STUDIES ON IMPROVEMENT OF CHINESE WHEAT CULTIVARS FOR RESISTANCE AGAINST AN ENDEMIC VIRUS USING TRANSGENESIS AND VIRUS-WHEAT INTERACTIONS AT THE MOLECULAR AND ULTRASTRUCTURAL LEVELS

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed .... [redacted] ..... Date....31/5/01....

(Director of Studies)

Declaration

I hereby declare that this work has not previously been accepted in substance for any other award and is not being currently submitted in candidature for any other degree. I further declare that except where stated the work presented in thesis is original and was performed by the author at the Scottish Crop Research Institute.

Signed: [redacted]

Date: 31/10/01

ABSTRACT

The present study was aimed at the improvement of Chinese wheat cultivars for resistance to *Soil-borne wheat mosaic virus* (SBWMV) by transgenesis, and an examination of virus movement by studying the complementation of movement proteins (MPs) from *Tobacco mosaic virus* (TMV) and SBWMV using a TMV-based virus vector.

A number of SBWMV coding sequences, including the functional coat protein (CP) gene, dysfunctional MP gene, and functional and dysfunctional replicase complex sequence, were cloned into an expression vector pUbi35S and the expression of those viral genes under the control of maize ubiquitin-1 promoter was tested in transfected tobacco protoplasts.

Parameters for the bombardment of immature embryos using the biolistic gun were optimised through transient expression of a chimeric β-glucuronidase (*uid*A) gene. Transgenic wheat for the model variety Bob White and three Chinese semi-winter wheat cultivars were obtained by microprojectile bombardment of pre-cultured immature embryos using a chimeric *bar* gene as the selectable marker. Plants were regenerated under phosphinothricin (PPT) selection. The transgenic nature of the regenerated wheat plants was demonstrated by Southern hybridization analysis. Expression of viral CP gene was confirmed from most CP-transgenic wheat by RT-PCR testing, while only one CP-transgenic plant produced CP at a detectable level. The meiotic stable transmission of transgenes in several transgenic lines was confirmed by polymerase chain reaction (PCR) analysis of R1 progenies. *ELISA* testing of several selected transgenic lines mechanically inoculated with *Chinese wheat mosaic virus* (CWMV), a new member of *Furovirus* genus, revealed that most of the lines were infected by the virus, while some plants from several lines appeared not to be infected.

A new selection approach based on the phosphomannose isomerase (*pmi*) gene as the selectable marker, and mannose as the selective agent, was developed to produce transgenic wheat plants for both Bob White and Chinese wheat varieties. The introduction of SBWMV CP gene into Chinese wheat cultivars was accomplished using the mannose selection system, and Southern analysis confirmed the integration of transgenes in the genome of transgenic wheat.

An expression vector was constructed by cloning the putative SBWMV MP coding sequence into a TMV-based vector in which the native TMV MP gene was rendered defective by open reading frame shift. When using this chimeric virus to infect tobacco plants, it was found that the 37 kDa protein (p37) encoded by SBWMV could be functionally complementary to the 30 kDa of TMV MP, this also confirmed that the p37 is the MP of SBWMV.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

[α-^{32} dNTP]	α-^{32}P-labelled deoxynucleotide
$AlCl_3$	aluminum chloride
AMV	*Alfalfa mosaic virus*
ATP	adenosine-5'-triphosphate
BAP	6-benzylaminopurine
BaYMV	*Barley yellow mosaic virus*
BNYVV	*Beet necrotic yellow vein virus*
bp	base pair(s)
BSA	bovine serum albumin
BSMV	*Barley stripe mosaic virus*
$CaCl_2$	calcium chloride
CaMV	*Cauliflower mosaic virus*
cDNA	complementary DNA
CMV	*Cucumber mosaic virus*
CP	coat protein
CPMV	*Cowpea mosaic virus*
CTAB	cetyltrimethylammonium bromide
CTP	cytidine-5'-triphosphate
$CuSO_4$	cupric sulfate
CW	cell wall
CWMV	*Chinese wheat mosaic virus*
CymRSV	*Cymbidium ringspot virus*
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
DTT	dithiothreitol
dTTP	deoxythymidine-5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraaceticacid (disodium salt)
ELISA	enzyme-linked immunosorbent assay
EtoH	ethanol
$FeSO_4$	ferrous sulfate
GFP	green fluorescent protein
GTP	guanosine-5'-triphosphate
GFLV	*Grapevine fan leaf virus*
GRV	*Groundnut rosette virus*
GUS	β-glucuronidase
HCl	hydrochloric acid
H_3BO_3	boric acid
hr	hour (s)
kb	kilobase/kilobase pairs
kDa	kilodalton
kg	kilogram
KH_2PO_4	potassium phosphate
KI	potassium iodide
KNO_3	potassium nitrate
LB	Luria-Bertani
MDMV	*Maize dwarf mosaic virus*
mg	milligram
$MgSO_4$	magnesium sulfate
ml	millilitre
mm	millimetre
mM	millimolar
$MnSO_4$	manganese sulfate
mRNA	messenger RNA
MS	Murashige-Skoog (medium)

MW	molecular weight
NAA	α-naphthaleneacetic acid
$NaCl_2$	sodium chloride
Na_2EDTA	disodium ethylenediaminetetraaceticacid
NaOH	sodium hydroxide
NH_4NO_3	ammonium nitrate
$NiCl_2$	nickel chloride
ORF	open reading frame
PAP	pokeweed antiviral protein
PTA-ELISA	plate-trapped antigen enzyme-linked immunosorbent assay
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMMoV	*Pepper mild mottle virus*
PMSF	phenylmethylsulfonyl
PPT	phosphinothricin
PVP	olyvinylpyrrolidone
PVX	*Potato virus X*
PVY	*Potato virus Y*
RIP	ribosome inactivating protein (s)
RCNMV	*Red clover necrotic mosaic virus*
RNA	ribonucleic acid
RSV	*Rice stripe virus*
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reactions
SBWMV	*Soil-borne wheat mosaic virus*
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
TAE	Tris/acetic acid/EDTA buffer

Taq	*Thermus aquaticus*
T-DNA	transferred DNA from Ti plasmid
TEMED	*N'N'N'N'* tetramethyl ethylene diamine
TGMV	*Tomato golden mosaic virus*
Ti-plasmid	tumour-inducing plasmid of *A. tumefaciens*
TMV	*Tobacco mosaic virus*
Tris	tris (hydroxymethyl) methylamine
TSWV	*Tomato spotted wilt virus*
TuMV	*Turnip mosaic virus*
2.4-D	2,4-dichlorophenoxy-acetic acid
UTP	uridine-5'-triphosphate
UV	ultraviolet
WSSMV	*Wheat spindle streak mosaic virus*
$ZnSO_4$	zinc sulfate

CHAPTER 1

INTRODUCTION

1. 1 Wheat production

Wheat (*Triticum aestivum* L.) is one of the three major food crops in the world, accounting for over one-quarter of global cereal production (Morris and Bryce, 2000). Its annual production approached 584 million metric tonnes by 1996 (FAO, 1996). Wheat is a staple food for about 36% of the world's population (Oerke *et al.*,1994), while more than 15% of wheat is being used as animal feed (Morris and Bryce, 2000). China is one of the major wheat producers in the world along with the USA and India. In China, wheat is grown on more than 28.9 million hectares of arable land and it is the staple food for more than half of the 1.2 billion population.

During the past several decades the global yield and quality of wheat have been increased substantially by the use of varieties with higher yield potential and the intensive use of fertilisers to maximise yield. However, wheat production will not meet the global increase demand for food and feed, especially in developing countries. In 1993 the world population was 5.7 billion, and will double in 44 years if it continues to grow at the currently annual growth rate of 1.6%. In addition, the demand for cereals for animal feed is projected to double in developing countries between 1993 and 2020 (Pinstrup-Andersen, 2000). To keep pace with this enormous growth of world population and increased demand for animal feed, wheat production will have to be increased greatly in the next several decades. However there is unlikely to be a great increase in cropped area in the future. In fact the process of urbanisation and

industrialisation is leading to the area of arable land declining in most Asian countries. In addition, the annual increase in yields of major cereals is likely to slow down during 1993-2000 in both developed and developing countries (Pinstrup-Andersen, 2000).

Wheat production in China has increased dramatically over the past several decades. By 1996, the wheat yield of China had increased by 243% to more than 109 million tonnes (FAO, 1996) compared to the production of wheat in 1969. However, domestic production of wheat could not meet the increasing demand both for food and animal feed in China, therefore more than 11.8 million tonnes of wheat had to be imported in 1990, representing 8.2% of world wheat imports (OECD, 1993).

Wheat yield is continuously threatened by many diseases caused by insect pests and pathogens such as fungi, bacteria and viruses. From 1988 to 1990, the annual crop losses in wheat in the principal wheat-growing regions of the world were estimated at about 52% of total yield; of this 16.7% was due to disease, 11.3% to animal pests and 23.9% to weeds. Therefore preventing these losses could make a great contribution to increasing wheat production.

1.2 Soil-borne wheat mosaic (SBWM) and wheat spindle streak mosaic (WSSM) diseases

Wheat (*Triticum aestivum* L.) is susceptible to infection by many viruses. *Soil-borne wheat mosaic* and *Wheat spindle streak mosaic viruses* cause two serious diseases of winter wheat in most winter wheat growing regions of the world. They have a significant impact on wheat production in the USA as well as in both China and Europe. The disease caused by SBWMV was first described in Illinois, USA in 1919 (McKinney,1923), and has been found successively in north and south America, in southern and eastern Europe and in eastern Asia. The diseases caused by SBWMV and WSSMV can be devastating. Total yield

loss of some susceptible cultivars has been reported during the 1920s (McKinney, 1937). Kucharek and Walker (1974) have reported losses of 50% in Florida and losses of 45% have been reported in Nebraska (Palmer *et al.,* 1975). The severity of disease caused by SBWMV may vary with wheat cultivar and environmental conditions. In 1984, Shirako and co-workers reported that a deletion mutant of Nebraska SBWMV can cause more severe symptoms than the wild-type virus on infected plants (Shirako and Brakke, 1984b). Chen and colleagues studied the effect of cultivation temperature on spontaneous deletion in *Soil-borne wheat mosaic virus* RNA2 and found the symptoms also became more serious when plants were infected with the second passage isolate of Oklahoma SBWMV from diseased plants in the glasshouse (Chen *et al.*, 1994).

Since the first report of disease caused by WSSMV in Canada by Slykuis (1960) it has become spread worldwide. WSSMV is an important virus which can cause high losses of quality and yield. Subsequent studies have been made to evaluate the effect of the disease on the grain yield of wheat (Cunfer *et al.*, 1988; Bays, 1985; Nguyen, 1980). It has been reported that yields of wheat growing in infected fields may be reduced by 25-50% (Slykhuis, 1976). Co-infection by SBWMV and WSSMV can often occur, which greatly increases symptom severity and crop loss (Chen, 1993; Rubies, 1987).

In the past two decades, diseases caused by soil-borne viruses have occurred successively in central and eastern China and have become increasingly important in winter wheat growing regions of China (Fig. 1, Chen, 1993a). The yield losses of wheat varied from 10% to 30% in infected areas and even approached 70% in some instances (Hou *et al.*, 1985, Chen, 1993b). According to Chinese crop protection authorities, losses nationwide in wheat due to pathogens were estimated as 1.9% for all rust diseases, 0.5% for *Fusarium spp* and 3.4% for *Erysiphe graminis* (Oerke *et al.*, 1994).

1.3 Symptoms of the disease caused by SBWMV and WSSMV

The symptoms of the disease caused by SBWMV include the appearance of a green and yellow mosaic on the leaves of infected plants (Fig. 2), especially on the youngest leaves, and the plants are usually severely stunted (Brakke, 1971). The disease is more severe in low areas of the wheat-growing field and affected sites in the field appear as green or yellow patches. The severity of symptoms depends on a combination of variety, strain of virus and weather conditions (Brakke, 1971).

Like SBWMV, WSSMV can cause a mosaic disease of autumn- or winter-sown wheat which is characterised by a chlorotic or necrotic spindle-shaped streak in the leaves (Slykhuis, 1976).

1.4 Transmission of soil-borne virus disease

It is known that both viruses are transmitted by a fungal vector, *Polymyxa graminis* which is soilborne, and an obligate parasite of many cereals and other grasses. SBWMV is transmitted by the fungal vector via zoospores, being released either from resting spores or from intracellular sporangia of *Polymyxa graminis* during infection of plant roots. Preventing infection of roots from *Polymyxa graminis* in the infested field is a formidable problem because the fungus can survive as resting spores in the soil for many years. Once a field is infested with viruliferous fungus there is no way to eradicate the virus. Therefore the only simple and economical method to control SBWMV and WSSMV is by growing resistant wheat cultivars in the infested fields.

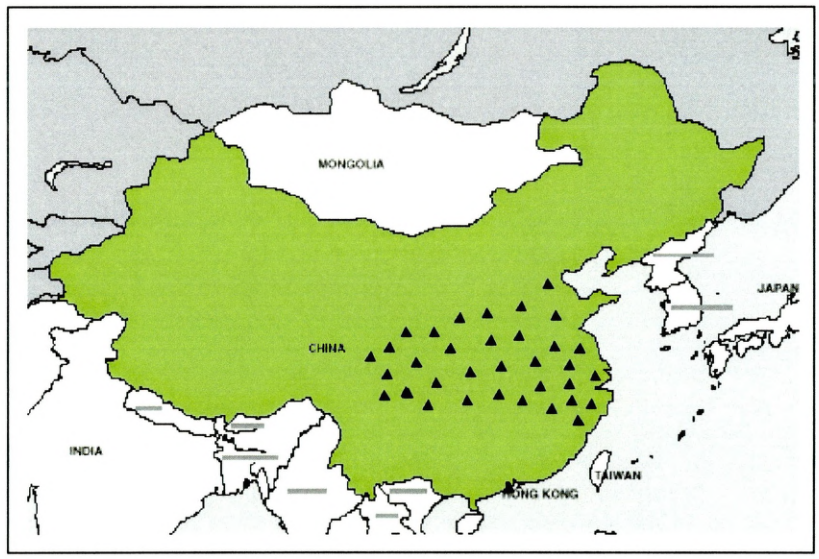


Figure. 1. Distribution of soil-borne viruses in China (Chen, 1993).

Figure. 2. Leaf symptoms of diseased wheat plants infected with *Soil-borne wheat mosaic virus* (adapted from a University of Nebraska NebGuide Publication)

1.5 *Wheat spindle streak mosaic virus* (WSSMV)

Wheat spindle streak mosaic virus is a member of *Bymovirus* genus. The type species of this genus is *Barley yellow mosaic virus. Bymoviruses* have various features in common with *Furoviruses,* such as containing two species of ssRNA and in the functional organisation of the polyprotein. Unlike other viruses that infect wheat, WSSMV has extremely long filamentous particles, more than 2000 nm in length (Slykhuis, 1976). Molecular studies revealed that WSSMV contains two RNAs, designated RNA1 and RNA2. The molecular weight (MW) of the RNA1 and RNA2 are 2.6 x 10^6 and 1.4 x 10^6 and the coat protein MW is 33 x 10^3 kDa (Usugi *et al.*, 1989).

1.6 *Soil-borne wheat mosaic virus* (SBWMV)

SBWMV along with nine other viruses were first proposed to be in a separate group named as furovirus by Shirako and Brakke in 1984 and this was approved in 1987 by the International Committee on Taxonomy of Viruses (Brunt and Richards, 1989)

Soil-borne wheat mosaic virus is classified as the type member of the *Furovirus* (*fun*gus-transmitted *ro*d-shaped) genus (Brunt, 1991; Shirako and Wilson, 1994). The virion contains two linear positive-sense ssRNAs, which are separately encapsulated in two rigid rod-shaped particles, around 20 nm in diameter. The larger is 281-300 nm in length, contains RNA1, and is designated as virion I. The smaller one is 138-160 nm in length, contains RNA2, and is designated as virion II (Brakke *et al.*, 1965; Brakke, 1977). Wild-type Nebraska isolate RNA1 has 7099 nucleotides (nt) and RNA2 contains 3593 nt (Shirako and Wilson, 1993). But RNA2 is variable in length, depending on the growth conditions and subsequent serial inoculations (Chen, 1995; Shirako, 1984). Both RNAs of the type virus of this group are capped at their 5'-termini and have a tRNA-like structure at the 3'-termini, without polyadenylation (Brunt, 1994).

Information about SBWMV has accumulated rapidly during the last decade. In 1993, Shirako and Wilson sequenced both RNAs of the Nebraska isolate of SBWMV and elucidated its genome organization (Fig. 3).

The genome of SBWMV RNA1 is 7099 nt in length. Sequence analysis showed that there are three possible open reading frames (ORFs) in SBWMV RNA1. The first ORF codes for a 150 kDa protein which starts from nucleotides 102-104 (an AUG) and terminates with a UGA at nucleotides 4062-4064. Sequence similar to ORF1 of SBWMV has been found in several other viruses, such as *Barley stripe mosaic virus* (BSMV) encoding a 130 kDa protein (Gustafson *et al.*, 1989) and tobacco mosaic virus (TMV) encoding a 126 kDa protein. Comparisons with the N-terminal region of the TMV 126 kDa protein and Sindbis virus nspl suggest that this region might be associated with an RNA capping enzyme activity. Meanwhile an NTP-binding helicase motif has been found at amino acids 1027-1034 of the deduced ORF1 polypeptide chain.

ORF2 starts at nucleotide 4185 and terminates with a UAA at nucleotides 5586-5588 and encodes the replicase. *In vitro* translation showed that the ORF1 stop codon UGA can be partially suppressed; therefore, a readthrough product, a 220 kDa protein can be generated. This polyprotein is probably cleaved post-translationally, yielding several viral proteins which may produce a replication complex (Shirako *et al.*, 1993).

The last ORF, 983 nuclotides long, starts at nt 5653 and terminates at nt 6636 with a UAG, followed by a 463 nt 3'-untranslated region (UTR). ORF3 encodes a 37 kDa cell-to-cell transport protein which is closely related to the equivalent movement proteins (MPs) of TMV and dianthoviruses (Shirako et al., 1993).

SBWMV RNA2 is 3593 nucleotides long, with three possible ORFs, found by analysis of start and stop codons in the RNA2 sequence. The first ORF starts with an AUG at nts 334-336 and stops with a UAG at nts 862-864; it

encodes a 19 kDa capsid protein. *In vitro* translation of wild-type viral RNA2 resulted in three proteins of 84 kDa, 28 kDa, and 19 kDa. The 28 kDa protein is considered to be associated with virus replication or virus persistence in nature. The second ORF coding for a 54 kDa protein starts at nt 1141 and stops at nts 2596-2598 with a UAA codon. The function of 54 kDa protein is unknown. An 84 kDa protein can be produced by readthrough of the ORF1 UGA stop codon. Studies on spontaneous deletions in RNA2 from virions isolated from plants with prolonged infection, or successively mechanically-inoculated plants, revealed that this readthrough region may be required for fungus transmission (Shirako *et al.*, 1984a; Hsu *et al.*, 1985a; Shirako *et al.*, 1986). The last ORF encodes a cysteine-rich protein of 19 kDa from nt 2665 and stops at nts 3187-3189 with a UAG. Similar proteins have also been found in several other plant viruses; BNYVV and BSMV, but the function of these proteins is unknown at present.

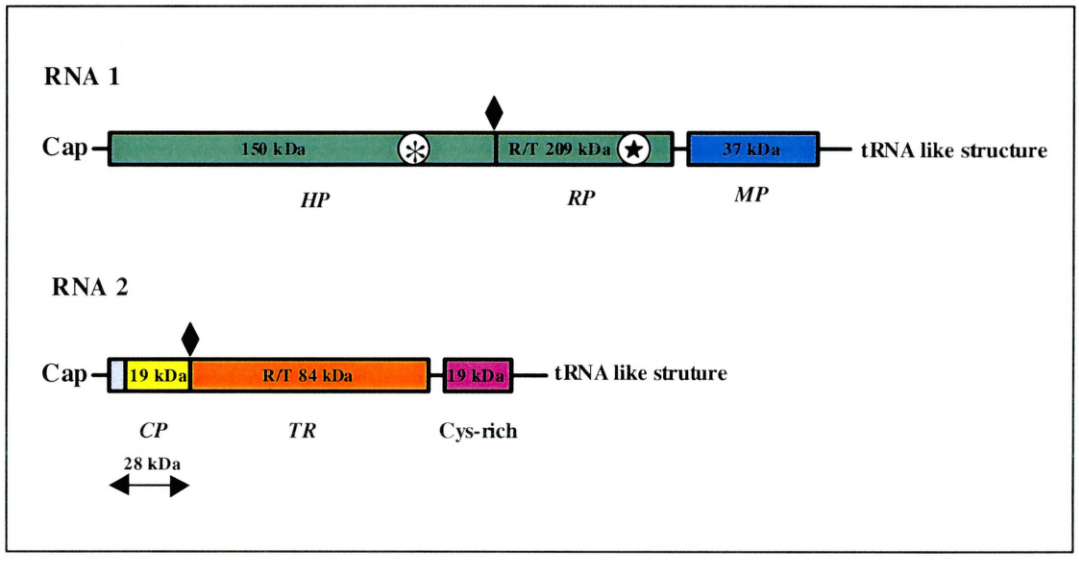


Figure. 3. Genome organisation of soil-borne wheat mosaic virus. R/T: Reading-through position, *HP*: Helicase coding region, *RP*: Replicase coding region, *MP*: Movement protein coding region, *CP*: Coat protein coding region, *TR*: Transmission-related protein coding region, Cys-rich: Cysteine-rich protein coding region.

1.7 *Chinese wheat mosaic virus* (CWMV)

A soil-borne virus disease of winter wheat has been known in China for many years. It was presumed that this disease was caused by infection of *Soil-borne wheat mosaic virus* alone or co-infection with *Wheat spindle streak mosaic virus* or *Wheat yellow mosaic virus*. In Shandong province this virus-related disease had occurred over the past two decades, which seriously damaged wheat production. Two viruses were identified as responsible for this wheat disease: a rod-shaped virus previously described as *Soil-borne wheat mosaic virus* based on its serological relationship with an isolate from Nebraska USA, and *Wheat yellow mosaic virus* (Ye *et al.*, 1999). Recently, the soil-borne wheat mosaic virus infecting winter wheat in Shandong province, China has been characterized. Complete nucleotide sequence and partial amino acid sequence analysis have shown that this Chinese virus is related to, but different from soil-borne wheat mosaic virus because the nucleic acid identities are only 75% in RNA1 and 63% in RNA2, while the amino acid sequences share from 62% (19 kDa RNA2 product) to 84% (RNA1, replicase) identity. The properties of this Chinese virus suggested it should be regarded as a new member of genus *Furovirus*, and it was therefore named as *Chinese wheat mosaic virus* (Diao *et al.*, 1999).

1.8 Natural resistance to SBWMV and WSSMV

Several resistant wheat cultivars have been identified by extensive screening trials in different countries, and some varieties expressing resistance to SBWMV and WSSMV have been developed in the USA as well as in other countries (Fouchard *et al.*, 1995; Rubies-Autonell *et al.*, 1985; Haufler *et al.*, 1986). Attempts have been made to address the mechanism of crop resistance to SBWMV and WSSMV. Early studies, conducted by Miyake (1938) indicated that a single dominant gene controls the expression of resistance to SBWMV. In contrast, the studies by Nakagawa (1960) showed that at least three plant genes

are involved in the resistance reaction to green and yellow strains of SBWMV. In 1966, Shaalan found, by analysis of the progeny obtained by crossing a resistant cultivar (Ottowa) with a susceptible cultivar (Bison), that resistance to SBWMV was controlled by two genes, a partially dominant gene which contributed resistance to virus and a second gene which modified the function of the first gene. Studies by Modawi *et al.* (1982) and Merkle *et al.* (1983) on the inheritance of field resistance to SBWMV implied that the resistance was dominant and monogenically inherited. In 1986, Lommel *et al.* found that some hard red winter cultivars, which are considered to be resistant to SBWMV, can be infected with virus and show typical symptoms when grown in soil infested with both SBWMV and WSSMV. The severity of the disease can be increased to the same level as in SBWMV-susceptible varieties, which indicates that environmental conditions can influence the level of resistance in resistant wheat cultivars. It is now believed that resistance to SBWMV and WSSMV may be due to resistance to virus infection and multiplication, rather than resistance to vector fungus infection (Haufler *et al.*, 1986). To date, the mechanism by which resistant wheat cultivars naturally expressed resistance to these two viruses is less well characterized. In addition, resistance traits are often linked to yield or grain quality traits which makes it difficult to transfer resistance to new cultivars by conventional breeding methods. So far, no genes conferring resistance to SBWMV and WSSMV have been transferred to commercial wheat cultivars.

1.9 Strategies for control of viral diseases

1.9.1 Conventional methods to control SBWMV and WSSMV

To some extent, virus-induced crop losses can be minimized by cultural methods (appropriate planting time, varietal rotation, increasing fertilizer application) combined with the use of fungicide and resistant cultivars (Brunt and Richards, 1989). Delayed crop sowing may help some plants escape infection by fungal spores in the infested field. This is assumed to be due to sub-optimal

conditions for fungus infection (Kendal *et al.*,1988). Varietal rotation can reduce the disease risk caused by monoculture and unbalanced rotation of cereals, it is considered to reduce the effective "titre" of field inoculum but it cannot eliminate the occurrence of disease (Brunt *and* Richards, 1989). Increasing fertiliser application might promote infected plants to outgrow the virus, however, this can increase the susceptibility of the crops to attack by harmful organisms. For example, the importance of aphids and powdery mildew has increased because of the enhanced application of nitrogen fertilisers, which makes wheat a more susceptible host. This not only raises the severity of infection, but can also increase the damage done by each organism. Although applying fungicides, such as methyl bromide and formaldehyde, to infested fields can greatly diminish the incidence of SBWMV and WSSMV, it is not an economically or environmentally sustainable or acceptable means, especially in undeveloped countries, for the effective and reliable control of fungal diseases on a large scale. Therefore the more effective method to control the virus disease is to develop and grow virus resistant wheat cultivars. The developments of modern molecular biology and plant transformation techniques allowed us to genetically improve economically important crops for enhanced resistance to many pathogenic diseases, thus creating virus resistant wheat cultivars that could contribute significantly to reducing the losses in wheat production in the world.

1.9.2 Genetically engineered resistance to plant viruses

Plants can be engineered to become resistant to virus disease by expressing some viral sequences in transgenic plants (Hamilton, 1980). Several strategies for engineering virus resistance in plants have been developed. These include coat-protein (CP) mediated resistance or protection, movement-protein mediated resistance, the expression of replicase or satellite RNA sequences, use of dysfunctional DNA or RNA sequences, and the use of ribosome inactivating protein. Amongst these, coat-protein mediated resistance or protection has been the most successful approach applied to generate virus-

resistant crop plants. This is based on a naturally-occurring mechanism of protection against virus. The phenomenon that a plant, systemically infected by one strain of a particular virus producing a mild symptom, may become resistant to infection by the same or other related strain of the same virus was referred to as "cross-protection" (Fraser, 1992). The concept of pathogen-derived resistance (PDR) was first postulated by Sanford and Johnston (1985). They suggested the possibility of engineering pathogen resistance by expressing genes derived from the pathogen itself in susceptable plants. Powell-Abel *et al.* (1986) first demonstrated the use of viral CP genes to produce virus-resistant plants. Since then, virus resistance has been achieved by transferring and expressing particular viral genes from many plant species (Fitchen *et al.*, 1993; Wilson, 1993).

1.9.2.1 Coat protein-mediated virus resistance or protection

Most plant virus genomes encode a coat protein in which the virus genomes are encapsidated. Apart from their protective function, virus coat proteins also have many other functions involved in virus life cycle and virus-plant interaction. Powell-Abel *et al.* (1986) first reported that transgenic tobacco plants transformed with the coat protein gene of *Tobacco mosaic virus* (TMV) showed no systemic or delayed disease development when challenged with purified TMV. Since then, coat protein-mediated resistance has been successfully applied to many crops like tobacco, tomato, alfalfa, cucumber, potato, papaya, sweet corn, rice and sugar beet against viruses from different genera such as tobamoviruses and potyviruses (Beachy *et al.*, 1990; Gonsalves *et al.*, 1993; Hackland *et al.*, 1994; Stark *et al.*, 1989). In cereals, Hayakawa *et al.* (1992) reported that the expression of *Rice stripe virus* (RSV) CP gene in rice conferred resistance to this planthopper-transmitted virus, while transgenic maize expressing the coat protein gene of *Maize dwarf mosaic virus* (MDMV) was shown to be resistant to MDMV infection (Murry *et al.*, 1993).

Although coat protein-mediated protection has been achieved for many crops the mechanism involved remains unclear. A number of different mechanisms relating to different host-virus systems have been advanced. One possible explanation for this phenomenon is that excessive coat protein accumulated in the infected cells interrupts the many aspects of virus life cycle through inhibiting the uncoating of challenge virus; therefore the plant was protected from virus infection or showed a delayed symptom development (Kahl and Winter, 1995). This possible mechanism is supported by the fact that coat protein-mediated resistance can be overcome by direct inoculation with viral RNA (Powell-Abel *et al.*, 1986; Loesch-Fries *et al.*, 1987; Van Dun *et al.*, 1987). However this mechanism is not applicable for all viruses since some transgenic plants with undetectable coat protein have been found to be resistant to virus to some extent (Stark *et al.*, 1989; Farinelli *et al.*, 1993). This indicated that the mechanism of resistance conferred by viral CP gene might differ for different viruses. The evidence that resistance in some transgenic plants expressing viral CP gene could not be overcome by corresponding viral RNAs implied that CP-mediated protection may occur at a different stage of challenge virus infection. Although multiple mechanisms may exist for CP-mediated protection, in most cases the consensus is that the endogenous coat proteins inhibit disassembly of a challenge virus, leading to the protection of plant from virus infection (reviewed by Reimann-Philipp and Beachy, 1993; Hackland *et al.*, 1994).

So far, coat protein-mediated resistance or protection has been reported for more than 35 viruses, representing 15 viral taxonomic group (reviewed by Palukaitis and Zaitlin, 1997). However, there are no reports of successful resistance to viruses by transfer of viral coat protein genes into wheat plants.

1.9.2.2 Movement protein-mediated virus resistance

Movement of virus, leading to systemic infection of plants, was considered to be controlled by one or more virus-encoded movement proteins (MPs) and its

interaction with host plant proteins. This hypothesis was first confirmed by Deom and co-workers (Deom *et al.*, 1987). In their experiments, a construct containing the MP coding sequence of the L strain of TMV was transformed into *Nicotiana tabacum* cv. Xanthi. Molecular analysis showed the integration and expression of the viral MP sequence in transgenic plants. Furthermore, inoculation of transgenic plants with a mutant of TMV L, LS1, caused large lesions on the leaves and chlorotic symptoms in the upper leaves; however, only small lesions on the leaves of inoculated wild-type plants were found and no symptoms developed in their upper leaves. Wolf *et al.* (1989) and Berna *et al.* (1991) reported that MP can localise to some plant cell fractions and leads to changes in the characteristics of plasmodesmata. This confirmed the hypothesis that viruses move from cell-to-cell *via* plasmodesmata. Cooper *et al.* (1995) showed that a defective movement protein of TMV conferred resistance to multiple viruses in transgenic plants, whereas the functional analogue increased susceptibility. The mechanism of resistance conferred by the dysfunctional movement protein is unknown.

1.9.2.3 Replicase-mediated virus resistance

Expression of viral replicase complex in transgenic plants can also confer a high level of resistance to the virus or related strains. In 1990, Golemboski *et al.* transformed tobacco with a cDNA clone of a 54 kDa protein, the putative replicase protein of TMV, and found the plants highly resistant to both TMV virus and viral RNA, and the resistance was also observed against closely-related strains of TMV. Since then this strategy has been successfully used to develop virus resistant plants. The transferred replicase-derived sequences encoded either functional or dysfunctional proteins, in some cases even no proteins. The expression of the replicase gene of *Cymbidium ringspot virus* (CymRSV) in transgenic *Nicotiana benthamiana* led to high resistance to both CymRSV and virus RNA (Rubino *et al.* 1993). Transgenic *N. benthamiana* expressing the 200 kDa replicase gene of *Cowpea mosaic virus* (CPMV) exhibited resistance to

CPMV (Sijen *et al.*, 1994). Tobacco transformed with a deleted version of the *Cucumber mosaic virus* (CMV) replicase (2a protein) gene, encoding a truncated 70 kDa protein was resistant to CMV and CMV RNA infection (Anderson *et al.*, 1992). Replicase-mediated resistance has been achieved for 14 viruses representing 10 plant virus taxons (reviewed by Palukaitis and Zaitlin, 1997).

Different mechanisms might exist for engendering replicase-mediated resistance to different viruses. Studies using protoplasts derived from tobacco plants transformed with the 54 kDa region of TMV replicase gene suggested the expression of 54 kDa protein in the transgenic plant might be critical in eliciting a plant resistance response. The protein-mediated resistance has also observed in transgenic tobacco plants with the CMV 2a protein (Carr *et al.*, 1993) and in transgenic tobacco plants with the AMV replicase gene (Brederode *et al.*, 1995). However, there is evidence that expression of the 54 kDa region of *Pepper mild mottle virus* (PMMoV) replicase gene is not essential in induction of resistance (Tenllado *et al.*, 1995). This RNA-related resistance has also been found to be effective against CymRSV and PVX (Rubino and Russo, 1995; Mueller *et al.*, 1995). The studies on transgene copy number, level of mRNA and protein level in transgenic tobacco plants expressing different levels of resistance to cucumber mosaic virus suggested that translatability of the transgene and possibly expression of the transgene protein itself facilitates replicase-mediated resistance to CMV (Wintermantel and Zaitlin, 2000).

1.9.2.4 RNA-mediated virus resistance

Most cases of RNA-mediated resistance to virus infection were discovered from the studies of coat protein-mediated resistance. In the case of *Tomato spotted wilt virus* (TSWV), tobacco plants transformed with a translationally defective TSWV CP gene exhibited a resistance level similar to those of CP-expressing plants, and the phenotype of resistance in both cases was identical (De Haan *et*

al., 1992). This suggested the presumed CP-mediated resistance was possibly conferred by viral RNA molecules. RNA-mediated resistance has also been observed in transgenic tobacco and potato plants, expressing either translationally defective or antisense viral coat protein sequences. The levels of resistance of these transgenic plants were similar to those in plants transformed with translationally competent gene constructs (Kawchuk *et al.*, 1990; Van der Vlugt *et al.*, 1992).

RNA-mediated resistance was also demonstrated in transgenic tobacco plants transformed with an antisense TMV CP-gene constructs or tomato golden mosaic virus (TGMV) antisense replicase gene, and the levels of resistance were directly correlated with the level of transgene mRNA (Powell-Abel *et al.*, 1989; Day *et al.*, 1991). Jan *et al.* (1998) studied the effect of transgene length and non-target DNA sequence on RNA-mediated virus resistance using transgenic *Nicotiana benthamiana*. *Nicotiana benthamiana* plants were transformed by *Agrobacterium* with constructs containing a range of nucleocapsid (N) gene (from 28 to 400 bp) of *Tomato spotted wilt virus* (TSWV) alone or either fused to jellyfish green fluorescent protein (GFP) gene or fused to *Turnip mosaic virus* (TuMV) coat protein gene. Results showed that the full length of the N gene sequence in transgenic tobacco is not required for transgene silencing and virus resistance, instead the ineffective short transgene fragments can also confer TSWV resistance when fused to either GFP or TuMV CP gene. They concluded that a critical length of TSWV (236-387bp) N gene rather than full length of coding sequence is required for RNA-mediated TSWV resistance in transgenic tobacco plants (Jan *et al*,. 1998).

Multiple mechanisms may exit for the RNA-mediated resistance. These include the methylation of transgene DNA (Matzke *et al.*, 1993) and cytoplasmic activity which targets specific RNA sequences for inactivation (Lindbo *et al.*, 1993; Müeller *et al.*, 1995).

1.9.2.5 Alternative strategies for virus resistance

Other strategies explored to achieve virus tolerance or resistance include the expression of satellite RNA sequences and the use of ribosome inactivating proteins (RIPs).

Satellite RNAs are small components of some RNA viruses without significant homology to the genome of their dependent virus. They require a helper virus for their replication and propagation within the virus. Harrison *et al.* (1987) showed that expressing a *Cucumber mosaic virus* (CMV) satellite RNA gene in transgenic tobacco plants significantly inhibited the replication of challenge virus, leading to a much lower level of accumulation of virus, whereas the satellite RNA was replicated to high levels upon the infection.

Ribosome inactivating proteins (RIP) are commonly found in plants, fungi, and bacteria. By now more than 70 RIPs have been isolated which are classified into three groups based on their properties (Wang and Tumer, 2000). Recently RIPs were used as a source of resistance for plant protection. Transgenic potato and tobacco plants expressing cDNA clones of pokeweed antiviral protein (PAP) or variant PAP exhibited resistance against a broad-spectrum of viruses (Lodge *et al.* 1993). Resistance to TMV, PVX and the soil-borne fungus *Rhizoctonia solani* was achieved by transformation and expression of PAPII from *Phytolacca americana*, a less toxic PAP, in tobacco plants (Wang *et al.*, 1998). Less is known about the mechanism by which RIPs inhibit viral infection.

1.10 Plant Transformation Techniques

The advent of plant transformation technology has provided a powerful tool in plant breeding for genetic improvement of crops for enhanced resistance to plant pathogens and pests. A technique of gene transfer, which introduces foreign genes into the recipient cells, is the prerequisite for achieving transgenic plants. The first attempt to genetically transform a plant was by De Block with tobacco in 1984 (De Block *et al.*, 1984). Since then, several methods for plant transformation have been developed. To date, transformation has been reported from more than 120 different species in at least 35 families, including most major crops.

1.10.1 *Agrobacterium*-mediated transformation

Among gene transfer methods, *Agrobacterium tumefaciens*-mediated transformation and microprojectile bombardment approaches represent the most successful and widely used techniques (Potrykus, 1991; Christou 1996). *Agrobacterium*-mediated transformation was first developed successfully to transfer foreign genes into plants and marked the breakthrough in gene transfer to plants. This system is based on the fact that the soil bacterium *Agrobacterium tumefaciens* can deliver a portion of its megaplasmid (Ti plasmid) into host plant cells and the transferred T-DNA can be stably integrated into the plant nuclear genome.

Since the first report of successful *Agrobacterium*-mediated transformation and plant regeneration in tobacco (Horsch *et al.*,1984), the *Agrobacterium* transformation system has been applied extensively for the recovery of transgenic dicotyledonous species including important crops such as cotton (Firoozabady *et al.*, 1987), potato (Sheerman *et al.*, 1988), soybean (Hinchee *et al.*, 1988), sugarbeet (Lindsey *et al.*, 1990), sunflower (Everett *et al.*, 1987), tomato (Fillatti *et al.*, 1987), papaya (Fitch *et al.*, 1993) and cassava (Li *et al.*, 1996).

Monocotyledonous plants, particularly the graminaceous species, were considered to be out of the *Agrobacterium* host range (Bevan, 1984; De Cleene, 1985). However, remarkable progress has been made in monocot. transformation by the *Agrobacterium*-mediated transformation approach in recent years. Transgenic plants have been generated from asparagus (Byterbier *et al.*, 1987), maize (Gould *et al.*, 1991; Ishida *et al.*, 1996), rice (Hiei *et al.*, 1994; Aldemita and Hodges, 1996; Rashida *et al.*, 1996) and barley (Tingay *et al.*, 1997). More recently, Cheng reported the first successful transformation of spring wheat (cv. Bob White) by use of the *Agrobacterium*-mediated transformation system (Cheng *et al.*, 1997).

1.10.2 Direct gene transfer to protoplasts

Following the development of the *Agrobacterium* transformation system, many efforts have been made to attempt to directly introduce naked DNA into plant protoplasts. Transformation techniques have been developed to transfer genes into protoplasts by use of polyethylene glycol (PEG) or electroporation (Potrykus *et al.*, 1985; Lorz *et al.*,1985; Uchimiya *et al.*, 1986). Although these transformation techniques are applicable for both dicotyledonous and monocotyledonous species, and no biological vector is required, the successful application of these methods for the recovery of transgenic plants needs competent protoplasts for plant regeneration. The establishment and maintenance of embryogenic cell cultures, which are needed to provide the necessary competent protoplasts for regeneration and genetic transformation in monocots., is time-consuming, labour-intensive and technically demanding. Furthermore, it has been reported that the regeneration capacity declined during cultivation in cereal suspension (Jähne *et al.*, 1991a) and plant regeneration from long-term cultured protoplasts may lead to undesirable somaclonal variation and regeneration of sterile plants (Datta *et al.*, 1992). Therefore, these disadvantages may limit the use of these techniques for plant transformation.

1.10.3 Electroporation of plant tissues

The achievement in transformation of protoplasts by electroporation has stimulated several groups to attempt to transform various tissue explants by electroporation, such as embryogenic calli (D'Halluin *et al.*, 1992; Arencibia *et al.*, 1995), immature embryos (D'Halluin *et al.*, 1992; Songstad *et al.*,1993; Klöti *et al.*, 1993; Ke *et al.*, 1996), mature embryos (Xu and Li, 1994), suspension cultures (Yang *et al.,* 1993; Laursen *et al.,* 1994) and other tissues (Dekeyser *et al.*, 1990).

Several authors have reported the regeneration of fertile transgenic plants from maize and rice by use of this method (D'Halluin *et al.*, 1992; Xu and Li, 1994; Laursen *et al.,* 1994). In their experiments, partial enzymatic digestion or mechanical wounding of plant tissue was often used before electroporation (D'Halluin *et al.*, 1992; Xu and Li, 1994; Laursen *et al.,* 1994).

1.10.4 Microprojectile bombardment

The microprojectile bombardment technology was first conceived by Sanford *et al.* in 1987, and has become the second most widely-used method for plant genetic transformation. It employs high velocity metal particles to deliver foreign genes into any plant cells and tissues (McCabe and Christou, 1993). It involves the acceleration of DNA-coated microprojectiles, by means of an explosion of gunpowder (Christou *et al.*, 1991) or burst of gas (Finer *et al.*, 1992) to a high velocity to penetrate the plant cell walls. The main advantage of this technique over other gene delivery methods is that it allows targeting to a wide range of plant species, explants, cell types and cellular compartments to which foreign DNA can be transferred. Although several versions of particle bombardment instruments have been developed and being used in different laboratories the most commonly used gene gun is the Biolistic PDS-1000/He System built by Bio-Rad laboratory (Figure 4).

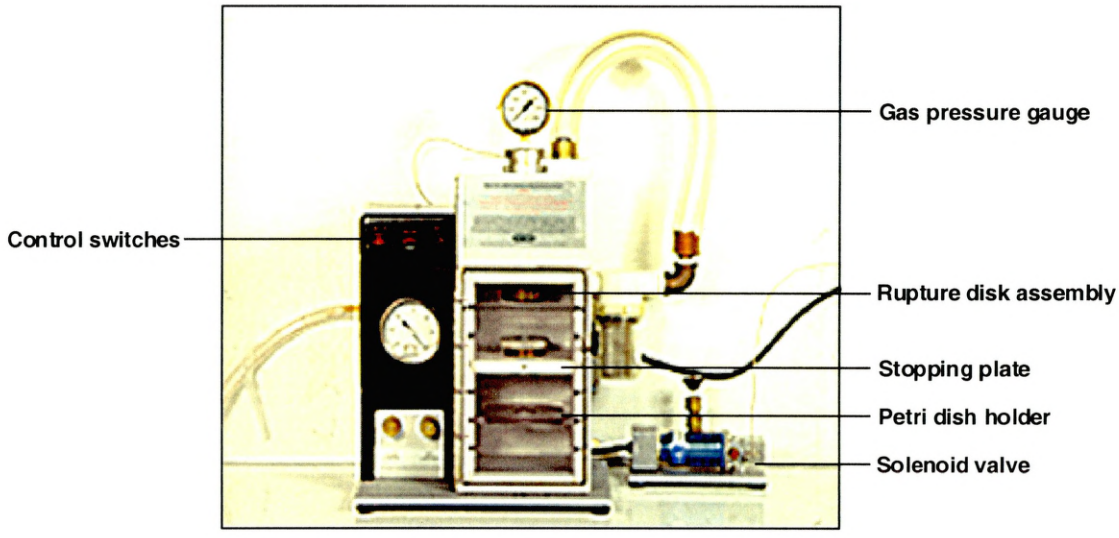


Figure. 4. The Biolistic PDS-1000/He System.

The development of the microprojectile bombardment technique has strongly influenced monocotyledonous species transformation, especially in cereals, in which the particle bombardment method has been primarily used for the transformation of major crops. This technique significantly reduced the time required for the generation of transgenic plants, and greatly facilitated the process of transformation of cereal plants. Transgenic plants made *via* microprojectile bombardment have been recovered from the most important cereals including maize (Gordon-Kamm *et al.*, 1990; Fromm *et al.*, 1990), rice (Christou *et al.*, 1991; Cao *et al.*, 1992), wheat (Vasil *et al.*, 1992, 1993; Weeks *et al.*, 1993; Jähne *et al.*, 1995; Nehra *et al.*, 1994; Takumi *et al.*, 1996; Altpeter *et al.*, 1996; Barro *et al.*, 1997), barley (Wan and Lemaux, 1994; Ritala *et al.*, 1994; Jähne *et al.*,1994), and sorghum (Casas *et al.*, 1993), as well as from major dicotyledonous crops (Christou, 1996).

1.11 Wheat transformation

Wheat, as a principal cereal crop, is an important target for the application of genetic manipulation techniques. However, due to the difficulty in initiation and maintenance of efficient *in vitro* culture systems from which fertile plants can be regenerated at a high frequency, and its large genome (more than 10^{10} base pairs) and its hexaploidy, genetic transformation of wheat was considered to be difficult in the past. In the past two decades, sustained effort has been devoted to establish an efficient tissue culture system from which high-frequency regeneration can be obtained by:

1). detecting the callus response of different wheat explants such as leaf (Ahuja *et al.*, 1982; Zaghmout *et al.*, 1993; Zamora *et al.*, 1983), all parts of the young seedlings (Bartok *et al.*, 1990; O'Hara *et al.*, 1978; Trione *et al.*, 1968), apical meristem (Wernicke *et al.*, 1984; Simmonds *et al.*, 1992), root (Chin *et al.*, 1977; Shimada *et al.*, 1969; Nabors *et al.*, 1983), inflorescence (Ozias-Akins *et al.*, 1982; Maddock *et al.*, 1983; Rajyalakshml *et al.,* 1988), mature and immature embryo (Chin *et al.*, 1977; O'Hara *et al.*, 1978; Ozias-Akins *et al.*, 1983; Shimada *et al.*, 1978; Ahloowalia *et al.*, 1982; Iglesias, *et al.*, 1994), anther and microspore (Wang *et al.*, 1973; Chu *et al.*, 1975; Ozias-Akins *et al.*, 1983; Feng *et al.*,1988).

2). optimising medium composition (Chu, 1978; Feng *et al.*,1988; Lucketteral *et al.*, 1991).

3). detecting genotypic effects (Ahloowalia et al., 1982; Carman *et al.*, 1987; Mathias *et al.*, 1986, 1988; Papenfuss *et al.*, 1987; Sears *et al.* 1982; Fennell *et al.*, 1996).

Although significant achievements in the regeneration of plants from wheat tissue culture have been obtained from previous studies and successful regeneration of plants from various explants of wheat have been reported by

numerous researchers, efficient plant regeneration from wheat tissue culture still remained difficult due to the lack of reliable and reproducible culture procedures either for establishment and maintenance of embryogenic cell culture or for regeneration of fertile plants from protoplast culture. Thus, this important limitation has impeded the development of genetic transformation of wheat.

Wheat was the last of the major cereal crops to be transformed. The successful recovery of fertile transgenic plants from bombarded regenerable embryogenic callus was first reported by Vasil *et al.* (1992) who employed the particle bombardment technique. The plasmid pBARGUS containing a selectable marker, the *bar* gene, driven by the *Adh1* promoter with the *Adh1* intron1, and a reporter gene (*gus*) driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter with the same intron as the *bar* gene were delivered into long-term regenerable embryogenic calli of two spring wheat cultivars by particle bombardment. Phosphinothricin (PPT) was used as the selective agent. Molecular analysis showed that the *bar* gene was integrated into the transgenic plants which were obtained by selection with the herbicide Basta®. Analysis of R1 and R2 progenies from transgenic lines indicated that *bar* gene was inherited following a Mendelian segregation pattern. Although there are some disadvantages such as difficulties of identification, selection and maintenance of long-term type C callus, and problems associated with the maturity and fertility of R_0 plants maintained in this system (Biady, 1992), this achievement in the recovery of transgenic wheat pioneered the possibility of wheat manipulation by applying recombinant DNA technology.

Weeks and co-workers (1993) reported a high frequency of regeneration of transgenic wheat after particle bombardment of cultured immature embryos of the highly-embryogenic wheat cultivar Bob White, and a transformation frequency which was increased to 0.1-0.2%. In the same year, Vasil also reported the production of transgenic plants from bombardent of cultured immature embryos or 1-2 month-old embryogenic calli (Vasil *et al.*, 1993). Since then, immature embryos have been widely used for the regeneration of fertile

transgenic wheat and in the last 5 years many groups have reported the regeneration of fertile transgenic plants from particle bombardment of immature embryos of different wheat cultivars (Weeks *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994; Zhou *et al.*, 1995; Zhang *et al.*, 1996; Altpeter *et al.*, 1996; Barro *et al.*, 1997). Regeneration of transgenic wheat with improved properties has been reported by Altpeter *et al.* (1996) and Barro *et al.* (1997), respectively. This demonstrated that the functional properties of wheat can be improved by genetic manipulation. In the first report, immature embryos of the spring wheat cultivar Bob White were cotransformed with pAHC 25 and pHMV 1AX1 by microprojectile bombardment. The plasmid pHMV 1AX1 contains a gene coding for high molecular weight glutenin (HMW-G) which is known to be associated with good bread-making quality. In some transgenic lines the amount of HMW-GS 1AX1 protein was increased to 70% of total HMW-GS proteins. The same constructs (pHMV 1AX1 and pAHC 25) were used for transformation of two near-isogenic wheat lines, L88-6 and L88-31, in the second report. Nine transgenic lines containing either subunit gene 1AX1 or/and the 1DX5 gene were obtained. The transformation with 1AX1, or 1AX1 plus 1D5, resulted in a stepwise increase in dough elasticity.

Recently, a report showed the transformation of immature embryos and embryogenic calli by the *Agrobacterium*-mediated transformation method and the regeneration of transgenic fertile plants (Cheng et al., 1997). This work was done using the model wheat variety Bob White transformed with the *nptII* (neomycin phosphotransferase II) and *gus* genes. Application of this method to agronomically important wheat varieties is still desirable.

Remarkable progress in wheat transformation has been achieved in the last several years. Particle bombardment has provided the decisive technological breakthrough for genetic manipulation of wheat. There is no doubt that the development of particle bombardment technology and the application of this technique will greatly speed up the process of wheat breeding and improvement. Bliffeld *et al.* (1999) reported the expression of antifungal barley-

seed class II chitinase in transgenic wheat plants led to the increased resistance to infection with the powdery mildew fungus *Erysiphe graminis*. Transgenic wheat plants expressing the cDNA of antifungal protein KP4 from *Ustilago maydis*-infecting virus under the control of maize ubiquitin promoter showed resistance to *U. maydis* (Clausen *et al.*, 2000).

Although transformation of wheat by either direct gene transfer or *Agrobacterium*-mediated methodology had already been the object of several studies, to date there have been no reports on successful transferring the sequences of SBWMV into wheat cultivars.

Positive complementation of movement protein genes between related or unrelated viruses has implied the virus encoded-movement function might play a dominant role in determining the host ranges. Study in this field might have great value for understanding the virus movement protein contribution to the virus–host interaction.

1.12 Virus movement proteins and virus movement

1.12.1 Virus movement proteins

In general movement of virus in plants has two essential prerequisites: first, the virus must encode the functions that facilitate the movement of virus, second, the presence of a pathway that allows the virus to move either from cell to cell or between different tissue or organs. This had been proven by the identification of the movement proteins from many viruses and the characterisation of the functional structure of plant cell.

Movement proteins (MPs) have been identified from many families and genera of plant viruses. Development of local necrotic lesions caused by TMV is influenced by the 30 kDa protein. The mutant TMV V23, derived from the U1 strain by replacement of its native 30 kDa gene with U2 30 kDa protein gene,

induced local lesions similar to those caused by the U2 strain on the inoculated Xanthi-nc leaves, and further experiments using V23 and U2 to infect the transgenic Xanthi-nc tobacco expressing the 30 kDa gene induced similar size lesions to those caused by U1 strain (Nejidat *et al.*, 1991). The elegant experiment conducted by Holt and his colleagues (1991) demonstrated that the movement of TMV in tobacco plants requires a 30 kD protein encoded by the virus, which is known as the movement protein of TMV. When the 30 kDa protein of an infectious cDNA clone was substituted by a defective version, the resulting virus was limited in the infected cell and incapable of movement from cell to cell. Furthermore, transgenic tobacco plants expressing the TMV movement protein lead to the complementation of movement function of a MP-defective TMV strain. This has provided the direct evidence that the movement protein is essential to the virus movement from cell to cell. Further evidence came from the experiments on the complementation of movement function from unrelated viruses. It has been found that transgenic tobacco plants expressing TMV MP enable the MP-defective cucumber mosaic virus (CMV) to move from cell to cell (Cooper *et al.*, 1996). Replacement of native MP of *Barley stripe mosaic virus* with the TMV MP lead to the movement of the modified virus in *Nicotiana benthamiana* and *Chenopodium amaranticolor* (Solovyev *et al.*, 1996).

It has been found that MPs have different domains that attribute to the virus movement at different levels. Berna *et al.* (1991) have found that a stretch of 19 amino acids at the C-terminus of TMV 30 kDa movement protein is indispensable in locating the MP to the cell wall fraction. In contrast, such a binding region seems to locate at N-terminus of *Alfalfa mosaic virus* (AMV) MP from 13 to 77 amino acids. It was found that the N-terminal sequence of TMV MP was required for virus movement function. Deletion of three amino acids of the N-terminus altered the movement function of TMV MP (Gafny *et al.*, 1992). Recently, independent experiments with MP of TMV and *Cowpea mosaic virus* (CPMV) demonstrated that the domains responsible for exploiting plasmodesmata channels were located at the opposite terminus of two MPs (Waigmann *et al.*, 1994, Lekkerkerker *et al.*, 1996). Different MP domains have

also been identified from *Red clover necrotic mosaic virus* (Giesman-Cookmeyer *et al.*, 1993). The difference in MP domains of different viruses might reflect the different models of virus movement from cell to cell.

1.12.2 Virus cell- to-cell movement

It is believed that plant viruses move from cell to cell through the intercellular channels, plasmodesmata. Two possible modes of movement of virus progeny from cell to cell through the plasmodesmata have been proposed based on the studies on movement protein of TMV and *Cowpea mosaic virus*. Cytological studies on the TMV-infected and TMV MP-transgenic plants provided a direct visual evidence that movement proteins accumulate in the plasmodesmata (Tomenius *et al.*, 1987; Atkins *et al.*, 1991a). Virus capable of replication in non-host plant cells following entry and their inability to move from the infected to adjacent cell suggested that the cellular components may play an important role in determining virus movement from cell to cell. However, little is known about how the host plant cells recognize and interact with the specific pathogenic virus, resulting in the progressive virus infection. Doubtless, virus movement from cell to cell is dependent on the interaction between the virus- encoded function and the plant cellular components.

Biochemical and cytological studies on tissues infected by TMV and other viruses have led to the description of two possible distinct mechanisms by which either the virus genome or virion pass the cell wall through plasmodesmata. In TMV and some other viruses using the TMV-like movement mechanism, viral genomes were transported through plasmodesmata in the form of a nucleoprotein complex. Non-specific ssRNA or ssDNA binding properties of movement proteins have been characterised from TMV and other viruses including CMV, *Alfalfa mosaic virus* (AMV) and *Red clover necrotic mosaic virus* (RCNMV; Osman *et al.*, 1992; Schoumacher *et al.*, 1992; Giesman-Cookmeyer and Lommel, 1993; Li and Palukaitis, 1996). It is proposed that the events

required for the intracellular transport of nucleoprotein involve the interaction between the viral genome and MP, and in some cases, other viral proteins such as replication complex, as well as interactions between the nucleoprotein transport complex and intracellular trafficking system (Carrington *et al.*, 1996). The modified TMV fused with the jellyfish (*Aequorea victoria*) green fluorescent protein (GFP) gene remain infectious and the virus was found to move in the form of long filaments in transfected tobacco protoplasts and in infected leave tissue. Recent experiments suggested that the cytoskeleton system might provide a possible functional network that facilitates the movement of nucleoprotein complex within the host cell (Heinlein *et al.*, 1995; Elliot *et al.*, 1997).

Some viruses exploit cell walls to form tubule structures. The virus is transported in the form of virus particles which pass through cell walls via plasmodesmata using tubule structures. Storms *et al.* (1995) found the expression of nonstructural movement (NSm) protein of *Tomato spotted wilt virus* in transfected *Nicotiana rustica* protoplasts induced tubular structures in the cell wall. Furthermore, electron microscopy studies have provided visible evidence that virions can be transported between adjacent cells since particles of *Cowpea mosaic* and *Grapevine fan leaf viruses* have been found in the tubular structures (Van Lent *et al.*, 1991; Ritzenthaler *et al.*, 1995). Although the mechanism by which viruses exploit the cell wall to form such tubular structures is unknown, some experimental data suggested that the formation of the tubular structures might be virus-dependent rather than dependent on host factors (Storms, 1995).

1.12.3 Virus long distance movement

Virus long-distance movement in host plants requires the interaction between the viral-encoded products and host functions which apparently differ from those involved in movement through mesophyll cells. Some viruses capable of

replication and movement from cell to cell were defective in long distance movement (Carrington *et al.*, 1996). Apart from movement protein, the CP has been found to be involved in the long distance movement of some viruses (Dawson *et al.*, 1988; Saito *et al.*, 1990; Xiong *et al.*, 1993; Vaewhongs and Lommel, 1995; Carrington *et al.*, 1996). However, this hypothesis is not applicable to all viruses examined. The cell to cell and long distance movement of *Groundnut rosette virus* (GRV) does not require virus-encoded coat protein. To date, no consensus model exists for the mechanism of CP-mediated virus long distance movement. Very recently, Ryabov and his colleagues reported that protein encoded by ORF3 of GRV can functionally complement to the CP of TMV, hence the hybrid virus can induce the systemic infection of inoculated *Nicotiana benthamiana*. This implied that the GRV ORF3-coded protein might function as a kind of virus long distance movement factor which can facilite movement of an unrelated viral RNA in a nonviron form (Ryabov *et al.*, 1999).

The development of virus-based vectors for the expression of foreign genes has greatly facilitated the manipulation of RNA viruses at the molecular level. A virus-based expression vector was first developed by Ahlquist and Janda in 1984. Using this vector, named pPM1, infectious *Brome mosaic virus* (BMV) RNAs were first synthesised *in vitro* from a full-length cDNA clone derived from BMV (Ahlquist *et al.,* 1984). Since then, based on the same strategy many plant and animal RNA viruses have been successfully expressed *in vitro*. Now *in vitro* transcription has become a routine technique and has been extensively used for genetic analysis of virus RNA genomes. A TMV-based expression vector, p30B, developed by Dawson (1998) has been successfully used for expression of functional proteins, such as the green fluorescent protein (GFP) or pharmaceutical proteins (Shivprasad *et al.*, 1999). This vector was constructed by cloning a recombinant TMV sequence containing the first two ORFs of TMV U1 genome and the last ORF of TMV U5 genome into the vector pUC19 which contains the bacteriophage T7 promoter. The presence of multiple cloning sites allows the subcloning of foreign genes into this vector and their

expression under the control of the upstream TMV U1 subgenomic promoter adjacent to the 3' end of second ORF of U1.

1.13 *Tobacco mosaic virus*

Tobacco mosaic virus (TMV), as the type member of the *Tobamovirus* genus, is probably the best-characterised plant RNA viruses. TMV has rod-shaped virus particle of approximately 300 x 18 nm in size (Dawson and Lehto, 1990). The virion contains a linear positive single-stranded RNA (ssRNA) of about 6400 nucleotides. The RNA is helical and fully coated with about 2000 units of a single structural protein to form a straight tube with a central hole of 4 nm in diameter. TMV is spread worldwide, and can cause significant crop losses. The infected plants develop mosaic symptoms on their leaves and the plants are stunted, especially in young plants. TMV has a very broad host range and can be easily transmitted mechanically to infect susceptible species although no natural vectors have been found to transmit this virus. To some extent, the damage caused by TMV can be reduced by employing some measures such as cultural practices, by applying chemical or using mild strains for cross protection, but the most effective method to control the TMV is by growing resistant cultivars.

1.13.1 Genome organisation of tobamoviruses

The genome organisation of tobamoviruses has been elucidated based on the entire sequence analyses of two typical strains, TMV-U1 and TMV-L (Goelet *et al.*, 1982; Ohno *et al.*, 1984). The tobamovirus genomes comprise four open reading frames (ORFs) and some ORFs overlap other ORFs in some strains (Fig. 5). The first ORF encoding a 126 kDa protein product starts at 70 nt and ends at 3420 nt with an amber stop codon (UAG). The second ORF is from 70 nt to 4920 nt and encodes a 184 kDa protein by readthrough of the stop codon

of first ORF. The third ORF starts at 4900 nt and ends at 5700 nt which encode a 30 kDa polypeptide product required for virus movement within infected plants. There is a short overlap range from 8 to 23 nucleotides between the second and third ORF. The last ORF encode a 17.5 kDa virus coat protein. In some tobamoviruses, there is overlap by 2 or 26 nucleotides between the 30 kDa and 17.5 kDa ORFs. There is no overlap between the 30 kDa and 17.5 kDa ORFs in the U1, OM L, and O strains. The *in vitro* translation of viron RNA suggested that the 126/183 kDa proteins are translated from genomic RNAs, whereas others are thought to be translated from subgenomic mRNAs (Knowland *et al.*, 1974). Accumulated evidence indicated that the virus movement from cell-to-cell and long-distance movement depend on the interaction between the virus and components of the host plant.

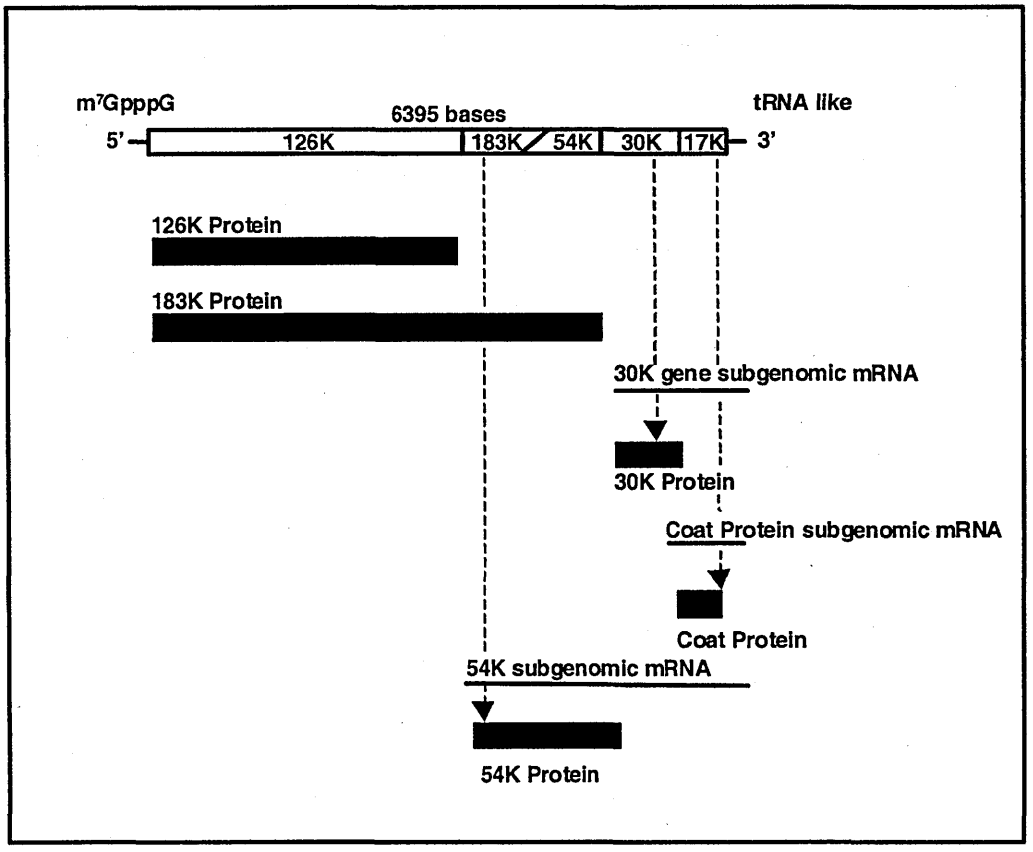


Figure. 5. Genome organization of tobamovirus (adapted from Dawson and Lehto, 1990)

1.14 Aims of this work

Soil-borne virus disease has recently been becoming increasingly important in winter wheat growing regions in China. The causal viruses are transmitted by a fungal vector, *Polymyxa graminis*, and at the moment there is no economically and environmentally acceptable measure available for control of this fungus, which make it very difficult to control this important virus disease. Development of virus resistant wheat cultivars by means of genetic transformation could contribute a great deal in the improvement of wheat production and quality. Virus movement protein plays a pivotal role in determining viral pathogenicity. Studies on possible complementation of movement proteins either from related or from unrelated viruses might lead to the insight understanding for the process of virus infection. This might make possible the eventual development of practical techniques for conferring resistance to crops for economically important virus diseases. With a view to achieving this, the aims of this work were:

- Development of expression vectors for wheat transformation and test of their expression in transfected tobacco protoplasts.

- Establishment of an efficient biolistic-mediated transformation system by transient expression of GUS in the model spring wheat cv. Bob White.

- Stable transformation of Bob White and Chinese wheat cultivars using the designed constructs containing functional viral CP and dysfunctional viral MP coding sequences.

- Development and use of the mannose selection system for transformation of Bob White and Chinese wheat cultivars.

- Analysis of transgenic plants.

- Construction and use of a TMV-based expression vector for the study of functional complementation of viral movement proteins.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Enzymes

2.1.1 Restriction enzymes

All of the restriction endonucleases used belonged to the type II restriction enzyme system, which were known to be able to bind specifically to, and cut double-stranded DNA at specific recognition sites that are generally a palindrome of four to six nucleotides in length with an axis of rotational symmetry. Cleavage by these enzymes may generate blunt-ended or cohesive ends with 5'-phosphate and 3'-hydroxyl radicals. Digestions were performed at 37 °C for one hour or over night. The enzymes used were supplied by Boehringer Mannheim, UK, Life Technologies, UK, and Promega Corporation, UK. The restriction sites and cleavage positions are shown as following from 5' to 3'.

*Apa*I	(GGGCC/C)	*Bam*HI	(G/GATCC)	*Bst*NI	(CC(A/T)GG)
*Eco*RI	(G/AATTC)	*Eco*RII	(CC(A/T)GG)	*Hin*dIII	(A/AGCTT)
*Kpn*I	(GGTAC/C)	*Pst*I	(CTGCA/G)	*Xho*I	(C/TCGAG)
*Pme*I	(GTTT/AAAC)				

2.1.2 Modified enzymes

2.1.2.1 Phosphatase

Phosphatase cleaves the 5'-phosphate groups from a nucleic acid strand. Calf intestinal alkaline phosphatase (Pharmacia Biotech Ltd, UK) was used to prevent vector religation to reduce the background in gene cloning work.

2.1.2.2 Reverse transcriptase

Reverse transcriptase is RNA-dependent DNA polymerase that uses an RNA template with a primer to produce the DNA. Avian myeloblastosis virus reverse transcriptase (AMV/RT, Promega Corporation, UK) is one of the most commonly-used enzymes and was used for first strand DNA synthesis in RT-PCR.

2.1.2.3 Deoxyribonuclease I

Deoxyribonuclease I, purified from bovine pancreas, degrades single stranded- and double stranded-DNA to produce 3'-hydroxyl oligonucleotides. *RQ1* RNase-free Deoxyribonuclease I is a preparation of Deoxyribonuclease I without detectable RNase activity. This enzyme was used to eliminate DNA from RNA samples for RT-PCR. The supplier was Promega Corporation, UK.

2.1.2.4 T4 DNA ligase

T4 DNA ligase can catalyze the joining of two DNA strands with 3'-hydroxyl and 5'-phosphate termini. The enzyme (Life Technologies Ltd, UK) was used to clone DNA fragments with either staggered or blunt-ended termini into plasmid vectors.

2.1.2.5 T4 DNA polymerase

T4 DNA polymerase is a DNA-dependent DNA polymerase with associated 3'-5 exonulease activity. The enzyme was used to blunt DNA ends for cloning purposes. The supplier was Life Technologies Ltd, UK.

2.1.2.6 T4 polynucleotide kinase

Polynucleotide kinase (PNK) can catalyze the transfer of the γ-phosphate group from ATP to the 5'-terminus of polynucleotides or mononucleotides. The enzyme was used to add the phosphate group to the DNA fragment prior to cloning. The supplier was Promega Corporation, UK.

2.1.2.7 *Taq* DNA polymerase

Taq DNA polymerase is a thermostable DNA-dependent DNA polymerase with 5' exonuclease activity and an optimum reaction temperature of approximately 74 °C. This enzyme lacks 3' exonuclease activity, therefore it cannot remove an incorrectly inserted base (proofreading function) of 3' end of extending chain and generally add a single dATP to the 3' end. It was used to amplify the target DNA sequence in the polymerase chain reaction using sequence-specific primers. The supplier was (Pharmacia Biotech Ltd, UK).

2.1.2.8 *Pfu* DNA polymerase

Pfu DNA polymerase is a DNA-dependent DNA polymerase and purified from bacterium *Pyrococcus furiosus.* Unlike *Taq* DNA polymerase, it has 3' exonuclease activity and can be used to amplify a long DNA fragment in high fidelity PCR. The supplier is Promega Corporation, UK.

2.1.2.9 AMV reverse transcriptase

AMV reverse transcriptase is purified from *Avian myeloblastosis virus* and is an RNA-dependent DNA polymerase with both 5'exoribonuclease and 3' exoribonuclease activity. This enzyme was used to drive the synthesis of first strand cDNA from mRNA or virus RNA in RT-PCR. When the reverse transcription and subsequent DNA amplification were performed in one tube with same buffer, following the reverse transcription, the AMV reverse transcriptase enzyme must be inactivated at 94 °C for 2 minutes to obtain high yields of amplification product using thermophilic DNA polymerase such as *Tfl* polymerase. The supplier was Promega Corporation, UK.

2.1.2.10 *Tfl* DNA polymerase

Tfl DNA polymerase is purified from bacterium *Thermus flavus*, and is a DNA-dependent DNA polymerase with 5' exonuclease activity. This enzyme possesses reverse transcriptase activity under certain conditions. This enzyme was used for synthesis of second strand cDNA from first strand cDNA and subsequent DNA amplification in RT-PCR. The supplier was Promega Corporation, UK.

2.2 DNA manipulation

2.2.1 Extraction and precipitation of DNA

To remove contaminants in DNA samples, an equal volume of phenol/chloroform (1:1) was added and the mixture was vortexed thoroughly for 1 minute. After centrifugation at 14000 rpm for 3 minutes, the upper aqueous phase was transferred to a clean tube. To precipitate DNA, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added to the

DNA solution. After gentle mixing by pippeting, the samples were placed at –70 °C for 30 minutes. The DNAs were pelleted by centrifugation at 14000 rpm for 10 minutes. After removals of supernatant, the pellets were washed with 70% ethanol, air dried and dissolved in an appropriate volume of water or TE.

2.2.2 Restriction digestion of DNA

Plasmid DNAs were digested with appropriate restriction enzymes depending on the target specific site of DNA sequences. For single digestion, the restriction enzyme was added at a ratio of 10 units/µg DNA and the reaction performed in an appropriate reaction volume (from 10 to 100 µl) with 1 x incubation buffer as recommended by the supplier, by incubating the mixture at recommended temperature (in most cases at 37 °C) for 1 hour. For double digestion, a compatible incubation buffer was chosen according to the supplier's recommendation. After digestion, the DNA was either visualised by gel electrophoresis or extracted with phenol/chloroform and precipitated with ethanol for subsequent manipulation.

2.2.3 Isolation of DNA fragments from agarose gel

The DNA fragments generated by restriction digestion or PCR were fractionated on an agarose gel with different concentration of agarose depending on the length of fragment to be resolved. The DNA fragment was recovered using microconcentrators (MICROCON®, Amicon Inc., USA). The stacked centrifugal device was assembled and about 150 mg gel pieces were placed into the gel nebulizer. After adding 100 µl 40% isopropanol into the gel nebulizer, the stack was centrifuged at 13000 rpm for 10 minutes. The micropure and gel nebulizer were discarded after centrifugation, and the microcon inserted into a new tube. After adding 450 µl H_2O to the microcon, the samples were spun at 13000 rpm

for 8 minutes. To recover the DNA fragment, the inverted microcon was inserted into a new tube and then centrifuged at 3000 rpm for 3 minutes.

2.2.4 DNA Dephosphorylation

To achieve high recombination efficiency, the digested vector was dephosphorylated prior to cloning event to prevent its self-ligation. Following restriction digestion, 1 unit alkaline phosphatase (Boehringer Mannheim GmbH, Germany) was added into the same tube, and the sample was further incubated at 37 °C for 30 min without changing the reaction buffer conditions.

2.2.5 DNA phosphorylation

In some cases, restriction adaptors were added to the blunt-ended DNA fragment for cloning purpose. Cohesive termini are usually not phosphorylated and phosphate groups must be added at 5 ' end of termini using polynucleotide kinase. A 20 µl reaction mixture containing DNA and adaptors was set up by adding 1x PNK buffer, 1 mM spermidine, 0.5 µM EGTA, 1 mM ATP and 8 units T4 polynucleotide kinase (Promega Corporation, UK). The mixture was incubated at 37 °C for 1 hour. The enzyme was removed by phenol/chloroform extraction and the DNA was recovered by ethanol precipitation.

2.2.6 Generation of blunt-ended DNA termini

To meet the specific requirement for blunt ends, protruding termini, usually generated from restriction digestion, need to be removed or filled in. To remove a 3'-overhang, a 10 µl reaction mixture was made up by adding 1x T4 DNA Polymerase buffer and 5 units of T4 DNA Polymerase (2.5units/µl, Pharmacia

Biotech Ltd, UK). The mixture was incubated at 37 °C for 5 minutes followed by further incubation at 70 °C for 10 minutes to inactive the enzyme. To fill in 5'-overhangs, reactions were performed in a total 10 µl containing 1x reaction buffer, 2 units of Klenow polymerase (Promega Corporation, UK) and 40 µM each dNTP (dATP, dCTP, dGTP, dTTP). To reduce the 3'-5' exonuclease activity of Klenow polymerase, the mixtures were incubated at 12 °C for 15 minutes. After incubation, the mixtures were subsequently incubated at 70 °C for 10 minutes to denature the enzyme.

2.2.7 Agarose gel electrophoresis

100 ng of miniprep plasmid was digested with a selected restriction enzyme and appropriate digestion buffer as recommended by enzyme supplier in a 10 µl final volume mix. A 1% agarose gel was prepared by melting 400 mg agarose (IBI, USA) in 40 ml 1x TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA pH 8.0) buffer using microwave heating and the gel solution after cooling to 50 °C was poured into the gel electrophoresis tank. After the digestion was completed, 1 µl 10x loading buffer (4.2% bromophenol blue, 4.2% xylene cyanol, 60% glycerol) was added into the tube and the samples were loaded into the wells of gel adjacent to lanes carrying 1Kb DNA ladder (Promega, USA) or DNA marker VIII (Promega, USA). Electrophoresis was carried out with 1x TBE buffer at 60 V for 40-60 min. depending on the size of DNA fragment to be resolved. After the electrophoresis was completed, the gel was stained with 0.5 µg/ml ethidium bromide and examined under UV light.

2.2.8 DNA sequencing

The samples for sequence analysis were prepared using the ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit following the supplied protocol

(*PE* Applied Biosystems, UK). Thermal cycling was carried out with the GeneAmp PCR System 2400. 10 μl mixtures including 4 μl terminator-ready reaction Mix, 250 ng plasmid DNA and 1.6 pmol primer were placed into 0.2 ml GeneAmp tubes, and the reaction were performed for 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 64 °C for 4 minutes. Extension products were purified by ethanol precipitation according to manufacture's instruction. For each reaction, 2 μl of 3M sodium acetate (pH 4.6) and 50 μl of 95% ethanol were added into a 1.5 ml microcentrifuge tube. The entire 20 μl of reaction from each tube were transferred to the microcentrifuge tube containing the mixture of ethanol and sodium acetate. After briefly vortexing, the tubes were placed at room temperature for more than 15 min and then centrifuged at 13000 rpm for 30 min. After complete removal of ethanol solution, the pellet was washed with 250 μl of 70% ethanol twice, and then the pellet was dried at 90 °C for one min. The samples were sent to the Sequencing laboratory of the Scottish Crop Research Institute for analysis by ABI PRISM™ 373 DNA sequencer. The sequence data were analyzed using the University of Wisconsin GCG program package.

2.3 Bacterial strains

2.3.1 DH5α

Escherichia coli DH5α is a recombination deficient suppressing strain, which is free of endonuclease I that allows preparation of plasmid DNA of high quality. The existence of the φ80d*lac*ZΔM15 marker provides α-complementation of the β-galactosidase gene existed in the pUC or similar vector, which can be used for blue/white screening of colonies on bacterial plates containing Bluo-gal or X-gal. This strain was used for amplification of some recombinant plasmid DNA. The supplier was Life Technologies Ltd, UK.

2.3.2 XL1 Blue

Epicurian Coli® XL-1 Blue is an endonuclease and recombination deficient strain with high transformation efficiency. The strain was used for preparation of some recombinant plasmid DNA. The supplier was Stratagene Ltd, UK.

2.3.3 HB101

Escherichia coli HB101 competent cells were used to amplify some recombinant plasmid DNA. The HB 101 strain is a high efficiency competent cell which can be used for cloning in some vectors that do not require α-complementation for blue/white screening. The supplier was Promega Corporation, UK.

2.4 Plasmid DNA preparation

2.4.1 Mini-preparation of plasmid DNA

Small amounts of plasmids were prepared using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA). One loop of transformant cells from an individual colony was inoculated into a 13.5 ml sterile test tube (Sterilin, UK) containing 5 ml LB ampicillin (50 μg/ml) liquid culture solution, and the sample was incubated overnight at 37 °C with shaking at 310 rpm. Minipreps were carried out following manufacturer's instruction. To collect the bacteria, the bacterial cultures were centrifuged for 5 minutes at 5000 rpm. Minipreps were carried out following manufacturer's instruction.

2.4.2 Large-scale preparation of plasmid DNA

Large-scale preparation of plasmid DNA was performed using JETSTAR plasmid maxiprep kit (Ams Biotechnology, UK). One loop of transformant cells

from an individual colony was transferred into a 13.5 ml sterile test tube (Sterilin, UK) containing 5 ml LB ampicillin (50 µg/ml) liquid culture solution and the sample was incubated at 37 °C for 4 hours with shaking at 310 rpm. The culture was poured into the flask containing 400 ml LB ampicillin (50 µg/ml) liquid culture solution, and incubated overnight under the same conditions. To harvest bacterial cells, the culture was centrifuged at 6000 rpm and 4 °C for 15 minutes. Maxipreps were carried out following manufacturer's instruction. Plasmid DNA was dissolved in an appropriate volume of sterile distilled water and then stored at –20 °C.

2.5 Genomic DNA extraction from plant tissues

2.5.1 Mini-preparation of genomic DNA from wheat for PCR

This method was based on a simple method of preparing plant samples for PCR by Wang *et al.* (1993b). Fresh leaf from wheat plants was ground in 0.5 N NaOH with a micropestle. For this, 10 µl 0.5 N NaOH was added to every milligram of tissue. 5 µl of the extract was transferred to a new tube containing 245 µl 100 mM Tris-HCl pH 8.0 and mixed thoroughly. After boiling for 5 minutes, the mixture was placed on ice for 5 minutes and 5 µl of sample was directly used in 25 µl of PCR reaction mixture. The samples were stored at –20 °C.

2.5.2 Isolation of genomic DNA from wheat for Southern analysis

This protocol to isolate genomic DNA from wheat plant is a modification of the cetyltriethylammoniumbromide (CTAB) extraction method described by Murray and Thompson (1980). The protocol is suitable for the isolation of large-scale amounts of DNA from many different plant species. Fresh leaves were collected and ground to a fine powder in liquid nitrogen using a mortar and pestle. The

powder was transferred to a pre-cooled disposable plastic tube (15 ml) with a punctured screw cap. The screw cap was taped with Micropore™ tape and the sample was lyophilised for 24 hours. After distributing the powder into 2.2 ml large Eppendorf tubes, 800 µl of extraction buffer (50 mM Tris-HCl, pH 8.0, 700 mM NaCl, 10 mM EDTA, 1% (w/v) CTAB, 1% (v/v) β-mercaptoethanol) was added to each tube and the sample was mixed with a plastic spatula until complete rehydration of the powder was achieved. The mixture was incubated in a water bath at 56 °C for 15 minutes. After cooling down to room temperature, 800 µl of chloroform/octanol (24:1) was added to each tube and mixture was mixed well by inverting tubes. After centrifuging samples for 5 minutes at 14000 rpm at room temperature, the upper aqueous phase was transferred to a new tube without disturbing the interphase. Re-extraction was performed by mixing the extract with 80 µl of CTAB stock solution (10%) and 800 µl of chloroform/octanol (24:1) and centrifuging at 14000 rpm at room temperature for 5 minutes. To precipitate DNA, 1 ml of precipitation buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% (w/v) CTAB) was added to the re-extracted solution and the sample was mixed gently. The mixture was incubated at room temperature for 20 minutes and then spun for 10 minutes at 14000 rpm and room temperature. After discarding the supernatant, the pellet was dissolved in 400 µl of 1 N NaCl. To degrade RNA from DNA sample, 2 µl of 10 mg/ml RNase were added and the sample was incubated at 37 °C for 30 minutes. The DNA was precipitated by adding 1 ml of absolute ethanol. After keeping at –70 °C for 30 minutes, the DNA was pelleted by spinning down for 10 minutes at 14000 rpm at room temperature. The supernatant was removed and the pellet was washed with 70 % ethanol once, air dried and then dissolved in 50 µl sterile distilled water. The DNA samples were stored at – 20 °C.

2.6 RNA extraction

2.6.1 RNA extraction from transfected tobacco protoplasts

The protoplasts were collected by centrifugation. 600 µl of solution D (5 M guanidinium isothiocyanate, 0.5% sarkosyl, 25 mM Na-citrate, 50 mM Tris-HCl pH 7.6, 2 mM EDTA, 1 mM β-mercaptoethanol) were added to each tube and vortexed to fully resuspend them. Following centrifugation at 3000 rpm for 5 seconds, 40 µl of 3 M NaOAc (pH 4.0) and 1 µl of 10 mg/l tRNA were added to each tube, and the sample was mixed by vortexing. 600 µl of water-saturated phenol and 150 µl chloroform/Isoanylalcohol (24:1) were added to each tubes. The tubes were placed on ice for 15 minutes following vortexing for 1 minute. The samples were spun down at 14000 rpm at 4 °C for 10 minutes. The upper aqueous phase was transferred into a new tube containing 600 µl of isopropanol and vortexed briefly. The samples were placed at –20 °C for 30 minutes and centrifuged at 14000 rpm at 4 °C for 15 minutes. All liquids were removed completely from the tubes, and the pellets were resuspended with 100 µl TE buffer (10 mM Tri-HCl pH 8.0, 1 mM EDTA). For RNA re-precipitation, 10 µl of 3 M NaOAc (pH 4.8) and 300 µl of 100% ethanol were added, and the mixture was kept at –20 °C for 30 minutes. The samples were centrifuged at 14000 rpm for 10 minutes and the pellets were washed twice with 80% ethanol. The RNA pellets were resuspended in 36.5 µl of TE buffer, 2.5 µl RNasin (Promega), 1.0 µl of 25 mM $MgCl_2$ and 5.0 µl RQ1 DNase (Promega). The samples were vortexed for 3 minutes and incubated at 37 °C for 15 minutes. Following incubation, 200 µl of phenol/chloroform (1:1) and 150 µl TE were added into the tubes. The samples were vortexed briefly and placed on ice. After centrifugation at 14000 rpm for 5 minutes, the upper layers were transferred into new tubes containing 20 µl of 3M NaOAc (pH 4.8) and 200 µl of chloroform/isoamyl alcohol (24:1). After centrifugation at 14000 rpm for 2 minutes, the upper layer was transferred to a new tube containing 500 µl of 100% ethanol. The samples were

placed at –20 °C for 20 minutes. RNA was recovered by centrifugation at 14000 rpm for 5 minutes and then resuspended in 10 µl sterile water.

2.6.2 RNA extraction from wheat plants for RT-PCR

Total RNA was extracted from wheat leaves using The SV Total RNA Isolation System (Promega Corporation, UK). This system is suitable for the preparation of purified and intact total RNA from many organisms and tissues. Fresh wheat leaves (100 mg) were collected and ground with a mortar and pestle under liquid nitrogen to a fine powder. The powder was immediately transferred into a pre-weighed Eppendorf tube containing 175 µl of SV Lysis Buffer (4 M GTC, 0.01 M Tris-HCl pH 7.5, 0.97% β-mercaptoethanol) and mixed thoroughly by inversion. The ratio of tissue mass to SV Lysis Buffer should be made to approximately 30 mg/175 µl. To this, 350 µl of SV RNA Dilution Buffer was added. After inverting the tube 3-4 times, the mixture was incubated in a water bath at 70 °C for 3 minutes. The sample was centrifuged for 10 minutes at 13000 rpm and room temperature. The cleared lysate was transferred into a new microcentrifuge tube and mixed with 200 µl 95% ethanol by pipetting 3-4 times. The mixture was transferred to the Spin Column Assembly and spun at 13000 rpm and room temperature for 1 minute. The flowthrough was discarded and 50 µl of DNase incubation solution (40 µl Yellow Core Buffer (0.0225 M Tris-HCl pH 7.5, 1.125 M NaCl and 0.0025% yellow dye), 5 µl 0.09 M $MnCl_2$, 5 µl DNase I) were added onto the membrane of the Spin Basket. After incubation for 15 minutes at room temperature, 200 µl of SV DNase Stop Solution (2 M guanidine isothiocyanate, 4 mM Tris-HCl pH 7.5, 57% ethanol) was added into the Spin Basket. After adding 900 µl of SV RNA Wash Solution (60 mM potassium acetate, 10 mM Tris-HCl pH 7.5, 60% ethanol), the Spin Basket was centrifuged at 13000 rpm for 1 minute, then the Spin Basket was washed by adding 250 µl of Wash Solution and centrifuging for 2 minutes. To elute the RNA, the spin basket was inserted into the elution tube. After adding 100 µl of nuclease-free water, the

Spin Basket Assembly was centrifuged at 13000 rpm for 1 minute. The purified RNA sample was stored at –20 °C.

2.7 Molecular analysis techniques

2.7.1 PCR analysis

The PCR was used to confirm the presence of genes of interest either in recombinant DNA or in transgenic plants. PCR was carried out using *Ready To Go*™ PCR beads (Amersham Pharmacia Biotech. USA) in a 25 µl reaction containing 100 ng DNA template, 1.5 units *Taq* DNA polymerase, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µl of each dNTP (dATP, dCTP, dGTP, dTTP) and 1 µM of each primer. The reaction conditions varied depending on the content and the length of primers and the length of target DNA fragment. In general, the reaction started at 94 °C for 30 seconds for denaturation of DNA template followed by 35 cycles of amplification reaction. After denaturation at 94 °C for 1 minute, the annealing reactions were performed at different temperatures ranging from 50 to 64 °C depending on the melting temperature of primers to be used. The extension was at 72 °C for 1 to 2 minutes based on 1 minute for 1 kb DNA fragment to be synthesised. After 35 cycles of amplification, the final elongation was carried out at 72 °C for 5 minutes. The amplified PCR products were fractionated by agarose gel electrophoresis.

2.7.2 RT-PCR analysis

RT-PCR was used to examine the expression of transgenes in transfected protoplasts or transgenic plants and to detect RNA virus movement in infected plants. The RT-PCR was carried out using an "Access RT-PCR System" kit

(Promega Corporation, UK) following the supplier's instruction. This system permits the reverse transcription (RT) and polymerase chain reaction (PCR) to be accomplished in one tube with an optimal single buffer, which to a large extent reduces the potential contamination usually encountered in normal RT-PCR reactions. The system uses AMV reverse transcriptase from *Avian myeloblastosis virus* for first strand cDNA synthesis, and the thermostable *Tfl* DNA Polymerase from *Thermus flavus* for second strand cDNA synthesis and DNA amplification. The reaction was made up to 50 µl in diethyl pyrocarbonate-treated water containing 0.1 µg total RNA, 5 units AMV reverse transcriptase, 5 units T*fl* DNA polymerase, 1x AMV/*Tfl* reaction buffer, 0.2 mM each dNTP, 50 pmol each primer and 1 mM $MgSO_4$. The standard reaction conditions were reverse transcription at 48 °C for 45 minutes, 94 °C for 2 minutes for AMV RT inactivation and RNA/cDNA/ primer denaturation followed by 40 cycles of 94 °C for 30 seconds for denaturation, 60 °C for 1 minute for annealing and 68 °C for 2 minutes for extension, and finally at 68 °C for 7 minutes for final extension. The RT-PCR products were fractionated on a 0.8% agarose gel to detect the expected amplified fragments.

2.7.3 Southern blot hybridization analysis

The Southern blot hybridization technique is widely used in many aspects of genomic DNA analysis. It allows the efficient transfer of DNA from agarose gel to a membrane and detection of the DNA fragment of interest after hybridization with either radioactive or nonradioactive probes. This technique was used to confirm the presence of transgenes in transgenic wheat plants.

2.7.3.1 DNA sample preparation and agarose gel electrophoresis

To prepare the DNA samples, 20 μg genomic DNA from transgenic plants were digested with appropriate restriction enzymes at 37 °C overnight. The same amount of digested genomic DNA from nontransgenic plants was used as the negative control and 5-10 pg of digested plasmid as positive control. The digested DNA samples were loaded into the slots of a 0.8% agarose gel. The gel electrophoresis was performed in an appropriate gel tank with TAE buffer (0.04 M Tris pH 7.8, 2 mM EDTA) at 30 V overnight. After electrophoresis, the gel was stained with 0.5μg/ ml ethidium bromide, visualised on a Vilber Lourmat transilluminator emitting UV light at 302 nm and images were recorded using a Mitsubishi video copy processor.

2.7.3.2 Southern blotting

The gel was incubated in 0.25 M HCl for 10 minutes on a shaker at room temperature until the bromophenol blue indicator included in samples turned to yellow. Then the gel was incubated in denaturation buffer (500 mM NaOH, 1.5 M NaCl) for 30 minutes on a shaker at room temperature. Two washes of 15 minutes each were performed by incubating the gel in neutralisation buffer on a shaker at room temperature. The capillary blot assembly was set up using the following procedures. The tray was filled with 20 x SSC solution (3 M NaCl, 0.3 M tri-sodium citrate) and covered with a clean glass plate. A paper bridge was made up by placing a piece of 3 MM Whatman filter paper on the surface of glass plate, with both ends of this filter paper soaked in the blotting buffer. The gel was placed on the wick avoiding trapping any air bubbles beneath it, and the rest of filter paper on the platform was covered with Parafilm strips. A gel-sized Hybond N^+ Nylon membrane (Amersham International plc, UK), pre-wetted with 2 x SSC, was placed on the top of the gel and the bubbles beneath the membrane were removed smoothly by rolling a glass pipette over it. Two of the filter papers same size as the membrane were placed on the top of membrane.

A layer, approximately 5 cm high, of paper towels was put on the top of gel-membrane-filter paper. After covering with a glass plate on the top of the paper towels, a 1 kg weight was put on the top of the plate. The transfer was performed at room temperature overnight.

After blotting, the stack was disassembled carefully and the membrane was washed with 2x SSC. The wet membrane was placed with the DNA-linked side up on the top of a filter paper to air dry. To fix the DNA blot, the dry membrane was placed onto the UV transilluminator (302 nm) with the DNA side facing the UV source and exposed for 5 minutes.

2.7.3.3 Labelling of radioactive DNA probe

Labelling of DNA probe was performed using *Ready-To-Go* DNA Labelling Beads (-dCTP) from Amersham Pharmacia Biotech UK Limited. The DNA fragment was denatured by heating for 2-3 minutes at 100 °C and then placed on ice for two minutes. The reaction mixture was made up to 50 µl by adding 25 ng denatured DNA fragment, 5 µl [α-^{32}P] dCTP (3000Ci/mmol) and distilled water. After mixing gently by pipetting several times, the mixture was centrifuged briefly to remove air bubbles. The mixture was incubated at 37 °C for 30 minutes and then purified with ProbeQuant™ G-50 Micro Column to remove unincorporated nucleotides.

2.7.3.4 Southern hybridisation with radioactive probe

The Southern blot membrane was placed in a hybridization tube and 25 ml of pre-hybridization solution (5 x SSPE (0.72 M NaCl, 0.04 M Na_3PO_4, 0.005 M EDTA pH 7.7), 5 x Denhardt's solution (0.1% (w/v) bovine serum albumin, 0.1% (w/v) Ficoll™, 0.1% (w/v) polyvinylpyrrolidone), 0.5% (w/v) SDS) were added to the tube avoiding trapping air bubbles between the membrane and glass tube.

Herring sperm DNA was used as a blocking agent to reduce non-specific binding of radiolabelled probe to the membrane. After heating to 100 °C for 5 minutes and chilling on ice, 50 µl of blocking DNA (10 µg/µl) was added to the pre-hybridization solution. Pre-hybridization was performed by incubating the tube at 65 °C rotating in the hybridization oven for 1 hour. After pre-hybridization, the pre-hybridization solution was replaced by 25 ml of fresh pre-hybridization solution. To denature the labelled probe, the probe DNA was heated to 100 °C for 5 minutes and immediately pipetted into the hybridization solution. The hybridisation was carried out by incubating the tube for more than 12 hours at 65 °C rotating in the hybridisation oven. Following hybridisation, the hybridisation solution was poured off and the filter was rinsed with 30 ml of low stringency wash solution containing 2 x SSPE and 0.1% (w/v) SDS. The filter was washed by incubating at 65 °C for 15 minutes with 1 x SSPE and 0.1% (w/v) SDS in the oven. After pouring off the wash solution, the filter was washed with high stringency wash solution (0.1 x SSPE, 0.1% (w/v) SDS) by incubating at 65 °C for 10 minutes. During incubation, the filter was checked with a radioactivity monitor for background activity. After washing, the filter was drained on a piece of Whatman 3 MM filter paper and wrapped with Saran wrap.

2.7.3.5 Molecular imager and autoradiography

The radioactive blots were first checked with the storage phosphorimaging system of Bio-Rad. This system is much more sensitive for the detection of the radioactive signal compared to the X-ray film autoradiography. It allows examination of the result of Southern hybridisation prior to autoradiography and determination of the optimal exposure time for X-ray film autoradiography. After molecular image analysis, the filters were exposed to X-ray film in a cassette at –70 °C for 2-3 weeks depending on the intensity of the signals.

2.7.4 Western blot analysis

2.7.4.1 SDS Polyacrylamide Gel Electrophoresis (PAGE)

The glass-plate sandwich was assembled according to the manufacturer's instruction. Ten ml of 15% resolving gel were prepared by adding 2.45 ml water, 2.5 ml gel buffer (1.5 M Tris-HCl pH 8.8, 0.4% SDS), 5.0 ml acrylamide/bis acrylamide stock solution (30%/0.8%, Severn Biotech Ltd, UK), 75 μl freshly prepared 10% ammonium persulphate (APS) and 5 μl N,N,N',N'-tetramethylethylenediamine (TEMED). The acrylamide solution was poured into the gap between the glass plates and covered with water- saturated butanol. The gel was left in a vertical position at room temperature for one hour for the polymerization reaction. After pouring off the butanol layer, the top of gel was washed with deionized water to remove the unpolymerized acrylamide. Remaining water was removed with the edge of a paper towel. Ten ml of 5% stacking gel solution was prepared by adding 5 ml water, 2.5 ml gel buffer (Tris-HCl pH 6.8, 0.4% SDS), 1.67 ml acrylamide/bis acrylamide stock solution, 100 μl freshly prepared 10% APS and 10 μl TEMED. After inserting a clean comb, the stacking gel was poured to overlay the resolving gel without trapping any air bubbles. The gel was kept in a vertical position at room temperature for around 30 minutes. After polymerisation was complete, the comb was removed carefully and the gel was washed with deionized water.

2.7.4.2 Samples preparation and gel electrophoresis

Leaf samples (100 mg) were homogenized by grinding in liquid nitrogen with a pestle and mortar. The powder was transferred to a 1.5 ml tube containing 300 μl SDS-PAGE sample buffer (100 mM Tris-HCl, pH 8.2, 1 mM EDTA, 1% SDS, 10 mM DTT) and the samples were suspended by vortexing gently. After centrifugation at 14000 rpm for 2 minutes, the supernatant was transferred to a new Eppendorf tube. The loading samples were made by mixing 20 μl

supernant and 5 μl 5x loading buffer (313.7 mM Tris-HCl, pH 6.9, 15% SDS,10 mM EDTA, 324.1 mM DTT, 0.01% Bromphenol blue). Before loading the samples into the slots, the mixtures and an appropriate molecular weight marked were incubated at 100 °C for 5 minutes, and then the samples and marker were loaded into the gel wells in a determined order.

Gel electrophoresis was performed in a running buffer containing 25 mM Tris pH 8.8, 192 mM glycine and 0.1% SDS at 80 V for the first 40 minutes and then at 120 V for several hours until the bromophenol blue reached the end of gel.

2.7.4.3 Western blot

The gel was equilibrated in transfer buffer containing 25 mM Tris-HCl pH 8.3, 0.192 M glycine and 20% methanol for 15 minutes. The nitrocellulose membrane and four 3 MM filter papers were cut exactly to the size of the gel and soaked in transfer buffer. The blotting apparatus was assembled according to the manufacturer's instruction (Mini Trans-Blot Electrophoretic Transfer Cell, BioRad). The transfer sandwich was set up in the order of filter paper, gel, membrane, filter paper, avoiding trapping any air bubbles by softly rolling a clean glass pipette from the middle of the sandwich to the outside. The transfer sandwich was placed in the cassette and then put into the transfer tank containing the transfer buffer. The transfer was performed at 30 V overnight. The transfer apparatus was disassembled and gel was stained with Coomassie Brilliant Blue solution containing 0.05% Coomassie Brilliant Blue G250, 50% (v/v) methanol and 10% (v/v) acetic acid, then destained with Coomassie Brilliant Blue solution containing 50% (v/v) methanol and 7% (v/v) acetic acid to check the efficiency of transfer.

2.7.4.4 Immunodetection of the proteins

Following SDS-PAGE and western blotting, the protein of interest was detected using a specific antiserum. To block the membrane, it was incubated at room temperature for 30 minutes with gentle shaking in TBST (20 mm Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 plus 5% non-fat milk powder. To bind primary antibody, the membrane was incubated for 1 hour at room temperature with gentle agitation with TBST containing polyclonal antiserum (kindly provided by Dr. J. Sherwood, Oklahoma State University, Oklahoma, USA) against SBWMV p37 protein 1000 x diluted. After three washes in TBST for 10 minutes each, the membrane was transferred to TBST containing anti-rabbit IgG/alkaline phosphatase conjugate (Sigma, USA) used at 5000 x dilution and incubated for 30 minutes with gentle agitation. After three washes with TBST of 10 minutes each to remove unbound secondary antibody and brief repeated rinsing with TBS (TBST without Tween 20), the colour reaction was performed by incubating the membrane in Western Blue® Stabilised Substrate for alkaline phosphatase until the desired intensity of band was obtained, then washing the membrane with tap water to stop the reaction.

2.8 Electron Microscopy

For electron microscopy tests, samples were prepared according to Roberts (1986). Leaves were cut into small pieces c. 0.1 cm^2, and then transferred into a 0.5 ml Eppendorf tube. After adding 2-5 mg Carborundum powder and 2-5 drops of water, the samples were ground thoroughly using a motorised micro mortar at 2000-10000 rpm. The samples were centrifuged at 12000 rpm for 2 minutes. One drop of virus extract was placed on the grid. After draining by touching the edge of the grid with filter paper, one drop of stain solution (2% sodium phosphotungstate pH 7.0) was added onto the surface of the grid. After leaving for 10-30 seconds, the grid was drained again with filter paper and air dried. Virus particles were detected using transmission electron microscopy (TEM) at a magnification of 45,000x.

2.9 Cloning of DNA into plasmid vector

2.9.1 DNA ligation

For cloning a DNA fragment into plasmid vector, following restriction digestion with appropriate enzyme(s), the digested plasmid was generally dephosphorylated following the procedures as described in 2.2.4. 10 µl of ligation mix was assembled in a 0.2 ml tube with 50 ng dephosphorylated plasmid DNA, 150 ng isolated insert DNA, 1 unit T4 DNA Ligase and 1 µl 10 x T4 ligase buffer (Boehringer Mannheim GmbH, Germany) and 2 µl H_2O. The ligation was performed at 12 °C overnight.

2.9.2 Transformation of *E. coli*

DH5α was used for routine cloning procedures. Cells were thawed on ice and then 100 µl of competent cells was transferred into a 15 ml sterile tube. 5 µl ligation products was added into the tube and mixed well by pipetting. Cells were incubated on ice for 30 min. After heat treatment at 42 °C for 45 seconds, the mix was placed on ice for 2 min. 900 µl SOC liquid culture solution (2% bacto tryptone, 0.5% yeast extract, 2.5 mM KCl, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added into the tube which was then incubated at 37 °C for 16-18 hr. Both 10 µl of transformant culture and the remainder of the culture concentrated by centrifuging into a volume of 10 µl were spread onto LB ampicillin plates (50 µg/ml ampicillin, 1% bacto tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) and incubated at 37 °C overnight. Transformants were visualised as individual white colonies on the plates.

2.9.3 Quick screening transformants

Individual colonies were streaked onto the ampicillin agar plates and the samples were incubated at 37 °C overnight. The cells were transferred into the Eppendorf tube containing 40 µl cell lysis buffer (100 mM NaCl, 20 mM Tris-HCl , pH8.0, 10 mM EDTA, 0.84% bromophenol blue) and incubated for 5 minutes at room temperature. After adding 40 µl phenol/chloroform, the mixture was vortexed thoroughly and centrifuged at 14000 rpm for 10 minutes. The upper aqueous layer containing plasmid DNA was analysed by electrophoresis on a 0.8% agarose gel.

2.10 Stable transformation of wheat

2.10.1 Plant materials

Spring wheat (*Triticum aestivum* L.) cv. Bob White and three Chinese semi-winter wheat cultivars (Yangmai 93-111, Yangmai 94-141 and Yanmai 2980) were grown in the glasshouse at 18 °C day and 14 °C night under 12 hr photoperiod supplemented with 400 W plant irradiation luminaire (SON-T AGRO 400W Philips, Belgium) when the light intensity was under 5000 lx during the day. All the plants were watered every two days and fertilised once a week with 0.4 g/l Vitax general purpose fertiliser (Clydeside Trading Ltd., UK).

2.10.2 Excision of immature embryos

Wheat spikes were harvested and then sterilised with 70% ethanol for 3 minutes followed by two washes with sterile distilled water. Immature caryopses were removed from the spikelet using a fine forceps. Immature embryos (1.5-2.0 mm in length) were isolated carefully under a stereo dissecting microscope using 1.0 ml volume syringe with an appropriate needle.

2.10.3 Culture medium

MS medium (Murashige & Skoog, 1962) supplemented with 2.0 mg/l 2.4-D, 500 mg/l glutamate, 100 mg/l casein hydrolysate, 30 g/l maltose was used for induction of somatic embryogenesis from immature embryo culture. The medium, designated as SMS, was prepared by mixing 500 ml of filter-sterilised, double-strength, MS Medium (pH 5.6, Duchefa, The Netherlands) with 500 ml autoclaved double-strength 0.4% Gelrite (Duchefa, The Netherlands) to solidify the medium with final concentration of 0.2% Gerlite.

2.10.4 Culture conditions

The immature embryos were placed onto the SMS culture medium with the scutellum side up. The cultures were incubated in the dark at 25 °C for 4 days.

2.10.5 Bombardment of immature embryos

2.10.5.1 High osmotic treatment

After 6 days culture on the induction medium, 35-40 immature embryos were transferred to the centre (20 mm in diameter) of a 30 mm Petri dish (Sterilin, UK) containing plasmolysing medium (SMS plus 20% maltose) for high osmotic treatment. The embryos were incubated in the dark for 4 hours under the same culture conditions as used for cell proliferation prior to the bombardment.

2.10.5.2 Preparation of DNA coated particle solution

Gold particles (1.0 μm in diameter, BioRad, USA) were coated with plasmid following the protocol of Iglasias (1994). 50 mg gold particles were suspended in 1 ml 50% glycerol (Sigma) and then autoclaved. 2.5 M $CaCl_2$ and 0.1 M spermidine stock solutions were sterilised with a syringe filter (0.2 μm, 25 mm, NALGENE® Brand Products, Nalge Company, Rochester, New York, USA) and

stored at –20 °C. 50 µl aliquots of gold particles were mixed with 5 µg each plasmid (1 µg/µl), 50 µl stock $CaCl_2$ and 20 µl stock spermidine in a 1.5 ml Eppendorf tube while the tube was vortexed vigorously. The mixture was vortexed for another 3 minutes and then centrifuged at 14000 rpm for 5 seconds. After removal of the supernatant, DNA coated gold particles were washed with 70% ethanol once and then resuspended in 100 µl absolute ethanol.

2.10.5.3 Loading DNA coated particle on microcarriers

The microcarriers and rupture discs were soaked in 100% ethanol for 10 minutes at room temperature. After draining on a sterile filter paper and drying, the microcarriers were placed in the microcarrier holder and 10 µl DNA coated particle solution were loaded on the center of the microcarriers.

2.10.5.4 DNA delivery

Cultured and osmotically pre-treated immature embryos were bombarded using the Dupont helium-driven Biolistic particle delivery system (PDS 100) with DNA-coated gold particles under the optimal gene delivery conditions obtained from transient expression experiments.

2.10.6 Post-bombardment culture

After bombardment, the samples were kept on the high osmotic medium and incubated in the dark for 14-16 hours under the same conditions, and then transferred onto the same medium as used for cell proliferation for further culture.

2.10.7 Histochemical GUS assay

Transient GUS expression in targeted tissues was assayed by immersing two days post-bombardment immature embryos in X-Gluc buffer (1mg X-Gluc in 1 ml phosphate buffer pH 7.0), made up according to Gillian *et al.* (1995), at 37 °C for 12-24 hr. The staining buffer was removed from incubations, the samples were washed with water twice and kept in 70% ethanol. To determine the number of cells or cell clusters which transiently express GUS from bombarded immature embryos under the different shooting parameters, embryos were divided into two sections by eye and the blue spots were counted from one section of each of the bombarded embryos under a stereo dissecting microscope. One blue spot or blue cell cluster represented one GUS expression event. The total number of GUS expression events from all of the bombarded immature embryos was obtained by doubling the resultant scores.

2.10.8 Recovery culture of post bombardment immature embryos

Bombarded immature embryos were transferred on to the SMS medium as used for somatic embryogenesis and cell proliferation. After one week of culture, the calli were transferred on to the selection medium for further culture.

2.10.9 Plant regeneration under the PPT selection

After recovery culture, the calli were transferred on to the selection medium SMS5 (MS supplemented with 30 g/l maltose, 2.0 mg/l 2.4-D) containing 5 mg/l phosphinothricin (PPT) for further somatic embryogenesis and cell proliferation under selection. The calli were transferred on to the same selective medium for every two weeks and the calli were subcultured under selection for 6 weeks.

All the calli obtained from bombarded immature embryos were transferred on to the regeneration medium RMS5 (MS supplemented with 20 g/l sucrose, 2.0 mg/l 6-BA , 0.5 mg/l KT and 5.0 mg/l PPT) for plant regeneration under PPT selection. The calli were transferred onto the same medium for every two weeks.

After culture on selective regeneration medium for 6-8 weeks, regenerants around 2-3 cm in length were transferred to round plastic containers (Greiner, Germany) containing selective rooting medium RMS3 (1/2 MS supplemented with 10 g/l maltose, 0.1 mg/l KT, 0.1 mg/l NAA, and 3.0 mg/l PPT). After 4-6 weeks, rooted plantlets were transferred to soil and grown to maturity under glasshouse conditions.

CHAPTER 3

CLONING SBWMV CODING SEQUENCES INTO AN EXPRESSION VECTOR AND TESTING VIRAL GENE EXPRESSION IN TRANSFECTED TOBACCO PROTOPLASTS

3.1 Introduction

To generate the constructs for wheat transformation, a number of SBWMV genes, including viral coat protein gene, movement protein gene and replication complex gene, have been cloned into the vector pUbi35S (Fig. 6). Plasmid pUbi35S, containing the maize ubiquitin-1 promoter (Christensen *et al.*, 1992) followed by ubiquitin intron1 and CaMV 35S terminator at its 3' region, was constructed and kindly provided by X. Ye, Institute of Plant Sciences, Swiss Federal Institute of Technology, Zurich, Switzerland. The maize ubiquitin-1 promoter and its intron1 have been chosen because of its high efficiency in driving gene expression in monocot. plant species. In addition, the presence of a unique *Bam*HI site in the multiple cloning sites enables easy insertion of any foreign DNA fragments containing the same restriction site into this vector.

Protoplast technology has been widely used as an experimental tool in many fields of genetic manipulation of plants. One of the important application of this technology is to analyse the function of plant gene promoters by transient expression of reporter genes in transformed cells. Protoplasts can be easily isolated in a large quantity from a variety of plant species by enzymatic digestion, and leaf tissue is commonly used as a source material for protoplast isolation (Blackhall *et al.*, 1994). Protoplasts can be promoted to uptake DNA from medium either by chemical treatment, e.g. using polyethylene glycol (PEG), or by the use of the electric pulses (electroporation) (Fromm *et al.*, 1986).

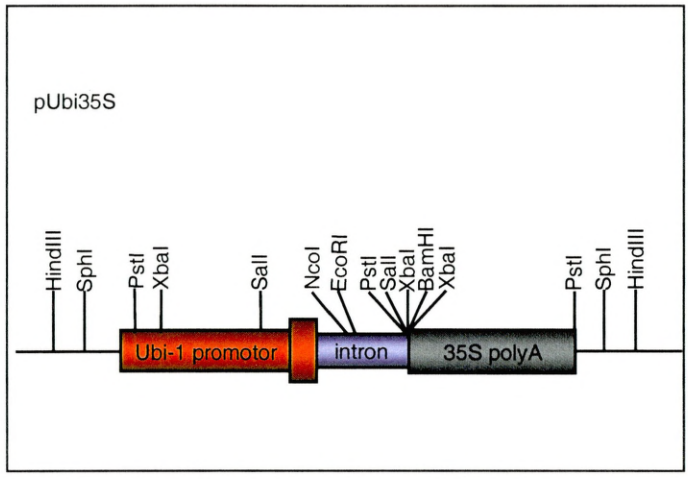


Figure 6. Cloning vector for generating wheat transformation constructs.

To test the expression of viral genes driven by the maize ubiquitin-1 promoter, protoplasts have been isolated by enzymic digestion of tobacco leaves and transfected with different constructs via PEG treatment. The expression of genes was detected by analysis of transcripts produced from the introduced gene constructs.

This chapter describes the construction of a variety of plasmids by subcloning the SBWMV coding sequences into a suitable expression vector and functional tests of viral gene expression in tobacco protoplasts by transient expression studies.

3.2 Materials and methods

3.2.1 Isolation of plasmids containing the cDNA of SBWMV coding sequences

Plasmids containing the cDNA of different coding sequences of SBWMV Oklahoma isolate were prepared using the Wizard® Plus SV Minipreps DNA Purification System (Promega, UK). The *E. coli* strains harbouring the plasmids containing full length cDNA clones of SBWMV RNA 2 and partial cDNA clone of SBWMV RNA 1(Oklahoma isolate) were obtained from Dr. J. P. Chen of Scottish Crop Research Institute, UK. The preparation was carried out as described in section 2.4.1. The plasmids were checked for their concentration on a 1% agarose gel.

3.2.2. Cloning SBWMV viral coding sequences into pUbi35S

3.2.2.1 DNA fragment preparation by PCR

3.2.2.1.1 Primer design

A number of SBWMV coding sequences were amplified by PCR and cloned into the vector pUbi35S. Several sets of primers with different length and base composition were designed to target the specific viral DNA sequences according to the published data. A *Bam*HI restriction site, 5'-ggatcc-3', along with two additional single bases of "c" and "g" were added to the end of all primers for cloning purposes (5'-cgggatcc-3'). Table 1 illustrates the details of primers designed to target viral-coding sequences and the details describing their construction are in the following results section.

3.2.2.1.2 Amplification conditions

PCRs were carried out in 25 µl volume containing 100 ng template DNA using *Ready To Go*™ PCR beads (*Amersham Pharmacia Biotech.* USA) fol-

lowing the manufacturer's instructions. The reaction conditions varied depending on the content and the length of primers and the length of target DNA fragment. In most cases, amplification started at 94 °C for 1 minute for denaturation followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and elongation at 72 °C for 1 minute, with 5 minutes at 72 °C for final elongation.

Table 1. PCR primers used for amplification of SBWMV viral genes and resultant constructs.

3.3.1.1	PCR primers (Forward/reverse)	Description of resultant constructs
	5'-cgggatccCCAAAGGTTACTACACCCTTGC/ 5'-cgggatccTTAACTCGAACCTTCCCATTTC	**pUbiCP1 &pUbiCP2**
	*Bam*HI 334 843 862 cgggatcc CCA AAG GTT ACT ACA ... TGC GTT ATG GGG ... CTT GAA ... AGT GGT TCG AGT TGA GGT TTC CAA TGA TGT ... ACG CAA TAC CCC ... GAA CTT ... TCA CCA AGC TCA ATT cctagggc *Bam*HI M M ORF1 aa 1-176 Target: SBVMV Coat Protein Gene	

3.3.1.3	PCR primers (Forward/reverse)	Description of resultant constructs
	5'-cgggatccATGGGCTGACAGGATGTCTAAGG/ 5'-cgggatccCTACACTATCAAAATTACCAAAC	**pUbiMP1 & pUbiMP2**
	*Bm*HI 5653 5560 5571 5575 6615 6635 cgggatcc ATG GGC TGA CAG GAT GTC TAA GGT ... TTA ... AGT TTG GTA ATT TTG ATA GTG TAG TAC CCG AGT GAC CTA CAG TTT CCA ... AAT ... TCA AAC CAT TAA AAC TAT CAC ATC cctagggc *Bam*HI M M ORF3 aa 1-317 Target: SBWMV Movement Protein Gene	

3.3.1.4	PCR primers (Forward/reverse)	Description of resultant constructs
	5'-cgggatccATGATGAATGAGTTGGTCATTTATCG/ 5'-cgggatccTTACCATTCAAAGTTCCTATCCACCC	**pUbiFRP1 & pUbiFRP2**

*Bam*HI 3090 3115 5563 5587

cgggatcc ATG ATG AAT GAG TTG GTC... CGT AAT ... AGG GTG GAT AGG AAC TTT GAA TGG TAA

TAC TAC TTA CTC AAC CAG ... GCA TTA ... TCC CAC CTA TCC TTG AAA CTT ACC ATTcctagggc

*Bam*HI

M M ORF1 aa 997-1828

Target: SBWMV Replicase Gene

3.3.1.5	PCR primers (Forward/reverse)	Description of resultant constructs
	5'-cgggatccATGATGAATTAGTTGGTCATTTAACG/ 5'-cgggatccTTACCATTCAAAGTTCCTATCCACCC	**pUbiDFRP1 & pUbiDFRP2**

*Bam*HI 3090 3099 3113 3115 5563 5587

cgggatcc ATG ATG AAT TAG TTG... TAA CGT AAT ... AGG GTG GAT AGG AAC TTT GAA TGG TAA

TAC TAC TTA CTC AAC ... ATA GCA TTA ... TCC CAC CTA TCC TTG AAA CTT ACC ATTcctagggc

*Bam*HI

M M ORF1 AA 997-1828

Target: SBWMV Replicase Gene

Capital letters in PCR primers represent viral sequences; others are additional sequences added in primers. Red capital letter indicates the mutated site nucleotides introduced by PCR.

3.2.2.1.3 Restriction enzyme digestion of PCR products

PCR products were purified with S-400 HR MicroSpin™ Columns (*Amersham Pharmacia Biotech.,* USA) to remove the excess primers following the supplied instructions, and collected in a clean 1.5 ml Eppendorf tube. To generate protruding termini, purified PCR products were digested in a final volume of 40 µl containing 10 units *Bam*HI and buffer recommended by the

enzyme supplier. Digestion was performed at 37 °C for 1hr. After extraction with phenol/chloroform, the upper aqueous phase was transferred into a clean tube and then purified with S-400 HR MicroSpin™ Columns. After precipitation with ethanol, the DNA pellet was resuspended in 10 µl H_2O.

3.2.2.1.4 Digestion and dephosphorylation of vector pUbi35S

10 µl pUbi35S (1µg/µl) was digested at 37 °C for 1 hr in a 100 µl final volume containing 50 units *Bam*HI (Boehringer Mannheim GmbH, Germany), and buffer as recommended by enzyme supplier. Following the digestion, dephosphorylation was carried out as described in Chapter 2. After extraction with phenol/chloroform, the dephosphorylated plasmid was precipitated as described previously with sodium acetate and absolute ethanol, then dissolved in 50 µl H_2O.

3.2.2.1.5 Ligation

10 µl of ligation mixture was assembled in a 0.2 ml tube with 50 ng *Bam*HI-digested dephosphorylated pUbi35S, 150 ng *Bam*HI-digested PCR product, 1 unit T4 DNA ligase and 1 µl of 10 x T4 ligase buffer (Boehringer Mannheim GmbH, Germany) and 2 µl H_2O. The ligation was performed at 12 °C overnight.

3.2.2.1.6 Transformation and clone selection

100 µl of *E. coli* HB101competent cells were mixed with 5 µl of ligation mix. Transformation and selection was performed following the same procedures as described previously in Chapter Two.

3.2.2.1.7 Quick screening transformants

To identy the clones containing the gene of interest, crude DNA samples were extracted from a number of ampicillin resistant clones using lysis buffer and then electrophoresed on a 0.8% agarose gel (Section 2.9.3). Plasmids were prepared from selected clones using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) (section 2.4.1) and recombinant DNA was subject to further analysis.

3.2.2.1.8 Analysis of recombinant plasmids

Recombinant plasmids were analysed by restriction digestion with appropriate enzymes, and the orientation of insert with respect to the promoter was determined by DNA sequencing.

3.2.3 Transient expression of SBWMV *CP*, *MP* and *RP* genes in tobacco protoplasts

3.2.3.1 Plant material

Tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) were grown in a growth chamber at 24 °C under 12 h photoperiod during the day and at 24 °C in the night respectively.

3.2.3.2 Media

Two kinds of medium were used for protoplasts preparation and culture. The composition of the media used at the final concentration of their individual ingredients is shown in Table 2.

Table 2. Composition of the media used for transfection of tobacco protoplasts

Media component	Media	T1	T2
Macroelements (mg/final concentration)			
NH_4NO_3		825	825
KNO_3		950	950
$CaCl_2$, $2H_2O$		220	220
$MgSO_4$, $7H_2O$		185	185
KH_2PO_4		85	85
Microelements (mg/l final concentration)			
$FeSO_4$, $7H_2O$			27.8
Na_2EDTA			37.2
H_3BO_3		1	1
$MnSO_4$ H_2O		1	1
$ZnSO_4$ $7H_2O$		0.1	0.1
$CuSO_4$ $5H_2O$		0.03	0.03
$AlCl_3$		0.03	0.03
$NiCl_2$ $6H_2O$		0.03	0.03
KI		0.01	0.01
Vitamins (mg/l final concentration)			
Myo-inositol		100	100
Thiamine HCl		1	1
Pyridoxine HCl		1	1
Nicotinic acid		1	1
Calcium pantothenate		1	1

Carbohydrates (g/l final concentration)		
Mannitol	80	80
Sucrose		20
Hormones (mg/l final concentration)		
NAA	3	3
BAP	1	1
Biotin	1	1
Others (mg/l final concentration)		
Cefotaxime	100	100
Tween 20	10	10

3.2.3.3 Protoplast preparation

The fully expanded leaves were collected and sterilised by incubation in 70% Domestos for 10 minutes at room temperature in a plastic container. The leaves were then washed with sterile water four times. The sections of the leaf between larger veins were excised following peeling with fine forceps and transferred into a 9 cm Petri dish containing 15 ml of enzyme solution (1 mg/l Cellulase, 0.5 mg/l Driselase, 0.2 mg/l Macerozyme in T1 medium). The plates were incubated at 25 °C overnight in the dark. Protoplasts were isolated from leaf debris by sieving through a 100 µ sieve into a new dish. 10 ml sieved protoplasts was transferred to a 12 ml sterile centrifuge tube and centrifuged at 400 rpm for 5 minutes. The supernatant was removed following centrifugation and the pellet was resuspended in 10 ml of T1 solution. 5 ml of resuspended protoplasts were layered onto 2.5 ml of 16% sucrose solution and centrifuged at 800 rpm for 5 minutes. The protoplast band at the

interface above the sucrose cushion was collected using a sterile plastic pipette and transferred to a new tube, combining the protoplasts from two interphases to 10 ml T1 medium.

3.2.3.4 Protoplast transfection

30 µg plasmid in 20 µl water were added into 200 µl purified protoplasts suspension and mixed carefully in a small sterile Petri dish. 200 µl of PEG (25% PEG 8000, 0.1M $Ca(NO_3)_2$, 0.45 M Mannitol, 10 mM MES (pH 6.0)) was added into the protoplasts sample and incubated for 20 minutes at room temperature. Then 800 µl of 0.275 M $Ca(NO_3)_2$ were added as two aliquots of 200 µl and one of 400 µl, mixing after each addition. Then 4 ml of $Ca(NO_3)_2$ solution was added into the tube and the sample was incubated for 20 minutes at room temperature. The sample was transferred to a tube and centrifuged at 400 rpm for 3 minutes. The supernatant was removed, and the pellet was resuspended with 5 ml of T2 medium. The protoplasts sample was transferred into a small dish and incubated at 25 °C for 24 hours under the light.

3.2.3.5 RNA extraction from transfected tobacco protoplasts

Total RNAs were extracted from tobacco protoplasts transfected with plasmids containing SBWMV coding sequences following the procedures described in section 2.6.1.

3.2.3.6 RT-PCR analysis

To examine gene expression at the RNA level, RT-PCR was performed using the "Access RT-PCR System" kit (Promega Corporation, UK) following the supplier's instruction. The reaction conditions varied depending on the content and the length of primers and the length of target DNA fragment.

3.3 Results and discussion

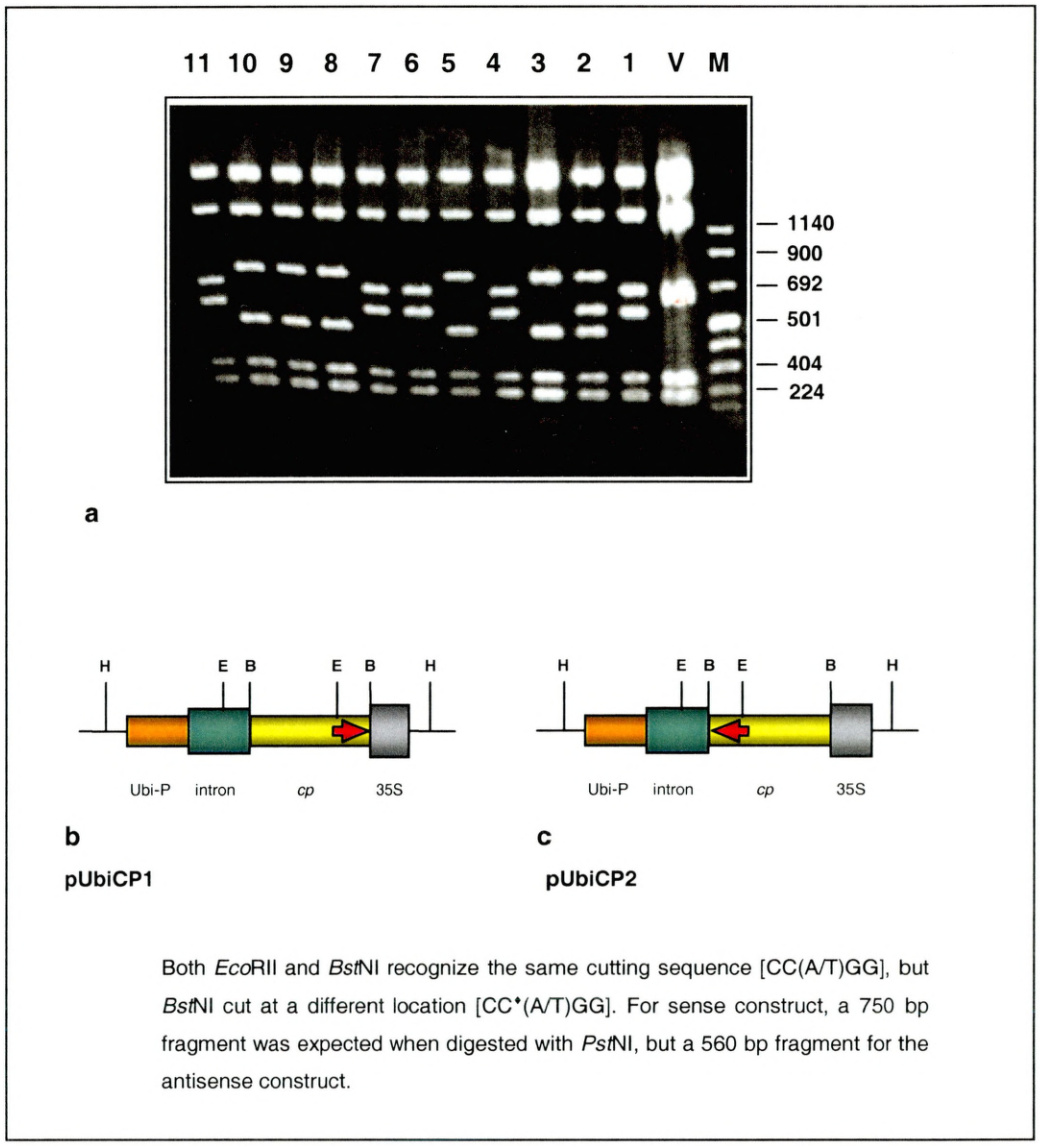
3.3.1 Construction of expression vectors

3.3.1.1 Construction of pUbiCP1 and pUbiCP2

PCR was used to amplify the SBWMV coat protein (CP) gene from one of four clones (pJC1A) which contained cDNA encoding full-length viral RNA 2 sequences and using primers as detailed in Table 1. The *Bam*HI restriction sites were added to both primers and the CP gene termination codon was changed from TAG to TAA to eliminate the possibility of read-through. PCR, DNA ligation and recovery of recombinant DNA were performed following the procedures as described in Materials and Methods. The recombinant DNAs were analysed by restriction enzyme digestion and DNA sequencing.

A 564 bp fragment, corresponding to the CP gene of SBWMV was obtained from all 11 clones when digested with *Bam*HI, which confirms that the CP gene was cloned into the vector pUbi35S. But, it was not possible to determine the orientation of the insert in the recombinant plasmids since the CP gene might be ligated into the vector in either orientation. Therefore, another enzyme had to be chosen for this analysis. Sequence data revealed that *Eco*RII digestion would distinguish between the two different orientations of the fragment. However, using *Eco*RII resulted in incomplete digestion because it could not cleave DNA when the 3' C residue is either 4-methylcytosine or 5-methylcytosine. It was only possible to determine the orientation of the insert by using *Bst*NI, an isoschizomer of *Eco*RII, which should give rise to two obvious different fragment patterns, because this enzyme has a single restriction site in both the CP gene and the upstream intron. Digestion of recombinant DNA from 11 clones resulted in two major fragment patterns when electrophoresed on a 1% agarose gel (Fig. 7a). Two types of these, e.g. in lanes 3 and 1, were consistent with sense and antisense cloning of the coat protein gene into pUbi35S respectively. The plasmid containing sense CP cDNA, which gives rise to an expected 750 bp fragment in *Bst*NI digests, was designated as pUbiCP1 (Fig. 7b), and the

antisense version with a 560 bp fragment as pUbiCP2 (Fig. 7c). In lane 2 the band pattern was identical to the pattern of lane 3, but one additional band around 570 bp appeared. Analysis has revealed that this clone resulted from the combined insertion of two sense CP fragments. This clone was not used for wheat transformation.



Both *Eco*RII and *Bst*NI recognize the same cutting sequence [CC(A/T)GG], but *Bst*NI cut at a different location [CC•(A/T)GG]. For sense construct, a 750 bp fragment was expected when digested with *Pst*NI, but a 560 bp fragment for the antisense construct.

Figure 7. Gel electrophoresis of *Bst*NI cleaved recombinant DNA and diagram of resultant constructs. a) M: DNA marker VIII (Promega, UK) V: Cloning vector pUbi35S, Lane 1-11: Different clones, **b)** Plasmid containing sense coat protein cDNA, **c)** Plasmid containing antisense coat protein cDNA, ubi-p: ubiquitin-1 promoter, *cp*: SBWMV coat protein coding sequence, 35S: 35S terminator, B: *Bam*HI, E: *Eco*RII, H: *Hin*dIII.

DNA sequencing reactions were carried out to confirm the desired constructs from four clones which had been identified as containing sense CP cDNA insert by restriction analysis. Figure 8 illustrates the sequencing primer, which was used for sequencing all of the constructs, starting the sequencing in the ubiquitin-1 intron in vector pUbi35S. The example of the sequencing data is shown in figure 9.

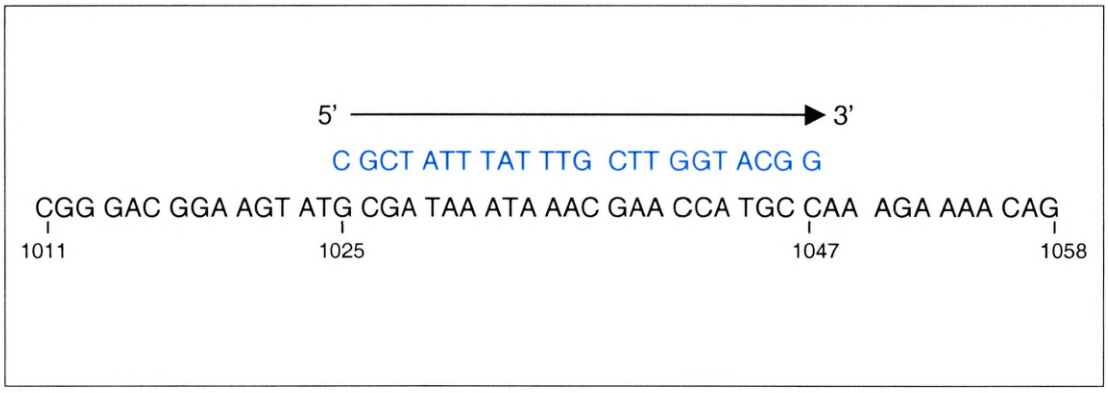


Figure 8. Sequencing primer and the partial sequence of Ubiquitin-1 intron. Blue capital letter; primer sequence; Black letter: partial sequence of Ubiquitin-1 intron in vector pUbi35S.

Comparison of the resulting nucleotide and derived amino acid sequences with published SBWMV coat protein DNA and corresponding amino acid sequences revealed that only one of the sense clones precisely matched the published amino acid sequence although two single nucleotide mismatches have been found in it. More nucleotide mismatches were found in the other three clones, these led to a number of amino acid changes in the derived amino acid sequences. This was possibly due to base misincorporations during the process of PCR amplification. The construct containing the correct insert of coat protein gene was used for wheat transformation.

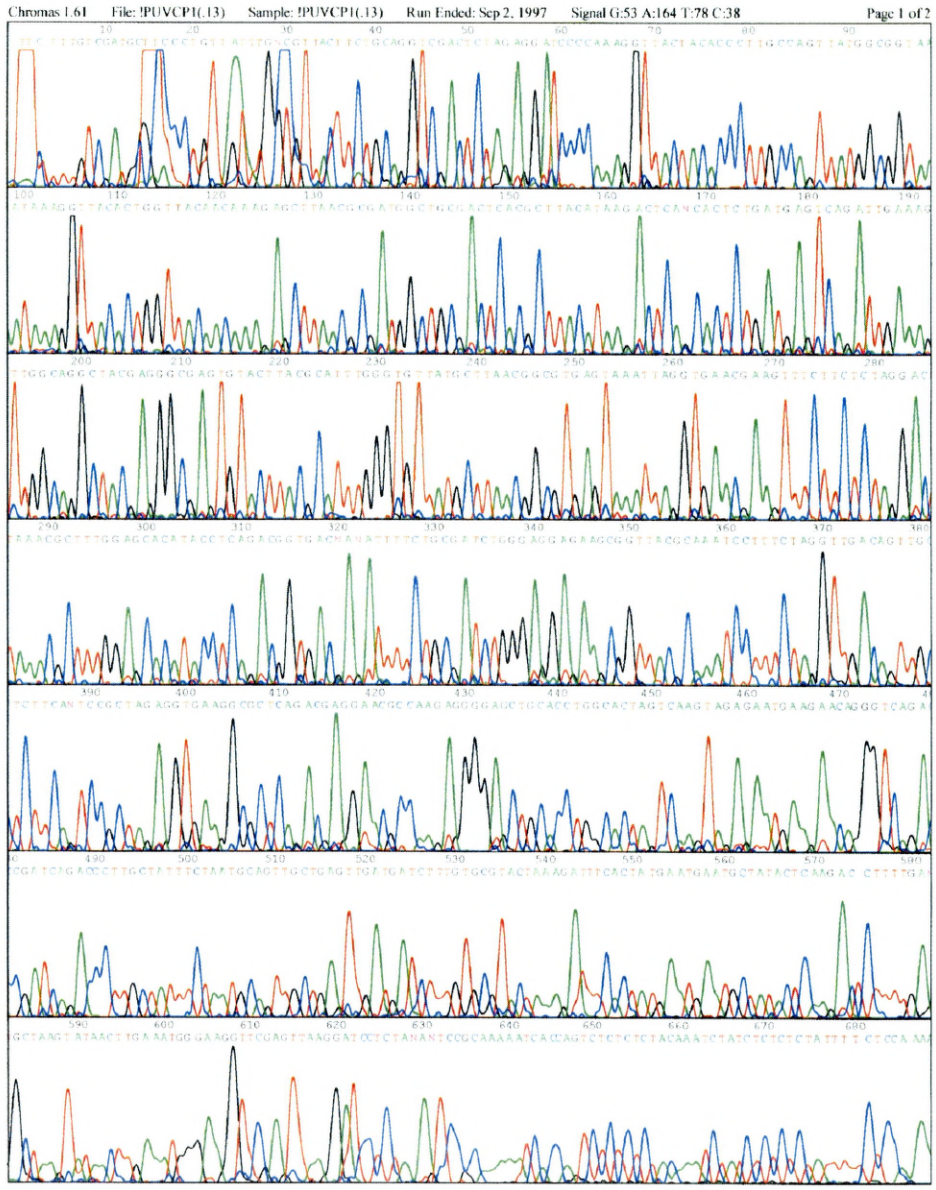


Figure 9. Result of DNA sequencing of clone pUbiCP. The clone was partially sequenced from the 5' end of CP gene, using the fluorescence-based dideoxynucleotide chain termination method (Sanger et al., 1977). The sequencing reaction was performed using the ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit, and run the gel on the ABI PRISM™ 373 DNA sequencer.

3.3.1.2 Construction of pUbiCPbar1 and pUbiCPbar2

The complex constructs containing both viral coat protein gene sequences and a selectable marker *bar* gene encoding phosphinothricin acetyltransferase were generated by subcloning *Hin*dIII restriction fragments from pUbiCP1 and pUbiCP2 into the single *Hind*III site of pAB1 (Fig. 10; kindly provided by B. Roland, Institute of Plant Sciences, Swiss Federal Institute of Technology, Zurich Switzerland).

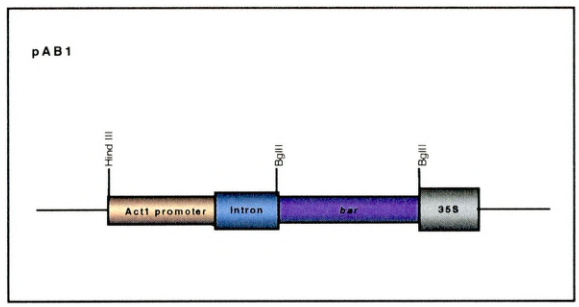
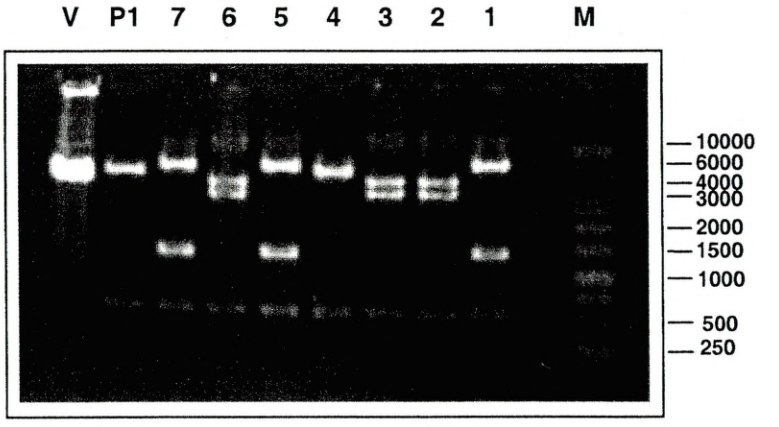


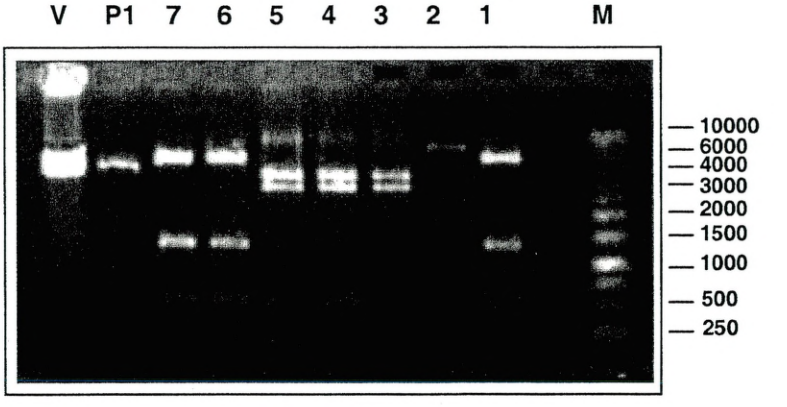
Figure 10. Plasmid used for generating complex constructs.

SBWMV coat protein expression cassette (promoter-insert-terminator) containing both orientations of insert were isolated from pUbiCP1 and pUbiCP2 using *Hin*dIII, and then ligated with *Hin*dIII-digested, dephosphorylated vector pAB1. After transformation with two kinds of ligation mix and selection of *E. coli* (as described in Materials and Methods), recombinant DNAs obtained from individal bacterial transformants were analysed by restriction enzyme digestion. *Bam*HI digestion of recombinant DNAs obtained from 7 clones of each transformation resulted in three identical fragment patterns (Fig. 11). One of these, e.g. lane 2, 3 and 6 in figure 11a and lane 3, 4. and 5 in figure 11b, was consistent with sense or antisense cloning of the CP gene with expression cassette being inserted into pAB1 in an anti-clockwise direction relative to the cloning vector. Gel electrophoresis also demonstrated that

the coat protein expression cassette derived from pUbiCP1 and pUbiCP2 were integrated into the *Hin*dIII site of cloning vector pAB1 in both clockwise and anti-clockwise direction in these two cloning experiments. The plasmid containing the sense CP cDNA which was inserted into pAB1 in anti-clockwise direction was designated as pUbiCPbar1 (Fig. 12) and the antisense CP cDNA version as pUbiCPbar2 (Fig. 12).



a



b

Figure 11. Gel electrophoresis of recombinant DNA digested with *Bam*HI. a) *Bam*HI cleaved recombinant DNA which include sense CP cDNA. M: 1 Kb DNA marker (Promega, UK), V: Cloning vector pAB1, P1: pUbiCP1, Lane 1-7: Different clones **b)** *Bam*HI cleaved recombinant DNA which include antisense CP cDNA. M: 1 Kb DNA marker (Promega, UK), V: Cloning vector pAB1, P2: pUbiCP2, Lane 1-7: Different clones.

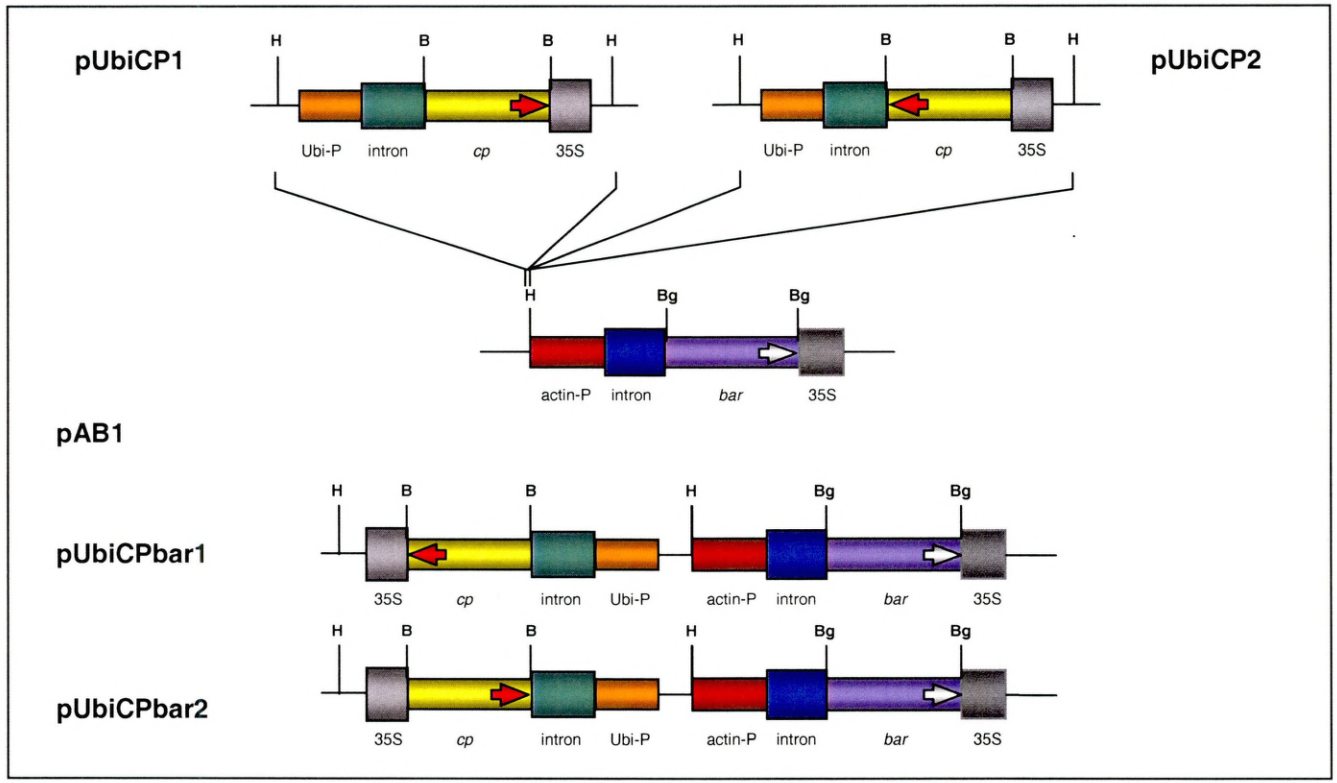
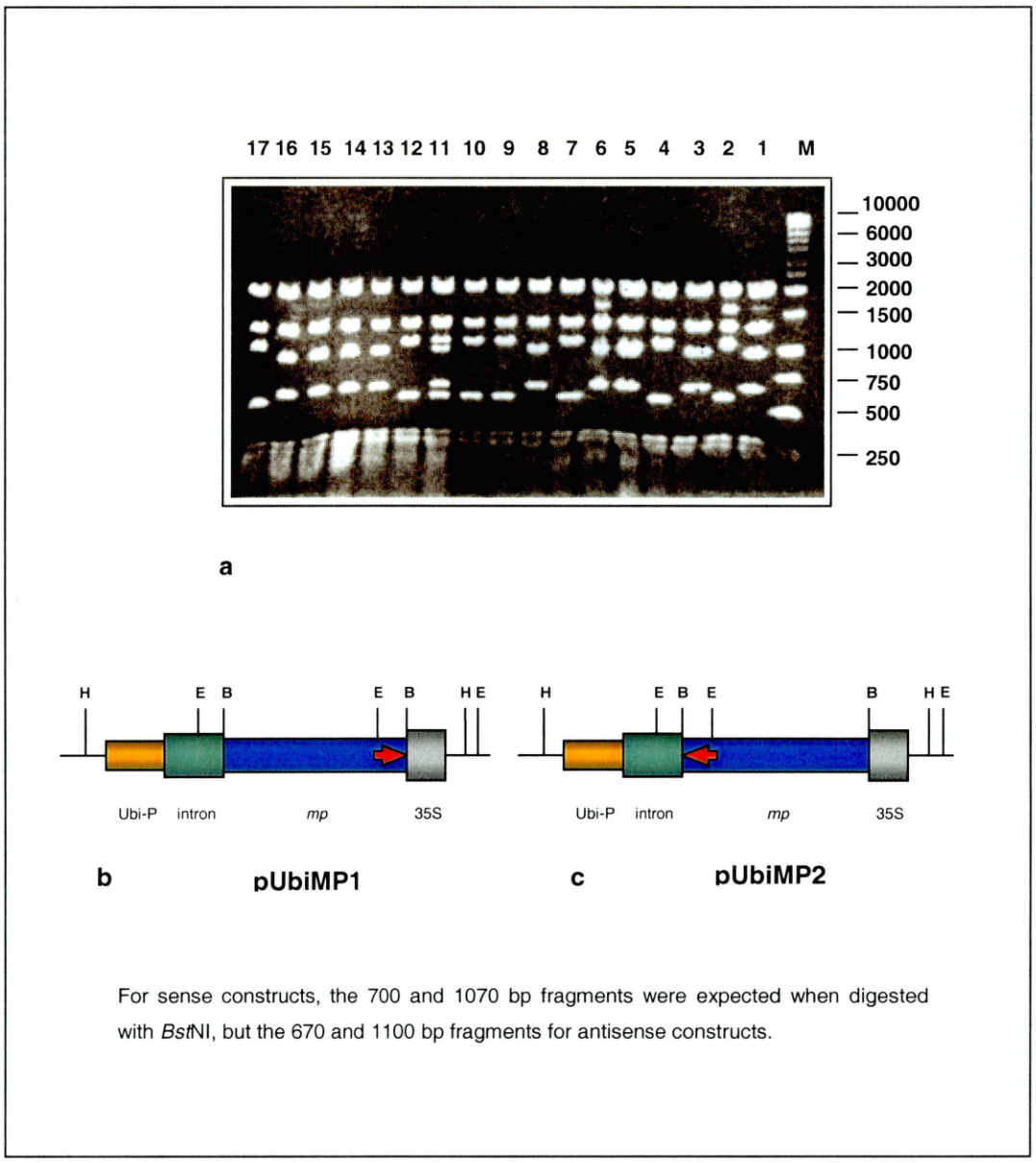


Figure 12. Construction of complex expression vectors pUbiCPbar1 and pUbiCPbar2. The CP expression cassette was released from pUbiCP1 and pUbiCP2, and then ligated into the *Hin*dIII site of pAB1.

3.3.1.3 Construction of pUbiMP1 and pUbiMP2

SBWMV movement protein (MP) gene was amplified by PCR reaction from pJC1A, which contains partial-length of viral RNA1 sequences from nt 1670-7098. *Bam*HI restriction sites were added to both forward and reverse primers. This fragment was designed to confer the transgenic plant resistance to SBWMV via RNA-mediated resistance mechanism, therefore mutations were introduced into viral MP coding sequences by designing a 5' primer with two single base changes, from C to G and A to T respectively, which lead to the formation of two stop codons immediately downstream of the start codon at the 5' end of MP coding sequences. PCR and DNA ligation were performed following the same procedures as used for construction of other transformation vectors. After transformation and selection of *E. coli*, plasmids were prepared using a Miniprep kit (as described in Materials and Methods). Restriction analysis and DNA sequencing were carried out to determine the orientation of the insert in recombinant DNA.

The recombinant DNAs from 17 clones were first digested with *Bam*HI. Digested products were separated on agarose gel. The expected 945 bp fragment, corresponding to the SBWMV MP coding sequence was observed from all 17 digests, which indicated the MP gene had been cloned into the vector. After digestion with *Bst*NI, four fragment patterns were observed when the digests were separated on a 1% agarose gel (Fig.13). Comparison of two patterns, e.g. lanes 3 and 4, revealed that the MP gene was cloned into the *Bam*HI site of cloning vector pUbi35S in different directions; however, determination of insert orientation in recombinant DNA could not be accomplished because the proximity of the bands derived from these two patterns made it difficult to identify the orientation of the insert depending only on the result of gel electrophoresis. As an alternative, DNA sequencing was conducted to confirm the orientation of sense and antisense MP cDNA in these two kinds of clones.



For sense constructs, the 700 and 1070 bp fragments were expected when digested with *Bst*NI, but the 670 and 1100 bp fragments for antisense constructs.

Figure 13. Gel electrophoresis of *Bst*NI digested recombinant DNA from 17 individual clones and diagram of resultant constructs. a) *Bst*NI digested recombinant DNAs. M: 1 kb DNA marker (Promega, UK); Lane 1-17: Different clones. b, c) Diagram of resultant constructs which contain sense or antisense MP cDNA, ubi-p: ubiquitin promoter, *mp*: SBWMV movement protein coding sequence, 35S: 35S terminator, B: *Bam*HI, E: *Eco*RII, H: *Hin*dIII.

Four clones (Figure 13, clone 9, 10 13 and 14) were sequenced. The sequencing primer was the same as used for CP-constructs analysis. Although only part of MP sequences, about 500 bp, was sequenced using this primer, it was enough to determine the orientation of the insert in the recombinant plasmids. Sequencing results revealed that clone 13 and 14 contain the sense insert of MP, while clones 9 and 10 contain the antisense of insert (Fig. 13a). Insertion of the sense MP cDNA into the vector resulted in pUbiMP1 (Fig.13b) and the antisense cDNA version as pUbiMP2 (Fig.13c).

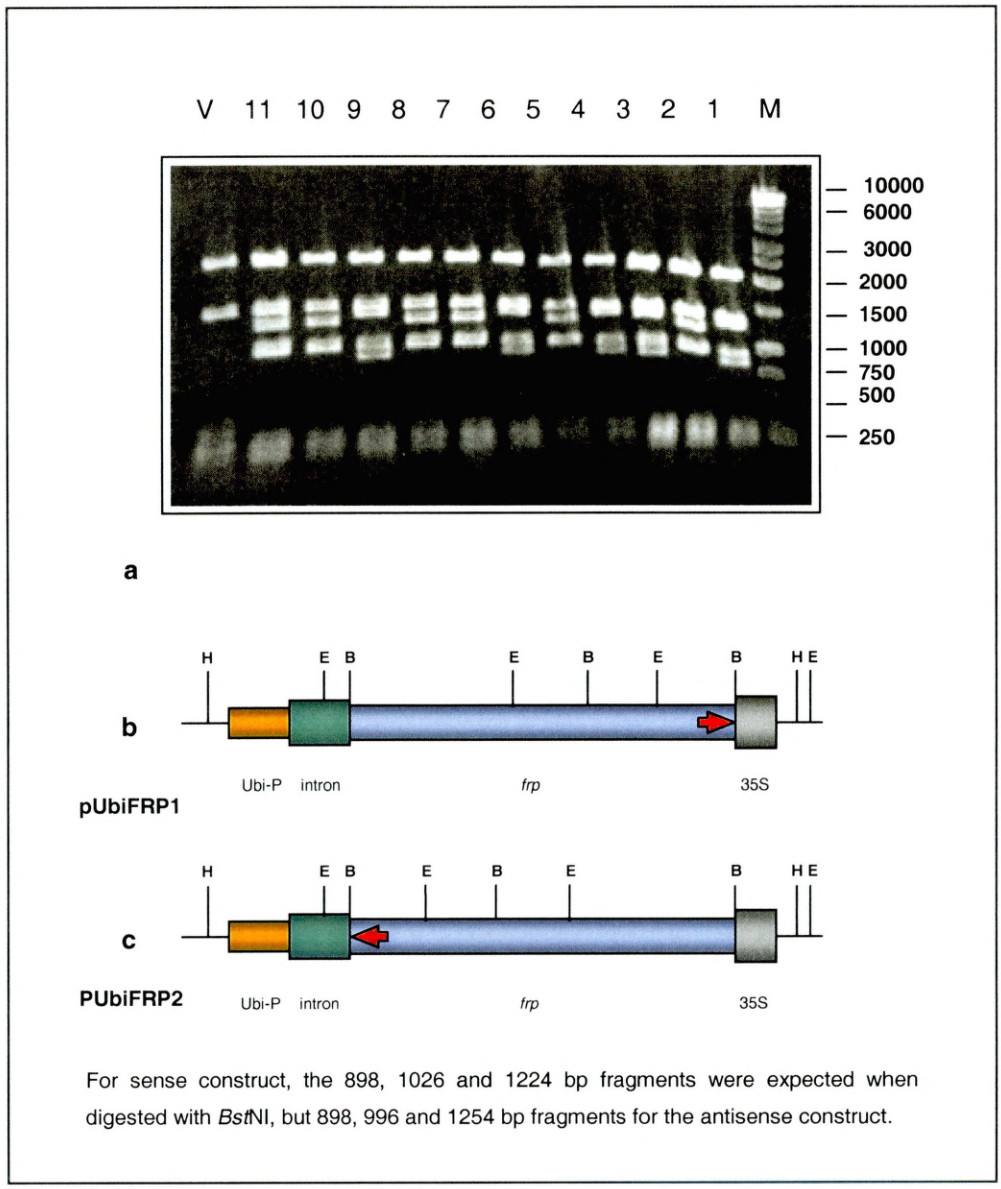
3.3.1.4 Construction of pUbiFRP1 and pUbiFRP2

Cloning of SBWMV replicase (RP) gene into the vector pUbi35S was first made possible by introducing a *Bam*HI site into the viral RP-coding sequence via PCR. The PCR programme used for the amplification of 2587 bp of the full RP coding region was the same as for amplification of other viral genes with the exception of the elongation phase of each cell cycle which was held at 72 °C for 2.5 minutes. Due to the existence of an internal *Bam*HI site in the amplified sequence (RP coding sequence), PCR-amplified RP fragments were partially digested using *Bam*HI prior to ligation with cloning vector. DNA ligation, transformation of *E. coli* and the selection of recombinant clones was conducted as described in Materials and Methods. Restriction mapping analysis showed that multiple fragments in different combination had been inserted into the vector, which made it difficult to identify the recombinant plasmids with the correct insert. Therefore, a second approach was used to produce RP vectors. To eliminate possible base misinsertions which frequently occur during polymerization, *pfu* DNA polymerase was used for the PCR. Because *pfu* DNA polymerase-generated PCR fragments are blunt-ended, for cloning purpose, the 5'-protruding end of vector pUbi35S generated by *Bam*HI digestion was filled in using Klenow fragment (Section. 2.2.6). Ligation, transformation and selection of *E. coli* were performed following the protocols as described in Materials and Methods. Plasmids were prepared from 11 clones which were presumed to contain recombinant DNA after a quick screening test. The cloning of functional replicase gene into the vector

was confirmed from all 11 resultant clones by *Bam*HI digestion analysis, which demonstrated the expected band patters. *Bst*NI digestion of those 11 clones resulted in two expected types of fragment patterns. (Fig.14a). DNA sequencing was used to confirm the orientation of the insert in recombinant DNAs, the plasmid containing the sense functional RP gene insert was designated as pUbiFRP1 (Fig.14b) and the antisense cDNA version as pUbiFRP2 (Fig.14c).

3.3.1.5 Construction of pUbiDFRP1 and pUbiDFRP2

The same strategy was used in the construction of dysfunctional SBWMV RP gene constructs. Two stop codons were introduced into the 5' end of viral RP coding sequence via changes of two nucleotides (at nt 3098 from G to T, at nt 3112 from T to A) by PCR reaction. PCR and DNA ligation were carried out following the same procedures as used for cloning the functional RP. After transformation and selection of *E. coli,* the resultant plasmids were analysed by restriction digestion, and 7 clones were found to have the cloned insert which gave rise to the expected fraction patterns when electrophoresis of *Bam*HI digests was conducted in an agarose gel. The expected two types of band patterns were obtained when using *Bst*NI digestion of those 7 clones. DNA sequencing was employed to confirm the constructs that contain sense or antisense dysfunctional viral RP gene. Insertion of sense dysfunctional RP fragment into the vector resulted into pUbiDRP1, and the antisense version as pUbiDFR2.



For sense construct, the 898, 1026 and 1224 bp fragments were expected when digested with *Bst*NI, but 898, 996 and 1254 bp fragments for the antisense construct.

Figure 14. Gel electrophoresis of *Bam*HI digested recombinant DNA and the diagram of resultant constructs. a) *Bam*HI cleaved DNAs. M: 1 kb DNA marker (Promega, UK); Lane 1-11: Different clones. **b, c)** Diagram of resultant constructs which contain the sense and antisense functional RP gene. ubi-p: ubiquitin promoter, *rp*: SBWMV replicase coding sequence, 35S: 35S terminator, B: *Bam*HI, E: *Eco*RII, H: *Hin*dIII.

3.3.2 Transient expression of SBWMV *CP*, *MP* and *RP* genes in tobacco protoplasts

RT-PCR was used to detect mRNA transcribed from constructs in tobacco protoplasts using the primers as used for the cloning work. When using 5 μl of total RNA (0.1μg) in 50 μl of RT-PCR, a 564 bp RT-PCR product corresponding to the CP encoding gene was detected from the CP RNA sample and positive control plasmid, while a negative PCR result was obtained from the same reaction mixture without reverse transcriptase (Fig. 15). However, the expected amplification products corresponding to the 983 bp MP coding sequence and 2595 bp RP complex sequences were undetectable, even though the amount of total RNA used was increased 5 times in those subsequent RT-PCR tests (data no shown).

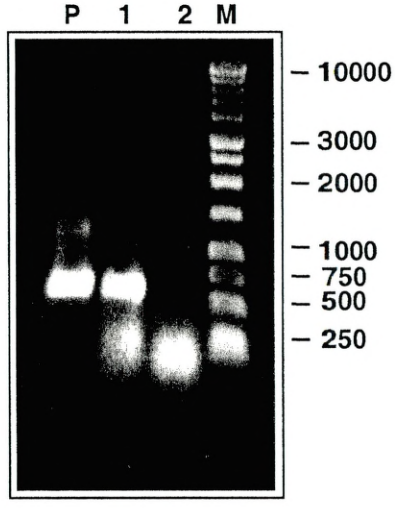


Figure 15. RT-PCR amplification of the 564 bp cDNA of SBWMV CP gene from total RNA extracted from pUbiCP1- transfected tobacco protoplasts. M) 1kb DNA maker (Promega, UK); 1) Reaction using CP primers with reverse transcriptase; 2) Reaction using CP primers without reverse transcriptase; P) pUbiCP1 positive control.

Direct gene transfer to tobacco protoplasts is a simple and effective method in functional testing of a gene of interest, with promoter and termination sequences. To test the activity of maize ubiquitin-1 promoter and the expression of the viral genes driven by this promoter, transient expression study of the SBWMV viral genes were conducted by transfecting tobacco protoplasts via the polyethylene glycol (PEG) mediated gene transfer technique with designated constructs containing the sense viral genes for subsequent integrative transformation of wheat. RT-PCR is the most sensitive technique, which was widely used to determine transcript presence, to clone cDNA products and to estimate the expression level of target genes. RNAs were extracted from transformed tobacco protoplasts and the reverse transcription polymerase chain reaction (RT-PCR) was used to detect the expression of viral genes in transfected tobacco protoplasts.

Since the first report on the use of maize *Ubiquin-1* promoter in transient and stable transformation of cereals (Christensen *et al.*, 1992; Toki *et al.*, 1992; Uchimiya *et al.*, 1993), this promoter has been successfully used in driving expression of a number of genes in transgenic monocotyledonous species, including several cereals (Wilmink *et al.*, 1995; McElory and Brettell, 1994; Cornejo *et al.*, 1993; Weeks *et al.*, 1993; Rolfe and Tobin, 1991). The expression of viral CP gene under the control of maize Ubiquitin-1 promoter in tobacco protoplasts demonstrated that the monocotyledonous plant promoter can effectively direct the expression of the viral gene in dicotyledonous plant species, similar to the results obtained from electroporated tobacco protoplasts with plasmid pUBI-CAT (Christensen *et al.*, 1992). However, failure to reverse transcribe the defective viral MP gene was found in this experiment. This was possibly due to the poor stability of mRNA transcribed from the defective DNA sequences in which two stop codons were introduced directly downstream of the start codon.

Although RNA stability is of importance in the control of gene expression, much less is known of the process of mRNA breakdown. There is evidence that the presence of an in-frame stop codon could significantly lower the steady-state mRNA level, resulting in a lower rate synthesis of the

cognate protein. It has been found that a mutant form of the soya bean kunitz trypsin inhibitor gene containing in-frame stop codons can be transcribed at the same rate as the control gene, but the steady-state mRNA levels were suppressed, which resulted in the decrease of protein accumulation by at least 100-fold. Furthermore, complete suppression of gene expression, due to the existence of an in-frame stop codon, in transgenic plants has been reported. When a lectin gene from *Phaseolus vulgaris* containing a stop codon due to a frame shift was introduced into transgenic plants, the mutant gene was not expressed at detectable levels, whereas, the gene repaired by *in vitro* mutagenesis was fully active, and the steady-state mRNA level was estimated to be increased by at least 40-fold (Robinson *et al.*, 1993). However, in contrast, Lindo and Dougherty (1992) transformed tobacco plants with tobacco etch virus (TEV) CP modified by introduction of three stop codons immediately downstream of the AUG start codons. It has been found such transgenic plant lines express an untranslatable TEV CP mRNA and 40% of transgenic plant lines expressing this RNA were highly resistant to TEV infection.

No amplification product was generated from the RT-PCR reaction using the RNA template derived from the protoplasts transfected with plasmid pUbiRP, but a 2595 bp specific fragment was amplified from the pUbiRP1 positive control reaction using the same pair of primers. It is not clear whether this is due to the failure of expression of the replicase gene in transfected tobacco protoplasts, or the long mRNA preventing reverse transcriptase extending the primer over a long distance. To detect the presence of mRNA, new primers might be required to allow the reverse transcription of internal partial sequence of the transcripts.

3.4 Conclusions

This chapter describes the generation of gene constructs for wheat transformation by the subcloning of a number of SBWMV coding sequences into a suitable expression vector, and tests of gene expression using transfected tobacco protoplasts.

The use of the maize ubiquitin-1 promoter to drive viral gene expression would provide the best promoter to express those genes in transgenic wheat plants. The unique cloning site in this vector allowed the target genes with the same sticky ends to be inserted into the vector in two different orientations in one cloning reaction. A variety of SBWMV coding sequences, which encode either functional or dysfunctional products, were successfully cloned into the vector pUbi35S by introducing PCR-generated defined sequences. Recombinant DNAs were analysed by restriction digestion with appropriate enzymes and DNA sequencing used to confirm the correct insert and their orientation.

The application of protoplasts for transient expression has proven to be a very important tool to detect the gene expression under the control of specific promoter. RT-PCR is a very sensitive and specific means for analysing the transcriptional activity of genes. Transient expression experiments were performed to verify that viral genes could be transcribed into RNA in tobacco protoplasts. Amplification of the expected product by RT-PCR from total RNA derived from protoplasts transfected with CP construct indicated that the expression of functional CP gene in tobacco protoplasts was achieved. But, the expression of dysfunctional MP gene and both functional and dysfunctional replicase genes were undetectable, this is possibly due to the nature of the genes tested and the technical limitation of the RT-PCR technique.

CHAPTER 4

TRANSIENT EXPRESSION EXPERIMENTS AND STABLE TRANSFORMATION OF WHEAT UNDER PHOSPHINOTHRICIN SELECTION

4.1 Introduction

4.1.1 Transient gene expression

Transient expression of transgenes following bombardment provides a simple and convenient tool to study gene expression driven by a certain promoter or to evaluate conditions for optimal gene transfer using the biolistic device. Unlike stable expression of transgenes, transient expression allows the transcription and translation of the introduced DNA without it being incorporated into the genome of host cells. Therefore, it has been widely used for the study of the regulation of gene expression and for optimising the parameters for particle bombardment of certain tissues.

A number of reporter genes including β-glucuronidase (GUS), anthocyanin, and luciferase (Luc) are available for transient expression study. These genes normally encode enzymes that can be easily assayed with good sensitivity in plant extracts (Russell and Fromm, 1995). Amongst these, GUS and anthocyanin have been commonly used for *in situ* detection of gene expression.

The factors influencing transient expression following particle bombardment include biological and bombardment physical parameters. The physiological conditions of the recipient cells could affect their competency for transient and stable expression of the transgene. It has been shown that

osmotic treatment of target tissues both before and after bombardment enhanced transient expression and stable transformation frequency (Sanford *et al.*, 1993). Similar results were also observed from the treatment of embryogenic maize cells and embyrogenic soybean tissue (Vain *et al.*, 1993a). Other physiological factors, including the pre-culture of tissues before the bombardment (Seki *et al.*, 1991) and the age of the cells (Sanford *et al.*, 1993), can significantly influence transient expression and stable transformation efficiency.

The combination of each optimal physical condition will significantly increase the transient expression and stable transformation efficiency. For a selected tissue, it is necessary to optimise the particle bombardment process to maximize gene delivery with minimizing effect on the vigour of the target tissue. In general, using the Sanford-type bombardment device, the most common parameters that need to be examined are the size of the particle, the burst pressure (gas pressure at which the rupture disk bursts), the flying distance of the DNA coated particle, and the amount of DNA per shot.

In monocotyledonous plants, immature embryos have been frequently used for the study of gene transfer by the particle bombardment approach (Christou, 1992; Kartha *et al.*, 1989; Klein *et al.*, 1988; Reggiardo *et al.*, 1991; Sautter *et al.*, 1991; Taylor and Vasil, 1991; Wan and Lemaux, 1994; Weeks *et al.*, 1993). In the experiments reported here, several bombardment parameters including the working pressure, working distance, the amount of DNA per bombardment were examined by transient expression of GUS in bombarded wheat immature embryos prior to the stable transformation of wheat with the designed constructs.

4.1.2 Wheat transformation

Wheat is the most widely cultivated and important food crop in the world (Vasil, 1999). Conventional plant breeding has achieved considerable success in improving yield and quality of wheat during the past several

decades. For example, the innovation and implementation of the "Green Revolution" has led to the enormous increase in wheat production world-wide. However, wheat production has been affected by many diseases caused by pathogens and pests. Recently, one of the most important fungal transmitted viruses, *Soil-borne wheat mosaic virus*, has become increasingly important in the winter wheat growing regions. Although several virus-resistant cultivars have been recognised and some achievements have been made in developing virus-resistant cultivars by conventional plant breeding techniques, the available resistance genes from the natural gene pool were limited and not characterised. In addition, traditional plant breeding might alter other useful traits already present in the genome of some specific varieties. Therefore, it is difficult to breed wheat for virus resistance by conventional plant breeding systems.

Genetic manipulation of plants heavily depends on successful tissue culture techniques with which fertile transgenic plants can be regenerated. To develop a wheat tissue culture system, many efforts have been made to attempt to establish regenerable tissue culture of wheat using a variety of explants. Among the different explants of wheat, immature embryos and inflorescences have proven to be the most favourable cell resource from which entire plants can be regenerated via somatic embryogenesis.

Early wheat transformation experiments involved transient expression of reporter genes in protoplasts of *Triticum monococcum* or *Triticum aestivum* transformed by direct DNA delivery using osmotic or electric shock (Ou-Lee *et al.*, 1986, Hauptmann *et al.*, 1987, Oard *et al.*, 1989). Although protoplast-derived stably transformed callus lines were obtained in some experiments, no transgenic plants were recovered in these studies (Lörz *et al.*, 1985, Hauptmann *et al.*, 1988, Marsan *et al.*, 1993, Zhou *et al.*, 1993, Chamberlain *et al.*, 1994, Müller *et al.*, 1996). He *et al.* (1994) reported the recovery of transgenic wheat plants from transformed protoplasts, but no transgenic progeny were obtained.

Plant biotechnology provides considerable scope for crop improvement. *Agrobacterium tumefaciens*-mediated transformation systems and the microprojecticle bombardment approach are the most successful and widely-used techniques for stable genetic transformation of crops. Application of the *Agrobacterium tumefaciens*-mediated transformation technique has resulted in the recovery of fertile transgenic plants of most dicotyledonous species used, and more recently of some monocotyledonous species, including the model spring wheat cultivar Bob White.

The success of recovery of fertile transgenic wheat plants relied on the improvements in tissue culture technology with which plants could be regenerated with high frequency, and the invention of reliable and efficient DNA delivery techniques. The first report of recovery of fertile transgenic wheat plants was from regenerable embryogenic callus bombarded with DNA coated gold particles using a microprojectle bombardment apparatus. The herbicide-resistant plants were regenerated under PPT selection (Vasil *et al.*, 1992). Since then there have been many reports of some wheat varieties, mainly spring varieties, transformed with a number of different genes driven by different promoters. The transformation frequencies were rather lower in early transformation experiments. This was improved by using highly regenerable scutellar cells of the immature embryos as target tissues (Weeks *et al.*, 1993). Altpeter *et al.* (1996) achieved recovery of transgenic plants in 8-9 weeks after bombardment and the production of R3 homozygous seed in less than one year. Production of transgenic wheat plants by bombardment of immature embryos has now become routine in many laboratories. Recently, transgenic wheat plants with improved functional properties such as producing high amount of HMW-GS 1Ax1 protein have been achieved (Altpeter *et al.*, 1996).

Despite many attempts towards creating pathogen-resistance crops, fungal and virus resistance has so far not been successfully engineered in wheat (Vasil and Vasil, 1999). To date, there has been only one report of recovery of transgenic wheat plants transformed with a gene encoding the coat protein (CP) of *Barley yellow mosaic virus* (BaYMV, Karunaratne *et al.*,

1996). Although BaYMV does not infect wheat, expression of BaYMV CP gene in transgenic plants might cross-protect against some serologically-related viruses such *as Wheat spindle streak mosaic virus* (WSSMV).

With the recently developed tools of genetic engineering of plants, it is possible to engineer wheat for SBWMV resistance using a virus-derived resistance strategy. In this study, a spring wheat cultivar and several Chinese wheat varieties were transformed by microprojectle bombardment of immature embryos using the designed plasmids. Possible strategies tested include the expression or production of SBWMV components in transgenic plants to confer SBWMV resistance in transgenic plants via CP-mediated or RNA-mediated protection mechanisms.

This chapter describes the optimisation of gene delivery parameters by transient expression of the GUS gene on bombarded immature embryos of wheat and the stable transformation of wheat with constructs containing SBWMV CP and MP genes and molecular characterization of the transgenic plants.

4.2 Materials and methods

4.2.1 Plant materials

Wheat cv. Bob White and three Chinese wheat cultivars were grown in the glasshouse under the conditions as described in section 2.10.1.

4.2.2 Transient expression of GUS on bombarded immature embryos

Immature embryos (1.5-2 mm) were isolated from sterilised spikes (section 2.10.2), and then placed on SMS medium (section 2.10.3) and cultured for one week. After further culture on SMS20 medium for 4 hours, the embryos were subjected to bombardment with pUbiGUS under various conditions. The DNA-coated microcarriers (gold particles 1.0 μm in diameter) were prepared as described in section 2.10.5.2. Following the bombardment, explants were incubated at 25 °C in the dark overnight, and then transferred onto SMS medium for further culture under the same conditions. Transient expression of the β-glucuronidase (*gus*) gene was visualised two days after bombardment (section 2.10.7). GUS-expressing cells and cell clusters were detected as blue-coloured foci under an inverted microscope.

Three parameters including the acceleration pressure, distance between the stop screen and the target explants, and the amount of gold particles per shot were evaluated with other parameters fixed as recommended by the manufacturer's instructions. For each treatment, 25-30 pre-cultured embryos were bombarded, with 4 replicates.

4.2.3 Stable transformation of wheat under PPT selection

Stable transformation of model variety Bob White and three Chinese wheat cultivars was carried out by particle bombardment of pre-cultured immature embryos with gold particles coated with plasmids containing the sense CP and MP genes under the optimal gene delivery parameters obtained from

transient expression experiments. Recovery of transgenic plants was carried out following the procedures as described in Section 2.10.9.

4.2.4 Molecular characterization of transgenic plants and virus resistance testing

Putative transgenic plants were analysed for the presence of the transgene and its expression at the RNA or protein level. For virus resistance tests, seeds harvested from transgenic plants were sent to Prof. Mike Adams' laboratory of the Plant Pathology Department, IACR-Rothamsted, Harpenden, Herts, England. Transgenic plants were mechanically inoculated with *Chinese wheat mosaic virus* (CWMV) inoculum and *ELISA* was performed using a monoclonal antibody against CWMV CP to detect possible virus infection in the inoculated plants.

Virus was extracted by first grinding wild type CWMV infected wheat leaves in 50 mM K_2HPO_4 buffer (pH 7.0), and then mixed with 2x GKP buffer (100 mM glycine, 100 mM K_2HPO_4, pH 9.2, 2% Bentonite, and 2% Celite) at the ratio of 1:1. At the 2-leaf stage, wheat plants were inoculated by rubbing leaves with the virus extract. After inoculation, plants were kept in the dark for 5 days at 15 °C, and then grown in a glasshouse at 15 °C with supplemented light for 15 hr each day. Four weeks after inoculation, the plants were tested by *ELISA* using an antiserum against CWMV.

4.3 Results

4.3.1 Optimisation of gene delivery conditions

To optimise gene delivery conditions, transient expression was carried out by bombardment of immature embryos using pUbiGUS under different parameters. Plasmid pUbiGUS (6.8 kb) which bears a chimeric *gus* (*uidA*) gene controlled by maize ubiquitin-1 promoter and its intron1 and 35S

terminator at the 3' end was used for transient expression experiments (Fig. 16). This plasmid was constructed and supplied by X. Ye, Institute of Plant Sciences, Swiss Federal Institute of Technology, Zurich, Switzerland.

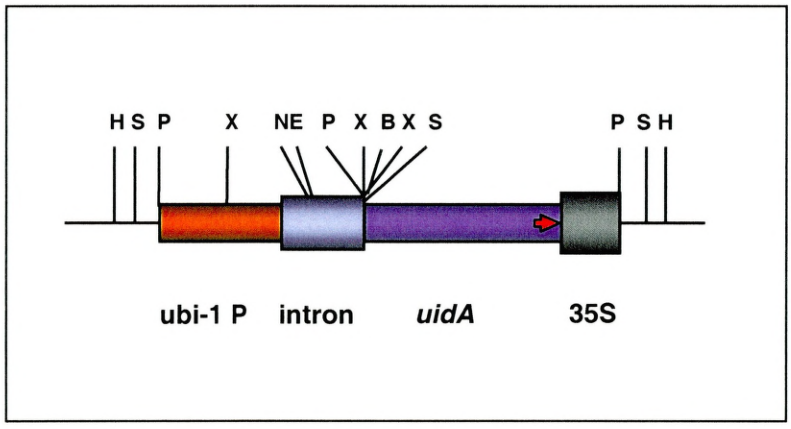


Figure 16. Plasmid used for transient expression experiments. ubi-1 p: ubiquitin-1 promoter, *uidA*: β-glucuronidase coding sequence, 35S: 35S terminator, B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII, N: *Nco*I, P: *Pst*I, S: *Sph*I, X: *Xba*I.

Optimal parameters were determined according to the number of cells and cell clusters expressing GUS activity in bombarded immature embryos. The following parameters were tested:

Working distance:

The influence of gold particle delivery distance, ranging from 7 to 13 cm, was tested. Higher transient expression of GUS (120 blue spots/embryo) was observed in immature embryos bombarded at a delivery distance of 10 cm, on the other hand, shorter (7 cm) or longer (13 cm) working distance yielded lower levels of transient expression (Fig. 17a). Representative GUS transient expression from bombarded immature embryo is shown in Fig.18.

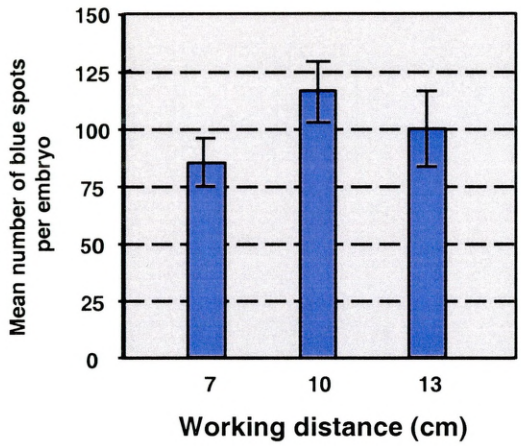
Acceleration pressure:

The working pressure was evaluated by bombardment of immature embryos with 650, 950, 1100 and 1300 psi ruptures (Fig. 17b). Transient expression levels increased from 90 to 120 blue spots per embryo when the pressure increased from 650 to 1100 psi. However, a decrease of GUS expression was found when the pressure exceeded 1100 psi. The maximum expression of GUS activity (120 blue spots/embryo) was achieved with a working pressure of 1100 psi.

The amount of gold particles per shot:

The amount of gold particles per shot can significantly influence transient expression. In this experiment, variable amounts of gold particles (250, 500, 750, 1000 and 1250 μg per shot) were used to bombard target tissues. Although the transient expression efficiency did not significantly vary among the amount of gold particles from 500 to 1000 μg per shot (Fig. 17c), increasing the amount of gold particles can cause remarkable tissue damage. An optimum of 500 μg gold particle per shot was found from this experiment.

Considering the number of transient expression cells and the extent of tissue damage, the optimal conditions for DNA delivery were 500 μg DNA-coated gold particles per shot in combination with 10 cm working distance and 1100 psi helium pressure.



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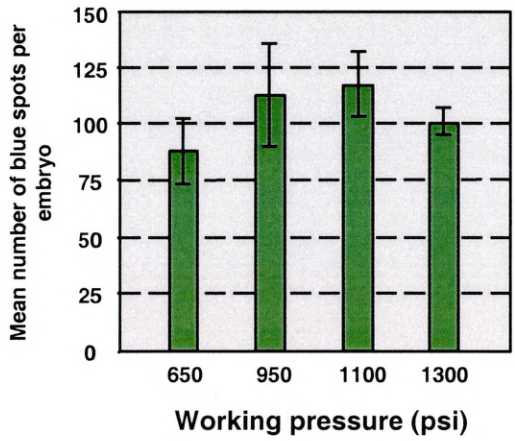

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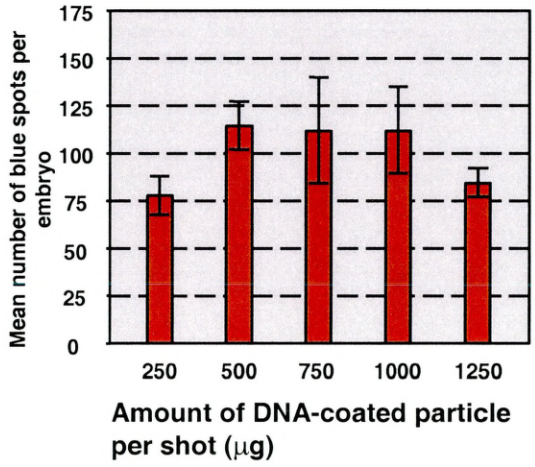


C

Figure 17. The influence of different bombardment parameters on transient expression. Bars = standard error from 4 replications.

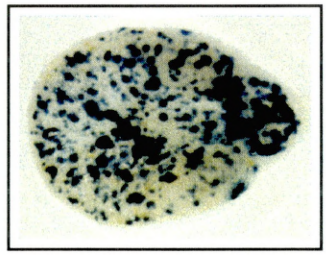


Figure 18. GUS transient expression on bombarded immature embryo of cv. Bob White

4.3.2 Stable transformation and recovery of transgenic wheat plants

Immature embryos from the spring wheat cultivar Bob White and three semi-winter Chinese wheat varieties, Yangmai 93-111, Yangmai 94-141, and Yanmai 2980 were used for stable transformation via microprojectile bombardment techniques. In most cases, microprojectiles were coated with plasmids pAB1 containing the *bar* gene under the control of the rice actin-1 promoter and a second plasmid bearing a SBWMV viral coding sequence (CP gene, MP gene, or replicase coding sequence) driven by a maize ubiquitin-1 promoter and its intron. In some experiments, microprojectles were coated with a complex construct (pubiCPbar) containing the selectable *bar* gene and viral CP gene. The experimental conditions (particle bombardment parameters, pre- and post-bombardment treatments of target immature embryos) obtained from transient GUS expression experiments resulting in the highest number of GUS expressing on the bombarded scutella tissue, were chosen for the stable transformation experiments. After one-week 'recovery culture' on the non-selection medium SMS, a selection scheme was initiated. This proceeded with 4-6 weeks culture on the selection medium containing 5 mg/l PPT. Fifteen independent experiments with different numbers of immature embryos from individual varieties were performed. After two or three weeks culture on the selection medium, a large number of distinct somatic embryos appeared from most of the callus derived from Bob White immature embryos (Fig. 19), whereas, only a few somatic structures were found from the cultured immature embryos of all of three Chinese wheat cultivars.

Upon transfer onto the selection medium containing 5 mg/l PPT for regeneration, in most cases about 70% of the somatic embryos from Bob White were regenerated to plantlets, whereas, the regenerants derived from the cultured calli of Chinese cultivars varied from 10-15% on average (Fig. 20-21). Lower regeneration frequencies were observed from Bob White and Chinese varieties under the selection conditions compared to the control experiments with bombarded explants cultured on the non-selection medium. A large number of somatic embryos were arrested from further development under the selection. This implied that PPT selection strongly interferes with plant regeneration from wheat immature embryo culture.

Following 4-6 weeks culture on the selection regeneration medium, the surviving regenerants, with or without roots, were transferred to a flask with medium containing 3 mg/l PPT for root formation. Only a fraction of regenerants grew vigorously with strong roots under selection, while the majority of regenerants were stunted in growth and became necrotic or chlorotic and eventually died after remaining on the same medium for further culture. The rooted plantlets that survived selection were transferred to soil and grown under glasshouse conditions. Although some of the transgenic plants exhibited a normal phenotype identical to wild type wheat plants, many fewer tillers and leaves were obtained from most of T_0 transgenic plants which made it difficult to get enough leaf material for further analysis. Therefore, the transgenic plants were grown in the glasshouse for about 4 months to allow the setting of normal seeds. Nearly half of the putative transgenic Bob White plants were completely sterile and a few plants showed partial fertility. Most of the Chinese plants produced normal seeds although 3 of the CP-transgenic Yangmai 93-111 plants were sterile. Upon transplanting into soil, several transgenic Bob White and Chinese wheat plants failed to grow normally and could not be maintained.

In total, 6750 immature embryos from Bob White and three Chinese cultivars were bombarded. Two hundred and five regenerants were screened by PCR.

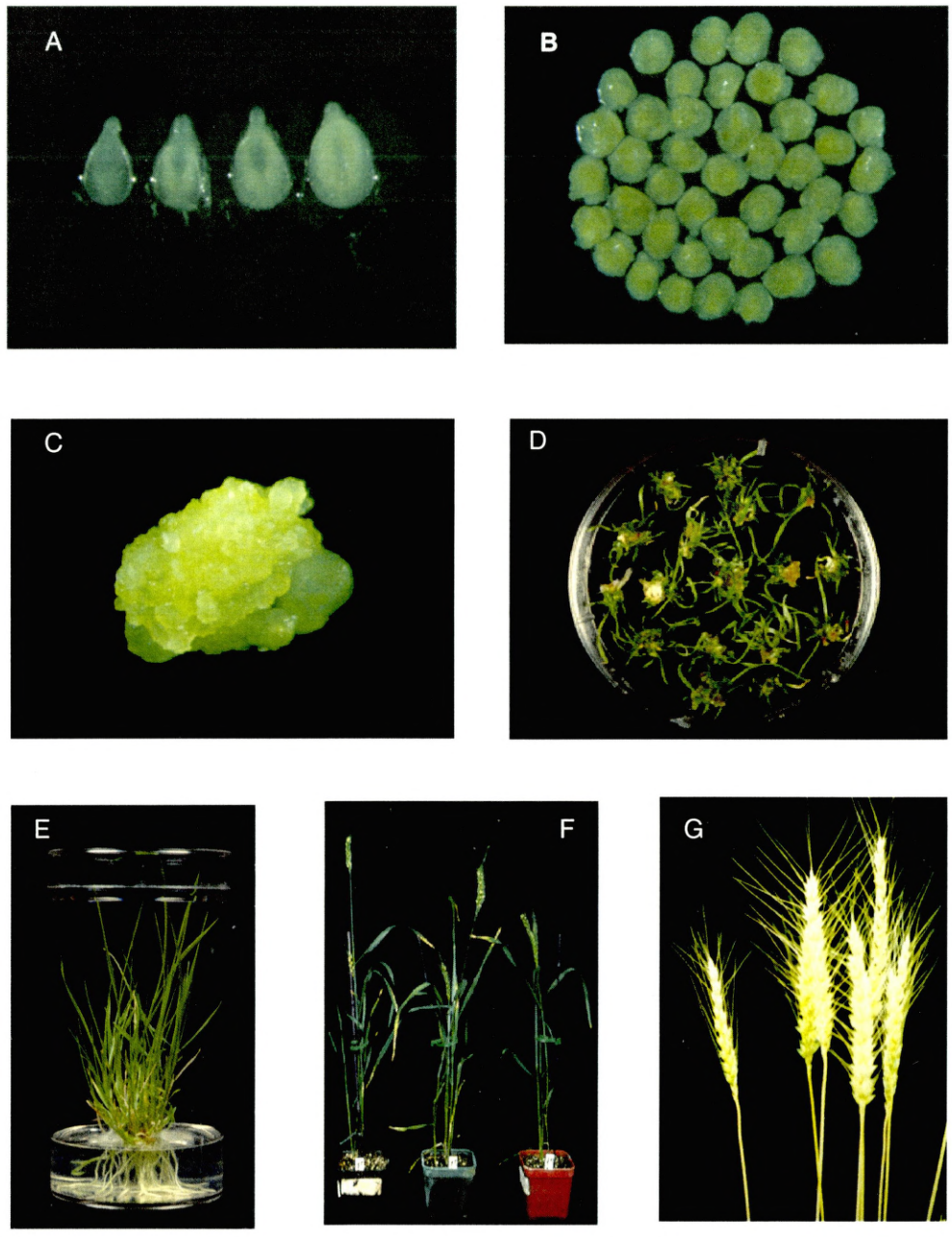


Figure 19. Transformation of wheat (*Triticum aestivum* L. cv. Bob White) by microprojectile bombardment of immature embryos. A) Freshly isolated different size of immature embryos (1-2 mm); B) Somatic embryogenesis from scutellar tissue of immature embryos after 1 week culture on somatic embryogenesis induction medium (SMS); C) Somatic embryogenesis and cell proliferation on the callus 4 weeks after bombardment with pAB1 and pUbiCP coated microprojectiles and cultured on the somatic embryogenesis induction medium in presence of 5 mg/l PPT; D) Plant regeneration under PPT selection (5mg/l) 4 weeks after transfer onto the regeneration selection medium; E) Root development of transgenic regenerant 6 weeks after transfer onto selective rooting medium containing 3 mg/l PPT; F) Independent transgenic T_0 lines growing in the soil 4 months after particle bombardment; G) Mature transgenic plants in the glasshouse.

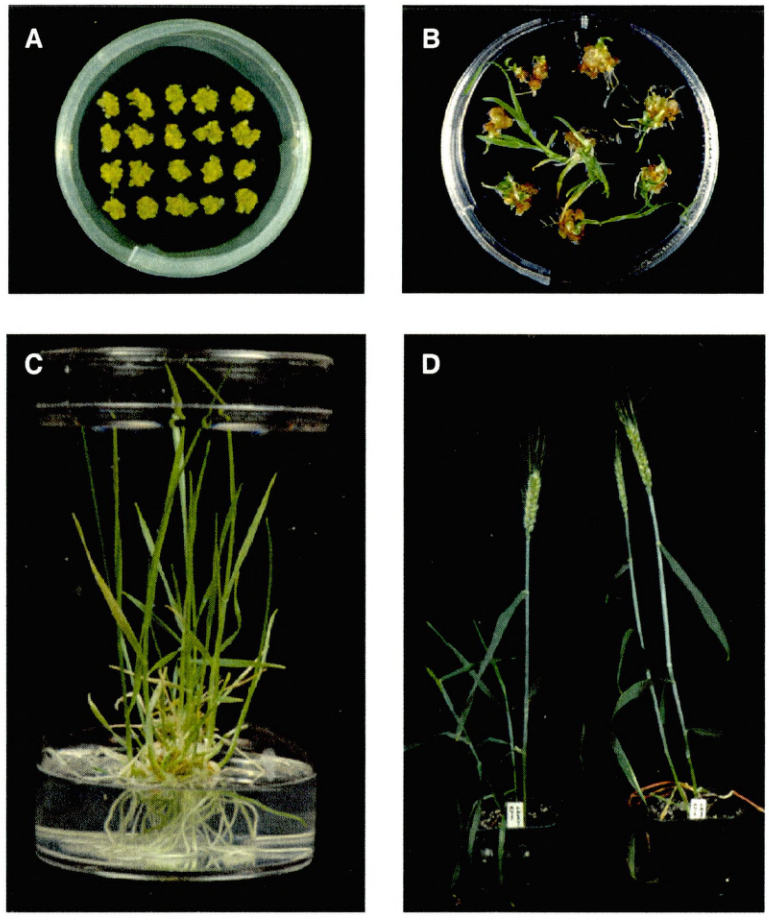


Figure 20. Transformation of Chinese wheat cultivar (*Triticum aestivum* L. cv. Yangmai 93-111) by microprojectile bombardment of cultured immature embryos. A)Embryogenic callus of Yangmai 93-111 cultured on the somatic embryogenesis medium (SMS) containing 5 mg/l PPT 5 weeks after bombardment with pAB1 and pUbiCP coated microprojectiles; B) Plant regeneration from callus derived from microprojectile bombarded immature embryos 6 weeks after transfer onto the selective regeneration medium in presence of 5 mg/l PPT; C) Root development of transgenic regenerant 4 weeks after transfer onto rooting medium (RTM) in presence of 3 mg/l PPT; D) Transgenic Yangmai 93-111 R_0 plant growing in soil 4 month after bombardment of immature embryos.

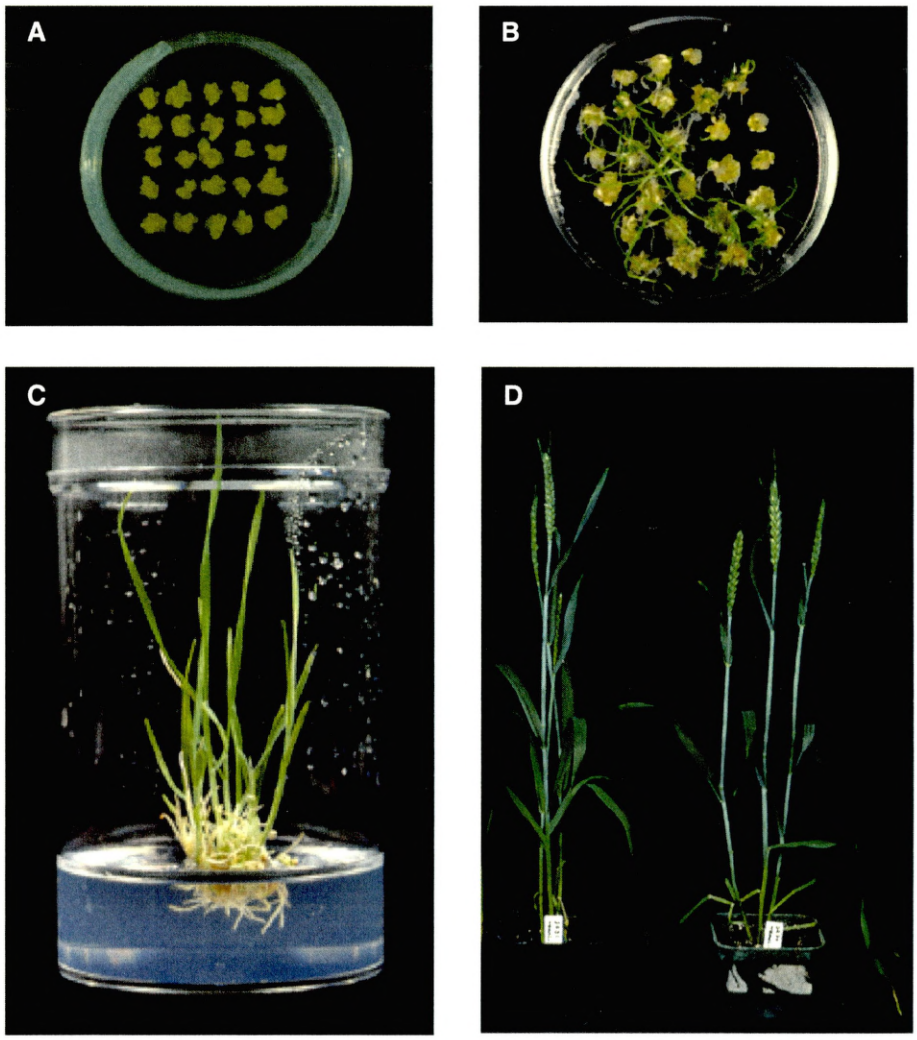


Figure 21. Transgenic Chinese wheat plant (*Triticum aestivum* L. cv. Yanmai 2980) by microprojectile bombardment of cultured immature embryos. A) Embryogenic callus of Yangmai 2980 cultured on the somatic embryogenisis medium (SMS) containing 5 mg/l PPT 4 weeks after bombardment with pAB1 and pUbiMP coated microprojectiles; B) Plant regeneration from callus derived from microprojectile bombarded immature embryos 4 weeks after transfer onto the selective regeneration medium in presence of 5 mg/l PPT; C) Root development of transgenic regenerant 3 weeks after transfer onto the rooting medium (RTM) in presence of 3 mg/l PPT; D) Transgenic Yangmai 2980 T_0 plant growing in soil 4 months after bombardment of cultured immature embryos.

4.3.3 PCR analysis of putative transgenic plants

A PCR screening of 205 putative transgenic plants obtained from transformation experiments was performed with primers designed to amplify a 275 bp internal *bar* gene fragment. Forty-four plants were found to be *bar* gene positive. The analysis of the PCR amplification products demonstrated the presence of a band of the same size as expected in all of the *bar* positive plants (Fig. 22). To detect whether the viral gene was present in the transgenic plants, the *bar*-positive transgenic plants were analysed by PCR using primers designed for amplification of viral CP and MP genes. The 650 bp full SBWMV CP gene sequence was amplified from 8 of 12 *bar*-positive transgenic Bob White plants cotransformed with pUbiCP and pAB1, 4 out of 5 *bar*-positive Yangmai 93-111 transformed with pUbiCP and pAB1, 6 out of 8 *bar*-positive Yangmai 93-111 transformed with pUbiCPbar, 2 out of 3 *bar*-positive Yangmai 94-141 transgenic plants transformed with pUbiCP and pAB1 respectively. Eight Bob White lines were confirmed to contain the *bar* gene, and the 945 bp SBWMV MP gene was amplified from 6 *bar* positive plants. Two *bar*-positive transgenic Yangmai 94-141 and 3 *bar*-positive transgenic Yanmai 2980 were MP PCR positive. Representative results of this analysis are shown in the figures 23-24. The plants which showed a negative result for PCR test are considered to be the escapes.

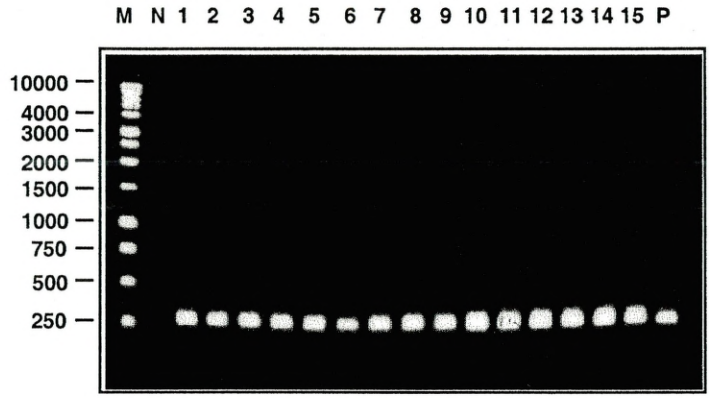


Figure 22. Amplification of *bar* gene from independent transgenic wheat lines. M) 1kb DNA marker (Omega, UK); N) Negative control; 1-8) Eight independent *bar* transgenic Bob White plants transformed with pAB1 and pUbiCP; 9-12) Four independent *bar* transgenic Yangmai 93-111 plants transformed with pAB1 and pUbiCP; 13-14) Two independent *bar* transgenic Yangmai 94-141 plants transformed with pAB1 and pUbiCP; 15) One *bar* transgenic Yanmai 2980 plants transformed with pAB1 and pUbiMP; P) positive control pAB1.

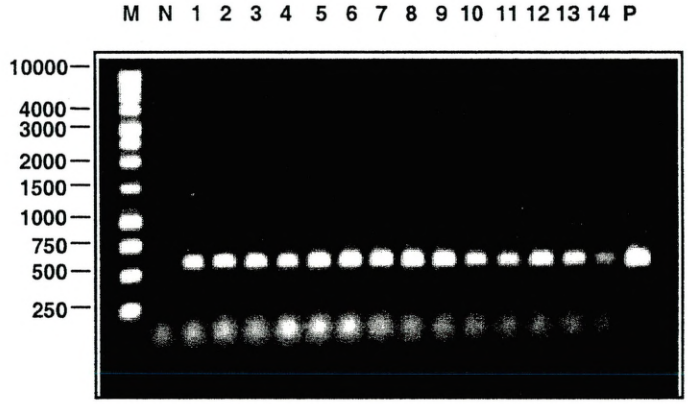


Figure 23. Amplification of SBWMV CP gene from independent transgenic wheat lines transformed with pUbiCPbar or, pAB1 and pUbiCP. M) 1kb DNA marker (Promega, UK); N) Negative control; 1-8) Eight independent transgenic Bob White plants transformed with pUbiCP and pAB1; 9-12) Four independent transgenic Yangmai 93-111 plants transformed with pUbiCPbar; 13-14) Two independent transgenic Yangmai 94-141 plants transformed with pUbiCP and pAB1; P) positive control pUbiCP.

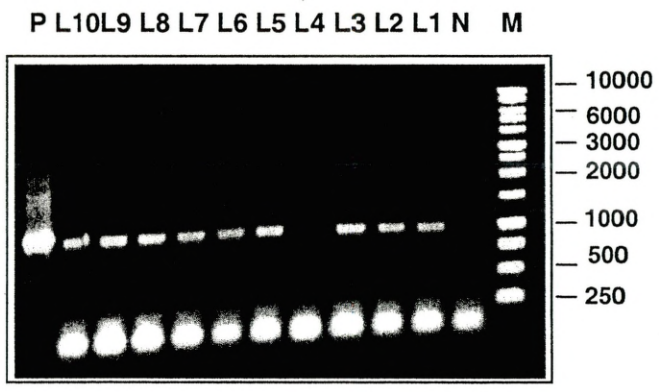


Figure 24. Amplification of SBWMV MP gene from independent transgenic wheat lines. M) 1Kb DNA marker; N) Negative control; L1-3) Three independent MP transgenic Yangmai 2980; L4) Non-transgenic plant (escape); L5-8) Four independent MP transgenic Bob White plants; L9-10) Two independent MP transgenic 93-111 plants; P) Positive control pUbiMP.

4.3.4 Verification of putative transgenic plants by southern analysis

Southern blot hybridization is the most widely used method to analyse transformed plants. It allows confirmation of integration of a given foreign DNA fragment in the genome of a transgenic plant and determination of the number of copies of integrated DNA sequences. This can be achieved by digestion of genomic DNA with appropriate restriction enzymes that either cut out the whole inserted fragment or cleave once within the target sequences.

Because the amount of genomic DNA obtained from most of the putative transgenic lines was very limited, only 11 *bar* PCR-positive lines were selected and analysed for the presence of the *bar* gene by Southern blot. Other *bar* positive lines were only tested for the presence of viral genes by Southern analysis. Genomic DNAs were extracted from *bar*-positive plants. The genomic DNA from a wild type plant was used as a negative control, while the pAB1 used for transformation acted as a positive control. Approximately 20 µg DNA was digested overnight with 10 units of *Bgl*II in a total volume of 30 µl. The digested DNA was separated by agarose gel

electrophoresis, and blotted onto a Nylon membrane, which was hybridised with a radiolabelled *bar* gene probe. The *bar* probe was generated by PCR with a pair of primers designed to amplify a 275 bp internal fragment of the *bar* gene sequence. This probe should give little or no endogenous signal from the plant genome. Following hybridization, blots were washed to high stringency and analysed by autoradiography. A single band (560 bp) corresponding to the *bar* gene was present for 9 of the 11 Bob White transgenic line analysed. A very weak band of the same size was found from line 3 and 9. This was apparently due to the insufficient input of DNA for this analysis. Hybridization to the positive control was detected, while no signal was found from the negative control as expected. The high hybridization background observed from this Southern blot was possibly due to the incomplete restriction digestion of genomic DNA. Representative results from this analysis are shown for 11 putative transgenic Bob White plants in Figure 25.

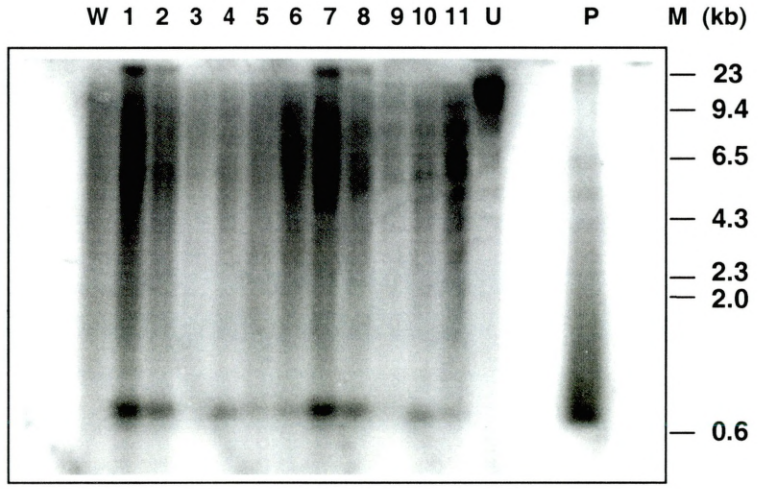


Figure 25. Southern analysis of *bar* putative transgenic Bob White plants. W) Genomic DNA from wild type Bob White as a negative control; 1-7) Seven putative transgenic Bob White plants transformed with pAB1 and pUbiCP; 8-11) Four putative transgenic Bob White plants transformed with pAB1 and pUbiMP; U) Undigested genomic DNA from plant 1; P) Plasmid pAB1 as a positive control; M) λ DNA/*Hin*d III Markers (Promega, UK).

To determine the presence of SBWMV MP coding sequence in the wheat plant genome, Southern analysis was performed using *Bam*HI- and *Eco*RI-digested genomic DNA from putative transgenic plants (Fig. 26). The blot was hybridised with the entire MP gene isolated from plasmid pUbiMP and radiolabelled. Plasmid pUbiMP cut with *Bam*HI and *Eco*RI, respectively, were used as the positive control. *Bam*HI digestion will release the intact MP coding sequence, the intensity of this band may provide information about the occurrence of multiple plasmid insertions, and the rearrangement of inserts resulting in bigger or smaller bands will be determined by the analysis of additional bands. *Eco*RI cut only once in the intron of the transformation vector. After high stringency washing, a hybridising fragment corresponding to the 945 bp viral MP gene was detected from two Chinese transgenic lines (line 1 Yanmai 2980, line 6 Yangmai 93-111) and four Bob White lines (line 2-5). Those were previously confirmed to contain both the *bar* and viral MP genes by PCR analysis. This indicated that the intact coding sequences were inserted into the genome of transgenic plants. The occurrence of several additional bands with different molecular weight was observed from most *Bam*HI-digest samples, this was possibly due to the incomplete restriction digestion of genomic DNA or the presence of several rearranged MP coding sequences. The detection of several hybridisation bands from all *Eco*RI-cut samples suggested the insertion of multiple MP coding regions. The detection of two and three bands in *Eco*RI-digested DNA from line 6 (C21709) and line 5 (B21704) suggested the insertion of 3 and 2 coding regions, respectively. Line 3 (B21702) and line 4 (B21703) exhibited the same banding pattern with four bands at approximately size of 7.0, 5.7, 5.0 and 4.3 kb respectively, which implied that these two lines originated from the same transformation event. The detection of several MP-hybridizing fragments in line 1 (C2980-1) suggested the presence of multiple MP coding regions in this transgenic line. Strong hybridisation signals were obtained from both positive controls (Fig.26)

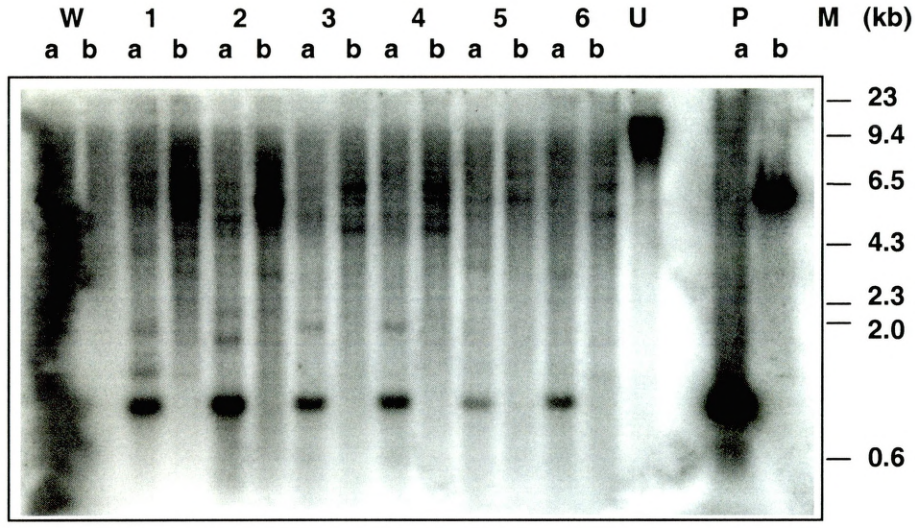
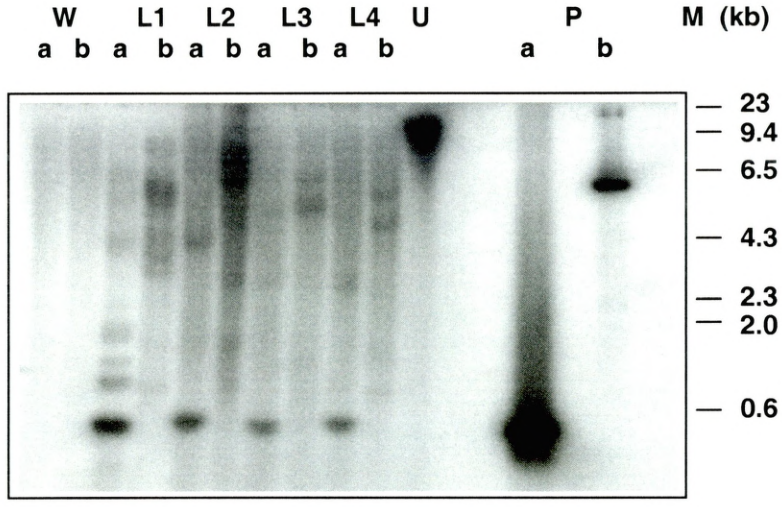


Figure 26. Southern analysis of MP putative transgenic plants transformed with pAB1 and pUbiMP. W) Genomic DNA from wild type wheat plant as a negative control; 1) Transgenic Yanmai 2980; 2-5) Four transgenic Bob White plants; 6) Transgenic Yangmai 94-141; U) Undigested DNA from transgenic Yanmai 2980; P) Positive control pUbiMP; a, b) DNA digested using *Bam*HI and *Eco*RI respectively; M) λ DNA/*Hin*d III Markers (Promega, UK).

A Southern blot of *Bam*HI- and *Eco*RI-digested genomic DNA from CP putative transgenic plants was also generated and probed with a radiolabelled CP gene sequence generated by digestion of plasmid pUbiCP with *Bam*HI. *Bam*HI- and *Eco*RI-digested plasmid pUbiCP was used as the positive control, while the genomic DNA from wild type plants was used as the negative control. *Bam*HI can cut out the entire CP gene from the genomic DNA which can be used to confirm the integration of intact insert, while *Eco*RI cut only once in the intron of the vector and was able to show the copy number of the insert in the different lines. Southern hybridization revealing the expected 564 bp hybridizing band corresponding to the CP coding sequence was observed from all 12 *Bam*HI-digest DNA samples in two Southern blots (Fig 27 A, B). In the first Southern blot, the additional weak bands in *Bam*HI digest of genomic DNA from all lines indicated the incomplete DNA digestion. Two or three hybridization bands were observed from *Eco*RI-cut samples which indicated multiple copies of insertion in the genome of transgenic plants. In the second Southern blot, the variation of hybridization signal from different *Eco*RI-cut samples suggested that different copy number of CP coding sequence may be inserted into the genome of different transgenic plants. Detection of a weak band of 5.0 kb and 3.5 kb in *Bam*HI digested samples in T3 and T4 was possibly due to incomplete restriction digestion (Fig. 27B). The detection of one band from *Eco*RI-digested samples of three Yangmai 93-111 transgenic lines (Fig. 27B, T 3-5) transformed with a complex construct pUbiCPbar in the second blot revealed that only one copy of the CP gene was inserted into the genome of transgenic plants, while two or three hybridization bands found in T7 and T8 suggested the presence of two or three copies of CP gene insert in these lines. Table 3 shows the details of the transformation experiments.



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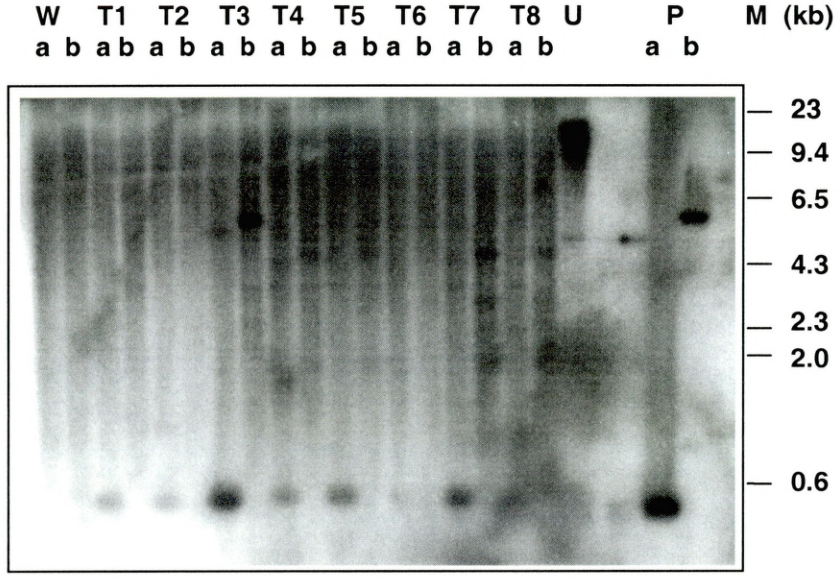


B

Figure 27. Southern analysis of CP putative transgenic plants. W) Genomic DNA from wild type wheat plant as a negative control; L1-2) Two independent transgenic Bob White transformed with pAB1 and pUbiCP; L3-4) Two independent transgenic Yangmei 94-141 transformed with pAB1 and pUbiCP; T1-2) Two independent transgenic Bob White transformed with pAB1 and pUbiCP; T3-6); Four transgenic Yangmei 93-111 transformed with pUbiCPbar; T7-8); Two transgenic Yangmai 93-111 transformed with pAB1 and pUbiCP; U) Undigested DNA from transgenic line T3; P) Positive control pUbiCP; a, b) DNA digested using *Bam*HI and *Eco*RI respectively; M) λ DNA/*Hin*d III Markers (Promega, UK).

Table 3. Analysis of transgenic wheat plants generated from 15 independent experiments

Cultivars	Plasmids	Bombarded embryos	Number of regenerants tested	PCR +ve			Southern +ve		
				Bar	CP	MP	*bar*	CP	MP
Bob White	pUbiCP pAB1	1500	38	12	8		6/7	4/4	
Bob White	pUbiMP pAB1	1050	47	8		6	3/4		4/4
93-111	pUbiCP pAB1	900	25	5	4		nt	2/2	
93-111	pUbiCPbar	1200	35	8	6		nt	4/4	
93-111	pUbiMP pAB1	750	25	4		2	nt		1/1
94-141	pUbiCP pAB1	600	25	3	2		nt	2/2	
2980	pUbiMP pAB1	750	10	4		3	nt		1/1
Total		6750	205	44	20	11	9	12	6

PCR refers to the number of transgenic plants being *bar* or *bar* and CP/MP positive in the respective analysis with *bar* or viral coding sequence specific primers, while CP and MP transgenic plants refer to the number of transgenic plants being Southern positive with specific probes. nt: not tested.

4.3.5 RT-PCR analysis of CP and MP in transgenic plants

Transgenic plants confirmed by Southern analysis were analysed for the expression of CP or MP gene by RT-PCR analysis using primers designed to amplify full length CP and MP coding sequences. Total RNAs were isolated from leaf tissue sampled from PCR positive progenies of all Southern positive transgenic lines. RT-PCR was carried out under standard conditions (section 2.7.2). The expected amplification product corresponding to a 564 bp full length CP coding sequence was detected from 8 out of 12 CP transgenic plants. Amongst these, 3 lines were from Chinese variety Yangmai 93-111 transformed with pUbiCPbar, 2 from Bob White transformed with pUbiCP and pAB1, 2 Yangmai 93-111 and 1 Yangmai 94-141 transformed with pUbiCP and pAB1. Representative results for RT-PCR analysis of CP transgenic plants is shown in figure 28. The expected amplification product corresponding to the entire viral MP coding sequence (945 bp) was undetectable from all six MP transgenic plants (data not shown).

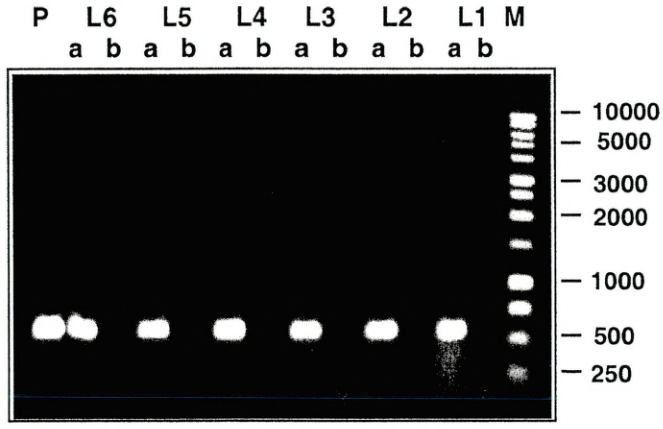


Figure 28. RT-PCR analysis of CP transgenic plants. L1-3) Three independent Yangmei 93-111 transformed with pUbiCPbar; L4-5) Two transgenic Yangmai 93-111 transformed with pUbiCP and pAB1; L6) One transgenic Yangmai 94-141 transformed with pUbiCP and pAB1; M) I kb DNA marker (Promega, UK); P) Positive control pUbiCP; a) With reverse transcriptase; b) Without transcriptase.

4.3.6 Analysis of transgene transmission in transgenic progenies

To study the transmission of the selectable *bar* gene and the viral CP and MP gene in transgenic progenies, several transgenic lines were selected. Transgenic plants were screened for the presence of either bar gene and/or viral genes by PCR. Table 4 shows the results of this PCR test.

Table 4. Results of PCR test for transgenes in R1 progenies of transgenic plants and statistical test

Transgenic lines	Plasmids	*bar+*	X^2(df) P	*cp+*	X^2(df) P	*mp+*	X^2(df) P
BW4330	pUbiCP pAB1	7/10	0.133(1) 0.715	7/10	0.133(1) 0.715		
C93-111-1	pUbiCP pAB1	nt		5/6	0.222(1) 0.637		
C2901	pUbiCPbar	nt		8/13	1.265(1) 0.262		
C2902	pUbiCPbar	nt		1/7	13.762(1) <0.001		
C2980-1	PUbiMP PAB1	10/14				12/14	0.857(1) 0.355
B21701	PUbiMP PAB1	nt				7/10	0.133(1) 0.715
B21703	PUbiMP PAB1	nt				6/9	0.333(1) 0.564

nt: not tested

PCR revealed that transgenes, either selectable marker gene or viral gene, were transferred into the next generation. Statistical analysis showed that 6 of the 7 lines tested did not differ significantly in the inheritance of either bar or viral genes in R1 progenies from Mendelian segregation for a single insertion site.

In line C2902, the segregation ratio of CP was significantly different from the expected Mendelian model. This was possibly due to the population analysed being too small.

4.3.7 Western blot and *ELISA* analysis of CP transgenic plants

Eight CP-transgenic plants were first analysed for the presence of SBWMV CP by Western blot analysis. Using a rabbit polyclonal antiserum which reacted against all SBWMV isolates from different sources, no SBWMV CP was detected in any of the 8 transgenic lines, while the antiserum reacted to the protein with molecular weight of 19 kDa from positive control samples derived from frozen leaves of SBWMV infected plants. Strong cross reactions were found in this Western blot because several bands with high molecular weight were observed in all samples, including the sample from the wild type plant as the negative control (data not shown). An effort was made to concentrate CP by using a commercial concentration gradient gel (Bio-Rad, UK), however this also failed to detect the CP in all transgenic plants. This result indicated that CP-transgenic plants might contain an undetectable level of CP or no CP.

Alternatively, *ELISA* was performed to detect the expression of CP in transgenic plants using three monoclonal antibodies (MAbs) SCR 132, SCR 133 and SCR 134. These three MAbs react with different SBWMV isolates and SCR 132 and SCR 133 might recognise the epitopes in N-terminus and C-terminus, respectively (Chen *et al.*, 1997). The result of this analysis showed that only one (C2906) out of eight lines tested was positive, which exhibited an absorbance value 13 times greater than the negative control

when using SCR 132 for the reaction. Relatively weak reactions with SCR 133 and SCR 134 were obtained from the same transgenic line which resulted in nearly 4 times and 1.7 times absorbance value of the control. The absorbance obtained in the plate-trapped antigen (PTA)-ELISA of different transgenic plants is shown in the Table 5.

Table 5. Enzyme-linked immunosorbent assay (*ELISA*) results of eight CP-transgenic lines

Line	SCR132		SCR133		SCR134	
	A	B	A	B	A	B
PC	2.540	2.548	2.911	2.521	0.591	0.680
NC	0.095	0.099	0.094	0.105	0.148	0.157
1	0.106	0.101	0.114	0.116	0.156	0.151
2	0.087	0.100	0.103	0.110	0.155	0.158
3	0.080	0.083	0.091	0.093	0.136	0.146
4	0.087	0.090	0.094	0.095	0.144	0.172
5	0.158	0.164	0.126	0.128	0.141	0.148
6	1.213	1.227	0.400	0.416	0.248	0.262
7	0.082	0.088	0.098	0.097	0.124	0.140
8	0.089	0.088	0.104	0.109	0.140	0.152

L1) B3405; L2) B4330; L3) C93-1; L4) C2901; L5) C2902; L6) C2906; L7) C62902; L8) C62909; NC) Wild type wheat plant as negative control; PC) Virus infected wheat plant as positive control; A.B) Two replicates; SCR132-134) Three monoclonal antibodies.

4.3.8 Virus resistance test

9 transgenic lines, which produced enough seeds at the time of this test, were selected for virus challenge experiments. This work was done by Mr. Diao in the Plant Pathology Department, IACR-Rothamsted, England. For each line, eight to ten plants were mechanically inoculated with inoculum made from CWMV infected wheat leaves. *ELISA* was performed to detect possible virus infection of inoculated plants. The results showed that most of the plants were susceptible to virus infection (Table 6). The plants that exhibited resistance to infection were tagged to allow setting seeds for further analysis. Seeds from susceptible plants, together with those from resistant plants will be tested under field conditions.

Table 6. *ELISA* test of transgenic wheat plants for resistance to *Chinese Wheat Mosaic Virus* (CWMV)

Lines/ control	Introduced genes	Cultivars	No. of inoculated plants	No. of plants of *ELISA* positive
B3405	CP + *bar*	Bob White	10	9
B4330	CP + *bar*	Bob White	10	10
C93-1	CP + *bar*	Yangmai 93-111	8	6
C2906	CP + *bar*	Yangmai 93-111	10	8
C2980-1	MP + *bar*	Yanmai 2980	9	8
B21701	MP + *bar*	Bob White	9	9
B21703	MP + *bar*	Bob White	10	9
B21704	MP + *bar*	Bob White	9	9
C93-21709	MP + *bar*	Yangmai 93-111	8	8
Control 1	-	Bob White	14	14
Control 2	-	Yangmai 93-111	6	5
Control 3	-	Yanmai 158	10	5

4.4 Discussion

4.4.1 Transient expression

In this chapter transient expression of *gus* in the bombarded immature embryos was first described in the initial transformation experiments. Three parameters were evaluated for their influences on gene delivery and the optimal parameters were obtained based on the highest transient expression rate.

The optimum conditions for the biolistic-based transformation of plants depend on the type of tissue to be bombarded (Sanford, 1993). In wheat, the immature embryo has been the most common explant used for the study of gene transfer (Weeks *et al.*, 1993; Nehra *et al.*, 1994). Previous studies have revealed that Bob White, a spring wheat cultivar, was the most favourable variety for biolistic transformation, therefore, this model variety was chosen for the transient expression experiment as well as for subsequent stable transformation with viral genes.

It has been shown that treatment of target tissue prior to bombardment can significantly increase transformation rate. Osmotic treatment of embryogenic cells pre- and post-bombardment resulted in a two-three fold increase in transient GUS expression events (Vain *et al.*, 1993b. Highest transient expression of GUS was also obtained with 4-6 hours pre-bombardment treatment, followed by 72 hours post-bombardment incubation on a 1.0M osmoticum medium (0.5M mannitol + 0.5 M sorbitol). In the experiments reported here, the precultured immature embryos were treated on plasmolysing medium containing 200g/l sucrose for 4 hours prior to bombardment, followed by 16-18 hours post bombardment incubation on the same medium in the dark.

Heiser (1995) has pointed that out several physical parameters including the particle size, acceleration pressure and particle flying distance had major effects on the efficiency of microprojectile bombardment-based

transformation. The experiments performed here showed that, with other parameters fixed, the variation of working pressure and working distance can significantly influence the transient expression rate. Higher transient expression of GUS (120 blue spots/embryo) was obtained when the embryos were placed at a position 10 cm from the macrocarrier, this was consistent with Sanford's observations. He found 1000 psi acceleration pressure to be optimal or nearly optimal for most applications of the helium-driven system (Sanford *et al.*, 1993). A decrease in the rate of transient GUS expression when increasing the working distance was observed in these experiments. Higher rupture pressure allowed the particles with greater velocities to reach the underlying cell layers, which might increase the transient expression events. It has been found that increasing rupture burst pressure from 1200 psi to 1800 psi would significantly affect the ability of particles to penetrate into maize embryo tissue. But too high a working pressure will greatly damage the surface layers of the target tissue, consequently resulting in lower transient and stable transformation rates. The amount of DNA-coated particles per shot also has a major influence on rates of transient expression (Sanford *et al.*, 1993). The highest transient expression rate was observed when using 500 μg DNA-coated particle particles to shoot target embryos. There was no significant difference in terms of the average number of GUS expression cells or cluster when using the amounts of gold particles between 500 and 1000 μg. However, transient expression events were dramatically decreased when bombarding embryos with 1250 μg gold particles, This was possibly due to the greater damage caused by high intensity of particles. From these experiments, tthe optimal gene delivery parameters were determined as: a) working pressure, 1100 psi; b) working distance, 10 cm; c) 500 μg of DNA coated gold particles.

Although transient expression experiments had been routinely conducted aiming at achieving stable transformation at high efficiency, and

many different protocols had been published, there is no unique protocol applicable to all of species to be transformed. In addition even using the same variety, optimal conditions for transient gene expression and stable transformation obtained in a lab under certain conditions might differ from the result of others. Altpeter *et al.* (1996) reported that 30 μg of gold particles per bombardment was optimal for high rate of regeneration and stable transformation although 53% more transient expression events were obtained with 100 μg particles. However up to 583 μg of gold particles per shot have been previously used to generate transgenic wheat (Weeks *et al.*, 1993).

Although there is no direct correlation between the higher transient activity and efficient stable transformation, transient gene expression has proven to be an important guide to the development of an efficient system for the stable transformation of a given species.

4.4.2 Stable transformation of wheat

4.4.2.1 Transformation efficiency

Most protocols published for wheat transformation were based on the study of transformation of the cultivar Bob White which is favourable for tissue culture. In these experiments, transformation of this model variety was used as an aid to evaluate the transformation efficiency in a new environment. The protocol adopted for wheat transformation was first developed by Iglesias of Swiss Federal Institute of Technology, Zurich and then modified by Zhang and Zhao in the same laboratory (Zhang *et al.*, 1996; Zhao *et al.*, 1996). This modified protocol had been successfully used by the same group for introducing several agronomically important traits, including fungal resistance proteins such as chitinase and RIP into Bob White as well as several Swiss wheat cultivars (Zhang *et al.*, 1996; Zhao *et al.*, 1997). Using this protocol to transform Bob White, the transformation frequency increased from 0.1 to 1% in term of numbers of immature embryos bombarded (Zhang *et al.*, 1996). In

Scotland when using this protocol to transform wheat, lower transformation frequencies were obtained in experiments from both Bob White and Chinese wheat cultivars. For Bob White, the average transformation frequency was about 0.8%, while it varied from 0.5 to 0.66% for Chinese varieties. The relative lower transformation frequency was possible due to the suboptimal conditions for growing wheat in Scotland. Although the wheat plants were grown in a special glasshouse, correct temperature and humidity regimes in the glasshouse could not be maintained consistently because of the changeable weather. Furthermore, application of fungicide and insecticide to control powdery mildew infection and insects during the summer season made it very difficult to supply a high quality of immature embryos all year round. Consequently, transformation frequency varied from one experiment to the other. The quality of the explant material had a significant influence on the transformation frequency. It was found that the ability of transformed cells surviving selection depend on the quality of the callus, which is directly related to the condition of the donor plants (Ozias-Akins and Vasil, 1982).

In general, two selection strategies were commonly used for the selection of transgenic wheat plants, i.e. lower and higher selection pressures. Lower selection pressure normally results in a large number of escape plants. Analysis of a large population of putative transgenic plants is time consuming and costly. However this strategy might be applicable to those species unfavourable in tissue culture resulting in improved transformation efficiency. Higher doses of the selective agent might help to eliminate untransformed plants. Therefore this strategy can be used to select transformed cells from some species with high regenerative capacity. But, high concentration of selective agent in the culture medium might have a negative effect on the quality of transformants (Schuurink and Louwerse, 2000). In the experiments reported here, 5 mg/l of PPT was used for callus induction and plant regeneration, and 3 mg/l of PPT for the rooting stage. The overall transformation frequency was comparable to that of previous reports by other groups. Becker *et al.* (1994) selected transgenic plants with 5 mg/l PPT, with a transformation frequency of 1.1%, while a transformation frequency of 0.5-2.5% was achieved by Nehra *et al.* (1994).

4.4.2.2 Integration and expression of transgene in transgenic plants

PCR and Southern analysis of transgenic wheat plants regenerated from biolistic transformation experiments, followed by selection in the presence of PPT, provided the evidence for the integration of both selectable marker gene and viral coding sequences into the genome of a model variety and three Chinese wheat cultivars. A contransformation frequency of 66-75% was observed by PCR analysis of transgenic plants. Similar co-transformation frequencies have been reported for biolistic transformation of plants using transgenes located on two individual or the same plasmids for wheat (Zhang *et al.*, 1996), rice (Cooley *et al.*, 1995; Nayak *et al.*, 1997) and barley (Wan and Lemaux, 1994). The use of two separate plasmids containing the selectable marker gene or gene of interest to transform wheat may allow the generation of transgenic wheat plants which carry the marker and other genes at unlinked loci, thus enabling the production of marker-free transgenic plants through genetic segregation. However, little is known about how the transgenes integrate into the genome of transgenic plants.

Despite poor resolution of restriction fragments in some Southern blots, the analysis of 12 CP and 6 MP transgenic plants has revealed that the entire CP and MP coding sequences were integrated into the genome of transgenic plants. Evidence of multiple copies of MP gene was observed from all MP-transgenic lines with one line having up to 7 copies of insert. In CP-transgenic plants the copy number varied from 1 to 3 in different lines. A similar result was reported by Srivastava *et al.* (1996). They found that relatively lower copy number of inserts (1-5) was integrated into the six transgenic plants derived from three different cultivars. Barro *et al.* (1998) reported low-copy numbers of insert and simple integration patterns to be prevalent in their transgenic plants. Incomplete digestion of genomic DNA led to difficulty during the analysis of DNA by Southern blotting. The poor quality of genomic DNA mainly resulted in difficulty in restriction digestion. Removal of contaminants from genomic DNA was performed by phenol/chloroform extraction; however no improvement was achieved in subsequent Southern

blotting. Alternative procedures for isolation of purified genomic DNA are required in further studies.

In this study, the integration patterns of MP coding sequence is more complex than those of CP coding region in transgenic plants. It was unclear whether this was due to the difference of stability between these two genes. The functional CP gene, which was expected to be expressed at RNA and protein level, was transformed into wheat plants. However, due to mutation there would be no protein expressed for MP gene. Register *et al.* (1994) found the integration patterns of *uid*A gene being more complex than those of selectable marker gene such as the *bar* gene after analysing the transgenic plants transformed with a complex construct pBARGUS. However little difference was observed by Barro *et al.* (1998) in the complexity of integration patterns between selectable and non-selectable marker genes. In their experiments, the *uid*A gene and the *bar* gene introduced into wheat plants were located on two separate plasmids. They concluded that such contrasting results might attribute to the inherent difference between the constructs used for their experiments.

Fourteen of 31 transformed plants did not set seeds. Compared to previously published results, the frequency of sterile plants was quite high. Among the 14 sterile transgenic plants 5 plants flowered, but did not set any seeds when cultured in the rooting medium. After transferring into soil, another 9 plants either did not flower or did not set any seeds. The phenotype of these 9 plants were abnormal compared to the wild type wheat plants. These plants exhibited a very stunted morphology with very small leaves. It was unclear whether this was due to the integration and expression of viral CP and MP genes. It was difficult to compare this result to previously published results since no other transformation study had been conducted with this virus. But there is evidence that the expression of certain transgenes in transgenic plants leads to abnormal morphology. Transformation of potato with CP gene of *Potato virus Y* resulted in one plant exhibiting a very stunted morphology with small and very hairy leafs, and no tubers being formed from this and several other lines (Van der Vlugt, 1993). Ye (1997) found that the

phenotype of the three *Bacillus subtilis SacB*-transgenic ryegrass plants accumulating HMV levan-type fructans, due to the transgenic expression of chimeric *SacB* genes, differed from the control plants. These three transgenic plants were stunted and approximately 2/3 the size of the control plants. Another factor, which may potentially contribute to the high degree of morphological disorder in these experiments, was somaclonal variation. This somoclonal variation may be due to the prolonged tissue culture in the presence of synthetic auxins, and to the long period of exposure to the selective agents (Schuurink *et al.*, 2000), since many studies have shown that extensive periods of tissue culture often result in genetic mutation that impact negatively on regenerated plants, with partial or complete sterility of regenerated transgenic plants (Christou, 1996). Reduced fertility in transgenic plants has been reported for transgenic maize (Gordon-Kamm *et al.*, 1990), wheat (Vasil *et al.,* 1992) oat (Somers *et al.*, 1992), and rice (Qu *et al.*, 1996). Another factor, which might be responsible for the reduced fertility, is the state of donor plants which can significantly affect the tissue culture behaviour. Donor plants grown under suboptimal conditions might lead to variation of transgenic plant in agronomic performance. It has been found that certain antibiotics can cause complete sterility in transgenic plants. When using kanamycin or G418 to select *nptII* transgenic rice, almost all the transgenic plants were completely sterile (Qu *et al.*, 1996). This seems not to be the case here because there were no serious fertility problem for transgenic wheat from most of wheat transformation work in which PPT was used to select for *bar* transgenic plants.

PCR screening of four CP and three MP transgenic lines revealed that most lines showed Mendelian segregation of transgene, but one line transformed with the CP gene showed less than the expected proportion. The distorted segregation of transgene in transgenic progenies might be associated with a variety of factors [genetic variation, disruption of endogenous genome, chimerism origination (regenerant containing both transformed and non-transformed cells), and inherent instability, Barro *et al.*, 1998]. But it was difficult to draw any conclusions from this line based on analysis of such small population.

RT-PCR analysis of the CP-transgenic plants identified the presence of full-length transcript for viral CP from 8 out of 12 CP-transgenic plants analysed. Failure to detect CP transcripts from five lines indicated that transgene silencing might occur at the transcriptional level (TGS). The reasons for triggering transgene silencing in those lines are not clear. The mechanisms of transgene silencing in plants have been recently reviewed by Fagard and Vaucheret (2000). They pointed out TGS could occur in *cis* because of the presence of neighbouring endogenous silencing elements or because of interaction of transgene repeats which create a silencing structure. In the experiments here, the ubiquitin-1 promoter was used to drive expression of viral CP and MP genes. This promoter has been previously used to drive gene expression at much higher levels than other promoters such as rice *Act*1, CaMV 35S and maize *Adh* promoters (Cornejo *et al.*, 1993). Furthermore, transgenic wheat was also produced using the ubiquitin-1 promoter to drive a selectable marker gene or an antifungal gene (Vasil *et al.*,1993; Weeks *et al.*, 1993; Clausen et al., 2000). It has been found that methylation was often associated with gene silencing. Although it is unclear whether methylation is the cause or a consequence of gene silencing, much evidence suggests TGS correlates mainly with methylation of the promoter sequence, whereas, posttranscriptional gene silencing is correlated with methylation of coding sequences (Fagard and Vaucheret, 2000). It is not clear whether the ubiquitin-1 promoter in the CP gene silencing lines were methylated which may block the transcription from CP coding sequence. It has been found that the methylation pattern of introduced genes affects reporter gene transient expression levels in wheat tissue and barley cell lines (Graham and Larkin, 1995; Rogers and Rogers, 1995). The methylation of adenine residues at *dam* sites in different promoters resulted in different enhancement of expression levels (Baga, *et al.*, 1999). The gene silencing in the four CP-transgenic lines (L1, L2, L3, and T7, see page 108) might be due to the insertion of multiple transgenes since it has been suggested that degree of methylation and gene silencing appear to be correlated with copy number. This is not always the case, however but would be particularly serious when using direct gene transfer techniques such as particle bombardment to transform plants, which generally lead to the insertion of

multiple copies of transgene at a single locus (Baga et al., 1999). Further molecular analysis of those four lines using Northern blotting technique is needed to detect any possibly aberrant or truncated transcriptional products because only full length of CP transcripts would be detected in the RT-PCR test. The lack of expression of viral MP gene at RNA level in transgenic plants was possibly due to the existence of inframe stop codons in the MP coding sequence which would reduce the stability of the transcribed products.

Eight CP-transgenic plants, which had been confirmed by Southern analysis, were tested for the production of SBWMV CP by PAT-ELISA and Western blot analysis of total proteins derived from leaf samples. Although Western blotting is an extremely sensitive analytical technique which is capable of detecting as low as 50 ng antigen in crude protein extracts (Scott *et al.*, 1988), no CP was detected from the transgenic lines analysed by Western blot with a polyclonal antibody, suggesting the absence of CP in the protein samples or that the accumulation of this protein in transgenic plants was extremely low. However, when using the more sensitive technique *ELISA* to detect the CP from transgenic plants with the specific MAbs, one line strongly reacted with one of three MAbs, suggesting the production of viral CP in this plant. From this result, it is suggested that failure to detect CP by Western blotting in that CP-expression line was possibly due to low expression of this protein in transgenic plant or due to the protein degradation. It was not clear why no CP could be detectable from other lines. Plants were transformed with the same transgene, but did not express to the same level in individual lines, reflecting the complexity of transgene integration and expression controlled by host plant machinery. The insertion and expression of foreign genes may alter the genome structure or interrupt the normal function of endogenous genes which might stimulate host plant defense systems, which result in the variation of transgene expression. Little is known about how the transgene or its product activate the plant defense system.

RT-PCR analysis of transgenic plants with an integrated CP gene identified the presence of full length transcripts for CP, but no CP were produced in most of those transgenic plants, which might indicate problematic post-transcriptional processing. As post-transcriptional silencing is mainly correlated with coding sequences, therefore failure of translation of CP in those transgenic plants might result from the formation of aberrant forms of mRNA which prevent the initiation of translation. Further sequencing of cDNA from aberrant transcripts may answer this question.

Despite the absence of detectable levels of CP in most CP-transgenic plants, 4 CP-transgenic lines including one line expressing CP and 5 MP-transgenic lines were challenged with *Chinese wheat mosaic virus* (CWMV). *ELISA* testing of all wheat plants which were inoculated with virus revealed the accumulation of CWMV in most of plants analysed. However several plants from different lines seemed not to be infected because no virus could be detected from those plants. It was not clear if these plants were resistant to virus infection or not because the inoculation of the susceptible varieties also resulted in several plants that did not become infected. All the inoculated plants have set seeds which will be used for further analysis. These analyses will include detecting the amount of virus accumulated in different infected lines which may exhibit different degrees of resistance, and testing the transgenic plants under natural inoculation conditions under which transgenic plants may respond differently to the fungus inoculation. Moreover, plants will be tested for possible resistance to *Soil-borne wheat mosaic virus*, inoculum of which was not available when the original resistance tests were made.

CP-mediated resistance has become a general method to achieve virus protection in plant. CP genes from more than 35 viruses, representing 15 viral taxonomic groups have been engineered into many different plant species (Palukaitis and Zaitlin, 1999), The expression of virus CP genes resulted in various degree of resistance in different plant/virus combinations. Extreme resistance to virus infection has been reported for tobacco plants expressing *Alfalfa mosaic virus* CP gene (van Dun *et al.*, 1987; Tumer *et al.*,

1987), while several authors reported the expression of viral CP conferred no protection for transgenic potato and rice (Jongedijk *et al.*,1993; Klöti, 1996). At the moment, the mechanisms underlying the CP-mediated resistance is not fully understood. In addition, the expression of transgene cannot always be predicted. Therefore, successful protection of transgenic wheat plants to virus infection may still rely on the production of large number of independent transgenic lines from which the transgenic plants with desired transgenic traits can be identified.

CHAPTER 5

GENETIC TRANSFORMATION OF WHEAT BY MANNOSE SELECTION

5.1 Introduction

It is well known that selectable marker genes are essential for the recovery of transgenic plants in most plant transformation. Theoretically, the role of the selectable marker gene in gene transfer is to ensure that only the cells containing and expressing the selectable marker gene survive selection pressure applied on the selection medium, thus allowing plant regeneration from transformed cells under the selection. Joersbo and Okkels (1996) have proposed two kinds of selection strategies, currently used for the genetic transformation of plants. These two selections were termed as "negative selection" and "positive selection" respectively.

Early transformation experiments employed genes conferring resistance either to antibiotic or to herbicide to allow the transgenic cells to develop to transgenic plants. In these transformation systems, the transgenic cells acquired the ability to survive and develop to transgenic plants under the selection, whereas, the nontransgenic cells were killed by the selection agent. This phenomenon is termed as "negative selection". It is understandable that the transgenic cells are surrounded by non-transformed cells in the multicellular target tissue and the successful application of this selection approach with which the transgenic cells could be regenerated to plants, depends on the efficient expression of selectable marker gene and the interaction between the transgenic cells and the surrounding non-transformed cells. Unfortunately, little is known about such interactions. It is presumed that transformed cells acquire the possibility to proliferate and to develop to transgenic plants, whereas the selection agent will eradicate the non-

transformed cell. However, a large number of escapes obtained from most transformation work indicates this selection process is far more complicated than we thought.

Since the first recovery of transgenic callus line by the direct transfer of the neomycin phosphotransferase II (*nptII*) gene into protoplasts of *Triticum monococcum* (Lörz *et al.*, 1985), the *nptII* gene has been widely used as the selectable marker gene in the production of transgenic plants (Nehra *et al.*, 1994, Cheng *et al.*, 1997). Recoveries of *nptII*-expressing plants require kanamycin as the selective agent; however, most graminaceous species, including wheat have been found naturally resistant to kanamycin, and therefore this selection system is not applicable for all plant species. The use of geneticin or G418 as the selective agent has resulted in the production of transgenic wheat plants either by biolistic bombardment of immature embryos or the *Agrobacterium*-mediated transformation system. Although the use of many other selectable marker genes including hygromycin phosphotransferase gene (*hpt*), 5-enolpyruvylshikimate phosphate synthase (EPSPS) gene and glyphosate oxidoreductase (GOX) gene has allowed the recovery of transgenic wheat plants under antibiotic or herbicide selection, lower transformation frequencies have been obtained in most cases. Although an efficient selection system employing the *bar* gene, which confers resistance to phosphinothricin, has recently been developed and successful transformation of wheat using this system have been reported (Vasil *et al.*, 1993; Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.*, 1996; Takumi and Shimada, 1996), the presence and expression of a herbicide-resistance gene may be undesirable in the final product. Therefore, to meet this requirement, new selection methods need to be exploited.

A new selection strategy has been recently introduced into plant transformation which allows the transformed cells to metabolise a chemical while the nontransformed cells are starved under the selection, eventually losing viability. This was termed a "positive selection" strategy. Joersbo (1996) found that transformation of tobacco via *Agrobacterium*-mediated gene transfer using the *uidA* gene as the screenable and selectable marker

gene and the use of cytokinin glucuronides as the selective agent resulted in a higher transformation frequency compared to kanamycin selection. In this experiment, cytokinin glucuronides can be hydrolysed by the GUS to become the active cytokinin, and the release of the cytokinin promotes the regeneration of transgenic shoot from transgenic cells. Another newly-developed selection method was the application of mannose as the selection agent, which resulted in the production of transgenic potato and sugar beet (Joersbo *et al.*, 1998). The mannose selection strategy is based on the fact that the accumulation of non-metabolisable mannose-6-phosphate in nontransgenic cells results in the starvation of phosphate and ATP which interferes with cell proliferation. In contrast, the transgenic cells expressing the phosphomannose isomerase (*pmi*) gene can convert this product to fructose-6-phosphate which can be utilised as a carbon source. Joersbo and his colleagues (1999) have studied several parameters that may interact with mannose selection employed for the production of transgenic sugar beet. In this study, four saccharides (sucrose, glucose, fructose, and maltose) have been tested as counteragents to the mannose. It was found that all of these four carbohydrates could strongly interact with the phytotoxic effect of mannose. Glucose can completely counteract the mannose-induced growth inhibition. The use of maltose resulted in the highest transformation efficiency although the highest regeneration frequency was obtained by the use of sucrose. Other parameters including light intensity and the addition of phosphate also affect the mannose selection efficiency. Furthermore the mannose selection seems to be genotype independent. This indicated this selection system might be applicable to other plant species. Although to date the mannose selection system has only been successfully used to generate transgenic plants from a limited number of plant species such as potato, sugar beet and cassava (Zhang *et al.*, 1999), the application of this system to some other plants is still desirable.

This chapter describes the establishment of mannose selection system for wheat transformation and the transformation of wheat with SBWMV CP coding sequence by the use of this transformation approach.

5.2 Materials and methods

5.2.1 Plant material

The spring wheat cv. Bob White and three semi-winter cultivars were grown in the glasshouse under the conditions as described in Chapter 2, section 2.10.1.

5.2.2 Construction of pUbiMan

The 1.2 kilobase pairs (kbp) phosphomannose isomerase (*pmi*) coding sequence was isolated from plasmid pmanG19 and cloned into an expression vector pUbi35S. Plasmid pmanG19 is a Ti plasmid harbouring the *pmi* gene driven by a CaMV 35S promoter and the CaMV 35S terminator at it 3' end. These two plasmids were kindly provided by Dr. Ye, Institute of Plant Sciences, Swiss Federal institute of Technology Zurich, Switzerland.

Plasmid pmanG19 was digested by restriction enzymes *Xho*I, and electrophoresed on a 1% agarose gel. The 1.2 kb band corresponding to the entire *pmi* gene was excised from the gel (section 2.2.3). The fragment was blunt ended by the DNA polymerase I large (Klenow) fragment (section 2.2.6), The cloning vector pUbi35S was digested using *Bam*HI, and then blunt ended by the Klenow fragment (section 2.2.6). The DNA fragments were then ligated into the digested vector pUbi35S. The resulting plasmid was analysed by restriction digestion and DNA sequencing for the correct insert. The insertion of *mpi* gene into the vector pUbi35S resulted into pUbiMan.

5.2.3 Dose response of wheat immature embryo on mannose

To evaluate the effect of mannose on callus induction and somatic embryogenesis, wheat immature embryos were isolated (section 2.10.2) and cul-

tured on the induction medium. The induction medium was the same as used for the production of transgenic plants under PPT selection, but, supplemented with concentrations of 0, 1, 2, 3, 4, 5, 7, 9 g/l mannose (Sigma, UK). Three Petri dishes were used for each treatment, and the embryos were incubated at 25 °C in the dark. Somatic embryogenesis and callus induction were assessed after 14 days.

5.2.4 Stable transformation of wheat under mannose selection

Initially, the model variety Bob White was co-transformed, by particle bombardment of immature embryos, with plasmids pUbiGUS and pUbiMan. Immature embryos were isolated and then cultured on the induction medium (section 2.10.3) for one week to enable the cell proliferation and embryogenesis. After high osmotic treatment for 4 hr (section 2.10.5.1), embryos were subjected to the bombardment under the optimal physical conditions as obtained from the transient expression experiments (Chapter 4). 5 µl of DNA-coated gold particle solution was used for each bombardment containing 1 µg of each plasmid. Recovery culture of the bombarded embryos and the plant regeneration were the same as described for the transformation of wheat under PPT selection, but with the exception of the use of 5-7 g/l of mannose throughout all selection procedures. At each step, a small number of samples was tested for GUS expression by histochemical GUS assay (section 2.10.7). The putative transgenic plants were analysed by PCR.

Stable transformation of Bob White and Chinese wheat cultivars were conducted using the plasmids pUbiCP and pUbiMan following the same procedures as stated above. The putative transgenic plants were analysed by PCR and Southern blot.

5.2.5 Histochemical GUS activity assay

Two days after bombardment, some bombarded calli were stained with GUS solution to examine the efficiency of bombardment, while others were kept on the selection medium to allow plant regeneration. To screen the transgenic plants, leaves were excised from regenerants and stained with GUS solution.

5.2.6 Analysis of putative transgenic plants

PCR was used to amplify both *pmi* and *uidA* genes from GUS positive plants. Crude DNA samples were isolated from leaf of putative transgenic plants using the alkali extraction method (section 2.5.1). For amplification of the 466 bp internal sequence of *pmi* gene, the primers (Forward 5' AAACGCTTTGGCGAACTG 3'; Reverse 5' ACAGACCGCTGTCTTCCG 3') were designed and used for PCR. PCR was started at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 1 minute, 58 °C for 1 minute and 72 °C for 1 minute. For amplification of the 460 bp long internal sequence of *uidA* gene, the primers (Forward 5' ACACCGATACCATCAGCGAT 3'; Reverse 5'TCACCGAAGTTCATGCCAGT 3') were employed. PCR conditions were the same as used for amplifying the *pmi* gene with the exception of 55 °C for 1 minute for annealing. Amplified products were visualized after electrophoresis in 1% agarose gel.

Total cellular DNA was isolated from leaf material from glasshouse-grown putative transgenic plants of the Chinese cultivars. The genomic DNAs were digested using *Bam*HI and *Eco*RI respectively, and then hybridised with (^{32}P) dATP-labeled *pmi* probe (PCR-amplified 466 bp fragment of *pmi* gene) (section 2.7.3). To determine the presence of *cp* gene in the *pmi* positive plants, DNA samples were cut using *Eco*RI, and then subjected to hybridisation with *cp* probe (PCR-amplified 537 bp full length of *cp* gene).

5.3 Results

5.3.1 Construction of pUbiMan

Plasmids were isolated from 18 clones using using the Wizard® Plus SV Minipreps DNA Purification System (Promega, USA). The plasmids were checked by *Xho*I digestion. After electrophoresis on a 1% agarose gel, 13 clones were found to give rise to two expected fragments of 1.2 and 4.8 kb. To determine the orientation of the insert in the recombinant plasmids, these 13 clones were further checked by *Sph*I digestion. Electrophoresis of digests revealed that the *pmi* gene was cloned into the vector in two opposite orientations, which resulted in the sense and antisense constructs. A large number of plasmids was prepared from three clones containing the sense construct and the plasmids were checked again by digestion with *Sph*I and *Hin*dIII respectively (Fig. 29a). The resultant construct was named as pUbiMan (Fig. 29b).

5.3.2 Dose response experiment

To detect the minimum mannose dosage for suppression of callus induction and somatic embryogenesis from immature embryos, the embryos were exposed to different concentrations of mannose. Table 3 shows the results of this experiment.

The results showed that 1-2 g/l mannose led to callus induction at the control level. When cultured on the medium containing 3 g/l mannose, nearly 55% of embryos were able to grow, but the induced calli were apparently smaller in size than those from control samples and those cultured on medium containing 1-2 g/l mannose. The callus induction rate dramatically decreased when increasing the mannose from 3 to 5 g/l. No callus induction was found from immature embryos cultured on the medium containing 7 or 9 g/l mannose (Figure 30).

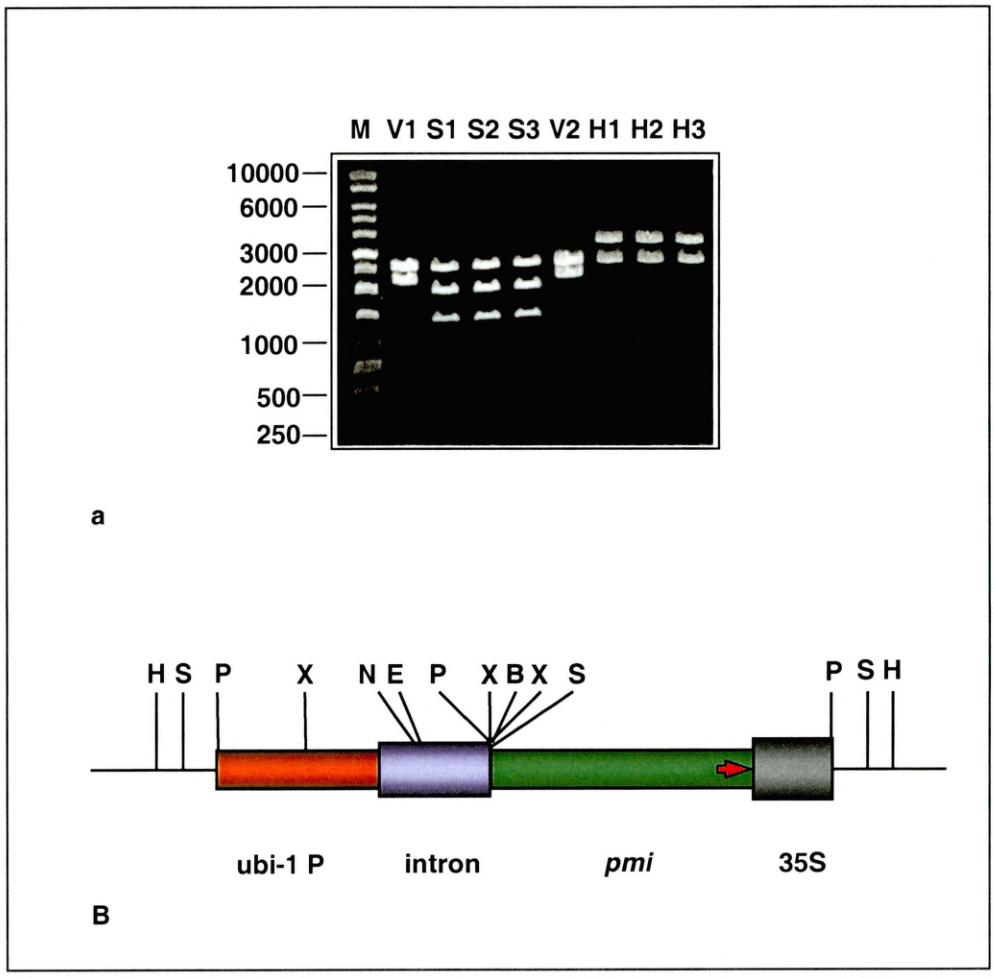


Figure 29. **Gel electrophoresis of *Sph*I and *Hin*dIII cleaved recombinant DNA and diagram of resultant construct pUbiMan**. M: I kb DNA marker Promega, UK) V1: Cloning vector pUbi35S cut by *Sph*I, S1-3: Three different clones cut by *Sph*I, H1-3: Three same clones cut by *Hin*dIII, V2: Cloning vector pUbi35S cut by *Hin*dIII, ubi-1 p: ubiquitin-1 promoter, *pmi*: phosphomannose isomerase coding sequence, 35S: 35S terminator, B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII, N:*Nco*I, P:*Pst*I, S: *Sph*I, X: *Xba*I.

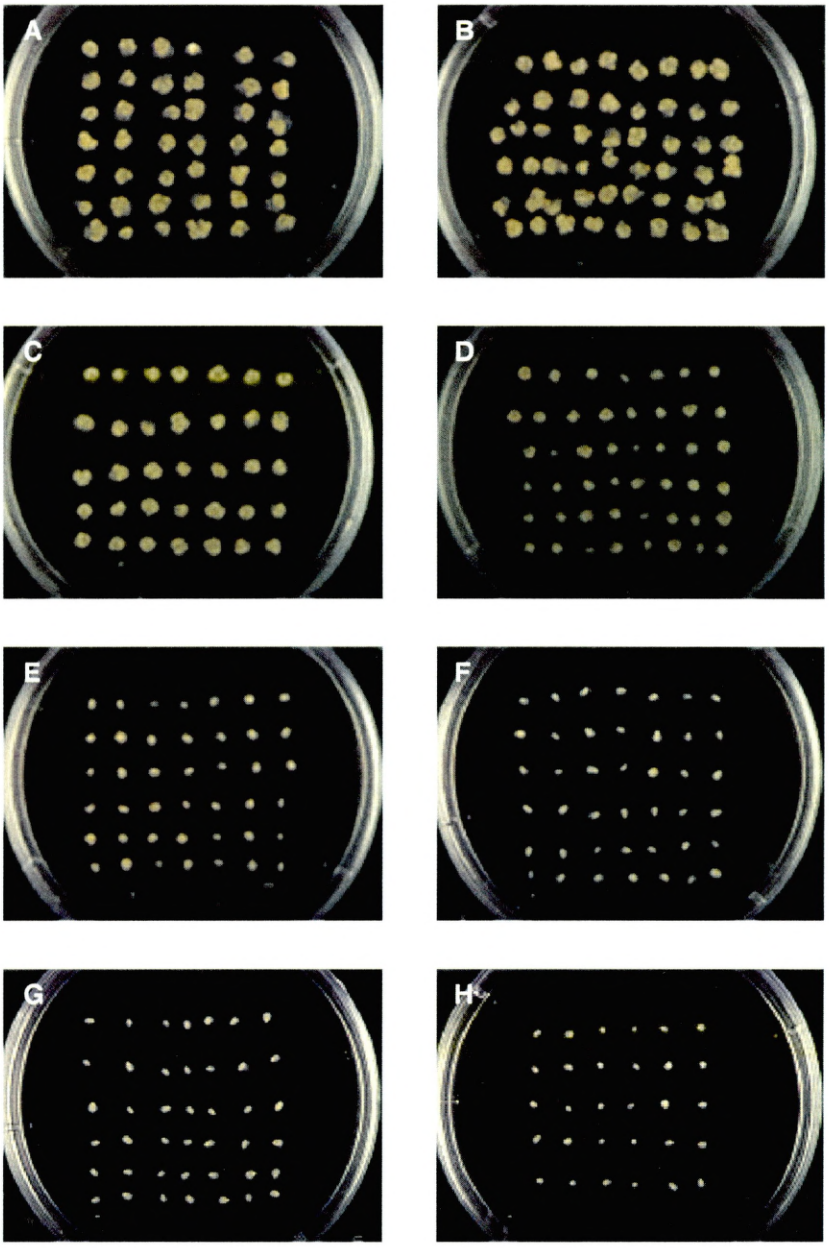


Fig. 30. Dose response of wheat immature embryos with mannose. Embryos were cultured on SMS medium for two weeks with different concentrations of mannose. A) Control, B) 1 g/l, C) 2 g/l, D) 3 g/l, E) 4 g/l, F), 5 g/l, G) 7 g/l, H), 9 g/l.

Table 7. The effect of different concentrations of mannose on callus induction and embryogenesis of wheat immature embryos

Mannose concentration (g/l)	No. of grown embryos / No. of embryo cultured	Callus induction (%)
0	120/120	100
1	125/125	100
2	120/120	100
3	65/120	54.2
4	20/120	16.7
5	6/120	5
7	0/125	0
9	0/100	0

5.3.3 Transformation of wheat with pUbiGUS and pUbiMan

The cultivar Bob White was chosen for transformation as it has shown high regeneration capacity and transformation frequency under PPT selection from a number of previous transformation experiments (Vasil *et al.*, 1993; Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.*, 1996a; Takumi and Shimada, 1996). Stable transformation of wheat was carried out with plasmid pUbiMan and pUbiGUS. Three independent experiments were performed using different selection schemes. The procedure described was used for the first and second transformation experiments.

Wheat immature embryos were isolated and cultured on callus induction medium SMS without mannose for one week. After 4 hours osmotic pre-bombardment treatment on SMS20 medium (SMS with 20% maltose), the embryos were bombarded with plasmid DNA-coated gold particles under the optimal DNA delivery conditions. After keeping on the same medium for 12-14 hours, the bombarded embryos were transferred onto the culture medium containing 5 g/l mannose. Transient expression of GUS on bombarded embryos was visualised by staining a small number of the bombarded embryos two days post-bombardment (Fig 31A). The calli were transferred onto the same selection medium to culture every two weeks. During the selection culture, several calli from each plate were stained in GUS solution, small blue sector can be observed from some calli which indicated the stable expression of *uidA* gene in the transformed sector (Fig. 31B). After six weeks of culture on the selection medium, the calli were transferred onto the selection regeneration medium REM containing 5 g/l mannose (MS medium supplemented with 20 g/l sucrose, 2 mg/l 6-BA, 0.5 mg/l KT) for plant regeneration. The cultures were kept at 25 °C under a 12 hour photoperiod for 4-6 weeks. The cultures were transferred onto new selection-regeneration medium every two weeks. Shoot formation was generally observed from all of the calli after 4-6 weeks culture on this medium (Fig. 31C). The vigorous-growing regenerants with or without roots were transferred onto the rooting medium (1/2 MS supplemented with 10 g/l maltose, 0.1 mg/l KT and 0.1 mg/l NAA, and 5 g/l mannose). A small piece of leaf was cut from the plantlets and subjected to histochemical GUS activity assay (Fig. 31E). For PCR analysis, a small amount of DNA was isolated from leaves using the alkaline extraction method during the rooting culture. After 4-6 weeks culture on the rooting medium, the rooted plantlets (Fig. 31D) were taken out, and transferred into soil and grown to maturity under glasshouse conditions. Pollen were collected from putative transgenic plants and stained in GUS solution (Fig. 31F). Expression of GUS gene was observed from the mature seeds of transgenic plants (Fig. 31G). In the third experiment, after two weeks culture on the callus induction medium containing 5 g/l mannose, the calli were transferred onto the medium containing 7 g/l mannose followed by the same procedure as used in the first and second experiments with exception of using of 7 g/l

mannose in the regeneration medium. In this case, the relevant lower regeneration frequency, but higher transformation frequency were observed as the result of applying higher selection pressure. Table 8 shows the results of these three independent experiments.

Table 8. Transformation of wheat with *uidA* and *pmi* genes

Experiment	Bombarded Embryos	Tested Regenerants	GUS^+ Plants	PCR		Transformation Frequency (%)
				$uidA^+$	pmi^+	
1	98	45	1	1	1	1.02
2	102	40	1	1	1	0.98
3	203	75	6	6	6	2.96

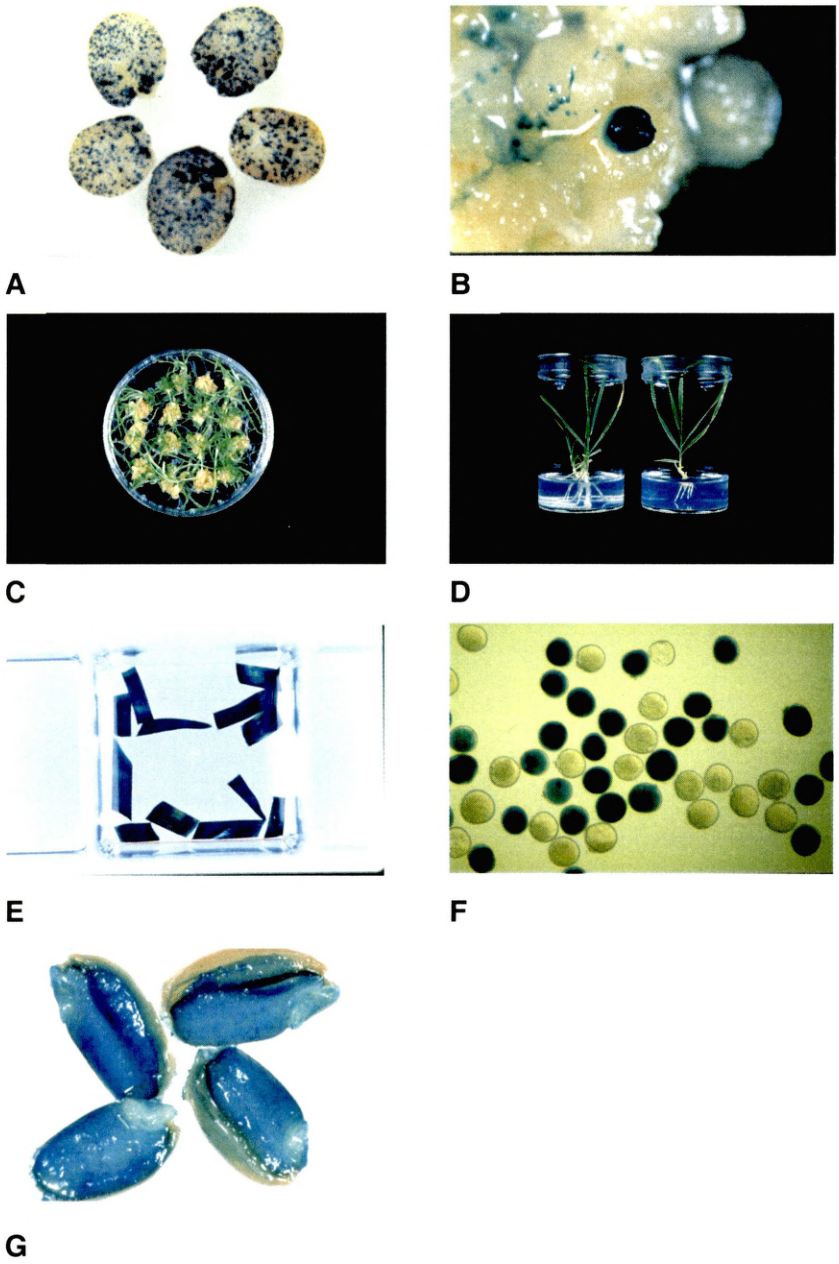


Figure 31. Transformation of wheat (*Triticum aestivum* L. cv. Bob White) with pUbiMan and pUbiGUS. A); β-glucuronidase (GUS) transient expression from immature embryos 2 days after bombardment with pUbiGUS and pUbiMan projectiles, B) Gus expression of sector in callus after two weeks cultured on the selection medium C) Plant regeneration from callus three weeks after culture on the selection regeneration medium containing 7 g/l mannose; D) Root development of transgenic regenerants 6 weeks after transfer onto selective rooting medium containing 5 g/l mannose; E) GUS expression in leaves of transgenic wheat plant; F) GUS expression in pollen of transgenic wheat plants showing the transgene segregated in 1:1 ratio; G) GUS expression in mature transgenic wheat seeds.

5.3.3.1 PCR analysis of GUS transgenic plants

The GUS-positive transgenic plants obtained from the first experiments were analysed by PCR. DNA was extracted from leaf materials using the method for small amount tissues (see materials and methods). PCRs were performed under different conditions with two sequence specific primers respectively. Two plasmids pubiMan and pUbiGus were used as the positive control. A predicted 466 bp band corresponding to the internal sequence of *uidA* gene and a 460 bp band correspoding to the internal portion of *pmi* gene were amplified from both GUS positive plants. Figure 32 shows an example of reaction products electrophoresed on an agarose gel. PCR screening of the other 7 GUS-positive plants obtained from the further two experiments showed the presence of transgenes in the transgenic plants (data not shown).

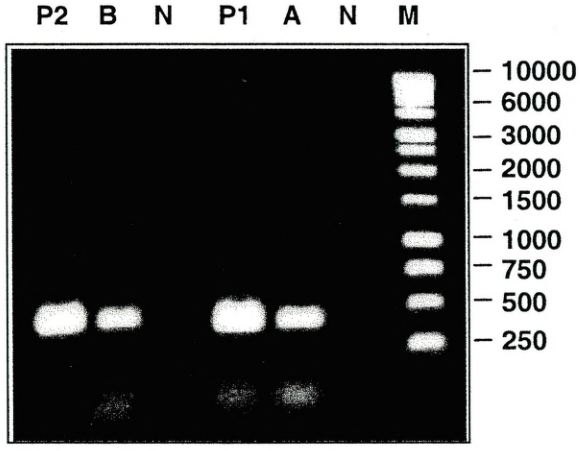


Figure 32. PCR analysis of transgenic wheat plant transformed with pUbiGUS and pUbiMan. M) 1kb DNA marker (Promega, UK); N) negative control; A) Amplification of the 466 bp internal fragment of *pmi* gene form transgenic line one; B) Amplification of the 460 bp internal fragment of *UidA* gene from transgenic line one; P1) pUbiMan positive control; P2) pUbiGUS positive control.

5.3.4 Transformation of wheat with pUbiMan and pUbiCP

5.3.4.1 Recovery of transgenic wheat plant

Following the success in introducing the *gus* gene into wheat plants, two Chinese wheat cultivars Yangmai 93-111 and Yangmai 94-141 were co-transformed with pUbiMan and pUbiCP via microprojectle bombardment of pre-cultured immature embryos. The experimental conditions (bombardment parameters, pre- and post-bombardment treatment of immature embryos, tissue culture medium for callus induction, plant regeneration and rooting and the selection scheme) are the same as used for the production of GUS transgenic plants as described above.

There was no significant difference in callus induction between cv. Bobwhite and those two Chinese cultivars, but the regeneration frequencies of Chinese wheat were lower than that of Bob White (Fig.33). When transferred onto the medium for regeneration, approximately 60 % of the calli were morphogenic, and differentiated green plantlets. Multiple regenerants were obtained from most of the differentiated calli, but only one or two plantlets grow vigorously.

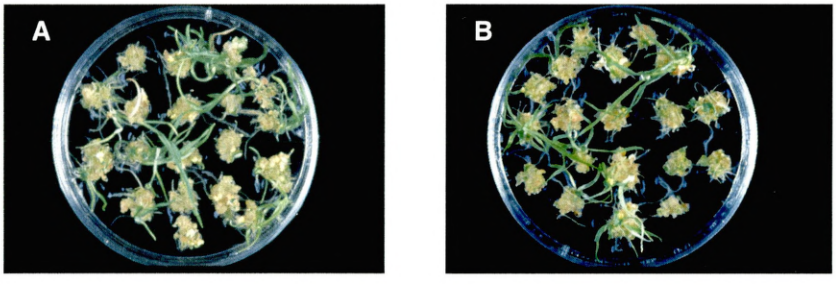


Figure. 33. Plant regeneration under mannose selection. A) Yangmai 93-111, B) Yangmai 94-141, Calli were cultured on selection regeneration medium containing 7 g/l mannose for three weeks.

In total, 1600 immature embryos were bombarded. Eleven *mpi*-transgenic lines were obtained. Amongst these transgenic lines, six plants were confirmed to contain the SBWMV *cp* gene.

5.3.4.2 Molecular analysis of transgenic wheat plants

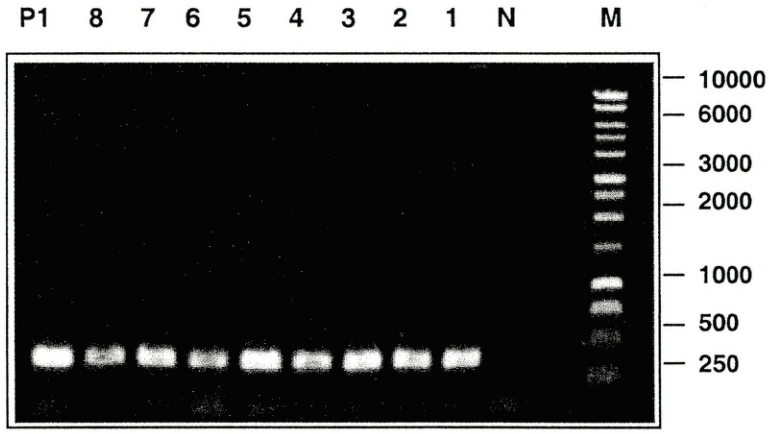
Putative transgenic wheat plants were first subjected to a PCR screening with both *pmi* and SBWMV *cp* gene primers. The analysis of the PCR amplification products obtained with *pmi* primers revealed the presence of the expected band from 11 out of 85 putative transgenic plants. The *pmi* PCR positive plants were then screened with viral *cp* primers. PCR analysis revealed that 6 transgenic wheat plants, 1 Yangmai 94-141 and 5 Yangmai 93-111, were *cp* positive. An average cotransformation frequency of 33% and 62% were observed from these two Chinese wheat cultivars, respectively. Representative results of this PCR analysis are shown for 8 plantlet regenerated from calli derived from immature embryos of Yangmai 93-111 (Fig. 34).

The transgenic nature of wheat plants was confirmed by Southern hybridisation of undigested and digested total cellular DNA samples with a radiolabelled *mpi* gene probe. The results are shown for the 1 transgenic Yangmai 94-141 and 5 transgenic Yangmai 93-111 lines. *Eco*RI was chosen for the digestion of genomic DNA although it will cut once only in the ubiquitin-1 intron of the entire plasmid. Different hybridisation patterns were observed from all of the samples with exception of that of line 2 and 3 being identical. A strong hybridisation signal was obtained from the undigested DNA sample derived from line 1. A band of 6 kb corresponding to the entire plasmid pubiMan was observed from the line 2, 3 and 7. Multiple fragments were hybridised with the probe, resulting in the appearence of complex band patterns from lane 1, 2, 3, and 7, which suggested that more copies of *pmi* gene might insert into the genome of the transgenicn plants (Fig. 35A).

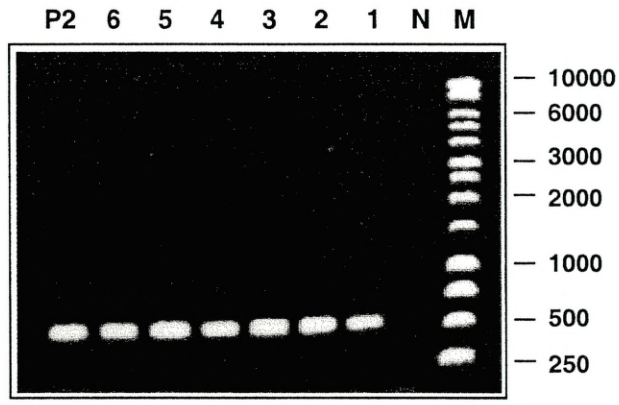
To confirm the presence of *cp* gene in the *pmi* Southern-positive transgenic plants, DNA samples were digested with *Bam*HI and *Eco*RI re-

spectively and then hybridized with the SBWMV *cp* probe generated by PCR amplification. *Bam*HI cuts the entire *cp* gene from genomic DNA which was used to confirm the insertion of full length of viral CP coding sequence into the genome of transgenic plants, while *Eco*RI cuts only once within the *cp* gene which will reveal the copy number of insert and its integration complexity. Hybridization signals corresponding to the full length SWVMV *cp* gene (the expected 564 bp band hybridising to the *cp* probe) were observed in the Southern hybridisation analysis using *Bam*HI-digested DNA samples, (Fig. 35B). Strong hybridisation signal was observed from the line one (Yangmai 94-141) to line three (two transgenic Yangmai 93-111), but weak signal was found from another two transgenic Yangmai 93-111 lines (Line 5 and 6). No signal was found from the third transgenic line (Line 3) although it was previously found positive in PCR test. The PCR positive result from this line was possibly caused by the contamination of DNA sample. Hybridization of *Eco*RI-digested DNA from Line 1 resulted in a three-band pattern, but apparently different from that of hybridisation with lane 2 and 3 samples. This suggested that the *cp* gene was integrated into the genome of different plants at different loci with multi insertions or partial copies of this transgene. The band pattern was identical between the Line 2 and Line 3 when digested with *Eco*RI, which suggested that these two transgenic lines might originate from one transformation event. Incomplete digestion of genomic DNA was observed from this Southern blot which was indicated by the appearance of an additional band of about 5 kb in the Line 2 and 3. No hybridisation band was found from the DNA samples of nontransgenic plant as the negative control.

Detailed information on transformation of Chinese wheat cultivars with the SBWMV *cp* gene under mannose selection is provided in table 9.

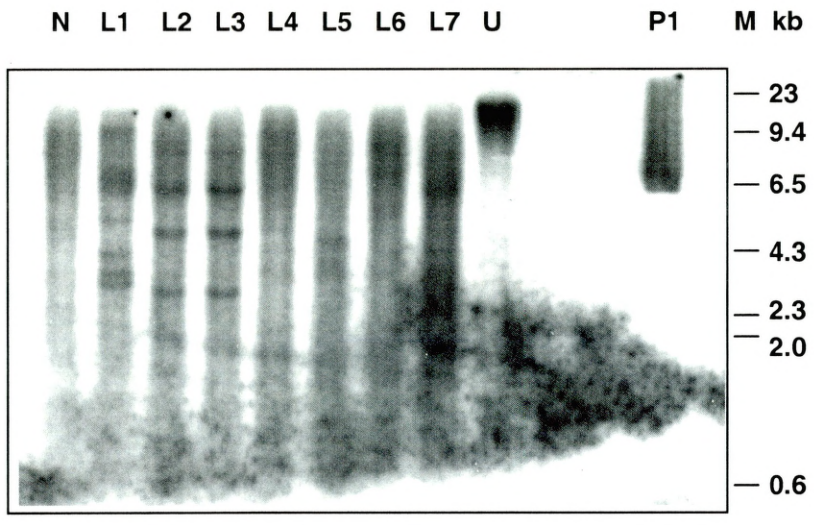


A

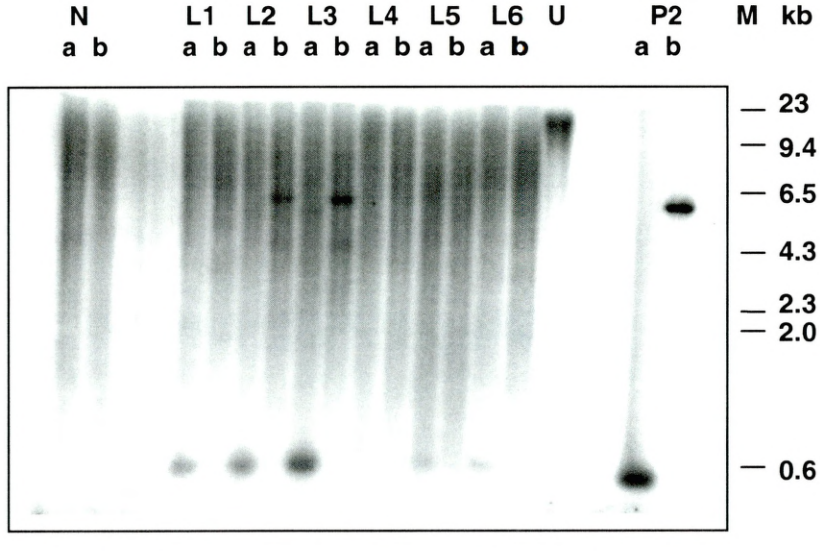


B

Figure 34. PCR analysis of putative transgenic plants transformed with pUbiMan and pUbiCP. A) Amplification of the 460 bp internal fragment of *pmi* from 8 transgenic plants (Yangmai 93-111); M) 1kb DNA molecular marker (Promega, UK); N) Negative control; 1-8) 8 independent Yangmai 93-111 transgenic lines; P1) Positive control pUbiMan; B) Amplification of 564 bp of SBWMV CP from 6 transgenic plants; 1-5) Five independent transgenic Yangmai 93-111; 6) One transgenic Yangmai 94-141; P2) Positive control pUbiCP.



A



B

Figure 35. Southern analysis of transgenic plants transformed with pUbiMan and pUbiCP. A) Southern hybridization analysis of transgenic Chinese wheat cvs. using *mpi* probe; L1) Transgenic Yangmai 94-141; L2-6) 5 transgenic Yangmai 93-111; L7) One transgenic Bobwhite transformed with pUbiGUS and pUbiMan; P1) Positive control pUbiMan digested with *Eco*RI; B) Southern hybridization analysis of transgenic Chinese wheat cvs. using *cp* probe; L1-6) Six transgeic lines as above-mentioned in A; P2) Positive control pUbiCP; M) λ DNA/*Hin*d III Markers (Promega, UK); a,b) DNA digestion using *Bam*HI and *Eco*RI respectively.

Table 9. Transformation of Chinese wheat cultivars with pUbiMan and pUbiCP

cv.	No. of Bombarded embryos	No. of regenerants tested	PCR		Southern	
			pmi+	*cp+*	*pmi+*	*cp+*
Yangmai 93-111	1250	65	8	5	5	4
Yangmai 94-141	350	20	3	1	1	1
Total	1600	85	11	6	6	5

5.4 Discussion

A reproducible protocol for the production of transgenic wheat, based on microprojectile bombardment of immature embryos was established. It employed the *pmi* gene as the selectable marker gene and mannose rather than antibiotics or herbicides as the selection agent to allow the recovery of transgenic wheat plants.

For wheat, immature embryos have proved to be the most efficient tissue source for genetic transformation. A simple and fast tissue culture method and high regeneration potential, compared to that of many other explants previously used for plant regeneration, have made it possible to produce multiple or numerous plants from individual embryos of some favourable cultivars. In addition possible single cell-based somatic embryogenesis permits the generation of uniformly transformed plants as observed in many transformation experiments. Since the first achievement of production of fertile transgenic wheat via direct bombardment of cultured immature

embryos (Vasil *et al.*, 1993), this methodology has been widely used for the generation of transgenic wheat plants, and a number of important genes have been introduced and stably expressed, mainly in spring wheat cultivars and in limited winter varieties with high regeneration potentials (Weeks *et al.*, 1993; Nehra *et al.*, 1994; Becker *et al.*, 1994; Zhou *et al.*, 1995; Zhang *et al.*, 1996; Altpeter *et al.*, 1996; Barro *et al.*, 1997).

Recovery of transgenic plants relies on the application of a certain selectable marker which allows the selection of transformed cells by their ability to survive and grow in the presence of the selection agent. Strategies currently used for the selection of transgenic plants are generally based on the expression of a gene encoding an enzyme, which confers resistance to an antibiotic or herbicide.

At the moment, the most commonly used selectable marker gene for wheat transformation is *bar* gene which encode for phosphinothricin acetyl-transferase (PAT) conferring the transgenic cells resistance to the herbicide Basta and other related chemicals. This approach has clearly opened many possibilities for the genetic modification of wheat, however, challenges still remain in terms of achieving high transgenic efficiency. To date recovery of fertile transgenic plants from tissue culture unfavourable, but agronomically important wheat cultivars is still problematic.

A new procedure based on a *pmi* gene as selectable marker and mannose as the selective agent has been recently developed, which resulted in the recovery of transgenic plants in potato and sugar beet (Joersbo *et al.*, 1998), and more recently the production of transgenic cassava (Zhang *et al*, 2000). This technique was demonstrated to be particularly useful for the transformation of recalcitrant species such as sugar beet (Joersbo *et al.*, 1998). Therefore, this mannose-based selection approach may offer alternatives and advantages over existing selection systems currently used for the generation of transgenic wheat, especially in the recalcitrant wheat species. In the experiments reported here, mannose selection resulted in transformation frequencies of around 1% from the first two independent experiments

using a model wheat variety. A selection pressure of 5 g/l mannose might be strong enough to inhibit the further development of somatic embryos at early development stages, but it was not high enough to prevent regeneration of plants from somatic embryos at their later development stages. The stepwise increase in concentration of mannose from 5 to 7 g/l resulted in much higher transformation frequency in the third experiment. Improved rooting was found when using mannose selection instead of PPT selection, which is in accordance with the study on potato transformation showing dramatically improved root formation using mannose rather than kanamycin as the selection agent (Joersbo *et al.*, 1998).

Molecular analysis of transgenic plants revealed that both viral *cp* gene and the selectable marker *pmi* were incorporated into the genome of Chinese wheat cultivars with 62% of cotransformation frequency which is similar to that of transformation of wheat under PPT selection. The multiple bands found from Southern blot analysis revealed that multiple copies of transgene might be integrated into the genome of some transgenic plants, which has been previously reported from most transformation experiments using the microprojectile bombardment-based approach.

In this experiment, mannose selection resulted in the production of a large number of escape plants. This suggested the selection pressure might not be tight enough to allow only those transgenic cells or tissue to regenerate into the plants. However, too high a selective pressure will significantly affect the potential of plant regeneration, especially for those tissue culture-unfavourable species. Therefore, one selection procedure optimal for the production of transgenic plants from a favourable cultivar may be not suitable to those cultivars with poor tissue culture response of the same plant species. Prolonged selection culture on the selection medium may help to eradicate the non-transgenic plants, but a long period of culture in the presence of synthetic auxins and selective agents will lead to the morphological changes of regenerated plants due to somaclonal variation. In addition, tissue culture can also lead to variation of agronomic performance of the regenerants.

Therefore, prolonged tissue culture should be avoided in term of eradicating the escape plants.

There were no previous reports on successful transformation of wheat using the mannose selection approach. The results presented here demonstrated that genetic transformation of wheat has been accomplished by microprojectile bombardment of cultured immature embryos followed by mannose selection. Although this transformation protocol may need to be improved to achieve much higher transformation frequency in Chinese wheat cultivars, this new technique might provide a most promising method not only for the genetic engineering of agronomically important, but transformation-recalcitrant wheat varieties, but also for the transformation of other plant species. There are no doubts that reports of successful transformation of other plant species by applying this selection strategy will appear in the near future.

CHAPTER 6

COMPLEMENTATION OF *SOIL-BORNE WHEAT MOSAIC VIRUS* 37 kDa MOVEMENT PROTEIN (MP) TO *TOBACCO MOSAIC VIRUS* 30 kDa MP

6.1 Introduction

Plant viruses normally encode one or several movement protein(s), which exploit and modify the plasmodesmata between cells, enabling the viral genome or in some cases virus particles to move from cell to cell resulting in systemic infection in infected plants. The 30 kDa movement protein of tobamoviruses is encoded by a single gene of their genome (Deom *et al.*, 1987), whereas some other viruses, such as hordeiviruses (Petty *et al.*, 1990; Jackson *et al.*,1991), benyviruses (Gilmer *et al.*, 1992) and potexviruses (Beck *et al.*, 1991), have three genes encoding three movement proteins which are required for transporting the viral genome from cell to cell.

Virus-encoded movement proteins have been identified from a number of plant viruses. Evidence suggested that the movement proteins from different viruses might function by different mechanisms. It is clear that the 30 kDa protein encoded by TMV facilitates the movement of the viral genome from cell to cell through modified intercellular channels, plasmodesmata, and the 17.5 kDa coat protein is required for the long distance movement of virus via vascular tissues (Hilf and Dawson, 1993). Sequence analysis has revealed that a short element in the N-terminal region of the 37 kDa protein encoded by SBWMV RNA I shares similarity to the equivalent parts of transport proteins of tobamoviruses and tobraviruses (Koonin *et al.*, 1991), and SBWMV 37 kDa is closely related to the movement proteins of dianthovi-

ruses (Shirako and Wilson, 1993), therefore this 37 kDa protein has been proposed to function as a movement protein.

There is evidence to show that the MPs from different plant viruses are often interchangeable (Ryabov *et al.*, 1998). This has provided a valuable tool to study the function of movement proteins from related and unrelated viruses aiding in understanding virus-host interactions. To study the function of the 37 kDa protein encoded by SBWMV RNAI, TMV was used as a vector to express this foreign gene. The third ORF of SBWMV RNA I was introduced into TMV vector p30B. Using this hybrid vector, it was possible to determine whether SBWMV MP could support the systemic spread of a variant TMV which itself had a defective MP.

6.2 Materials and methods

6.2.1 Plant material

Nicotiana benthamiana plants were grown in the glasshouse at 24 °C and 70% relative humidity, and wheat plants (cv. Galahad) were grown in a growth chamber at 18 °C day and 14 °C night under 12 hr photoperiod with 70% relative humidity.

6.2.2 Construction of TMV-based expression vectors

The SBWMV MP coding sequence was amplified by PCR and cloned into the plasmid p30B to generate a chimeric vector containing the TMV 30 kDa MP gene and 37 kDa MP gene of SBWMV. In a second construct the native TMV MP gene was rendered defective by frame shift to produce a vector harbouring a defective TMV MP gene and functional MP coding sequence of SBWMV. Table 10 shows the details of the TMV-based constructs used in the research.

Table 10. TMV-based expression vectors

Plasmids	Description	References
p30B	Expression vector containing cDNA of TMV	Lacomme and Santa Cruz, 1999
p30BGFP	p30B carrying a cDNA encoding the GFP	Lacomme and Santa Cruz, 1999
p30BMP	p30B carrying a cDNA encoding the SBWMV movement protein	in this thesis
p30BDMP	p30B carrying a cDNA encoding the SBWMV movement protein, but with defective 30 kDa TMV MP coding sequence	in this thesis

6.2.2.1 Construction of p30BMP

6.2.2.1.1 Amplification of SBWMV MP gene by PCR

A set of primers was designed to amplify the SBWMV movement protein coding sequence. For cloning purposes, *Pme* I *and Xho* I restriction sites were added to the 5' end of forward and reverse primers respectively. Both forward and reverse primers are 33 mers (forward 5' CCGTTTAAAC ATGGGCTCACAGGATGTCTAAGG3'; reverse 5'CGCTCGAGCTACCA TATCAAAATTACCAAAC 3'. To enable expression of SBWMV MP in this hybrid expression vector, a start codon ATG was introduced directly upstream of the viral MP coding sequence, and a stop codon TAG directly downstream of the coding sequence. PCR was carried out in 25 µl volume containing 100 ng pJC1A (cDNA clone of SBWMV RNA II) using *Ready To Go*™ PCR beads (*Amersham Pharmacia Biotech.,* UK) following the manufacturer's instructions. The amplification started at 94 °C for 1 minute for denaturation followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and elongation at 72 °C for 1 minute, with 5

minutes at 72 °C for final elongation. PCR products were purified with S-400 HR MicroSpin™ Columns.

6.2.2.1.2 Restriction enzyme digestion and clean-up of PCR products

Purified PCR products were digested in a final volume of 40 µl containing 10 units of *Pme*I and *Xho*I and buffer as recommended by the enzyme supplier. Digestion was performed at 37 °C for 1hr. After extraction with phenol/chloroform, the digested products were purified with S-400 HR MicroSpin™ Columns. The purified PCR products were recovered by ethanol precipitation.

6.2.2.1.3 Digestion and dephosphorylation of vector p30B

The expression vector p30B was obtained from Dr. Santa Cruz of the Virology Department, Scottish Crop Research Institute, UK. 5 µl of p30B (1µg/µl) was digested at 37 °C for 2 hr in a 40 µl final volume, containing 20 units of two enzymes, *Pme*I and *Xho*I, and buffer as recommended by enzyme supplier. To dephosphorylate digested-p30B, 1 unit alkaline phosphatase (Boehringer Mannheim GmbH, Germany) was directly added into the same tube and the reaction was performed following the procedures as described in section 2.2.4. After extraction with phenol/chloroform, the upper aqueous phase was transferred into a clean tube and then purified with S-400 HR MicroSpin™ Columns. The dephosphorylated linear plasmid was precipitated as described previously with sodium acetate and absolute ethanol, then dissolved in 10 µl of H_2O.

6.2.2.1.4 DNA ligation

Ligation of *Pme*I: *Xho*I-digested dephosphorylated p30B with *Pme*I :*Xho*I-digested PCR products was performed using the procedures as described previously (section 2.9.1).

6.2.2.1.5 Transformation of *E. coli,* clone selection and minipreparation of recombinant plasmids

Ligation products had been transformed into three strains of *E. coli* competent cells, being D5Hα, XL1 blue and HB101, and the transformants were selected on LB agar selection medium (section 2.9.2). Several clones were cultured and a small amount of plasmid was prepared from the selected clones using the Wizard® Plus SV Minipreps DNA Purification System.

6.2.2.1.6 Analysis of recombinant DNA

The recombinant DNAs were analysed by restriction enzyme digestion and DNA sequencing.

6.2.2.2 Construction of p30BDMP

6.2.2.2.1 *Apa*I digestion of p30BMP

5 µg of p30BMP was digested at 37 °C for 2 hr in a 20 µl final voume containing 20 units of *Apa*I. After digestion, 1 µl of digest was checked on a 1 % agarose gel to confirm complete digestion.

6.2.2.2.2 Blunting *Apa*I-digested p30BMP and DNA religation

*Apa*I-digested p30BMP was blunt-ended using T4 DNA polymerase (section 2.2.6), and then religated with T4 DNA ligase (section 2.9.1).

6.2.2.2.3 Transformation of *E. coli* , clone selection and plasmid minipreparation

E. coli strain HB101 was transformed with ligation production and the recombinants were selected for antibiotic resistance (section 2.9.2). A small amount of plasmid was produced from several selected clones using the Wizard® Plus SV Minipreps DNA Purification System (section 2.4.1).

6.2.2.2.4 Analysis of recombinant DNA

The recombinant DNAs were analyzed by restriction enzyme digestion and sequencing. DNA sequencing start from the upstream of the insert (40 nucleotides before *Pme*I site). The sequencing primer is 5'-CGG AAT TCA TAT GTC TTA CAG TAT CAC TAC-3'.

6.2.3 *In vitro* transcription and agarose gel electrophoresis

Plasmids p30B, p30BGFP (Lacomme and Santa Cruz, 1999), p30BMP and p30BDMP were linearised downstream of the TMV cDNA using *Kpn*I and then used for an *in vitro* transcription reaction. The *in vitro* transcription reactions were carried out using Ambion message Machine™ *In Vitro* Transcription Kit (Austin, Texas, USA) following the manufacturer's instruction. The mixtures were assembled in a 10 µl volume containing 1 µl 10X reaction buffer, 5 µl ribonucleotide mix (15 mM ATP, CTP, UTP; 3 mM GTP and 12 mM Cap Analog), 1 µl 10X enzyme mix, 0.5 µl linearized template DNA. The samples were incubated at 37 °C for one hour. After *in vitro* transcription, 1 µl of the transcribed products were loaded onto a 0.8% agarose gel to check RNA concentration.

6.2.4 Plant inoculation with *in vitro* transcripts

Nicotiana benthamiana plants were inoculated at the 4-5-leaf stage. Leaves were dusted with Carborundum and two leaves of each plant were inoculated with 2 µl of *in vitro* transcripts. The inoculated leaves were rinsed briefly with tap water and then the plants were kept in a growth chamber (12 hour light 28 °C/12 hour dark 18 °C).

6.2.5 Inoculation of plants with sap made from virus infected *Nicotiana benthamiana*

Leaves of infected *Nicotiana benthamiana* in which symptoms developed were removed, and ground in 0.1 M potassium phosphate buffer (pH 7.0) in a mortar. The procedure for inoculation of *Nicotiana benthamiana* was the same as described in section 2.1.1 with the exception of the use of 5 μl inoculum. Five μl of inoculum was used to inoculate wheat plants (3 leaf stage) and then plants were placed into a growth chamber (12 hour light 22 °C /12 hour dark 18 °C).

6.2.6 Western blot analysis

The expression of the movement protein gene from different hybrid TMV vectors in infected tobacco plants was examined by Western blot analysis using a polyclonal antiserum against 37 kDa MP of SBWMV (J. Sherwood, Oklahoma State University) and another polyclonal antiserum against 30 kDa MP of TMV (S. Santa Cruz, SCRI). The virus MPs from crude protein extracts were separated on polyacrylamide gels (section 2.7.4.2), and then electrically blotted onto a nitrocellulose membrane (section 2.7.4.3). The MPs were probed with the above-mentioned polyclonal antibodies.

6.2.7 Fractionation of infected tobacco leaf tissue

The method used to enrich the subcellular fractions of *Nicotiana benthamiana* was as described by Donald *et al.* (1993). One gram of infected *N. benthamiana* leaves was ground into a fine powder in liquid nitrogen using a pestle and mortar. The powder was resuspended by vigorous mixing in 4 ml of protein extraction buffer (400 mM sucrose, 100 mM Tris, 10 mM KCl, 5 mM $MgCl_2$, 10% Glycerol, 10 mM β-mercaptoethanol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl (PMSF), pH 7.5). The suspension was then filtered through a Nylon mesh (500 µm pore size). The cell wall fraction (CW) was derived from debris trapped on the Nylon mesh. To remove chlorophyll from debris, the cell wall fraction was washed with extraction buffer supplemented

with 2% Triton-X 100 by repeated cycles of centrifugation (10,000x g) and resuspension (4 times) until the pellet became white. The pellet was drained and resuspended in 500 µl of Laemmli sample buffer containing 9 M urea to release cell wall protein.

6.3 Results

6.3.1 Construction of p30BMP

To generate the expression vector, the SBWMV MP gene was cloned into the TMV-based vector p30B. The p30B was the most effective vector for expressing foreign viral sequences. The presence of a double TMV subgenomic promoter enables the cloning of heterologous viral genes to produce foreign protein products. The resultant constructs were analysed by restriction digestion and DNA sequencing.

To produce plasmid p30BMP, 61 clones obtained from transformation of *E. coli* D5Hα were screened and plasmids were prepared from 16 selected clones. To determine whether the recombinant plasmids contained the correct insert, (the MP gene of SBWMV), plasmids were double digested with *Pme*I and *Xho*I. However, agarose gel electrophesis of digests showed that the expected 549 bp of insert was not found and that partial deletion of the cloning vector occurred from all of the clones derived from the transformants (data not shown). In a second experiment, the ligation products were transformed into another *E. coli* strain (XL1 blue), but, the deletion happened again. It was only possible to generate plasmid p30BMP by transforming a third *E. coli* strain HB 101. Plasmids were isolated from eight different clones, and then digested using *Pme*I and *Xho*I. Electrophoresis of digests showed that a 946 bp fragment corresponding to the MP gene was cloned into the vector. The plasmids from clones 1 and 2 were digested by *Hin*dIII which showed the expected pattern when electrophoresed on a 1% agarose gel (Fig. 36). One of these two clones was confirmed to contain the correct insert by DNA sequencing. The plasmid bearing the SBWMV MP cDNA was designated as p30BMP (Fig. 37).

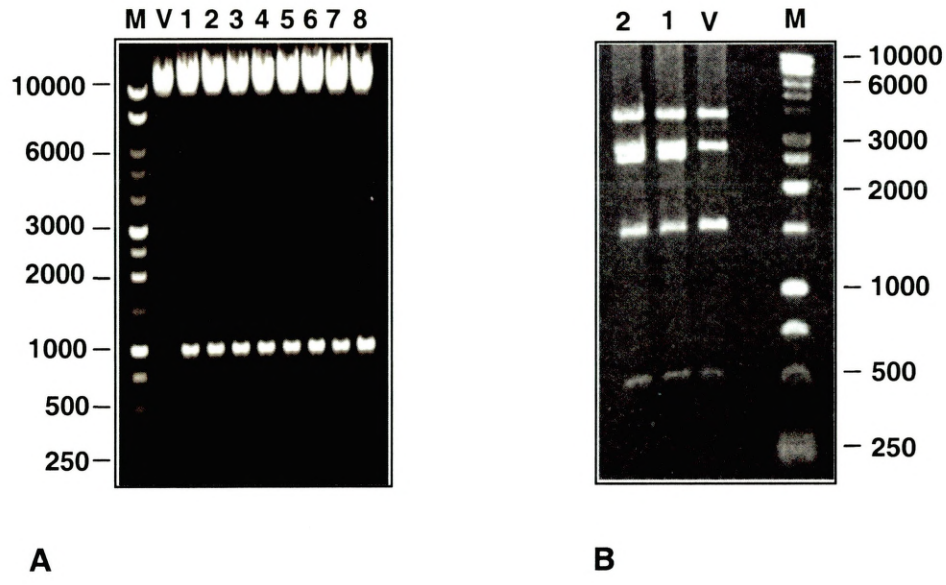


Figure 36. Analysis of plasmids using *Pme*I/*Xho*I (A) and *Hin*d III (B). 1-8) Eight different clones; V) Vector p30B; M) 1 kb DNA marker (Promega, UK).

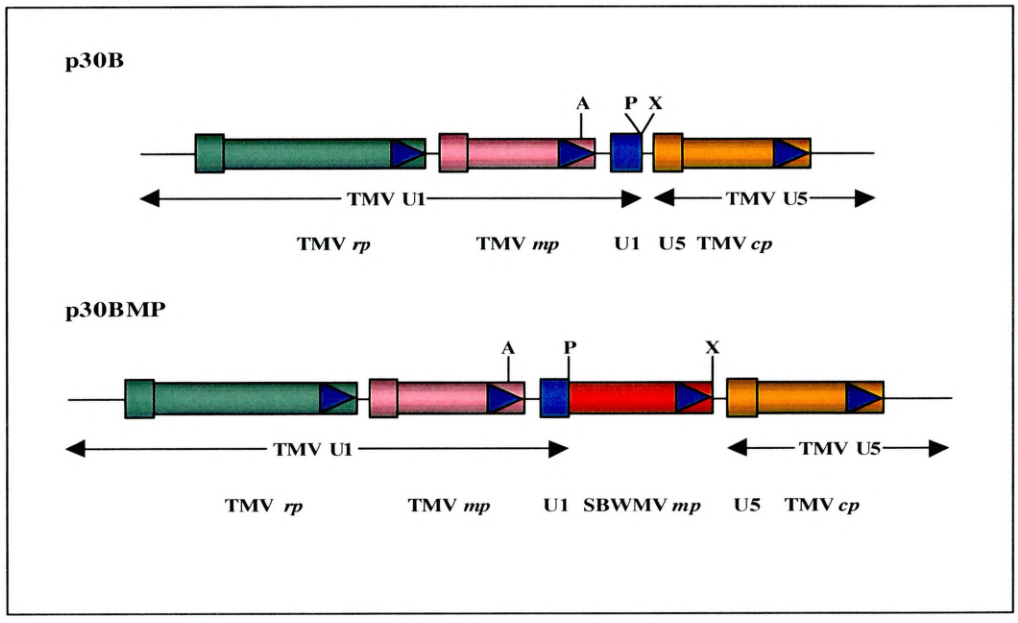
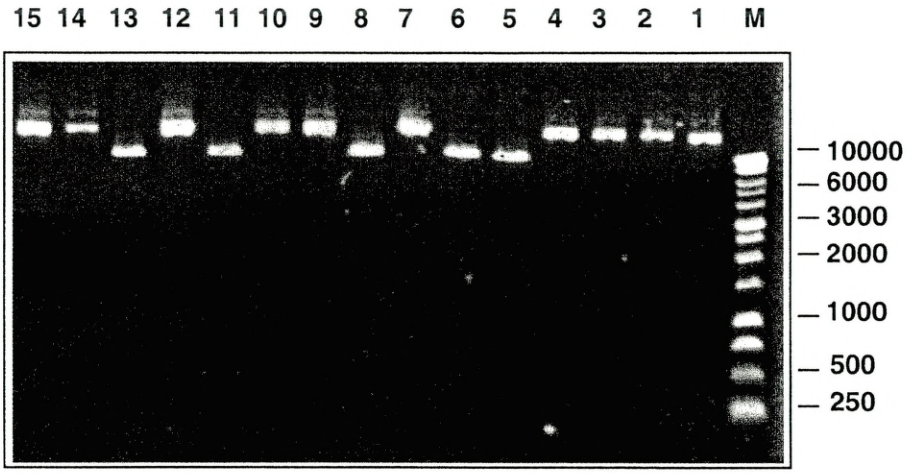


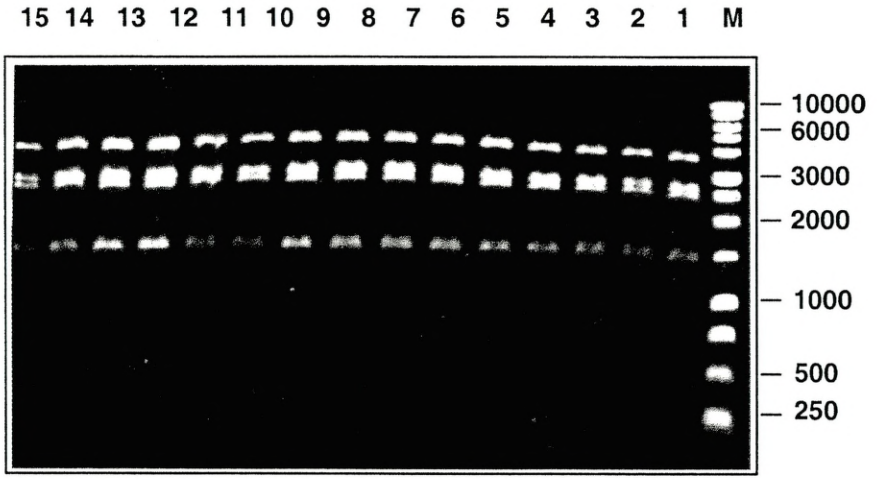
Figure 37. SBWMV movement protein coding sequence was fused to the vector p30B to generate construct p30BMP. TMV U1: Sequence from tobacco mosaic virus U1 strain, TMV U5: Sequence from tobacco mosaic virus U5 strain, U1: U1 promoter, U5: U5 promoter, *rp*: RNA dependent RNA polymerase, *mp*: Movement protein coding sequence, *cp*: Coat protein coding sequence, A: *Apa* I, P: *Pme* I, X: *Xho*I.

6.3.2 Construction of p30BDMP

The presence of a single *Apa*I site near the 3' end of the TMV movement protein coding sequence provided a rapid and simple way to mutate the native TMV MP gene. The p30BMP was cut by *Apa*I digestion, after blunt-ending using T4 DNA polymerase, the plasmid was religated using T4 ligase. After transformation and selection, 15 resultant clones were screened by *Apa*I digestion. Electrophoresis revealed that 10 clones were not cut by *Apa*I, which indicated the absence of this restriction site in the mutant clones (Fig. 38A). When the mutant clones were digested with *Hin*dIIII, the same patterns were observed as those of p30BMP digested with same enzyme (Fig. 38B). The vector harbouring the mutant TMV movement protein and the functional SBWMV movement protein genes was designated as p30BDMP.



A



B

Figure 38. Restriction digestion of resultant plasmids. Samples were digested with A) *Apa*I; B) *Hin*d III; 1-15) Fifteen clones; M) 1 kb DNA molecular marker (Promega, UK).

6.3.3 Symptoms of infection by mechanical inoculation of *in vitro* transcripts

To test possible complementation of virus movement protein (MP) from a different virus, *Nicotiana benthamiana* plants were inoculated with *in vitro* transcripts transcribed from p30B, p30BGFP, p30BMP (TMV + SBWMV MP) and p30BDMP (TMV with defective native MP + SBWMV functional MP). For each treament, two plants were inoculated. The inoculated plants were incubated in a growth chamber at 34 °C for 8 days. Typical symptoms of tobacco mosaic virus appeared at the same time in all the inoculated plants, whereas no symptom development was observed on control plants inoculated with water. Most of the inoculated leaves became yellow and dry, and upper leaves were distorted. The plants inoculated with p30B, p30BGFP, p30BMP and p30BDMP were stunted compared to the control plant. Under UV light, both inoculated leaves and upper leaves showed fluorescence from the p30BGFP-infected *N. benthamiana,* which indicated the spread of infection.

In the first experiment, the circular plasmid p30BDMP was used as the template for *in vitro* transcription. More severe mosaic symptoms were found from the systemic leaves of plant infected by p30BMP using linearised DNA as the template than those of plants inoculated with circular p30BDMP. In a second experiment all four vectors were linearised by *Kpn*I digestion and used as template for *in vitro* transcription, there was no significant difference in the symptoms induced on systemic leaves. Figure 39 shows the systemic symptoms developed on inoculated plants.

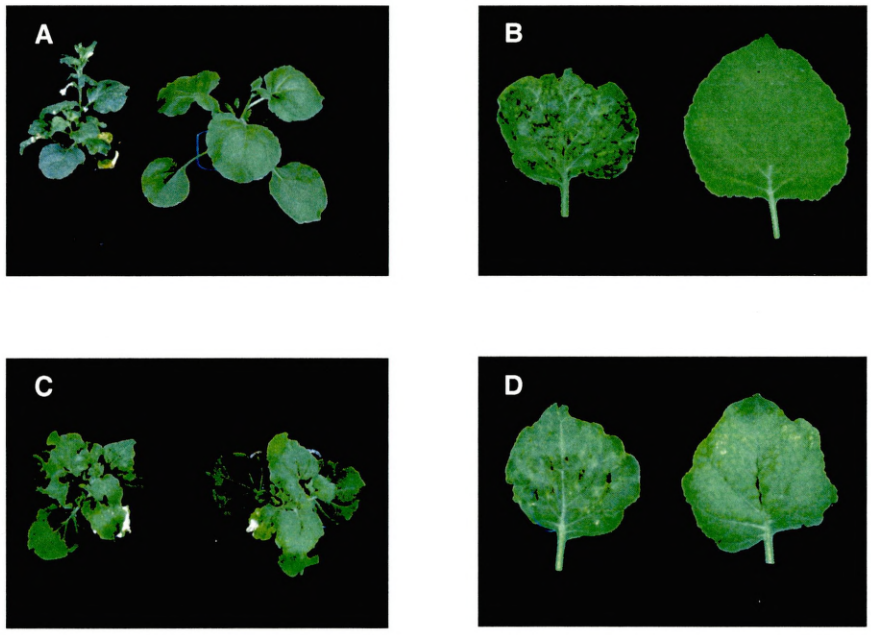


Figure 39. Symptom development in *Nicotiana benthamiana* 8 days after mechanical inoculation with *in vitro* transcripts. Inoculation was carried out as described in Materials and Methods (section 5.2.4). A) Plant inoculated with transcripts derived from p30B (left) and control plant (right); B) Systemically infected leaf from p30B-inoculated plant (left) and leaf from control plant (right); C) Plants inoculated with transcripts from p30BMP (left) and p30BDMP (right). D) Systemically infected leaves from p30BMP- and p30BDMP-inoculated plants.

6.3.4 RT-PCR analysis of multiplied viruses from plants inoculated with p3BMP and p30BDMP *in vitro* transcripts

To probe virus multiplication in inoculated leaves and possible movement of progeny virus throughout the plant, RT-PCR was carried out using total RNA isolated from inoculated and systemic leaves of plants inoculated with *in vitro* transcripts from p30BMP and p30BDMP. Primers were designed to detect the specific movement protein genes. The primers used for detecting the SBWMV-MP sequence from the hybrid expression vector were the same as used for cloning work described before (Chapter 3). Primers were also designed to detect the TMV MP gene from the viral RNA present in infected-plants. The forward primer was a 18-mer (5'GGAGGTGTGAGCGTGTGT3') and the reverse primer a 19-mer (5'CGACAGTAGCCTCCGAATC3'). RT-PCR was performed to amplify the partial sequence of the TMV movement protein gene, covering the unique *Apa*I restriction site (Chapter 2, section 2.8.9).

RT-PCR products were subjected to electrophoresis on an 0.8% agarose gel. A band of 530 bp, corresponding to the target TMV MP gene sequence was amplified from all of the samples. Apart from this major band, several weak bands of high molecular weight were also found from positive control and all RNA samples, these possibly due to the non-specific amplification of DNA sequences (Fig. 40).

6.3.5 *Apa*I digestion of RT-PCR Products

To determine whether the mutation in TMV MP gene is present in progeny virus, RT-PCR products from the RNAs derived from inoculated and systemically infected leaves of tobacco plants infected with p30BMP and p30BDMP were analysed by *Apa*I digestion. For p30BMP inoculated plants, the digestion gave rise in both inoculated and systemic leaves to the same band pattern as that of *Apa*I digested p30BMP plasmid, however, this enzyme did not cut the RT-PCR products from RNAs derived from p30BDMP inoculated and systemic infection leaves (Fig. 41). It was clear that failure of digestion with

*Apa*I was due to the absence of this restriction site in the RT-PCR products. This result indicated this hybrid TMV vector with a defective native TMV MP gene was able to move in the infected plants and that movement of this virus was therefore conferred by the SBWMV MP.

6.3.6 Sequence analysis of RT-PCR products

Although *Apa*I digestion of RT-PCT products indicated the presence of the mutated site in the virus progeny from both infected and systemic leaves, this still needed to be confirmed by direct evidence. Sequence analysis of RT-PCR products was performed to identify the mutated site. RT-PCR products derived from RNAs of p30BDMP inoculated *Nicotiana benthamiana* were sequenced from both directions with the same primers as used for the above RT-PCR analysis. Comparison with equivalent portion of the native TMV movement protein gene showed the absence of the *Apa*I site, GGGCC'C, in the RT-PCR products, which verified the persistence of mutation in virus progeny. Figure 42 and 43 show the full sequence of TMV MP gene and partial sequence of defective TMV movement protein gene, respectively.

6.3.7 Inoculation of tobacco plants with sap from leaves of p30B, p30BGFP, p30BMP and p30BDMP systemically infected plants.

To determine whether the mutated virus progeny multiplied from systemic infection remained infective, healthy plants were inoculated with sap inoculum. Inocula were prepared by grinding systemically infected leaves of p30BMP- and p30BDMP-infected-tobacco plants in 0.1 M potassium phosphate buffer pH 7.0. The sap from plants inoculated with buffer was used as negative control, while sap from p30B and p30BGFP systemically infected leaves was used as the positive control. Plants were inoculated as described in section 2.12. After inoculation, plants were kept in growth cabinet at 28 °C. Mosaic symptoms developed on all inoculated plants except the negative control within 10 days (Fig. 44). The p30BGFP-inoculated plants, showing typical symptoms, were checked under UV light to ensure successful mechanical inoculation.

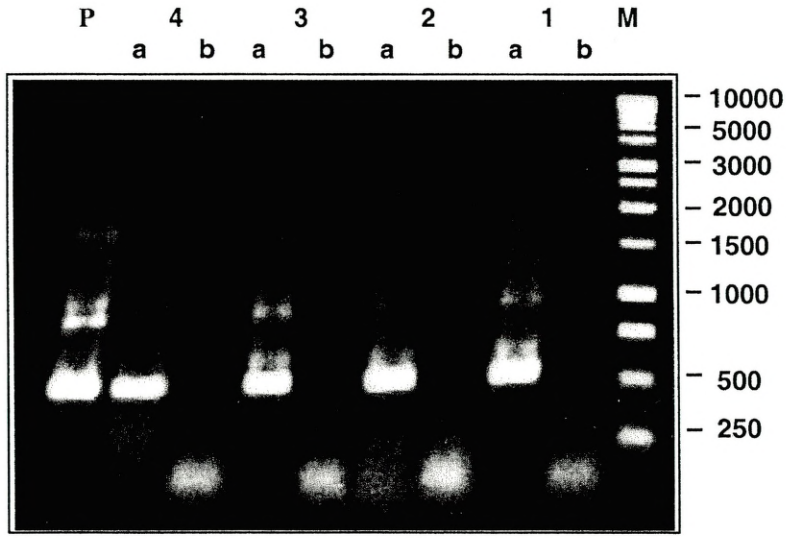


Figure 40. RT-PCR analysis of multiplied virus from inoculated plants using primers designed to amplify partial sequence of TMV MP gene. RT-PCR using 1) RNA sample from p30B-inoculated leaf; 2) RNA sample from systemically infected leaf of p30BMP-infected plant; 3) RNA sample from p30BDMP-inoculated leaf; 4) RNA sample from systemically infected leaf of p30BMDP-infected plant; a) With *Avian myeloblastosis virus* reverse transcriptase (AMV/RT+); b) Without *Avian myeloblastosis virus* reverse transcriptase (AMV/RT-); P) Plasmid p30DMP; M) 1 kb DNA molecular marker (Promega UK).

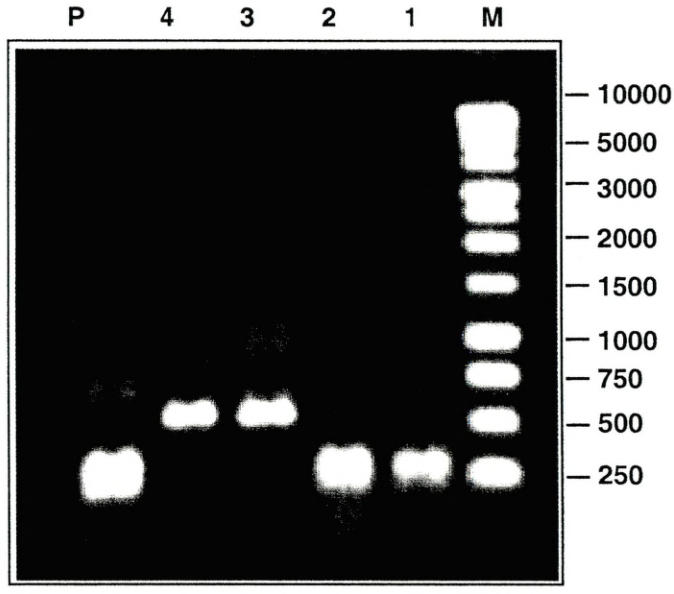


Figure 41. *Apa*I digestion of RT-PCR products. M: 1 kb DNA molecular marker (Promega UK); 1: product from RNAs of p30BMP inoculated leaf; 2: product from RNAs of systemically infected leaf of p30BMP infected plant; 3: product from RNAs of p30BDMP inoculated leaf; 4: product from RNAs of systemically infected leaf of p30BDMP infected plant; p: product from plasmid p30BMP.

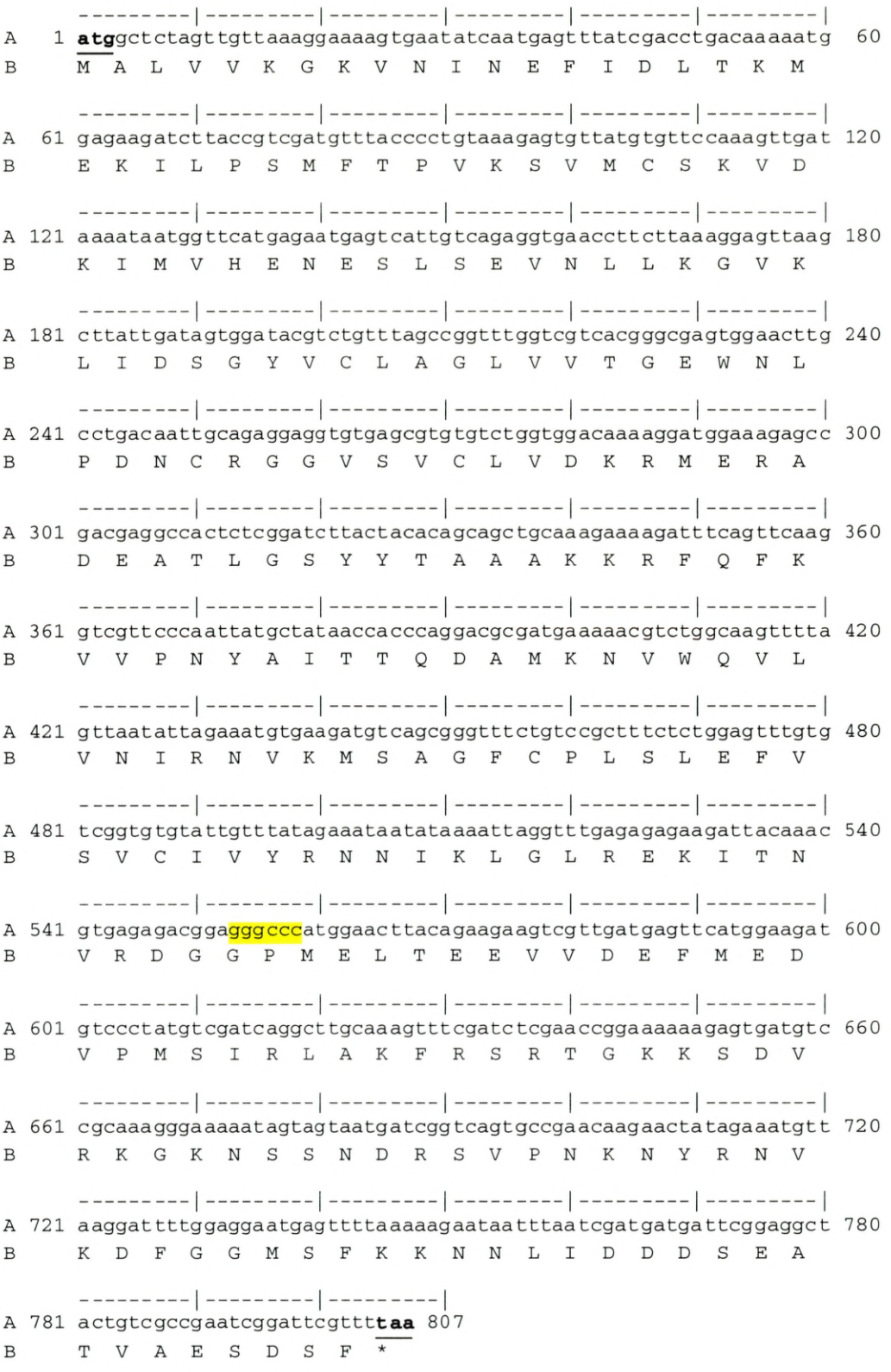


Figure 42. Nucleotide and coding sequences for movement protein of TMV U1 strain. A) nucleic acid sequence; B) amino acid sequence; **atg**: start codon; **taa**: stop codon; the *Apa*I restriction site was denoted in yellow.

(A)

5′ ⟶ 3′

306 CGCCACTCTC GGATCNTACT ACACAGCAGC TGCAAAGAAA AGATTTCAGT 355

356 TCAAGGTCGT TCCCAATTAT GCTATAACCA CCCAGGACGC GATGAAAAAC 405

406 GTCTGGCAAG TTTTAGTTAA TATTAGAAAT GTGAAGATGT CAGCGGGTTT 455

456 CTGTCCGCTT TCTCTGGAGT TTGTGTCGGT GTGTATTGTT TATAGAAATA 505

506 ATATAAAATT AGGTTTGAGA GAGAAGATTA CAAACGTGAG AGACGGAGCA 559

560 TGGAACTTAC AGAAGAAGTC GTTGATGAGT TCATGGAAGA TGTCCCTATG 609

610 TCGATCAGGC TTGCAAAGTT TCGATCTCGA ACCGGAAAAA AGAGTGATGT 659

660 CCGCAAAGGG AAAAATAGTA GTAGTGATCG GTCAGTGCCT AACAAGAACT 709

710 ATAGAAATGT TAAGGATTTT GGAGGAATGA GTTTTAAAAA GAATAATTTA 759

760 ATCGATGATG ATTCGGAGGC TACTG 784

(B)

5′ ⟶ 3′

303 GACCGCCACT CTCGGATCNT ACTACACAGC AGCTGCAAAG AAAAGATTTC 352

353 AGTTCAAGGT CGTTCCCAAT TATGCTATAA CCACCCAGGA CGCGATGAAA 402

403 AACGTCTGGC AAGTTTTAGT TAATATTAGA AATGTGAAGA TGTCAGCGGG 452

453 TTTCTGTCCG CTTTCTCTGG AGTTTGTGTC GGTGTGTATT GTTTATAGAA 502

503 ATAATATAAA ATTAGGTTTG AGAGAGAAGA TTACAAACGT GAGAGACGGA 552

553 GCATGGAACT TACAGAAGAA GTCGTTGATG AGTTCATGGA AGATGTCCCT 606

607 ATGTCGATCA GGCTTGCAAA GTTTCGATCT CGAACCGGAA AAAAGAGTGA 656

657 TGTCCGCAAA GGGAAAAATA GTAGTAGTGA TCGGTCAGTG CCTAACAAGA 706

707 ACTATAGAAA TGTTAAGGAT TTTGGAGGAA TGAGTTTTAA AAAGAATAAT 756

757 TTAATCGATG ATGATTCGGA GGCTACTGC 785

Figure 43. Partial sequence of RT-PCR product derived from RNA extracted from p30BDMP inoculated plants. RNA from A) inoculated leaves; B) systemic infection leaves; 5'⟶ 3') sequencing direction; the number indicated the sequencing region equivalent to the part of nucleotide sequence of TMV MP gene; the resultant mutation site by ORF shift is denoted in yellow; the newly-created stop codon resulting from the *Apa*I frame shift is denoted in purple.

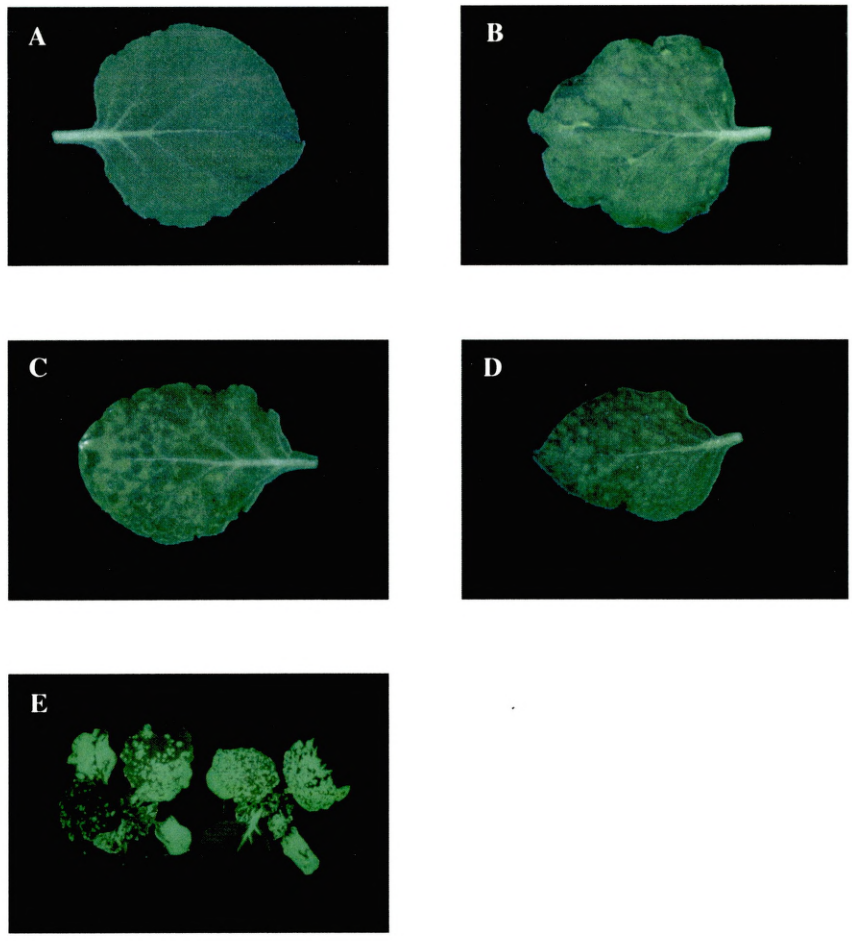


Figure 44. Mosaic symptom development in leaves of *Nicotiana benthamiana* 8 days after mechanical inoculation with sap made from healthy and virus infected tobacco. Sap from: A) healthy *Nicotiana benthamiana* leaves; B) p30B-infected plant leaves; C) p30BMP-infected plant leaves; D) p30BDMP-infected plant leaves; E) p30BGFP-infected plant leaves, plant checked under UV light (λ 366 nm).

6.3.8 Electron microscopy

Sap samples for electron microscopy were prepared from tobacco leaves systemically infected with p30BDMP following the procedures as described in section 2.8. After negative staining, virus was detected using the transmission electron microscope. Typical tobacco mosaic virus particles were found from the tested samples (work of I. Roberts, SCRI; pers. commmun.). This indicated that the functional replacement of the TMV MP gene with the SBWMV MP gene did not change the morphology of TMV virions.

6.3.9 Western blot analysis

For Western blot analysis, samples were prepared from the leaves of p30BMP- and p30BDMP-infected plants, and viral proteins separated by SDS-PAGE as described in Chapter 2. After transfer to nitrocellulose membranes, the proteins were probed with a polyclonal antibody against SBWMV 37 kDa MP. Results showed that the SBWMV movement protein might be detectable (shown as a 37 kDa band relative to the molecular weight standards) from the samples derived from infected leaves inoculated with p30BMP or p30BDMP *in vitro* transcripts. Several non-specific bands were also found in the same western blot, and because of that it was not possible to draw a definite conclusions in this experiment. However, no 37 kDa proteins were detected from either the positive control (frozen SBWMV infected wheat leaves) or systemic leaves from both infected plants.

Viral movement protein is mostly located in the cell wall fraction therefore it is normally difficult to extract MP from plant tissue using commonly used protein extraction methods. To improve the Western blot analysis attempts were made to enrich the movement proteins in extraction by fractionation of p30BMP and p30BDMP infected tobacco leaves following the procedures as described in section 6.2.7. 10 µl of extract was electrophoresed on a 10-20% SDS polyacrylamide gradient gel. Protein extracted from p30BGFP inoculated-tobacco was used as the positive control for the 30 kDa

TMV movement protein. Since neither the 37 kDa SBWMV movement protein nor fresh SBWMV-infected wheat leaves were available, a sample extracted from wheat plants infected by a SBWMV French isolate, provided by the Central Science Laboratory, was used as the positive control for the 37 kDa SBWMV MP. For both Western analyses, leaf extract from non-infected tobacco was used as the negative control.

A high intensity band of 37 kDa was detected in the cell wall fractions of all of p30BMP- and p30BDMP- infected plants when probed with antiserum against SBWMV 37 kDa movement protein (Fig. 45). The same size band was found from the positive control although the intensity of band was much less than those obtained from p30BMP and p30BDMP infected plants. Several bands of lesser intensity can be seen from both positive control and virus infected samples, but the pattern of these bands was different. No positive signal was detected from the cell wall fractions of healthy plants.

Using the antiserum against 30 kDa TMV MP, a protein with a molecular weight of 30 kDa was seen on Western blot in the cell wall fractions derived from p30BMP inoculated and systemically infected leaves and from the positive control p30BGFP infected plant, while, as expected, this band cannot be seen in extracts from healthy and p30BDMP infected plants (Fig. 46). A lower band with a molecular weight of approximately 27 kDa was found in the cell wall fractions from p30BDMP inoculated and systemic leaves. A strong non-specific cross reaction was found in this Western blot analysis when using polyclonal anterium against TMV 30 kDa MP as a major band was observed from all of the samples tested. Attempts were made to remove the cross-reaction by cross-absorption with sap made from healthy tobacco plants, but no improvement was seen in the subsequent Western analysis (data not shown).

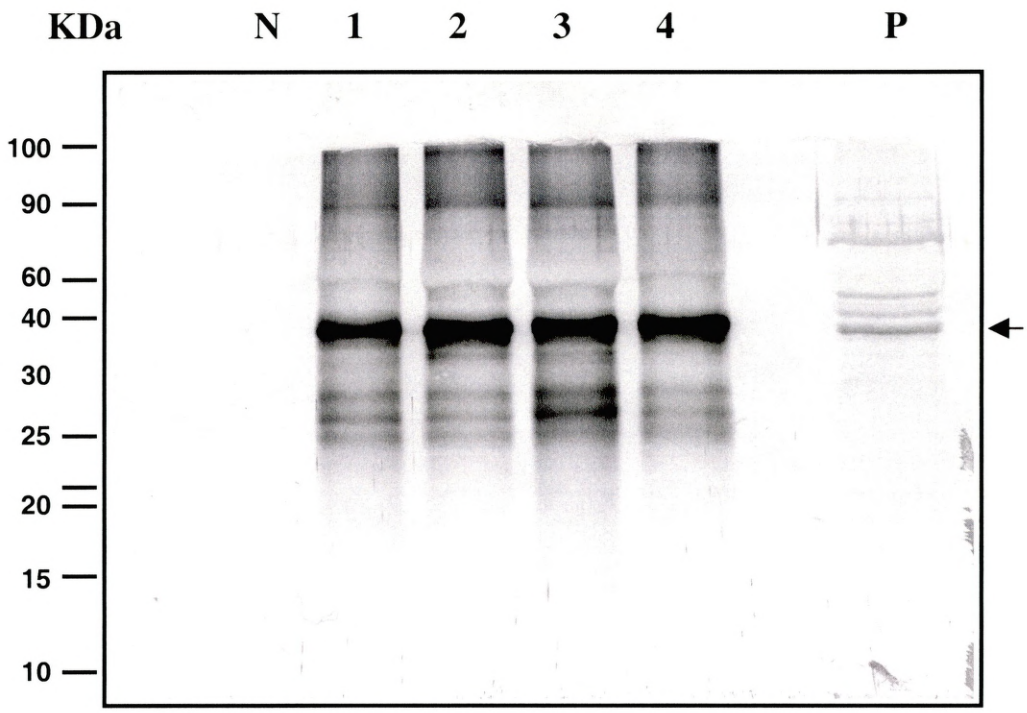


Figure 45. Western blot analysis of subcellular fractions of p30BMP and p30BDMP infected plants using antiserum against 37 kDa SBWMV movement protein. N) Negative control; 1) p30BMP-inoculated leaf sample; 2) p30BMP systemic infection leaf sample, 3) p30BDMP-inoculated leaf sample, 4) p30BDMP systemic infection leaf sample, P) Positive control leaf infected with French isolate of SBWMV (from Central Science Laboratory, UK).

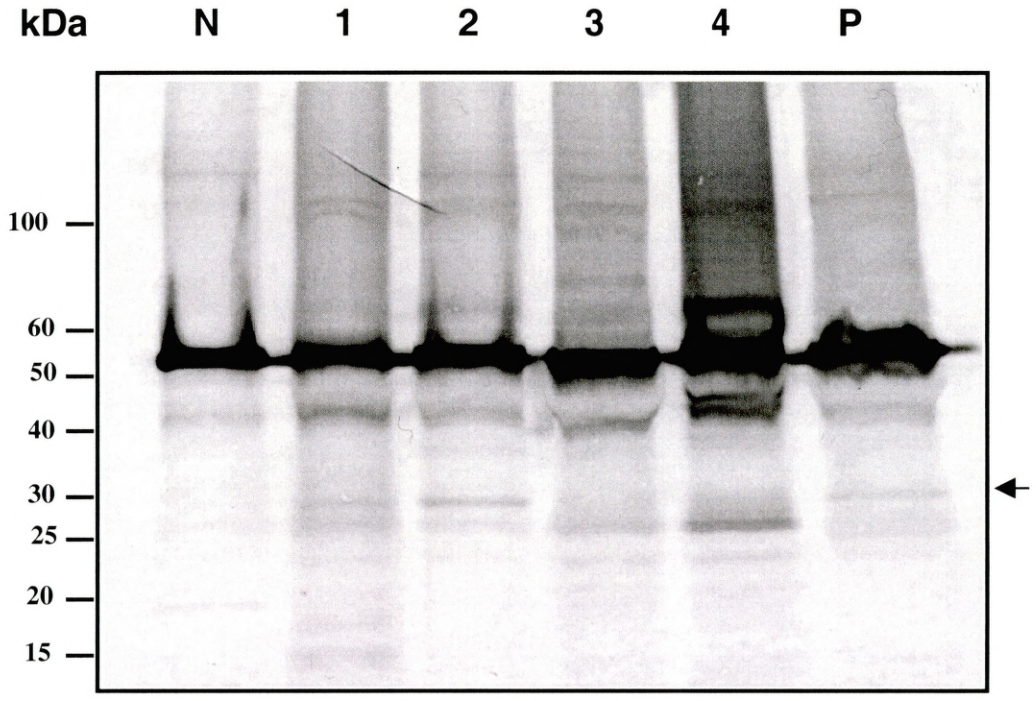


Figure 46. Western blot analysis of subcellular fractions of p30BMP and p30BDMP infected plants using antiserum against 30 kDa TMV movement protein. N) negative control (non-infected *Nicotiana B.*); 1) leaf inoculated with p30BMP; 2) p30BMP systemic infection leaf; 3) leaf inoculated with p30BDMP; 4) p30BDMP systemic infection leaf; P) Positive control (p30B inoculated leaf).

6.3.10 Inoculation of wheat plant with sap made from *Nicotiana benthamiana* infected by p30B, p30BGFP, p30BMP and p30BDMP

To determine if the hybrid TMV containing SBWMV 37 kDa MP gene is able to infect wheat plants, a non-host for TMV, wheat plants were mechanically inoculated with sap made from *N. benthamiana* inoculated with infectious transcripts of p30B, p30BGFP, p30BMP and p30BDMP (see section 6.2.5). No mosaic symptoms developed on any of the inoculated plants. Under UV light, no green fluorescent spots can be found from the plants inoculated with inoculum derived from p30BGFP infected *Nicotiana benthamiana*.

6.4 Discussion

A virus can become infectious to non-host plants with the help of related or unrelated viruses. This was proposed to be due possibly to the complementation of virus movement function between different viruses (Malyshenko *et al.*, 1989). Accumulating evidence has shown that movement proteins (MPs) from some very different taxonomic groups are able to complement each other. *Barley stripe mosaic virus* (BSMV) and TMV belong to two different taxonomic groups and sequence analysis has revealed that there is no sequence similarity between the MPs of these two viruses. BSMV containing TMV MP cDNA replacing the native BSMV MP gene was able to infect inoculated *N. benthamiana* leaves, but it failed to infect *Nicotiana benthamiana* systemically. When this hybrid BSMV was used to inoculate barley, a systemic host of BSMV, no infection was found. This was possibly due to fundamental difference in transport systems of these two viruses (Solovyev *et al.*, 1996).

In this study, a recombinant TMV-based vector was used to examine the possible functional complementation of movement by the SBWMV 37 kDa protein. Like p30B, both recombinant TMV vectors, containing the insert of full length SBWMV MP cDNA clone (p30BMP) and the same vector with the defective native TMV MP gene (p30BDMP), can cause systemic infection in inoculated *N. benthamiana*, but failed to infect wheat plants when inocu-

lated with infectious sap. This result indicated that the TMV MP could be functionally replaced by the SBWMV MP in the hybrid virus that was able to systemically infect *N. benthamiana*. TMV MP is defective due to a frame shift mutation introduced by digestion with *Apa*I and religation, hence the mutant TMV fails to induce any visible symptoms on inoculated *N. benthamiana* which is the natural host for TMV. Direct evidence for this resulted from the inoculation of the *N. benthamiana* with mutant p30BGFP *in vitro* transcript. It was found that the mutant virus was restricted in the original inoculated cells which showed individual green fluorescent spots under UV light (Wright *et al.*, 2000).

To confirm the defective TMV 30 kDa MP from virus genome of both p30BDMP inoculated and systemically infected leaves, RT-PCR products derived from total RNAs isolated from p30BDMP-inoculated and systemically infected leaves were analysed by restriction digestion and DNA sequencing. Failure of *Apa*I digestion of the RT-PCR products derived from both RNAs indicated the conservation of the mutation in amplified viruses. Sequence analysis of RT-PCR products derived from viruses extracted from both inoculated and systemically infected tobacco leaves also provided direct evidence, that the systemic infection by p30BDMP was not due to spontaneous mutation occurring in progeny virus. Electron microscopy revealed that the insertion of SBWMV 37 kDa movement protein coding sequence in TMV did not alter particle morphology.

Zhu found that TMV with a replacement of the native TMV MP coding sequence by SBWMV MP failed to infect *N. benthamiana,* and no local lesions developed in inoculated leaves (Zhu, personal communication). This was presumed to be due to a coincident defect in the RNA dependent RNA polymerase (RdRp) of the TMV genome resulting from this replacement. The removal of TMV MP coding sequence will unavoidably change the context of the C-terminus of TMV replicase because the replicase gene of TMV overlaps with that of 30 kDa MP. Therefore failure of functional complementation between 30 kDa MP of TMV and 37 kDa MP of SBWMV seems to be due to the defect in the replicase gene. There is evidence that in some cases repli-

case-associated proteins are associated with virus movement function (Traynor *et al.*, 1991; Weiland and Edwards, 1996; Deom *et al.*, 1997). However in other cases, expression of movement function seems to be independent of replicase-associated protein. Zhu also found when using the same recombinant TMV to inoculate Xanthi-nc, which is a local lesion host of TMV, no symptoms developed in inoculated leaves. However, numerous local lesions developed when using the same amount of *in vitro* transcripts to inoculate the TMV MP-transgenic Xanthi-NN plant. This was unlikely to be due to recombination between the virus and host plant that restored the wild type RNA since subsequent inoculation on the non-transgenic Xanthi-NN with sap derived from symptom developed leaves of inoculated transgenic Xanthi-NN plants resulted in no symptom development in the inoculated leaves. From this result it was clear that endogenous TMV 30 kDa MP could support the replication of recombinant TMV and local spread of infection and 30 kDa MP played a crucial role in determining host range.

Sequence analysis has revealed that MP of SBWMV has sequence similarity to that of TMV. However, the complementation of SBWMV and TMV MPs seems not to be due to the sequence homology. Since Zhu also observed that modified *Red clover necrotic mosaic virus* (RCNMV) containing MP of SBWMV was incapable of infecting barley, a natural host for these two viruses, although the MPs of SBWMV and RCNMV were found to have sequence similarity (X. Zhu, personal communication).

The results presented here demonstrate that the SBWMV MP can efficiently substitute the MP of TMV and also confirmed that the 37 kDa protein encoded by the third ORF of SBWMV RNA I functions as the movement protein of this virus. The complementation of cell-to-cell movement between related or unrelated plant viruses has been studied for many years however the mechanism underlying this phenomenon is not yet clear. To date, no consensus model exists for the mechanism of MP complementation from different viruses tested. It is generally believed that apart from the MPs, other virus encoded functions (CP, RNA dependent RNA polymerase) host factors

and the interactions between viral functions and host factors may together contribute to host specificity.

CHAPTER 7

SUMMARY AND FURTHER WORK

The research aims described in this thesis concern firstly the development and improvement of transgenic resistance in Chinese wheat cultivars against *Soil-borne wheat mosaic virus* and related viruses, and secondly the understanding of the mechanism by which virus spreads in infected plants through studying functional complementation of viral movement proteins between *Soil-borne wheat mosaic virus* and *Tobacco mosaic virus*, which may aid the development of the new strategies for conferring virus resistance in wheat.

Over the past two decades a soil-borne virus has become an increasingly important pathogen in winter-wheat growing regions in China, and has caused serious damage in wheat production. This might be attributed to the intensified monocultivation of the susceptible varieties and the spread of the transmission vector, *Polymyxa graminis*. Improvement of virus resistance by conventional breeding had little success and, furthermore, the available sources of natural resistance against SBWMV are very limited. Therefore, alternative strategies for conferring virus resistance need to be exploited.

At the start of this research sequence data of the Chinese virus isolate were not available. Therefore the cDNA clone of an American SBWMV isolate was employed to design transformation constructs. Near the end of this project, the sequence of the Chinese virus isolate had become available. Comparison of complete genomic RNA sequences between the Chinese and American isolates suggests that the Chinese virus represents a distinct species of *Furovirus*

genus, but closely related to *Soil-borne wheat mosaic virus*. However, expression of the American viral sequences in Chinese wheat may still confer resistance against infection with the homologous Chinese virus.

Initial work focused on the construction of expression vectors with viral coding sequences from the American isolate. The use of a suitable vector (pUbi35S) possessing a unique cloning site has facilitated the cloning of viral coding sequences. Thereby, a number of transformation constructs, containing either the functional CP gene, or dysfunctional MP gene and functional or dysfunctional replicase complex sequences were constructed. The constructs were tested for the expression of viral genes under control of the *Ubi-1* promoter in transfected tobacco protoplasts prior to transforming wheat plants. RT-PCR analysis demonstrated the expression of a functional CP gene at the RNA level. However, no expression products were detected from protoplasts transfected with constructs containing dysfunctional MP gene and functional and dysfunctional replicase coding sequences. Successful expression of CP gene in the same vector demonstrated the efficient function of this promoter in driving gene expression. Therefore, the failure of expression of other viral genes may be due to the specific characteristics of those genes. Further studies should be undertaken to compare the effect of functional and dysfunctional MP coding sequences on gene expression, and also the expression of the replicase gene might be detectable using specific primers to amplify an internal fragment of the gene.

Introducing SBWMV CP and MP genes into several Chinese wheat cultivars as well as a model spring wheat cv. Bob White was accomplished by particle bombardment of pre-cultured immature embryos. The average transformation frequencies were relatively lower for both cv. Bob White and Chinese wheat cultivars compared with those published previously (Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994; Altpeter *et al.* 1996). This was possibly due to the poor state of explants and the difference of *in vitro* response in different genotypes, because the physiological condition and the

developmental state of the explant, along with the genotype had been found to be the most significant factors in determining *in vitro* response (Vasil, 1999). Increasing transformation frequency could be achieved by growing the plants in optimal greenhouse conditions and by improving tissue culture protocols for Chinese wheat cultivars from which fertile plants could be regenerated at a high frequency.

PCR and Southern analysis of transgenic plants regenerated from biolistic transformation experiments, followed by effective selection in the presence of PPT, provided evidence for the integration of both selectable marker gene and viral coding sequences into both Bob White and three Chinese wheat cultivars. Southern blotting also revealed that both CP and MP genes were inserted into the host genomic DNA, either with one or multiple copies. PCR analysis of R1 progenies from several selected CP- and MP-transgenic lines demonstrated that both CP and MP genes were inherited in a Mendelian fashion from most of the transgenic lines analysed.

Analysis of the CP-transgenic wheat lines showed that most lines expressed CP-specific RNA transcripts. Failure to detect CP gene expression in other CP-transgenic lines might be due to the CP coding sequence being inserted at a site with a low level of expression, or, the insertion of CP coding sequence altering the host genome structure, resulting in transcriptional gene silencing. Western blotting analysis of several CP-transgenic plants showed that no viral CP could be detected from any transgenic lines analysed. It is not known whether this was caused by inefficient protein extraction, or improper folding of the CP, resulting in decreased stability of the protein. Although no CP was detectable from the CP-transgenic plants by Western blotting, the more sensitive *ELISA* approach showed one line was found to produce and accumulate CP. To test whether transgenic plants were resistant to virus infection, CP- and MP-transgenic plants were mechanically inoculated with *Chinese wheat mosaic virus*. *ELISA* testing of the inoculated plants has shown that virus was not accumulated in some transgenic plants from several CP- and

MP-transgenic lines. It remains to be tested whether this was due to the failure of technical inoculation, or the expression of viral coding sequences conferring resistance against CWMV infection in these transgenic plants. The transgenic plants have yet to be tested for possible resistance to SBWMV.

Public concerns over genetically modified crops have stimulated scientists to develop new selection strategies from which transgenic plants can be produced without undesirable traits. A new selection approach based on the phosphomannose isomerase (*pmi*) gene as the selectable marker, and mannose as the selective agent was recently developed. Using this approach, recovery of transgenic plants has been achieved from a limited number of crop species (Donaldson *et al.*, 1998; Joerbo *et al.*, 1998; Zhang *et a*l., 1999). However, there is no report on the production of transgenic wheat using this selection system. In Chapter 5, work was focused on the development of the mannose selection approach for genetic transformation of wheat. Using this approach, *uid*A gene and SBWMV CP gene were successfully transformed into Bob White and Chinese wheat cultivars, respectively. Presence of both *pmi* and *uid*A genes in transgenic Bob White plants was confirmed by PCR testing and the expression of *uid*A gene was demonstrated by GUS histochemical assay. PCR and Southern analysis of transgenic plants regenerated from particle bombarded immature embryos of Chinese wheat cultivars, followed by selection in presence of mannose, provided evidence for the integration of both *pmi* and CP genes. Although a large number of escape wheat plants were obtained in this experiment, results demonstrated the successful transformation of wheat by applying this most promising selection system. Further refinement of this protocol is required so as to regenerate transgenic wheat plants with a high efficiency.

Virus movement protein(s) play an important role in determining pathogenicity and virulence. The understanding of virus MPs and the mechanisms by which they function may lead to the development of new practical strategies for virus resistance in plants. For example, the expression of

mutant virus MP in transgenic plants may interfere with the movement function of invading virus, leading to plant resistance to infection by this virus. The complementarity of movement proteins from different viruses provides a very useful tool to study the function of movement proteins from related and unrelated viruses. Sequence analysis of the third ORF of SBWMV RNA I suggested the 37 kDa protein encoded by this ORF may function as the virus MP (Shirako *et al.*, 1993). The results in Chapter 7 demonstrated that p37 could be functionally complementary to the 30 kDa TMV MP, confirming that the p37 is the MP of SBWMV.

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