Theor Appl Genet (1995) 91:598–602

B. Campion · B. Bohanec · B. Javornik

Gynogenic lines of onion (*Allium cepa* L.): evidence of their homozygosity

Received: 4 January 1995 / Accepted: 24 February 1995

Abstract Haploid induction via gynogenesis offers the possibility of using doubled haploid (DH) inbred lines in onion breeding. A first DH line that originated from the open-pollinated (OP) cultivar 'Dorata di Parma' was obtained after overcoming difficulties associated with the haploidy of the regenerants. Spontaneous chromosome doubling occurs seldom in onion. The first DH line obtained was cloned and selfed to produce sufficient seeds for genetic studies. The homozygosity of the DH gynogenic line was revealed on the basis of the low standard deviations of the bulb traits polar diameter, shape index and weight with respect to those of the S_1 line or the OP cultivar. In the DH line, moreover, segregation of RAPD and alpha esterase markers was not noted. Out of four primers revealing polymorphism at 16 ge-netic loci in the OP cultivar 'Dorata di Parma', none produced polymorphism in the DH gynogenic line. The *Est-1* locus, homozygous in 22 plants (*Est-1*$^{1/1}$ in 3 and *Est-1*$^{2/2}$ in 19) and heterozygous (*Est-1*$^{1/2}$) in 11 plants of the OP cultivar, always carried the same alleles in the DH line. We also tested genetic stability during micropropagation of a second halpoid line obtained via gynogenesis from var. 'Senshyu Yellow'. Seventeen plants of this line were tested to detect changes occurring during the tissue culture process. Again no polymorphism was observed. The high genetic homogeneity observed in the two gynogenic lines of onion could be related to the absence of the callus phase during the gynogenic process.

Key words Gynogenesis · Isozyme · RAPD · Agronomic evaluation · Gametoclonal variation · Genetic homogeneity

Communicated by Y. Gleba

B. Campion (✉)
Istituto Sperimentale per l'Orticoltura, Via Paullese 28, 20075 Montanaso Lombardo (Lodi), Italy

B. Bohanec · B. Javornik
University of Ljubljana, Biotechnical Faculty, Agronomy Department, Jamnikarjeva 101-61111 Ljubljana, Slovenia

Introduction

Cultivated onion, *Allium cepa* L., is a biennial allogamous vegetable crop of major importance in which inbreeding is fairly well-tolerated (Jones and Davis 1994). In spite of this, the development of inbred lines for hybrid seed production remains a demanding and time-consuming task. The production of a large number of homozygous lines in a short time through the use of tissue culture techniques would thus be of interest for breeding purposes.

Four research groups working on this species (Campion and Azzimonti 1988; Campion and Alloni 1990; Muren 1989; Keller 1990; Smith et al. 1991) have reported successful cases of gynogenesis obtained in vitro; further studies have been focused on the improvement of haploid induction through the in vitro culture of unpollinated ovaries and flowers (Campion et al. 1992). More recently, a fifth group (Doré and Marie 1993) has demonstrated that gynogenic plants of onion can also be induced in planta after fertilization with irradiated pollen. A high degree of homogeneity and genetic stability is supposed to be the major attribute of doubled haploid (DH) lines.

The use of tissue culture to produce plants from microspores or megaspores is frequently associated to the induction of genetic changes, a phenomenon termed gametoclonal variation (Evans et al. 1984; D'Amato 1985; Ziauddin and Kasha 1990). Mutational events arise either before chromosome doubling, originating useful agronomic variants of the R_0 generation, or after chromosome doubling. In the latter case they are always undesirable since they cause segregation of the selfed R_1 generation (Oono 1981). The genetic variation present in DH lines should therefore be assessed before their use in plant breeding either by considering morphological

traits or by analysing biochemical or molecular markers.

Genetic marker analysis has been used to study the degree of genetic change in plants regenerated in vitro, such as pea (Cecchini et al. 1992), sugar beet (Sabir et al. 1992) and wheat (Brown et al. 1993), as well as to evaluate doubled haploids obtained through androgenesis in barley (Finnie et al. 1991), potato (Singsit and Ozias-Akins 1993) and maize (Murigneux et al. 1993). In onion, reports on genetic marker analysis are limited to the use of isozyme (Peffley and Mangum 1990; Loaiza-Figueroa and Weeden 1991) or random amplified polymorphic DNA (RAPD) markers, the latter having been useful in a genetic analysis of *Allium* species (Wilkie et al. 1993).

The aim of our work was to acquire precise information on the homogeneity of a gynogeneic DH line of onion. From this line it was rather difficult to obtain a sufficient quantity of R_2 seeds as most of the gynogenic regenerants we have produced have a strong tendency to remain haploid (Campion et al. 1994). The DH line of 'Dorata di Parma' described in this paper is the only one available so far that has generated a considerable number of R_2 plants. Agronomic performance in this line was studied to estimate its morphological uniformity. RAPD markers and the estimate its morphological uniformity. RAPD markers and the esterase isoenzymes were used to assay the genetic stability of the DH line together with a second micropropagated haploid onion line.

Materials and methods

Experimental material and origin of the gynogenic lines

Genetic materials used in the field experiments were 480 plants of the open pollinated (OP) 'Dorata di Parma', a long-day onion cultivar; 480 plants of an S_1 line produced by selfing 1 plant randomly chosen; and 480 plants of a DH gynogenic line obtained from the same OP population through ovule culture (Campion and Alloni 1990). Biochemical and RAPD analyses were performed on 33 plants of 'Dorata di Parma', and RAPD and isozyme analyses were carried out on 31 and 33 plants, respectively, of the R2 gynogenic line and 17 plants of a haploid gynogenic line (Ro progeny) obtained from the short-day OP 'Senshyu Yellow' through flower-bud culture (Campion et al. 1992). The gynogenic line of 'Dorata di Parma' doubled spontaneously its chromosomes during plant development, which started in vitro and continued in the greenhouse (Campion and Azzimonti 1988). The R_2 generation was obtained by two cycles of seed production through self-pollination. The haploid 'Senshyu Yellow' was micropagated in vitro and transplanted to soil for bulb development.

Morphological evaluation

OP, S_1, and DH line seeds were sown in February in the greenhouse, and plants were transplanted in April into the open field in a randomized complete block design. The experimental plot consisted of 120 plants and was replicated four times. At harvest, for each individual bulb, polar and equatorial diameter, their ratio (= shape index) and weight were recorded. The evaluation of the variability among genotypes was based on the analysis of variance and on Duncan's multiple range test. Intravarietal variability was quantified by calculating the standard deviation (SD) on the total number of bulbs of all replications.

Isozyme analysis

Approximately 200 mg of leaf tissue, sampled from the first leaves that sprouted out from the onion bulbs, was crushed in 200 µl of extraction buffer consisting of 15% (w/v) sucrose, 50 m*M* Tris-HCl, pH 7.1, in 0.5% (v/v) Triton X-100. The extracts were centrifuged at 26,500 *g* for 5 min at 4 °C, and the supernatant was immediately loaded onto gels. Stacking gels consisted of 24.6 g/l acrylamide and 6.15 g/l bisacrylamide, 4.0 g/l Triton X-100, 700 mg/l ammonium persulfate, 0.6 mg/l TEMED in 0.07 *M* Tris, pH 7.8. Resolving gel consisted of 57.8 g/l acrylamide and 2.2 g/l bisacrylamide, 0.37 g/l ammonium persulfate, 0.37 ml/l TEMED, 2.00 g/l Triton X-100 in 0.07 *M* Tris, pH 7.8. As electrode buffer, 1 g/l Tris and 5.52 g/l barbitone, pH 7.3, was used. Electrophoresis was carried out at 10 °C for 3 h at a constant voltage of 225 V. Alpha esterases were stained according to Wendel and Weeden (1989).

RAPD analysis

Total DNA was isolated from 100 mg of fresh leaf tissue using a modified CTAB extraction procedure (Saghai-Maroof et al. 1984). The DNA concentration was estimated by a mini-DNA fluorometer (Hoefer, TKO 100).

The 25-µl polymerase chain reaction (PCR) mixture contained approximately 40 ng genomic DNA, 200 µM each of dATP, dGTP, dCTP and dTTP, 200 n*M* primer (Operon Technologies), 4 m*M* $MgCl_2$ and 0.5 units of *Taq* polymerase with 10 × incubation buffer (Boehringer Mannheim). Amplifications were performed in a Perkin-Elmer/Cetus Thermal Cycler 480 under the following temperature conditions: preliminary 5 min denaturation of DNA at 95 °C followed by the addition of the enzyme and then a total of 48 cycles of 1 min at 94 °C, 1 min at 37 °C and 90 s at 72 °C. The amplification was completed with an incubation at 72 °C for 10 min, followed by a 4 °C soak until recovery.

The PCR products were separated by electrophoresis in 1.4% agarose gel and observed under UV light after staining with ethidium bromide. Molecular DNA marker no. 6 (Boehringer Mannheim) was used.

Twenty different decamer primers (Operon Technologies, Alameda, Calif., USA) were used in the PCR reaction to amplify DNA fragments from the OP 'Dorata di Parma': OPA-01, OPA-13, OPA-16, OPA-17, OPA-18, OPA-19, OPA-20; OPB-08, OPB-10, OPB-11, OPB-12, OPB-15, OPB-16, OPB-17, OPB-18, OPB-20; OPM-01, OPM-02, OPM-03, OPM-04. Those revealing the highest polymorphism of markers were used for a further analysis of variability carried out for the other genotypes. Only reproducible RAPD bands were scored by their presence/absence in the electrophoretic profile. Expected heterozygosity was calculated from the observed RAPD bands including both monomorphic and polymorphic loci. Allelic frequencies (f1 and f2) were calculated assuming the dominant (present)/recessive (absent) nature of the RAPD markers as $H = 1 - (f1^2 + f2^2)$.

Results

Field trail

Visual observation of the plants grown under field conditions revealed that the gynogenic and S_1 material had a higher homogeneity than the OP population of 'Dorata di Parma'.

Statistical analyses of the most important morphological traits are summarized in Tables 1 and 2. The

analysis of variance revealed highly significant differences among the three genotypes for the mean of polar diameter, shape index and bulb weight (Table 1). The equatorial diameter was the only character for which the three means differed, and only at $P \leq 0.05$.

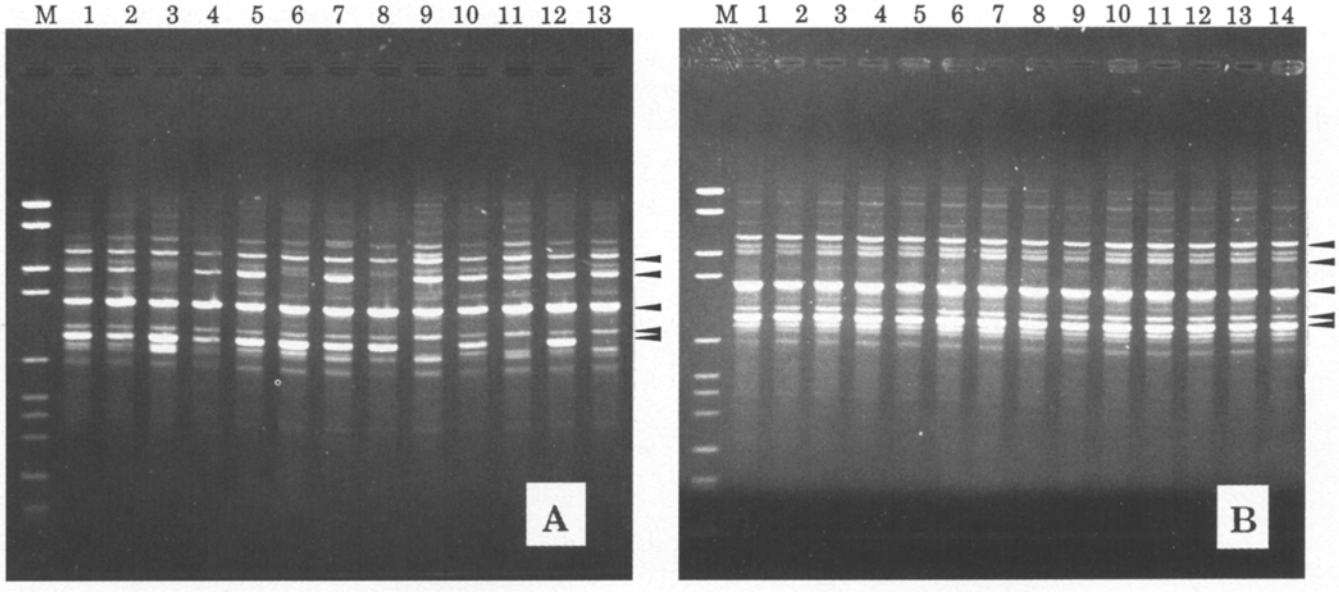
Of the three genotypes tested, the mean and the SD of the DH line were the lowest for all four traits considered (Table 2). In the case of bulb weight, S_1 and 'Dorata di Parma' did not statistically differ at the $P \leq 0.01$ level.

Table 1 Intergenotypical variance among the means of four morphological traits evaluated in an OP population of 'Dorata di Parma' and in S_1 and DH gynogenic lines

	Polar diameter	Equatorial diameter	Shape index	Weight of bulbs
Mean square	1.468	0.667	0.028	1,579
F values	35.41**	9.04*	132.35**	33.89**

*,** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

Fig. 1A, B RAPD amplification products for primer OPA-19. **A** Thirteen bulbs originated from open-pollinated 'Dorata di Parma; **B** fourteen bulbs originated from DH line from 'Dorata di Parma'. *Lane M* contains size markers. Arrowheads indicate scorable bands



RAPD and isozyme analysis

The 4 primers OPA-19 (5'CAAACGTCGG'3), OPA-20 (5'GTTGCGATCC'3), OPB-11 (5'GTAGACCCGT'3) and OPM-02 (5'ACAACGCCTC'3) revealed the highest level of polymorphism in the OP 'Dorata di Parma'.

Primers OPA-20, OPA-19, OPB-11 and OPM-02 generated 5, 7, 8 and 8 scorable amplified PCR products, respectively. Figure 1 shows the RAPD bands generated by OPA-19 in the OP 'Dorata di Parma' and in the DH line. In the OP line, 3 out of 5 amplified products of OPA-19 were polymorphic, 4 out 7 for OPA-20, 5 out of 8 for OPB-11 and 4 out of 8 for OPM-02. The DH and the haploid line were homomorphic when several bulbs of the two genotypes were scored with RAPD markers or were analysed on the basis of esterase alleles.

At the Esterase locus *Est-1*, the alleles $Est\text{-}1^1$ and $Est\text{-}1^2$ were present. In the OP 'Dorata di Parma', allele $Est\text{-}1^{1/1}$ was present in 3 bulbs and $Est\text{-}1^{2/2}$ in 19 bulbs; 11 bulbs were heterozygous, showing the banding pattern of both alleles $Est\text{-}1^{1/2}$. The two gynogenic lines tested were homozygous at the *Est-1* locus: the DH extracted from 'Dorata di Parma' had the allele $Est\text{-}1^{1/1}$, while the haploid line from 'Senshyu Yellow' had the allele $Est\text{-}1^2$.

Results of RAPDs and esterase analysis are presented analytically in Table 3.

Table 2 Mean and standard deviation of four morphological traits evaluated in three genotypes

	Genotype	Polar diameter	Equatorial diameter	Shape index	Weight of bulbs
Mean[a]	Dorata di Parma	4.21 a	5.12 a	0.83 b	88.4 a
	S_1	3.87 a	4.54 ab	0.87 a	61.6 b
	DH line	3.03 b	4.33 b	0.71 c	49.6 b
Standard deviation	Dorata di Parma	0.784	0.940	0.093	37.73
	S_1	0.682	0.868	0.111	27.38
	DH line	0.509	0.766	0.060	18.62

[a] Values not sharing a common letter are significantly different at $P \leq 0.01$

Table 3 Frequencies of RAPD and isozyme markers and heterozygosity estimations in three genotypes: the OP 'Dorata di Parma', the DH line originated from 'Dorata di Parma' and the micropropagated haploid line originated from 'Senshyu Yellow'

Locus defined by specific markers	OP Dorata di Parma			DH Dorata di Parma[a]		Haploid Senshyu Yellow[a]	
	Marker Present	Absent	H[b]	Marker Present	Absent	Marker Present	Absent
A19-1	31	0	0.000	31	0	17	0
A19-2	25	6	0.493	0	31	17	0
A19-3	30	1	0.295	31	0	17	0
A19-4	31	0	0.000	31	0	17	0
A19-5	20	11	0.482	31	0	0	17
A20–1	22	8	0.499	31	0	17	0
A20-2	24	6	0.494	31	0	17	0
A20-3	30	0	0.000	31	0	17	0
A20-4	27	5	0.478	31	0	17	0
A20-5	32	0	0.000	31	0	17	0
A20-6	32	0	0.000	31	0	17	0
A20-7	29	3	0.425	0	31	0	17
B11-1	33	0	0.000	31	0	17	0
B11-2	32	1	0.287	31	0	17	0
B11-3	33	0	0.000	31	0	17	0
B11-4	5	28	0.146	0	31	17	0
B11-5	27	6	0.489	31	0	17	0
B11-6	33	0	0.000	31	0	17	0
B11-7	1	32	0.030	0	31	0	17
B11-8	9	24	0.251	0	31	0	17
M2-1	5	28	0.146	0	31	0	17
M2-2	33	0	0.000	31	0	17	0
M2-3	22	11	0.488	31	0	0	17
M2-4	17	16	0.423	31	0	0	17
M2-5	33	0	0.000	31	0	17	0
M2-6	13	20	0.344	0	31	0	17
M2-7	33	0	0.000	31	0	17	0
M2-8	33	0	0.000	31	0	17	0
Est-1 Alleles 1/1 1/2 2/2				1/1 1/2 2/2		1/1 1/2 2/2	
Frequency 3 11 19			0.333	35 0 0		0 0 17	
Sum			0.199				

[a] All values of H were 0.000
[b] H was calculated as described in Materials and methods

The calculated value of heterozygosity for the OP 'Dorata di Parma' was 0.199, while the two gynogenic lines did not show any sign of heterozygosiy.

Discussion

Morphological analysis of field performance showed that the OP 'Dorata di Parma' and the derived gynogenic DH line were statistically different for most important agronomical traits considered. A progressive decrease in plant vigour, based on the mean values of morphological traits, and from the OP 'Dorata di Parma' to the DH line was expected. However, the loss of bulb weight in the homozygous line was within tolerable limits, and therefore such a line could be easily used for breeding purposes. The degree of variability found within each genotype was measured by SD values. For all traits, the DH line showed the lowest SD values, indicating the highest degree of morphological homogeneity. The standard deviation of the equatorial diameter values of the three genotypes was quite similar. This was expected, since this character is known to be influenced by environmental conditions (Dowker and Fennell 1974; McCollum 1971).

The results obtained by the biochemical and molecular analyses supported those obtained in the field trial. RAPD profiles and the frequency of esterase alleles of the DH line were uniform among all bulbs analysed, indicating that the DH line is genetically stable after selfing (R_2 progeny).

RAPD analysis of the OP cultivar 'Dorata di Parma' revealed the existence of high polymorphism. The estimated heterozygosity (H) was in fact equal to 0.199. Wilkie et al. (1993) measured the degree of polymorphism among six *Allium cepa* cultivars using 6 primers that generated 57 scorable bands. Among these bands, only 7 were polymorphic, indicating a lower variability among their onion cultivars than that found among individuals of the OP 'Dorata di Parma'. Comparison between the two RAPD profiles generated with the primers OPA-19 and OPA-20 that were common in the two experiments, that of Wilkie et al. (1993) and the one here described, showed more bands in our PCR banding patterns.

Results described for some plant species, but particularly for tobacco (Kumashiro and Oinuma 1985; Werns-

man 1992), show how haploids that originate from female gametopyte have fewer 'genetic penalties' than those originating from microspores. Furthermore, the gynogenic process induced in onion does not pass through a phase of proliferation of the callus but occurs through direct plant formation from a reduced cell of the embryo sac (Campion and Alloni 1990; Compion et al. 1992). On the basis of these considerations it is possible to predict that a high genetic stability is a characteristic of most gynogenically produced onion DH lines.

Although our investigation was limited to two lines, we could demonstrate, for the first time in onion, the obtaining of homozygous gynogenic lines that remain genetically stable after selfing and that have acceptable agronomical characteristics.

Acknowledgements The authors thank Prof. F. Salamini for critical revision of the manuscript. This research was supported for one-third by the Italian 'Ministero per le Risorse Agricole, Alimentari e Forestali' in the framework of the research project 'Development of Advanced Technologies in Agricultural Plants'.

References

Brown PTH, Lange FD, Kranz E, Lörz H (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Mol Gen Genet 237:311–317

Campion B, Alloni C (1990) Induction of haploid plants in onion (*Allium cepa* L.) by in vitro culture of unpollinated ovules. Plant Cell Tissue Org Cult 20:1–6

Campion B, Azzimonti MT (1988) Evolution of ploidy level in haploid plants of onion (*Allium cepa* L.) obtained through in vitro gynogenesis. In: Riggs TJ, Astley D, Brewster JL, Dawson PR, Maude RB (eds) Proceedings of the Eucarpia 4th Allium Symp. Institute of Horticultural Research, Wellesbourne Warwick, 85–89

Campion B, Azzimonti MT, Vicini E, Schiavi M, Falavigna A (1992) Advances in haploid plant induction in onion (*Allium cepa* L.) through in vitro gynogenesis. Plant Sci 86:97–104

Campion B, Perri E, Azzimonti MT, Vicini E, Schiavi M (1994) Gynogenic lines of onion (*Allium cepa* L.): spontaneous and induced chromosome doubling. Plant breed (in press)

Cecchini E, Natali N, Cavallini A, Durante M (1992) DNA variation in regenerated plants of pea (*Pisum sativum* L.). Theor Appl Genet 84:874–879

D'Amato F (1985) Cytogenetics of plant cell and tissue cultures and their regenerates. In: Conger BV (Ed) CRC Critical Reviews in Plant Sciences, vol 3, Issue 1. CRC Press, Inc. Boca Raton, Fla., pp 73–112

Doré C, Marie F (1993) Production of gynogenetic plants of onion (*Allium cepa* L.) after crossing with irradiated pollen. Plant Breed 111:142–147

Dowker BD, Fennell JFM (1974) Heritability of bulb shape in some North European onion varieties. Ann Appl Biol 77:61

Evans DA, Sharp WR, Medina-Fihlo HP (1984) Somaclonal and gametoclonal variation. Am J Bot 71:759–774

Finnie SJ, Forster BP, Chalmers KJ, Dyer AF, Waugh R, Powell W (1991) Genetic stability of microspore-derived doubled haploids of barley: a cytological, biochemical, and molecular study. Genome 34:923–928

Jones HA, Davis GN (1944) Inbreeding and heterosis and their relation to the development of new varieties of onions. US Dep Agric Tech Bull 874:1–28

Keller J (1990) Culture of unpollinated ovules, ovaries, and flower buds in some species of the genus *Allium* and haploid induction via gynogenesis in onion (*Allium cepa* L). Euphytica 47:241–247

Kumashiro T, Oinuma T (1985) Comparison of genetic variability among anther-derived and ovule-derived doubled haploid lines of tobacco. Jpn J Breed 35:301–310

Loaiza-Figueroa F, Weeden N (1991) Effects of seed increase procedures on isozyme polymorphism in *Allium*. FAO/IBPGR Plant Genet Resources Newsl 83/84:1–3

McCollum GD (1971) Heritability of onion bulb shape and size: estimates from half-sib families. J Hered 62:101

Muren R (1989) Haploid plant induction from unpollinated ovaries in onion. HortScience 24:833–834

Murigneux A, Barloy D, Leroy P, Beckert M (1993) Molecular and morphological evaluation of doubled haploid lines in maize. 1. Homogeneity within DH lines. Theor Appl Genet 86:837–842

Oono K (1981) In vitro methods applied to rice. In: Thorpe TA (ed) Plant tissue culture, Academic Press, New York, pp 273–298

Peffley EB, Mangum PD (1990) Introgression of *A. fistulosum* L. into *A. cepa* L.: cytogenetic evidence. Theor Appl Genet 79:113–118

Sabir A, Newbury HJ, Todd G, Catty J, Ford-Lloyd BV (1992) Determination of genetic stability using isozymes and RFLPs in beet plants regenerated in vitro. Theor Appl Genet 84:113–117

Saghai Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018.

Singsit C, Ozias-Akins P (1993) Genetic variation in monoploids of diploid potatoes and detection of clone specific random amplified polymorphic DNA markers. Plant Cell Rep 12:144–148

Smith BM, Godwin RM, Harvey E, Werner CP (1991) Gynogenesis from whole flower buds in bulb onions (*Allium cepa* L.) and leeks (*Allium porrum* L.). J Genet Breed 45:353–358

Wendel JF, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Dioscorius Press, Portland, Ore., pp 5–45

Wernsman E (1992) Varied roles for the haploid sporophyte in plant improvement. In: Stalker HT, Murphy JP (eds) Plant breeding in the 1990s. C.A.B. Int, Wallingforde, UK, pp 461–484

Wilkie S, Isaac P, Slater R (1993) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor Appl Genet 86:497–504

Ziauddin A, Kasha KJ (1990) Genetic stability in haploid cell cultures. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 12: haploids in crop improvement. Springer, Berlin Heidelberg New York, pp 83–98