TRANSIENT GENE EXPRESSION IN SUGARCANE PROTOPLASTS AFTER ELECTROPORATION AND POLYETHYLENE GLYCOL TREATMENT.

Pedro Molina, Eduardo Menéndez, Ariel Arencibia, Alejandro Fuentes, Julio Alfonso, Yamilet Coll, Virginia Greenidge, Sergio Pérez and Guillermo Selman-Housein.

Center for Genetic Engineering and Biotechnology, P.O.Box 6162, Cubanacán, C.P. 10600, Ciudad Habana. CUBA.

Recibido en mayo de 1992. Aprobado en agosto de 1993.

Key words: Transformation, Sugarcane protoplasts, electroporation, PEG.

SUMMARY

The conditions for genetic transformation of sugarcane (*Saccharum officinarum L.*) protoplasts from commercial varieties POJ 2878 and B 4362 were stablished. Protoplasts from both varieties showed transient expression of β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) genes when they were transformed with plasmids pBI221.1 and pCaMVICN either by electroporation or polyethylene glycol (PEG) treatment. For electroporation, optimal results were obtained using a capacitance of 660 μF with an electrical field strength of 600 v/cm. Using PEG, the best results were obtained at concentration of 20%.

RESUMEN

Se establecieron las condiciones para la transformación genética de protoplastos de caña de azúcar a partir de las variedades comerciales POJ 2878 y B 4362. Los protoplastos de ambas variedades mostraron actividad transitoria de los genes de la β-glucuronidasa (GUS) y cloranfenicol acetiltransferasa (CAT) después que fueran transformados con los plasmidios pBI221.1 y pCaMVICN mediante la electroporación o tratamiento con polietilen glicol (PEG). Para la electroporación los resultados óptimos fueron obtenidos usando 660 μF de capacitancia con un campo eléctrico de 600 v/cm. El tratamiento con polietilen glicol al 20% mostró los mejores resultados.

INTRODUCTION

Agriculturally important species of gramineous monocots, including sugarcane, have been difficult to modify genetically by means of current genetic engineering techniques using *Agrobacterium*, because with few exceptions, many species of this family are not readily susceptible to infection by *A.tumefaciens*. For this reason, direct gene transfer into plant cell or protoplasts was developed (Potrykus *et al.*, 1985; Paszkowski *et al.*, 1984; Fromm *et al.*, 1985 and Chen *et al.*, 1987).

Instead of particle bombardment becoming more and more popular as a tool for monocots transformation, current methods for obtaining transgenic plants by electroporation and PEG treatments maintain its relevance on behalf of the high frecuency of transformation that is possible to obtain using these methodologies when efficient approaches for the protoplast culture and plant regeneration are available. For example, protoplast electroporation has been successfully used for obtaining transient expression and stable integration of foreing genes in different species of gramineous monocots: *Triticum monococcum* (Hauptmann *et al.*,1987; Ou-Lee *et al.*, 1986), Zea mays (Fromm *et al.*,1985), *Pennicetum purpureum* and *Panicum maximum* (Hauptmann *et al.*,1987), *Oryza sativa* and *Sorghum bicolor* (Ou-Lee *et al.*,1986). On the other hand, PEG-treatment has been successfully used for obtaining transgenic rice plants (Uchimiya, 1986 and Datta *et al.*, 1990).

On sugarcane, only few transformation reports have been made using electroporation or PEG treatment. Hauptmann *et al.* (1987) reported that protoplasts from one cell line of sugarcane expressed chloramphenicol acetyltransferase activity following electroporation, however culture conditions were suboptimal during these experiments. Later, Hauptmann *et al.* (1988), using a plasmid that confers resistance to methotrexate, reported the obtainment of methotrexate resistant colonies after electroporation, but southern blot analysis showed no hybridization with specific DNA probe. In 1987 Chen *et al.*, following PEG-induced uptake of linearised DNA, reported transformation of protoplasts from Formosa sugarcane commercial variety F 164 until formation of kanamycin resistant colonies.

Sugarcane is one of the most important crop in tropical countries. For this reason, it is desirable to develop methods for the obtainment of new varieties

using genetic engineering methodologies. In this work, we established the conditions for transformation of protoplasts from two varieties commercially important in Cuba.

MATERIALS AND METHODS

Plant material and cell culture

Two or three month-old established embryogenic callus culture of sugarcane (*Saccharum officinarum L.*) varieties POJ 2878 and B 4362 were used. Two grams (fresh weight) callus were put in 50 ml liquid basal MS medium (Murashige and Skoog, 1962) supplemented with 500 mg/l casein hydrolizate, 2 mg/l 2,4-dichlorophenoxyacetic acid, 100 mg/l myo-inositol, 0.8 mg/l thiamine-HCl, 3% sucrose and maintained in darkness, shaken at 100 rpm at 25°C. The cell suspensions were observed with a microscope for weekly subculture (dilution 1:2 in the same medium) until embryogenic cell clumps appeared. After aproximately 9 weeks subculture (more than 80% of embriogenic cell clusters), the cellular suspensions were filtered through 500 and 300 μm mesh stainless steel filters every three weeks. Dilution was calculated in each subculture in relation to doubling time (approximately 50 h).

Preparation of plasmids

We used the plasmids pBI221.1 (Jefferson *et al.*, 1987) which contain the *gus* gene under the control of 35S promoter from CaMV and polyA region of the *Agrobacterium nos* gene, and pCaMVICN containing the *cat* gene under the same above mentioned regulatory signals (Klein *et al.*, 1988). *E. coli* plasmids were isolated using an alkaline lysis procedure (Maniatis *et al.*, 1982) following CsCl gradient purification. The plasmid concentration were determined spectrophotometrically and verified by agarose gel electrophoresis (Maniatis *et al.*, 1982).

Protoplasts isolation

Twenty milliliters of cell suspension in exponential growth phase (three days after subculture) were sedimented by centrifugation at 650 rpm for 10 min and resuspended in 40 ml of $CaCl_2$ (0.24 M). After 30-40 min, the cells were collected by centrifugation and resuspended in 40 ml of the enzyme solution consisting of 2% (w/v) cellulase R10 (Yakult Honsha Co. LTD., Nishinomiya Hyogo, Japan) 0.1% (w/v) pectolyase Y23 diluted in CPW salts (Power and Chapman, 1985), pH 8. The enzyme-cell mixture was incubated with slow shaking (about 25 rpm) at 25°C for 4-5 h. Released protoplasts were separated from undigested cells by filtration through 100 μm and 50 μm mesh sieves filters and purified by sucrose-mannitol gradient centrifugation. The protoplasts were washed twice with CPW-13M solution (CPW salts, 13% mannitol) and collected by centrifugation at 650 rpm for 10 min.

Protoplasts transformation by electroporation

Washed protoplasts were resuspended in the electroporation medium containing 10 mM Hepes, 0.5 M mannitol, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.2. Approximately 500 μl volumen, containing $3 \div 5 \times 10^5$ protoplasts, 20 μg of plasmid DNA and 25 μg sonicated Calf Thymus carrier DNA were placed in a dispossable cuvette (Biorad, path length 0.4 cm). The DNA-protoplasts mixture was placed on ice for 10 min, mixed and electroporated at different capacitances and voltages in an electroporator of exponential pulse EPE-010 (CIGB, Cuba). After electroporation the samples were placed on ice for 10 min, then at room temperature for 15 min, added to 4.5 ml of protoplasts culture medium modified by Chen *et al.* (1987) and cultured onto 60 ml petri dishes in the dark at 28°C until GUS activity determination.

Protoplasts transformation by PEG-treatment

We used the procedure of Chen *et al.* (1987) with few modifications. One milliliter of purified protoplasts (1×10^6) resuspended in protoplast culture medium was heat shocked at 37°C for 10 min and then placed for 10 seconds on ice and another 10 min at room temperature, then protoplasts were mixed with 20 μg of plasmid DNA and 1 ml, 0.5 ml or 0.25 ml of 40% (w/v) PEG 6000 in F solution: 0.368 g/l KCl, 8 g/l NaCl, 0.125 g/l Na_2HPO_4, 18.4 g/l $CaCl_2$, pH 5.8 (Chen *et al.*, 1987) were added in dependence of the final PEG concentration to be tested. After 30 min at 25°C with gently homogenization, the incubation mixture was diluted by stepwise addition of 10 ml of F solution containing 11 mM glucose (2 ml aliquots over 25 min). The protoplasts were cultured in the dark at 28°C.

Transient expression assays

Twenty four hours after the transformation process, protoplasts viability in samples were determinated by staining with fluorescein diacetate (FDA) according to Power and Chapman (1985). The protoplasts were harvested by centrifugation and resuspended in specific buffer for each assay. β-glucuronidase activity was performed by fluorescent method following the procedure of Jefferson (1987). Fluorescence emission was measured using an Ultra-Micro-Analytical-System (CIE, CUBA). Chloramphenicol acetyltransferase assays were performed according to Fromm *et al.* (1986). Total protein concentration in each sample was determinated according to Bradford's results (1976).

RESULTS AND DISCUSSION

a.- Electroporation

Our aim was to establish optimal electroporation conditions for the transformation of sugarcane protoplasts pulsing the plasmid pBI221.1.

All our initial experiments were done with protoplasts from a cell suspension of sugarcane variety POJ 2878. The protoplasts were electroporated using three level of capacitance: 440, 660 and 880 μF, in combination with voltages between 400 and 700 V/cm. In previous experiments (data not shown) it was found that field strength lesser than 300 V/cm did not give satisfactory results which is in agreement with the results of Hauptman *et al.* (1987) who stated that the cell sensitivity to electrical discharges may be influenced by factors such as: degree of vacuolation, cell size and membrane composition. These considerations explain the need for an increased voltage in order to obtain transient expression in protoplasts of *Gramineae* whose cells are small and whose cytoplasme is dense.

The figure 1 shows that increase in electrical field strength results in a gradual decrease of the protoplast viability. This decrease is more accentuated in the treatment with the highest capacitance. Since gene expression is rather unlikely when the viability is lower than 20%, a compromise has to be found between the pulse discharge used and the resulting protoplast viability.

When a capacitance of 440 μF was used, the GUS expression levels were four times higher than the control values. However, when higher values of capacitance were applied (660, 880 μF) in combination with differents field strength up to 40 times the values of the controls were reached (figure 2). The combinations 660 μF with 600 V/cm and 880 μF with 500 V/cm gave the highest expression levels. It must be pointed out

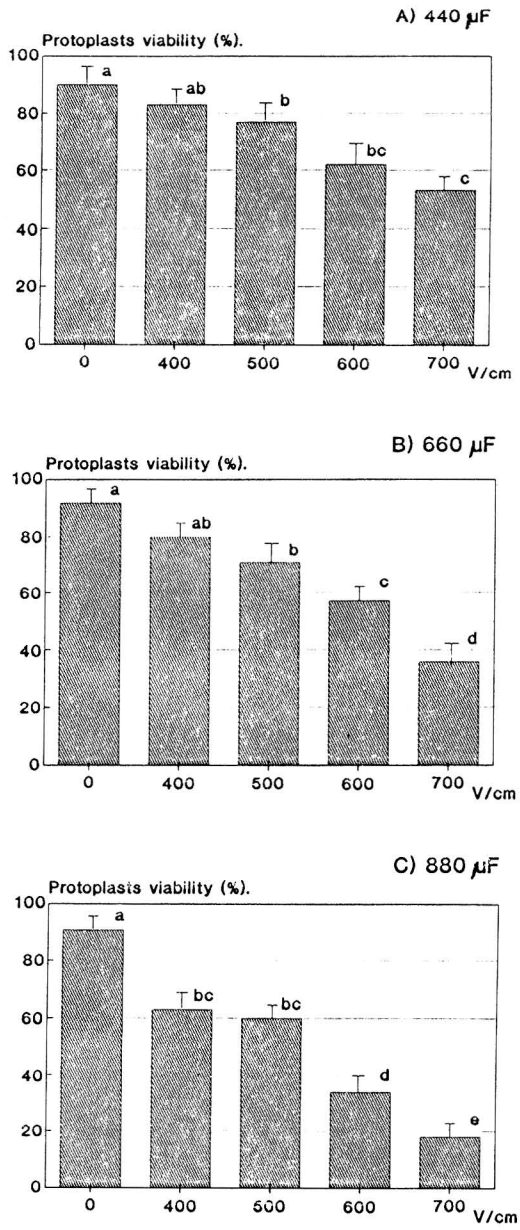

Fig. 1. Effect on the field strengh on survival of POJ-2878 protoplasts. The viavility was determinated by staining with fluorescein diacetate 24 h after electroporation using various voltages and capacitances. Each point is based on mean of at least 200 protoplasts from 3 independent experiments.

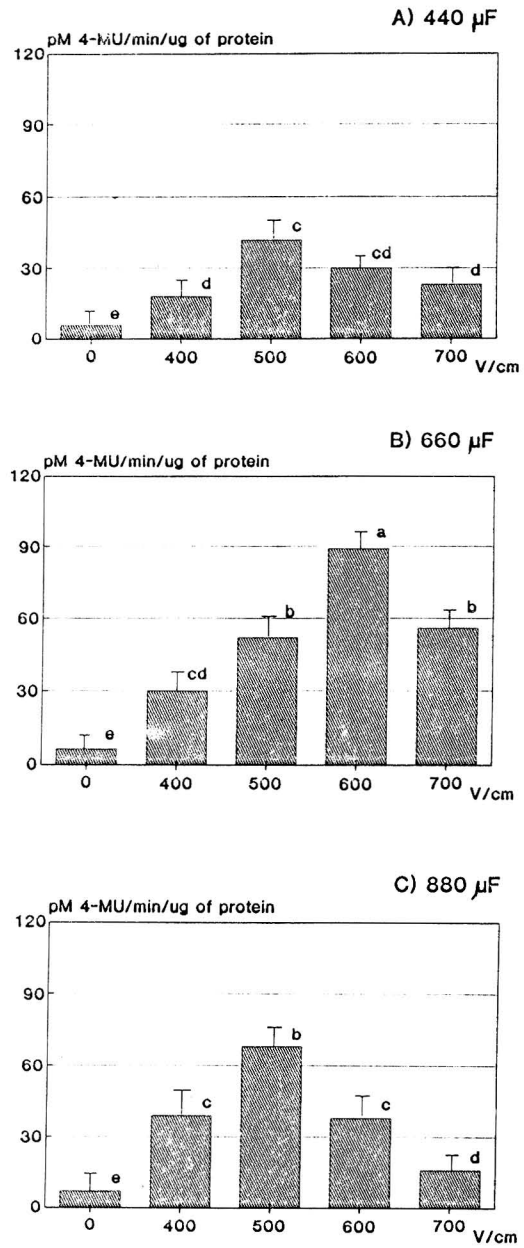

Fig. 2. Transient β-glucuronidase activity in protoplasts of POJ-2878 electroporated with 20 μg of plasmid pBI221.1 using various voltages and capacitances after 24 hours culture period. Each point is an average from at least 3 independent experiments.

that with both treatments the viability of the protoplasts after electroporation was similar, about 50-60%.

The same experiments were repeated with the sugarcane variety B 4362, but leaving out the capacitance of 440 μF since this gave unsatisfactory results in our previous experiments. As shown in figure 3, the best GUS expression levels were obtained at the same discharges as for variety POJ 2878.

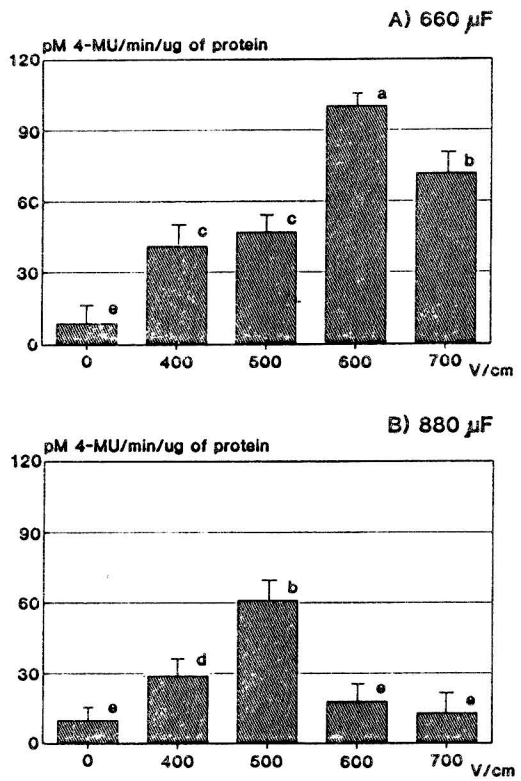

Fig. 3. Transient β-glucuronidase activity in protoplasts of B-4352 electroporated with 20 μg of plasmid pBI221.1 using various voltages and capacitances after 24 h culture period. Each point is an average from at least 3 independent experiments

Our results showed that sugarcane protoplasts can be transformed by electroporation and that there is no significant difference between the two varieties tested. The best transformation values were obtained using a combination of 600 V/cm and a capacitance of 660 μF.

b.- PEG- Treatment

Different final concentrations of PEG 6000 were tested (10, 14 and 20%). The experimental results showed that using the plasmid pBI221.1 in both varieties the transient GUS activity increased directly proportional to the tested PEG concentration, so at 20% of PEG in the transformation mixture the fluorescence values were seven times higher than in control treatments (figure 4).

Others concentrations of PEG in the mixture highers than 20% are toxic to protoplasts. (Data not shown)

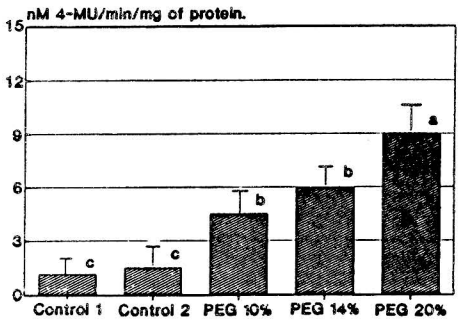

Fig. 4. Transient β-glucuronidase activity in protoplast of B-4362 var. incubated in the presence of polyethylene glycol at 10, 14 and 20% with pBI221.1. Each point is an average of 3 independent experiments.

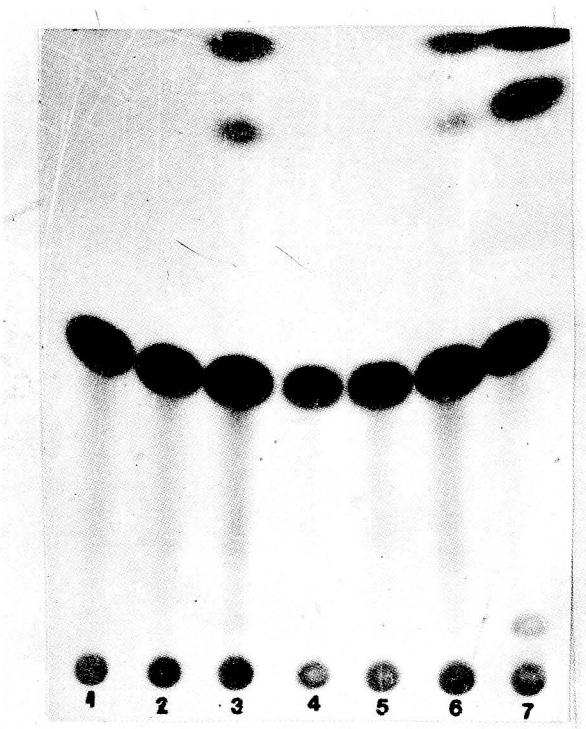

Fig. 5. Chloramphenicol acetyltransferase activity in electroporated and polyethylene glycol transformed protoplasts from B-4362 var. **Line 1**: Protoplasts without treatment; **Line 2**: Protoplast PEG-treated without plasmid DNA; **Line 3**: Protoplasts PEG-treated with 20 μg of plasmid DNA; **Line 4**: Protoplasts not electroporated and incubated with 20 μg of plasmid DNA; **Line 5**: Protoplasts electroporated at 600 V/cm and 660 μF without plasmid DNA; **Line 6**: Protoplast electroporated at 600 V/cm and 660 μF with plasmid DNA; **Line 7**: Bacterial standar equivalent to 10^7 cell/ml.

c.- Transient expression of CAT

In order to confirm our results, we performed a group of experiments at the optimized electroporation (600 V/cm and 660 μF) and PEG-treatment (20% PEG) conditions using the plasmid pCaMVICN. The CAT determinations clearly showed the validity of our previously obtained results (figure 5).

The electroporation and PEG-treatment are useful tools for the evaluation of gene regulation and construction proof by transient expression assay. These methods could be useful means for plant transformation, including sugarcane, when efficient approaches for the cell culture and plant regeneration are well established.

REFERENCES

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254

BOWER, R. and R. BIRCH (1990). Competence for gene tranfer by electroporation in a sub-population of protoplast from uniform carrot cell suspension culture. *Plant Cell Reports* **9**:386-389

CHEN, W.H.; M.A. GARTLAND; M.R. DAVEY; R. STOTAK; J.S. GARTLAND; B.J. MULLIGAN; J.B. POWER and E.C. COCKING (1987). Transformation of sugar cane protoplast by direct uptake of a selectable chimeric gene. *Plant Cell Reports* **6**:297-301.

DATTA, S.K.; A. PETERHANS; K. DATTA and I. POTRYKUS (1990). Genetically engineered fertile indica-rice recovered from protoplasts. *Bio/Technology* **8**:736-739.

FROMM, M.; L.P. TAYLOR and V. WALBOT (1985). Expression of genes transfered into monocot and dicot plant cells by electroporation. *Proc. Natl. Acad. Sci. USA* **82**:5824-5828.

FROMM, M.; L. P. TAYLOR and V. WALBOT (1986). Stable transformation of maize after gene transfer by electroporation. *Nature* **319**:791-793.

HAUPTMANN, R.M.; P. OZIAS-AKINS; V. VASIL; Z. TABAEIZADEH; S.G. ROGERS; R.B. HORSCH and I.K. VASIL (1987). Transient expression of electroporated DNA in monocotyledoneus species. *Plant Cell Reports* **6**:265-270.

HAUPTMANN, R.M.; V. VASIL; P. OZIAS-AKINS; Z. TABAEIZADEH; S. G. ROGER; R.T. FRALEY; R.B. HORSCH and I.K. VASIL (1988). Evaluation of selectable markers for obtaining stable transformants in the Gramineae. *Plant Physiol.* **86**:602-606.

JEFFERSON, R.A. (1987). Assaying chimeric genes in plants. The GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**:387-405.

KLEIN, T.M.; M. FROMM; A. WEISSINGER; D. TOMES; S. SCHAAF; M. SLETTEN and C.J. SANFORD (1988). Transfer of foreing genes into intact maize cells with high-velocity microprojectiles. *Proc. Natl. Acad. Sci. USA* **85**:4305-4309.

MANIATIS, T.; E.F. FRITSCH and J. SAMBROOK (1982). In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, USA.

MURASHIGE T. and F. SKOOG (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum* **15**:473-497.

NEGRITIU, I.; R. SHILLITO; I. POTRYKUS; G. BIASINI and F. SALA (1987). Hibrid genes in the analysis of transformation conditions. *Plant Molecular Biology* **8**:363-373.

OU-LEE, T.M.; R. TURGEON and R. WU (1986). Expression of a foreing gene linked to either a plant-virus or a Drosophila promoter, after electroporation of protoplast of rice, weat and sorghum. *Proc. Natl. Acad. Sci. USA* **83**:6915-6819.

PASZKOWKI, J.; R. SHILLITO; M. SAUL; V. MANDAK; T. HOHN; B. HOHN and I. POTRYKUS (1984). Direct gene transfer to plants. *The EMBO J.* **3**:2717-2722.

POTRYKUS, I.; M. SAUL; J. PETRUSKA; J. PASZKOSKY and R. SHILLITO (1985). Direct gene transfer to cells of a graminaceous monocot. *Mol. Gen. Genet.* **199**:183-188.

POWER, J.B. and J.V. CHAPMAN (1985). In: Dixon RA (ed) *Plant Cell Culture: a practical approach*, IRL Press Ltd, Oxford, England, pp 37-66.

UCHIMIYA, H.; T. FUCHIMI; H. HASHIMOTO; K. SYONO and Y. SUGAWARA (1986). Expression of a foreing gene in callus derived from DNA treated protoplast of rice. *Mol. Gen. Genet.* **204**:204-207.