MOLECULAR CHARACTERIZATION OF TRANSGENIC SWEET POTATOES (*IPOMOEA BATATAS* (L.) Lam.) FOLLOWING GENETIC TRANSFORMATION WITH VIRAL COAT PROTEIN AND GUS GENES

BY

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Declaration

I, Mutegi Rosemary Wanja declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award.

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Dedication

I gratefully dedicate this thesis to my beloved parents Justus and Joyce Mutegi for their undying love, encouragement, support and prayers throughout my journey towards achieving this goal.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>X-gluc</td>
<td>5-Bromo-4chloro-3-indoly1 β-D glucuronidase</td>
</tr>
<tr>
<td>aad</td>
<td>Adenyltransferase</td>
</tr>
<tr>
<td><em>Gus</em></td>
<td>Beta -D- glucuronidase</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl Trimethyl Ammonium Bromide</td>
</tr>
<tr>
<td>CP-MR</td>
<td>Coat protein mediated resistance</td>
</tr>
<tr>
<td>CP</td>
<td>Coat protein</td>
</tr>
<tr>
<td>CI</td>
<td>Cylindrical inclusion protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HC-pro</td>
<td>Helper component proteinase</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee of Taxonomy of Viruses</td>
</tr>
<tr>
<td>CIP</td>
<td>International Potato Center</td>
</tr>
<tr>
<td>KBC</td>
<td>KARI Biotechnology Centre</td>
</tr>
<tr>
<td>KARI</td>
<td>Kenya Agricultural Research Institute</td>
</tr>
<tr>
<td>KSP</td>
<td>Kenyan Sweet Potato</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>Mv</td>
<td>Mosaic virus</td>
</tr>
<tr>
<td>NPT II</td>
<td>Neomycin Phosphotransferase type II</td>
</tr>
<tr>
<td>Nos</td>
<td>Nopaline synthase</td>
</tr>
<tr>
<td>Nia</td>
<td>Nuclear inclusion protein a</td>
</tr>
<tr>
<td>Nib</td>
<td>Nuclear inclusion protein b</td>
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</table>
Ori Origin of replication
Pi-Pro Papain like proteinase
PCR Polymerase Chain Reaction
P-e35s Promoter for 35s RNA from cauliflower mosaic virus
P-FMV Promoter from figwort mosaic virus
pMON Plasmid MONSANTO
RNA Ribonucleic acid
SSC Saline sodium citrate
SPCSV Sweet potato chrolothic stunt virus
SPFMV-CP Sweet potato feathery mottle virus coat protein
SPFMV Sweet potato feathery mottle virus
SPLV Sweet potato latent virus
SPMMV Sweet potato mild mottle virus
SPVD Sweet potato virus diseases
P3 Third protein
T-DNA Transfer-DNA
Vpg Viral protein genome linked
ABSTRACT

Sweet potato (*Ipomoea batatas* (L.) Lam) is a major crop that ranks seventh among food crops in annual production in the world. The most widespread virus infecting sweet potato is the sweet potato feathery mottle virus (SPFMV). Yield loss caused by the virus can be as high as 80%. Efficient methods to control the virus are not available and conventional breeding programs to introgress genetic resistance into the cultivated germplasm have had very limited success. Breeding for resistance through genetic engineering offers an alternative solution for the control of SPFMV. Collaborative efforts between Monsanto Company in the USA and the Kenya Agricultural Research institute (KARI) succeeded in introducing SPFMV coat protein gene in an attempt to enhance resistance to SPFMV in the sweet potato. Molecular analysis of transgenics as well as expression studies haven’t been carried out. In order to do this, the following work was carried out to confirm the presence, expression and the number of copies of the transgene inserted using an *Agrobacterium tumefaciens* mediated system. Kanamycin painting showed that leaves of transgenic plants failed to show visible damage. Those of the non-transformed control plants treated with kanamycin exhibited severe necrosis, chlorosis and bleaching. There were no differences in antibiotic resistance at 1.5% kanamycin concentration between the transgenic lines. These transgenic sweet potato plants grew normally and formed storage roots after three months in the screen house. There were no morphological differences between the untransformed control plants and the transgenic plants when they were grown in the screen house. Leaves of the freshly sprouting shoots from harvested storage roots of transgenic plants also showed kanamycin resistance suggesting that the antibiotic resistance gene was transmitted to their vegetatively propagated progenies through storage roots. Up to eighty five percent of the transgenic sweet potato explants analyzed histochemically had a characteristic blue staining on the entire surface. Wounded (cut) regions on the leaf reacted more intensively, suggesting higher enzymatic activity. The roots displayed intense blue color in the whole segment tested indicating strong gene activity. Amplified DNA fragments of 450bp were produced in 65% of the samples tested using coat protein specific primers and DNA extracted from putative transgenic plants. This 450bp fragment was the size of the coat protein gene that had been inserted in the sweet potato using *Agrobacterium* as a vector. No amplification occurred with the DNA from control plants and in two putative transformants. The transgene in these two
putative transformants might have been silenced or undergone some positional effects. Southern blot analysis revealed that the plasmid DNA had a single copy insert. This positive control sample produced a light diffuse band indicating a single copy insert. There were no bands detected in the other transformants. This was attributed to the insensitive detection method used. From this study, simple efficient and cost effective methods of confirming transformability in transgenics have been developed which are being applied in other transgenic crops. Some sweet potato lines characterized in this study were also shown to have integrated the transgene stably in their genome, which is likely to reduce yield losses if the gene will be translated to functional proteins by the plant cell ribosomes.
1.1 Sweet potato

Sweet potato (*Ipomoea batatas* (L. Lam)) is a herbaceous perennial vine that is cultivated as an annual food crop in the world. *Ipomoea batatas* originated from a cross between the ancestors of *Ipomoea trifida* and another wild *Ipomoea* species in Central or North-South America, probably in the region between the Yucatan Peninsula of Mexico and the mouth of the Orinoco river in Venezuela, 500 years ago (Huang and Sun, 2000; Jarret and Austin, 1994). Sweet potato is a dicotyledonous plant producing both tuberous and fibrous roots (Austin, 1987). The flowers of the sweet potato are complete with a compound superior pistil, five separate stamens attached to the corolla with petals united into a trumpet or bell shaped corolla. The corolla is usually white at the margin and pink to purple in the throat. Seeds have a hard seed coat and develop within a capsule. The plant usually sets few viable seeds. Many genotypes do not usually flower while others are sterile and most are self-incompatible (Bashaaba *et al.*, 1995). Sweet potato is a hexaploid with 90 chromosomes while other *Ipomoea* species have 30 chromosomes.

The sweet potato is an important crop in Sub-Saharan Africa grown largely by smallholder farmers for household consumption and sale to local and urban populations. It is one of the most important high yielding staple crops in the world with an annual production of 133 million tons (CIP, 1999a). Worldwide, sweet potato currently ranks as the seventh most important food crop on a fresh weight basis, fifth in the developing countries after rice, wheat, maize, and cassava (CIP, 1999b). In Kenya, it is ranked as the third most important root crop with an annual production of 550 000 tons (Lenne, 1991). Production is concentrated in regions around Lake Victoria. The species is adaptable to a wide range of environmental conditions. Its
presence in the tropical areas where a high proportion of the world's poorest people live together with its rich nutritional content and biomass provide an enormous potential for managing malnutrition and enhancing food security in the developing World (CIP, 1996, Sinclair et al., 1997). Similarly, because of its large genetic diversity (Zhang et al., 1998, 2000) and the accompanying diversity in phenotypic and morphological traits (Woolfe, 1992), the crop has great potential for further development to accommodate specific uses. For example, to make french fries, liquor, snacks or animal feed. In addition, the crop has a high solar fixing efficiency (Alexander, 1989). The sweet potato can be cultivated by farmers with limited resources because it requires few inputs in terms of fertilizer, manure, labour, irrigation and matures fast (three months). The storage roots can be harvested and stored over a long period of time, an important factor for families with little income. In some areas, up to three harvests can be achieved per year (Karyeija et al., 1998a).

In spite of the crop's robustness, farmers in sub-Saharan Africa experience very low production levels (Qaim, 2001). Some of the constraints for sweet potato production include the widespread use of low yielding and late maturing traditional varieties and the subsequent subdivision of land holdings into smaller uneconomical units coupled with other post harvest problems like fungi, rats and nematodes. Also, there is limited recognition of sweet potato potential and also lack of policies and programmes for improvement leading to over reliance on cereals. Out of these, the key constraints to sweet potato production are diseases of which viruses are a major cause (Geddes, 1990). These include the sweet potato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV), sweet potato mild mottle virus (SPMMV), sweet potato latent virus (SPLV), cucumber mosaic virus (CMV), sweet potato caulimovirus (SPCa-LV) and sweet potato chlorotic fleck virus (SPCFV).
Viral diseases can cause yield reduction of up to 98% thereby constraining production (Ngeve and Bouwkamp, 1991; Gibson et al., 1997; Gutierrez et al., 2003). Efficient methods to control these viruses are not available and conventional breeding programs to confer genetic resistance have had very limited success. The SPVD diseases are mainly caused by the sweet potato feathery mottle virus (SPFMV), which has a cosmopolitan distribution (Karyeija et al., 1998a). The threat by SPFMV is of particular importance as the use of low input cropping systems coupled with the vegetative nature of sweet potato propagation, lends itself to both the accumulation and distribution of viruses in planting material (Geddes, 1990). SPFMV causes external necrotic lesions, cracking and internal corking making the tubers unpalatable and thus lowering their market value (Clark and Moyer, 1988; Ames et al., 1996). The economic damage has been shown to be more serious in East Africa where sweet potato plays a critical role as the main source of starch besides maize.

Breeding for resistance through genetic engineering has the potential to offer a solution into reducing losses caused by SPFMV attacks. Collaborative efforts between Monsanto Company in the U.S.A and Kenya Agricultural Research Institute (KARI) succeeded in introducing the SPFMV coat protein gene into sweet potato (KARI, 1999, 2000 & 2001). Transgenic sweet potato lines were introduced in Kenya and approved trials were carried out in 2001 and 2002 by KARI. However, these plants were not characterized to determine the integrity and copy number of the inserted DNA sequences. In addition the plants contained the UidA gene encoding the reporter enzyme β-Glucuronidase. The expression of the Gus gene in transformed tissues of sweet potato was also not established.
This research was designed to confirm the presence, expression and copy number of the coat protein and Gus gene at molecular level through kanamycin painting, histochemical GUS assays, Polymerase chain reaction and Southern blot analyses.

1.1 Hypotheses

i. The introduced SPFMV coat protein gene was stably integrated in the sweet potato genome.

ii. There is normal expression of the introduced gene in the sweet potato plant tissues both in laboratory and screen house conditions.

iii. There were single gene copy inserts of the transgene in transgenic sweet potato plants.

1.2 Research objectives

Using locally transformed and Monsanto transformed sweet potato, the objectives of the study were.

➢ To characterize transgenic sweet potatoes using kanamycin painting, histochemical staining techniques, PCR and nucleic acid hybridization.

➢ To undertake gene expression studies on transgenic sweet potatoes under laboratory and screen house conditions.

➢ To determine the copy numbers of the inserted gene.

1.3 Justification

As we approach the beginning of the 21st century, all evidence points to an increasing worldwide food deficit (Alexander, 1989). Seventy percent of the World nutritional needs are derived from plants sources including the sweet potato. The sweet potato is one of the few highly nutritious and drought tolerant crops cultivated by smallholder farmers in Kenya. SPFMV has been identified as one of the major impediments to sweet potato crop production. In dealing with this problem, the International Potato Center (CIP), which has the mandate for research on the crop for
developing countries, has identified viral resistance via gene transfer as opposed to conventional breeding as a priority for Africa (Anon, 1995). Conventional breeding involves introgression of a large number of genes of unknown value linked to the trait of interest unlike genetic engineering which involves the transfer of one or few genes of known function. In addition, the precise manner in which genetic engineering controls the nature and expression of the transferred DNA offers greater confidence for producing the desired outcome compared with traditional breeding.

Application of this esoteric technology is likely to lead to yield increases, which would in turn have a positive contribution to the economy of the country. For instance, since sweet potatoes have a high nutritive value, in this era of HIV/Aids whose one major management strategy is good nutrition, they become handy in its management and that of other malnutrition related diseases. The crop has also a great potential in soil fertility improvement since it acts as a cover crop, reducing soil erosion and enhancing potency of microorganisms. Efficient sweet potato production will also lead to poverty alleviation through increased income of the smallholder farmers when well engaged in its production. In addition, development of protocols for confirming transformability will help save on costs incurred during the process because one can determine the success in the calli stage instead of spending lots of money and time to realize later that the plants are not transformants.

This study was necessary to characterize the transgenic sweet potato so as to confirm the insertion of the transgene, determine the level of expression of the novel protein in different sweet potato tissues and the copy number of the inserted coat protein gene. It was also important to develop and optimize protocols for confirming transformability.
CHAPTER TWO

LITERATURE REVIEW

2.1 Sweet potato utility and production

The sweet potato is a perennial plant belonging to the family Convovulaceae, the Morning glory (Austin, 1987). The family contains about 55 genera (Watson and Dallwitz, 2000). The genus has over 500 species with ploidy levels ranging from 2x to 6x (Ozias-Akin and Jarret, 1994). Sweet potato is the only Ipomoea species of economic importance as a food crop (Onwueme and Charles, 1994). It is grown as an annual crop in more than 100 countries worldwide (Woolfe, 1992). The sweet potato is an important food security crop grown in Kenya mainly by women for household consumption and as a source of family cash income (Carey et al., 1997, Hijmans et al., 2001).

Factors contributing to sweet potato's popularity in the developing world include its adaptability to adverse environmental conditions such as drought, ability to grow well in marginal soils, lack of definable maturity, a short growing season allowing it to be harvested year round, and the fact that the plant lends itself to low input agricultural systems. In this, the crop fulfills an important food security function for households because, under adverse climatic conditions and low input regimes, it yields higher amounts of food energy and micronutrients per hectare than any other crop (Scott and Ewell, 1992, Abaad and Ducreux, 1997).

In the developing countries the sweet potato is an important staple food and its fresh roots and leaves provide nutrition predominantly for the poorest groups of the population. The sweet potato is also a high-energy food and provides over 90% of nutrients per calorie required for most people. In addition it is a valuable source of the
following useful ingredients in the growth and development of animals: carbohydrates for ATP (energy) production, vitamin A for synthesis and regeneration of visual eye pigments, vitamin B for co-enzymes in carbohydrate metabolism and vitamin C for construction of intercellular ‘cement’ for bones, cartilage and the skin. It is a good source for minerals such as iron for haemoglobin and cytochromes (Woolfe, 1992). It also contains calcium necessary for the skeleton, muscle contraction, blood coagulation, casein, membrane permeability, and nerve and muscle activity. Further, it is a source of potassium required for cell division, protein synthesis, growth factors, kidney function and phosphorus for muscle, skeletal, ATP, nucleic acids and bone formation. Its sodium content is essential for muscle activity and active transport (FAO, 1997; Woolfe, 1992). The sweet potato can be utilized as a tropical salad, staple or vegetable food, sweet dessert, fast food (e.g. French fries), liquor, snack or basic industrial raw material. In addition to being used for human consumption, sweet potato is also widely used as an animal feed (CIP, 2000).

Kenya’s annual sweet potato production was estimated at 550 000 tons with an average of over 13 tons per hectare in 1990 (Lenne, 1991). In 1999, production was estimated at 452 700 tons with a mean of 9 tons per hectare (FAO, 1997) indicating a decrease in yield. Sweet potato production throughout Africa is plagued by unique problems such as pests and diseases, which are further aggravated by environmental stresses and lack of access to agronomic technologies. Since the levels of natural resistance to diseases and pests are generally low in the sweet potato, the continued use of conventional breeding technologies is unlikely to solve these problems (Qaim et al., 2000). Innovative biotechnologies can play a crucial role in the engineering of pre-adapted varieties of the sweet potato to make them more productive through enhanced disease and pest resistance.
2.2 Virus diseases of the sweet potato

Viruses induce several plant diseases among them tobacco mosaics and necrosis, alfalfa mosaics and wound tumors as well as sweet potato diseases. Sweet potato production is seriously threatened by viruses, which cause damage to both roots and leaves causing severe crop losses annually. Worldwide, at least nineteen different viruses have been described in sweet potato but only eleven of these have been recognized by the International Committee of Taxonomy of Viruses (ICTV) (Cohen and Loebenstein, 1991). In developing countries, the use of low input agricultural production systems has facilitated both the accumulation and dissemination of viruses in the sweet potato, with infected planting materials becoming the primary source and host of all viruses. In the crop, viral diseases are readily spread from one cropping cycle to the next by sprouts produced from diseased roots, vine cuttings from the previous years' harvest, as well as transfer by insect vectors (Clark and Moyer, 1988; Karyeija et al., 1998b). Unlike living organisms, viruses are packages of molecules that cannot reproduce autonomously and cannot be easily eliminated by application of chemical substances.

2.3 Complex virus diseases of sweet potato

Multiple virus infections are common in sweet potato (Chung et al., 1986; Clark et al., 1998; Cohen et al., 1995; Di Feo et al., 2000; Rossel and Thottappily 1988; Scheafers and Terry, 1976) and synergistic interactions are often involved. The most common of these disease complexes known as sweet potato virus disease (SPVD) is caused by simultaneous infection with SPFVMV and SPCSV (Ngeve and Bouwkamp 1991; Winter et al., 1992). This type of disease complex was first described around 1940 in Uganda, Burundi, Rwanda and Eastern Belgian Congo.
(Hansford, 1944). The disease is characterized by chlorosis, small-deformed leaves and severe stunting (Hahn-Terry and Leuschner, 1981; Mukiibi, 1977; Carey et al., 1999). SPVD is the most serious disease of sweet potato in Africa (Geddes, 1990) and it's the most important virus disease of sweet potato globally (Carey et al., 1999).

2.4 Synergism between sweet potato viruses

When two unrelated viruses infect a host plant, they may interact to increase or decrease the amount of one of the two (Matthews, 1991). Synergism is thus recognized when one virus enhances the replication of a second unrelated virus, leading to increased titres and more severe symptoms in a host plant (Vance et al., 1995; Pruss and Inouye, 1997a; Pruss et al., 1997b; Karyeija et al., 2000; Hull, 2002). In some cases, both of the co-infecting viruses may benefit from the synergism (Scheets, 1998; Fondong et al., 2000). Synergism has also been shown to occur between viruses and their satellite virus (Sanger et al., 1994) or between viruses and viroids (Savenkov and Valkonen, 2001).

When SPFMV alone infects the sweet potato, mild or no symptoms are observed and in symptomless leaves the SPFMV titres are below the detection limits of most serological tests. Although DNA or RNA probes can detect SPFMV in symptomless leaves, the signals are just slightly above the background and not as clear as testing symptomatic leaves. Resistance to SPFMV as been observed to be mostly overcome when sweet potatoes are co-infected with SPCSV forming SPVD (Karyeija, 1999, Aritua et al., 1998a, 1998b). The best synergisms are those where a potyvirus induces an increase in the titres and/or pathogenicity of another unrelated virus (Poolpol and Inouye, 1986; Vance et al., 1995; Pruss et al., 1997b; Scheets, 1998; Abaad and Moyer, 1992). The mechanism of synergism may vary, in some
cases; the helper virus may aid another virus movement (Barker, 1989) thereby enabling it to invade tissues it otherwise could not have invaded. In other cases, viral replication and accumulation are enhanced. In the potyvirus mediated synergism, it has been shown that the central region of the HC-pro (Helper component proteinase) is the mediator of synergism, and that the same region also suppresses the host RNA silencing mechanism suggesting that these two phenomenon are linked (Kasschau and Carrington, 1998; Anandalakshmi et al., 1998; Brigneti et al., 1998).

2.5 Sweet potato feathery mottle virus (SPFMV)

The sweet potato feathery mottle virus is the most thoroughly characterized within Ipomoea, occurring in all sweet potato growing areas (Karyeija et al., 1998a; Revers et al., 1996) in Africa. Other synonyms include sweet potato chlorotic leaf spot virus, sweet potato internal cork virus, sweet potato russet crack virus and sweet potato virus A. The virus was first described in 1945 by Delittle and Halter in the United States. In East Africa (Kenya, Uganda and Tanzania) it was described by Sheffield in 1957 under the name sweet potato virus. It is a member of the genus Potyvirus of the family Potyviridae (Shukla et al., 1994; Pringle, 1999; Van Rogenmortel, 2000) the largest genus and family of plant viruses to date.

The potyviruses are distinguished by their characteristic pinwheel like cylindrical inclusions formed by viral proteins in the cytoplasm of infected cells (Edwardson, 1992; Riccah, 1989). The genus has 180 definite or possible members. Like other definite potyviruses, the SPFMV (a retrovirus) virion is an elongate flexous filamentous rod with a monopartite, positive sense and single stranded RNA molecule of about 1060 bases (Fig 1) (Sakai et al., 1997, Shukla et al., 1994). This is larger than the average 970 bases of a potyvirus genome (van Rogenmortel, 1990).
The virion is between 830-850nm in length and between 11 to 13 nm in diameter. The genome has a covalently attached protein (VPg) at the 5’end and a poly A tail at the 3’ end (Rossel and Thottapilly, 1988). The genome has a single open reading frame that encodes one large polyprotein precursor that is autocatalytically cleaved into ten functional polypeptides (Fig 2, Table 1). The sweet potato feathery mottle virus has numerous copies of coat protein that are exceptionally large (38k Da) as compared to other potyviruses. This is largely due to the insertion of a contiguous sequence at the 5’ end of the CP cistron (Abad and Moyer, 1992). The SPFMV is not seed transmitted in the sweet potato (Wolters et al., 1990) but it is transmitted by the aphid Myzus persicae in a non-persistent, non-circulative and stylet borne manner (Riccah, 1989). Aphids acquire viral particles after brief feeding periods on an infected host and they retain the virus for less than an hour. Because of the brief retention of the virus, aphids normally carry viruses for relatively short distances although occasionally strong winds can blow them over great distances.

Fig. 1: Particle morphology of sweet potato feathery mottle virus. The virus has an elongate flexous filamentous rod with a monopartite single stranded RNA molecule (Shukla et al., 1994)
Fig. 2. Genome organization of the sweet potato feathery mottle virus

The genome contains one large open reading frame. The vertical lines represent cleavage sites of the polyprotein. Names of the final protein products are indicated as boxes. The mature polyviral proteins are PI proteinase, helper component proteinase (HC-Pro), the third protein (P3), 6-kDa protein1 (6K1), cylindrical inclusion protein (CI), 6-kDa protein 2 (6K2), nuclear inclusion protein a (Nia), nuclear inclusion protein b (Nib), and the coat protein (CP). The 5' and 3' are at the extreme right and left end of the genome respectively. The RNA genome is 3' polyadenylated and has a viral protein (Vpg) linked to the 5' end.

(Settumba, 2004)
Protein Function

P₁ (aseline protein) - The gene is associated with genome amplification, cell to cell movement and virus host interaction (Lai, 1998) - Has also been linked to symptom development and host range - Has been implicated as a suppressor of virus induced silencing upon infection (Kasschau and Carrington, 1998)

HC-pro (Helper component proteinase) - Important for virus transmission by aphids - Also associated with post-transcriptional gene silencing (PTGS) as a counter defensive strategy in potyviruses (Kasschau and Carrington, 1998)

P₃ (third protein) - Associated with inclusion bodies suggesting that it is involved in virus replication (Kasschau et al., 1997)

C₁ protein with a conserved RNA helicase domain - Also involved in genome replication (Shukla et al., 1994)

Nia (Nuclear Inclusion protein a) - It is involved in host genotype specific long distance movement (Schaad et al., 1997)

Nib - Thought to be the RNA replicase (RNA dependent polymerase responsible for virus multiplication) (Murphy et al., 1999)

CP (coat protein) - It encapsidates the viral genome and is also involved in virus transmission by aphids, cell to cell movement and virus spread (Atreya et al., 1992, 1995; Rojas et al., 1997). It represents 95% of the virion by weight and has an extremely N-terminal sequence linked to a conserved core domain (Shukla et al., 1994)

Table 1: Functions of proteins found in Sweet Potato Feathery Mottle Virus

2.5.1 Transmission of SPFMV

The transmission of the virus is dependent on the HC-pro and the composition of the particle CP. There are two molecular strategies regulating the interaction between viruses and their vectors. The capsid strategy in which the virus CP interacts directly with the vector and the helper strategy in which at least one non-structural viral gene product (HC-pro) is required for successful transmission (Fraser, 1992). The coat protein is associated with aphid transmission in the capsid strategy. In this
strategy, a motif of the coat protein directly binds to the vector’s receptor (Froissart et al., 2002). The interaction between the HC-pro and CP of the virus and with structures in the mouthparts of the vectors has been demonstrated (Blanc et al., 1998). Existence of non-transmissible isolates with functionally active HC-pro suggested the involvement of CP in the process of another determinant of transmission. A highly conserved motif of three amino acids Asp-Ala-Gly (DAG), located in the N-terminus of the potyviruses CP has also been demonstrated to be associated with transmission (Atreya et al., 1992, 1995)

2.5.2 Infection cycle of sweet potato feathery mottle virus

Potyviruses enter their hosts via the stylet of an aphid. The acquisition of the virus may take seconds and loss of virus transmissibility occurs after a short time. Virus acquisition by aphids is dependent on an N-terminal amino acid motif (Asp-Ala-Gly) in the coat protein (Shukla et al., 1994) as well as the N-terminal motives (Lys-Ile-Thr-Cys) (Atreya et al., 1992; Blanc et al., 1998; Sasaya et al., 2000) and Pro-Thy-Lys (Peng et al., 1998) in the non-structural helper component (HC-Pro). Upon feeding on the plant the aphid regurgitates some saliva and by this process inoculates the plant with the virus (Martin et al., 1997). The virus then starts to disassemble and after being recognized by the host cell as an endogenous mRNA, it is simultaneously translated by a process known as “co-translational disassembly”. By the time the virus has fully disassembled, the first viral proteins have already been produced and are ready to start replicating the viral RNA. The large open reading frame (ORF) in their genome is then translated into a single poly-protein, which is then autocatalytically digested into the functional proteins. All potyvirus proteins have been shown to have multiple functions (Kekerainen et al., 2002). (Fig 2, Table
1. In addition, cleavage intermediates may have multiple functions and interactions with other viral or host proteins are required for different functions.

Upon infection, potyviruses can cause the complete shut down, or up regulation of several host genes (Escaler et al., 2000, Aranda et al., 1996). Viral replication is restricted to a narrow zone of cells at the infection front (Aranda et al., 1996). It occurs in tight association with membranous structures (Schaad, et al., 1997), probably by a multimeric complex of viral and host proteins that specifically recognize and mediate replication of viral RNA but not host RNA. After infection of the inoculated cell, the virus moves to neighboring cells and into the vascular tissue where it spreads throughout the plant following the source sink stream (systemic or long distance movement). The mechanism of movement is not yet understood but several proteins are involved. The traditional view is that transport between cells requires active processes and receptor like interactions between the virus and the host cell plasmodesmata (Beachy, 1997, Carrington, 1999). Both HC-Pro and CP have been shown to increase the size exclusion limit of plasmodesmata in mesophyll cells (Rojas et al., 1997). However, many movement proteins (mps) are also suppressors of host cell defense responses. There is also increasing evidence that a multitude of plant encoded mRNAs travel through the phloem. Therefore, it is possible that some viral movement proteins facilitate cell to cell or systemic movement of viruses by suppressing cell-to-cell communication of plant defence responses rather than by actively mediating transport through the plasmodesmata (Carrington, 1999). The potyvirus translation strategy is thought as a down side since all proteins have to be produced in equimolar amounts. Their amounts are dictated by the protein that is required in the highest molarity and this is the coat protein. This leads to the
accumulation of huge amounts of redundant proteins and this is probably the cause of typical inclusion bodies found in potyvirus infected cells.

2.5.3 Symptoms of SPFMV in sweet potato

SPFMV has great economic impact as a component of SPVD (Karyeija et al., 1998a). The host range is narrow and is mostly limited to plants from the family Convovulaceae and especially to the genus Ipomoea (Nakashima et al., 1993). The range of symptoms associated with SPFMV infection differ according to the plant genotype, environment and the virus isolate (Matthews, 1991, Atreya et al., 1992). These symptoms, host range and serology have been used to group the SPFMV isolates into two strains; the common strain (C) and the russet crack strain (RC) (Moyer and Kennedy, 1978; Cali and Moyer, 1981). Leaf symptoms of the virus (SPFMV) are mild and transient, but they may include chlorotic spots, vein feathering of the leaf midrib and vein clearing particularly on older leaves. Root symptoms include internal necrosis and external cracking depending on the cultivar and the virus isolate (Clark and Moyer, 1988; Mori et al., 1995). Data on yield losses caused by SPFMV in developing countries are scarce as the focus has mainly been on the distribution and benefits of using virus free planting material as opposed to accessing the yield damages resulting from SPFMV attacks (Karyeija et al., 1998b).

Accurate assessments of yield losses have also been hindered by lack of good methodology. This is because SPFMV is most often symptomless by visual screening, thus increasing the spread of the disease by the use of infected planting material from one planting season to the next (Clark and Moyer, 1988). Gibson et al (1997) have shown that yield losses resulting from SPFMV are more than 50%.
The virus has been shown to diminish tuberous root yields in sweet potato cultivars constituting a serious constraint in sweet potato production.

2.6 Control of sweet potato viral diseases

Unlike other pathogens, viruses are obligate intracellular parasites utilizing precursors and the cell biosynthetic machinery to support their propagation in plant tissues. Methods used in the control of plant viruses include use of chemicals that can sometimes, effectively control the spread of circulatively transmitted viruses and especially the colonizing vectors (Antignus, 1987). This is due to the long feeding periods that are required for acquisition together with inoculation phases. This method is not suitable in non-persistent, non-circulative transmission system where the transmission cycle is very short and can be affected by the application of insecticides since the vectors will have transmitted the virus before they die (Thottapilly, 1992). Furthermore, vector movement may increase following spraying with insecticides leading to increased levels of virus spread (Berger, 2001). This was a method of choice for a long time. However, its negative effects like toxicity to the environment and the destruction of the non target organisms combined with pesticide resistance development, have led to a support for more environmentally friendly means of control. More so, cost and safety are immediate considerations for pesticide usage by smallholder sweet potato growers.

Phytosanitation is concerned with the eradication of sources of infestation and inoculum. This important approach includes maintenance of tidy fields, the disposal of unwanted crops and crop residues and elimination of weeds or volunteers that harbor pests. Farmers in particular, usually select cuttings relatively unaffected by diseases to use as parents of the next crop and regard this as a main control method.
for virus diseases (Gibson and Aritua, 2002). Roughing, a practice, which involves removing and destroying plants as they become infected, can also reduce development of virus population and/or inoculum sources (Dent, 1995, Bashaaba et al., 1995; Aritua et al., 1998a, 1998b). However, some sweet potato farmers do not rogue for fear that this would cause a proportional loss in yields, perhaps due to lack of knowledge on the cause and spread of viruses. The destruction of surviving crops in old and sometimes abandoned fields can conceivably reduce sources of infection and virus incidence.

Besides its agronomic benefits, crop rotation provides a cheap and effective means of crop protection against viruses (Dent, 1995). This is particularly effective against pests, which are relatively host specific. However, crop rotations have become less popular as agriculture has intensified and greater emphasis has been placed on continuous cropping.

The two most effective strategies for the control of viral diseases in sweet potato are the use of virus-free planting material and the breeding of resistant cultivars. Although the development of 'healthy' plants through a combination of virus indexing and meristem tip culture has been employed worldwide in sweet potato production, it is not 100% effective as no single assay exists to detect all viruses and the sweet potato is often subject to multiple infections (Moyer and Salazaar, 1989). The use of virus-resistant sweet potato cultivars developed through traditional breeding methods is still the most reliable form of disease control (Karyeija et al., 1998a; Berger, 2001), although the strategy has had very limited success up to now. This is because resistant cultivars are subject to re-infection in the field by new and potentially more virulent strains of the virus. Also the virus resistance only slows down but does not prevent the spread of the virus in vegetatively propagated crops.
(Fargette od Vie, 1995). Furthermore, it has been noted that although there are some sweet potato land races with a certain degree of resistance, their use in breeding programs has been unsuccessful as the trait is negatively correlated with yield performance (Qaim, 2001). In addition, it is also difficult to use conventional breeding on polyploid species such as the sweet potato, which has a low seed set and is self-incompatible (Thompson et al., 1997).

With the development of techniques for the introduction of foreign genes into plants, a number of different approaches have become available for the development of resistance to viral diseases. Using genetic transformation, varying levels of resistance have been engineered into crops with the expression of viral replicase genes, antisense RNA’s and viral coat protein genes (Berger, 2001). Of these strategies, coat protein-mediated resistance conferred by viral coat protein transgenes has been the most successful because of its ubiquitous control of a variety of viral diseases in a number of different transgenic crops (Qaim, 2001).

2.7 Coat Protein Gene transfer approaches

Genetic engineering of crops involves the use of the natural gene transfer mechanisms of Agrobacterium, a common soil bacterium. A modified transfer-DNA (T-DNA) vector is constructed in which the desired DNA fragment is inserted between the T-DNA border regions (specific 25 bp direct repeat regions) of Agrobacterium. This vector is transferred into Agrobacterium and the virulence gene products of Agrobacterium actively recognize, excise, transport and integrate the T-DNA region into host plant genomes. The natural host range of Agrobacterium however limits this approach in some crops, especially the cereals and other monocotyledonous species. For such crops, alternative approaches such as direct
uptake of naked DNA into protoplasts or tissues using electroporation or particle gun bombardment are available (Bendahmane and Beachey 1999, Bendahmane et al., 1999). In this case, a plasmid or linearized DNA with the gene of interest is fixed to tungsten or gold particles (micro carriers), which are delivered to host cells at high speed so as to penetrate the nucleus of the plant cell (Kahl, 2001).

The co-transfer of a selectable marker gene along with the gene of interest allows the preferential growth of the transformed cells in cell culture. Successive manipulations of the chemical composition of the culture medium, especially the plant hormones allow the regeneration of complete plants. The ease with which genetically engineered plants can be developed varies markedly between crops, and even between different genotypes within crop species. Despite this, genetically engineered plants have been recovered in virtually all crop plants (Bendahmane et al., 1999).

*Agrobacterium* mediated transformation of plant tissue is preferred over other DNA delivery techniques since the transgenic plants that result have a low transgene copy number, minimal rearrangements and higher transformation efficiency (Pawlowski and Somers 1996; Gelvin, 1998). Wenck *et al.* (1997) and Kononov *et al.* (1997) demonstrated that plasmid backbone sequences beyond the borders of the T-DNA could also be integrated along with the genes of interest. Experiments by Kononov *et al.* (1997) demonstrated that plasmid backbone sequence could be integrated into the host genome coupled with either the right or left border sequences, or as an independent unit unlinked from the T-DNA. Matzke and Matzke (1998) state that backbone sequences that join T-DNA appear to be especially deleterious for gene expression, an observation supported by the author’s finding that backbone fragment separated from T-DNA have been found associated with unstably expressed transgenes.
2.7.1 Nature of transferred DNA

The amount of DNA transferred is usually less than 10 kb but can be very large even up to 100kb, which is minuscule compared to the genome size of crop plants (Gelvin, 1998). The sequence of the DNA region to be transferred is either known or can be deduced from the component DNA fragments. In cases where the technology has reached the point of transferring genes for genetic improvement of crops, the nature of all encoded functions is well understood. In addition, the manner in which the transgenes are intended to be expressed in the resulting plant can be controlled by the use of appropriate cis-regulatory sequences. In particular, promoters can be selected that either allow constitutive gene expression or limit gene expression to only specific cell types or in response to specific environmental stimuli. The translational fusion of specific signal sequences to the peptide-coding region can target expression to particular subcellular or extracellular locations.

2.7.2 Coat protein mediated resistance (CP-MR) for SPFMV disease control

Coat protein–mediated resistance (CP-MR) is a form of genetically engineered resistance, whereby the expression of viral coat protein genes in transgenic plants induces a cross “protection-like” phenomenon that confers resistance to the virus from which the gene was initially derived, as well as to related viral strains (Mori et al., 1995). Coat protein–mediated resistance is characterized by few systemically infected plants, reduced sites of infection, less viral accumulation in infected plants and reduced severity of disease symptoms (Baulcombe, 1996; Beachy, 1997). It is largely unclear what the molecular mechanisms of CP-MR are, but two working theories have been proposed. The re-encapsidation theory proposes that endogenously
produced viral coat protein subunits in the plant prevent the virus from uncoating by binding to viral RNA as its own protein subunits are removed outside the cell. The second theory proposes that the endogenously produced coat protein subunits bind putative cellular receptors in the plant cell, thus hindering or preventing viral entry or disassembly (Murray, 1980). Although SPFMV and SPCSV in combination are responsible for sweet potato virus disease, the virus resistance mechanism has been shown to be based on the SPFMV coat protein gene (Beachy, 1997, Qaim et al., 2000).

2.7.3 Coat protein gene integration into sweet potato plant genome

Transformation events are genetically equivalent to small addition or deletion mutations and the transgenic plants initially regenerated are hemizygous for the inserted DNA and therefore segregate upon self-pollination or outcrossing (Conner and Christey 1994). The DNA fragment intended for transfer may become integrated as a single discrete copy or as repeated regions on the same tandem sequence on the same spot or different spots. In addition, insertion may occur at one or more integration sites (Jorgensen, 1987; Christey and Sinclair, 1992; Devos and Gale, 2000). At any site of incorporation into plant chromosomes, the transferred genes may integrate in a complete, a truncated, or a rearranged manner (Deroles and Gardner, 1988; Christey and Sinclair, 1992). In some instances, *Agrobacterium*-mediated transformation can result in complex integration patterns, which may include vector sequences outside the T-DNA region (Wolters et al., 1990). The preferred integration event is the insertion of one complete intact DNA fragment as a single locus (Czernilofsky et al., 1986). This is a relatively common event for the T-DNA transfer events associated with *Agrobacterium*-mediated transformation,
whereas direct DNA transfer approaches often result in long concatamers of the transferred DNA (Czernilofsky et al., 1986). Although the intact nature of the transferred DNA cannot be guaranteed prior to any transformation event, the nature of transgene integration can be accurately determined by Southern blot analysis although its activity is dependent on precise location of regulator genes in the cell. Complex integration patterns can also be effectively analyzed by fluorescence in situ hybridization on extended DNA fibres (Wolters et al., 1990).

2.8 Variability in transgene expression

Until 1995, it was generally assumed that sequences between the left and right borders of the T-DNA were the only transgenic elements transferred to the recipient host. Plants transformed independently with the same plasmid will commonly have different levels of expression, a phenomenon that is not always correlated with copy number (Gelvin, 1998). Instead, differential expression of transgenes has been attributed to some “positional effects” whereby the position of the T-DNA integration site in the host genome affects the level of transgene expression. However, other research has indicated that factors in addition to, or other than, the position of the site of integration contribute to the level of transgene expression (Gelvin, 1998). This is particularly true of the variable arrangements that transgene sequences may take in the host genome.

T-DNA can integrate into the host genome in patterns other than as a single copy at a single site. Multiple copies in direct or inverted repeats and other complex patterns may also occur. The presence of multimeric T-DNA inserts, especially
inverted repeat structures, is strongly linked to the phenomenon of transgene silencing (Baulcombe, 1996).

2.9 Advantages of genetic engineering technology.

Biotechnology helps to bring forth innovations that could not be achieved using conventional research tools alone. Genetic engineering ensures repeated transfer of new genes into existing cultivars or elite lines in plant breeding programmes. This contributes more efficiently to development of new cultivars without many generations of hybridization and selection to recover agronomically useful lines. The transfer of single discrete genes can be readily achieved via genetic engineering. This overcomes the problem of linkage drag in traditional plant breeding which is associated with the transfer of many unwanted and undefined closely linked genes (Bruce et al., 2003; Machuka, 2004).

Genetic engineering ensures that the germplasm base is extended from related wild species to any source of DNA such as other plants, microorganisms and animals. This provides new opportunities to complement the existing genes in the declining genepools of traditional breeding. In addition, new gene formulations can be specifically designed and constructed using the tools of molecular biology. This may involve the use of promoter regions that target gene expression at a desired magnitude, time or location in plants and animals as well. It is also possible to target the specific ‘knock out’ of discrete gene function through antisense or gene silencing approaches (Machuka, 2004, Fagard and Vaucheret, 2000).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant tissue culture

Tissue culture is a technique through which a plant part is cultured on nutrient medium under sterile conditions with the purpose of obtaining growth. Any plant part can theoretically be cultured e.g. leaf tissue, stem cylinders, auxiliary buds or even single cells. The material used is routinely referred to as explant material. The term tissue culture is normally used as a blanket term to cover cultivation of any plant part, whether through its organs, tissue or cells. In references to tissue culture, the term \textit{in-vitro} is very often used. \textit{In-vitro} means in culture and the term is used in contrast to the term \textit{in-vivo} which means as in living environment.

3.2 Plant materials used in the study

### Experiments

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>Kanamycin painting</th>
<th>Gus assays</th>
<th>PCR</th>
<th>Southern blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local sweet potato varieties</td>
<td>CPT 560</td>
<td>CPT 560</td>
<td>CPT 560</td>
<td>CPT 560</td>
</tr>
<tr>
<td></td>
<td>KSP 36</td>
<td>KSP 36</td>
<td>KSP 36</td>
<td>KSP 36</td>
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<tr>
<td></td>
<td>KSP 20</td>
<td>KSP 20</td>
<td>KSP 20</td>
<td>KSP 20</td>
</tr>
</tbody>
</table>

Table 2: Codes and sources of sweet potato lines used in each experiment.

#### 3.3 Explant preparation and culture

Plant tissues, collected from the field or greenhouse were disinfected before being placed on culture medium. The most common disinfectants for this purpose include 0.5% sodium hypochlorite (equivalent to about 10% in common commercial bleaches), 70% alcohol and 10% hydrogen peroxide. Others include 7% saturated solution of calcium hypochlorite, 1% solution of bromine water and 0.2% mercuric chloride solution (Cassells, 1997). When using the heavy metal containing solutions such as mercuric chloride, precautions for minimizing health and safety risks must be taken. The type of disinfectant used, the concentration and the amount of exposure...
time vary depending on the tissue and how difficult it is to disinfect (Miller and Murashige, 1976).

3.4 In-vitro culture and maintenance of sweet potato stock plants

Young vigorously growing, healthy and disease free materials 2-3 months old were collected from the KARI screen house into sampling bags and transported into the laboratory. Stems were cut about 10 cm long with intact nodes. They were washed thoroughly in running water with Teepol Tm (detergent) to remove soil and plant debris. The stems were cut into small pieces of about 2-3 cm each having a node. They were washed with Tween-20 in order to lower the surface tension of water to allow the disinfectants to penetrate to the inside of the explants. The plant pieces were transferred to the lamina flow cabinet and soaked in 40% Jik commercial bleach (Jik household bleach containing 3.5% sodium hypochlorite m/v) for 20 minutes with occasional shaking. The pieces were then transferred to 70% ethanol for 6 minutes, and then rinsed four times in distilled water. Two percent Jik commercial bleach was added to the final rinse. Some were kept overnight and others planted on media the same day to check whether the incubation time influenced contamination level. Using a sharp slant cut the damaged part of each stem was cut off. The pieces were planted in universal bottles containing semi-solid sweet potato media. The sweet potato media used contained 4.17g of Murashige and Skoog media (1962), calcium panthothenate (0.002g), Gibberellic acid (0.02g), ascorbic acid (0.1g), calcium nitrate (0.1g), putresine HCL (0.02g), L-Arginine HCL (0.1g), sucrose (30g) and phytagel (2.5g). All ingredients except the phytagel were dissolved in distilled water and the volume adjusted to one litre. The pH was corrected to 5.6 using 1.0 M HCL (hydrochloric acid) or 1.0 M NAOH (sodium hydroxide). Monitoring of the pH was carried out
using a pH meter. Phytagel was then added and the mixture boiled with constant stirring to dissolve the phytagel.

The combined medium mixture was sterilized by autoclaving at 121 pounds per square inch (psi) pressure for 15 minutes. The inoculated culture bottles were incubated in the growth room and examined periodically for growth and contamination (Plate 1). The cultures were maintained at 27°C at a light intensity of 3000-10000 flux and at 100% relative humidity at a 16hr photoperiod (16hrs light and 8hrs dark). Any plant that showed contamination was discarded. The plants were finally micropropagated into kilner jars for 3-5 weeks and then used for kanamycin painting, histochemical assays and molecular characterization. Plants to be planted in the screen house were not surface sterilized. They were transplanted to pots containing a mixture of manure and sterile soil (1:3) and maintained at 26°C under 16 photoperiod for two months (Plate 2).

### 3.5 Gene construct used in sweet potato transformation

Coat protein gene constructs designated pMON 54929 and a *uidA* gene construct designated pMON 54975 had been used in transformation of the sweet potato lines that were used in this study. The constructs contained 300bp right and left border sequences. The right border acted as the initial point of DNA transfer into plant cell whereas the left border was to delimit the T-DNA transfer and also to act as the end point of DNA transfer into plant cells (Figure 3; Table 3). The pMON 54929 construct contained a 950bp coat protein gene from sweet potato feathery mottle virus (SPFMV) under the control of an enhanced 35S cauliflower mosaic virus promoter with a duplicated enhancer region and another promoter from figwort mosaic virus used to derive the expression of *uidA* gene. The *uidA* gene from *E. coli* was for
encoding a β-D glucuronidase or GUS protein. The construct had an intron from *Solanum tuberosum*, which was present to increase the level of gene transcription. It also contained neomycin phosphotransferase antibiotic selectable marker gene (nptII). The enzyme confers resistance to selected aminoglycoside antibiotics such as kanamycin, paramomycin, neomycin and gentamycin. The genetic elements are as shown in Table 3, Fig 3.
Fig. 3. The physical map of one of the gene constructs used in sweet potato transformation. Shown are the right and the left border regions, SPFMV coat protein gene, different promoters and the selectable marker gene. Relevant restriction sites are indicated. Source: Monsanto Company, USA.
<table>
<thead>
<tr>
<th>Genetic Element</th>
<th>Size (Kb)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Border</td>
<td>0.03</td>
<td>A 25-nucleotide sequence that acts as the initial point of DNA transfer into plant cells originally isolated from pTiT37 (Fagard and Vaucheret 2000).</td>
</tr>
<tr>
<td>P-FMV</td>
<td>0.67</td>
<td>The 35S promoter from a modified Figwort mosaic virus used to drive expression of uidA gene (Tempe and Schell, 1997; Sanger et al., 1990).</td>
</tr>
<tr>
<td>GUS:uidA</td>
<td>1.81</td>
<td>The uidA gene from E. coli encoding a β-D-glucuronidase or GUS protein. Modified to have a NcoI site and Kozac sequence at the start codon.</td>
</tr>
<tr>
<td>ST.LS1*INT</td>
<td>0.2</td>
<td>Intron from Solanum tuberosum. Present to increase the level of gene transcription</td>
</tr>
<tr>
<td>GUS1</td>
<td>1.42</td>
<td>Beta glucuronidase coding sequence from E coli</td>
</tr>
<tr>
<td>E9 3'</td>
<td>0.63</td>
<td>The 3' end of the pea (Pisum sativum) rbcS E9 gene which provides the polyadenylation sites for the uidA gene (Beck et al., 1982)</td>
</tr>
<tr>
<td>PNOS (Nos Poly A)</td>
<td>0.012</td>
<td>The nptII promoter from Tn5. Site of Poly A addition for nopaline synthase (Beck et al., 1982).</td>
</tr>
<tr>
<td>NptII</td>
<td>0.80</td>
<td>The neomycin phosphotransferase II gene was obtained from Tn5. This enzyme confers resistance to selected aminoglycoside antibiotics and is used as a plant selectable marker (Beck et al., 1982). The promoter for this gene is only active in bacteria hosts.</td>
</tr>
<tr>
<td>NOS 3'</td>
<td>0.26</td>
<td>The 3' non-translated region of the nopaline synthase gene from the Ti plasmid of Agrobacterium, which terminates transcription and directs polyadenylation (Fraley et al., 1983). Contains Poly A site.</td>
</tr>
<tr>
<td>P-e35S</td>
<td>0.62</td>
<td>The cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985) with the duplicated enhancer region (Kay et al., 1985) used to drive expression of the SPFMVCP gene.</td>
</tr>
<tr>
<td>SPFMVCP</td>
<td>0.95</td>
<td>The coat protein of the SPFMV obtained from R. Beachy, Scripps Institute, La Jolla, CA (Mori et al., 1995) in sense or antisense orientation.</td>
</tr>
<tr>
<td>E9 3'</td>
<td>0.63</td>
<td>The 3' end of the pea (Pisum sativum) rbcS E9 gene which provides the polyadenylation sites for the SPFMVCP gene</td>
</tr>
<tr>
<td>Left border</td>
<td>0.03</td>
<td>A 25-nucleotide sequence that delimits the T-DNA transfer and acts as the endpoint of DNA transfer into plant cells. It was originally isolated from Ti plasmid PtiA6 (Beck et al., 1982).</td>
</tr>
<tr>
<td>ori-V</td>
<td>0.40</td>
<td>Origin of DNA replication, originally isolated from plasmid RK2; permits plasmid replication in E. coli. Functional only when the trfA protein is present in the same cell.</td>
</tr>
<tr>
<td>Rop</td>
<td>0.2</td>
<td>Coding region for repressor of primer. Also known as rom</td>
</tr>
<tr>
<td>Ori-322</td>
<td>0.63</td>
<td>Origin of replication isolated from the plasmid pBR322; permits plasmid replication in E. coli. This is the minimum known sequence required for a functional ori. Sequence downstream of this region is known to affect copy number</td>
</tr>
<tr>
<td>Aad</td>
<td>0.79</td>
<td>The bacteria gene (aad) encoding the Tn7 3' adenyltransferase conferring spectinomycin and streptomycin resistance to bacterial cells that carry the plant vector. (Coding region for Tn7 adenyltransferase (AAD)(3') (Murray and Thompson, 1980).</td>
</tr>
</tbody>
</table>

Table 3: Summary of the Genetic Elements in Sweet Potato Feathery Mottle Virus gene construct used in transformation
3.6 Verification of the presence of the transgene

3.6.1 Selectable marker genes

Selectable genes encode proteins that render the transformed plant resistant to phytotoxic agents (GaluN and Breiman, 1997). Such agents are added to the culture medium converting it to a selective medium. The tissues or plant organs are transferred to selective medium shortly after the transformation process. The plant tissue to be transformed must be sensitive to the phyotoxin. The presence of selective gene eliminates this sensitivity. The most commonly used antibiotic selectable marker is the gene coding neomycin phosphotransferase II (nptII). This gene encodes for the enzyme neomycin phosphotransferase, which detoxifies several aminoglycoside antibiotics such as kanamycin, paramomycin, gentamycin and neomycin (Gray, 1993).

3.6.1.1 Kanamycin painting

Two months after culture of plantlets the surface of the fully expanded leaves of the CPT 560 control lines were wetted with different concentrations of kanamycin solutions to establish a killing curve for selection. These concentrations were 0 g/l, 5 g/l, 10 g/l, 15 g/l, and 20g/l. Observations were made each day for seven days and the level of bleaching was scored on a scale of 0-6 as follows.

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no bleaching observed on leaf</td>
</tr>
<tr>
<td>1</td>
<td>10% of leaf bleached</td>
</tr>
<tr>
<td>2</td>
<td>25% of leaf bleached</td>
</tr>
<tr>
<td>3</td>
<td>50% of leaf bleached</td>
</tr>
<tr>
<td>4</td>
<td>75% of leaf bleached</td>
</tr>
<tr>
<td>5</td>
<td>100% fully bleached leaf</td>
</tr>
<tr>
<td>6</td>
<td>Dead leaf</td>
</tr>
</tbody>
</table>
After seven days, the surface of fully expanded leaves of each transgenic plant and untransformed control plants were wetted with 15g/l kanamycin solution. This was the highest concentration of kanamycin that bleached most leaves of the untransformed CPT 560. The assumption was that if the non-transformed CPT 560 were bleaching at 15g/l kanamycin solution, the same concentration should not bleach the transgenic plants if they were expressing the selectable marker gene.

### 3.6.2 Screenable marker genes

Reporter genes are coding sequences that upon expression in the transgenic plant provide a clear indication that genetic transformation did take place. In order to be useful, reporter genes should express a feature (commonly an enzyme activity) that does not exist in the host plant (Martin et al., 1992). Therefore, the most commonly used reporter genes were derived from bacteria, insects or jelly fish. For instance, an \textit{E. coli} gene (termed \textit{uid A} or \textit{Gus A}) encodes \(\beta\)-glucuronidase (GUS) enzyme. GUS 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 (De Block, 1993; Jefferson et al., 1987). The \textit{Gus} gene gives the plant the ability to produce \(\beta\) glucuronidase enzyme. The substrate for this enzyme is 5-bromo-4-chloro-3 \(\beta\) –D-indolyl glucuronide (X-gluc). Other screenable markers include Green Fluorescent Protein (\textit{gfp}) gene that encodes the green fluorescent protein (GFP) which is detected by irradiating UV light on the tissues or cells (Chalfie et al., 1994, Ahlandsberg et al., 1999)
3.6.2.1 Histochemical GUS analysis

Histochemical GUS assays using 5-Bromo-4-chloro-3-indolyl β-D glucuronide (X-gluc) as a substrate was employed to determine whether plants expressed the screenable Gus marker gene after transformation (Jefferson, et al., 1987). The assays were used to assess stable expression of the Gus gene in kanamycin resistant lines of the transgenic sweet potatoes. Young vigorously growing leaves, stems and tubers from the transformed and non-transformed plants were obtained and fixed by submerging in a 10mM solution of 2-N-Morpholino ethane sulfonic acid (MES) for one hour. The fixative (MES) contained 10mm 2-N- Morpholino ethane sulfonic acid, 100mM formaldehyde and 500mM mannitol all dissolved in distilled water and pH adjusted to 5.6. They were then rinsed four times in a 50mm solution of sodium dihydrogen orthophosphate (NaH₂PO₄ (pH 7.0)). After the final rinse the explants were flooded with 1mM solution of 5 Bromo-4-Chloro-3-Indolyl β-D-Glucuronidase (X-Gluc) in sodium phosphate buffer pH 7.0. They were incubated overnight at 37°C and then examined microscopically for blue staining after soaking the plant tissues in 70% ethanol to remove excess chlorophyll.

3.7 Molecular analysis

The plant cell membrane has to be disrupted to release cell contents so as to release the DNA for molecular analysis. Detergents lyse the cell membrane by solubilising membranous material such as lipid molecules (Dellaporta et al., 1983). The most commonly used detergent is Cetyl Trimethyl Ammonium Bromide (CTAB). In addition to DNA, the cell lysate has significant amounts of proteins, carbohydrates and RNA which have to be removed. The standard way to deproteinise the cell extract
is to treat with phenol, chloroform and isoamyl alcohol. These organic solvents precipitate proteins but leave the nucleic acids, DNA and RNA in aqueous solution. The aqueous solution of nucleic acids is removed by pipetting after spinning. If the protein content is great, the preparation may be deproteinised using pronase or proteinase K before the phenol extraction step. The process breaks down polypeptide chains to smaller units more easily removed by phenol (Hames and Rickwood, 1996). After deproteinisation, RNA is removed with ribonuclease (RNAseA) which degrades the RNA. To get rid of carbohydrates, the DNA preparation is treated with alcohol which precipitates the DNA out of the solution. The precipitated DNA is harvested either by centrifugation or by use of a glass rod.

3.7.1 DNA extraction

One hundred milligrams of leaf tissue was weighed and ground in a pre-chilled mortar in liquid nitrogen to obtain a fine powder. (Grinding with liquid nitrogen bursts up the plant cell walls, allowing the DNA to go into the Cetyl Trimethyl Ammonium Bromide (CTAB) solution. The freezing with liquid nitrogen allows tough plant material to be ground easily, and minimizes the action of enzymes present in the cell that can break down the DNA. The CTAB solution contained: - 2g CTAB, 28 ml 5M sodium chloride, 4ml 0.5M EDTA, 10ml 1M Tris HCL pH 4, 1g Polyvinyl pyrocollidone (PVP), 85µl β-mercaptoethanol and 58 ml distilled water. All the ingredients were dissolved in 100ml distilled water and the volume adjusted to 100ml. CTAB whose salts lyse cell membrane wall was dissolved after salt was added.

Ground sweet potato leaves were transferred into an Eppendorf tube, seven hundred microlitres of fresh CTAB buffer was added and the mixture was vortexed.
The mixture was then maintained at 65°C for 45 min with stirring of the tubes every 15 minutes. The PVP in the buffer helps bind up polysaccharides and polyphenols present in the plant that could co-precipitate with DNA. Seven hundred microlitres of chloroform:isoamyl:alcohol (24:1) was added to each tube. The mixture was vortexed briefly and gently to avoid shearing the DNA. The chlorophyll and most of the other pigments get transferred into the Chloroform (CI) layer because the pigments are non-polar and thus dissolve in the highly non-polar chloroform. The mixture was then spun at 14,000 revolutions per minute (rpm) in a tabletop Eppendorf centrifuge to pellet the cell debris. After centrifugation there were three layers; the green CI layer with pigments, a whitish middle layer of polysaccharides and a transparent aqueous layer on top that contained the DNA. The aqueous layer was then removed and transferred into a newly-labeled eppendorf tube. Care was taken to avoid carrying over the interphase material. Fifty microlitres of 10% CTAB was then added, vortexed briefly and gently. Seven hundred microlitres of chloroform:isoamyl alcohol was again added and the mixture centrifuged at 14,000 rpm for 5 min. The top aqueous layer was removed and transferred into a newly-labeled eppendorf tube. Five hundred microlitres of ice-cold isopropanol was added to each tube and the mixture was turned over several times. The tubes were then allowed to stand at 4°C for 30 minutes to allow the DNA to precipitate. The mixture was then spun at 14,000 rpm for 20 minutes to pellet the precipitated DNA. The supernatant was then poured carefully taking care not to loose the DNA pellet. The tubes were then left to air dry for 2 minutes.

3.7.2 DNA washing

One milliliter of 70% ethanol (EtOH) was added prior to spinning of the tubes at 14,000 rpm for 30 minutes to pellet the DNA. The supernatant (EtOH) was
carefully poured out and the pellet was again washed with 95% EtOH and spinned at 14,000 for 30 minutes. Ethanol was carefully poured out and the tubes were turned upside down in a laminar flow hood and left to air dry overnight. EtOH removes traces of CTAB buffer and salts that may have co-precipitated with the DNA. DNA is highly insoluble in alcohol, but other compounds like salts are not and they dissolve in 70% alcohol. The DNA was dissolved in 150 µl of TE buffer (10mmTris-HCL, 1mm EDTA pH 8.3). Two microlitres of RNAse (10mg/ml) was also added to degrade the RNA. The DNA was then incubated at 37°C for one hour. DNA was quantified using a Beckman DU-65 spectrophotometer and the quality was checked by running agarose gels.

**3.7.3 Quantification and quality control of DNA**

**3.7.3.1 UV quantification of DNA**

Fifteen microlitres of each sample was added to 735 microlitres of sterile double distilled water and vortexed to give a 1:50 dilution. A Beckman DU-65 spectrophotometer was used to read the optical density at 260 and 280nm (OD 260/280) so as to determine the concentration of DNA in µg/ml and also to determine the purity of the extracted DNA. DNA has been shown to absorb UV light at 260 nm and one optical density (OD) at 260nm is equivalent to 50 ng (Sambrook *et al.*, 1989). Therefore DNA concentration was calculated as follows:

\[
\text{DNA concentration (ng/µl)} = \frac{\text{OD}_{260} \times 50 \text{ (dilution factor)} \times 50\text{µg/ml}}{1000}
\]

(Sambrook *et al.*, 1989)
The ratio $\frac{OD_{260}}{OD_{280}}$ was used to determine the purity of the DNA samples. It has been shown that if the ratio is between 1.8 and 2.0 the absorption is due to nucleic acids. A ratio less than 1.8 indicates that there may be proteins or other UV absorbers in the sample. A ratio higher than 2.0 indicates that samples may be contaminated with chloroform or phenol (Rojas, 1997). After quantification, samples that had a ratio less than 1.8 and higher than 2.0 were reprecipitated with ethanol.

3.7.3.2 Agarose gel electrophoresis

Agarose is a galactose based polymer, widely used in analytical and preparative electrophoretic separation of linear nucleic acids in the size range above 100 bp. DNA was applied to an agarose gel and after exposing to an electric field it migrated towards the anode since nucleic acids are negatively charged. The migration was inversely proportional to the length of the fragment. A molecular weight fragment ladder was placed in a lane alongside the experimental samples to determine the size of the separated fragments.

3.7.3.2.1 Gel preparation and electrophoresis

One gram of agarose powder was weighed and dissolved in 100 ml of TBE. [(Tris Borate EDTA)(54g Tris base, 27.5g Boric acid 20ml 500mm EDTA)] buffer by slowly boiling in a microwave oven. The mixture was covered to avoid evaporation. The agarose was allowed to cool down to 60°C and Ethidium Bromide was added to the gel at a concentration of 1mg/ml. Care was taken because Ethidium Bromide is a mutagen. Ethidium Bromide intercalates between the DNA making it visible under a UV trans-illuminator. While the agarose was cooling, the gel tray was prepared by placing tape across the ends of the tray to avoid leakage and so that the tray could
accommodate the desired thickness of the gel. The agarose was then poured into the tray and the combs were inserted. The gel was allowed to solidify for 30 minutes before removing the tape, and then the tray was placed in the gel rig containing TBE buffer. The combs were removed and 20 μl of each DNA sample containing 2 μl of loading solution (50mm EDTA, 50Mmm NaCl, 5ml glycerol and 0.01g Bromophenol blue) was loaded to the wells of the gel to the top. A lane of molecular weight marker lambda DNA (digested with EcoRI and HindIII restriction enzymes) was included in each side of the gel to aid in determination of the quality and quantity of the DNA samples. DNA was mixed with the loading solution in order to make the solution sink in the gel wells. The samples were run at 100 mA for 10 minutes and the amperage was then reduced to 25 mA, constant current until the bromophenol blue in the loading solution had migrated almost to the end of the gel. Resolution was improved by recirculating the buffer every 20 minutes. The gel was then removed from the rig, placed onto a UV transilluminator and photographed using a digital camera.

3.8 Polymerase chain reaction (PCR)

Polymerase Chain Reaction, (Mullis et al., 1987) is a method of producing an extremely large number of copies of specific DNA sequence from a mixture without having to clone it.

3.8.1 Standard PCR Assay

Commercially acquired left and right primers that bound to the 5’ and 3’ ends respectively of the transgene were used. The sequence of the coat protein forward primer was 5’GGATGGTGATGAGCAAGTGACATA3’ and that of the reverse primer was 5’ACCCCTCATTCCTAAGAGGTTA 3’. The sequence of the non inverted forward primer was 5’GCCCTGCACAGTTAGTGACTAA 3’ and that of the
reverse primer was 5'TCAGAGATCCATTCCAGACCTT 3'. PCR was performed in 0.2 ml tubes in 50 μl reactions containing 5.0 μl of the 50mM MgCl₂, 1μl of the 10 mM dNTPs (an equimolar mixture of dATP, dCTP, dGTP and dTTP), 0.5 μl of the 5 units/μl Taq DNA polymerase (Amplitaq Gold, Applied Biosystems, USA), 5.0 μl forward and reverse primers, 2pmols each, 5.0μl 10x PCR Buffer, 5μl of DNA template, 5ng/μl each and 23.5 μl double distilled water. A control consisting of 50 μl of the master mix without sample DNA was included in each series of the reaction. After initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C- 1 min; primer annealing at 55°C -30sec, DNA synthesis at 72°C -60 sec, were run with a final extension step of 10 mins at 72°C. Samples were then kept on hold at 4°C before running the gel. Following amplification, 15 μl of each reaction mix was mixed with 5μl of loading solution and was separated on 1.5% agarose gel (100volts at constant current), stained with ethidium bromide and visualized using Uv transilluminator. One 100-bp molecular marker with fragment lengths of 12000, 5000, and 2000...... down to 100 bp was used to evaluate the molecular weights of the amplicons. The DNA Polymerase enzyme, dNTPs, PCR buffer and MgCl₂ were from Applied Biosystems Company, New Jersey, USA. The primers were from Invitrogen Company, Chicago, USA.

3.9 Southern blot analysis.

Named after its inventor Edward Southern, Southern blotting is a procedure for analysis of DNA sequences complementary to a radioactive or non-radioactive probe (Southern, 1975).
3.9.1 Preparation of the glycerol stock of bacteria containing gene construct

Luria Bertani (LB) medium (10g/l tryptone, 5g/l yeast extract, 10g/l NaCl) containing 100 μg/ml spectinomycin, 21.5 μg/ml chloramphenical and 50μg/ml kanamycin (SCK) was prepared and poured in petri-dishes. Agrobacteria containing the pMON 54929 and pMON 54975 constructs were subcultured onto the LB plates. The plates were incubated at 28°C for 3 days. One colony of the bacteria was inoculated into 2mls of the LB broth containing the antibiotics and incubated for 2 days at 28 °C. Four hundred microlitres of the bacteria suspension was inoculated into a 2ml tube containing 200μl of sterile glycerol and 400μl of LB medium with the three antibiotics. The tube contents were mixed well and frozen at -80°C.

3.9.2 Probe preparation

Frozen Agrobacterium was streaked onto Luria Bertani (LBSCK) plates and maintained in an incubator at 29°C for 2 days. On the 3rd day one loopful of bacteria was transferred onto a culture tube containing Yeast Extract Peptone (YEPSCK) medium (Appendix 2). The bacteria in culture tubes were maintained in a shaker at 29°C for 6 hours. Four milliliters of the bacterial culture was added to 50 ml of ABSCK medium in sterile flask and was shaken overnight. The Agrobacterium was now ready for plasmid DNA extraction.

Plasmid DNA was extracted using the Wizard™ mini prep kit (Promega, Madison, USA) according to the manufacturer’s instructions. The DNA was separated using 1% agarose gel to check the quantity and the purity of the DNA before labeling. Labeling was done using Digoxygenin (DIG, Roche Applied Science, Mannheim, Germany) dNTPS in a standard PCR assay as mentioned earlier. Thereafter the DIG-labeled CP probe was extracted using the QIAquick Gel Extraction Kit Protocol (Qiagen, Hilden,
USA) according to the manufacturer’s instructions. The probe was then purified using ethanol/lithium chloride precipitation.

3.9.3 Purification of DIG-labeled CP fragments using ethanol/lithium chloride.

All the CP samples were combined into one eppendorf tube and the volume was taken (150μl). The labeled nucleic acid was precipitated with 0.1 volume (15 μl) of 4M Lithium chloride (LiCl) and 3 volumes of chilled absolute ethanol (450 μl). The preparation was mixed and centrifuged at 14000rpm. Ethanol was poured off and the pellet was washed with 100μl of ice cold 70% EtOH. The pellet was dried for 30 minutes and then resuspended in 50 μl of TE buffer.

3.9.4 Estimating the yield of DIG-labeled CP probe using a dot blot

An accurate quantification of DIG- labeled DNA probe obtained in the labeling reaction is the most important for optimal and reproducible results in various membrane hybridization techniques.

The newly labeled DNA probe and the DIG labeled control DNA were diluted to a final concentration of 1ng/μl as shown in Table 4 and Table 5.
<table>
<thead>
<tr>
<th>Tube number</th>
<th>Volume of DNA (µl)</th>
<th>Volume of dilution buffer (µl)</th>
<th>Dilution factor</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>20</td>
<td>5x</td>
<td>1ng</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>20</td>
<td>2x</td>
<td>500 pg</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5</td>
<td>2x</td>
<td>250 pg</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>5</td>
<td>2x</td>
<td>125 pg</td>
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<td>8</td>
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<td>12</td>
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<td>3 pg</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>4</td>
<td>3x</td>
<td>1 pg</td>
</tr>
</tbody>
</table>

Table 4: Serial dilutions of control DNA that were used to estimate the concentration of labeled probe used for hybridization.

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Volume of DNA (µl)</th>
<th>Volume of dilution buffer (µl)</th>
<th>Dilution factor</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>45</td>
<td>10x</td>
<td>10x</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>45</td>
<td>10x</td>
<td>100x</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5</td>
<td>2x</td>
<td>200x</td>
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<td>5</td>
<td>5</td>
<td>2x</td>
<td>400x</td>
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<td>5</td>
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<td>5</td>
<td>2x</td>
<td>800x</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>5</td>
<td>2x</td>
<td>1600x</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>12</td>
<td>3.4x</td>
<td>5400x</td>
</tr>
</tbody>
</table>

Table 5: Serial dilutions of probe DNA. The probe was blotted against the control labeled DNA at different concentrations so as to determine the probe concentration. The diluted probe DNA and the control DNA were spotted 1 µl to 1µl on a nylon membrane. The DNA was then fixed to the membrane by baking at 120 °C in an oven.
3.9.5 Detection of labeled probes

The membrane was transferred to a plastic tray containing 100 ml of washing buffer (0.1 M Maleic acid, 0.15 M NaCl, Appendix 1) and incubated for 2 minutes at room temperature with shaking to equilibrate the membrane. The washing buffer was discarded and 100 ml of the blocking solution (10x Blocking Solution diluted 1:10 with Maleic Acid Buffer, Appendix 1) was added to the tray and incubated for 30 minutes with shaking. This step prevents non-specific attraction of antibody to the membrane. The blocking solution was poured out and 20 ml of the antibody solution (Anti-digoxigenin-AP diluted 1:5000 in Blocking solution, Appendix 1) was added and incubated for 30 minutes with shaking. The membrane was then washed twice with 100 ml portions of the washing buffer (Appendix 1), equilibrated for 3 minutes in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5 Appendix 1). Two hundred microlitres of the NBT/BCIP stock solution was added to 10 ml of detection buffer to make the colour substrate solution. The membrane was covered completely with the colour substrate solution for 16 hours in the dark. When the colour reaction had produced spot of the desired intensity the reaction was stopped by rinsing the membrane for 5 minutes with Tris-EDTA (TE) buffer (Appendix 1). This preparation was used to determine the probe concentration for hybridization. The membrane was then dried and photographed using a digital camera.

3.9.6 Restriction digestion of DNA samples for Southern transfer

Ten micrograms of DNA from each sample together with the plasmid were digested with EcoRI and Bam H1 restriction endonuclease enzymes (Sigma, St. Louis, MO, USA). EcoRI nicks DNA at the GA and AG sites while Bam HI nicks
DNA at the GG sites. The enzyme cut next to the transferred gene as well as at many places on the plant’s chromosomes where the DNA sequence was recognized by the restriction enzyme. Some of the segments generated were expected to contain the transgene inserted in the genomic DNA of the transgenic plant.

3.9.7. Southern transfer and probe hybridization

The fragments generated by restriction digest of the genomic DNA were loaded onto an agarose gel and separated by electrophoresis. The gel was then soaked in 200 ml denaturation solution (0.4 M NaOH, 1M NaCl) for 5 minutes. This converts all DNA in the gel to the single stranded form necessary for hybridization. The gel was then submerged in 200 mls neutralization solution (1M NH₄ OAc) for 15 minutes to neutralize it. A large sheet of nylon membrane was placed on top of the gel and a stack of Whatman filter papers were placed on top of the nylon membrane. A 500g weight was placed on top of the gel (Fig 4). After overnight transfer, the nylon membrane was baked in an oven at 120°C for 30 minutes to fix the DNA. The blot was placed in a hybridization bottle containing 20 ml of the prehybridization solution (Appendix 1) for 10 hours. The hybridization oven was set at 42°C. The prehybridization solution was discarded and the hybridization solution containing the DIG-labeled probe was added to the bottle and left overnight in the oven at 39°C. The membrane was washed twice for 15 minutes with 2 times wash solution (Appendix 1) and 0.5 times wash solution (Appendix 1). The membrane was then developed using NBT/BCIP as described earlier.
Fig 4: **Blotting apparatus for DNA transfer** (Sambrook, *et al.*, 1989)
CHAPTER FOUR

RESULTS

4.1 *In vitro* culture and propagation of sweet potato stock plant in the laboratory

Both the transformed (KBC/2002/4, KBC/2002/5, KBC/2002/6, KBC/2002/8, KBC/2002/9 pMON/2000/53, pMON/2000/61, pMON/2000/86, pMON/2000/94, pMON/2000/160, pMON/2000/173, pMON/2000/228, pMON/2000/245, pMON/2000/283, pMON/2000/300 and pMON/2000/336) and untransformed negative control (CPT 560, KSP 20 and KSP 36) plants were cultured and maintained in the laboratory with minimal contamination. The stock plants were maintained in culture being examined periodically for growth and contamination. Any plant that was contaminated was discarded. ‘Clean’ plants were propagated into kilner jars every 3 weeks (Plate 1). Stock plants established *in-vivo* were not contaminated and were maintained in the screen house for three months (Plate 2). In the experiment to compare the rate of contamination between the explants incubated in 2% Jik commercial bleach at different time intervals, it was observed that leaving the explants in 2% commercial bleach overnight reduced the rate of contamination by half (Table 6).
Plate 1: Stock plants of KSP 36, KSP 20, CPT 560 and transformed plants established *in-vitro* growing in the growth room: A. Stocks established *in-vitro* in universal bottles from where they were subcultured in kilner jars. B. Stock plants established *in-vitro* in kilner jars.

Plate 2: Stock plants of KSP 36, KSP 20, CPT 560 and transformed plants established *in-vivo* growing in the screen house. Each tray was maintained for each of the different lines.
Sterilization treatment | No. of explants initiated in the laboratory | No. of explants contaminated and discarded
---|---|---
Explants put in media immediately after final rinse with 2% commercial bleach | 46 | 27

Explants kept in 2% Jik overnight | 46 | 44

Table 6: Effects of different incubation times of stem explants in 2% Jik commercial bleach on the level of contamination

The univariate analysis of variance at p=0.05 showed that the difference in the results of the two treatments were highly significant (p=0.001) with overnight incubation having the lowest level of contamination (59 %) and immediate planting being less successful (96 %) (Table 6). This showed that overnight incubation of explants in 2% Jik was more effective than immediate planting.

4.2 Kanamycin painting

Researchers at the Monsanto Company St Louis, USA and KARI succeeded in regenerating and transforming the sweet potato lines variety CPT 560 that were characterized in this study (unpublished results). The gene constructs that were used in the transformation process had been based on the antibiotic selectable marker nptII which breaks down aminoglycoside antibiotics such as kanamycin, neomycin, gentamycin and paramomycin (Njagi, 2004). This experiment was therefore carried out to determine the lowest concentration of kanamycin solution that would bleach the non-transformed CPT 560. The assumption was that since the non-transformed CPT 560 did not have the antibiotic resistance gene, the leaves were expected to bleach at a concentration which was not expected to bleach the transformed plants if they had integrated the selectable marker gene. In the experiment, leaf explants of different
sweet potato lines were subjected to varying concentrations of kanamycin. The lowest concentration found to cause total bleaching of the nontransformed CPT 560 was 1.5g/l of kanamycin solution (Figure 5; Plate 3). The leaves of transgenic lines painted with this kanamycin concentration failed to show visible damage although those of the untransformed control plants treated with kanamycin exhibited severe necrosis, chlorosis and bleaching. At 0% there was no change to the explants on the 7th day for both the transformed and non-transformed lines. At 0.25% explants of the non-transformed CPT 560 and KSP 36 showed early signs of bleaching by the 7th day but the transformed lines did not bleach. At 0.5% and 1% kanamycin concentration explants from the non-transformed lines started to bleach on the 5th day but had not bleached fully by the 7th day. At 1.5% the non-transformed lines were fully bleached by the 7th day. For the transformed leaf explants there were no signs of bleaching observed at all kanamycin concentration i.e 0%, 0.25%, 0.5%, 1%, 1.5% and 2% (Plate 4, Plate 5). There were no differences in antibiotic resistance at 1.5% kanamycin concentration between the transgenic lines.

These transgenic sweet potato plants grew normally and formed storage roots after three months in the screen house. There were no morphological differences between the untransformed control plants and the transgenic plants during the entire growth period in the screen house.
Fig. 5: Effect of Kanamycin on untransformed CPT 560 landrace. The lowest concentration found to cause total bleaching of the non-transformed CPT 560 was 1.5% kanamycin solution. Most leaves had a score 5 which represented 100% bleached leaf. At 2% kanamycin concentration, all the leaves necrosed whereas at 0.5%, 20% of the leaves had bleached and at 1% only 30% of the leaves had bleached on the seventh day. At 0% there was no change on the leaf tissue on the seventh day.
Plate 3: Kanamycin painting of non-transformed CPT 560. Numbers represent kanamycin concentrations. At 0%, there was no change to the explants on the 7th day. At 1.0% kanamycin concentration the explant showed early signs of bleaching on the 7th day. At 1.5% kanamycin concentration, explants had fully bleached on the 7th day of incubation. At 2.0% the explants had bleached before the 7th day.
Plate: 4 *In-vitro* leaf tests for resistance of transgenic sweet potato plants to kanamycin on the seventh day. Line 8 (KBC/2002/008) and line 9 (KBC/2002/009) are transformed whereas CPT 560 and KSP 36 are not transgenics.
Plate 5A & B: In-vivo (in-planta) leaf painting tests for resistance of transgenic sweet potato plants to kanamycin on the seventh day: The transgenic lines have the coat protein gene and the selectable marker (Npt II) under the control of P-e35S promoter. In the screen house, non-transformed CPT 560, KSP 20 and KSP 36 that were painted with 1.5% kanamycin concentration exhibited severe necrosis, chlorosis and bleaching. Leaves of the transformed plants KBC/2002/008 and KBC/2002/009 painted with 1.5% kanamycin concentration did not undergo necrosis or chlorosis.
4.3. Histochemical GUS assays

Transient GUS expression assays were performed on pMON 54929 and pMON 54975 transformed explants to verify that the transgene had been integrated into the transgenics and was being expressed. The assumption was that since the same transformation process had been used for both the coat protein and Gus gene, the coat protein and Gus gene must have been introduced into the transformed plants at identical rates. Out of the 14 transformed explants assayed, 12 explants showed characteristic blue staining on the entire surface (Plate 6, 7 & 8). Transformed pMON/2000/53, pMON/2000/173 and the non-transformed control plants did not exhibit any blue color. The Gus gene in the two transgenic lines might have been silenced or there might have been some positional effects due to random integration of the transgene. It was also observed that the wounded (cut) regions on the leaf reacted more intensively, suggesting higher enzymatic activity (Plate 6B). The root and stem explants of the 12 transgenic lines displayed intense blue color in the whole segment tested (Plate 7 & 8) indicating strong gene activity.


4.4 DNA isolation, quantification and purity checking

DNA isolation using the protocol of Doyle and Doyle (1990) yielded DNA from sweet potato. Spectrophotometric analysis of the DNA isolated from transgenic and control plants showed that the DNA extracted was of good quality since most of the samples had ratios of OD \( \frac{260}{280} \) of between 1.8 and 1.98. The concentration of the DNA in most samples ranged between 61-113 μg/μl (Table 7). After quantification, it was found out that DNA from KBC/2002/008, KBC/2002/009, Pmon/2000/86, pMON/2000/300 and CPT 560 was of low purity and low concentration. This showed that the DNA was contaminated with either proteins, polysaccharides or phenolic
compounds. These samples were therefore precipitated with absolute ethanol to remove the contaminants.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>OD</th>
<th>DNA [µg/ml]</th>
<th>OD Ratio 260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBC/2002/004</td>
<td>2.090</td>
<td>1.106</td>
<td>104</td>
</tr>
<tr>
<td>KBC/2002/004</td>
<td>1.959</td>
<td>0.995</td>
<td>98</td>
</tr>
<tr>
<td>KBC/2002/006</td>
<td>1.959</td>
<td>0.995</td>
<td>98</td>
</tr>
<tr>
<td>KBC/2002/008</td>
<td>0.28</td>
<td>0.140</td>
<td>10</td>
</tr>
<tr>
<td>KBC/2002/009</td>
<td>0.430</td>
<td>0.259</td>
<td>22</td>
</tr>
<tr>
<td>pMON/2000/53</td>
<td>0.085</td>
<td>1.082</td>
<td>104</td>
</tr>
<tr>
<td>pMON/2000/61</td>
<td>1.359</td>
<td>0.697</td>
<td>68</td>
</tr>
<tr>
<td>pMON/2000/86</td>
<td>0.508</td>
<td>0.290</td>
<td>25</td>
</tr>
<tr>
<td>pMON/2000/94</td>
<td>1.332</td>
<td>0.672</td>
<td>67</td>
</tr>
<tr>
<td>pMON/2000/160</td>
<td>2.251</td>
<td>1.148</td>
<td>113</td>
</tr>
<tr>
<td>pMON/2000/173</td>
<td>1.416</td>
<td>0.717</td>
<td>71</td>
</tr>
<tr>
<td>pMON/2000/245</td>
<td>1.275</td>
<td>0.659</td>
<td>64</td>
</tr>
<tr>
<td>pMON/2000/283</td>
<td>0.219</td>
<td>0.138</td>
<td>64</td>
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<tr>
<td>pMON/2000/300</td>
<td>0.710</td>
<td>0.476</td>
<td>11</td>
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<td>KSP 20</td>
<td>1.986</td>
<td>1.021</td>
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<tr>
<td>KSP 36</td>
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<td>0.659</td>
<td>64</td>
</tr>
<tr>
<td>CPT 560</td>
<td>0.122</td>
<td>0.084</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 7: Spectrophotometric results of DNA isolated from transgenic and control plants. The concentration of the DNA in most samples ranged between 61-113 µg/ml.
After quantification and reprecipitating DNA samples that had ratios lower than 1.8 and higher than 2.0, the DNA samples attained a higher concentration and better purity after dissolving in 25μl TE buffer (Table 8).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>OD</th>
<th>DNA [μg/ml]</th>
<th>OD Ratio 260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>KBC/2002/008</td>
<td>1.41</td>
<td>0.697</td>
<td>69</td>
</tr>
<tr>
<td>KBC/2002/009</td>
<td>1.416</td>
<td>0.72</td>
<td>72</td>
</tr>
<tr>
<td>pMON/2000/86</td>
<td>1.281</td>
<td>0.659</td>
<td>64</td>
</tr>
<tr>
<td>pMON/2000/300</td>
<td>1.21</td>
<td>1.01</td>
<td>99</td>
</tr>
<tr>
<td>CPT 560</td>
<td>0.132</td>
<td>0.081</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 8: Quantification results of DNA samples after reprecipitation. The DNA samples became 'clean' and of higher concentration.
Gel electrophoresis of the isolated DNA showed that the DNA was 'clean' and of high molecular weight. The DNA did not contain RNA and it was not degraded in all the samples (Plate 9).
4.5. Polymerase chain reaction

The presence of the transgene in putative transformed plants was confirmed by PCR amplification of a 450bp fragment of the coat protein gene in the DNA extracted from putative transgenic plants. DNA from non-transformed control plants was not expected to possess this fragment whereas DNA from the *Agrobacterium* was expected to possess the 450bp fragment. Detection of the transgene from the transformed plants was done using forward and reverse primers as described in materials and methods. A 450bp single band was obtained in 7 transgenic plants and the *A. tumefaciens* (Plate 10). No amplification occurred with the DNA from control plants and in the case of KBC/2002/008 and pMON/2000/300. The transgene in these two transgenic plants might have been silenced or undergone some positional effects resulting in unstable integration.
Plate 10: Gel electrophoresis showing the results of PCR amplification of DNA extracted from 9 transformed, 2 control plants and from *Agrobacterium tumefaciens*. Seven out of the 9 transformed plants showed a characteristic 450 bp bands, which is absent in the control plants (lane 12 and 13) and in the 2 putative transformant lane 5 and 7 (KBC/2002/008 and pMON/2000/300 respectively). Lane 1-100bb MWM, Lane 2-KBC/2002/004, Lane 3-KBC/2002/005, Lane 4-KBC/2002/006, Lane 5- KBC/2002/008, Lane 6-KBC/2002/009, Lane 7-pMON/2000/300, Lane 8-pMON/2000/94, Lane 9-pMON/2000/245, Lane 10-pMON/2000/336, Lane11- 54975 construct, Lane12- KSP 36, Lane13- CPT 560, Lane14- 100bp MWM
4.6. Probe preparation

*Agrobacterium* cultures were maintained at 28°C for 3 days before plasmid DNA extraction. On the third day, the colonies had grown and separated into single colonies which were required for DNA extraction (Plate 11).

![Plate 11: *Agrobacterium* plates for plasmid DNA extraction on the third day](image)

4.6.1 Plasmid extraction

The plasmid DNA extracted using the Wizard T\(^{\text{TM}}\) Miniprep Kit (Promega, Madison, USA) was separated on 1% agarose to check the quality and quantity of the plasmid DNA before labeling. Agarose gel electrophoresis showed that the extracted plasmid DNA was of good quality and of high molecular weight (Plate 12).
Plate 12: Agarose gel electrophoresis of extracted plasmid DNA. DNA is of high quality and high molecular weight. Lane 1 & 6 – 1 kb molecular weight marker
Lane 2, 3, 4 & 5 - Plasmid DNA
4.6.2 DIG labeled PCR products

It was observed that the DIG labeled PCR products and the non-labeled PCR products migrated as separate bands of approximately 450bp on the electrophoretic gel (Plate 13). The labeled products migrated slowly on the gel than the non-labeled products because the presence of DIG in the DNA made it heavier. The intensity of the stained DIG labeled probe was equal to that of the non-labeled PCR products.

Plate 13: DIG-labeled PCR products. The DIG- labeled plasmid DNA was of high molecular weight than the non-labeled PCR products and therefore migrated slower on the gel. Lane1-5 Plasmid DNA amplified with DIG labeled dNTPs, Lane 6 Plasmid DNA amplified with non-labeled dNTPs.
4.7 Restriction digestion of genomic DNA

DNA from transgenic plants, one control line and from the plasmid was digested with EcoRI and BamH1 restriction endonucleases. In this case, BamH1 was the rare cutter where as EcoRI was a frequent cutter. The enzymes cut next to the transferred gene and other places on the plants chromosomes where the DNA sequences were recognized by the enzyme. This resulted into a smear after separating the products in a 1% agarose gel (plate 14).

Plate 14: Gel electrophoresis of restricted samples. DNA samples in lane 3 & 4 were not sufficient to be restricted and be visible on gel despite quantification. Lane 1-CPT 560, Lane 2-Plasmid DNA, Lane 3 pMON/2000/94, Lane 4 pMON/2000/336, Lane 5-KBC/20002/004, Lane 6-KBC/20002/009.
4.8 Estimating the yield of DIG labeled DNA using a dot blot

An accurate quantification of the DIG-labeled DNA probe is the most important aspect for optimal and reproducible results in membrane hybridization. The labeled probe and the control DNA were blotted in a nylon membrane in a 1 is to 1 μl volume to estimate the probe concentration. The yield was determined after detection as in section 3.9.5. It was observed that probe DNA with a final dilution 1600x was equivalent to labeled control DNA, which had a final concentration of 3pg/μl (Plate 15). The probe concentration was 3pg which was equivalent to 4.8ng/μl (1600x3=4800pg=4.8ng). Best calorimetric signal was obtained with dilution of 1600x (Plate 15). Therefore the probe that was used for hybridization was diluted 1600x with hybridization buffer.
Plate 15: Dot blot of purified DIG-labeled probe.
The control DNA and the labeled probe were applied in a dilution series of 1 dot, approximately µl. The concentrations were ranging from 1000pg to 1pg. The total DNA was diluted two fold between steps.
4.9 Southern blot

Southern blot hybridization analysis was done so as to determine the copy number of the coat protein gene within the sweet potato genome. The southern results produced one very faint diffuse band of approximately 450 bp in the plasmid DNA (Plate 16). These results established that the plasmid had a single copy insert. All the other test plants did not show any band. This was because of the insensitive detection method used after hybridization.

Plate 16: Southern blot analysis of the transformed plants and the control DNA hybridized with DIG labeled coat protein probe. Only the plasmid restricted DNA showed one very faint diffuse band. There was no band observed in all the other lines because of the insensitivity of the detection method. Lane1-CPT 560 (negative control), Lane2-Plasmid DNA (positive control), Lane 3-pMON/2000/94, Lane 4 pMON/2000/336, Lane 5-KBC/2002/004, Lane 6-KBC/2002/009,
CHAPTER FIVE

DISCUSSION

In this study, simple, fast and cost effective protocols have been developed for confirming transformation in sweet potato. Experiments revealed that the lowest kanamycin concentration that bleached explants of the non-transformed CPT 560 was 1.5%. The leaves of transgenic sweet potato lines failed to show visible damage suggesting that they were expressing the selectable marker gene. It was also observed that the leaves of the freshly sprouting shoots from harvested storage roots of transgenic plants also showed kanamycin resistance suggesting that the antibiotic resistance gene was transmitted to their vegetatively propagated progenies through storage roots. Since sweet potatoes are commonly propagated using storage roots, the use of genetically engineered sweet potato may be suitable for the practical breeding of this plant species.

Transient GUS expression assays were done to determine whether the explants were expressing the uid A gene and to determine the expression levels in different tissues. Optimization of this protocol was an important aspect in this study because it can be used to confirm the success of transformation process before spending a lot of money and time to confirm later that no transformation has taken place. This is because, especially when coat protein is used in transformation, explants take a long period (over 9months) to form leafy transgenic plants that can allow PCR testing (Njagi, 2004). Eighty five percent of the explants tested gave positive GUS results. The transformation efficiency in plants has been shown to be affected by several factors e.g inclusion of acetosyringone, age of plant, age of leaf and differences
between the *Agrobacterium* strains used (Otani *et al.*, 1998). The characteristic blue color covered the entire surface of leaves tested in transformed plants except for pMON/2000/53, pMON/2000/173 and the non-transformed control plants. The transgene in these two transgenics might have been silenced or undergone some positional effects resulting into random integration and hence was not expressed in the tissues/explants tested. It was also observed that the wounded (cut) regions on the leaf reacted more intensively, suggesting higher enzymatic activity. Similar results on wound inducible activity were reported by Landridge *et al.*, (1989). These results are also in accordance with those of Sanger *et al.*, (1990) who observed that the activity of the nptII gene driven by a promoter was increased in wounded leaves but not in roots. Roots and stem explants of the 12 transgenic lines displayed intense blue color in the whole segment tested indicating strong gene activity. These results are in accordance with those of Saito *et al.*, (1991). This group when studying the regulation of the mannopine synthase promoter also observed a stronger activity in roots than in leaves, as well as an increase of activity after wounding and when under physiological stresses. Gelvin *et al.*, (1998) showed that plants transformed independently with the same plasmid will commonly have different levels of expression, a phenomenon that is not always correlated with copy number. It has also been shown that differential expression of transgenes has been attributed to some “positional effects” whereby the position of the T-DNA integration site in the host genome affects the level of transgene expression. However, research by Goldberg and Brakke (1987) has also indicated that factors other than the position of the site of integration contribute to the level of transgene expression. This is particularly true of the variable arrangements that transgene sequences may take in the host genome.
PCR analysis has effectively been used in numerous studies for screening transgenic plants (Calinet et al., 1998, Sonoda et al., 1999 Okanda et al., 2001). In this study, PCR results have provided preliminary evidence for the presence of the SPFMV coat protein gene in the transgenics. Sixty five percent of the plants tested showed the characteristic 450bp fragment. Transformed plants that gave negative PCR results might have had the coat protein gene silenced or integrated in a promoter and consequently suppressed. It has been shown that variable expression of transgenes or gene silencing is a ubiquitous phenomenon in transgenic plants whether produced by direct DNA uptake or Agrobacterium-mediated transformation. Gene silencing can result from interactions between multiple copies of transgenes and related endogenous genes. This is associated with homology-based mechanisms that act at either the transcriptional or post transcriptional level (Matzke and Matzke, 1998). Silencing that results from the impairment of transcription is often associated with cytosine methylation and or chromatin condensation (Fagard and Vaucheret, 2000) while post-transcriptional silencing (co-suppression) involves enhanced RNA turn over in the cytoplasm (Matzke and Matzke, 1998; Beyer et al., 2002). A third category of silencing has also been proposed which involves positional effects where flanking plant DNA and or unfavorable chromosomal location exert a silencing effect on the transgene (Matzke and Matzke 1998; Beyer and Potrykus, 2001). This type of silencing reflects the epigenetic state of host sequences flanking the insertion site or the tolerance of particular chromosome regions to insertion to foreign DNA.

Southern blot analysis was done to determine the copy number of the inserted coat protein gene. In this experiment, the positive control sample produced a very light diffused band of approximately 450bp. This showed that the plasmid had a single copy insert. There were no bands in all other samples which was due to the
Insensitive detection method used. Chemiluminescent detection method eg CPD Star and exposure of membranes to X-ray could have been used so as to detect weak signals.

Recommendations

Further molecular analysis such as northern blot analysis need to be done to determine whether the transgene is being transcribed into mRNA and to determine the levels at which such transcription occur. This is important because the success of genetic engineering by coat protein mediated resistance has been shown to rely on transcription of transgene into mRNA. There is also need to carry out a western blot to determine whether the mRNA will be translated by the plant cell ribosomes into functional proteins. It is important that Southern blots be repeated using a more sensitive detection method like the use of CDP-Star or use of X-ray so as to recognize weak signals. Subsequently, the transgenics need to be challenged with the SPFMV to determine their resistance to the viral disease.
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Appendices

Appendix 1: buffers and solutions

Buffers for DNA extraction

CTAB extraction buffer
2g CTAB
28 ml 5M sodium chloride
4ml 0.5M EDTA
10ml 1M Tris HCL pH 4
1g polyvinyl pyrocallidone (PVP)
85µl β-mercaptoethanol
58 ml distilled water

TBE Buffer (Tris Borate EDTA)
54g Tris base
27.5g Boric acid
20ml 500mm EDTA)

Chloroform:isoamyl Alcohol 24:1

TE buffer 10mm Tris-Hcl
1 mm EDTA, Ph 8.0

Loading solution
50mm EDTA
50Mmm NaCl
5ml glycerol
0.01g Bromophenol blue

Buffers for Southern transfer

Denaturing solution 0.4 M NaOH
1M NaCl

20XSSC NaCl 175.3g
Na Citrate.2H2O 88.2g
pH 7.4

Pre-hybridization solution Formamide 50mls
Blocking reagent 5g
5XSSC 25mls
N- Lauroly sarcosine 1ml of 10% stock
SDS 0.2 mls of 10% stock

Hybridization solution Formamide 50mls
Blocking reagent 5g
5XSSC 25mls
N- Lauroly sarcosine 1ml of 10% stock
SDS 0.2 mls of 10% stock
Probe 4.8ng

Wash buffer Maleic acid buffer
Tween 20
Buffers for detection of hybridization probes on a blot

Meleic Acid buffer  0.1 M MALEIC ACID 
                     0.15 M NaCl: 
                     pH  7.5 

Blocking Solution   10x Blocking Solution diluted 1:10 
                    with Maleic Acid Buffer. 

Washing Buffer      0.1 M Maleic acid 
                    0.15 M NaCl: 

Antibody solution   Anti-digoxigenin-AP 
                    diluted 1: 5 000 
                    (150 Mu/ML) in Blocking solution. 

Detection Buffer    0.1 M Tris-HCl 
                    0.1 M NaCl 
                    pH 9.5 (20 C) 

TE buffer           10mm Tris-Hcl 
                    1 mm EDTA, Ph 8.0 

Colour Substrate Solution 200 μl of NBT/BCIP 
(Cat. No. 1 681 451- Sigma)  stock solution added to 10ml of Detection Buffer 

Appendix 2: culture media 

Sweet potato media 

Murashige and Skoog media 4.17g 
Calcium panthothenate 0.002g 
Gibberellic acid 0.02g 
Ascorbic acid 0.1g 
Calcium nitrate 0.1g 
Putresine HCL 0.02g 
L-Arginine HCL 0.1g 
Sucrose 30g 
Phytage1 2.5g 

LBSK media          Tryptone 5g 
                    Yeast 2.5g 
                    Nacl  5g 
                    Bacto Agar 7.5g 
                    Spectinomycin 2mls 
                    Chloramphenical 1ml 
                    Kanamycin 1ml 

YEPSCK              Peptone 5g 
                    Yeast extract 5g
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.5g</td>
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<tr>
<td>Spectinomycin</td>
<td>2mls</td>
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<tr>
<td>Chloramphenical</td>
<td>1ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1ml</td>
</tr>
</tbody>
</table>

**AB buffer**

- K$_2$HPO$_4$.3H$_2$O 39.33g
- K$_2$HPO$_4$
- NaHPO$_4$

**AB Salts**

- NH$_4$Cl 10g
- MgSO$_4$.7H$_2$O 12.5g
- KCl 1.5g
- CaCl$_2$.2H$_2$O
- FeSO$_4$.7H$_2$O 0.0455g

**ABSCK Media**

- Glucose water 450 mls
- AB salts 25 mls
- AB buffer 25 mls
- Spectinomycin 2mls
- Chloramphenical 1ml
- Kanamycin 1ml