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Molecular marker analysis of 24- and 25-chromosome plants obtained from *Solanum tuberosum* L. subsp. *andigena* (2*n* = 4*x* = 48) pollinated with a *Solanum phureja* haploid inducer

Y. Samitsu and K. Hosaka

Abstract: Clones with 24 or 25 chromosomes were obtained by pollinating an Andean cultivated tetraploid potato (*Solanum tuberosum* subsp. *andigena* clone 94H94, 2*n* = 4*x* = 48) with the *Solanum phureja* haploid-inducer clone 1.22. Their genetic composition was analyzed in an RAPD assay using 135 decamer primers and in an RFLP assay using 45 single-copy DNA probes. In total, 22 RAPD and 20 RFLP markers were found to be specific to *S. phureja*. None of these markers were found in the 24- and 25-chromosome clones. RFLP genotypes for the 45 RFLP loci were further determined for each clone. Genotypes of the 24-chromosome clones were characterized using two alleles randomly selected from four alleles of the parental tetraploid clone for almost all RFLP loci. Five 25-chromosome clones had extra alleles for all of the RFLP loci of chromosomes 4, 8, 10, 11, and 12, respectively, suggesting primary trisomy for one of these chromosomes. Clones with genotypes showing double reduction were also identified. Therefore, the obtained clones likely originated from random samples of female gametes, and hence are euhaploids or aneuhaploids of *S. tuberosum* subsp. *andigena*, strongly supporting parthenogenesis to be a primary mechanism for haploid induction in potato.

Key words: *Solanum tuberosum* subsp. *andigena*, RFLP, RAPD, haploid, trisomic.

Résumé : Des clones à 24 ou 25 chromosomes ont été obtenus en fécondant une pomme de terre tétraploïde cultivée dans les Andes (*Solanum tuberosum* ssp. *andigena* clone 94H94, 2*n* = 4*x* = 48) avec le clone 1.22 inducteur d'haploïdie du *S. phureja*. Leur composition génétique a été analysée à l'aide de 135 amorces RAPD et à l'aide de 45 sondes RFLP à simple copie. Au total, 22 marqueurs RAPD et 20 marqueurs RFLP étaient spécifiques du *S. phureja*, mais aucun de ces marqueurs n'était présent chez les clones à 24 ou à 25 chromosomes. Les génotypes à 45 locus RFLP ont été examinés plus en détail chez chaque clone. Les génotypes des clones à 24 chromosomes étaient composés de deux allèles choisis aléatoirement parmi les quatre allèles du clone tétraploïde parental pour presque tous les locus RFLP. Cinq clones à 25 chromosomes possédaient des allèles additionnels pour tous les locus RFLP des chromosomes 4, 8, 10, 11 et 12, respectivement, suggérant ainsi une trisomie primaire pour l'un de ces chromosomes. Des clones dont les génotypes indiquaient une double réduction ont également été identifiés. Conséquemment, les clones obtenus provenaient vraisemblablement d'un échantillonnage aléatoire des gamètes femelles du *S. tuberosum* ssp. *andigena*, lesquels étaient ainsi euhaploïdes ou encore aneuhaploïdes. Ces observations supportent fortement l'hypothèse de la parthénogenèse comme mécanisme primaire pour l'induction haploïde chez la pomme de terre.

Mots clés : *Solanum tuberosum* subsp. *andigena*, RFLP, RAPD, haploïde, trisomique.

[Traduit par la Rédaction]

Introduction

Potato (*Solanum tuberosum* L. subsp. *tuberosum*) is a highly heterozygous tetraploid (2*n* = 4*x* = 48) with tetrasomic inheritance. One of the strategies to simplify genetic and breeding studies is the use of haploids. Hougas and Peloquin (1957) first obtained a haploid of *S. tuberosum*. Superior *Solanum phureja* "haploid-inducer" clones were developed that induce high frequencies of haploid plants and have selection markers to distinguish possible haploid plants or embryos from true hybrid plants or embryos (Peloquin and Hougas 1959; Hermsen and Verdenius 1973). This method of haploid induction, which has often been applied in potato breeding, revealed thousands of haploid *S. tuberosum* (e.g., Kotch and Peloquin 1987) used for genetic analyses of various traits (Matsubayashi 1979; De Maine 1984; Kotch et al. 1992) and for transferring wild species germplasm into diploid parental lines (Hermundstad and Peloquin 1985).

Parthenogenesis has generally been accepted as a mechanism for haploid induction in potato (von Wangenheim et al.

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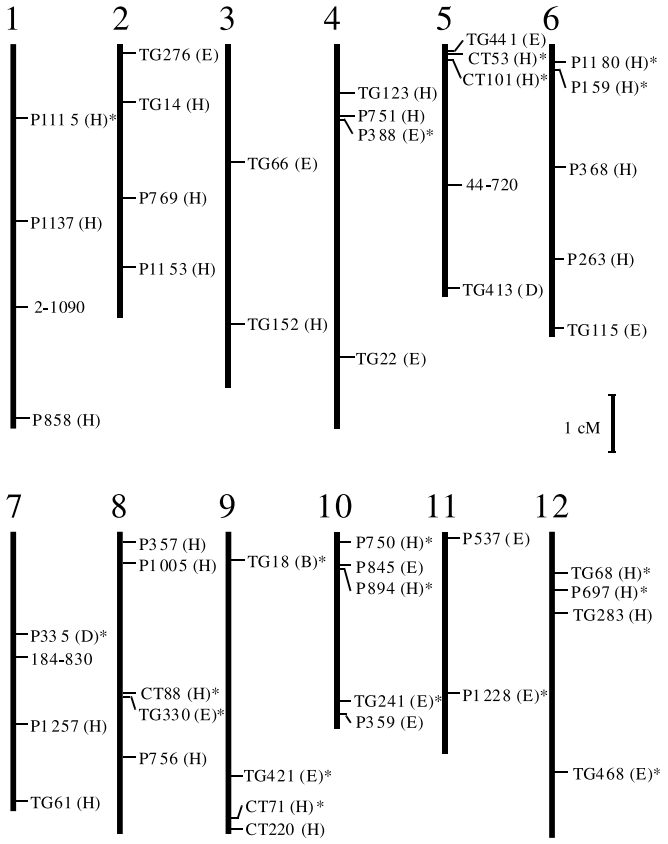
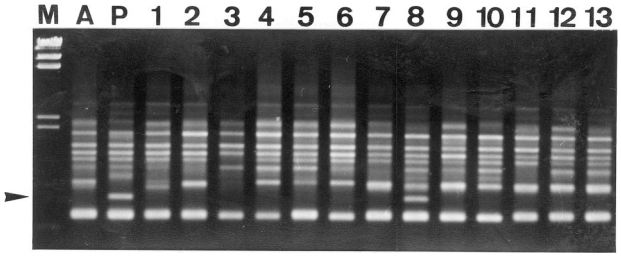
Fig. 1. A potato genetic map showing approximate locations of RAPD and RFLP markers used in this study. RAPD markers are indicated by a primer number hyphenated with the size of the marker band. All others are RFLP markers shown with restriction enzymes in parentheses. B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII. Asterisks indicate RFLP markers that generated *S. phureja* 1.22-specific markers.



1960; Ross 1986). Plants obtained accordingly may be aneuhaploid, because of a high incidence of chromosomal irregularities during meiosis (Hermsen 1969; Hermsen et al. 1970). Clulow et al. (1991), who found haploid clones with a high proportion of root-tip cells containing 25 or 26 chromosomes and who also detected *S. phureja* haploid inducer specific RFLP bands in the haploid clones, proposed an additional mechanism for haploid formation through egg cells fertilized by pollen from *S. phureja*, followed by preferential elimination of the *S. phureja* chromosomes. Such aneusomatic haploid clones expressed leaf isozymes and tuber patatin specific to the *S. phureja* haploid inducer (Clulow et al. 1993). Even in a true euhaploid clone, Wilkinson et al. (1995) observed by genomic in situ hybridization (GISH) chromosome segments of the *S. phureja* haploid inducer in *S. tuberosum* complements.

Considering the aforementioned examples of chromosomal variations, it is important to conduct a detailed analysis on the genetic composition of 2*n* = 24 plants of *S. tuberosum*. In this study, we examined both 24- and 25-chromosome clones from a cross between the Andean tetraploid cultivated potato, *S. tuberosum* subsp. *andigena*, and a haploid inducer *S. phureja* clone 1.22, which is widely

Fig. 2. RAPD banding patterns obtained using primer No. 184 (5′-AGTCGTCCCC-3′). The *S. phureja* 1.22-specific band is indicated by an arrowhead. M, lambda DNA *Hin*dIII digests; A, 94H94; P, 1.22; lanes 1–7 (all 2*n* = 25): 94H93-1 and 95H133-21, -67, -78, -81, -82, and -88; lanes 8–10 (all 2*n* = 36): 95H133-11, -95, and -20; lanes 11–13 (all 2*n* = 24): 95H133-1, -2, and -4.



used in North America. Our aims were (*i*) to clarify whether or not *S. phureja* germplasm is incorporated in these clones, (*ii*) to reveal the origin of the extra chromosome in 25-chromosome clones, and (*iii*) to discuss the most likely mechanism for haploid formation.

Materials and methods

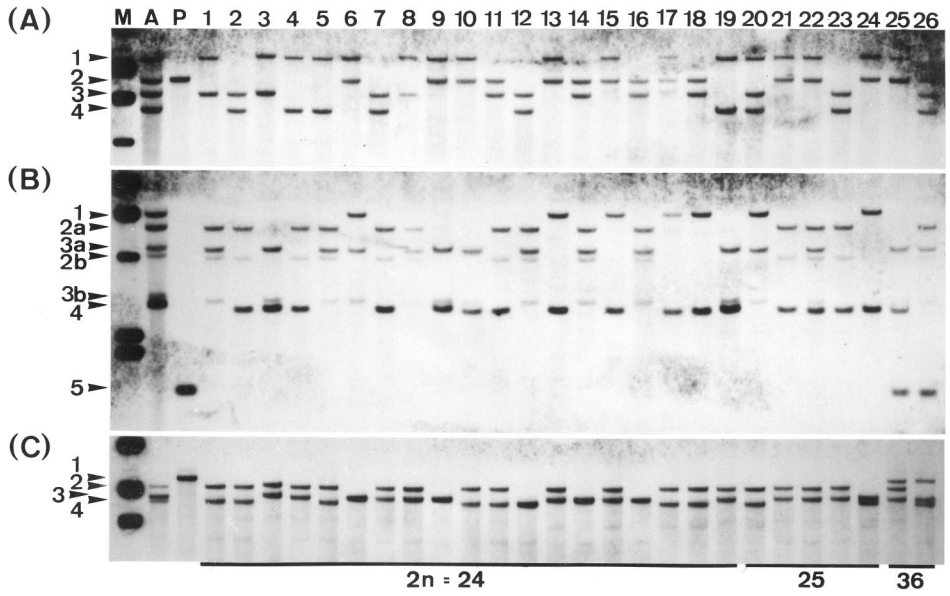
Plant materials

The scab-resistant clone 94H94 (*S. tuberosum* subsp. *andigena*, T-AY-20) had green stems and selfed progeny did not show any red pigment in their hypocotyls. The *S. phureja* haploid inducer, clone 1.22, had dark-red stems and produced a pink or red color in the hypocotyls of true hybrid plants. Clone 94H94 was emasculated and pollinated with the pollen of a haploid inducer in an insect-free screenhouse. Seeds were germinated after soaking in 2000 ppm gibberellic acid GA3 for two days. Plants were separated on the basis of green vs. pink hypocotyl color. For establishing ploidy levels, plastids in guard cells of the stomata were counted (Frandsen 1968). Cell-spread preparations of root-tip meristems from putative diploid and triploid plants were then used for chromosome counting using the method of Yamada et al. (1997).

RAPD and RFLP analysis

The procedures for DNA isolation and detection of RAPD and RFLP markers were described previously (Hosaka and Hanneman 1998). RFLP probes prefixed with "TG" or "CT" were single-copy tomato probes obtained from S.D. Tanksley, Cornell University, Ithaca, N.Y., and were localized on tomato or potato genetic maps (Tanksley et al. 1992). Probes prefixed with "P" were single-copy DNA probes selected from a random genomic DNA library of *S. phureja* 1.22 (Hosaka and Spooner 1992), which have been localized on this potato genome (Hosaka and Hanneman 1998; Hosaka 1999). RFLP bands were evaluated not only for presence or absence, but also for band intensity. We therefore adjusted 5 µg of the DNA digests to assess band intensity equal among samples by Southern-hybridizing with multiple-copy probe P1120.

Fig. 3. RFLP banding patterns obtained using probes P357 (A), P894 (B), and P159 (C). M, lambda DNA *Hin*dIII digests; A, 94H94; P, 1.22; lanes 1–19 (all 2*n* = 24): 95H133-1, -2, -5, -8, -13, -16, -17, -18, -25, -27, -31, -33, -34, -35, -37, -39, -40, -41, and -42; lanes 20–24 (all 2*n* = 25): 94H93-1, 95H133-21, -67, -81, and -88; lanes 25 and 26 (all 2*n* = 36): 95H133-11 and -95. See text for explanation of patterns.



Results

Haploid induction

From 374 pollinations with pollen of the *S. phureja* haploid inducer 1.22, *S. tuberosum* subsp. *andigena* clone 94H94 set 187 berries containing 261 seeds. All seeds were sown (family 95H133), from which 184 plants germinated. Eighty-eight plants had pale-green hypocotyls and 82 had pink hypocotyls. The remaining plants died precociously. The following season, ploidy levels of 162 clones were assessed on the basis of plastid numbers in stomata guard cells, revealing 66 diploid, 11 triploid, and 85 tetraploid clones. A year later, we established accurate chromosome numbers in mitotic cell spread preparations for clones mostly having diploid or triploid guard cells. Ultimately, 63 clones of 2*n* = 24, 6 clones of 2*n* = 25, 8 clones of 2*n* = 36, and one clone of 2*n* = 38 were obtained. Among them, 53 clones having pale-green hypocotyl and diploid-like stomata were either 2*n* = 24 or 25. In the following analysis, clones of the family 95H133 with 2*n* = 24 or 25 and an additional clone with 2*n* = 25 (94H93-1) that had been previously induced from 94H94 by clone 1.22, were used.

RAPD analysis

Parental clones 94H94 and 1.22 were compared using 135 decamer primers. Twenty-two primers produced 22 reliable bands that were specific to the 1.22 clones. Three of them were marker bands that had been prevoiusly mapped on the clone 1.22 genome (Hosaka 1999; Fig. 1). The map locations of the other band markers are unknown. These 1.22-specific bands were scored for presence or absence in three clones of 2*n* = 24 (95H133-1, -2, and -4), seven clones of 2*n* = 25 (94H93-1, 95H133-21, -67, -78, -81, -82, and -88), and three clones of 2*n* = 36 (95H133-11, -20, and -95) (Fig. 2). None of 1.22-specific bands were found in the 24- and 25-chromosome clones, but were segregating for presence or absence among the 36-chromosome clones.

RFLP analysis

In a preliminary experiment, 45 RFLP probes were selected as strictly single-copy DNA probes showing multiple bands in 94H94 in at least one of the *Bam*HI-, *Dra*I-, *Eco*RI-, or *Hin*dIII-digested genomic DNAs. These probes covered all potato chromosomes (Fig. 1). Segregation of RFLP bands and band intensity were analyzed in 19 clones of 2*n* = 24, 5 clones of 2*n* = 25, and 2 clones of 2*n* = 36, and compared with the parental clones (Fig. 3; Table 1).

In Fig. 3A, four different RFLP bands observed in 94H94 were segregating in the progeny. Assuming each band was controlled by one RFLP allele in the same RFLP loci, the bands 1, 2, 3, and 4 in sequence of decreasing fragment size could be represented as alleles 1, 2, 3, and 4. Thus, the RFLP genotype of 94H94 is presented as being 1234. The RFLP genotype of 1.22 could be 22 because of the double intensity of band 2. The genotypes of 24-chromosome clones were all represented by random combination of two of the four alleles. Among the 25-chromosome clones, 94H93-1 (lane 20) showed three bands, corresponding to alleles 1, 3, and 4. The other 25-chromosome clones showed two bands. In the 36-chromosome clones, 95H133-11 (lane 25) showed bands 1 and 2, the latter with a higher intensity. Thus, its genotype could be 122. The genotype of 95H133-95 (lane 26) was presented as 234.

In Fig. 3B, six different bands were observed in the banding pattern of 94H94. However, two sets of two bands (band 2*a* and 2*b*, and bands 3*a* and 3*b*) displayed cosegregation. Band 5 was a 1.22-specific band detected only in the 36-chromosome clones. Genotypes of 24- and 25-chromosome clones were all represented by random combination of two of four alleles of 94H94 except for that of 95H133-67 (lane

Table 1. Estimated RFLP genotypes for each RFLP locus (or probe) in 94H94, 1.22, and the 2*n* = 24, 25, and 36 progenies.

		Chromosome 1			Chromosome 2				Chromosome 3	
Plant	2*n*	P1115	P1137	P858	TG276	TG14	P769	P1153	TG66	TG152
94H94	48	1134	1123	1123	1122	1122	1112	1122	1123	1122
1.22	24	12	22	11	11	11	11	11	11	12
95H133-1	24	11	12	11	12	11	12	12	12	12
95H133-2	24	13	23	13	11	12	11	12	12	11
95H133-5	24	14	13	23	12	12	11	12	23	11
95H133-8	24	13	13	23	12	11	11	11	12	11
95H133-13	24	14	12	12	22	12	11	12	23	11
95H133-16	24	13	23	13	12	12	12	12	13	12
95H133-17	24	14	11	11	11	11	12	11	12	11
95H133-18	24	13	11	12	12	11	12	12	23	11
95H133-25	24	14	11	11	12	12	11	11	12	11
95H133-27	24	14	12	11	12	12	12	12	13	12
95H133-31	24	34	13	23	12	11	12	12	23	11
95H133-33	24	13	13	13	22	12	12	12	11	12
95H133-34	24	13	23	13	12	11	12	11	23	11
95H133-35	24	34	13	23	12	11	11	11	12	11
95H133-37	24	11	11	12	22	12	11	11	12	12
95H133-39	24	34	13	23	12	11	11	11	12	11
95H133-40	24	11	12	12	12	11	12	12	11	12
95H133-41	24	13	13	13	22	12	11	12	13	11
95H133-42	24	14	12	12	12	12	12	22	13	22
94H93-1	25	13	13	13	12	11	12	12	13	12
95H133-21	25	34	13	13	12	12	11	11	13	11
95H133-67	25	13	11	12	12	12	11	12	13	11
95H133-81	25	13	13	23	12	11	12	12	13	12
95H133-88	25	11	23	23	12	12	11	12	11	12
95H133-11	36	123	123	123	111	111	112	112	112	112
95H133-95	36	114	112	123	112	111	111	111	123	122

Note: Figures in genotypes indicate allele numbers that correspond to RFLP bands in order from larger bands.

22), which showed a three-allele banding pattern with a genotype of 234.

The parental clone 94H94 did not always show four different bands, but occasionally displayed two or three bands. In those cases, band intensities and segregation patterns in the progeny were considered to estimate RFLP genotypes. For example in Fig. 3C, three bands were detected in 94H94. Its genotype was estimated to be 2334 because the intensity of band 3 was high and the 33 genotype was often observed in haploid progeny.

Likewise, RFLP banding patterns for each clone with all probes used could be estimated as shown in Table 1. In total, 20 bands distributed on 10 chromosomes were specific to 1.22 (Fig. 1) and none of these markers were found in 24- or 25-chromosome clones. All 24- or 25-chromosome clones displayed two-allele banding patterns, the alleles of which could be estimated by random selection of four alleles of 94H94, except for the following cases. As shown in Fig. 3C, 95H133-33 (lane 12) showed a 44 genotype, which could not be expected by random selection of two of the four 94H94 alleles, 2, 3, 3, and 4. Similar cases were found in 95H133-31 for TG123 (chromosome 4), in 95H133-34 for TG115 (chromosome 6), and in 95H133-13 for P1228 (chromosome 11) (Table 1). The 25-chromosome clones 94H93-1 and 95H133-21, -67, -81, and -88 showed three-allele banding patterns with all probes of chromosomes 8, 12, 10, 4, and 11, respectively. Three alleles were all different in at least one probe of the chromosomes in these clones, with the exception of 95H133-88, where the third allele was the same as one of the other two alleles in each of two probes used for chromosome 11 (Table 1).

Genotypes of 36-chromosome clones in all RFLP loci of all chromosomes were shown by combination of randomly selected two 94H94 alleles and one of two 1.22 alleles (Table 1).

Discussion

None of the molecular markers specific for the *S. phureja* haploid inducer were detected in the 24- and 25-chromosome clones. Hence, we could not find any evidence that a chromosome or a chromosome segment of a *S. phureja* haploid inducer was introgressed into the 24- or 25-chromosome clones derived from *S. tuberosum* subsp. *andigena*. Clulow et al. (1991) found *S. phureja* specific RFLP markers in haploid clones of 'Pentland Crown' induced by using various *S. phureja* haploid inducers (but not 1.22). Misoo et al. (1997) found *S. phureja* specific RAPD markers in euhaploid and aneusomatic clones of 'Chijiwa' by pollinating with *S. phureja* haploid inducer 460, which is

Chromosome 4				Chromosome 5				Chromosome 6				
TG123	P751	P388	TG22	TG441	CT53	CT101	TG413	P1180	P159	P368	P263	TG115
1234	1112	2344	1223	1222	1235	1344	1234	1222	2334	1122	1122	1223
14	11	15	33	22	44	23	44	23	11	22	11	11
14	12	44	22	12	35	14	23	12	24	12	22	13
34	12	24	22	12	25	44	34	12	24	12	12	22
23	12	44	22	12	23	14	23	12	23	22	11	22
12	12	24	23	12	12	34	14	12	23	22	11	23
14	12	24	23	22	15	34	14	22	24	12	12	23
12	12	34	12	12	23	14	14	22	33	12	12	12
12	12	34	23	22	35	14	23	12	23	22	11	22
24	11	23	13	22	35	14	23	12	23	22	12	13
14	11	23	13	12	12	34	24	22	33	12	12	12
23	11	23	12	12	12	34	23	12	24	12	12	23
11	12	44	22	12	23	14	13	12	24	12	12	23
24	11	24	13	22	13	13	24	22	44	11	22	13
23	11	24	23	12	25	44	14	12	23	12	12	11
14	12	44	23	12	23	14	12	22	33	12	12	12
13	12	24	13	22	35	14	23	22	23	12	12	23
14	12	44	23	12	23	14	13	22	33	12	12	12
34	11	23	22	12	23	14	13	12	24	12	12	23
13	11	23	13	22	35	14	14	12	24	12	12	23
34	11	23	12	12	23	14	23	12	23	12	11	22
24	11	34	13	12	12	34	24	12	24	12	12	23
23	11	34	23	12	25	44	13	12	23	12	12	23
12	12	34	23	12	12	34	12	12	23	12	22	13
123	112	234	123	12	23	14	12	12	23	12	12	23
14	12	44	23	22	15	34	23	12	34	12	12	23
113	112	144	223	222	345	124	344	223	123	122	112	112
134	111	124	233	222	145	234	134	123	134	112	112	123

in contrast to our observation. The number of markers used in this study might not be sufficient to detect small segments intogressed from the *S. phureja* haploid inducer. However, it seems more likely that the difference is due to the genotypes of female or male parents, or its combination. We used *S. tuberosum* subsp. *andigena* as a tetraploid female parent, whereas Clulow et al. (1991) and Misoo et al. (1997) used *S. tuberosum* subsp. *tuberosum*. Different haploid inducer clones were also used.

Almost all RFLP genotypes in 24-chromosome clones could be explained by two out of four random parental alleles. Exceptions were found in four genotypes with one of the four parental alleles being duplicated. These genotypes possibly resulted from "double reduction," a special case of chromatid exchange involving a quadrivalent formation and a crossover between the centromere and the locus concerned in which the two sister chromatids with the same allele are distributed to the same pole and are included in one gamete (Mather 1936). The sporadic occurrence of double reduction in *S. tuberosum* has been evidenced by examining the segregation of isozyme loci (Haynes and Douches 1993).

We obtained five 25-chromosome plants, 94H93-1 and 95H133-21, -67, -81, and -88, with an extra chromosome 8, 12, 10, 4, and 11, respectively, of the tetraploid parental clone 94H94. As it is likely that these extra chromosomes are not partial segments, but whole chromosomes; not duplicated chromosomes, but different chromosomes from the first or second homologous chromosomes. We assume that these 25-chromosome clones are primary trisomics. Hermsen (1969) and Hermsen et al. (1970) obtained hyperploids with 25–28 chromosomes from tetraploids (*S. tuberosum* subsp. *andigena*, *S. tuberosum* subsp. *tuberosum*, and colchicine-doubled *Solanum chacoense*) pollinated with *S. phureja* haploid inducers. These hyperploids have been considered to originate from unfertilized egg cells with aneuploid chromosome numbers resulting from chromosomal irregularities during meiosis in autotetraploids (Hermsen 1969).

In conclusion, the 24- and 25-chromosome clones in this study are possibly euhaploid and aneuhaploid clones from *S. tuberosum* subsp. *andigena*, likely originated from random samples of female gametes. Clones with 36 chromosomes are apparently triploid hybrids between *S. tuberosum* subsp. *andigena* and *S. phureja*. Therefore, we strongly support the parthenogenesis pathway as a haploid induction mechanism. Although the preferential elimination hypothesis of *S. phureja* chromosomes (Clulow et al. 1991) could be denied for the present parental combination, it is necessary to con-

Table 1. *(concluded)*.

		Chromosome 7			Chromosome 8					Chromosome 9			
Plant	2*n*	P335	P1257	TG61	P357	P1005	CT88	TG330	P756	TG18	TG421	CT71	CT220
94H94	48	2333	1123	1222	1234	1112	2234	1244	1123	1112	2223	1124	1112
1.22	24	13	11	22	22	11	11	33	13	13	11	13	11
95H133-1	24	23	12	22	13	12	24	24	13	12	23	12	11
95H133-2	24	23	13	12	34	11	24	24	13	12	23	12	11
95H133-5	24	23	12	12	13	12	34	12	13	11	23	12	11
95H133-8	24	33	23	22	14	12	34	12	13	11	22	11	11
95H133-13	24	23	12	22	14	12	24	24	23	11	22	24	12
95H133-16	24	23	11	12	12	12	34	12	12	11	23	14	12
95H133-17	24	23	13	22	34	11	22	44	12	12	23	14	12
95H133-18	24	33	12	22	13	12	24	24	13	12	23	12	11
95H133-25	24	33	12	22	12	12	22	44	12	12	23	24	12
95H133-27	24	33	12	22	12	12	22	44	12	11	22	14	12
95H133-31	24	33	23	22	23	11	23	14	12	11	22	24	12
95H133-33	24	33	23	22	34	11	24	24	23	12	22	14	12
95H133-34	24	33	23	22	12	12	23	14	12	11	22	14	12
95H133-35	24	23	12	22	23	12	34	12	13	11	22	24	12
95H133-37	24	23	11	12	12	12	34	12	23	12	23	11	11
95H133-39	24	23	12	22	23	12	34	12	13	11	22	24	12
95H133-40	24	33	11	12	12	12	34	12	13	11	22	12	11
95H133-41	24	23	13	12	23	11	24	24	13	12	23	12	11
95H133-42	24	33	12	22	14	12	24	24	23	11	23	11	11
94H93-1	25	23	12	12	134	111	223	144	122	12	22	14	12
95H133-21	25	23	12	22	12	12	23	14	23	11	22	12	11
95H133-67	25	23	13	22	12	12	34	12	13	11	22	14	12
95H133-81	25	23	12	12	34	11	24	24	23	12	22	12	11
95H133-88	25	23	12	12	12	12	34	12	13	12	22	14	12
95H133-11	36	333	123	122	122	112	134	123	113	111	123	134	112
95H133-95	36	133	123	122	234	111	122	344	123	111	122	124	112

Note: Figures in genotypes indicate allele numbers that correspond to RFLP bands in order from larger bands.

duct the same type of analysis for haploids induced from *S. tuberosum* subsp. *tuberosum* to draw a more general conclusion for a haploid induction mechanism in potato.

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Chromosome 10					Chromosome 11		Chromosome 12			
P750	P845	P894	TG241	P359	P537	P1228	TG68	P697	TG283	TG468
1234	1234	1234	1122	1223	1222	1123	1235	1133	1122	2345
55	44	55	33	22	22	14	24	23	11	12
23	14	23	12	23	12	23	13	13	12	45
23	34	24	12	12	12	12	35	11	22	35
12	13	34	12	23	22	13	12	13	12	24
13	34	24	12	12	22	13	35	11	12	45
24	12	23	12	12	22	33	35	13	12	24
13	24	13	22	13	22	13	12	33	11	24
13	34	24	11	22	22	13	13	13	12	35
13	14	23	12	23	12	12	15	13	12	24
12	13	34	12	12	12	13	23	13	12	45
12	13	34	12	12	22	12	13	13	12	24
14	34	24	12	12	12	13	25	13	12	35
23	14	23	12	23	12	12	35	13	12	35
14	23	14	12	23	12	23	23	13	12	45
23	14	23	12	23	22	23	12	33	11	24
24	12	14	12	12	12	12	13	13	12	24
23	14	23	12	23	22	23	12	33	11	24
14	23	14	22	13	22	13	25	13	12	24
14	23	14	12	12	22	23	35	11	22	35
23	14	34	12	23	12	23	25	13	12	45
34	12	13	11	22	22	13	23	33	12	23
13	34	24	12	23	12	23	125	133	112	245
123	134	234	122	223	12	12	15	13	12	45
13	34	24	12	23	12	23	13	13	12	35
23	14	14	12	12	122	113	35	11	12	24
125	134	345	223	123	222	124	235	113	122	145
135	144	235	123	223	122	112	225	133	112	223

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