Polyploidization in leaf callus tissue and in regenerated plants of dihaploid potato

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Abstract. Cytological studies on leaf callus cells and regenerated potato plants suggest that it may be possible to utilize somatic chromosome doubling to obtain tetraploids from outstanding dihaploid breeding clones. The ploidy levels found in callus-derived plants were diploid, tetraploid, and octaploid, but the proportion of these was dependent on the donor genotype. L_1 and L_3 germ layers were studied in more than 300 plants; periclinal ploidy chimerism, an undesirable feature of colchicine doubling, was not found.

Leaf callus was more efficiently induced using NAA than 2, 4-D as an auxin source in the Murashige and Skoog medium. A high proportion of dividing cells in young calli were polyploid. The frequency of doubled and octaploid plants regenerated was significantly dependent on donor genotype. The extent of polyploidization was marginally higher after callus growth on a medium containing 2, 4-D than in a medium containing NAA. In some genotypes the chromosome numbers of regenerated plants were variable, being less than tetraploid (mixohypotetraploid). After tuber propagation, the original ploidy level was maintained although mixohypotetraploidy persisted.

In a few somatically doubled clones, male fertility was tested and found to be satisfactory with respect to seed-setting.

Introduction

Cytological studies on callus cultures and callus-derived plants have been reported for tobacco [9], barley [5, 7], *Lycopersicon peruvianum* [15] and dihaploid potato (*Solanum tuberosum*) [6, 13, 14]. The ploidy levels of regenerated tobacco [9], barley [7], and *L. peruvianum* [15] plants suggested the occurrence of spontaneous chromosome doubling. In the conventional breeding of tetraploid potato varieties via dihaploids, an effective method of doubling dihaploid breeding clones is desirable. At present, selected argonomic dihaploid clones are doubled by colchicine treatment according to the procedure described by Ross et al. [10]. Perclinal chimerism and genotypic differences in response to doubling are the main disadvantages of this method.

Chromosome doubling, which occurs spontaneously in tissue culture, could be exploited. This investigation was prompted by the fact that,

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although this in vitro form of doubling appears to be promising [6], there has been no intensive karyological study on calli originating from the young leaf tissue or on regenerated plants of dihaploid potato. The effects of several factors on doubling rate were also investigated.

Materials and Methods

Materials. Callus was induced in 50 dihaploid clones of *S. tuberosum*. Their origins have been described elsewhere [11]. The dihaploid genotypes H^2 140, H^2 258, and H^2 494 were used because of their predisposition to callus induction, capacity for regeneration, and for their differences in doubling rate observed in earlier studies [6].

Culture conditions. The youngest fully expanded leaves of six-week-old plants were used. The leaves were sterilized for 1 min in 70% alcohol, 7 min in a mixture of 0.1% $HgCl_2$ and 0.1% SDS (sodium dodecylsulphate) solution, and washed three times in sterilized water; they were cut into 0.5 cm^2 pieces and placed on the callus-initiating medium (see below), and incubated in darkness at 25 °C. Callus occurred after 1–2 weeks. Calli were subcultured on fresh medium every three weeks. In the first transfer to fresh medium, the callus was removed from the residual leaf tissue. After 6–8 weeks or six months, the calli were transferred to the regeneration medium (see below) and incubated at 25 °C with 16 h light.

The two culture media used for callus induction and growth were developed by Behnke [1] and differed only in auxin composition. The basal Murashige and Skoog (MS) medium [8] was supplemented with 5 mg/l NAA (α-naphthalene-acetic acid) (medium *a*) or 4.8 mg/l 2, 4-D (2, 4-dichlorophenoxyacetic acid) (medium *b*). The regeneration medium was MS medium supplemented with 2 mg/l zeatin riboside [1].

Regenerated shoots were transferred to basal MS medium for root initiation and finally transplanted in soil. Regenerated plantlets were referred to as LC_1 plants (LC = leaf callus culture) and tuber progenies derived from these as LC_2 plants.

Leaf callus cytology. The ploidy level in leaf callus cells was investigated according to the procedure described by Sopory and Tan [14].

Determination of ploidy levels in LC_1 plants. Previous experience [6] has shown that the ploidy level of the whole potato plant can be reliably established from one germ layer (viz. L_1 germ layer) by scoring the number of chloroplasts in stomatal guard cells [3]. This simple procedure enabled the ploidies of all LC_1 plants to be tentatively characterized. Chromosome counts of root-tip cells (L_3 germ layer) provided additional evidence of ploidy levels for 21 of 45 dihaploids and in all the 155 polyploidized LC_1

plants obtained; precise chromosome counts were made on 20 dihaploid and doubled LC_1 plants and 54 LC_2 plants. Some genotypes developed roots during callus proliferation on medium *a*. The chromosome numbers of these roots were also established. In all cases, the root tips were stained with acetic orcein [16].

Results

Callus induction

The responses shown by the three genotypes on the two induction media (Table 1) indicated NAA to be more efficacious (at $p = 0.05$) than 2, 4-D.

Table 1. Callus induction in three dihaploid genotypes of potato on media *a* and *b*: percentages of leaf pieces with callus formation based on 108 pieces cultured

	Replication	$H^2$494	$H^2$140	$H^2$258	Average
Medium *a* (5 mg/l NAA)	1	89.5%	76.9%	91.7%	87.7%
	2	99.0%	84.3%	85.2%	
Medium *b* (4.8 mg/l 2, 4-D)	1	73.5%	75.0%	75.0%	74.3%
	2	66.7%	98.0%	57.4%	

Genotypic differences in response, however, were not significant. Callus induction of leaf tissue on medium *a* was further tested for 47 dihaploid genotypes. Callusing was obtained in each case.

Effect of auxin source during the callus growth phase and the influence of genotype on frequency of doubled LC_1 plants

The genotypes $H^2$140 and $H^2$258 and the callus-sustaining media *a* and *b* were used to study the effects of genotype and auxins on the incidence of doubling among LC_1 plants. Dihaploid, tetraploid, and octaploid plants were obtained (Table 2). Chromosome counts on root-tip cells confirmed the ploidies of LC_1 plants ascertained from stomatal chloroplast numbers. Doubled and octaploid LC_1 plants of both genotypes could be recognized by changes in their morphological appearances. Doubled LC_1 plants were characterized by larger and round leaves which were dark green. Octaploid LC_1 plants had greyish-green leaves, showed abnormal growth, and no tubers or flowers were formed. Cytological analysis (Table 2) showed that chromosome doubling was more frequent among LC_1 plants from $H^2$258 than from $H^2$140 on both media. Within either medium, the proportion of polyploids among the regenerants of the two clonal genotypes differed significantly (medium *a*: $X_1^2 = 17.5$, $p < 0.001$; medium *b*: $X_1^2 = 14.5$, $p < 0.001$). When both media were considered, the extent of polyploidization was, however, similar for both genotypes ($H^2$258: $X_1^2 = 2.8$, $p > 0.05$; $H^2$140: $X_1^2 = 3.4$, $p > 0.05$). The frequency of octaploids was significantly higher in $H^2$258

Table 2. Ploidy levels of regenerated LC_1 plants of H²140 and H²258, after callus induction and growth on media *a* and *b*: from a single callus, ten plants were regenerated and investigated

Genotype	Medium *a*			Medium *b*		
	2x[a]	4x[b]	8x[b]	2x	4x	8x
H² 258	3	7	0	1	9	0
	1	8	1	0	10	0
	0	10	0	0	5	5
	0	10	0	0	2	8
	1	9	0	0	10	0
c	5	44	1	1	36	13
d	5	26	2	2	15	0
H²140	8	2	0	3	7	0
	1	9	0	5	5	0
	6	4	0	0	10	0
	0	10	0	7	3	0
	9	1	0	0	8	2
c	24	26	0	15	33	2
d	3	9	0	4	7	0

[a] Ploidy level was established from the number of chloroplasts in stomatal guard cells and additionally in a number of plants from the somatic chromosome number of root-tip cells.
[b] Ploidy level was based on both the number of chloroplasts in stomatal guard cells and the somatic chromosome number of root-tip cells.
[c] Ploidy level of LC_1 plants regenerated after 6–8 weeks of callus growth on medium *a* or *b*.
[d] Ploidy level of LC_1 plants regenerated after six months of continuous growth on medium *a* or *b*.

($X_1^2 = 6.0$, $p < 0.05$) especially when 2,4-D was used as the auxin source (H² 258: $X_1^2 = 11.0$, $p < 0.001$). Doubling during the callus phase was dependent on donor genotype (medium *a*: $X_1^2 = 17.9$, $p < 0.001$; medium *b*: $X_1^2 = 11.2$, $p < 0.001$). Auxin source was not significant. The ploidies of ten LC_1 plants from a single six- to eight-week-old callus were studied. From the type of plants regenerated, the calli could be classified into four groups, namely, those yielding:

(1) only diploid LC_1 plants,
(2) diploid and tetraploid LC_1 plants,
(3) only tetraploid LC_1 plants, and
(4) tetraploid and octaploid LC_1 plants.

In studies on LC_1 plants of other genotypes, the group yielding only diploids was frequently represented.

Plants have been regenerated in 26 different genotypes thus far and doubled LC_1 plants have been detected in at least nine clones. Only a limited number of LC_1 plants from six-month-old calli were studied (Table 2);

dihaploids, tetraploids, and a few octaploids were found. The incidence of doubling among LC_1 plants was higher in H^2 140 than in H^2 258. Octaploidy was insignificant.

Cytological studies on callus cells, LC_1 and LC_2 plants

Ploidy level of callus cells. Four different clones were studied at the callus stage. The ploidy levels of four-week-old callus cells could only be approximated. Three or four calli were investigated per clone (Table 3). Of 15 calli

Table 3. Ploidies of dividing cells in four-week-old calli of four genotypes grown on medium *b*: ploidy was established by chromosome counts

Clone	Cells	Calli	Ploidy level of cells			Number of calli with only diploid cells
			2x	4x	> 4x	
$H^2$140	53	4	18 (34%)	29 (55%)	6 (11%)	0
$H^2$409	35	3	8 (23%)	27 (77%)	0	0
$H^2$411	100	4	58 (58%)	34 (34%)	8 (8%)	0
Spe$H^3$69, 1382/14	47	4	35 (74%)	12 (26%)	0	1

studied, diploid as well as tetraploid cells were detected in 14. In 'young' calli of the genotypes H^2 140 and H^2 411, dividing octaploid cells were also present. Considerable variation in the frequency of dividing polyploidized cells was found among the different genotypes.

Ploidy level of callus roots. A total of 37 roots resulting from rhizogenesis in genotypes $H^2$432 and Spe$H^3$69, 1382/14 were studied. As with callus cells, ploidy level was only approximated. In $H^2$432, 23%, and in Spe$H^3$69, 1382/14, 35% of the roots were tetraploid. Most of the calli gave both diploid and tetraploid roots. In clone $H^2$432, one octaploid root was found.

Chromosome numbers in root-tip cells of LC_1 and LC_2 plants. Chromosome counts made on root-tip cells of LC_1 plants showed that at the 'tetraploid' level the chromosome number is frequently variable within and between roots. The instability was limited to mixohypotetraploidy. This phenomenon was commonly detected in LC_1 plants of genotypes $H^2$140 and $H^2$258. Therefore, 53 tuber-propagated LC_2 plants, originating from 20 different LC_1 plants, were investigated with respect to their chromosome constitutions. It was found that ploidy levels of LC_1 parents and those of all their LC_2-progeny plants were the same. These results support earlier observations [6] that ploidy chimerism was not present. At the diploid level, all regenerated and tuber-propagated plants showed an euploid chromosome number. In 'tetraploid' LC_2 plants, mixohypotetraploidy was still present. The chromosome numbers in root-tip cells of clone $H^2$258 varied from 43 to 48, and in clone $H^2$140, from 45 to 48. In two LC_2-progeny plants, originating

from the same LC_1 plant of H^2258, cells with a maximum of 43 chromosomes were found. The doubled LC_2 plants of H^2140 possessed predominantly 46 chromosomes.

Crossing results of somatically doubled clones

Flowering time of doubled LC_2 plants was delayed in the same way as colchicine-doubled clones. The somatically doubled genotypes H^2140, H^2258, and USW5295.7 were crossed with 4x females to test male fertility. H^2140 was male-sterile at the diploid and tetraploid levels and there was no seed set. The doubled clones H^2258 and USW5295.7 showed a regular seed set in such crosses (43.5 seeds per berry and 43.4 seeds per berry). Seed set is important in respect to utilizing the doubled plant as a breeding parent in practical breeding.

Discussion

Callus induction was shown to be significantly higher for both genotypes when NAA was used as an auxin source in the medium (Table 1). Polyploidization was found more frequently in plants regenerated from calli grown on the medium containing 2, 4-D as reflected in a higher frequency of polyploidized LC_1 plants, including a significantly larger number of octaploid LC_1 plants of genotype H^2258 (Table 2).

The detection of diploid and tetraploid callus-derived roots and callus cells (Table 3) in nearly all the calli studied is consistent with observations reported for barley anther calli [5, 7]. However, unlike that of the barley callus, the basal ploidy involved in this study was diploid. In studies on LC_1 plants regenerated from a single callus, plants of the same ploidy were frequently found (Table 2). This could be explained by assuming that there was selection for cells of a particular ploidy level which were capable of regeneration. Therefore culture of many calli per genotype is necessary to increase the probability of detecting somatically doubled LC_1 plants.

In the present study, the identical ploidy level of LC_1 plants and their tuber-propagated progeny LC_2 plants indicated that ploidy chimerism was absent, and this confirmed an earlier finding [6]. In over 300 LC_1 individuals studied, ploidy chimerism was not found, which was at variance with observations reported for *L. peruvianum* plants [15] regenerated from anther and internode calli. In the latter plants, chromosome doubling was also found frequently, but it was associated frequently with ploidy chimerism. Mixohypotetraploidy detected in doubled LC_1 plants was still present in LC_2 plants after tuber propagation. The same was found in early and later developmental stages of anther-derived barley plants [7]. The genotypes H^2140 and H^2258 which have been studied intensively showed regeneration of (mixo)hypotetraploid plants, while, in barley [7] and tobacco [2, 4], mixoploid plants were regenerated. The origin of chromosome doubling in

callus-derived plants has been discussed elsewhere [see 1–3]. In potato, it is difficult to study the origin of aneuploidy because of small chromosome size. In barley, the instability in chromosome number within a plant was caused by chromosomal abberations such as inversions and translocations. In dividing potato callus cells, bridges have been observed [14] which could be one of the causes of the hypotetraploidy found in LC_1 plants.

Mixohypotetraploid and aneuploid LC_2 plants with predominantly 43 and 46 chromosomes could be used as sources for the induction and selection of hypoploid dihaploids. Additional intensive cytological studies on LC_1 plants of other genotypes may lead us to understand the variation in predisposition of genotypes to (mixo)hypotetraploidy.

The presence of octaploid cells in the callus was established and the regeneration of octaploid LC_1 plants was therefore not surprising, although the influence of genotype and auxin source (Table 2) was evident. In doubled LC_1 plants of the genotypes H^2258 and USW5295/7, male fertility was unimpaired. The importance of this character has to be studied in different somatically doubled genotypes. The influence of (mixo)hypotetraploidy on characters such as flowering and fertility has to be compared with reduced fertilities commonly found in colchicine-doubled plants [10]. After one cross-cycle, especially with a doubled LC_1 plant as the male parent, aneuploidy is usually eliminated in the progeny.

The results reported herein give rise to optimisim that somatic doubling of dihaploid breeding clones via leaf tissue culture can be an adjunct to the colchicine method. An important consideration is a short callus phase to minimize aneuploidy.

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