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Agrobacterium-mediated transformation of potato cv. Mnandi for resistance to the potato tuber moth (*Phthorimaea operculella*)

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Abstract

Potato tuber moth (*Phthorimaea operculella*) is a major insect pest of potatoes during field cultivation and post harvest storage, causing high annual losses in potato production areas. The *Bacillus thuringiensis* gene (*cry1Ia1*) was introduced into the South African potato cultivar Mnandi with *Agrobacterium*-mediated transformation. The *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pSPUD5 carrying the *cry1Ia1* gene under the transcriptional control of the $(ocs)_3$mas promoter and the *nptII* gene (as selectable marker) was used to transform *in vitro* potato explants. *In vitro* potato explants were infected with *Agrobacterium* LBA4404, cultured on the pre-culture medium with 50 μM acetosyringone, co-cultivated on pre-culture media for two days, and then regenerated on medium containing MS basal medium, 0.27 μM NAA, 0.58 μM GA_3, 9.12 μM zeatin and 50 mg l^{-1} kanamycin. The regeneration frequency of 15% obtained with the potato cultivar Mnandi was an indication that this cultivar is sensitive to transformation with *Agrobacterium.* Verification of stable integration of the *cry1Ia1* gene was confirmed by PCR and Southern blot hybridization procedures. The level of resistance to the potato tuber moth was investigated using leaf and tuber bio-assays which involved feeding studies of potato tuber moth larvae on the leaves and tubers of putatively transformed plants. Seven transformed lines expressed potato tuber moth resistance during the leaf and tuber assays. The Southern blot hybridization analysis verified that one to three copies of the *cry1Ia1* gene was integrated into the genome of five transformed lines.

Keywords: *Agrobacterium tumefaciens*; *Bacillus thuringiensis*; *Phthorimaea operculella*; Potato cultivar Mnandi; Potato tuber moth; Transformation

1. Introduction

Potato tuber moth (*Phthorimaea operculella* Zeller) is one of the most damaging Lepidoptera pests of potatoes in the field and during postharvest storage. *P. operculella* is the most widely distributed of the potato tuber moth complex, and is a world-wide pest occurring wherever potatoes are cultivated (Ghislain et al., 2003). In South Africa potatoes are planted throughout the year in 14 production areas, where the highest production areas are in the Sandveld, Eastern Free State, Limpopo and Mpumalanga (Theron and Mienie, 2003). Cultivation takes place either under dry land or under irrigation conditions, depending on the production area. The annual average yield loss due to potato tuber moth is estimated at 8%, but during the 2007 season the yield losses in the Eastern Free State were as high as 40% (Visser, 2007). It is especially destructive where potatoes are grown under dry land conditions during warmer seasons (Visser, 2005). There is currently no registered insecticide, including Bt sprays, against the potato tuber moth under storage conditions.

Potato is tetraploid and therefore conventional breeding for disease resistance is difficult, while applied molecular genetics has become a popular tool to engineer pest resistance in potatoes. The most popular method for genetically transforming

Abbreviations: Amas, mannopine synthase activator; Aocs, octopine synthase activator; B, *Bacillus thuringiensis*; bp, base pair; DIG, digoxygenin; GA_3, gibberellic acid 3; NAA, naphthalene acetic acid; D, optical density; PCR, polymerase chain reaction; Pmas, mannopine synthase promoter; CTAB, cetyltrimethylammonium bromide.

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dicotyledonous plants is by means of *Agrobacterium*-mediated gene transfer (Mullins et al., 2006). Several reports describe the introduction of resistance to the potato tuber moth in potato by means of *Agrobacterium*-mediated transformation. Li et al. (1999) used the *Bacillus thuringiensis (Bt)-cryV* gene (now known as *cry1Ia1*) to transform a North African potato cultivar Spunta for resistance to the larvae of potato tuber moth. The resulting transgenic lines were screened for insecticidal activity and all the lines were effective against potato tuber moth (Mohammed et al., 2000). Five of these *Bt*-Spunta lines were tested against two potato tuber moth species in Peru and high levels of *cry1Ia1* expression and mortality of both *P. operculella* and *Symmetrischema tangolias* were observed (Lagnaoui et al., 2000). Douches et al. (1998, 2001) transformed different potato cultivars with the *cry1Ia1* (coleopteran and lepidopteran specific) and *cry3A* (coleopteran specific) genes. In these reports, they state that the *cry3A* gene is more effective against the Colorado potato beetle than the *cry1Ia1* gene.

The potato cultivar Desiree was genetically modified with the *cry1Ba* gene to resist attack from the Colorado potato beetle, potato tuber moth and the European corn borer. The resulting transgenics exhibited complete protection of the leaves against all three insect larvae as well as Colorado potato beetle adults (Naimov et al., 2003). Potato plants transformed with the *cry1Ac9* gene inhibited the growth of *P. operculella* larvae on foliage of greenhouse plants. Selected lines were evaluated with Southern analysis and the transformed plants contained two to five copies of the *cry1Ac9* gene (Davidson et al., 2004). Davidson et al. (2006) reported on the performance of these potato lines in a scaled-up field trial using infestation by a wild population and artificial infestation by a laboratory colony of potato tuber moth. The transgenic potato lines proved to be resistant to potato tuber moth.

In the current study, we report the successful transformation of the South African potato cultivar Mnandi with the *Bt-cry1Ia1* gene for resistance against the potato tuber moth using *Agrobacterium*-mediated transformation techniques.

2. Materials and methods

2.1. Plant material

All media, buffers and supplies were sterilized by autoclaving at 121 °C and one atmosphere pressure for 20 min or by filter sterilization (pore size 0.22 μM). Virus free *in vitro* potato plants of cultivar Mnandi were obtained from the Agricultural Research Council–Vegetable and Ornamental Plant Institute's *in vitro* gene bank. Plants were micropropagated by means of nodal cuttings and maintained on MS (Murashige and Skoog, 1962) basal medium (MS including vitamins, 30 g l^{-1} sucrose, pH 5.7 and solidified with 7 mg l^{-1} Duchefa™ plant agar). Three different types of explants (leaves, petioles and stems) were cut from three to four week old *in vitro* plants and used as donor material in the transformation experiments. Leaf explants were obtained by slicing 3–5 mm off the tip and base of each leaf. Petiole explants were prepared by cutting each petiole at the node (destroying the auxiliary bud) and retaining 3–5 mm of the base of the leaf. Stem explants consisted of 10 mm internodal segments.

2.2. Agrobacterium-mediated transformation

Genetic modification of the cultivar Mnandi was performed using a disarmed *Agrobacterium tumefaciens* strain LBA4404 containing pBI121, a derivative of the plasmid pBIN19, harbouring the *cry1Ia1*/GUS gene fusion under the control of the $A(ocs)_3$AMasPmas promoter (Mohammed et al., 2000). Little or no GUS expression was present. The *cry1Ia1* 2200 bp gene is a codon-modified gene derived from the common soil bacterium *B. thuringiensis (Bt)* kurstaki (Tailor et al., 1992) (Fig. 1).

A. tumefaciens cultures were inoculated in 25 ml yeast peptone extract medium containing 50 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin. The *Agrobacterium* was cultured on a shaker at 27 °C for 36–48 h. The cells were harvested by centrifugation (3300 rpm, 4 °C, 25 min) and the pellet was resuspended in 25 ml MS liquid medium. The optical density (OD) of the cell suspension was measured in the visible spectrum at 620 nm with a spectrophotometer (1 OD at 620 nm = 5 × 108 cells/ml). If the OD reading was higher than 0.8, the cell suspension was diluted 1:2 with MS liquid medium.

The *Agrobacterium*-mediated transformation protocol was adapted from that of Murray et al. (1998) and Li et al. (1999). Leaf, stem and petiole explants were used in the transformation study. The explants were pre-incubated on CP3 medium (MS basal medium, 0.27 μM naphthalene acetic acid (NAA), 0.58 μM GA_3 and 9.12 μM zeatin), CP5 medium (MS basal medium, 0.9 μM 2,4D and 2.28 μM zeatin). CP3 and CP5 were supplemented with 50 μM acetosyringone, (CP3AS and CP5AS respectively). After 48 h the explants were immersed for 10, 20 or 30 min in the *A. tumefaciens* cell suspension and excess fluid was removed from the dipped explants by blotting on sterile blotting paper. The explants were transferred back onto the pre-culture medium (CP3, CP5, CP3AS or CP5AS) and were co-cultivated for 2, 4 or 5 days at 24 ± 2 °C, 16 h photoperiod, with a light intensity of 30 μmol m^{-2} s^{-1} (Osram™ cool white fluorescent tubes). The potato explants were transferred every 10–14 days to PSM1 (CP3 medium with 30 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime) or PSM3 (MS basal medium, 0.58 μM GA_3, 2.28 μM zeatin with 30 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime) regeneration medium. After 60 days the explants on PSM1 medium were transferred to PSM2 (MS basal medium, 0.58 μM GA_3, 5.69 μM zeatin with 30 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime) and the explants on PSM3 remained on the medium for shoot induction. The 90 mm Petri™ dishes were sealed with Micropore tape^{3M} to promote gas exchange. Regenerated shoots were transferred to MS basal medium containing 30 mg l^{-1} kanamycin to initiate root formation.

2.3. PCR analysis

Plant genomic DNA was isolated from seventeen putative transgenic *in vitro* Mnandi lines using the cetyltrimethylammonium

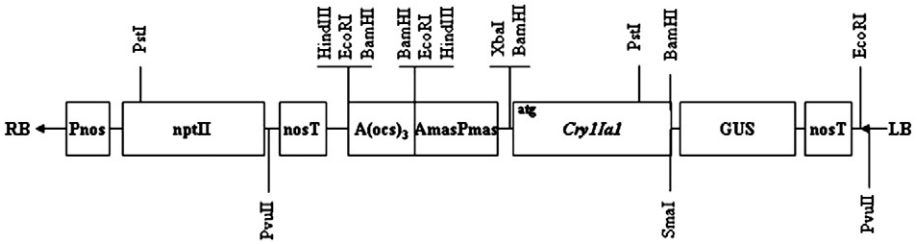

Fig. 1. The *cry1Ia1/GUS* gene fusion was cloned under control of the viral $(ocs)_3$mas promoter consisting of the trimer octopine synthase upstream activating sequence (Aocs) joined to a mannopine synthase activator (Amas) and mannopine synthase promoter (Pmas) (Ni et al., 1995). The kanamycin resistance gene, *nptII*, under control of the nopaline synthase promoter and polyadenylation signals was included as plant selectable marker.

bromide (CTAB) method described by Edwards et al. (1991). Polymerase chain reaction (PCR) was performed on 50 ng DNA template using a MJ Research PTC-200 Programmable Thermal Controller (MJ Research Inc.). Each reaction consisted of: 1× PCR buffer, 3.5 mM $MgCl_2$, 0.24 μM of each dNTP, 0.24 μM of each primer, 0.75 Units *Taq* DNA polymerase (Takara) and 50 ng DNA. *Cry1Ia1* specific primers were used during PCR to amplify the expected 2,200 bp fragment. The sequences of the primers were as follows: Cry1Ia1.1: 5′-CTCTCGATGTG-CAGCTGCTTG-3′ and Cry1Ia1.2: 5′-GCAGCGATAAGCT-GAAG-AACC-3′. An annealing temperature of 50 °C was used according to the recommended temperature for the amplification of the *Bt* gene. The PCR reaction commenced at 94 °C for 1.5 min for initial denaturation and was followed by 35 amplification cycles (15 s at 94.5 °C, 20 s at 50 °C and 30 s at 72 °C) and final extension step of 72 °C for 2 min. The products were visualised under ultra violet illumination after staining of the gel in ethidium bromide at a final concentration of 0.5 μg/ml for 20 min and sized by comparison to Molecular Weight Marker VI (Roche Diagnostics Ltd.).

2.4. Leaf and tuber bio-assays

Five plantlets of each PCR positive lines were *ex vitro* acclimatised in the greenhouse for the leaf assay. No-choice leaf bio-assays were performed by removing four leaves from separate individual plants of each ‘Mnandi’ line. Untransformed ‘Mnandi’ lines were used as controls. The leaves were placed in a 90 mm Petri™ dish on filter paper moistened with water. Three one day old potato tuber moth larvae were placed on each leaf and the larvae were allowed to feed. The filter paper was kept moist to prevent the leaves from drying out. The following criteria were used to evaluate the feeding damage by the larvae after seven days: no damage, minimal damage (<20% of the entire leaf area had feeding damage) and severe damage (>20% of the entire leaf area had feeding damage).

The tuber assay consisted of potato tubers that were harvested from the greenhouse plants after four month growth and stored at 4 °C. Four medium sized potato tubers were selected from each line for use during no-choice and free-choice tuber trials. Untransformed ‘Mnandi’ tubers were used as controls. The tubers of each line were placed randomly in an open 90 mm Petri dish. The no-choice assay consisted of four potato tuber moth larvae that were placed on each tuber. The larvae were compelled to feed on the tubers where they were placed on. The Petri™ dishes with the tubers and larvae were placed in an enclosed insect proof cage (450 × 450 × 350 mm). The free-choice assay consisted of a Petri™ dish filled with pupae that would hatch within 36–48 h, placed in the centre of an enclosed insect cage between the Petri™ dishes containing the tubers. Once the moths emerged from the pupae they were free to lay their eggs on any potato tuber inside the cage. The newly hatched larvae could then feed on those potato tubers. The moths died approximately seven days after emergence. The cage containing the larvae and tubers was incubated at ambient temperature in the dark. The potato tuber moth larvae were allowed to feed for seven to ten days before visual assessment of feeding damage on the tubers.

2.5. Southern blot analysis

Southern blot hybridizations were used to determine the stable integration and copy number of the *Bt-cry1IA1* gene of seven putatively transformed lines into the potato genome. One gram fresh leaf material was grounded to a fine powder and genomic DNA was extracted using the DNeasy plant maxi kit (Qiagen™). The genomic DNA of the untransformed ‘Mnandi’ line was also isolated and was used as a negative control and positive control (pSPUD1 plasmid was added to untransformed ‘Mnandi’ DNA). Twelve micrograms of genomic DNA from each line was digested with 100 Units each of the restriction enzymes *Bam*HI and *Xba*I according to standard procedures (Sambrook et al., 1989). *Bam*HI cuts on both sides of the transgene between the T-DNA borders of the transformation vector. *Xba*I was selected because it has a single recognition sequence and cuts once between the T-DNA borders and randomly in the transgenic ‘Mnandi’ plant genome. The fragments were separated overnight by electrophoresis (40 V) at 7 °C on a 1% agarose gel 150 mm × 200 mm. Capillary transfer, using 20 × SSC [3 M NaCl, 0.3 M Sodium citrate, pH 7.0] for transfer of the DNA fragments from the gel to a nylon membrane (Osmonics Magnacharge nylon transfer membrane, Amersham Biosciences Inc.), was performed. After overnight transfer the membrane was subjected to hybridization overnight at 42 °C in digoxygenin (DIG) Easy HYB solution (Roche Diagnostics Ltd.). Post-hybridization washes were performed as outlined in the DIG Wash and Block Buffer Set (Roche Diagnostics Ltd.). The protocol consisted of washing the membrane 2–5 min in 100 ml Stringency Washing Buffer I (2 × SSC and 0.1% SDS) at room temperature, and 2 × 15 min in 100 ml

Stringency Washing Buffer II (0.5 × SSC and 0.1% SDS) at 65 °C in a hybridization oven. The probe was prepared and purified from the *cry1Ia1* gene (2200 bp) construct during a PCR reaction containing DIG-dUTP (alkali-labile) using the PCR DIG Probe synthesis kit (Roche Diagnostics Ltd.). Hybridization was detected using the DIG Wash and Block Buffer Set and the DIG Luminescent Detection Kit for nucleic acids as set out in the user manual (Roche Diagnostics Ltd.).

3. Results

3.1. Agrobacterium-mediated transformation

The combination of the pre-culture medium CP3AS, 30 min explant infection time with the *A. tumefaciens* cell suspension, a co-cultivation period of 24 h and the selection media PSM1 and PSM2 were effective in the successful transformation of the cultivar Mnandi. Small amounts of calli developed on the explants (24/113, 21.2%) using this protocol, and the callus was hard granular and green with only a few shoots being produced (2/113, 1.8%) (Fig. 2). The explants died after selection using the other protocols.

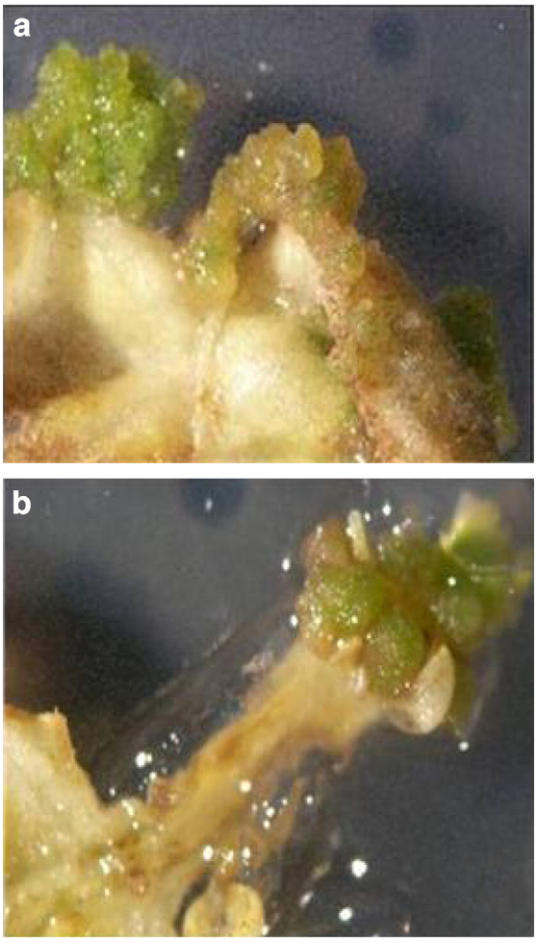

Fig. 2. Green and rough regenerable callus development from the cut edges of the (a) leaf explant and (b) stem explant after transformation with *Agrobacterium tumefaciens* on CPS3 selection medium (027 μM NAA, 0.58 μM GA_3, 5.69 μM zeatin and 30 mg l^{-1} kanamycin).

The explants were scored three months after transformation. Only two explants (2/113, 1.8%) produced shoots three months after transformation. Six months after transformation more explants (15/113, 13.3%) produced shoots. A total of seventeen shoots (MN1, MN3.1, MN3.2, MN4, MN5, MN6, MN7.1, MN 7.2, MN8, MN9, MN10.1, MN10.2, MN10.3, MN10.4, MN11, MN12 and MN13) were obtained after selection on PSM3 medium and with a transformation frequency of (17/113) 15%. The untransformed 'Mnandi' control explants developed shoots equally well in all three protocols, and there were no significant differences in the regeneration frequency and response of the control explants. Transformed cells were able to survive on MS basal medium with 30 mg l^{-1} kanamycin, which is the adequate minimum concentration for selection.

3.2. PCR analysis

During the PCR analysis the MN− (untransformed 'Mnandi' negative control) yielded no amplification products, as expected. Fourteen putatively transgenic lines tested positive for the insertion of the *cry1Ia1* gene into the potato genome, as indicated by the PCR amplification of a 2,200 bp fragment (Fig. 3). This corresponds to the expected size of the *cry1Ia1* gene. These lines were MN3.1, MN3.2, MN4, MN5, MN6, MN7.1, MN7.2, MN8, MN10.1, MN10.2, MN10.3, MN10.4, MN12 and MN13.

3.3. Leaf and tuber bio-assays

Only two transgenic lines (MN5 and MN6) exhibited no feeding damage on the leaves after one week. Little damage was observed on MN3.2, MN4, MN10.1, MN10.3 and MN10.4. The leaves of line MN13 dried out and no results could be taken for this line. The MN− control exhibited a large amount of feeding damage, as expected, as well as MN1, MN3.1, MN7.2, MN9 and MN11. After a further seven days the following lines still exhibited resistance to potato tuber moth larvae during the leaf assay, since the larvae that fed on these lines did not survive: MN3.2, MN4, MN5, MN6, MN8, MN10.1, MN10.3, MN10.4 and MN12. The minimal feeding damage observed after the first seven days can be due to the initial feeding of the larvae on the leaves. The leaves of lines MN−, MN1, MN3.1, MN7.1, MN7.2, MN9 and MN11 showed feeding damage and the larvae were still alive on these lines.

The results that were obtained during the no-choice and free-choice tuber assays clearly shows that the untransformed tubers were damaged by larval feeding, whereas the tubers of 7/16 (MN10.2 died in the greenhouse) transgenic lines did not show any evidence of feeding by the larvae (Fig. 4).

Evidence of larval feeding was excreta discharged from the larval entry point on the surface of infested tubers and the shrivelled appearance of the tubers. These symptoms were evident for untransformed (MN−) and non-resistant transformed tubers which, when cut open, revealed larval feeding. For both the free- and no-choice tuber assays the same lines exhibited 100% resistance to the potato tuber moth larvae. These lines

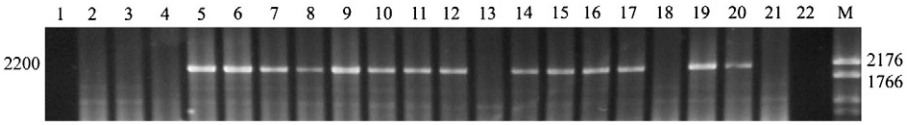

Fig. 3. PCR amplification of the 2155 bp *cry1Ia1* gene in putatively transformed 'Mnandi' plants. *Lane 1*: H_2O control; *Lanes 2–3*: MN− (negative control); *Lanes 4–20*: MN1, MN3.1, MN3.2, MN4, MN5, MN6, MN7.1, MN7.2, MN8, MN9, MN10.1, MN10.2, MN10.3, MN10.4, MN11, MN12, and MN13; *Lane 21*: MN− (negative control); *Lane 22*: H_2O control; *Lane M*: molecular weight marker VI (MVI) (Roche Diagnostics Ltd.).

were MN4, MN5, MN6, MN10.1, MN10.3, MN10.4 and MN13. The transformed 'Mnandi' lines expressing tuber moth resistance for both the leaf and tuber assays were MN4, MN5, MN6, MN10.1, MN10.3, MN10.4 and MN13. (Fig. 4).

The lines were classified into two groups: the lines exhibiting 100% resistance as 'resistance' and the rest of the lines as 'susceptible'. Since the data from the two samples is not normally distributed, a non-parametric method (the Wilcoxon Rank Sum test) to test for equality of medians between the two samples was used. The null hypothesis was that the medians of the two samples are the same, and the alternative hypothesis is that the medians of the two samples are different. According to the significantly low p-value of the statistical test when comparing the median of the two groups, it was a good classification. Based on the resulting p-value of 0.0003997 ($\ll 0.05$), the null hypothesis can be rejected and the conclusion is that the two groups are significantly different (Fig. 5).

3.4. Molecular analysis

The lines analysed were MN4, MN5, MN6, MN10.1, MN10.3, MN10.4 and MN13. Sharp bands formed where the *Bt*-probe hybridized to the digested DNA of the *Bt* transgenic lines (Fig. 6). *Bam*HI digestion of the positive control and *Bt*-'Mnandi' transgenic lines yielded a single fragment of approximately 2,200 bp. This corresponds to the *Bt*-fragment of 2,200 bp, as expected. No bands were observed in the negative controls. This was an indication that the *Bt-cry1Ia1* gene was integrated into genomes of the above mentioned 'Mnandi' transgenic lines, confirming the PCR results.

The restriction digestions of the *Bt*-transformed lines with *Xba*I yielded different banding patterns for each line, indicating the insertion of between one to three copies into the genomes of various transgenics (Fig. 4). The *Xba*I digestions yielded one DNA fragments of approximately 19,329 bp for MN5, one DNA fragment of approximately 9,300 bp for MN13, two

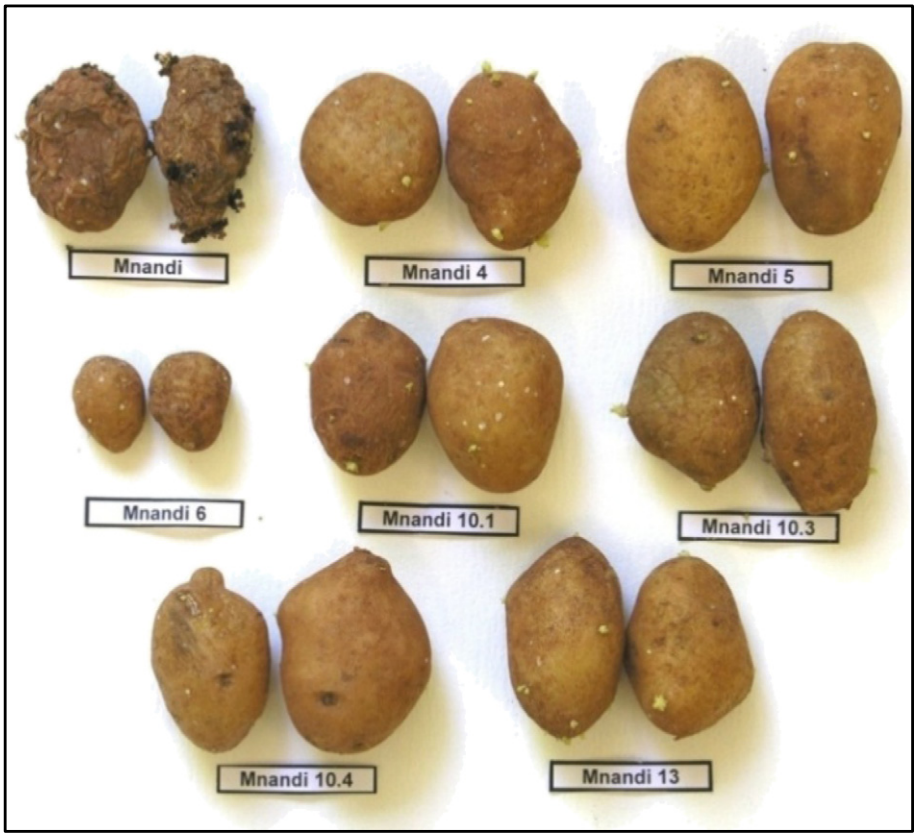

Fig. 4. The transformed 'Mnandi' lines expressing tuber moth resistance. The tubers of seven *Bt*-transformed lines (MN4, MN5, MN6, MN10.1, MN10.3, MN10.4 and MN13) exhibiting no larval feeding damage whereas larval damage on the MN− line (top left tubers appearing shrivelled) can be seen.

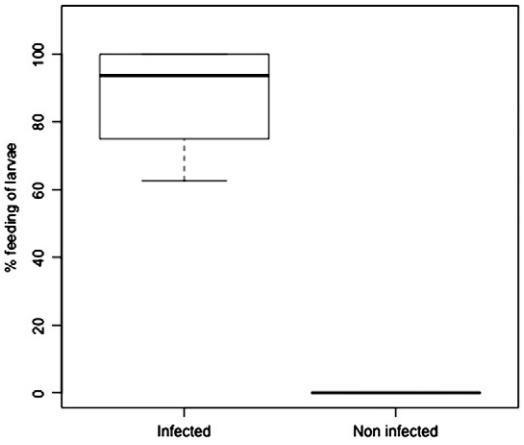

Fig. 5. The percentage of tubers of the different lines infected with tuber moth larvae. For both the free- and no-choice tuber assays the same lines exhibited 100% resistance to the potato tuber moth larvae. These lines were MN4, MN5, MN6, MN10.1, MN10.3, MN10.4 and MN13. The median of the larvae on susceptible tubers was significantly higher than the median of the larvae on resistant tubers (p-value = 0.0003997).

DNA fragments of approximately 7,743 bp and 5,526 bp for MN4, and three DNA fragments of approximately 19,329 bp, 7,743 pb and 4,400 bp for MN6.

The *Xba*I digested DNA profiles for MN10.1, MN10.3 and MN10.4 were similar since two DNA fragments of approximately 10,500 bp and 9300 bp were yielded (results not showed). This was an indication that these lines are clones from the same transformation event and these lines were renamed to MN10.

After the verification and evaluation of the 'Mnandi' lines, stable transformation frequency was calculated at 31% (7/16 lines). Out of sixteen putatively transformed lines only five lines were stably transformed: MN4, MN5, MN6, MN10 and MN13. Only one copy of the gene was integrated in the genome of two lines, MN5 and MN13.

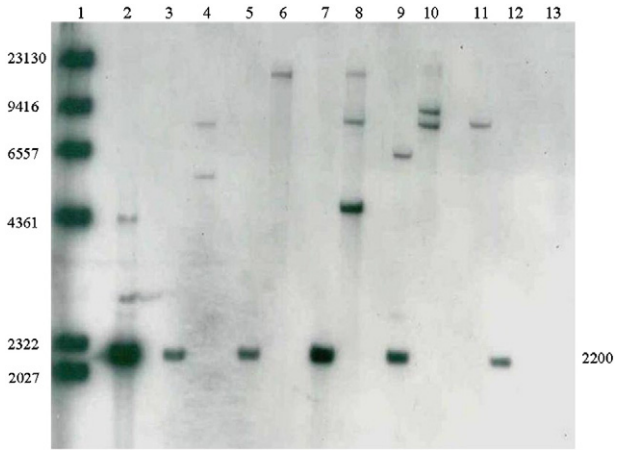

Fig. 6. Southern blot analysis of the 'Mnandi' transgenic potato lines MN4, MN5, MN6, MN10, MN13 and the untransformed line MN−. *Lane 1*: DIG labelled molecular marker II (MII), *Lane 2*: positive control for *Bt* fragmant, (pSPUD plasmid added to untransformed 'Mnandi' DNA and digested with *Bam*HI); *Lanes 3, 5, 7, 9, 12*: transformed 'Mnandi' DNA (MN4, MN5, MN6, MN10.1 and MN13) digested with *Bam*HI; *Lanes 4, 6, 8, 10, 11*: transformed 'Mnandi' DNA (MN4, MN5, MN6, MN10.1 and MN13) digested with *Xba*I; *Lane 13*: undigested DNA from untransformed 'Mnandi' line.

4. Discussion

Efficient *A. tumefaciens* mediated transformation must be followed by reproducible regeneration of whole plants. The combination of the *Agrobacterium* and the selection agent kanamycin resulted in a low survival rate of the putatively transformed 'Mnandi' explants. Visser (1991) reported that the regeneration efficiency can decrease by 5–100 fold after *Agrobacterium*-mediated transformation.

The combination of the plant growth regulators NAA, zeatin and GA_3 has previously been used in various potato transformation experiments to optimize direct organogenesis (Banerjee et al., 2006; De Block, 1988; Ducreux et al., 2005; Kumar, 1995). Results from our study indicated that a high zeatin level result in a high regeneration frequency. Combining a low level of NAA (0.27 μM) with a high level of zeatin (5.69 μM) controlled excessive callus proliferation and development. The use of zeatin in the callus medium induces shoot regeneration and elongation, thus resulting in callus development in a shorter time preventing somaclonal variation in regenerated plants (Beaujean et al., 1998).

Genotypic differences between cultivars play an important role in the reaction of tissues *in vitro*. Some varieties are more amenable to transformation than others and the relatively low transformation frequency of 15% obtained with the potato cultivar Mnandi was an indication that this cultivar is not easily transformed by *Agrobacterium*. Higgins et al. (1992) showed that there are differences in the early transformation phases between different genotypes under standard conditions. Different parameters such as acetosyringone concentration, STS and *A. tumefaciens* strains were tested on several cultivars. Only a specific combination of these parameters was successful for a specific genotype in order to obtain a high number of transformed plants (Higgins et al., 1992). There are numerous reports of successful transformation of various potato cultivars especially among the European cultivars Desiree, Bintje, Pentland Squire, Maris Peper and the American cultivar Russet Burbank (Beaujean et al., 1998; De Block, 1988; Higgins et al., 1992; Hoekema et al., 1989; Kumar, 1995; Newell et al., 1991; Visser, 1991; Wenzler et al., 1989). These studies indicated that these genotypes are amenable to *Agrobacterium*-mediated transformation.

Liu et al. (1995) reported on the transformation of a wild potato species. The *Agrobacterium* strains LBA4404 and GV2260 were used and the transformation frequencies were compared with each other. With the GV2260 strain a transformation efficiency of 11% was achieved in comparison with 1% transformation efficiency with LBA4404. The transformation frequency of 'Mnandi' can be increased with the use of a more virulent *Agrobacterium* strain. AGL1 is a more virulent *Agrobacterium* strain due to the presence of a virulent tumour inducing plasmid (Lazo et al., 1991). However, Davidson et al. (2004) suggest that LBA4404 is more suitable for potato transformation than the *Agrobacterium* strain AGL1 since LBA4404

and AGL1 have the same transformation efficiencies, but a higher number of plants transformed with AGL1 have abnormal appearances. These results were confirmed by Nadolska-Orczyk et al. (2007). The suitability of an *Agrobacterium* strain for the transformation of potatoes must be determined not only by transformation efficiency, but also the potato genotype, *Agrobacterium* strain, features of the vector, the transformation method and phenotypic evaluation of greenhouse plants.

The time of co-cultivation has proven to be an important factor, which can be varied to increase transformation efficiency. The co-cultivation time should be sufficient for the bacteria to attach to the plant, activate the *vir*-genes and induce the T-DNA to be transferred (Higgins, 1992). Most transformation protocols for potato use a co-cultivation period of 48 to 72 h (De Block, 1988; Beaujean et al., 1998; Davidson et al., 2004; Gustafson et al., 2006). In our study, a two day co-cultivation with 50 μM acetosyringone was the most effective for transformation. Acetosyringone in the co-cultivation medium assists in the activation of the *vir* genes and induces production of the T-DNA. *Agrobacterium* recognizes the acetosyringone signal when active growing cells are wounded and activate the plant cell transformation response (Stachel et al., 1985). The addition of acetosyringone in the co-cultivation medium resulted in an increase in transformation frequency of tomato and this appears to have a similar effect during potato transformation (Davis et al., 1991).

The level of resistance to potato tuber moth was investigated during the leaf and tuber bio-assays. The fatal effect of the *Bt* toxin on larvae protects the leaves and tubers from high infestation and damage (Arnone et al., 1998). Although the level of resistance varied between lines, the bio-assays demonstrated the effectiveness of the expressed transgene product during infestation. The results indicated that more lines (9/16) demonstrated resistance in the leaf bio-assay than in the tuber bio-assays (7/16). This can be due to higher expression levels of the *cry* gene product in the foliage than in the tubers. The variation in the level of resistance was also observed in the potato cultivars ND5873 and Spunta transformed with the *Bt cry1Ac* gene (Estrada et al., 2007). The other possibility for this observation could be that some tuber-specific genes interfere with the expression of the *cry* gene when inserted into certain positions in the genome (Li et al., 1999). This study shows that high efficiency of the promoter and high enough expression of the *cry1Ia1* gene can be obtained to control potato tuber moth. Ni et al. (1995) reported that the $(ocs)_3$mas promoter results in stronger expression of transgenes than the 35S promoter in *Arabidopsis thaliana,* and Mohammed et al. (2000) reported the effective control of potato tuber moth in potato when both the $(ocs)_3$mas and CaMV35S promoters were used.

In this study, we were able to identify five transgenic potato lines of the cultivar Mnandi with resistance to the potato tuber moth. These lines were shown to have one to three copies of the *cry* gene by Southern blot analysis. Similar results were achieved by Estrada et al. (2007) where one to two copies were observed. The results confirm the value of using genetic engineering to develop transgenic potato plants with improved resistance to *P. operculella*. The effectiveness of controlling potato tuber moth should be verified in further studies, such as field trials.

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