Development of transgenic lines from a male-sterile potato variety, with potential resistance to *Tecia solanivora* Povolny

Desarrollo de líneas transgénicas de una variedad androestéril de papa, potencialmente resistentes a *Tecia solanivora* Povolny

Emy Shilena Torres[1, 2], Javier Torres[1, 2], Claudia Moreno[1], and Rafael Arango[1, 2]

ABSTRACT

Male sterility is a very important characteristic for environmental safety in genetically modified (GM) plants, particularly in the release of transgenic plants in the centers of origin or high biodiversity areas. In order to contribute to the development of environmentally safe agricultural technologies that allow the proper use of transgenic potato crops in Colombia, this project developed transgenic potato *cry1Ac* of *Bacillus thuringiensis* (Bt), lines that are potentially resistant to *T. solanivora*, from the male-sterile variety Pastusa Suprema (PS) (*Solanum tuberosum* ssp. andigena). Modifications were made to the *Agrobacterium tumefaciens* mediated-transformation protocol which allowed the genetic transformation of leaves of *in vitro* plants, with transformation efficiencies of 22 and 37%. Cry1Ac protein levels in transgenic leaves ranged from 88 to 639 ng mg^{-1} of fresh leaf tissue, suggesting a better potential plant resistance. This is the first report on transgenic lines with potential resistance to *T. solanivora* from a male-sterile variety of *S. tuberosum* ssp. andigena.

Key words: *Solanum tuberosum*, *Agrobacterium tumefaciens,* genetic transformation, *Bacillus thuringiensis*, Guatemalan potato moth.

RESUMEN

La androesterilidad es una característica de importancia en la bioseguridad ambiental de las plantas modificadas genéticamente para la liberación de éstas en los centros de origen o en áreas de alta biodiversidad de los cultivos. Como una contribución al desarrollo de tecnologías agrícolas medioambientalmente seguras, que permitan el uso adecuado de cultivos transgénicos de papa en Colombia, en este trabajo se desarrollaron líneas transgénicas de papa con el gen *cry1Ac* de *Bacillus thuringiensis* (Bt), potencialmente resistentes a *T. solanivora*, a partir de la variedad androestéril, Pastusa Suprema (PS) (*Solanum tuberosum* ssp. andigena). Modificaciones realizadas con *Agrobacterium tumefaciens* mediante el protocolo que permitieron la transformación genética de hojas de plántulas *in vitro*, con eficiencias de transformación de 22 y 37%. En las plántulas transgénicas se obtuvieron niveles de expresión de la proteína Cry1Ac, con valores entre 88 a 639 ng mg^{-1} de tejido foliar fresco, lo cual sugiere un buen potencial de resistencia de las plantas. Este es el primer reporte de líneas transgénicas, con resistencia potencial a *T. solanivora*, a partir de una variedad androestéril de *S. tuberosum* ssp. andigena.

Palabras clave: *Solanum tuberosum*, *Agrobacterium tumefaciens,* transformación genética, *Bacillus thuringiensis*, polilla guatemalteca de la papa.

Introduction

Potatoes (*Solanum tuberosum* L.) are grown in 125 countries and consumed by more than one billion people worldwide, and are the fourth most important food crop, after wheat, rice and maize. Its annual production is more than 320 million t and constitutes an important dietary source of fiber, carbohydrates, protein, vitamins C, minerals and trace elements (Mullins *et al.,* 2006; FAOSTAT, 2010). In Colombia, this crop ranks fourth in agricultural production, with 2.2 to 3.0 million t per year, and the ninth in size, with about 128,701 ha (Agronet, 2009).

Tecia solanivora Povolny, commonly known as the guatemalan potato moth, is one of the principal entomological pests of economic importance for the potato; found mainly in Central and South America, and in parts of southeast Europe (Child, 2004; EPPO, 2006;). This insect has become a serious pest in Colombian agriculture, as it attacks tubers in the field and in storage, causing losses of up to 100% of total production (Osorio *et al.,* 2001). To control the moth, farmers use indiscriminate applications of chemical pesticides which contribute to the selection of resistant insects, mortality of non-target species and destruction of natural enemies. Additionally, these substances generate pollution and adversely affect human health (Arévalo, 2003).

Received for publication: 11 February, 2011. Accepted for publication: 29 June, 2012.

[1] Faculty of Sciences, Universidad Nacional de Colombia. Medellin(Colombia). estorresv@gmail.com
[2] Plant Biotechnology, Corporación para Investigaciones Biológicas (CIB). Medellin (Colombia).

Integrated pest management programs (IPMP) of the potato have not had enough success in the field, due to serious difficulties in implementing the management practices for this pest. Genetically modified potato plants carrying *Bacillus thuringiensis* (Bt) genes constitute a viable alternative to produce plants which are resistant to moths (Meiyalaghan *et al.,* 2006; Estrada *et al.,* 2007*;* Kumar *et al.,* 2010).

In previous studies, the transformation of Colombian potato varieties (*S. tuberosum* ssp. andigena) has been reported (Valderrama *et al*., 2007). Potato lines of Colombian varieties Diacol Capiro and Parda Pastusa expressing 0.02 to 17 μg g^{-1}, *Cry1Ac* protein in tubers and fresh tissue were shown to be able to cause 87 to 100% mortality of *T. solanivora* larvae under laboratory and greenhouse conditions (Torres, 2010; Valderrama *et al*., 2007).

Although these transgenic potato lines represent a promising future for control of the Guatemalan potato moth, in general, the release of genetically modified (GM) organisms for agricultural production has generated considerable debate, particularly among the general public, biotechnologists, international food safety organizations and government officials (Nap *et al.,* 2003). The concerns are related to the possible risk that GM plants may present to the environment. Among the main biological risks that have been cited are: horizontal gene transfer mainly of antibiotic resistance, possible adverse effects in the host organism, negative effects on non-target species, insect resistance, increased invasive weeds, and impact on biodiversity (Conner *et al*., 2003; Chaparro, 2011). One of the most important concerns is associated with the potential risk of gene flow from GM crops to neighboring plants of the same or related species, which may generate plants that invade agricultural or natural habitats and adversely affect biodiversity (Conner *et al*., 2003).

As part of the evaluation of the biosafety of transgenic potato plants for release in centers of origin and diversity, studies have been conducted to measure the flow of genes from wild to cultivated potatoes (Celis *et al.,* 2004). A solution for the problem of possible gene flow is the use of male-sterile transgenic potato lines. Celis *et al.* (2004) suggested the use of male-sterile varieties as a basis for field testing in the Andean region. They report the use of a transgenic male-sterile potato variety called revolution (*S. tuberosum* ssp. tuberosum × *S. tuberosum* ssp. andigena) that is resistant to *Globodera pallida.*

In Colombia, the only male-sterile potato variety that has been produced by conventional breeding is the so called Pastusa Suprema variety. This variety was the result of the interspecific hybridization of a clone of *Solanum stoloniferum* 230490 × *Solanum phureja* (Juz. *et*. Buk) var. Yema de huevo, as the maternal parent and *Solanum tuberosum* ssp. andigena var. Parda Pastusa as the paternal parent. This new variety has high resistance to *Phytophthora infestans,* the causal agent of gout and also has high commercial potential in Colombian markets (Segura *et al.,* 2006; López and Chaparro, 2007; Ñústez, 2011).

The present study reports of development of transgenic potato lines of this male-sterile variety with potential resistance to *T. solanivora*. For this purpose, an *Agrobacterium* transformation system for the variety was optimized, using a transformation vector carrying a cassette containing the *Cry1Ac* gene of *Bacillus thuringiensis.*

Materials and methods

Plant material

Plantlets of *S.tuberosum* ssp. andigena variety Pastusa Suprema were provided by Universidad Nacional de Colombia, Bogotá. The plantlets were aseptically micropropagated using internodal cuts in polypropylene vials containing a solid propagation medium (MP) consisting of Murashige and Skoog salts (MS) supplemented with 2.0 mg L^{-1} of D-pantothenic acid, 0.4 mg $L^{-1}$of thiamine, mg L^{-1} of myo-inositol, 20.0 g L^{-1} of sucrose, 1.7 g L^{-1} of phytagel (pH 5.7) and 12 μM of silver thiosulfate (STP) sterilized by filtration (Rodriguez *et al.,* 2000). The plant material was grown at an average temperature of 20±2°C, relative humidity of 60-70%, light intensity of 3,000 lux and a photoperiod of 12 h. The plantlets were subcultured every month, and the leaves were used for transformation experiments and kanamycin selection.

Transformation vector and *Agrobacterium*

The p1AcPRD vector was obtained from University of Ottawa, under a material transfer agreement (MTA). This plasmid carries two constructs: a selection marker cassette containing the *nptII* gene under regulation of the nos *promoter* and nos *terminator,* and a cassette of interest, which carries a sybnthetic *cry1Ac* gene together with the 2X CaMV35S promoter and the nos terminator (Fig. 1).

The vector was transferred to the *Agrobacterium tumefaciens* LBA4404 strain by the method described by Hofgen and Willmitzer (1988). The transformed *Agrobacterium* strain was grown in 50 mL of liquid LB medium supplemented with 100 mg L^{-1} of kanamycin and 25 mg L^{-1} of streptomycin, for 16 h at 28°C in an orbital shaker at 200 rpm

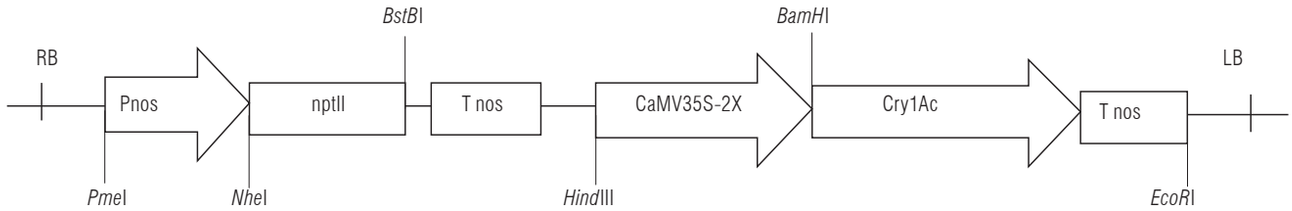

FIGURE 1. Linear map of the cassette containing *npt*II and *cry1Ac* genes. RB, right border of the T-DNA region; LB, left border of the T-DNA region; CaMV35S-2X, *Cauliflower mosaic virus* 2 x promoter; P nos, nopalin sintetase promoter; T nos, nopalin sintetase terminator; nptII, neomycin fosfotransferase gene; *cry1Ac*, *B. thuringiensis cry1Ac* gene.

and cryopreserved at -70°C for use in the transformation experiments.

Determination of the optimal concentration of kanamycin for the selection of transformed explants

To estimate the optimum concentration of kanamycin for the selection of transformed explants, its effect on callus and shoot formation and viability was evaluated as follows: leaves approximately 5x5 mm in size from 4-week-old *in vitro* plantlets were cut and placed in basal and apical contact with the beam with a regeneration medium (RM) which consisted of MS salts supplemented with 3% sucrose (w/v), 0.05% casein enzymatic hydrolyzate, 1 mg L^{-1} of GA_3, 40 mg L^{-1} of ascorbic acid, 1 mg L^{-1} of IAA and 3 mg L^{-1} of zeatine riboside ZR (Rodríguez *et al.,* 2000) supplemented with different concentrations of kanamycin (0, 25, 50, 100 and 150 mg L^{-1}). For each concentration of kanamycin, 6 petri dishes with 10 leaf explants were used. The explants were incubated in the dark for 15 d at 20±2°C to allow callus formation. Afterwards, during eight weeks, under a photoperiod of 12 h, the explants were transferred every 15 d to fresh RM medium with the corresponding concentration of kanamycin. The number of viable explants and/or calli and shoots was then determined.

The evaluation of the response variables was done under an experimental design of randomized blocks with 30 experimental units. The explants were considered as viable when they remained green with no signs of necrosis or chlorosis, and as non-viable when the explants were necrotic or chlorotic in appearance.

Transformation and plant regeneration

For the plant transformation experiments, 50 mL of AB liquid medium which consisted of 5 g L^{-1} glucose; 50 mL of Buffer AB (20x) (60 g L^{-1} K_2HPO_4, 20 g L^{-1} NaH_2PO_4, 6 g L^{-1} $MgSO_4 \cdot 7H_2O$); and 50 mL of AB salts (20x) (3 g L^{-1} KCl, 20 g L^{-1} NH_4Cl, 6 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1} $CaCl \cdot 7H_2O$ and 0.72 g L^{-1} NaFe-EDTA supplemented with 50 mg L^{-1} of kanamycin were inoculated with 100 µL of aLBA4404 strain culture carrying the p1AcPRD vector and grown to an optical density (OD_{600}) of 0.6. The culture was then centrifuged at 1,000 g_n for 30 min at 4°C, the supernatant was discarded and the bacterial pellet was kept on ice for the explant co-culture.

The transformation experiments were carried out following the protocol described by Trujillo *et al.* (2001) with some modifications generating two new protocols. In the first protocol (Trujillo modified 1), acetosyringone was added to 100 µM final concentration to AB medium MS salts (supplemented with 1% sucrose) and RM media were used in the activation, infection and co-cultivation steps respectively. In the second protocol (Trujillo modified 2), the bacterial infection medium MIB which consisted in 50 mL AB salts, 5 g glucose, 0.24 g NaH_2PO_4, 3.9 g de MES (Sigma-Aldrich, St. Louis, MO) and 100 µM acetosyringone finally adjusted to pH 5.6), MIB medium (Gelvin *et al.,* 1989; Ramírez *et al.,* 2009) was used as the infection medium and the infection time was prolonged for 1 h. Transformation controls were treated in the same manner but without *Agrobacterium*.

The transformation experiments were conducted under a completely randomized design where each treatment (transformation protocol) was done with five experimental units. Each experimental unit was a petri dish with 10 leaf explants of the Pastusa Suprema variety. Response variables evaluated were: callus formation, shoot regeneration and root formation.

Molecular characterization

PCR analysis. Total genomic DNA of the *in vitro* plantlets (corresponding to the independent putative transformants) and control plants was extracted using the protocol described by the International Potato Center (CIP) (Ghislain *et al.*, 1997). PCR was done to detect the *cry1Ac* gene by specific amplification of a 766 pb fragment. The primers used were: CRYC1 (5'-ATCTTCACCTCAGCGTGCTT-3') and CRYC2 (5'-GGCACATTGTTGTTCTGTGG-3') (Valderrama *et al.,* 2007). Each PCR reaction was adjusted to a final volume of 25 µL containing 1X PCR buffer (50 mM of

KCl, 10 mM of Tris-HCl, pH 8.3), 1.5 mM of $MgCl_2$, 0.2 mm of dNTPs, 0.4 µM of each primer, 1.5 U of Taq polymerase and 100-200 ng of genomic DNA.

DNA amplifications were performed in an iCycler™ (Bio-Rad, Hercules, CA) thermocycler using the following temperature profile: an initial step of 94°C for 3 min, 25 amplification cycles of 1 min, denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C, followed by an additional extension cycle of 10 min at 72°C. The PCR products (5 µL) were mixed with 6X DNA Loading Dye and ran in a 1% (w/v) agarose gel at 70 V for 45 min. The DNA bands were observed using a UV transiluminator.

DAS-ELISA assay. For this assay, extracts of leaves of *in vitro* plantlets were prepared. The presence of the Cry1Ac protein was detected using an ELISA kit Bt-Cry1Ab/1Ac® (Agdia, Elkhart, IN). The ELISA protocol was performed following the manufacturer's instructions. Readings were done at 650 nm in an ELISA Bio-Rad (Model 680) Microplate-Reader (Molecular Devices, Sunnyvale, CA). To quantitate the amount of Cry1Ac protein in µg mL^{-1}, a standard curve was constructed using the control provided in the kit. To determine the percentage of Cry1Ac (% Cry-1Ac), the total protein concentration was estimated using the Bradford method (Bradford, 1976). The OD was read in each microplate and the ratio of µg of Cry1Ac protein per µg of total protein was estimated for each transgenic line. Additionally, we calculated: µg Cry1Ac per mg of fresh leaf tissue.

Statistical analysis

Results were analyzed using SAS® 9.1 statistical software (SAS Institute, 2003). The association between each concentration of kanamycin and response variables (callus formation, shoot regeneration and viability) was determined by logistic regression analysis.

The effect of transformation protocols on callus formation, regeneration of shoots and root formation was determined by one-way ANOVA and Duncan test. Likewise, for each modified protocol, the regeneration frequency and the transformation efficiency were calculated. The first was calculated as the number of explants that formed shoots divided by the total number of treated explants and the second as the number of PCR and DAS-ELISA positive plantlets divided by the total number of treated explants. To determine significant differences between the percentages of Cry1Ac protein expression of transformed plantlets, an ANOVA and a Duncan test for comparison of means were performed.

Results and discussion

Effect of kanamycin on explant regeneration and viability

Callus formation was found to be decreased with increasing concentrations of kanamycin. Callous formation of leaf explants exposed to 0 and 25 mg L^{-1} of kanamycin was 100 and 94%, respectively, while at 50, 100 and 150 mg $L^{-1,}$ callous formation was 70, 5 and 0%, respectively (Fig. 1). The effect of kanamycin on shoot regeneration was observed on leaf explants cultured in kanamycin concentrations equal to or greater than 50 mg L^{-1}. The percentage of shoot regeneration at this concentration was 0% (Fig. 1). Additionally, 99% of the explants were not viable at 50 mg L^{-1} of kanamycin. This was evidenced by the calli not being able to produce shoots and by the appearance of the explants, which became oxidized and necrotic. (Fig. 2).

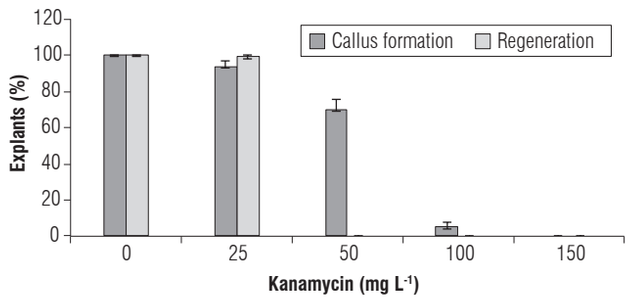

FIGURE 2. Effects of kanamycin concentrations on the callus formation and regeneration of explants. The vertical bars indicate standard deviation.

The Pastusa Suprema variety was able to tolerate levels of kanamycin of 25 mg L^{-1}, with explants remaining green at this dose, with many calluses and high percentages of shoot regeneration at week 3 of the culture . Furthermore, concentrations equal to or above 50 mg L^{-1} were toxic to the explants, drastically inhibiting shoot formation.

Analysis of these data allowed for the selection of the concentration of 100 mg L^{-1} of kanamycin for the selection of transformed leaf explants. Higher antibiotic concentrations were not used because they could exert strong selection pressure and cause the loss of transformed explants by oxidation due to necrosis or chlorosis of non-transformed tissues (Quissen *et al.,* 2009).

The effect of kanamycin on callus formation and regeneration has been extensively studied in *S. tuberosum*. In most studies, concentrations above 50 mg L^{-1} have been used and 100 mg L^{-1} is the most common concentration used for the selection of transformed leaf explants (De Block, 1988; Tavazza *et al.,* 1988 Wenzler *et al.,* 1989; Visser *et al.,* 1991; Beaujean *et al.,* 1998; Trujillo *et al.,* 2001; Banerjee *et al.,* 2006). The toxic effect of kanamycin observed at these

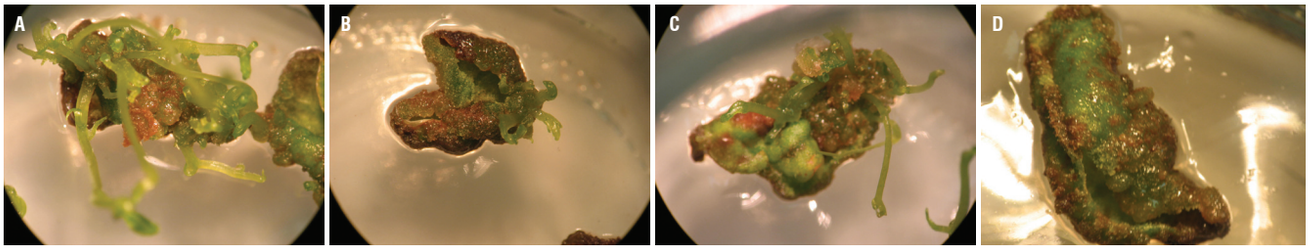

FIGURE 3. Regeneration of transformed Pastusa Suprema leaf explants in regeneration medium RM media supplemented with kanamycin 100 mg L^{-1} A, Calli produced 3 weeks after transformation. B, Regeneration of shoots at 5 weeks after transformation. C, Stems grown at 6 weeks. D, Regeneration after 8 weeks. Source photographs: Torres, E.S.

TABLE 1. Regeneration and transformation frequency obtained in the transformation protocols.

Treatments	% Explants with callus (mean±SD)	% Explants with shoots (mean±SD)	% Shoots with roots (mean±SD)	% Regeneration frequency[a]	% Transformation efficiency[b]
Trujillo	9.0±2.9	0	0	0	0
Trujillo M1	38.0±4.0	28.0±4.0	28.0±4.0	28	22
Trujillo M2	52.0±3.3	44.0±4.6	44.0±4.6	44	37

[a] Regeneration frequency was calculated as the number of explants that formed shoots divided by the total number of treated explants. [b] Transformation efficiency was calculated as the number of PCR, DAS-ELISA positive seedlings divided by the total number of treated explants. Trujillo M1, acetosyringone in AB medium MS salts and regeneration medium for activation, infection and co-cultivation steps respectively. Trujillo M2, the bacterial infection medium MIB and acetosyringone prolonged 1 h.

concentrations is explained by its direct action on mitochondria and chloroplast ribosomes of plant cells, resulting in inhibition of protein synthesis. This causes failures in the synthesis of chlorophyll evidenced by chlorosis and growth inhibition (Brasileiro and Aragão, 2001).

Transformation and regeneration of the potato lines

Comparison of transformation protocols showed that the method by Trujillo *et al.* (2001) produced 9% callus formation but no shoot regeneration. Friable calli were produced but they became oxidized. The Trujillo modified 1 protocol gave 38% callus formation and 28% shoot regeneration frequency. When Trujillo modified 2 Protocol was used, 52% of explants formed callus and 44% regenerated shoots (Tab.1 and Fig. 3). In both protocols, all regenerating shoots were transferred to a MP medium supplemented with kanamycin, in order to form rooted plantlets. Statistical analysis showed that callus formation, shoot formation and root formation differed significantly in the modified protocols with respect to the original protocol by Trujillo *et al.* (2001), with Trujillo modified 2 being significantly better.

The results show that the protocol published by Trujillo *et al.* (2001) is less efficient for the transformation of PS. However, the success of this protocol for transformation of other varieties of *S. tuberosum* ssp. andigena (Diacol Capiro and Parda Pastusa, among others) suggests an influence by the genotype on the regeneration efficiency, and transformation (Dale and Harnpson, 1995; Heeres *et al.,* 2002). This is especially important when considering that PS is an interspecific hybrid, unlike the Parda Pastusa and Diacol Capiro varieties, and therefore there may have a marked influence from the genotype. The results of this study, together with other reports in the literature, favor the dependency relationship between genotype and transformation, although others claim that transformation is independent of genotype (De Block, 1988; Kumar *et al.,* 1995).

The large difference between the transformation efficiency of the original protocol of Trujillo *et al.* (2001) and the modified protocols is probably due to the addition of acetosyringone to all the culture media used for transformation, as this substance acts as a transcriptional activator of the *vir* genes, which encode proteins responsible for transfer of T-DNA into the plant genome (Stachel *et al.,* 1986; Gelvin, 2006). This is in agreement with reports by Hamdi *et al.* (2003) who had higher rates of transformation and regeneration when acetosyringone was added at a concentration of 10 mg L^{-1} to a co-culture medium during *A. tumefaciens* mediated genetic transformation of the *S. tuberosum* var. Desirée. Henzi *et al.* (2000) were also able to observe increases in the efficiency of transformation of *Brasica oleracea* L. var. Italics, with the addition of acetosyringone to the infection medium.

Moreover, the difference between the frequencies of transformation in the Trujillo modified 1 and Trujillo modified 2 protocols might have been influenced by the parameters: time of infection and infection medium, since these were the basic differences between the two protocols.

The infection medium MIB (Gelvin *et al.,* 1989) used in Trujillo modified 2 contains substances that activate the

virulence of *Agrobacterium:* glucose and acetosyringone (Doty *et al.,* 1993; Gelvin, 2003; Gelvin, 2006; Higgins *et al.,* 1992), and compounds which act as buffers or pH stabilizers: sodium dihydrogen phosphate (NaH_2PO_4), morpholino ethanesulfonic acid (MES) (Gelvin, 2006).

Glucose may also be involved in stimulating the virulence of *Agrobacterium*. Monosaccharides such as glucose, fucose, arabinose and acids such as D-glucuronic acid and D-galacturonic have been identified as inducers of *vir* genes of *Agrobacterium*. The *Chv*E gene is required for the induction of the *vir* genes by sugars (Doty *et al.,* 1993; He *et al.,* 2009). Peng *et al.* (1998) and Ramírez *et al.* (2009) reported that an important condition for achieving high *vir* gene induction is the presence of glucose in the infection medium. Glucose is generally substituted for sucrose in the activation and infection media. However, this substance is not as effective as the co-inducer of *vir* genes (Gelvin, 2006).

NaH_2PO_4 and MES can affect *vir* gene expression due to the interactions that occur between the pH, temperature and inorganic phosphate levels (Gelvin, 2006). In some cases, the environment surrounding wounded plant cells is inhibitory to bacteria because of acid exudates of plant cells (Gelvin, 2006). It is necessary to ensure that *Agrobacterium* is in an environment or culture medium with good buffering capacity, to help facilitate transformation (Kanemoto *et al*., 1989).

The MES concentration appears to be critical to determine the transformation efficiency. Ramírez *et al.* (2009) used the culture media MIB, PIM2 (Aldemita and Hodges, 1996) and Gelvin-Liu (Gelvin and Liu, 1994) for infection and co-cultivation in the genetic transformation of the tomato var. Unapal-Arreboles. These media differed only in the concentration of MES (20, 75 and 50 mM, respectively). Transient expression of the β-glucuronidase gene (GUS) used was increased when the MIB medium was used.

For the influence of the time of infection in the frequencies of transformation, it has been reported that successful transformation of explants can depend on the time of exposure with the inoculum; if it is very long, high-mortality of plant cells occurs due to bacterial infection and if it is very short, only transient expression might be observed (Ramírez *et al.,* 2009). Most research in potato transformation (*S. tuberosum* spp.) proposes inoculation times between 2 and 15 min (Gustafson *et al.,* 2006; Trujillo *et al*., 2001). However López and Chaparro (2007) inoculated explants for 30 min, while others, like De Block (1988), performed a single inoculation and co-culture for 2 d. Finally, others, like Hamdi *et al.* (2003) and as in the present study, had success using 1 h of infection in darkness.

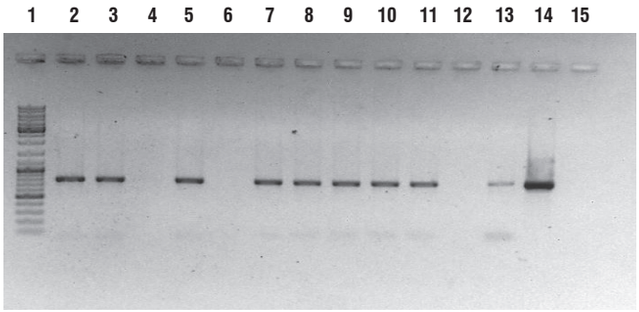

FIGURE 4. Amplification of the *cry1Ac* gene by PCR in putative transformed plantlets. Lane 1, 100 bp molecular weight marker; lanes 2-11, putative transformed plants; lane 12, negative control (non-transformed plantlet); lane 13, positive control (transgenic line 40.7 a DC); lane 14, p1AcPRD vector; lane 15, absolute control (no DNA mix).

Molecular characterization

The presence of the *cry1Ac* gene was evaluated by PCR in 38 putative transformants, showing the expected band of 766 bp corresponding to the amplification product of the *cry1Ac* gene in 31 plants. The non-transformed control plant was PCR negative (Fig. 4).

All 31 PCR positive lines were tested by ELISA to determine Cry1Ac protein expression. All lines expressed Cry1Ac protein. The Cry1Ac protein percentage ranged between 2.5 and 6.4% of the soluble proteins extracted from the leaf tissue of *in vitro* plants, corresponding to expression levels between 88 and 639 ng mg^{-1} of fresh-leaf tissue (Tab. 2).

TABLE 2. Bt transgenic lines of the Pastusa Suprema variety with the highest Cry1Ac protein expressions.

Plants grown in kanamycin	Cry1Acng protein / mg of leaf tissue
PS 40.10	614
PS 40.13	573
PS 40.14	575
PS 40.17	492
PS 40.18	491
PS 40.23	469
PS 40.24	463
PS 40.27	639

According to the PCR and DAS-ELISA analysis, the transformation protocols Trujillo modified 1 and Trujillo modified 2 were effective for regenerating transgenic potato shoots. Transformation efficiencies were 22 and 37%, respectively. The difference between these efficiencies

confirms the effectiveness of the changes made in the Trujillo modified 2 protocol to induce *Agrobacterium* virulence and consequently increase the number of transgenic shoots. The obtained transformation efficiencies are higher than those reported by López and Chaparro (2007), who obtained initial transformation efficiencies of 31.0±2.5%, expressed in the formation of callus and a final eficiency of 30%, based on the GUS assay.

The difference in the level of expression of Cry1Ac protein in the transgenic plants obtained in this study (88 to 639 ng mg^{-1} of leaf tissue) can be explained by the fact that such variation in expression is common between populations that are independently transformed with the same transgene (Meiyalaghan *et al*., 2006; Estrada *et al*., 2007). This unpredictability of transgene expression is usually attributed to differences in the integration site of the T-DNA into the plant genome (Conner and Christie, 1994; Davidson *et al*., 2004) and or differences in the number of gene copies (Hobbs *et al*., 1993). Therefore, further work is required for the determination of the number of *cry1Ac* gene copies by Southern blot or real time PCR, in order to relate the differences found in the *cry1Ac* gene expression with the gene copy number.

These results are similar to those obtained in other studies of genetic transformation of plants with cry genes, including transgenic lines with cry genes under the regulation of the CaMV35S promoter (Meiyalaghan *et al*., 2006; Estrada *et al*., 2007; Valderrama *et al*., 2007; Jacobs *et al*., 2009; Kumar *et al*., 2010).

The levels of protein expression *in vitro* in our study (639 ng mg^{-1} of leaf tissue) are comparable with those reported by Torres (2010) on the expression of the Cry1Ac protein in DC variety *in vitro* plantlets (to 620 ng/mg of leaf tissue), also under the control of the CaMV35S 2X promoter. Nevertheless, they are superior to those obtained by Estrada *et al*. (2007) in greenhouse plants, resulting from the transformation of the potato ND5873 line with the *cry1Ac* gene under regulation of the CaMV35S promoter-1X (up to 0.58 ng/mg of leaf tissue). This difference in Cry1Ac expression may be due, among other factors, to the variety used for each study, the transformation vector (the promoter having great influence), the plant tissue and the growth condition (*in vitro* or greenhouse) (Valderrama *et al*., 2007; Torres, 2010; Vanegas *et al*., 2010). Of the above factors, the most important factor is probably the use of the 2X CaMV35S promoter. The results of several studies suggest greater activity of the CaMV35S 2X promoter for protein expression compared to the single promoter (1X) (Torres, 2010; Vanegas *et al*., 2010). In a study on Cry1Ab protein expression in potato tubers, it was found that the amount of this protein was three to four times higher under the CaMV35S 2X promoter compared to the single promoter (Cañedo *et al*., 1998).

The high Cry1Ac protein expression in the leaves of the transgenic plantlets obtained in this study could suggest a potential high expression in tubers. Valderrama *et al*. (2007), who also transformed varieties of potato *S. tuberosum* ssp. andigena with the *cry1Ac* gene under regulation of the 2X CaMV35S promoter, found Cry1Ac protein expression levels between 0.02 and 17.00 ng g^{-1} of tuber tissue. Additionally, Torres (2010) reported increased levels of Cry1Ac protein expression in fresh tuber tissue (up to 1528 ng/mg of tuber tissue) as compared to the levels obtained in *in vitro* leaves (210-620 ng/mg tissue). Other studies have also reported a correlation between the Cry protein expression in leaves and tubers (Cañedo *et al*., 1998; Estrada *et al*., 2007; Jacobs *et al*., 2009; Kumar *et al*., 2009; Meiyalaghan *et al*., 2006).

Since high levels of *cry1Ac* gene expression were obtained in the PS transgenic lines developed in this study (88 to 639 ng mg^{-1}), it is possible that the resistance to *T. solanivora* of these lines is good, considering that tubers from transgenic plants that express similar quantities of Cry1Ac protein in leaf tissue have shown between 80 and 100% larval mortality (Valderrama *et al*., 2007; Torres, 2010).

Conclusions

In this study, two protocols were optimized for the genetic transformation of the male-sterile Colombian potato variety Pastusa Suprema. These protocols allowed the transformation of this variety with transformation efficiencies 22 to 37% better than other protocols previously reported by other authors. The availability of these protocols allows the possibility of introducing other agronomically important genes, such as those conferring resistance to other pests and/or abiotic stress.

The transgenic Pastusa Suprema male-sterile variety provides a basis for solving the problem of gene flow in potato in the Andean region. The possibility of using these lines in national markets will require safety assessments of the phenotypic characteristics of substantial equivalence and productivity as well as biosafety trials under greenhouse and field conditions. This is the first report to our knowledge on the development of *cry1Ac* transgenic potato lines potentially resistant to *T. solanivora*, developed from a male-sterile variety.

Acknowledgements

We want to thank the Colombian Ministerio de Agricultura y Desarrollo Rural (MADR) for financial support that helped to carry out this study. We thank Professor Guillermo Correa of the National University of Colombia, Medellin and Wilmer Cuervo for statistical advice.

Literature cited

Aldemita, R.R. and T.K. Hodges. 1996. *Agrobacterium tumefaciens*-mediated transformation of japonica and indica rice varieties. Planta 199(4), 612-617.

Agronet. 2009. Oferta agropecuaria ENA - Cifras 2009. Boletines estadísticos. In: MADR and CCI, http://www.agronet.gov.co/www/docs_agronet/201046112648_RESULTADOS_ENA_2009.pdf; consulted: March, 2012.

Arévalo, A. 2003. Análisis de la problemática en Colombia de la polilla guatemalteca de la papa *Tecia solanivora*. pp. 168-173. In: II Taller Nacional de *Tecia solanivora*. Cevipapa. Bogota.

Banerjee, A.K., S. Prat, and D. Hannapel. 2006. Efficient production of transgenic potato (*S. tuberosum* L. sp. *andigena*) plants via *Agrobacterium tumefaciens* mediated-transformation. Plant Sci. 170, 732-738.

Beaujean, A., R.S. Sangwan, A. Lecardonnel, and B.S. Sangwan-Norreel. 1998. *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced intermodal explants: an efficient protocol of transformation. J. Exp. Bot. 49, 1589-1595.

Bradford, M.M. 1976. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72, 248-254.

Brasileiro, A.M.M. and J.F.L. Aragão. 2001. Marker genes for *in vitro* selection of transgenic plants. J. Plant Biotechnol. 3(3), 113-121.

Cañedo, V., J. Benavides, A. Golmirzaie, F. Cisneros, M. Ghislain, and A. Lagnaoui. 1998. Assesing Bt-transformed potatoes for potato tuber moth. pp. 161-170. In: *Phthorimaea operculella* (Zeller) management. Program Report. International Potato Center. Lima.

Celis, C., M. Scurrah, S. Cowgill, S. Chumbiauca, J. Green, J. Franco, G. Main, D. Kiezebrink, R.G. Visser, and H.J. Atkinson. 2004. Environmental biosafety and transgenic potato in a centre of diversity for this crop. Nature 432, 222-225.

Chaparro, A. 2011. Cultivos transgénicos: entre los riesgos biológicos y los beneficios ambientales y económicos. Acta Biol. Colomb. 16(3), 231-252.

Conner, A.J. and M.C. Christey. 1994. Plant breeding and seed marketing options for the introduction of transgenic insect-resistant crops. Biocontrol. Sci. Technol. 4(4), 463-473.

Conner, A.J., T.R. Glare, and J.P. Nap. 2003. The release of genetically modified crops into the environment: II. Overview of ecological risk assessment. Plant J. 33(1), 19-46.

Dale, P.J. and K.K. Harnpson. 1995. An assessment of morphogenic and transformation efficiency in a range of varieties of potato (*Solanum tuberosum* L.). Euphytica 85, 101-108.

Davidson, M.M., M.F. Takla, J.M. Jacobs, R.C. Butler, S.D. Wratten, and A.J. Conner. 2004. Transformation of potato (*Solanum tuberosum*) cultivars with a *cry1Ac9* gene confers resistance to potato tuber moth (*Phthorimaea operculella*). Crop Hortic. Sci. 32(1), 39-50.

De Block, M. 1988. Genotype-independent leaf transformation of potato (Solanum tuberosum) using *Agrobacterium tumefaciens*. Theor. Appl. Genet. 76(5), 767-774.

De Block, M. and D. Debrouwer, 1993. Engineered fertility control in transgenic *Brassica napus* L.: histochemical analysis of anther development. Planta 189, 218-225.

Doty, S.L., M. Chang, and E.W. Nester. 1993. The chromosomal virulence gene, *chvE*, of *Agrobacterium tumefaciens* is regulated by a LysR family member. J. Bacteriol. 175(24), 7880-7886.

EPPO. 2006. *Tecia solanivora*. EPPO Bulletin. In: http://www.eppo.org/QUARANTINE/insects/Tecia_solanivora/DS_Tecia_solanivora.pdf; consulted: March, 2012.

Estrada, M., K. Zarka, S. Cooper, J. Coombs, and D.S. Douches. 2007. Potato tuberworm (Lepidoptera: Gelichiidae) resistance in potato lines with the *Bacillus thuringiensis cry1Ac* gene and natural resistance. HortScience 42(5), 1306-1311.

FAOSTAT. 2010. Producción de productos alimentarios y agrícolas. http://faostat.fao.org/; consulted: March, 2012.

Gelvin, S.B. and C.N. Liu. 1994. Genetic manipulation of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant plant species. pp. 1-13. In: Gelvin, S.B. and R. Schiperoort (eds.). Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Gelvin, S.B. 2003. *Agrobacterium*-metiated plant transformation: the Biology behind the "GENE-Jockeying" tool. Microbiol. Mol. Biol. R. 67(1), 16-37.

Gelvin, S.B. 2006. *Agrobacterium* virulence gene induction. pp. 77-86. In: Wang, K. (ed). Methods in molecular biology. Agrobacterium protocols II. Totowa, Humana Press, NJ.

Gelvin, S.B., R. Scholperoort, and D.S. Verma. 1989. Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Ghislain, M., D. Zhang, and M.R. Herrera. 1997. Protocolos de laboratorio de biología molecular. Tipificación genética. 2nd ed. International Potato Center, Lima.

Gustafson, V., S. Mallubhotla, J. Macdonnell, M. Sanyal-Bagchi, B. Chakravarty, G. Wang-Pruski, C. Rothwell, P. Audy, D. Dekoeyer, M. Siahbazi, B. Flinn, and S. Regan. 2006. Transformation and plant regeneration from leaf explants of *Solanum tuberosum* L. cv. Shepody. Plant Cell Tiss. Org. 85(3), 361-366.

Hamdi, M., C. Rouvière, J. Rojas, and P. Jardin. 2003. Optimisation de la transformation génétique de la ponme de terre par *Agrobacterium tumefaciens*. Biotechnol. Agron. Soc. Environ. 7(3), 183-188.

He, F., G.R Nair, C.S. Soto, Y. Chang, L. Hsu, E. Ronzone, W. F. De Grado, and A.N. Binns. 2009. Molecular basis of ChvE function in sugar binding, sugar utilization, and virulence in *Agrobacterium tumefaciens*. J. Bacteriol. 191(18), 5802-5813.

Heeres P., M. Schippers-Rozenboom, E. Jacobsen, and R. Visser. 2002. Transformation of a large number of potato varieties: genotype dependent variation in efficiency and somaclonal variability. Euphytica 124, 13-22.

Henzi, X., M. Christey, and D. Mcneil. 2000. Factors that influence *Agrobacterium* rhizogenes-mediated transformation of broccoli (*Brassica oleracea* L. var. *Italica*). Plant Cell Rep. 19(10), 994-999.

Higgins, E., J. Hulme, and R. Shields. 1992. Early events in transformation of potato by *Agrobacterium tumefaciens*. Plant Sci. 82(1), 109-118.

Hobbs, S.L.A., T.D. Warkentin, and C.M. Delong. 1993. Transgene copy number can be positively or nagatively associated with transgene expression. Plant Mol. Biol. 21(1), 17-26.

Höfgen, R. and L. Willmitzer. 1988. Storage of competent cells for *Agrobacterium* transformation. Nucleic. Acids. Res. 16(20), 9877.

Jacobs, J.M., M.F. Takla, L.C. Docherty, C.M. Frater, N.P. Marckwick, S. Meiyalaghan, and A.J Conner. 2009. Potato transformation with modified nucleotide sequences of the *cry9Aa2* gene improves resistance to potato tuber moth. Potato Res. 52(4), 367-378.

Kanemoto, R.H., A.T. Powell, D.E. Akiyoshi, D.A. Regier, R.A. Kerstetter, and E.W. Nester. 1989. Nucleotide sequence and analysis of the plant inducible locus pinF from *Agrobacterium tumefaciens*. J. Bacteriol. 171(5), 2506-2512.

Kumar, M., V. Chimote, R. Singh, G.P. Mishra, P.S. Naik, S.K. Pandey, and S.K. Chakrabart. 2010. Development of Bt transgenic potatoes for effective control of potato tuber moth by using *cry1Ab* gene regulated by GBSS promoter. Crop Prot. 29(2), 121-127.

Kumar, A. 1995. *Agrobacterium* mediated transformation of potato genotypes. pp. 121- 128. In: Gartland K.M.A and M.R. Davey (eds.). Methods in molecular biology. *Agrobacterium* protocols. Vol. 44. Humana Press, Totowa, NJ.

López, A. and A. Chaparro. 2007. Propuesta de un sistema de transformación de plantas de papa (*Solanum tuberosum* sp. *andigena* var. Pastusa Suprema) mediado por *Agrobacterium tumefaciens*. Agron. Colomb. 25(1), 16-25.

Mc Partlan, H.C. and P.J. Dale. 1994. An assessment of gene transfer by pollen from field-grown transgenic potatoes to nontransgenic potatoes and related species. Transgenic Res., 3(4), 216–225.

Meiyalaghan, S., J.M. Jacobs, R.C. Butler, S.D. Wratten, and A.J. Conner. 2006. Transgenic potato lines expressing *cry1ba1* or *cry1ca5* genes are resistant to potato tuber moth. Potato Res. 49(3), 203-216.

Mullins, E., D. Milbourne, P. Carlo, M. Barbara, D. Prestwich, and C. Meade. 2006. Potato in the age of biotechnology. Trends Plant Sci. 11(5), 254-259.

Nap, J.P., P.L.J. Metz, M. Escaler, and A.J. Conner. 2003. The release of genetically modified crops into the environment: I. Overview of current status and regulations. Plant J. 33(1), 1-18.

Niño, L. 2004. Revisión sobre la Polilla de la Papa *Tecia solanivora* en Centro y Suramérica. Rev. Latinoam. Papa (Suppl.) 4-22.

Ñústez, C.E. 2011. Variedades colombianas de papa. Universidad Nacional de Colombia, Bogota.

Osorio, P., E. Espitia, E. Luque. 2001. Reconocimiento de enemigos naturales de *Tecia solanivora* (lepidoptera: gelechiidae) en localidades productoras de papa en Colombia. Rev. Colomb. Entomol. 27(3), 177-185.

Peng, W.T., Y.W. Lee, and E.W. Nester. 1998. The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein are broadened by a high level of the sugar binding protein ChvE. J. Bacteriol. 180(21), 5632-5638.

Quissen, R., Y. Oliveira, M. Pileggi, F. Cuquel, and M. Quoirin. 2009. Selective agent and *A. tumefaciens* overgrowth-control antibiotics in *Eucalyptus camaldulensis* cotiledonary culture. Braz. Arch. Biol. Techn. 52(6), 1485-1492.

Ramírez, H., Z. Lentini, and F. Vallejo. 2009. Evaluación y selección de un protocolo vía *Agrobacterium* para la incorporación de resistencia al cogollero en la variedad de tomate Unapal-Arreboles. Acta Agron. 58(2), 61-68.

Rodríguez, E., C. Trujillo, S. Orduz, S. Jaramillo, R. Hoyos, and R. Arango. 2000. Estandarización de un medio de cultivo adecuado para la regeneración de tallos a partir de hojas, utilizando dos variedades colombianas de papa (*Solanum tuberosum* L). Rev. Fac. Nal. Agron. Medellin 53(1), 887-899.

SAS Institute. 2003. Introductory guide for personal computers. Versión 9.1. Cary, NC.

Segura, M., M. Santos, and C.E. Ñústez. 2006. Desarrollo fenológico de cuatro variedades de papa (*Solanum tuberosum* L.) en el municipio de Zipaquirá - Cundinamarca. Fitotecnia Colomb. 6(2), 33-43.

Schmuelling, T., H. Roehrig, S. Pilz, R.Walden, and J. Schell, 1993. Restoration of fertility by antisense RNA in genetically engineered male sterile tobacco plants. Mol General Genet. 237, 385-394.

Scurrah, M., C. Celis, S. Chumbiauca, A. Salas, and R.G. Visser. 2008. Hybridization between wild and cultivated potato species in the Peruvian Andes and biosafety implications for deployment of GM potatoes. Euphytica 164(3), 881-892.

Stachel, S.E., E.W. Nester, and P. Zambryskr. 1986. A plant cell factor induces *Agrobacterium* vir gene expression. Proc. Natl. Acad. Sci. 83(2), 379-383.

Stewart, C.N., M.D. Halfhill, and S.I. Warwick, Transgene introgression from genetically modified crops to their wild relatives. Nat. Rev. Genet. 4, 806-817.

Tavazza, R., M. Tavazza, R.J. Ordas, G. Ancora, and E. Benvenuto. 1988. Genetic transformation of potato (*Solanum tuberosum*): an efficient method to obtain transgenic plants. Plant Sci. 59, 175-181.

Torres, J.M. 2010. Evaluación biológica y molecular de plantas de papa (*Solanum tuberosum*) mejoradas genéticamente con el gen *cry1Ac* de *Bacillus thuringiensis* en condiciones de invernadero. M.Sc. thesis. Faculty of Sciences, Universidad Nacional de Colombia. Medellín, Colombia.

Trujillo, C., E. Rodríguez, S. Jaramillo, R. Hoyos, S. Orduz, and R. Arango. 2001. One step transformation of two Andean potato cultivars (*Solanum tuberosum* L. sp. *andigena*). Plant Cell Rep. 20(7), 637-641.

Valderrama, A.M., N. Velásquez, E. Rodríguez, A. Zapata, M. Zaidi, I. Altosaar, and R. Arango. 2007. Resistance to *Tecia solanivora* (Lepidoptera: Gelechiidae) in three transgenic andean varieties of potato expressing *Bacillus thuringiensis* Cry1Ac protein. J. Econ. Entomol. 100(1), 172-179.

Vanegas, P.A., J.T. Blanco, and A. Chaparro. 2010. Expresión de la proteína Cry1Ac en tejidos de líneas transgénicas de papa (*Solanum tuberosum* spp. *andigena*) var. Diacol capiro. Acta Biol. Colomb. 15(2), 101-114.

Visser, R.G. 1991. Regeneration and transformation of potato by *Agrobacterium tumefaciens*. pp. 1-9. In: Lindsey, K. (ed.). Plant tissue culture manual: Fundamentals and applications. Kluwer Academic Publishers, Dordrecht, The Netherland.

Wenzler, H., G. Mignery, G. May, and W. Park. 1989. A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. Plant Sci. 63, 79-85.