# Transgenic Expression of Malaria Surface Antigens Under the Control of Phaseolin Promoter

By

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A Thesis Submitted in Partial fulfillment of
the Requirement for the degree of
Master of Philosophy
in
Biology

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September 2004

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# Statement

The construction of pBI/Phas/MP42 and pBI/Phas/MP19 and their plant transformations were performed by W.K. Ng. The construction of pET-MP42 for bacterial induction and the preparation of rabbit anti-His-$MSP1_{42}$ serum were prepared by O. S. Lau. All the other experimental work reported in this thesis was performed by the author, unless specially stated otherwise in the text.

W. L. W. CHAN

# Acknowledgements

I sincerely thank Prof. S. S. M. Sun for supervising my Mphil studies, especially his long-term guidance, his advice on my future career and the thesis review. His valuable experience sharing and comments give positive influence on my attitude.

Also, I truly thank Prof. L. Jiang, my co-supervisor, for his guidance and advice on my project, especially the protein-targeting constructs and the GUS fusion constructs; Prof. H. M. Lam and Prof. Y. S. Wong, other committee members, for giving me advice on my research and reviewing the thesis; and Prof. W. K. Yip for being my external examinar and reviewing the thesis.

I thank Prof. S.P. Chang who provided the native $MSP1_{42}$ cDNA, Prof. W.K.K. Ho for the monoclonal antibody Mab5.2, Prof. Z.D. Wu who provided the malaria-infected human serum, Prof. M.C. Fung for bringing that serum to our lab, Danny Ng and On Sun Lau for their previous works which paved the way to my study on this project.

I thank all my labmates: Dr. Daming Chen, Fiona Chan, Rebacca Chan, Yuk Wah Chan, Stanley Cheung, Kenneth Chik, Maggie Chong, Smile Duan, William Ho, Tobi Kwok, Juon Juon Lee, Trista Leung, Melinda Liu, Dr. Qiaoquan Liu, Steffi Long,

Cathy Luk, Ruby Ng, Eric Suen, Annual Tam, Jennifer Wei, Gundum Wong, Dr Changjie Yan, Pauline Yip, Dr. Abby Yu, Angela Yu, Marilyn Yu, Ronald Yu, Dingyuan Yuan, Junjun Zheng, and many others, for their longtime support, care and advice on my project.

Also, thanks to Mr. Shu Wa Ngai, Hung Kin Man and Bondy Lai for the planting work in the registered greenhouse and experimental field; and the members of AoE, G92, G93, G94, G95, G96, 387, Team A and Team C in Biology department for their help.

Finally, special thanks to my family and my boy friend Ming Ho who have given me a lot of support during my studies. Moreover, I give my Lord thanks, as without him, this work would not be possible.

# Abstract

Malaria is one of the major health problems in the world, especially in many developing countries and *Plasmodium falciparum* causes the majority of infection. The efficacies of fragments 42-kDa and 19-kDa at the C terminus ($MSP1_{42}$ & $MSP1_{19}$) of merozoite surface protein 1 (MSP1) of *P. falciparum* in inducing protective immunity were suggested as two leading malaria vaccine candidates. However, in a previous work, our group was not successful in detecting the mRNA or protein of $MSP1_{42}$ in transgenic plants. To improve expression, the destabilizing signals in mRNA were avoided and codon usage was optimized, through sequence modification. The expression of the modified genes was detected, firstly, in transient expression study by using β-glucuronidase (GUS) as a reporter gene and then in transgenic *Arabidopsis* in the present study. Transgenic *Arabidopsis* with homozygous transgenes were generated subsequently and the expression of the modified $MSP1_{42}$ and $MSP1_{19}$ were detected at RNA level but not protein level.

Further optimization of transgene expression was attempted by fusing the modified MSP1 cDNAs either with protein-targeting sequences, BP-80, α-TIP and RMR or GUS. These two sets of expression constructs, driven by the seed-specific phaseolin promoter, were studied using tobacco as a model plant system. Transient assay of the GUS fusion constructs was performed before plant transformation and the constructs without phaseolin signal peptide could give protein expression. As glycosylation of the GUS protein with signal peptide in the secretary pathway will inactivate the GUS activity, modified GUS gene was made. Expression of all these constructs in plant was detected by northern and western blotting, amounting to 0.4% of total extractable seed protein, except constructs RMR or GUS without phaseolin

signal peptide. This level of protein expression represents a significant improvement in producing the MSP1 protein in plant system. The transgene expression of the malaria surface antigen by the protein-targeting or GUS fusion strategy in this study provides a novel approach in designing constructs for expressing foreign proteins in plant system.

# 摘要

瘧疾是世界上其中一種主要的疾病，尤其在發展中的國家，情況更爲嚴重，而原生動物惡性瘧疾蟲 (*Plasmodium falciparum)* 引致最嚴重的瘧疾感染。而可用來誘發保護免疫反應的惡性瘧疾蟲的一號裂殖孢子表面蛋白 $MSP1_{42}$ 和 $MSP1_{19}$是兩種領先的瘧疾疫苗侯選者。

可是，我們已往未能成功在植物中表達它們的核糖核酸 (mRNA) 和蛋白質。因此，我們嘗試透過修改其互補去氧核糖核酸 (cDNA) 避免那些不穩定的轉錄序列信號以及優化它的密碼子選擇，從而改善其表達。修改了的基因，其表達首先在本研究中利用β-葡萄甘酸酶(GUS)作爲報告基因，測得其瞬間表達，及後在轉基因的擬南芥菜亦被測量到。之後我們在純合轉基因的擬南芥菜中測量得到那些已被修改了的 $MSP1_{42}$ 和 $MSP1_{19}$的核糖核酸，但卻未能測量到其蛋白質。

透過把修改了的一號裂殖孢子表面蛋白的去氧核糖核酸與蛋白質靶向序列 BP-80, α-TIP and RMR 或 β-葡萄甘酸酶的融合去進一步優化轉基因的表達。我們把這兩組由菜豆蛋白基因訊號肽調控的表達載體，導入植物模型系統煙草中作研究。在導入植物之前，我們利用瞬間表達去測試 β-葡萄甘酸酶融合的載體，而我們發現沒有菜豆蛋白基因訊號肽的載體可有蛋白質的表達。而且，由於有蛋白基因啓動子的β-葡萄甘酸酶在分泌途徑中的糖基化作用會使β-葡萄甘酸酶的活動變得不活躍，因此我們準備了修改了的 β-葡萄甘酸酶 。

所有這些載體在植物的表達，可利用北方印跡和韋斯頓印跡去測定。除了 RMR 或沒有菜豆蛋白基因訊號肽的 β-葡萄甘酸酶外，我們成功檢測到基因的表達，而蛋白量約佔總種子可提取蛋白的 0.4%。這蛋白的含量表示了惡性瘧疾

蟲的一號裂殖孢子表面蛋白在植物的產量有顯著改善。而且，在這項研究中，惡性瘧疾蟲的一號裂殖孢子表面蛋白經過與蛋白質靶向或 β-葡萄甘酸酶的融合後的轉基因表達結果，爲我們提供了設計在植物系統中生產外來蛋白載體的創新方法。

# List of Abbreviations

| | |
|---|---|
| +ve | Positive |
| BCA | Bicinochoninic acid |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| CaMV | Cauliflower mosaic virus |
| cDNA | Complementary DNA |
| CTAB | Cetyltrimethylammonium bromide |
| DIG | Digoxigenin |
| DNA | Deoxyribonucleic acids |
| dNTP | Deoxyribonucleotide triphosphate |
| DTT | Dithiothreitol |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetra-acetic acid |
| EK | Enterokinase |
| GUS | β-Glucuronidase |
| GUSN | β-Glucuronidase without its start codon, ATG |
| His | Histidine |
| Kb | Kilobases |
| KD | Kilodalton |
| MGUSN | Modified β-Glucuronidase without its start codon, ATG |
| MP42/19 | Modified native cDNA encoding $MSP1_{42/19}$ |
| mRNA | Messenger ribonucleic acid |
| MS | Murashige and Skoog medium |

| | |
|---|---|
| MSP-1 | Merozoite surface protein-1 |
| MW | Molecular weight |
| Nos | Nopaline synthase |
| NPTII | Neomycin phosphotransferase II |
| OD | Optical density (Absorbance) |
| OFP | Orange fluorescent protein |
| P42/P19 | Native cDNA encoding $MSP1_{42/19}$ |
| PAGE | Polyacrylamide gel electrophoresis |
| PCR | Polymerase chain reaction |
| Phasp | Phaseolin promoter |
| Phast | Phaseolin terminator |
| PVDF | Polyvinylidene difluoride |
| R1 | First generation of R0 |
| R2 | Second generation of R0 |
| R3 | Third generation of R0 |
| RNase | Ribonuclease |
| rNTP | Ribonucleotide triphosphate |
| R0 | The generation of plants to be transformed |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SDS | Sodium dodecyl sulfate |
| SP | Signal peptide |
| TAE | Tris-acetate/EDTA |
| t-DNA | Transfer DNA |
| TE | Tris-EDTA |
| TEMED | N,N,N,N-tetramethylethylenediamine |

| | |
|---|---|
| Ti plasmid | Tumor-inducing plasmid |
| -ve | Negative |
| WT | Wild type |
| X-gluc | 5-bromo-4-chloro-3-indolyl-β-glucuronic acid |

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# Chapter 1 General Introduction

Malaria is one of the major health problems in the world, especially in many developing countries. It is caused in human by four species of *Plasmodium* protozoa parasite and *P. falciparum* is the species that causes the majority of infection. Between 300-500 million people in the world are infected with the disease. The global resurgence of malaria, because of the spread of drug-resistant *P. falciparum*, brings further burden to the world, especially the developing countries. This burden intensifies our efforts to develop a malaria vaccine, especially inexpensive and affordable ones.

Using plants as expression system for vaccine production is an attractive option because of the low production cost, safety to human and functional antigen production. It will more desirable if plants can serve as edible vaccine, as costs of purification, manufacturing and administration will be greatly reduced.

Merozoite surface protein 1 (MSP1) of *P. falciparum* was found to protect the *Aotus* monkeys from a lethal challenge with the parasites (Chang *et al,* 1996). Both the two C-terminal fragments, $MSP1_{42}$ & $MSP1_{19,}$ of MSP1 are potential candidates for vaccine production, because they were shown to be functional antigens against the parasites.

Our group has been trying to use plants as bioreactor to produce these two leading malaria antigens, $MSP1_{42}$ & $MSP1_{19.}$ However, codon usage problem and destabilizing signals in the mRNA of MSP1 in the plant system have resulted in expression of undetectable to low level of truncated mRNA and undetectable protein of $MSP1_{42}$ in transgenic plants. Modification of the cDNAs was then carried out.

Subsequent studies of the modified $MSP1_{42}$ by our group revealed that recombinant $MSP1_{42}$ could be produced as 0.2% of total soluble protein. This expression level represents a great improvement as only 0.0035% of $MSP1_{19}$ was detected by another group (Ghosh *et al.*, 2002).

In this study, further optimization was attempted using the strategies of fusing the $MSP1_{42}$ and $MSP1_{19}$ with protein-targeting signals or GUS. We reason that target foreign proteins to appropriate compartments may enhance their stable accumulation. Thus Peabp80 (BP-80), α-tonoplast intrinsic protein (α-TIP) and ReMember-RingH2 membrane protein (RMR) were used in the chimeric constructs. BP-80 can target a foreign protein to the globoid portion of the protein storage vacuole (PSV) via the Golgi; α-tonoplast intrinsic protein (α-TIP) directs a protein to the PSV, bypassing the Golgi; RMR targets the protein to the crystalloid region of the protein storage vacuole.

As GUS protein can be expressed stably in plant system, we also reason that fusion of a target protein with GUS may also provide stabilizing effect to the protein. In addition, GUS serves as a marker at the same time to indicate the introduction of the transgene in the plant system. Since glycosylation of the GUS protein in the secretory pathway inactivates its GUS activity, modified GUS gene was thus made.

Transient assay by using particle bombardment was firstly used to test the transcribability and translatability of the GUS fusion constructs before their transformation into tobacco.

# Chapter 2 Literature Review

## 2.1 Malaria

Malaria is one of the major health problems in the world, with 102 countries being affected and nearly ten percent of the global population suffering from it annually (Shi *et al.*, 1996). The World Health Organization (WHO) estimated that 300-500 million cases of malaria with 1.5-2.7 million deaths occur each year. The disease is concentrated in the developing countries, for example, in sub-Saharan Africa alone, more than 150 million clinical cases with up to 2 million deaths every year (Moorthy and Hill, 2002). The bulk of malaria-related morbidity and mortality in an endemic setting is concentrated in children below the age of five, for instance, in Africa, million of children die from this disease every year (Miller *et al.*, 2002).

The control of malaria becomes less successful because of the occurrence of drug-resistance parasite. This brings economic burdens to many countries (Oaks *et al.*, 1991). Therefore, new drugs, especially inexpensive and affordable one, and more practical formulations of existing drugs/compounds are badly needed.

## 2.2 History of Malaria

Before 1950, malaria caused a major death in the central highlands of the African island nation of Madagascar, but in the late 1950s, malaria virtually disappeared as an aggressive insecticide spray was carried out and a victory in the battle against malaria achieved in 1970s. However, the disease reinvaded the highlands in the early 1980s as the spraying program stopped. This caused another boom of death as the older population had lost the partial immunity they once had and the younger one did not have any immunity to the disease (Oaks *et al.*, 1991). Then, by the use of antimalarial drugs, such as chloroquine, with outstanding properties against malaria and the slow speed of drug resistance developed by the parasites, malaria had been controlled for many years later. However, nowadays, malaria parasite has developed resistance to the antimalarial drugs to certain extent (Ridley, 2002). Therefore, developing an effective antimalarial drug or vaccine is urgent in need.

## 2.3 Malaria parasite

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium* which has a complex life cycle between the mosquito vector and the human host. These are four species of single-cell highly specific *Plasmodium* protozoa parasites: *P.*

*falciparum, P. vivax, P. ovale,* and *P. malaria* that cause malaria in humans, of which *P. falciparum* is the most pathogenic, accounting for the majority of infections and the most fatal (Oaks *et al.*, 1991; Wipasa *et al*, 2002).

## 2.4 Life cycle

The life cycle of malaria parasite is complex and can be divided into three phases: the liver phase, blood phase, and the mosquito phase. (Oaks *et al.*, 1991; Miller *et al.*, 2002; Moorthy *et al.*, 2002)

**Liver stage (pre-erythrocytic stage)**

The malaria parasites, in the form of sporozoites with about 5-20 in number are injected by the malaria parasite carrier female mosquito into the human host through each biting, usually into the subcutaneous tissue. Then, the sporozoites migrate through bloodstream within minutes and enter hepatocytes in which the sporozoites multiplies and differentirates into a liver-stage trophozoites (Moorthy *et al.*, 2002).

**Blood stage (asexual blood stage)**

In liver, each schizont which differentiates from a trophozoite during hepatocyte lysis releases 10,000 –30,000 merozoites which are able to invade other red blood cells. Afterward, cycles of red blood cell invasion with red blood cell lysis is triggered continuously until the death of the human host or control by the immune system of the

host (Miller *et al.*, 2002; Moorthy *et al.*, 2002).

**Mosquito stage (sexual blood stage)**

Some merozoites will differentiate into gametocytes which will be ingested by an anopheline mosquito. After, in the female mosquito, the gametocyte develops into oocyst and then sporosite which swims to the salivary glands to complete the life cycle. If the infected mosquito bites on a human host, the sporosite will be injected to the human host and another infection will be caused (Moorthy *et al.*, 2002).

## 2.5 Potential use of malaria vaccine

The idea of malaria vaccine is feasible as its potential use was supported by several studies, including for example, diversity of immune responses in humans to malaria antigens (Richie and Saul, 2002); stimulation of protective immunity in mice by the injection of X-irradiated sporozoites (Nash *et al.*, 1967); passive transfer of human immunity by vaccination with potential candidate, γ-globulin purified from human serum (Cohen, 1961); different extents of protection of the rodents by using recombinant protein of malaria antigen (Chang *et al.*, 1996; Kumar *et al.*, 2000; Stowers and Chen *et al.*, 2002; Stowers and Kennedy *et al.*, 2002); and protection of mice from blood-stage challenge with sporozoites by the use of malaria DNA vaccines (Gardner *et al.*, 1996).

There are many potential targets for vaccination because of the complex life cycle of the malaria parasite. For instance, antibodies to sporozoites and interferon gamma can respectively prevent invasion of hepatocytes and inhibit the development of liver stage; antibodies to merozoites can prevent the invasion of red blood cells; and antibodies to gametocytes can prevent development into sporozoites (Oaks *et al.*, 1991; Holder, 1999). As asexual blood stage is the only stage of the parasite life cycle that causes clinical symptoms and death in human host, it is thus a potential candidate for vaccination. Among these potential candidates, P. *falciparum* merozoite surface protein 1 (MSP1) is the most studied.

## 2.6 Merozoite Surface Protein 1 (MSP1)

The merozoite surface protein 1 (MSP1) is naturally immunogenic. Immunization of nonhuman primates with native or recombinant MSP-1 was found to develop various degrees of resistance to the challenge of *P. falciparum* (Egan *et al*, 1997). MSP1, the major surface membrane-associated protein expressed by merozoites during the asexual erythrocytic stage, is a relatively abundant protein which has been found in all *Plasmodium* species examined.

MSP1 is synthesized as a high molecular mass precursor protein, with 195 kDa, which is proteolytically processed into four smaller fragments (Wiser *et al*, 1996; Shi

*et al*, 1996; Wu *et al*, 2000). The proteolytic processing of MSP1 is a two-step procedure characterized by primary and secondary processing events. The primary processing occurs at, or just before, the terminal merozoite differentiation and results in the formation and release of a noncovalent polypeptide complex with four fragments of approximately 83, 30, 38 and 42 kDa (Egan *et al*, 1997). These discrete products reside on the surface of the merozoite, but most of these fragments disappear at the time the merozoite invades the erythrocyte (Shi *et al*, 1996).

The secondary processing results in the shedding of a soluble MSP1. The 42 kDa C-terminal fragment which is directly attached to the merozite surface via a glycoslphosphatidylinositol anchor is further processed into 33 and 19 kDa fragments at the time of merozoite invasion, i.e. during subsequent contact with an uninfected erythrocyte. The 33 kDa fragment is shed from the merozoite surface prior to erythrocyte invasion and the remaining glycosylphosphatidylinositol-anchored 19 kDa fragment which contains two epidermal growth factor-like modules is carried into the erythrocyte upon merozoite invasion (Egan *et al*, 1997).

## 2.7 Potential use of MSP1

The *P. falciparum* MSP1 is a blood stage antigen which has been studied extensively. Although MSP1 is polymorphic, its cysteine-rich carboxy-terminus

shows only four single amino acid changes among the strains. Also, nonhuman primates could be protected from the challenges of P. falciparum by vaccination with MSP1 produced by parasite culture (Chang et al, 1996) and an immune response to MSP1 is expected to stop merozoite invasion of erythrocytes — the developmental stage of the parasite that causes clinical malaria. (Marriott *et al*, 2000). Therefore, it is a potential vaccine candidate.

However, a correct expression system of the MSP1 should be used for the production of a functional antigen because the sequence encoding the protective MSP1 epitopes, which are disulfide-dependent conformation, may play an important role in immunity (Chang *et al,* 1996; Wu *et al*, 2000). For example, by the use of recombinant MSP1 polypeptides produced by a bacterial expression system, no level of immunity to *P. falciparum* infection was determined when compared to those produced by parasite (Chang *et al,* 1996; Wu *et al*, 2000).

## 2.8 Significance of MSP1 C-terminal fragments

### 2.8.1 Significance of $MSP1_{42}$

The 42-kDa fragment of the C terminal of MSP1 ($MSP1_{42}$) contains a cluster of cysteine residues which are highly conserved among the divergent *Plasmodium* species and these cysteine residues contribute to the disulfide bonds which participate

in conformation epitopes. These conformational epitopes of $MSP1_{42}$, gp-195-specific, are critical for the induction of parasite growth-inhibitory antibodies (Chang *et al*, 1992). For example, MSP1 specific antibody was produced in *Aotus* monkeys after immunization with BVp42, a baculovirus recombinant polypeptide based on the MSP1 (Chang *et al,* 1996), and an inhibitory activity of anti- $MSP1_{42}$ antibodies from rabbit serum against malaria parasites was observed (Pang *et al*, 2002).

The immunogenicity and protective efficacy of a baculovirus recombinant polypeptide based on the MSP1 has been evaluated in *Aotus lemurinus griseimembra* moneys. Immunization of *Aotus* monkeys with BVp42 resulted in high antibody titers against the immunogen as well as parasite MSP1 with closely approximate the antgenicity and immunogenicity of the native MSP1 C-terminal processing fragment (Chang *et al,* 1996). Also, high-titer antibody against $MSP1_{42}$ epitopes was triggered in rabbits which were vaccinated with the recombinant $MSP1_{42}$ produced by virus-silkworm expression system (Pang *et al,* 2002).

These findings suggest that $MSP1_{42}$ contains the sequence encoding the essential epitopes which can be recognized by the antibody and can trigger the immune response in the tested animals. Therefore, $MSP1_{42}$ is a potential candidate for malaria vaccine production.

### 2.8.2 Significance of $MSP1_{19}$

The C-terminal 19-kDa fragment ($MSP1_{19}$), the only fragment, remaining on the merozoite surface after the secondary processing of the 42-kDa fragment at the C-terminal region during erythrocyte invasion, is an ideal target for blocking parasite invasion into the erythrocyte (Shi *et al*, 1996).

The 19-kDa fragment is highly conserved, with only four amino acid differences in 96 residues (Egan *et al*, 1997). $MSP1_{19}$ contains two cysteine-rich epidermal growth factor (EGF)-like domains, each of which consists of 45-50 amino acids contributing to the disulfide bond pattern in the critical conformational epitopes (Burghaus *et al*, 1993; Morgan *et al*, 1999). Other research study also demonstrated that the antigen of the yeast-expressed recombinant 19-kDa fragment of *P. falciparum* MSP-1 processes a majority of the protective epitopes of the C-terminal 42-kDa region (Shi *et al*, 1996) and these reduction-sensitive epitopes can be recognized by the monoclonal or polyclonal antibodies which can stop the red blood cell invasion (Donnell *et al*, 2001). For example, natural immune response was found in human who lived in the malaria affected areas and showed immunization to the recombinant protein $MSP1_{19}$ produced by parasites (Shi *et al*, 1996). Also, high levels of antibodies to $MSP1_{19}$ were elicited in the *Aotus* monkeys which were vaccinated with that recombinant protein produced by yeast (Kumar et al, 2000).

## 2.9 Production of MSP1 C-terminal fragments

Many different types of systems have been used for the production of the recombinant proteins for vaccination, such as in bacteria (*Escherichia coli, Salmonella typhi)*, baculovirus, baculovirus-silkworm, parasites, yeast, the milk of transgenic mice, mammalian cells, tobacco and *Arabidopsis* (Chang et al, 1992; Burghaus et al, 1993; Chang et al, 1996; Shi et al, 1996; Egan et al, 1997; Pan et al, 1999; Kumar et al, 2000; Wu et al, 2000; Stowers et al, 2002; Pang et al, 2002; Ghosh et al, 2002; Lau, 2003). Different degrees of protein expression were found. Also, inhibition of parasite growth, rise of antibody levels and immunity responses were detected in tested individuals.

## 2.10 Plants as bioreactor

Producing desired recombinant proteins by transgenic plants benefits in several ways. First, the cost of plant growing is low. Second, scale up is feasible in planting. Therefore, a bulk quantity of the protein can be produced economically. Third, the ease in harvesting, transporting, storing and processing is also an advantage.

There are several other potential advantages by producing proteins in plants over microbial fermentation, animal-cell cultures and transgenic animals, including for example, limited capacity for production of the accurate post-translational

modification of eukaryotic proteins in bacterial fermentation; substantial costs involved in bacterial fermentation and animal-cell culture; and public and ethical concerns risen by the use of transgenic animals (Kusnadi et al, 1997; Oscar et al,1995).

Successful productions of materials like carbohydrates, lipids and proteins, such as high-value pharmaceutical polypeptides, industrial enzymes, in plants had been demonstrated (Oscar *et al*, 1995; Hood *et al*, 1999; Mercenier *et al*, 2001), for example, production of the B-subunit of heat-labile toxin in potato amounting to 1.8% of total soluble protein (Hugh *et al,* 1998); Norwalk virus capsid protein expressed in transgenic tobacco and potato with orally immunogenic in mice (Mason *et al*, 1996); and hepatitis B surface antigen expressed in transgenic tobacco and immune response in mice fed with the hepatitis B surface antigen transgenic potato tubers (Mason *et al*, 1992; Richter *et al*, 2000).

Vaccine production by the use of plants has potential advantages over the traditional methods, for example, eliminating the chance of infection to innate toxic as subunit of the pathogen or toxin is produced in plant; avoiding pathogen contamination, as no known human or animal pathogens that are infections to plants; formulating multi-component vaccines by breeding the seed of transgenic lines expressing different proteins; and producing oral vaccine by expressing the desired

protein in the commonly consumed food plants (Streatfield *et al*, 2001).

## 2.11 Expression of MSP1 C-terminal fragments in transgenic plants

The expressions of MSP1 C-terminal fragments in transgenic plants were studied previously (Ng, 2001; Ghosh et al., 2002; Lau, 2003).

Expressing $MSP1_{42}$ and $MSP1_{19}$ in tobacco and *Arabidopsis* were performed by our group previously (Ng, 2001; Lau, 2003). Initially, the cDNA, encoding $MSP1_{42}$ of the *P. falciparum* Uganda Palo Alto (FUP) strain, was cloned and expressed using the *Phaseolin* promoter. However, only a very low amount of mRNA of $MSP1_{42}$ was detected in some transgenic *Arabidopsis* lines by RT-PCR and no signal was observed in all lines by northern blot analysis. Also, no mRNA was detected in all transgenic tobacco lines by both RT-PCR and northern blot analysis. And no protein or positive result was detected by both western blot and particle bombardment using GUS fusion to the C-terminal of $MSP1_{42}$ cDNA. These suggested that $MSP1_{42}$ could not be expressed in transgenic plants at the protein level, as well as at the RNA level (Ng, 2001).

The failure of gene expression of $MSP1_{42}$ may be due to several reasons. First, within the intron of the $MSP1_{42}$ sequence, the AT-richness of transgene may

facilitate pre-mRNA splicing in plants. Second, the presence of ATTTA pentamer in $MSP1_{42}$ is a mRNA destabilizing sequence causing instability of the reporter transcripts (Ohem-Takagi *et al.*, 1993). Third, the AATAAA/AATAAA like-motifs in $MSP1_{42}$ as polyadenylation signals (Joshi, 1987), will cause premature polyadenylation. Fourth, the occurrence of A+T in third degenerate base in the $MSP1_{42}$ sequence may cause codon usage problem. In addition, the presence of codons in *flgp42-His* cDNA that are rarely used in plants. These four factors may have caused the failure of the gene expression at both transcriptional and translational levels.

Therefore, the $MSP1_{42}$ was modified previously in our laboratory to decrease the AT content of the sequence from 70.6% to 50%. All 9 ATTTA pentamers as well as 24 AATAAA/ AATAAA-like motifs were avoided. Moreover, the modified $MSP1_{42}$ is having 81.2% codons in which G/C is at the third degenerate base. Furthermore, codon usage was designed on the basis of plant genes (Ng, 2001).

ATGTGGAGCTGGAAGTGCCTCCTCTTCTGGGCTGTCCTGGTCACAGCCACACTCTGC
ACCGCGGCGATATCTGTCACAATGGATAATATCCTCTCAGGATTTGAA**AATGAA**TAT
GATGTTATATATTTAAAACCTTTAGCTGGAGTATATAGAAGCTT**AAAAAA**ACAAATT
G**AAAAAA**ACATTTTTTACATTTAATTTAAATTTGAACGATATCTTAAATTCACGTCTT
AAGAAACGAAAATATTTCTTAGATGTATTAGAATCTGATTTAATGCAATTTAAACAT
ATATCCTCA**AATGAA**TACATTATTGAAGATTCATTTAAATTATTGAATTCAGAAC**AA**
**AAAA**ACACACTTTTAAAAAGTTACAAATA**TATAAA**AGAATCAGTAGAAAATGAT**ATT**
**AAA**TTTGCACAGGAAGGTATAAGTTATTATGAAAAGGTTTTAGCGA**AATATA**AGGAT
GATTTAG**AATCAATTAA**AAAAGTTATCAAAGAAGAAAAGGAGAAGTTCCCATCATCA
CCACCAACAACACCTCCGTCACCAGCAAAAACAGACGAACAAAAGAAGGAAAGTAAG
TTCCTTCCATTTTTAACAAACATTGAGACCTTATAC**AATAAC**TTAGTT**AATAAA**ATT
GACGATTACTT**AATTAA**CTTAAAGGCAAAGATTAACGATTGTAATGTTGAAAAAGAT
GAAGCACATGTTAA**AATAAC**TAAACTTAGTGATTTAAAAGCAATTGATGACAA**AATA**
**GA**TCTTTTTAAAAACCATAACGACTTCGAAGC**AATTAA**AAAATT**GATAAA**TGATGAT
ACG**AAAAAA**GATATGCTTGGCAAATTACTTAGTACAGGATTAGTTCAAAATTTTCCT
AATAC**AATAAT**ATCAA**AATTAA**TTGAAGGAAAATTCCAAGATATGTTAAACATTTCA
CAACACCAATGCGT**AAAAAA**ACAATGTCCAGAAAATTCTGGATGTTTCAGACATTTA
GATGAAAGAGAAGAATGTAAATGTTT**ATTAAA**TTACAAACAAGAAGGTGATAAATGT
GTTGAAAATCCAAATCCTACTTGTAACGAA**AATAAT**GGTGGATGTGATGCAGATGCC
AAATGTACCGAAGAAGATTCAGGTAGCAACGGAAAGAAAATCACATGTGAATGT**ACT**
**AAA**CCTGATTCTTATCCACTTTTCGATGGTATTTTCTGCAGTCATCATCATCATCAT
CATTAGTAG

**Figure 2-1 Nucleotide sequences of malaria surface protein 1, 42- and 19-kDa ($MSP1_{42}$ & $MSP1_{19}$) fragment cDNAs of the *Plasmodium falciparum* Uganda Palo Alto (FUP) strain**

Nucleotide sequences of $MSP1_{42}$ cDNA (1122 bp) and $MSP1_{19}$ (303 bp, underlined) showing the AT-richness of coding region, the presence of ATTTA destabilizing sequence (boxed) and putative polyadenylation signals AATAAA or AATAAA-like motifs (bolded).

ATGGCTATCTCTGTGACCATGGACAACATCCTCAGCGGATTCGAGAACGAGTACGACGTTA
TCTACTTGAAGCCTCTCGCTGGAGTGTACAGGAGCTTGAAGAAGCAGATCGAGAAGAACAT
CTTCACCTTCAACCTCAACTTGAACGACATCTTGAACAGCAGGCTCAAGAAGAGGAAGTAC
TTCTTGGACGTGCTTGAGTCTGACCTCATGCAGTTCAAGCACATCTCCAGCAACGAGTACA
TCATCGAGGACAGCTTCAAGCTCCTCAACAGCGAGCAGAAGAACACCCTTCTCAAGAGCTA
CAAGTACATCAAGGAGAGCGTGGAGAACGACATCAAGTTCGCTCAGGAGGGTATCAGCTAC
TACGAGAAGGTTCTCGCCAAGTACAAGGATGACCTTGAGAGCATCAAGAAGGTGATCAAGG
AGGAGAAGGAGAAGTTCCCAAGCAGCCCACCAACCACTCCTCCAAGCCCAGCTAAGACCGA
CGAGCAGAAGAAGGAGAGCAAGTTCCTTCCATTCTTGACCAACATCGAGACCTTGTACAAC
AACCTCGTGAACAAGATCGACGACTACCTCATCAACCTCAAGGCTAAGATCAACGACTGCA
ACGTTGAGAAGGATGAGGCTCACGTTAAGATCACTAAGCTCAGCGATCTCAAGGCTATCGA
TGACAAGATCGATCTCTTCAAGAACCACAACGACTTCGAGGCTATCAAGAAGCTCATCAAC
GATGATACCAAGAAGGACATGCTCGGTAAGCTCCTCAGCACCGGACTCGTTCAGAACTTCC
CTAACACCATCATCAGCAAGCTCATCGAGGGAAAGTTCCAAGACATGCTCAACATCAGCCA
ACACCAGTGCGTGAAGAAGCAATGCCCAGAGAACTCTGGATGCTTCAGACACTTGGATGAG
AGGGAGGAGTGCAAGTGCCTCCTCAACTACAAGCAAGAGGGTGACAAGTGCGTTGAGAACC
CAAACCCTACTTGCAACGAGAACAACGGTGGATGCGATGCTGATGCCAAGTGCACCGAGGA
GGACAGCGGTAGCAACGGTAAGAAGATCACCTGCGAGTGCACTAAGCCTGACTCTTACCCA
CTCTTCGACGGTATCTTCTGCAGCTAA

**Figure 2-2 Nucleotide sequence of malaria modified *flgp42-His* cDNA**

Nucleotide sequence of the *flgp42-His* cDNA showing the modified bases (red).

After modification, the expression of the $MSP1_{42}$ and it fragment, $MSP1_{19}$, in plants was markedly improved at both transcriptional and translational levels (Lau, 2003).

In the study of expressing $MSP1_{42}$ and $MSP1_{19}$ in transgenic tobacco, the highest level of expression of $MSP1_{19}$ of total soluble protein was at 0.0035% (Ghosh *et al.*, 2002), while 0.2% was found for $MSP1_{42}$ in which the protein was fused with

AFVY, a phaseolin signal peptide (Lau, 2003). The low expression level of the $MSP1_{19}$, as 0.0035% of total soluble protein (TSP), under the control of 35S promoter of cauliflower mosaic virus, in tobacco plants was explained by Ghosh *et al.* (2002) due to the large number of cysteine residues in the $MSP1_{19}$ protein and also the presence of disulfide bridges which cause the degradation or destabilization of the protein molecule in the highly reducing condition of cytoplasm (Bosch *et al.*, 1994; Florack *et al.*, 1994).

For our group, to improve the expression, the $MSP1_{42}$ was fused with lysine rich protein (LRP) or a phaseolin-targeting signal (AFVY), a necessary determinant for the vacuolar sorting (Frigerio *et al.*, 1998; Frigerio *et al.*, 2001), and these constructs was transformed into *Arabidopsis* or tobacco (Lau, 2003). The $MSP1_{42}$ was detected by western blot analysis in both types of constructs and the expression level of $MSP1_{42}$-AFVY was found to be expressed at a higher level than the LRP- $MSP1_{42}$ (Lau, 2003). Also, the expression level of the $MSP1_{42}$, at 0.2%, was much improved when compared with that of Ghosh *et al.* (2002) at 0.0035%. This suggests that expressiing of MSP1 protein and its fragments in plants should be stabilized by cellular compartment sorting or fusion with a protein which can be expressed stablily in a plant system, such as the lysine rich protein.

## 2.12 Phaseolin and its sorting signal

Phaseolin, a homotrimeric soluble glycoprotein that is synthesized during seed development and accumulates in protein storage vacuoles of protein bodies, is the major storage protein in bean (*Phaseolus vulgaris*) (Sturm *et al.*, 1987; Frigerio *et al.*, 1998). This major storage protein of the French bean was isolated (Sun *et al.*, 1981) and the complete nucleotide sequence was determined (Slightom *et al.*, 1983). The expression of phaseolin gene and its promoter had been studied. The phaseolin gene was found to express at a higher level in the seeds of transformated tobacco plants and the phaseolin promoter functions in the embryonic tissue in tobacco seeds (Sengupta-Gopalan *et al.*, 1985). The phaseolin promoter has been utilized in expressing foreign protein in plants because of its strong and seed-specific expression (Hoffman *et al.*, 1987; Altenbach *et al.*, 1989; Altenbach *et al.*, 1992 and).

The vacuolar sorting of phaseolin was shown to be very efficient in tobacco leaf cells while the sequence Ala-Phe-Val-Tyr (AFVY), a highly hydrophobic sequence, at the C-terminal of the phaseolin was used. However, the protein will be secreted by Golgi-mediated secretion if the protein expression is at a high level, a saturation level. On the other hand, the phaseolin without AFVY will be secreted completely from the cells and accumulates in the extracellular medium (Frigerio *et al.*, 1998).

Also, in a previous study, we found that the fusion construct of $MSP1_{42}$ with AFVY showed a higher protein expression level, at 0.2% of the total soluble protein (TSP) over the $MSP1_{42}$ without the AFVY signal (Lau, 2003). This level is much higher then the previous report on 0.0035% TSP obtained by Ghosh *et al.* (2002). Thus AFVY appears to be capable of directing foreign proteins to plant vacuole for stable accumulation.

## 2.13 Protein Targeting Sequences

Accurate foreign protein sorting is important for the success of the target protein production in plant cells because soluble enzymes, which may digest the target protein, are present in all subcellular compartments, such as cytosol, the lumen of the endoplasmic reticulum (ER), cisternae of the Golgi apparatus and cell wall. Therefore, directing the target proteins to a certain cellular compartment for stable accumulation may be one of the critical factors for enhancing the yield of recombinant protein production.

Both lytic vacuoles and protein storage vacuole (PSV) present in plant cells. Plant seeds are generally rich in protein with a large percentage of the soluble proteins stored in the protein storage vacuole, so sorting the target protein to this compartment may help improve protein production (Jiang and Sun, 2002).

Specific sequences derived from the transmembrane domain (TMD) and cytoplasmic tail (CT) are required for the integral membrane protein sorting to specific vacuoles. The peabp80 (BP-80), α-tonoplast intrinsic protein (TIP) and ReMember- RingH2 membrane protein (RMR) were used in this study.

**(a) Peabp80 (BP-80)**

PeabpP80, consisting of a large luminal domain, a single TMD and a CT, contains a peptide sequence with very high degree of conservation in the *Arabidopsis* family. However, it is unclear if all the peabp80 homologues are involoved in sorting proteins to the lytic vacuole.

Peabp80 TMD alone was found to be sufficient in membrane anchoring, for example, in lytic prevacuolar targeting in tobacco cells (Jiang and Roger, 1998) but not sufficient to accumulate the foreign protein in the prevacuole. Whereas, CT of BP-80 has a dominant role for localization because trans-Golgi network (TGN) and prevacuole targeting information does not residue within the TMD. When the CT works together with TMD in a fusion protein, the protein was found to be directed via the Golgi to lytic vacuole, in vegetative plant cells, and globoid in the PSV in plant seeds (Jiang and Rogers, 1998; Saint-Jore-Dupas et al, 2004).

**(b) α-tonoplast intrinsic protein (α-TIP)**

α-tonoplast intrinsic protein (α-TIP), which presents in the membrane of PSV, serves as an indicator of PSV. The intact α-TIP, with α-TIP TMD and CT were found to target a reporter protein to tonoplast (Jiang and Rogers, 1999).

α-TIP CT with BP-80 TMD will target protein, by passing through Golgi, to PSV with the same colocalization with the intact α-TIP protein (Jiang and Rogers, 1998) and this targeting sequence was used in the presnet study of $MSP1_{42}$ and $MSP1_{19}$ and is simplifyly named as α-TIP.

**(c) ReMember-RingH2 membrane protein (RMR)**

ReMember-RingH2 membrane protein (RMR), which is predicted from a tomato EST clone with high similarity to that of the *Arabidopsis* protein, was identified by Jiang *et al.* (2000). RMR was present exclusively in the membrane fraction and remained intact. The reporter protein which was fused with TMD and CT of RMR was found to be trafficked to the protein storage vacuole via the Golgi (Saint-Jore-Dupas et al, 2004).

# Chapter 3 Materials and Methods

## 3.1 Introduction

*Arabidopsis* and tobacco were used as model plants for expressing $MSP1_{42}$ and $MSP1_{19}$ cDNAs. The expression of the modified $MSP1_{42}$ or $MSP1_{19}$ cDNA was tested in transgenic *Arabidopsis,* while those in either protein targeting fusion constructs or β-glucuronidase (GUS) fusion constructs were tested in transgenic tobacco.

Transient assay by using particle bombardment was used to test for transient expression of $MSP1_{42}$ or $MSP1_{19}$ cDNA fused with reporter β-glucuronidase (GUS) gene.

## 3.2 Chemicals and enzymes

All the chemicals used were of reagent grade or molecular grade (unless otherwise specified) and were purchased from Sigma Chemical Co. or Bio-Rad Co. (unless otherwise specified) and the enzymes used were from New England Biolab unless specific stated.

## 3.3 Cloning

### 3.3.1 $MSP1_{42}$ and $MSP1_{19}$ constructs

Two chimeric gene constructs containing modified $MSP1_{42}$ and $MSP1_{19}$ in phaseolin cassette were prepared by Ng, early (Ng, 2001).

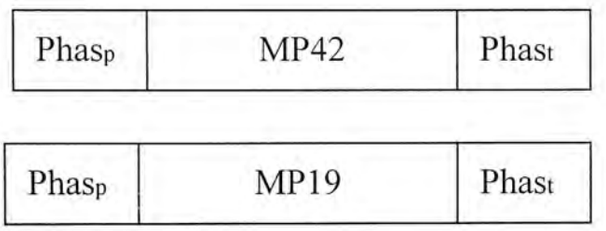



**Figure 3-1 Constructs of $MSP1_{42}$ and $MSP1_{19}$ in Phaseolin cassette**
In these constructs, phaseolin promoter (Phasp) and terminator(Phast) were used to drive the expression of modified $MSP1_{42}$ (MP42) and modified $MSP1_{19}$ (MP19)

### 3.3.2 Protein targeting fusion constructs

Altogether eight chimeric constructs, 1) MP42, MP42 with protein targeting signals: 2) BP-80, 3) α-TIP and 4) RMR (MP42-BP-80; MP42-α-TIP and MP42-RMR); 5) MP19, MP19 with protein targeting signals: 6) BP-80, 7) α-TIP and 8) RMR (MP19-BP-80; MP19- α-TIP and MP19-RMR) were included in this study. The target gene MP42 and MP19 were PCR amplified, using pTZ.Phas/MP42 as a template, for the introduction of 5'NdeI and 3' AccI sites. The primers sequences are as shown in Figure 3-8. A 50μl PCR reaction mixture containing 40ng DNA template, 1X Pfu buffer (Strategene), 0.2mM dNTP, 0.5μM 5' primer, 0.5μM 3' primer and 2.5units Pfu DNA polymerase (Strategene, 2.5u/μl) was prepared for each of the target gene. The PCR

conditions were as follows: 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, followed by 1 cycle of 72°C for 10 minutes. Then, the PCR product was used for agarose/ TAE gel electrophoresis.

The PCR products were cloned into pGEM-T vector (Promega) in order to increase the restriction digestion efficiency by AccI and NdeI.

To amplify the target genes:

**MP42:** primers LNspM42 + RANM42 using pTZ/Phas/MP42 as template

**MP42-BP-80:** primers LNspM42 + RASM42 using pTZ/Phas/MP42 as template

**MP42-αTIP:** primers LNspM42 + RASM42 using pTZ/Phas/MP42 as template

**MP42-RMR:** primers LNspM42 + RASM42 using pTZ/Phas/MP42 as template

**MP19:** primers LNspM19 + RANM42 using pTZ/Phas/MP42 as template

**MP19-BP-80:** primers LNspM19 + RASM42 using pTZ/Phas/MP42 as template

**MP19-αTIP:** primers LNspM19 + RASM42 using pTZ/Phas/MP42 as template

**MP19-RMR:** primers LNspM19 + RASM42 using pTZ/Phas/MP42 as template

A stop codon was included at the 3' end of MP42 and MP19, but not for those constructs containing the protein targeting genes. The target gene was then excised using NdeI and AccI and was cloned into pBK/Phas/SP vector which contains a phaseolin promoter cassette and a phaseolin signal peptide.

Protein targeting sequences BP-80, α-TIP and RMR were PCR amplified as mentioned above for the introduction of AccI site at both ends. The PCR products were cloned into pGEM-T vector for efficient restriction digestion by AccI.

To amplify the protein targeting gene:

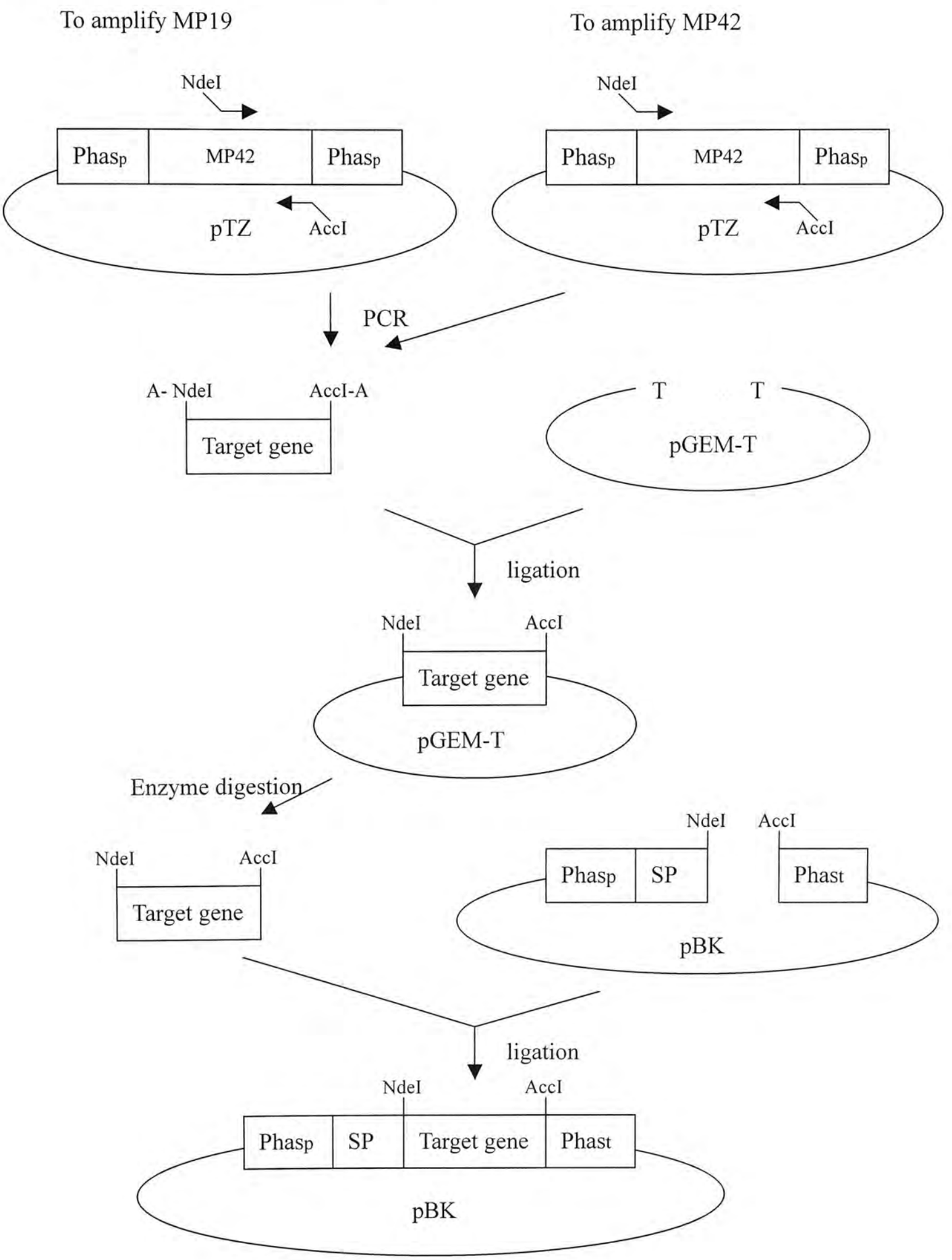
**BP-80:** primers Acc491-L + RABP-80 using PSB130/Gt1/SP/hG-CSF/TMD/BP80 (from Prof. Jiang, Biology Department, CUHK) as template

**α-TIP:** primers Acc491-L + RAαTIP using PSB130/Gt1/SP/hG-CSF/TMD/αTIP (from Prof. Jiang, Biology Department, CUHK) as template

**RMR:** primers Acc727-L + RARMR using PSB130/Gt1/SP/hG-CSF/TMD/ RMR (from Prof. Jiang, Biology Department, CUHK) as template

The protein targeting gene with the MP42 or MP19 was cloned in frame by AccI sites into the pBK/Phas/SP vector containing phaseolin promoter cassette as well as a phaseolin signal peptide. The orientation of the protein targeting gene in the pBK/Phas/SP vector was checked by PCR as mentioned above using 5' primer of MP42 or MP19 and 3' primer of the protein targeting gene.

**(a) For Phas/SP/Mp42 and Phas/SP/MP19:**



**(b) For Phas/SP/MP42 and Phas/SP/MP19 with protein targeting signal:**

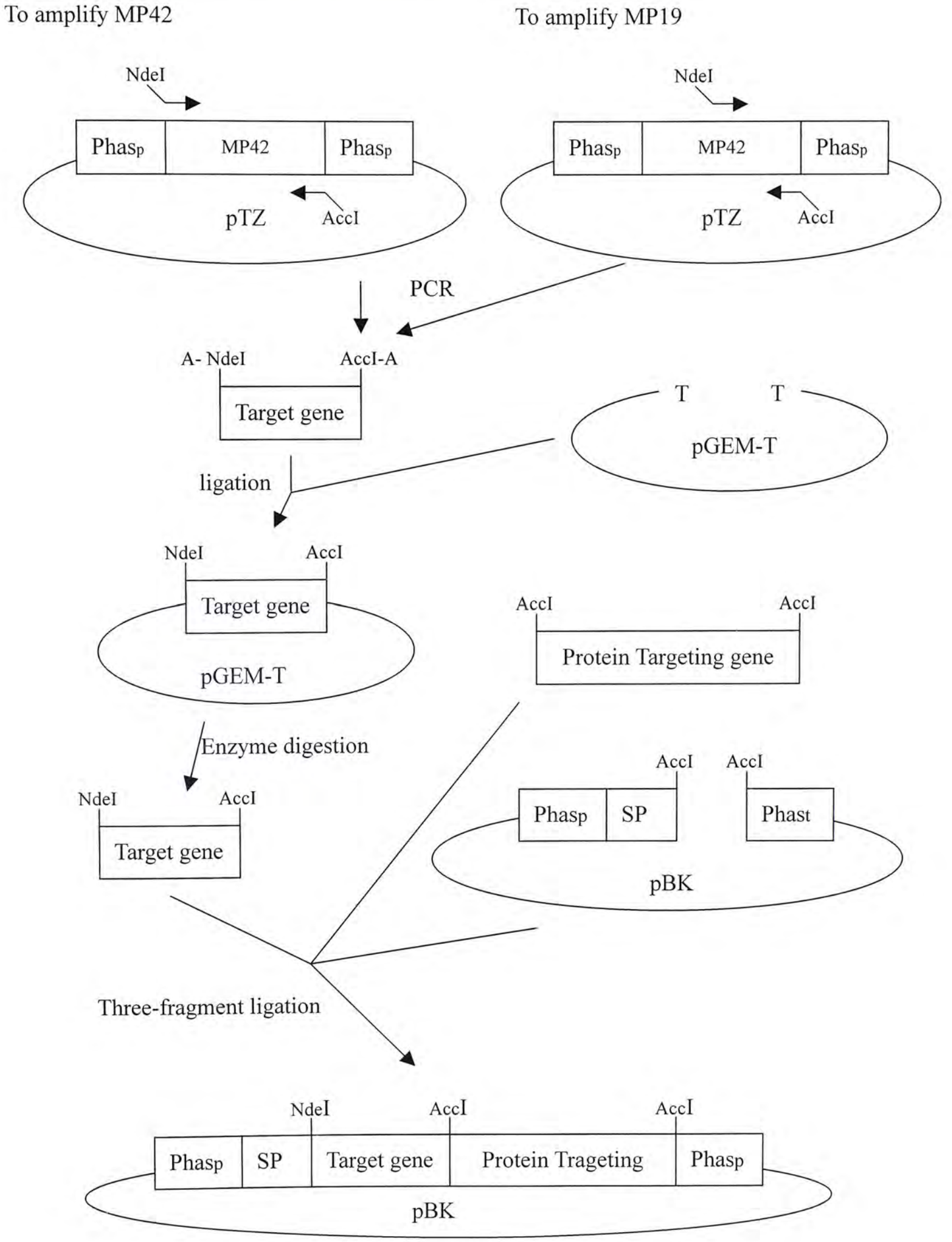


**Figure 3-2 (a and b) General scheme for the construction of protein targeting constructs**

Target gene represents either MP42 or MP19. $Phas_P$ = phaseolin promoter; SP = phaseolin signal peptide; and $Phas_T$ = phaseolin terminator. Protein targeting gene represents the inclusion of α-TIP, BP80 or RMR.

### 3.3.3 Gus fusion Constructs

**(a) Particle bombardment**

All together six constructs were included in this part. The β-glucuronidase (GUS), with the start codon excluded, was fused with the 1) modified and 2) unmodified $MSP1_{42}$ and 3) modified and 4) unmodified $MSP1_{19}$, and with GUS 5) with and 6) without a start codon as positive control and negative control, respectively.

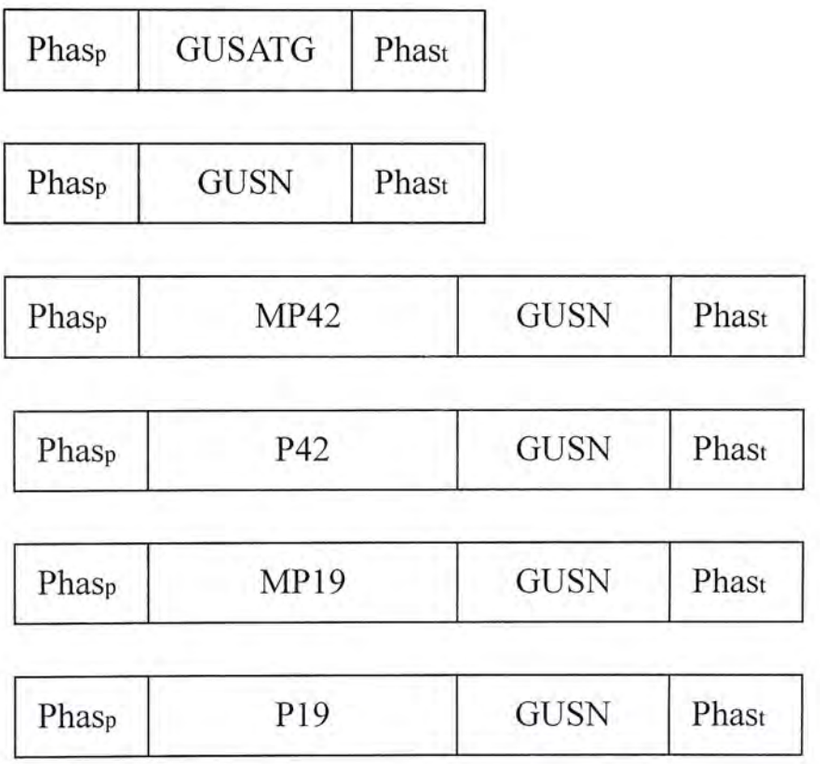



**Figure 3-3 The $MSP1_{42}$ and $MSP1_{19}$ GUS fusion constructs used in particle bombardment**

A total of six constructs, using GUS as a reporter gene, in single and in fusion with MP42 and MP19, in studying the expression of modified MP42 and MP19. GUSATG, in which the GUS gene retained its ATG; GUSN, without the ATG codons.

**Cloning of pTZ/Phas/MP19::GUSN**

pTZ vector with phaseolin promoter and terminator was prepared from the digestion of pTZ/phas/MP19 by AccI while, MP19 fragment was prepared from the digestion of pTZ/phas/MP19 by AccI and PstI. Moreover, the GUS gene without ATG (GUSN) was prepared from the digestion of pTZ/phas/MP42 DNA fragment by AccI and Pst I.

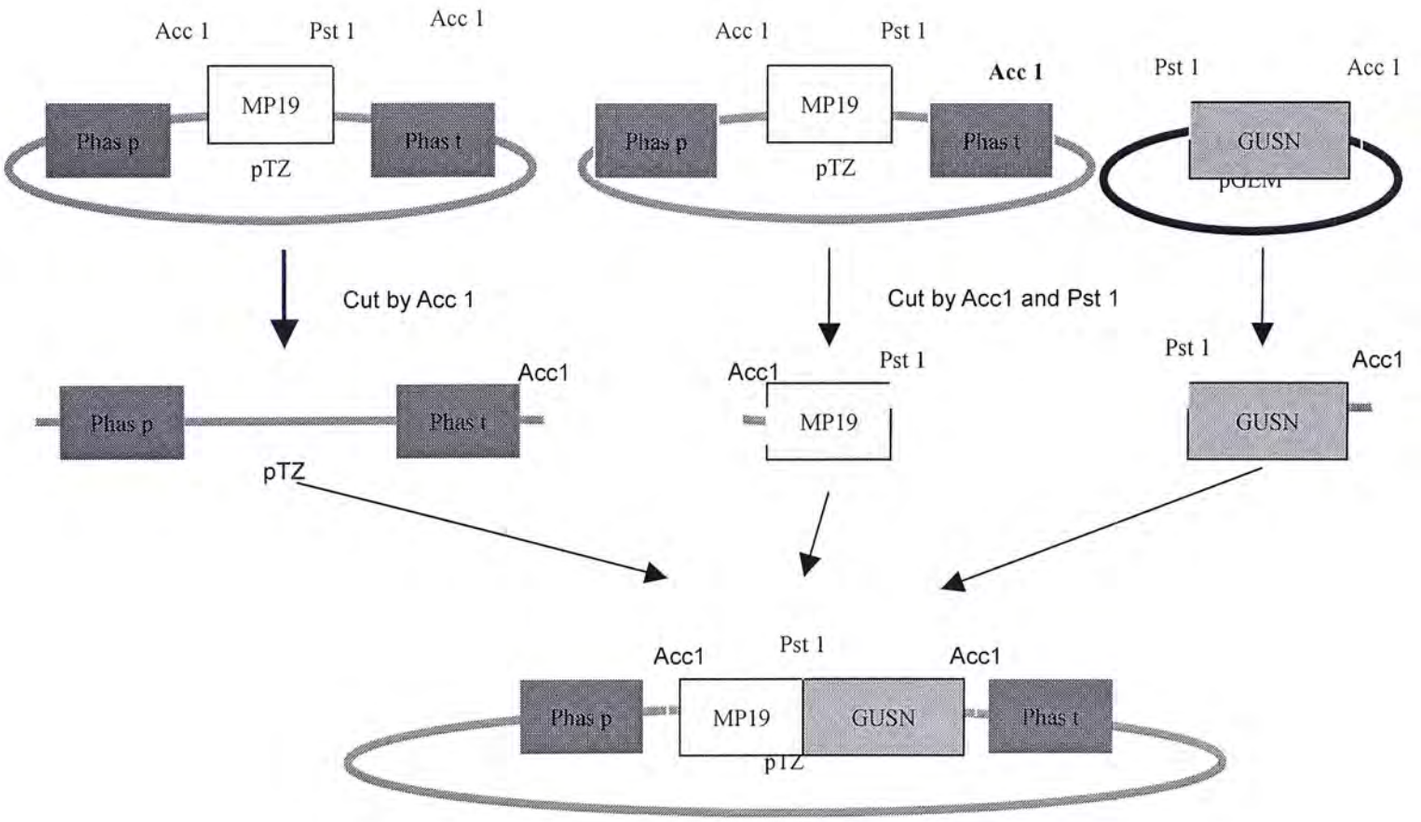



**Figure 3-4 Construction scheme of Phas/ pTZ/ MP19::GUSN for particle bombardment**

Phasp indicates phaseolin promoter while Phast, phaseolin terminator.

**(b) GUS fusion constructs for plant transformation**

In this part, altogether six chimeric constructs, including target genes MP42 and MP19: 1) Phas/MP42-EK::GUSN; 2) Phas/MP19-EK::GUSN; 3) Phas/SP/MP42-EK::GUSN; 4) Phas/SP/MP19-EK::GUSN; 5) Phas/SP/MP42-EK::GUSN-AFVY and 6) Phas/SP/MP19-EK::GUSN-AFVY, were prepared.

For the first two constructs, Phas/MP42-EK::GUSN, Phas/MP19-EK::GUSN, the MP42 and MP19 gene was PCR amplified as mentioned in 3.3.2 to introduce a 5'AccI site and 3'enterokinase (EK) plus a PstI site. The PCR products were cloned into pGEM-T vector for increasing the efficiency of AccI digestion.

To amplify the MP42 and MP19 gene, the following experiments were carried out:

**MP42:** Primers LANM42 + RPEKM42 using pTZ/Phas/MP42 as template

**MP19:** Primers LANM19 + RPEKM42 using pTZ/Phas/MP42 as template

The constructs were cloned by three fragment ligation. After cloning into pGEM-T vector, the MP42 and MP19 gene was excised by AccI and PstI with the removal of the 3' stop codon of the gene. Also, GUSN (GUS without ATG) was released from pTZ/Phas/MP42::GUSN (from Ng, W. K.) under the digestion by AccI and PstI. The pTZ

vector with Phaseolin cassette was prepared by enzyme digestion with AccI on pTZ/Phas/MP42::GUSN (from Ng, W. K.). Then, three-fragment ligation was performed.

For constructs Phas/SP/MP42-EK::GUSN and Phas/SP/MP19-EK::GUSN, the MP42 and MP19 gene were PCR amplified as mentioned in section 3.3.2 to introduce 5'NdeI site and 3'enterokinase (EK) site plus PstI.

To amplify the MP42 and MP19:

**MP42:** Primers LNspM42 + RPEKM42 using pTZ/Phas/MP42 as template

M**P19:** Primers LNspM19 + RPEKM42 using pTZ/Phas/MP42 as template

The PCR products were cloned into pGEM-T vector. A three-fragment ligation was performed using the MP42 and MP19 which were excised by NdeI and PstI from the pGEM-T vector, the GUSN (GUS without ATG) which was released from pGEM-T/P-GUSN-A (from Ng, W. K.) by PstI and AccI, and the pBK/Phas/SP vector which contains Phaseolin casetter together with Phaseolin signal peptide which was prepared by digestion with NdeI and AccI on pBK/Phas/SP/MP42 (section 3.3.2).

For the constructs with AFVY, namely Phas/SP/MP42-EK::GusN-AFVY and Phas/SP/MP19-EK::GusN-AFVY, GUSN (GUS without ATG) was PCR amplified as

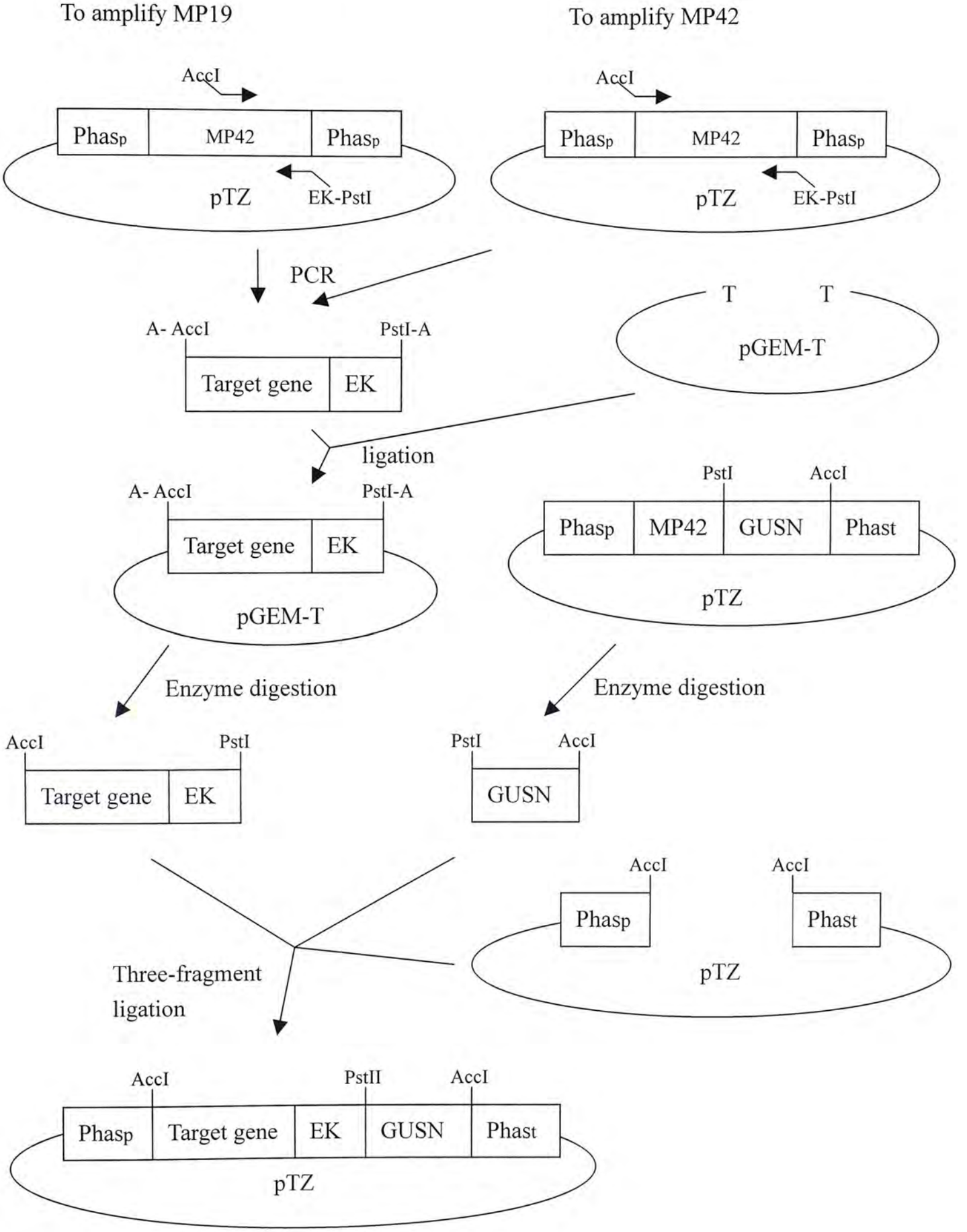
mentioned in section 3.3.2 to introduce a 5'PstI site and 3' enterokinase (EK) site plus AccI.

To amplify the GUS gene, the following experiments were carried out:

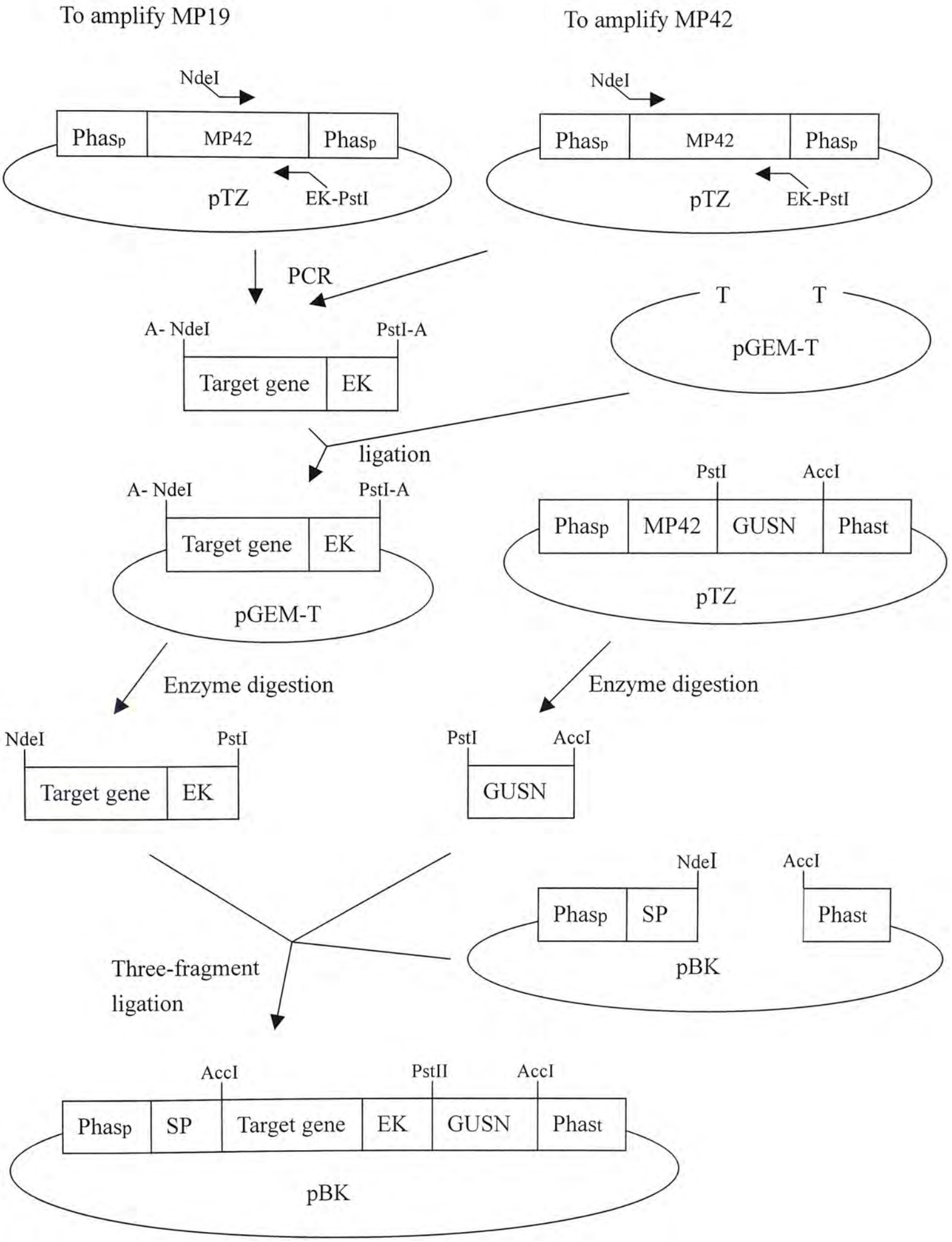
**EK::GUSN-AFVY:** Primers LPEKGus + RAAFVYGus using pBI121 as template

The PCR products of amplified GUS were cloned into pGEM-T vector, the EK::GUSN-AFVY was excised from the pGEM-T vector by PstI and AccI, whereas MP42 and MP19 were excised by NdeI and PstI with the removal of the 3' stop codon from the pBK/Phas/SP/MP42 and pBK/SP/MP19 (section 3.3.2), respectively. Then, three-fragment ligation was performed to ligate the MP42 or MP19 with EK::GUSN-AFVY and pBK/Phas/SP vector, containing the Phaseolin casetter together with Phaseolin signal peptide, which was prepared by digestion with NdeI and AccI of the pBK/Phas/SP/MP42 (section 3.3.2).

**(a) For Phas/MP42-EK::GUSN and Phas/MP19-EK::GUSN**



**(b) For Phas/SP/MP42-EK::GUSN and Phas/SP/MP19-EK::GUSN**



**(c) For Phas/SP/MP42-EK::GUSN-AFVY and Phas/SP/MP19-EK::GUSN-AFVY**

To amplify EK::GUSN-AFVY

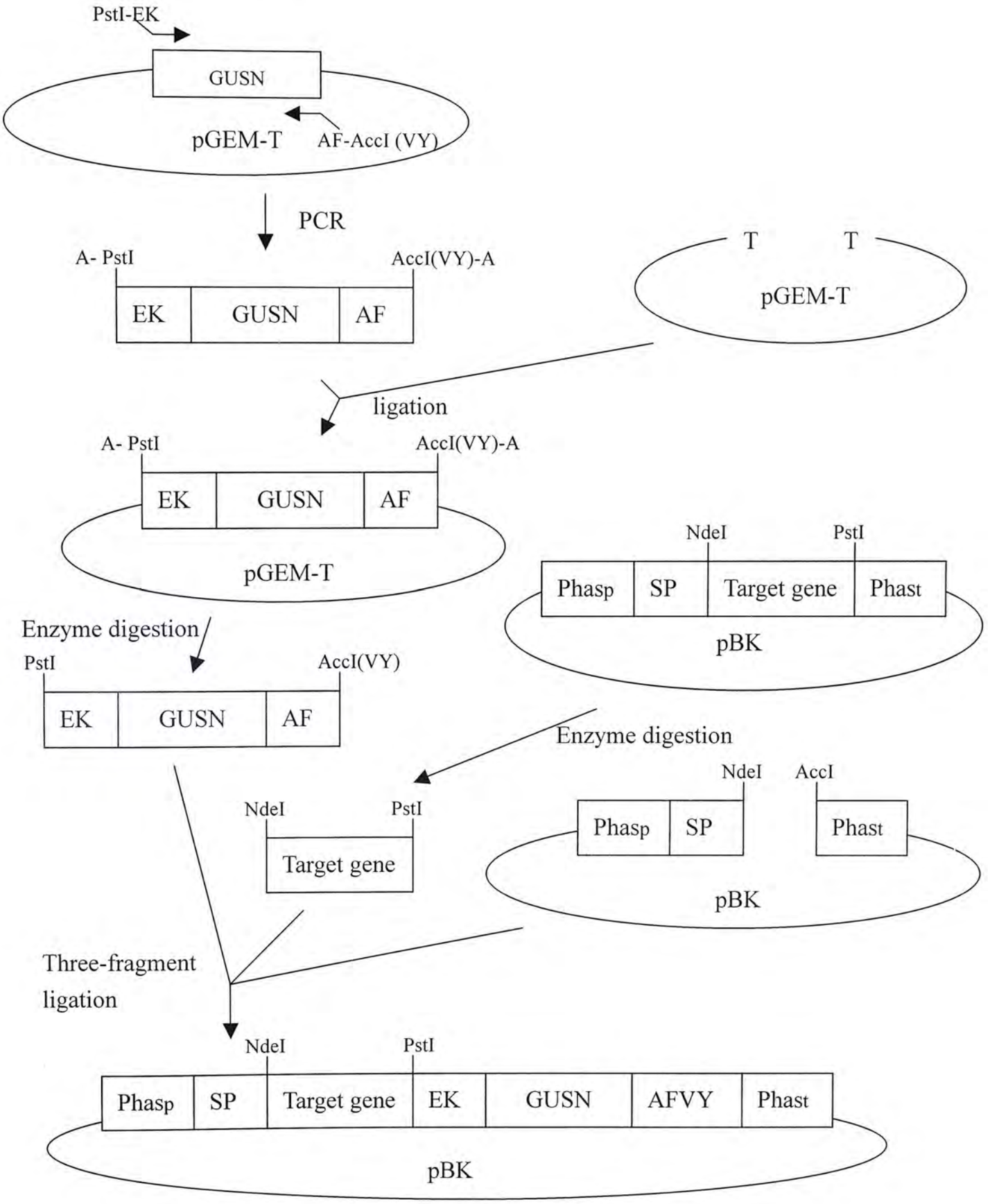


**Figure 3-5 (a-c) General schemes for the contruction of GUS fusion constructs**

Target gene represents either MP42 or MP19 while $Phas_P$ = phaseolin promoter; SP = phaseolin signal peptide; $Phas_T$ = phaseolin terminator; EK = enterokinase site; GUSN = β-glucuronidase without a start codon.

**(c) Modified GUS fusion constructs**

In this part, two chimeric constructs, including target genes MP19: 1) Phas/SP/MP19-EK::MGUSN; and 2) Phas/SP/MP42-EK::MGUSN-AFVY, were prepared.

MGUSN (modified GUS without ATG) was PCR amplified as mentioned in 3.3.2 to introduce a nucleotide mutation, i.e. Asn-358 to Ser.

To amplify the MGUSN and MGUSN-AFVY, the following experiments were carried out:

**EK::MGUSN:** Primers LPEKGus + RmGUS and Primers LmGUS + RGUSACC#, in two separate reactions, using pBI121 as template for first round PCR

**EK::MGUSN-AFVY:** Primers LPEKGus + RmGUS and Primers LmGUS + RAAFVYGus, in two separate reactions, using pBI121 as template for first round PCR

PCR products, 0.5µl from each of the two reaction mixtures was mixed and used as template for the second round PCR. The PCR amplified EK::MGUSN and EK::MGUSN-AFVY were cloned into pGEM-T vector and excised from the pGEM-T vector by PstI and AccI and the following cloning steps were as mentioned in section 3.3.3b and Figure 3-5c.

## 3.4 Transfer of chimeric genes into *Agrobacterium* binary vector

The chimeric gene constructs (sections 3.3.1 and 3.3.2) were excised by HindIII and ligated into the *Agrobacterium*-binary vector pBI121 carrying a neomycin phosphotransferase II (NPTII) selectable marker and β-glucuronidase (GUS), a screenable marker gene. Whereas, Chimeric gene constructs (section 3.3.3b) were excised by HindIII and ligated into the *Agrobacterium*-binary vector pSUN1 carrying a neomycin phosphotransferase II (NPTII) selectable marker and a orange fluorescent protein (OFP) screenable marker gene.

### 3.4.1 Cloning of pSUN1

The GUS gene driven by 35S promoter in pBI121 was replaced by the orange fluorescent protein (OFP, Dr. Wan, CUHK). OFP was PCR amplified to introduce a 5' BamHI and a 3' SacI site, respectively. This was done by Dr Wan's lab, Biochemistry Department. The PCR product was cloned into pGEM-T vector.

The cloning and construction of pSUN1 was as follow:

**OFP:** 5' OFP- Bam HI + 3' OFP- SacI, the PCR amplified step was done by Dr Wan's lab in Biochemistry Department

After cloning into pGEM-T vector, OFP was excised by BamHI and SacI. The DNA fragment was then cloned into pBI221. The construct pBI221/35S/OFP was used to perform particle bombardment and for orange fluorescent detection. The OFP was excised by BamHI and SacI digestion and was cloned into pBI121 by using these enzyme sites.

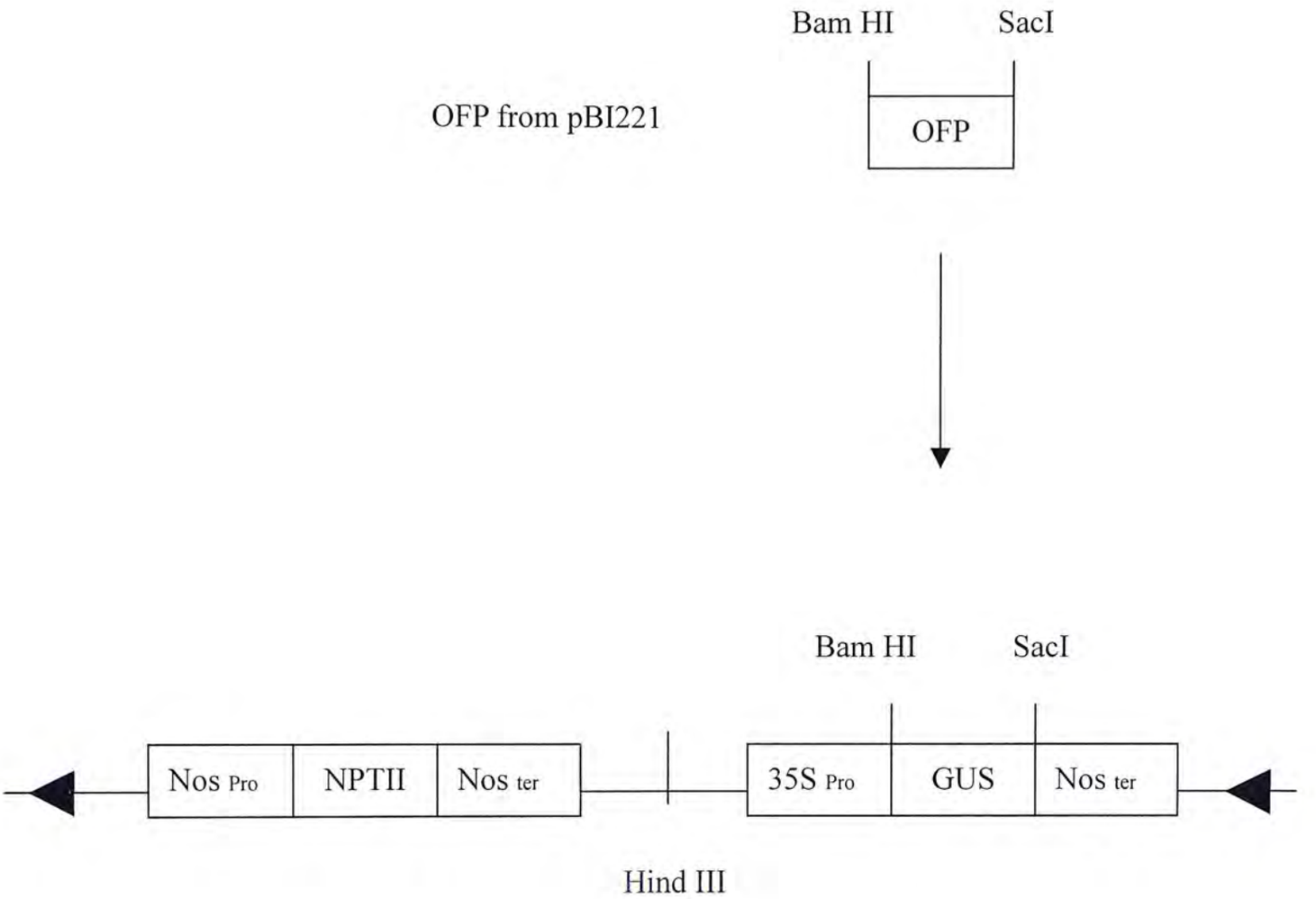
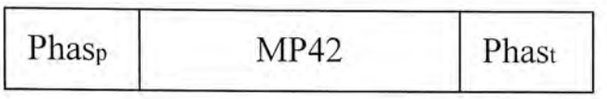



**Figure 3-6 Construction of pSUN1**

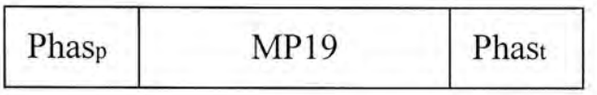
The coding sequence of orange fluorescent protein (OFP) was used to replace the GUS gene in pBI221. Nos $_{Pro}$ = Nos promoter; Nos $_{ter}$ = Nos terminator; NPTII = neomycin phosphotransferase II, a selectable marker amd GUS = β-glucuronidase.

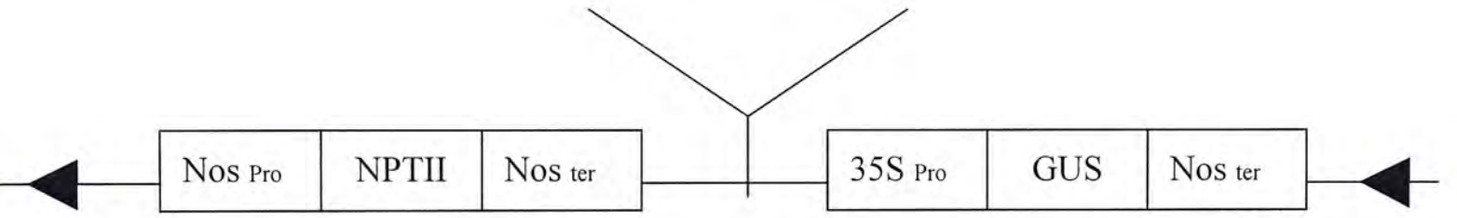
**(a) MP42 and MP19 constructs**

(1) Phas/MP42




(2) Phas/MP19



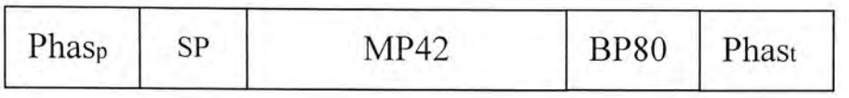

Hind III

(These constructs were cloned by Ng, W. K.)

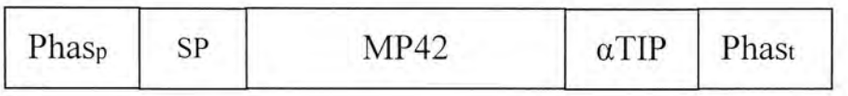
**(b) Protein targeting constructs**

(1) Phas/SP/MP42

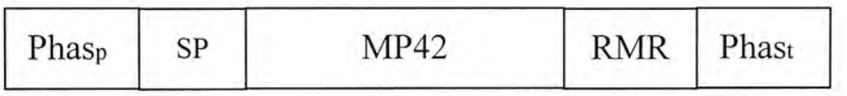

(2) Phas/SP/MP42-BP80



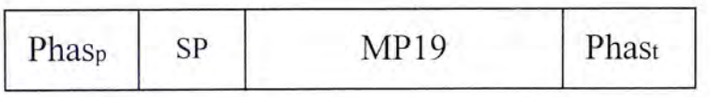

(3)Phas/SP/MP42-αTIP



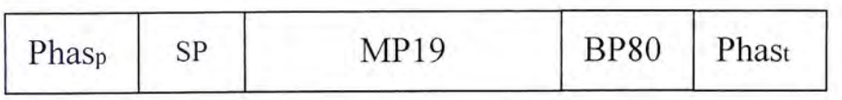

(4)Phas/SP/MP42-αTIP



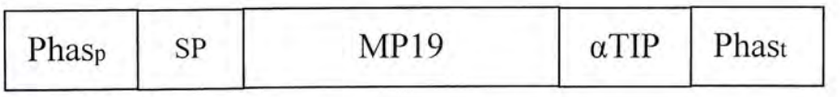

(1) Phas/SP/MP19



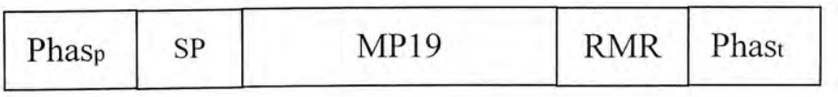

(2) Phas/SP/MP19-BP80

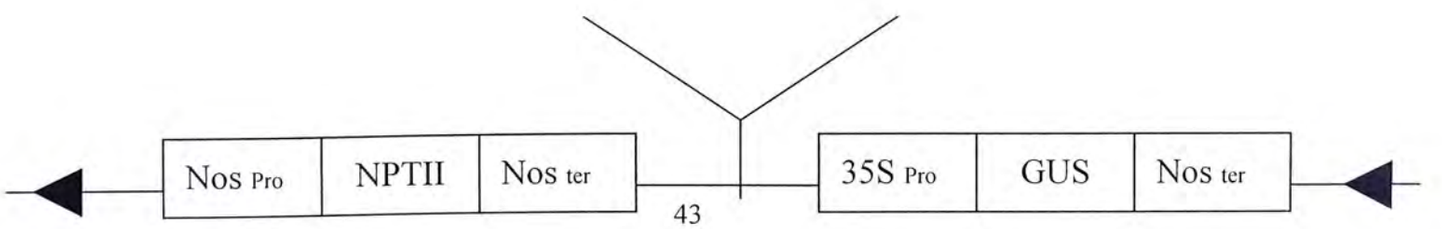



(7)Phas/SP/MP19-αTIP




(8)Phas/SP/MP19-RMR



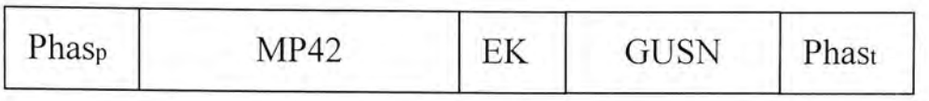





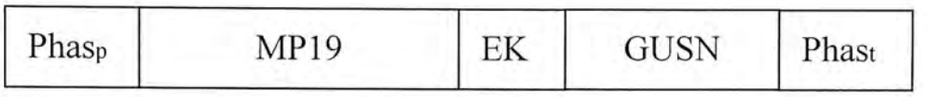

Hind III

**(c) GUS fusion constructs**

(1) Phas/MP42-EK::GUSN



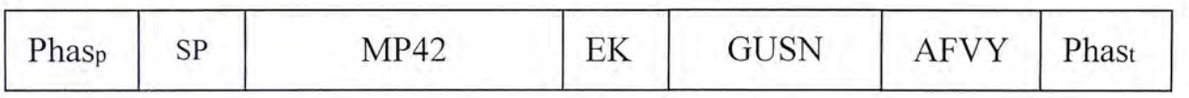

(2) Phas/MP19-EK::GUSN



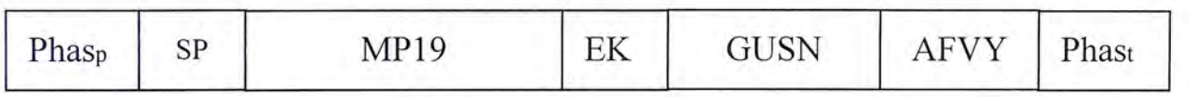

(3) Phas/sp/MP42-EK::GUSN

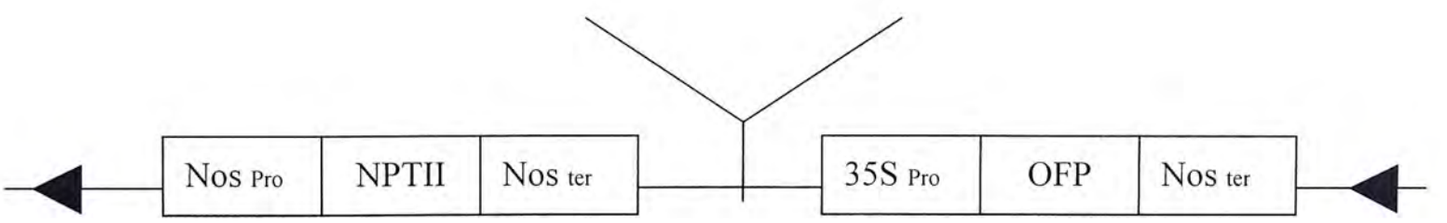

(4) Phas/sp/MP19-EK::GUSN


(5) Phas/sp/MP42-EK::GUSN




(6) Phas/sp/MP19-EK::GUSN




Hind III

**Figure 3-7 (a-c) Schematic summary of the construction and transfer of chimeric genes into *Agrobacterium* binary vector: pBI121 or pSUN1**

### 3.4.2 Primer sequence

LANM42: 5' AGTCTACCATGGCTATCTCTGTG 3'

LNspM42: 5' CCATATGCCACTTCACTCGCTATCTCTGTGACTATGG 3'

LANM19: 5' AGTCTACCATGGGAAAGTTCCAAGACATG 3'

LNspM19: 5' CCATATGCCACTTCACTCGGAAAGTTCCAAGACATGC 3'

RANM42: 5' CGTATACCATGGTTAGCTGCAGAAGATACCGT 3'

RASM42: 5' CGTATACGAGCTCGCTGCAGAAGATACCGT 3'

RPEKM42: 5' CGCTGCAGGGCTTGTCGTCGTCGTCGCTACAGAAGATACCGTCGAA 3'

RAAFVYGus: 5' A**GTATACAAATGC**TTGTTTGCCTCCCTGCTGC 3'

LPEKGus: 5' TTCTGCAGCGACGACGACGACAAGTTACGTCCTGTAGAAACCCCA 3'

LmGUS: 5' CGGCTTTAGCCTCTCTTTAG 3'

RmGUS: 5' CTAAAGAGAGGCTAAAGCCG 3'

3' RGUSACC#: 5' GGTATACTCATTGTTTGCCTCCCTGCTGC 3'

Acc 491-L: 5' CGTATACACTAAAACTGCCAGTCAGGC 3'

Acc 727-L: 5' CGTATACTGGTTGATCCCAAGTTTTGAG 3'

RAαTIP: 5' CGTATACTCAGTAATCTTCAGTTGCCAAAG 3'

RABP-80: 5' CGTATACTCAACCTCTTTGATGATTGACA 3'

RARMR: 5' CGTATACTCAACAGTCTGGAAGCGAGTTTG 3'

**Figure 3-8 Sequence of primers used in the chimeric gene construction**
A total of 14 primer sequences were synthesized for the amplification and cloning. AccI site: underlined; PstI site: grey solid boxed ; EK site: boxed; AFVY: bolded.

## 3.5 Bacterial strains

*E. coli* DH5α was used for chimeric gene construction. *Agrobacterium tumefaciens* GV3101/pMP90 (Koncz and Schell, 1986) and LBA4404/pAL4404 (Hoekema et al., 1983) were employed in *Arabidopsis* and tobacco transformation, respectively.

## 3.6 Particle bombardment

### 3.6.1 Plant materials

The cotyledons of developing fresh snow bean, from local store, were removed from the bean pods and used for particle bombardment.

### 3.6.2 Microcarrier Preparation and Coating DNA onto microcarrier

For microcarrier preparation, 40mg gold particles with diameter of 1μm were weighed and washed with 0.8ml freshly prepared 70% ethanol. After, the mixture was vortexed for 3-5 minutes. Then it was allowed to stand for 15 minutes and pelleted by spinning for 5 seconds. The supernatant was discarded and the pellet was washed three times with 0.8ml sterile distilled water, 1 minute each, then settled for 1 minute by 2 seconds brief centrifugation. Finally, 50% glycerol was added to bring the microparticle concentration to 60mg/ml.

For bombardment, 33.3μl (2mg) microcarriers was used and 3.33μl plasmid DNA

(1μg/μl), 33.3μl $CaCl_2$ (2.5M) and 13.3μl spermidine (0.1M) were added into the microcarriers while vortexing vigorously for 2-3 minutes. Then the microcarriers were allowed to settle for 1 minute and spun for 2 seconds. The supernatant was discarded and 93.3μl 70% ethanol was added and discarded without disturbing the pellet. Then, 93.3μl 100% ethanol was added and then removed without disturbing the pellet. Finally, the pellet was resuspended in 32μl 100% ethanol thoroughly by vortexing at low speed for 2-3 seconds. The preparation was sufficient for 4 bombardments. For each bombardment, 6μl aliquot was transferred to the center of a macrocarrier and allowed to air dry before bombardment. The conditions for bombardment were listed in Table 3-1.

The cotyledons used for the experiment were placed onto 0.8% bactoagar and bombarded using the biolistic PDS-1000/He system according to procedures described by Sanford (Sanford, 1987). After the bombardment, the cotyledons were incubated for 20-24 hours at room temperature and GUS staining was performed afterward.

**Table 3-1: Parameters used in particle bombardment**

| Parameters | Conditions |
|---|---|
| Micromarriers | 1μm gold |
| Vacuum | 27 inches Hg |
| Helium pressure | 1,100 psi |
| Target distance | 9cm |

### 3.6.3 GUS assay

GUS assay was done by following standard protocol (Jefferson, 1987). Snow beans received particle bombardment were placed into a falcon tube containing the GUS staining solution (100mM Na-phosphate buffer, pH7.0, 0.1% Triton X-100, 1mM EDTA, 0.5mM $K_3Fe(CN)_6$, $K_4.Fe(CN)_6$ and 0.5mg/mL X-gluc) and incubated at 37°C. After overnight incubation, 70% ethanol was added to remove chlorophyll. Then, relative intensities of blue-dot development among the cotyledons in each individual test were recorded under the observation of dissection microscope.

## 3.7 Transgenic expression in *Arabidopsis thaliana*

### 3.7.1 Plant materials

*Arabidopsis thaliana* ecotype *Columbia-0* was used in the study. Surface sterilization of the seeds was performed by shaking the seeds in Clorox solution (sodium hypochlorite, 5.25%) for 3 minutes followed by 3 minutes washing in sterilized distilled water for 3 times. Then, the sterilized seeds were plated onto MS medium (4.3g/L MS salts (Gibco), 2% sucrose, 1x B5 vitamin, 0.8% bacto-agar, pH5.7). The seeds were firstly placed in cold chamber at 4°C for 2 days and then in 22°C growth chamber with a light (daylight 4000k lux) & dark cycle of 16 hours and 8 hours, respectively. The geminated plantlets were collected and further grown on a new MS medium for 2-3 weeks before being transferred into soil.

### 3.7.2 *Agrobacterium* transformation

This was done by Ng, W. K. (Ng, 2001) with standard procedures.

### 3.7.3 Vacuum infiltration *Arabidposis* transformation

The method was based on Bechtold *et al.* (1993) with modification by Lam H.M. (Personal communication, Chinese University of Hong Kong). A single colony of GV3101/pMP90 containing the target gene (section 3.3.1) was cultured with shaking in 5

ml LB medium (10g/L NaCl, 10g/L bacto-tryptone and 5g/L yeast extract) containing 50mg/l rifampicin, kanamycin and 25mg/L gentamycin at 28°C. On the second day, 1ml starter culture was sub-cultured in 500ml YEP (10g/L bacto-peptone, 10g/L yeast extract and 5g/L NaCl) medium containing the same antibiotics mentioned above until $OD_{500} > 2$. The culture was then centrifuged at 7000rpm for 10 minutes and resuspended in 1L infiltration medium (2.2g/L MS salts, 1x B5 vitamins, 50g.L sucrose, 0.5g/L MES, 0.044μM benzylaminopurine, and 0.02% silwet$_{TM}$ L-77, pH5.7).

The resuspended cultured was then poured into a 1L beaker and placed into a vacuum chamber. The plants, as mentioned in 3.6.1, were then inverted with the silique soaking into the bacterial solution. Vacuum was applied and the plants were allowed to stay under vacuum for 10 minutes with bubbles starting to form. At the end of the treatment, vacuum was released and the plants were drained briefly. Then the plants were set upright using the hollow cylinders made by transparencies and they were allowed to grow for seed collection. The seeds so harvested are the $R_1$ seeds.

### 3.7.4 Selection of successful transformants

Surface sterilization of the $R_1$ seeds (100μl roughly 2500 seeds) was performed with a procedure which was mentioned in 3.6.1 except that the seeds were plated onto the MS medium containing 50mg/L kanamycin. The incubation and treatment of the seeds were the same as mentioned in section 3.6.1 and the successful transformants which grew

into green plantlets were selected and further grew on a new MS medium for 2-3 weeks before transfering into soil. To prevent cross fertilization, the individual transformant was wrapped by hollow cylinder made of overhead transparency. The seeds so harvested were the $R_2$ seeds.

### 3.7.5 Selection for homozygous plants

About 50 $R_2$ seeds were sterilized as mentioned in 3.6.1 and plated on a selection medium containing kanamycin. The $R_2$ plants showing 3 to 1 survival to death ratio in a Chi-square analysis were grown for seed collection ($R_3$). A further screening on the $R_3$ seed was carried out and those survived in the selection medium were selected as homozygous line for further expression analysis.

## 3.8 Transgenic expression in tobacco

### 3.8.1 Plant materials

Leaves of about 1 month old wild type tobacco (*Nicotiana tabacum* L. *cv Xanthin. nc*) grown in a 24°C growth chamber with a 16 hour photoperiod were used for *Agrobacterium*-mediated transformation.

### 3.8.2 *Agrobacterium* transformation

The Freeze-frost method was used for transformation of the *pBI121* vector carrying chimeric gene constructs into *Agrobacterium tumefaciens* LBA4404/pAL4404.

#### 3.8.2.1 Preparation of *Agrobacterium tumefaciens* LBA4404/pAL4404 competent cells

On day 1, 10ml LB containing streptomycin (25mg/L) and *Agrobacterium* LBA4404/pAL4404 starter culture were grown at 28°C for 2 days. On day 3, 400μl of the *Agrobacterium* culture was subcultured into 40ml LB and allowed to grow for 5 hours at 28°C. Then, the *Agrobacterium* cells was spun down at 4800rpm for 5 min and washed with 4ml sterilized distilled water. This was followed by a spin and the cells were resuspended in 4ml LB with aliquot of 500μl.

Transformation

First, an aliquot of 500μl *A. tumefaciens* LBA4404/pAL4404 competent cells was thrawn on ice with the addition of 5μl plasmids which contained the chimeric gene constructs for 5 minutes. Then, the mixture was incubated with liquid nitrogen for 5 minutes followed by an incubation at 37°C for 5 minutes. After, one ml LB was added and the culture was incubated at 28 °C for 4 hours. Finally, the culture was spun at 5000 rpm, resuspended in 100μl LB, and spreaded on a agar plate with streptomycin (25mg/L)

and kanamycin (50mg/L). Colonies which developed on the plate at 28°C after 36 hours were selected for subsequent mini-prep (Promega kit) and PCR checking (section 3.3.2).

### 3.8.3 Leaf discs method for tobacco transformation

A single colony of *Agrobacterium tumefaciens* LBA4404 containing the target gene (3.3.2 and 3.3.3b) was cultured in 3 ml LB medium containing 50 mg/l kanamycin and 25 mg/l streptomycin at 28 °C with shaking until $OD_{620} > 1$. The bacterial culture was spun down and resuspended in inoculation medium. The wash was repeated for 2 times and finally the cells were resuspended in 3 ml MS liquid medium. Leaves from the wild type tobacco were cut into small pieces (~ 0.7x0.7 $cm^2$) with razor blade. The cut leaf explants were then submerged in diluted (1:10) *Agrobacterium* culture, 18 ml inoculation medium with 2 ml resuspended *Agrobacterium* culture, for 10 minutes. After blotting dry on sterile filter paper, the explants were placed on co-cultivation medium.

After 4 days co-cultivation at 24 °C with a 16 hour photoperiod, leaf explants were transferred onto shooting medium and incubated in growth chamber under the same conditions as above for callus induction and shoot formation. The leaf explants were transferred to fresh medium every 16-20 days. The regenerated shoots (1 to 3 cm in height) were cut off and inserted into rooting medium for rooting. Finally, the regenerated tobaccos were transferred to soil and grown in certified greenhouse or experimental field.

**Table 3-2: Composition of tissue culture media**

| | MS liquid medium | Co-cultivation medium | Shooting medium | Rooting medium |
|---|---|---|---|---|
| MS salts g/L (Gibco) | 4.3 | 4.3 | 4.3 | 4.3 |
| 1x B5 vitamin | + | + | + | + |
| Sucrose g/l | 20 | 20 | 20 | 20 |
| BA mg/l | 1 | 1 | 1 | - |
| NAA mg/l | 0.1 | 0.1 | 0.1 | - |
| Phytagel mg/l | - | 0.9 | 0.9 | 0.9 |
| Kanamycin mg/l | - | - | 100 | 50 |
| Carbenicilin mg/l | - | - | 300 | 200 |

(BA = N6-benzyladenine, NAA = Naphthaleneacetic acid and all medium is at pH 5.7)

### 3.8.4 GUS staining

GUS staining was done by following a standard protocol (Jefferson, 1987). Regenerated tobacco leaf disc was placed into an eppendorf tube containing the GUS staining solution (100mM Na-phosphate buffer, pH7.0, 0.1% Triton X-100, 1mM EDTA, 0.5mM $K_3Fe(CN)_6$, $K_4.Fe(CN)_6$ and 0.5mg/mL X-gluc) and incubated at 37°C. After overnight incubation, the GUS staining solution was removed and 70% ethanol was added to remove chlorophyll. The stained plant materials were placed in 50% glycerol for long time storage.

## 3.9 DNA analysis

### 3.9.1 Genomic DNA extraction

Genomic DNA was isolated from *Arabidopsis* or tobacco leaves using the CTAB protocol (Doyle *et al.*, 1990) and was suspended in 50 μl $ddH_2O$. DNA concentrations were determined with a spectrophotometer by $OD_{260}$ measurement and were checked by gel electrophoresis in a 1% agarose/ TAE (0.04M Tris-acetate and 1 μM EDTA) gel.

### 3.9.2 Genomic PCR

By using genomic DNA as template, PCR as mentioned in 3.3.2 was performed to detect the presence of the transgene in the regenerated tobacco except that a 25μl reaction mixture containing 0.5μg genomic DNA was used. Then, 5μl PCR products were then taken for agarose/ TAE gel electrophoresis.

### 3.9.3 Southern Blot

Genomic DNA (15 μg) was digested overnight with BamHI, separated on 1% agarose/ TAE gel, at 60 V for 4 hours, and then transferred to nylon membrane (positively charged, Roche) using VacuGeneXL Vacuum blotting System (Pharmacia Biotech). Hybridization and detection was performed according to the method described in the DIG Nucleic Acid Detection Kit (Roche) with single strand DIG-labeled DNA probes

(modified $MSP1_{42 \text{ or } 19}$) which was prepared using DIG DNA labeling Kit (Roche) by PCR amplification using LANm42 or LANm19 and DIG-labeled dUTPs.

## 3.10 RNA analysis

### 3.10.1 RNA extraction

Total RNA was extracted from the developing silique of *Arabidopsis* and developing toabacco seeds by the method as described by Altenbach *et al.* (1989). The concentration and the quality of the extracted RNA was checked by a spectrophotometer and electrophoresis in 1% agarose/ formaldehyde gel.

### 3.10.2 Northern Blot

Total silique RNA and tobacco RNA, with 6 μg was first separated in 1% agarose/ formaldehyde gel and then transferred to nylon membrane (positively charged, Roche) using VacuGeneXL Vacuum blotting System (Pharmacia Biotech). Hybridization and detection was performed according to the method described in the DIG Nucleic Acid Detection Kit (Roche) with single strand DIG-labeled DNA probes (modified $MSP1_{42 \text{ or } 19}$).

## 3.11 Protein analysis

### 3.11.1 Protein extraction

Seed total protein extraction

Total seed protein was extracted from 0.04g mature dry seeds by grinding the seeds into powder and mixing with 0.4ml protein extraction buffer [50mM Tris-HCl pH 8.0, 0.1M NaCl, 10mM EDTA and 1% SDS]. The homogenate was then centrifuged at 14,000 rpm for 15 minutes at 4°C. The clear supernatant was transferred to a new eppendorf tube and saved as total protein extract. The step of centrifugation was repeated once as this may help to prevent lipid contamination. The protein concentration was determined by the Lowry method using the DC protein assay (Bio-Rad) with bovine serum albumin (BSA) as a standard.

Protein from soluble and membrane fractions

Cell soluble protein (CS) was extracted from 0.04g mature dry seeds by grinding the seeds into powder and mixing with 0.5ml protein extraction buffer [50mM Tris-HCl pH 8.0, 0.1M NaCl and 10mM EDTA]. The homogenate was then centrifuged at 14,000 rpm for 15 minutes at 4°C. The clear supernatant was transferred to a new eppendorf tube and saved as seed soluble protein. 0.3ml protein extraction buffer [50mM Tris-HCl pH 8.0, 0.1M NaCl, 10mM EDTA and 1% SDS] was added to the pellet and mixed. The homogenate was then centrifuged at 14,000 rpm for 15 minutes at 4°C. The clear

supernatant was transferred to a new eppendorf tube and saved as seed insoluble cell membrane protein (CM). The protein concentration was determined by the Lowry method using the DC protein assay (Bio-Rad) with bovine serum albumin (BSA) as a standard.

### 3.11.2 Western Blot

For tricine-SDS-PAGE (Laemmli, 1970), total seed protein (50 μg) was mixed with equal volume of 2x sample loading buffer (0.5M Tris-HCl, pH6.8, 10% SDS, 0.2M EDTA, trace amount bromophenol blue) with or without β-mercaptoethanol (4%). For samples with β-mercaptoethanol, they were further incubated at 99°C for 10 minutes. All samples were separated by 16.5% tricine-SDS-PAGE. After electrophoresis, the gel was either stained in Coomassie blue solution (1 g/L Coomassie brilliant blue G-250, 1% methanol) or transferred to membrane for immuno-detection. Stained gel was destained in destaining solution (methanol : 100% glacial acetic acid : water, 20 : 6 : 55).

### 3.11.3 Western blot analysis

After tricine-SDS-PAGE, protein samples were blotted onto PVDF membrane (Bio-Rad) using mini-Trans-blot system (BioRad) as described in the user manual. Electro-transfer was performed at constant voltage (100 V) for 60 minutes in transfer buffer (48mM Tris, 39mM Glycine, 20% methanol). The efficiency of the transfer was

monitored by staining the transferred gel as in section 3.5.9.

The transferred membrane was first blocked in 0.2% blocking solution (1X TBS, 0.2% AURORA blocking reagent, 0.1% Tween20) at room temperature for 1 hour and was then incubated with 3.3 μl of anti-$MSP1_{42}$ serum as primary antibody (Lau, 2003) in 10 ml of 0.2% blocking solution at room temperature for 1 hour. After washing the membrane three times in 0.2% blocking solution, the membrane was incubated in 0.2% blocking solution containing alkaline phosphatase (AP)-conjugated goat anti-rabbit immuno-globulin G (Bio-Rad) at a 1:3000 dilution for 1 hour. The membrane was further washed by 0.2% blocking solution, and the reactive protein bands were detected by incubation with the enzyme colour substrate NBT-BCIP (Bio-Rad).

# Chapter 4 Results

## 4.1 Transient expression of $MSP1_{42}$ and $MSP1_{19}$

### 4.1.1 Construction of the GUS fusion constructs

Four Gus fusion constructs, pTZ/Phas/P42::GUSN, pTZ/Phas/MP42::GUSN, pTZ/Phas/P19::GUSN and pTZ/Phas/MP19::GUSN were tested for transient expression by particle bombardment with a positive control, pTZ/Phas/GUS and a negative control: pTZ/Phas/GUSN. GUSN without a start codon was used for the fusion constructs except for the positive control.

Schematic diagrams of the constructs are shown in Figure 4.1.1-1 and restriction maps for the construction of pTZ/Phas/MP19::GUSN is shown in Figure 4.1.1-2. In addition, DNA sequencing was performed to further confirmed the fidelity of the constructs (data not shown).

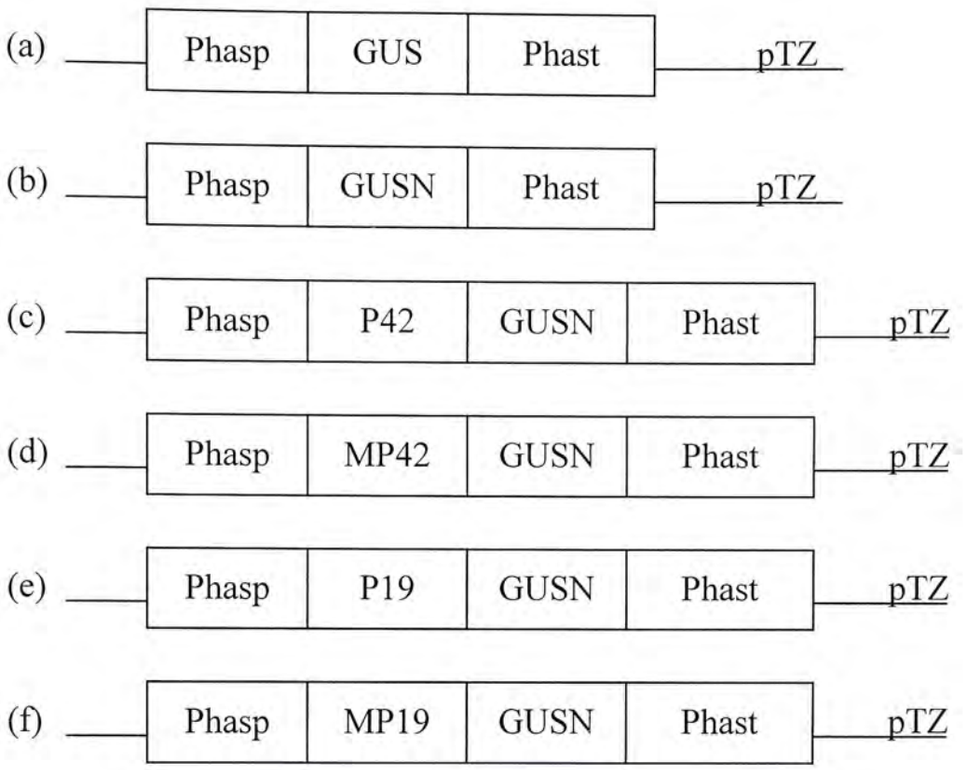
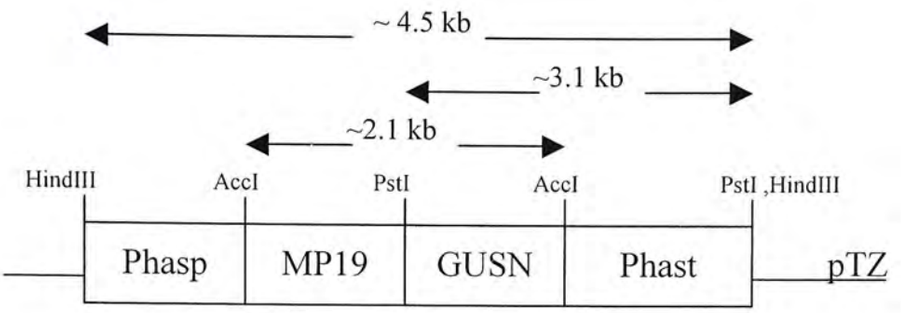



**Figure 4.1.1-1 (a-f) GUS fusion constructs used in the transient assay**

(a) pTZ/Phas/GUSATG, positive control; (b) pTZ/Phas/GUSN, negative control. (c) pTZ/Phas/P42::GUSN, unmodified P42; (d) pTZ/Phas/MP42::GUSN, modified P42; (e) pTZ/Phas/P19::GUSN, unmodified P19; (f) pTZ/Phas/MP19::GUSN, modified P19

(a)




(b)

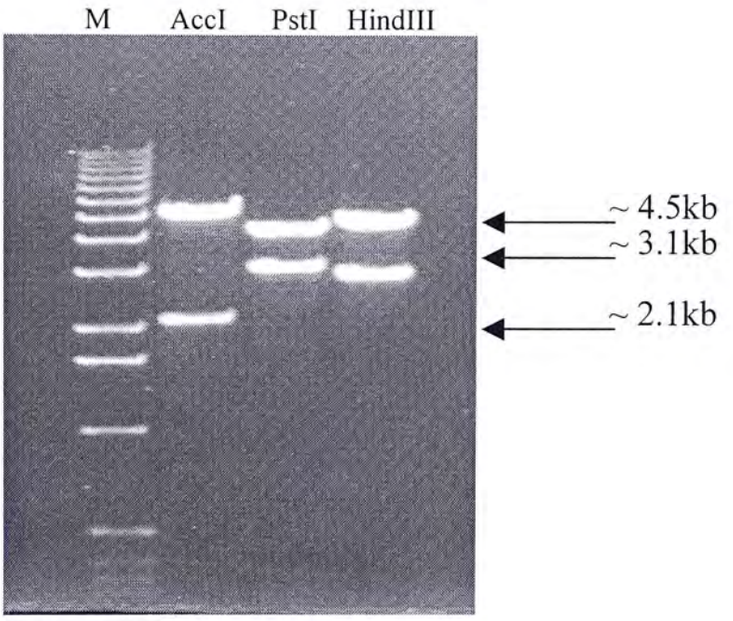



**Figure 4.1.1-2 Construction of the pTZ/Phas/MP19::GUSN**

(a) Structure of the pTZ/phas/MP19::GUSN showing the position of different enzyme sites and the fragment sizes. (b) Restriction digestion of the recombinant plasmids by different enzymes as indicated. The arrows indicate the expected sizes of the bands. Key: M: 1 Kb plus DNA ladder (Invitrogen).

### 4.1.2 Particle Bombardment

Totally, Four bombardment tests were performed. The number of snow bean cotyledons showing blue dot development with different relative intensities was counted. The results were shown in Table 4.1.2-1 a-d and bar charts of Figure 4.1.2-1 a-d. Photographs were taken to illustrate the different levels of blue dot development by the six constructs in Figure 4.1.2-2 a-f.

All cotyledons of positive control gave blue dot development with more or less even distribution but at all intensity levels (from 5+ to 1+). The level of expression by the modified genes was greater than that of the unmodified ones because, firstly, over 80% of the cotyledons bombarded with pTZ/Phas/MP42::GUSN gave positive results including a few gave distinctly high intensity, but no blue dot was found in the cotyledons of pTZ/Phas/P42::GUSN. Secondly, greater number of cotyledons of pTZ/Phas/MP19::GUSN gave blue dot development than the pTZ/Phas/P19::GUSN.

**Table 4.1.2-1 a-d: Transient expression of MSP1 as GUS fusion in bean cotyledons**

Tables a to d show the results of Test 1 to 4, respectively. Cotyledons shown one or more blue dots were regarded as positive results. Key: + shows the relative intensity of blue dots (5+, maximum)

(a)

| Constructs | Dish No. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| pTZ/Phas/GUS | 1 | 2 | 3 | 5 | 3 | 3 | 0 | 16 |
| | 2 | 1 | 3 | 4 | 2 | 5 | 0 | 15 |
| pTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 16 | 16 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 16 | 16 |
| pTZ/Phas/P42::GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 16 | 16 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| pTZ/Phas/MP42::GUSN | 1 | 0 | 1 | 0 | 5 | 8 | 2 | 16 |
| | 2 | 1 | 2 | 3 | 1 | 5 | 4 | 16 |

(b)

| Constructs | Dish No. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| pTZ/Phas/GUS | 1 | 2 | 3 | 3 | 5 | 3 | 0 | 16 |
| | 2 | 4 | 2 | 3 | 6 | 1 | 0 | 16 |
| pTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| pTZ/Phas/P42::GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| pTZ/Phas/P19::GUSN | 1 | 0 | 1 | 0 | 2 | 7 | 5 | 15 |
| | 2 | 0 | 0 | 1 | 1 | 11 | 3 | 15 |
| pTZ/Phas/MP42::GUSN | 1 | 1 | 3 | 2 | 0 | 8 | 1 | 15 |
| | 2 | 1 | 2 | 3 | 1 | 4 | 4 | 15 |
| pTZ/Phas/MP19::GUSN | 1 | 0 | 4 | 1 | 3 | 7 | 0 | 15 |
| | 2 | 2 | 2 | 3 | 4 | 5 | 0 | 16 |

(c)

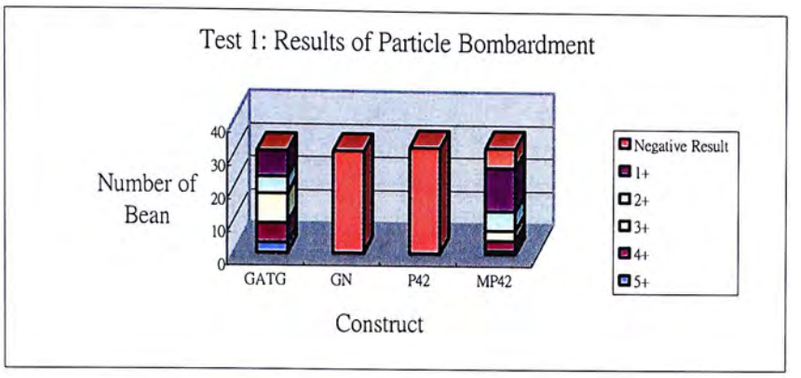
| Constructs | Dish No. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| pTZ/Phas/GUS | 1 | 2 | 1 | 3 | 4 | 5 | 0 | 15 |
| | 2 | 2 | 1 | 2 | 4 | 5 | 1 | 15 |
| pTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| pTZ/Phas/P19::GUSN | 1 | 0 | 2 | 0 | 2 | 7 | 4 | 15 |
| | 2 | 0 | 0 | 1 | 4 | 9 | 1 | 15 |
| pTZ/Phas/MP19::GUSN | 1 | 2 | 3 | 4 | 3 | 1 | 2 | 15 |
| | 2 | 3 | 2 | 4 | 3 | 2 | 1 | 15 |

(d)

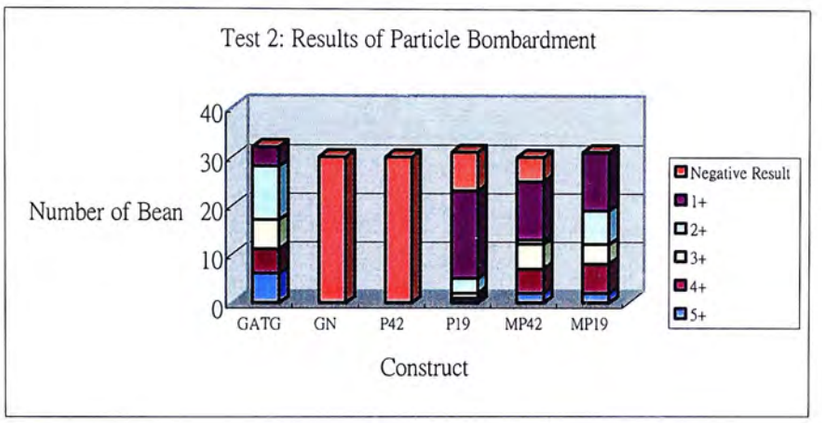
| Constructs | Dish No. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| pTZ/Phas/GUS | 1 | 3 | 4 | 3 | 3 | 2 | 0 | 15 |
| | 2 | 4 | 3 | 3 | 4 | 1 | 0 | 15 |
| pTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 16 | 16 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| pTZ/Phas/P42::GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| pTZ/Phas/P19::GUSN | 1 | 1 | 0 | 1 | 0 | 9 | 4 | 15 |
| | 2 | 2 | 1 | 1 | 2 | 5 | 4 | 15 |
| pTZ/Phas/MP42::GUSN | 1 | 2 | 2 | 1 | 5 | 4 | 1 | 15 |
| | 2 | 1 | 1 | 2 | 3 | 6 | 2 | 15 |
| pTZ/Phas/MP19::GUSN | 1 | 1 | 3 | 1 | 6 | 1 | 3 | 15 |
| | 2 | 1 | 3 | 3 | 1 | 7 | 1 | 16 |

In summary, pTZ/Phas/P19::GUSN, pTZ/Phas/MP42::GUSN and pTZ/Phas/MP19::GUSN can be expressed while the modified shorter sequence pTZ/Phas/MP19::GUSN give better expression.

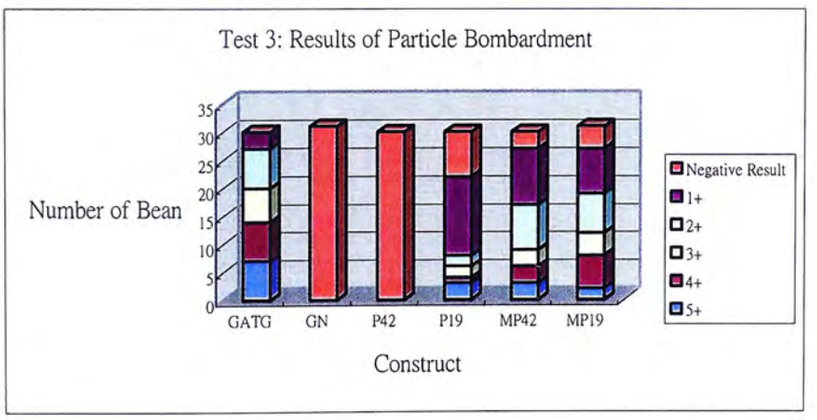
(a)


Test 1: Results of Particle Bombardment
Number of Bean
40
30
20
10
0
GATG
GN
P42
MP42
Construct
Negative Result
1+
2+
3+
4+
5+

(b)


Test 2: Results of Particle Bombardment
Number of Bean
40
30
20
10
0
GATG
GN
P42
P19
MP42
MP19
Construct
Negative Result
1+
2+
3+
4+
5+

(c)


Test 3: Results of Particle Bombardment
Number of Bean
35
30
25
20
15
10
5
0
GATG
GN
P42
P19
MP42
MP19
Construct
Negative Result
1+
2+
3+
4+
5+

(d)

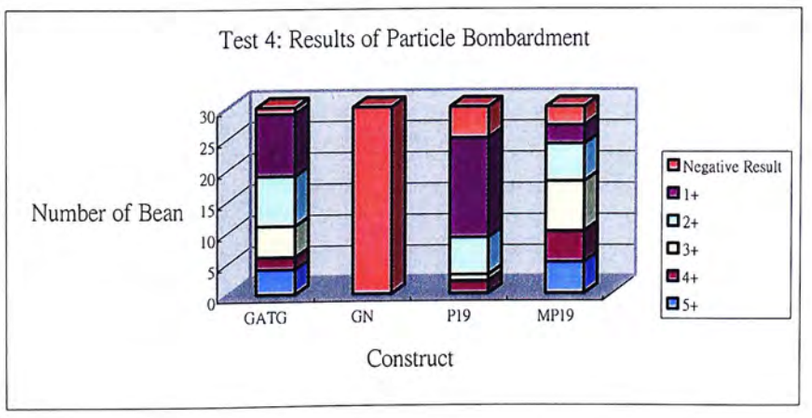

Test 4: Results of Particle Bombardment
Number of Bean
30
25
20
15
10
5
0
GATG
GN
P19
MP19
Construct
Negative Result
1+
2+
3+
4+
5+

**Figure 4.1.2-1 a-d Particle bombardment and transient expression of MSP1 GUS fusions**

Number of cotyledons showing different relative intensities are shown in bar charts in different colours as indicated. Key: GATG: Phas/GUS (GUS with ATG, a positive control); GN: Phas/GUSN (GUS without ATG, a negative control); P42: Phas/P42::GUSN; MP42: Phas/MP42::GUSN; and P19: Phas/P19::GUSN; MP19: Phas/MP19::GUSN

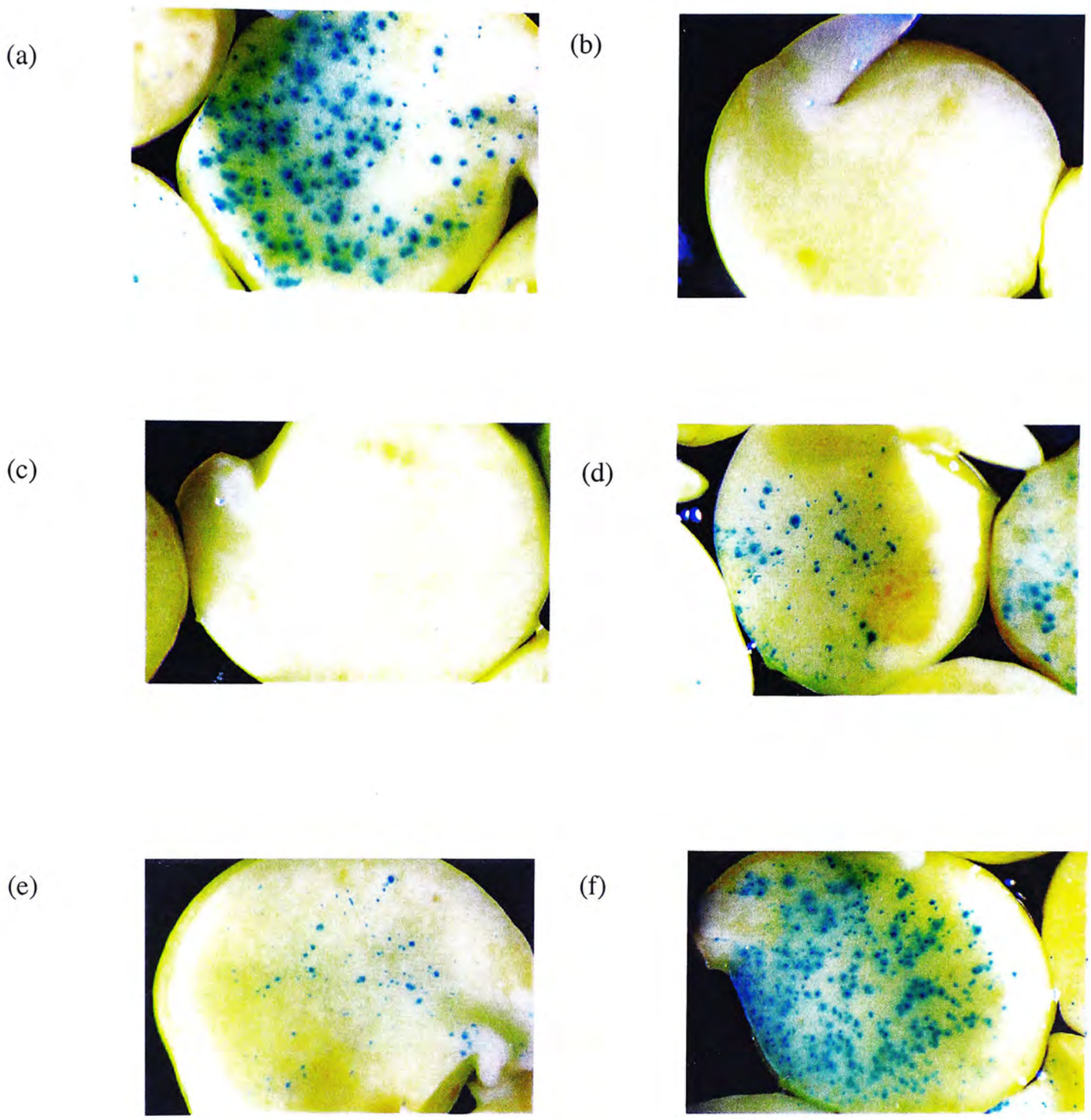



**Figure 4.1.2-2 a-f. Photographs illustrating the different levels of blue dot development in particle bombardment**

(a) pTZ/Phas/GUSATG, positive control; (b) pTZ/Phas/GUSN, negative control; (c) pTZ/Phas/P42::GUSN, unmodified P42; (d) pTZ/Phas/MP42::GUSN, modified P42; (e) pTZ/Phas/P19::GUSN, unmodified P19; (f) pTZ/Phas/MP19::GUSN, modified P19

## 4.2 Transgenic analysis of $MSP1_{42}$ and $MSP1_{19}$ expression

### 4.2.1 $MSP1_{42}$ and $MSP1_{19}$ constructs and transformation

$MSP1_{42}$ (MP42) and $MSP1_{19}$ (MP19) were used to construct chimeric genes under the control of phaseolin promoter and terminator.

Diagrams of the constructs are shown in Figure 4.2.1-1

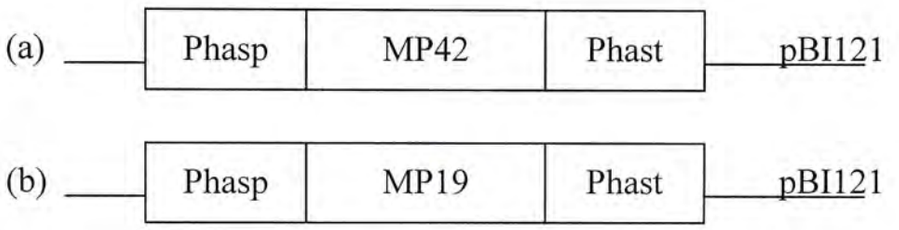



**Figure 4.2.1-1 $MSP1_{42}$ and $MSP1_{19}$ chimeric constructs in vector pBI121.** (a) Phas/MP42 and (b) Phas/MP19. Phasp: Phaseolin promoter; Phast: Phaseolin terminator.

The two chimeric constructs pBI/Phas/MP42 and pBI/Phas/MP19 were transformed into *A. tumefaciens* GV3101/pMP90 for *Arabidopsis* transformation.

Construct diagrams are shown in Figure 4.2.1-1

### 4.2.2 Selection of transgenic plants

After *Arabidopsis* transformation, first generation transgenic plants (R1) harboring the two chimeric gene constructs were obtained through kanamycin medium selection.

To select heterozygous lines, the self-fertilizied seeds (R2) of the R1 transgenic plants, based on the Mendel's law for monohybrid cross, were further screened for 3:1 survival to dead ratio on kanamycin medium and Chi-square statistical test was used to determine the credibility of the survival ratio.

Seven out of fourteen lines of R2 seeds of $MSP1_{42}$ (Table 4.2.2-1a) and eleven out of twenty-four lines of R2 seeds of $MSP1_{19}$ (Table 4.2.2-1b) were screened and identified as heterozygous.

Homozygous lines (R3) of the two chimeric constructs were selected by screening the fertilized seeds of the R2 plants on kanamycin medium. All the seeds of homozygous lines survived on the selection medium and the selected homozygous lines were used in subsequent molecular analysis (Table 4.2.2-2).

Sixteen homozygous lines were collected for $MSP1_{42}$ and eight lines for $MSP1_{19}$.

**Table 4.2.2-1 a and b: Selection of R2 *Arabidopsis* lines by Chi-square analysis**

R2 progenies giving roughly 3 to 1 survival to death ratio were assumed to be heterozygous lines. The validity of data was analyzed using Chi-square test at P value of 0.05 (chi-square = 3.84). "+" means P value > 0.05 (chi-square < 3.84) indicating that the difference between the observed and predicted results was insignificant and thus selected.

(a) pTZ/Phas/MP42

| Plant | Total no. | No. survived | No. died | Chi-square |
|---|---|---|---|---|
| 1.1 | 81 | 47 | 34 | - |
| 1.2 | 96 | 96 | 0 | - |
| 2.2 | 126 | 91 | 35 | + |
| 2.3 | 101 | 79 | 22 | + |
| 2.4 | 96 | 84 | 12 | - |
| 3.3 | 95 | 49 | 46 | - |
| 4.1 | 102 | 19 | 83 | - |
| 4.2 | 105 | 78 | 27 | - |
| 5.1 | 102 | 83 | 19 | + |
| 5.2 | 111 | 110 | 1 | - |
| 5.3 | 108 | 81 | 27 | + |
| 6.3 | 108 | 82 | 26 | + |
| 7.7 | 115 | 93 | 22 | + |
| 8.1 | 89 | 68 | 21 | + |

Key: "+" stands for positive result; "-" stands for negative result

(b) pTZ/Phas/MP19

| Plant | Total no. | No. survived | No. died | Chi-square |
|---|---|---|---|---|
| 1.1 | 117 | 79 | 38 | + |
| 1.2 | 93 | 73 | 20 | + |
| 1.3 | 84 | 38 | 46 | - |
| 1.4 | 101 | 46 | 55 | - |
| 2.1 | 106 | 77 | 29 | + |
| 2.2 | 110 | 89 | 21 | + |
| 2.3 | 107 | 92 | 15 | - |
| 2.4 | 103 | 88 | 15 | - |
| 2.5 | 127 | 99 | 28 | + |
| 2.6 | 105 | 49 | 56 | - |
| 3.1 | 98 | 59 | 39 | - |
| 3.2 | 90 | 62 | 28 | + |
| 3.3 | 95 | 70 | 25 | + |
| 3.4 | 86 | 18 | 68 | - |
| 3.5 | 96 | 64 | 32 | - |
| 5.1 | 108 | 106 | 2 | - |
| 5.2 | 102 | 15 | 87 | - |
| 5.3 | 84 | 64 | 20 | + |
| 5.4 | 105 | 89 | 16 | - |
| 5.5 | 107 | 84 | 23 | + |
| 9.2 | 104 | 89 | 15 | - |
| 9.3 | 95 | 65 | 30 | + |
| 10.1 | 102 | 73 | 29 | + |
| 10.2 | 88 | 47 | 41 | - |

Key: "+" stands for positive result; "-" stands for negative result

**Table 4.2.2-2: Selection of homozygous transgenic R3 *Arabidopsis* lines**

R3 transgenic lines homozygous in gene insertion all survived antibiotic selection

| **Homozygous transgenic R3 *Arabidopsis*** | |
|---|---|
| Phas/MP42 | Phas/MP19 |
| 2.3.1 | 2.5.2 |
| 2.3.2 | 3.3.1 |
| 2.3.3 | 3.3.2 |
| 2.3.4 | 3.3.3 |
| 2.3.5 | 5.3.6 |
| 2.3.11 | 9.3.2 |
| 5.3.3 | 10.1.4 |
| 5.3.8 | 10.1.8 |
| 6.3.1 | |
| 6.3.2 | |
| 7.7.3 | |
| 7.7.4 | |
| 7.7.7 | |
| 8.1.3 | |
| 8.1.7 | |
| 8.1.12 | |

### 4.2.3 Southern analysis

Southern analysis was carried out to confirm the integration of the transgene in the plant genome. The copy number of the integrated transgene was estimated through the blotting. For this analysis, the genomic DNA of the homozygous lines was extracted and digested with BamHI which cuts only one time on the T-DNA vector backbone, but not the transgene.

The leaves of sixteen homozygous lines of $MSP1_{42}$ and eight of $MSP1_{19}$ were collected for genomic DNA extraction and Southern blot analysis using $MSP1_{42}$ and $MSP1_{19}$ as a probe, respectively.

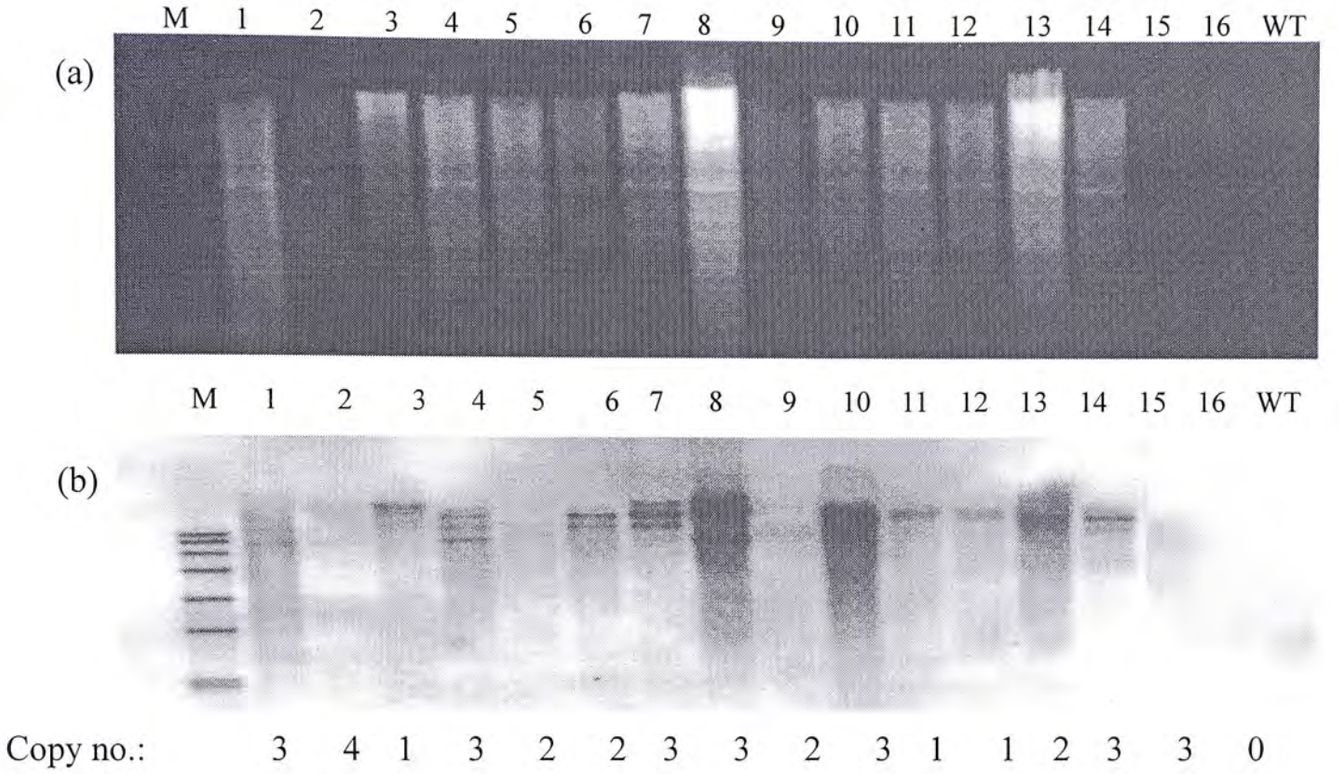



**Figure 4.2.3-1 Southern analysis of Phas/MP42 transgenic *Arabidopsis***

(a) Genomic DNA (15μg) was digested with BamHI and separated by gel electrophoresis. (b) The resolved DNA was blotted onto a nylon membrane for hybridization. Key: WT: wild type Col-o; M: DNA Molecular Weight Marker VII, DIG-labeled (Roche). **1:** transgenic line 2.3.1; **2:** 2.3.2; **3:** 2.3.3; **4:** 2.3.4; **5:** 2.3.5; **6:** 2.3.11; **7:** 5.3.3; **8**: 5.3.8; **9:** 6.3.1; **10:** 6.3.2; **11:** 7.7.3; **12:** 7.7.4; **13:** 7.7.7; **14:** 8.1.3; **15:** 8.1.7; and **16:** 8.1.12. The estimated copy number of the transgene in each line was also shown in (b).

### 4.2.4 Northern analysis

Northern blotting with total silique RNA was carried out to study the transgene expression at transcriptional level (Figures 4.2.4-1 and 4.2.4-2). MSP1 transcript of most of the transgenic lines (14 out of 16) was detected with the expacted band size, 1.2 Kb for the $MSP1_{42}$ construct and of eight transgenic lines with the expected size, 0.3 Kb for the $MSP1_{19}$ construct.

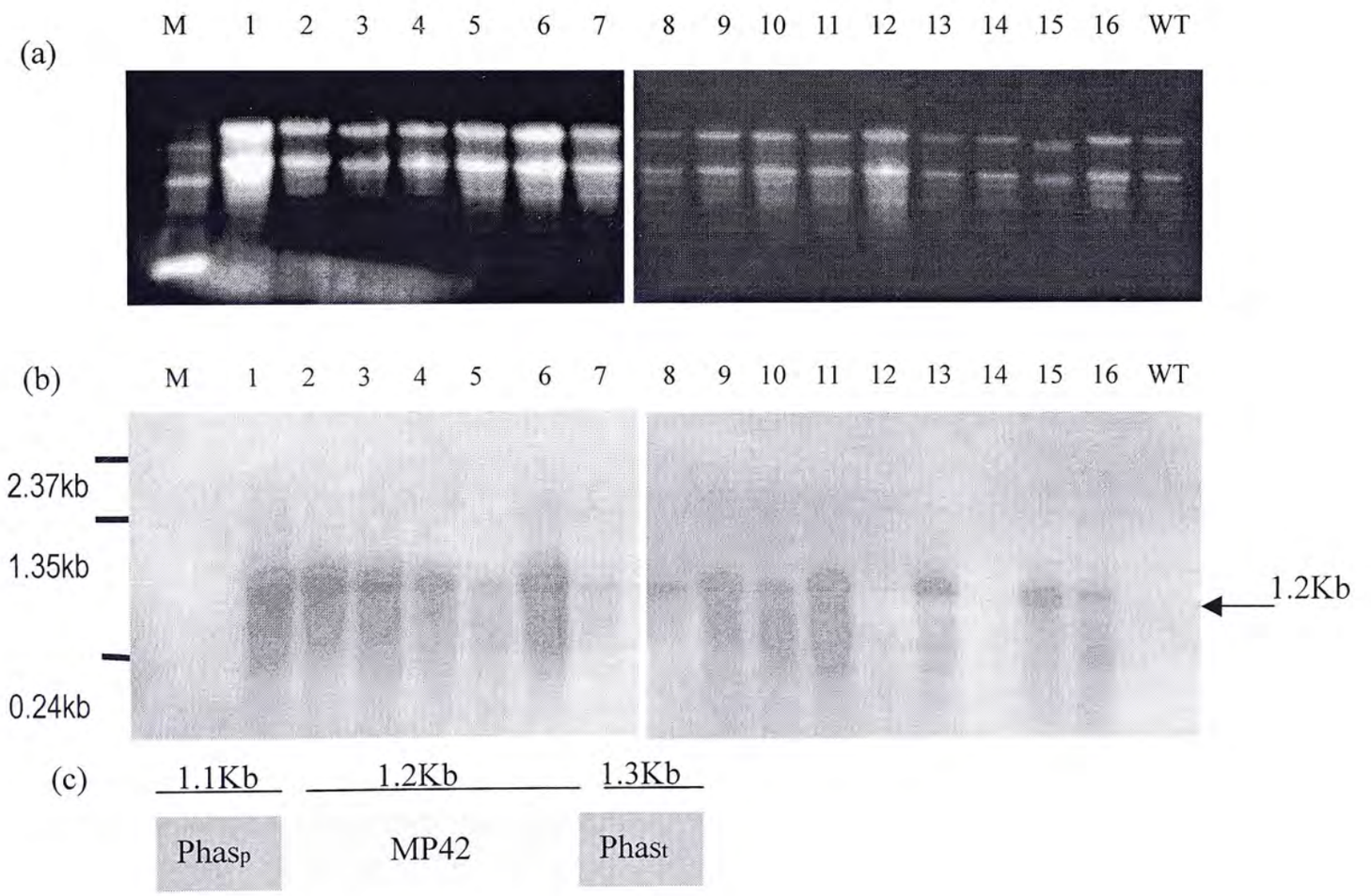
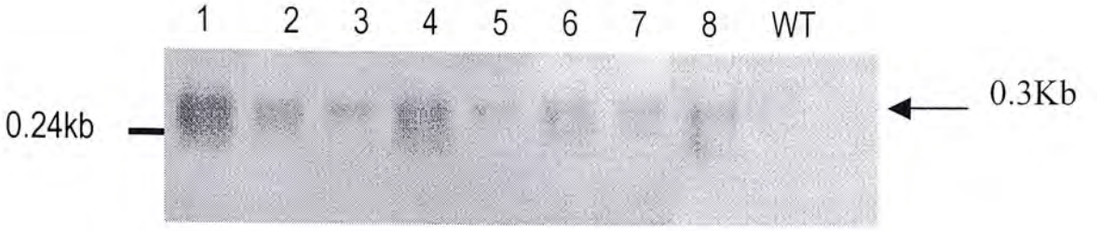



**Figure 4.2.4-1 Northern analysis of MP42 the transgenic *Arabidopsis.***
(a) Total siliques RNA (8µg/lane) was resolved in 1% RNA denaturating gel; (b) The resolved RNA was blotted onto a nylon membrane for hybridization and detection; and (c) Diagram showing the size of the chimeric gene fragments. **Key: M:** 0.24-9.5Kb RNA ladder (Invitrogen); **WT:** Wild type; **1:** Transgenic line 2.3.1; **2:** 2.3.2; **3:** 2.3.3; **4:** 2.3.4; **5:** 2.3.5; **6:** 2.3.11; **7:** 5.3.3; **8**: 5.3.8; **9:** 6.3.1; **10:** 6.3.2; **11:** 7.7.3; **12:** 7.7.4; **13:** 7.7.7; **14:** 8.1.3; **15:** 8.1.7; and **16:** 8.1.12

(a)




(b)

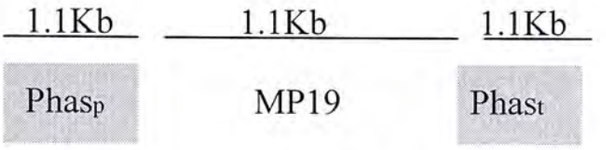



**Figure 4.2.4-2 Northern analysis of the MP19 transgenic *Arabidopsis*.**

(a) The resolved RNA was blotted onto a nylon membrane for hybridization and detection and (b) Diagram showing the size of the chimeric gene fragments. **Key: M:** 0.24-9.5Kb RNA ladder (Invitrogen); **WT:** Wild type; **1:** transgenic line 2.5.2; **2:** 3.3.1; **3:**3.3.2; **4:**3.3.3; **5:** 5.3.6; **6:** 9.3.2; **7:** 10.1.4;and **8:** 10.1.8

### 4.2.5 Western analysis

Recombinant protein was detected by western blotting analysis. For this detection, all of the transgenic were shown to give positive signal in the Northern analysis (Figures 4.2.4-1 and 4.2.4-2). The expected sizes of the $MSP1_{42}$ and $MSP1_{19}$ recombinant proteins are 42Kda and 19Kda, respectively. However, no comparable protein band was detected for both the $MSP1_{42}$ and $MSP1_{19}$ constructs (Figures 4.2.5-1 and 4.2.5-2).

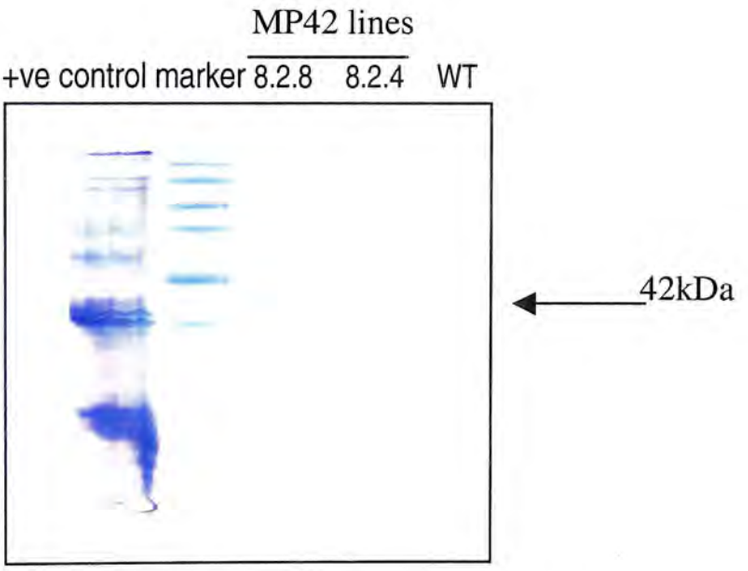



**Figure 4.2.5-1 Western analysis of the MP42 transgenic *Arabidopsis***
Total seed soluble protein (50$\mu$g/lane) resolved in 12.5% SDS-PAGE without β-mercaptoethanol was blotted onto PVDF membrane (Bio-Rad) and reacted with monoclonal antibody MAb5.2 as primary antibody and alkaline phosphatase conjugated anti-mouse IgG as secondary antibody. Protein sample from MP42-AFVY was included as a positive control.

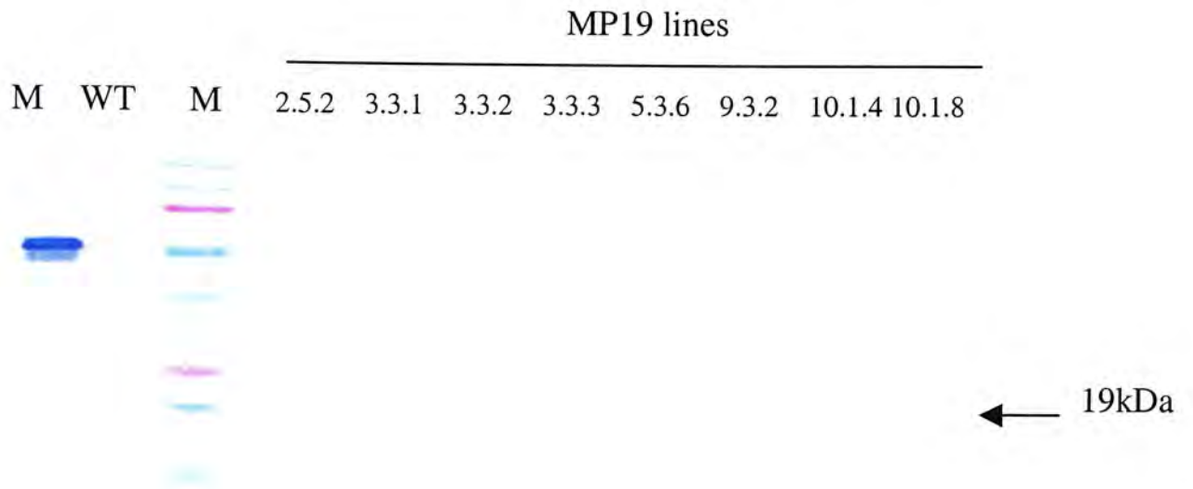



**Figure 4.2.5-2 Western analysis of the MP19 transgenic *Arabidopsis***

Total seed soluble proteins (50$\mu$g/lane) resolved in 12.5% SDS-PAGE without β-mercaptoethanol was blotted onto PVDF membrane (Bio-Rad) and reacted with monoclonal antibody MAb5.2 as primary antibody and alkaline phosphatase conjugated anti-mouse IgG as secondary antibody. Protein sample from denatured LRP/MP42 (line 1.2.8) was included as a positive control with a calculated MW of 61.6kDa. The arrow shows the position of the expected $MSP1_{19}$ protein

# 4.3 Expression of the protein-targeting and GUS-fused modified MSP1 constructs

### 4.3.1 Construction of the fusion constructs

#### (A) Protein-targeting constructs

In an attempt to target the MP42 and MP19 to protein storage vacuole for accumulation and deposit, three targeting sequences, BP-80, α-TIP and RMR were introduced to their C-terminal ends. All these constructs were driven by the phaseolin promoter with its signal sequence.

Eight chimeric gene constructs including pBI/Phas/SP/MP42, pBI/Phas/SP/MP19, pBI/Phas/SP/MP42-BP80, pBI/Phas/SP/MP19-BP80, pBI/Phas/SP/MP42-α-TIP, pBI/Phas/SP/MP19-αTIP, pBI/Phas/SP/MP42-RMR and pBI/Phas/SP/MP19-RMR were thus constructed (Figure 4.3.1-1) and introduced into LBA4404 for Tobacco transformation.

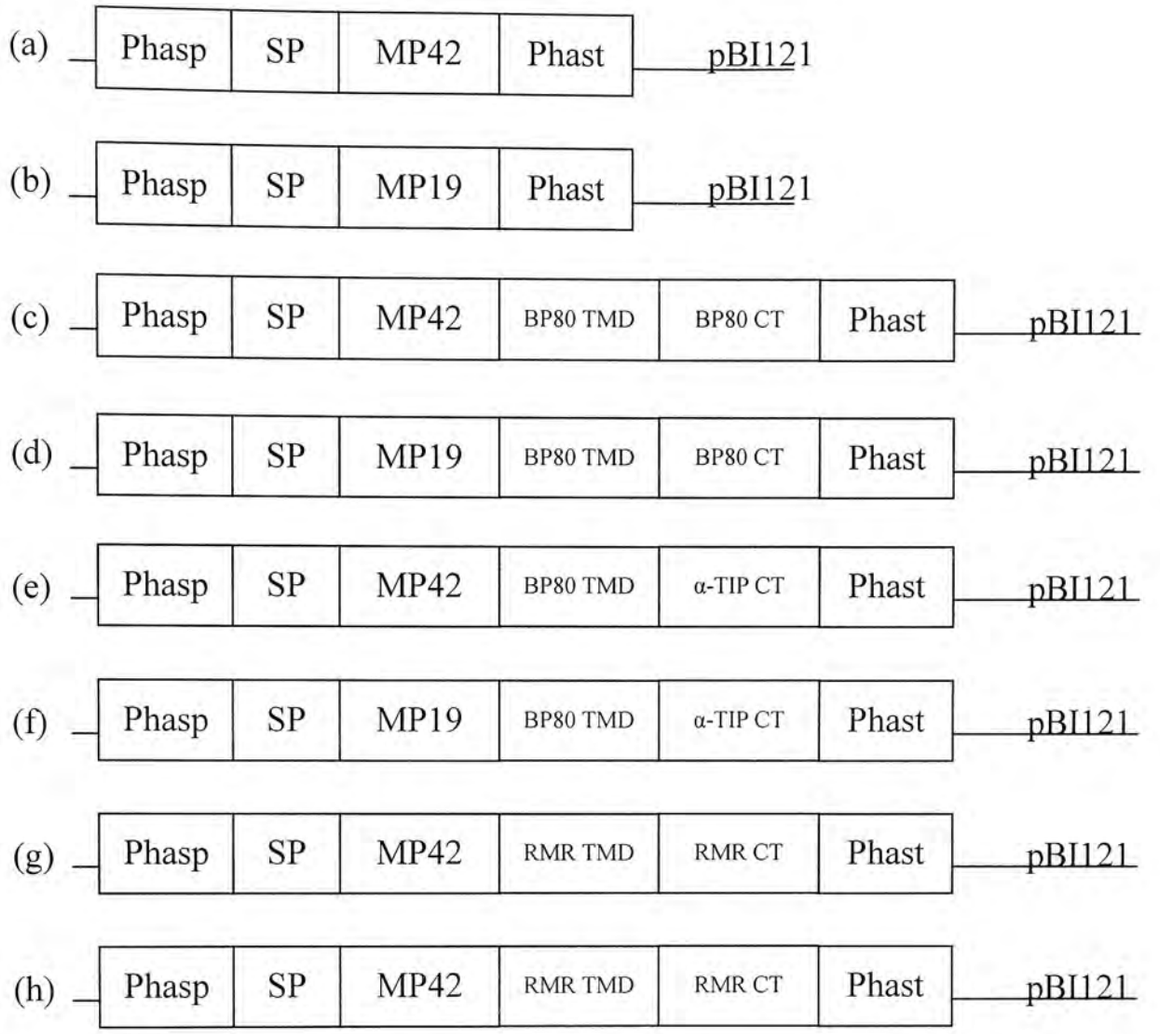



**Figure 4.3.1-1 Protein-targeting constructs**
a and b: controls ; c and d: α-TIP constructs; e and f: BP-80 constructs; and g and h: RMR constructs

These constructs were checked for expected restriction fragments as shown in Figures 4.3.1-2 to -5. HindIII was used to release the phaseolin cassette and Xba I, which cuts only one time in the vector backbone and in the phaseolin promoter, was used to check the orientation of the cassette. As the restriction sites at both ends of the protein targeting construct are the same, i.e. AccI sites, using PCR to check its orientation after cloning was done by using the 5' primer specific for $MSP1_{42}$ or $MSP1_{19}$ and the 3' primer specific for the protein targeting sequence. Finally, DNA sequencing was performed to confirm the fidelity of the constructs (data not shown).

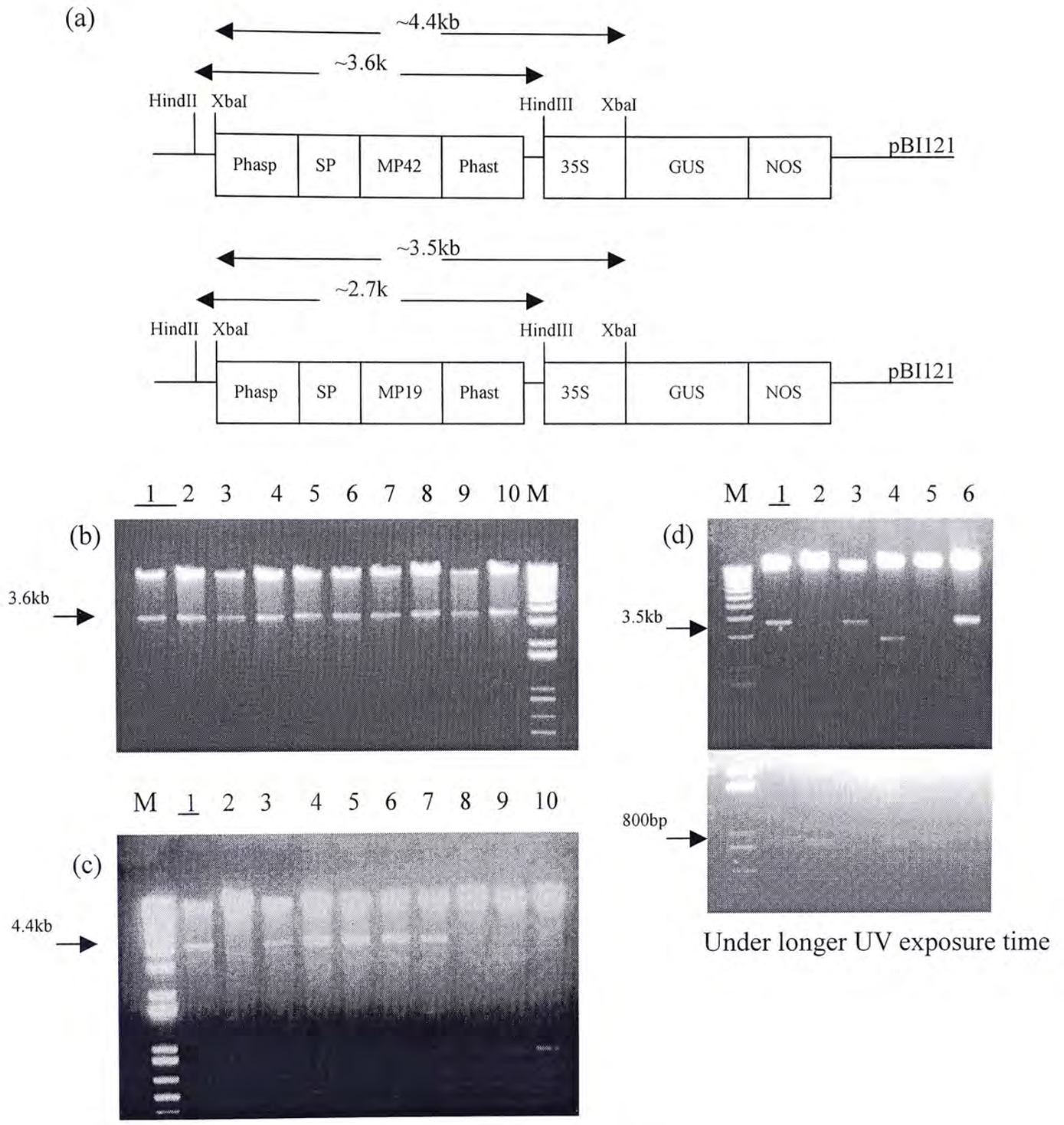



**Figure 4.3.1-2 The Phas/SP/MP42 and Phas/SP/MP19 constructs**

(a) Structure of the Phas/SP/MP42 and Phas/SP/MP19 constructs in pBI121 showing the position of the HindIII abd XbaI sites and the fragment sizes; (b) Restriction digestion of the plasmid Phas/SP/MP42 by HindIII and (c) by XbaI; (d) Restriction digestion of the plasmid Phas/SP/MP19 by XbaI. Key: lane M: 1Kb plus DNA ladder (Invitrogen); lanes 1-10 and 1-6: the colony numbers; Arrow indicates the band position and size; and the underlined colony numbers denoted from *Agrobacterium* LBA4404 that used for transformation.

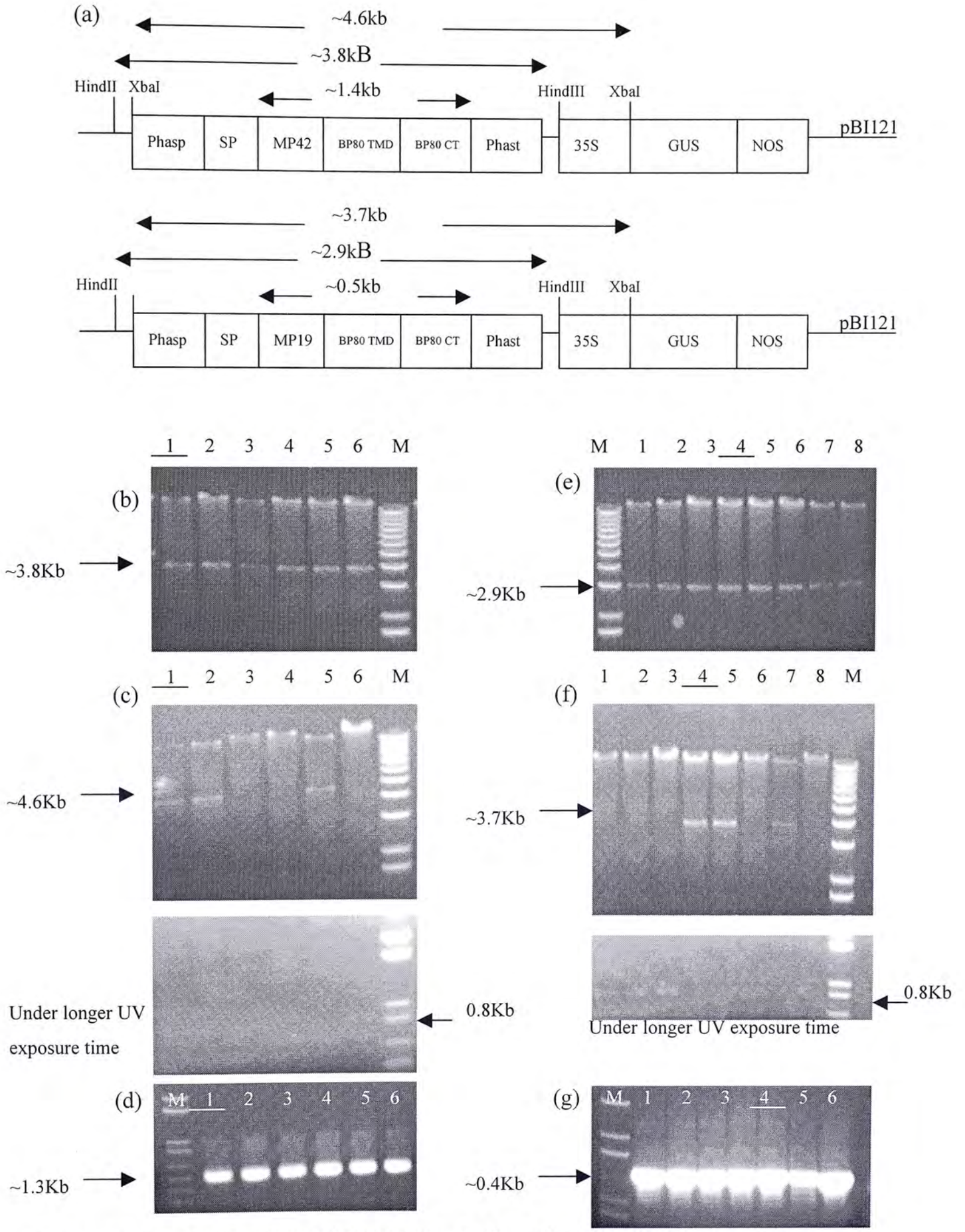
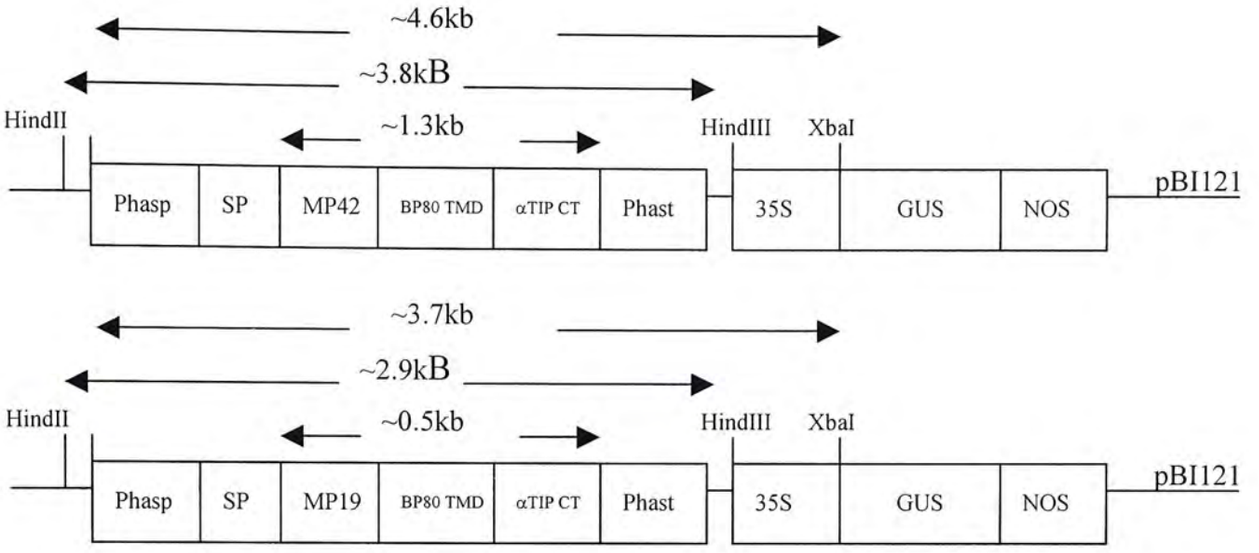



**Figure 4.3.1-3 The Phas/SP/MP42-BP80 and Phas/SP/MP19-BP80 constructs**

(a) Structure of the Phas/SP/MP42-BP80 and Phas/SP/MP19-BP80 constructs showing the position of the HindIII and XbaI sites, and the fragment sizes. (b)

restriction digestion of the plasmid Phas/SP/MP42-BP80 by HindIII and (c) by XbaI; (d) PCR amplification of MP42- BP80 in the Phas/SP/MP42-BP80 construct using primers LNspM42 and RABP-80. (e) Restriction digestion of plasmid Phas/SP/MP19-BP80 by HindIII and (f) by XbaI; (g) PCR amplification of MP19-BP80in the Phas/SP/MP19-BP80 using primers LNspM19 and RABP-80. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); other lanes with numbers show the number of colonies; arrows show the band positions and sizes; the underlined colony indicated DNA from *Agrobacterium* LBA4404 that used for transformation.

(a)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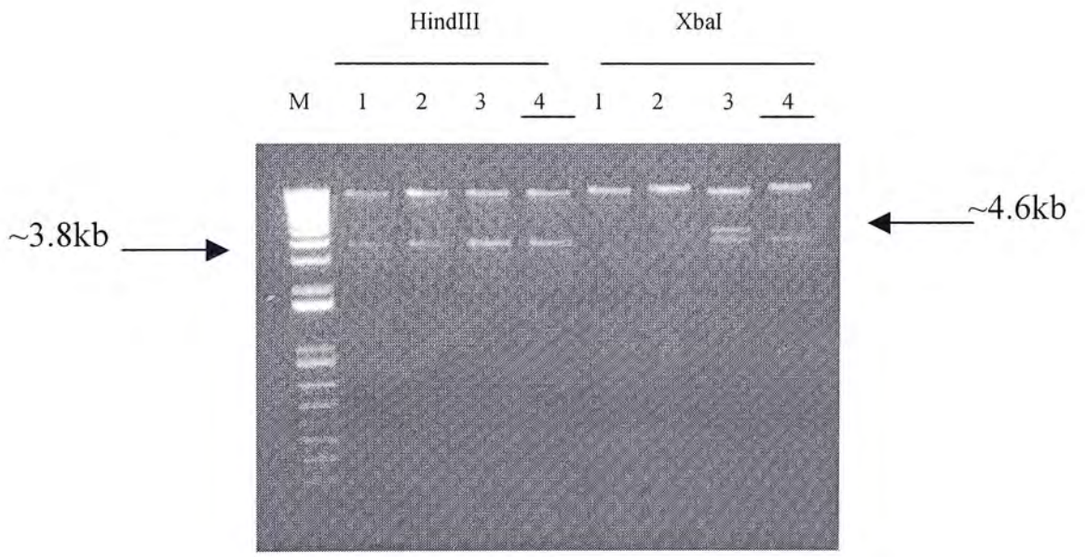
~3.8kB
~1.3kb
HindII
HindIII
XbaI
Phasp
SP
MP42
BP80 TMD
αTIP CT
Phast
35S
GUS
NOS
pBI121
~3.7kb
~2.9kB
~0.5kb
HindII
HindIII
XbaI
Phasp
SP
MP19
BP80 TMD
αTIP CT
Phast
35S
GUS
NOS
pBI121

(b)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XbaI
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~3.8kb
~4.6kb

(c)

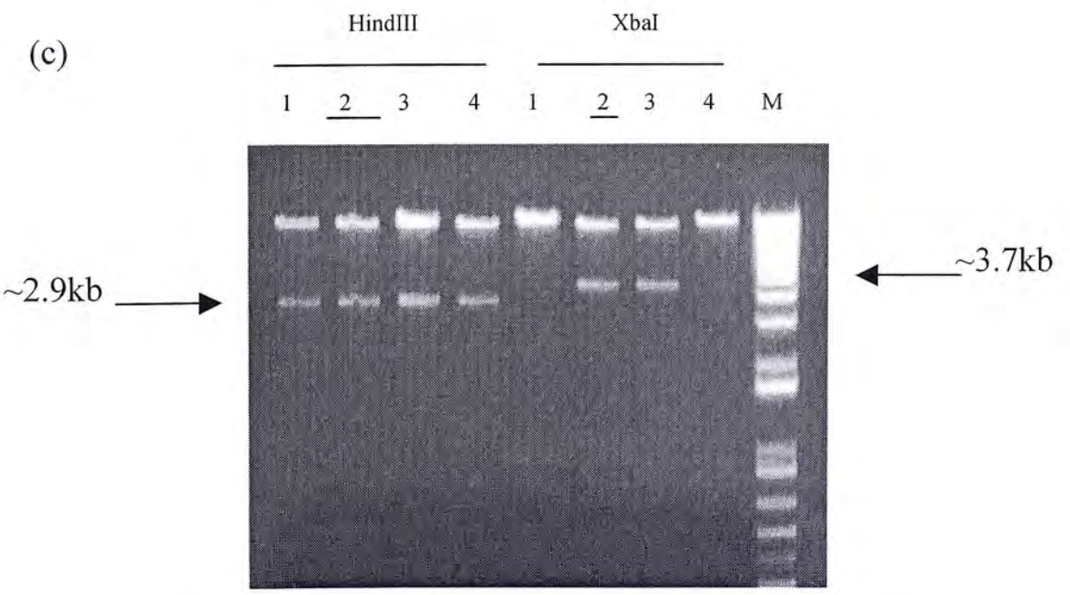
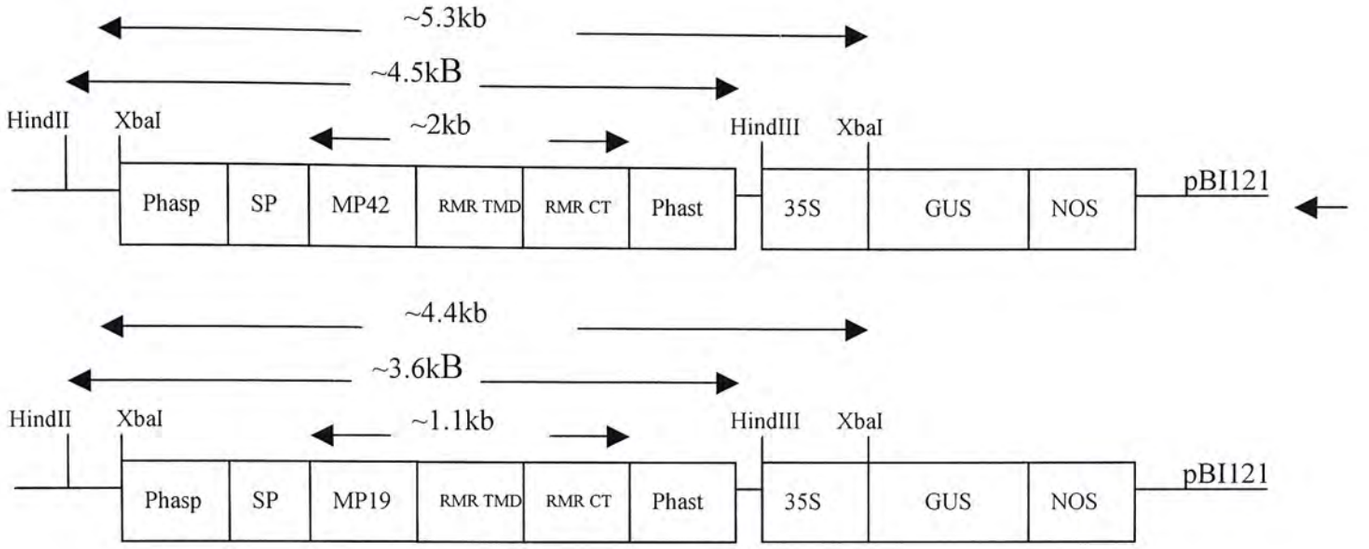

HindIII
XbaI
1 2 3 4 1 2 3 4 M
~2.9kb
~3.7kb

**Figure 4.3.1-4 The Phas/SP/MP42-α-TIP and Phas/SP/MP19-α-TIP constructs**

(a) Structure of the Phas/SP/MP42-α-TIP and Phas/SP/MP19-α-TIP constructs showing the position of the HindIII and XbaI sites and the fragment sizes; (b) restriction digestion of the plasmid Phas/SP/MP42-α-TIP by HindIII or XbaI; (c) of Phas/SP/MP19-α-TIP by HindIII or XbaI. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); lanes 1-4 show the colony numbers and the arrows show the position and size of DNA bands; and colonies underlined denoted those chosen for *Agrobacterium* LBA4404 transformation.

(a)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~2kb
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XbaI
HindIII
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Phasp
SP
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RMR TMD
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Phast
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~3.6kB
~1.1kb
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MP19
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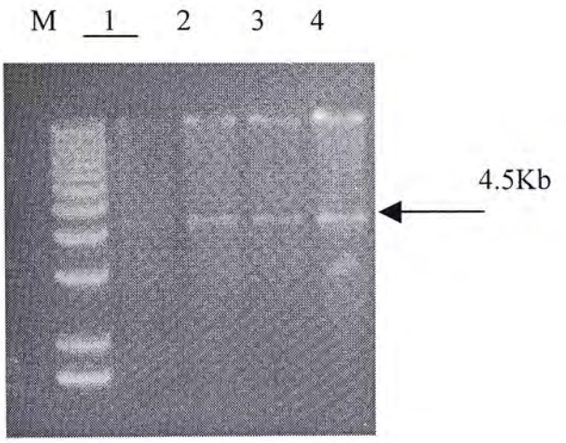
Phast
35S
GUS
NOS
pBI121

(b)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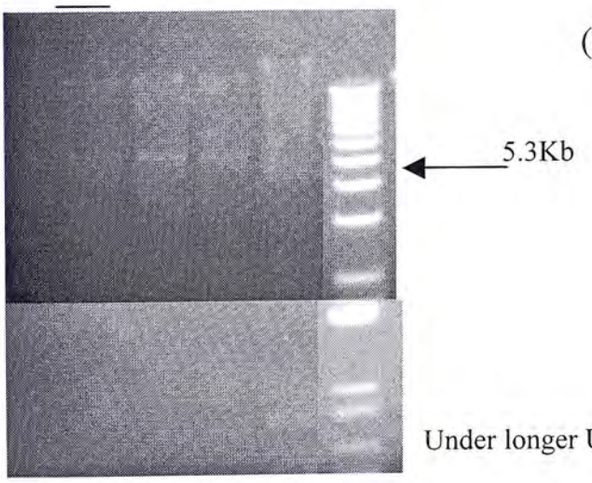
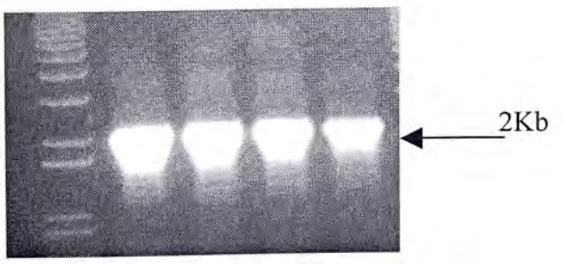
4.5Kb

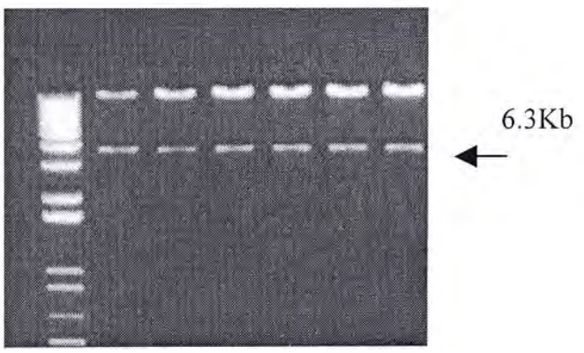
(c)


1 2 3 4 M
5.3Kb
Under longer UV exposure time

(d)


M 1 2 3 4
2Kb

(e)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6.3Kb

(f)

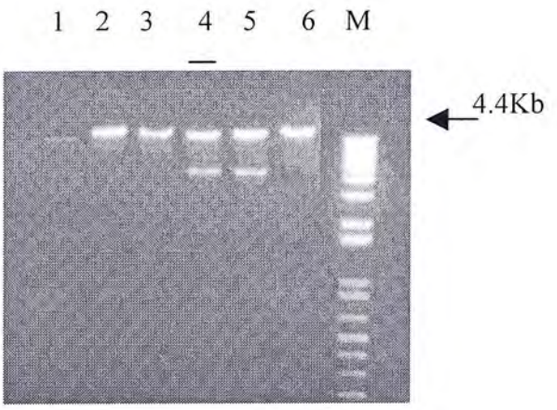

1 2 3 4 5 6 M
4.4Kb

**Figure 4.3.1-5 The Phas/SP/MP42-RMR and Phas/SP/MP19-RMR constructs**

(a) Structure of the Phas/SP/MP42-RMR and Phas/SP/MP19-RMR constructs showing the position of HindIII and XbaI sites and the fragment sizes; (b) restriction digestion of plasmid Phas/SP/MP42-RMR by HindIII or (c) by XbaI (d) PCR amplification of the MP42-RMR in Phas/SP/MP42-RMR using primers LNspM42 and RARMR; (e) restriction digestion of plasmid Phas/SP/MP19-RMR by HindIII and (f) by XbaI. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); lane 1-4 and 1-6 show the colony numbers; the arrows show the band positions and sizes; and the colonies underlined denoted those chosen for *Agrobacterium* LBA4404 transformation.

**(B) GUS fusion constructs**

**B1. Constructs for transient assay**

We hypothesized that GUS fusion may stabilize the expression of the MSP1 transgene at protein level of the transgene in fusion with GUS showed positive expression in our earlier transient assay (section 4.1.2). Therefore, six chimeric GUS fusion gene constructs, namely pTZ/Phas/MP42::EK-GUSN, pTZ/Phas/MP19::EK-GUSN, pBK/Phas/SP/MP42::EK-GUSN, pBK/Phas/SP/MP19::EK-GUSN, pBK/Phas/SP/MP42::EK-GUSN-AFVY and pBK/Phas/SP/MP19::EK-GUSN-AFVY were constructed (Figure 4.3.1-6) and tested for transient expression by particle bombardment before being transformed into tobacco via *Agrobacterium* (LBA 4404)

The major restriction sites for the $MSP1_{42}$ GUS fusion constructs are shown in Figure 4.3.1-7. AccI was used to release the $MSP1_{42}$/GUS fragment from the pTZ/Phas/MP42::EK-GUSN for verification whereas incomplete digestion by NdeI and PstI was used for checking the presence of the $MSP1_{42}$ gene and GUS gene in the constructs of pBK/Phas/SP/MP42::EK-GUSN and pBK/Phas/SP/MP42::EK-GUSN-AFVY. DNA sequencing was also performed to further confirmed the fidelity of the constructs (data not shown).

In these chimeric gene constructs, the modified $MSP1_{42}$ (MP42) and $MSP1_{19}$

(MP19) cDNAs were fused with the GUS coding sequence without translation start codon (GUSN). The two coding sequences were linked by the enterokinase cleavage sequence (EK) for fusion separation of the two proteins. Some of the constructs (Figure 4.3.1-6 c-6) contained the phaseolin signal sequence (SP) while others also contained the Phaseolin vacuole targeting tetrapeptide (AFVY) signal (Figure 4.3.1-6 e-f). All the fusion constructs were driven by the phaseolin promoter (Phasp) and terminator (Phast), in either pTZ or pBK vectors.

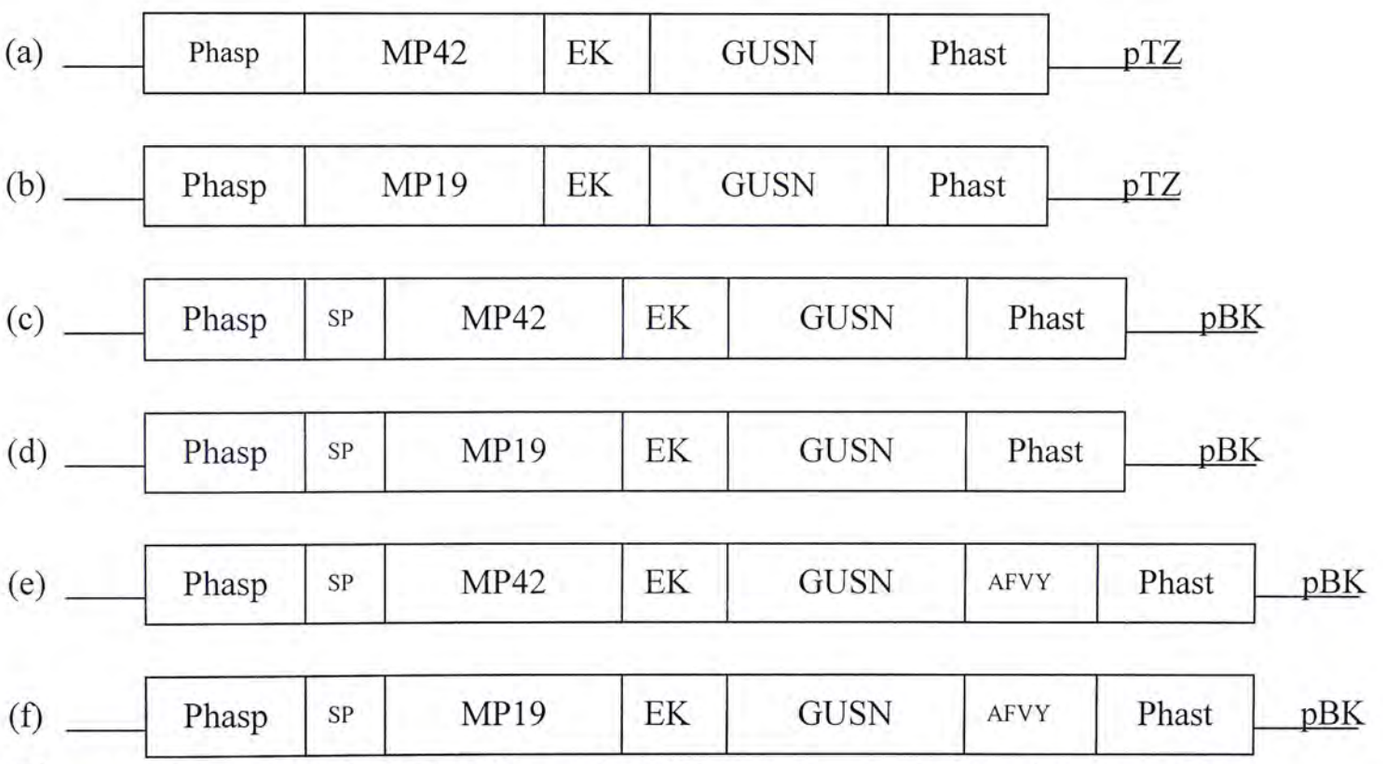
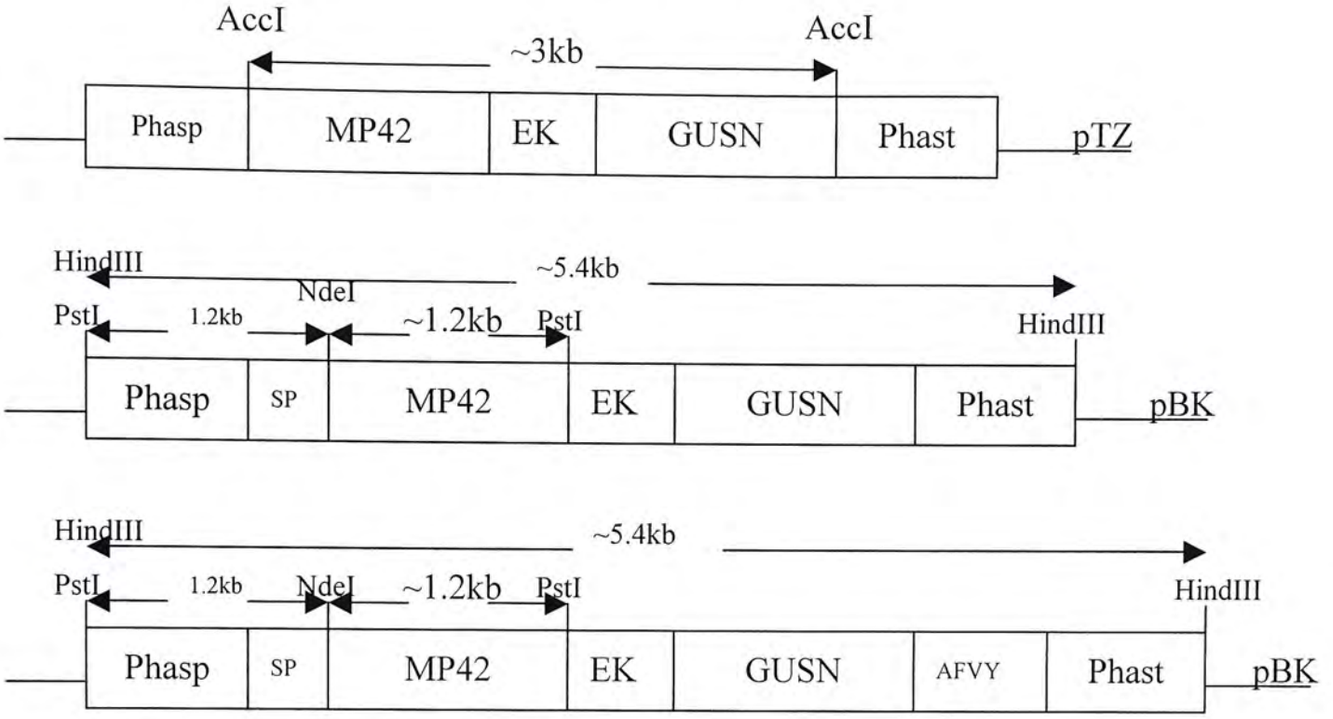



**Figure 4.3.1-6 Modified MSP1 and GUS fusion constructs for transient expression**

(a) pTZ/Phas/MP42::EK-GUSN; (b) pTZ/Phas/MP19::EK-GUSN; (c) pBK/Phas/SP/MP42::EK-GUSN; (d) pBK/Phas/SP/MP19::EK-GUSN; (e) pBK/Phas/SP/MP42::EK-GUSN-AFVY; and (f) pBK/Phas/SP/MP19::EK-GUSN-AFVY. Phasp=Phaseolin promoter; Phast= Phaseolin terminator; EK= enterokinase cleavage sequence; GUSN= GUS coding sequence without ATG; SP= Phaseolin signal sequence; and AFVY= Phaseolin targeting sequence.

(a)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AccI
Phasp
MP42
EK
GUSN
Phast
pTZ
HindIII
~5.4kb
NdeI
PstI
1.2kb
~1.2kb
PstI
HindIII
Phasp
SP
MP42
EK
GUSN
Phast
pBK
HindIII
~5.4kb
PstI
1.2kb
NdeI
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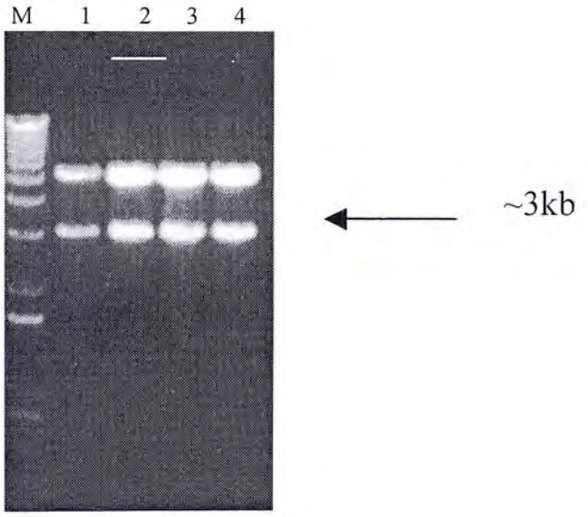
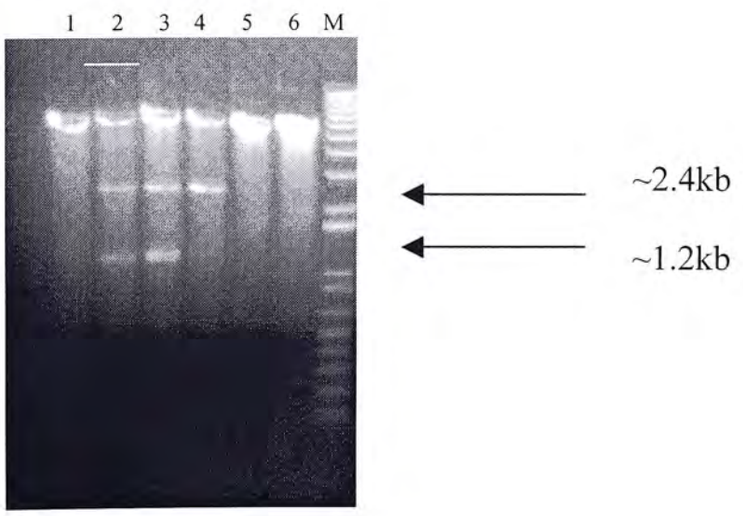
HindIII
Phasp
SP
MP42
EK
GUSN
AFVY
Phast
pBK

(b)


M 1 2 3 4
~3kb

(c)


1 2 3 4 5 6 M
~2.4kb
~1.2kb

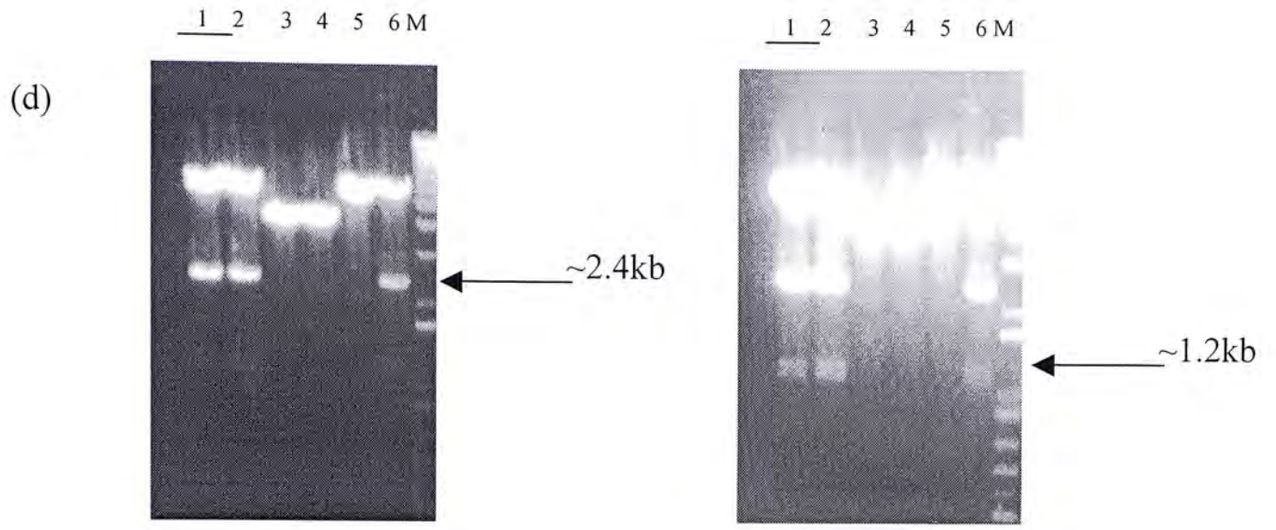
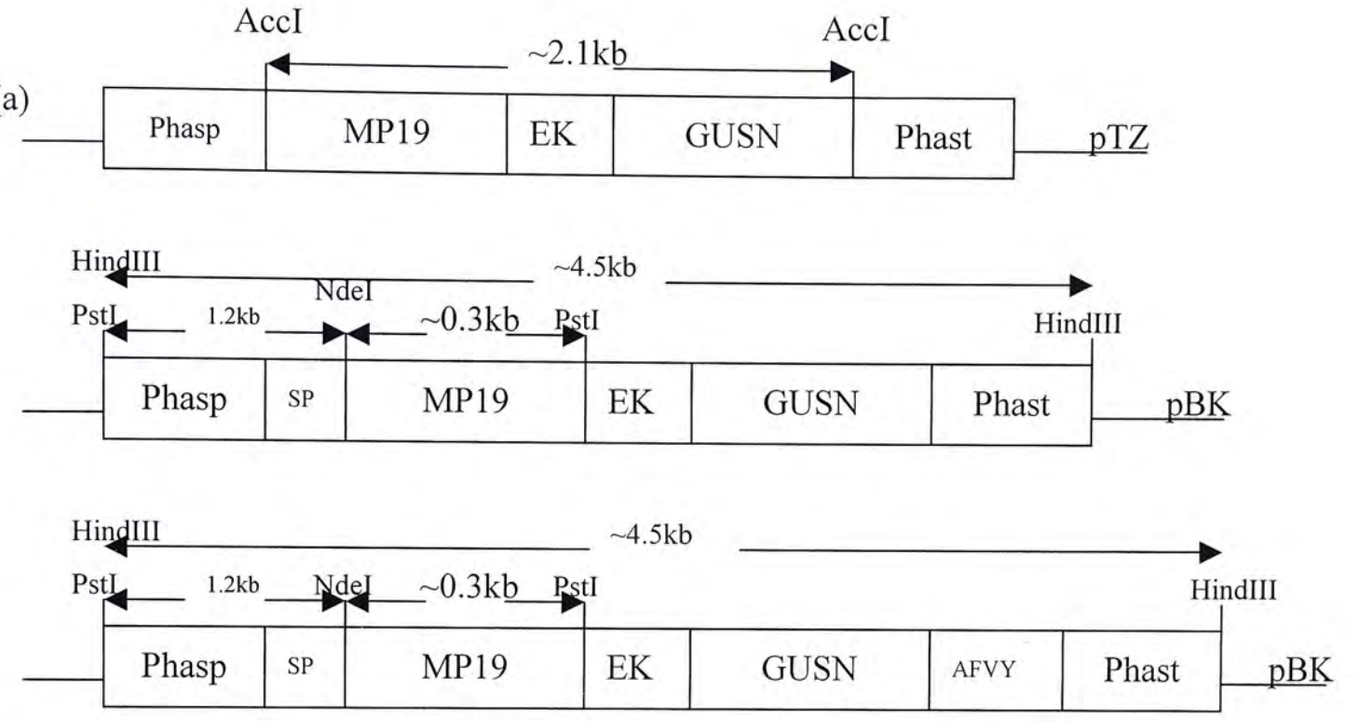



**Figure 4.3.1-7 Cloning of the MSP1$_{42}$ and GUS fusion constructs**
(a) Diagram of the constructs pTZ/Phas/MP42::EK-GUSN, pBK/Phas/SP/MP42::EK-GUSN and pBK/Phas/SP/MP42::EK-GUSN-AFVY; (b) restriction digestion of the plasmid pTZ/Phas/MP42::EK-GUSN by AccI; (c) restriction digestion of the plasmid pBK/Phas/SP/MP42::EK-GUSN by NdeI and Pst; (d) restriction digestion of pBK/Phas/SP/MP42::EK-GUSN-AFVY by NdeI and PstI; arrows indicate the expected sizes of the bands on the gels. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); lanes 1-4 and 1-6 represent colony numbers; colonies underlined denote those chosen for particle bombardment and further cloning.

The major restriction sites for the MSP1$_{19}$ GUS fusion constructs are shown in Figure 4.3.1-8. AccI was used to release the MSP1$_{19}$/GUS fragment from the pTZ/Phas/MP19::EK-GUSN whereas incomplete digestion by NdeI and PstI was used for checking the presence of the MSP1$_{19}$ and GUS genes in the constructs of pBK/Phas/SP/MP19::EK-GUSN and pBK/Phas/SP/MP19::EK-GUSN-AFVY. In addition, DNA sequencing was performed to further confirmed the fidelity of the constructs (data not shown).

(a)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PstI
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HindIII
Phasp
SP
MP19
EK
GUSN
Phast
pBK
HindIII
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PstI
1.2kb
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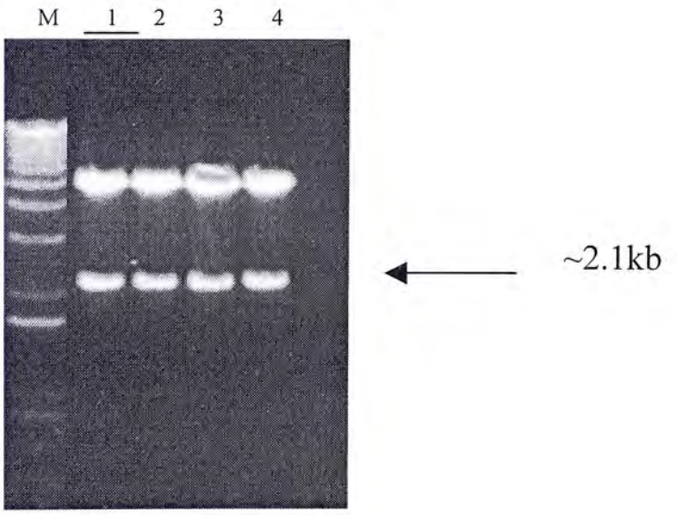
HindIII
Phasp
SP
MP19
EK
GUSN
AFVY
Phast
pBK

(b)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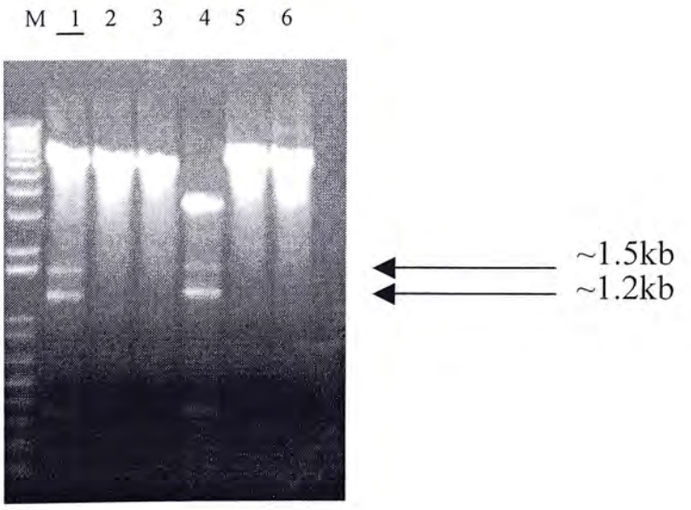
1
2
3
4
~2.1kb

(c)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2
3
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5
6
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~1.2kb

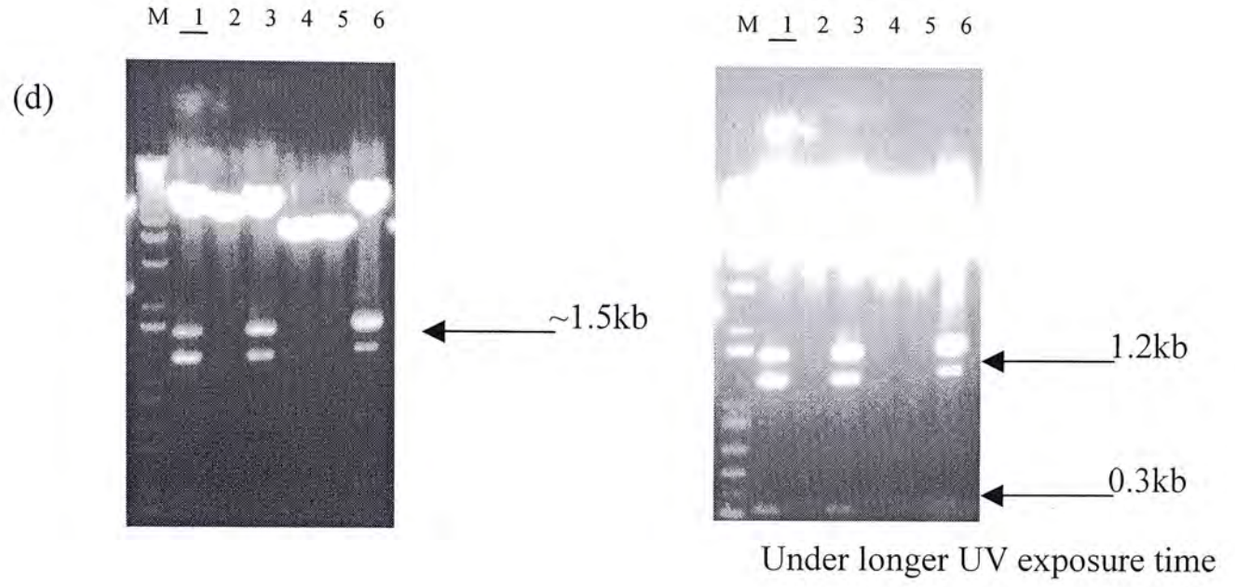



**Figure 4.3.1-8 Cloning of the $MSP1_{19}$ and GUS fusion constructs**

(a) Diagram of the constructs pTZ/Phas/MP19::EK-GUSN, pBK/Phas/SP/MP19::EK-GUSN and pBK/Phas/SP/MP19::EK-GUSN-AFVY; (b) restriction digestion of pTZ/Phas/MP19::EK-GUSN by AccI; (c) Restriction digestion of the plasmid pBK/Phas/SP/MP19::EK-GUSN by NdeI and PstI;. (d) restriction digestion the plasmid pBK/Phas/SP/MP19::EK-GUSN-AFVY by NdeI and PstI; arrows indicate the expected sizes of the bands found on the gels. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); lane 1-4 and 1-6 represent coloniy numbers;.colonies underlined denote those chosen for the particle bombardment and further cloning.

**B2. Modification of GUS sequence**

Then enzymatic activity of GUS may be inactivated due to N-linked glycosylation of its one or two potential sites in the endoplasmic reticulum (ER) where oligosaccharide transferase is found in the lumenal side (Iturriaga *et al.*, 1989). Yan *et al.* (1997) modified a potential glycosylation site of GUS by mutation (Asn-358 to Ser) and were able to prevent the inactivation of GUS activity. In the present study, as the phaseolin signal peptide was used in most of the GUS fusion constructs, it is possible that the signal sequence will direct the GUS fusion protein into ER where in the GUS activity could be inactivated.

Therefore, mutation (Asn-358 to Ser) was introduced into the GUS gene constructs in which phaseolin signal peptide was present so as to test by transient assay if inactivation of GUS activity was due to N-linked glycosylation. $MSP1_{19}$ was used for the study because $MSP1_{19}$ gave much higher expression than $MSP1_{42}$ in the transient assay.

Two chimeric gene constructs with the modified GUS (MGUSN), pBK/Phas/SP/MP19::EK-MGUSN and pBK/Phas/SP/MP19::EK-MGUSN-AFVY, were constructed (Figure 4.3.1-9) and used for transient expression by particle bombardment.

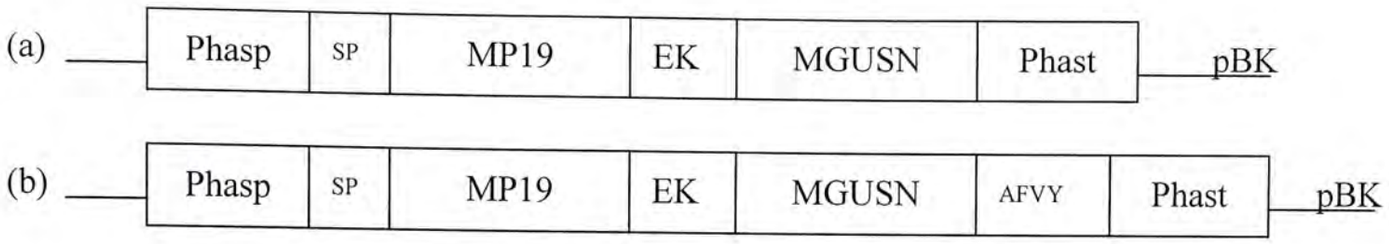
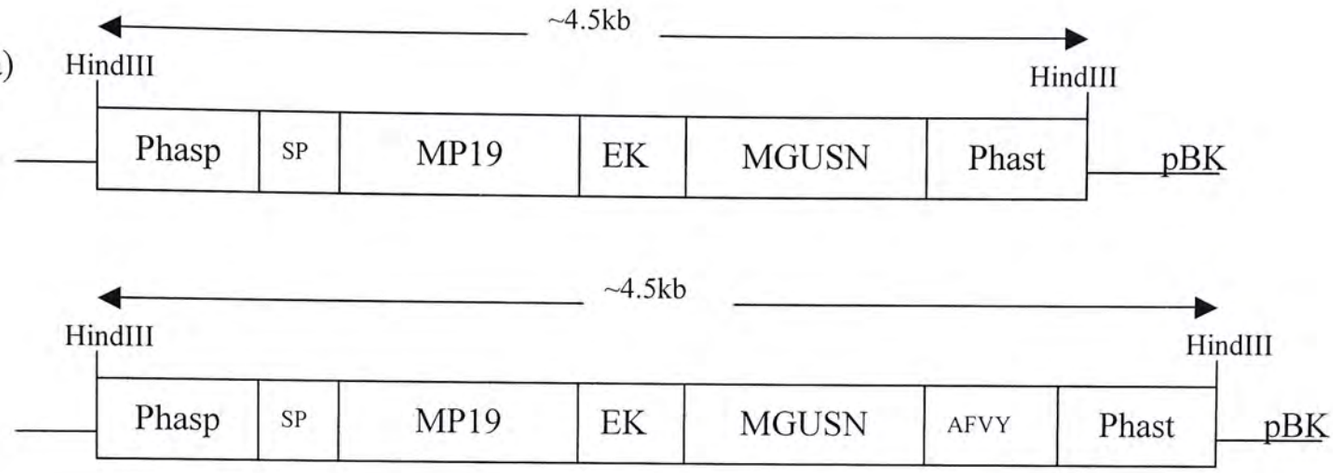



**Figure 4.3.1-9 Modified GUS-fusion constructs used for transient assay**
(a) pBK/Phas/SP/MP19::EK-MGUSN and (b) pBK/Phas/SP/MP19::EK-MGUSN-AFVY. MGUSN= modified (Asn-358 to Ser) GUS without translation start codon (ATG).

The major restriction sites for the modified GUS (MGUS) fusion constructs are shown in Figure 4.3.1-10. HindIII was used to release the phaseolin cassette from plasmid pBK/Phas/SP/MP19::EK-MGUSN and pBK/Phas/SP/MP19::EK-GUSN-AFVY for verification.. DNA sequencing was performed to further confirmed the mutation site of the GUS gene in the constructs (data not shown).

(a)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HindIII
Phasp
SP
MP19
EK
MGUSN
Phast
pBK
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HindIII
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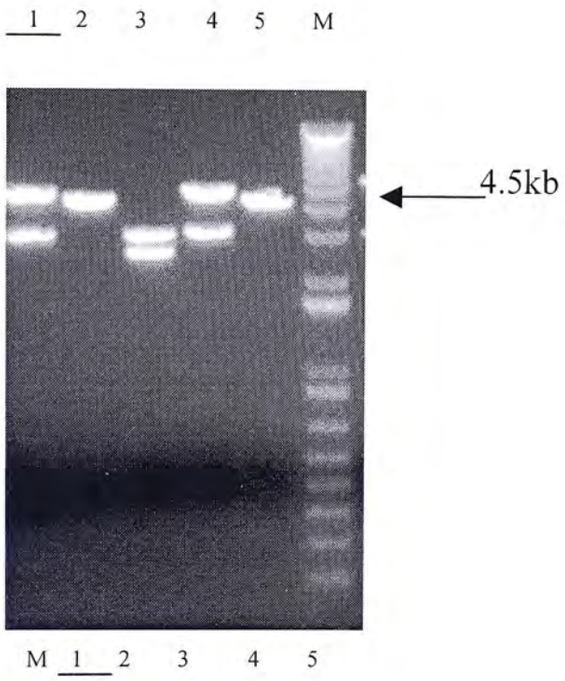
EK
MGUSN
AFVY
Phast
pBK

(b)


1 2 3 4 5 M
4.5kb
M 1 2 3 4 5

(c)

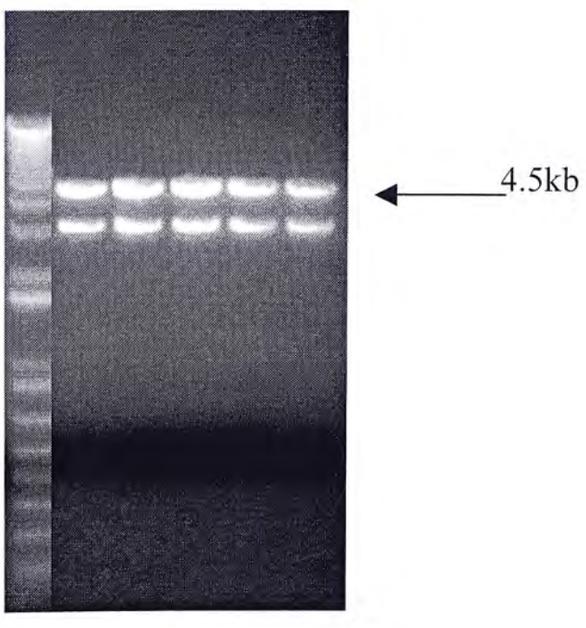

4.5kb

**Figure 4.3.1-10 Cloning of the Phas/SP/MP19::MGUSN and Phas/SP/MP19::MGUSN-AFVY constructs**

(a) Diagram of the Phas/SP/MP19::MGUSN and Phas/SP/MP19::MGUSN-AFVY constructs showing the position of the HindIII sites and the size of their franking fragments; (b) restriction digestion of the plasmid Phas/SP/MP19::GUSN by HindIII; and (c) of Phas/SP/MP19::GUSN-AFVY. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); lanes 1-5 show the colony numbers; arrows show the expected band positions and sizes; colonies underlined denote those chosen for the particle bombardment.

## B3. Constructs for tobacco transformation

For expression of the modified MSP1 and GUS fusion proteins in tobacco, the three phaseolin promoter-directed MP42/GUSN and three MP19/GUS fusion constructs prepared for transient expression assay (B1. of section 4.3.1) were excised from the pTZ and pBK vectors and inserted into the pSUN1 vector, which is a modified pBI121 *Agrobacterium* binary vector in which the GUS marker gene is replaced by an orange fluorescent protein (OFP) gene as marker.

The six chimeric gene constructs are pSUN1/Phas/MP42::EK-GUSN, pSUN1/Phas/MP19::EK-GUSN, pSUN1/Phas/SP/MP42::EK-GUSN, pSUN1/Phas/SP/MP19::EK-GUSN, pSUN1/Phas/SP/MP42::EK-GUSN-AFVY and pSUN1/Phas/SP/MP19::EK-GUSN-AFVY (Figure 4.3.1-11). They were introduced into *Agrobacterium* LBA4404 for tobacco transformation.

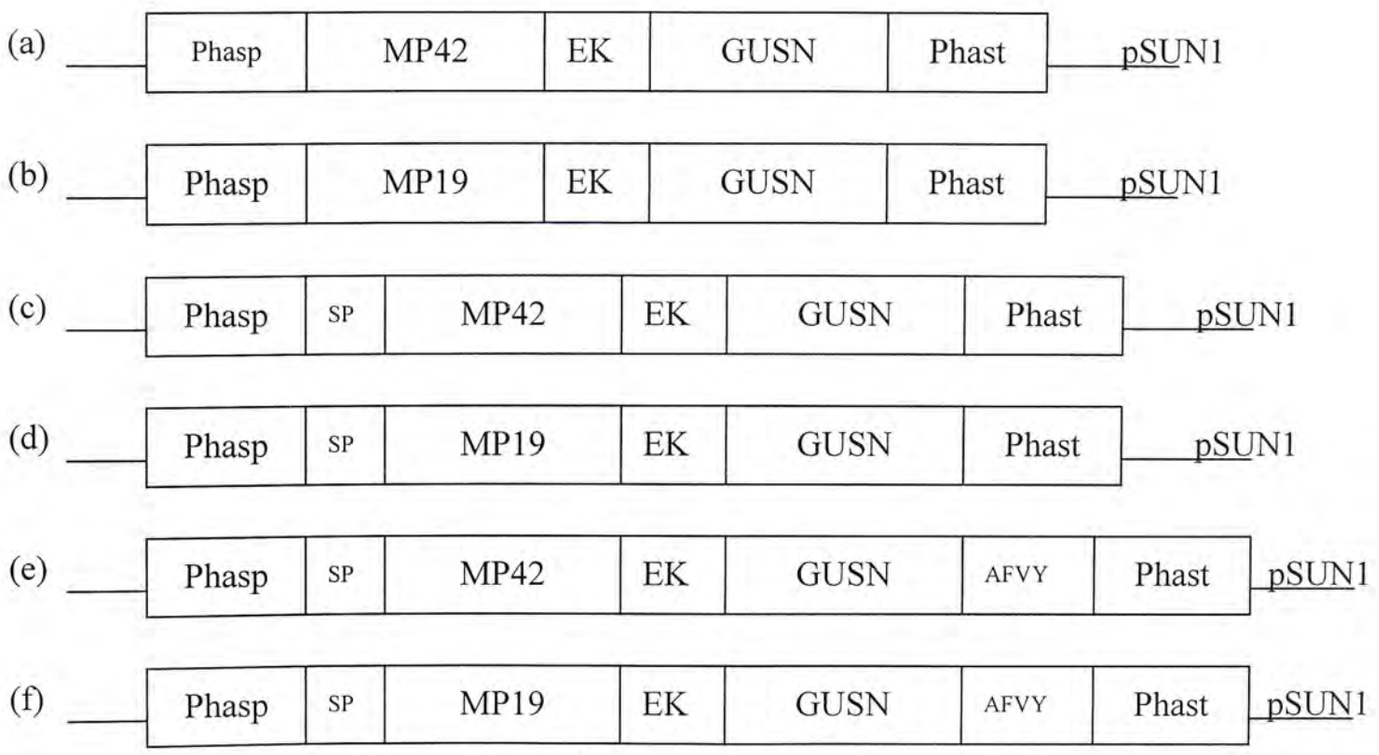
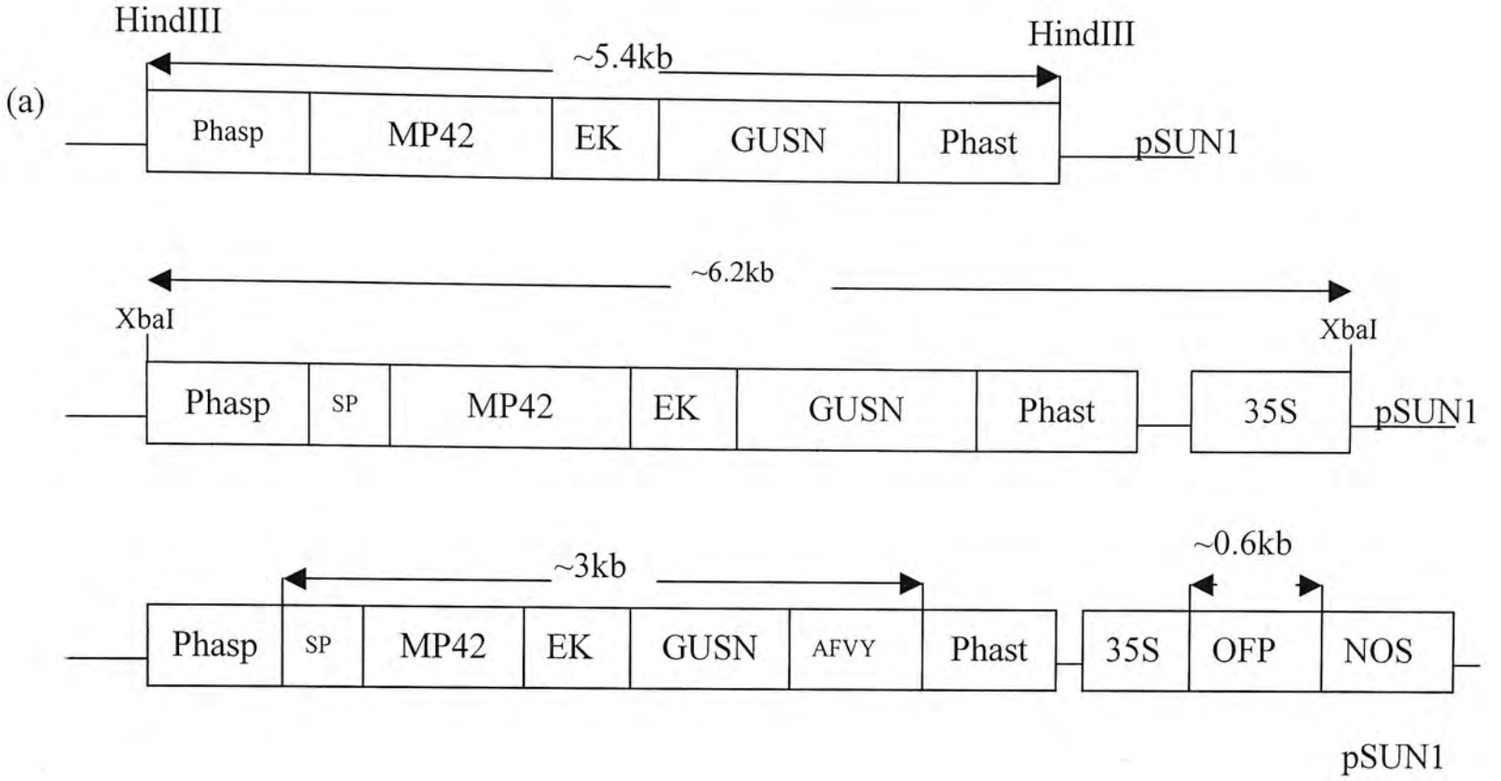


**Figure 4.3.1-11 Diagram of MSP1 and GUS fusion constructs for tobacco transformation**

(a) pSUN1/Phas/MP42::EK-GUSN; (b) pSUN1/Phas/MP19::EK-GUSN; (c) pSUN1/Phas/SP/MP42::EK-GUSN; (d) pSUN1/Phas/SP/MP19::EK-GUSN; (e) pSUN1/Phas/SP/MP42::EK-GUSN-AFVY and (f) pSUN1/Phas/SP/MP19::EK-GUSN-AFVY. pSUN1 is a modified pBI121 in which the GUS is preplaced by an orange fluorescent protein (OFP) as marker protein.

The major restriction sites for the $MSP1_{42}$ GUS fusion constructs in pSUN1 are shown in Figure 4.3.1-12. To verify the constructs, HindIIII was used to release the phaseolin cassette from pSUN1/Phas/MP42::EK-GUSN; XbaI was used to check the presence of the phaseolin cassette as well as its orientation in constructs pSUN1/Phas/SP/MP42::EK-GUSN; and PCR with corresponding primers was used to amplified the $MSP1_{42}$/GUSN and orange fluorescent protein (OFP) in pSUN1/Phas/SP/MP42::EK-GUSN-AFVY. DNA sequencing was performed to further confirm the fidelity of the constructs (data not shown).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MP42
EK
GUSN
Phast
pSUN1
~6.2kb
XbaI
XbaI
Phasp
SP
MP42
EK
GUSN
Phast
35S
pSUN1
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EK
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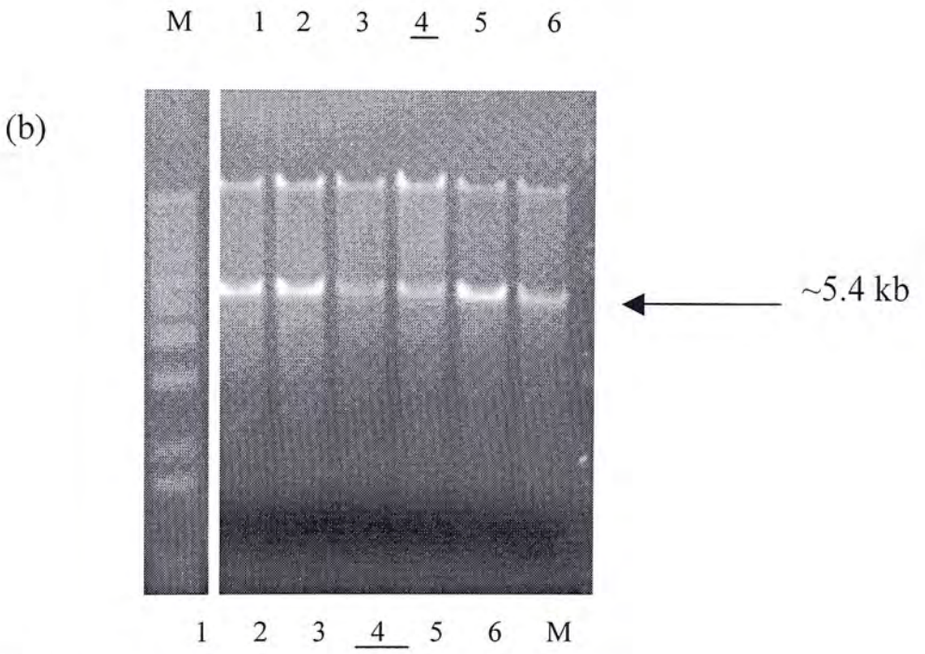
AFVY
Phast
35S
OFP
NOS
pSUN1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(b)
~5.4 kb
1 2 3 4 5 6 M
(c)
~6.2 kb

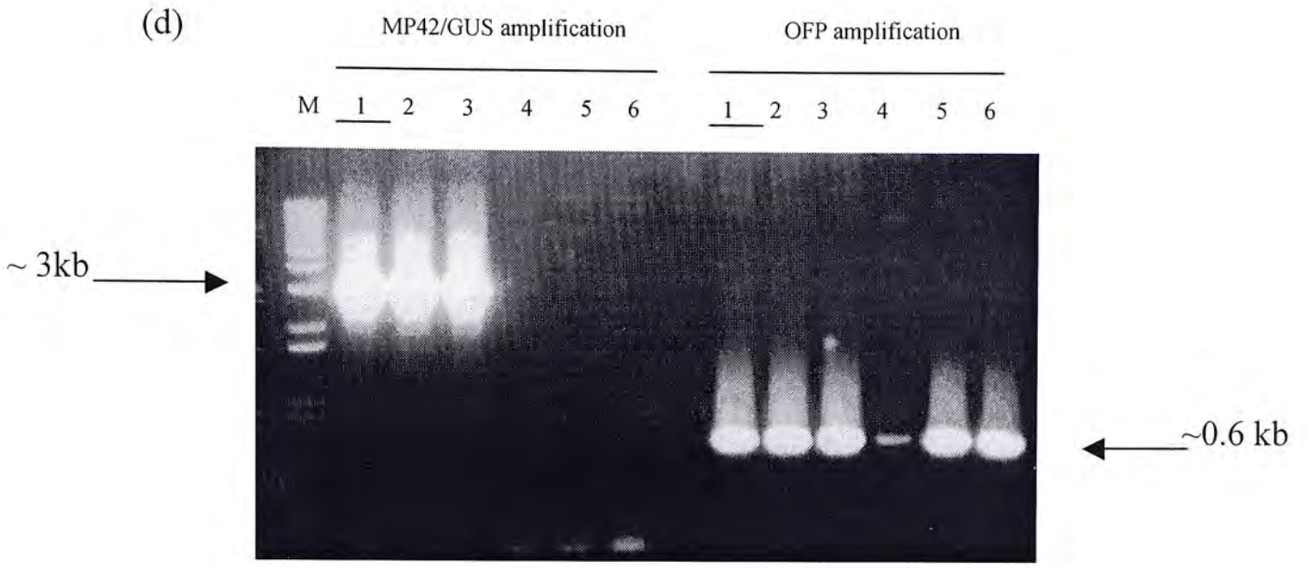


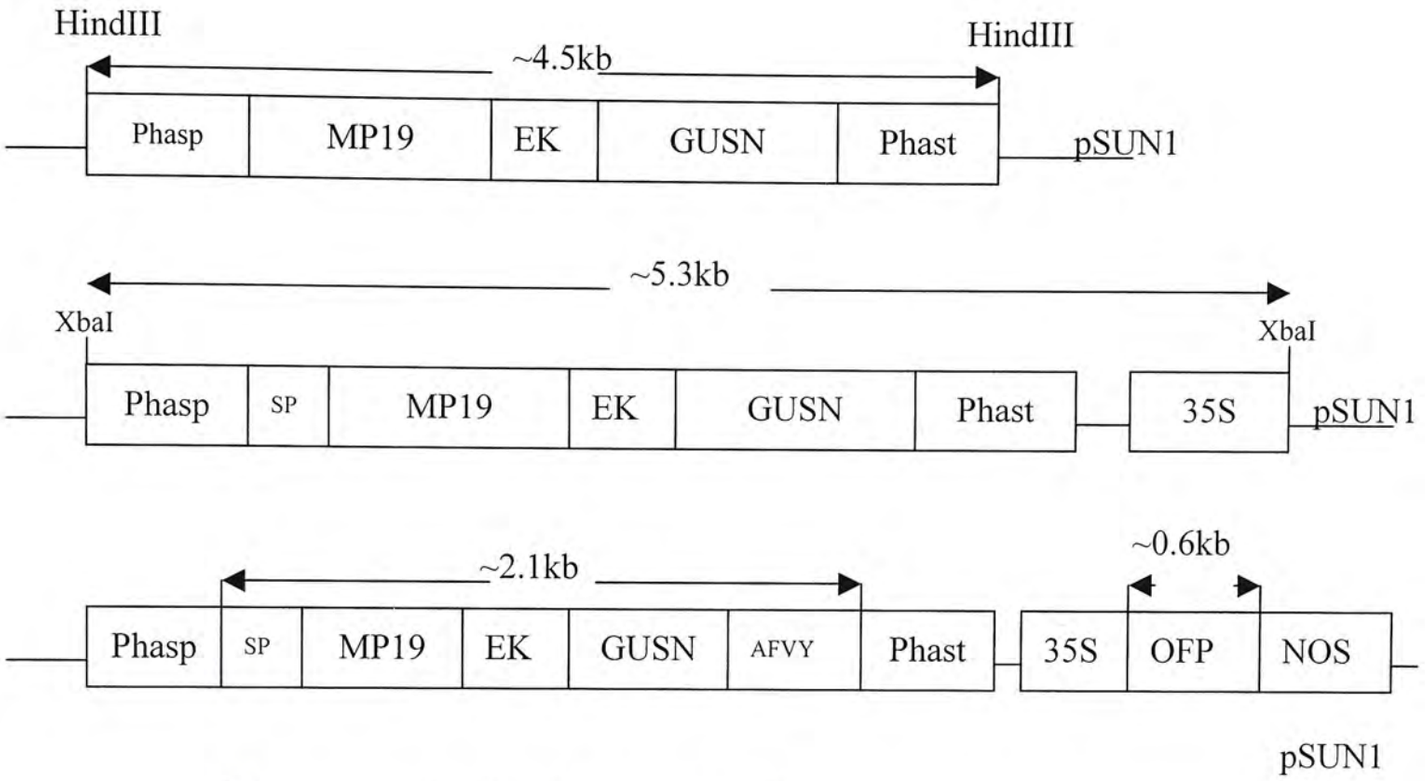

**Figure 4.3.1-12 Cloning of the $MSP1_{42}$ and GUS fusion constructs for tobacco transformation**

(a) Diagram of the plasmids pSUN1/Phas/MP42::EK-GUSN, pSUN1/Phas/SP/MP42::EK-GUSN and pSUN1/Phas/SP/MP42::EK-GUSN-AFVY; (b) restriction digestion of pSUN1/Phas/MP42::EK-GUSN by HindIII;. (c) restriction digestion of pBK/Phas/SP/MP42::EK-GUSN by XbaI; (d) PCR amplification of MP42/GUSN and OFP fragments in pBK/Phas/SP/MP42::EK-GUSN-AFVY; arrows indicate the sizes of the bands. Key: M: 1 Kb plus DNA ladder (Invitrogen); lanes 1-6 represent colony numbers; colonies underlined denote those chosen for introduction into *Agrobacterium* LBA 4404 for tobacco transformation.

The major restriction sites of the $MSP1_{19}$ and GUS fusion constructs in pSUN1 are shown in Figure 4.3.1-13. To verify the constructs, HindIII was used to release the phaseolin cassette from pSUN1/Phas/MP19::EK-GUSN; XbaI was used for checking the presence as well as the orientation of the phaseolin cassette in the constructs of pSUN1/Phas/SP/MP19::EK-GUSN; and PCR with corresponding

primers was used to amplify the $MSP1_{19}$/GUSN and orange fluorescent protein (OFP) in the pSUN1/Phas/SP/MP19::EK-GUSN-AFVY. DNA sequencing was performed to further confirmed the fidelity of the constructs (data not shown).

(a)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GUSN
Phast
35S
pSUN1
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MP19
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GUSN
AFVY
Phast
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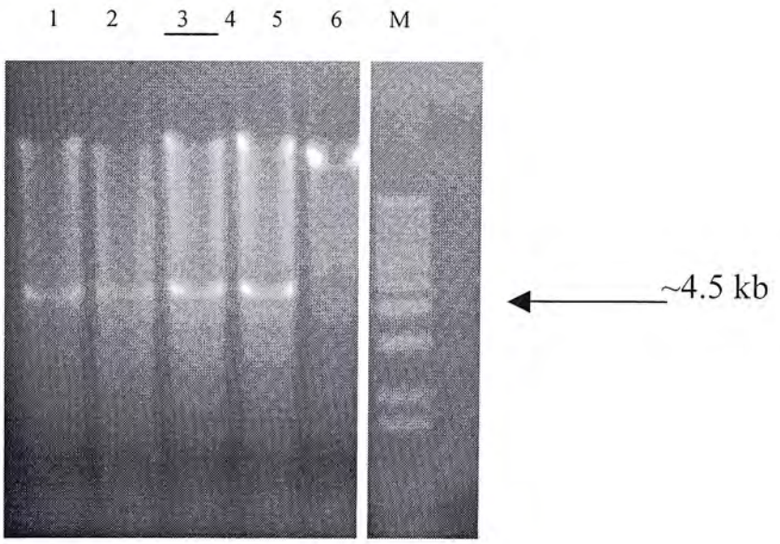
OFP
NOS
pSUN1

(b)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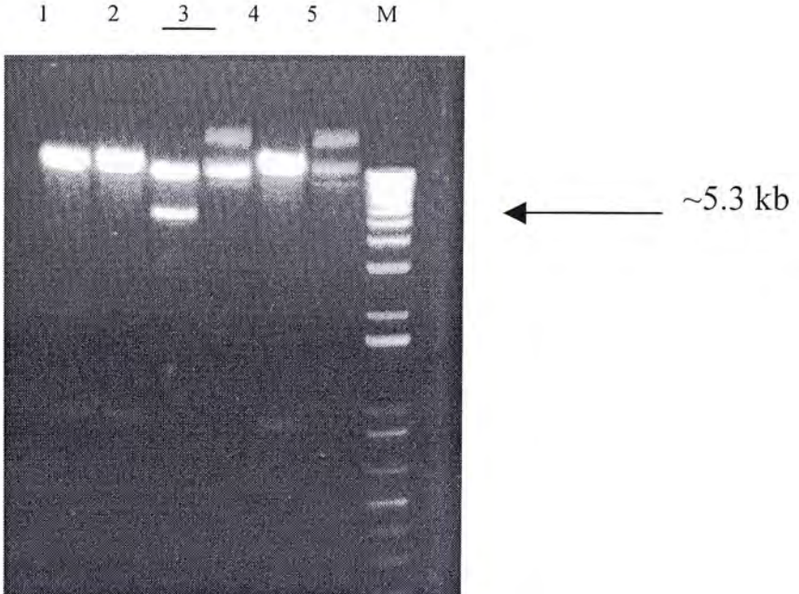
(c)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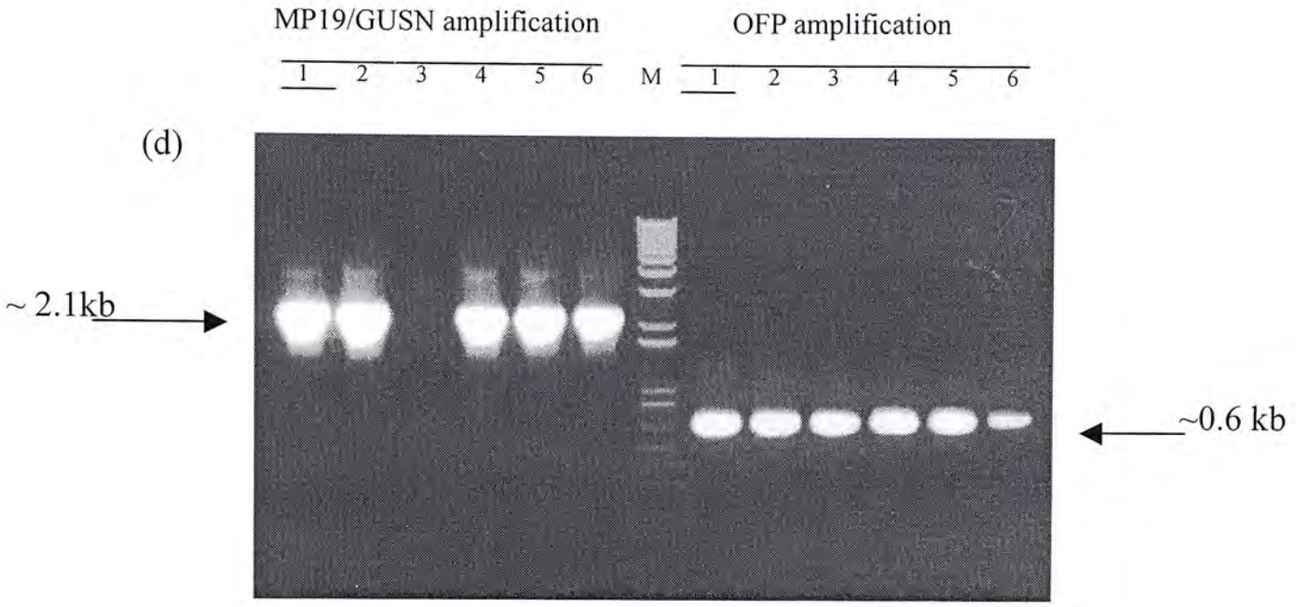



**Figure 4.3.1-13 Cloning of the $MSP1_{42}$ GUS fusion constructs for tobacco transformation**

(a) Diagram of the pSUN1/Phas/MP19::EK-GUSN, pSUN1/Phas/SP/MP19::EK-GUSN and pSUN1/Phas/SP/MP19::EK-GUSN-AFVY; (b) restriction digestion of pSUN1/Phas/MP19::EK-GUSN by HindIII.; (c) restriction digestion of pBK/Phas/SP/MP19::EK-GUSN by XbaI; (d) PCR amplification of MP19/GUSN and OFP in pBK/Phas/SP/MP19::EK-GUSN-AFVY. The arrows indicate the sizes of the bands found on the gels. Key: lane M: 1 Kb plus DNA ladder (Invitrogen); lane 1-6 represent colony numbers; colonies underlined denoted those chosen for introduction into *Agrobacterium* LBA 4404 for tobacco transformation.

## 4.4 Transient assay of GUS fused MP42 and MP19 constructs by particle Bombardment

### 4.4.1 The GUS fusion constructs

GUS fusion may stabilize the expression of the modified MSP1 gene at protein level as demonstrated by our earlier transient assay study (section 4.1.2). Constructs for transient assay were thus made (section 4.3.1B) and studied.

In the study, the number of snow bean cotyledons showing blue dot development with different relative intensities was counted and the results were shown in Tables 4.4.1-1 a–b and in bar chart Figures 4.4.1-1a-b. Photographs were also taken to illustrate the different levels of blue dot development by the six constructs (Figure 4.4.1-2 a-f and Figure 4.4.1-3 a-f).

Constructs Phas/MP42::EK-GUSN and Phas/MP19::EK-GUSN were found to express and show blue dot development with different intensities, but not for the Phas/SP/MP42::EK-GUSN, Phas/SP/MP19::EK-GUSN, Phas/SP/MP42::EK-GUSN-AFVY and Phas/SP/MP19::EK-GUSN-AFVY, which showed no blue dot development.

**Table 4.4.1-1a: Transient expression of the modified $MSP1_{42}$ constructs**

The particle bombarded-cotyledons showing one or more blue dots were regarded as positive results. Key: + indicates the relative intensity of blue dots (5+, maximum)

| | Dish no. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| PTZ/Phas/GUSATG | 1 | 1 | 1 | 1 | 2 | 7 | 1 | 13 |
| | 2 | 2 | 0 | 2 | 0 | 6 | 3 | 13 |
| PTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 13 | 13 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| PTZ/Phas/MP42::GUSN | 1 | 1 | 0 | 1 | 2 | 3 | 7 | 14 |
| | 2 | 0 | 0 | 0 | 2 | 5 | 7 | 14 |
| PTZ/Phas/MP42-EK::GUSN | 1 | 0 | 0 | 0 | 1 | 1 | 11 | 14 |
| | 2 | 0 | 0 | 1 | 1 | 2 | 11 | 15 |
| PTZ/Phas/SP/MP42-EK::GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 14 | 14 |
| PTZ/Phas/SP/MP42-EK::GUSN-AFVY | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |

**Table 4.4.1-1b: Transient expression of the modified $MSP1_{19}$ constructs**

The particle bombarded-cotyledons showing one or more blue dots were regarded as positive results. Key: + indicates the relative intensity of blue dots (5+, maximum)

| | Dish no. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| PTZ/Phas/GUSATG | 1 | 1 | 0 | 1 | 1 | 6 | 5 | 15 |
| | 2 | 0 | 1 | 2 | 1 | 7 | 2 | 15 |
| PTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| PTZ/Phas/MP19::GUSN | 1 | 0 | 0 | 1 | 0 | 6 | 8 | 15 |
| | 2 | 0 | 1 | 2 | 2 | 3 | 7 | 15 |
| PTZ/Phas/MP19-EK::GUSN | 1 | 0 | 2 | 2 | 3 | 5 | 3 | 15 |
| | 2 | 1 | 2 | 2 | 3 | 6 | 2 | 16 |
| PTZ/Phas/SP/MP19-EK::GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| PTZ/Phas/SP/MP19-EK::GUSN-AFVY | 1 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 15 | 15 |

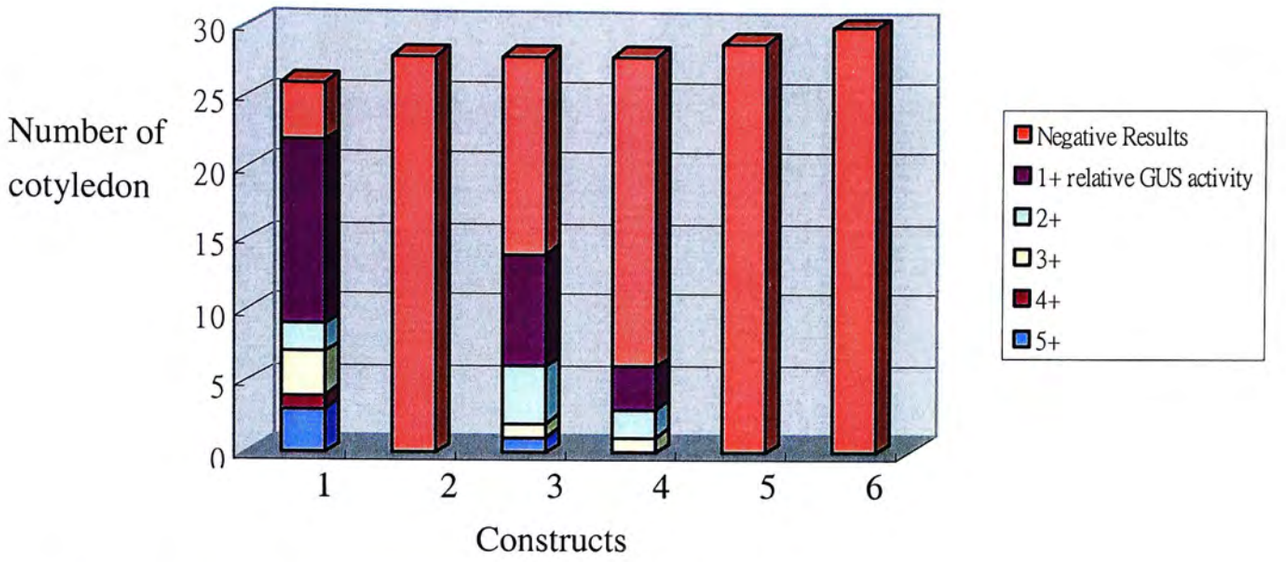



**Figure 4.4.1-1a Transient expression of the MP42 constructs**

Constructs 1: pTZ/Phas/GUS; 2: pTZ/Phas/GUSN; 3: pTZ/Phas/MP42::GUSN; 4: pTZ/Phas/MP42-EK::GUSN; 5: pBK/Phas/SP/MP42-EK::GUSN and 6: pBK/Phas/SP/MP42-EK::GUSN-AFVY. The relative intensities of blue dot in cotyledons are represented by different colours in the bar chart (5+=max. relative intensity).

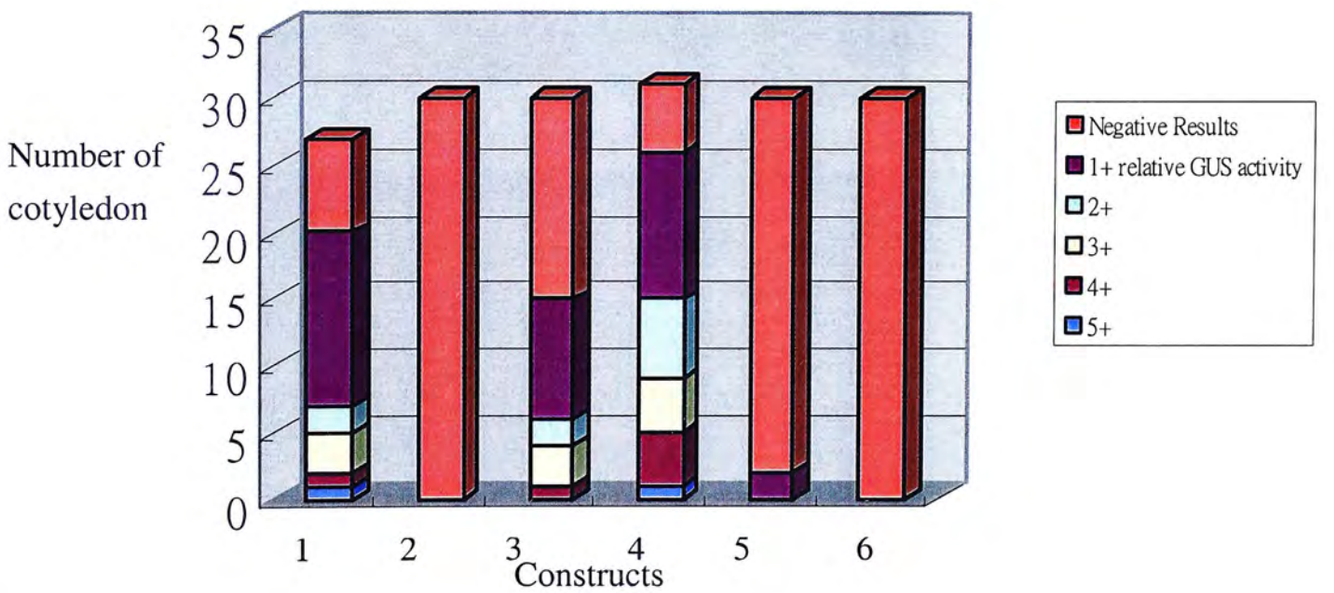



**Figure 4.4.1-1b Transient expression of the MP19 constructs**

Constructs 1: pTZ/Phas/GUS; 2: pTZ/Phas/GUSN; 3: pTZ/Phas/MP19::GUSN; 4: pTZ/Phas/MP19-E::GUSN; 5: pBK/Phas/SP/MP19-EK::GUSN and 6: pBK/Phas/SP/MP19-EK::GUSN-AFVY. The relative intensities of blue dot in cotyledons are represented by different colours in the bar chart (5+= max. relative intensity).

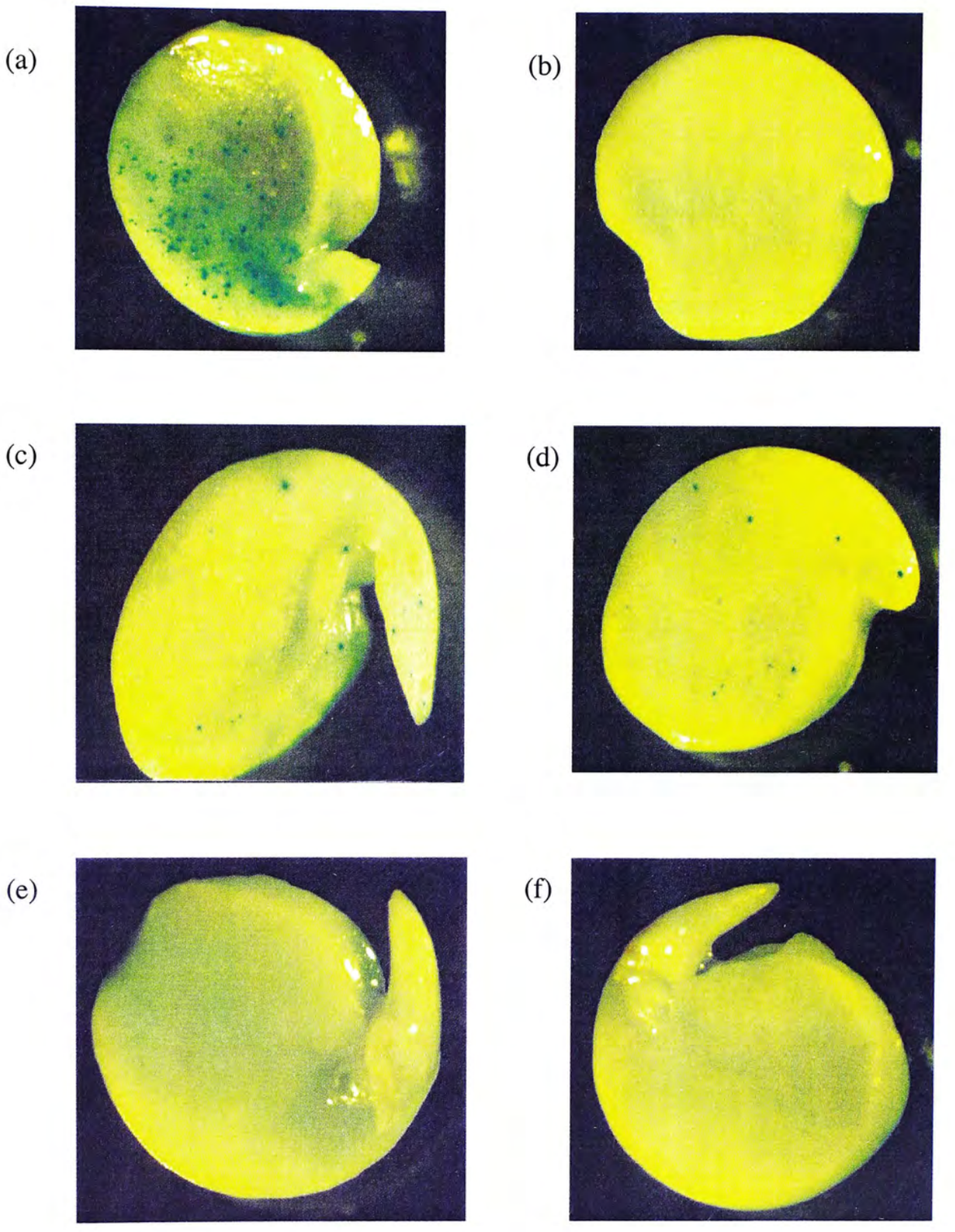



**Figure 4.4.1-2 a-f Photographs illustrating the different levels of blue dot development in particle bombardment of MP42 and GUS fusion constructs**

Constructs (a) pTZ/Phas/GUSATG, positive control; (b) pTZ/Phas/GUSN, negative control; (c) Phas/MP42::GUSN; (d) Phas/MP42-EK::GUSN; (e) Phas/SP/MP42-EK::GUSN; and (f) Phas/SP/NP42-EK::GUSN-AFVY

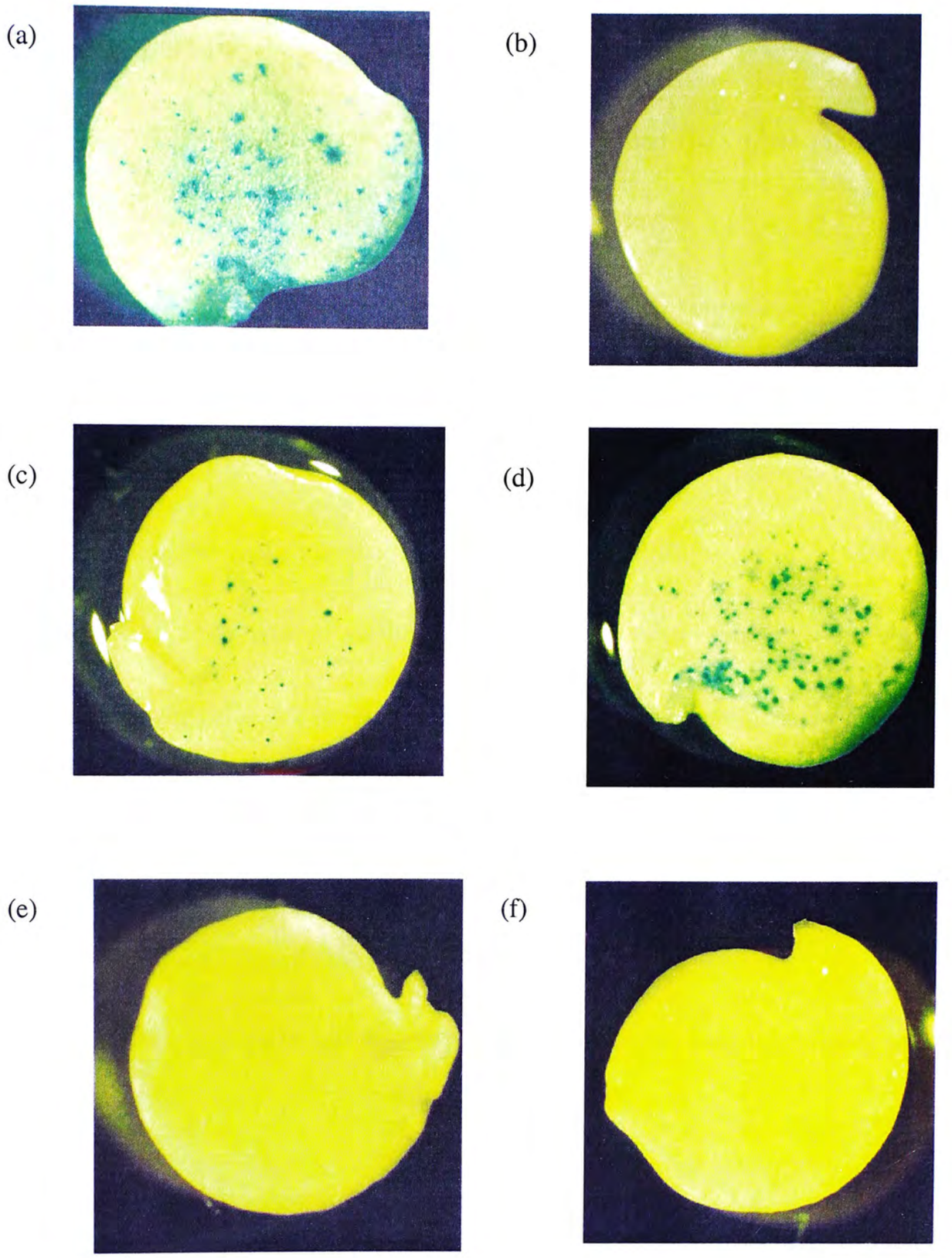



**Figure 4.4.1-3 a-f Photographs illustrating the different levels of blue dot development in particle bombardment of MP19 and GUS fusion constructs**

Constructs (a) pTZ/Phas/GUSATG, positive control; (b) pTZ/Phas/GUSN, negative control; (c) Phas/MP19::GUSN; (d) Phas/MP19-EK::GUSN; (e) Phas/SP/MP19-EK::GUSN; and (f) Phas/SP/NP19-EK::GUSN-AFVY

### 4.4.2 Modification of GUS sequence

The two chimeric gene constructs with the modified GUS (Ans-358 to Ser, MGUSN), pBK/Phas/SP/MP19::EK-MGUSN and pBK/Phas/SP/MP19::EK-MGUSN-AFVY, as detailed in their construction in section 4.3.1B2, and three controls, pTZ/Phas/MP19::EK-GUSN (GUS without Asn-358 to Ser modification), pTZ/Phas/GUSN (GUS without ATG translation initiation codon as negative control)and pTZ/Phas/GUS (GUS with ATG codon as positive control) (Figure 4.4.2-1) were used in particle bombardment. The number of snow bean cotyledons showing blue dot development in different relative intensities was count. The results were shown in Tables 4.4.2-1 and as bar charts in Figure 4.4.2-2. Photographs were also taken to illustrate the different levels of blue dot development by the five constructs (Figure 4.4.2-3).

Constructs Phas/GUS and Phas/MP19::EK-GUSN were found to express and develop blue dots of different intensities, but not in Phas/GUSN, Phas/SP/MP19::EK-MGUSN, and Phas/SP/MP19::EK-MGUSN-AFVY.

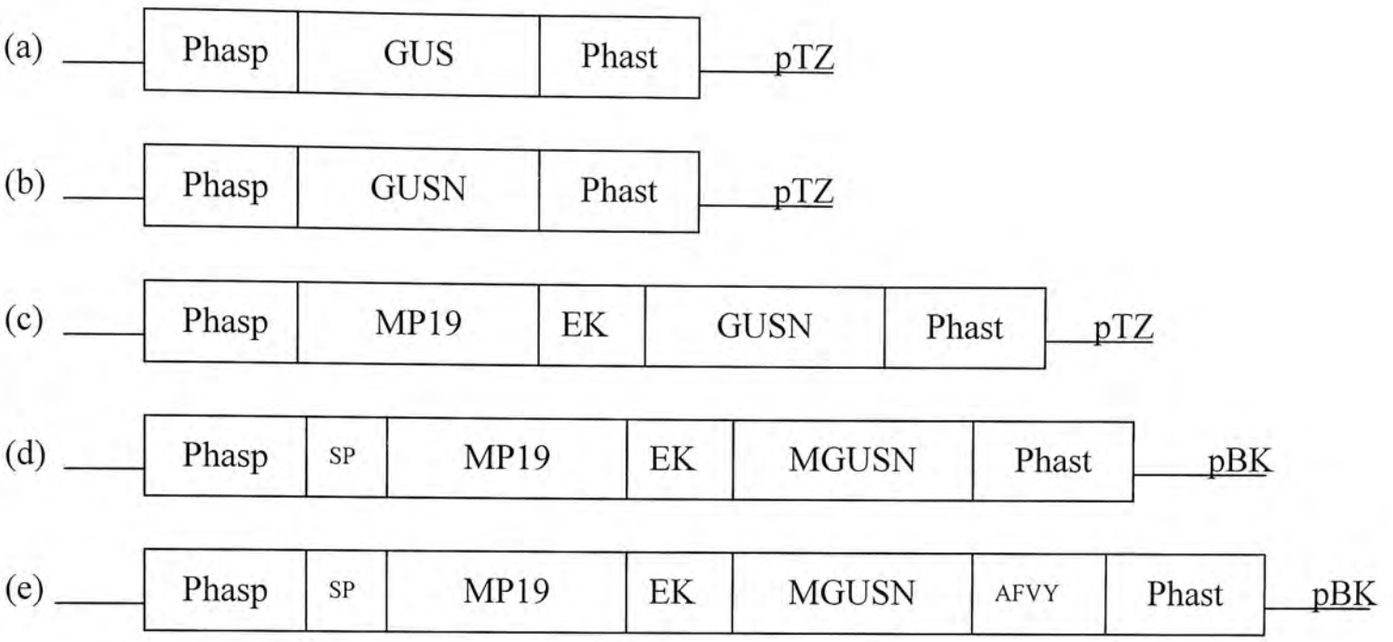



**Figure 4.4.2-1 Constructs with modified GUS for transient expression assay**

Constructs (a) Phas/GUS (with ATG as a positive control); (b) Phas/GUSN (without ATG as a negative control); (c) Phas/MP19-EK::GUSN; (d) Phas/SP/MP19-EK::MGUSN; and (e) Phas/SP/MP19-EK::MGUSN-AFVY. The GUS coding sequence without the translation initiation codon (GUSN) was sequence modified (Asn-358 to Ser) to give MGUSN.

**Table 4.4.2-1 Transient expression of the MP19 and modified GUS fusion constructs**

Particle bombarded cotyledon showing one or more blue dots were regarded as positive results. Key: + shows the relative intensity of blue dots (5+, maximum)

| | Dish no. | Positive Results | | | | | Negative Results | Total |
|---|---|---|---|---|---|---|---|---|
| | | 5+ | 4+ | 3+ | 2+ | 1+ | | |
| PTZ/Phas/GUSATG | 1 | 1 | 1 | 2 | 1 | 3 | 10 | 18 |
| | 2 | 3 | 2 | 1 | 4 | 3 | 4 | 17 |
| PTZ/Phas/GUSN | 1 | 0 | 0 | 0 | 0 | 0 | 19 | 19 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 20 | 20 |
| PTZ/Phas/MP19::GUSN | 1 | 0 | 0 | 2 | 1 | 2 | 11 | 16 |
| | 2 | 1 | 1 | 1 | 1 | 4 | 8 | 16 |
| PTZ/Phas/SP/MP19-EK::MGUSN | 1 | 0 | 0 | 0 | 0 | 0 | 18 | 18 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 17 | 17 |
| PTZ/Phas/SP/MP19-EK::MGUSN-AFVY | 1 | 0 | 0 | 0 | 0 | 0 | 17 | 17 |
| | 2 | 0 | 0 | 0 | 0 | 0 | 16 | 16 |

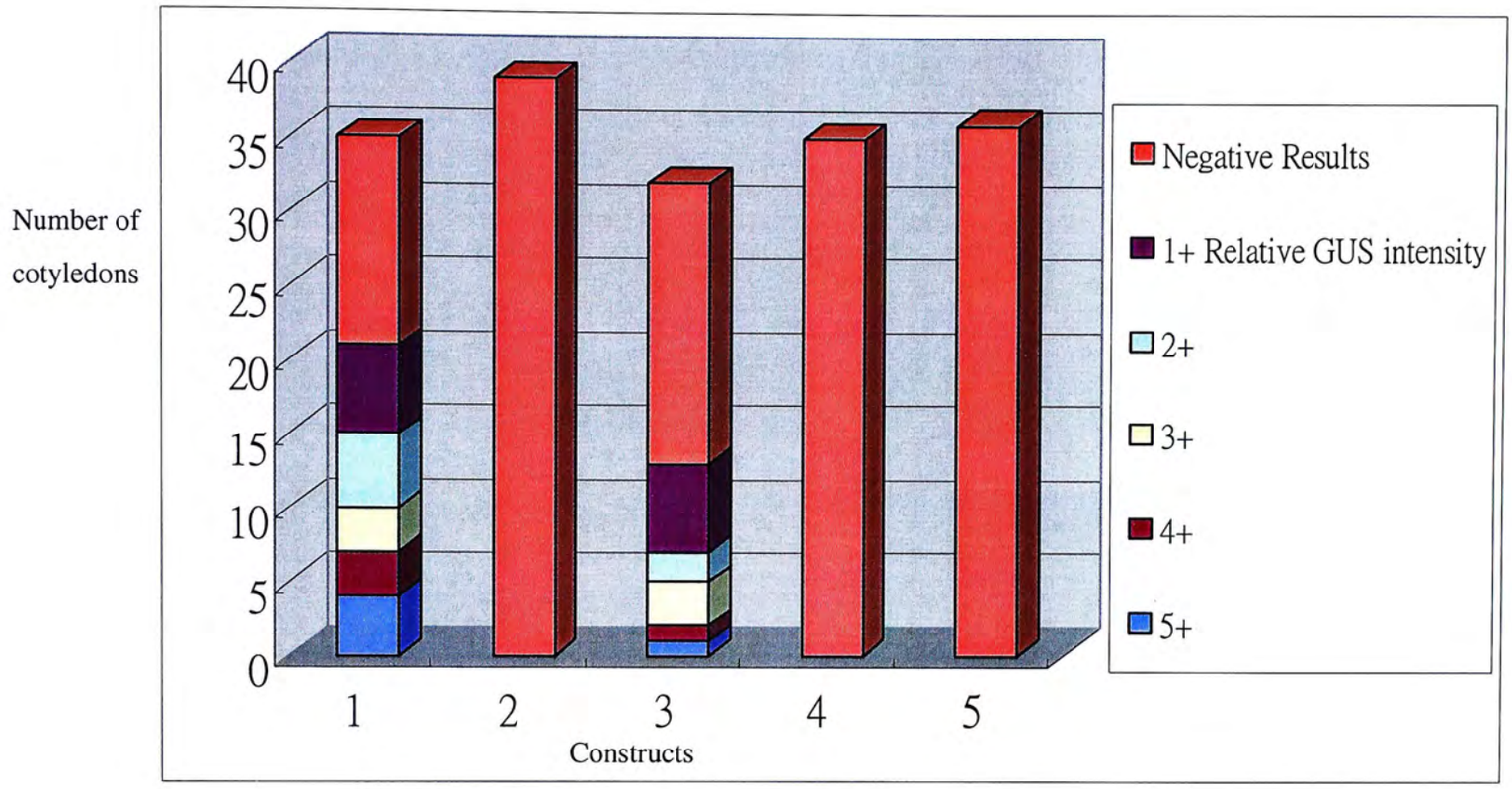



**Figure 4.4.2-2 Transient expression of the MP19 and modified GUS fusion constructs**

Constructs 1: pTZ/Phas/GUS; 2: pTZ/Phas/GUSN; 3: pTZ/Phas/MP19-EK::GUSN; 4: pBK/Phas/SP/MP19-EK::MGUSN; and 5: pBK/Phas/SP/MP19-EK::MGUSN-AFVY. The relative intensities of blue dot in cotyledons are represented by different colours in the bar chart (5+= max. relative intensity).

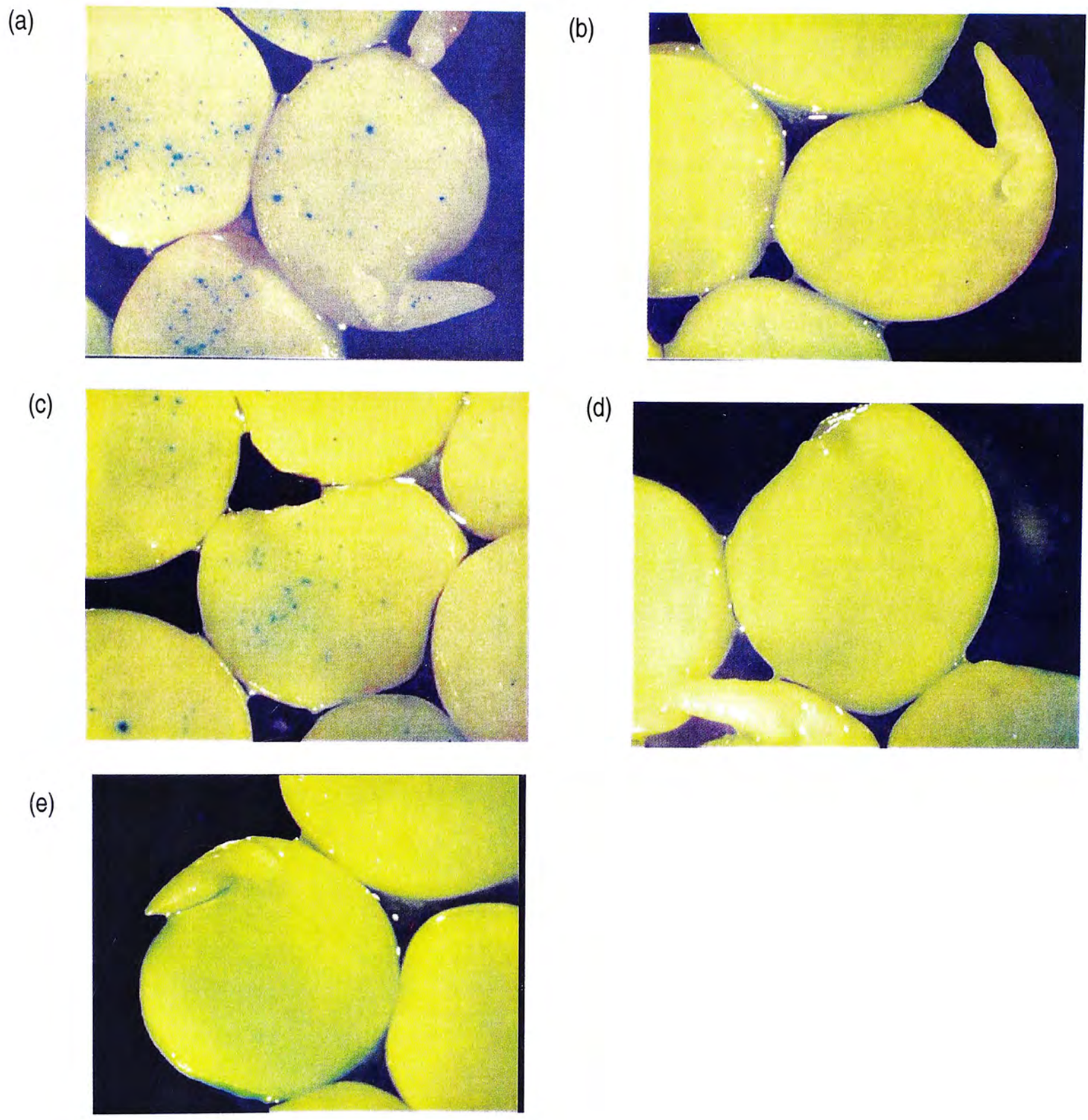



**Figure 4.4.2-3 Photographs illustrating the transient expression of the modified GUS fusion constructs**

Snow bean cotyledons were bombarded with the following constructs (a) positive control: Phas/GUS; (b) negative control: Phas/GUSN; (c) Phas/MP19-EK::GUSN; (d) Phas/SP/MP19-EK::MGUSN; and (e) Phas/SP/MP19-EK::MGUSN-AFVY.

## 4.5 Generation of transgenic tobacco

### (A) Protein targeting constructs

First generation transgenic plants (R1) were selected out on kanamycin shooting medium after tobacco transformation of the protein targeting chimeric constructs Phas/SP/MP42, Phas/SP/MP42-BP80, Phas/SP/MP42-αTIP, Phas/SP/MP42-RMR, Phas/SP/MP19, Phas/MP19-BP80 and Phas/MP19-RMR (Figure 4.3.1-1). The selected transgenic plants were then transferred to rooting medium and the leaves of the plants were collected for GUS staining (Figure 4.5-1 and.4.5-2).

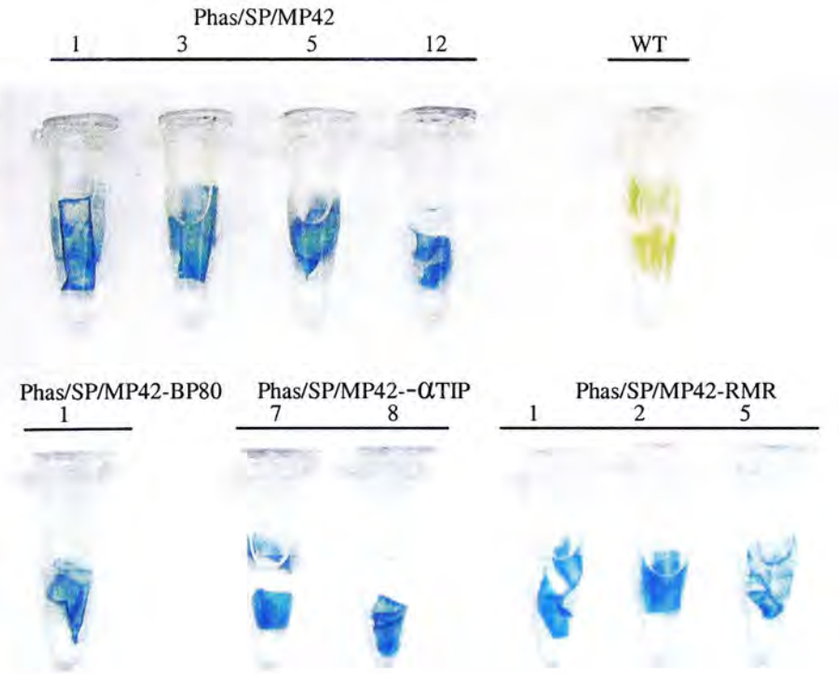



**Figure 4.5-1 Integration of chimeric gene constructs into tobacco plant genome-GUS staining of tobacco plants transformed with the NP42 protein targeting constructs**

Leaves from tobacco plants transformed with Phas/SP/MP42, Phas/SP/MP42-BP80, Phas/SP/MP42-α-TIP, or Phas/SP/MP42-RMR and wild type plants (WT, Control) were GUS stained. Numbers indicate the line numbers of transgenic plants harboring

the respective gene constructs.

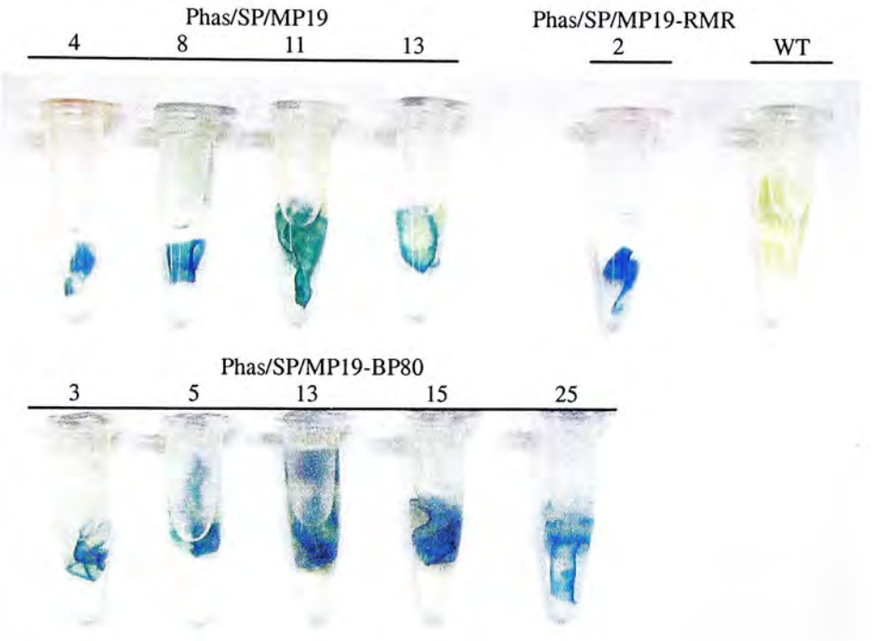



**Figure 4.5-2 Integration of chimeric gene constructs into tobacco plant genome -GUS staining of tobacco plants transformed with the MP19 protein constructs**
Leaves from tobacco plants transformed with Phas/SP/MP19, Phas/SP/MP19-BP80, or Phas/SP/MP19-RMR and wild type plants (WT, control) were GUS stained. Numbers indicate the line numbers of transgenic plants harboring respective gene constructs.

**(B) GUS fusion constructs**

First generation transgenic plants (R1) were selected out on kanamycin shooting medium after tobacco transformation for each of the chimeric constructs pSUN1/Phas/MP42::EK-GUSN, pSUN1/Phas/MP19::EK-GUSN, pSUN1/Phas/SP/MP42::EK-GUSN, pSUN1/Phas/SP/MP19::EK-GUSN, pSUN1/Phas/SP/MP42::EK-GUSN-AFVY, pSUN1/Phas/SP/MP19::EK-GUSN-AFVY (Figure 4.3.1-11). The selected transgenic plants were then transferred to rooting medium and the leaves of the plants were collected for orange fluorescent detection.

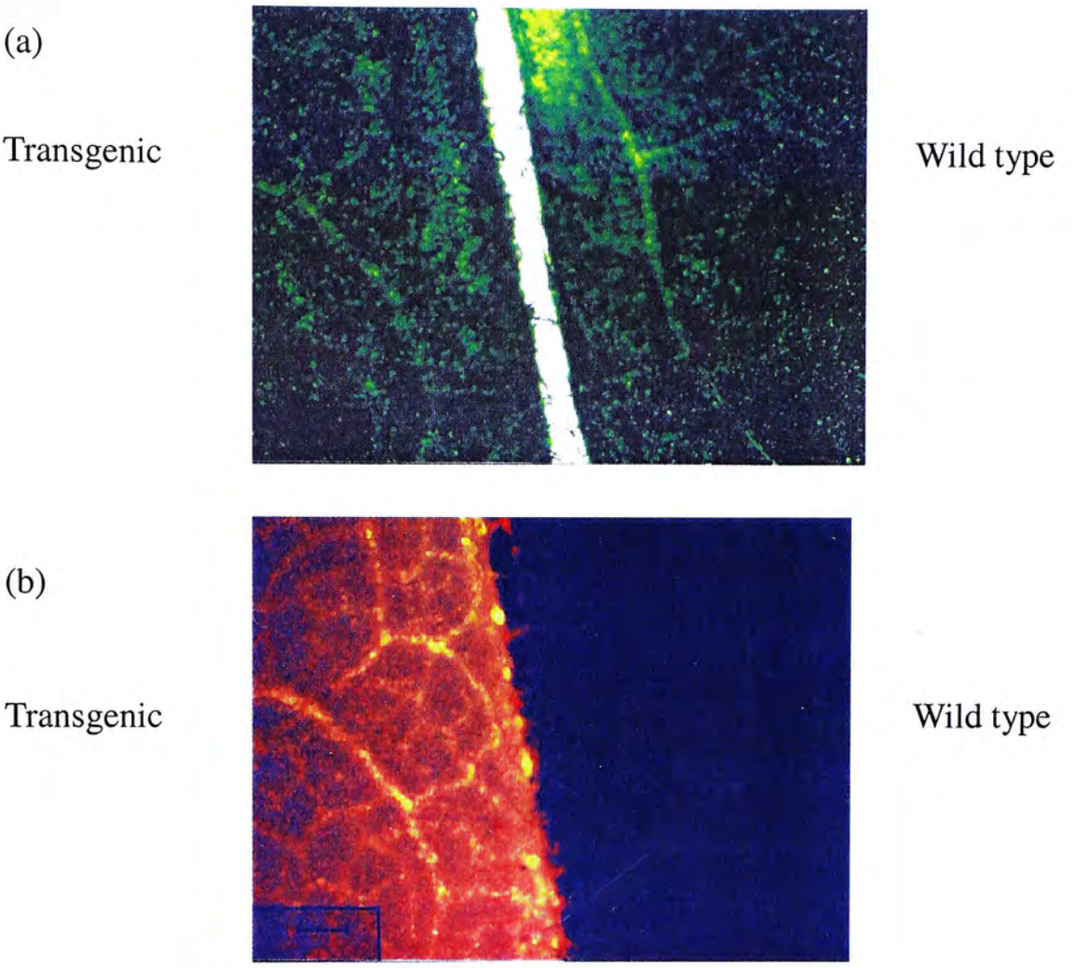
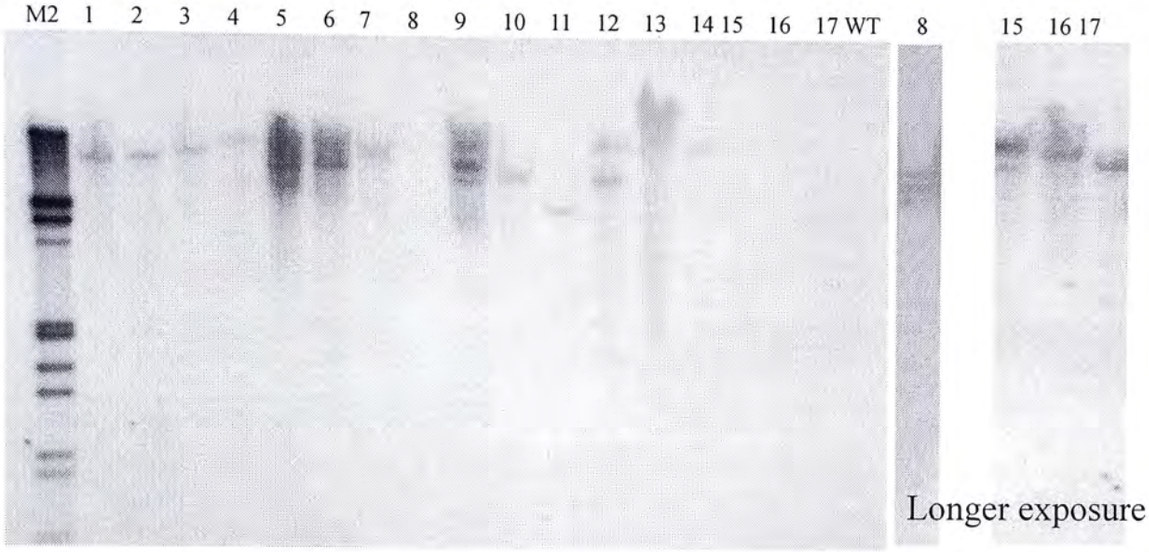



**Figure 4.5-3 Microscopic view of transgenic and wild type tobacco**

Leaves from tobacco plants transformed with MP42 and MP19 GUS fusion constructs were collected and viewed under fluorescent microscope using orange fluorescent filter. (a) under light field (b) under orange fluorescent filter

## 4.6 Southern analysis

The integration of MP42 and MP19 in the plant genome was confirmed by Southern blot analysis. Genomic DNA of the lines was extracted and digested with BamHI which cuts only one time in the T-DNA backbone, but not in the transgene. MP42 or MP19 specific probe was used to detect the transgene sequence. The copy number of the transgene integration were estimated through the hybridization results. From this analysis, transgenic plants were selected from each constructs for further analysis. A total of 35 transgenic lines were selected (Table 4.6-1).



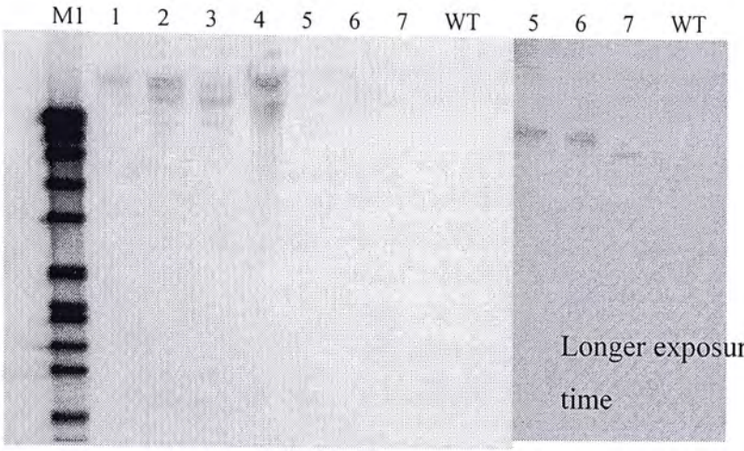

Copy no.: 1 1 1 1 3 2 2 3 1 1 2 1 1

Key: Lane M1 and M2= molecular markers; WT, wild type; number in front of construct= lane number; number after construct = number of transgenic lines for the constructs

| Lane | Sample | copy no. |
|---|---|---|
| 1: | Phas/SP/MP42 | 1 |
| 2: | Phas/SP/MP42 | 3 |
| 3: | Phas/SP/MP42 | 5 |
| 4: | Phas/SP/MP42 | 12 |
| 5: | Phas/SP/MP42-αTIP | 7 |
| 6: | Phas/SP/MP42-αTIP | 8 |
| 7: | Phas/SP/MP42-BP80 | 1 |
| 8: | Phas/SP/MP42-RMR | 1 |
| 9: | Phas/SP/MP42-RMR | 2 |
| 10: | Phas/SP/MP42-RMR | 5 |
| 11: | Phas/MP42::EKGUSN | 2 |
| 12: | Phas/MP42::EKGUSN | 4 |
| 13: | Phas/MP42::EKGUSN | 6 |
| 14: | Phas/MP42::EKGUSN | 15 |
| 15: | Phas/SP/MP42::EKGUSN | 7 |
| 16: | Phas/MP42::EKGUSN-AFVY | 15 |
| 17: | Phas/MP42::EKGUSN-AFVY | 10 |
| WT: | Wild Type | |

**Figure 4.6-1 Southern analysis of MP42 transgenic tobacco**

Genomic DNA (15μg) was digested with BamHI, was seperated by gel electrophoresis and blotted onto a nylon membrane for hybridization and detection. The lines (boxed) were used for further Northern and Western analysis.

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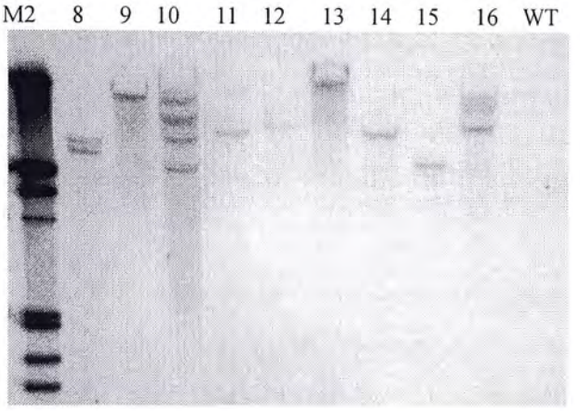


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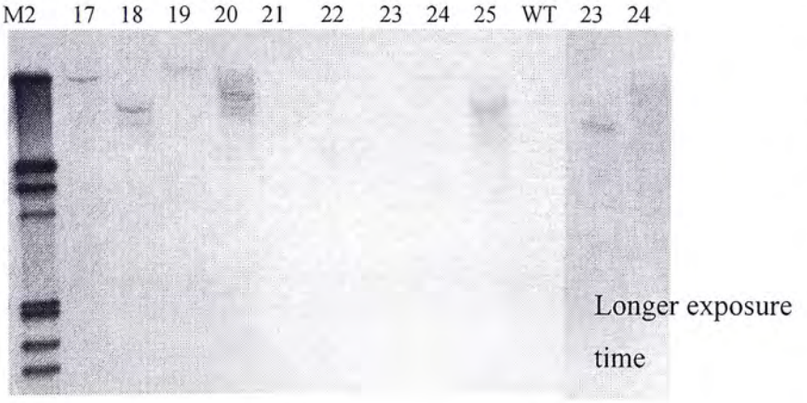



Copy no.: 1 2 1 5 0 0 1 0 >2 1

Key: Lane M1 and M2= molecular markers (Roche) ; WT, wild type

Number in front of construct= lane number; number after construct = number of transgenic lines for the constructs

1: SP/ MP19 2

2: SP/ MP19 3

3: SP/ MP19 4

4: SP/ MP19 8

5: SP/ MP19-RMR 2

6: SP/ MP19-EK::GUSN 2

7: SP/ MP19-EK::GUSN 4

8: SP/ MP19 4

9: SP/ MP19 11

10: SP/ MP19 13

11: SP/MP19 14

12: SP/ MP19-BP80 3

13: SP/ MP19-BP80 5

14: SP/ MP19-BP80 13

15: SP/ MP19-BP80 15

16: SP/ MP19-BP80 19

17: SP/ MP19-BP80 21

18: SP/ MP19-BP80 25

19: SP/ MP19-BP80 28

20: SP/ MP19-BP80 30

21: SP/ MP19-BP80 32

22: SP/ MP19-RMR 6

23: SP/ MP19-RMR 12

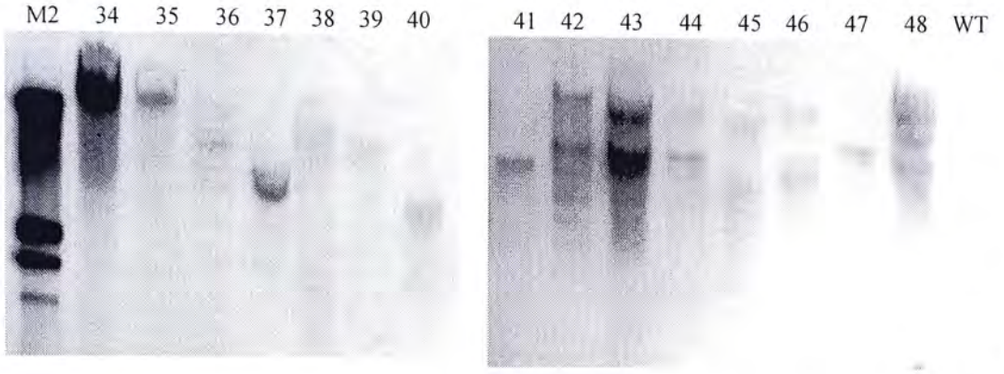
24: MP19-EKGN 6

25: MP19- EKGN 8

WT: Wild Type

M1: DNA Molecular WeightMarker VII

M2: DNA Molecular WeightMarker III




34: Phas/MP19::EKGN 12

35: Phas/MP19::EKGN 14

36: Phas/SP/MP19::EKGN 2

37: Phas/SP/MP19::EKGN 4

38: Phas/SP/MP19::EKGN 7

39: Phas/SP/MP19::EKGN 11

40: Phas/SP/MP19::EKGN 12

41: Phas/SP/MP19::EKGN 17

42: Phas/SP/MP19::EKGN 23

43: Phas/SP/MP19::EKGN –AFVY 1

44: Phas/SP/MP19::EKGN –AFVY 2

45: Phas/SP/MP19::EKGN –AFVY 7

46: Phas/SP/MP19::EKGN –AFVY 5

47: Phas/SP/MP19::EKGN –AFVY 11

48: Phas/SP/MP19::EKGN –AFVY 16

WT: Wild Type

M1: DNA Molecular WeightMarker VII

M2: DNA Molecular WeightMarker III

**Figure 4.6-2 Southern analysis of MP19 transgenic tobacco**

Genomic DNA (15ug) was digested with BamHI, was seperated by gel electrophoresis and blotted onto a nylon membrane for hybridization and detection. The lines (boxed) were used for further northern and Western analysis.

**Table 4.6-1: Transgenic lines selected from constructs for further analysis**

| Protein-targeting constructs | | GUS fusion constructs | |
|---|---|---|---|
| $MSP1_{42}$ | $MSP1_{19}$ | $MSP1_{42}$ | $MSP1_{19}$ |
| Phas/SP/MP42<br>Lines 1, 3, 5, 12 | Phas/SP/MP19<br>Lines 4, 8, 11, 13 | Phas/MP42-EK::GUSN<br>Lines 4, 6, 15 | Phas/MP19-EK::GUSN<br>Line 8 |
| Phas/SP/MP42-BP80<br>Line 1 | Phas/SP/MP19-BP80<br>Lines 3, 5, 13, 15, 25 | Phas/SP/MP42-EK::GUSN<br>Line 7 | Phas/SP/MP19-EK::GUSN<br>Lines 2, 6, 7, 11, 12 |
| Phas/SP/MP42-α-TIP<br>Lines 7, 8 | | Phas/SP/MP42-EK::GUSN<br>-AFVY<br>Line 15 | Phas/SP/MP19-EK::GUSN<br>-AFVY<br>Lines 2, 5, 7, 11, 16 |
| Phas/SP/MP42-RMR<br>Lines 1, 2, 5 | Phas/SP/MP19-RMR<br>Line 2 | | |

## 4.7 Northern analysis

### (A) Protein-targeting constructs

The transgene expression of the protein-targeting constructs Phas/SP/MP42, Phas/SP/MP42-BP80, Phas/SP/MP42-α-TIP, Phas/SP/MP42-RMR, Phas/SP/MP19, Phas/SP/MP19-BP80, and Phas/SP/MP19-RMR, at transcriptional level was studied by northern blotting analysis using developing tobacco seed RNA and MP42 or MP19 specific probe (Figures 4.7-1, 4.7-2, 4.7-3 and 4.7-4). Transcripts of expected size were detected in all the constructs under study.

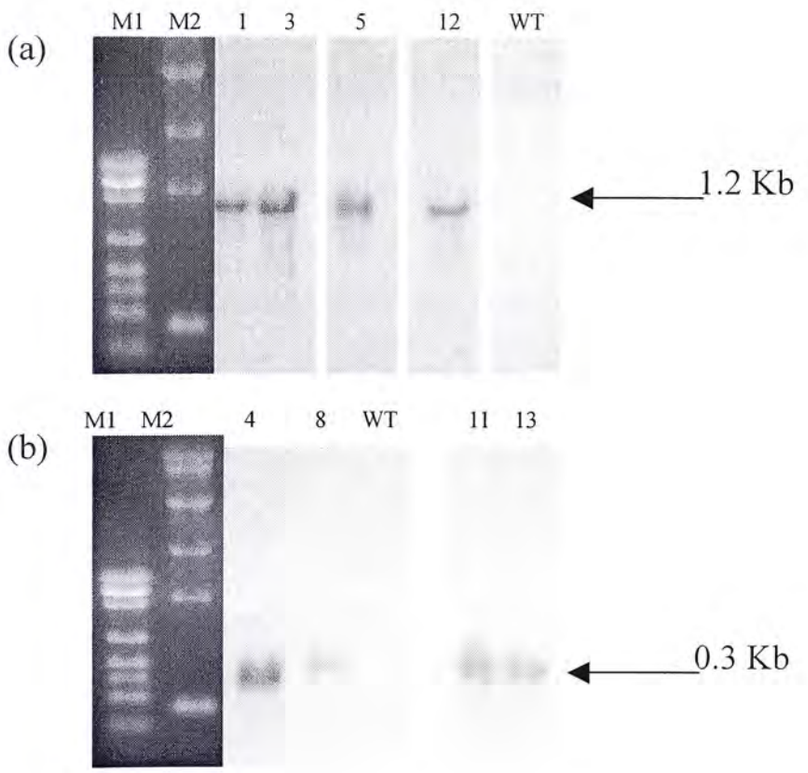



**Figure 4.7-1 Northern analysis of transgenic Phas/SP/MP42 and Phas/SP/MP19 tobacco plants**

Total developing tobacco seed RNA (8 μg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and

detection with MP42 or MP19 specific probe. (a) construct Phas/SP/MP42 and (b) construct Phas/SP/MP19. Key: WT: wild type; numbers represent the line numbers of each of the construct; M1: 0.16-1.77kb RNA ladder (Invitrogen); M2: 0.24-9.5Kb RNA ladder (Invitrogen); arrows show the expected sizes of the transcripts.

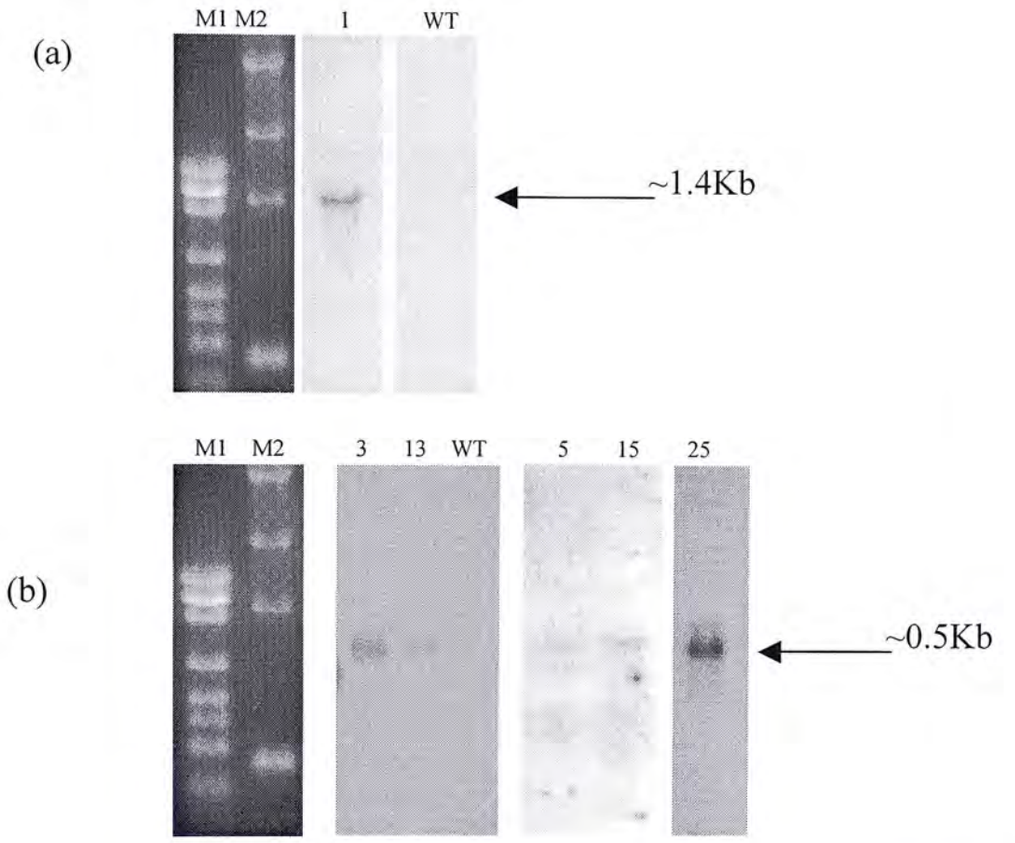



**Figure 4.7-2 Northern analysis of transgenic tobacco with BP80 constructs**
Total developing tobacco seed RNA (8 μg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and detection with MP42 or MP19 specific probe. (a) Construct Phas/SP/MP42-BP80 and (b) construct Phas/SP/MP19-BP80. Key: WT: wild type; numbers represent the line numbers of each construct; M1: 0.16-1.77Kb RNA ladder (Invitrogen); M2: 0.24-9.5kb RNA ladder (Invitrogen); arrows show the expected sizes of the transcripts.

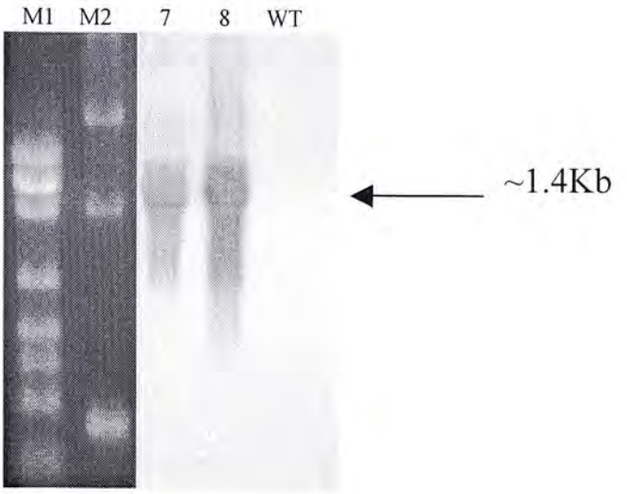



**Figure 4.7-3 Northern analysis of transgenic tobacco of α-TIP constructs**

Total developing tobacco seed RNA (8μg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and detection with MP42 specific probe. Key: WT: wild type; numbers represent the line numbers of the Phas/SP/MP42-αTIP construct; M1: 0.16-1.77Kb RNA ladder (Invitrogen); M2: 0.24-9.5kb RNA ladder (Invitrogen); arrows show the expected size of the transcript.

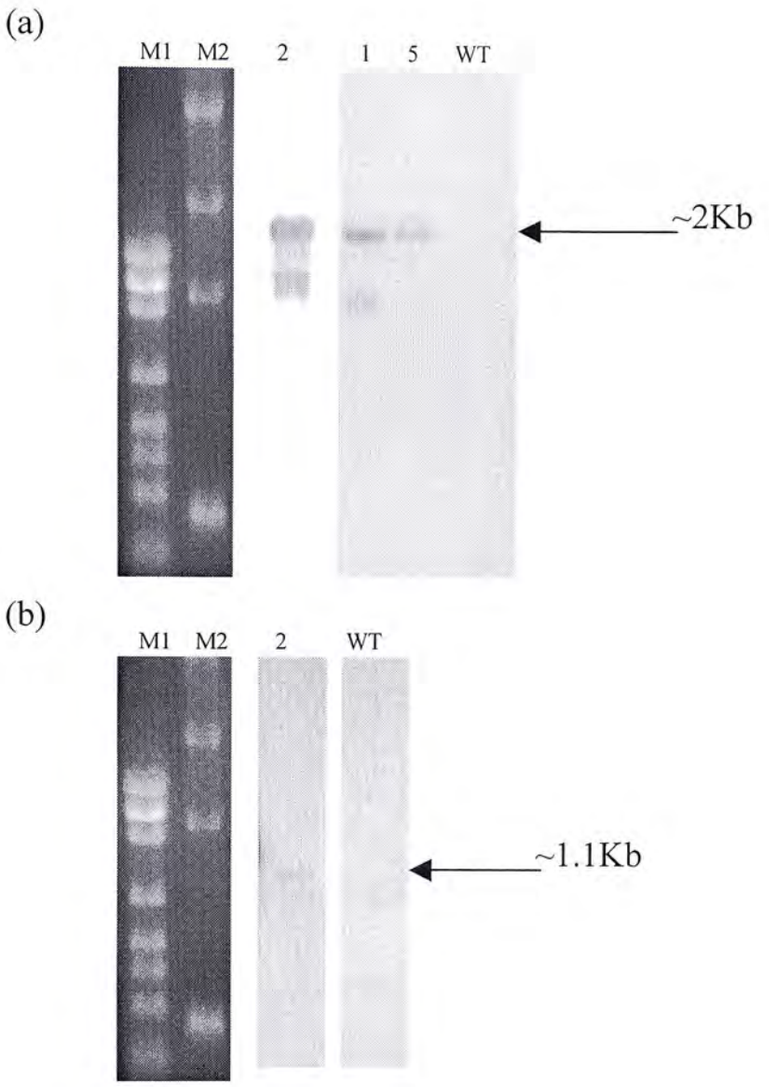



**Figure 4.7-4 Northern analysis of transgenic tobacco with RMR constructs** Total developing tobacco seed RNA (8µg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and detection with MP42 or MP19 specific probe. (a) Construct Phas/SP/MP42-RMR and (b) Construct Phas/SP/MP19-RMR. Key: WT: wild type; numbers represent the line numbers of each construct; M1: 0.16-1.77Kb RNA ladder (Invitrogen); M2: 0.24-9.5kb RNA ladder (Invitrogen); arrows show the expected sizes of the transcripts.

**(B) GUS fusion constructs**

Northern blotting analysis using total developing seed RNA and MP42 or MP19 specific probe was carried out to study the transgene expression of constructs Phas/MP42::EKGUSN, Phas/SP/MP42::EK-GUSN, Phas/MP19::EK-GUSN, Phas/SP/MP19::EK-GUSN, and Phas/SP/MP19::EK-GUSN-AFVY at mRNA level (Figure 4.7-5 and 4.7-6). Transcripts with expected size were detected.

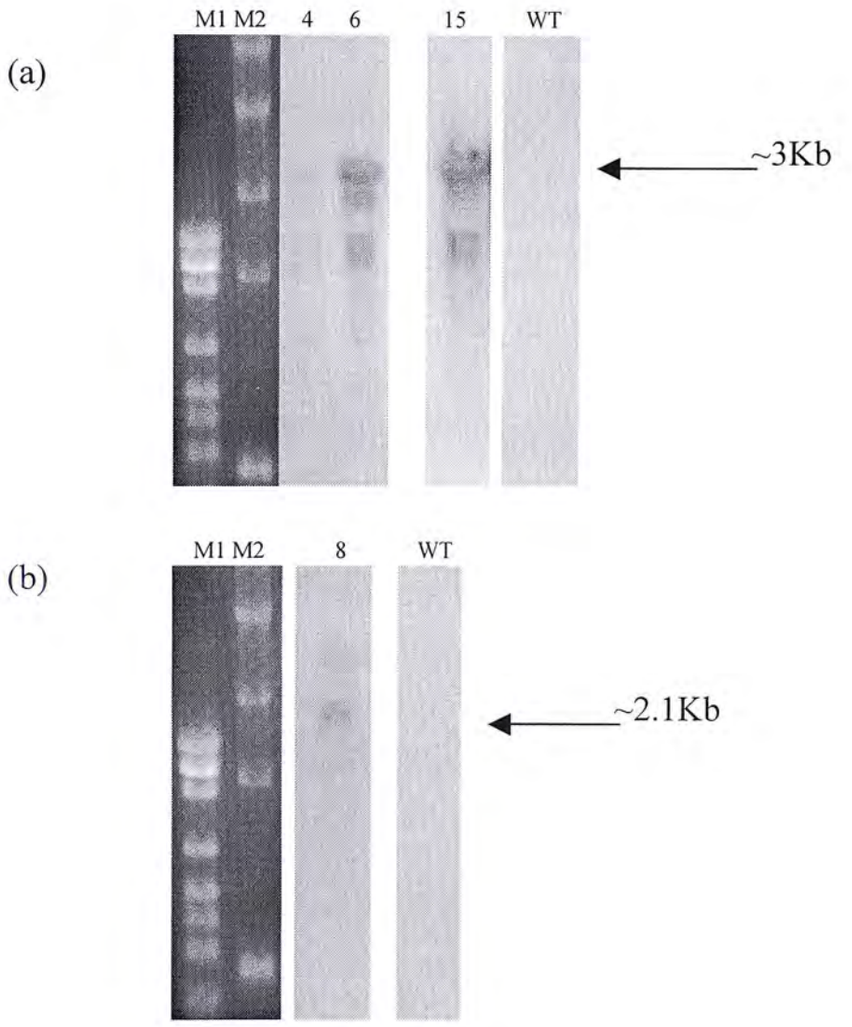



**Figure 4.7-5 Northern analysis of transgenic Phas/MP42::EK-GUSN and Phas/MP19::EK-GUSN tobacco plants**

Total developing tobacco seed RNA (8μg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and

detection with MP42 or MP19 specific probe. (a) construct Phas/MP42::EK-GUSN and (b) construct Phas/MP19::EK-GUSN. Key: WT: wild type; numbers represent the line numbers of each of the construct; M1: 0.16-1.77Kb RNA ladder (Invitrogen); M2: 0.24-9.5Kb RNA ladder (Invitrogen); arrows show the expected sizes of the transcripts.

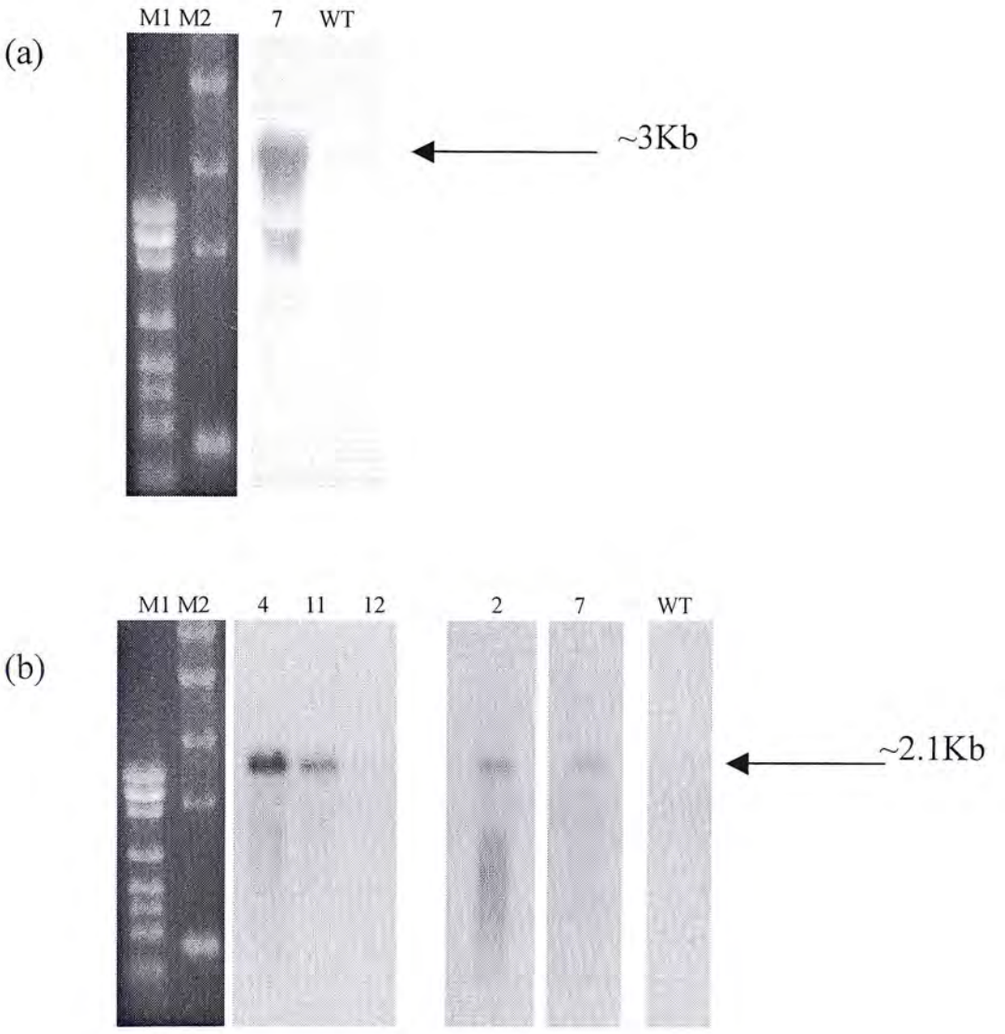



**Figure 4.7-6 Northern analysis of transgenic Phas/SP/MP42::EK-GUSN and Phas/SP/MP19::EK-GUSN tobacco plants**

Total developing tobacco seed RNA (8μg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and detection with MP42 or MP19 specific probe. (a) Construct Phas/SP/MP42::EK-GUSN and (b) Construct Phas/SP/MP19::EK-GUSN. Key: WT: wild type; numbers represent the line numbers of each construct; M1: 0.16-1.77kb RNA ladder (Invitrogen); M2: 0.24-9.5kb RNA ladder (Invitrogen); arrows show the expected sizes of the transcripts.

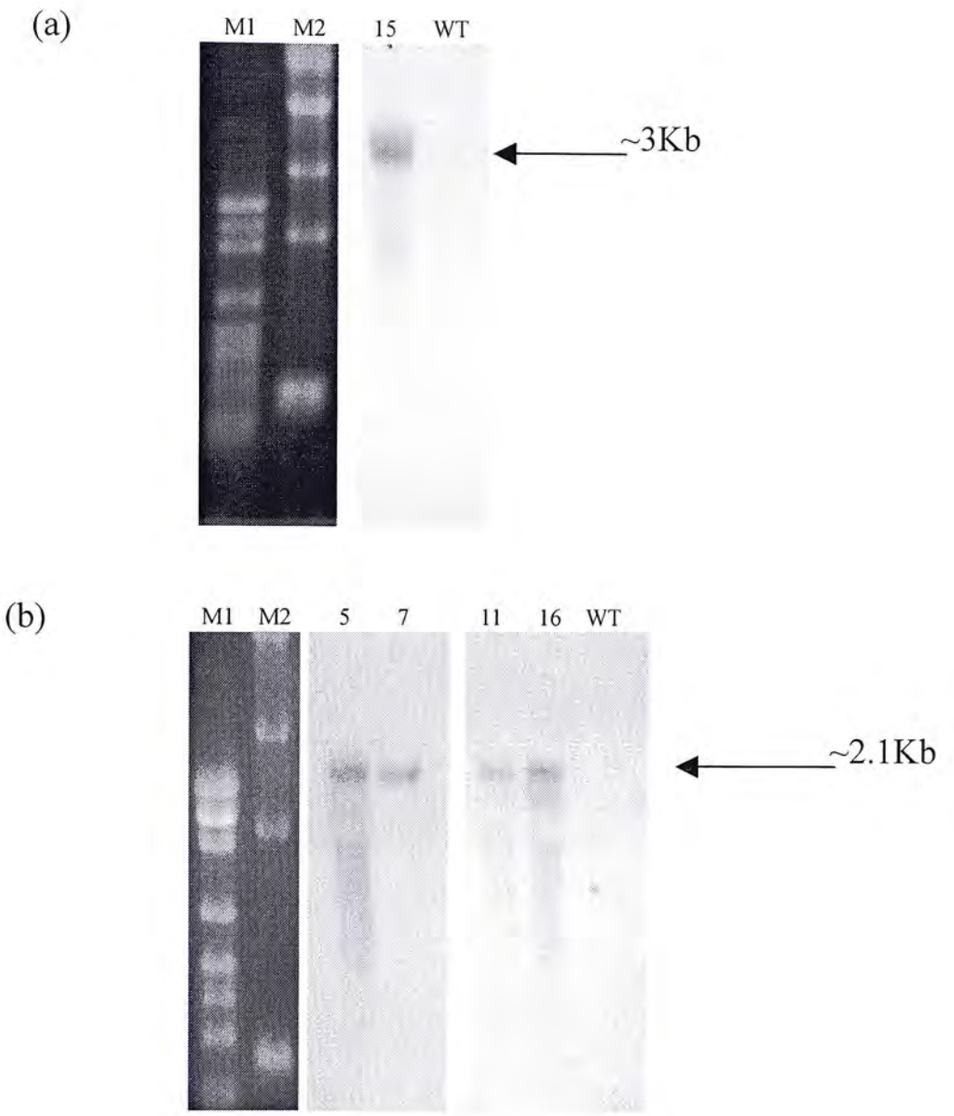



**Figure 4.7-7 Northern analysis of transgenic Phas/SP/MP42::EK-GUSN-AFVY and Phas/SP/MP19::EK-GUSN-AFVY tobacco plants**

Total developing tobacco seed RNA (8μg) was resolved in 1% RNA denaturating gel and the resolved RNA was blotted onto a nylon membrane for hybridization and detection with MP19 specific probe. (a) construct Phas/SP/MP42::EK-GUSN-AFVY and (b) construct Phas/SP/MP19::EK-GUSN-AFVY. Key: WT: wild type; numbers represent the line numbers of each construct; M1: 0.16-1.77Kb RNA ladder (Invitrogen); M2: 0.24-9.5Kb RNA ladder (Invitrogen); arrows show the expected sizes of the transcripts.

## 4.8 Western analysis

### (A) Protein-targeting constructs

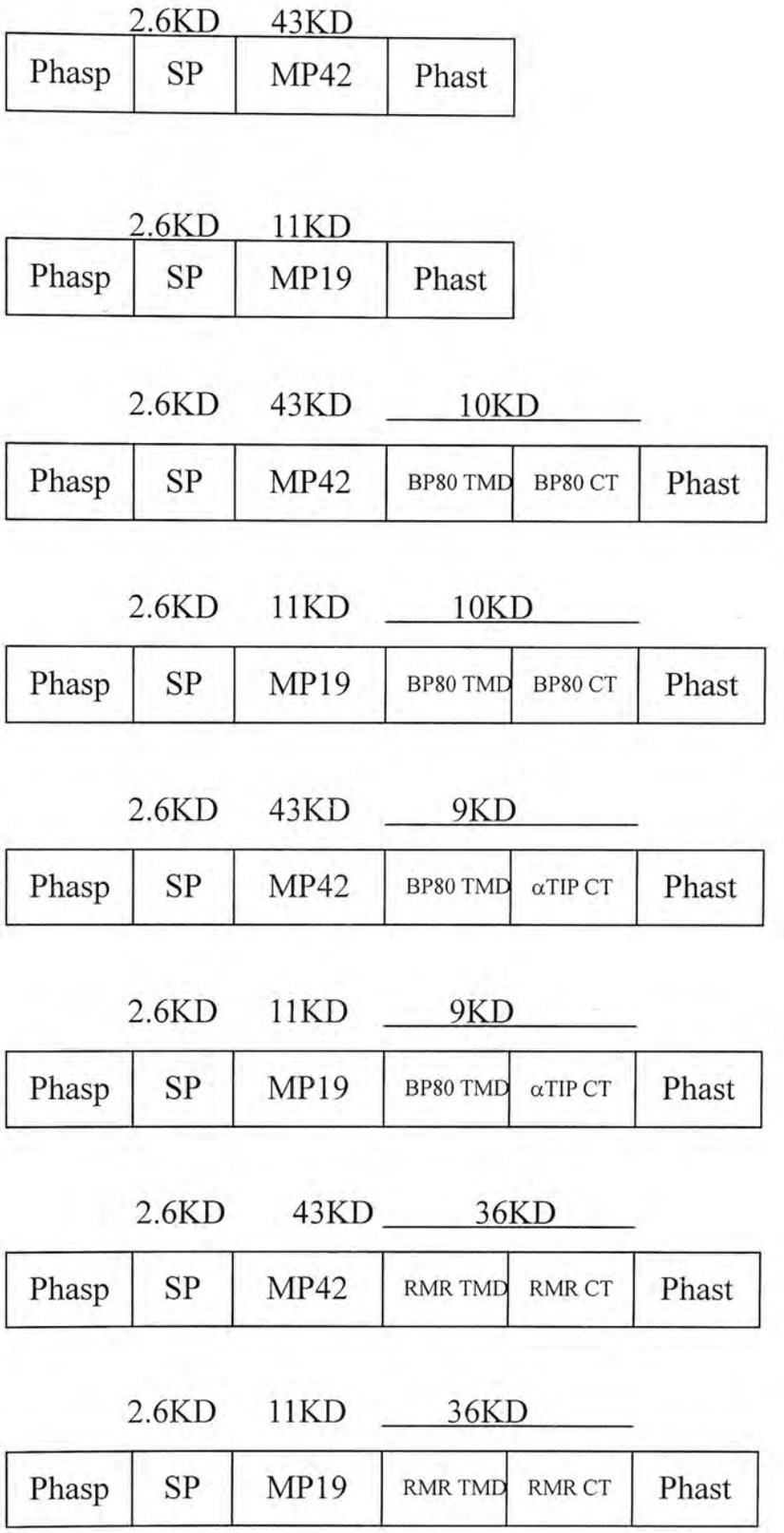
Western blotting analysis was used to detect the expression of recombinant target protein in transgenic tobacco seeds. All of the lines used for western analysis had been shown to give positive results in the northern analysis (Figures 4.7-1, 4.7-2, 4.7-3 and 4.7-4).

Fifty μg of seed total protein from different lines of each construct was used for the western blot and varying amounts of purified His-MP42 were also included, so as to estimate the recombinant protein production.

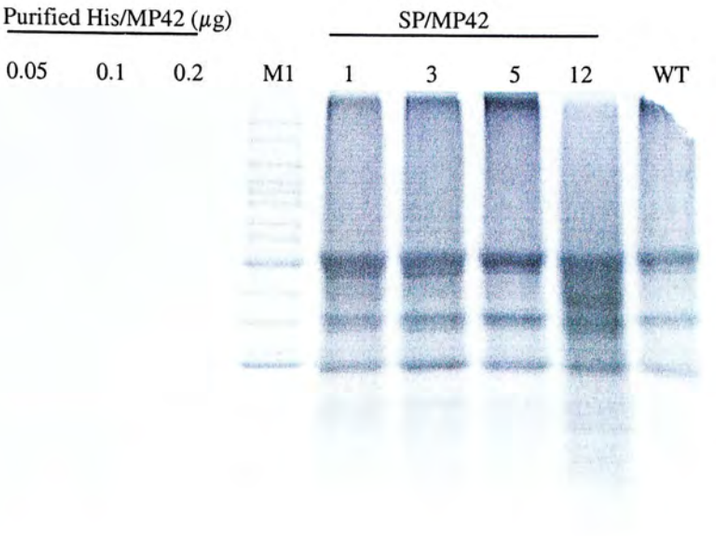
As shown in Figure 4.8-1, the highest expression of the recombinant target protein was found for construct Phas/SP/MP42-α-TIP, amounting to 0.4% of the total seed protein. For the BP80 constructs, the expression of the recombinant protein was estimated as 0.04% and 0.06% in the Phas/SP/MP42-BP80 and Phas/SP/MP19-BP80 transgenic seeds, as 53KD and 19KD proteins, respectively. About 0.1% recombinant protein with expected size (43KD) was found for construct Phas/SP/MP42 containing only the phaseolin signal peptide, while multiple protein bands with different intensities were detected for the Phas/SP/MP19 construct.

No recombinant protein was detected in the RMR constructs.

(a)

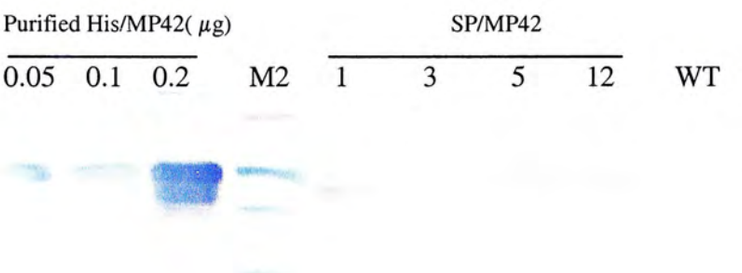

2.6KD
43KD
Phasp
SP
MP42
Phast
2.6KD
11KD
Phasp
SP
MP19
Phast
2.6KD
43KD
10KD
Phasp
SP
MP42
BP80 TMD
BP80 CT
Phast
2.6KD
11KD
10KD
Phasp
SP
MP19
BP80 TMD
BP80 CT
Phast
2.6KD
43KD
9KD
Phasp
SP
MP42
BP80 TMD
αTIP CT
Phast
2.6KD
11KD
9KD
Phasp
SP
MP19
BP80 TMD
αTIP CT
Phast
2.6KD
43KD
36KD
Phasp
SP
MP42
RMR TMD
RMR CT
Phast
2.6KD
11KD
36KD
Phasp
SP
MP19
RMR TMD
RMR CT
Phast

(b-1)




Coomassie blue staining of Phas/SP/MP42

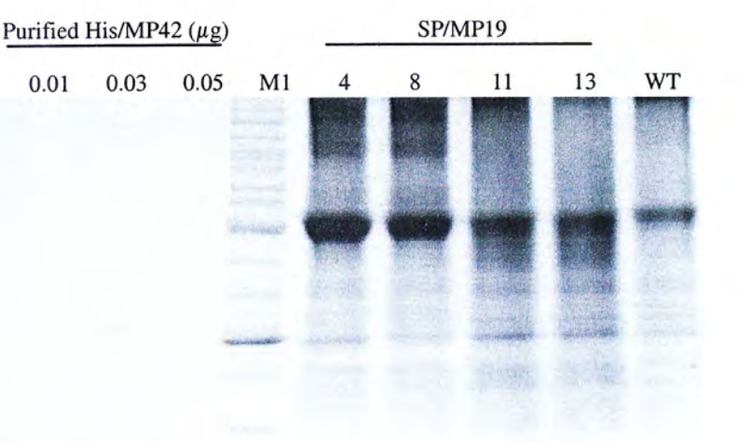
(b-2)




~43 KD

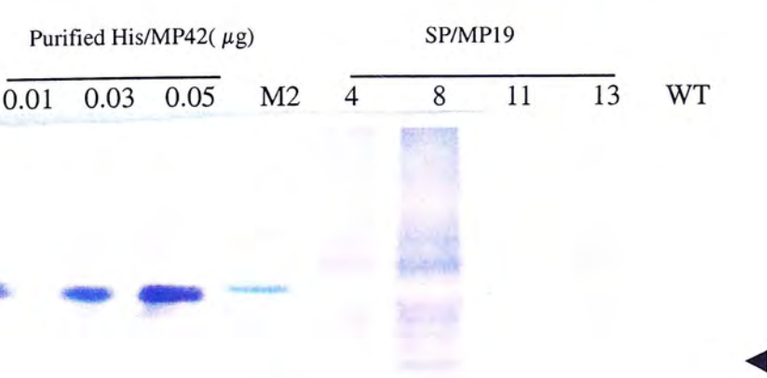
Western blotting of Phas/SP/MP42

(c-1)




Coomassie blue staining of Phas/SP/MP19

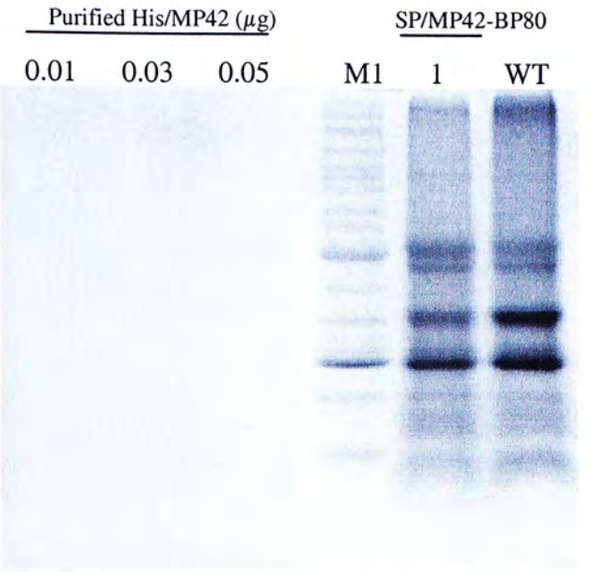
(c-2)



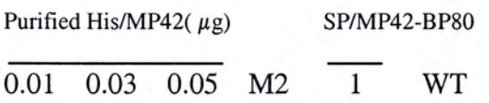

Western blotting of Phas/SP/MP19

~11KD

(d-1)



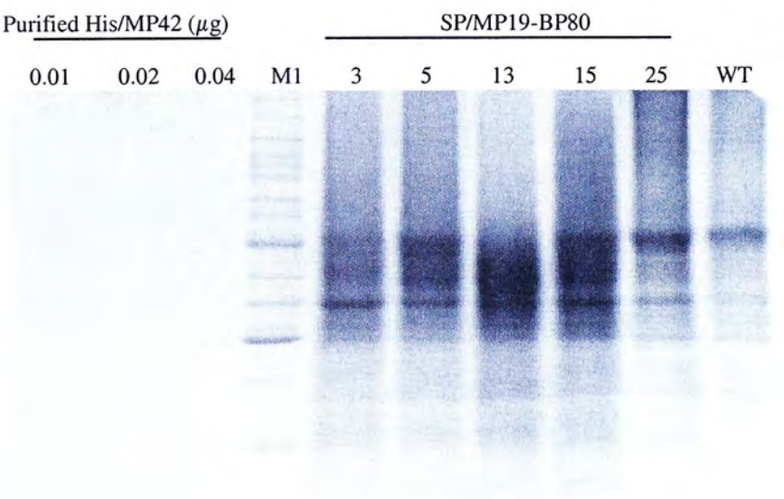

Coomassie blue staining of Phas/SP/MP42-BP80



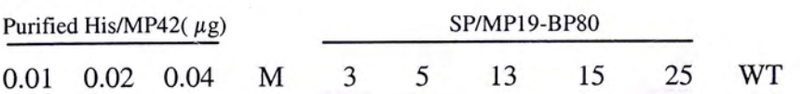

(d-2)


Western blotting of Phas/SP/MP42-BP80

(e-1)

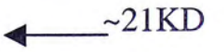



Coomassie blue staining of Phas/SP/MP19-BP80

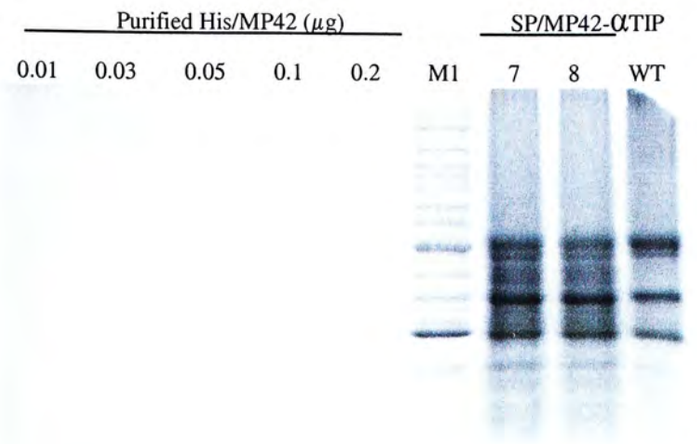



(e-2)

Western blottingof Phas/SP/MP19-BP80

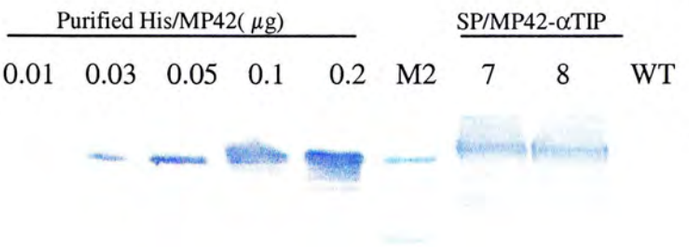


(f-1)



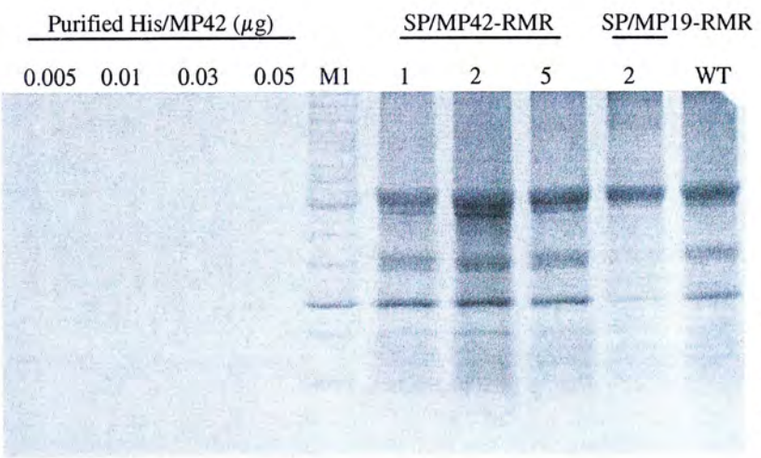

Coomassie blue staining of Phas/SP/MP42-αTIP

(f-2)



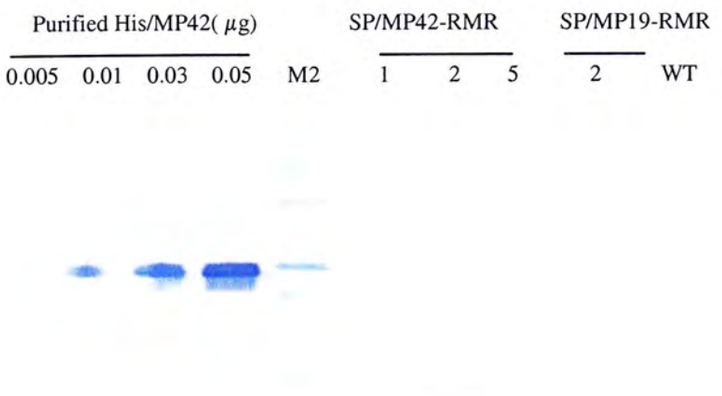

Western blotting of Phas/SP/MP42-αTIP

(g-1)




Coomassie blue staining of Phas/SP/MP42-RMR and Phas/SP/MP19-RMR

(g-2)




Western blotting of Phas/SP/MP42-RMR and Phas/SP/MP19-RMR

**Figure 4.8-1 Western analysisof tobacco plants transformed with protein-targeting constructs**

(a) The calculated MW of proteins; (b-2 to g-2) total seed proteins (50μg) was resolved in 16.5% tricine gel with β-mercaptoethanol and the sample was boiled before loading for the MP42 constructs but not for MP19 constructs, blotted onto PVDF membrane, and was detected using anti-$MSP1_{42}$ serum as primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as secondary antibody; (b-1 to g-1) Coomassie blue stained. The numbers represent line numbers of each construct. Purified protein His-$MSP1_{42}$, as 47.8KD protein, was included as a positive control with concentration as indicated; (b) SP/MP42; (c) SP/MP19; (d) SP/MP42-BP80; (e) SP/MP19-BP80; (f) SP/MP42-αTIP; and (g) SP/MP42-RMR and SP/MP19-RMR. Key: WT, wild type; M1, Bench Mark Protein Ladder (Invitrogen); M2, Precision Plus Protein Dual Standards (Bio-Rad). Arrows indicate major proteins reacted with the MSP1 antibody.

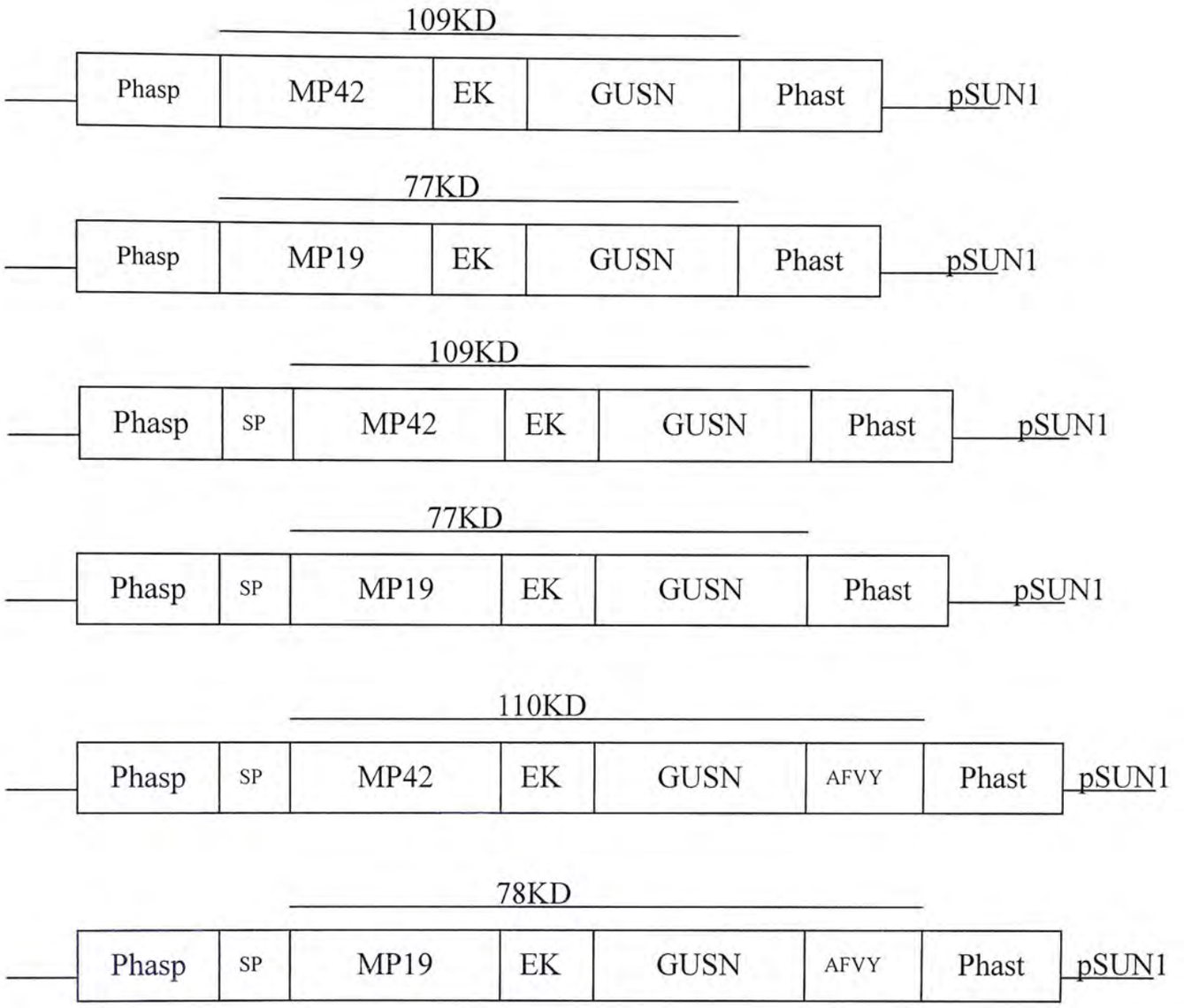
**(B) GUS fusion constructs**

For the GUS fusion constructs, altogether six constructs, Phas/MP42-EK::GUSN, Phas/MP19-EK::GUSN, Phas/SP/MP42-EK::GUSN, Phas/SP/MP19-EK::GUSN, Phas/SP/MP42-EK::GUSN-AFVY and Phas/SP/MP19-EK::GUSN-AFVY, were studied. All lines used in the analysis had been shown to give positive mRNA signals in northern analysis (Figures 4.7-5, 4.7-6 and 4.7-7).

Results revealed that recombinant target proteins were detected in the constructs with phaseolin signal peptide. Around 0.04% and more than 0.02% of the total seed protein of transgenic Phas/SP/MP42-EK::GUSN and Phas/SP/MP19-EK::GUSN tobacco seeds were 109KD protein and 77KD protein, respectively. The expression of Phas/SP/MP42-EK::GUSN-AFVY and Phas/SP/MP19-EK::GUSN-AFVY, with proteins of 109KD and 77KD, respectively, was estimated to yield around 0.16% and more than 0.04% as total seed protein, respectively.

No recombinant protein was detected in the constructs without the phaseolin signal peptide, i.e. Phas/MP42-EK::GUSN and Phas/MP19-EK::GUSN.

(a)


109KD
Phasp
MP42
EK
GUSN
Phast
pSUN1
77KD
Phasp
MP19
EK
GUSN
Phast
pSUN1
109KD
Phasp
SP
MP42
EK
GUSN
Phast
pSUN1
77KD
Phasp
SP
MP19
EK
GUSN
Phast
pSUN1
110KD
Phasp
SP
MP42
EK
GUSN
AFVY
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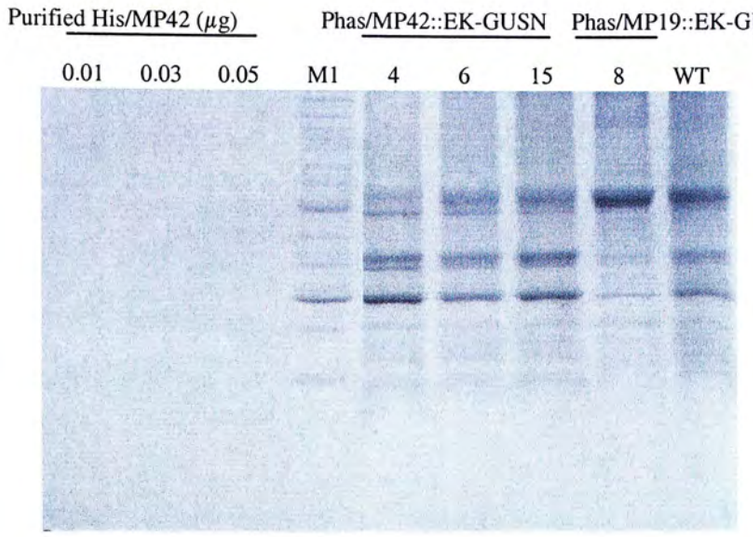
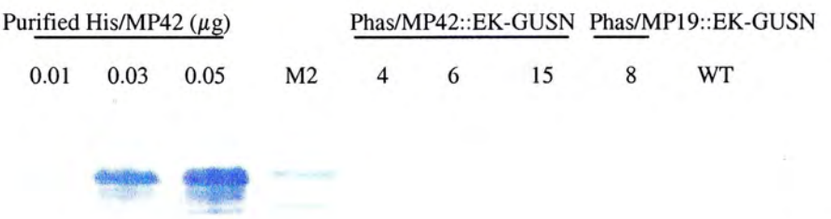
SP
MP19
EK
GUSN
AFVY
Phast
pSUN1

(b-1)



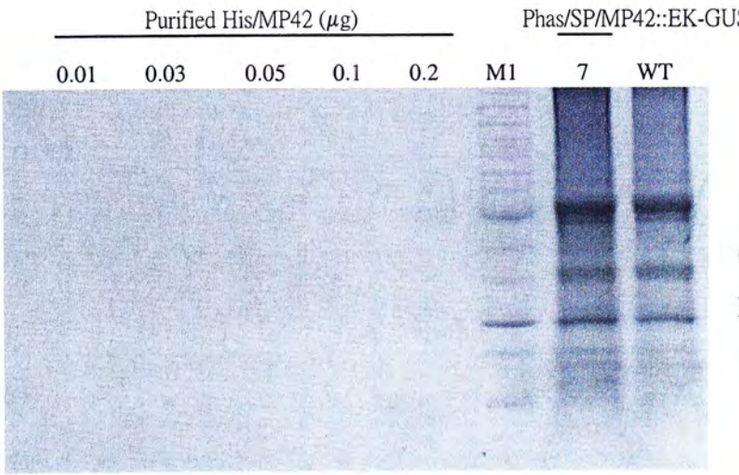

Coomassie blue staining of Phas/MP42::EK-GUSN and Phas/MP19::EK-GUSN

(b-2)



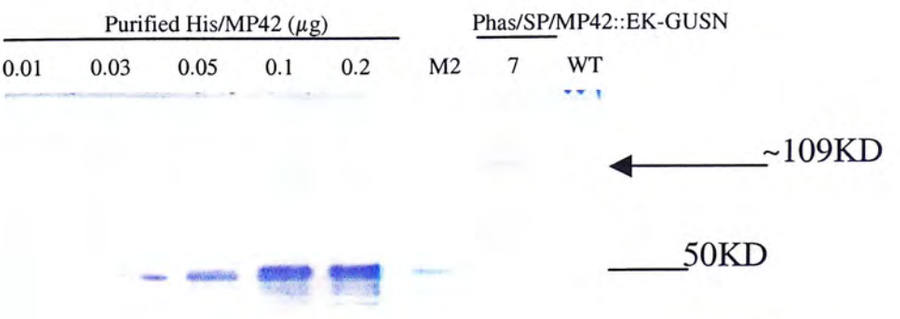

Western blotting of Phas/MP42::EK-GUSN and Phas/MP19::EK-GUSN

(c-1)



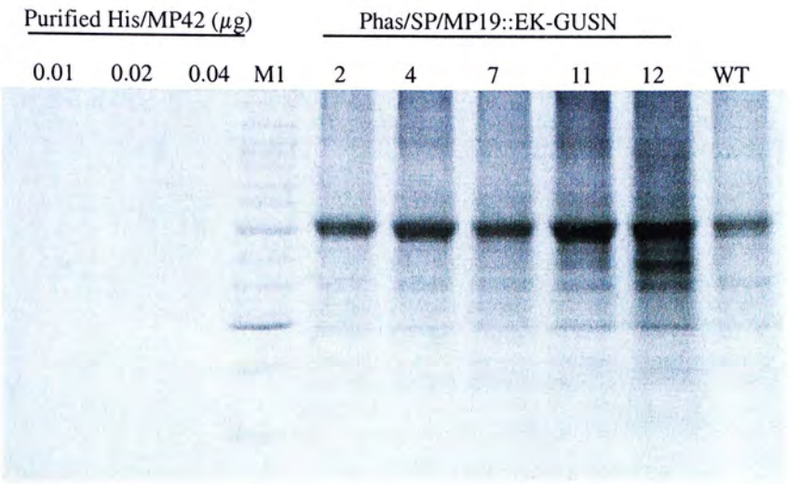

Coomassie blue staining of Phas/SP/MP42::EK-GUSN

(c-2)



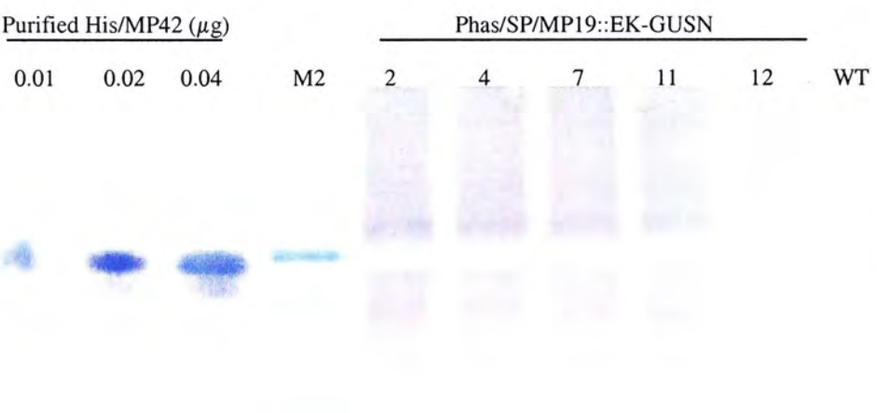

Western blotting of Phas/SP/MP42::EK-GUSN

(d-1)



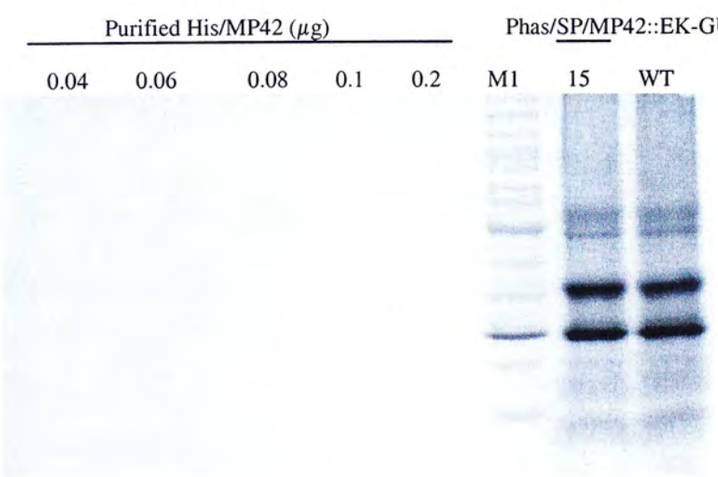

Coomassie blue staining of Phas/SP/MP19::EK-GUSN

(d-2)



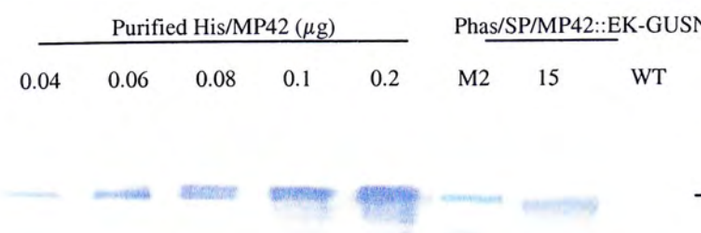

Western blotting of Phas/SP/MP19::EK-GUSN

(e-1)




Coomassie blue staining of Phas/SP/MP42::EK-GUSN -AFVY

(e-2)




Western blotting of Phas/SP/MP42::EK-GUSN-AFVY

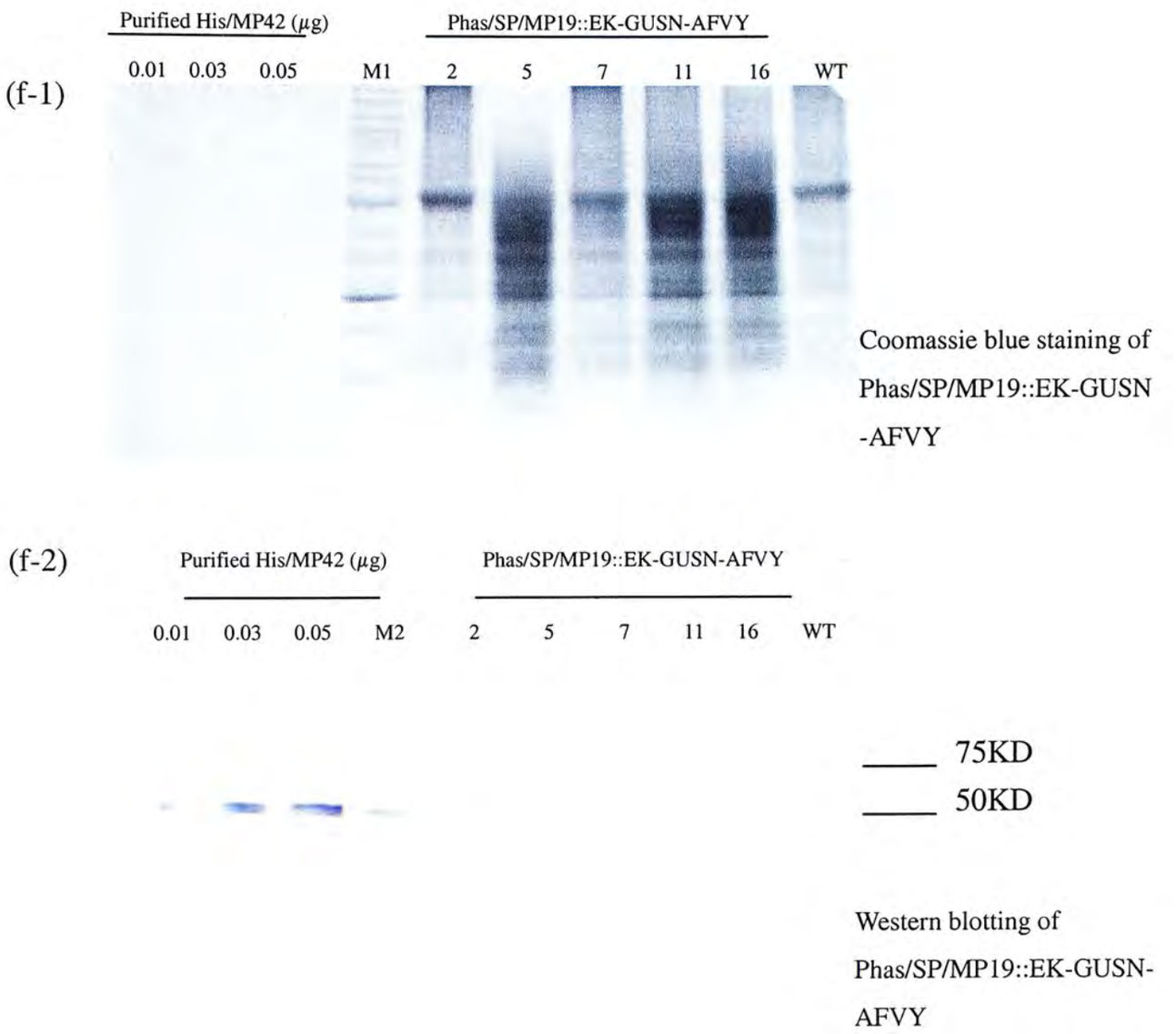



**Figure 4.8-2 Western analysis of transgenic tobacco with GUS fusion constructs**
(a) The calculated MW of the intact recombinant proteins; (b-2 to f-2) Total seed protein (50$\mu$g) was resolved in 16.5% tricine gel with β-mercaptoethanol with the sample boiled for MP42 construct but not the MP19 constructs, blotted onto PVDF membrane, and detected using anti-$MSP1_{42}$ serum as primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as secondary antibody and (b-1 to f-1) Coomassie blue stained. The numbers represent the line numbers of each construct. Purified protein His-$MSP1_{42}$ was included as a positive control with concentration as indicated; (b) SP/MP42::EKGUSN and SP/MP19::EK-GUSN; (c) SP/MP42::EK-GUSN; (d) SP/MP19::EK-GUSN; (e) SP/MP42::EK-GUSN-AFVY; and (f) SP/MP19::EK-GUSN-AFVY. Key: WT, wild type; M1, Bench Mark Protein Ladder (Invitorgen); and M2, Precision Plus Protein Dual Standards (Bio-Rad).

## 4.9 Detection of MP42 and MP19 in soluble and insoluble fraction of transgenic tobacco seeds

To find out the localization of the recombinant target protein in the transgenic cells, the lines giving the highest recombinant protein expression of each construct, Phas/SP/MP42 (line 12), Phas/SP/MP42-αTIP (line 8), Phas/SP/MP42-BP80 (line 1), were used for this studied (Figure 4.8-1 and 4.8-2).

The recombinant proteins were mainly detected in the cell membrane (CM) fraction of the $MSP1_{42}$ constructs and the constructs with α-TIP (Figure 4.9-1(2); Figure 4.9-2(1)) and AFVY (Figure 4.9-2(3) gave relatively high expression. However, cell soluble (CS) fraction of $MSP1_{19}$ constructs contains more recombinant protein than the CM fraction (Figure 4.3-3).

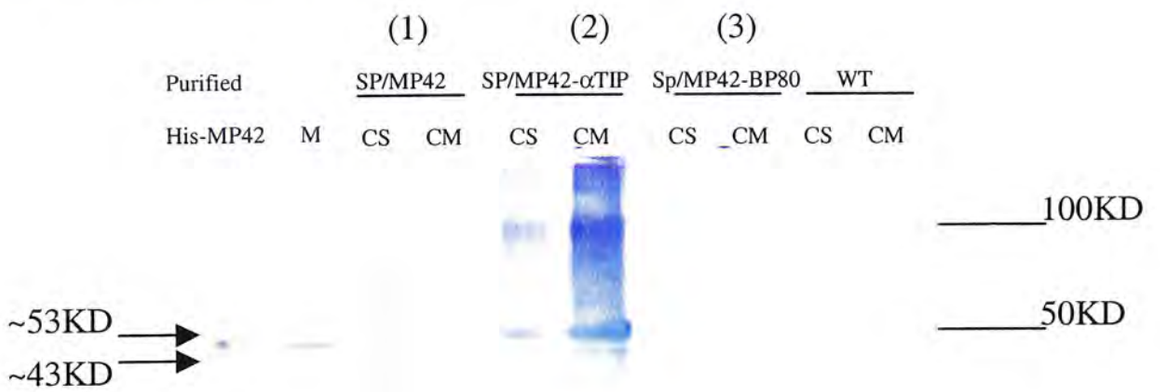



**Figure 4.9-1 Detection of MP42 in soluble and insoluble fractions, with or without targeting sequence, in transgenic tobacco seeds**

Constructs (1) Phas/ SP/MP42 (line 12); (2) Phas/SP/MP42-αTIP (line 8); and (3) Phas/SP/MP42-BP80 (line 1) were studied and as indicated in gel lanes, 1, 2 and 3, respectively. Soluble seed proteins (50$\mu$g), as CS fraction, and insoluble seed proteins (50$\mu$g), as CM fraction, were resolved in 16.5% tricine gel with β-mercaptoethanol, after boiling in dissociating buffer, blotted onto PVDF membrane, and detected using anti-$MSP1_{42}$ serum as primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as secondary antibody. Purified protein His-$MSP1_{42}$ (50$\mu$g) was included as a positive control. Key: WT, wild type and M, Precision Plus Protein Dual Standards (Bio-Rad).

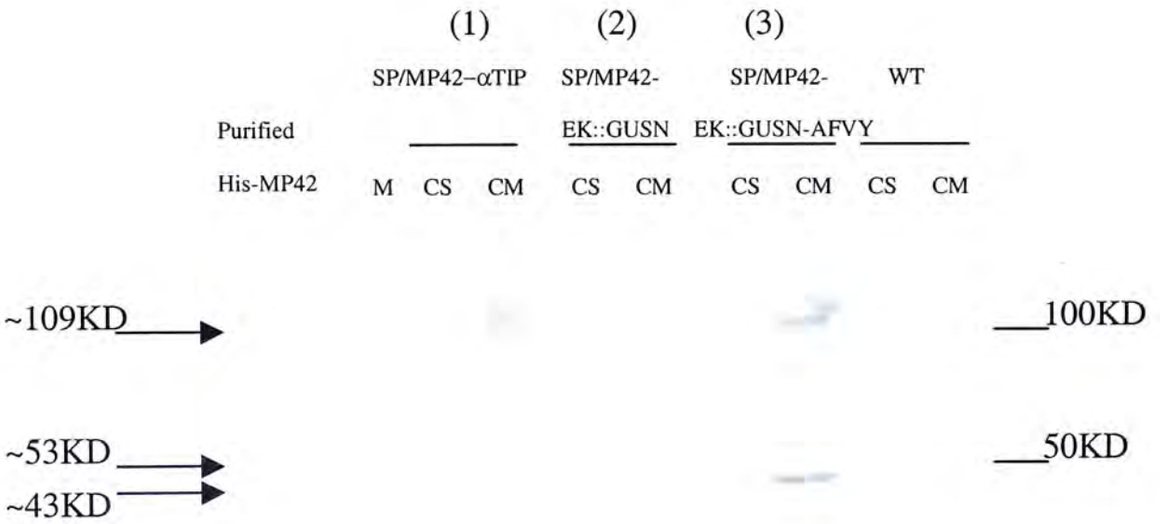



**Figure 4.9-2 Detection of MP42 in soluble and insoluble fractions, with targeting sequences and/or GUS fusion, in transgenic tobacco seeds**

Constructs (1) Phas/ SP/MP42-αTIP (line 8); (2) Phas/SP/MP42-EK::GUSN (line 7); and (3) Phas/SP/MP42-EK::GUSN-AFVY (line 15) were studied and as indicated in gel lanes 1, 2 and 3, respectively. Soluble seed proteins (50$\mu$g), as CS fraction, and insoluble seed proteins (50$\mu$g), as CM fraction, were resolved in 16.5% tricine gel with β-mercaptoethanol. After boiling in dissociation buffer, blotted onto PVDF membrane, and was detected using anti-$MSP1_{42}$ serum as primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as secondary antibody. Purified protein His-$MSP1_{42}$ (0.03$\mu$g) was included as a positive control. Key: WT, wild type and M, Precision Plus Protein Dual Standards (Bio-Rad).

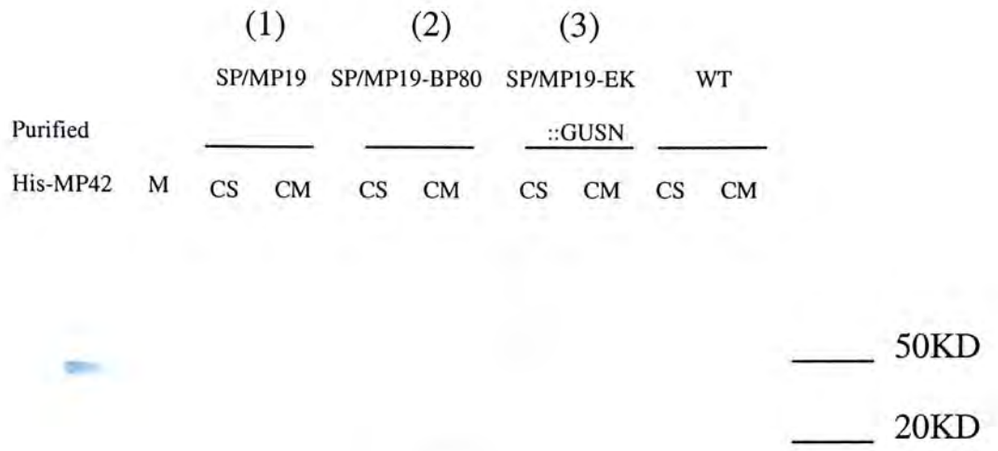



**Figure 4.9-3 Detection of MP19 in soluble and insoluble fractions, with or without targeting sequence or GUS fusion in transgenic tobacco seeds**

Constructs (1) Phas/ SP/MP19 (line 8); (2) Phas/SP/MP19-BP80 (line 25); and (3) Phas/SP/MP19-EK::GUSN (line 2) were studied and as indicated in gel lane 1, 2 and 3, repectively. Soluble seed proteins (50$\mu$g), run as CS fraction, and insoluble seed proteins (50$\mu$g), run as CM fraction, were resolved in 16.5% tricine gel with β-mercaptoethanol, after boiling with dissociation buffer, blotted onto PVDF membrane, and detected using anti-$MSP1_{42}$ serum as primary antibody and alkaline phosphatase conjugated anti-rabbit IgG as secondary antibody. Purified protein His-$MSP1_{42}$ (0.03$\mu$g) was included as a positive control. Key: WT, wild type and M, Precision Plus Protein Dual Standards (Bio-Rad).

# Chapter 5 Discussion

In this study, the significant findings are, firstly, the transcript of the previously non-transcribable native $MSP1_{42}$ cDNA can be detected after sequence optimization of the cDNA for plant expression. Secondly, by fusing the transgene with sequence for protein targeting or β-glucuronidase (GUS), production of recombinant $MSP1_{42}$ and $MSP1_{19}$ proteins were improved.

In a previous study performed by our group, the expressions of $MSP1_{42}$ in tobacco and *Arabidopsis* were not satisfactory because only trace amount or no mRNA was detected in the transgenic plants and no recombinant protein was detected by western analysis. We reasoned that the native $MSP1_{42}$ cDNA may be not stable or suitable for plant expression. The $MSP1_{42}$ cDNA was thus plant-optimized (Ng, 2001).

Before transformation of the optimized $MSP1_{42}$ cDNA into plants, transgene expression of the modified cDNA was studied by transient assay using particle bombardment (Results 4.1). In the transient assay, both modified and unmodified $MSP1_{42}$ and $MSP1_{19}$ cDNA were fused separately with a GUS reporter gene. In the fusion, the start codon (ATG) of GUS was removed, so that GUS expression (blue dot development) totally depends on the read through of the $MSP1_{42}$ and $MSP1_{19}$ cDNA

during translation. Besides, to prevent technical errors in comparing the relative intensities of blue-dot development, such as variation of incubation conditions and qualities of different batches of beans bought in different times, the relative intensities were compared among the cotyledons in each individual tests.

In the transient expression study, firstly, GUS expression was found for the modified $MSP1_{42}$ and $MSP1_{19}$ cDNA constructs, i.e. Phas/MP42 and Phas/MP19, suggesting that elimination from the native $MSP1_{42}$ cDNA the AT-rich nucleotides, the ATTTA pentamer, the AATAAA/AATAAA like-motifs, and the A+T nucleotide in the third degenerate base that are not favourable in plant system, enhances the stability of transcripts.

Secondly, the shorter MSP1 cDNA, namely $MSP1_{19}$, showed better gene expression than the longer $MSP1_{42}$, as greater number of cotyledons bombarded with the modified $MSP1_{19}$ cDNA gave blue dot development than the unmodified one.

Therefore, the transient assay demonstrated that the optimized $MSP1_{42}$ cDNA allows the expression of MSP1 protein in plant systems and the $MSP1_{19}$ cDNA will give a greater recombinant protein expression in transgenic plants.

Based on the transient assay, the modified $MSP1_{42}$ and $MSP1_{19}$ cDNA were transformed into *Arabidopsis* (Results 4.2). The expression of $MSP1_{42}$ and $MSP1_{19}$ transgenes was greatly improved as transcripts were detected by northern analysis

(Results 4.2.4). However, no recombinant protein could be detected by western analysis (Results 4.2.5). This is inconstant with the positive results from transient assay (Results 4.1).

It is possible that for some unknown reason that the $MSP1_{42}$ and $MSP1_{19}$ mRNAs were not translated in the plant system. However, it is more likely that proteins produced are unstable for accumulation, either digested by proteases or because the $MSP1_{42}$ and $MSP1_{19}$ proteins, which contain a large number of cysteine residues and disulphide bridges, may not be stable in the reducing environment of the cytosol. It has been reported that the expression of secretary proteins containing a large number of cysteine residues and disulphide bridges in cytoplasm was very low (Bosch *et al.*, 1994; Florack *et al.*, 1994). GUS is a stable protein and in fusion it may stabilize the $MSP1_{42}$ and $MSP1_{19}$ expression, as blue-dot development could be detected in the transient assay but not in the transgenic *Arabidopsis* when no GUS fusion was included in the constructs.

Therefore, sequence modification alone may not be sufficient for stable accumulation of recombinant $MSP1_{42}$ and $MSP1_{19}$ proteins in transgenic plants. Further optimization are required.

We hypothesize that by incorporating protein targeting sequences (Literature review, 2.13) or fusing GUS gene with the proteins, the expressed $MSP1_{42}$ and

$MSP1_{19}$ recombinant proteins may be stabilized and enhanced. The protein targeting sequences, α-TIP and BP-80, suggested to direct foreign proteins to the membrane of the protein storage vacuole of the tobacco seeds (Jiang and Rogers, 1998 and 1999) were used in this study; whereas RMR, which is isolated from *Arabidopsis* , may target the foreign proteins to the membrane of the protein storage vacuole.

In addition, GUS fusion may stabilize the $MSP1_{42}$ and $MSP1_{19}$ expression, it was included in making fusion constructs. Several considerations were made on incorporating the GUS gene with MSP1. Firstly, as GUS may interfer the anti-malaria function, so an enterokinase (EK) site was inserted between the MSP1 gene and the GUS gene for later removal of the GUS from the MSP1 proteins. Secondly, as the expression of $MSP1_{42}$ showed a marked improvement by adding the phaseolin targeting signal, AFVY (Lau, 2003), so this signal was also incorporated in one of the GUS fusion constructs. Thirdly, GUS at the same time can serve as a reporter gene in transient assay. When the GUS fusion constructs were tested for expression by particle bombardment before plant transformation, the expression of the $MSP1_{42}$ and $MSP1_{19}$ through fusion with GUS was observed in a short time.

By transient expression assay of the GUS fusion with $MSP1_{42}$ and $MSP1_{19}$ (Results 4.4.1), protein expression, as evident by blue-dot development, could be detected for constructs Phas/MP42-EK::GUSN and Phas/MP19-EK::GUSN, but not

for Phas/SP/MP42-EK::GUSN, Phas/SP/MP19-EK::GUSN, Phas/SP/MP42-EK::GUSN-AFVY and Phas/SP/MP19-EK::GUSN-AFVY. The unexpressed GUS gene should not be due to any mutation in the sequences of $MSP1_{42}$ cDNA, $MSP1_{19}$ cDNA, the phaseolin signal peptide, the phaseolin targeting signal (AFVY) and the EK site, as DNA sequencing was performed in the cloning steps to ensure the sequences were correct. These DNA fragments were cloned in frame, so the unexpression should not be because of frame shift. Furthermore, the GUS cDNA used in the cloning of Phas/MP42-EK::GUSN, Phas/MP19-EK::GUSN, Phas/SP/MP42-EK::GUSN and Phas/SP/MP19-EK::GUSN were excised from the same clone, Phas/MP42::GUSN, and the GUS gene could be expressed in the former two constructs but not the latter two in the transient assay (Results 4.4.1), so the unexpressed GUS in constructs Phas/SP/MP42-EK::GUSN and Phas/SP/MP19-EK::GUSN should not be because of the fidelity of the GUS gene.

By grouping the constructs into two categories, namely GUS expressed or not expressed, the unexpressed GUS constructs Phas/SP/MP42-EK::GUSN, Phas/SP/MP19-EK::GUSN, Phas/SP/MP42-EK::GUSN-AFVY and Phas/SP/MP19-EK::GUSN-AFVY share a common sequence of phaseolin signal peptide, which was not present in the constructs that allowed GUS expression. It thus suggests that the phaseolin signal peptide may be the cause of failed expression. The

presence of signal peptide had been shown to translocate GUS to the endoplasmic reticulum (ER) where the GUS was modified by glycosylation and lost its enzymatic activity (Iturriaga *et al.*, 1989). There are two potential sites in GUS for glycosylation, at position 358 to 360 and 423 to 425. By using the modified GUS reporter gene, in which one of the two potential glycosylation sites was mutated (Asn-358 to Ser), the inactivation of GUS enzymatic activity was prevented (Yan *et al.*, 1997). Therefore, mutation of the GUS gene by changing Asn-358 to Ser was done in my study and transient assay of the constructs with mutated GUS (MGUSN) was performed (Results 4.4.2).

After mutating the GUS genes in the $MSP1_{42}$ and $MSP1_{19}$ constructs containing phaseolin signal peptide, however, transient assay still showed no GUS expression as no blue dot was observed (Results 4.4.2). This may be due to several reasons. First, the $MSP1_{42}$ and $MSP1_{19}$ cDNA for some reason could not be expressed at translational level in the presence of phaseolin signal peptide. As the start codon of the GUS gene was removed, so GUS could not be expressed. Second, only one of the two potential glycosylation sites in the GUS gene was mutated, and this may not be sufficient to prevent enzymatic inactivation. Third, the recombinant protein of $MSP1_{42}$ and $MSP1_{19}$ in fusion with GUS may fold in a form which inactivates the enzymatic activity of GUS; therefore, GUS may be translated, but lost its enzymatic

activity. This can be studied by using anti-β-glucuronidase antibody in the western analysis of the transgenic tobacco seeds.

For transgenic expression of the protein targeting and GUS fusion constructs in transgenic tobacco seeds, by northern analysis, transcripts were detected in all protein-targeting and GUS fusion constructs (Results 4.7), demonstrating that the transgene expression of $MSP1_{42}$ and $MSP1_{19}$ can be greatly improved at transcriptional level after the codon usage was made more plant based.

Expression of the $MSP1_{42}$ and $MSP1_{19}$ proteins was detected by western analysis and their expression levels were found to vary according to different protein-targeting sequences or GUS fusion. Among all of the constructs, $MSP1_{42}$ with α-TIP showed the highest protein expression, amounting to 0.4% of the total seed protein. The second highest protein expression was $MSP1_{42}$ fusion with GUS and AFVY, around 0.16% of total seed protein. $MSP1_{42}$ with phaseolin signal peptide resulted in 0.1% expression level, while the rest of the constructs, ranged from 0.02% to 0.04% of the total seed protein. The RMR constructs did not give any expression at the protein level (Results 4.8). The protein expression level at 0.4% of total seed protein was 2-fold higher than the 0.2% expression levels obtained for rhG-CSF in tobacco seed (Lee, 2002)

The seed proteins extracted from $MSP1_{42}$ transgenic seeds were in denatured

forms for the western analysis because the anti-$MSP1_{42}$ rabbit serum used as primary antibody can recognize both linear and conformational epitopes on the $MSP1_{42}$ protein. After denaturation, the $MSP1_{42}$ recombinant protein could be separated and linearized. Therefore, the expressed recombinant protein could be observed as a sharp band in comparing with the purified $MSP1_{42}$ protein in western analysis. However, the proteins extracted from the $MSP1_{19}$ transgenic seeds should not be denatured by treatment with boiling and β-mercaptoethanol, because the anti-$MSP1_{42}$ rabbit serum could only recognize the conformational epitope on the C-terminal of $MSP1_{42}$, that is the $MSP1_{19}$ fragment. As a result, a complex protein pattern, possibly due to the formation of dimmers, trimmers and higher mass of $MSP1_{19}$ mainly around 40KD and 60KD, were observed in the western blotting of the $MSP1_{19}$ transgenic seeds. Therefore, the expression of the fusion constructs of $MSP1_{19}$ should amount to more than 0.02% and 0.04%, as only the intensity of the major bands was used in the quantitation (Results 4.8).

The recombinant proteins produced by the constructs $MSP1_{42}$ with α-TIP and with BP-80 were round 52 KD, indicating that the protein-targeting sequences were not cleaved off during protein synthesis and targeting. Proteins at around 110KD were detected in the $MSP1_{42}$/GUS fusion constructs Phas/SP/MP42-EK::GUSN and Phas/SP/MP42-EK::GUSN-AFVY, suggesting that the recombinant protein consists

of $MSP1_{42}$ and GUS. For the AFVY construct, a major protein band at around 42KD was detected in the western analysis, suggesting that the protein was $MSP1_{42}$, resulting from processing. In order to further study the subcellular location of the recombinant proteins in plant cell, confocal study need to carry out to subcellularly locate the proteins.

For the $MSP1_{19}$ constructs, as the protein used for western blotting was not denatured, recombinant proteins of various sizes, due to protein association, were detected. These immuno-signals all came from the $MSP1_{19}$ protein as no signal was detected from the wild type. Thus, diffused bands were generally observed for the $MSP1_{19}$ constructs. For the BP-80 construct, two relatively sharper and clearer bands were detected at around 19KD and 22KD. The 19KD was the size of the native form $MSP1_{19}$ while the 22KD protein may be the glycosylated form of the 19KD protein. This could be confirmed by performing experiment on deglycosylation.

Signal peptide plays a critical role in expressing recombinant proteins in plants. For example, firstly, for those constructs without the phaseolin signal peptide, no protein was detected in the transgenic *Arabidopsis* (Results 4.2.5) although mRNA was detected (Results 4.2.4). Secondly, the GUS fusion constructs without phaseolin signal peptide were also not expressed at protein level (Results 4.8), although transcript was detected (Results 4.7). Once phaseolin signal peptide was incorporated,

either in the protein-targeting or GUS fusion constructs, the transgene could be expressed at protein level (Results 4.7). Moreover, the protein expression level of the $MSP1_{42}$ and $MSP1_{19}$ constructs containing only the phaseolin signal peptide, at around 0.1% of total seed protein, was found to be higher than most of the constructs fused with protein-targeting sequences or GUS, generally at around 0.04% of total seed protein. It is possible that the $MSP1_{42}$ and $MSP1_{19}$ proteins without a signal peptide will end up in the cytosol where they would be degraded. For the lower protein expression by the BP-80 construct in comparing with constructs containing only phaseolin signal peptide, it may due to the limited amount of BP-80 membrane anchoring domain on the membrane of protein storage vacuole to accept the targeting protein.

For the constructs fused with RMR, no protein was detected. The RMR gene could be expressed in tobacco culture cell lines in the studies by other group. The reason for this difference is unclear at this time.

Besides, the protein expression levels may be proportionally related to the copy numbers which were usually ranged from one to three in tobacco transformation and the RNA expression levels. For example, SP/MP42, SP/MP19, SP/MP19-BP80 and SP/MP19::EKGUSN-AFVY gave higher protein expression levels when the integration copy numbers increased. However, the protein expression levels may also

vary amount the plants carrying the same copy number of target genes of the same constructs because of the differences in the integration positions of target gene in the tobacco plant genome.

The recombinant proteins were generally found in the cell membrane fraction. This may be due to the presence of the α-TIP and BP-80 targeting sequences which direct the protein to the membrane of the proteins storage vacuole.

As a whole, by the use of protein-targeting sequences, the expression of $MSP1_{42}$ and $MSP1_{19}$ can be enhanced up to 0.4% of total seed protein. Also, by fusion with GUS or with just the phaseolin signal peptide, $MSP1_{42}$ and $MSP1_{19}$ can also be expressed at protein level (up to 0.1%). Thus by using an integrated approach combining codon optimization, target protein stabilization by fusion with a stable and highly expressed protein, and protein targeting for vacuolar storage, the expression of target protein can be significantly enhanced for recombinant production and through this strategy, $MSP1_{42}$ and $MSP1_{19}$ can be expressed in transgenic seeds with significant amounts, an improvement step in our effort to develop an inexpensive malaria vaccine.

# Chapter 6 Conclusion

Optimization of cDNA sequence for plant expression is important, but may be only limited to transcriptional level. Further optimization, such as incorporating signal peptide, protein-targeting signal and fusion protein may be necessary in enhancing protein expression and accumulation.

Through integrating these optimizations, malaria surface antigen MSP1, previously could not be expressed at mRNA level, now could be expressed at protein level, up to 0.4% as total seed protein. Further work, such as confocal microscopy to study the subcellular localization of the recombinant protein in plant cells and to test the antigenicity of the plant derived malaria surface antigens ($MSP1_{42}$ and $MSP1_{19}$) are important in this line of research and development.

# References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