

Transformation of Potatoes with the Potato Leafroll Virus Coat Protein Gene.

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PREFACE

The experimental work described in this thesis was carried out at the ARC-Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria, South Africa under the supervision of Dr W.A. Cress, Dr J.T. Burger and Professor J. van Staden.

This study represents original work and has not otherwise been submitted in any form for any degree or diploma to any other University.



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ABSTRACT

Potato leafroll virus (PLRV) is one of the most destructive potato viruses in South Africa. In order to establish resistance against PLRV in commercial potato cultivars, the coat protein (CP) gene of the virus was previously isolated, cloned and subcloned into the plant expression vector pBI121 in both the sense and antisense orientations (BURGER, unpublished results). The pBI121 constructs containing the PLRV-CP gene were subsequently transferred to *Agrobacterium tumefaciens* LBA 4404 in a triparental mating process with the helper plasmid pRK2013. Two *A. tumefaciens*- mediated transformation methods for potatoes were investigated, viz. vacuum infiltration and leaf disk transformation. In addition, optimal transformation and regeneration conditions were identified for potato cultivars Late Harvest and BP₁. In total, 27 transgenic potato lines containing the PLRV-CP, β -glucoronidase (GUS) and *nptII* (neomycin phosphotransferase II) transgenes were generated under kanamycin selection. Transgenic plants grown in the glasshouse appeared to be phenotypically normal, and no differences in ploidy level in comparison to non-transformed plants could be established.

Stable transgene insertion into the genome of the transgenic plants was verified using PCR and Southern blot analysis. Expression of the GUS transgene was investigated using a fluorometric assay (JEFFERSON *et al.* 1987), and it was found that orientation of the inserted PLRV-CP gene upstream from the GUS gene had a direct influence on the levels of GUS expression. The expression of the PLRV-CP gene was analysed using DAS-ELISA and immunoblot detection. Coat protein could not be detected in either assay. RNA dot blots were used successfully to show PLRV-CP expression in transgenic potato plants at the mRNA level.

ABBREVIATIONS

ARC	Agricultural Research Council
ARC-Roodeplaat	ARC-Roodeplaat Vegetable and Ornamental Plant Institute
bp	base pairs
BA	6-benzylaminopurine
BSA	bovine serum albumin
CP	coat protein
CPMR	coat protein-mediated resistance
DIG	digoxigenin
ELISA	enzyme linked immuno-sorbent assay
g	gram
GA ₃	gibberellic acid
GUS	β-Glucuronidase
IAA	3-indolylacetic acid
kD	kilo Dalton
l	litre
LH	Late Harvest
μg	microgram
μl	microlitre
mA	milliamperes
mg	milligram
ml	millilitre
MU	methyl umbelliferone

MUG	4-methyl umbelliferyl β -D glucoronide
NAA	naphthalene acetic acid
ng	nanogram
PCR	polymerase chain reaction
PLRV	potato leaf roll virus
rpm	revolutions per minute
U	unit
V	volt
$^{\circ}\text{C}$	degrees Celsius

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CHAPTER ONE.

GENERAL INTRODUCTION AND LITERATURE REVIEW.

1.1. Cultivation of Potatoes.

The cultivated potato (*Solanum tuberosum* L.) was imported into Europe from South America in the 16th century (HUISMAN *et al.* 1992). Potatoes were subsequently accepted as a major food source and were cultivated extensively throughout Europe. However, cultivated potatoes were derived from wild species in South America, resulting in a very narrow genetic base associated with a lower disease resistance. After the blight epidemics in 1845-1846, genetic transfer was initiated by the introduction of wild *Solanum* species into breeding programmes (VISSER 1989). Today, broadening the genetic diversity of potatoes proceeds in a planned and collaborative manner (INTERNATIONAL POTATO CENTRE 1984).

In South Africa, the potato is the largest cultivated vegetable crop, and during the period July 1993 to June 1994, 1.31 million tonnes of potatoes were produced (ABSTRACT OF AGRICULTURAL STATISTICS 1995). This corresponds to a gross value of R691 629 000 (ABSTRACT OF AGRICULTURAL STATISTICS 1995). Although the potato was traditionally an European crop, it is now grown extensively in tropical and sub-tropical regions of the world. Developing countries are expanding potato production for several reasons, the most important of these is that a potato crop produces more edible protein per hectare per unit time than practically any other crop (INTERNATIONAL POTATO CENTRE 1984). However, virus degradation of tubers is a major constraint

on potato production, especially in third world countries where virus-free production programmes may be inadequate and where a warm climate favours the multiplication of aphids and other virus vectors (BRANDOLINI *et al.* 1992). As the emphasis in South African agriculture shifts towards addressing the needs of the small scale farmer, the importance of cultivating disease-free potatoes in South Africa can only be re-emphasised. Three potato cultivars (BP₁, Vanderplank and Up-to-date) have been accepted as well adapted to the harsh South African environment, and account for 90% of potatoes presently cultivated in South Africa (POTATO BOARD INFORMATION BROCHURE 1992).

1.2. Potato Leafroll Virus.

The most widespread and destructive viral diseases of potato are potato leafroll virus (PLRV), potato virus X (PVX) and potato virus Y (PVY) (INTERNATIONAL POTATO CENTRE 1982). PLRV is found commonly in all countries where potatoes are grown, either in single virus field infections or in mixed infections with PVX and/or PVY (BEEMSTER & ROZENDAAL 1972; INTERNATIONAL POTATO CENTRE 1982). In terms of production, PLRV infection of a plant causes it to produce smaller tubers, and yield losses can amount to 90% (INTERNATIONAL POTATO CENTRE 1982).

Symptoms of PLRV infection depend on viral strain, potato cultivar and environmental conditions. Primary symptoms, caused by current-season infection, appear mainly in young leaves at the top of the plant. Leaves tend to stand upright, are pale yellow and are often rolled. With many cultivars these leaves are tinged purple or red. Symptoms

may not be seen in cases where late infection has occurred. Tubers of highly sensitive cultivars develop necrosis of the vascular system, which is also referred to as net necrosis. Symptoms of secondary infection, which develop in plants grown from infected tubers, are more serious for the whole plant, although they are less pronounced at the top of the plant. Plants are stunted and erect, with leathery, stiff older leaves often tinged purple on the abaxial surface. Younger leaves are pale (BEEMSTER & ROZENDAAL 1972; INTERNATIONAL POTATO CENTRE 1982).

PLRV is transmitted by aphids, principally *Myzus persicae* (INTERNATIONAL POTATO CENTRE 1982). PLRV is confined to the phloem tissue of the plant, and in ultrastructural studies the greatest number of virus particles were found in the cytoplasm of the phloem parenchyma and companion cells (HARRISON 1984). The virus induces necrosis in the phloem tissue, which in turn causes thickening of the infected cells. Callose is deposited in sieve tube elements which obstructs the transport of assimilates. Consequently the external symptoms such as leaf rolling and purple colour development become apparent (HARRISON 1984; VAN DEN HEUVEL 1991).

PLRV is a member of the luteovirus group (HARRISON 1984; VAN DEN HEUVEL 1991). It has a single positive-strand RNA genome and an isometrical particle morphology (ROWHANI & STACE-SMITH 1979). PLRV particles have a diameter of about 23 nm (PETERS 1967 cited in VAN DEN HEUVEL 1991) and consist of one main protein species, the coat protein, with a molecular mass of approximately 26 kD as determined by SDS-polyacrylamide gel electrophoresis (ROWHANI & STACE-SMITH 1979). A determination of the complete nucleotide sequence of the PLRV

genome revealed that it consists of 5987 nucleotides (MAYO *et al.* 1989). It consists of 6 open reading frames (ORF), arranged in two gene clusters separated by a small non-coding region (MAYO *et al.* 1989) (Fig.1.1). Open reading frame 4 encodes the coat protein and was shown to be expressed via a 2.3 kb subgenomic RNA (TACKE *et al.* 1990).

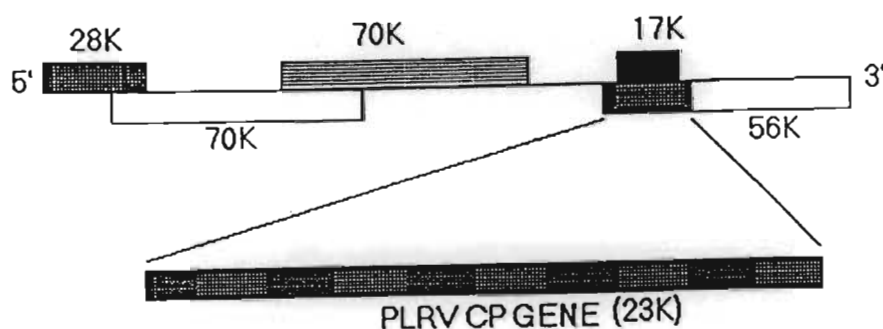


Fig.1.1. The genome organisation of PLRV (MAYO *et al.* 1989).

PLRV can be controlled by selecting healthy plants and removing plants with secondary symptoms during cultivation. Growing potatoes in areas where aphids are few also helps to control PLRV. In addition, spraying with systemic insecticides may help to control aphid spread. Although the virus can be eliminated from tubers by heat treatment, planting resistant cultivars is most probably the most efficient manner of controlling the disease (HARRISON 1984; INTERNATIONAL POTATO CENTRE 1982).

1.3. Introducing PLRV resistance into potatoes.

Conventional Potato Breeding.

Producing cultivars with resistance to major plant viruses has traditionally been a goal of many potato breeding programmes. Breeders try to incorporate one of three types of resistance in new varieties: resistance to infection, in which only a small percentage of plants in a field become infected; hypersensitivity, where the potato variety is so sensitive to the virus that the virus is restricted to the site of infection and no systemic infection occurs; or extreme resistance, equivalent to immunity (WIERSEMA 1972). In the case of PLRV, breeding for resistance to infection has been used.

Unfortunately, not much is known about PLRV resistance, and it appears that the available resistance is multigenic and not dominant (ROSS 1986 cited in BRANDOLINI *et al.* 1992). Usually, a resistant clone can be generated by crossing unrelated resistant parents, if they are sexually compatible, and this resistance can be increased by intercrossing resistant types. Unfortunately, in the past these resistant clones have been of poor quality (WIERSEMA 1972). This can be improved by outcrossing with high-quality varieties. However, quality is also determined by many genes, so the breeder must combine two polygenic traits. This is a slow, time consuming process which requires large breeding populations (HUISMAN *et al.* 1992).

An attractive commercial goal for potato breeding is the ability to isolate and introduce in a single step, viral resistance into many elite potato varieties without compromising

existing agronomic characteristics (WILSON 1993). Molecular breeding techniques offer this possibility, and a great deal of research world-wide has been implemented for potatoes (HUISMAN *et al.* 1992).

Molecular Breeding.

SANFORD & JOHNSTON (1985) first proposed creating virus-resistant crops by genetically engineering them to express part of a viral genome or a virus-associated sequence. This idea evolved from the practice, referred to as cross-protection, of using mild, symptomless or attenuated viral strains to protect field crops such as tomato, apple or citrus against closely related but very pathogenic viral strains (SEQUEIRA 1984 cited in WILSON 1993). Over the last ten years, many different approaches based on Sanford and Johnston's idea have been proposed, with varying degrees of success. These include coat protein-mediated resistance (CPMR), replicase-mediated resistance, satellite RNAs, antisense RNA, ribozymes, antibodies, defective interfering RNAs, antiviral and virus movement proteins (VAN DEN ELZEN *et al.* 1989; BEACHY *et al.* 1990; HUISMAN *et al.* 1992; WILSON 1993; ZAITLIN 1993). The approach implemented in the present study is CPMR.

Coat protein-mediated resistance.

The first report of coat protein-mediated resistance (CPMR) was published in 1986 by POWELL-ABEL and co-workers. In this study, the coat protein (CP) gene of tobacco mosaic virus (TMV) was transformed into tobacco cells using an attenuated strain of *Agrobacterium tumefaciens*. Regenerated plants expressed TMV mRNA and CP.

Transgenic plants inoculated with TMV showed delayed disease development. Subsequently, the CPMR concept has been widely applied to other viruses and plant families. Different levels of resistance have been obtained, ranging from the development of fewer necrotic lesions to a reduced rate of systemic disease spread and a reduced accumulation of virus particles in infected transgenic plants compared to control plants (VAN DEN ELZEN *et al.* 1989). Since 1986, there has been an explosion of work on CPMR. In a 1990 review, BEACHY and co-workers listed eight virus/plant examples. More recently, ZAITLIN (1993) reported a count of CPMR studies involving 22 viruses from 14 virus groups. This was linked to a wide variety of dicotyledonous plant species, and it is presumed that as soon as the technical difficulties associated with monocotyledonous transformation and regeneration protocols are resolved, CPMR for these crops will also be possible (WILSON 1993).

The mechanism of CPMR is not fully understood, but many models have been proposed. Understanding of CPMR is made more difficult by the fact that each study has unique aspects. These aspects include the precise sequence of the viral CP gene construct used, the positional effects of random transgene insertion in the plant, the transgene copy number, the site of virus replication in the plant, the secondary effects of the transformation process on the plant cells and the general stress responses of the host to the disease (WILSON 1993). In early CPMR studies it appeared that the amount of functional CP produced in the transgenic plants was directly correlated to the efficacy of CPMR, especially as high concentrations of virus inoculum overcame CPMR (POWELL ABEL *et al.* 1986; LOESCH-FRIES *et al.* 1987; VAN DUN *et al.* 1987). Originally it was believed that transgenic coat protein interfered with uncoating of the

infecting virus, for example it was shown that in tobacco protoplasts expressing the CP gene from TMV, uncoating of the viral particles and thus viral replication, was inhibited (WU *et al.* 1990). However, it was shown that resistance was obtained in some cases when naked RNA was used as inoculum, for example in a study for potato virus S (MACKENZIE & TREMAINE 1990). Furthermore, CPMR studies have been implemented where intentionally truncated, antisense or non-expressing CP genes have been transformed into plants, for example cucumber mosaic virus (CUOZZO *et al.* 1988), PVX (HEMENWAY *et al.* 1988), PVY (FARINELLI & MALNOE 1993; SILVA-ROSALES *et al.* 1994) and PLRV (KAWCHUK *et al.* 1991). In all cases various degrees of protection against the relevant virus were obtained. It thus appears that in some cases a functional protein is not required for resistance, but that some form of direct RNA-RNA interaction between the transgene mRNA and the infecting virus provides the resistance (WILSON 1993).

It has also been shown that plants expressing the CP gene of one virus exhibit "broad spectrum" resistance to infection by another virus. For example, STARK & BEACHY (1989) transformed the CP gene from soybean mosaic potyvirus into tobacco, and the resulting plants were resistant to infection by two serologically unrelated potyviruses, PVY and tobacco etch virus. However, the plants were not resistant to infection by TMV, the type member of the tobamovirus group. STARK & BEACHY (1989) suggested that CPMR requires structural as well as sequence homology between the protecting and the challenging virus CP.

It appears that CPMR is also involved with the interference of viral spread in the

infected plant and with later events in the virus replication cycle (WILSON 1993). WISNIEWSKI and co-workers (1990) studied the systemic spread of TMV in CP transformed (CP+) and non-transformed (CP-) tobacco plants. These authors showed that viral spread in the CP+ plants occurred essentially as in CP- plants but at a reduced rate. Furthermore, they found that when stem tissue with a leaf attached from a CP+ plant was grafted onto a CP- plant, the systemic spread of the virus was prevented. These findings indicated that overall resistance to TMV is due to prevention of the systemic spread of the virus in plants transformed with the CP gene and to protection at the initial infection stage. In a related study, CLARK *et al.* (1990) used tissue-specific promoters to investigate CPMR to TMV. Their findings indicated that the level of CP in epidermal cells is significant for protection to TMV in mechanically inoculated tobacco plants.

Engineering CPMR to PLRV in potatoes.

The approach implemented in the present study is the introduction of coat protein-mediated resistance to PLRV in South African potato cultivars. The CP gene from PLRV has been transformed previously into various potato cultivars, namely Russet Burbank (KAWCHUK *et al.* 1990; 1991), Désirée (VAN DER WILK *et al.* 1991), Pentland Squire and various lines from the Scottish Crop Research Institute breeding programme (BARKER *et al.* 1992; 1994). CPMR to PLRV does not give the plant immunity to the virus, but provides a degree of resistance to virus replication. In some cases the virus concentration in CP-transgenic plants was only 1% of that reached in susceptible, control plants (KAWCHUK *et al.* 1991; BARKER *et al.* 1994), as

determined by ELISA analysis. Resistance to PLRV was not directly proportional to the amount of CP produced in transgenic plants. KAWCHUK *et al.* (1990) were able to detect PLRV CP in most transgenic lines, but BARKER *et al.* (1992) only detected CP sporadically and VAN DER WILK *et al.* (1991) did not detect CP at all. The probable conclusion from these results is that protection against PLRV is mediated by CP gene transcripts in the mRNA form, rather than the protein translated from these transcripts. This hypothesis is further supported by the observation that resistance can be conferred by transformation with antisense PLRV CP sequences (KAWCHUK *et al.* 1991; VAN DER WILK *et al.* 1991).

Furthermore, enhanced resistance to PLRV multiplication in potatoes has been shown by combining the effects of host genes and transgenes (BARKER *et al.* 1994). In this study, potato clones which had showed host gene-mediated resistance to PLRV were transformed with the PLRV CP gene. The effects of the host derived resistance genes and the CP gene appear to be additive as these transgenic lines showed greater resistance to PLRV than either the non-transgenic, resistant clones or the transgenic susceptible clones.

1.4. Transformation and regeneration of potatoes.

The relative difficulty involved with breeding makes the potato one of the prime targets for improvement using applied plant molecular genetics (VAYDA & BELKNAP 1992). A very important step in the creation of better potato lines is the process in which the novel gene is transferred into the plant.

Practical methods of plant transformation can broadly be divided into two categories, viz. indirect and direct transfer (SCHAFF 1991). *Agrobacterium*-mediated gene transfer is considered to be an indirect method as the gene of interest has first to be transferred to the bacterium before transformation of plant cells can take place. Direct gene transfer methods occur when the foreign DNA is delivered directly into the plant cell. The direct approach is concerned either with converting the plant cells to protoplasts, and then stimulating fusion by electroporation or chemical treatment (calcium phosphate or polyethylene glycol), or by subjecting intact plant tissue to a shower of high velocity particles coated with the DNA. Another method involves microinjection of the DNA directly into the plant nucleus (SCHAFF 1991). By far the most popular method for transforming dicotyledonous crops, including potato, is using *Agrobacterium tumefaciens* (VAYDA & BELKNAP 1992).

Agrobacterium tumefaciens.

A. tumefaciens, a gram-negative bacterium, is the causal organism of the widespread crown gall disease of plants (SMITH & TOWNSEND 1907 cited in HOOYKAAS & SCHILPEROORT 1992). Development of crown galls is dependent on the genetic transfer from *A. tumefaciens* to the plant cells of a section of DNA (the T-DNA) present on a large extrachromosomal plasmid, called the Ti (tumour inducing) plasmid (VAN LAREBEKE *et al.* 1975). Furthermore, Southern blotting of an octopine Ti plasmid has shown that two segments of the Ti plasmid are independently transferred to plant cells, and integrated into the plant DNA during tumour induction. One segment, the left transferred DNA (T_L-DNA) was oncogenic to plant cells, while the other, the right transferred DNA (T_R-DNA), was not (BEVAN *et al.* 1983). Sequencing of these regions

showed that the T-DNA is surrounded by a conserved 24 bp direct repeat, thought to be a recognition signal for the transfer of the T-DNA found between these regions. It was also found that the copy number of the T-DNA in transformed plant lines is usually low. Through mutagenesis studies, it was established that T-DNA genes are involved in the production of a variety of opines and phytohormones (OOMS *et al.* 1981). In this way, wild type *A. tumefaciens* is capable of genetically commandeering plant cells to produce compounds that the bacteria can uniquely use as a carbon/nitrogen source.

Genetic colonisation of plant cells by *A. tumefaciens* and subsequent crown gall development proceeds in a set manner (HOOYKAAS & SCHILPEROORT 1992). Firstly, the plant cells must be wounded in order to allow for entrance of the bacterium and to make available compounds that induce its virulence system. The bacteria multiply in the wound sap and attach to the plant cell walls. Subsequently, the T-DNA is transferred and expressed even before integration into the plant nucleus. After integration, T-DNA expression is maintained at a stable level, depending on the site of integration. It has been suggested that T-DNA integrates preferentially in potentially transcriptionally active areas in the plant nucleus (HOOYKAAS & SCHILPEROORT 1992).

Attachment of *A. tumefaciens* to plant cell walls is dependent on the products of two genes present on the bacterial chromosome viz. *chvA* and *chvB* (DOUGLAS *et al.* 1985). The *chvB* gene codes for a 235 kDa protein involved in the formation of a cyclic β -1,2 glucan, while there is evidence that the *chvA* gene codes for a transport protein located in the bacterial inner membrane necessary for transport of the β -1,2 glucan into the

periplasm. Another 24 genes are involved in tumourigenity and are present in the *vir* region of the octopine Ti plasmid. The genes are present in eight operons called *virA* to *virH*, and except for *virA* and *virG*, are not usually expressed during normal growth (HOOYKAAS & SCHILPEROORT 1992). The virulence system becomes activated when the bacteria are present near wounded plant cells and sense plant cell exudates. STACHEL *et al.* (1985) identified these factors as plant phenolics, in particular acetosyringone, α -hydroxyacetosyringone and various lignin precursors.

The *vir* system has been reviewed by HOOYKAAS & SHILPEROORT (1992) and ZUPAN & ZAMBRYSKI (1995). The *virA*-protein, which is present in the bacterial inner membrane, senses and becomes activated by phenolic compounds and in turn activates the *virG*-protein, which is a DNA-binding activator protein. In this way the remaining *vir* genes become activated (Fig.1.2 Step 1). Two proteins, *virD1* and *virD2* encoded by the *virD* operon, together determine endonuclease activity capable of recognising and nicking the T-DNA border sequences at a precise location. It is probable that the nick site acts as a starting point for DNA synthesis in the 5' to 3' direction. Single stranded (ss) T-DNA strands will then be released by displacement (Fig.1.2 Step 2). It is believed that this process is evolutionarily related to other bacterial systems capable of producing ss-DNA, for example in conjugation. The right border is more efficient as a starting point for DNA synthesis as there is an enhancer present adjacent to the right sequence. The product of the gene *virC1* binds to this enhancer. Furthermore, the *virD2* protein remains covalently attached to the 5' end of the ss T-DNA, making this terminus less vulnerable to attack by exonucleases and ensuring that the 5' side is the leading end. The method in which the T-DNA is

transferred into the plant cell is still unknown, but it may be that the virE2 protein is involved as it is capable of binding ss-DNA, thus coating the T-DNA strand (Fig.1.2 Step 3). Furthermore, binding of virE2 protein unfolds and extends the ss DNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. Binding of the virD2 and virE2 proteins to the ss T-DNA is termed the T-complex.

Subsequently, the T-complex must exit the bacterial cell (Fig.1.2 Step 4), passing through the bacterial inner and outer membranes and bacterial cell wall and entering the plant cell through the plant cell wall and membrane (Fig.1.2 Step 5). However, very little is known about these processes. The products of the *virB* operon are essential for virulence and as they are found in the membrane, it has been suggested that they may form a structure through which T-DNA is delivered into a plant cell. Once inside the plant cell, the T-complex targets to the plant cell nucleus and crosses the nuclear membrane (Fig.1.2 Step 6). It is thought that the virD2 and virE2 proteins are both involved in mediating this process, as both have homology to a nuclear localization signal (NLS). Furthermore, it has been shown that some plant cells produce a NLS-binding protein, also thought to be involved in nuclear uptake. Integration of the T-DNA into the plant chromosome has yet to be characterized (Fig.1.2 Step 7).

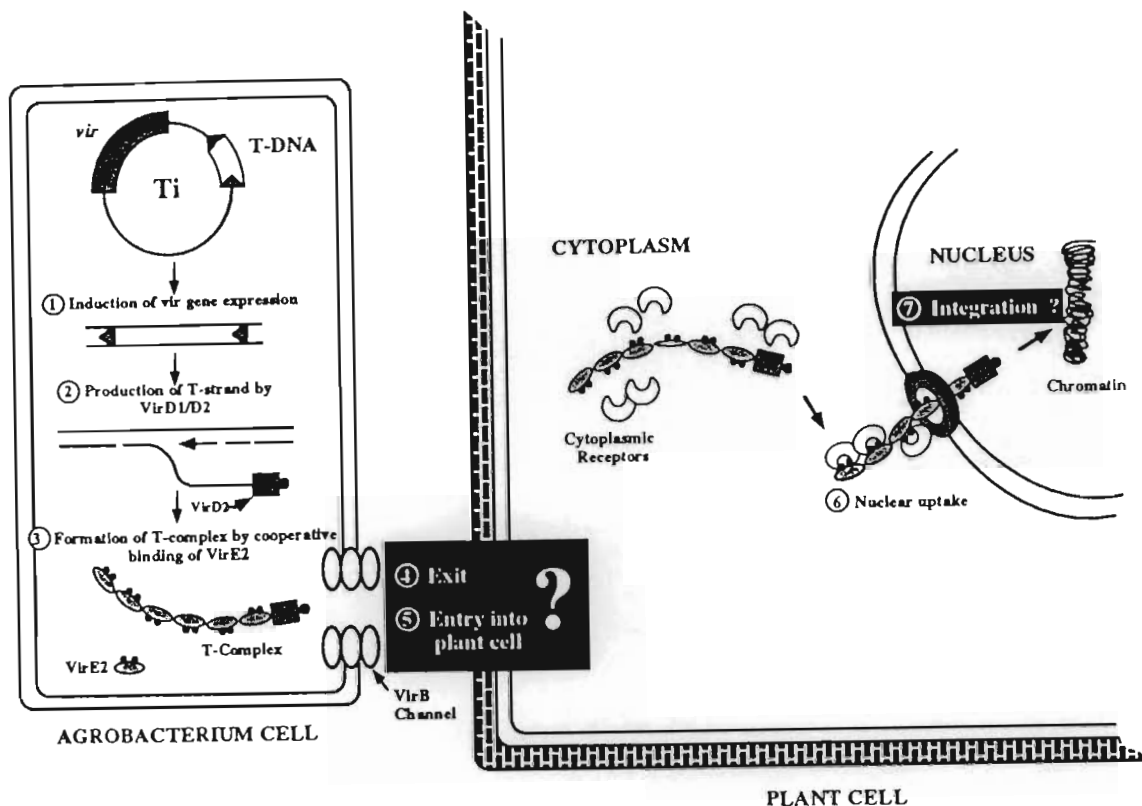


Fig.1.2. Basic steps in the transformation of plant cells by *A. tumefaciens* (ZUPAN & ZAMBRYSKI 1995). Individual steps are discussed in more detail in the text.

As it was firmly established that no physical linkage between the T-region and the rest of the Ti plasmid was necessary for T-DNA transfer to occur, it became possible to manipulate the Ti plasmid for use in plant genetic engineering (HOOYKAAS & SCHILPEROORT 1992). Two types of expression vectors have been developed for use in plant transformation studies, viz. co-integrative and binary systems (ARMITAGE *et*

al. 1988). In the co-integrative system, new genes are introduced between artificial T-DNA borders already present on the Ti plasmid via homologous recombination (ZAMBRYSKI *et al.* 1983). In the binary system, the new genes are cloned into plasmids between artificial T-DNA borders, which are subsequently introduced into an *A. tumefaciens* strain already harbouring a Ti plasmid with an intact *vir* region but lacking T-DNA (HOEKEMA *et al.* 1983). The recombinant plant expression vector is transferred from *E. coli* into *A. tumefaciens* via a triparental mating process. This process is mediated by conjugation "helper" plasmids, specific for either system, which provide mobilisation (*mob*) and transfer (*tra*) functions in *trans*. The *mob* and *tra* gene products act on the origin of transfer (*oriT*) and activation site (*bom*) contained in the plant expression vector, and this plasmid is subsequently transferred into *A. tumefaciens* (ARMITAGE 1988).

Many plant expression vectors for use in *Agrobacterium tumefaciens*-mediated transformations have been created and are available commercially. These vectors contain a bacterial origin of replication and a bacterial selectable marker so that the vector can be manipulated in bacteria. Furthermore, the vector contains a plant selectable marker to allow for selection of transformed cells, and a multiple cloning site which contains unique restriction enzyme recognition sequences, often flanked on the 5' side by a plant promoter (commonly the cauliflower mosaic virus 35S promoter) and on the 3' side by a plant polyadenylation signal sequence (SCHAFF 1991). Selectable marker genes can be *npIII*, *bar*, *hpt*, *uidA* which code respectively for kanamycin resistance, phosphinothricin resistance, hygromycin resistance and β -glucuronidase (HOOYKAAS & SCHILPEROOT 1992). β -Glucuronidase (GUS) has become a popular plant reporter

gene and is used widely in plant transformation studies (DE BLOCK 1993). GUS can be measured quantitatively using umbelliferyl derivatives, releasing umbelliferone after enzyme activity which can be measured fluorometrically. Alternatively, histochemical staining for the reporter enzyme can be done using 5-bromo-4-chloro-3-indolyl derivatives, which release a compound after enzymatic activity that is quickly converted into blue pigmentation by oxygen (JEFFERSON *et al.* 1987).

The plant expression vector used in the present study is the plasmid pBI121. This plasmid contains downstream from the right T-DNA border, the gene encoding neomycin phosphotransferase II (*nptII*), which gives the transformed plant cells resistance to the antibiotic kanamycin, driven by the promoter of the nopaline synthetase (NOS) gene from a wild type *A. tumefaciens* strain and terminated by the polyadenylation signal of the NOS gene. Downstream from this is the cauliflower mosaic virus 35S (CaMV35S) promoter cloned into the polylinker site upstream from the *uidA* or GUS gene, which is followed by the NOS terminator and the left T-DNA border.

Plant transformation by A. tumefaciens.

Apart from a historical reason (*Agrobacterium* was the first DNA delivery system available), this system has a variety of advantages over direct DNA transformation methods in that it is simple, efficient and inexpensive. In addition, plants are produced with a limited number of transgenes and insertion is usually a precise event, with the DNA between the two defined border sequences being completely and exclusively transferred (DE BLOCK 1993).

Figure 1.3 is a representation of the scheme implemented for *A. tumefaciens*- mediated transformation of plant tissue (adapted from DE BLOCK 1993). In this scheme the tissue culture of the transformed cells and their further development is very important. An explant (leaf, hypocotyl, stem, root or tuber disk) is cut from an *in vitro* plant or seedling, during which time wounding sites on the plant tissue are produced. A preculture is performed for one to several days on callus inducing medium. A feeder layer, consisting of suspension culture cells, may be used during the preculture period. Explants are subsequently inoculated with triparentally mated *A. tumefaciens* cells. Explants and *A. tumefaciens* undergo a period of co-cultivation during which gene transfer takes place, and where acetosyringone may be introduced to induce expression of the *vir* genes. After this period, explants are transferred to callus or shoot inducing medium which contains a selective agent (kanamycin, phosphinothricin or hygromycin) which kills or inhibits the growth of untransformed cells, and an antibiotic (carbenicillin or cefotaxime) which eliminates *A. tumefaciens* from the culture. Callus produced from the explants may still be chimeric; consisting of transformed and non-transformed cells, and so shoots regenerated from these cells may be transformed, chimeric or non-transformed (escapes). The transgenic shoots are multiplied and rooted *in vitro* before being hardened off for growth in the glasshouse. Transgenic plants are subsequently screened for phenotypic abnormalities and subjected to molecular assays to confirm stable transgene insertion, to determine copy numbers of inserted transgenes and evaluate transgene expression. Where applicable, seeds are produced by self crossing and transgene expression is studied in subsequent generations. Successful transformation

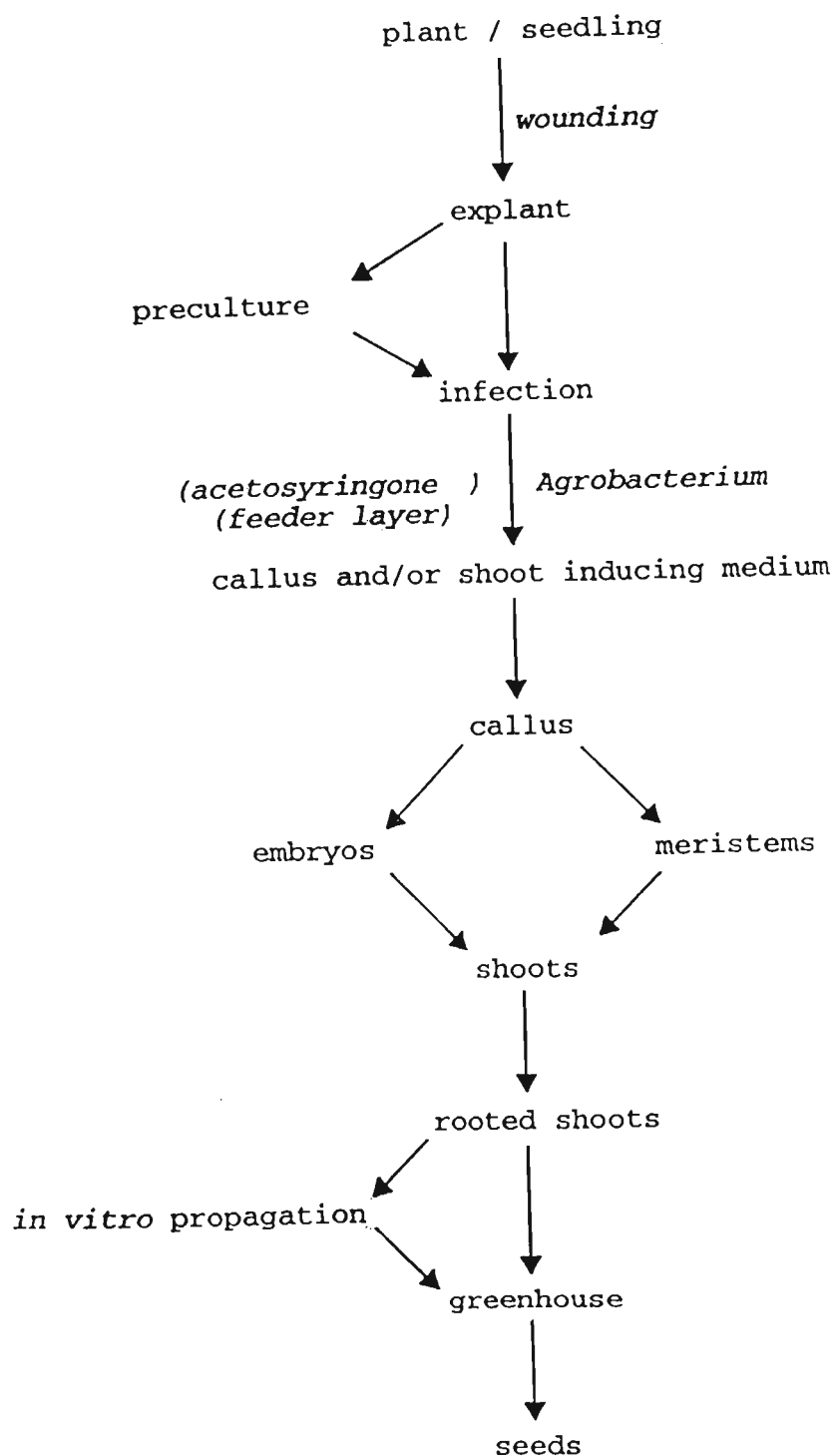


Fig.1.3. Schematic diagram of the steps involved in *Agrobacterium tumefaciens*-mediated transformation of explants (adapted from DE BLOCK 1993).

using this scheme is dependent on the susceptibility of the explants to *A. tumefaciens*, the ability to select for transformed cells *in vitro* and the regeneration potential of the selected cells (DE BLOCK 1993).

Recently a new method for *in situ* transformation by *A. tumefaciens* has been reported (BECHTOLD *et al.* 1993). This approach is based upon vacuum infiltration of a suspension of *A. tumefaciens* cells into *Arabidopsis* plants, with the idea of inserting the T-DNA region into the nucleus of the reproductive cells, so that progeny plants will contain the transgenes of interest. Upon characterization these plants were found to have transformed and untransformed sectors. An average of five transgenic plants per inoculated plant was obtained from the progeny of the infiltrated plants. Molecular analysis showed that the transgenics were hemizygous (the transgene was present in only one copy in one chromosome) and that different numbers of the T-DNA had been inserted. The advantage of using this method is that it is quick and suitable for generating a large number of independently transformed lines. However, it is probable that the amount of endogenous *A. tumefaciens* in the plant cells is quite high. BECHTOLD and co-workers (1993) speculated that this method could be adapted for the transformation of other crop species.

Examples of potato transformation.

At least three different methods have been used successfully to produce transgenic potato plants, using as an explant source either leaf disks, internodal stem segments or minituber disks (VAYDA & BELKNAP 1992). Although this is a cultivar dependent choice and often researcher-dependent (VISSER 1991), there are many reports of

transformation of some cultivars using more than one type of explant source.

Slices of minitubers (potato tubers produced *in vitro*) were used in the transformation of European cultivars including Bintje and Désirée by *Agrobacterium tumefaciens*-mediated gene transfer (SHEERMAN & BEVAN 1988; STIEKMA *et al.* 1988; OTTAVIANI & HANISCH TEN CATE 1991). In addition, cultivars Lemhi Russet and Russet Burbank (ISHIDA *et al.* 1989; SNYDER & BELKNAP 1993) and 11 leading Japanese cultivars (ISHIGE *et al.* 1991) were transformed using minitubers as the explant source. The transformation of the cultivars Maris Bard, Désirée (OOMS *et al.* 1987) and Russett Burbank (NEVELL *et al.* 1991) were obtained by infecting stems segments of *in vitro* grown plantlets with *Agrobacterium*. Leaf disk transformation was successful for cultivars Bintje, Berolina, Désirée and Russet Burbank (DE BLOCK 1988), Désirée (TAVAZZA *et al.* 1988; TRINCA *et al.* 1991), Russet Burbank and a diploid line ADX262-9 (AN *et al.* 1986), line FL1607 (WENZLER *et al.* 1989), line NDD272-2 (SHAHIN & SIMPSON 1986), three leading New Zealand potato cultivars (CONNER *et al.* 1991) and the Brazilian cultivar Mantiqueira (FILHO *et al.* 1994).

Using these various methods of transformation, transgenic potatoes with many different agriculturally important traits have been developed (VAYDA & BELKNAP 1992). Transgenic potatoes expressing the gene for an endotoxin (*cryIII*) from *Bacillus thuringiensis* demonstrated dramatic resistance to the Colorado potato beetle (*Leptinotarsa decemlineata*) (FRAYLEY 1991 cited by VAYDA & BELKNAP 1992). A great deal of research has been implemented in engineering potatoes for viral resistance, and this has been achieved by Monsanto for PVX and PVY (LAWSON *et*

al. 1990). As previously discussed, this has also been achieved for PLRV (KAWCHUK *et al.* 1991; VAN DER WILK *et al.* 1991; BARKER *et al.* 1994). As potatoes are susceptible to several bacterial diseases, for example tuber soft rot, blackleg and bacterial ring rot, expression of an antibacterial protein in potatoes may reduce the incidence or severity of these disease conditions. Potatoes have been engineered to express the antibacterial proteins cecropin and lysozyme, and decreased maceration by the tuber soft rot pathogen (*Erwinia carotovora*) was observed (TRINCA *et al.* 1991; HASSAN *et al.* 1993). In addition, the starch content of transgenic potato tubers has been modified by adding additional copies of an enzyme involved in carbohydrate metabolism (VAYDA & BELKNAP 1992). Herbicide-resistant transgenic potatoes have also been generated by expressing the *bar* gene from *Streptomyces hygroscopicus* (DE BLOCK 1988; FILHO *et al.* 1994).

Regeneration from leaf disks.

Of interest to the present study is transformation of potatoes using leaf disks as the explant source. The advantages of using leaf explants over tuber explants are that vigorously growing *in vitro* material is available at all times during the year and transformation of potato genotypes that produce no or few tubers is possible (VISSER 1991). However, a major disadvantage of using leaf explants is that because of a callus phase, somaclonal variation may occur (VISSER 1991). This can be kept in check by keeping the callus phase as short as possible. In most published procedures, callus formation from the cut edges of potato leaf disks is promoted by the presence of approximately 10 μ M cytokinin and 1 μ M auxin, shoot initiation by the removal of auxins, and shoot development by the addition of gibberellins at approximately 10 μ M

(HULME *et al.* 1992). The published procedures for potato regeneration can be classified into one-step methods where a single culture medium is used for all these phases, two-step methods where callus initiation takes place on one medium and shoot formation and development on another, and three-step methods where different media are used for each of the three phases. In an example of the one-step method high regeneration frequencies for cultivar Désirée were obtained when transformed leaf explants were cultured on a medium containing auxins, cytokinins and gibberellins (TAVAZZA *et al.* 1988). In the two step regeneration methods, explants were cultured on medium containing auxins, cytokinins and gibberellins for two to three weeks followed by a similar period where the auxins were removed from the medium (WHEELER *et al.* 1985; WENZLER *et al.* 1989). DE BLOCK (1988) described a three-step method, used successfully in transforming four potato cultivars, as being genotype independent. In this case, two days of preculture in liquid medium without plant growth regulators during which time the explants could be infected with *A. tumefaciens*, was followed by three weeks of callus initiation in the presence of auxins and cytokinins. Shoot initiation was prompted by the removal of auxin for a two week period and shoot development by addition of gibberellins. HULME *et al.* (1992) assessed the regeneration of plants from leaf explants of eight cultivars using a number of published one-, two- and three-step methods. A so called genotype-independent method was identified as being the most rapid and efficient. In this method, leaf explants were subjected to an overnight pulse in liquid medium containing high levels of cytokinin and auxin, followed by a two-step regeneration medium to which the ethylene inhibitor, silver thiosulphate was added. In the present study, this regeneration approach was compared to a two-step potato regeneration medium already in use at the

1.5. The Aim of the Present Study.

The aim of the present study was two-fold.

The first objective was to transform the potato cultivars Late Harvest and BP₁ with the PLRV-CP gene using *A. tumefaciens*-mediated transformation, and to regenerate transgenic plantlets. The PLRV-CP gene was previously isolated, cloned and sequenced (Patent 91/8773 1991). The CP gene was further manipulated to be cloned directionally into the Xba I and Sma I sites downstream from the CaMV 35 S promoter in the plant expression vector pBI121, in both the sense and anti-sense orientations (BURGER, unpublished results), as outlined in Fig.1.4 and Fig.1.5. Both constructs will be used in triparental matings with the helper plasmid pRK2013 to transform the LBA 4404 strain of *Agrobacterium tumefaciens* according to the method of ARMITAGE (1988), and will be used in potato transformation experiments. Optimal transformation, both by inoculation of leaf disks and vacuum infiltration of plantlets *in vitro*, and regeneration procedures will be investigated and identified. Transgenic shoots will be regenerated and rooted under kanamycin selection, before being hardened off for growth and observations on phenotypic stability in the glasshouse.

Secondly, transgenic plants will be analyzed for stable transgene insertion and

expression. Initially, transgene insertion into the plant genome will be demonstrated using PCR on small scale genomic DNA extracts with transgene-specific primers. These results will be verified with Southern blot analysis (SAMBROOK *et al.* 1989), and the transgene copy numbers determined. Expression of the marker gene GUS in transgenic potato plants will be shown using both the fluorometric and histochemical GUS assays (JEFFERSON *et al.* 1987). Expression of the PLRV CP gene in transgenic plants will be investigated at both the protein and mRNA levels. Proteins will be extracted and separated using SDS-Poly Acrylamide Gel Electrophoresis according to the method of LAEMMLI (1970), and subsequently subjected to Western blot analysis (SAMBROOK *et al.* 1989). Alternatively, the levels of coat protein in the transgenic plants will be detected using ELISA. Northern blot analysis will be used to determine the extent of mRNA transcription from the PLRV-CP gene (SAMBROOK *et al.* 1989). Previously published studies on PLRV CPMR (KAWCHUK *et al.* 1991; VAN DER WILK *et al.* 1991; BARKER *et al.* 1992) have only been successful at uniformly detecting PLRV-CP expression at the mRNA level, and a similar scenario will be investigated in the present study.

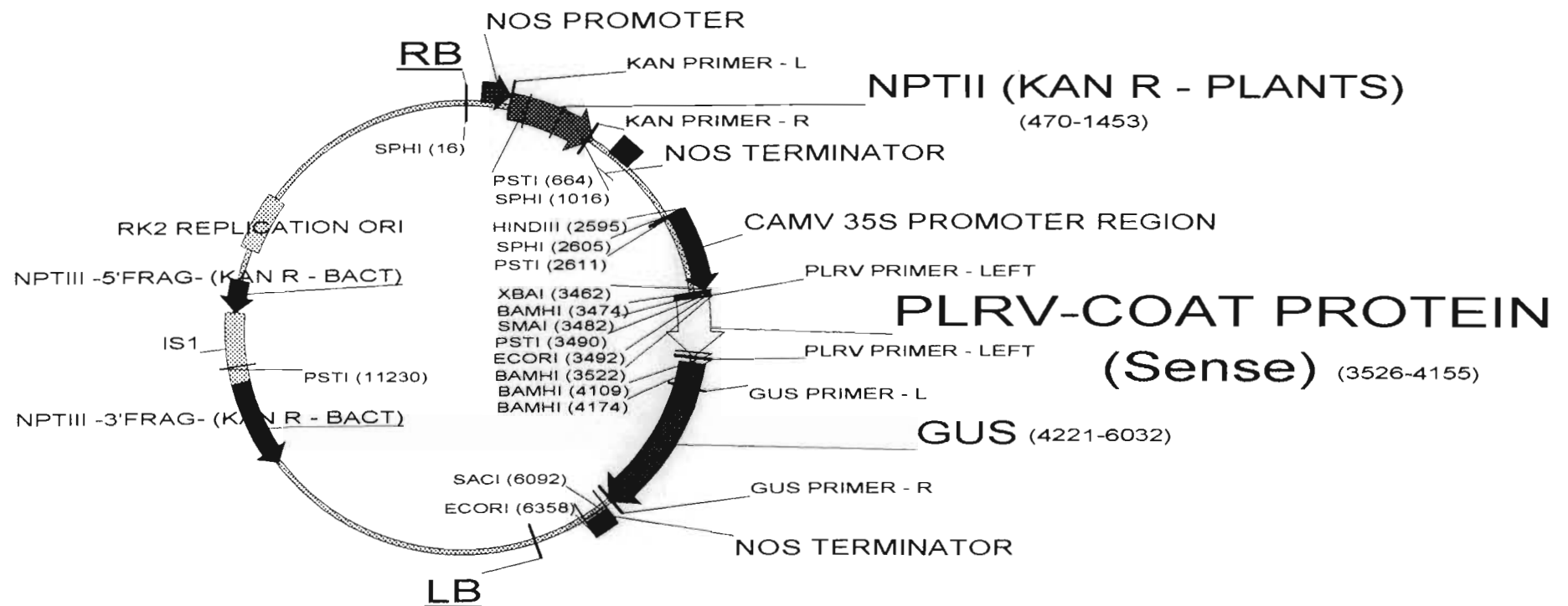


Fig.1.4. Plant expression vector pBI121 containing the PLRV-CP gene in the sense orientation (15489 bp).

BACT= bacteria; GUS= β -Glucoronidase; KAN R= kanamycin resistance gene; LB= left border; nptII= neomycin phosphotransferase II;

PRIMER LEFT (L)= 5' primer for PCR; PRIMER RIGHT (R)= 3' primer for PCR; RB= right border.

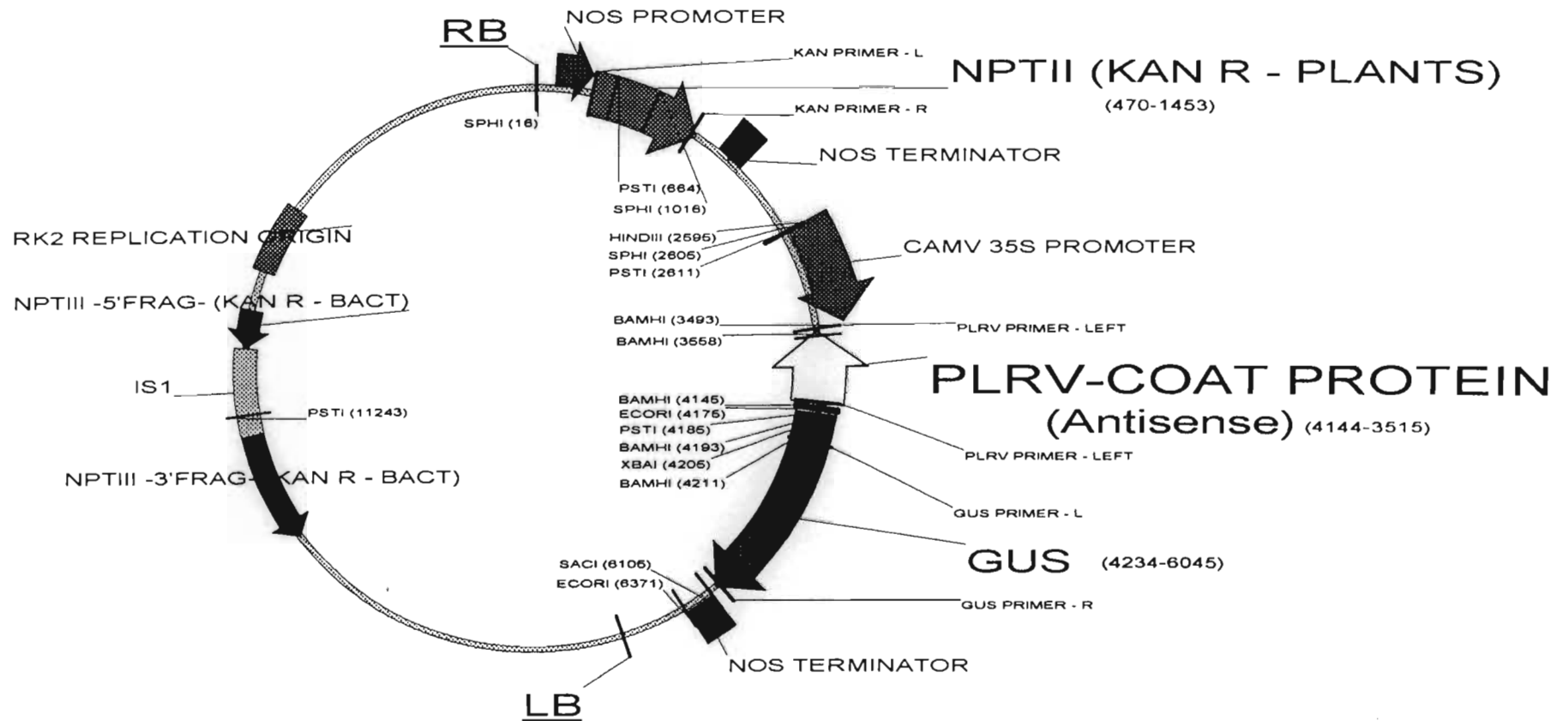


Fig.1.5. Plant expression vector pBI121 containing the PLRV-CP gene in the antisense orientation (15502 bp).

BACT= bacteria; GUS= β -Glucoronidase; KAN R= kanamycin resistance gene; LB= left border; nptII= neomycin phosphotransferase II;

PRIMER LEFT (L)= 5' primer for PCR; PRIMER RIGHT (R)= 3' primer for PCR; RB= right border.

CHAPTER TWO.

MATERIALS AND METHODS.

All media, buffers and supplies were sterilized by autoclaving at 121°C and 1 atmosphere pressure for twenty minutes or by filter-sterilization (pore size 0.22 µm, Millipore). Manipulations of both bacteria and plants *in vitro* were performed using sterile techniques. All formulations of buffers and media are outlined in the Appendix.

2.1. *Agrobacterium tumefaciens*.

The following bacterial strains were used in triparental matings:

- 1) The recipient strain *Agrobacterium tumefaciens* LBA 4404, which is rifampicin resistant.
- 2) The donor strain *E. coli* HB 101 which contains one of six binary plasmids, viz.
 - pBI121
 - pBI121-16, pBI121-15, pBI121-14 or pBI121-1, which all contain the PLRV-CP gene in the sense orientation
 - pBI121-13, containing the PLRV CP gene in the antisense orientation.

All three *E. coli* strains are kanamycin resistant.

- 3) The strain which donates the helper plasmid during triparental mating, *E. coli* HB 101 (pRK2013), which is kanamycin resistant.

Triparental Mating.

The triparental mating procedure was performed using the method of ARMITAGE (1988). All bacterial strains were streaked out from bead cultures stored at -70°C , onto solidified Luria-Bertani (LB) medium containing the relevant antibiotics at $100\text{ }\mu\text{g.ml}^{-1}$ kanamycin for *E. coli* HB 101 (pBI121), $50\text{ }\mu\text{g.ml}^{-1}$ kanamycin for *E. coli* HB 101 (pRK2013) and $100\text{ }\mu\text{g.ml}^{-1}$ rifampicin for *A. tumefaciens* LBA 4404. *E. coli* and *A. tumefaciens* cultures were grown at 37°C and 28°C respectively. Single colonies of all three strains were grown on a shaker in liquid LB medium, supplemented with the relevant antibiotics, for 36 hours in the case of *A. tumefaciens* and overnight for the *E. coli* strains. Cultures were then mixed in a 1:1:1 ratio in an eppendorf tube, and spread evenly onto LB plates. The plates were incubated at 28°C for 24 to 36 hours until a lawn of bacterial growth could be visualised. A loopful of this growth was subsequently streaked out on selective plates consisting of LB + $100\text{ }\mu\text{g.ml}^{-1}$ rifampicin + $100\text{ }\mu\text{g.ml}^{-1}$ kanamycin, in order to select for successful transformants. As a control measure, *A. tumefaciens* LBA 4404 and the two *E. coli* HB 101 strains were also streaked out onto the selective medium. The presence of GUS activity in positive colonies [*A. tumefaciens* LBA 4404 (pBI121) (pRK2013)] was confirmed using the small-scale GUS fluorometric assay outlined in paragraph 2.7.1.

Agrobacterium quick screen.

In order to verify that the transgenes were present in the *A. tumefaciens* colonies, DNA was extracted using the "quick screen" method (AN *et al.* 1992) and subjected to PCR

analysis with transgene specific primers. The PCR conditions are outlined in paragraph 2.6.

A. tumefaciens cells were grown overnight in liquid LB supplemented with 100 $\mu\text{g.ml}^{-1}$ rifampicin and 100 $\mu\text{g.ml}^{-1}$ kanamycin. The cells were collected by centrifugation at 12000 rpm for 5 minutes and 4 °C. The pellet was resuspended in 0.1 ml ice-cold, freshly prepared Quickscreen solution I containing 4 mg.ml^{-1} lysozyme. After 10 minutes incubation at room temperature, 0.2 ml freshly prepared Quickscreen solution II was added to the samples and mixed well. The samples were incubated at room temperature for 10 minutes. Following this time, 30 μl of phenol: solution II (1:2) was added to the samples which were vortexed and 150 μl 3M sodium acetate (pH 4.8) was subsequently added to the samples. The samples were incubated at -20 °C for 15 minutes. Samples were centrifuged at 12000 rpm for 5 minutes at 4 °C. The supernatant was removed, added to 1 ml ice-cold 99% ethanol and incubated at -20°C for 15 minutes. The precipitated DNA was pelleted by centrifugation. The pellet was resuspended in 0.5 ml 0.3M sodium acetate, pH 7.0, and the DNA was precipitated by addition of 1 ml 99% ethanol. The DNA was collected by centrifugation, washed in 70% ethanol and resuspended in 50 μl TE buffer before being subjected to PCR analysis.

Growth of *A. tumefaciens* for plant transformations.

A. tumefaciens strain LBA 4404, into which pBI121 or one of the pBI121-PLRV CP constructs had been triparentally mated, was grown in liquid LB medium supplemented with 100 $\mu\text{g.ml}^{-1}$ kanamycin and 100 $\mu\text{g.ml}^{-1}$ rifampicin on a shaker at 28 °C for 36

hours. The antibiotics were removed from the medium by centrifugation (3300 rpm, 4 °C, 25 minutes) of the cell suspensions. The supernatant containing the antibiotics was discarded and the pellet was resuspended in LB only. This bacterial suspension was subsequently used in plant transformation experiments (VISSER 1991).

2.2. Potato tissue culture.

In vitro derived leaves from virus free potato plantlets (cultivars Late Harvest and BP₁) were used for leaf disk transformation and regeneration experiments. The plantlets were maintained on MS medium containing 1.5 mg.l⁻¹ silver thiosulphate (STS). The medium was dispensed into bottles prior to autoclaving. Bottles were used for the plantlets in order to minimise ethylene build-up. All potato tissue culture vessels were placed in the growth room at 26 °C and 16 hours light/ 8 hours dark photoperiod.

Regeneration from leaf disks.

Three different regeneration treatments were compared, in order to ascertain which medium gave the highest regeneration rate for the cultivars Late Harvest and BP₁ respectively. These were the R1AT two step method (VENTER pers. comm.), a three step method which included an overnight pulse with high levels of cytokinin and auxin (HULME *et al.* 1992), and the three step method without the pulse treatment.

A two-step potato leaf disk regeneration medium was previously in use at R.V.O.P.I. (VENTER, pers. comm.). Using this approach, potato leaves produced from 4 to 6 week

old *in vitro* plantlets with the apical and basal 3mm removed, were placed on plates containing the regeneration medium R1AT. Explants were subcultured onto fresh medium once a week, and when callus production could be visualised on the cut surfaces of the leaves, the leaves were transferred onto R1AT medium without the auxin component in order to further stimulate shoot production. Shoots were rooted on Osborne medium (VENTER, pers. comm.).

A three-step so called genotype independent potato regeneration method was developed by HULME and co-workers (1992). In this method, leaf explants were prepared as above, and were floated overnight in a liquid pulse medium. Explants were subsequently placed on callus induction medium for 2 weeks, after which they were transferred to shoot induction medium, and sub-cultured to fresh medium every week.

2.3. Potato transformations.

Leaf disk transformation.

The protocol used was adapted from DE BLOCK 1988, VISSER 1991 and HIGGINS 1992.

Potato leaves, produced as for the regeneration experiments, were pre-incubated on MS plates containing 1.5 mg.l^{-1} STS for 48 hours in the growth room at 16 hours light/ 8 hours dark and 26 °C. Following this, the leaves were immersed in the *A. tumefaciens* cell suspensions for varying time periods, blotted on sterile filter paper and replaced onto

the MS plates for two or more days, during which time co-cultivation occurred. In some experiments, 10 mg.l⁻¹ 3'5'dimethoxy-4'hydroxyacetophenone (acetosyringone) was added to the bacterial cell suspensions.

After co-cultivation, the potato leaves were placed onto regeneration medium to which kanamycin (50 µg.ml⁻¹) and cefotaxime (250 µg.ml⁻¹) were added. Regeneration on various kanamycin concentrations was tested in order to determine the optimum concentration. The leaves were subcultured onto fresh medium once a week, and were placed in the growth room at 26 °C and 16 hours light /8 hours dark. When callus production was seen at the cut edges, the leaves were placed onto medium with the antibiotics but with the auxin (NAA) removed. Regenerated shoots were rooted on Osborne medium.

Transformation by vacuum infiltration.

An adaption of the protocol developed by BECHTOLD and co-workers (1993) for *Arabidopsis* was used. *In vitro* potato plantlets (apical bud and roots removed) or nodal sections were submerged in *A. tumefaciens* cell suspensions and a vacuum drawn for 2, 10 or 20 minutes. Explants were placed on MS medium for 3 to 4 days during which time co-cultivation was allowed to occur. After this time explants were placed on selection medium consisting of Osborne supplemented with 25 µg.ml⁻¹ kanamycin and 250 µg.ml⁻¹ cefotaxime.

2.4. Hardening off of transgenic potato plantlets.

Transgenic plantlets were removed from *in vitro* culture and planted into potting soil in small pots. The pots were covered with clear plastic bags and placed in the glasshouse at 20 °C. Plantlets were gradually exposed to the atmosphere by removing the bags for short periods until the plants were strong enough to remove the bags completely. Plants were watered once a week with a 5 g.l⁻¹ Multifeed^R solution.

After a 4 month growth period in the glasshouse, the transgenic potato plants died back and the tubers were harvested. Tubers were stored at 4 °C. In order to stimulate shoot production from these tubers, the tubers were incubated in a solution consisting of 7 parts chlorohydrin, 3 parts ethylene dichloride and 1 part carbontetrachloride for 48 hours. Following this, the tubers were incubated at 25 °C until shoot production could be seen at which stage the tubers were planted out.

2.5. Determination of ploidy level.

The abaxial epidermis of young leaves was peeled away from the leaf, and placed on a microscope slide in 1 to 2 drops of a potassium iodide solution. The sample was covered by applying a coverslip, and the epidermis was viewed under the 400X objective of a Nikon Microphot-FX photomicroscope. Ten representative guard cells were selected and the number of chloroplasts per cell were counted.

2.6. Verification of transgene insertion.

PCR Analysis.

In order to verify that transgenes had been integrated into the genome of the transgenic plants, PCR was performed on genomic DNA samples using transgene specific primers. Small-scale DNA extractions were done by an adaption of the method of EDWARDS *et al.* (1991). Small leaf disks (approximately 1 cm²) were ground in eppendorf tubes in the presence of "super-quick" buffer and carborundum, and incubated at 60 °C for 10 minutes. DNA was extracted with chloroform:isoamylalcohol (24:1) and precipitated with 2 volumes 99% ethanol and 0.1 volumes 3M sodium acetate, pH 5.2. The DNA was collected by centrifugation and the pellet was washed in 70% ethanol. The DNA was resuspended in 100 µl TE buffer. DNA concentrations were determined using a Sequoia-Turner fluorometer. PCR was performed on 30 ng DNA templates under the following conditions: 10 mM Tris-HCl (pH 8.8); 50 mM KCl; 0.1 % Triton-X; 1.5 mM MgCl₂; 100 µM each dNTP and 0.5 µM of each primer. The thermostable DNA polymerase DyNAzyme™ II (Finnzymes Oy) was used to amplify the gene of interest at a concentration of 0.5U per 10 µl reaction volume. PLRV-CP, neomycin phosphotransferase II (*nptII*) and GUS specific primers were synthesised (U.C.T. Department of Biochemistry). Only the left primer of the PLRV-CP gene was needed to amplify the PLRV-CP gene as insertion of this primer sequence had been accidentally duplicated during cloning (BURGER, pers. comm.). The sequence of the primers were as follows:

PLRV-CP-left: 5'-GCAGGATCCTAATGAGTACGG-3'

GUS-left: 5'-GGTGGGAAAGCGCGTTACAAG-3'

GUS-right: 5'-GTTTACGCGTTGCTTCCGCCA-3'

nptII-left: 5'-GAGGCTATTCGGCTATGACT-G-3'

nptII-right: 5'-ATCGGGAGCGGCGATACCGT-A-3'

The annealing temperature was deduced from the following equation: $T_m = 4(G+C) + 2(A+T)$. In the case of the GUS primers, it was found that the annealing temperature had to be increased to 70 °C in order to prevent non-specific amplification of the GUS gene (BURGER, unpublished results). The annealing temperatures used were 60 °C and 64 °C for the *nptII* and PLRV-CP genes respectively. A PCR program was designed as follows:

Stage 1:

Step 1: Dissociation of DNA strands at 94.5 °C for 30 seconds

Step 2: Primer annealing at 70 °C/ 60 °C/ 64 °C for 30 seconds, depending on the primer

Step 3: Primer elongation at 72 °C for 45 seconds

Stage 2:

Step 1: Dissociation of DNA strands at 94.5 °C for 30 seconds

Step 2: Primer annealing at 70 °C/ 60 °C/ 64 °C for 30 seconds, depending on the primer

Step 3: Primer elongation at 72 °C for 5 minutes

Samples were subjected to 35 cycles of Stage 1 and 1 cycle of Stage 2 using the Hybaid Omnigene PCR machine, the PCR products were resolved in a 0.8% agarose gel,

visualised by ethidium bromide staining and sized by comparison to molecular markers.

Analysis by Southern Blotting.

In order to perform Southern blotting for confirmation of gene insertion and determination of the copy number of the transgenes, a very pure large scale plant genomic DNA extraction was required. The genomic DNA was subsequently cut with various restriction enzymes, the DNA fragments were separated by electrophoresis and subsequently transferred onto a nylon membrane. DNA probes were labelled either by nick translation and incorporation of ^{32}P -dCTP, or by random primed labelling and incorporation of DIG-11-dUTP. The membranes were subsequently probed with the labelled DNA, and hybridization of the probe to the target DNA was detected.

DNA Extraction (RICHARDS 1989).

DNA was extracted from glasshouse plants. Fresh young leaf tissue (2.5 - 10 grams) was harvested and ground in liquid nitrogen with carborundum in an ice cold mortar and pestle. The ground leaf material was transferred to a SS34 centrifuge tube to which 2 - 4 ml of DNA extraction buffer was added per gram of leaf tissue. A 10 % Sarkosyl solution was added to a final concentration of 1 % before the samples were incubated in a hot water bath at 55 °C for 1 to 2 hours. Following this, the samples were centrifuged in a Beckman centrifuge at 7000 g for 10 minutes at 4 °C in order to pellet the debris. The supernatant was removed, strained through sterile cheesecloth, and placed in a clean tube to which 0.6 volumes of isopropanol was added. If a precipitate was not formed immediately, the tubes were incubated at -20 °C for 30 minutes. The

samples were centrifuged at 8000g for 15 minutes at 4 °C. The pellet was resuspended in 4 ml TE, 4.8 g of cesium chloride was added and mixed until it had dissolved. The tubes were incubated on ice for 30 minutes, followed by centrifugation at 8000g for 10 minutes at 4 °C. A 10 mg.ml⁻¹ ethidium bromide solution (250µl) was added to the supernatant in a covered tube. The tubes were incubated on ice for 30 minutes followed by centrifugation at 8000g for 10 minutes at 4 °C. The refractive index of the samples was read on an Abbe refractometer and if necessary, the refractive index of the samples was adjusted to between 1.388 and 1.392. The supernatant, with no debris present, was transferred to Beckman quick seal tubes and sealed. The samples were centrifuged using a NVT 90 rotor in a Beckman Ultracentrifuge at 80 000 rpm and 20 °C for 4 hours or at 60 000 rpm and 20 °C for 16 hours. The DNA bands were visualised using UV light (350nm) and collected using a syringe. The ethidium bromide was removed from the DNA sample by extraction with NaCl-saturated isopropanol. The DNA was precipitated by adding 2 volumes sterile distilled water and 3 volumes isopropanol and the sample was mixed well by inversion of the tube. The DNA was pelleted by centrifugation at 12 000 rpm and 4 °C for 10 minutes. The pellet was washed in 70% ethanol, dried and resuspended in 100 µl TE buffer. The concentration of the DNA was determined by reading a 2 µl sample on the Sequoia-Turner fluorometer. The readings were confirmed by running a 500 ng sample on a 0.8 % agarose gel, and comparing the intensity of the DNA smear in the gel under UV light with the intensity of a DNA standard.

Southern Blotting.

Ten micrograms of plant genomic DNA were cut with various restriction enzymes at 37 °C overnight. Plasmid DNA was also cut in order to provide a positive control.

DNA fragments were separated by electrophoresis in a 0.8% agarose gel in 1 X TAE buffer and visualised by ethidium bromide staining. The gel was photographed and the DNA fragments were immediately transferred from the gel onto a nylon membrane (Hybond N+ or Boehringer Mannheim's positively charged nylon membrane) using the capillary transfer system as outlined by SOUTHERN (1975). Prior to blotting, the gel was subjected to a number of treatments.

Firstly, the gel was slowly agitated in 250 mM HCl for 5 minutes in order to depurinate the DNA and thus improve the efficiency of transfer, and then rinsed in distilled water. Secondly, the gel was agitated twice in denaturation solution for 15 minutes. This treatment was followed by a rinse in distilled water. Thirdly, the gel was neutralized by 2 washes in neutralization solution for 15 minutes each. The transfer procedure was subsequently performed overnight using 20 X SSC buffer. Following transfer, the DNA was fixed onto the membrane by baking the membrane at 80 °C for 90 minutes. The membranes were loosely wrapped in foil and stored before hybridization at room temperature.

Labelling of probe with ^{32}P dCTP.

Double-stranded DNA fragments, prepared by PCR, were labelled using the Amersham Nick translation kit N5000. The Amersham nick translation protocol was modified so that the following were mixed in an eppendorf:

0.5-1 µg template DNA	x µl
Nucleotide/buffer solution	5 µl
^{32}P -dCTP (10 µCi.µl ⁻¹)	2.5 µl

Klenow/DNase I solution 2.5 μ l

The solution was made up to 25 μ l with dH₂O.

The contents of the tube were stirred gently, and incubated for 2 hours at 15 °C. Following this time, the labelled probe was purified from the sample mix through a Sephadex G50 pasteur column, and the amount of incorporated radioactivity was measured using a 1219 Rackbeta liquid scintillation counter. The amount of probe to be used in hybridization experiments was subsequently calculated. At least 10-20 ng probe was used per ml, with a specific activity greater than 10⁹ counts per minute (cpm) per μ g of DNA was used (SAMBROOK *et al.* 1989).

Labelling of probe with DIG-11-dUTP.

DIG-11-dUTP molecules were incorporated into double-stranded DNA fragments, prepared by PCR, through random primed labelling according to the DIG Manual (BOEHRINGER MANNHEIM 1993). The DNA fragments were first denatured at 95 °C for 10 minutes, followed by immediate cooling on ice. The following reagents were mixed in an eppendorf:

1 μ g template DNA	x μ l
Hexanucleotide mix	2 μ l
dNTP labelling mix	2 μ l
Klenow enzyme	1 μ l

The solution was made up to 20 μ l with dH₂O.

The tubes were incubated at 37 °C for 20 hours. EDTA (2 μ l) was added to terminate

the reaction, and the DNA was precipitated with 0.1 volumes 4M LiCl and 2.5 volumes 99% ethanol. The DNA was precipitated by centrifugation at 12000rpm for 15 minutes at 4°C, the pellet was washed in 70% ethanol, dried and resuspended in 50 µl TE. The yield of DIG-labelled probes was estimated by dotting various concentrations of the DIG-11-dUTP labelled probe onto a Hybond N⁺ membrane, and comparing the amount of DIG present to control labelled DNA using the Colourimetric Detection System.

Hybridization of ³²P labelled probe.

Prehybridization buffer was made up as follows (SAMBROOK *et al.* 1989):

7.5 ml	20 X SSC (final concentration 6 X SSC)
2.5 ml	50 X Denhardt's solution (final concentration 5 X Denhardt's solution)
1.25ml	10% (w/v) SDS (final concentration 0.5% SDS)

The buffer was made up to 25 ml with dH₂O.

Herring sperm DNA, which had been denatured by boiling at 95 °C for 5 minutes and immediately cooled on ice, was added to a final concentration of 100 µg.µl⁻¹. Ten ml prehybridization buffer was added per 100 cm² membrane in a hybridization bottle, and prehybridization occurred for at least one hour at 65 °C in a GFL 7601 hybridization oven.

Following the prehybridization treatment, the probe which had been denatured by boiling at 95 °C for 5 minutes and cooled on ice, was added directly to the prehybridization buffer, and hybridization was allowed to proceed overnight at 65 °C. The following day, blots were washed twice for 15 minutes at 65 °C in 2 X SSC, 0.1% SDS. The amount

of radioactivity on the blot was monitored as this was an indication of probe binding. As soon as the level of background radioactivity was low, the washing steps were stopped. If levels were still high, a more stringent wash (0.1 X SSC, 0.1% SDS) was applied.

Hybridization of DIG labelled probe.

Prehybridization buffer for use with DIG-labelled probes was made up as follows (BOEHRINGER MANNHEIM 1993, NEUHAUS-URL & URL 1993):

62.5 ml	20 X SSC (final concentration 5 X SSC)
25 ml	10% (w/v) DIG Blocking reagent (final concentration 1%)
2.5 ml	10% (w/v) N-lauroylsarcosine (final concentration 1%)
5 ml	10% (w/v) SDS (final concentration 0.2%)
125 ml	formamide (final concentration 50%)

The buffer was made up to 250 ml with dH₂O.

Ten ml prehybridization buffer was added per 100 cm² membrane in a hybridization bottle, and prehybridization occurred for at least one hour at 42 °C in the hybridization oven.

Hybridization buffer was made by adding labelled DNA to prehybridization buffer at a concentration of 25 ng.ml⁻¹ (BOEHRINGER MANNHEIM 1993). Prehybridization buffer was discarded, hybridization buffer was added to the bottle, and hybridization was allowed to proceed at 42 °C overnight. The following day, blots were washed twice for 5 minutes at room temperature in 2 X SSC, 0.1% SDS in order to remove the unbound probe. The blots were subsequently washed twice in 0.1 X SSC, 0.1% SDS for 20

minutes at 68 °C, before detection of probe binding took place.

Detection of binding of the ³²P labelled probe.

Membranes were sealed in thick plastic bags in 2 X SSC as it was imperative that the membrane should not dry out. Hyperfilm™ (Amersham) was applied to the membrane, and the membrane was placed between 2 intensifying screens in an autoradiography cassette. The cassette was incubated in the -70 °C freezer for 1-14 days, at which time the Hyperfilm™ was developed for detection of probe binding.

Detection of binding of the DIG labelled probe.

Blots were firstly incubated in DIG buffer 1 for 1 minute. The blots were subsequently blocked by gentle agitation in DIG buffer 2 for one hour at room temperature. Anti-DIG antibody conjugated to alkaline phosphatase was diluted 1:10000 in DIG buffer 2, and added to the blots after blocking. The blots were gently agitated for 30 minutes. Following probing with the antibody, blots were washed four times for 20 minutes each wash in DIG buffer 1 + 0.3% Tween-20^R. Subsequently, the blots were incubated in DIG buffer 3 in order to activate the alkaline phosphatase. The chemiluminescent substrate for alkaline phosphatase, CDP-Star™, was diluted 1:100 in DIG buffer 3 and poured over the blots. The blots were then incubated in a sealed plastic bag for 5 minutes. The blots were removed from the bag, the excess liquid removed by light blotting, and the blots sealed in a fresh bag. The blots were exposed to Hyperfilm™ for various times, and the film was developed for detection of probe binding.

2.7. Verification of transgene expression.

2.7.1. GUS assays.

Histochemical assay (JEFFERSON et al. 1987).

Putative transgenic plant tissue was incubated overnight at room temperature in X-Gluc buffer. When green tissue was used, the stained tissue was decolourised by incubation of the sample in 70% ethanol overnight. Production of the blue precipitate was visualised and recorded using a Nikon Mikrophot-FX photomicroscope.

Small-scale fluorometric assay.

Putative transgenic plantlets and bacteria were evaluated for GUS expression by incubating either small pieces of plant material or a loop of bacteria in GUS-extraction buffer containing 1 mM methyl umbelliferyl glucuronide (MUG) for one hour at 37 °C, or overnight at 28 °C. If GUS activity was present, the sample fluoresced under UV light.

Fluorometric assay (JEFFERSON et al. 1987).

Crude extracts were prepared as follows: GUS extraction buffer (100µl) and 5 mg polyvinyl pyrrolidone were added to a 100 mg leaf sample in an eppendorf. A sterile glass rod was used to grind the tissue. The extracts were centrifuged at 12 000 rpm for 5 minutes at 4 °C, the supernatant removed and used in the assay.

The protein concentrations of the leaf extracts was determined using the Bradford macroassay method (Bio-rad Protein Assay Instruction Manual, 1986). This method is discussed under paragraph 2.7.2. A protein standard curve was constructed, which was used to determine the protein concentration of the leaf extracts.

The fluorescence of the leaf extracts were measured in order to determine the extent of GUS activity in the sample. The GUS reaction buffer contains the substrate 4- methyl umbelliferyl β -D glucuronide (MUG), which is cleaved to form the fluorescing product methyl umbelliferone (MU). A MU standard curve was constructed by first diluting a 10 mM MU stock solution to make the following standard solutions : 0.0; 0.1; 0.2; 0.3; 0.5; 0.8; 1.0; 5.0; 8.0 and 10 μ M MU. The fluorescence of these standards was read by the fluorometer set at an excitation wavelength of 360 nm, and the values used to construct a standard curve using Curfit™ (Curve fitter-PC, Interactive Microware Inc.). In order to measure the fluorescence of the tissue extracts, 40 μ l of the different tissue extracts were added to 500 μ l of GUS reaction buffer and mixed thoroughly. The reaction mixture was incubated at 37 °C, 70 μ l volumes were removed and added to stop buffer at 5 minute intervals until the reaction had run for 30 minutes. Fluorescence of the stopped reactions was read on the fluorometer. Using the standard graphs, the amount of MU produced in μ mol MU per mg protein was calculated and plotted against time for each plant sample.

2.7.2. Detection of Coat Protein (CP) expression.

Two methods were employed in order to detect CP expression in the PLRV-CP transgenic potato plants, viz. DAS-ELISA and immunoblot analysis.

DAS-ELISA.

A double-sandwich enzyme linked immuno-sorbent assay (DAS ELISA) was performed. Anti-PLRV IgG (RSA strain) was diluted 1:1000 in coating buffer, and 200 µl of this solution was added to each well of a Nunc ELISA plate. The ELISA plate was incubated overnight at 4 °C. The following day, the ELISA plate was washed 3 times with washing buffer. A leaf from each transgenic plant was rapidly ground in a roller press, and the liquid plant sap was diluted 1:10 with sample conjugate buffer. Two hundred microlitres of the dilutes plant sap were added to each well in the ELISA plate and the plate was incubated overnight at 4 °C. On day 3, the contents of the ELISA plate were shaken out, and the plate was washed three times with washing buffer. Anti-PLRV IgG conjugated to alkaline phosphatase was diluted 1: 1000 in sample conjugate buffer, and 200 µl were added to each well. The ELISA plate was incubated for 4 hours at 37 °C, washed 3 times in wash buffer before 200 µl of substrate buffer were added to each well. After a 1 hour incubation at room temperature, the absorbance of the contents of each well was read at 405nm using the Titertek^R Multiskan photometer.

Immunoblot Analysis.

Extraction of plant proteins.

Protein extraction was performed according to the method of FARINELLI and co-workers (1992). One gram of young fresh leaf material was broken up in liquid nitrogen, and further ground into a powder by adding carborundum. The ground up leaf material was transferred to tubes and 2 ml of protein extraction buffer were added. The samples were subsequently incubated at 100 °C for 5 minutes. The samples were centrifuged at 12 000 rpm and 4 °C for 10 minutes. The supernatant was aliquoted into 1.5 ml eppendorf tubes, the tubes were shock frozen in liquid nitrogen and stored at -70 °C.

Determination of Protein Concentration.

The Bradford Macroassay was used to determine the total concentration of plant proteins (Bio-rad Protein Assay Instruction Manual, 1986). A Bovine Serum Albumin (BSA) standard was diluted to form solutions of the following concentrations in $\mu\text{g}.\text{ml}^{-1}$: 20, 40, 60, 80, 100. Bio-rad reagent (5 ml) and distilled water (990 μl) was added to 10 μl of these solutions, and after a period of 5 minutes to 1 hour, the OD_{595} was measured against a reagent blank. The OD_{595} values were plotted against the concentration of the BSA standards using the Curfit™ (Curve fitter-PC, Interactive Microware, Inc.) program, and a standard curve was constructed. In order to determine the protein concentration of the plant samples, 10 μl of the plant protein extracts were added to 990 μl distilled water and 5 ml Bio-rad reagent. After a period of 5 minutes to an hour, the OD_{595} values of the plant protein samples were read using a spectrophotometer. The

concentrations of the plant protein extracts were then read off from the standard curve.

Dot Blot

A number of transgenic plants were screened for the production of PLRV coat protein. Known protein concentrations were applied to a nitrocellulose membrane (Hybond C) using the Bio-rad Dot Blot™ apparatus, and the membrane probed with the relevant antibodies as described below.

SDS-PAGE Gels (LAEMMLI 1970).

Each plant protein sample was mixed with treatment buffer to give a concentration of 50 µg per 30 µl. Samples were placed in boiling water for 3 minutes. Following this, the samples were loaded onto a gel consisting of a 12 % resolving gel and a 4% stacking gel. Proteins were separated by electrophoresis at 20 mA. Two gels were run : one gel was stained so that the protein bands could be visualised and the molecular weights determined by comparison to Rainbow markers (Amersham), the other gel was used for Western blotting.

Western Blotting.

After electrophoresis, proteins from the PAGE gels were electro-blotted in transfer buffer at 50 mA overnight onto a nitrocellulose membrane (Hybond C) using the Bio-rad Electro Eluter. All membranes were blocked by gently agitating the membranes for 1 hour or longer at 37°C in blocking buffer. Following this, the membranes were probed firstly with anti-PLRV rabbit IgG diluted 1:1000 in blocking buffer for at least one hour at 37 °C. Secondly, the membranes were probed with goat anti-rabbit antiserum

conjugated to alkaline phosphatase. Following probing, membranes were washed twice by agitation for 5 minutes in washing buffer. Visualisation of antibody binding occurred by developing the blot using the DIG Colourimetric Detection System (Boehringer Mannheim).

Staining of SDS-PAGE gels.

The gel was gently agitated in the Coomassie Blue protein stain for 30 to 45 minutes at 37 °C. Following staining, the gel was incubated firstly in destain solution I and then in destain solution II (SAMBROOK *et al.* 1989). Gels were stored in a plastic bag containing destain solution II.

2.8. Verification of transgene transcription.

In order to identify transgene expression at the RNA level, Northern blot analysis was performed. Special care was taken when working with RNA in order to minimise contamination by RNases: sterile gloves were always worn, all glass and plasticware was soaked in a 2% abSolve™ (DuPont) solution before use and double distilled water was used in buffer formulations.

Plant RNA extraction.

Total RNA from PLRV-CP transgenic plants was extracted using the method developed by VERWOERD and co-workers (1989). Leaf pieces (approximately 1cm²) were collected in sterile eppendorf tubes and immediately frozen in liquid nitrogen. The leaf

material was ground using a sterile, precooled glass bar and 500 µl RNA extraction buffer (preheated to 80 °C) was added to each tube. The samples were homogenized by vortexing for 30 seconds before 250 µl chloroform: isoamylalcohol (24:1) was added and the samples were re-homogenized. The tubes were centrifuged at 12000 rpm, 4 °C and 5 minutes, and the upper aqueous layer was removed and mixed with 1 volume of a 4M LiCl solution. The RNA was allowed to precipitate overnight and collected the following day by centrifugation (12000 rpm, 4 °C, 5 minutes). The pellet was dissolved in 250 µl sterile double-distilled water, 0.1 volumes 3M sodium acetate (pH 5.2) was added and the RNA was precipitated with 2 volumes 99% ethanol. After centrifugation (12000 rpm, 4 °C, 5 minutes), the pellet was washed in 70% ethanol and resuspended in sterile double-distilled water. The optical density of the RNA sample was read at 260 nm using a Beckman spectrophotometer in order to determine the RNA concentration (RNA concentration = $OD_{260} \times \text{dilution factor} \times 40 \mu\text{g.ml}^{-1}$).

Northern blot.

Total plant RNA (10 µg) was separated on a 1% agarose/formaldehyde denaturing gel (SAMBROOK *et al.* 1989). RNA isolated from PLRV was included as a positive control. Two gels were prepared per analysis so that one gel could be stained using ethidium bromide for RNA sizing and the other gel could undergo Northern blotting. The gels were prepared by dissolving 1% agarose in a 1 X MOPS solution and cooling to 60 °C before 40% formaldehyde was added to a final concentration of 6%. The gel was poured, allowed to set and run in a fume hood. Samples to be loaded on the gels were prepared as follows:

RNA solution (10 µg)	x	µl
10 X MOPS buffer	2	µl
40% formaldehyde	3.5	µl
formamide	10.0	µl

The samples were heated at 65 °C for 15 minutes and immediately cooled on ice before 2 µl of loading buffer was added to each sample. The samples were loaded onto the gel and the RNA species were separated by electrophoresis at 60 V. One gel was stained with ethidium bromide and the RNA bands sized by comparison to RNA molecular weight markers (Bethesda Research Laboratories). Capillary transfer of RNA from the agarose/formaldehyde gel onto a nylon membrane (Hybond N+ or Boehringer Mannheim's positively charged nylon membrane) was performed using the same method as the Southern blots, but no depurination and denaturalisation steps were included.

Probe labelling.

Double stranded DNA probes were used. Probe labelling was done as for the Southern blots, and ³²P-dCTP was incorporated into the probe. At least 100 ng of probe was used with a specific activity of 2 X 10¹⁰ cpm per µg of DNA (SAMBROOK *et al.* 1989).

Hybridization.

Prehybridization buffer was made up as follows (SAMBROOK *et al.* 1989):

7.5 ml	20 X SSC (final concentration 6 X SSC)
2.5 ml	50 X Denhardt's solution (final concentration 5 X)
1.25ml	10% (w/v) SDS (final concentration 0.5% SDS)
12.5ml	formamide (final concentration 50%)

The buffer was made up to 25 ml with dH₂O.

Prehybridization, hybridization of single stranded DNA probes to mRNA targets and washing of membranes were performed as for Southern blot analysis, but the hybridization temperature was set at 42 °C.

Detection of probe binding.

Detection of probe binding was done as for Southern blot analysis.

RNA dot blots.

In order to evaluate relative transgene transcription in the different transgenic potato lines, various amounts of total plant RNA was applied to a nylon membrane (Hybond N⁺). The membrane was probed as for the Northern blots.

CHAPTER THREE.

PRODUCTION OF TRANSGENIC POTATO LINES.

Introduction.

In order to produce transgenic potato lines, it was first necessary to transfer the plant expression vectors containing the PLRV-CP gene to *A. tumefaciens* strain LBA 4404 in a triparental mating process. Subsequently, the presence of the binary plasmids in *A. tumefaciens* was confirmed using a GUS assay and PCR analysis.

In order to develop a potato leaf disk transformation system, optimal regeneration from leaf disks was identified, and kanamycin tolerance experiments were performed. Pilot transformation experiments were conducted with the plant expression vector pBI121 in order to identify the best transformation conditions, before transformation experiments with the PLRV-CP gene began. Vacuum infiltration of stem sections in an *A. tumefaciens* suspension was also investigated as an alternative transformation method. Transgenic lines were produced, hardened off for growth in the glasshouse and analysed for phenotypic abnormalities and ploidy instability.

The materials and methods used to produce the transgenic potato lines are outlined in Chapter Two.

Results and Discussion.

3.1. *Agrobacterium tumefaciens*.

Successful triparentally mated *A. tumefaciens* cells containing one of the six binary plasmids viz. pBI121, pBI121-1, pBI121-13, pBI121-14, pBI121-15 or pBI121-16 were selected for kanamycin resistance and thus neomycin phosphotransferase II activity on LB medium supplemented with 100 $\mu\text{g.ml}^{-1}$ rifampicin and 100 $\mu\text{g.ml}^{-1}$ kanamycin at 28 °C. Colonies that grew on this medium had acquired kanamycin resistance from the respective pBI121 based plasmid. Colonies were subsequently tested for GUS activity using the small-scale GUS fluorometric assay (Fig.3.1). In this assay, fluorescence of a well under UV light indicates the presence of GUS activity.

Results from the kanamycin selection of triparentally mated cells and GUS assay are outlined in Table 3.1. Colonies that were selected on LB containing 100 $\mu\text{g.ml}^{-1}$ kanamycin and 100 $\mu\text{g.ml}^{-1}$ rifampicin are designated Km^+ and colonies that tested positive in the GUS assay are designated GUS^+ .

The reason for the discrepancy between the number of colonies that grew on kanamycin-containing medium and the number of colonies that demonstrated GUS activity may be that gene rearrangements or deletions have occurred during the mating process.

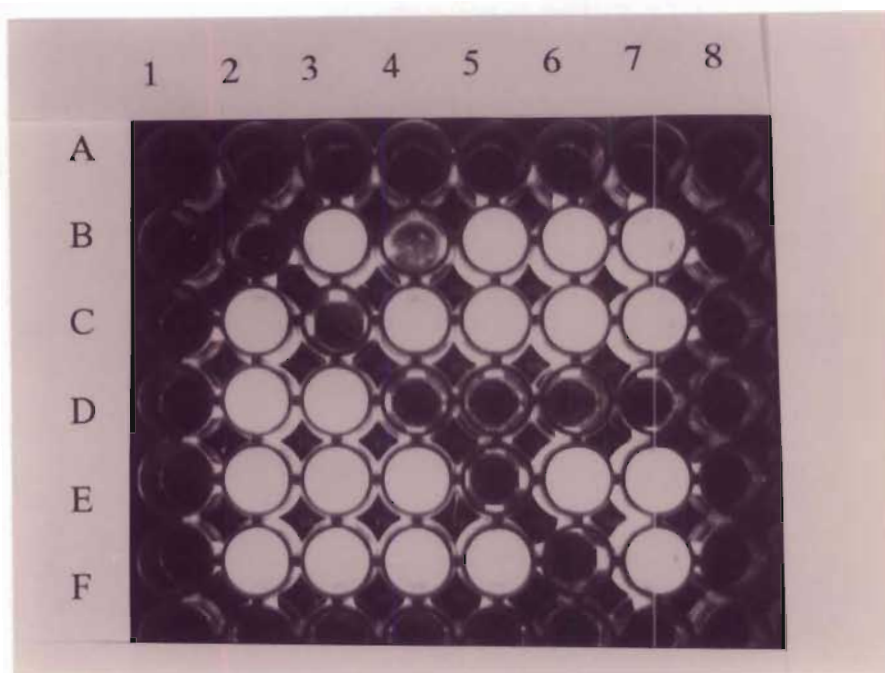


Fig.3.1. Small-scale GUS fluorometric assay of transgenic *A. tumefaciens* strains carried out in a Nunc ELISA plate.

Wells containing buffer only (blank wells) are found in row A, column 1 and B2, C3, D4, D5, D6, D7, E5 and F6. The positive control [*E. coli* HB 101 (pBI121)] was placed in well B3, and the negative control (*A. tumefaciens* LBA 4404) was placed in well B4. The remaining fluorescing wells contain cells from *A. tumefaciens* strains containing either pBI121, pBI121-1, pBI121-13, pBI121-14, pBI121-15 or pBI121-16, which demonstrates GUS activity.

Table 3.1. Number of colonies produced in triparental matings with various binary plasmids.

PLASMID	NO. OF COLONIES KM ⁺	NO. OF COLONIES GUS ⁺
pBI121	4	4
pBI121-13	10	5
pBI121-1	5	5
pBI121-14	6	5
pBI121-15	5	4
pBI121-16	5	3

PCR analysis using the *Agrobacterium* quick screen method (AN *et al.* 1992) was performed in order to show that the binary vector had been co-integrated into *A. tumefaciens*. PCR reactions using either the PLRV-CP gene, GUS or neomycin phosphotransferase II (*nptII*) specific primers were conducted, and the results are presented in Fig.3.2.

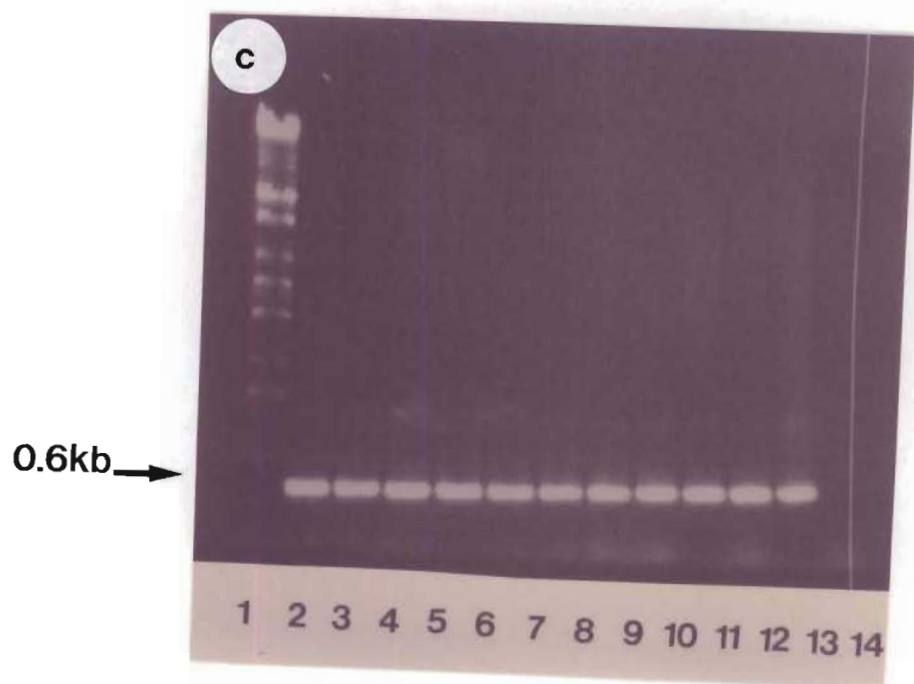
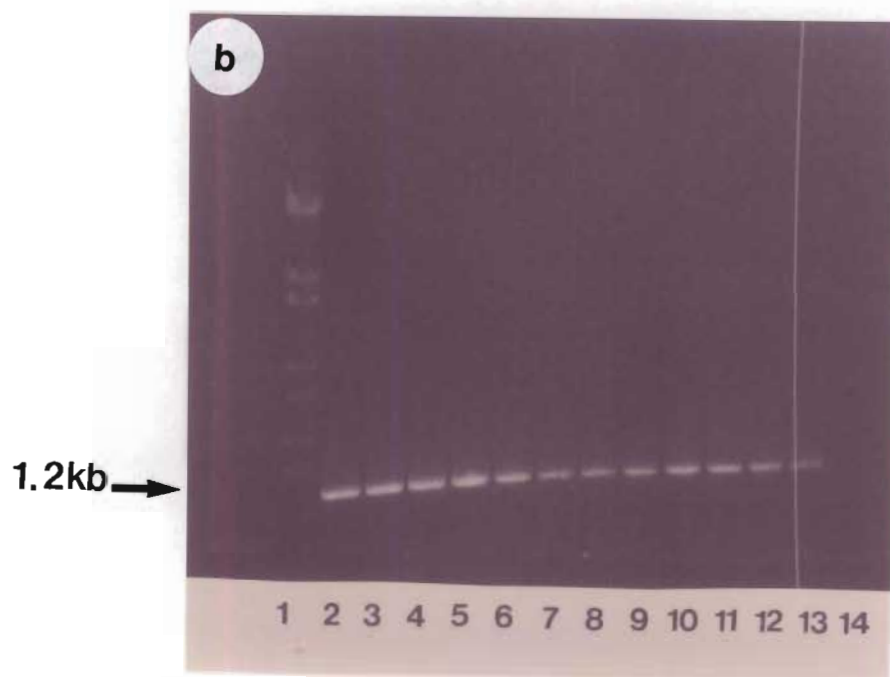
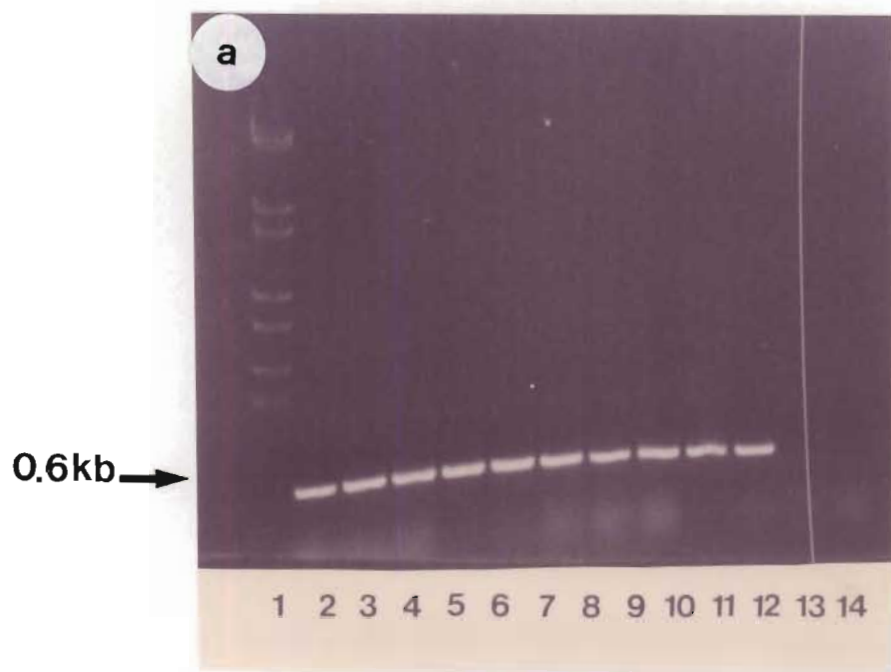


Fig. 3.2. PCR analysis of transgenic *A. tumefaciens* colonies generated by triparental matings.

(a) PCR analysis using the palindromic primer specific for the PLRV-CP gene. DNA amplified by PCR corresponds to the size of the PLRV-CP gene (0.6 kb).

(b) PCR analysis using primers specific for the GUS gene. DNA amplified by PCR corresponds to the size of the GUS gene (1.2 kb).

(c) PCR analysis using primers specific for the *nptII* gene. DNA amplified by PCR corresponds to the size of the *nptII* gene (0.6 kb).

DNA samples were loaded on the gel in the following order:

(1) Sty I marker consisting of DNA fragments of the following sizes in kb:

19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.

(2) *A. tumefaciens* LBA 4404 (pBI121-16) (pRK2013) 3

(3) *A. tumefaciens* LBA 4404 (pBI121-16) (pRK2013) 5

(4) *A. tumefaciens* LBA 4404 (pBI121-15) (pRK2013) 2

(5) *A. tumefaciens* LBA 4404 (pBI121-15) (pRK2013) 3

(6) *A. tumefaciens* LBA 4404 (pBI121-14) (pRK2013) 2

(7) *A. tumefaciens* LBA 4404 (pBI121-14) (pRK2013) 6

(8) *A. tumefaciens* LBA 4404 (pBI121-13) (pRK2013) 3

(9) *A. tumefaciens* LBA 4404 (pBI121-13) (pRK2013) 4

(10) *A. tumefaciens* LBA 4404 (pBI121-1) (pRK2013) 2

(11) *A. tumefaciens* LBA 4404 (pBI121-1) (pRK2013) 5

(12) pBI121-16

(13) *A. tumefaciens* LBA 4404

(14) H₂O

Two representative colonies were chosen for each binary plasmid. All samples analyzed showed amplification of DNA corresponding to the size of the gene: 0.6 kb in the case of the PLRV-CP and *nptII* genes and 1.2 kb in the case of the GUS gene. Water controls showed no gene amplification, and amplification of the GUS and *nptII* genes were only seen for the pBI121 sample. However, the GUS gene was amplified from the *A. tumefaciens* LBA 4404 sample (the negative control), while this was not the case in PCR reactions with the other 2 primer sets. The presence of the GUS gene in the negative control can probably be explained as non-specific gene amplification by the GUS primers. However, the results of the PCR analysis do indicate the presence of the PLRV-CP, GUS and *nptII* genes, thus indicating the presence of the binary plasmid in the transgenic *A. tumefaciens* strains.

3.2. Regeneration from leaf disks.

Regeneration from leaf disks using the R1AT two-step procedure.

The R1AT two-step procedure was previously used at ARC-Roodeplaat to regenerate shoots indirectly from potato leaf disks (VENTER, pers. comm.). In this procedure, leaves approximately 1 to 1.5 cm in length were removed from 4-6 week old *in vitro* grown potato plantlets. Three mm sections from the tip and base were cut off and the leaf was placed adaxial side facing upwards onto R1AT medium in petri dishes. Leaf explants were sub-cultured onto fresh medium weekly. As soon as callus production could be visualised at the cut surfaces of the leaves (5-8 weeks depending on the cultivar), the leaves were transferred to R1AT medium without auxin (designated R1AT-). Production of buds from the callus occurred over a period of at least 7 weeks. Sometimes the callus or meristemoid structures

did not develop further but remained lumpy. As soon as emerged shoots were 1 cm in length or longer, they were excised from the callus mass and rooted on Osborne medium. At least one shoot per leaf was produced for both cultivar Late Harvest (LH) and BP₁ (VENTER, pers. comm.). In addition, it was found that LH regeneration occurred in a shorter time period than for BP₁. As the R1AT two-step regeneration procedure showed a fairly good regenerative response (more than 60% of explants produced shoots), it was decided to use this regeneration medium in potato leaf disk transformations. The two R1AT-based media were adapted to include the ethylene inhibitor silver thiosulphate (STS) as it was reported by HULME and co-workers (1992) that incorporation of STS into regeneration media had a beneficial effect on plantlet regeneration from potato leaf tissue. However, DE BLOCK (1988) found that potato shoots generated on regeneration medium containing STS were difficult to root. This was not found to be a problem for the cultivars used in this study. In addition, it was shown that the presence of STS during the co-cultivation period with *A. tumefaciens* and the early regeneration phase increased the number of transformed foci (or centres) for three potato cultivars (HIGGINS 1992). A possible reason for the beneficial effect of STS is that ethylene is thought to play a role in the induction of pathogen defence responses in plants, and interference with the ethylene response may help to overcome some resistance in the plant to *A. tumefaciens*. It was also found by other potato researchers at ARC-Roodeplaat that incorporation of STS into tissue culture media increased the surface area of the *in vitro* leaves (VENTER, pers. comm.)

Optimisation of shoot regeneration from leaf disks.

VISSER (1991) reported that *A. tumefaciens*-mediated transformation lowered the regeneration efficiency of potatoes 5 to 100 fold. Although leaves incubated on R1AT showed a fairly good regenerative response, it was decided to investigate other published regeneration protocols in an attempt to develop an optimal regeneration procedure. HULME and co-workers (1992) assessed a number of procedures and developed a so called genotype independent method for regeneration of potato plants from leaf disks. In this procedure, leaf explants were subjected to an overnight pulse of high cytokinin and auxin (10 mg.l^{-1} NAA and 10 mg.l^{-1} BA), a 7 to 10 day treatment on a callus induction medium with moderate amounts of growth regulators (0.02 mg.l^{-1} IAA and 2.25 mg.l^{-1} BA) followed by shoot regeneration on an auxin free medium containing 2.25 mg.l^{-1} BA and 4.8 mg.l^{-1} GA₃. Leaves were subcultured to fresh medium weekly, and STS was included in all solid media.

In a preliminary experiment, the exact method of HULME *et al.* (1992) was implemented for cultivars BP₁ and LH as well as the existing R1AT two-step method in order to compare the response of the two cultivars. After a 12 week culture period, explants from both cultivars and both treatments produced shoots.

Following this experiment, three treatments for the cultivars BP₁ and LH were implemented:

A: R1AT for 5 weeks until callus production occurred, followed by 7 weeks on NAA-free medium;

B: an overnight pulse, callus induction for 2 weeks followed by 10 weeks shoot initiation

treatment

C: callus induction for 2 weeks followed by 10 weeks shoot initiation.

The third treatment was included in order to evaluate the influence of the pulse on shoot regeneration. Results presented in Table 3.2 were obtained after 12 weeks.

Table 3.2: Comparison of three different regeneration treatments for potato cultivars LH and BP₁. Treatment A, B and C are outlined in the text.

CULTIVAR	TREATMENT	% EXPLANTS PRODUCING CALLUS	% EXPLANTS PRODUCING SHOOTS
LH	A	85	30*
LH	B	95	15*
LH	C	100	100**
BP ₁	A	100	0
BP ₁	B	83	0
BP ₁	C	80	0

* one shoot produced per explant

** average number of shoots produced per explant is 8.95

From these results it appears that the method from HULME *et al.* (1992) without the pulse treatment is optimal for cultivar LH, both in number of responding explants and in number of shoots produced per explant. In this experiment, no shoot production was visualised for BP₁ explants 12 weeks after initiation of the experiment, although shoot production was seen at this time in the initial experiment. However, the BP₁ explants did show good callus production (ranging from 80 to 100% of the explants) and it is probable that shoot production would have occurred had the duration of the experiment extended beyond 12 weeks. In addition, the results of this experiment confirms the previously seen trend that BP₁ regeneration is slower than LH.

Following this experiment it was decided that either regeneration treatment A or C, ie the two-step R1AT approach or the two-step method excluding the pulse treatment adapted from HULME *et al.* (1992), would be implemented in further transformation procedures.

3.3. Antibiotic tolerance experiments.

In order to establish the optimum kanamycin concentration at which selection of transformed cells could occur, kanamycin tolerance regeneration experiments were carried out for both leaf disks and plantlets. Kanamycin concentrations ranging from 0 to 200 µg.ml⁻¹ were added to the rooting medium and the two R1AT-based media. Kanamycin is a deoxystreptamine aminoglycoside capable of binding ribosomal components in the cell and thus inhibiting protein synthesis (SAMBROOK *et al.* 1989). For both cultivar LH and BP₁, 50 µg.ml⁻¹ kanamycin was the minimum concentration at which regeneration was suppressed from leaf

disks, and 25 $\mu\text{g.ml}^{-1}$ kanamycin was the minimum concentration at which rooting was suppressed.

VISSER (1991) recommended using 250 $\mu\text{g.ml}^{-1}$ cefotaxime in regeneration media in order to eliminate *A. tumefaciens* from the tissue cultures following the co-cultivation period. In tandem with the kanamycin tolerance experiments, the influence of cefotaxime on regeneration from leaf disks was investigated. All leaf explants for both cultivars produced callus when incubated on the two-step R1AT media containing 250 $\mu\text{g.ml}^{-1}$ cefotaxime over the time of the experiment (8 weeks). Thus the use of cefotaxime as an anti-bacterial agent did not adversely affect the regenerative response of potato leaf disks, and may in fact stimulate regeneration, although this was not directly investigated in the present study. Cefotaxime has been shown to stimulate callus growth, embryogenesis and regeneration in wheat and barley (BORRELLI *et al.* 1992). Cefotaxime exerts its antibacterial effect by specifically binding to the penicillin binding proteins of the inner membrane of the bacterial envelope (MATHIAS & BOYD 1986), and does not have a molecular structure like that of any known plant growth regulator. A possible explanation may be that cefotaxime is converted by the plant cell metabolism to an unknown compound with growth regulator- like activity.

3.4. Pilot transformation experiments.

HIGGINS (1992) reported that a number of factors influenced *A. tumefaciens*-mediated transformation of potato leaf disks. These included preculturing of the explants, varying inoculation and co-cultivation times and the influence of acetosyringone on gene transfer.

Using *A. tumefaciens* LBA 4404 (pBI121) (pRK2013) and the R1AT regeneration medium, the influence of these factors was investigated in pilot transformation experiments of potato cultivars LH and BP₁ in order to establish an optimal protocol.

VISSER (1991) suggested pre-culturing potato leaf disks for 2 days on MS medium prior to inoculation with *A. tumefaciens* and subsequent transformation. This approach was routinely implemented for all transformation experiments in the present study.

Results of pilot experiments for cultivar LH are summarized in Tables 3.3 and 3.4. Results were taken 10 weeks after the beginning of an experiment. In the case of varying dip times, a 2 day co-cultivation time was implemented and a 20 minute dip period was used when investigating the influence of co-cultivation time.

Table 3.3: The influence of *A. tumefaciens* LBA 4404 (pBI121) (pRK2013) dip times and a two-day co-cultivation period on subsequent callus formation from leaf disks.

Results were taken 10 weeks after the initiation of the experiment.

DIP TIME (minutes)	% EXPLANTS PRODUCING CALLUS
2	0
5	12.5
10	40
15	28
20	42.85

Table 3.4: The influence of co-cultivation time of *A. tumefaciens* LBA 4404 (pBI121) (pRK2013) with leaf disks after a 20 minute dip period on subsequent callus formation.

Results were taken 10 weeks after the initiation of the experiment.

CO-CULTIVATION PERIOD (days)	% EXPLANTS PRODUCING CALLUS
0	10
2	10
5	68
7	55

From these results it appears that optimal regeneration, as scored by callus production, was obtained using a 20 minute dip time and 5 day co-cultivation period. These results were obtained with cultivar LH and extrapolated for BP₁. Unfortunately large percentages of the explants were contaminated by mite infestation in the growth rooms and therefore the shoot production from the callus could not be scored. However, a few explants escaped the mite contamination and some shoots were generated from the callus (Fig.3.3). Shoots were subsequently rooted on Osborne medium containing kanamycin (Fig.3.4). Nine LH and two BP₁ putative transgenic lines were generated. Molecular analyses of transgenic potato plants will be discussed in Chapter 4.

The influence of acetosyringone on transformation was also investigated. Acetosyringone is a stimulus for Ti plasmid *vir* gene induction, and thus plays a role in transfer of T-DNA from *A. tumefaciens* to the plant cell. Acetosyringone was added to the *A. tumefaciens* cell suspensions at a concentration of 10 mg.l⁻¹ (VISSER 1991). At this concentration, acetosyringone did not increase callus production in comparison to callus production from explants that had not been exposed to the acetosyringone. Alternatively, acetosyringone may be added directly to the regeneration media, but this approach was not investigated in the present study. HIGGINS (1992) found that acetosyringone decreased the number of potato leaves that had no transformed foci, thus increasing the frequency of gene transfer.

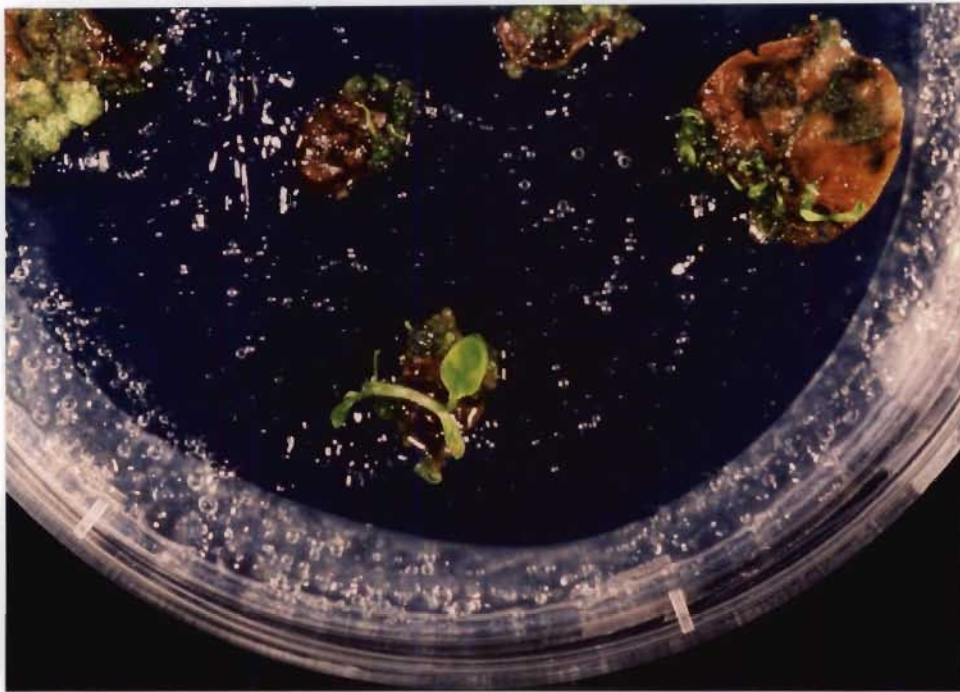


Fig.3.3. Indirect shoot regeneration from potato leaf disks (cv Late Harvest) cultured on R1AT- medium containing 50 $\mu\text{g.ml}^{-1}$ kanamycin and 250 $\mu\text{g.ml}^{-1}$ cefotaxime.

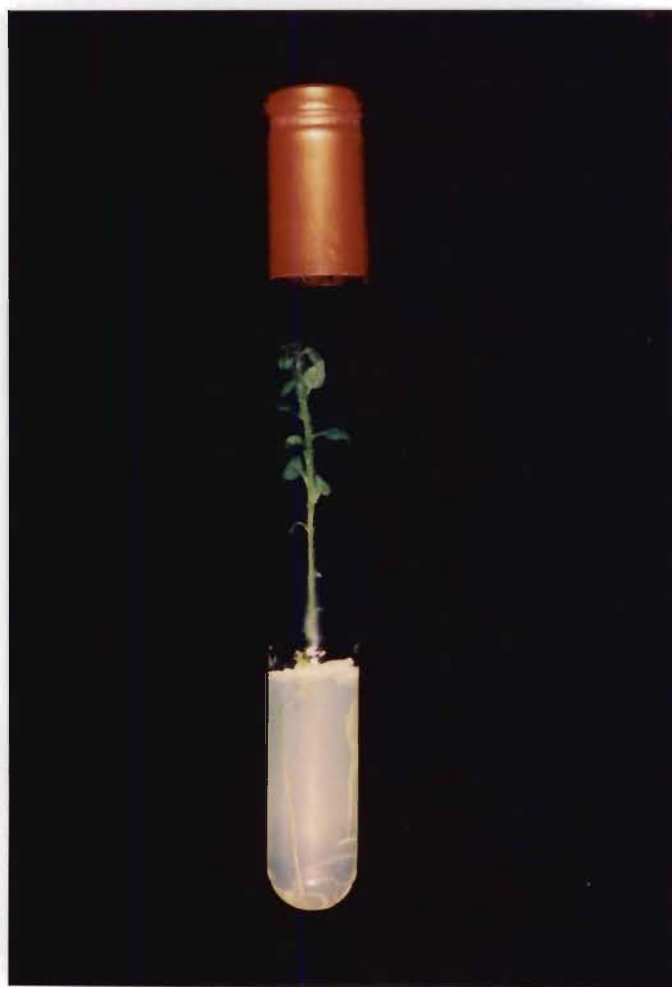


Fig.3.4. Rooting of a transgenic potato plantlet (cv Late Harvest) on Osborne medium containing $25\text{ }\mu\text{g.ml}^{-1}$ kanamycin and $250\text{ }\mu\text{g.ml}^{-1}$ cefotaxime.

3.5. Leaf disk transformation with PLRV-CP constructs.

Potato leaf disk transformations.

Leaf disks from cultivars LH and BP₁ were transformed with the sense and antisense PLRV-CP constructs (pBI121-16 and pBI121-13) using the optimised transformation conditions and either the R1AT 2-step regeneration procedure or the 2-step regeneration procedure adapted from HULME *et al.* (1992). Unfortunately many of the explants were contaminated by the mites. However, some of the explants placed on the R1AT regeneration media survived, from which 24 putative transgenic plantlets could be regenerated. The results of the molecular analyses of these plants are presented in Chapter 4.

Transformation by vacuum infiltration.

Transformation by vacuum infiltration was investigated as a method to improve transformation frequency and reduce the regeneration period. In an initial experiment, nodal sections from *in vitro* plantlets were submerged in flasks containing *A. tumefaciens* and a vacuum was drawn for various time periods. Nodal sections were rooted on Osborne medium containing 25 µg.ml⁻¹ kanamycin and 250 µg.ml⁻¹ cefotaxime. More than 60% of putatively transformed explants died, but 7.7% of plantlets subjected to 2 minutes infiltration rooted, 12.5% from the 10 minute treatment rooted and 29 % from the 20 minute treatment rooted. In subsequent transformations of LH and BP₁ plantlets a 20 minute infiltration period was used. When plantlets, with the apical bud and roots removed, were used as starting material, 72% survived the infiltration process and the co-cultivation period. These plantlets were subsequently cut into nodal sections and transferred to Osborne medium containing 25 µg.ml⁻¹ kanamycin and

250 $\mu\text{g}.\text{ml}^{-1}$ cefotaxime. Of the LH lines, 22.2% rooted while 17.6% of the BP₁ lines rooted. This amounted to a total number of 20 putative transgenic lines. Evaluation of transgene insertion in these lines will be discussed in Chapter 4.

3.6. Hardening off of transgenic potato plants.

Transgenic plantlets were hardened off in a glasshouse (Fig.3.7) and back-up *in vitro* plantlets were maintained. Notes were made on the general phenotypic appearance of the glasshouse potato plants and the number of tubers produced. On the whole, PLRV-CP transgenic plants appeared similar to plants that had not been exposed to a tissue culture phase, except for a few discrepancies in leaf morphology which included epinasty, dwarfism and the absence of leaf petioles. However, it could not be determined whether these abnormalities were due to somaclonal variation, positional effects of the inserted gene or to general glasshouse conditions. All plants produced tubers, which were harvested and subsequently induced to sprout. Plants from the second generation (T₂) were subjected to molecular analyses.

3.7. Determination of ploidy level.

The ploidy level of the PLRV CP-transgenic potato lines was determined in order to ascertain the degree of genetic stability of the plants. If somaclonal variation had occurred during the tissue culture stages, this may be seen as an increase or decrease in the ploidy level. The traditional method for determining the ploidy of a plant involves counting the number of chromosomes in a cell taken from a root tip squash. This method is difficult and time



Fig.3.7. Transgenic potato plants (cv Late Harvest) in the glasshouse.

consuming. A much more efficient method involves counting the number of chloroplasts found in the epidermal guard cells, as the number of chloroplasts present can be taken as an indication of the ploidy level. This method has been used for many crops, including melons, tobacco, tomato and potato (KOORNNEEF *et al.* 1989).

Table 3.5 outlines the average number of chloroplasts found in the guard cells of the abaxial epidermis of transgenic potato lines. Ten guard cells pairs (surrounding one stomatal pore) were randomly selected and the number of chloroplasts in each pair were counted, as illustrated in Fig. 3.6.

The "rule of thumb" when evaluating ploidy levels of potato plants using this method is that dihaploid plants have 10 to 15 chloroplasts per guard cell, triploid plants 15 to 20 chloroplasts and tetraploid plants 20 to 24 chloroplasts per guard cell pair. A tetraploid number was expected but the average number for all lines, including non-transformed potatoes, fell in the triploid range. KOORNNEEF and co-workers (1989) found that bigger leaves on older tomato plants tended to have higher numbers of chloroplasts per guard cell pair, and it may be that age has an influence on the number of chloroplasts present for potatoes as well. However, it appears that the transgenic lines analyzed here are genotypically and phenotypically comparable to non-transformed plants, thus suggesting that the transgenic plants are genetically stable.

Table 3.5 : Average number of chloroplasts found per guard cell pair from 10 guard cell pairs selected randomly from each PLRV-CP transgenic potato line.

POTATO LINE	AVE. NO. OF CHLOROPLASTS
Non-transformed LH	17.3
LH 16.2	16.5
LH 16.3	16.8
LH 13.1	15.3
LH 13.2	17.0
LH 13.3	18.2
LH 13.4	17.7
Non-transformed BP ₁	18.8
BP ₁ 16.5	17.4
BP ₁ 16.8	17.7
BP ₁ 16.9	17.6
BP ₁ 16.11	17.8
BP ₁ 16.12	18.2

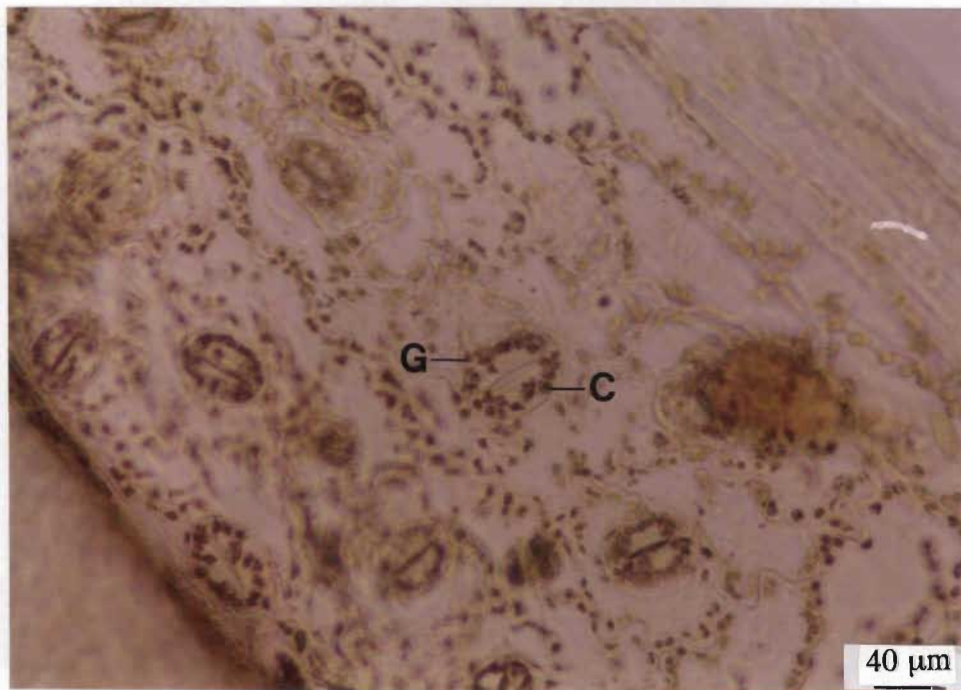


Fig.3.6. Abaxial epidermis of a transgenic potato line stained with potassium iodide, showing stomatal pores and guard cells. Chloroplasts in the guard cells are stained dark brown.

C= chloroplast; G= guard cell

CHAPTER FOUR

MOLECULAR ANALYSES OF TRANSGENIC POTATO LINES.

Introduction.

The transgenic potato lines generated either by leaf disk transformation or by vacuum infiltration were subjected to a number of molecular analyses. It was first necessary to verify stable transgene insertion into the plant genome, and this was shown for all three transgenes using PCR analysis. In addition, the presence of the PLRV-CP gene was confirmed using Southern blot analysis. Secondly, it was necessary to verify that the transgenes were expressed in the regenerated plants. This was investigated at the protein level using a fluorometric assay in the case of the GUS gene. In the case of the PLRV-CP gene, protein production was investigated using DAS-ELISA and immunoblot analysis. Expression at the mRNA level was investigated using Northern blot analysis.

The materials and methods used for the molecular analyses are outlined in Chapter Two.

Results and Discussion

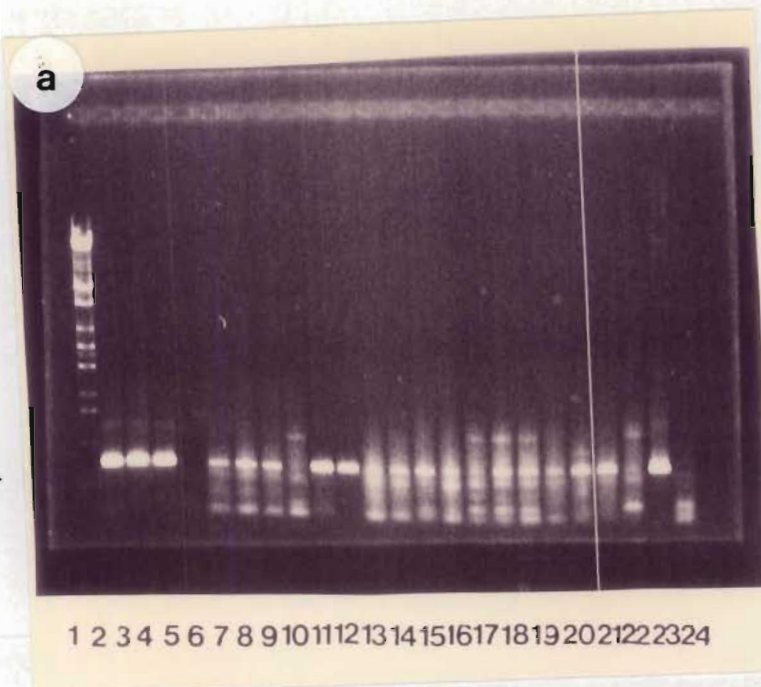
4.1. Verification of transgene insertion.

PCR analysis

PCR analysis of transgenic plantlets generated by leaf disk transformation.

Twelve of the twenty-four putative PLRV-CP transgenic potato lines generated by leaf disk transformation were randomly selected for PCR analysis. The 12 selected lines consisted of 4 LH lines transformed with the antisense orientation of the PLRV-CP gene (LH 13.1; LH 13.2; LH 13.3; LH 13.4), 2 LH lines transformed with the sense orientation of the PLRV-CP gene (LH 16.2; LH 16.3), 1 BP₁ line transformed with the antisense PLRV-CP gene (BP₁ 13.6) and 5 BP₁ lines transformed with PLRV-CP in the sense orientation (BP₁ 16.5; BP₁ 16.8; BP₁ 16.9; BP₁ 16.11; BP₁ 16.12). Small-scale genomic DNA extractions were made from leaf material, and the DNA from each individual line was used in 3 different PCR reactions with the PLRV-CP, GUS and *nptII* primers respectively. The PCR products were separated using gel electrophoresis, visualised by ethidium bromide staining and sized by comparison to the Sty I DNA marker. Results obtained are outlined in Fig. 4.1.

0.6kb →



1.2kb →



0.6kb →

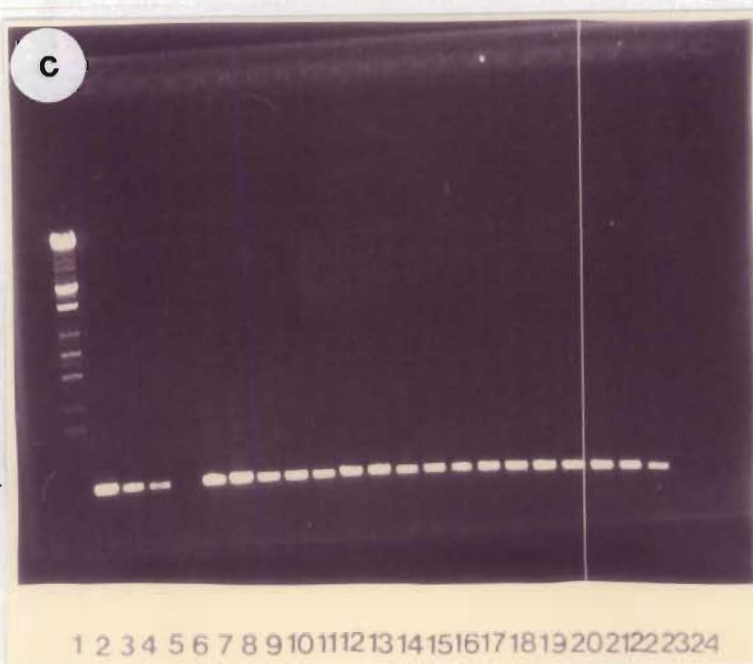


Fig.4.1. PCR analysis of transgenic potato plantlets generated by leaf disk transformation.

- (a) PCR analysis using the palindromic primer specific for the PLRV-CP gene. DNA amplified by PCR corresponds to the size of the PLRV-CP gene (0.6 kb).
- (b) PCR analysis using primers specific for the GUS gene. DNA amplified by PCR corresponds to the size of the GUS gene (1.2 kb).
- (c) PCR analysis using primers specific for the *nptII* gene. DNA amplified by PCR corresponds to the size of the *nptII* gene (0.6 kb).

DNA samples were loaded on the gel in the following order:

- (1) Sty I DNA marker, consisting of DNA fragments of the following sizes in kb: 19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.
- (2) LH 13.1
- (3) LH 13.2
- (4) LH 13.3
- (5) LH 13.4
- (6) LH 16.2
- (7) LH 16.3
- (8) Non-transformed LH
- (9) LH transformed with pBI121
- (10) Blank well
- (11) BP₁ 13.6
- (12) BP₁ 16.5
- (13) BP₁ 16.8
- (14) BP₁ 16.9
- (15) BP₁ 16.11
- (16) BP₁ 16.12
- (17) Non-transformed BP₁
- (18) BP₁ transformed with pBI121
- (19) pBI121-13
- (20) H₂O control.

For all three sets of reactions, a DNA band was not visualised for the water control samples, indicating that there was no contaminating DNA in the PCR chemicals. All of the PLRV-CP transgenic lines showed amplification of the PLRV-CP, GUS and *nptII* genes as bands corresponding to the size of the gene were visualised (0.6 kb in the case of the PLRV-CP and *nptII* genes, and 1.2 kb in the case of the GUS gene). Reactions with the PLRV-CP specific palindromic primer showed no DNA band for the negative controls (untransformed material and plants transformed with pBI121), and a band was seen for the positive control (pBI121-13). However, in the case of the GUS primers, DNA was amplified in the untransformed samples, even when the annealing temperature in the PCR reaction was increased to 70°C. The same result was obtained for tomato and tobacco, and it appears that non-specific DNA amplification occurs when the GUS primers are used. When PCR was performed with *nptII* primers, the untransformed LH DNA showed no DNA amplification, whereas a band was visualised for the untransformed BP₁ sample. It is possible that this band was due to minute amounts of contaminating DNA in the PCR tube, or to a contamination introduced during the DNA extraction.

PCR Analysis of transgenic plantlets generated by vacuum infiltration.

Twenty putative transgenic lines were generated by vacuum infiltration. Small-scale genomic DNA extractions were made from leaf material, and the DNA from each individual line was used in 3 different PCR reactions with the PLRV-CP, GUS and *nptII* primers respectively. The PCR products were separated using gel electrophoresis, visualised by ethidium bromide staining and sized by comparison to the Sty I DNA marker. Results obtained are outlined in Fig.4.2.

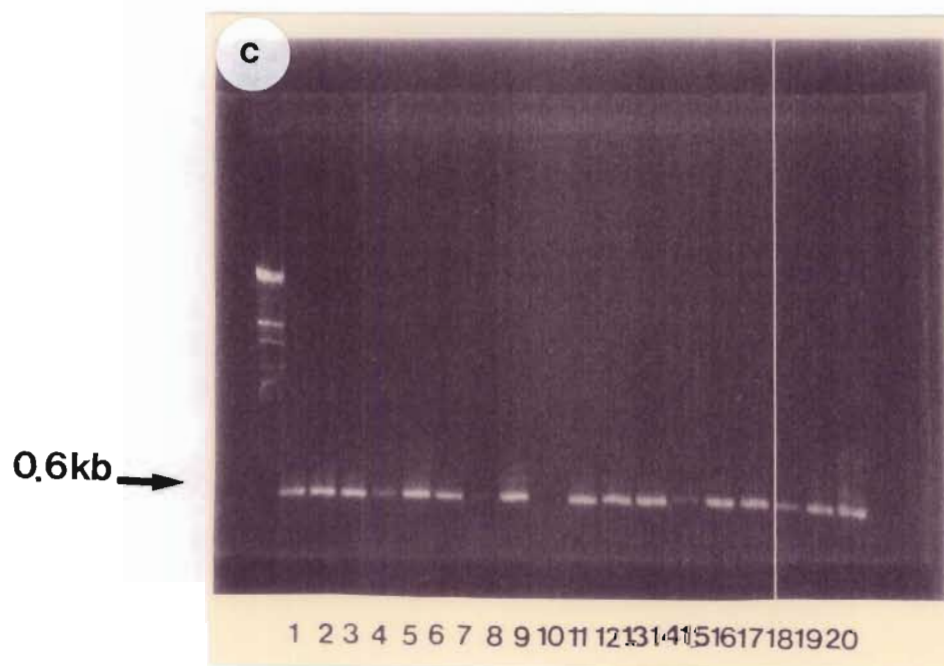
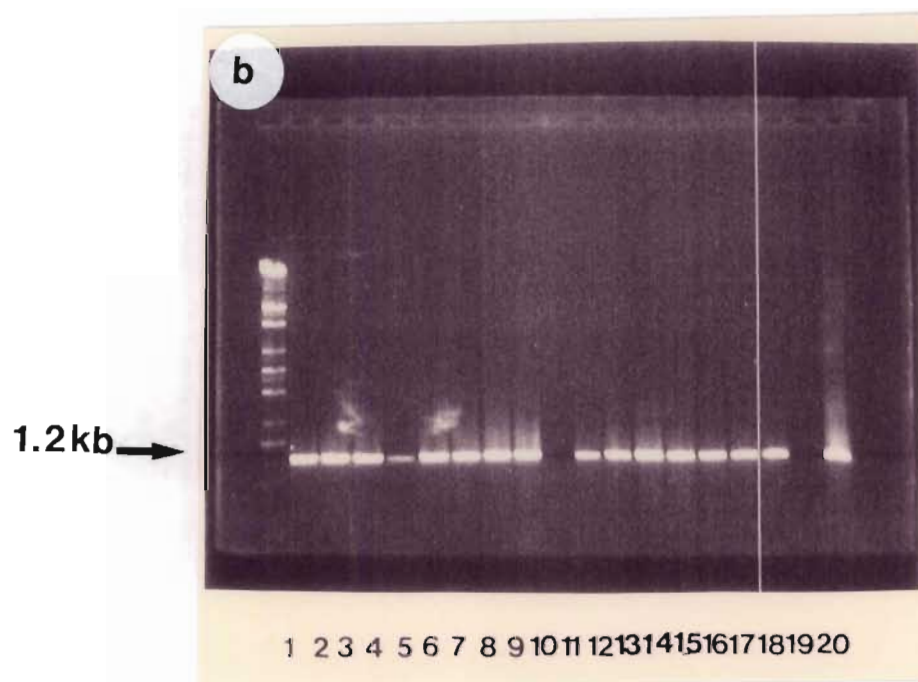
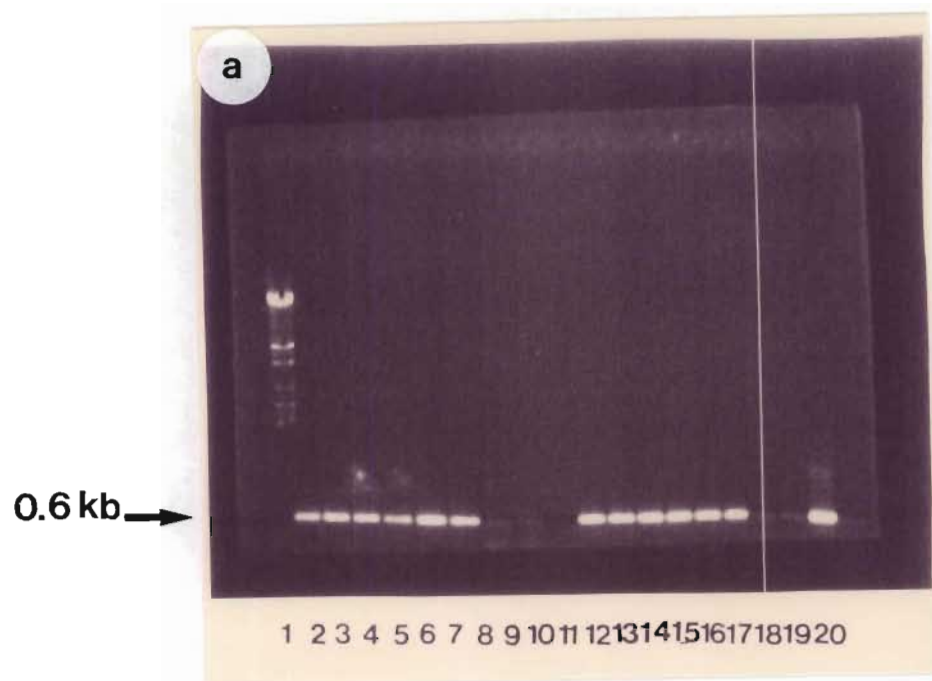


Fig.4.2. PCR analysis of transgenic potato plantlets generated by vacuum infiltration.

- (a) PCR analysis using the palindromic primer specific for the PLRV-CP gene. DNA amplified by PCR corresponds to the PLRV-CP gene (0.6 kb).
- (b) PCR analysis using primers specific for the GUS gene. DNA amplified by PCR corresponds to the GUS gene (1.2 kb).
- (c) PCR analysis using primers specific for the *nptII* gene. DNA amplified by PCR corresponds to the *nptII* gene (0.6 kb).

DNA samples were loaded on the gel in the following order:

- (1) Sty I DNA marker, consisting of DNA fragments of the following sizes in kb: 19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.
- (2) LH V16.2.1
- (3) LH V16.2.2
- (4) LH V16.2.4
- (5) LH V15.2.1
- (6) LH V14.1.1
- (7) LH V14.1.2
- (8) LH V14.1.3
- (9) LH V14.1.4
- (10) LH V13.3.1
- (11) LH V13.3.2
- (12) LH V13.3.3
- (13) LH V16.1.3
- (14) LH V16.1.4
- (15) LH V16.1.5
- (16) LH V13.3.1
- (17) LH V16.3.2
- (18) LH V16.4.1
- (19) LH V16.4.2
- (20) LH V13.3.1
- (21) LH V13.3.2
- (22) Non-transformed BP₁
- (23) pBI121-13
- (24) H₂O

As can be seen in Fig.4.2., only three of the putative transgenic lines showed amplification of all 3 transgenes, viz. LH V 16.2.1; LH V 16.2.2 and LH V 16.2.4. In the case of potato lines which did not show amplification of any of the 3 transgenes, it is possible that gene transfer did not take place, resulting in the production of "escapes". When only one or two transgenes were visualised in a line by PCR analysis, it is possible that gene rearrangements or deletions occurred during the gene transfer process and subsequent integration into the plant genome. These potato lines were discarded, and the 3 lines that showed amplification of all 3 transgenes were subjected to Southern blot analysis in order to confirm transgene insertion.

Southern blot analysis.

Southern blot analysis was used to verify the results of the PCR analysis. In addition, information generated from Southern blots was used to determine the number of inserted copies of the PLRV-CP gene.

The pBI121-13 and pBI121-16 plasmids, which contain the PLRV-CP gene, were used as the positive control. In order to provide an accurate control, the amount of plasmid (and therefore PLRV-CP gene) corresponding to the number of genome copies in 10 µg potato DNA had to be calculated. The calculations were as follows:

The constant amount of DNA present in a tetraploid potato cell is 8.4 pg (BENNETT & SMITH 1976). Ten micrograms of potato genomic DNA was used in the Southern blot analysis, so the number of genome copies in 10 µg is 1.2×10^6 .

1pg DNA = 0.965×10^6 kb (BENNETT & SMITH 1976)

so 1 kb of DNA = 1.03×10^6 pg

The size of pBI121-13/pBI121-16 is 15.5 kb,

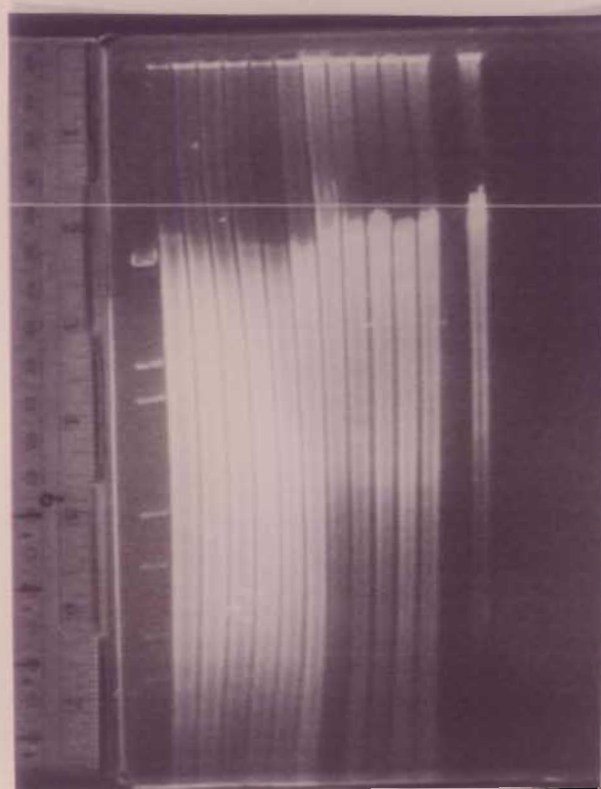
so 15.5 kb of DNA = 1.6×10^5 pg

The mass of 1.2×10^6 copies of pBI121-13/pBI121-16 is therefore 19.2 pg.

Presuming only one PLRV-CP transgene was incorporated into the tetraploid potato cell from which an individual transgenic line was subsequently regenerated, 19.2 pg of pBI121-13 or pBI121-16 represents the number of PLRV-CP gene molecules present in 10 µg of potato genomic DNA.

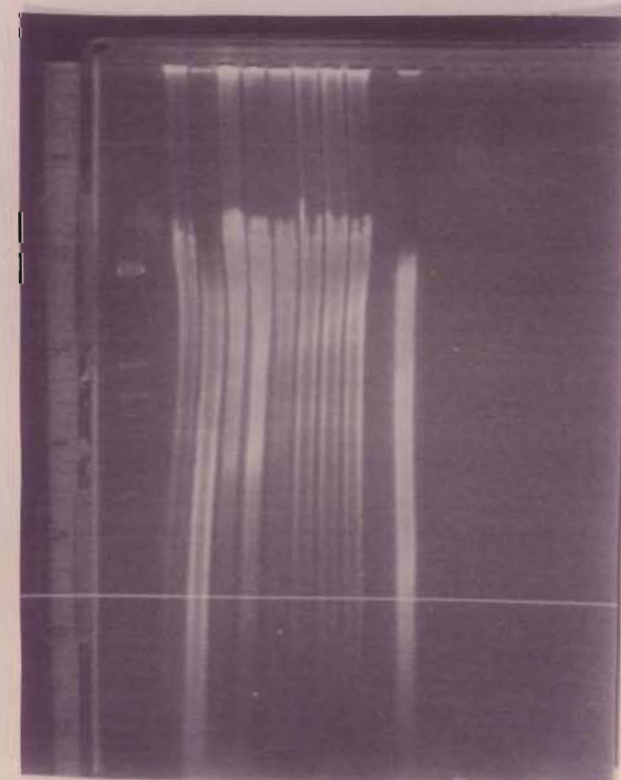
Potato genomic DNA (10 µg) was cut with one of two restriction endonucleases (Fig.4.3), depending on the orientation of the inserted PLRV-CP gene in the line. The enzymes were chosen in order to distinguish between the inserted PLRV-CP gene, and the gene present in *A. tumefaciens*. It is possible that the PCR analysis had amplified DNA from endogenous *A. tumefaciens* which had remained after the tissue culture phase, thus creating a false positive result. Genomic DNA from plants containing the PLRV-CP gene in the sense orientation (Fig.1.4) was cut with PstI, whereas genomic DNA from plants containing the PLRV-CP gene in the antisense orientation was cut with EcoRI (Fig.1.5). Restriction digestion of pBI121-16 will produce a fragment 7.77 kb in size, which will hybridize to the PLRV-CP gene probe. Restriction digestion of pBI121-13 will produce a fragment 13.3 kb in size, which will hybridize to the PLRV-CP probe. In both cases, if the transgene has inserted into the plant genome, bands for the plant samples should be of different sizes in comparison to the plasmid controls.

a



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

b



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig.4.3. Visualisation of restriction digestion of transgenic potato genomic DNA.

Genomic DNA (10 µg) was cut with PstI or EcoRI, the DNA fragments were separated by gel electrophoresis and visualised by ethidium bromide staining.

(a) Gel showing LH samples.

DNA samples were loaded on the gel in the following order:

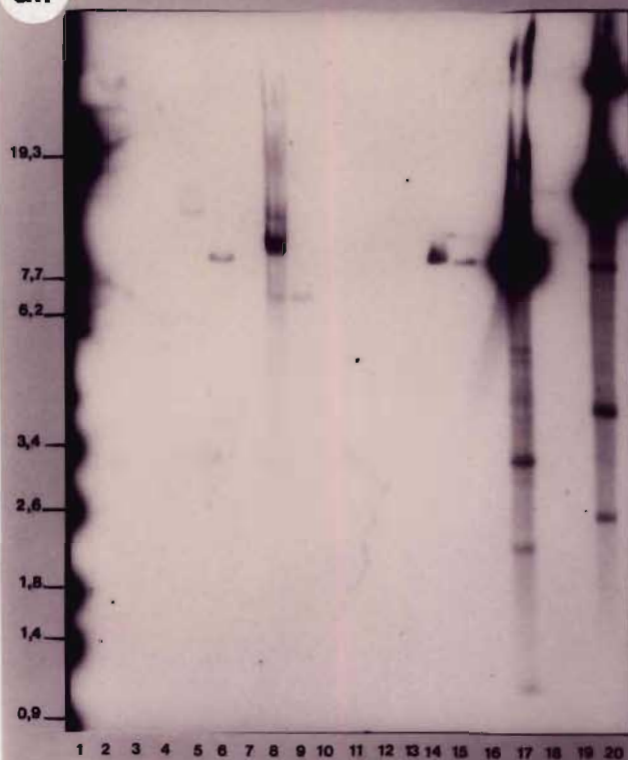
- (1) Sty I DNA marker, consisting of DNA fragments of the following sizes in kb: 19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.
- (2) Non-transformed LH digested with EcoR1
- (3) LH 13.1 digested with EcoR1
- (4) LH 13.2 digested with EcoR1
- (5) LH 13.3 digested with EcoR1
- (6) LH 13.4 digested with EcoR1
- (7) Empty lane
- (8) LH 16.2 digested with Pst1
- (9) LH 16.3 digested with Pst1
- (10) LH V16.2.1 digested with Pst1
- (11) LH V16.2.3 digested with Pst1
- (12) LH V16.2.4 digested with Pst1
- (13) Empty lane
- (14) Non-transformed LH + 192 pg pBI121-16 digested with Pst1
- (15) 19.2 pg pBI121-16 digested with Pst1
- (16) Empty lane
- (17) 19.2 ng pBI121-16 digested with Pst1.

(b) Gel showing BP₁ samples.

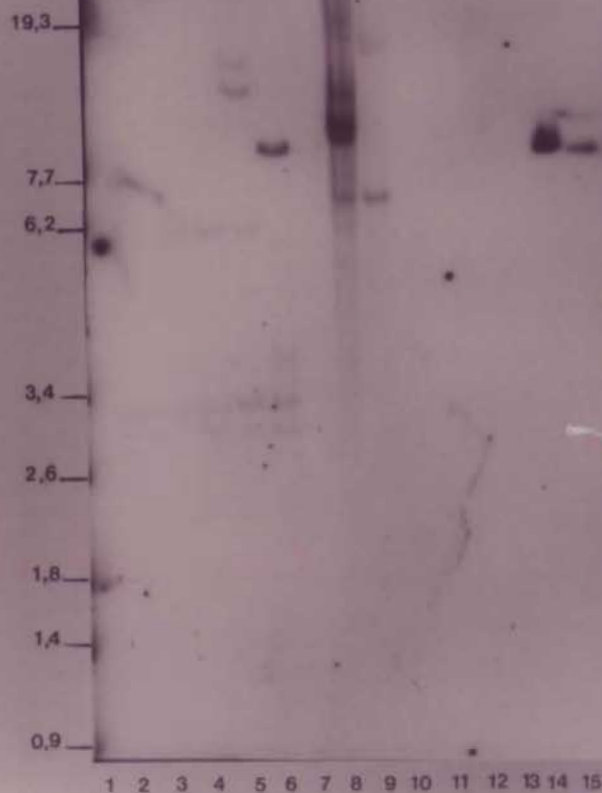
DNA samples were loaded on the gel in the following order:

- (1) Sty I DNA marker, consisting of DNA fragments of the following sizes in kb: 19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.
- (2) Empty lane
- (3) Non-transformed BP₁ digested with Pst1
- (3) BP₁ 13.6 digested with EcoR1
- (4) BP₁ 16.5 digested with Pst1
- (5) BP₁ 16.8 digested with Pst1
- (6) BP₁ 16.9 digested with Pst1
- (7) BP₁ 16.11 digested with Pst1
- (8) BP₁ 16.11 digested with Pst1
- (9) BP₁ 16.12 digested with Pst1
- (10) Empty lane
- (11) Empty lane
- (12) Non-transformed BP₁ + 192 pg pBI121-13 digested with EcoR1
- (13) Empty lane
- (14) 19.2 pg pBI121-16 digested with Pst1
- (15) Empty lane
- (16) 19.2 ng pBI121-16 digested with Pst1
- (17) Empty lane.

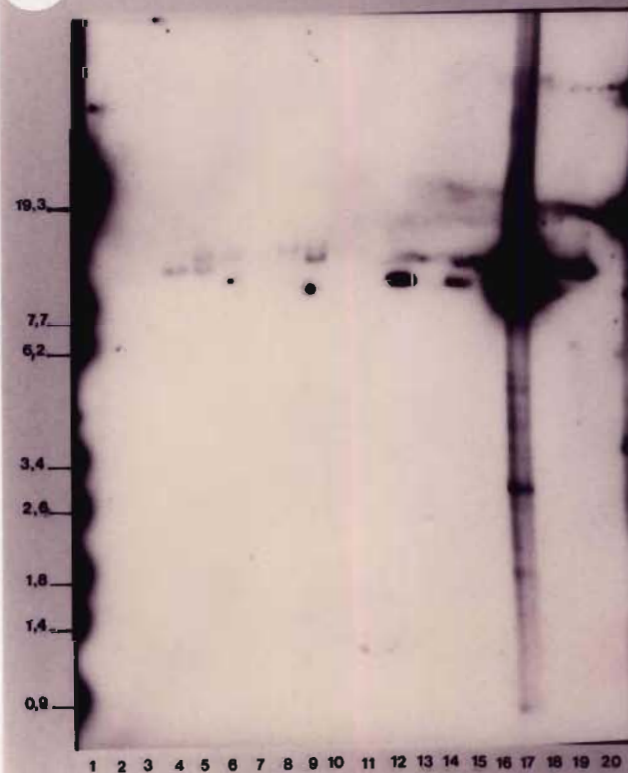
a.i



a.ii



b.i



b.ii

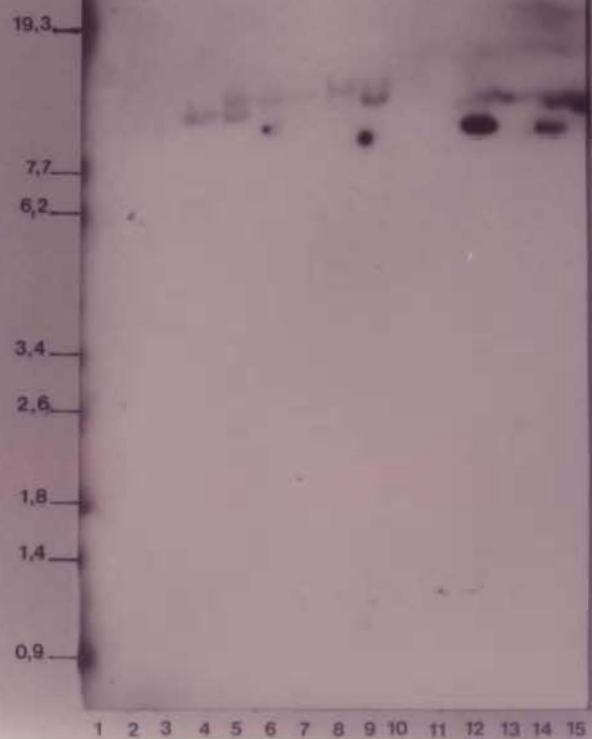


Fig. 4.4. Southern blot analysis of PLRV-CP transgenic potato lines.

PLRV-CP DNA was labelled with DIG-dUTP and used to probe a Boehringer Mannheim positively charged nylon membrane onto which the genomic DNA from Fig.4.3 had been transferred and fixed by baking at 80°C for 90 minutes. Blots were exposed to Hyperfilm^R for 15 minutes (i) or 60 minutes (ii).

(a) Blots showing LH samples

DNA samples were loaded in the following order:

- (1) Sty I DNA marker, consisting of DNA fragments of the following sizes in kb: 19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.
- (2) Non-transformed LH digested with EcoR1
- (3) LH 13.1 digested with EcoR1
- (4) LH 13.2 digested with EcoR1
- (5) LH 13.3 digested with EcoR1
- (6) LH 13.4 digested with EcoR1
- (7) Empty lane
- (8) LH 16.2 digested with Pst1
- (9) LH 16.3 digested with Pst1
- (10) LH V16.2.1 digested with Pst1
- (11) LH V16.2.3 digested with Pst1
- (12) LH V16.2.4 digested with Pst1
- (13) Empty lane
- (14) Non-transformed LH + 192 pg pBI121-16 digested with Pst1
- (15) 19.2 pg pBI121-16 digested with Pst1
- (16) Empty lane
- (17) 19.2 ng pBI121-16 digested with Pst1
- (18) 19.2 pg pBI121-13 digested with EcoR1
- (19) Empty lane
- (20) 19.2 ng pBI121-13 digested with EcoR1.

(b) Blots showing BP₁ samples.

DNA samples were loaded in the following order:

- (1) Sty I DNA marker, consisting of DNA fragments of the following sizes in kb: 19.329; 7.743; 6.223; 4.254; 3.472; 2.69; 1.882; 1.489; 0.925.
- (2) Empty lane
- (3) Non-transformed BP₁ digested with Pst1
- (3) BP₁ 13.6 digested with EcoR1
- (4) BP₁ 16.5 digested with Pst1
- (5) BP₁ 16.8 digested with Pst1
- (6) BP₁ 16.9 digested with Pst1
- (7) BP₁ 16.11 digested with Pst1
- (8) BP₁ 16.11 digested with Pst1
- (9) BP₁ 16.12 digested with Pst1
- (10) Empty lane
- (11) Empty lane
- (12) Non-transformed BP₁ + 192 pg pBI121-13 digested with EcoR1
- (13) Empty lane
- (14) 19.2 pg pBI121-16 digested with Pst1
- (15) Empty lane
- (16) 19.2 ng pBI121-16 digested with Pst1
- (17) Empty lane
- (18) 19.2 pg pBI121-13 digested with EcoR1
- (19) Empty lane
- (20) 19.2 ng pBI121-13 digested with EcoR1.

The PLRV-CP gene, used as a probe in the Southern hybridization experiments, was labelled either with ^{32}P -dCTP or DIG-dUTP in separate hybridization experiments. It was found that the DIG fluorometric detection system using the alkaline phosphate substrate CDP-Star^R was more sensitive than detection of ^{32}P irradiation. In addition, results using the DIG system were obtained after 15 minutes exposure to Hyperfilm^R (Fig.4.4.a.i and Fig.4.4.b.ii) whereas results were obtained after 2 weeks exposure using ^{32}P . However, detection with the DIG system using CDP-Star^R was so sensitive that 19.2 ng of both the pBI121-1 and pBI121-13 plasmids loaded as a positive control (corresponding to 1000 copies of the PLRV-CP gene) resulted in over-exposure and detection of non-specific hybridization after only 15 minutes (lane 17 and 20 in Fig.4.4.a.i and Fig.4.4.b.i). Subsequently, these lanes were blocked off and the blots were re-exposed to Hyperfilm^R for 60 minutes, in order to see the fainter bands (Fig.4.4.a.ii and Fig.4.4.b.ii).

The number of bands obtained in Fig.4.4, which are an indication of probe binding, are listed as follows: LH 13.1 - 2; LH 13.2 - 3; LH 13.3 - 5; LH 13.4 - 3; LH 16.2 - 3; LH 16.3 - 2; LH V16.2.1 - 0; LH V16.2.3 - 1; LH V16.2.4 - 1; BP₁ 13.6 - 0; BP₁ 16.5 - 2; BP₁ 16.8 - 1; BP₁ 16.9 - 1; BP₁ 16.11 - 2 and BP₁ 16.12 - 2. In order to confirm that the number of bands visualised corresponds to the presence of separate PLRV-CP gene insertions in the different transgenic potato lines, it would be necessary to re-digest the genomic DNA with a different restriction endonuclease and repeat the Southern blot analysis. The same number of bands would be expected. Unfortunately there was insufficient genomic DNA remaining for an additional digestion. However, it appeared that the digestions had run to completion (Fig.4.3) as smears were visualised for each

sample, and thus the number of bands produced can be taken as a fairly good indication of copy numbers. No bands were seen for the non-transformed BP₁ sample (lane 3, Fig.4.4.b), whereas one very faint band of about 3 kb was seen for the non-transformed LH sample (lane 2, Fig.4.4.a.ii). Detection of this band was most probably due to non-specific hybridization. All the LH samples that had this band also had additional bands, and these additional bands represented copies of the inserted PLRV-CP gene. A band of 13.3 kb was obtained for one copy of pBI121-13 (19.2 pg) digested with EcoR1 (Lane 18, Fig.4.4.a.i and Fig.4.4.b.i), and a band of 7.7 kb was obtained for one copy of pBI121-1 (19.2 pg) digested with PstI (lane 15, Fig.4.4.a.ii and lane 14, Fig.4.4.b.ii). No bands of these sizes were obtained for any of the transgenic plant samples, indicating that the bands seen for the transgenic plant samples represented the PLRV-CP gene inserted into the plant genome, and not the PLRV-CP gene present in endogenous *A. tumefaciens*. One of the transgenic lines generated by vacuum infiltration (LH V16.2.1) did not have a band, and it is likely that the positive result obtained in the PCR analysis was due to the presence of endogenous *A. tumefaciens* in the DNA extraction. In addition, only a very faint band of approximately 14 kb was obtained for LH V16.2.3 and LH V16.2.4 (lane 11 and 12, Fig.4.4.a.ii). As these plants were generated from axillary buds vacuum infiltrated in a *A. tumefaciens* suspension, they are most probably chimeras as it is unlikely that the bacteria infected every cell in the bud. If they are chimeras, this would explain why the bands are so light, as the transgenes would not be present in every cell.

From the results of the Southern blot analysis, it thus appears that vacuum infiltration is a less effective transformation method for potatoes in comparison to leaf disk

transformation, as results of the PCR analysis were confirmed using Southern blot analysis in the case of the plants produced by leaf disk transformation. However, the results from the PCR analysis for only two out of the three plants generated by vacuum infiltration were confirmed by Southern blot analysis.

4.2. Verification of transgene expression.

Kanamycin resistance.

Transgenic plants were regenerated using selection on media containing kanamycin. In general, if a putative "kanamycin-resistant" explant is capable of a further regenerative response, for example the production of callus or roots, on medium containing kanamycin, the tissue is considered to be kanamycin resistant (VISSER 1991). Kanamycin was included in all rooting media, and the plantlets thus generated were taken as producing neomycin phosphotransferase II.

Expression of GUS.

GUS expression in pBI121 transgenic potato lines.

Pilot transformation experiments were conducted using *A. tumefaciens* LBA 4404 (pBI121) (pRK2013) in order to establish an optimal transformation protocol. Nine LH and 2 BP₁ putative transgenic shoots were produced, which rooted *in vitro* on Osborne

medium containing kanamycin. These putative transgenic lines were subjected to the small-scale GUS fluorometric assay (Fig. 4.5). Four out of the nine LH lines tested positive for GUS activity. The discrepancy between the number of plantlets that showed resistance to kanamycin and expression of the GUS transgene may be attributed to the production of escapes. These are plantlets generated from a cell which was not transformed by *A. tumefaciens*, but which was tolerant to the kanamycin present in the regeneration medium, and thus capable of regeneration. However, it is unlikely that escapes were produced as control leaf explants not exposed to the *A. tumefaciens* and placed on regeneration medium containing $50 \mu\text{g} \cdot \mu\text{l}^{-1}$ kanamycin did not regenerate. Alternatively, the GUS gene may be present although unexpressed. It has been reported that non-expression of introduced traits in transgenic plants following selection for a linked selectable marker gene is not due to the absence of the transgene, but rather to its inactivation, usually by methylation (FINNEGAN & McELROY 1994).

The histochemical GUS assay was also performed on transgenic LH potato lines in order to verify GUS expression. In all transgenic plants tested the results of both the histochemical and the small-scale fluorometric assays corresponded to one another. Figure 4.6 is an example of the histochemical GUS assay performed on a cross section through a root of a transgenic LH line. GUS expression was found predominantly in the vascular tissue, and to a lesser degree in the root cortex. In the plant expression vector pBI121 the GUS gene is under control of the nominally constitutive Cauliflower Mosaic Virus 35S (CaMV 35S) promoter. GUS expression in the transgenic potatoes was thus found to be tissue specific, and corresponded to GUS expression in the vascular tissue of transgenic tobacco (BENFY & CHUA 1989).

Both BP₁ transgenic lines tested positive for GUS activity using the small-scale GUS fluorometric assay (results not shown).

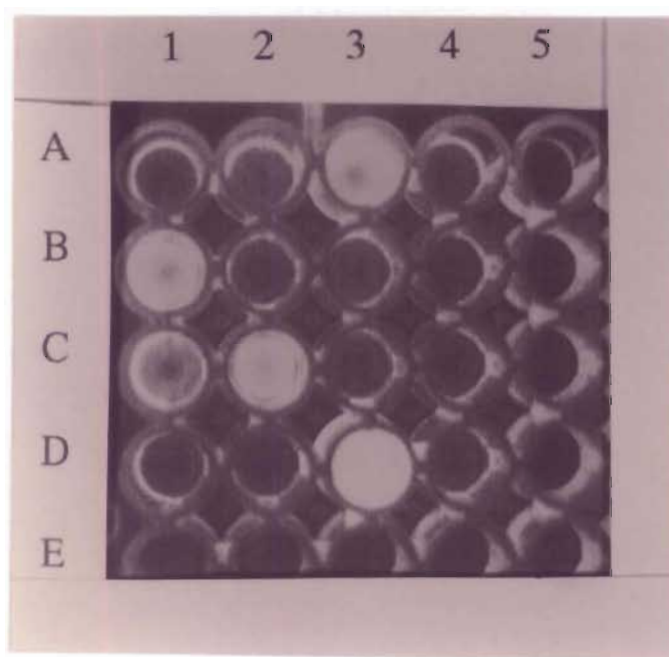


Fig.4.5. Small-scale GUS assay of pBI121 transgenic potato lines (cv Late Harvest).

Blank wells are found in A1; B2; C3; D4 and D1. D2 contains the negative control (non-transformed leaf material) and D3 contains the positive control [*A. tumefaciens* LBA 4404 (pBI121) (pRK2013)]. The remaining wells contain macerated leaf material from individual transgenic potato lines. Fluorescence of a well indicates the presence of GUS activity.



Fig.4.6. Cross section through a root from a pBI121 transgenic potato plant (cv Late Harvest) incubated in X-Gluc buffer. Blue colouration indicates the presence of GUS activity.

GUS expression in PLRV-CP transgenic potato lines.

It was found that the small-scale GUS fluorometric assay was not sensitive enough to detect GUS activity in the PLRV-CP transgenic potato lines. It was then decided to use the full-scale fluorometric assay (JEFFERSON *et al.* 1987).

As GUS catalyses the cleavage of MUG to MU, the amount of MU produced by a tissue sample in reaction buffer can be taken as an indication of the extent of GUS activity. A protein standard curve was constructed of absorbance readings at 595 nm plotted against BSA concentration (Fig.4.7). Using the software program Curfit™, a best fit linear curve was constructed with the values depicted in Fig.4.7, which was subsequently used to determine the protein concentrations of the plant samples. In addition, a MU standard curve of MU concentration plotted against fluorescence was constructed (Fig.4.8), which was then used to determine the MU concentration of the plant samples at set times using Curfit™. The amount of MU produced in μmol per mg plant protein was calculated and plotted against time (Fig. 4.9-4.12). There appears to be a certain amount of background MU present in potatoes, since most samples at time=0 showed MU production of about $2 \mu\text{mol.mg}^{-1}$ (Fig. 4.9-4.12). In the non-transformed extracts these levels remain constant over time, and are most probably due to endogenous MU and not endogenous GUS activity (Fig.4.9c and Fig.4.11a). Plants transformed with the antisense PLRV-CP gene (designated 13) or the pBI121 construct showed increased MU production over time (Fig. 4.10 and 4.11), whereas plants transformed with the sense PLRV-CP gene (designated 16) showed stable MU presence over time, or only slightly increased MU production (Fig.4.9 and 4.12). This result was expected because of the presence of a translational stop codon (TAG) at the 3' end of the PLRV-CP gene

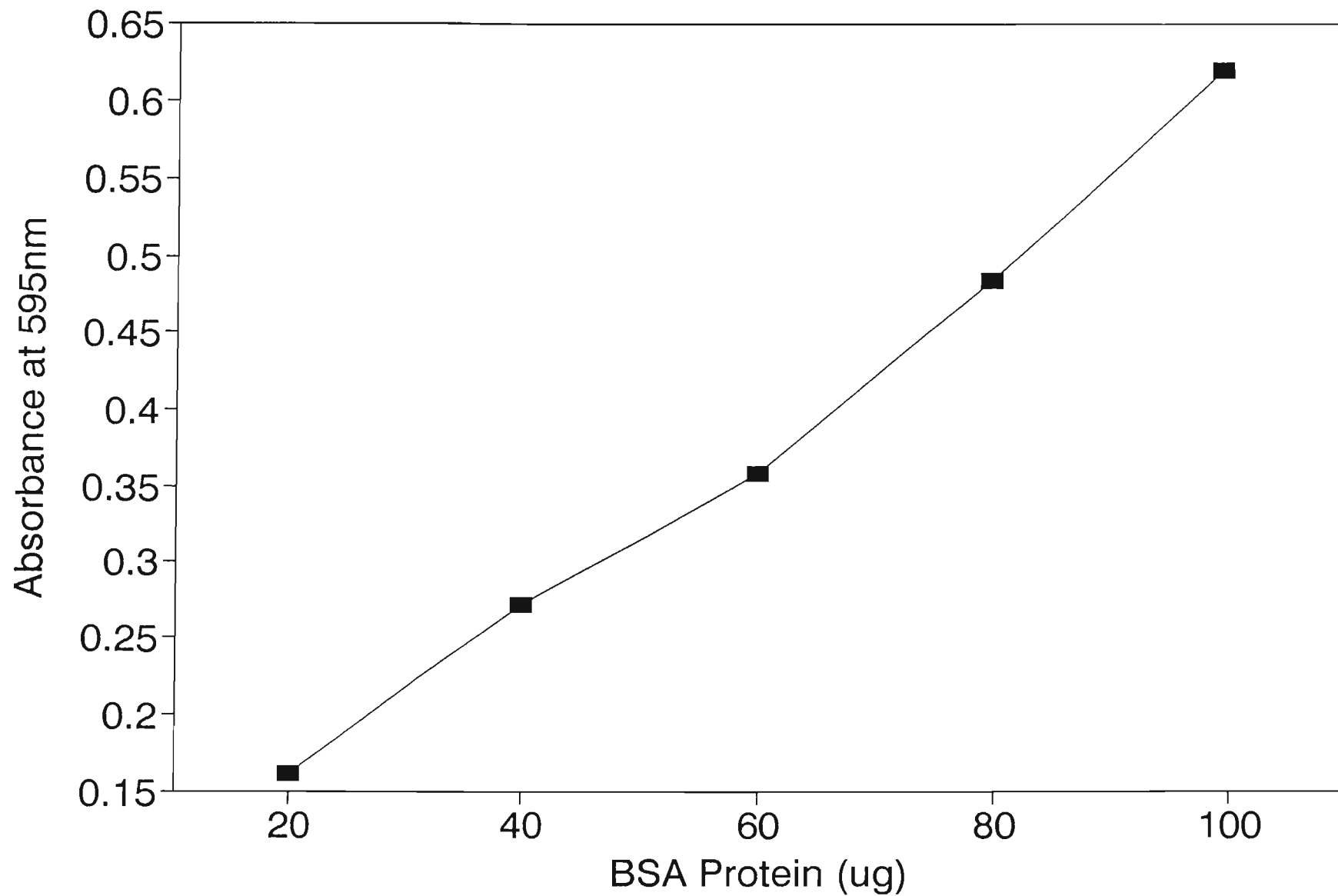


Fig.4.7. Protein standard curve of A_{595} plotted against BSA concentration for use in the GUS assay.

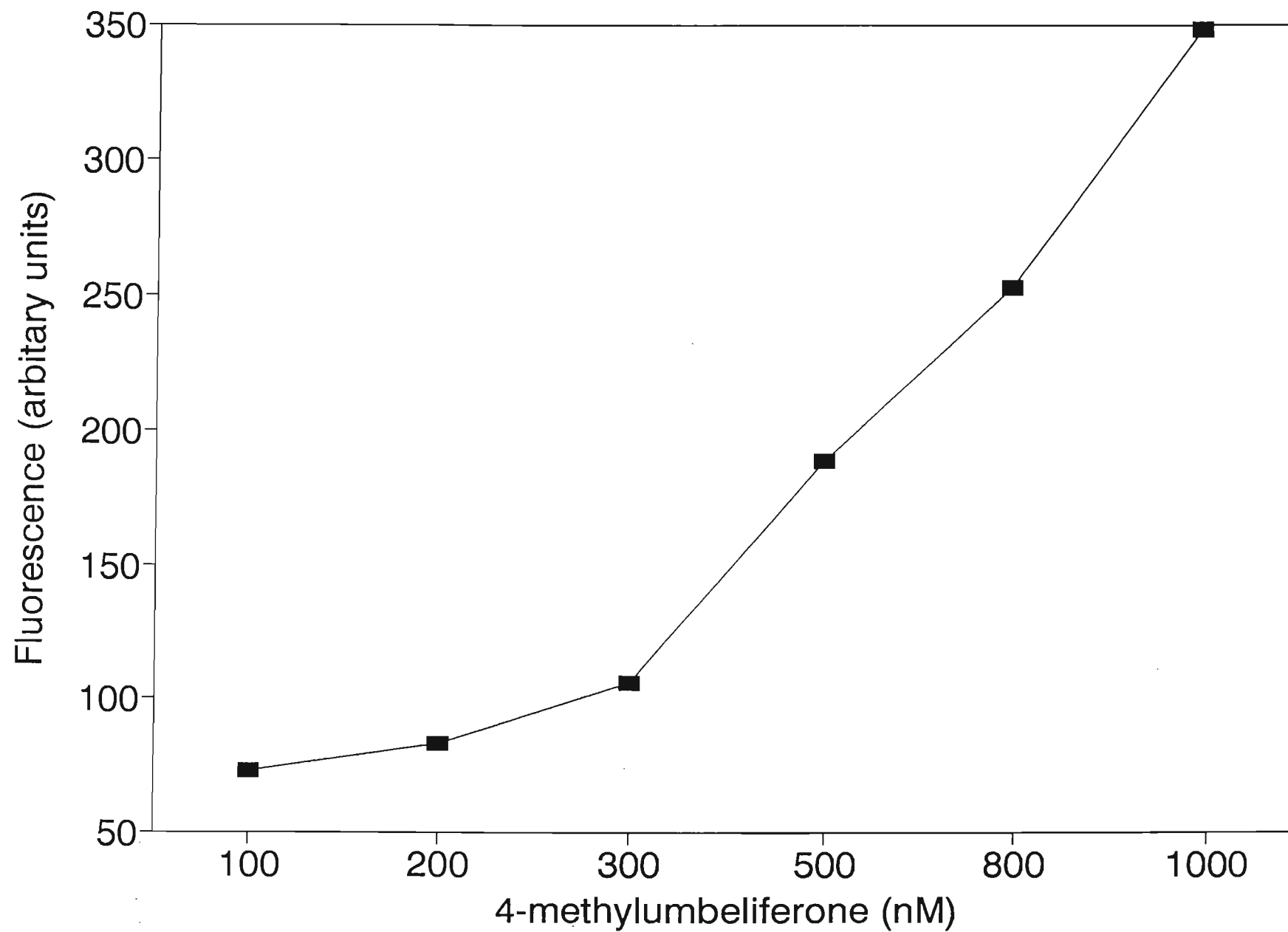


Fig.4.8. MU standard curve of fluorescence plotted against MU concentration.

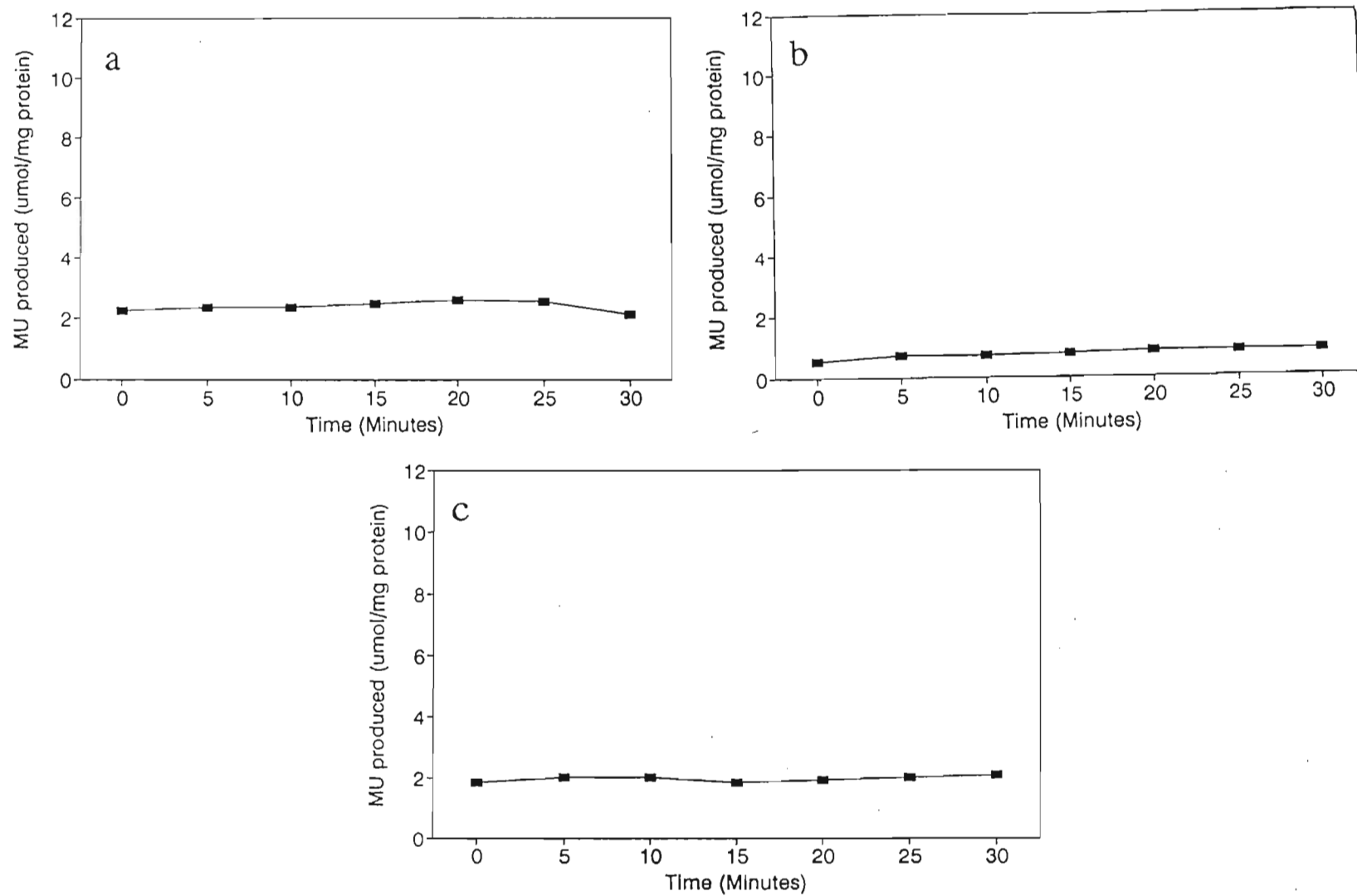


Fig.4.9. The amount of MU produced by cleavage of MUG over time for transgenic potatoes.

- (a) LH 16.2
- (b) LH 16.3
- (c) Non-transformed LH

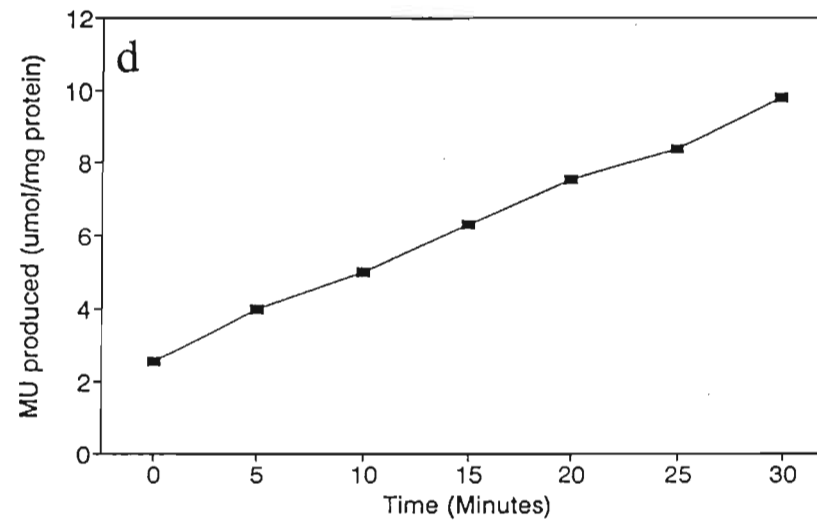
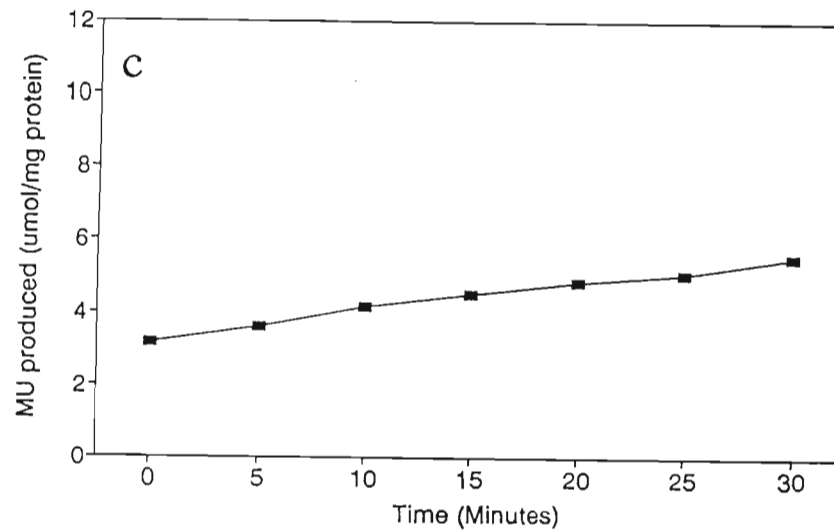
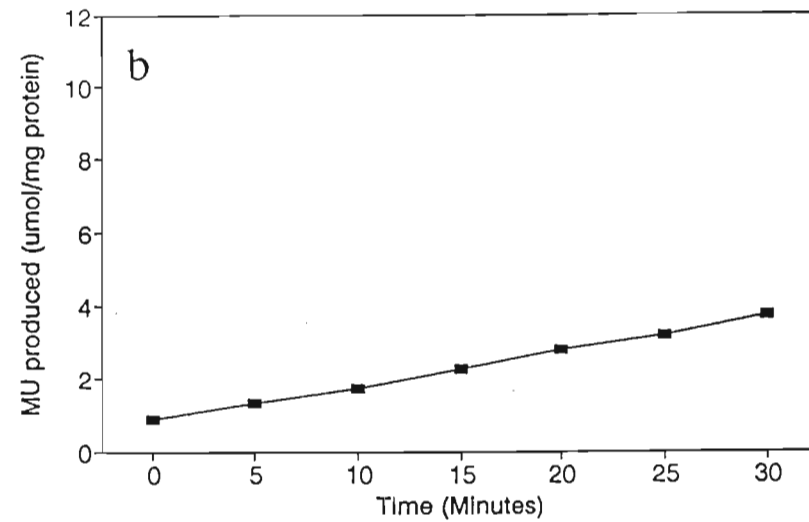
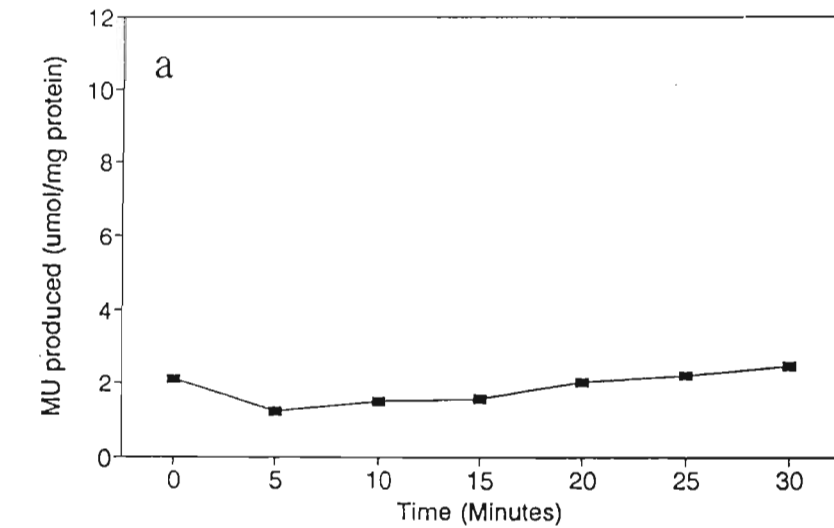


Fig.4.10. The amount of MU produced by cleavage of MUG over time for transgenic potatoes.

(a) LH 13.1

(b) LH 13.2

(c) LH 13.3

(d) LH 13.4

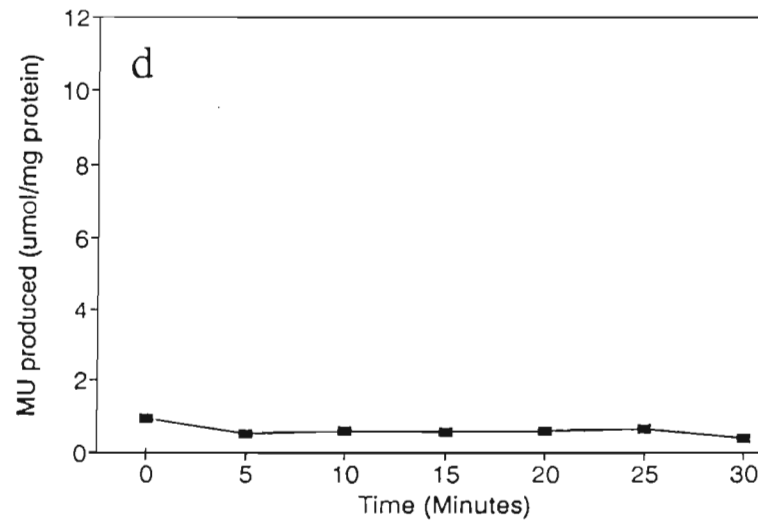
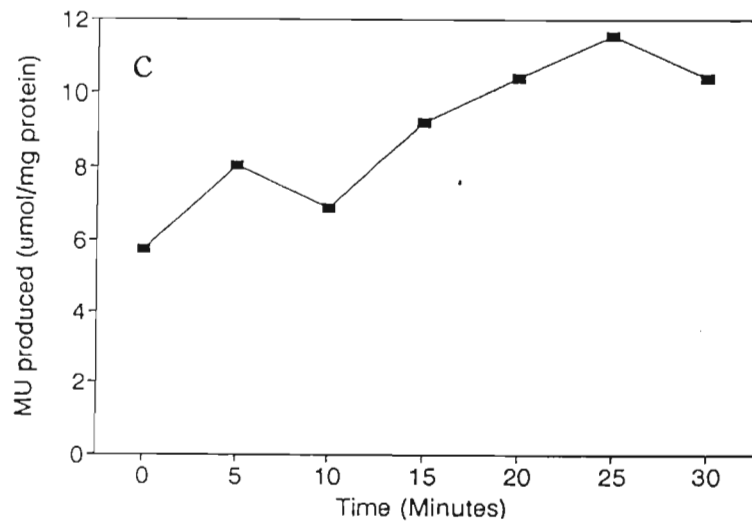
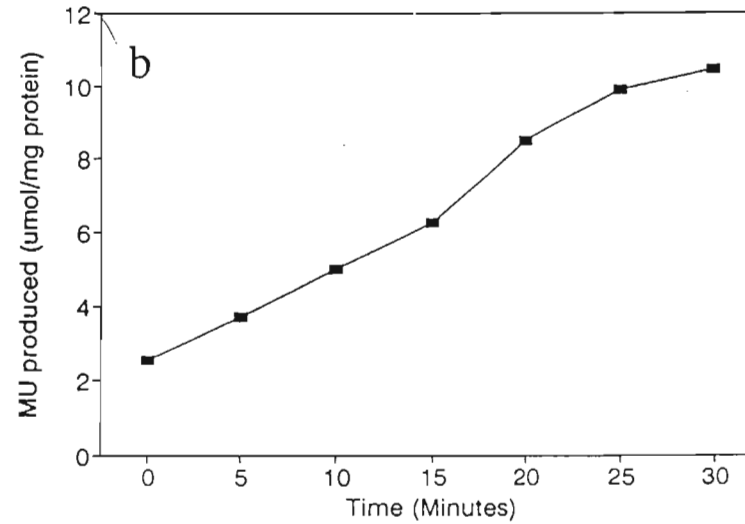
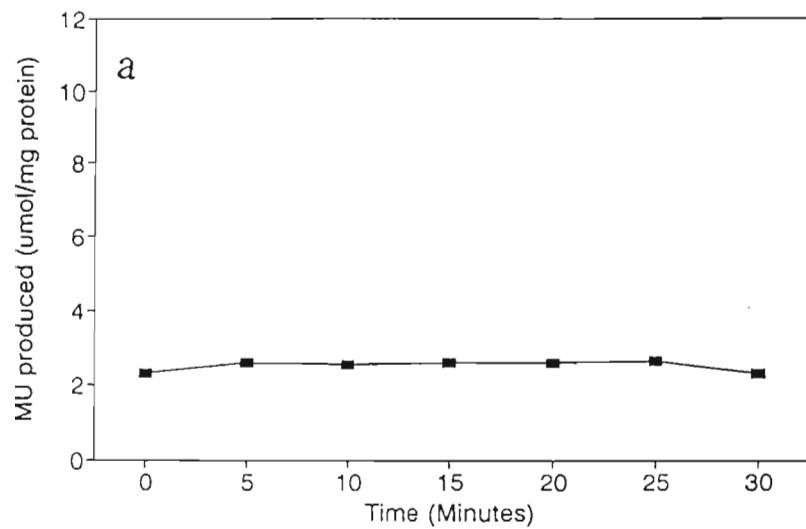


Fig.4.11. The amount of MU produced by cleavage of MUG over time for transgenic potatoes.

- (a) Non-transformed BP₁
- (b) BP₁ 121.3
- (c) BP₁ 13.6
- (d) BP₁ 16.5

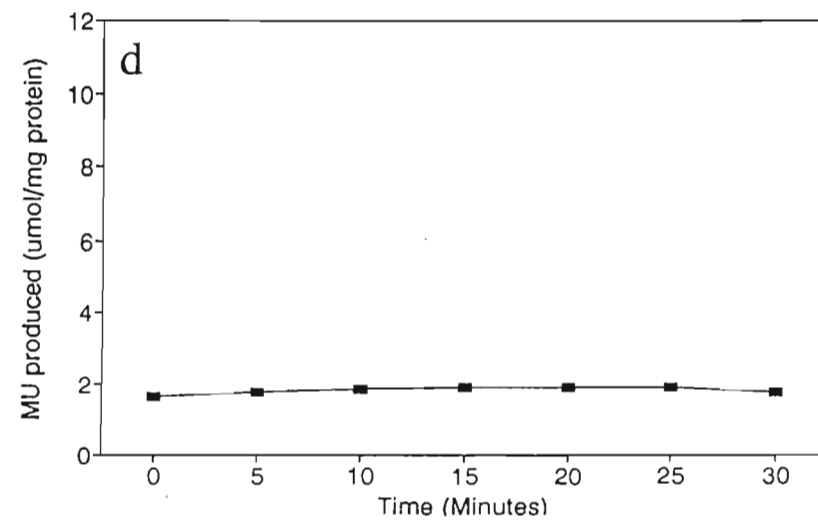
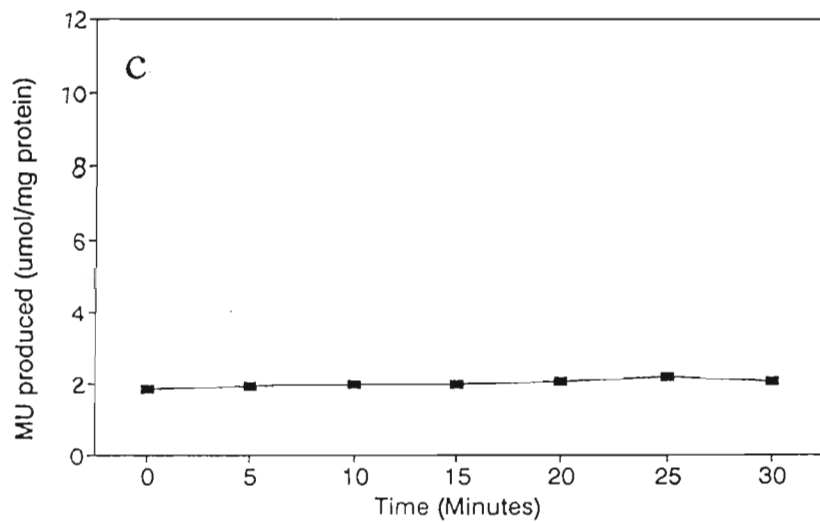
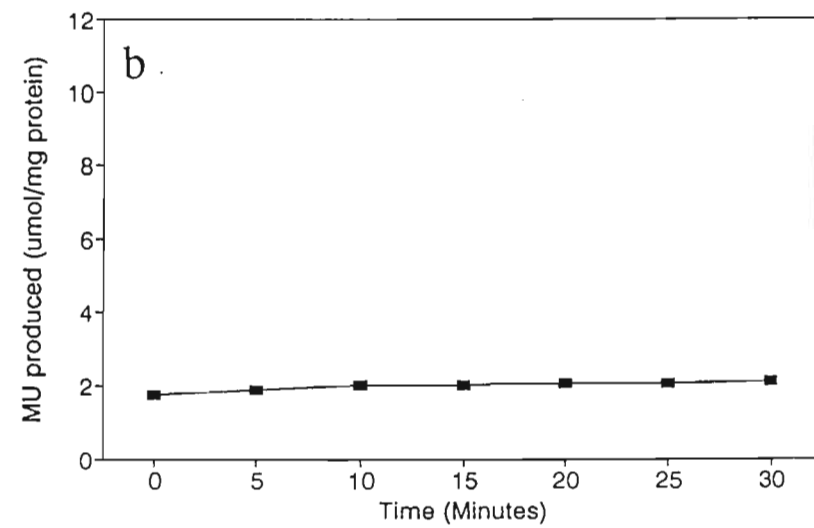
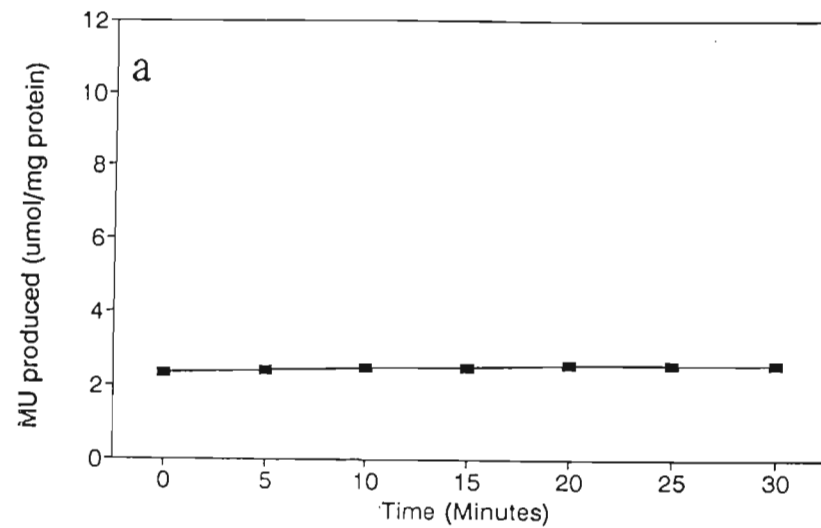


Fig.4.12. The amount of MU produced by cleavage of MUG over time for transgenic potatoes.

(a) BP₁ 16.8

(b) BP₁ 16.9

(c) BP₁ 16.11

(d) BP₁ 16.12

(BURGER, unpublished results). As the PLRV-CP gene was inserted between the CaMV35S promoter and the GUS gene, transcription of the PLRV-CP gene and the GUS gene on a single mRNA transcript will take place in transgenic plants. However, only the first gene on a single mRNA transcript is translated into a protein as the 80S ribosome moving along a mRNA transcript will dissociate when it reaches a stop codon (FRIEFELDER 1987). Therefore, in the sense-PLRV-CP transgenic plants the PLRV-CP gene is translated but not the GUS gene. In the anti-sense PLRV-CP transgenic plants the PLRV-CP is in the anti-sense orientation and thus the translational start and stop codons will not be recognised. The GUS gene is recognised as the first gene on the mRNA and is translated into a protein. GUS protein will thus be present in the antisense PLRV-CP transgenic plant cells, and increased MU production will be obtained in the assay.

Expression of PLRV coat protein.

DAS-ELISA.

The double antibody sandwich enzyme linked immuno-sorbent assay (DAS-ELISA) was performed on the PLRV-CP transgenic lines in an attempt to detect coat protein. In this assay, leaf extracts are probed with antibodies raised against PLRV particles, which are capable of binding to PLRV coat protein particles. Antibody binding is detected by an enzymatic reaction and the absorbance is read at 405 nm. Absorbance readings greater than 0.1 represents the presence of PLRV coat protein. Absorbance readings ranged between 0.002 and 0.02 for the transgenic lines, whereas a reading of 0.735 was obtained for PLRV-infected leaves (the positive control). It thus appears that PLRV coat

protein production can not be detected in transgenic lines using DAS-ELISA. These results indicate, however, that DAS-ELISA will be a useful tool in resistance trials since "false-positive" readings from endogenous coat protein in the transgenic plants will not be obtained.

Immunoblot Analysis.

In order to determine the concentration of the plant protein samples, a protein standard curve was constructed of absorbance readings at 595 nm plotted against BSA concentration (Fig.4.13). Using the software program CurfitTM, a best fit linear curve was constructed using the values depicted in Fig.4.13, which was subsequently used to determine the protein concentrations of the plant samples. Immunoblot analysis in the form of a dot blot was conducted initially. Development of the dot blot showed varying degrees of purple colour development for all samples, including the non-transformed negative control plants. As this result did not give any information, it was decided to proceed to SDS-PAGE gels.

Two identical SDS-PAGE gels were run for both the LH and the BP₁ samples, and 50 µg of the total protein sample was loaded for each sample on both of the gels. One gel was stained so that the protein bands could be visualised and their molecular weights determined by comparison to Rainbow markers; the other gel was used for Western blotting. Staining of the proteins in the first gel occurred successfully. A band corresponding to 26 kD, the size of the coat protein was obtained for the PLRV sample, as was expected (Fig.4.14a and Fig.4.15a). Bands were also seen in this region for the plant samples, including the non-transformed plant. The 26 kD band from the virus

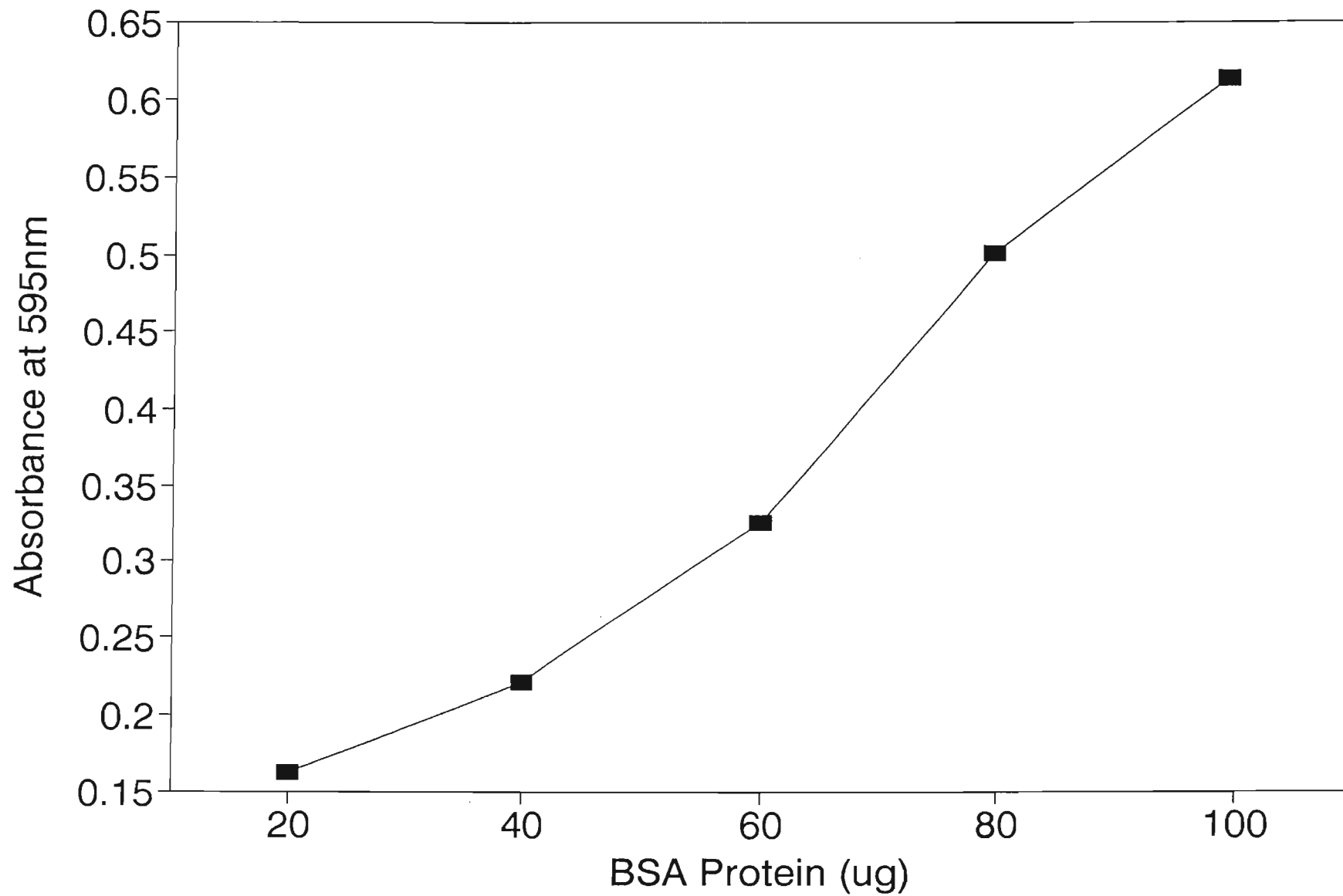


Fig.4.13. Protein standard curve of A₅₉₅ plotted against BSA concentration for use in immunoblot assays.

sample and a smaller band, most probably due to viral degradation, showed colour development and thus antibody-binding in the Western blot (Fig.4.14b and Fig. 4.15b), whereas no antibody-binding could be detected for the proteins from both transformed and non-transformed plants. However, the plant protein sample (50 µg) spiked with PLRV virus (500 ng) did show antibody binding in the form of a single band, which indicates that the viral coat protein can be detected amongst plant proteins at a concentration of 1%. It may be possible that the PLRV coat protein is expressed in transgenic plants, but at such a low level that it could not be detected in this study using either DAS-ELISA or immunoblot analysis.

Other workers have found this to be the case even though increased resistance to PLRV was established in transgenic potato lines. So it appears that resistance to PLRV is not directly proportional to the amount of CP produced. KAWCHUK *et al.* (1990) were able to detect PLRV-CP in most transgenic lines, but BARKER *et al.* (1992) only detected PLRV-CP sporadically and VAN DER WILK *et al.* (1991) did not detect CP at all. The probable conclusion from these results is that protection against PLRV is mediated by PLRV-CP gene transcripts binding to complementary viral mRNA during viral replication. In order to investigate gene expression at the mRNA level, it was decided to implement Northern blot analysis.

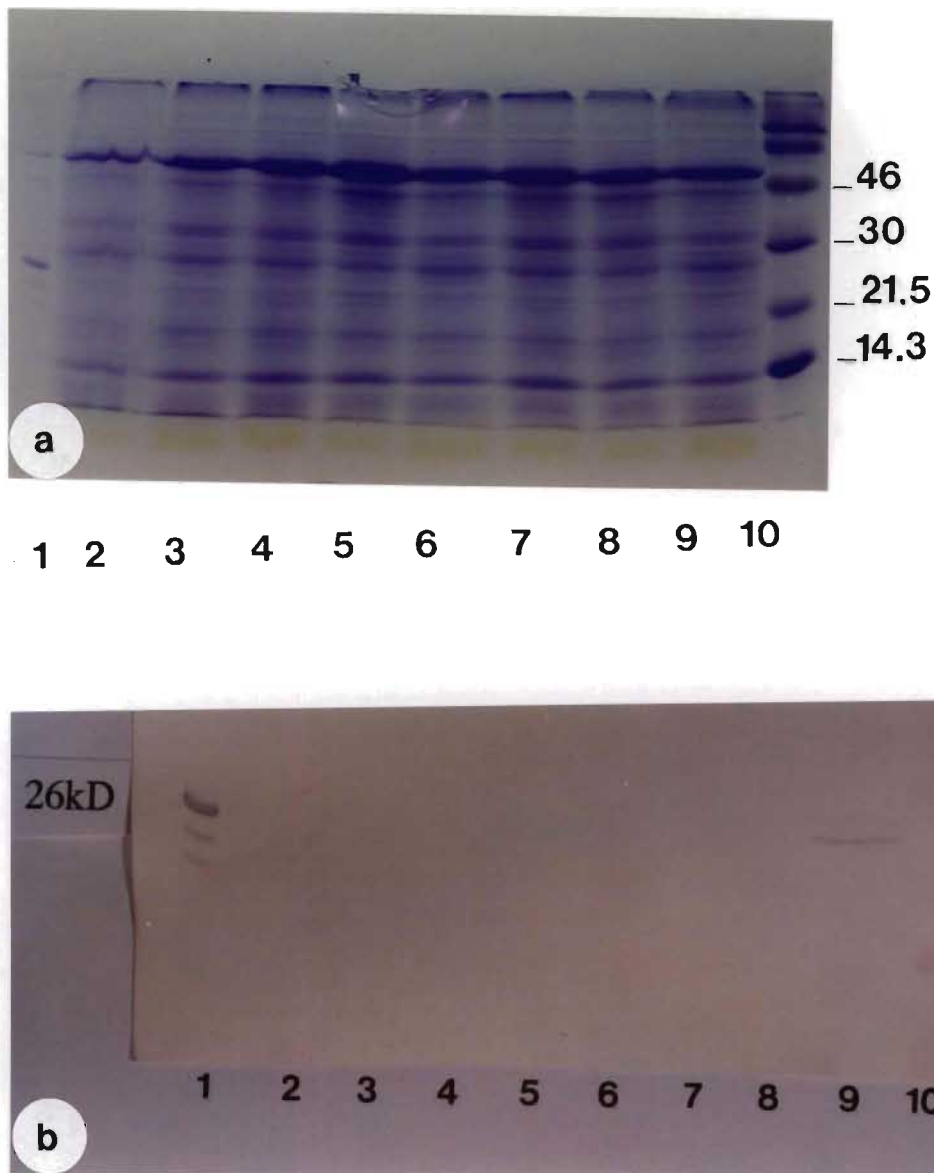


Fig. 4.14: Immunoblot analysis of transgenic LH lines.

(a) SDS-PAGE gel of proteins extracted from LH transgenic lines.

(b) Immunoblot of proteins extracted from LH transgenic lines and probed with anti-PLRV IgG.

Protein samples were loaded in the following order:

(1) PLRV (500 ng); (2) LH 16.3; (3) LH 16.2; (4) LH 13.4; (5) LH 13.3; (6) LH 13.2; (7) LH 13.1; (8) Untransformed LH; (9) Untransformed LH + 500 ng PLRV; (10) Rainbow protein markers, consisting of proteins of different masses in kD: 200; 97.4; 69; 46; 30; 21.5; 14.3.

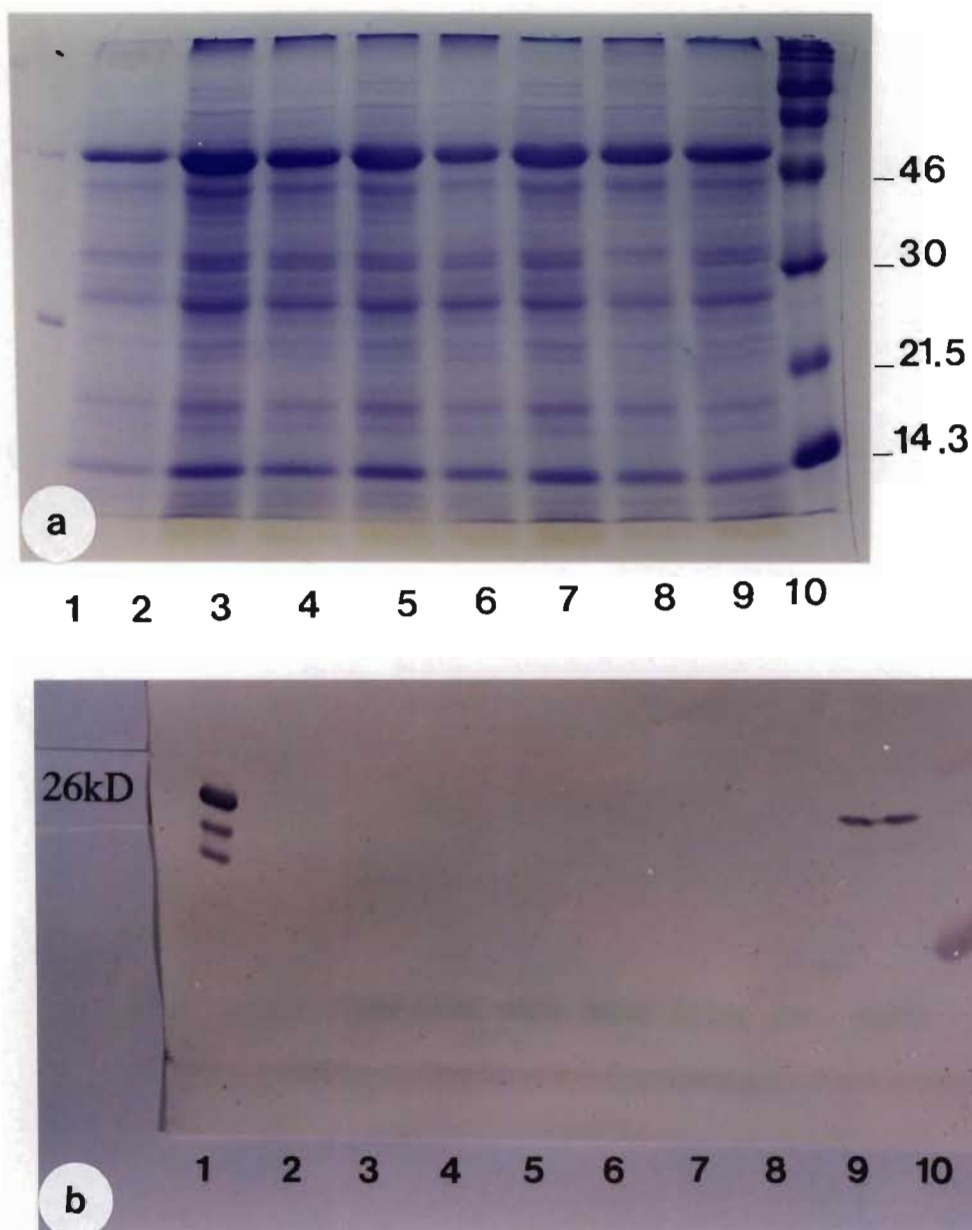


Fig. 4.15: Immunoblot analysis of transgenic BP₁ lines.

(a) SDS-PAGE gel of proteins extracted from BP₁ transgenic lines.

(b) Immunoblot of proteins extracted from BP₁ transgenic lines and probed with anti-PLRV IgG.

Protein samples were loaded in the following order:

(1) PLRV (500 ng); (2) BP₁ 16.12; (3) BP₁ 16.11; (4) BP₁ 16.9; (5) BP₁ 16.8; (6) BP₁ 16.5; (7) BP₁ 13.6; (8) Untransformed BP₁; (9) Untransformed BP₁ + 500 ng PLRV; (10)

Rainbow protein markers, consisting of proteins of different masses in kD: 200; 97.4; 69; 46; 30; 21.5; 14.3.

Transcription of the PLRV-CP gene.

As PLRV coat protein could not be detected in the PLRV-CP transgenic potato plants, it was decided to investigate expression of this transgene at the mRNA level by using Northern blot analysis. It was not necessary to implement Northern blot analysis for the GUS and *npII* transgenes as expression had been shown at the protein level.

Total RNA was successfully extracted from transgenic plants using a small-scale method (VERWOERD *et al.* 1989) and the amount of RNA was quantified by A_{260}/A_{280} readings on a spectrophotometer. In order to verify that the extracted RNA was not degraded, 2 μ g samples of total RNA from each line were separated on a 1.0% agarose gel (Fig.4.16). All samples were not degraded, and RNA fragments of between 3 kb and 0.2 kb were seen. Only transgenic LH potato plants were used in this experiment as there was insufficient BP₁ leaf material available for an RNA extraction.

The PLRV-CP gene, labelled with 32 P-dCTP, was used as a probe in the Northern hybridization experiments. As it was a double-stranded DNA probe, the same probe could be used in Northern hybridizations with RNA extracted from both the transgenic plants with the PLRV-CP in the sense and antisense orientations. Initially, an experiment was performed using 10 μ g of RNA from the different lines in both a dot blot and a Northern blot hybridization. However, no hybridization was visualised, and it was decided to repeat the experiment using only a dot blot and increased amounts of total RNA (20 μ g, 50 μ g and 100 μ g). After hybridization, washing and one week of exposure to Hyperfilm^R, a result was obtained (Fig.4.17).

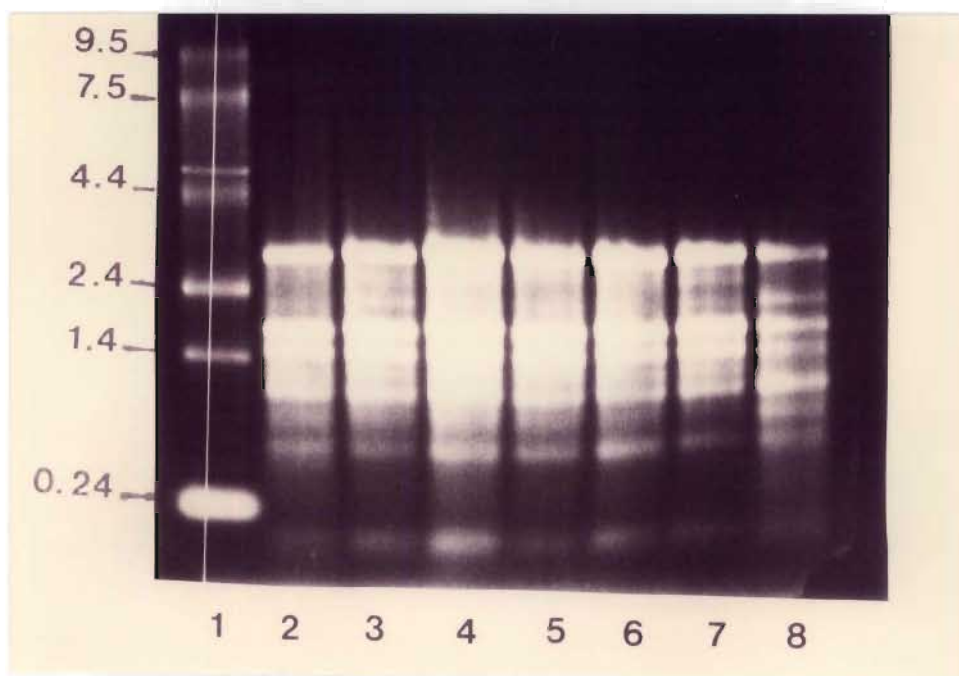


Fig.4.16. Total RNA extracted from various trasngenic potato lines, and separated on a 1.0% agarose gel.

RNA was loaded on the gel in the following order:

1) RNA marker, consisting of RNA fragments of the following sizes in kb:

9.5; 7.5; 4.4; 2.4; 1.4; 0.24.

2) Non-transformed LH

3) LH 13.1

4) LH 13.2

5) LH 13.3

6) LH 13.4

7) LH 16.2

8) LH 16.3

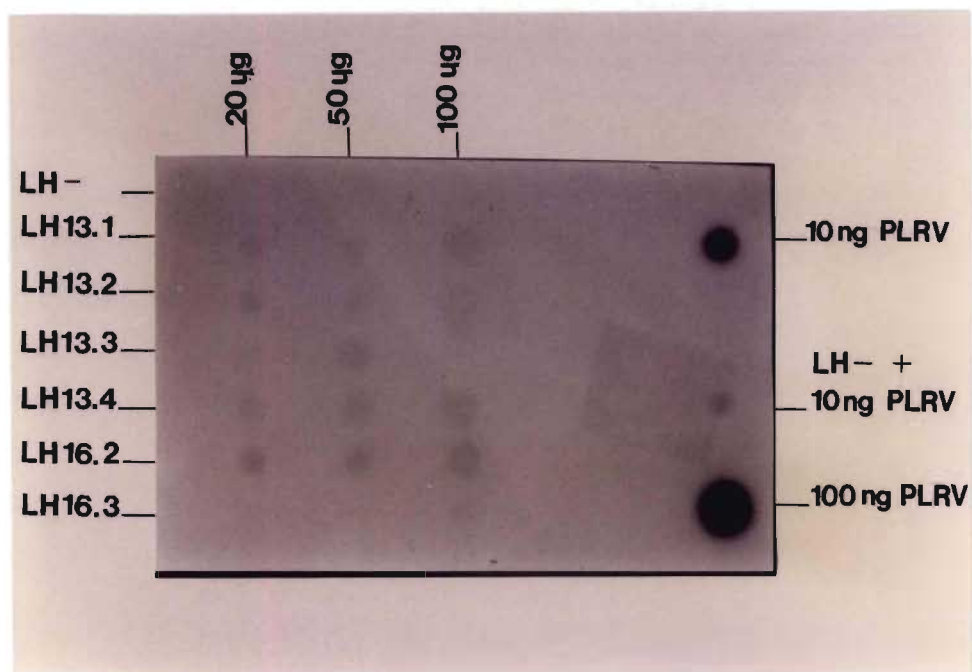


Fig.4.17. Dot Blot of various amounts of total RNA extracted from PLRV-CP transgenic potato plants showing hybridization to a PLRV-CP probe.

LH- = Non-transformed LH

No hybridization was visualised for the non-transformed samples (the negative control), and hybridization was clearly seen for the PLRV RNA samples (the positive control). For lines LH 13.1, LH 13.2, LH 13.4 and LH 16.2, hybridization was visualised for the 20 µg RNA samples, whereas hybridization was only seen in the 50 µg LH 13.3 RNA sample. In addition, hybridization was only faintly visualised for 100 µg LH 16.3 RNA.

The results of this experiment correlate to those obtained by previous workers (KAWCHUK *et al.* 1991; VAN DER WILK *et al.* 1991; BARKER *et al.* 1992), viz. that expression of the PLRV-CP gene in transgenic potato plants can only be identified at the mRNA level, and not at the protein level.

CHAPTER FIVE.

CONCLUSIONS.

This study forms part of a project aimed at introducing resistance to potato leafroll virus in South African potato cultivars through molecular breeding. The coat protein-mediated resistance approach was selected, and the coat protein gene from potato leafroll virus was isolated, cloned and sub-cloned into a plant expression vector (BURGER, unpublished results). The aim of this study was to transfer the PLRV-CP gene into potato explants via *Agrobacterium tumefaciens*-mediated transformation, to regenerate transformants and to verify transgene insertion and expression in the transgenic plants.

Transformation and regeneration was successful, and 24 transgenic potato lines were generated for cultivars Late Harvest and BP₁ using the leaf disk transformation method. Transformation by vacuum infiltration was also investigated, but was found not to be as successful as only two of the three putative transgenic lines were shown by Southern blot analysis to contain the PLRV-CP gene. Further optimization of this method is needed, but it still is an attractive proposition as it is a less labour-intensive and thus more cost-effective transformation method for potatoes.

The leaf disk transformation method can now be extended to other potato cultivars, by first identifying optimal regeneration conditions. In addition, the technology now also exists for the insertion of other transgenes into South African potato cultivars.

The next important step in this project is to investigate the degree of resistance to PLRV in PLRV-CP transgenic potato lines in glasshouse and field trials. It has already been shown that coat protein-mediated resistance in potatoes to PLRV does not induce immunity to the virus, but provides a degree of resistance to virus replication (KAWCHUK *et al.* 1991; VAN DER WILK *et al.* 1991; BARKER *et al.* 1992), and it is necessary to show if this applies to the transgenic lines generated in this study. Furthermore, it has been shown in previous studies that levels of resistance to PLRV could not be correlated to increased levels of accumulated coat protein in PLRV-CP transgenic potato lines. The consensus is that CPMR to PLRV operates at the mRNA level, viz. that the PLRV-CP mRNA transcribed from the transgene binds to the complementary viral mRNA strand during viral replication in the plant cell (KAWCHUK *et al.* 1991; VAN DER WILK *et al.* 1991; BARKER *et al.* 1992). It has been shown in this study that PLRV coat protein could not be detected in the transgenic potato lines using either DAS-ELISA or immunoblot analysis, whereas PLRV-CP mRNA was detected. It will be interesting to correlate these results to the results of the resistance trials, when they become available.

As PLRV is a low titre virus and is phloem-bound, it is envisaged that the transgenic potato plants will be infected with viliferous aphids carrying PLRV, and that PLRV levels in the plants will be monitored using DAS-ELISA at set intervals. It has already been shown that endogenous PLRV coat protein in transgenic potato plants can not be detected using DAS-ELISA, thus making DAS-ELISA a useful tool in glasshouse resistance trials. Potato lines which perform well in glasshouse resistance trials will be released in field trials designed according to the regulations of the South African

Committee on Genetic Experimentation (SAGENE). In addition, potato plants which appeared phenotypically normal in glasshouse trials may show abnormalities under field conditions, and it will be necessary to investigate this.

It may be possible that the PLRV-CP transgenic potato plants generated in this study will show inactivation of the PLRV-CP gene under field conditions. Reports of transgene silencing in plants have been summarised in a recent review article (FINNEGAN & McELROY 1994). These include inactivation of novel transgenes, *trans*-inactivation of independently transferred transgenes and co-suppression of an introduced transgene by the endogenous gene. Although the mechanisms of transgene inactivation are currently unknown, it results in events which include chromatin restructuring, DNA methylation and the inhibition of mRNA processing, transport, export or translation (FINNEGAN & McELROY 1994). Even though the glasshouse transgenic plants tested in this study showed expression of the PLRV-CP at the mRNA level, inactivation of the transgene will have to be analyzed in field resistance trials. It is likely that each transgenic potato line will react differently, as each line represents a different insertion event. FINNEGAN & McELROY (1994) suggested a number of potential methods to avoid transgene inactivation, one of which included screening for plants with single copy transgene insertion events. Transgenic potato plants generated in this study had one to five inserted copies of the PLRV-CP gene, and it will be interesting to see in resistance trials if an increase in copy number can be correlated to a loss of resistance, or to less resistance in comparison to transgenic potato lines containing only one copy of the inserted transgene.

APPENDIX

2% (V/V) abSolve™. (DuPont)

Add 20 ml abSolve™ concentrate to 1l double distilled water.

Agarose gel loading buffer.

50% Glycerol

50% 1 X TAE buffer

0.25% (w/v) Bromophenol blue

Antibody coating buffer for DAS-ELISA.

1.59 g.l⁻¹ Na₂CO₃

2.93 g.l⁻¹ NaHCO₃

0.2 g.l⁻¹ NaN₃

pH 9.6

Blocking Buffer for Western blots.

10 mM Tris, pH 7.5

150 mM NaCl

3% (w/v) Sigma milk powder

0.1% (v/v) Tween-20

Callus induction medium (HULME *et al.* 1993).

MS stocks 1-6

20 g.l⁻¹ sucrose

2.25 mg.l⁻¹ 6-benzylaminopurine (BA)

0.0175 mg.l⁻¹ 3-indolylacetic acid (IAA)

1.5 mg.l⁻¹ STS

7.5 g.l⁻¹ agar

pH 5.8

Coomasie Blue Protein Stain.

0.125% (w/v) coomasie blue in water

50% (v/v) methanol

10% (v/v) acetic acid

Denaturation Solution.

0.5M NaOH

1.5M NaCl

50 x Denhardt's solution.

1% (m/v) Bovine serum albumin

1% (m/v) Ficoll

1% (m/v) Polyvinyl pyrrolidone

Depurination Solution.

0.25M HCl

Destain Solution I.

50% (v/v) methanol

10% (v/v) acetic acid

Destain Solution II.

5% (v/v) methanol

7% (v/v) acetic acid

DIG Buffer 1.

150 mM NaCl

100 mM maleic acid, pH 7.5

The pH was adjusted with solid NaOH.

DIG Blocking reagent stock solution.

10% (w/v) DIG blocking reagent in DIG buffer 1, dissolved by several 30 second heat pulses in the microwave, and autoclaved.

DIG Buffer 2.

Blocking reagent stock solution was diluted 1:10 in DIG buffer 1.

DIG Buffer 3.

100 mM Tris, pH 9.5

100 mM NaCl

50 mM MgCl₂

Buffer 3 was prepared from corresponding stock solutions in order to prevent precipitation of MgCl₂.

DNA Extraction Buffer (RICHARDS 1989).

100 mM Tris, pH 7.5

100 mM EDTA

250 mM NaCl

100 µg.ml⁻¹ proteinase K, which is added after autoclaving

0.5M EDTA Stock Solution.

Dissolve 93.05 g EDTA in 400ml water by vigorous stirring. Adjust the pH to 8.0 by adding NaOH.

10 mg.ml⁻¹ EtBr.

Add 1g EtBr to 100ml, stir well and store in a foil-wrapped bottle.

GUS reaction buffer. (1 mM MUG in GUS-extraction buffer)

Dissolve 18 mg 4-methyl umbelliferyl β-D-glucuronide (MUG) in 50 ml GUS-extraction buffer.

GUS-extraction buffer for fluorometric assays.

50 mM NaPO₄

10 mM EDTA

0.1% (w/v) Triton-X 100

0.1% (v/v) Sarcosyl

10 mM 2-mercaptoethanol

4M LiCl.

Dissolve 4.238 g LiCl in 100 ml water.

Luria-Bertani Medium.

10 g.l⁻¹ Tryptone

5 g.l⁻¹ Yeast Extract

10 g.l⁻¹ NaCl

15 g.l⁻¹ agar (if LB medium is solidified)

10 X MOPS solution.

Add 4.18 g MOPS to 80 ml water, adjust pH to 7.0 with NaOH.

1.66 ml 3M sodium acetate

2 ml 0.5M EDTA

Adjust to a final volume of 100 ml. Filter the solution and store at room temperature in a foil-wrapped bottle.

MS Stock Solutions. (MURASHIGE & SKOOG 1962)

MS stock solutions were prepared according to the following formulations:

STOCK SOLUTION	SALTS/VITAMINS	MS-STOCK SOLUTION (g.l ⁻¹)	AMOUNT OF STOCK ADDED (ml.l ⁻¹)
1	NH ₄ NO ₃	33.0	50
	KNO ₃	38.0	
2	MgSO ₄ .7H ₂ O	37.0	10
	MnSO ₄ .4H ₂ O	2.23	
	ZnSO ₄ .7H ₂ O	0.86	
	CuSO ₄ .5H ₂ O	0.0025	
3	CaCl ₂ .2H ₂ O	44.0	10.0
	KI	0.083	
	CoCl ₂ .6H ₂ O	0.0025	
4	KH ₂ PO ₄	17.0	10.0
	H ₃ BO ₃	0.62	
	Na ₂ MoO ₄ .2H ₂ O	0.0250	
5	FeSO ₄ .7H ₂ O	2.785	10.0
kept in dark	Na ₂ -EDTA	3.725	
6	Inositol	10.0	10.0
kept in dark	Glycine	0.2	
	Nicotinic acid	0.05	
	Pyridoxine-HCl	0.05	
	Thiamine-HCl	0.01	

MS Medium.

MS stocks 1-6

20 g.l⁻¹ sucrose

7.5 g.l⁻¹ agar

pH 5.8

Neutralization Soutlion.

0.5M Tris, pH 7.5

3M NaCl

Osborne Medium.

MS stocks 1-6

25 g.l⁻¹ sucrose

0.1 mg.l⁻¹ gibberellic acid (GA₃)

0.02 mg.l⁻¹ 1-naphthalene acetic acid (NAA)

7.5 g.l⁻¹ agar

pH 5.8

Phosphate Buffered-Saline (PBS).

0.2 g.l⁻¹ KH₂PO₄

2.9 g.l⁻¹ Na₂HPO₄12H₂O

8.76 g.l⁻¹ NaCl

0.2 g.l⁻¹ NaN₃

0.2 g.l⁻¹ KCl

Potassium iodide solution.

2 g potassium iodide

1 g iodine

Dissolve in 200 ml water.

Protein extraction Buffer (FARINNELLI *et al.* 1993)

80 mM Tris, pH 6.8

10 % (v/v) glycerol

2 % (w/v) SDS

2 % (v/v) 2-mercaptoethanol

Pulse medium (HULME *et al.* 1992).

MS stocks 1-6

20 g/l sucrose

10 mg/l 6-benzylaminopurine (BA)

10 mg/l 1-naphthylacetic acid (NAA)

pH 5.8

Quickscreen solution I.

50 mM glucose

10 mM EDTA

25 mM Tris, pH 8.0

Quickscreen solution II.

1% (w/v) SDS

0.2M NaCl

R1AT Regeneration medium.

MS stocks 1-6

20 g.l⁻¹ sucrose

2 mg.l⁻¹ zeatin, added after autoclaving

0.02 mg.l⁻¹ 1-naphthalene acetic acid (NAA)

0.02 mg.l⁻¹ gibberellic acid (GA₃)

7.5 g.l⁻¹ agar

pH 5.8

1.5 mg.l⁻¹ STS, co-autoclaved, was added for transformation experiments.

Resolving gel.

1.5 M Tris, pH 8.8

10% (w/v) SDS

30% (v/v) acrylamide stock consisting of 30% (w/v) acrylamide and 0.8% (w/v) BIS-acrylamide

0.005% (w/v) ammonium persulphate

0.005% (v/v) Temed

RNA Extraction buffer (VERWOERD *et al.* 1989).

0.1 M LiCl

100 mM Tris, pH 8

10 mM EDTA

1% (w/v) SDS

This buffer is mixed in 1:1 ratio with equilibrated phenol (pH 8.0) before use in RNA

extractions.

Sample Conjugate Buffer for DAS-ELISA.

2% (w/v) polyvinyl pyrrolidone

0.2% (w/v) egg albumin

0.05% (v/v) Tween-20^R

Make up to 1l in PBS.

Shoot induction medium (HULME *et al.* 1992).

MS stocks 1-6

20 g.l⁻¹ sucrose

2.25 mg.l⁻¹ 6-benzylaminopurine (BA)

4.85 mg.l⁻¹ gibberellic acid (GA₃)

1.5 mg.l⁻¹ STS

7.5 g.l⁻¹ agar

pH 5.8

Silver Thiosulphate Stock Solution (STS).

A 1.5 mg/ml stock solution of STS was prepared by combining in a 1:1 ratio a 33.3 mg.ml⁻¹ silver nitrate and a 238.2 mg.ml⁻¹ sodium thiosulphate stock solution.

3M Sodium Acetate, pH 5.2.

Dissolve 40.8 g sodium acetate.3H₂O in about 80ml water. Adjust pH to 5.2 with glacial acetic acid, and adjust volume to 100ml.

20 X SSC.

3M NaCl

0.3M sodium citrate

pH 7.0

Stacking gel.

0.5 M Tris, pH 6.8

10% (w/v) SDS

30% (v/v) acrylamide stock consisting of 30% (w/v) acrylamide and 0.8% (w/v) BIS-acrylamide

0.005% (w/v) ammonium persulphate

0.005% (v/v) Temed

Stop buffer. (0.2 M Na₂CO₃)

Dissolve 2.12 g Na₂CO₃ in distilled water and make up to 100ml.

Substrate Buffer.

97 ml.l⁻¹ diethanolamine

0.2 g.l⁻¹ NaN₃

pH 9.8

Store in bottle wrapped in foil.

"Super-quick" DNA Extraction buffer (EDWARDS *et al.* 1991).

200 mM Tris, pH 7.5

250 mM NaCl

25 mM EDTA

0.5% (w/v) SDS

50 X TAE.

0.04M Tris

0.001M EDTA

1 X TAE working solution is made up by diluting 50 X TAE.

TE buffer (pH 7.4/7.6/8.0).

10 mM Tris

1 mM EDTA

Transfer Buffer for Western Blots.

25 mM Tris, pH 8.3

192 mM Glycine

20% Methanol

Treatment Buffer.

0.5M Tris, pH 6.8

10% (w/v) SDS

20% (v/v) glycerol

10% (v/v) 2-mercaptoethanol

0.02% (w/v) bromophenol blue

1M Tris Stock Solution.

Dissolve 121,1 g Tris in 1l water, and adjust the pH to the required value with 1M HCl.

Washing buffer for Western blots.

10 mM Tris, pH 7.5

150 mM NaCl

0.1% Tween-20

Washing buffer for DAS-ELISA.

PBS + 0.05% Tween 20

X-Gluc buffer (JEFFERSON *et al.* 1987).

0.05 % (w/v) 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc)

10 mM EDTA

0.1 % (v/v) Triton-X 100

100 mM NaPO₄

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