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High frequency of tetraploidy in *Agrobacterium*-mediated transformants regenerated from tuber discs of diploid potato lines

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Summary. Variations in the ploidy level of 69 transgenic potato (*Solanum tuberosum* L.) plants regenerated from the tuber discs of 17 diploid lines were studied: 24 plants (35%) were diploid, the other 45 plants (65%) were tetraploid. Seventy-eight control regenerants obtained without *Agrobacterium* inoculation showed a relatively low tendency to tetraploidization (35%). The results obtained suggested that chromosome doubling occurred frequently in diploid potato lines during the tissue culture process for regeneration. Putative somaclonal changes in *in vitro*-formed tuber proteins were detected in three out of six transformants by electrophoretic analysis.

Introduction

Genetic transformation offers a great potential to improve crops, especially clonal-propagated ones such as potato. Since potato (*Solanum tuberosum* L.) cultivars are tetraploid (2n=4x=48), breeding programs aiming at incorporating the valuable agronomic characters of diploid potato lines have been carried out. Tetraploid potato plants can thus be obtained via somatic cell fusion between selected diploid lines (Wenzel et al. 1979; Debnath and Wenzel 1987). Therefore, transformation of diploid potato lines is expected to be a valuable method for the breeding of improved potato varieties.

According to the results published in the literature, the appearance of tetraploidy in regenerated transformants derived from diploid potato lines varied from 15 to 100 % (de Vries-Uijtewaal et al. 1989; Visser et al. 1989a, b; van der Leij et al. 1991). Recent progress in tuber disc regeneration system has enabled to obtain direct regeneration with negligible callus formation, which may confer genetic stability during the tissue culture process for transformation (Sheerman and Bevan, 1988). However, this system has not been applied to diploid potato lines.

Before genetic manipulation studies are carried out, the frequency of undesirable somaclonal variation should be checked. This report describes a high frequency of tetraploidy in plants regenerated from tuber discs of diploid potato lines. Somaclonal variations in tuber proteins were studied by two-dimensional gel electrophoresis.

Materials and methods

Plant materials. Twenty-two diploid potato lines (Table 1a, b) were used. Tubers of 20 lines (P88-P123, P162) were obtained from the Potato Breeding Laboratory, Hokkaido National Agricultural Experiment Station, Hokkaido, Japan. Two lines (P86 and P87) were kindly provided by Dr S. Kuroda, Shikoku National Agricultural Experiment Station, Kagawa, Japan. *In vitro* shoot cultures were maintained and aseptic tubers were induced as described previously (Imai et al. 1992).

Transformation. Agrobacterium tumefaciens harboring a Ti-plasmid vector containing fusion genes (1): 35S promoter of cauliflower mosaic virus (P_{35S}) / neomycin phosphotransferase II (NPT II) coding gene (Ishige et al. 1991); or (2): P_{35S} / hygromycin phosphotransferase (HPT) coding gene (kindly provided by Dr A. Kato, Hokkaido National Agricultural Experiment Station) were used for transformation. *Agrobacterium* was inoculated on discs of *in vitro*-formed tubers and transformants were selected as described previously (Ishige et al. 1991). Transformants were checked twice for their ability to form roots from the cut surface into a medium containing kanamycin 100 mg l^{-1} or hygromycin 10 mg l^{-1} (Ishige et al. 1991). Integration of the gene coding for antibiotic resistance was confirmed by Southern blot analysis using the coding region probes of NPT II or HPT genes. To avoid the decrease in the regeneration ability, only young tubers (within 5 weeks after harvest) were used. Transformant clones were randomly chosen for ploidy analysis using one, two or three clones for each combination of original diploid line / antibiotic resistance marker. Non-transgenic plants regenerated from tuber discs (Table 1b) were used as a control.

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Table 1. Variations in ploidy level of potato plants regenerated from tuber discs of diploid lines.
a) Transformants obtained after co-cultivation with *Agrobacterium tumefaciens.*

Line	Identity	Kanamycin selection					Hygromycin selection				
		Shoot[a]	Transformant				Shoot[a]	Transformant			
			Obtained[b]	Tested[c]	2x	4x		Obtained[b]	Tested[c]	2x	4x
P 86	AH84.4568/6[d]	5	3	2	0	2	13	7	3	1	2
P 87	H75.1207/7[d]	5	1	1	1	0	2	1	0		
P 88	W624209-H10	0					0				
P 89	W702206-1	22	2	0			13	6	3	1	2
P 90	W774212-H7	6	4	3	1	2	6	4	4	1	3
P 91	W672211-1	0					0				
P 92	W774213-H1	8	4	3	1	2	2	1	1	0	1
P 93	WB792202-1	13	1	0			7	2	2	1	1
P 94	W662249-1	4	0				0				
P 95	W794215-H36	0					3	0			
P113	W822226-3	73	6	3	1	2	36	6	2	1	1
P114	W822227-1	27	2	2	2	0	12	4	3	3	0
P115	W822228-5	35	15	3	2	1	9	3	3	1	2
P116	W822229-1	40	10	3	2	1	1	0			
P117	W822229-5	14	3	3	1	2	1	1	1	1	0
P118	W822230-1	15	2	2	1	1	1	0			
P119	W822296-8	76	9	2	0	2	18	6	3	0	3
P120	W832236-1	3	1	1	0	1	0				
P121	W832222-6	13	5	3	0	3	4	1	1	1	0
P122	W832222-7	13	7	3	1	2	20	5	3	0	3
P123	W832222-8	14	8	3	0	3	8	6	3	0	3
	Total	386	83	37	13	24	156	53	32	11	21

[a] Total number of regenerated shoots.
[b] Total number of transformants obtained judging from root-forming ability into a medium containing either antibiotic.
[c] Total number of transformants analyzed for ploidy level.
[d] Cited from Debnath et al. (1986).

Table 1. b) Control regenerants without co-cultivation with *A. tumefaciens.*

Line	Tested[e]	2x	4x
P 90	13	7	6
P113	19	14	5
P115	12	9	3
P118	19	10	9
P162	15	11	4
Total	78	51	27

[e] Total number of non-transformed control shoots analyzed for ploidy level.

Determination of ploidy level. The ploidy level was determined by the number of chloroplasts in the stomatal guard cells of *in vitro* - grown plants as described by Mattheij et al. (1992) without alcohol treatment. The number of chloroplasts in 15 stomatal guard cells of at least two independent leaves per plant was counted. To confirm the tetraploidy, chromosomes in the root tip cells were counted for the clones with a large number (14-16) of chloroplasts.

Analysis of tuber protein and DNA. Electrophoretic patterns of *in vitro*- formed tuber proteins were compared between the original diploid (P90) and six clones of its transformant (Ag 637, 713, 715, 751, 752 and 753). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out as described previously (Imai et al. 1992).

DNA was extracted from *in vitro*- grown plantlets of eight transformants (Ag637, 713, 714, 715, 731, 732, 751 and 752) and their original diploids (P90 and P118). About two grams of stems and leaves were homogenized with a buffer (5 ml / g initial wt.) containing 0.1M Tris-HCl (pH 8.0), 0.05M Na_2EDTA, 0.5M NaCl, 0.01M 2-mercaptoethanol. The homogenate was degraded by adding 1.2% (final conc.) SDS and following incubation at 70 °C for 15 min. Thereafter 5M potassium acetate (1.8 ml / g initial wt.) was added to the mixture and kept in wet ice for 20 min. Cell debris were removed by centrifugation at 26000 *g* for 15 min. Crude DNA was separated by the addition of equal volumes of 2-propanol and collected by coiling round a bamboo stick. The crude DNA was digested with proteinase and RNase for 2 h at 37 °C and further purified as described previously (Ishige et al. 1991).

Results and discussion

The relation between the ploidy level and the chloroplast number in stomatal guard cells was confirmed by counting the root tip cell chromosomes using several diploid lines and tetraploid cultivars (data not shown). Based on these data, the ploidy of the plants was determined from the chloroplast number as follows: 6-8 for diploid, and 10-15 for tetraploid. These results were consistent with those reported in greenhouse-grown plants (Mattheij et al. 1992). The twenty-two lines used in this study (Table 1a, b) were all confirmed to be diploid by chloroplast counts (not shown).

Regeneration frequency of transformants and their ploidy levels are summarized in Table 1a. The observation of regenerated plants based on their root-forming ability into a medium containing either antibiotic was effective to confirm the integration of the gene coding for antibiotic resistance. All the plants selected by this method harbored the coding gene based on Southern blot analysis (Fig. 1). The antibiotic-resistant phenotype was expressed stably over a period of one year of subculture.

Overall ratio of 2x : 4x transformants was about 1 : 2 (Table 1a), irrespective of the antibiotics used for selection (kanamycin or hygromycin). No transformants above the hexaploid level were detected. The ploidy level of the 78 control regenerants obtained without co-cultivation with *Agrobacterium* showed a relatively low tendency to tetraploidization: 51 diploid plants (65%) and 27 tetraploid plants (35%) (Table 1b).

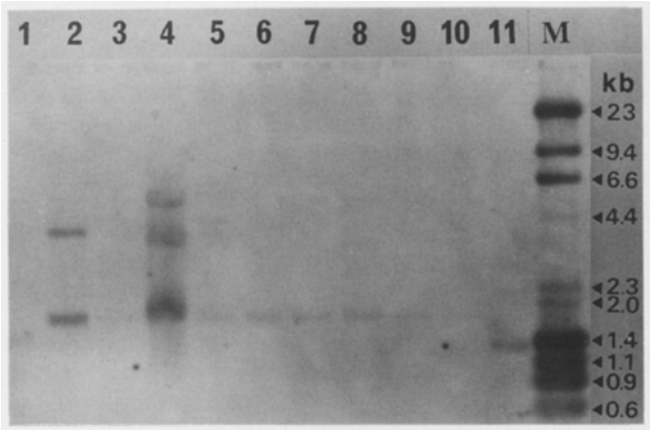

Fig. 1. Genomic Southern analysis of kanamycin-resistant transformants. Five micrograms of *Bam* H I-digested total DNA was separated on a 0.7% agarose gel and blotted onto a nylon membrane. The blot was probed with a digoxigenin-labeled NPT II coding gene fragment and detected by chemiluminescent method. Lane 1: non-transformed original line P90 (2x) (negative control). Lanes 2-10: transformants obtained from tuber discs. The ploidy level and the original diploid line of the transformants are indicated in parentheses. Lane 2: Ag714 (2x/P90); lane 3: Ag713 (4x/P90); lane 4: Ag715 (4x/P90); lane 5: Ag776 (4x/P92); lane 6: Ag739 (2x/P122); lane 7: Ag717 (2x/P113); lane 8: Ag721 (2x/P114); lane 9: Ag725 (2x/P116); lane 10: Ag731 (2x/P118). Lane 11: NPT II gene fragment (positive control). Lane M: digoxigenin-labeled markers. The approximate sizes of the markers are indicated on the right-hand side.

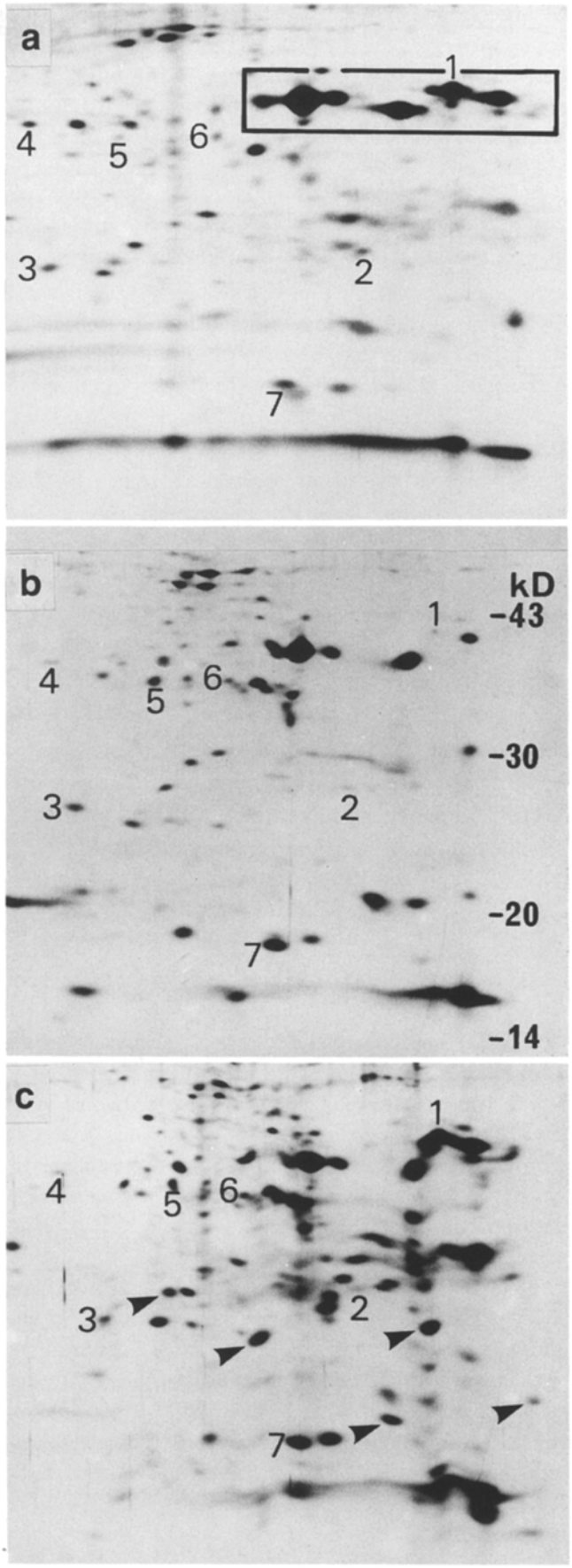

Fig. 2. (In the right column) Two-dimensional electrophoretic patterns of microtuber proteins. Anode is on the right-hand side (isoelectric focusing) and the bottom side (SDS-PAGE). (a): Original diploid line P90, two transformed lines (b): Ag752 (4x) and (c): Ag637 (2x). The numbers indicate seven representative spots of those commonly found in (a), (b) and (c). Polypeptide spots of patatin, a major potato tuber protein, are boxed in (a). The arrowheads indicate additional spots found in Ag637.

According to the results published in the literature, the appearance of tetraploidy in several transformed root clones derived from diploid or monoploid potato lines or in regenerated transgenic plants varied from 15 to 100 % (de Vries-Uijtewaal et al. 1988, 1989; Visser et al. 1989a, b; van der Leij et al. 1991). In these studies, the regeneration systems were different from those used in our study: (1) transformation by *Agrobacteria* carrying an Ri-plasmid and (2) regenerating from stem tissue. The results of our experiments in which we used a relatively wide range of diploid lines also indicate a tetraploidization tendency in the plants regenerated from tuber discs. In addition, chromosome doubling has often been observed in plants regenerated from protoplasts of diploid potato lines. (Debnath and Wenzel 1987; Waara et al. 1991). Based on these data, in the plants regenerated from *in vitro* cultures of diploid lines, it appears that chromosome doubling occurs frequently during the tissue culture process for regeneration (cell proliferation and following differentiation), in contrast to the transformants derived from tetraploid potato cultivars, which remained mostly tetraploid (Hänisch ten Cate et al. 1988; Stiekema et al. 1988; De Block 1988). In conclusion, the ploidy level of the transformants derived from diploid potato lines is important since diploid transformants with appropriate markers for selection can be used as source material for somatic cell fusion, unlike tetraploid transformants.

Putative somaclonal changes in tuber proteins were observed in three (Ag637, 752 and 753) out of six transgenic plants by 2D-PAGE. The spots of patatin, a major potato tuber storage protein, near spot 1 were faint in Ag752 (4x) (Fig. 2b). Identical changes were observed in Ag753 (4x) (not shown). The other spots showed the same intensity as that of the original diploid line i.e. P90 (cf. Fig. 2a). In the case of Ag637 (2x), five additional spots were observed (Fig. 2c, indicated by arrowheads). Since *in vitro* tubers were induced after ten or more subcultures, it is assumed that the changes in the 2D-PAGE patterns may not be transient and further analyses should be carried out to confirm the stability of the changes.

Electrophoretic patterns of Ag713 (4x), Ag715 (4x) and Ag751 (4x) were similar to that of the original diploid line P90 (not shown), regardless of chromosome doubling.

Southern hybridization analysis using a probe of the patatin promoter region (Aida et al. 1991) did not reveal any variations among eight transformants (data not shown) including the case of Ag752 which exhibited an altered 2D-PAGE pattern of patatin polypeptides. Further investigations should be carried out to analyze the variations in the patatin genes.

Evaluation of transgenic potato plants has been carried out mainly in relation to the expression of introduced foreign genes or phenotypic analysis of greenhouse-grown plants (de Vries-Uijtewaal et al. 1989; Visser et al. 1989a; Ishige et al. 1991; van der Leij et al. 1991). Recently, Dale and McPartlan (1992) have reported on the field performance of transgenic potato plants obtained from *Agrobacterium*- inoculated tuber discs compared with two control groups. As a result, the values for the agronomic characters of three groups were in the order of (1): shoot cuttings, (2): non-transgenic plants regenerated from tuber discs and then (3): transgenic plants. The results suggested that two factors affected plant performance: variation induced during the tissue culture process and foreign DNA insertion. By applying the methods described in this study, it may be possible to select transgenic potato plants with valuable agronomic characters and few somaclonal changes before field evaluation in breeding programs.

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