식물학회지 제36권 제1호 (1993)
Korean J. Bot. 36(1) : 105~112

Selection of Glyphosate-Resistant Clones from MNNG-treated Mesophyll Protoplasts of Haploid Tobacco Plants

Sung, Soon-Kee, Sang-Bong Choi and Kwang-Woong Lee
(Department of Biology, Seoul National University, Seoul)

반수체 담배의 엽육 원형질체로부터 MNNG 처리에 의한 Glyphosate 저항성 클론의 선별

成 淳 基·崔 相 烽·李 光 雄
(서울대학교 자연과학대학 생물학과)

ABSTRACT

Selection of glyphosate-resistant clones from MNNG-treated mesophyll protoplasts of haploid tobacco and their differentiation were studied. The protoplasts were treated with 0.1 to 100 μg/mL *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 30 min when they expanded to oval shapes. After the treatment, the protoplasts in 4-16 cell stages were transferred to the selective medium containing 1 mM glyphosate for the selection of the glyphosate-resistant colonies. The efficiency of the cell division of the protoplasts in the selective medium decreased as the MNNG concentrations increased. Optimal MNNG concentration for induction of the glyphosate-resistant clones was 10 μg/mL and mutation frequency was 2.66×10^{-6}. The stability of the glyphosate-resistance of the clones was examined by prolonged subculture in the medium with 1 mM glyphosate, and the resistant clones were survived more than 10 months. Among them one clone has been proliferating and greening and the others were proliferating without greening or greening with slower proliferating.

INTRODUCTION

Glyphosate (N-[phosphonomethyl]glycine) is a broad spectrum, nonselective herbicide which is highly effective, yet poses minimal environmental risk (Franz, 1985). The primary action mechanism of the glyphosate is a competitive inhibitor of 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) with respect to phosphoenolpyruvate (PEP) in the shikimate pathway in plants and microorganisms (Mousdale and Coggins, 1985; Hollander-Czytko and Amrhein, 1987), thus it prevents growth by blocking the production of aromatic amino acids in plants and bacteria (Boocock and Coggins, 1983).

Mutants resistant to glyphosate have been isolated in several bacteria and known to be the result of mutations at the *aro*A locus encoding EPSPS protein, which are not inhibited by glyphosate (Comai *et al.*, 1983; Kishore *et al.*, 1986). Recently, protoplast cultures from several species of Solanaceae have been successfully used in studies on mutagenesis and selection for various biochemical mutants (Grandbastien *et al.*, 1985). The haploid protoplast culture system in *Nicotiana tabacum* is an ideal target for mutagenesis. In addition to the one-step achievement of homozygosity, haploid is particulary advantageous in expressing phenotyps for recessive mutations, which can be easily lost in a heterogeneous diploid background (Hung, 1992). Also, most of the reported variants selected in mesophyll protoplast cultures have been regenerated into fertile plant (Bourgin, 1978; Bourgin *et al.*, 1979).

The selection of the glyphosate insensitive mutants is prerequisite to investigate the cellular and molecular me-

chanism of glyphosate insensitive EPSPS protein. It is required to monitor the screening conditions such as composition of the culture medium, the effect of mutagen, strength of the selection pressure, ploidy level and cell cycle stage of the population (Negrutiu, 1990). A number of plant cell culture lines were selected for their ability to grow in the presence of normally lethal concentrations of glyphosate (Nafziger *et al.*, 1984; Smart *et al.*, 1985; Steinrucken *et al.*, 1986; Dyer *et al.*, 1988). Any of these cell lines did not have glyphosate insensitive form of EPSPS, just representing elevated levels of EPSPS activity (Hollander-Czytko *et al.*, 1988; Goldsbrough *et al.*, 1990). These cell lines resulted from the glyphosate-adapted cells.

We established haploid tobacco plants via anther culture, and isolated mesophyll protoplasts from the haploid plants to investigate the effect of a chemical mutagen (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) on cell division and further development. In the present study, we induced glyphosate-resistant colonies from haploid mesophyll protoplasts of *Nicotiana tabacum* on a selective medium with a lethal concentration of glyphosate.

MATERIALS AND METHODS

Haploid plant induction. Immature flower buds were obtained from tobacco (*Nicotiana tabacum* L. cv. Xanthi) and incubated for 3-7 days at 4℃. The cold treated buds were surface sterilized in 70% ethanol for 10 s, 2% sodium hypochlorite solution for 10 min and washed five times with sterile distilled water. The surface-sterilized buds were dissected and anthers were removed without filament, and placed to induce androgenesis on Nitsch and Nitsch medium (1969) containing 20 g/L sucrose, 100 mg myo-inositol, 0.04 mg/L kinetin, 10 mg/L IAA, 0.5% charcoal, 0.9% agar, pH 5.7. The induced seedlings were replated on MS medium (Murashige and Skoog, 1962), and whole grown plants were transferred to pots.

Chromosome counting. Root tips were obtained from the regenerated plants and incubated on 1% monobromonaphthalene (v/v) solution for 2 h and fixed with ethanol : acetic acid = 3 : 1 (v/v) for 24 h at 4℃ and hydrolysed with 1 N HCl for 10 min at 60℃. Lysed root tips were stained with aceto-carmine and squashing, and the chromosome number was counted.

Protoplast isolation and culture. The fully expanded young leaves of haploid plant were surface-sterilized in 70% ethanol for 10 s, 0.5% sodium hypochlorite solution for 10 min and washed five times with sterile distilled water. After lower epidermis of the sterilized leaves was removed by peeling, the leaves were incubated in cell and protoplast washing (CPW) solution (Fearson *et al.*, 1973) containing 0.5% macerozyme R10, 1.5% cellulase R10 (Yakult Honsha) and 11% mannitol. After incubation in the washing solution on a low speed reciprocal shaker (30 rpm) for 6-7 h at 26℃, the incubation mixture was passed through 145 μm mesh. Protoplasts were collected by centrifugation at 100 *g* for 2 min, and pellets were run on a 21% sucrose gradient centrifugation at 500 *g* for 2 min. The intact protoplasts were washed three times by low speed centrifugaton in the CPW 11M solution (CPW salts, 11% mannitol). Washed protoplasts were resuspended in MS medium supplemented with 100 mg/L myo-inositol, 2 mg/L α-naphthaleneacetic acid (NAA), 0.5 mg/L 6-benzylaminopurine (BAP), 30 g/L sucrose, 9% mannitol (w/v), pH 5.7 (referred as MSP 9M). The protoplasts were cultured at a density of 5×10^4 protoplasts/mL in liquid MSP 9M medium, distributed in aliquots of 4 mL per Petri dish (diameter = 60 mm). Incubation was carried out in the dark at 26℃.

Observation of nuclear division. The liquid cultured protoplasts were removed at several time intervals in aliquots of 1 mL in eppendorf tubes and mixed with one drop of 4',6-diamidino-2-phenylindole (DAPI) stock solution (1 mg/mL in distilled water). After a few minutes, the protoplasts were observed by the phase contrast and fluorescence microscopy (Nikon Epi-Fluorescence) using Nikon UV-2 filter.

Mutagenesis procedure. When more than half of the protoplasts were expanded (about 36-48 h after culture), they were collected and incubated in filter sterilized 0.25 M $CaCl_2 \cdot 2H_2O$ (pH 5.7) containing 0.1 to 100 μg/mL of MNNG for 30 min in the dark at 26℃. After treated with MNNG, protoplasts were washed twice with CPW 9M solution (CPW salts, 9% mannitol) and cultured in the same protoplast culture conditon as descrived previously.

Growth measurements and determination of the selective level of glyphosate concentration. MSP medium (MSP 9M without mannitol) containing glyphosate (*N*-[phosphonomethyl]glycine mono-isopropylamine salt, Monsanto) in concentrations of 0, 0.15, 0.25, 0.5, 0.75, 1.0 and 1.5 mM were prepared. Fifty microcolonies of about 30 mg (fr wt) and the same number of callus about 300 mg (fr wt) were placed on the medium described above. After four weeks, the increment of fr wt for each treatment was determined and plotted against each glyphosate concentrations.

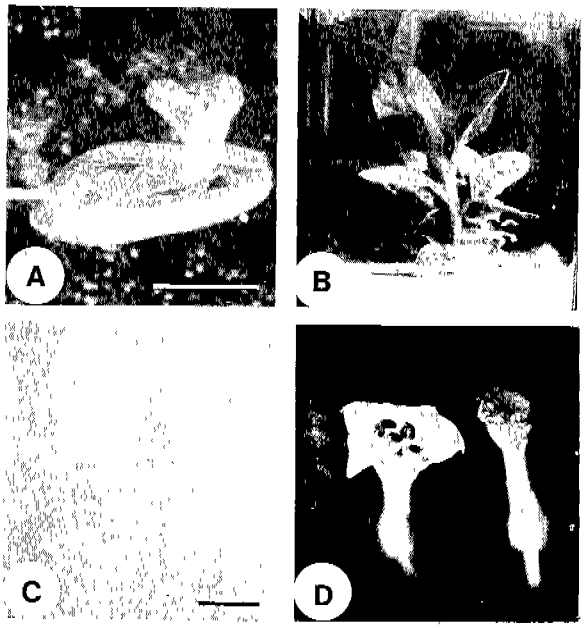

Fig. 1. Induction of haploid plants from anther culture of *N. tabacum* L. cv. Xanthi (2n=48). A, Excised anthers in 2-3 weeks after culture on Nitsch and Nitsch medium (1969) showed the microspore-derived embryo (bar=1.2 mm); B and C, Replating onto MS basal medium (1962) and the chromosome numbers (n=24) of regenerated plants (bar=10 μm); D, Flower and seeds of haploid tobacco plants induced from anther culture.

Selection of glyphosate-resistant clones. When approximately 50% of the protoplast-derived cells treated with MNNG had divided at least once, between 9 and 13 days after culture, they were collected by a low speed centrifugation at 100 *g*. Pelleted cells were resuspended with modified T7 medium (Grandbastien *et al.*, 1985) containing 1 mM glyphosate, 0.5 mM glutamine, 3 mM *N*-morpholinoethanesulfonic acid (pH 5.7) and 7% (w/v) mannitol, without Tween 80. The protoplast-derived cells were cultured at a density of approximately 1.5×10^4 divided cells/mL with 0.8% low gelling temperature agar. Incubation was carried out in tightly closed and transparent plastic boxes with 70% relative humidity under the light condition of 30 $\mu E\cdot m^{-2}\cdot s^{-1}$ and 16 h photoperiod at 26℃. Four weeks after culture, osmotic pressure of the culture medium was reduced by addition of 4 mL of the T7 liquid medium containing 4% mannitol and 1 mM glyphosate. Cells were cultured by the agarose-bead culture method (Paszkowski *et al.*, 1984) in the 30 $\mu E\cdot m^{-2}\cdot s^{-1}$ and 16 h photoperiod at 26℃. When putative resistant colonies reached a diameter of 1 mm, they were individually transferred onto solidified MSP medium containing 1 mM glyphosate, and subcultured at every 4 weeks.

RESULTS

Haploid plant induction and characterization. Haploid tobacco plants were induced via anther culture. About three weeks after culture, the anther sac opened and microspore-derived embryos came out (Fig. 1A). Explants induced from the embryos were transferred to hormone-free MS medium and grew into the whole plant (Fig. 1B). Chromosome numbers (n=24) were confirmed from the root tips of these explants (Fig. 1C) and the other well developed plants were transferred to pots. These plants showed self-incompatibility which is another feature of the haploid plant (Fig. 1D).

Observation of early protoplast development and nuclear division. Protoplast development was related to the senescence and growth condition of the mother plants. Morphological change of protoplasts according to culture time was not consistent in each expriment. However, there was a considerable relationship between morphological change of protoplast and its nuclear division. When nucleus was read to divide, the shape of protoplasts changed from spherical to oval, and the nucleus was surrounded by chloroplasts (Fig. 2).

Survival of protoplast-derived cells after MNNG treatment. In this experiment we used wide ranges of MNNG concentration (0.1, 10 and 100 μg/mL). At high concentration (100 μg/mL) treated, most of the protoplasts were killed (Fig. 3). From the results of this experiment, it was determined that the proper level of MNNG would be below 20 μg/mL. As the concentration of MNNG increased, cell division was decreased and its rate was delayed (Table 1), and there were no relationships between the mutation frequency and increasing of the MNNG concentration (Table 2).

Determination of the selective level of glyphosate concentration. To determine the glyphosate concentration of the selective medium for glyphosate-resistant clones, we examined the lethal concentration of glyphosate for microcolony and callus of wild tobacco cells. LD_{50} (the concentration of glyphosate at which the increment of the fr wt was reduced to 50% compared to control) was 0.15 mM for microcolony and 0.25 mM for callus (Fig. 4). Because too high selective level of glyphosate concentration may cause somatic variation during culture, and too

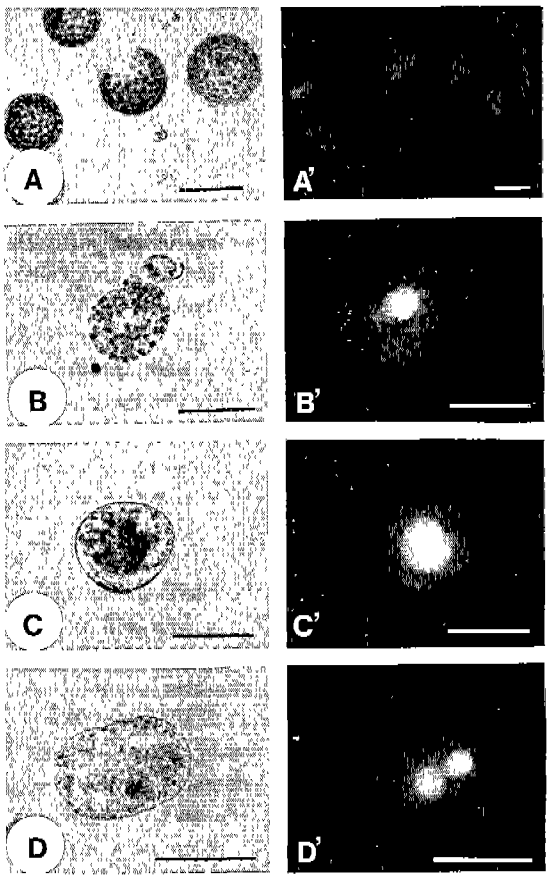

Fig. 2. Protoplast development in liquid culture. It was observed with phase-contrast microscope (left) and staining with 4',6-diamidino-2-phenyl-indole (right). A, Freshly isolated mesophyll protoplasts; B, 36-48 h after culture, loss of spherical shape, expanding, and migration of its chloroplasts to nucleus; C, 3 days after culture, immediately after nuclear division; D, 4-5 days after culture, immediately after cell division. All bars=40 μm.

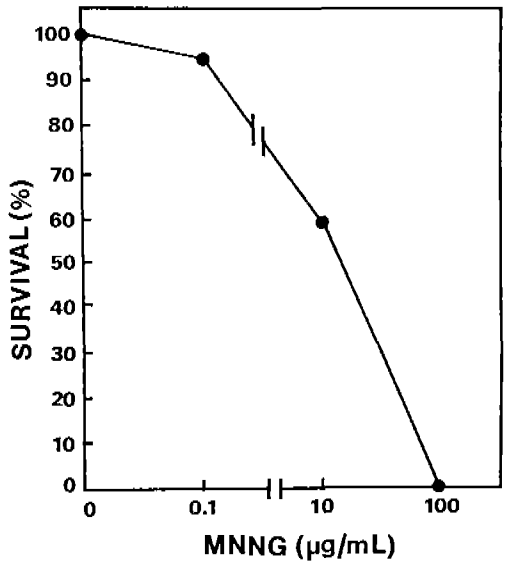

Fig. 3. Survival curve for protoplast-derived cells after MNNG treatment. Division efficiency was measured at 2 weeks after culture. Protoplasts were grown in MS medium (1962) supplemented with 2 mg/L NAA and 0.5 mg/L BAP.

Table 1. Effect of MNNG treatment on the survival of haploid tobacco mesophyll protoplast-derived cells

	MNNG treatment (μg/mL for 30 min at 26℃)						
	0	5	10	15	20	25	30
Culture time (d) after MNNG treatment	9	9	9	11	11	13	13
% of divided cells[a]	48.2	22.9	19.2	37.5	25	25	20
% of colony with							
2 cells	56.1	80	100	50	80	100	100
4 cells	25.0	20	—	50	20	—	—
8 cells	18.9	—	—	—	—	—	—

[a]No. of divided cells/No. of protoplasts MNNG-treated.

low selective level has no selection effects, the selective level was determined as 1 mM glyphosate.

Sclection of glyphosate-resistant colonies from MNNG-treated protoplasts. *N. tabacum* protoplasts were treated with several concentrations of MNNG when the protoplasts expanded (Fig. 5A). A decline in the division efficiency of the protoplasts was detected with increased concentration of MNNG (Fig. 3 and Table 1). After MNNG treament, glyphosate-resistant colonies were selected at 4-16 cell stages (Fig. 5B). Selection was done by their ability of greening and growing on the selective medium containing 1 mM glyphosate. After one week culture on the selective medium, most of the protoplast-derived cells were showed plasmolysis (Fig. 5C). Green colonies appeared after 2 month culture on the selective medium (Fig. 5D). The presumed resistant colonies were transferred onto solidified agar medium containing 1 mM glyphosate and subcultured every 4 weeks to preclude epigenic variants (Fig. 5E, 5F). The stablity of glyphosate-resistance was studied by a prolonged subculture on the

Table 2. Induced mutation frequencies[a] for glyphosate-resistance clones with increasing MNNG treatment

MNNG-treated (μg/mL)	No. of protoplasts	No. of resistant colonies	Resistant clonies per 1×10^6 protoplasts
0	2.5×10^6	0	0
5	1.75×10^6	0	0
10	2.63×10^6	7	2.66
15	2.63×10^6	2	0.76
20	1.75×10^6	0	0

[a]Protoplasts were incubated for 30 min with MNNG and allowed to multiply during 9-13 days until three division cycles had occurred. Cells were then plated for selection of 1 mM glyphosate, and resistant clones were counted after 3 months of culture.

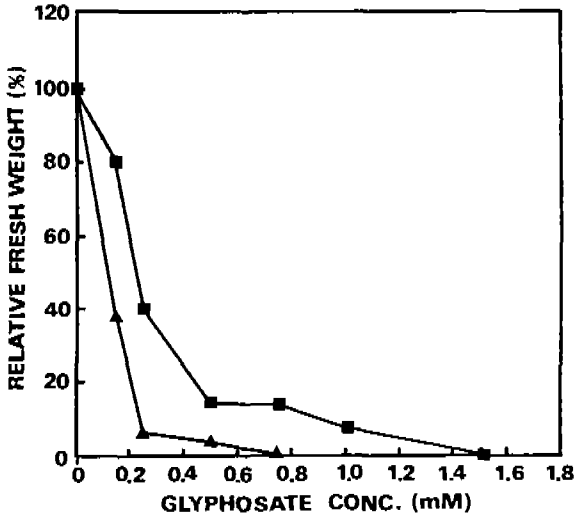

Fig. 4. The level of glyphosate tolerance from wild tobacco callus (■) and microcolony (▲). Cells were grown on the growth medium containing various glyphosate concentration. The relative increment of fresh weight of the cells were plotted as glyphosate concentration at 28 days after culture.

selective medium. All of these resistant colonies have been surviving more than 10 months. However, there were several patterns of callus growth in the resistant colonies with prolonged subculture (Fig. 6). One callus has been proliferating and greening among them, whereas the others proliferating without greening, and greening with slower proliferating.

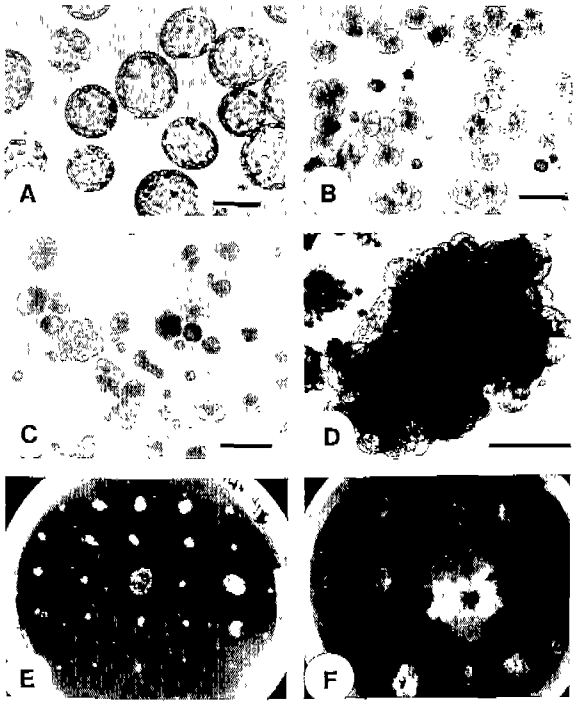

Fig. 5. Selection and culture of glyphosate-resistant clones. A, Protoplasts at 36-48 h after culture (bar=50 μm); B, Protoplast-derived cells after MNNG treatment and 9-13 days of incubation in culture medium (bar=150 μm); C, Protoplasts-derived cells on selective medium (1 mM glyphosate) at one week after culture (bar=180 μm); D, Yellow and green colony developed from a single protoplast-derived cell at 12 weeks after culture on the selective medium (bar=600 μm); E and F, The presumtive resistant colonies were transferred onto solidified agar medium containing 1 mM glyphosate, and subcultured every 4 week-intervals.

DISCUSSION

The control of several screening parameter such as expression time, optimal density at selection, and composition of selective medium has been achieved by simulation and reconstruction experiments of mutant selection (Bourgin *et al.*, 1980; Negrutiu and Muller, 1981). Unfortunately, there were no reports on the induction and selection of the glyphosate-resistant mutants using protoplasts. We examined the whole sequence of selection steps such as protoplast isolation, mutagen treatment and culture of the selected variants.

The nuclear division according to the changes of protoplast shape at early protoplast development was also examined. When the haploid mesophyll protoplasts were los-

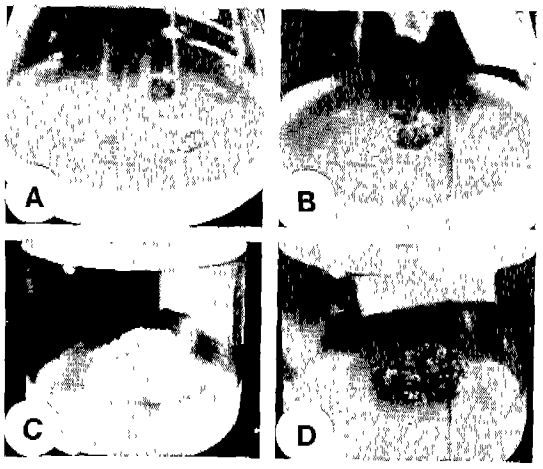

Fig. 6. Various glyphoaste-resistant clones from prolonged subculture on the selection medium after mutagenesis of haploid tobacco protoplasts. A, Calluses greening and proliferating; B, Calluses puffing without proliferating and greening; C, Calluses proliferating without greening; D, Calluses greening with slow proliferating.

ing of the spherical shape and expanding the size, and chloroplasts migrated to nucleus (about 36-48 h after culture), in phase contrast and DAPI staining observation, it showed that the protoplasts were prior to the nuclear division and assumed to be in S phase of cell cycle. MNNG is considered as a very potent mutagen and it acts primarily on replicating DNA (King, 1984). Thus we treated MNNG when the shape of protoplasts changed from spherical to oval, and the nucleus was surrounded by chloroplasts. It has already been mentioned that protoplasts were synchronized to a large extent in G_0 in certain species. In the case of *N. plumbaginifolia,* leaf protoplast have been reported to enter S and G_2 phase from 20 h after culture and maximum after 36 h (Magnien *et al.*, 1982). Similar conclusions can be drawn from data on tobacco protoplasts (Galbraith *et al.*, 1981).

Most experiments on mutagenesis showed increased frequency of variants with increased mutagen doses (Sung, 1976; Weber and Lark, 1980). High dose of mutagen, however, induce abnormalities and affect on the redifferentiation ability. Caboche and Muller (1980) have demonstrated that most protoplast-derived colonies showed reduced regeneration ability at increased dose of mutagen like nitrosoguanidine and gamma-irradiation. The treatment at high concentration of MNNG for a short time killed protoplasts more readily than treatment at low concentration for a long time in carrot suspension culture (Sung, 1976). However, the physiological condition between protoplast and cell suspension were different from each other, and the half life time of MNNG is 7.5 h at neutral pH (King, 1984). We fixed the treatment time at 30 min and varied the MNNG concentration. A wide range of concentrations were tested in the preliminary experiment. In our experiment, 10 μg/mL of MNNG was optimum for induction of the glyphosate-resistant clones. The stablity of glyphosate-resistance was examined by a prolonged subculture on the selective medium. All of these resistant colonies were survived more than 10 months. However, several patterns of callus growth were found when the resistant colonies were in prolonged subculture (Fig. 6). The growth patterns of the prolonged calluses on the selective medium showed that calluses were puffing without proliferating, greening with slower proliferating, and proliferating without greening.

Mesophyll protoplasts of tobacco provided a suitable material for the *in vitro* selection of various biochemical mutants (Carlson, 1973; Bourgin 1978). Although relatively high protoplast densitiy is required to allow colony formation (Nagata and Takebe, 1971), the treatment of mutagen and addition of selective agent have diminished survivability of the cells. To overcome this problem, appropriate defined media have been developed for the culture of protoplast-derived cells from tobacco (Caboche, 1980; Muller *et al.*, 1983), and the plating efficiency of the subcultured cells was independent on cell density at least from 1 cell/mL to 2×10^4 cells/mL. In the present study, we employed the low cell-density culture medium (Caboche, 1980; Grandbastien *et al.*, 1985) and applied agar-block culture method (Paszkowski *et al.*, 1984). To improve the contact between the cells and selective agent, the mutagenized protoplasts have been transferred to liquid medium containing glyphosate, and then molten with agar containing glyphosate. Osmotic pressure of the culture medium was reduced by addition of liquid medium containing half concentration of mannitol. It was observed that the colony formation was less efficient in liquid medium than in agar-block medium. It is believed that toxic substances released from dead cells diffused more easily in liquid medium than in agar-block medium.

Glyphosate-resistant calluses were inoculated on the regeneration medium with the addition of glyphosate. All of resistant calluses did not regenerate shoots in the presence of 1 mM glyphosate. It is our opinion that in addition to the choice of optimum mutagen doses for mutant screening, the occurrence, extent, and effect of secondary lesions on differentiation should be considered.

적 요

반수체 담배의 엽육 원형질체로부터 돌연변이 유발원의 일종인 *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)을 처리하여 glyphosate 저항성 클론을 선별하고 이들 클론의 분화를 확인하였다. 원형질체를 배양하여 이들이 타원형을 취하고 팽창하는 시기에 0.1-100 μg/mL의 MNNG를 30분간 처리하였다. 2-4회의 세포 분열 후 이들 원형질체 유래 세포를 1 mM glyphosate를 포함하는 선별배지에서 배양하였다. 0.1-100 μg/mL의 MNNG 처리구에서, MNNG 처리농도의 증가에 따라 원형질체의 세포분열능은 감소하였다. Glyphosate 저항성 클론의 유도에서, 최적의 MNNG 처리농도는 10 μg/mL 이였고 돌연변이 빈도는 2.66×10^{-6} 이였다. 선별된 클론들로부터 glyphosate 저항성의 안정성을 조사하기 위하여 이들 클론을 1 mM glyphosate를 포함하는 배지에서 장기간 배양하였다. 선별된 저항성 클론들은 10개월 이상 생존하였다. 캘러스 수준에서 이들의 생장 양상은 대부분 녹화가 수반되지 않는 분열만이 계속되거나, 녹화는 가능하나 분열능이 미약한 클론들이였고, 한 클론만이 정상적인 녹화와 분열능을 나타내었다.

ACKNOWLEDGMENTS

This work was supported by a grant from S.N.U. Daewoo Research Fund to K.-W. Lee (91-06-2063), and in part by a grant of Korea Science and Engineering Foundation through Research Center for Cell Differentiation to him (92-5-2).

REFERENCES

Boocock, M.R. and J.R. Coggins. 1983. Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. *FEBS Lett.* **154**: 127-133.

Bourgin, J.P. 1978. Valine-resistant plants from *in vitro* selected tobacco cells. *Mol. Gen. Genet.* **161**: 225-230.

Bourgin, J.P., Y. Chupeau and C. Missionier. 1979. Plant regeneration from mesophyll protoplasts of several *Nicotiana* species. *Physiol. Plant.* **45**: 288-292.

Bourgin, J.P., M.C. Hommel and C. Missionier. 1980. Expression of resistance to valine in protoplast-derived cells of tobacco mutants. *In*, Plant cell culture: results and perspectives: F. Sala, B. Parisi, R. Cella and O. Cifferi (eds.). Elsevier, Amsterdam, pp. 161-167.

Caboche, M. 1980. Nutritional requirements of protoplast-derived, haploid tobacco cells grown at low densities in liquid medium. *Planta* **149**: 7-18.

Caboche, M. and J.F. Muller. 1980. Use of a medium allowing low cell density growth for *in vitro* selection experiments; isolation of valine resistant clones from nitrosoguanidine mutagenized cells and gamma-irradiated tobacco plants. *In*, Plant cell culture: results and perspectives: F. Sala, B. Parisi, R. Cella and O. Cifferi (eds.). Elsevier, Amsterdam, pp. 133-138.

Carlson, P.S. 1973. The use of protoplasts for genetic research. *Proc. Natl. Acad. Sci. USA* **70**: 598-602.

Comai, L., L.C. Senand D.M. Stalker. 1983. An altered *aro*A gene product confers resistance to the herbicide glyphosate. *Science* **221**: 370-371.

Dyer, W.E., S.C. Weller, R.A. Bressan and K.M. Herrman. 1988. Glyphosate tolerance in tobacco (*Nicotiana tabacum* L.). *Plant Physiol.* **88**: 661-666.

Fearson, E.M., J.B. Power and E.C. Cocking. 1973. The isolation, culture and regeneration of petunia leaf protoplasts. *Dev. Biol.* **33**: 130-137.

Franz, J.E. 1985. Discovery, development and chemistry of glyphosate. *In*, The Herbicide Glyphosate: E. Grossman and D. Atkinson (eds.). Butterworths, London, pp. 3-17.

Galbraith, D.V., T.J. Mauch and B.A. Shields. 1981. Analysis of the initial states of plant protoplast development using 33258 Hoechst: reactivation of the cell cycle. *Physiol. Plant.* **51**: 380-386.

Goldsbrough, P.B., E.M. Hatch, B. Huang, W.G. Kosinski, W.E. Dyer, K.M. Herrmann and S.C. Weller. 1990. Gene amplification in glyphosate tolerant tobacco cells. *Plant Sci.* **72**: 53-62.

Grandbastien, M.A., J.P. Bourgin and M. Caboche. 1985. Valine-resistance, a potential marker in plant cell genetics. II. Optimization of UV mutagenesis and selection of valine-resistant colonies derived from tobacco mesophyll protoplasts. *Genetics* **109**: 409-425.

Hollander-Czytko, H. and N. Amrhein. 1987. 5-enolpyruvylshikimate-3-phosphate synthase, the target enzyme of the herbicide glyphosate, is synthesized as a precursor in a higher plant. *Plant Physiol.* **83**: 229-231.

Hollander-Czytko, H., D. Johanning, H.E. Meyer and N. Amrhein. 1988. Molecular basis for the overproduction of 5-enolpyruvylshikimate 3-phosphate synthase in a glyphosate-tolerant cell suspension culture of *Corydalis sempervirens*. *Plant Mol. Biol.* **11**: 215-220.

Hung, B. 1992. Genetic manipulation of microspores and microspore-derived embryos. *In Vitro Cell Dev. Biol.* **28**: 53-58.

King, P.J. 1984. Mutagenesis of cultured cells. *In*, Cell Culture and Somatic Cell Genetics of Plants: Vasil, I.K. (ed.). Academic Press, New York, pp. 547-552.

Kishore, G.M., L. Brundage, K. Kolk, S.R. Padgette, D. Rochester, Q.K. Huynh and G. della-Cioppa. 1986. Isolation, purification and characterization of a glyphosate tolerant mutant *E. coli* EPSP synthase. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **45**: 1506.

Magnien, E., X. Dalschaert and P. Faraoni-Sciamanna. 1982.

Transmission of a cytological heterogeneity from the leaf to the protoplast in culture. *Plant Sci. Lett.* **25**: 291-303.

Mousdale, D.M. and J.R. Coggins. 1985. Subcellular localization of the common shikimate pathway enzymes in *Pisum sativum* L. *Planta* **163**: 241-249.

Muller, J.F., C. Missionier and M. Caboche. 1983. Low density growth of cells derived from *Nicotiana* and *Petunia* protoplasts: influence of the source of protoplasts and comparison of the growth-promoting activity of various auxines. *Physiol Plant.* **57**: 35-41.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-497.

Nafziger, E.D., J.M. Widholm, H.C. Steinrucken and J.L. Killmer. 1984. Selection and characterization of a carrot cell line tolerant to glyphoste. *Plant Physiol.* **76**: 571-574.

Nagata, T. and I. Takebe. 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. *Planta* **99**: 12-20.

Negrutiu, I. 1990. *In vitro* mutagenesis. *In*, Plant Cell Line Selection: Philip, J.D. (ed.). VCH Publishers, Inc., New York, pp. 19-38.

Negrutiu, I. and J.F. Muller. 1981. Culture conditions of protoplast-derived cells of *Nicotiana sylvestris* for mutant selection. *Plant Cell Rep.* **1**: 14-17.

Nitsch, J.P. and C. Nitsch. 1969. Haploid plant from pollen grains. *Science* **163**: 85-87.

Paszkowski, J., R.D. Shillito, M. Saul, V. Mandak, B. Hohn and I. Potrykus. 1984. Direct gene transfer to plants. *EMBO J.* **3**: 2717-2722.

Smart, C.C., D. Johanning, G. Muller and N. Amrhein. 1985. Selective overproduction of 5-enolpyruvylshikimic acid 3-phosphate synthase in a plant cell culture which tolerate high dose of the herbicide glyphosate. *J. Biol. Chem.* **260**: 16338-16346.

Steinrucken, H.C., A. Schulz, N. Amrhein, C.A. Porter and R.T. Fraley. 1986. Overproduction of 5-enolpyruvylshikimate-3-phosphate synthase in a glyphosate tolerant *Petunia hybrida* cell line. *Arch. Biochem. Biophys.* **244**: 169-178.

Sung, Z.R. 1976. Mutagenesis of cultured plant cells. *Genetics.* **84**: 51-57.

Weber, G. and K.G. Lark. 1980. Quantiative measurement of the ability of different mutagens to induce an inherited change in phenotype to allow maltose utilization in suspension cultures of soybean. *Genetics* **96**: 213-222.

(Received February 22, 1993)