Induction, Selection and Characterization of Lysine-Resistant Mutants of Potato Using Protoplast Culture System

By SHIGERU KURODA

Department of Crop Breeding, Shikoku National Agricultural Experiment Staion
(Zentsuji, Kagawa, 765 Japan)

Introduction

Efficient and reproducible techniques to induce and select different biochemical mutants from plant cells would provide a valuable tool for plant sciences and breeding. Although an increasing number and wide spectrum of the mutants have been isolated in vitro cell culture systems of higher plants, much yet remains to be done in their routine selection, characterization and mutagenic treatment. Recent successful achievements in obtaining mutant plants derived from protoplasts have proved that the protoplast culture systems would be one of the effective tools in crop mutation and selection programs. The systems, being simple and efficient in terms of practical use, provide statistical interpretations because they involve an ability to produce a large size of homogeneous populations of haploid or diploid protoplast per mutagenic treatment and regenerate fertile plants from variants[2,5,6,8,9].

A study was undertaken to evaluate methodological advantages of the protoplast culture systems in inducing and selecting mutants. This paper presents mutagenic effects of the ultraviolet (UV)-irradiation on mesophyll protoplasts from a dihaploid line of potato, *Solanum tuberosum* L., efficiency of the selection of resistant protoplasts to the medium containing toxic concentration of L-lysine which is one of the important protein amino acids, and phenotypic expression at the plant level with regard to the trait under selection.

UV-irradiation treatment in protoplast culture

Taking into account the fact that UV-irradiation on tobacco protoplasts increases mutation rates and that mutagenesis is generally more efficient in case where haploid rather than diploid protoplasts are employed[9], a homozygous dihaploid clone (AH 84.4568)[4] of potato was chosen for the protoplast culture experiments to induce mutations. This clone was provided by Prof. G. Wenzel, Institute for Resistance Genetics, Federal Republic of Germany.

Mesophyll protoplasts were enzymatically isolated from the upper half of the shoot cultured in vitro, rinsed and plated on a V-KM medium[1] at a density of 10^5 protoplasts per m*l* in plastic petri dishes. Immediately after plating, with the purpose of mutagenic treatment, lids of the dishes were removed and protoplasts were exposed to UV light by a Heraeus 306 A lamp (major emission at 366 nm) at an incident dose rate of 16 μW/mm^2/sec during 2 to 30 min. To prevent photoreactivation, UV-treated protoplasts were kept in dark for at least 3 days after the UV-irradiation. UV dose effects were evaluated by counting number of the divided protoplasts, or survivals, after 2 weeks of the irradiation. Dose-response of the protoplasts to the UV-irradiation is indicated in

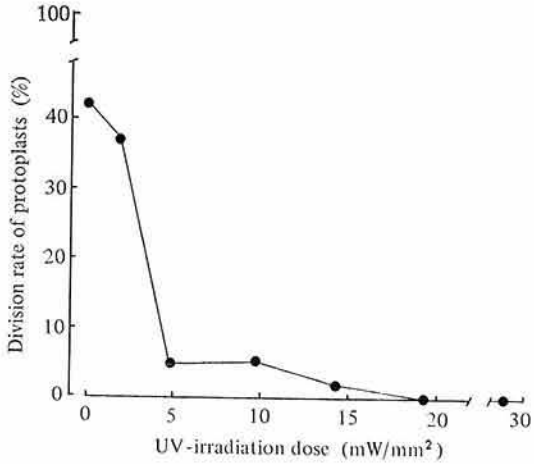

Fig. 1. Division rate of potato mesophyll protoplasts irradiated by several doses of UV light
Division rates were calculated on the basis of the divided protoplasts against the total number of protoplasts.
Each point in the figure corresponds to the mean value of the three replicates.

Fig. 1. The control protoplasts which were not exposed to the UV-light showed 42% of the division rate. In accordance with the increased dose of irradiation, the division rate of protoplasts decreased considerably. A statistical analysis of the data from the three replicates showed that the relationship between divisions of irradiated protoplasts and UV doses could nearly fit in a single exponential curve, and that the lethal dose was in the order of 19.2 mW/mm² (20 min). UV doses in 50% and 70% relative survival to the control were estimated at approximately 3.8 mW/mm² (4 min) and 2.9 mW/mm² (3 min), respectively.

Selection of lysine resistant cells from mutagenized protoplasts

To test the toxicity of lysine to protoplasts without UV-irradiation, protoplasts were inoculated into the V-KM medium containing concentration of 0.14 to 3.4 mM lysine and grown for 2 weeks. The division rate of protoplasts remarkably reduced 2 weeks later with an increasing lysine concentration. The range varied from 40% division in the control without lysine to 0% at 1.3 mM lysine. At the level of 0.54 mM of lysine, the division rate was 4%. The data indicate that lysine has a toxicity and 0.54 mM lysine inhibits the growth of approximately 90% of the protoplast population, as indicated in percentage of the control. These results have brought about two methodological problems in selecting lysine resistant cells: (1) in case where the surviving cells at 0.54 mM lysine were transferred 2 weeks later to the newly-made medium containing 0.54 mM lysine, the cells, being aggregated with dead protoplasts, generally turned brown and hardly continued to further grow, while the surviving cells that were replated on the non-lysine medium continued to grow; and (2) the surviving cells, though only a few, showing below 1% of division rate of protoplasts did not continue to grow even under the condition of non-addition of lysine to the medium. However, it was confirmed that these methodological problems could be dissolved by making a device so that the division rate of protoplasts was maintained at over 1% at a minimum, in other words, over 2.5% of relative survivals to the control under the treatment with both UV and lysine, and the surviving cells were replated on the nonselective medium after 2 weeks.

With the purpose of determining the optimum treatment for an induction and selection of lysine resistant protoplasts associated with UV exposure, the follow-up experiments were designed. Protoplasts were plated on the medium containing 0.54 mM lysine and irradiated by one of the several doses of UV light. Under the UV-irradiation of 2.9 mW/mm² for 3 min, the protoplasts irradiated showed 1 to 2% divisions in the three replicates, where the above device could be well contrived. This dose of the treatment was routinely applied to the follow-up experiment, accordingly.

With the purpose of testing regeneration abilities of the mutagenized protoplasts, three different conditions were given as follows: (1) treatment without both lysine and UV (control); (2) treatment with 0.54 mM lysine

alone (L); and (3) treatment with both 0.54 mM lysine and UV for 3 min (L+UV). The protoplasts subjected to these three conditions grew and formed calli after an approximately 2-month period. In order to test abilities of shoot regeneration from callus, 20 calli each were selected in two different manners: one was random selections in the control; and the other was selections in accordance with the size of calli in the other conditions, under which there existed a great variation of callus size. After an additional 2-month period, the calli from which plantlets were regenerated were 6 in number in the control, 4 in the L and 1 in the L+UV treatment. The plantlets from each callus were grown for propagation by in vitro shoot tip cultures with an MS medium[7] which contained 1% sucrose without lysine and growth hormones. This MS medium was used at an interval of 3 weeks for 6 months in order to remove remaining influences of the different conditions in the selection of each regenerated plant line. The plant lines which did not show any epigenetic or physiological changes were used for the testing at plant level, as described hereafter.

Phenotypic expression of the selected trait at plant level

Lysine-resistance of the regenerated plant lines under the in vitro selection conditions was evaluated with shoot tips (2 cm long) grown for 1 month on the MS medium containing 1 to 80 mM lysine (Table 1). Shoot explants both from the control line and the L line were unable to grow in the presence of 30 mM lysine with small differences among the intra- and inter-lines. No variation could be seen between these two lines and their parental strain in terms of the degree of resistance to lysine. Whereas, shoot explants from the L+UV line did grow under the same level of concentration of lysine, but not at the higher level of lysine concentration. This result indicates that the explants from the L+UV line had slightly higher resistance to lysine than the other lines and parental strain. The phenotypes of the resistant plants appeared normal, while those plants were a dihaploid ($2n = 2x = 24$). Some of the calli that were derived from the lysine resistant protoplasts selected under the L+UV conditions had much lower capabilities in differentiating into plants. This deteriolation manifested physiological disturbance in the mutant cells, which might have resulted in the lowering of differentiating abilities. Such a loss in potato mutant cells might have been caused by a similar mechanism which was found in asparagus cells in the course of selection for methionine and tryptophan analog resistance[3]. It was estimated that a population size of the protoplasts which had a capacity of cell division accounted to approxi-

Table 1. Lysine-resistance as expressed in terms of the concentration of lysine in shoot explants derived from potato mesophyll protoplasts treated with or without lysine (indicated by L ; 0.54 mM) and UV light (UV; 2.9 mW/mm², 366 nm)

Shoot explant	Lysine concentration (mM)									
	0	1	5	10	15	20	30	40	50	80
Parental strain	+++	+++	+++	+++	++	±	−	−	−	−
Plant lines derived from protoplasts										
Control	+++	+++	+++	+++	+	±	−	−	−	−
L	+++	+++	+++	+++	++	±	−	−	−	−
L+UV	+++	+++	+++	+++	++	+	±	−	−	−

Growth level of shoot explants: 5−7 cm (+++); 2−5 cm (++); 0.5−2 cm (+); less than 0.5 cm (±); death (−).

mately 6×10^4 per petri dish. Since this magnitude of population was subjected to the selection in the three replicates, the mutation frequency caused by UV-irradiation (2.9 mW/mm^2, 366 nm) was estimated at 1.8×10^{-5} per plated active protoplast at a minimum, though a spontaneous mutation rate could not be separated. It is therefore concluded that a UV-irradiation teratment would be effective as a mutagenesis in the protoplast culture system.

In the course of a series of this experiment, it was also found that lethal doses of lysine for a protoplast, a callus (data not shown) and a shoot tip in the control line were approximately 0.54, 2.0 and 30.0 mM, respectively. This result implies that a protoplast has a higher sensitivity to lysine in comparison with a callus and a shoot tip.

Characterization of the selected plant lines

To analyze amino acids of the selected lines, plants 5 to 8 cm tall in each line and the parental strain sampled from the agar medium were dried at −10°C using a freeze dryer and crushed. A part of the dry matter was hydrolyzed in 6N HCl under N_2 for 24 and 48 hr and dried, while the other part was extracted in 70% ethanol, followed by centrifugation at 150 g for 10 min and dried. The residues were then dissolved in lithium citrate buffer, and were subjected to the physiological fluid amino acid analysis with a Hitachi model 835 amino acid analyzer.

In the amino acid analysis of plant hydrolyzates (Table 2), the L+UV line, in comparison with the control line and parental strain, showed much higher concentrations of the asparatic acid, the glutamic acid and the total amino acid, while lower concentration of the glycine. The lysine level which the selection was based on, however, was slightly higher (5.5% more) in the L+UV line than in the control line. On the other hand, there was a great variation among the control, L and L+UV lines in amino acid concentrations in plant hydrolyzates, as indicated in Table 2. This variation was

Table 2. Amino acid contents of acid hydrolyzates in plant lines derived from potato mesophyll protoplasts treated with or without lysine (L ; 0.54 mM) and UV light (UV ; 2.9 mW/mm^2, 366 nm)

(n mol/mg dry wt)

Amino acid	Parental strain	Plant lines derived from protoplasts		
		Control	L	L+UV
Asp	313[1] (100)	377 (120)[2]	427 (136)	495 (158)
Thr	83 (100)	85 (102)	92 (111)	95 (114)
Ser	116 (100)	117 (101)	111 (95)	107 (92)
Glu	322 (100)	427 (133)	608 (189)	657 (204)
Gly	268 (100)	248 (93)	214 (80)	189 (71)
Ala	148 (100)	148 (100)	156 (105)	162 (109)
Val	113 (100)	115 (102)	120 (106)	128 (113)
Met	12 (100)	12 (100)	12 (100)	13 (108)
Ile	85 (100)	86 (101)	89 (105)	90 (106)
Leu	150 (100)	150 (100)	153 (102)	162 (108)
Tyr	51 (100)	51 (100)	55 (108)	53 (104)
Phe	75 (100)	75 (100)	77 (103)	81 (108)
Lys	108 (100)	109 (101)	114 (106)	115 (106)
His	48 (100)	48 (100)	50 (104)	57 (119)
Arg	152 (100)	155 (102)	149 (98)	174 (114)
Pro	9 (100)	9 (100)	9 (100)	10 (111)

1): Values represent means of amino acid contents in the samples hydrolyzed for 24 and 48 hr.
2): In parentheses: Percentages of the parental strain.

Table 3. Concentrations of free amino acids with characterestic variation in plant lines derived from potato mesophyll protoplasts treated with or without lysine (L) and UV light (UV)

(n mol/mg dry wt)

Free amino acid	Parental strain	Plant lines derived from protoplasts		
		Control	L	L+UV
Asp	10[1] (100)	11 (110)[2]	14 (140)	17 (170)
Glu	152 (100)	274 (163)	484 (318)	497 (327)
Asn (+Gln)[3]	306 (100)	387 (126)	449 (147)	545 (178)
Ser	30 (100)	34 (113)	27 (90)	19 (63)
Gly	110 (100)	106 (96)	65 (59)	20 (18)

1): Values represent means of the two replicates.
2): In parentheses: Percentages of the parental strain.
3): A peak of glutamine could not be fully separated from that of asparagine, though a very low level of glutamine was contained in each sample.

closely associated with concentrations of free amino acids, which are shown in Table 3, but not with variations in kind and amount of structural proteins that compose the potato tissues. Therefore, the L+UV line is a biochemical mutant that is characterized by not only a higher resistance to exogenous lysine, but also an increased level of certain free amino acids like asparagine and glutamic acid accompanied by a decreased level of free amino acids such as glycine and serine.

When shoot tips in the control line were grown on the MS medium containing both 30 mM lysine having an inhibitory effect of plant growth and 0.3 or 1.0 mM glycine, the growth of shoot tips was clearly accelerated. This result indicates that the glycine which can be metabolized from serine acts as a detoxication agent for exogenous lysine.

It was also found that the plants in the L+UV line had a significantly higher level of urea than those in the control line, and had cross-resistance to exogenous urea.

It is concluded that the increased frequency of resistant mutants derived from the UV treatment indicates an effective induction and selection of newly resistant protoplasts and that the protoplast culture system would be a good tool for mutagenesis and selection for biochemical mutants.

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