70°C for 30 minutes, centrifuged at 8,500 x g for 15 minutes, washed with 70% ethanol and vacuum dried. DNA was redissolved in 3.5 mL of TE containing 0.1% Sarkosyl and purified in CsCl-ethidium bromide gradients as described by Sambrook et al. (1989).

2.3.2.4 Maxipreparations of plasmid DNA by QIAGEN-tip 500

Maxipreparations of plasmid DNA were made by QIAGEN-tip 500 described in Plasmid Maxi Protocol, Qiagen (1993). Up to 500 mg of plasmid DNA can be obtained from 100 mL of E. coli LB culture for high copy number plasmids (pBS, pUC, etc.) using QIAGEN-tip 500.

2.3.3 M13 single-stranded (ss) DNA

Fifty μL of overnight culture of E. coli and 12.5 μL of helper phage M13K07 (1.3 x 10^{11} pfu / mL) were added into 5 mL of 2x YT and incubated at 37°C overnight under the selection with 35 μg / mL Kanamycin. 1.5 mL culture was transferred to a microfuge tube and cells were removed by centrifugation at 13,000 rpm for 3 minutes. Phage virions were precipitated by mixing 1 mL of the supernatant with 250 μL of PEG / NaCl solution (25% polyethylene glycol 6,000, 2.5 M NaCl) and kept on ice for 1 hour, then pelleted by centrifugation at 13,000 rpm for 3 minutes and resuspended in 400 μL of STE. The ssDNA (2-5 μg) was extracted with phenol: chloroform,
precipitated with ethanol, dissolved in 20 μL of TE buffer and stored at -20°C.

2.3.4 Purification of segment 5 of RRSV by electroelution

RRSV genomic dsRNA was separated on a 5.5% polyacrylamide gel (acrylamide: bisacrylamide 19:1) (Section 2.7.2) or a 1% agarose gel (Section 2.7.1) using 1x TBE buffer. The fifth (on PAGE) or the second (on agarose gel) slowest migrating band (S5) was excised and put into a length of thin dialysis tubing. The dialysis tubing was then filled with 800 μL 0.25x TBE and electroeluted in 0.25x TBE at 100 to 200 V (<40 mA) for 1 to 2 hours. This dsRNA solution was extracted once with hot phenol after incubating with phenol at 65°C for 10 minutes, once with phenol: chloroform: IAA (25: 24: 1) and precipitated with ethanol and dissolved in 1x TE.

2.3.5 Isolation and purification of plant DNA

2.3.5.1 Isolation and purification of plant DNA from fresh leaf material

About 1 g of fresh leaves of Indica rice cv. Chinsurah Boro II was ground to powder with acid washed sand in liquid N₂ and mixed with 1 mL CTAB isolation buffer (Section 2.1.8.1) which was preheated to 60-65°C. Samples were incubated at 60-65°C for 30 minutes with occasional gentle swirling then extracted once with chloroform: isoamyl alcohol (24:1). The nucleic acids were precipitated by mixing with an equal volume of isopropanol and spinning at 10,000 x g for 5 minutes. The pellet was
washed with 70% ethanol and air dried and redissolved in 400 μL of 1M NaCl. Nucleic acids were then treated with RNase A at 100 μg/mL at 65°C for 10 minutes and extracted once with chloroform: isoamyl alcohol (24:1). Plant DNA was precipitated with 2 volume of ethanol, washed with 70% ethanol, vacuum dried and dissolved in 50 μL of sterile distilled water and stored at -20°C.

2.3.5.2 Isolation and purification of plant DNA from freeze-dried leaf material

Freeze-dried leaf material of Indica rice cv. Chinsurah Boro II (0.07g) was ground to powder with acid washed sand in liquid nitrogen, mixed with 600 μL of 1x CTAB extraction buffer (Section 2.1.8.1) and incubated at 56-65°C for 20 minutes. The sample was extracted with chloroform: IAA (24:1). The aqueous phase was mixed with 0.1 volume of 10% CTAB solution, 0.7 M NaCl and extracted with chloroform: IAA (24:1), then mixed with 1x CTAB precipitation buffer (Section 2.1.8.1) and kept at room temperature for 20 minutes. DNA was pelleted by centrifugation at 5,000 x g for 10 minutes and dissolved in 400 μL of 1 M NaCl. DNA was then treated with RNase A at 100 μg/mL at 65°C for 10 minutes and extracted once with chloroform: isoamyl alcohol (24:1). Finally, DNA was precipitated with 2 volume of ethanol, washed with 70% ethanol, vacuum dried and dissolved in 85 μL of sterile distilled water.
2.3.6 Small scale RNA isolation from plant leaf tissue

About 0.2 g of fresh leaves of Indica rice cv. *Chinsurah Boro II* was ground to powder with acid washed sand in liquid N₂ and mixed with 300 µL RNA isolation buffer (Section 2.1.8.5). Samples were then mixed with 250 µL of 3M ammonium acetate / EDTA (Section 2.1.8.5) and 400 µL of chloroform : isoamyl alcohol (24:1), and shaken for 20 minutes on a mixer. The supernatant (300 µL) was recovered by spinning at 10,000 x g at 4°C for 10 minutes. The RNA was precipitated by mixing with 900 µL of cold 3.6 M LiCl-EDTA (Section 2.1.8.5), keeping on ice for 15 minutes and spinning at 10,000 x g for 7 minutes. The RNA pellet was dissolved in 100 µL of TE buffer and extracted once with 100 µL of phenol / chloroform, precipitated with an equal volume of isopropanol and 0.3M sodium acetate (pH 5.5), washed with 70% ethanol, air dried, dissolved in 40 µL of sterile distilled water, and stored at -20°C.

2.4 Enzyme reactions

2.4.1 Restriction endonuclease digestion

DNA samples were hydrolysed with appropriate restriction endonuclease enzymes in suitable buffers according to the manufacturer’s instructions. Ten to 20 units of enzyme was used for hydrolysing of 1 to 2 µg of DNA at an appropriate temperature for 2 hours or overnight.

2.4.2 Dephosphorylation of vector DNA
Calf intestinal alkaline phosphatase (CIP) was used to remove 5' phosphate groups from vector DNA, increasing the efficiency of recombinant plasmid formation. Vector DNA was treated with CIP at the concentration of 1 unit per μg DNA in 1x CIP buffer (Section 2.1.8.2) for 1 h at 37°C. Reactions were terminated by adding EDTA to 5 mM and heating at 75°C for 15 minutes, followed by phenol: chloroform extraction and ethanol precipitation.

2.4.3 Ligation of DNA molecules

Suitably cut insert DNA with CIP treated vector DNA was ligated in 1x ligation buffer (Section 2.1.8.2) in the presence of 1 mM ATP and one unit of T4 DNA ligase at 16°C overnight. The ratio of insert DNA to vector DNA was from 1:1 to 1:2. Ligated DNA was precipitated with ethanol, dissolved in sterile distilled water and used for transformation by electroporation (Section 2.6.1).

2.4.4 Polymerase chain reaction (PCR)

PCR reactions were assembled in a 20 μL volume by mixing 20 ng of template DNA with 2 μL 10x PCR buffer (Section 2.1.8.2), 2 μL of 2 mM dNTP (2 mM each of dATP, dCTP, dGTP and dTTP), 2 μL of 10 pmol / μL each primer, 0.4 μL AmpliTaq polymerase (8 units / μL) and water, loaded into capillary tubes and placed in a FTS-1S Fast Thermal Sequencer (Corbett, Australia).
A PCR program of 32 cycles was used for cloning cDNA end sequences of RRSV S5 as follows: cycle 1 had a denaturation step at 95°C for 20 seconds; cycles 2 to 21 had steps at 95°C for 30 seconds, 55°C for 30 seconds and 70°C for 60 seconds; 10 further cycles of 95°C for 30 seconds and 70°C for 60 seconds followed by a final step at 70°C for 10 minutes. A PCR program of 37 cycles was used for other PCR analyses as follows: cycle 1 had a denaturation step at 95°C for 20 seconds; 35 cycles of denaturation step at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes; a final step at 70°C for 10 minutes, at 30°C for 2 minutes.

2.4.5 Reverse transcription polymerase chain reaction (RT-PCR)

Plant RNA (100 - 200 ng) (Section 2.3.5) was diluted to 7.3 μL and mixed with 1 μL of S5RTPR (100 ng / μL) (Section 2.1.4). Samples were heated at 65°C for 5 minutes, spun and chilled on ice. The reverse transcription reactions were assembled in 10 μL volume by mixing RNA / primer mix with 1 μL of 10x PCR buffer (Section 2.1.8.2), 0.5 μL 20 mM dNTPs, 0.1 μL RNase inhibitor (33 units / μL) and 0.1 μL reverse transcriptase (200 units / μL) (superscript), and incubated at 42°C for 1 hour.

PCR reactions were then assembled in 20 μL volume by mixing 10 μL of reverse transcription reactions with 0.5 μL of S5RTPR primer (100 ng / μL) (Section 2.1.4), 1 μL of S5RTPF primer (100 ng / μL) (Section 2.1.4), 0.1 μL AmpliTaq DNA polymerase (8 units / μL), 1 μL 10 x PCR buffer (Section
2.5 Synthesis of cDNA from RRSV dsRNA

2.5.1 Synthesis of cDNA library from RRSV dsRNA

The cDNA from RRSV dsRNA was synthesised using M-MLV H-reverse transcriptase (Superscript™, BRL) according to the manufacturer's protocol with minor modifications. First strand cDNA was synthesised by mixing 2.5 μg RRSV dsRNA with 1.25 μg of random hexamer in 22 μL of water, denaturing at 96°C for 8 minutes and snap cooled on ice for 1 minute. Denatured dsRNA and primers were mixed with 8 μL 5x Reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂), 4 μL 0.1 M DTT, 2 μL 10 mM dNTP stock (10 mM each dATP, dCTP, dGTP and dTTP, pH7.0) and 2 μL [α-³²P] dCTP (10 μCi / μL). The reaction was incubated at 45°C for 2 minutes, mixed with 2 μL (200 units) of M-MLV H- reverse transcriptase and incubated at 45°C for 1 hour. For the second strand cDNA synthesis, first strand cDNA reaction in 40 μL was mixed with 183.6 μL water, 64 μL 5x second strand buffer [94 mM Tris-HCl, pH 6.9, 453 mM KCl, 23 mM MgCl₂, 750 mM β-NAD, 50 mM (NH₄)₂SO₄], 6 μL 10 mM dNTP stock, 12 μL 0.1 M DTT, 1 μL [α-³²P] dCTP (10 μCi / μL), 4 μL E. coli DNA ligase (7.5 U / μL), 8 μL E. coli DNA polymerase I (10 U / μL) and 1.4 μL E. coli RNase H (2 U / μL), and incubated at 16°C for 2 hours. 5 μL T4 DNA polymerase (3 U / μL) was added and incubated continuously at 16°C for a further 5 minutes. The
reaction was stopped by adding 10 μL of 0.5 M EDTA. The cDNA was purified by passing through a SizeSelect (Sephacryl S-400) spun chromatography column and ligated with EcoRI adaptors, purified again by passing through a SizeSelect (Sephacryl S-400) spun chromatography column and cloned into phagemid pUC119 to produce a library of cDNA clones.

2.5.2 Synthesis of cDNA from RRSV dsRNA to obtain end sequences

To obtain the end sequences of RRSV S5, two pairs of primers (S55S / S55R and S53S / S53R) (Section 2.1.4) were used for cDNA synthesis from dsRNA. First strand cDNA was synthesised as described in Section 2.5.1 by using 750 ng RRSV dsRNA and 500 ng each primer S55R and S53S in a 20 μL volume of reaction. After 1 hour incubation at 42°C, 2.5 μL of 10x RNase H buffer (200 mM HEPES-KOH, pH 7.8, 10 mM DTT, 100 mM MgCl₂, 500 mM KCl) and 2 μL RNase H (0.9 U / μL) was added and incubated for 30 minutes at 37°C. The cDNA was purified with "Geneclean II Kit" (Section 2.1.6). First strand cDNA was A-tailed with "DNA Tailing Kit" (Section 2.1.6) according to the manufacturer’s protocol. The cDNA was amplified by PCR reaction (Section 2.4.4) using 5 μL first strand cDNA and 20 pmol of each primer (S55S, S55R, S53S and S53R) (section 2.1.4). PCR products were purified with Wizard™ PCR preps purification system (Section 2.1.6), ligated into pGEM-T vector using a pGEM-T vector system I (Section 2.1.6) and transferred into E. coli JM109 by electroporation (Section 2.6.1).
2.6 Bacterial transformation

2.6.1 Transformation by electroporation

Competent cells of *E. coli* JPA101 and JM109 were prepared as previously described by Dower *et al.* (1988) and Miller *et al.* (1988). Ten mL of overnight culture of *E. coli* was transferred into 1 L of 2x YT medium and incubated at 37°C for 2 to 3 hours to an OD_{600} of 0.5 to 0.6. Cells were chilled on ice and collected by centrifugation at 5,000 x g for 10 minutes at 4°C, then washed once with 500 mL of cold sterile distilled water (4°C), once with 250 mL of cold sterile distilled water (4°C) and once with 10 mL of cold 10% sterile glycerol (4°C). Competent cells were resuspended in 3 mL of cold 10% sterile glycerol and aliquoted into 40 μL and stored at -70°C.

To transform cells, ligated plasmid DNA was precipitated with ethanol and dissolved in 10 μL of sterile distilled water, mixed with 40 μL of thawed competent cells on ice, transferred into a cuvette and electroporated at 2.5 KV with a time constant of 3.7 msec. After electroporation, cells were mixed with 250 μL of cold 2x YT (on ice), transferred into a microfuge tube, kept at room temperature for a few minutes and incubated at 37°C for 30 minutes. Cells were mixed with 30 μL of each IPTG (20%) and X-Gal (20 mg/mL) and plated on LB medium with appropriate antibiotics. This protocol was routinely used for transformation of ligated plasmid DNA.

2.6.2 Transformation by heat shock
Competent cells of *E.coli* JPA101, JM109, AD202 and BL21 were prepared as described by Morrison (1979). Fifty μL of overnight culture in LB medium was diluted into 50 mL of SOBG medium (Section 2.1.1) in a 250 mL flask and incubated at 37°C to early exponential phase of growth. Cells were chilled on ice and collected by centrifuged at 5,000 x g for 10 minutes at 4°C, washed once with 20 mL cold 0.1 M MgCl₂, and resuspended in 2.5 mL of cold 50 mM CaCl₂ containing 14% glycerol and kept on ice for 1 hour. Competent cells were aliquoted into 200 μL and stored at -70°C.

To transform the cells, plasmid DNA (1 to 10 ng) was added to 200 μL of thawed competent cells and kept on ice for 5 to 7 minutes and shaken gently once or twice. This mixture was then heated for 100 seconds at 42°C, cooled for 5 minutes on ice, mixed with 1.8 mL of SOBG medium and incubated at 37°C for 30 minutes for phenotype expression. Cells were harvested and plated on selective media. This protocol was normally used for re-transformation of plasmid DNA.

2.7 Gel electrophoresis

2.7.1 Agarose gel electrophoresis

Total genomic dsRNA of RRSV, digested plasmid or plant DNA was separated on 1% agarose gels. An analytical agarose gel (100 x 150 x 5 mm) containing 80 mL of 1% agarose, 80 μg / mL ethidium bromide, was run in a horizontal system in 1x TBE (Section 2.1.8) buffer at 100 volts, 100 mA for 1 hour. A preparative gel was run similarly but was made with low
melting point agarose and run with 1x TAE buffer (Section 2.1.8.1) at 60 volts for 2 hours. Nucleic acids were visualised on a UV trans-illuminator (302 nm) and photographed with Polaroid film or a Gel Documentation System (NovaLine). A mini-horizontal gel system was also used. Samples were run in gels (100 x 65 x 5 mm) at 80 V, 50 mA for 1 hour. In such a system about 10 ng of DNA could be visualised by UV after ethidium bromide staining. All samples were prepared by the addition of SDS dye (Section 2.1.8.3) to 20% prior to electrophoresis.

2.7.2 Polyacrylamide gel electrophoresis of RRSV dsRNA

Total genomic dsRNA of RRSV was separated by electrophoresis in 5.5% polyacrylamide gels. A vertical gel (300 x 200 x 1.5 mm) was run in 1x TBE (Section 2.1.8.1) buffer at 200 V, 40 mA for 13 to 30 hours. Gels were prepared by adding 21 mL of a 40% acrylamide: bisacrylamide (19:1) stock solution, 30 mL of 10x TBE in 99 mL of distilled water. Polymerisation was initiated by the addition of 1.5 mL of 10% (w/v) ammonium persulphate and 150 µL of TEMED to a 150 mL acrylamide: bis mixture. Gels were stained and visualised as described in Section 2.7.1.

2.7.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of protein

Total proteins of RRSV particles, fusion protein of RRSV S5, total plant proteins or total brown planthopper proteins were separated by electrophoresis in 12% SDS-polyacrylamide gels which consisted of 12%
SDS-PAGE resolving gel and 5% SDS-PAGE stacking gel. Vertical gels were electrophoresed at 200 V for large gels (300 x 200 x 1.5 mm) (Bio-Rad) and at 100 V for mini-gels (150 x 100 x 3 mm) (Bio-Rad) in 1x Tris-glycine electrophoresis buffer (Section 2.1.8.4). The resolving gel (12%) was prepared by mixing 4.1 mL distilled water, 8.0 mL 30% acrylamide mix (29:1 Acrylamide : Bis), 7.5 mL 1 M Tris-HCl (pH 8.8), 0.2 mL of 10% SDS, 0.2 mL of 10% ammonium persulphate and 8 µL TEMED. The stacking gel (5%) was prepared by mixing 3.4 mL distilled water, 0.83 mL 30% acrylamide mix (29:1 Acrylamide : Bis), 0.63 mL 1 M Tris (pH 6.8), 50 µL of 10% SDS, 50 µL of 10% ammonium persulfate and 5 µL TEMED. Gels were stained with Coomassie Brilliant Blue (2.1.8), destained by soaking in destaining solution (Section 2.1.8.4), examined and photographed with Polaroid film or a Gel Documentation System (NovaLine).

Protein samples and Rainbow marker proteins were solubilized by heating at 100°C for 5 minutes in 1 x SDS-loading buffer. Twenty µL of each sample was loaded on each well of the gel. An equal volume of 1x SDS gel-loading buffer was loaded into wells that were unused.

2.7.4 Electrophoresis of DNA sequence reactions

The products of DNA sequencing reactions (Section 2.9) were resolved on 5% denaturing polyacrylamide gels containing 7 M urea. A 5% acrylamide gel mixture was made by dissolving 50.4 g urea in 45 mL distilled water at 65°C, and mixed with 12 mL of 10x TBE, 15 mL of a 40%
acrylamide: bis acrylamide (40: 1) stock and the volume adjusted to 120 mL; 840 µL of 10% (w/v) ammonium persulphate and 75 µL of TEMED were added to polymerise the gel. Gel mixture was poured into a IPC assembly (380 x 500 x 0.25 mm or 210 x 800 x 0.25 mm) of Sequi-Gen Nucleic Acid Sequencing Cell (Bio-Rad), and allowed to polymerize for 3 hours. Gel was pre-run in 1x TBE buffer for 1 hour to 45°C and run at a constant 2500V (38 x 50 cm) or 1,800 V (21 x 80 cm) at 50°C for 4 to 7 hours to allow resolution of the first 250 to 400 nucleotides from the point of initiation of a DNA chain termination sequencing reaction (Section 2.9). After electrophoresis, the glass plates were separated and the gel transferred to Whatman 3 MM paper, covered with "Glad Wrap", and dried at 80°C on a vacuum gel drier prior to overnight exposure to X-ray film (Kodak) at -70°C. X-ray films were developed with X-ray film developer, GEVAMATIC 60 (AGFA-GEVAERT, Germany).

2.8 Southern hybridization

2.8.1 Preparation of hybridization probes

2.8.1.1 Preparation of DNA probes

Radioactively-labelled hybridization probes were prepared by randomly-primed synthesis of DNA with *E. coli* DNA polymerase I (Klenow fragment) using a Multiprime DNA labelling systems (Amersham). Five µL DNA (100 ng) was denatured by heating for 3 minutes in a boiling water bath and chilled on ice. Denatured DNA was mixed with 10 µL buffer, 5 µL
primers, 23 µL sterile distilled water, 5 µL \([\alpha^{32}\text{P}]\text{dCTP}\) and 2 µL enzyme (Klenow), and incubated at 37°C for 30 minutes and kept on ice. Labelled probes diluted with 100 µL of water were purified by passing through a Sephadex G-50 column equilibrated with STE which had been sterilized by autoclaving. The probe (150 µL) was collected by spinning at 100 x g for 2 minutes and radioactivity monitored using a Geiger counter. The elutes obtained by the above method typically had a specific activity of \(10^7-10^8\) cpm / µg of DNA.

2.8.1.2 Preparation of dsRNA probes by 5’ end labelling of RRSV genomic dsRNA

Fifty ng of gel purified RRSV S5 in 5 µL of 1x TE was hydrolysed with 35 µL alkaline buffer (5 mM EDTA, 50 mM KOH) and incubated at 65°C for 8 minutes. The reaction mixture was then neutralised with 4.5 µL neutralization buffer (45 mM HCl, 22.5 mM Tris-HCl pH 7.0, 33.7 mM NaCl), precipitated with ethanol and sodium acetate, and dissolved in 25 µL of sterile distilled water. The dsRNA was incubated at 96°C for 5 minutes and kept on ice. Fifteen µL of \([\gamma^{32}\text{P}]\text{ATP}\) (10 µCi / µL) was vacuum dried for 15 minutes, then mixed with 25 µL of dsRNA, 5 µL of 10x PNK buffer, 18 µL of sterilised distilled water and 2 µL of PNK enzyme (10 U / µL) and incubated at 37°C for 30 minutes. The reaction was stopped by adding EDTA to a final concentration of 20 mM and extracted once with phenol: chloroform: IAA (25: 24: 1). Labelled dsRNA was mixed with carrier tRNA at a final
concentration 2 μg / μL, precipitated with ethanol and ammonium acetate, vacuum dried and redissolved in 25 μL of TE buffer.

2.8.2 Transfer of DNA to Hybond-N+ nylon filter (Southern, 1975)

For Southern blot hybridization analysis, DNA fragments were fractionated in 1% agarose gels (Section 2.7.1) and photographed. Gels were subsequently soaked in 0.25 M HCl for 5 minutes, denatured in 0.5 M NaOH, 0.5 M NaCl for 30 minutes, and neutralized by rinsing twice in 0.5 M Tris-HCl, pH 7.2, 2 M NaCl for 15 minutes. Gels were briefly rinsed in water each time before transferring into next solution. After a brief final rinse in 2x SSC (0.3 M NaCl, 0.03 M trisodium citrate) the denatured DNA was transferred to a Nylon membrane by blotting the gel with 20x SSC for 12 to 16 h. The filter was briefly rinsed in 2x SSC, air-dried and baked at 80°C in vacuum for 2 h.

2.8.3 Colony hybridization (Grunstein and Hogness, 1975; Grunstein and Wallis, 1979).

Recombinant plasmids were sometimes screened by hybridization to isolate clones with the desired insert. Nitrocellulose filter discs were placed on the surface of plates carrying colonies and left for 5 minutes. Bacterial colonies adsorbed to the filters were lysed and their DNA denatured by placing the filter on Whatman 3 MM chromatography paper soaked with 0.5 M NaOH, 0.5 M NaCl for 5 minutes. Filters were subsequently neutralised
for 5 minutes in 0.5 M Tris-HCl pH 7.2, 2 M NaCl and then rinsed for 5 minutes on 2x SSC (0.3 M NaCl; 0.03 M trisodium citrate). Nitrocellulose discs from all colony blots were air dried prior to baking at 80°C in a vacuum for 2 h.

2.8.4 Hybridization conditions

2.8.4.1 Hybridization of plasmid DNA / E. coli colony using labelled DNA probes

Southern blots (Section 2.8.2) and colony hybridizations (Section 2.8.3) were pre-hybridized in tubes containing 25 mL of Southern hybridization buffer (Section 2.1.B.1) for 1 hour in a hybridization oven at 65°C. Labelled DNA probes (Section 2.8.1.1) were denatured by heating in a boiling water bath for 5 minutes, chilled on ice and added to a hybridization mixture. Hybridization was at 65°C for 16 h. Southern blots were washed three times in 2x SSC at 25°C and monitored using a Geiger counter after washing. If the background reaction was greater than 100 counts per minutes the filters were washed once or twice in 0.1 x SSC containing 0.1% SDS. Colony hybridizations were washed at 65°C. Nitrocellulose filters were air-dried, but nylon membrane filters were covered with "Glad Wrap" to avoid complete drying. Filters were analysed on a PhosphorImager (Molecular Dynamics) or by autoradiography at -70°C with intensifying screens for times from a few hour to several days.
2.8.4.2 Hybridization of plasmid DNA using labelled RRSV dsRNA probes

Nylon membrane filters of Southern blots (Section 2.8.2) were pre-hybridized in tubes containing 25 mL of formamide hybridization buffer (Section 2.1.8.1) for 1 hour in a hybridization oven at 42°C. Labelled dsRNA probers (Section 2.8.1.2) diluted with 0.5 mL of water were denatured by heating at 96°C for 5 minutes then added to the hybridization tubes. Hybridization was at 42°C for 16 h. Filters were washed and exposed for phosphorimagery or autoradiography as described in Section 2.8.4.1.

2.8.4.3 Rice genomic Southern blots

Nylon membrane filters of rice plant DNA blots (Section 2.8.2) were pre-hybridised in 25 mL of Khandjian’s hybridization buffer (Section 2.1.8.1) for 3 hours at 42°C. Labelled DNA probes were denatured by heating in a boiling water bath for 5 minutes before adding into hybridization tubes. Southern blots were hybridised to the denatured probe for 16 hours at 42°C. Filters were briefly rinsed with, and then washed in 2 x SSC containing 0.05% SDS for 30 minutes at 42°C, and further washed in 0.1 x SSC containing 0.1% SDS, 0.1% sodium pyrophosphate for 1 to 2 hours at 65°C. Southern blots were exposed for phosphorimagery or autoradiography as described in Section 2.8.4.1.

2.8.5 Probe removal for rehybridization
Old probes were removed from nitrocellulose filters by soaking in 0.02 M NaOH at 58°C for 3 h, with 3 changes of NaOH. Filters were then soaked in 2x SSC for 3 h with 3 changes. If filters were still radioactive, they were washed further in 0.1x SSC. Filters were air dried and pre-hybridized before reprobing.

For Hybond nylon membranes, filters were soaked in 0.4 M NaOH for 30 minutes at 45°C and then transferred to 0.1x SSC containing 0.1% (w/v) SDS, 0.2 M Tris-HCl, pH 7.5, and incubated at 45°C for 30 minutes. Filters were then blot dried, sealed in plastic bags and pre-hybridized before reprobing.

2.9. DNA sequencing using chain termination (Sanger et al., 1977)

Restriction fragments were cloned into appropriate plasmids (Table 2.2) and dsDNA and ssDNA were prepared (Section 2.3.2.2; 2.3.3). The primers used were the M13 17-mer Sequencing Primer (Forward) (-20) #1211 and the 24-mer Revers Sequencing Primer (-48) #1233 (Section 2.1.4). The cDNA clones were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) using either 32P-end labelled primers or dye-labelled primers (Applied Biosystem, BRL). Reactions were done as described in the Taq Dye Primer Cycle Sequencing Kit (Applied Biosystem) or dsDNA cycle sequencing system (Life Technologies, Inc., BRL, 1993).
2.9.1 Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems)

Plasmid DNA for each reaction was diluted to 7 μL containing 500 to 750 ng of ssDNA or 1.0 to 1.5 μg of dsDNA and kept on ice. For each sequencing reaction, 0.5 μL of AmpliTaq DNA Polymerase (8 units / μL) was diluted and mixed with 1 μL of 5x buffer and 5.5 μL of distilled water. Reagent mixes were assembled in 4 Eppendorf tubes. Tube A contained 1.0 μL of each 5x buffer, d/ddNTP (A), primer (JOE) and diluted AmpliTaq DNA Polymerase. Tube C contained 1.0 μL of each 5x buffer, d/ddNTP (C), primer (FAM) and diluted AmpliTaq DNA Polymerase. Tube G contained 2.0 μL of each 5x buffer, d/ddNTP (G), primer (TAM) and diluted AmpliTaq DNA Polymerase. Tube T contained 2.0 μL of each 5x buffer, d/ddNTP (T), primer (ROX) and diluted AmpliTaq DNA Polymerase. The d/ddNTP mixes for cycle sequencing were prepared as Table 2.11.

Sequence reactions were assembled by mixing 4 μL of each reagent mixture A and C with 1 μL of DNA, and 8 μL of each reagent mixture G and T with 2 μL of DNA. The reactions were then sucked into capillary tube and loaded in the wells of FTS-1S Fast Thermal Sequencer (Corbett, Australia). The 33 cycle program was used for cycle sequencing as follows: 1 cycle had a denaturation step at 95°C for 1 minute 30 seconds; cycle 2 had an annealing step at 55°C for 60 seconds and an extension step at 70°C for 2 minutes; cycles 3 to 22 had steps at 95°C for 20 seconds, 55°C for 30 seconds and 70°C for 60 seconds; cycles 23 to 32 had steps at 95°C for 30 seconds and 70°C for 60 seconds; cycle 33 had a single step at 30°C for 1
minute. The Thermal Sequencer was stopped at cycle 1 and kept at 95°C for loading the sequencing reactions. The samples were denatured at 95°C for 3 minutes before starting cycle 1 of this program. This program took about 1.5 hours.

At the end of cycle sequencing, four reaction mixtures of each sample were transferred into an Eppendorf tube containing 80 μL of 95% ethanol and 1.5 μL of 3 M sodium acetate (pH 5.2). Samples were kept on ice for 10 to 15 minutes, and centrifuged at 13,000 rpm for 15 minutes, washed with 250 μL of 75% ethanol and spun for 5 minutes. The pellets were vacuum dried for 1 to 2 minutes and wrapped with aluminium foil and stored at -20°C before analysis.

2.9.2 Cycle sequencing using [γ-32P]-labelled primer [Taq Dye Primer Cycle Sequencing Kit (Applied Biosystem)]

Primers for each sample were end-labelled by mixing 1.5 μL of 1 pmol universal primer (Promega) (Table 2.12), 0.5 μL 10x Polynucleotide Kinase Buffer, 1 μL of 10 μCi / μL [γ-32P]ATP, 1 μL distilled water and 1 μL T4 Polynucleotide Kinase (1.5 U / μL) and incubated at 37°C for 30 minutes. Reactions were terminated by incubating at 90°C for 5 minutes, diluted with 3.5 μL of distilled water, spun for 2 seconds and kept on ice.

Preparation of plasmid DNA and diluted AmpliTaq DNA Polymerase was as described in Section 2.9.1. Sequencing reactions were assembled in 4 Eppendorf tubes. Each tube contained 2.0 μL of each 5x cycle sequencing
buffer, d/ddNTP, end-labelled primer, plasmid DNA and AmpliTaq DNA Polymerase dilution, in which tube A contained d/ddNTP (A), tube C contained d/ddNTP (C), tube G contained d/ddNTP (G) and tube T contained d/ddNTP (T). Preparation of d/ddNTP mixes for cycle sequencing was described in Table 2.11.

The program used for cycle sequencing was as described in Section 2.9.1. Upon completion, samples were transferred into Eppendorf tube containing 4 µL of sequencing stop solution (Section 2.1.8) and store at -20°C.

The DNA was denatured by heating in a boiling water bath for 5 minutes and chilling on ice prior to the loading 1 µL of sample onto a DNA sequencing gel (Section 2.7.3). Gels made with "Shark tooth" combs (with 5 mm between points) were used for loading the sequencing samples.

2.9.3 dsDNA Cycle Sequencing System Kit (Life Technologies, Inc., BRL, 1993)

Primers were end-labelled by mixing 1 µL of each 1 pmol primer (Table 2.12), distilled water, 5x polynucleotide Kinase Buffer, 10 µCi/µL [γ-32P]ATP and T4 Polynucleotide Kinase (1 U/µL) and incubating at 37°C for 30 minutes. Reactions were terminated by incubating at 55°C for 5 minutes, and spun for 2 seconds and kept on ice.

Sequence pre-reaction mixes were assembled by mixing 5 µL of [γ-32P]-labelled primer, 4.5 µL of 10x AmpliTaq Sequencing Buffer, 26 µL of
Table 2.11 Preparation of d / ddNTP mixes for cycle sequencing

<table>
<thead>
<tr>
<th>d / ddNTP stocks</th>
<th>A (μL)</th>
<th>C (μL)</th>
<th>G (μL)</th>
<th>T (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddATP</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ddCTP</td>
<td>-</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ddGTP</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>ddTTP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
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<td>dATP</td>
<td>6.25</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>dCTP</td>
<td>25</td>
<td>6.25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>diaza dGTP</td>
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<td>37.5</td>
<td>18.8</td>
<td>18.8</td>
</tr>
<tr>
<td>dTTP</td>
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<td>50</td>
<td>50</td>
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<tr>
<td>water</td>
<td>46.25</td>
<td>76.25</td>
<td>221.2</td>
<td>112.5</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>200</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>

Note: 2 mM dNTP and 5 mM ddNTP were used for preparing d / ddNTP mixes.
Table 2.12 Primer amount required for end labelling

<table>
<thead>
<tr>
<th>Primer Length (base pair)</th>
<th>ng of primer / pmol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>5.6</td>
</tr>
<tr>
<td>19</td>
<td>6.3</td>
</tr>
<tr>
<td>21</td>
<td>6.9</td>
</tr>
<tr>
<td>23</td>
<td>7.6</td>
</tr>
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<td>25</td>
<td>8.2</td>
</tr>
<tr>
<td>27</td>
<td>8.9</td>
</tr>
<tr>
<td>29</td>
<td>9.6</td>
</tr>
</tbody>
</table>

Notes:

* Estimated weight of 1 pmol obtained by multiplying the number of bases in the primer by 0.33 (ng / base of length).

* For the M13 / pUC Forward 23-base Sequencing primer, 1.5 μL of 5 ng / mL primer and 0.5 μL water were used.
Table 2.13 Weight of 50 fmol double-stranded DNA

<table>
<thead>
<tr>
<th>DNA Length (kb)</th>
<th>Vector</th>
<th>μg of dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>0.033</td>
</tr>
<tr>
<td>2.69</td>
<td>pUC18</td>
<td>0.09</td>
</tr>
<tr>
<td>4.11</td>
<td>pSPOR 1</td>
<td>0.14</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>7.25</td>
<td>M13mp19RF</td>
<td>0.24</td>
</tr>
<tr>
<td>8.8</td>
<td>pWE15</td>
<td>0.29</td>
</tr>
<tr>
<td>10.0</td>
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<td>0.33</td>
</tr>
<tr>
<td>15.0</td>
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<td>0.50</td>
</tr>
<tr>
<td>20.0</td>
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<td>0.66</td>
</tr>
<tr>
<td>25.0</td>
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</tr>
<tr>
<td>43.7</td>
<td>gt11</td>
<td>1.44</td>
</tr>
</tbody>
</table>

Notes:

* Estimated total weight of dsDNA was obtained by adding vector weight to approximate weight of appropriate size insert. Alternatively, the estimated total DNA size in kb was multiplied by 0.033 (μg / kb of length).

* For the pUC18 DNA control, 10 μL of 10 ng / μL DNA and 16 μL of H₂O were used.
template DNA (50 fmol) (Table 2.13) in distilled water, and 0.5 μL of
AmpliTaq DNA Polymerase (5 U/μL), spun and kept in wet ice.

For each sample, 2 μL of Termination Mix-A, C, G, or T was
transferred into 4 Eppendorf tubes labelled as A, C, G, and T. Then, 8 μL
prereaction mixes was added to each tube containing Termination Mixes-A,
C, G or T and mixed gently.

The program used for cycle sequencing was as described in Section
2.9.1. Samples were denatured and loaded on sequencing gels as
described in Section 2.9.2.

2.10 Computer aided sequence assembly and analysis

The sequences obtained from cDNA clones were assembled and
analysed using the programs of the University of Wisconsin Genetics
Computer Group (GCG) Version 7.0 (Deverex et al., 1984). Computer
searches for proteins containing similarities to the predicted proteins of
RRSV were made using GCG and Machintosh protein-toolbox plot software,
and ProteinPrediction (EMBL Heidelberg). The Wilber-Lipman program
(Wilber and Lipman, 1983) was used for rapid similarity searches of the
nucleic acid and protein data from GENBANK and EMBL databases. For
protein secondary structure predictions and hydrophilicity profiles, the guide
lines described by Chou and Fasman (1978a, 1978b) and Kyte and Doolittle
(1982) were adopted.
2.11 Northern hybridization

2.11.1 Transfer of RRSV dsRNA to Hybond-N+ nylon filter

For Northern blot analysis, RRSV genomic dsRNA was separated on a 1% agarose gel (Section 2.7.1) using 1x TBE buffer (Section 2.1.8) by loading 50 ng of dsRNA with 1x SDS-loading buffer (Section 2.1.8.1). To denature the dsRNA, the gel was soaked in 50 mM NaOH for 20 minutes and then washed with 20x SSC for 45 minutes and with 0.5x TBE for 10 minutes. The dsRNAs were electrotransferred (Towbin et al., 1979) onto a Hybond-N+ membrane (Amersham Australia) with 0.5x TBE buffer overnight at 30V, 40 mA at 4°C, using a mini trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, CA). After washing with 6x SSC (0.9 M NaCl, 90 mM tri-Sodium citrate, pH 7.0), the filters were air dried and baked in a vacuum at 80°C for 1.5 hour.

2.11.2 Northern hybridization of RRSV dsRNA using labelled cDNA probes

Nylon membrane filters (Section 2.11.1) were pre-hybridised in tubes containing 25 mL of Khandjian's hybridization buffer (Section 2.1.8.1) in a hybridization oven for 1 hours at 42°C. Labelled cDNA probes (Section 2.8.1.1) were denatured by heating in a boiling water bath for 5 minutes, and added into tubes. Hybridization was at 42°C for 16 h. Filters were washed twice in 2x SSC for 10 minutes at 25°C and monitored each time after washing. Filters were washed once or twice in 2x SSC containing 0.1%
SDS, 0.1% sodium pyrophosphate when more than 100 counts per minutes was detected. Filters were exposed for autoradiography as described in 2.8.4.1.

2.12 Expression of fusion protein of RRSV S5

2.12.1 Cloning RRSV S5 cDNA into a pGEX fusion expression vector, and transformation of E. coli

Clones pS5FL, pS5FLa, pS5H1+2, pS5H1+3, pS5H1 and pS5H2 (Table 2.7) were constructed from cDNA of RRSV S5 into a pGEX fusion protein vector (Table 2.2) and transformed into E. coli cell line JM109, whereas pS5FL and pS5H2 were also transformed into protease-negative strains, AD202 and BL21 (Table 2.1).

2.12.2 Isolation and purification of S5 fusion protein produced in E. coli

The method for isolation and purification of S5 fusion proteins was a modification of that described by Smith and Johnson (1988). E. coli was transformed with the appropriate recombinant plasmids (pS5FL, pS5FLa, pS5H1+2, pS5H1+3, pS5H1 or pS5H2) and grown overnight in 10 mL or 100 mL of 2x YT medium plus 50 mg / L ampicillin with or without 1% glucose at 28°C or 37°C. The following morning, the cultures were diluted 1:10 in 100 mL or 1 L of fresh 2x YT containing ampicillin with or without glucose and grown at 28°C or 37°C for 1 h. Isopropylthio-β-D-galactoside (IPTG) was then added to 0.1 mM for induction of transcription from the lac-
tac promoter. The cultures were grown at 28°C or 37°C for 3 h, after which the bacteria were harvested by centrifugation at 4500 x g for 5 minutes at 4°C. Bacteria were resuspended in 3 mL MTPBS (150 mM NaCl, 16 mM Na₂HPO₄·12H₂O, 4 mM NaH₂PO₄·2H₂O, pH 7.3), then Triton X-100 was added to 1%. Each sample was sonicated with four 1 minute (100W) pulses from a 9.5-mm in diameter probe using an Ultrasonic Homogenizer Labsonic-1510 (B. Braun Flexible Biotechnological Engineering). The lysates were centrifuged at 10,000 x g for 15 minutes at 4°C and the supernatants were incubated with 0.3 mL of pre-swollen 50% glutathione agarose beads at room temperature on a rotating platform. After absorption for 5 minutes, beads were collected by brief centrifugation at 500 x g and washed four times with 1 mL MTPBS. Fusion proteins were eluted from the agarose beads by competition for 2 minutes with free glutathione in 50 μL of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione. The yields of fusion proteins were quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

2.13. Antibody preparation

Purified protein (500 mg) from pS5H2 was emulsified with an equal volume of complete (for the first injection) or incomplete (for the subsequent three injections) Freund’s adjuvant and used for intramuscular injection of rabbits (Australian MZW strain). Rabbits were injected at two weekly intervals and blood was collected at 1, 2 and 3 week after the final injection.
Serum was separated by centrifugation at 7,700 x g for 10 minutes, mixed with 0.2 g/L NaN3 for long term preservation, aliquoted and stored at -20°C.

Polyclonal antisera raised against total viral proteins were gifts from Dr. Koganezawa (International Rice Research Institute (IRRI), Manila, Philippines) and Dr. Zuxun Gong (Shanghai Institute of Biochemistry, Academica Sinica, China). Antisera against GST-RRSV S9 was kindly provided by Dr NM Upadhyaya.

2.14 Immunoblot analysis of RRSV S5 protein

2.14.1 Protein isolation

2.14.1.1 Preparation of total proteins from purified RRSV particles

Purified RRSV viruses (Section 2.2) were mixed with an equal volume of 2x SDS loading buffer (Section 2.1.8.4) and denatured in a boiling water bath for 8 minutes. The denatured viral proteins were separated on 12% SDS-PAGE (Section 2.7.3).

2.14.1.2 Isolation of proteins from Indica rice plant material

About 0.5 g of healthy or infected rice leaves or roots (fresh or freezeed) were homogenised with acid washed sands in 0.5 mL of 1x SDS-PAGE sample buffer (50 mM Tris-HCl, pH 8.8, 2% SDS, 1% β-mercaptoethanol) at room temperature. Homogenized material was centrifuged at 13,000 rpm for 5-10 seconds and supernatants were mixed with an equal volume of 2x SDS loading buffer (Section 2.1.8.4) and
denatured in a boiling water bath for 5 minutes. The denatured proteins were separated on 12% SDS-PAGE (Section 2.7.3).

2.14.1.3 Isolation of proteins from brown planthoppers

Viruliferous or healthy brown planthoppers were frozen dried and stored at -20°C. Five brown planthoppers (viruliferous or healthy) were homogenised in an Eppendorf tube in 100 µL of 1x SDS-PAGE sample buffer (50 mM Tris-HCl, pH 8.8, 2% SDS, 1% β-mercaptoethanol) and centrifuged at 13,000 rpm for 10 seconds. Supernatants were mixed with an equal volume of 2x SDS loading buffer (Section 2.1.8.4) and denatured in a boiling water bath for 5 minutes. The denatured proteins were separated on 12% SDS-PAGE (Section 2.7.3).

2.14.2 Transfer of proteins to nitrocellulose filters

SDS-PAGE protein gels (Section 2.14.1) were soaked and shaken in the electrophoresis transfer buffer (Section 2.1.8.4) for 1.5 hour to remove SDS. Proteins were electrophoresed from the gel onto nitrocellulose in the electrophoresis transfer buffer (Section 2.1.8.4) for 3 hours at 100V, 150 mA or overnight at 30 V at 4°C. Nitrocellulose filters were stained lightly with Amido black (0.1% amido black in 45% methanol, 10% acetic acid) for 15 seconds and destained in water and photographed with Polaroid film 665.

Protein plots of RRSV were kindly provided by Dr W Kositratana from Thailand.
2.14.3 Immunoblot (Shirako and Ehara, 1986)

Nitrocellulose filters with transferred proteins [total proteins from purified virus particles of RRSV (Section 2.14.1.1), Indica rice (Section 2.14.1.2), brown planthoppers (Section 2.14.1.3) or GST-S5 fusion proteins from pS5H2] were soaked with blocking agent (5% nonfat milk powder in TBS-Tween pH 8.0), shaken for 1-2 hours at 37°C and incubated with antisera (antisera against total viral proteins or antisera against GST-S5 fusion protein from pS5H2) in heat sealed bags (10 μL of crude serum in 5 mL milk-TBS-Tween) for 2-3 hours at room temperature or overnight with shaking. Filters were washed 3 times for 15 minutes each with TBS-Tween. Proteins were probed with antibodies using 3 μL of alkaline phosphatase-conjugated goat anti-rabbit (Sigma) in 20 mL TBS-Tween buffer for 2 hours with shaking at room temperature. Filters were then washed 3 times with TBS-Tween (Section 2.1.8.4) and once with BCIP buffer (Section 2.1.8.4). Proteins immunoreacted to antibodies were visualised by incubating filters in 5 mL of chromogenic substrates containing 22 μL of BCIP (Section 2.1.6) and 16.5 mL of NBT (Section 2.1.6) in the dark and waiting for colour development. Reactions were stopped by washing with water. Filters were air-dried for storage.

2.15 Indica rice tissue culture

2.15.1 Growth condition for Indica rice
Indica rice, cultivar *Chinsurah Boro II* (Section 2.1.5) were planted in 15 cm pots. Plants were submerged in plastic boxes and grown in the Quarantine glasshouse and general glasshouse at CSIRO, Division of Plant Industry to produce immature embryos and seeds at 20°C (night) and 28°C (day) period. Insects were monitored and controlled by spreading 1.2 mL / L of Folimat or 1.2 g / L Orthene for aphids, 0.4 mL / L of Ambush for whitefly, and 0.3 mL / L of Avid and 0.4 mL / L of Apollo for two-spotted mite.

A base potting mix for rice was made by mixing soil (3 parts in volume), straw (1 parts in volume), Nitrum (567g / m3), calcium carbonate lime (533g / m3) and Superphosphate (500g / m3), and composted for a period of 12 to 18 months. This was mixed with 2 kg / m3 of calcium carbonate lime and 1 kg / m3 of Abaska. Asbaska contained 15.16% nitrogen (11.76% as ammonia and 3.40% as nitrate), 6.93% phosphorus (5.91% water soluble phosphorus, 1.02% citrate soluble phosphorus) and 5.19% potassium (Potassium Sulphate).

### 2.15.2 Surface sterilisation of Indica rice immature embryos

Immature seeds of Indica rice, cv. *Chinsurah Boro II* were collected when immature embryos were 0.5 to 1 mm long about 10 to 15 days after pollination and kept at 4°C overnight to increase callus formation during culture. About 100 immature seeds were surface sterilised in a 50 mL plastic container with 70% (v/v) ethanol for 30 seconds, then 50 mL of 1 to 2 % of sodium hypochlorite for 1 hour before rinsing 4 times with sterilised distilled
water. The immature seeds were then immersed in sterilised distilled water and left at room temperature (~25°C) overnight. The seeds were surface sterilised again with 50 mL of 1 to 2% of sodium hypochlorite for 1 hour and rinsed 4 times with sterilised distilled water in a laminar flow hood before use.

### 2.15.3 Initiation and maintenance of embryogenic calli of Indica rice

The immature embryos were cut out from immature seeds. About 10 immature embryos were put into each Petri plate, with scutellar surface upwards, on callus induction medium (MSC), a modified MS medium (Murashige & Skoog, 1962) (Table 2.9). After 3 weeks on MSC medium, the embryogenic primary calli were selected and subcultured on callus culture medium (R2C) (Table 2.9) which was modified from R2 medium (Ohira et al., 1973). The embryogenic calli were further selected by eye and subcultured every three weeks on R2C medium until 3 months.

### 2.15.4 Initiation and maintenance of embryogenic cell suspension of Indica rice

About 30 pieces of 3 month old secondary embryogenic calli or Indica rice cv. *Chinsurah Boro II* were selected and placed in a flask containing 10 mL of R2S liquid medium (Table 2.9). Flasks were placed onto a shaker at 80 rpm at 25°C. The cultures were selected by eye and
subcultured once every week in 10 mL of fresh R2S medium at first 3 weeks
or in 50 mL of fresh R2S after.

2.15.5 Isolation and culture of protoplasts of Indica rice

Protoplasts were isolated from 1 to 2 month-old embryogenic
suspension cells by incubating about 1 g suspension cells (3 to 5 days after
subculture) in a 10-cm plastic plate containing 10 mL protoplast enzyme
solution without shaking at 25°C for 3 to 4 hours in the dark. The protoplast
enzyme solution consisted of 1% cellulase RS (Section 2.1.7), 0.1%
Pectolyase Y-23 (Section 2.1.7), 0.1% Driselase (Sigma), 0.6 M mannitol, 5
mM CaCl₂ and 3 mM MES (pH 5.6), which was filter sterilized. The resulting
suspension was then filtered through 300 μm, 100 μm and 50 μm mesh
stainless-steel sieves and washed with an equal volume of protoplast
washing solution (0.6 M mannitol, 5 mM CaCl₂ and 3 mM MES, pH 5.6).
After centrifugation for 5 minutes at 100 × g, protoplasts were resuspended
in 1 to 2 mL of protoplast washing solution and loaded on the top of 5 mL of
0.6 M sucrose for purification of protoplasts. Protoplasts were collected after
centrifugation for 10 minutes at 40 × g, and washed with 5 mL of protoplast
washing solution. After spinning for 5 minutes at 100 × g, protoplasts were
resuspended in TBS buffer (pH 9.0) (3.63 g Tris, 876 mg CaCl₂, 8.78 g NaCl
and 50.50 g mannitol per litre) at 2x10⁶ protoplasts per millilitre for
electroporation.
2.15.6 Regeneration of Indica rice plants from embryogenic calli and suspension cells

Five to 11 month-old embryogenic calli and up to 3 month-old suspension cultures were used for regeneration of Indica rice plants on regeneration medium MSR (Table 2.9). The plantlets were subcultured to fresh MSR once and then to MSG (Table 2.9) for further growth.

2.15.7 Growth and development of Indica rice in soil

Plantlets about 10 to 15 cm high were transplanted into soil (Section 2.15.1) on the misting bench for 2 weeks and then transferred to glasshouse with a temperature at 20°C and 28°C period. Plants were maintained as described in Section 2.15.1 to mature stage.

2.16 Transformation of Indica rice

2.16.1 Transformation of protoplasts of Indica rice by direct gene transfer

Protoplasts were electroporated as described by Last et al. (1991). Five μg of DNA was mixed with 200 μL of protoplast suspension in TBS buffer and electroporated using a 24 μF buz-box (CSIRO, Division of Plant Industry, Australia) at 275 V, 100 ms delay, 5 ms pulse width and 3 pulses. Protoplasts were transferred back to original Eppendorf tube and kept on ice for 1 minute to recover, and washed with 600 μL of washing solution and spun at 100 x g for 5 minutes. Protoplasts were then resuspended in 1 mL of
protoplast culture medium R2P (Table 2.9) and cultured with nurse cells in 4 cm petri dishes for 48 hours at 24°C. The suspension cells of rice Oc line were used as nurse cells by putting 50 μL of cells inside a culture plate insert (0.45 mm, 12 mm diameter) (Millipore) in the center of the Petri dishes.

2.16.2 Particle bombardment of embryogenic calli and suspension cells

2.16.2.1 Preparation of tungsten and gold particles

100 mg of tungsten particles (with an average size of 1.1 micron) (Rhone-Poulenc, France), or 60 mg of gold particles (with an average size of 1.5 - 3.0 mm) (Aldrich) were sonicated 3 times for 3 minutes each before washing 3 times in 1 mL of 100% ethanol and 3 times in 1 mL of sterile distilled water, then resuspended in 50% glycerol (filter sterilised).

2.16.2.2 Preparation of target tissue

For bombardment, 2 to 5 month-old suspension culture cells (4 days after subculture) of Indica rice (Chinsurah Boro II), with cell clumps about 1 mm in size, were placed on a 5.5 cm diameter Whatman #1 filter paper in a funnel. Excess liquid was removed by vacuum filtration. Three to nine month old of embryogenic callus (2 weeks after subculture) of Indica rice (Chinsurah Boro II) were crushed to small cell clumps about 1 to 2 mm in size with a bent spatula.
Approximately 500 mg of suspension cells or crushed calli (about 100 to 150 pieces of calli) were placed in a 3 cm circle in the centre of a R2Os agar plate (Table 2.10) to form a target area and pressed partially into agar medium using a bent spatula. To increase transformation efficiency the target cells were kept on R2Os medium for 4 hours before bombardment and 16 hours after bombardment.

2.16.2.3 Bombardment using a particle inflow gun

Tungsten and gold particle mix was made by mixing an equal of volume of 100 mg / mL of tungsten particles and 60 mg / mL gold particles. 25 μL of particles were coated by sequentially mixing with 2 μL of each plasmid DNA (1 μg / μL) (for co-transformation), 25 μL of 2.5 M CaCl₂ and 5 μL of 0.1 M spermidine free base (Sigma) on ice for 20 minutes. The coated particles were spun down for 2 seconds, washed with 100 μL of 100% ethanol and resuspended in 34 μL of 100% ethanol.

Plates were bombarded with 5 μL of the coated mixed tungsten / gold particles under partial vacuum (-635 mm mercury), at a distance of 18 cm between loaded DNA and target cells, at a pressure of 900 to 1100 kPa, using a particle inflow gun (Finer et al., 1992).

2.16.2.4 Bombardment using a Bio-rad gun

Thirty five μL of 60 mg / mL gold particles were sonicated and coated by sequentially mixing with 10 μL of each plasmid DNA (1 μg / μL), 50 μL of
2.5 M CaCl$_2$ and 20 μL of 0.1 M spermidine free base (Sigma) on ice for 20 minutes. The coated particles were spun down for 2 seconds, washed with 200 μL of 100% ethanol and resuspended in 60 μL of 100% ethanol.

Plates containing embryogenic calli were bombarded with 10 μL of the coated gold particles under partial vacuum (635 mm of mercury), at a distance of 7 cm between loaded DNA (level 2 in gun chamber) and target cells (level 4 in gun chamber), at a 188 kpa rupture disk pressure, using a Model PDS-1000 / He Biolistic Particle Delivery System (Bio-rad).

### 2.16.3 Selection and culture of transformed cells of Indica rice

Bombarded suspension cells or calli were transferred from R2Os (Table 2.10) onto R2C medium (Table 2.9) 16 hours after bombardment and incubated for 7 days at 25°C under natural light before being transferred to the selection medium R2H30 (Table 2.10) containing hygromycin (Section 2.1.6) or R2B1 (Table 2.10) containing Bialaphos (Section 2.1.6). All bombarded cells were then subcultured every two weeks onto fresh R2H50 (Table 2.10) or R2B3 medium (Table 2.10) 3 to 4 times.

### 2.16.4 Regeneration of transformed calli

Calli that were possibly transformed after being bombarded with plasmids containing hph gene were transferred to MSRH30 (Table 2.10) to regenerate transgenic Indica rice plants (T0). The plantlets were subcultured to fresh MSRH30 once and then to MSG (Table 2.10) for further growth.
2.16.5 Transfer of transgenic rice into soil

Plantlets about 10 to 15 cm high were transplanted into soil on the misting bench for 2 weeks and then transferred to glasshouse. Putative transgenic plants were maintained in glasshouses as described in Section 2.14.1.

2.16.6 Analysis of transformed cells and transgenic plants of Indica rice

2.16.6.1 Histological staining for GUS expression

Transient (24 to 48 hours after bombardment) or stable expression of GUS (transformed calli or transgenic plants) was assessed by immersing suspension cells, calli or transgenic plant leaf tissue with X-Gluc staining solution (0.3% X-Gluc in DMSO, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3% Triton X-100, 100 mM sodium phosphate, pH 7.0) (Jefferson et al., 1987). After incubating at 37°C for 15 hours in darkness, staining was observed under a dissecting microscope. Blue expression units (BEUs) were counted and photographed.

2.16.6.2 GUS fluorescence assay

Electroporated protoplasts, 44-48 hours after incubation were collected by centrifugation at 55 x g for 5 minutes at room temperature. The protoplasts or bombarded suspension cells (24-48 hours after incubation)
were then lysed by sonication at 55 W for 5 seconds (for protoplasts) or at 100 W for 4 times of 1 minute (for suspension cells), in 250 μL (for protoplasts) or 500 mL (for suspension cells) of lysis buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl and 10 mM β-mercaptoethanol). The homogenates were then transferred to microfuge tubes and spun for 5 minutes at 4°C. Assay for GUS activity were performed as described (Jefferson, 1987).

### 2.16.6.3 Southern blot analysis of transgenic Indica rice plants

DNA was isolated from transgenic plants (Section 2.3.5.1, 2.3.5.2), separated on 1% agarose gel (Section 2.7.1), transferred onto Hybond Nylon membrane (Section 2.8.2) and hybridised with DNA probes (Section 2.8.4.3). DNA probes were made by labelling purified DNA fragment encoding *hph*, *gus* and RRSV S5 (Section 2.8.1.1).

### 2.16.6.4 Germination test of seeds of T₁ generation

Mature seeds (T₁ seeds) from each T₀ transgenic line were germinated on a 5.5 cm diameter Whatman #1 filter paper moistened with 1/2 MS liquid medium (Murashige and Skoog, 1962) containing 200 mg/L hygromycin. The number of germinated and non-germinated seeds was determined in 10 days.

### 2.16.6.5 PCR and RT-PCR analysis of T₁ transgenic rice plants
DNA or RNA was isolated from the leaves of \( T_1 \) transgenic plants (germinated from \( T_1 \) seeds) (Section 2.3.5.1; 2.3.6) and analysed using PCR (Section 2.4.4) or RT-PCR (Section 2.4.5).

2.1 Introduction

Rice ragged stunt virus (RRSV) is the pathogen that causes rice ragged stunt disease, it is the second most important virus disease of rice after rice tungro virus (Müller-Stoll Foundation, pers. comm.). RRSV was first reported in Indonesia (Trono et al., 1977) and the Philippines (Ling et al., 1978) and has been subsequently discovered in China, India, Japan, Malaysia, Sri Lanka, Taiwan and Thailand (Mills et al., 1983; Beersma & Mills, 1984). RRSV is transmitted by the brown planthopper (Nilaparvata lugens; Hibino et al., 1977) and has a segmented and encapsidated RNA genome comprised of five major and two minor structural segments and ten short RNA genome segments (Haynes et al., 1986; Beersma & Mills, 1984). On the basis of particle morphology and structure, the nature of the RNA genome segments and type of vector, RRSV has recently been classified as a member of the genus Oryzavirus in the family Reoviridae (Mayo & Varanasi, 1992).

The 10 dsRNA segments of the RRSV genome range in size from 2.56 x 10^5 to 0.79 x 10^6 and the nucleotide sequence of one segment (segment 9) has been determined (Oparkya et al., 1992; Kado et al., 1995). Little else is known of the genomic sequences or genomic organization of RRSV except for the 5' and 3' terminal sequences of all ten segments (Yen et al., 1992).
CHAPTER THREE: cDNA SYNTHESIS, CLONING AND SEQUENCING
OF RRSV SEGMENT 5

3.1 Introduction

Rice ragged stunt virus (RRSV) is the pathogen that causes rice ragged stunt disease. It is the second most important virus disease of rice after rice tungro virus (Rockefeller Foundation, pers comm). RRSV was first reported in Indonesia (Hibino et al., 1977) and the Philippines (Ling et al., 1978) and has been subsequently observed in China, India, Japan, Malaysia, Sri Lanka, Taiwan and Thailand (Milne et al., 1982; Boccardo & Milne, 1984). RRSV is transmitted by the brown plant hopper Nilaparvata lugens (Hibino et al., 1977) and has a single-shelled virus particle comprised of five major and two minor structural proteins and ten dsRNA genomic segments (Hagiwara et al., 1986; Boccardo & Milne, 1984). On the basis of particle morphology and structure, the number of dsRNA genomic segments and type of vector, RRSV has recently been classified as a member of the genus Oryzavirus in the family Reoviridae (Mayo & Martelli, 1993).

The 10 dsRNA segments of the RRSV genome range in M(r)s from $2.58 \times 10^6$ to $0.78 \times 10^6$ and the nucleotide sequence of one segment (segment 9) has been determined (Upadhyaya et al., 1995; Uyeda et al., 1995). Little else is known of the genomic sequences or genomic organization of RRSV except for the 5' and 3' terminal sequences of all ten segments (Yan et al., 1992).
In this chapter, I describe the purification of RRSV particles, the analysis of the RRSV genomic dsRNA, the cloning and sequencing of RRSV segment 5 (S5), and a comparison of its sequence with all the sequences in Genbank, including those of some other reoviruses.

3.2 Results

3.2.1 Purification of RRSV particles and RRSV dsRNA

Particles of RRSV were purified, as described in section 2.2, from infected rice plants which were mailed from Thailand. When the particle preparations were negatively stained with uranyl acetate and viewed under the electron microscope, a range of RRSV particles and contaminant bacteriophage particles were observed (Fig. 3.1). Approximately 5% of the RRSV particles had a diameter of approximately 63 nm and 6-7 truncated prism-like spikes on the outer surface (Fig. 3.1 A). These particles are similar to those reported by Milne (1980) and he described these as intact particles. About 20% of the RRSV particles appeared to be inner shells (approximately 47 nm diameter), penetrated by stain and devoid of spikes (Fig. 3.1B), and the remainder were at an intermediate stage of decomposition bearing between 1 and 5 spikes (Fig. 3.1C). Many of these particles were penetrated by stain which indicates that they have lost at least some of their genomic dsRNA, but whether this was before or after negative staining was not determined.
Fig. 3.1 Electron micrograph of negatively stained RRSV particles in purified preparation. (A) Intact particles: about 63 nm in diameter with six to seven spikes. (B) Degraded particles with spikes: approximately 53 to 57 nm in diameter with one or more spikes remaining. An inner shell structure of approximately 47 nm in diameter can also be detected. (C) Particles without spikes: empty viral particles with approximately 47 nm in diameter inner shell structure. (D) Bacteriophage particle.
It was found that extraction of RRSV deRNA directly from infected plants (as described in section 2.1.1) with sodium acetate and phenol chloroform extract deRNA in tissue effectively without losing the deRNA in 1% agarose or 0.8% agarose in one

degrees of purity. In approximately 0.5 times

3.2) but the

Segmentation of the position of the deRNA was

intermediate system 100 nM and the

bands in the

Table 3.4 shows a comparison of the size of RRSV determined by a

study with those determined for a Japanese isolate and the

Indonesian isolate (Kawano et al., 1984; Ikuta et al., 1991). This

comparison shows that there is some variation of the sequence segment size due to the gel system or methods used than variation between these workers.

3.3.2 Cloning of RRSV and sequencing of RRSV 95

deRNA was excised and cloned using an agarose gel. Then, it

and labelled with 32P using T4 polynucleotide kinase. The

labeled
It was found that extraction of RRSV dsRNA directly from infected plants (as described in section 2.3.1) was easier and gave greater yields than extracting the dsRNA from purified virus particles. Electrophoresis of this dsRNA in 1% agarose or 5.5% polyacrylamide gels gave different degrees of resolution. In agarose only 5 bands could be distinguished (Fig. 3.2) but these could be clearly resolved into 9 bands in acrylamide (Fig. 3.3). Segments 7 and 8 are not resolved under this condition. By comparing the positions of the bands with those of molecular weight markers, the sizes of the dsRNA in the bands in both systems were determined (Table 3.1) and, interestingly, found to differ. The two smallest bands in the acrylamide system (S9 and S10) had larger M(r)s than the size determined for the S9 / S10 comigrating band in agarose. Conversely, the M(r)s for the six largest bands in PAGE were smaller than the corresponding bands in agarose. Table 3.1 shows a comparison of the sizes of RRSV determined in this study with those determined for a Japanese (Uyeda et al., 1990b) and an Indonesian isolate (Kawano et al., 1984; Boccardo & Milne, 1984). This comparison shows that there is more variation of measured segment size due to the gel system or methods used than variation between virus isolates.

3.2.2 Cloning of RRSV and sequencing of RRSV S5

S5 dsRNA was excised and eluted from an acrylamide gel then 5' end labelled with $^{32}$P using T4 polynucleotide kinase. The radio-labelled
Fig. 3.2 Profile of genomic dsRNA of RRSV in 1% agarose gel. Lane RRSV: purified RRSV genomic dsRNA. Bands represented different segments are indicated on the left. Lane SPP-1: SPP-1 / EcoRI DNA molecular weight markers. Size of markers is indicated on the right.
Fig. 3.3 Profile of genomic dsRNA of RRSV in 5.5% PAGE. Electrophoresis was at 40 mA at 4°C in 2x TBE (Section 2.1.8). Bands representing different segments are indicated. Samples were subjected to 45 (RRSV 1) or 19 (RRSV 2) hours of electrophoresis. RRSV: Purified RRSV genomic dsRNA; SPP-1: SPP-1 / EcoRI DNA molecular weight marker.
Table 3.1 The size of RRSV dsRNA

<table>
<thead>
<tr>
<th>Segment</th>
<th>1% agarose</th>
<th>5.5% PAGE</th>
<th>Uyeda*</th>
<th>Boccardo*</th>
<th>Kawano*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>3.6</td>
<td>3.3</td>
<td>3.9</td>
<td>3.7</td>
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</tr>
<tr>
<td>S2</td>
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<td>3.3</td>
<td>3.8</td>
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<td>3.7</td>
</tr>
<tr>
<td>S3</td>
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<td>3.2</td>
<td>3.8</td>
<td>3.5</td>
<td>3.6</td>
</tr>
<tr>
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<td>3.1</td>
<td>3.8</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
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<td>2.6</td>
<td>2.1</td>
<td>2.8</td>
<td>2.7</td>
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</tr>
<tr>
<td>S6</td>
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<td>1.8</td>
<td>2.4</td>
<td>2.0</td>
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</tr>
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<td>1.7</td>
<td>2.0</td>
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</tr>
<tr>
<td>S8</td>
<td>1.7</td>
<td>1.7</td>
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<tr>
<td>S9</td>
<td>1.1</td>
<td>1.4</td>
<td>1.2</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>S10</td>
<td>1.1</td>
<td>1.35</td>
<td>1.2</td>
<td>0.7</td>
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</tbody>
</table>

RNA was then used to screen a library (kindly provided by Dr Upadhyaya, see Upadhyaya et al., 1995) of 1424 cDNA clones derived from RRSV dsRNA (all ten segments) using random primers. A total of 25 clones from this library gave strong hybridisation signals with the S5 probe (Fig. 3.4). These clones were digested with a range of different restriction enzymes and the restriction patterns used to order 16 of the clones into one continuous alignment. The restriction enzymes BspE1, HindIII, KpnI, SspI and XbaI were found to be the most useful for this purpose (Fig. 3.5). The contiguous alignment had an estimated size of 2.9 kb which is larger than the predicted size of S5 RNA from either the M(r) in acrylamide (2.1 kb) or the M(r) in agarose (2.6 kb). All 16 aligned clones and 9 unaligned clones were sequenced using the chain termination reaction (Section 2.9) with either γ-32P-labelled or dye-labelled primers. The sequences from these clones were assembled into one contiguous sequence, 2682 bp, (Fig. 3.6) using the GCG sequence analysis suite of programs (Devereaux et al., 1984); all regions were determined in both orientations (Fig. 3.7). The sequence of clone RR292 showed that it contained a double insert and that this was responsible for the discrepancy in size of S5 RNA and the contiguous alignment of cloned fragments by restriction mapping. Both 5' and 3' termini of the contiguous sequence was identical to that determined by Yan et al. (1992) by direct RNA sequencing. This was interpreted to mean that the contiguous sequence extended to the terminal nucleotide at both 5' and 3' ends of RRSV S5 RNA.
Fig. 3.4 Hybridization of RRSV S5-specific clones of RRSV using a 5' end labelled segment 5 dsRNA probe. One of S5-specific clones is indicated by an arrow.
Fig. 3.5 Restriction enzyme mapping of rice ragged stunt virus segment 5.
The cDNA clones of RRSV S5 containing EcoRI adaptors in pUC119, were
analysed with restriction enzymes BspEI, HindIII, KpnI, SspI and XbaI which
are indicated above the line. The number of nucleotides are indicated below
the line represented the full length of RRSV segment 5.
RR517  
RR518  
RR519  
RR299  
RR383  
RR215  
RR322S  
RR474  
RR541  
RR504  
RR532  
RR545  
RR443  
RR327  
RR322  
RR292  

predicted size of RRSV segment 5

<table>
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<th>500</th>
<th>1000</th>
<th>1500</th>
<th>2000</th>
<th>2500</th>
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<tbody>
<tr>
<td>KpnI</td>
<td>BspEI KpnI</td>
<td>HindIII</td>
<td>XbaI</td>
<td>SpeI</td>
</tr>
</tbody>
</table>

KpnI BspEI KpnI HindIII XbaI SpeI
Fig. 3.6. Nucleotide sequence of the positive sense strand of RRSV segment 5. The translational product of one ORF is shown below the sequence. The first initiation (ATG) and termination (TAA) codons are marked by asterisks. The conserved 5' and 3' terminal sequences of RRSV genome segments, hexanucleotides (GATAAA) and tetranucleotides (GTGC) are underlined. The pentanucleotide ---CCCCC--- at 5' terminus and hexanucleotide ---GGGGGG--- at the 3' terminus are shown in bold.
CCTTATGGTTCTGAGAGAATGCCGGCGTGTTTAGGATGTGGAAGATTGTACCCACAACAG
PYGSERSMPAACLGCGRLYQPQ
ATCCTCGCGAACTGTGCGCTCAAGTCAGATGTCCAGCTGCTTCTACATCGGATTGC
ILAKLCAQVRCPAWPSTSC
***
TTAATAGGAGTGAATATTTAATAGGTAATGTACGCTTTTGAAACGCGCGCACCAAAGTC
LIRSE*
CTCTCATGTATCTCACGCCGGTGTGAAGTTTTACGACCAGAGAAGGTTGTGTGTTTGAGCC
GAGAGCTGTGACCGCTCTATGCGGCCGATTTTCGTAATGTGGCTGAGTCCTTGCGCGGTTC
GTGACGTATGCGGCTAGCCCAATGAGAGCTGACAGAGGTGC
Fig. 3.7 Map of the fragments used to determine the nucleotide sequence of the cloned cDNA of rice ragged stunt virus segment 5. The numbers represent the assembled consensus sequence from all the above clones.
Repeated experiments were made to clone the 5' and 3' terminal region of RSV-5 RNA. The reverse transcription (RT-PCR) was performed with oligo(dT) primers annealed to the viral mRNA in the 5' terminal region of RSV-5 RNA. The products from this RT-PCR were cloned and sequenced to determine the terminal sequences.

Another RSV-5 RNA was kindly obtained from Dr. H. Oda. The other genomic RNA segments in both agarose and polyacrylamide gels were transferred to the nylon membrane. The arrows on the diagram represent the possible direction of the assignment of the cDNA clones. The probe encoded the radioactively labeled probe was generated from cloned RSV-5 RNA and hybridized with a nick-translated probe of the RSV genome. The probe reacted with the clone corresponding to 85 (Fig. 3.9). This probe hybridized with the correct sequence and the correct complementarity with the corresponding clone containing the viral genome. The continuous sequence is diagrammed on the right. It shows that there is little sequence homology between this segment and other segments of the RSV genome.

The 9.1 kilobase segment of RSV-5 RNA contains a single open reading frame (ORF) starting at nucleotide 28 and terminating at nucleotide 1047 and encoding a conserved sequence is present at nucleotides 297-298 and nucleotides 297-298 and nucleotides...
Repeated experiments were made to clone the 5' and 3' terminal region of RRSV segment 5. RRSV S5 RNA was used as a template for reverse transcription polymerase chain reaction (RT-PCR) with polyT primers and internal primers (Fig. 3.8). The products from this RT-PCR were cloned and sequenced to find out the nucleotide sequences corresponded to the desired region.

Although RRSV S5 RNA is clearly separated from the other genomic RNA segments on both agarose and polyacrylamide gels, there was the possibility of incorrect assignment of the cDNA clones. Therefore, a radioactively labelled probe was generated from clones RR443 and RR322, which in combination cover the entire contiguous sequence, and incubated with a Northern blot of the RRSV genome. The probe specifically hybridised with the RNA band corresponding to S5 (Fig. 3.9). This demonstrated that the contiguous sequence is derived from S5 and that there is little sequence homology between this segment and the other segments of the RRSV genome.

The contiguous sequence has a single open reading frame (ORF) starting at nucleotide 52 and terminating at nucleotide 2478 and encoding a 91 kDa polypeptide (Fig. 3.7). The first initiation codon has a unusual context (CXXAUGG) which differs from the highly conserved initiation context A/GXXAUGG proposed by Kozak (1987). However, two such conserved sequences are present at nucleotides 247-249 and nucleotides
Fig. 3.8 Map showing the positions of the sequences complementary to the primers used to clone the ends of RRSV S5. RRSV S5 RNA was used as a template for reverse transcription polymerase chain reaction with polyT primers and internal primers. A 20 mer internal primer S55R and a 15 mer polyT primer S55S (Section 2.1.4) were used for the 5' end cloning. A 18 mer internal primer S53S and a 14 mer polyT primer S53R (Section 2.1.4) were used for synthesis of the 3' end sequence.
predicted size of RRSV segment 5

500 1000 1500 2000 2500

S55S S55R  

S53S S53R
Fig. 3.9. RNA blot analysis of RRSV S5. Lane SPP-1: SPP-1 / EcoRI molecular weight marker. Lane RRSV: RRSV genomic dsRNA hybridized with $^{32}$P-labelled cDNA fragments of segment 1, 5, 6, 7, & 10. Lane RRSV S5: RRSV genomic dsRNA hybridized with $^{32}$P-labelled full length ORF from cDNA clone (pS5UC) of RRSV S5 (Chapter 4.2). The size of SPP-1 / EcoRI molecular weight marker is indicated on the left and bands represented different segments are indicated on the right.
696-701 regarding the possibility that it is not a true viral genome sequence.

Analysis of both the 5' and 3' terminal sequences of P3 indicated that the 3' terminal region
consists of a 25 base poly-U stretch followed by a 20 nucleotide sequence.

The putative leader sequence of RRSV S5 was extended using the PASTA program and the default parameter settings, with all parameters at the default level. No evidence of any recognition sequence was detected in any of the entries. The RRSV S5 sequence was compared in more detail using GCG programs, with the complete sequence of the CMV S5 for comparison. No exact identity was revealed.

The sequence encoded by the large ORF of RRSV 82 was also compared with the available acyl-CoA synthetase sequences of proteins encoded by those sequences (Table 3.2). Little or no significant similarity was found. This is in contrast to the S5
699-701 raising the possibility that it is involved in translational regulation. There are no large ORFs in any of the other 5 reading frames.

Analysis of both the 5' and 3' terminal sequences of RRSV S5 revealed an imperfect inverted repeat between the termini (Fig. 3.10). It consists of a 26 base region starting from the hexanucleotide ACUCUC at the 5' terminal region and the quasi-complementary sequence starting with UGAGAG at the 3' terminal region. Similar base pairing structures have been observed in the termini of all reoviruses.

3.2.3 Comparison of RRSV S5 sequence with sequences of other reoviruses

The nucleotide sequence of RRSV S5 was compared, using the GCG program FASTA and the default parameter settings, with all the nucleotide sequences available (July 1995) in GenBank. No substantial similarity was detected with any of the entries. The RRSV S5 sequence was also compared in more detail (using GCG programs, BESTFIT, GAP and FASTA with a variety of parameter settings) with the available sequences of other characterized plant and animal infecting reoviruses. These reoviruses were: WTV, RDV, RBSDV, RGDV, MRDV, BTV and REO 3. The amino acid sequence encoded by the large ORF of RRSV S5 was also compared with the available amino acid sequences of proteins encoded by these reoviruses (Table 3.2). Little sequence similarity was found. This is in contrast to the S5
Fig. 3.10 Nucleotide sequence of the terminal regions of the plus strand of RRSV segment 5. The segment-specific inverted repeat is oriented to indicate potential base-pairing interaction. Conserved 5' and 3' terminal sequences of RRSV genome segments, hexanucleotides (GATAAA) and tetranucleotides (GTGC) are marked by asterisks. The initiation codon (AUG), pentanucleotide ---CCCCC--- at 5' terminus and hexanucleotide ---GGGGGG--- at the 3' terminus are shown in bold. The numbers indicate the position of the nucleotides at the nucleotide sequence of RRSV genome segment 5 (refer to Fig. 3.6). Nucleotides 1 to 46 at 5' terminus and nucleotide 2630 to 2682 at 3' terminus of RRSV genome S5 are used for the indication of base-pairing, other part of the nucleotide sequence is not presented.
Table 3:2 Amino acid sequence identities (%) between BRSV sequence and the nucleotide sequence database.

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*****
GAUAAA GAA--- AAACACAU C----- - AC U
ACUCUC GC UCCCCCA GC G U
CGUAA GGAC------

****
UGAGAG CG GGGGGU UGCU G GGCUU CCUG------
CGUGGAGACAG AUGACC AU------ AUGCAG U C-

2682 2670 2660 2650 2640 2630
Table 3.2 Amino acid sequence identities (%) between RRSV segment 5 protein and the proteins encoded by other reoviruses deduced from the nucleotide sequence database

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Notes: ns: no sequence available. -: no such segment.
proteins of WTV and RDV which are very similar (52.2% identity; Hayashi and Minobe, 1990; Suzuki et al., 1990b).

The Heidelberg Protein predict program predicts the possible occurrence of α-helix, β-sheet and loops structural elements in proteins from their sequence. The patterns made up of these components can be used to identify secondary structure and possibly functional similarities. Using this program, similarly predicted structural patterns were observed for WTV P5, P6 and Pns10 with their counterparts RDV P5, P7 and Pns10 (see Fig. 3.11). Also, such similarities were detected between WTV P9 and RGDV P9; MRDV P6 and RBSDV P7; and among WTV P8, RDV P8 and RGDV P8; and WTV P11, RDV P9 and RGDV P10. However, this program showed no clear structural similarities between the protein encoded by RRSV S5 and that encoded by any of the other genomic dsRNAs of other reoviruses.

3.3 Discussion

The structure and size distribution of RRSV particles of the Thai isolate used in this work appears to be similar to those described for RRSV by Milne (1980) and Kawano et al. (1984). It has a single protein shell decorated with a symmetrical arrangement of 12 projections / spikes on its outer surface giving the particle an approximate diameter of 63 nm. This differs from the particles of phytoreoviruses and fijiviruses which have a double protein shell and a particle diameter of 70 - 80 nm (Streissle and
Fig. 3.11 Similarly predicted structural patterns of P5 proteins encoded by WTV and RDV using the Heidelberg program. H: α-helix, E: β-sheet; L: Loops.
Granados, 1968; Fukushi et al., 1962; Kimura and Shikata, 1968; Milne et al., 1973; Boccardo and Milne, 1975, 1981; Hatta and Francki, 1977; van der Lubbe et al., 1979). Viral particles of RRSV are similar to the subviral particles (SVPs) of Fijiviruses. Such SVPs have lost their spike caps and outer protein shells, but retain their spikes and inner protein shells.

The particles of RRSV prepared in this study appeared to be in different states. While some particles appeared to have 12 spikes, others had fewer spikes, and others had none. This indicates that the spikes are gradually lost from core of virus particles. This population of particle states is probably caused by degradation of the particles during transport between Thailand and Australia, as this normally took about 5 to 7 days, and the material was in poor condition when it arrived. No RRSV particles could be obtained from material that had been frozen before or after transport. However, unfrozen, fresh-looking infected rice plants arrived occasionally and yielded virus preparations such as shown in Fig. 3.1. This result is consistent with that of Kawano et al. (1983) who could not obtain RRSV particles from frozen materials.

Different M(r)s estimations were obtained for the RRSV dsRNA segments when 1% agarose or 5.5% polyacrylamide gels were used. Similar discrepancies are noticeable from the M(r)s determinations of RRSV by other workers (Boccardo and Milne, 1985; Kawano et al., 1984). The sequence data for segment 9 of RRSV (1132 bp) (Uyeda et al., 1995; Upadhyaya et al., 1995) and that for segment 5 (2682 bp) (this work, Fig.
3.6) show that the size determination made in 1% agarose gel / 1x TBE was more accurate.

The size of RRSV segment 5 fragment determined by restriction enzyme mapping of cDNA clones was larger than that calculated from the migration of S5 dsRNA in gels. This was because one clone (RR292) contained not only cDNA from S5 but also cDNA from another segment of RRSV. In fact, four clones contained inserts that were the S5 sequence and other RRSV segments. These non-S5 sequences were identified during the sequence alignment of the S5 clones.

The compiled nucleotide sequence of genome segment 5 of RRSV has a single large open reading frame (ORF) that encodes a 91 kDa protein. The S5s of two other plant-infecting reoviruses WTV and RDV have been shown from their nucleotide sequences to encode 90.53 kDa and 91.06 kDa proteins respectively, which are very similar; the encoded amino acid sequences are 52.2% identical (Suzuki et al., 1990; Hayashi and Minobe, 1990; Nuss & Dall, 1990). Despite the similarities in size of the S5 proteins encoded by RRSV, WTV and RDV, there is no convincing similarity either of their amino acid or nucleotide sequences. The protein encoded by segment 5 of WTV or RDV is a component of the particle outer shell. The lack of sequence homology of RRSV with that of WTV and RDV and the apparent lack of an outer shell of RRSV virus particles leaves the function of segment 5 of RRSV unresolved. Experiments to analyse this further are reported in Chapter 4.
An imperfect inverted repeat sequence was found in the terminal sequences of RRSV S5 (Fig. 3.10), as has also been found in some other RRSV segments (Upadhyaya, Li and Waterhouse, unpublished). Conserved complementarity motifs are also found in similar positions in genome segments of RDV, RGDV and WTV (Anzola et al., 1987; Kudo et al., 1991). It has been suggested that these regions are segment-specific and may play a significant role in molecular sorting and protein-RNA interaction during the replication and packaging of viral transcripts.

For a number of years RRSV has been regarded as sufficiently different in morphology, physico-chemical properties from other plant infecting reoviruses to warrant its classification into its own separate genus, Oryzavirus. The lack of sequence similarity of RRSV S5 with any other reovirus supports this classification.
CHAPTER FOUR: FUNCTIONAL ANALYSIS OF SEGMENT 5 OF RICE RAGGED STUNT VIRUS

4.1 Introduction

RRSV viral particles are composed of at least seven proteins, each presumably encoded by the different genomic dsRNA segments. The subviral locations of five of these proteins have been assigned (Hagiwara et al., 1986). The core particle consists of proteins of 145, 137 and 72 kDa while the spikes are composed of 47 and 37 kDa proteins. The location of the two minor components, 118 and 50 kDa proteins, is unknown.

There is limited information on the relationship of the RRSV segments to viral particle components. RRSV segment 9 (S9) was shown to encode a 39 kDa protein (Upadhyaya et al., 1995; Uyeda et al., 1995). In this chapter, I report experiments in which I expressed different portions of S5 encoded protein in bacteria, raised antisera against one of the protein fragments, and detected the presence of the S5 encoded protein in the RRSV virus particle. This virion protein was also detected both in infected plant material and in viruliferous brown planthoppers.

4.2 Results

4.2.1 Construction of clones for expression of RRSV S5 fusion proteins
Six fusion protein expression clones pS5FL, pS5H1, pS5H2, pS5H1+2, pS5H1+3 and pS5FLa (Table 2.7; Fig. 4.2A) were constructed for this study. A full length S5 ORF (pS5UC) (Fig. 4.1) was created by joining two large overlapping clones (RR443 and RR322) which was then subcloned into the EcoRI site of the kanamycin resistance plasmid, pJKKmf(-) (Kirschman and Cramer, 1988) to create pS5JK. The BamHI-EcoRI fragment (first hydrolysed with BamHI and then partially hydrolysed with EcoRI) or an EcoRI fragment from pS5JK was cloned into a BamHI-EcoRI-hydrolysed or an EcoRI-hydrolysed pGEX-3X vector (Smith and Johnson, 1988). This created pS5FL (Table 2.7, Fig. 4.2A) which contains a full length sense RRSV S5 ORF and pS5FLa (Table 2.7, Fig. 4.2A) which contains a full length antisense RRSV S5 ORF. There was no stop codon before the initiation codon AUG at 27 to 29 for constructs, pS5FL.

Different fusion protein clones carrying various hydrophilic regions were made to determine which combination could produce the greatest yields in *E. coli*. Using a hydrophilicity map of RRSV S5 protein (Fig. 4.2B), the protein can be divided into three hydrophilic regions (H1, H2 and H3) and two hydrophobic regions (N1 and N2). Clones pS5H1, pS5H1+2 and pS5H1+3 were constructed by various internal deletions within the ORF of pS5FL by hydrolysing with either SnaBI / Nrul, Xbal / SpeI, or SnaBI / Xbal, and religating the vector without the excised fragment at low DNA concentration.
Fig. 4.1 Construction of full length clone of RRSV S5. A clone, pS5UC, containing the full length ORF of RRSV S5 was constructed by ligating a BamHI / BspEI fragment from a clone RR443 into a BamHI / BspEI-hydrolysed clone RR322. B: BamHI; E: EcoRI; Bs: BspEI.
Fig. 4.2 Constructs for the expression of RRSV S5 protein. (A). The clones containing full length sense ORF (pS5FL), full length antisense ORF (pS5FLa), hydrophilic region (s) 1 and 2 (pS5H1+2), 1 and 3 (pS5H1+3), 1 (pS5H1) or 2 (pS5H2). Restriction enzymes SnaBl, XbaI, SpeI and NruI used for internal deletion were shown. (B). A hydrophilicity map of RRSV S5 protein was created using a Macintosh protein-toolbox plot software. The three regions termed hydrophilic regions H1, H2 and H3, and two regions termed hydrophobic regions N1 and N2 are shown underneath.
(A)

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(B)

Hydrophilicity

Scale = Kyte-Doolittle
Because these clones were constructed using convenient restriction enzyme sites, some contained both hydrophilic and hydrophobic regions. Construct pS5H1 contained hydrophilic region 1 and part of hydrophobic region 1, pS5H1+2 contained hydrophilic regions 1 and 2 and hydrophobic regions 1 and 2, and pS5H1+3 contained hydrophilic regions 1 and 3 and part of hydrophobic region 1 (Fig. 4.2A & B). The construct pS5H2, containing only hydrophilic region 2, was obtained by cloning the appropriate fragment from cDNA clone RR541 into pGEX-2T vector (Smith and Johnson, 1988).

Inferring from the nucleotide sequence, the expected sizes of fusion proteins from pS5FL, pS5H1+2, pS5H1+3, pS5H1 or pS5H2 are 117, 90, 76, 54.6 and 49 kDa respectively (Table 4.1).

4.2.2 Expression of RRSV S5 ORF in E. coli

The full length fusion protein (117 kDa), which includes a 91 kDa protein encoded by RRSV segment 5 and a 26 kDa GST protein derived from vector pGEX-3X, could not be detected in a culture of E. coli strain JM109 transformed with construct pS5FL or pS5FLa and kept overnight at 37°C, and analysed by SDS-PAGE (Fig. 4.3A). Therefore, plasmid pS5FL was transformed into two low proteinase E. coli lines (AD202 and BL21) and grown at 37°C or 28°C either in the present or absence of glucose. However, under none of these conditions, was the full length protein detected (Fig. 4.3A, 4.4; Table 4.2).
Fig. 4.3 Comparison of the expression of fusion proteins from RRSV S5 clones grown under different culture conditions using different cell lines. Proteins were separated in 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue. The fusion proteins expressed from pS5H2 and pS5FL were in *E. coli* JM109, AD202 and BL21 at 37°C in the absence (A) or presence (B) of 1% glucose. The sizes of the Rainbow coloured molecular weight markers are shown on the left. The expected size of full length fusion protein (S5 protein and GST protein) from pS5FL (117 kDa) and pS5H2 (49 kDa) are shown on the right.
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</table>
Fig. 4.4 Comparing expression of fusion proteins from RRSV S5 clones grown under different culture conditions and in different cell lines. Proteins were separated in 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue. The fusion proteins expressed from pS5H2 and pS5FL were in E. coli JM109, AD202 and BL21 at 28°C in the absence (A) or presence (B) of 1% glucose. The sizes of the Rainbow coloured molecular weight markers are shown on the left. The expected size of full length fusion protein (S5 protein and GST protein) from pS5FL (117 kDa) and pS5H2 (49 kDa) are shown on the right.
Table 4.1 Predicted size and yields of fusion proteins

<table>
<thead>
<tr>
<th>Clones</th>
<th>Encoded hydrophilic domain</th>
<th>Predicted protein size (kDa)</th>
<th>Size of RRSV S5 component (kDa)</th>
<th>Yield of Fusion proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS5FL</td>
<td>1, 2 and 3</td>
<td>117</td>
<td>91 (808 aa)</td>
<td>undetectable</td>
</tr>
<tr>
<td>pS5H1+2</td>
<td>1 and 2</td>
<td>90</td>
<td>64 (670 aa)</td>
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</tr>
<tr>
<td>pS5H1+3</td>
<td>1 and 3</td>
<td>76</td>
<td>50 (449 aa)</td>
<td>moderate</td>
</tr>
<tr>
<td>pS5H1</td>
<td>1</td>
<td>54.6</td>
<td>28.6 (269 aa)</td>
<td>low</td>
</tr>
<tr>
<td>pS5H2</td>
<td>2</td>
<td>49</td>
<td>23 (246 aa)</td>
<td>high</td>
</tr>
<tr>
<td>pS5FLa</td>
<td>1, 2 and 3</td>
<td>117</td>
<td>91 (808 aa)</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

Note: *E. coli* JM109 transformed with the indicated constructs was used for expression of the fusion proteins by culturing in the presence of 1% glucose at 28°C. The purified proteins were separated in 12% SDS-PAGE and stained with Coomssie Brilliant Blue 250.
Table 4.2 Comparison of *E. coli* cell lines, glucose and culture temperature on the yield of fusion proteins expressed from pS5FL and pS5H2

<table>
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</tr>
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</tr>
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<td>Absence</td>
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<td>AD</td>
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<td>pS5FL</td>
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<tr>
<td>pS5H2</td>
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<td>45</td>
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</table>

Note: The fusion proteins were expressed from pS5FL and pS5H2 in three *E. coli* cell lines at 28°C or 37°C in the presence or absence of 1% glucose. The stained protein gels were scanned and the amounts (in arbitrary units) and sizes of proteins estimated as described in Section 2.7.3.
A series of constructs pS5FL, pS5FLa, pS5H1, pS5H2, pS5H1+2 and pS5H1+3 which contained different portions of RRSV S5 were transformed into *E. coli* cell line JM109. Construct pS5H2 gave most protein, pS5H1+3 produced a moderate amount of protein and pS5H1 and pS5FL produced very little protein. There was no detectable protein produced from the constructs pS5HFLa, and pS5H1+2 (Fig. 4.5).

The highest yield of fusion protein (1 mg / 2 L culture) was obtained from construct pS5H2 (in *E. coli* JM109), containing hydrophilic region 2, when grown at 28°C in the presence of 1% glucose (Fig. 4.3, 4.4; Table 4.2). Under these conditions, *E. coli* cells produced more fusion protein (49 kDa) with fewer degradation products.

### 4.2.3 Antibody preparation and immunodetection of RRSV S5 protein

More than 50 mL of antisera was obtained from a rabbit injected four times (0.5 mg / injection) with the fusion protein produced by pS5H2. Antisera collected two weeks after the last injection reacted strongly in immunoblots with the fusion protein produced from constructs pS5H2 (Fig. 4.6).

Immunoblots of proteins from two different preparations of RRSV particles were probed with either the S5H2 antiserum or antiserum against total RRSV viral protein. As a control immunoblots of these preparations were also probed with an antiserum raised against a pGEX fusion protein containing the polypeptide encoded by RRSV S9. Ten or nine polypeptides
Fig. 4.5 Comparing expression of fusion proteins from various RRSV S5 clones. Proteins were separated in 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The fusion proteins expressed from pS5FL, pS5H2, pS5FLa, pS5H1+2, pS5H1+3, pS5H1 (Table 2.7; Fig. 4.2) and pGEX-3X (Table 2.2) grown in E. coli JM109 at 28°C in the presence of 1% glucose. The sizes of the Rainbow coloured molecular weight markers are shown on the left. The sizes of full length fusion proteins (S5+GST protein) from pS5FL (117 kDa), pS5H1+3 (76 kDa) and pS5H2 (49 kDa) are shown on the right.
Fig. 4.6 Immunoblot analysis of GST-S5H2 fusion protein with antisera against GST-S5H2. Protein produced from pS5H2 was separated in 12% SDS-PAGE gels and immunodetected with antisera. Total: total proteins extracted from *E. coli*, JM109 cells containing pS5H2 plasmid. GST-S5H2: purified GST-S5H2 protein.
were detected in the protein profiles of these virus preparations, never present by the antiserum against total PRSV 50 protein (Fig. 4.7, 4.8, Table 4.3).

The immunoblots probed with the GST-RSSV 50 antiserum gave two different banding patterns (summarized in Table 4.4). Three low abundance polypeptides were detected in the first virus preparation (Fig. 4.7, Table 4.4), and seven polypeptides were detected in the second extract (Table 4.4).

Antibodies raised against the GST-RSSV 50 antiserum specifically recognized the PRSV 50 antigen at 39 kDa protein in the first extract and with one of bands recognized by the GST-RSSV 50 antiserum in the second extract. It is noticeable that there were no antigens that are recognized by anti-GST antibodies.

The GST-RSSV 50 antiserum was then used for immunodetection of the PRSV 50 protein in viruliferous brown planthopper and infected rice plants. While the antiserum reacted with multiple proteins in healthy insect extracts, it specifically recognized two proteins (a minor one at 91 kDa and an abundant one of 64 kDa (Fig. 4.9), Table 4.4). The GST-RSSV 50 antiserum did not react with any proteins in healthy rice root extracts and only reacted with a 44 kDa protein in healthy rice leaf extracts. However, a faint band of ~31 kDa was detected in extracts from infected rice leaves (Fig. 4.9) and four bands (31, 57, 68 and 44 kDa) were detected in extracts from infected root tissue (Fig. 4.10, Table 4.4).

4.3 Discussion

Several proteins have been reported to exist in PRSV and variants (Table 4.3). The variation in the molecular weight reported for these proteins...
were detected in the protein profile of these virus preparations, when probed by the antiserum against total RRSV viral protein (Fig. 4.7-4.8; Table 4.3). The immunoblots probed with the RRSV S5 antiserum gave two different banding patterns (summarised in Table 4.4). Three low abundance polypeptides were detected in the first virus preparation (Fig. 4.7; Table 4.4), and seven polypeptides in the second preparation (Fig. 4.8; Table 4.4).

Antibodies raised against GST-RRSV S9 recognised the RRSV S9 encoded 39 kDa protein in the blots but it did not reacted with any of bands recognised by the GST-S5H2 antiserum. This demonstrates that there are no antigens that are recognized by anti-GST antibodies.

The GST-RRSV S5 antiserum was then used for immunodetection of the RRSV S5 protein in viruliferous brown planthoppers and infected rice plants. While the antiserum reacted to some proteins in healthy insect extracts, it specifically recognized two proteins (a minor one of 91 kDa and an abundant one of 44 kDa) (Fig. 4.9; Table 4.4). The GST-RRSV S5 antiserum did not reacted with any proteins in healthy rice root extracts and only reacted with a 44 kDa protein in healthy rice leaf extracts. However, a faint band of ~91 kDa was detected in extracts from infected rice leaves (Fig. 4.9) and four bands (91, 67, 55 and 44 kDa) were detected in extracts from infected root tissue (Fig. 4.10; Table 4.4).

4.3 Discussion

Several proteins have been reported to exist in RRSV viral particles (Table 4.3). The variation in the molecular weight reported for these proteins
Fig. 4.7 Immunoblot analysis of viral proteins of the first RRSV preparation. The viral proteins were separated in 10% SDS-PAGE gels and immunodetected with antisera. Marker: Rainbow coloured molecular weight markers, 14,300-20,000. Coomassie Blue: Purified virus particle stained with Coomassie Brilliant Blue. Total antisera: Viral preparation probed with antisera against total RRSV viral proteins. S5 antisera: Viral proteins probed with antisera prepared for GST-RRSV S5 fusion protein. S9 antisera: Viral proteins probed with antisera prepared for GST-RRSV S9 fusion protein. The sizes of the Rainbow coloured molecular markers (kDa) are shown on the left. The sizes of intact viral protein (91 kDa) and two small size products (67 and 44 kDa) which reacted with antisera against the GST-RRSV S5 fusion protein are shown.
Fig. 4.8 Immunoblot analysis of viral proteins of the second RRSV preparation. The viral proteins were separated in 10% SDS-PAGE gels and immunodetected with antisera. Marker: Rainbow coloured molecular weight markers, 14,300-20,000. Total antisera: Viral preparation probed with antisera against total RRSV viral proteins. S5 antisera: Viral proteins probed with antisera prepared for GST-RRSV S5 fusion protein. S9 antisera: Viral proteins probed with antisera prepared for GST-RRSV S9 fusion protein. The sizes of the Rainbow coloured molecular markers (kDa) is shown on the left. The size of intact viral protein (91 kDa) and two small size products (67 and 44 kDa) which immunoreacted with antisera against the GST-RRSV S5 fusion protein are shown.
Fig. 4.9 Immunoblot analysis of viral proteins of viruliferous brown planthoppers. Proteins from viruliferous and healthy brown planthoppers were separated in 12% SDS-PAGE and analysed with antisera prepared for GST-RRSV S5 fusion protein. Marker: Rainbow coloured molecular markers, 14,300-200,000. Viruliferous insect: Proteins from viruliferous brown planthoppers probed with antisera against the GST-RRSV S5 fusion protein. Healthy insect: Proteins from healthy brown planthoppers probed with antisera against GST-RRSV S5 fusion protein. The sizes of the Rainbow coloured molecular markers is shown on the left. The sizes of two proteins (91 and 44 kDa) which reacted with antisera against the GST-RRSV S5 fusion protein is shown on the right.
Fig. 4.10 Immunoblot analysis of viral proteins of infected rice plant. Total proteins from infected and healthy rice leaves or roots were separated in 12% SDS-PAGE gel and immunodetected with antisera prepared for GST-RRSV S5 fusion protein. Marker: Rainbow coloured molecular markers, 14,300-200,000. Infected leaf: Proteins from infected leaves. Healthy leaf: Proteins from healthy leaves. Infected root: Proteins from infected roots. Healthy root: Proteins from healthy roots. The sizes of the Rainbow coloured molecular markers are shown on the left. The size of four proteins (91, 67, 55, and 44 kDa) which immunoreacted with antisera against the GST-RRSV S5 fusion protein are shown on the right.
Table 4.8 Proteins detected in PRRV viral particles

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<th>Infected root</th>
<th>Healthy root</th>
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Note: Proteins detected in PRRV viral particles. Protein bands were visualized by Coomassie blue staining.
Table 4.3 Proteins detected in RRSV viral particles

<table>
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</tbody>
</table>

Note: Hagiwara: Hagiwara et al., 1986. Chen: Chen et al., 1989b. Lu: Lu et al., 1987. Virus 1 and 2: Virus preparation 1 and 2 in this study. Numbers in bold indicate the proteins that were in the largest concentrations major proteins. Proteins in this study were detected by antisera against total viral proteins of RRSV particles, however, proteins in other works were identified in Coomassie blue stained SDS-PAGE.
Table 4.4 Polypeptides recognised by antisera against GST-RRSV S5 in the protein profiles of two different RRSV preparations, viruliferous brown planthoppers and RRSV-infected rice leaf and roots

<table>
<thead>
<tr>
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<th>Root ##</th>
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<td>7</td>
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</tr>
</tbody>
</table>

Note: * Virus preparation 1 and 2. ** RRSV viruliferous brown planthoppers. # RRSV-infected rice leaf material. ## RRSV-infected rice root material.
may result from the use of the different gel systems and reference proteins. For example, Hagiwara et al. (1986) used the protein profile of RDV as their standard when evaluating the size of RRSV proteins.

From this study, the major RRSV virion proteins are 130, 67, 46, 44 and 39 kDa. These size estimates are closest to those determined by Hagiwara et al. (1986) and Chen et al. (1989). It is possible that the 130 kDa protein band in my study represents two proteins and that these proteins were separated in the gel systems of other workers as 145 and 137 kDa (Hagiwara et al., 1986) and 129 and 123 kDa (Chen et al., 1989). The 44 kDa protein in my study gave the strongest signal when probed with antiserum against whole virion proteins. However, this protein was only of moderate abundance as estimated by Coomassie Brilliant Blue staining. This suggests that the 44 kDa protein is the most antigenic virion protein of RRSV.

I intended to express the protein of entire ORF of RRSV S5 using the expression vector, pGEX and to produce antibodies against this fusion protein. However, it was difficult to obtain sufficient protein from this construct. Changing growth conditions or using proteinase deficient strains of E. coli made little difference. Therefore, different segments of the ORF were introduced into the pGEX plasmid. The yield of recoverable fusion proteins from clones was closely related to the overall hydrophilicity of the encoded fusion protein. Clones pS5H2 and pS5H1+3 which encode largely
hydrophilic protein yielded reasonable amounts, while the other clones produced little or no fusion protein.

Fusion protein from pS5H2 was used to raise an antiserum which specifically detected a 91 kDa protein in the RRSV particle protein profile. The size of this protein corresponds well to the inferred size of the protein encoded by the large ORF of RRSV S5. The antiserum detected some smaller proteins that are probably degradation products from the 91 kDa protein. Interestingly, Lu et al. translated RRSV S5 RNA in vitro and detected a 90 kDa protein and two smaller products (Lu et al., 1990).

The function of the viral proteins and the genomic segments from which they are translated are known for a few reoviruses. Using these data (Table 4.5 & 4.6) it is possible to predict the functions of the proteins encoded by the different segments of RRSV. Segments 3, 8, 5, 9, and 2 of RRSV are possibly the structural proteins ISL, ISS, OSL, OSS and SP respectively. The prediction that RRSV P5 is an outer coat protein is consistent with my data showing that it is present in virus particle preparations. However, it seemingly contradicts the proposals of Milne (1980) and Hagiwara et al. (1986) that RRSV does not possess an outer shell. I suggest that RRSV S5 encodes a protein that is present in the particle (probably in small amounts) and is packaged with the ISL, ISS and OSS into a single layer composed of four subunits rather than two layers each of two units (Fig. 4.11). This would have the effect of producing a particle with a single shelled appearance rather than the classic reovirus double shelled structure. This prediction could be tested by immuno-gold
labelling experiments using P5 specific antisera. I have sent my P5 antiserum to colleagues in China who will do such experiments.

<table>
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Fig. 4.11 Schematic representation of typical double-shelled particle and RRSV particle. (A). A typical double shelled particle. (B). A proposed RRSV particle. Five proteins, ISL, ISS, OSL, OSS and SP were labelled. The location of OSL and OSS was difference between these particles.
Table 4.6 Coding assignment of proteins of Reoviridae

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**REO 3:**

**BTV:**

**ROTA-A:**

**WTV:**

**RDV:**
CHAPTER FIVE: GENETIC TRANSFORMATION OF INDICA RICE

5.1 Introduction

Rice provides the staple food to more than a third of the world’s population, particularly in East and South East Asia (David, 1991) and Indica rice accounts for 80% of cultivated rice. Therefore, improvement of this crop by genetic engineering could be of profound importance. Genetic transformation of major cereal crops has been achieved recently, eg, wheat (Vasil et al., 1992; Weeks et al., 1993), sugarcane (Bower and Birch, 1992), maize (Rhodes et al., 1988; Fromm et al., 1990; Gordon-Kamm et al., 1990) and rice (Shimamoto et al., 1989). Indica rice transformation has been achieved by a few laboratories by directly transferring gene to protoplasts (Datta et al., 1990,1992; Peng et al., 1992), particle bombardment (Christou et al., 1991) or delivering genes by Agrobacterium (Chan et al., 1992). However, such transformation is still considered difficult. Besides target tissue, two factors which are crucial for Indica rice transformation are the use of suitable promoters and efficient selectable marker genes.

Several promoters have been used to control the expression of selectable marker and reporter genes in transgenic rice. These promoters include: CaMV35S promoter, the promoter of the cauliflower mosaic virus 35S RNA transcript (Cao et al., 1992; Christou et al., 1991; Shimamoto et al., 1989; Battraw and Hall, 1992; Meijer et al., 1991), RolC promoter, the promoter of the soil bacterium Agrobacterium rhizogenes harbouring Ri
plasmid ORF12 gene (RolC) (Matsuki et al., 1989), Emu promoter, a modified maize alcohol dehydrogenase 1 promoter and first intron (Chamberlain et al., 1994), Act1 promoter, the promoter of rice actin 1 gene (Cao et al., 1992; Zhang et al., 1991), and Ubi1 promoter, the promoter maize ubiquitin 1 gene (Toki et al., 1992). The Emu promoter gave GUS expression levels more than 20 times higher than from the CaMV35S promoter in rice protoplasts (Last et al., 1991). In a similar system, GUS expression levels obtained using the Act1 promoter were double that from the Adh1 promoter (McElroy et al., 1990). The Ubi1 promoter gave a 10 or 34 fold increase in transient gene expression when electroporated into maize protoplasts or in bombarded wheat cell suspensions when compared to a CaMV35S promoter or Adh1 promoter (Christensen et al., 1992; Taylor et al., 1993). In the above reports these promoters were not compared under identical conditions and there is no comprehensive comparison of these promoters for Indica rice transformation.

A number of selectable marker genes have been used for cereal transformation (see reviews by McElroy and Brettell, 1994; Vasil, 1994). These genes include the neomycin phosphotransferase II gene of E. coli (nptII) which confers resistance to the antibiotic kanamycin (Fromm et al., 1986; Lorz et al., 1985) or G418 (Potrykus et al., 1985), the hygromycin phosphotransferase gene of E. coli (hph) which confers resistance to hygromycin (Hauptmann et al., 1988; Horn et al., 1988; Shimamoto et al., 1989), the mouse dihydrofolate reductase gene (dhfr) of E. coli which
confers resistance to methotrexate (Hauptmann et al., 1988), the phosphinothricin acetyltransferase gene of *Streptomyces hygroscopicus* (*bar*) which confers resistance to the herbicide basta (glufosinate, bialaphos) (Fromm et al., 1990; Gordon-Kamm et al., 1990), and a mutant acetolactate synthase gene of *Arabidopsis thaliana* (*csr-1*) which confers resistance to sulfonylurea herbicides (Fromm et al., 1990). However, the *bar* gene, and to a lesser degree the *hph* gene, has been the most commonly used in recent years. To my knowledge a comparison of the efficacy of these selectable marker genes in Indica rice has not been reported.

In this chapter I describe the relative strengths of different promoters for transient gene expression in Indica rice, a comparison of the selectable marker genes, *hph* or *bar* (driven by the various promoters) for stable transformation, and a method for obtaining fertile transgenic Indica rice plants by particle bombardment using a particle inflow gun (Section 2.16.2.3) and a Bio-Rad gun (Section 2.16.2.4).

### 5.2 Results

#### 5.2.1 Callus, suspension cell and protoplast culture of Indica rice cv. *Chinsurah Boro II*

About 5% of the immature embryos isolated from plants grown in the glasshouse were contaminated with fungi and bacteria despite being sterilised with 1-2% of sodium hypochlorite for 1 h. This problem can be solved by rinsing the treated immature seeds in sterile distilled water then...
repeating the hypochlorite treatment. After 2 to 3 weeks initiation on MSC medium (Table 2.9), primary calli (Fig. 5.1B) were obtained from more than 70% of the immature embryos (Fig. 5.1A) of Indica rice, cv. Chinsurah Boro II. Most calli grew slowly and gradually became brown after subculture onto R2C medium, however, some calli continued to grow well (Fig. 5.1C). Careful selection and addition of AOA and PVP (Table 2.9) into the culture media were useful in reducing further browning. After 4 to 5 subcultures, about 5% of the original primary calli were uncoloured and growing rapidly. After a further 1 to 2 months of subculture, and visual selection, the embryogenic calli were of sufficient quality (rapidly growing, uncoloured and granular) to initiate suspension cultures or to be bombarded directly. Cell suspensions were obtained after 1 to 2 months culture in the liquid R2S medium (Table 2.9). The resulting embryogenic calli and suspension cells when transferred to MSG medium readily regenerated into whole plants of which 80%, derived from 9 or 11-month-old cultures, produced fertile seed in the glasshouse. 

5.2.2 Comparison of different promoters for gene expression in electroporated protoplasts and bombarded suspension cells

Plasmids with an Escherichia coli β-glucuronidase gene (gus) under the control of various promoters, pCaMV35S-gus, pEmu-gus, pRolC-gus and pAct1-gus (Table 2.3) were electroporated into protoplasts. Similarly, pCaMV35S-gus, pEmu-gus, pAct1-gus and pUbi1-gus (Table 2.3), were used to bombard suspension cells. With the exception of RolC, GUS
Fig. 5.1 Stages in the production of regenerated fertile transgenic plants of Indica rice from bombarded embryogenic calli. (A). Immature embryo of Indica rice cv. *Chinsurah Boro II*. (B). Primary calli derived from immature embryo. (C). Secondary calli. (D). Embryogenic calli bombarded with plasmids, p*Ubi1-gus* and p*Emu-hph* transiently expressing GUS. (E). Expression of GUS in stably transformed cell cluster 2 weeks after selection on medium containing 30 mg / L hygromycin B. (F). Callus line formed from *hph* resistant cell cluster 6 weeks to 2 months after selection on medium containing 30 or 50 mg / L hygromycin B. (G). Expression of GUS in a *hph* resistant callus line. (H). Plantlets regenerated from *hph* resistant callus line on medium containing 30 mg / L hygromycin B. (I). Transgenic Indica rice plants in the soil. (J). Expression of GUS in the leaves of a transgenic plant. (K). Fertile transgenic plants of Indica rice cv. *Chinsurah Boro II*. 
expression was detectable under the control of all the promoters tested. *RolC* has been reported to give expression only in vascular cells. The constitutive promoters *Act1* and *Ubi1* gave the highest expression levels (Fig. 5.2). The order of promoter strength (from strong to weak) in undifferentiated cells of Indica rice *Chinsurah Boroll* was *Ubi1>**Act1>*Emu>*CaMV35S>*RolC*. When compared to CaMV35S promoter, *Ubi1*, *Act1* and *Emu* gave 12, 8 or 2 fold higher expression levels respectively. The measured GUS activity was higher in electroporated protoplasts samples than in samples of bombarded suspension cells (Fig. 5.2). This is presumably because many more cells are transformed with DNA by the protoplast treatment than by bombarding the callus.

### 5.2.3 Comparison of *hph* and *bar* genes driven by various promoters for stable transformation

Embryogenic calli were bombarded with a mixture of *pUbi1-gus* and a range of plasmids containing either the *hph* or *bar* gene controlled by various promoters. Staining the calli for GUS activity one day after shooting gave more than three hundred blue expression units (BEUs) per Petri dish of calli bombarded with each plasmid combination (Table 5.1, Fig. 5.3 A, D, G & J). Although most of the BEUs diminished or disappeared, a few of the BEUs from calli bombarded with *pUbi1-gus* and any of the three *hph* containing plasmids gradually increased in size over 2 weeks of selection (Table 5.1, Fig. 5.3 B, E & H). After 4 weeks on selection media they
Fig. 5.2 Comparisons of promoter activity assessed by transient expression of gus gene in protoplasts and suspension cells of Indica rice. (A) In two separate experiments (experiment 1 blue bars; experiment 2 yellow bars), protoplasts were isolated from 1 to 2 month old embryogenic suspension cells of Indica rice cv. Chinsurah Boro II and electroporated with the gus gene constructs, pRolC-gus, pCaMV35S-gus, pEmu-gus, and pAct1-gus. After 48 hours incubation, protein was extracted from electroporated protoplasts and the GUS activity was measured. (B). The gus gene constructs containing various promoters, pCaMV35S-gus, pEmu-gus, pAct1-gus and pUbi1-gus, were used for particle bombardment of the embryogenic suspension cells of Indica rice cv. Chinsurah Boro II on two separate occasions, as described in Materials and Methods (experiment 1 solid bars; experiment 2 shaded bars). After 48 h incubation, protein was extracted and the GUS activity assayed.
**A**

GUS Activity (pmol 4-MU/min/mg protein)

<table>
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<tr>
<th>Promoters in GUS constructs</th>
</tr>
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<tbody>
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<td>Control</td>
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</tbody>
</table>

**B**

GUS activity (pmol 4-MU/min/mg protein)

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<th>Promoters in GUS constructs</th>
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<td>Control</td>
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Table 5.1 Numbers of blue expression units produced after bombarding embryogenic calli of Indica rice cv. Chinsurah Boro II with gus and either hph or bar genes

<table>
<thead>
<tr>
<th>Constructs</th>
<th>pCaMV</th>
<th>pEmu</th>
<th>pUbi1</th>
<th>pCaMV</th>
<th>pEmu</th>
<th>pAct1</th>
<th>pUbi1</th>
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</thead>
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<td>35S-hph</td>
<td>-hph</td>
<td>-hph</td>
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<td>35S-bar</td>
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<td>-bar</td>
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<th>4 weeks$^2$</th>
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<td>2.7</td>
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<tr>
<td></td>
<td>776</td>
<td>0.5</td>
<td>2.5</td>
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Note: Embryogenic calli were bombarded with the gus gene construct, pUbi1-gus together with either the hph gene constructs (pCaMV35S-hph, pEmu-hph & pUbi1-hph) or the bar gene constructs (pCaMV35S-bar, pEmu-bar, pAct1-bar & pUbi1-bar) using a particle inflow gun. They were transferred onto selection medium after being cultured on non-selection medium for 7 days as described in CHAPTER 2. Bombarded calli were stained with X-Gluc staining solution 1 day after bombardment, or 2 or 4 weeks after transfer onto selection media.

$^1$ The number of blue expression units (BEUs) is from 1 bombarded plate.

$^2$ Average number of BEUs is the mean from 4 bombarded plates.
formed nodular structures from the surface of the original calli (Table 5.1, Fig. 5.3 C, F & I). Bombardment of a mixture of \textit{pUbi1-gus} and \textit{pUbi1-hph} gave the most and largest BEUs after 4 weeks of selection, \textit{pEmu-hph} produced a similar number of smaller BEUs, and \textit{pCaMV35S-hph} gave the smallest size and number of BEUs. An increase in the size of the BEUs was seen in some cultures co-bombarded with \textit{gus} and \textit{bar} constructs, but the number and size increase of the BEUs was slight (Fig. 5.3K, Table 5.1). Furthermore, none of the BEUs developed into nodular structures (Fig. 5.3L).

### 5.2.4 Selection of stably transformed cell lines and regeneration of transgenic plants

Embryogenic calli were bombarded with the \textit{hph} gene constructs (\textit{pEmu-hph} & \textit{pUbi1-hph}) in combination with the \textit{gus} constructs (\textit{p4OCS\textDelta35SAcl-gus} & \textit{pUbi1-gus}). The calli yielded about 400 to 2000 BEUs 1 day after shooting (Table 5.2 & Fig. 5.1D). Without staining for GUS activity, the transformed cells or microcalli resulting from bombardment could not be identified under the dissecting microscope until at least one month after bombardment. However when a few plates of the target calli were stained for GUS activity after 2 weeks of selection, transformed cell clusters were observed (Fig. 5.1E). Out of the 26 hygromycin resistant cell lines resulting from co-bombardment with \textit{p4OCS\textDelta35SAcl-gus} or \textit{pUbi1-gus}, 17 lines were GUS positive (Fig. 5.1G, Table 5.3, 5.4) indicating a 65% co-
### Table 5.2 The correlation of the number of blue expression units (BEUs) and \textit{hph} resistance callus lines

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No of plates</th>
<th>No of BEUs per plate</th>
<th>No of \textit{hph} resistant callus lines</th>
<th>No of BEUs required for obtaining one \textit{hph} resistant callus line</th>
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<tbody>
<tr>
<td>3</td>
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<td>3</td>
<td>1497</td>
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<td>1365</td>
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<tr>
<td>10</td>
<td>7</td>
<td>392</td>
<td>6</td>
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<tr>
<td>Total</td>
<td>68</td>
<td>32</td>
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</table>

Note: Embryogenic calli were co-bombarded with \textit{pUbi1-gus} and either \textit{pUbi1-hph} (Experiments 3, 4, 9 and 10) or \textit{pEmu-hph} (Experiments 5-8) using either a particle inflow gun (Experiments 3-8) or a Bio-Rad gun (Experiments 9-10).
Table 5.3 Callus and genes used for stable transformation of Indica rice cv. *Chinsurah Boro II*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Callus age (month) / Callus line</th>
<th>No of plates bombarded</th>
<th>Selectable marker gene</th>
<th>Non-selectable gene</th>
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<tbody>
<tr>
<td>1</td>
<td>8.5 / 1</td>
<td>8</td>
<td>pEmu-hph</td>
<td>p4OCS35SAcl-gus</td>
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<tr>
<td>2</td>
<td>9 / 1</td>
<td>4</td>
<td>pEmu-hph</td>
<td>p4OCS35SAcl-gus</td>
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<tr>
<td>3</td>
<td>7.5 / 2</td>
<td>3</td>
<td>pUbi1-hph</td>
<td>pUbi1-gus</td>
</tr>
<tr>
<td>4</td>
<td>8 / 2</td>
<td>12</td>
<td>pUbi1-hph</td>
<td>pUbi1-gus</td>
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<tr>
<td>Total</td>
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<td>25</td>
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Note: The same set of experiments as in Table 5.2 were conducted in this study.
Table 5.4 Summary of stable transformation of Indica rice cv. *Chinsurah* Boro II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>hph- resistant callus lines</th>
<th>GUS(^*) embryogenic callus lines</th>
<th>No of GUS(^*) genic lines</th>
<th>Total plants</th>
<th>No of fertile plants</th>
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</thead>
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<td>1</td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>16</td>
<td>1 (1)(^2)</td>
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<tr>
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<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>17</td>
<td>6</td>
<td>26</td>
<td>1</td>
</tr>
</tbody>
</table>

Notes: 1 The same set of experiments as Table 5.3. 2 Number in bracket shows transgenic plant line expressing GUS when about 5 cm tall, but not at 20 cm tall.
transformation frequency. After 3 to 4 subcultures on selection media, fast growing callus lines could be distinguished among the bombarded calli (Fig. 5.1F). These callus lines were individually subcultured onto new selection medium for further growth. From 4 experiments and after 2 months of selection on R2H30 and R2H50 media, a total of 26 transformed callus lines were obtained from 25 bombarded plates (Table 5.3 & 5.4). Six of these 26 transformed lines regenerated into plants (a total of 26 plants) when placed onto regeneration medium, MSRH30 (Table 5.4; Fig. 5.1H) giving a regeneration frequency of about 23%. Of 6 lines co-transformed with gus and hph genes, 2 lines were GUS positive when the plants were about 5 cm tall. However, GUS activity of one line ceased as the plants matured (Fig. 5.1J). All the plants were transferred into soil (Fig. 5.1I) and grown in the glasshouse. Out of 26 plants, 18 plants were sterile and 8 plants were fertile (Fig. 1K, Table 5.4). The majority of the sterile plants were produced in experiment 1 which used foundation callus line 1 (Table 5.3 & 5.4). Eight transgenic plants derived from foundation callus line 2 were fertile (Table 5.3 & 5.4). The fertile plants were derived from 3 different transformed callus lines (pUbi1-hph). Southern analysis of at least one plant from five out of the six lines showed that they contained between one and five copies of the transgenes (Fig 5.4; Table 5.5). All five transgenic plant lines contained both the selectable and the non-selectable genes (Fig. 5.4, 5.5; Table 5.5), which were introduced on separate plasmids, thus giving a co-transformation frequency of 100%.
Fig. 5. Integration of the hph gene in hygromycin-resistant transgenic Indica rice plants. Southern blots of genomic DNA (4 μg / lane), hydrolysed with either BamHI/ SstI or BamHI alone, from 5 transgenic and 1 nontransgenic plants. The blot was hybridized with $^{32}$P-labelled hph from pUbi1-hph. Lane 1 in blot contains 25 pg of 6.9 kb BamHI / SstI-hydrolysed pUbi1-hph, this amount is equivalent to 1 gene copy per genome. Lane 2, Unhydrolysed DNA from non-transgenic rice plant; lanes 3-7: Unhydrolysed DNA from transgenic rice lines (TRL) Eh/RS5a 1-2-1 (see Chapter 6), Uh/Ugus 3-1-1, Uh/Ugus 3-3-1, Uh/Ugus 4-5-2 and Uh/RS5 5-3-6 (see Chapter 6); Lane 8: DNA from non-transgenic rice plant hydrolysed with BamHI / SstI; Lane 9-13: DNA from TRL Eh/RS5a 1-2-1, Uh/Ugus 3-1-1, Uh/Ugus 3-3-1, Uh/Ugus 4-5-2 and Uh/RS5 5-3-6, hydrolysed with BamHI / SstI; 14: DNA from non-transgenic rice plant hydrolysed with BamHI; Lanes 15-19: DNA from TRL Eh/RS5a 1-2-1, Uh/Ugus 3-1-1, Uh/Ugus 3-3-1, Uh/Ugus 4-5-2 and Uh/RS5 5-3-6, hydrolysed with either BamHI.
Fig. 5. 5 Integration of the gus gene in hygromycin - resistant transgenic Indica rice plants. Southern blots of genomic DNA (4 µg / lane), hydrolysed with either BamHI / SstI or BamHI alone, from 5 transgenic and 1 nontransgenic plants. The blots were hybridized with $^{32}$P-labelled gus from pUbi1-gus. Lane 1 in blot contains 25 pg of 6.9 kb BamHI / SstI-hydrolysed pUbi1-gus, this amount is equivalent to 1 gene copy per genome. Lane 2: DNA from non-transgenic rice plant hydrolysed with BamHI; Lanes 3-7: Genomic DNA from transgenic rice lines (TRL) Eh/mgus 1-5-1, Uh/Ugus 3-1-1, Uh/Ugus 3-3-1, Uh/Ugus 4-5-2 and Eh/mgus 1-12-1, hydrolysed with BamHI; Lane 8: Unhydrolysed DNA from non-transgenic rice plant; Lane 9-13: Unhydrolysed DNA from TRL Eh/mgus 1-5-1, Uh/Ugus 3-1-1, Uh/Ugus 3-3-1, Uh/Ugus 4-5-2 and Eh/mgus 1-12-1; Lane 14: DNA from non-transgenic rice plant hydrolysed with BamHI / SstI; Lanes 15-19: DNA from TRL Eh/mgus 1-5-1, Uh/Ugus 3-1-1, Uh/Ugus 3-3-1, Uh/Ugus 4-5-2 and Eh/mgus 1-12-1, hydrolysed with BamHI / SstI.
Table 5.5 Copy number of transgenes in transgenic Indica rice, cv. *Chinsurah Boro II*

<table>
<thead>
<tr>
<th>Transgenic plant line</th>
<th>Selectable marker gene</th>
<th>Non-selectable gene</th>
<th>Copies of <em>hph</em></th>
<th>Copies of <em>gus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Eh/mgus 1-5-1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>pEmu-<em>hph</em></td>
<td>p4OCSΔ35S</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Eh/mgus 1-12-1</td>
<td>pEmu-<em>hph</em></td>
<td>p4OCSΔ35S</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Uh/Ugus 3-1-1</td>
<td>pUbi1-<em>hph</em></td>
<td>pUbi1-<em>gus</em></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Uh/Ugus 3-3-1</td>
<td>pUbi1-<em>hph</em></td>
<td>pUbi1-<em>gus</em></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Uh/Ugus 4-5-2</td>
<td>pUbi1-<em>hph</em></td>
<td>pUbi1-<em>gus</em></td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: 1-5-1: plant regenerated from experiment 1 (first letter), callus line 5 (second letter) and was No1 plant (third letter) of this callus line.
5.3 Discussion

The choice of suitable plant tissue for genetic transformation is one of the most crucial aspects for achieving Indica rice transformation. Various rice tissues have been used for transformation by different research groups, including immature embryos (Christou et al., 1991; Li et al., 1993), embryogenic suspension cells (Cao et al., 1992) and embryogenic calli (Li et al., 1993). The prerequisites of material for transformation by particle bombardment are that: (1) the tissue is embryogenic and regenerable; (2) the material remains embryogenic despite repeated subculture and selection; and (3) large amounts of uniform material can be obtained for bombardment.

In my experiments, both direct bombardment of immature embryos and bombardment of initial callus from immature embryos of Chinsurah Boroll gave problems. Immature embryos from glasshouse grown plants harboured fungi and bacteria which were not eliminated by sterilisation. The majority of the callus tissue produced from the immature embryos, when placed on callus induction media, became brown and died. Suspension cells and embryogenic calli were therefore tested as target tissues. Embryogenic suspension cells of Chinsurah Boro II regenerated less than those from embryogenic callus and took longer to form plantlets after transfer onto regeneration medium. Bower and Birch (1992) also reported that embryogenic callus is a good target tissue for sugarcane transformation. Using embryogenic calli as the target tissue for particle
bombardment had the following advantages: (1) bacterial and fungal contamination was reduced; (2) the repeated, labour intensive, isolation of immature embryos was avoided; (3) it was easy to maintain embryogenic callus; and (4) more plantlets were obtained more rapidly from embryogenic callus than from suspension culture cells.

I have compared the *bar* and *hph* genes, and their respective selection agents, in transforming Indica rice from embryogenic callus. I have also compared the efficacy of these genes when controlled by different promoters. Embryogenic calli were bombarded with *hph* or *bar* containing plasmids, under the control of *Ubi1*, Emu or CaMV35S promoters together with a GUS expressing plasmid. Although all promoter / gene / selection combinations gave approximately the same number of BEUs one day after shooting, clear differences of growth rate of the whole calli and of the size of the BEUs was apparent after two and four weeks on selection media. In all combinations involving the *bar* gene, the calli grew poorly on Bialaphos containing media and few BEUs expanded over time. Those BEUs that did expand did not form nodular cell clusters. This suggests that the *bar* gene is less useful for Indica rice transformation than the other. The most rapid growth of hygromycin resistant calli and expansion of BEUs was observed when the *hph* gene was driven by the *Ubi1* promoter, Emu gave less growth and expression, and CaMV35S least. With all three promoters expanding BEUs formed nodular structures indicative of regeneration. The *Ubi1>*Emu>*CaMV35S ranking correlates with the relative levels of GUS expression
driven by these promoters in my transient and stable expression studies. From this it is inferred that the strongest promoter provides the most effective selection. Bower and Birch (1992) also demonstrated that strong promoters to drive expression of selectable marker genes produced effective selection. Furthermore, the data shows that only one hygromycin resistant callus line was produced for every 2000 to 4000 BEUs. However, this correlation between transient assay and stable transformation may vary with target material, different laboratories and bombardment instruments. My experiments showed that to obtain stably transformed cell lines, the bombardment process should be monitored using a gus reporter gene alongside the other gene constructs, and that if insufficient BEUs are obtained in any particular experiment it is prudent to repeat the experiment with new embryogenic calli rather than proceed with selection.

In conclusion, bombarding embryogenic callus with a selection gene plasmid and a gus expressing plasmid and staining at time intervals gives a clear indication of the likely efficacy of subsequently attempts to select for stably transformed lines, the hph gene / hygromycin B system selects much more efficacious for Indica rice transformation than the bar / Bialaphos system, the strong constitutive promoters Ubi1 and Emu controlling the hph gene give better selection, and a high frequency of fertile transgenic Indica rice plants can be obtained using these systems.
CHAPTER SIX: TRANSFORMATION OF INDICA RICE WITH RRSV S5 BASED GENES

6.1 Introduction

RRSV causes significant yield loss in Indica rice, especially in South East Asia, the annual worldwide losses are estimated at about US$150 million (Herdt, 1991). There is no natural resistance in rice against RRSV. The approach of breeding resistance to the brown planthoppers has been used in breeding programs, however, such resistance is easily circumvented by various biotypes of brown planthoppers (Ito et al., 1994; Wada et al., 1994; Nemoto and Yokoo, 1994).

The possibility of the protection of plants against viruses using introduced virus-derived genes offers considerable promise. This approach, suggested by Hamilton (1980), Gibbs (1982), and termed pathogen-derived resistance (PDR) by Sanford and Johnston (1985), provides a rational basis for designing resistance genes. The principal of this approach is that pathogen genes, when expressed at an inappropriate time and/or in an inappropriate place in a host organism, can render that host resistant to the pathogen. The virus-derived gene presumably disrupts the pathogen’s normal replication cycle, resulting in an abortive or attenuated infection.

One type of PDR protection is referred to as coat protein-mediated protection (Powell-Abell et al., 1986; Beachy et al., 1990; Farinelli & Malnoe, 1993). The viral coat protein expressed from an introduced gene in the host
plant possibly inhibits virus uncoating (Register and Beachy, 1988).

However, it has recently been suggested that viral RNA, not protein, may be the active molecule in this type of reaction. This has been investigated using non-translatable viral coat protein genes or replicase genes (Kawchuk et al., 1991; Lindbo & Dougherty, 1992; Carr et al., 1992; Anderson et al., 1992; Braun and Hemenway, 1992; Silva-Rosales et al., 1994; Smith et al., 1994; Leclerc and AbouHaidar, 1995).

Dougherty and his colleagues found that transgenic plants that resisted potato virus Y (PVY) had multiple PVY transgenes which appeared to be highly transcribed, yet accumulated small amount of the encoded protein (Smith et al., 1994). They proposed that PDR was caused by viral RNA, and that plant cells had a post transcriptional mechanism that controls the expression of genes. They proposed that when a certain level of the viral gene is exceeded, a system in the host cytoplasm is activated and specifically targets and degrades the transgene mRNA (Smith et al., 1994).

Expression of transgenes encoding unmutated or mutated viral replicase genes (Carr and Zaitlin, 1991; MacFarlane & Davies, 1992; Braun & Hemenway, 1992; Audy et al., 1994; Longstaff et al., 1993), dysfunctional viral movement protein (Lapidot et al., 1993), the Nla gene of potyviruses (Vardi et al., 1993; Maiti et al., 1993) and nucleoprotein (N) genes of tospoviruses (Gielen et al., 1991; De Haan et al., 1992; MacEnzie & Ellis, 1992; Pang et al., 1993, 1994; Prins et al., 1994; Vaira et al., 1995) have all been shown to confer resistance to the homologous virus.
So far, no effective resistance has been engineered using the PDR approach for reoviruses. Transgenic rice plants containing RRSV S9 have been obtained (Uyeda et al., 1995), but the plants showed no viral resistance.

Based on the available knowledge of particle structure and replication of plant reoviruses reviewed in Chapter 1, a number of possible strategies for engineering RRSV-resistance rice plants are described below:

**Sense expression of RRSV S5:**

RDRP within the reovirus core particles becomes active when the outer shell proteins have been removed. Although the virions of RRSV appears not to have an obvious double shell, S5 encodes a protein that appears to be located on the surface of RRSV particles (Chapter 4; Hagiwara et al., 1986). This protein is easily removed by adding MgCl₂ (Hagiwara et al., 1986). It is therefore possible that the removal of S5 protein may be involved with activating the RRSV RDRP. This suggests that expressing S5 protein in transgenic plant may interfere with RRSV replication by either preventing the uncoating of RRSV particles or by interfering with *de novo* viral particle assembly.

**RNA-mediated resistance:**
Transgenic plants exhibiting the RNA-mediated viral protection described by Smith et al. (1994) are often immune to viral infection. It may be possible to protect rice against RRSV using such a mechanism.

Expression of the antisense RNA of these structural protein genes in transgenic plants have proven effective in producing resistance (Day et al., 1991; Kawchuk et al., 1991; Bejarano and Lichtenstein, 1992; Leclerc & AbouHaidar, 1995). Therefore, expression of antisense RNA to RRSV structural genes in rice may also produce RRSV resistant plants.

Based on the strategies, in this chapter, I will describe how I constructed and expressed RRSV S5 in both sense and antisense orientations in Indica rice plants with the aim of producing RRSV-resistant transgenic Indica rice.

6.2 Results

Three promoters, Ubi1, CaMV35S and RolC, were selected for the construction of RRSV S5 based resistance genes. These were selected on the basis of the estimated strength of these promoters in undifferentiated cells of Indica rice Chinsurah Boro II (Chapter 5). These promoters have different expression specificities. The Ubi1 promoter is a strong promoter, the CaMV35S is weak, and the RolC is phloem specific. The expression cassettes containing either CaMV35S or RolC promoter were kindly provided by D. Llewellyn and M. Graham (Table 2.2).
6.2.1 Construction of pUbi1cas expression cassette

The expression cassette pUbi1cas, containing the strong promoter Ubi1, was constructed by first cloning the HindIII-BamHI promoter fragment (2012 bp) from pAHC27 (Table 2.3) into a HindIII-BamHI-digested pJKKm(-) to give a pUbi1JKm, then cloning a EcoRI-SacI fragment (269 bp) from pbar-NOS (a precursor of the pUbi1-bar construct which does not contain a Ubi1 promoter, Table 2.5) into a EcoRI-SacI-digested pUbi1JKm. The final construct, pUbi1cas, contains a maize ubiquitin 1 promoter (Ubi1) and nopaline synthase terminator (NOS). A multiple cloning site between the Ubi1 promoter and the NOS termination signal consists of four unique restriction enzyme sites, SacI, KpnI, SmaI and BamHI (Table 2.2; Fig. 6.1).

6.2.2 Construction of cDNA of RRSV S5 based resistance genes driven by CaMV35S, RolC and Ubi1 promoters and terminated by NOS 3' sequences

Five RRSV S5 based resistance genes were constructed during these studies (Table 2.8, Fig. 6.2). Full length RRSV S5 sequences were cloned into pBS-KS (Table 2.2), an EcoRI fragment, to give pS5BS. A BamHI-SalI fragment from pS5BS was cloned into BamHI-SalI-digested pGEM-11Zf(-) to create pS5GEM. These were subsequently used for the construction of RRSV S5 based resistance genes.

The construct pUbi1-S5 (Table 2.8; Fig. 6.2) which contains a full length RRSV S5 sense ORF driven by Ubi1 promoter and terminated by
Fig. 6.1 Plasmid map of plant expression vector, pUbi1cas. This construct contains a maize ubiquitin 1 promoter, *Ubi1*, and a nopaline synthase terminator, NOS. A polylinker contains restriction sites for *Bam*HI, *Sma*I, *Kpn*I and *Sac*I.
Plasmid map of plant expression vector, pUbi1cas
Fig. 6.2 Diagram of RRSV S5 based resistance gene constructs, driven by CaMV35S, RolC and Ubi1.
RRSV segment 5 based gene constructs

**p35S-S5**

Vector

<table>
<thead>
<tr>
<th>CaMV 35S promoter</th>
<th>Sense ORF</th>
<th>NOS 3'</th>
</tr>
</thead>
</table>

**p35S-S5a**

Vector

<table>
<thead>
<tr>
<th>CaMV 35S promoter</th>
<th>Antisense ORF</th>
<th>NOS 3'</th>
</tr>
</thead>
</table>

**pRoIC-S5**

Vector

<table>
<thead>
<tr>
<th>RoIC promoter</th>
<th>Sense ORF</th>
<th>NOS 3'</th>
</tr>
</thead>
</table>

**pRoIC-S5a**

Vector

<table>
<thead>
<tr>
<th>RoIC promoter</th>
<th>Antisense ORF</th>
<th>NOS 3'</th>
</tr>
</thead>
</table>

**pUbi1-S5**

Vector

<table>
<thead>
<tr>
<th>Ubi1 promoter</th>
<th>Sense ORF</th>
<th>NOS 3'</th>
</tr>
</thead>
</table>
NOS 3', was constructed by cloning a BamHI-SstI fragment from pS5GEM into BamHI-SstI-digested pUbi1cas.

The constructs pR oIC-S5 and pR oIC-S5a were constructed by cloning a BamHI-SalI-fragment or a PstI-SalI-fragment from pS5BS into BamHI-SalI-digested or PstI-SalI-digested pRoICcas (Table 2.8; Fig. 6.2).

The constructs p35S-S5 or p35S-S5a were created by cloning a EcoRI fragment into a EcoRI-digested pJ35SN vector (Table 2.8; Fig. 6.2).

6.2.3 Transformation of Indica rice plants with RRSV S5 based resistance genes

Embryogenic calli of Indica rice cv. Chinsurah Boro II were initiated from immature embryos, and subsequently selected and cultured on R2C medium (Table 2.9) as described in Chapter 5. Five to eight month old embryogenic calli were bombarded with tungsten or gold particles coated with a selectable marker gene plasmid, either pEmu-hph or pUbi1-hph together with one of five RRSV S5 based resistance gene constructs (Table 2.8; Fig. 6.2). Forty-five plates of embryogenic calli were bombarded in six individual experiments (Table 6.1), 24 individual hph-resistant callus lines were obtained (Table 6.2). Approximately one hph-resistant callus line was obtained from every 2 plates of embryogenic calli that were bombarded.

After transfer onto regeneration medium, MSRH30, 53 transgenic plants were regenerated from ten of these transformed callus lines (Table 6.2). In the glasshouse, most transgenic plants grew normally compared
Table 6.1 Callus and genes used for obtaining transgenic Indica rice cv. *Chinsurah Boro II* containing RRSV resistance genes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Callus age (month) /</th>
<th>Plates bombarded</th>
<th>Selectable gene</th>
<th>non-selectable gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus line</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.5 / 2</td>
<td>7</td>
<td>pEmu-(hph)</td>
<td>pRolC-S5a</td>
</tr>
<tr>
<td>2</td>
<td>8 / 2</td>
<td>7</td>
<td>pEmu-(hph)</td>
<td>pRolC-S5a</td>
</tr>
<tr>
<td>3</td>
<td>8.3 / 2</td>
<td>8</td>
<td>pEmu-(hph)</td>
<td>p35S-S5</td>
</tr>
<tr>
<td>4</td>
<td>8.3 / 2</td>
<td>9</td>
<td>pEmu-(hph)</td>
<td>p35S-S5a</td>
</tr>
<tr>
<td>5</td>
<td>5.3 / 3</td>
<td>7</td>
<td>pUbi1-(hph)</td>
<td>pRolC-S5</td>
</tr>
<tr>
<td>6</td>
<td>5.3 / 3</td>
<td>7</td>
<td>pUbi1-(hph)</td>
<td>pUbi1-S5</td>
</tr>
<tr>
<td>Total</td>
<td>5.3 / 3</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Experiments 1-4 were bombarded using a particle inflow gun; experiments 5-6 were bombarded using a Bio-Rad gun.
Table 6.2 Summary of stable transformation of Indica rice cv. Chinsurah Boro II with RRSV resistance genes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>hph resistant callus lines</th>
<th>Embryogenic transgenic lines</th>
<th>No of plants</th>
<th>No of fertile plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>3</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>10</td>
<td>53</td>
<td>47</td>
</tr>
</tbody>
</table>
with nontransgenic control plants derived from seeds. Forty-seven plants derived from 9 lines grew to maturity and were fertile (Table 6.2). However, plants derived from one hph-resistance callus line (experiment 5), grew until they were 20 to 30 cm tall and then turned yellow and ceased to grow and two other plants grew normally, but were sterile.

The amount of seed produced from different transgenic plant lines, even from different plants produced from the same callus line, varied (Table 6.3). One callus line gave only a single transgenic plant that produced 2 seeds, whereas other lines produced plants that gave an excess of 1000 seeds.

Generally speaking, transgenic plants that were regenerated after a short period of tissue culture produced more seeds. Most seeds produced from transgenic plants appeared normal, but two plants derived from two different callus lines, produced abnormally large seeds.

6.2.4 Analysis of transgenic Indica rice plants containing RRSV S5 based resistance genes

One or two plants from each of the nine transgenic lines were analysed by Southern blot for the presence of the hph gene. Different plants had between 1 and 8 copies of this gene (Fig. 6.3 & 6.4; Table 6.4). All of these plant lines were also analysed for the presence of the appropriate RRSV S5 based genes, and seven lines were found to contain 1 to 5 copies of RRSV S5 gene (Table 6.4; Fig. 6.5). The hph gene and RRSV S5 based
Fig. 6. 3 Integration of the *hph* gene in hygromycin - resistant transgenic Indica rice plants. Southern blots of genomic DNA (4 μg / lane), digested with either *EcoRI* / *Xhol* (Blot A) or *BamHI* (Blot B) alone, from 11 transgenic and 2 nontransgenic plants. The blots were hybridized with $^{32}$P-labelled *hph* from p*Ubi1-hph* hydrolysed with *BamHI* / *Saci* (Fig. 6.4). Lanes 1 and 2 in both blots contain 25 pg and 50 pg of 6.9 kb *EcoRI* / *Xhol*-digested p*Ubi1-hph*, these amounts are equivalent to 1 and 2 gene copies per genome respectively.

**(Blot A).** lane 3, Spp-1/*EcoRI* molecular weight markers; lane 4, Undigested DNA from non-transgenic rice plant; lanes 5-8: undigested genomic DNA from transgenic rice lines (TRL) TRL Eh/RS5a 2-8-1, Eh/RS5a 1-12-2, Eh/35S5 3-1-1 and Eh/RS5a 1-3-9; Lane 9, DNA from non-transgenic rice plant digested with *EcoRI* / *Xhol*; lanes 10-13, DNA from TRL Eh/RS5a 2-8-1, Eh/RS5a 1-12-2, Eh/35S5 3-1-1 and Eh/RS5a 1-3-9, digested with *EcoRI* / *Xhol*. **(Blot B).** Lanes 3 - 16 genomic DNA digested with *BamHI*. lanes 3 and 4, DNA from non-transgenic rice plants; lanes 5, TRL Eh/RS5a 2-8-1; lane 6, TRL Eh/RS5a 2-8-3; lane 7, TRL Eh/RS5a 1-12-2; lane 8, TRL Eh/35S5 3-1-1; lane 9, TRL Eh/35S5a 4-1-2; lane 10, TRL Eh/RS5a 1-3-9; lane 11, TRL Uh/US5 6-1-5; lane 12, TRL Uh/RS5 5-3-6; lane 13, TRL Uh/RS5 5-2-2; lane 14, TRL Uh/US5 6-1-6; lane 15, TRL Eh/mgus 1-5-1; lane 16, TRL Eh/mgus 1-12-1. TRL Eh/RS5a 2-8-1 and TRL Eh/RS5a 2-8-3, TRL Uh/US5 6-1-5 and TRL Uh/US5 6-1-6 are sibling lines derived from the same transgenic callus material.
Fig. 6.4 Diagram of hph based gene constructs driven by Ubi1 and Emu promoters.
**hph base gene constructs**

**pUbi1-hph**
- Vector
- Ubi1 promoter
- hph coding region
- NOS 3'
- Vector

**pEmu-hph**
- Vector
- Emu promoter
- hph coding region
- NOS 3'
- Vector

- HindIII
- BamHI, XhoI
- SacI
- EcoRI

- HindIII
- BamHI, BamHI
- XhoI
- SacI, EcoRI
Fig. 6.5 Integration of the RRSV S5 gene in transgenic Indica rice plants. Southern blot of genomic DNA (4 μg / lane), digested with either EcoRI/HindIII or SalI alone, from 4 transgenic and 1 nontransgenic plants. The blot was hybridized with $^{32}$P-labelled RRSV S5 from pS5UC hydrolysed with EcoRI (Fig. 4.1). Lane 1 in blot contains 20 pg of 5.7 kb EcoRI / HindIII-digested pS5UC, this amount is equivalent to 1 gene copy per genome; lane 2, Ssp-1/EcoRI molecular weight markers; lane 3: blank lane; lane 4, Undigested DNA from non-transgenic rice plant; lanes 5-8: undigested genomic DNA from transgenic rice lines (TRL) TRL Uh/US5 6-1-5, Uh/RS5 5-3-6, Uh/RS5 5-2-2 and Uh/US5 6-1-6; Lane 9, DNA from non-transgenic rice plant digested with EcoRI / HindIII; lanes 10-13, DNA from TRL Uh/US5 6-1-5, Uh/RS5 5-3-6, Uh/RS5 5-2-2 and Uh/US5 6-1-6, digested with EcoRI / HindIII; Lane 14, DNA from non-transgenic rice plant digested with SalI; lanes 15-18, DNA from TRL Uh/US5 6-1-5, Uh/RS5 5-3-6, Uh/RS5 5-2-2 and Uh/US5 6-1-6, digested with SalI.
Table 6.3 Fertility of transgenic plants in C. lanatus var. aconitifolius

<table>
<thead>
<tr>
<th>No. of seeds per plant</th>
<th>No. of transgenic plants</th>
<th>No. of transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 50</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50 to 100</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>100 to 500</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>500 to 1000</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1000 to 1500</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Note: There were no plants with more than 1500 seeds per plant. A subset of plants with more than 1500 seeds per plant were selected for further analysis; however, no consistent pattern was observed for different transgenic lines.
Table 6.3 Fertility of transgenic Indica rice cv. *Chinsurah Boro II*

<table>
<thead>
<tr>
<th>No of seeds per plants</th>
<th>No of transgenic plants</th>
<th>No of transgenic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 50 seeds</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>50 to 100 seeds</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>100 to 500 seeds</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>500 to 1000 seeds</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>1000 to 1900 seeds</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>9</td>
</tr>
</tbody>
</table>

Note: There is no relationship between “No of transgenic plants” and “No of transgenic lines”. For example, there are 5 transgenic plants having 50 seeds which belong to four different transgenic lines.
Table 6.4 Copy number of transgenes in transgenic Indica rice, cv. *Chinsurah Boro II* containing RRSV resistance genes

<table>
<thead>
<tr>
<th>Transgenic plant line</th>
<th>Selectable marker gene</th>
<th>Nonselectable marker gene of <em>hph</em></th>
<th>Copies of <em>hph</em></th>
<th>Copies of RRSV S5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eh/RS5a 1-2-1*</td>
<td>pEmu-<em>hph</em></td>
<td>pRo1C-S5a</td>
<td>8</td>
<td>Not tested</td>
</tr>
<tr>
<td>Eh/RS5a 1-3-9</td>
<td>pEmu-<em>hph</em></td>
<td>pRo1C-S5a</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Eh/RS5a 1-12-2</td>
<td>pEmu-<em>hph</em></td>
<td>pRo1C-S5a</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Eh/RS5a 2-8-1</td>
<td>pEmu-<em>hph</em></td>
<td>pRo1C-S5a</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Eh/RS5a 2-8-3</td>
<td>pEmu-<em>hph</em></td>
<td>pRo1C-S5a</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Eh/35S5 3-1-1</td>
<td>pEmu-<em>hph</em></td>
<td>pUbi1-S5a</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Eh/35S5a 4-1-2</td>
<td>pEmu-<em>hph</em></td>
<td>p35S-S5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Uh/RS5 5-2-2</td>
<td>pUbi1-<em>hph</em></td>
<td>pRo1C-S5</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Uh/RS5 5-3-6</td>
<td>pUbi1-<em>hph</em></td>
<td>pRo1C-S5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Uh/US5 6-1-5</td>
<td>pUbi1-<em>hph</em></td>
<td>pUbi1-S5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Uh/US5 6-1-6</td>
<td>pUbi1-<em>hph</em></td>
<td>pUbi1-S5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: *1-2-1*: plant regenerated from experiment 1 (first letter), callus line 2 (second letter) and was the first plant (third letter) of this callus line.
genes were introduced in separate plasmids, thus there was a co-transformation frequency of about 85%.

Interestingly, one hph resistance transgenic plant, Eh/35S 3-1-1 was shown to contain a single less-than-full length hph gene fragment (lane 12, Fig. 6.3). A transgenic rice plant with a similarly incomplete but functional hph gene has also been observed by Tada et al. (1990).

6.2.5 Analysis of transgene inheritance

T₁ progeny from 7 transgenic lines were used for PCR analysis. Two of them showed 1 : 1, another two lines showed 1 : 2 S5⁺ : S5⁻ segregation ratio of RRSV S5 (Table 6.5) and the remaining 3 lines exhibited a single copy or no RRSV S5 gene in their T₁ progeny.

Seven lines were selected to test the inheritance of functional Hph activity in their T₁ progeny plants by germination of seed on medium containing hygromycin. Three gave a 3 : 1 Hph⁺ : Hph⁻ segregation ratio (Table 6.5) indicating the inheritance of a single dominant locus for Hph activity. The remaining 4 lines showed either 1 : 1 or 1 : 2 Hph⁺ : Hph⁻ segregation ratio.

T₁ transgenic plants which contained hph gene by Southern blot analysis were used for RT-PCR to test the expression of RRSV S5 transgene. Expression of RRSV S5 transcript was detected in one of four T₁ lines (Table 6.5).
The seeds from all these transgenic lines have been sent to our collaborating laboratories in China, India and Thailand where RRSV and its insect vectors occur naturally. Progeny plants from these transgenic seeds will be challenged with RRSV to determine whether they are resistant to RRSV.

6.3 Discussion

As with other transgenic cereals, low fertility has been a major problem in transgenic Indica rice. However, in this study, 47 out of 53 transgenic plants regenerated from 9 different callus lines were fertile and most transgenic lines produced more than 500 seeds per plant. Plants obtained from short term tissue culture (about 6 months after initiation of callus from immature embryos) tended to produce more seeds per plant.

These fertile transgenic rice plants contained between 1 and 8 copies of both the *hph* and RRSV S5 genes. This range of transgene copy number is similar to that previously reported in rice (Schocher *et al.*, 1986; Zhang *et al.*, 1988; Kartzke *et al.*, 1990; Linn *et al.*, 1990; Tada *et al.*, 1990; Meijer *et al.*, 1991; Battraw and Hall, 1992; Peng *et al.*, 1992; Goto *et al.*, 1993; Cooley *et al.*, 1995). The co-integration frequency of the transgenes was approximately 85%, which is similar to previously published work (Schocher *et al.*, 1986; Goto *et al.*, 1993; Wan and Lemaux, 1994) although lower co-integration frequencies (20-30%) using co-transformation of unlinked genes
have also been reported (Christou and Swain, 1990; Peng et al., 1990; Rathore et al., 1993).

Preliminary results from PCR assay of T₁ plants and germination of T₁ seeds on hygromycin showed that both the hph gene and RRSV S5 based gene are transmitted to T₁ progeny plants. The segregation of hph gene in transgenic lines ranged from 1 : 1 to 3 : 1. The segregation of RRSV S5 based gene in some transgenic lines ranged from 1 : 2 to 1 : 1. However, some lines had low segregation ratios of the RRSV S5 transgene (eg. 1 : 18) and a couple of the lines had completely lost RRSV S5 transgene (eg. 0 : 9 or 0 : 10) in their progeny. Similarly unusual segregation ratios have been found in transgenic cereals by other workers (Wan and Lemaux, 1994; Somers et al., 1994) although the mechanism(s) responsible are unknown. Somers et al. (1994) suggested that the segregation ratio 1 : 1 may result from failure in transmission of the transgene through the male gametes, but this hypothesis was not tested experimentally. It would be very interesting to study the transgenic plants from my work that give unusual segregation ratios. Do transgenic progeny of these lines continue to give the same unusual segregation ratios in subsequent generations or do they at some point give and maintain classical ratios? What is the mechanism that causes these ratios - is it deletion or lethality of the transgene during meiosis?

Preliminary results showed that RRSV S5 transgene mRNA is expressed in the T₁ generation of at least one transgenic plant line. Plants
from this line are likely to be expressing the S5 encoding protein. The expression of this protein and its levels could be determined using the S5 antiserum described in Chapter 4. Such protein expressing plants may give resistance to RRSV by interfering with uncoating of RRSV particles as S5 encodes a large surface protein on the surface of the particle. The transgenic plant lines that do not appear to be expressing S5 mRNA but that do contain the S5 gene may also be useful for protecting the plant against RRSV. Lindbo and Dougherty (Lindbo et al., 1993; Dougherty et al., 1994) have shown that viral resistance can be obtained by a gene silencing mechanism. Transgenic plants containing viral transgenes, often with a high copy number, can involve a specific RNA degradation mechanism which degrades not only the transgene mRNA but also in-coming viral RNA of the same sequence. This results in high transcription rates (as measured from isolated nuclei) but little or no cytoplasmic transgene mRNA. The RRSV plants with no detectable RRSV mRNA may be the result of a similar mechanism and therefore the good candidates for possessing RRSV resistance.
Table 6.5 Segregation of RRSV S5 and *hph* genes in T<sub>1</sub> progeny of transgenic Indica rice plants

<table>
<thead>
<tr>
<th>Family</th>
<th>Copies of transgenes in T&lt;sub&gt;0&lt;/sub&gt; plants</th>
<th>PCR</th>
<th>Germination*</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eh/RS5a 1-2-1</td>
<td>8 : -</td>
<td>7 : 12 (1 : 1)</td>
<td>17 : 10 (2 : 1)</td>
<td>0 : 1</td>
</tr>
<tr>
<td>Eh/RS5a 1-3-9</td>
<td>5 : 3</td>
<td>10 : 13 (1 : 1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eh/RS5a 1-12-2</td>
<td>1 : 0</td>
<td>-</td>
<td>18 : 7 (3 : 1)</td>
<td>0 : 1</td>
</tr>
<tr>
<td>Eh/RS5a 2-8-3</td>
<td>2 : 1</td>
<td>4 : 8 (1 : 2)</td>
<td>15 : 10 (2 : 1)</td>
<td>0 : 1</td>
</tr>
<tr>
<td>Eh/35S5 3-1-1</td>
<td>1 : 1</td>
<td>1 : 18</td>
<td>20 : 5 (3 : 1)</td>
<td>-</td>
</tr>
<tr>
<td>Uh/RS5 5-2-2</td>
<td>8 : 5</td>
<td>0 : 10</td>
<td>15 : 5 (3 : 1)</td>
<td>-</td>
</tr>
<tr>
<td>Uh/RS5 5-3-6</td>
<td>2 : 2</td>
<td>0 : 9</td>
<td>15 : 14 (1 : 1)</td>
<td>-</td>
</tr>
<tr>
<td>Uh/US5 6-1-6</td>
<td>3 : 2</td>
<td>7 : 16 (1 : 2)</td>
<td>9 : 15 (1 : 1)</td>
<td>1 : 0</td>
</tr>
<tr>
<td>Control</td>
<td>0 : 25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: -: not tested.

*: T<sub>1</sub> seeds were germinated on Whatman #1 filter paper moistened with 1/2 MS liquid medium containing 200 mg / L hygromycin.
CHAPTER 7: SUMMARY, CONCLUSIONS AND FUTURE DIRECTION

Rice ragged stunt virus (RRSV) causes an important disease of rice, causing significant yield losses in rice in South East Asian countries. The principal approaches currently used to control RRSV are the use of cultivars resistant to the vector, insecticides, and various integrated pest management schemes which include the eradication of weeds and other virus sources together with adjusting the time at which planting occurs. These efforts have met with little success. Furthermore, there are no easily accessible natural resistance gene against RRSV that can be introduced into elite cultivars by conventional breeding. However, resistance has been achieved for a number of viruses, including rice stripe virus, by transforming plants with viral transgenes. Therefore, this approach may be also successful in protecting rice against RRSV.

The objective of this study was to transform Indica rice with RRSV genomic segment 5 (S5) based transgenes in an attempt to create RRSV resistance rice. This involved, firstly, the molecular analysis of RRSV S5, and then the transformation of the Indica rice cv. Chinsurah Boro II with RRSV S5 based gene constructs. The major findings of this project are summarised as follows:

7.1 Sequence analysis of RRSV S5

7.1.1 Summary
The complete nucleotide sequence of the genome segment 5 (S5) of a Thai isolate of rice ragged stunt virus (RRSV) was determined. The sequence of 2682 nucleotide contains a long open reading frame (ORF) initiating at nucleotide 52 and terminating at nucleotide 2478. This ORF encodes an 808 amino acid polypeptide with a Mr of 91 kDa.

An imperfect inverted repeat was found between the termini (or close to termini) of the RRSV S5 nucleotide sequence. Conserved complementarity motifs are also found in a similar position in the genome segments of other reoviruses. It has been suggested that these regions are segment-specific and are involved in molecular sorting and protein-RNA interaction which lead to the replication and packaging of viral transcripts (Anzola et al., 1987; Nuss and Dall, 1990).

Comparison of nucleotide and amino acid sequences of RRSV S5 with sequences in GenBank and EMBL databases, which include a number of plant and animal reoviruses, did not reveal any significant sequence similarities. The lack of sequence similarity with other reoviruses conforms the current status of RRSV being a separate reovirus genus, Oryzavirus.

7.1.2 Future direction

The sequencing of the other segments of the RRSV genome is in progress (N Upadhyaya and P Waterhouse, personal communication). The sequence of the entire RRSV genome may reveal some features in common between these segments, eg. segment-specific regions, information for the
coding assignment of encoded proteins and design of RRSV based resistance genes.

7.2 Expression of an RRSV S5 fusion protein

7.2.1 Summary

cDNA of RRSV S5 was translated into polypeptides using the pGEX vector. No detectable full length fusion protein was produced when the whole ORF was inserted into the pGEX vector and transformed into bacteria. Therefore, several segments of the ORF were selected and translated separately. It was found that the yield of fusion proteins from various clones was closely related to the ratio of hydrophilic and hydrophobic amino acids in each segment of the gene. The culture conditions and glucose concentration also affected the yield of fusion proteins. Clone pS5H2, which contained only a central portion of the ORF, encoding a strongly hydrophilic region, gave sufficient yields of fusion protein for a rabbit immunisation program. The fusion protein yield was greatest when the construct was grown in JM109 at 28°C in the presence of 1% glucose prior to induction. This work also demonstrated that E. coli cells containing RRSV S5 / pGEX constructs produced maximum yields when maintained on LB agar medium containing 1% glucose and incubated at 28°C.

7.3 Functional analysis of RRSV S5
7.3.1 Summary

Three proteins probably encoded by RRSV S5 were identified using antisera raised against the GST-RRSV S5 fusion protein. Proteins from two individual virus particle preparations, one viruliferous brown planthopper preparation and two infected rice plant preparations were probed with the antisera against RRSV S5 protein. Three proteins of 91, 67 and 44 kDa that reacted with antisera against RRSV S5 protein were found in immunoblot analyses of all three types of preparation (Table 4.4). This indicates that the three proteins (91, 67 and 44 kDa) are encoded by RRSV S5.

The nucleotide sequence of RRSV S5 determined in this study suggests that the 91 kDa protein is the primary translation product of RRSV S5 translating from the first initiation codon (nucleotide 52-54) even though it has an unfavourable Kozak's sequence context. It is possible that the 67 kDa protein is produced by translation of the S5 RNA initiating at position 699-701 which has a favourable Kozak's sequence. However, no good site for translation initiation could be found that would result in a 44 kDa product.

The particle structure and protein subviral locations of RRSV and other reoviruses (Milne, 1980; Hagiwara et al., 1986; Chapter 1), together with immunoblot analysis of RRSV S5, suggests that the proteins encoded by RRSV S5 may be located at the surface of viral particles, between the spikes.

7.3.2 Future directions
The nature of the 67 and 44 kDa proteins remains to be determined. Mutational changes of the initiation codons at nucleotides 52-54 and 699-701, followed by in vitro translation may help answer these questions.

The location of RRSV S5 protein needs to be further tested by immuno-gold labelling analysis of virus particles using antisera against RRSV S5 protein.

Fusion proteins encoded by other RRSV genomic segments should be produced and antisera raised against them. These should then be used to determine the architecture of the virions of RRSV particle and the segments responsible for the different structural proteins. Such a project is underway by Upadhyaya and Waterhouse.

7.4 Genetic transformation of Indica rice

7.4.1 Summary

An Indica rice transformation system was established using embryogenic calli of Indica rice cv. Chinsurah Boro II bombarded with both a selectable marker gene (hph) and a non-selectable gene. Indica rice transformation has been achieved by a few laboratories, however, it is still considered difficult. Two of the factors that influence Indica rice transformation, the use of suitable promoters and an efficient selectable marker gene, were assessed in this study.

Electroporating protoplasts and bombarding embryogenic callus with the gus gene under the control of various promoters showed that the order
of promoters strength (from strong to weak) in non-organised cells of Indica rice cv. *Chinsurah Boro II* was *Ubi1*-*Act1*-*Emu*-*CaMV35S*-*RolC*.

Bombardment of embryogenic callus with a selectable marker gene plasmid and a *gus* expressing plasmid followed by staining at time intervals gives a clear indication of the efficacy of the selection. The *hph* gene / hygromycin B selection system was much more efficacious for Indica rice transformation than the *bar* gene / Bialaphos system. The stronger constitutive promoters, *Ubi1* and *Emu* when used to control the *hph* gene gave a good selection and a high frequency of fertile transgenic Indica rice plants.

Five out of six transgenic plant lines analysed by Southern blot contained 1 to 5 copies of both *hph* and *gus* which were introduced on separate plasmids, giving a 100% co-integration. However, only one out of six of these transgenic lines continued the expression of the *gus* gene as the plants matured.

7.4.2 Future directions

A low frequency of co-expression of nonselectable and selectable genes in transgenic rice was obtained and similar results have been reported publications from other laboratories. The factors causing this gene silencing need to be further investigated.

7.5 Transformation of Indica rice with RRSV S5 genes
7.5.1 Summary

Plasmids were constructed by cloning the RRSV S5 ORF in both sense and antisense orientations under the control of various promoters, *Ubi1*, *Rocl* and *CaMV35S*.

Bombarding embryogenic callus with RRSV S5 based gene constructs and selection gene constructs, p*Ubi1-hph* and p*Emu-hph* produced 47 fertile transgenic plants from 9 transgenic plant lines.

Analyses of these transgenic plants by Southern blot showed that the *hph* and S5 genes co-integrated in more than 80% of the plants, and that they contained 1 to 8 copies of the *hph* and 1 to 5 copies of the S5 gene. It has been reported that the viral transgenes in transgenic plants, that are virus resistant, are highly transcribed (usually present at a high copy number) yet accumulate low levels of the transgene transcript (Lindbo *et al.*, 1993; Dougherty *et al.*, 1994). Therefore, transgenic rice plants containing RRSV S5 transgenes but for which no transcript could be detected are good candidates for possessing RRSV resistance.

Preliminary results from PCR assay of T1 plants and germination of T1 seeds on hygromycin showed that both the RRSV S5 gene and *hph* gene are transmitted to T1 progeny plants. The results from a RT-PCR assay confirmed that the RRSV S5 transgene mRNA, and therefore probably the S5 protein, is expressed in the T1 transgenic plants of at least one line. S5 protein is an outer surface protein of the RRSV particle, therefore its
expression in plants may interfere with the uncoating or replication of the virus

The seeds produced from these transgenic plants have been sent to our collaborators in China and India. The progenies of these transgenic plants will be challenged with RRSV.

7.5.2 Future directions

If any of the plants generated in this work show an increased resistance to RRSV, in glasshouse tests in India and China, such plants should be tested in the field. Different lines showing protection should be crossed with each other and evaluated for increased RRSV resistance. Also plants showing resistance should be selfed to produce plants homologous for the RRSV S5 genes. While the RRSV S5 gene constructs have the potential to produce RRSV resistant rice, genes derived from other genomic segments of RRSV should also be investigated for their capacity to protect rice against this virus.
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