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Somatic Hybrids by Electro-Protoplast Fusion between *N. tabacum* and *N. glutinosa*

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담배(*N. tabacum*)와 *N. glutinosa* 종간 원형질체 융합식물의 생성

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ABSTRACT

Protoplasts, isolated from leaf of *N. tabacum* NR^-/SR^+ and *N. glutinosa* were electrofused and divided with a plating efficiency of 30~35% in AAP1 9M medium. Green callus lines were selected in protoplast-derived colonies on $MSNO_3$ selection medium with 1.2mg/ml streptomycin sulfate on the basis of nitrate reductase proficiency and streptomycin resistance. Four putative hybrid plant lines regenerated from the green callus lines had intermediate morphology between that of parents with respect to floral shape, corolla length and ovate leaf blade. Zymograms of leaf peroxidase and esterase from these putative hybrid plant lines showed isozyme profiles derived from both parents and also, they exhibited additional and lost bands. Cytological analysis of two putative hybrid plant lines gave chromosome counts of 2n=66 in L22 and 2n=54 in L44 which were less than the expected number of *N. tabacum*(2n=48) and *N. glutinosa* (2n=24).

INTRODUCTION

Auxotrophic and drug resistant mutants have been useful for devising selection aimed at the recovery of a limited number of somatic hybrid colonies from the colonies derived from parental protoplasts or fused protoplasts. Mutants have been hybridized with other mutants to recover hybrids by genetic complementation(1, 2) or hybridized with species which have constraints of limited growth(3, 4). These methods can be only adapted to limited parental species which have markers of differential growth responses.

Hamill et al.(5) proposed an alternative approach which involves combining auxotrophic mutation and dominant resistant mutation in one species. Such construction of a double mutant of *Nicotiana tabacum* was reported which has nitrate reductase deficiency(NR^-) due to a recessive mutation at two loci in the nuclear genome, and streptomycin resistant(SR^+) due to a mutation in the chloroplast genome(5). It was hypothesised that a selection medium

containing nitrate as sole nitrogen source, and also containing streptomycin, would allow only recombinant genomes to grow as green colonies and thus be selected against a background of parental type colonies. We have previously reported the use of *Nicotiana tabacum* NR^-/SR^+, a double mutant for fusion studies with *Petunia inflata* and *P. hybrida* Monsanto(6, 7). In such intergeneric somatic hybridization the hybrid genome was unstable and limited gene transfer was proposed(6). As a test of this selection model of a double mutant, *N. tabacum* NR^-/SR^+ for interspecific hybridization, we attempted the somatic hybridization of *N. tabacum* NR^-/SR^+ with *N. glutinosa*. *N. glutinosa* has no selectable markers and its protoplast can be cultured under normal condition. These somatic hybrid plants is reported with using a multi-electrode chamber for protoplast electrofusion.

MATERIALS AND METHODS

Isolation, Culture and Fusion of Protoplasts

Protoplast isolation was carried out with the enzyme E0755(0.75% Cellulase 'Onozuka' R10, 0.05% Macerozyme R10, 13% mannitol) in the mesophyll cells of the fully expanded leaves in *Nicotiana tabacum* NR^-/SR^+ and *Nicotiana glutinosa*. After 16 hours of incubation protoplasts were collected and purified by centrifugation in CPW 13M and CPW 21S solution(8). For electrofusion the protoplasts of both species were washed twice in 11% mannitol with 2mM $CaCl_2$ $2H_2O$(pH 5.8) and a 1:1 mixture of the protoplasts, suspended at a density of 1×10^5/ml in the same washing solution, was pipetted into each well of a 25 well petri-dish. The multi-electro system had a 5mm distance between the parallel electrodes. Protoplasts were aligned in an AC field(100V/cm and 0.5 MHz) and subsequentely fused with DC pulse of 1 KV/cm for 2ms(9). Fusion-treated protoplasts were finally resuspended in AAP1 9M medium(MS medium modified by omitting inorganic nitrogen and by addition of 6 mM L-glutamine, 2mM L-aspartic acid, 1mM L-arginine and 0.1 mM glycine, 2mg/*l* NAA and 0.5mg/*l* BAP, 9% w/v mannitol)(10) so as to give a final density of 5×10^4/ml(10). Protoplasts were cultured at 27℃ with a continuous illumination of 2,000 lux(cool white fluorescent tubes).

Selection and Regeneration of Somatic Hybrid Plants

After 4 weeks protoplasts had divided to give small cell colonies which were subsequently transferred to $MSNO_3$ selection medium(MS medium modified by the addition of 38mM KNO_3 instead of the usual concentrations of NH_4NO_3 and KNO_3)(10) with 1.2mg/ml streptomycin. Whereupon colonies were maintained at a density of 1×10^3/ml. After 4 weeks green colonies which formed were transferred to the surface of the same selection medium solidified with 0.8%(w/v) agar. Selected green callus lines were transferred to regeneration medium, M/S medium with 3% sucrose, 1.0mg/*l* zeatin, 0.8% agar (pH 5.8) and regenerated shoots rooted on M/S medium lacking phytohormones.

Analysis of Regenerated Plants

Chromosome counts were performed by the pretreatment of excised roots in 0.05% colchicine at room temperature in the dark for 12~14 hours, fixation in acetic acid-alcohol(1:3) for 24 hours, and hydrolysis in 1N HCl at 60℃ for 10 min. These root tips were stained in Schiff's reagent(BDH) in the dark for 2~4 hours and squashed in 45% acetic acid.

For biochemical analysis, soluble protein extracts were prepared from leaf tissue. Tissue was ground in ice cold buffer which consisted of 50mM Trisma Base(pH 8.0), 100μM leucopeptin, 10mM $MgCl_2$. Electrophoresis of isozyme patterns were carried out in 9% polyacrylamide slab gel. Peroxidase isozymes were stained for in a solution of 50mM Trisma buffer pH 7.2, 200mM NaCl, 0.05% (w/v) 4-chloro-1-naphthol(sigma), 0.3%(w/v) H_2O_2 in 20% methanol. Esterase isozymes were analysed by the method of Smith(11).

RESULTS AND DISCUSSION

Response of the Protoplasts of *Nicotiana tabacum* NR^-/SR^+ and *N. glutinosa* on Selection medium

Protoplasts, isolated from leaf of double mutant plants of *N. tabacum* NR^-/SR^+ which has nitrate reductase deficiency(NR^-) and streptomycin resistant(SR^+), divided with a plating efficiency of 50~60% when cultured in amino acid medium, AAP1 9M medium(5, 12) containing

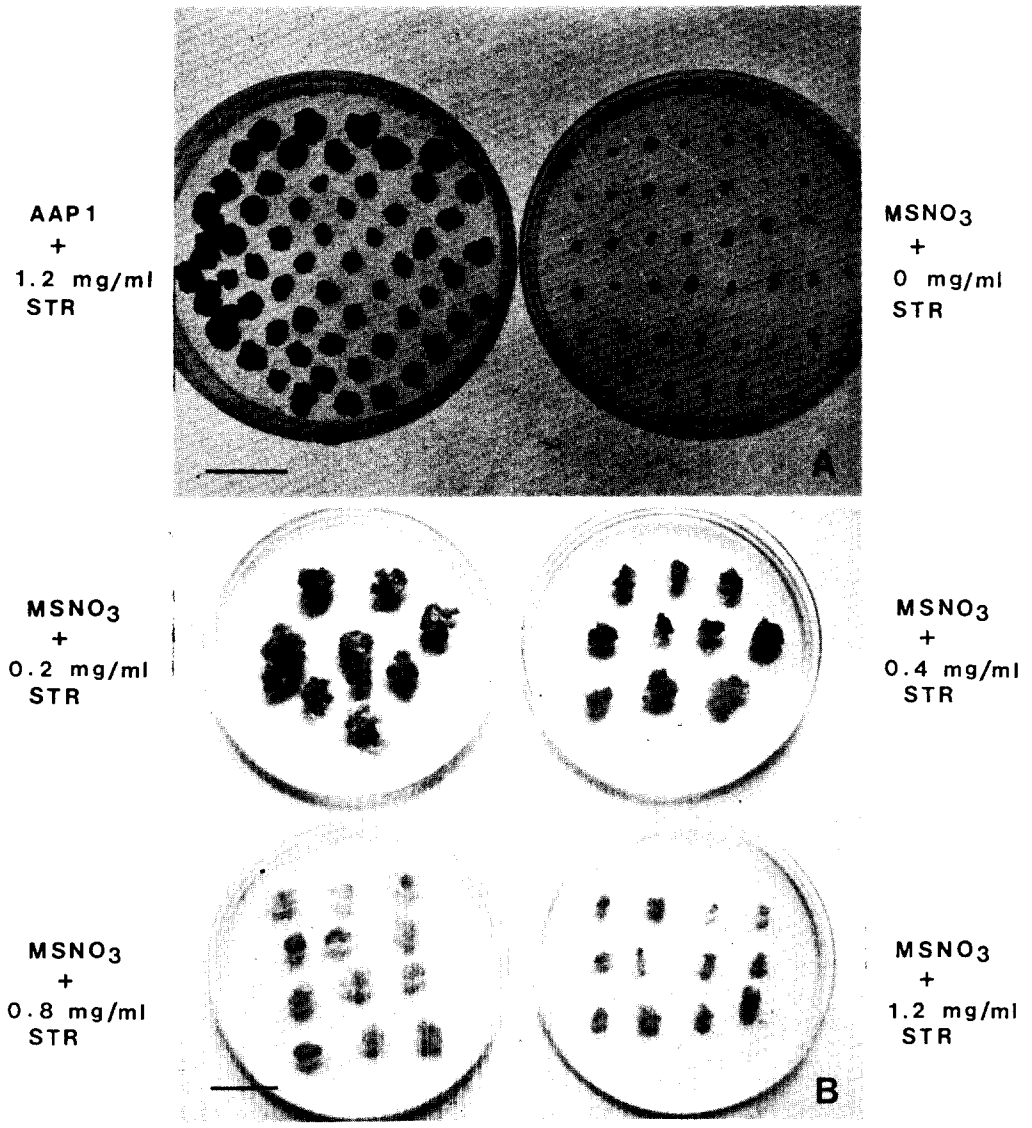

Fig. 1. Growth responses of the cells derived from the protoplasts of *N. tabacum* (A) and *N. glutinosa* (B) in AAP1 and $MSNO_3$ media including various concentrations of streptomycin sulfate(STR). All bars=2cm.

1.2mg / ml streptomycin sulfate and were capable of growth as colonies. However, double mutant cell colonies were not capable of growth in $MSNO_3$ medium because this mutant has nitrate reductase deficiency(8)(Fig. 1A).

The response of *N. glutinosa* colonies, derived from mesophyll protoplasts, was tested on $MSNO_3$ medium with and without streptomycin(Fig. 1B & Fig. 2). Cell colonies of *N. glutinosa* were capable of growth, but the rate of colony growth on $MSNO_3$ medium in which the only source of nitrogen was nitrate, was less on MS medium

considerably which contains the same hormone composition as $MSNO_3$ medium and ammonium nitrate as nitrogen source and also, cell colonies on $MSNO_3$ medium contanining streptomycin sulfate, 1.2mg / ml grew in yellow calli with incapability of chlorophyll synthesis. In this case as in Fig. 2, the protoplast derived colonies grew very slowly on $MSNO_3$ liquid medium with 1.2mg / ml of streptomycin and became brown after several weeks. It was suggested that fused cell between *N. tabacum* and *N. glutinosa* were grown in green calli, but colonies derived from *N. tabacum* were not dividing subsequently and colonies from *N. glutinosa* were cultured to limited growth of yellow callus.

After 2~3 days in culture, the majority of cells from the protoplasts of *N. tabacum* and *N. glutinosa*, and presumably heterokaryons divided in AAP1 medium. Fusion treated parental protoplasts of *N. tabacum* NR^-/SR^+ and *N. glutinosa* were estimated to give rise to cells with plating efficiencies of 30~35% on AAP1 medium(Fig. 3A & B).

Selection of Hybrid colonies

Colonies(20~40 cells), derived from fusion experiments involving leaf mesophyll protoplasts of *N. tabacum* and *N. glutinosa* were plated in selection medium composed of $MSNO_3$ with 1.2mg / ml streptomycin sulfate(Fig. 3C). After 4~6 weeks, green colonies were evident in those colonies derived from electrofused protoplasts, but not in their colonies from mixture control of non-fused protoplasts.

When the green colonies were cultured for a further period of time on $MSNO_3$ medium with 1.2mg / ml streptomycin sulfate, they grew as callus in Fig. 3D. Some of selected colonies show signs of organogenesis on MSZ medium containing 1.0mg / *l* zeatin and 0.8% agar. Four callus lines have undergone shoot regeneration and designed putative hybrid lines, L22, L31, L33 and L44(Fig. 4 A, B, C & D).

Confirmation and Analysis of Putative Somatic Hybrids

The floral morphology of all putative hybrid plant lines

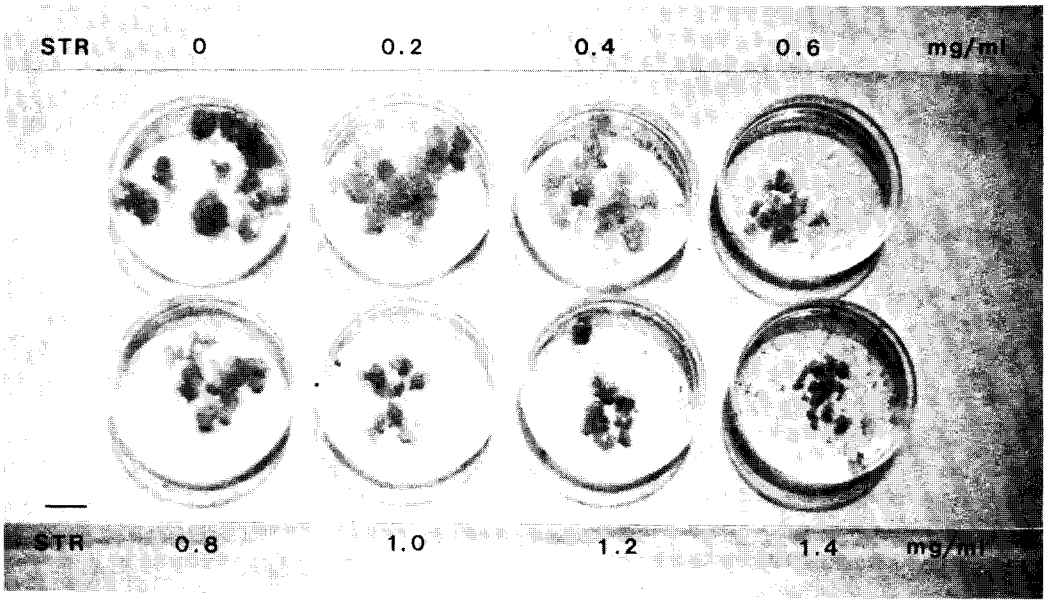

Fig. 2. Streptomycin responses of the protoplast-derived cells of ***N. glutinosa*** after 4 weeks of culture in liquid $MSNO_3$ 4.5M medium with various concentrations of streptomycin sulfate (STR). Bar=2cm.

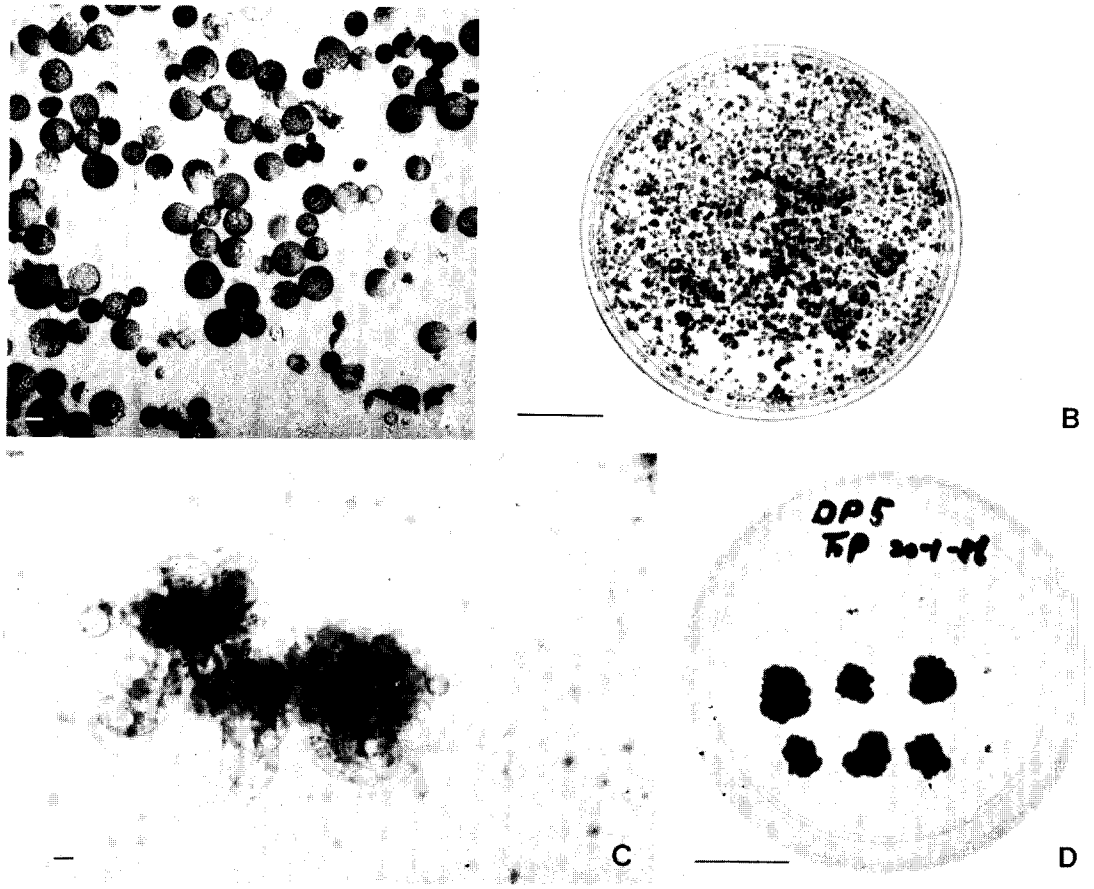

Fig. 3. Protoplasts and cell colonies in AAP1 and $MSNO_3$ media. A, mixed protoplasts of ***N. tabacum*** and ***N. glutinosa***. B, cell clusters in AAP1 medium from the electrofused protoplasts after 2 months of culture. C, cell colony. D, selected green callus lins in $MSNO_3$ medium. Bars=20μm in A & C, and Bars=2cm in B & D.

was intermediate between that of parents with respect to shape and corolla length, which are different for *N. tabacum* and *N. glutinosa*(Fig. 4 E & F). In leaf morphology these hybrid lines had ovate blade which was intermediate between that of parent, and also, they had long petiole and undulate margin which was similar as that of *N. glutinosa*. These results suggest the presence of both parental genomes in each putative somatic hybrid(6).

The banding patterns of leaf esterase and peroxidase were found to be different for *N. tabacum* and *N. glutinosa*. These differences can be used for the confirmation of putative hybrids since banding patterns can either be the sum of both parents, or could exhibit additional new bands. In analysis of leaf peroxidase, the putative hybrid plant lines(1.31 & 1.44, 1.22 & 1.33(not shown)) had both parental bands(Rf=0.27 in *N. tabacum*, Rf=0.39, 0.70 in *N. glutinosa* as in Fig. 5A). The banding patterns were not the same for two putative hybrid plant lines. 1.31 plant line lost three *N. tabacum* bands(Rf=0.47, 0.54, 0.87) and had one new band(Rf=0.05) and 1.44 line lost one *N. tabacum* band(Rf=0.87). In isozyme patterns of leaf esterase as in Fig. 5B, these hybrid lines

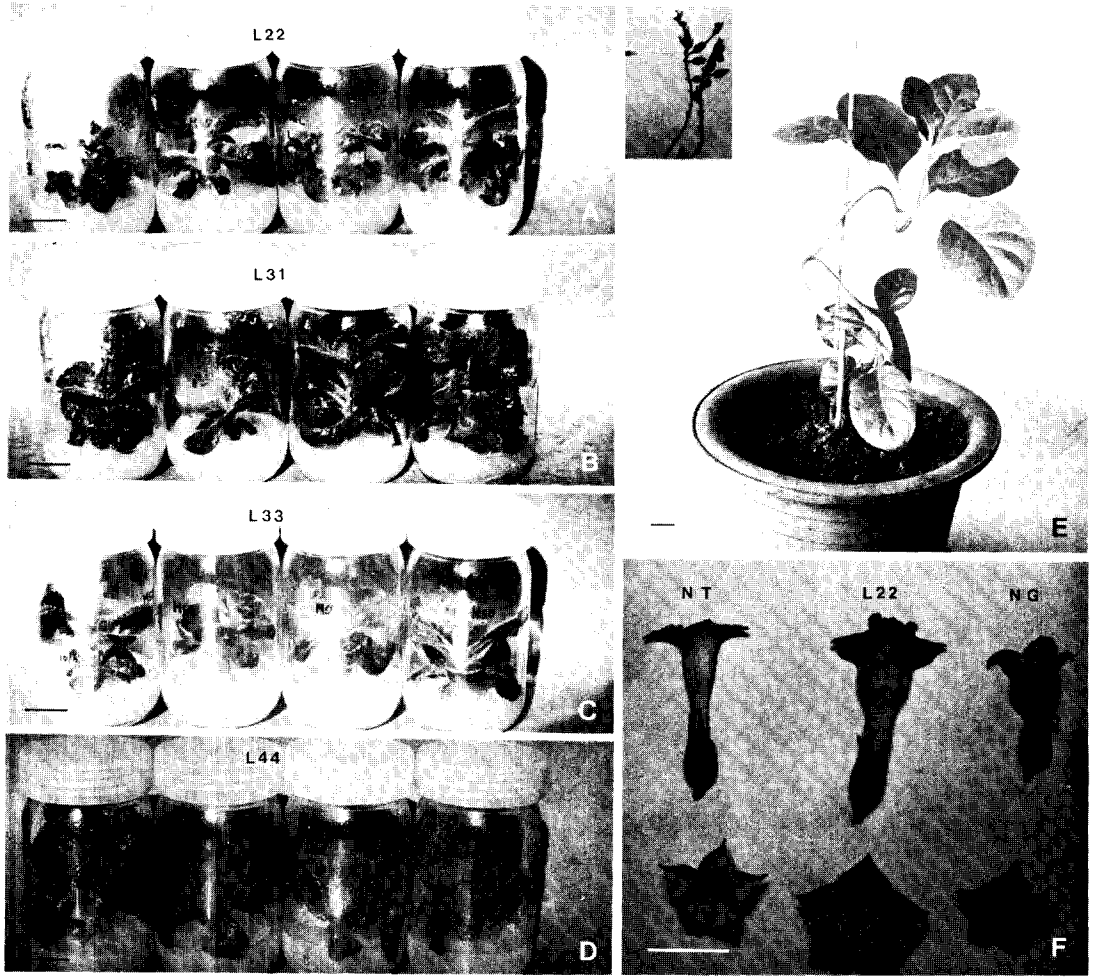

Fig. 4. Selected putative hybrid plant lines(A, B, C & D), leaf(E) and floral(F) morphology, and seed set(small area in E) of L22 hybrid plant between *N. tabacum* (NT) and *N. glutinosa* (NG). All bars=2㎝.

(L31 & L44, L22 &L33(not shown)) had both parental band, one band(Rf=0.76) in *N. tabacum*, two bands(Rf=0.78, 0.82) in *N. glutinosa*, and one additional band(Rf=0.63).

Zymograms obtained by polyacrylamide gel electrophoresis of peroxidase and esterase from these putative hybrid plant lines showed isozyme profiles derived from both parents and some variations in the number of bands may be related to loss of chromosomes during 8 month of culture(13, 14). The information gained from analysis of leaf esterase, leaf peroxidase and floral morphology from each of these putative somatic hybrid plant lines is regarded as providing sufficient evidence that these putative plant lines were somatic hybrids.

The hybrid nature of intergeneric somatic hybrids can occasionally so confirmed by the number and size of the chromosomes of both parents(15, 16). Cytological analysis of roots of two hybrid plant lines between *N. tabacum* NR^-/SR^1(2n=48) and *N. glutinosa*(2n=24) gave chromosome counts of 2n=66 in L22(Fig. 5C) and 2n=5

4 in L44. These results of lost chromosomes indicate that chromosomes of these hybrid plant lines were reconstructed in later cell divisions. Regardless of the mode of nuclear fusion, hybrid cells do not exhibit complete integration of the two parental sets of chromosomes for a number of cell generations an chromosome loss may br encountered

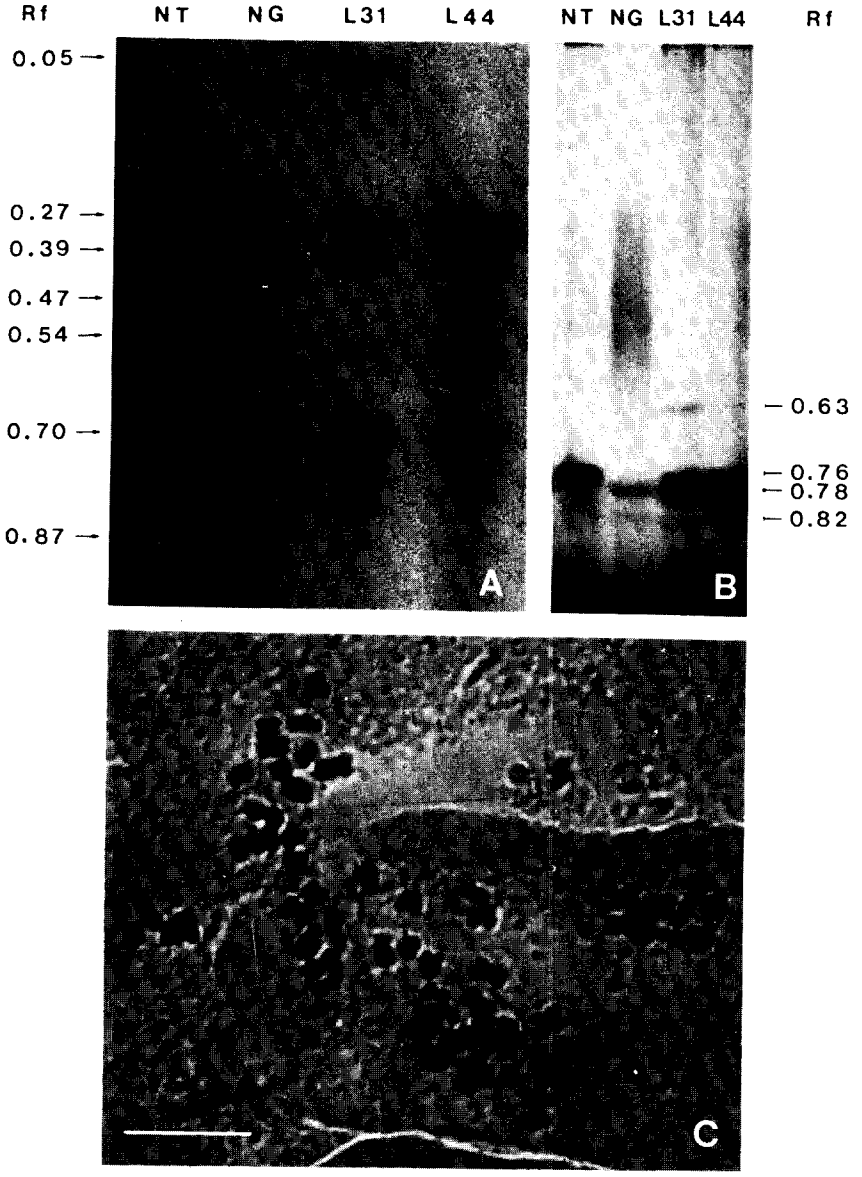

Fig. 5. Isozyme patterens of peroxidase(A) and esterase(B) of putative somatic hybrid lines(L31, L44) between *N. **tabacum***(NT) and *N. **glutinosa***(NG) with Rf value, and chromosomes(C), 2n=66 in L22 putative hybrid plant. Bar=10μm.

in long term of cell culture(16). While chromosome loss can be interpreted as incompatibility between distantly related species, it should be noted that even non-fused protoplasts may show chromosome aberrations in long term of cell culture(17). Growth of interspecific somatic hybrid plants obtained by means of protoplast fusion and aneuploid chromosome numbers of 2n=66 in L22 and 2n=54 in L44 may demonstrate that chromosome segregation affecting one parent does not interfere with the viability and may even be a desirable feature.

ACKNOWLEDGEMENT

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요 약

N. tabacum NR^-/SR^+과 *N. glutinosa*의 엽육세포로부터 원형질체를 분리하여 전기적으로 융합 및 배양하였으며 AAP1 9M 배지에서 이 원형질체의 plating efficiency는 30~35%였다. 분열 중인 소형 세포괴는 1.2mg/ml의 streptomycin이 포함된 $MSNO_3$ 배지에 치상하여 계속 분열을 유지하는 녹색 칼루스 계통을 선발하였다. 이 녹색 칼루스 계통에서 유래된 4계통의 식물들은 꽃의 형태, 화관의 길이와 엽신의 모양에서 양쪽 모식물체의 중간형을 보였고, 엽조직의 peroxidase와 esterase의 isozyme 분석에서도 양쪽 모식물체의 특성을 부분적으로 함께 나타냈으며 또한 결실되거나 새로운 isozyme band도 보였다. 이 융합체 추정계통들의 염색체 분석에서 L22계통에서 2n=66, L44계통에서 2n=54개가 조사되어 *N. tabacum*(2n=48)과 *N. glutinosa*(2n=24)의 융합체는 염색체의 부분적 감소현상을 보였다.

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