Genetics and Molecular Biology, 28, 3, 423-430 (2005)
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Research Article

Somaclonal variation in *Asparagus officinalis* plants regenerated by organogenesis from long-term callus cultures*

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Abstract

Somaclonal variation in plants regenerated by organogenesis from long-term cultured calluses of two diploid staminate genotypes of *Asparagus officinalis* cv. Argenteuil was characterized by plant phenotype, ploidy, meiotic behavior, pollen viability, fruit and seed set, and AFLP profiles. Phenotypic deviations from the donors were detected in foliage color, flower size, and cladode and flower morphology. Ploidy changes were observed in 37.8% of the 37 regenerants studied. Meiotic alterations in 12 out of 21 regenerants included laggards, dicentric bridges, micronuclei, restitution nuclei and polyads. Of the 408 AFLP markers screened in 43 regenerants and the donors, 2.94% showed polymorphism. High pollen viability was observed in the 22 regenerants analyzed. All crosses between one pistillate plant and 35 regenerants, as well as the controls, produced fruits and seeds; however, no plump seeds resulted in 35.3% of the crosses with regenerants, and no seeds germinated in 12.5% of those with apparently normal seeds. Fruit and seed set was similar in crosses with diploid regenerants with normal meiosis and the controls but was lower in crosses with diploid and polyploid regenerants with abnormal meiosis. Our results show that the regenerated plants exhibited conspicuous somaclonal variation that could be eventually exploited for *in vitro* selection systems.

Key words: AFLP, fertility, plant morphology, meiotic behavior, ploidy.

Received: October 14, 2004; Accepted: April 12, 2005.

Introduction

Garden asparagus (*Asparagus officinalis* L.) is a vegetable of high economic value. The main cause of asparagus crop decline in the world is the disease known as ‘crown and root rot’, caused by *Fusarium* spp. (Farr *et al*., 1989). Recovery of resistant asparagus cultivars through conventional breeding is hindered because *Fusarium* is ubiquitous in the soil, asparagus is perennial and dioecious, and disease resistance is subject to polygenic control (Lassaga *et al*., 1998). The application of *in vitro* selection techniques has facilitated the generation of disease resistant plants in other pathosystems (Crinò, 1997; Remotti, 1998). Many of these techniques exploit somaclonal variation that can arise or be detected during *in vitro* culture (Larkin and Scowcroft, 1981). However, before applying *in vitro* selection it is essential to identify the conditions which favor somaclonal variation and also to characterize such variation.

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Somaclonal variation in asparagus has mainly been investigated as an undesired phenomenon that may arise during mass propagation. For example, Kunitake *et al*. (1998) studied ploidy level and morphological variations while Raimondi *et al*. (2001) used a similar approach along with meiotic analysis and random amplified polymorphic DNA (RAPD) profiling to characterize somaclonal variation in plants regenerated by somatic embryogenesis. Both studies reported a low frequency of somaclones, presumably due to the mode of plant regeneration used, since embryogenesis is thought to involve stringent internal controls that may cause selection pressure against genetic changes (Swedlund and Vasil, 1985). In this sense, it has been suggested that organogenesis would favor the occurrence of somaclonal variation (Duncan, 1997).

In our laboratory we have developed an *in vitro* system consisting of long-term asparagus callus culture and plant regeneration through organogenesis to use in selection programs. The aim of the present study was to use asparagus phenotype, ploidy and fertility levels, meiotic behavior, and amplified fragment length polymorphism (AFLP) profiles of asparagus plants regenerated through

this protocol to induce and characterize somaclonal variation that could be potentially selectable *in vitro*.

Materials and Methods

Plant material

The explant tissue culture donors were two staminate clones (genotypes) of *Asparagus officinalis* L. cultivar (cv) Argenteuil, identified as clone numbers 265 and 357, that had been selected in a local breeding program for yield and spear tip compactness under high temperature conditions at harvest. Pistillate plants of a diploid population were used in crosses with clones 265 and 357 and their regenerants; pollen from staminate plants of the population was pooled and used in control crosses with the same pistillate plants.

In vitro culture

Spear sections about 2 mm long and devoid of lateral buds were excised from micropropagated 265 and 357 plants which were more than a year old and placed in tubes containing 7 mL of callus growth medium (CGM), consisting of Murashige and Skoog's (1962) callus growth basal medium (MS medium) supplemented with 30 gL^{-1} sucrose, 9 gL^{-1} agar, 4.65 µM kinetin and 6.79 µM of 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were kept in the dark at 26 °C and subcultured every 45 days. After nineteen months the callus sections were placed in tubes containing 7 mL of shoot primordia induction-shoot regeneration medium (SRM) consisting of MS salts supplemented with 5 mgL^{-1} of 2-isopentenyladenine (2iP), 1 mgL^{-1} indolacetic acid (IAA), 30 gL^{-1} sucrose and 5 gL^{-1} agar. After 90 days of culture in the dark at 26 °C, calluses with shoot primordia were cultivated for 90 days at 26 °C and a 16-h photoperiod in tubes containing 7 mL of shoot and mini-crown growth medium (MCM) consisting of MS salts supplemented with 1 mgL^{-1} each of thiamine, nicotinic acid, pyridoxin, calcium pantothenate and glycine, 100 mgL^{-1} myoinositol, 0.01 mgL^{-1} biotin, 0.2 mgL^{-1} kinetin, 0.2 mgL^{-1} α-naphthalenacetic acid (NAA), 0.1 mgL^{-1} benzylaminopurine (BA), 0.1 mgL^{-1} ancymidol, 30 gL^{-1} sucrose and 7 gL^{-1} agar. Developed shoots and minicrowns were transferred to tubes containing 7 mL of rooting medium (RM) consisting of MS salts supplemented with 0.04 mgL^{-1} thiamine, 1.32 mgL^{-1} ancymidol, 0.1 mgL^{-1} kinetin, 0.1 mgL^{-1} NAA, 70 gL^{-1} glucose and 8 gL^{-1} agar. Donor clones 265 and 357 were also micropropagated from axillary buds using appropriate media (*i.e.* SRM, MCM and RM sequentially). Plants with healthy and well-developed roots were transplanted to a sterilized mixture of soil, peat moss and perlite (3:1:0.5, v/v/v) and placed in a greenhouse at 26 °C under a 16-h photoperiod.

Plant phenotype

Plant height, cladode length and shape, foliage color, and flower morphology and size were compared with those of the respective donor clones and recorded for 38 regenerants (35 from clone 265 and three from clone 357).

Cytogenetic studies

For chromosome counts, spear tips were excised from 3 cm-long spears and processed by the method of Raimondi *et al*. (2001). Meiotic studies were carried out based on the methodology of Camadro (1992), with various flowers and stamens per flower being analyzed for each genotype.

Pollen viability

Pollen samples were collected and stored in gelatin capsules at 4 °C for the crossing work. An indirect estimation of viability was made shortly after collection by staining pollen from each sample with 2% w/v acetocarmine in 1:1 acid:glycerol solution (Marks, 1954) and using optical microscopy to determine the percentage of round, well-stained pollen in 200 pollen grains per sample.

Crosses

In two seasons, two pistillate plants of the diploid population grown in the field were pollinated with stored pollen. Thirteen flowers were pollinated per genotypic combination. Forty-eight hours after pollination, three of the 13 pollinated pistils were removed and fixed in formaldehyde/acetic acid solution (FAA, containing 8:1:1 (v/v/v) 70% ethanol: glacial acetic acid: 40% formaldehyde); the other 10 pollinated pistils were left *in situ* for fruit and seed set. Fixed pistils were processed in 8N NaOH for 4 h and thoroughly rinsed with tap water (Martin, 1958) before being mounted in a drop of glycerin on a glass slide for examination by ultraviolet (UV) microscopy. Pollen/pistil compatibility relations were determined for all genotypic combinations, *i.e.* two pistillate diploid plants pollinated by (a) the donor clones, (b) a 'pool' of pollen from the diploid population and (c) 35 regenerants. Ripened fruits were harvested and seeds recovered and stored at 4 °C. Seeds were sown in 100-mm-diameter Petri dishes, in a growth chamber at 26 °C under a 16-h photoperiod and the percentage of germination recorded 15 days later.

AFLP analysis

The method of Dellaporta *et al*. (1983) was used to extract DNA from tissue samples of the regenerants (34 derived from clone 265 and nine from clone 357) and their respective donor clones. The AFLP analysis was performed according to the method of Reamon-Büttner *et al*. (1998) except that for each sample 500 ng of DNA was digested for 3 h at 37 °C with 5 U *Eco*RI and 5 U *Mse*I. Amplification reactions were carried out with several combinations of primers with three selective nucleotides; reactions with selected primer combinations (E7-M20, E8-M17, E9-M18, E10-M22, E11-M16 and E12-M16) were duplicated (Table 1). Ten µL of tracking dye (98% formamide, 10 mM

EDTA pH 8.0, xylene cyanol and bromophenol blue) were added to each PCR reaction. Amplicons were denatured at 94 °C for 5 min and separated using 0.35 mm-thick 5% vertical polyacrylamide gel electrophoresis, the gels being run using 1X TBE at a constant 50 °C and 55-60W for approximately 2 h and stained with silver nitrate (Silver Sequence Staining Reagents; Promega). Permanent positive images of the gels were made using automatic processor compatible APC Film (Promega) and the images scored visually, with intense bands only being included in the analysis.

Results

Plant phenotype

Foliage color was green for all the regenerants except one derived from clone 357, which was greenish blue (glaucous) (Table 2). Aberrant flowers with a higher than normal number of stamens (some of which were adhered to the tepals, some tepals also being fused with the terminal cladodes) occurred in two diploid, one triploid and two tetraploid regenerants derived from clone 265. Flower size was much larger in the tetraploid regenerants as compared with the other regenerants and the donor clones.

Cytogenetic studies

Both donor clones and 23 out of 37 of the regenerants were diploid (2n = 2x = 20), although one mixoploid (20-30 chromosomes), four triploid and nine tetraploid regenerants also occurred (37.8%). Although floral buds were fixed from 38 regenerants and the donor clones (Table 2), many of them were not at an appropriate stage to study. Consequently, meiosis was studied in the donor clones and in 21 regenerants: 14 diploids, 13 from clone 265 and one from clone 357; two triploids from clone 265; and five tetraploids, four from clone 265 and one from clone 357. Meiosis was normal in both donor clones (not shown) and in nine out of the 14 diploid regenerants. The remaining regenerants exhibited several meiotic abnormalities (Table 2) such as laggards and dicentric bridges in meiosis I and/or II (Figure 1a) and bivalent migration in anaphase II and micronucleated sporads (2.9-38.1%) were also observed (Figure 1b).

Lack of cytokinesis in meiosis II in one or both cells was observed in two regenerants, along with triads in regenerant 8-I derived from callus 10 of clone 265, and with dyads, triads and monads in regenerant 6-I derived from callus 64 of clone 265. In regenerant 2-I (derived from callus 46 of clone 265) chromatid segregation occurred after prophase II and meiosis ceased prematurely at this point in 64.5% of meiocytes, resulting in a high percentage (52.7%) of dyads (Figure 1c). Another meiotic abnormality, *i.e.* sporads with five to seven microspores ('polyads'), was detected in seven regenerants (Table 2; Figure 1d). Microspores contained in these abnormal sporads were of heterogeneous size and frequently exhibited micronuclei.

Pollen viability

Pollen viability was 90% in both donor clones and oscillated between 60 and 90% in the regenerants (nine diploids, one triploid and four tetraploids; Table 2).

Crosses

All cross-pollinations were compatible, although fruit and seed set was low even when the pollinators were the donor clones or the plants from the population (control). This was attributed to the fact that the shoots of the pistillate plants were close to senescence, so these crosses were repeated in spring. All crosses set fruits and seeds; however, no plump seeds (an indication of seed viability) were obtained in 35% of crosses involving regenerants, and no seeds germinated in 12.5% of plants that produced apparently normal seeds (data not shown). Fruit and seed set was, on average, similar in control crosses and crosses with diploid regenerants with normal meiosis (5 to 6 fruits per plant, and 3-3.41 seeds/fruit) but it was lower in crosses with diploid regenerants with abnormal meiosis (3.4 fruits per plant, 2.5 seeds/fruit), and even lower in crosses with polyploid regenerants, all of which had abnormal meiosis (2.2 fruits per plant, 2.2 seeds/fruit) (Table 3). The percentage of seed germination followed a similar tendency, except that (as compared to the controls) it was also reduced in the crosses with diploid regenerants with normal meiosis (Table 3).

AFLP analysis

Our AFLP analysis of the donor clones and 43 regenerants (34 derived from clone 265 and nine from

Table 1 - Primers tested in the amplified fragment length polymorphism (AFLP) analysis of clones 265 and 357 and their respective regenerants.

Denomination[a]	Sequence
E7	5' GACTGCGTACCAATTCAAA 3'
E8	5' GACTGCGTACCAATTCAAC 3'
E9	5' GACTGCGTACCAATTCAAG 3'
E10	5' GACTGCGTACCAATTCACC 3'
E11	5' GACTGCGTACCAATTCAGA 3'
E12	5' GACTGCGTACCAATTCAGG 3'
E13	5' GACTGCGTACCAATTCATC 3'
E14	5' GACTGCGTACCAATTCATT 3'
M15	5' GATGAGTCCTGAGTAACAA 3'
M16	5' GATGAGTCCTGAGTAACAT 3'
M17	5' GATGAGTCCTGAGTAACCA 3'
M18	5' GATGAGTCCTGAGTAACCG 3'
M19	5' GATGAGTCCTGAGTAACCT 3'
M20	5' GATGAGTCCTGAGTAACGC 3'
M22	5' GATGAGTCCTGAGTAACTC 3'

[a] E = *Eco*RI + 3 primer; M = *Mse*I + 3 primer.

Table 2 - Phenotypic deviation from the donor clone, ploidy, meiotic behavior and pollen staining in regenerants derived from clones 265 and 357.

Donor clone	Callus N.	Regenerant	PD[a]	Ploidy (2n)	Meiotic behavior				Stained pollen (%)
					Type	Stage	Abnormalities		
							Type and % meiocytes[b]	N[c]	
265	04	1-I	none	2x = 20	normal[d]				-[e]
		8-C	none	3x = 30	abnormal	meiosis I meiosis II sporad	1 (46.6); 2 (11.4) 1 (70.4); 2 (18.5) 3 (38.1); 4 (8.8); 5 (19.5)	88 27 113	-
	05	2-C	none	2x = 20	normal				≈70
	10	7-C	none	3x = 30	-				-
		5-I	none	20-30	-				-
		8-I	1	4x = 40	abnormal	sporad	3 (38); 4 (4.8); 5 (26.4); 6 (2.9); 7 (6.3)	208	>90
	18	5-C	none	2x = 20	abnormal	sporad	3 (7.4)	136	≈90
		8-C	1	4x = 40	-				>90
	25	7-I	none	2x = 20	-				≈60
	26	5-I	none	2x = 20	normal				>90
		7-I	none	2x = 20	abnormal	meiosis I	1 and 2 (% not determined)	50	≈80
	35	5-C	none	3x = 30	-				≈70
		1-C	1	4x = 40	-				>90
	45	3-C	none	2x = 20	normal				-
		5-C	none	2x = 20	-				-
		5-I	none	2x = 20	normal				>90
	46	1-C	2	2x = 20	abnormal	sporad	3, 4, 5, 6 and 8 (% not determined)	50	>90
		2-C	none	2x = 20	abnormal	meiosis II	1 (5)	152	>90
		6-C	none	2x = 20	normal				>90
		8-C	1	4x = 40	-				-
		2-I	1	4x = 40	abnormal	meiosis I meiosis II sporad	1 (50); 2 (5) 1 (7.5); 2 (1); 9 (6.5); 10 (64.5) 3 (20); 4 (3.4); 5 (10.7); 8 (52.7)	20 93 205	-
		5-I	none	2x = 20	abnormal	meiosis II	1 and 2 (% not determined)	50	-
	47	8-C	2	3x = 30	abnormal	sporad	4 (6)	150	-
		7-I	1	-	-				-
	55	2-I	none	2x = 20	normal				>90
	56	2-C	none	2x = 20	normal				>90
		4-C	none	2x = 20	-				-
		5-C	none	2x = 20	-				-
		6-C	none	2x = 20	-				-
		6-I	none	2x = 20	-				>90
		8-I	none	2x = 20	-				>90
	64	4-I	1	4x = 40	-				≈70
		6-I	1, 2, 3	4x = 40	abnormal	meiosis I meiosis II sporad	1 (44.6) 1 (29.8); 7 (24.5) 3 (2.9); 5 (2); 6 (3.9); 8 (31.4); 11 (2)	56 57 102	-
	66	7-C	2	2x = 20	-				-
		8-I	1, 2, 3	4x = 40	abnormal	sporad	3 (12.7); 4 (6.1); 5 (24.6)	212	-
357	11	3-C	1, 4	4x = 40	abnormal	sporad	3 (12.9); 4 (5.7); 5 (19.2)	209	-
	17	6-I	none	2x = 20	normal				>90
		7-I	none	2x = 20	-				>90

[a]PD = Phenotypic deviations from the donor clone: 1 = larger flower; 2 = aberrant flowers; 3 = aberrant cladodes; 4 = glaucous foliage. [b]Meiotic abnormalities (with % of abnormal meiocytes between parenthesis): 1 = laggards; 2 = dicentric bridges; 3 = micronuclei; 4 = polyads; 5 = polyads and micronuclei in the same meiocyte; 6 = triads; 7 = chromosome segregation without further cytokinesis II (in one or both cells); 8 = dyads; 9 = bivalent migration in anaphase II; 10 = chromatid separation after prophase II and premature end of meiosis; 11 = monads. [c]N = number of meiocytes analyzed. [d]At least 100 meiocytes analyzed in each meiotic stage. [e]Sample not analyzed.

clone 357) resulted in a total of 408 amplified fragments; the number of fragments per plant and primer combination varied from 61 to 84 with an average of 68. All plants analyzed shared 279 fragments (68.38%), while another 117 fragments (28.68%) were polymorphic between donor clones. Clone 265 and its derived regenerants shared 51 fragments that were absent in the other plants, while 66 fragments appeared exclusively in clone 357 and its derived regenerants. The remaining 12 fragments (2.94%) showed variability between eight of the regenerants (six de-

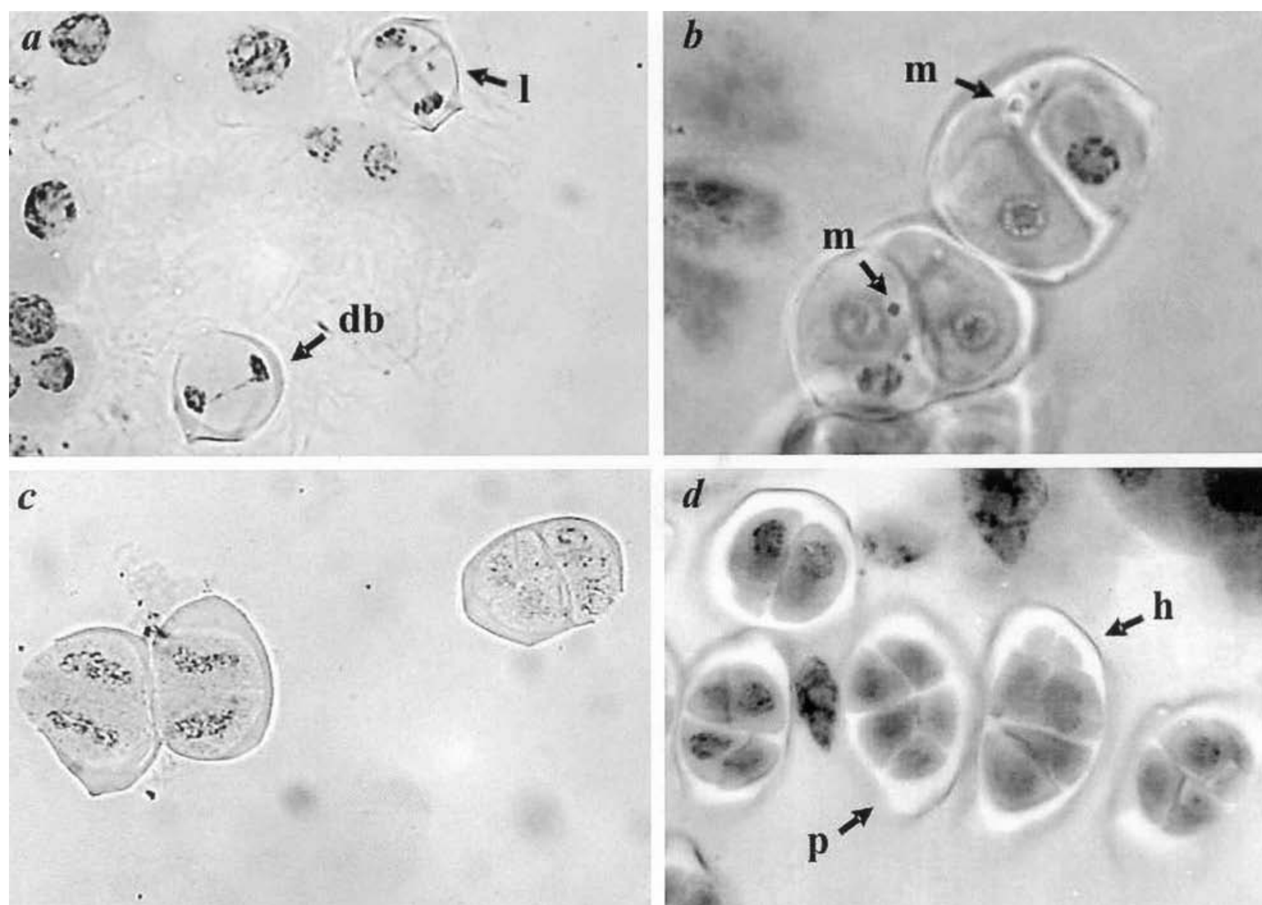

Figure 1 - Meiotic abnormalities of regenerants derived from calluses of clones 265 and 357. (*a*) Regenerant 5-I derived from callus 46 of clone 265, laggard (l) in cytokinesis I and dicentric bridge (db) in telophase I. (*b*) Regenerant 3-C derived from callus 11 of clone 357, sporads with micronuclei (m). (*c*) Regenerant 2-I derived from callus 46 of clone 265, two sporads with chromatid segregation in prophase II and premature end of meiosis (left) and normal tetrad (right). (*d*) Regenerant 8-I derived from callus 66 of clone 265, pentad (p) and hexad (h).

Table 3 - Fruit, seed set and percentage seed germination for clones 265 and 357 and their derived regenerants classified according to ploidy and meiotic behavior.

Genotype	Meiotic behavior	N.[a]	N. fruits[1]	N. seeds[2]	N. seeds/fruit	N. plump seeds[3]	N. plump seeds/fruit	N. germinated seeds[4]	%G[b]
'265'	normal	1	6	19	3.17	14	2.33	10	71.4
'357'	normal	1	5	15	3	11	2.2	8	72.7
2x regenerants	normal	8	5.125 (4, 4, 8, 4, 9, 5, 2, 5)	17.5 (16, 15, 25, 12, 37, 17, 5, 13)	3.41	10.25 (10, 13, 14, 6, 14, 12, 5, 8)	2	5.375 (2, 9, 5, 4, 8, 8, 2, 5)	52.44
	abnormal	5	3.4 (3, 6, 3, 3, 2)	8.4 (7, 14, 14, 5, 2)	2.47	5.8 (7, 9, 11, 2, 0)	1.7	2.4 (7, 0, 5, 0, 0)	41.38
3x and 4x regenerants	abnormal	6	2.17 (3, 2, 1, 2, 3, 2)	4.83 (5, 8, 4, 1, 6, 5)	2.23	0.5 (1, 0, 0, 0, 1, 1)	0.23	0.17 (1, 0, 0, 0, 0, 0)	34

[a]Number of genotypic combinations. [1,2,3,4]Average; between parenthesis are the values of each genotypic combination. [b]%G = % seed germination, based of the number of plump seeds.

rived from clone 265 and two from clone 357) and their respective donor clones (Table 4). None of these polymorphisms was shared by regenerants derived from different donor clones. Only two regenerants derived from the same callus (regenerants 6-I and 7-I, derived from callus 17 of clone 357) exhibited the same polymorphism in comparison with the donor clone (Table 4).

Discussion

Our results reveal conspicuous somaclonal variation in asparagus plants regenerated by organogenesis from long-term callus cultures. Phenotypic variants included aberrant flowers and cladodes, larger flower size and glaucous foliage. Flower abnormalities were similar to those found by Chang and Hung (1982) in terminal flowers of cv. UC 309 but in our case they occurred in all the flowers of a plant, indicating that they could be the result of mutations or modifications in the expression of the gene(s) involved in flower development. The increase in flower size in all tetraploid regenerants with respect to the donor clones and the rest of regenerants was probably due to the higher ploidy level, as has been widely observed in other species and organs (Blakeslee, 1941). The phenomenon of ploidy variation in plants regenerated from callus, observed in the present work, has previously been reported in asparagus (Kunitake *et al*., 1998; Raimondi *et al*., 2001) and other species (Larkin and Scowcroft, 1981; Singh, 1993; Duncan, 1997; Gupta, 1998). The proportion of regenerants that differed in chromosome number from the respective donor plant (37.8%) is much higher than that reported in similar studies (Larkin and Scowcroft, 1981; Singh, 1993; Duncan, 1997; Gupta, 1998), which could be ascribed to the fact that we used older calluses and longer subculture intervals in order to maximize somaclonal variation. Alternatively or additionally, it is feasible that ploidy differences resided in the explant (preexistent variation), as polysomatic species such as asparagus can exhibit endoreduplication in certain cell types (Bhojwani and Razdan, 1996). This could be reinforced by the fact that the explants were obtained from a highly differentiated tissue composed of several cell types. In our study, the occurrence of conspicuous ploidy variation among regenerants contradicts the evidence that morphogenic calluses generally bear the same chromosome number as the donor genotype (Gupta, 1998) and the idea that plant regeneration constitutes a selective instance against the chromosome number deviations observed during the callus phase (Bhojwani and Razdan, 1996; Singh, 1993). However, in asparagus, this selection against ploidy variation among regenerants could take place in long-term (10-year-old) stabilized callus cultures as found by Reuther (1990).

The mode of plant regeneration can have a great influence on the level of somaclonal variation (Duncan, 1997). Kunitake *et al*. (1998) detected little ploidy and phenotypic variation among somaclones obtained through somatic embryogenesis from asparagus genotypes. Similar results were obtained by Raimondi *et al*. (2001) on research with the same two clones as those used in our study, finding only two plants that were phenotypically different from the donor clone, three with ploidy variation and abnormal meiosis, and a lack of intraclonal polymorphisms in the RAPD markers. The much lower proportion of somaclonal variants found by Raimondi *et al*. (2001) in relation to our study could be attributed to callus age, *i.e.* seven months in their study compared to 19 months in our study. However, it is also feasible that somatic embryogenesis operated as a selection mechanism against chromosome and gene alterations, as observed in other work with asparagus (Araki *et al.* 1992).

The laggards and dicentric bridges in meiosis I and II, bivalent migration in anaphase II and sporads with micronuclei observed in our study have frequently been found in somaclonal variation studies (Larkin and Scowcroft, 1981; Singh, 1993; Bhojwani and Razdan, 1996; Gupta, 1998). Chromosome mispairing, gross chromosome rearrangements, ruptures in heterochromatic regions and transposon

Table 4 - Amplified fragment length polymorphism (AFLP) markers polymorphic among regenerants and their respective donor clones (265 and 357).

Donor clone	Callus	Regenerant	Polymorphism type	Markers[a]
265	10	8-I	Absence	E10-M22-4, E10-M22-38, E9-M18-3, E9-M18-5, E9-M18-7, E9-M18-32, E9-M18-41
			Presence	E9-M18-6, E7-M20-13
	18	5-C	Absence	E10-M22-4, E10-M22-38, E9-M18-3, E9-M18-5, E9-M18-32
	26	7-I	Absence	E10-M22-4, E10-M22-38, E9-M18-5, E9-M18-32
	60	1-I	Absence	E9-M18-29
	64	4-I	Absence	E9-M18-3, E9-M18-32
357	17	6-I	Presence	E10-M22-41
		7-I	Absence	E7-M16-18
			Presence	E10-M22-41

[a]Markers are denominated with the primer combination used plus the order of the marker in the lane (the higher the number the lower the molecular weight).

activation, among other phenomena, have been related to these abnormalities (Peschke and Phillips, 1992; Kaeppler *et al*., 1998; Cassells and Curry, 2001, among others). Some authors have suggested that the above phenomena could derive from a direct or indirect effect of 2,4-D (given its mutagenic character), oxidative stress, changes in methylation patterns, and/or a combination of these and other factors. This could be the case in the present work, as 2,4-D and a long-term culture period were used in the callus stage.

The other meiotic abnormality found in the regenerants was the production of microspores with unreduced chromosome numbers and while meiosis was normal in both donor clones, dyads, triads and/or monads were observed in four regenerants derived from '265' (one diploid and three tetraploids) derived from clone 265. Camadro (1992) observed similar variants in *A. officinalis, A. densiflorus* and *A. plumosus*, and hypothesized that the tendency towards these types of meiotic abnormalities is under genetic control as it is in potato and alfalfa. In our study, microspores with unreduced chromosome number could have originated from a mutational phenomenon, transposon excision or insertion (as Chen *et al*. (2002) found in *Arabidopsis*) or modifications in the regulation of gene(s) involved in the meiotic process.

The formation of polyads that we detected in one diploid, two triploid and four tetraploid regenerants has not previously been reported for somaclones, although Caetano-Pereira and Pagliarini (2001) found a spontaneous maize mutant that produces similar polyads to those detected by us. Polyad formation has been also described in *Solanum commersonii* (+) *S. tuberosum* somatic hybrids (Conicella *et al*. 1997) and in two mutants of *Arabidopsis thaliana*, *tam* (Magnard *et al*. 2001) and *atk1-1* (Chen *et al*. 2002), the *atk1-1* polyads occurring as a result of defective spindles as a consequence of the insertion of a *Ds* element in the *ATK-1* gene. The diploid regenerant (1-C, derived from callus 46 of clone 265) had a high percentage of viable pollen and fruit and seed set and a high percentage seed germination (45.5%) similar to the controls; on the other hand, the remaining two triploid and four tetraploid regenerants had very low fruit and seed set and mostly empty seeds (data not shown). Pollen staining in the regenerant with the highest percentage of polyads (8-I, derived from callus 10 of clone 265) was 90%, but when these regenerants were used in crosses some pollen grains were functional but almost all seeds were empty, indicating that in this material staining was inadequate for indirectly estimating pollen viability. Although the occurrence of meiotic abnormalities negatively affected fruit and seed set (Table 3) progenies were obtained from many crosses, and this constitutes a positive result in the context of a breeding program.

Although performed on a reduced scale, AFLP analysis revealed polymorphisms between regenerants and their respective donor clones. Although AFLP analysis is not as widely used in this type of study as RFLP or RAPD, Polanco and Ruiz (2002) used it to detected somaclonal variation in *Arabidopsis* and Vendrame *et al*. (1999) in pecan nuts. In asparagus, Raimondi *et al*. (2001) found no intraclonal polymorphisms for RAPD in 77 regenerants obtained by somatic embryogenesis from the same clones as were used in our study, but Raimondi *et al*. (2001) used a smaller number of markers (157 as against 408 in our study) and, analogously to what was previously discussed regarding ploidy variation, differences in callus age and/or mode of regeneration of somaclones could account for the differences in the results.

Six out of the 12 markers exhibiting intraclonal polymorphism were absent in two to four regenerants depending on the marker (Table 4). Only one of these regenerants derived from the same callus (regenerants 6-I and 7-I derived from callus 17 of clone 357), suggesting that both plants could have originated from a group of cells that shared that mutation. The remaining cases could be explained by independent mutations in the same sequences or, alternatively, a unique mutational event could have occurred in one of the several micropropagated plants that were used as explant sources. Since the origin of the explants was not documented, it was not possible to ascertain whether or not the calluses from which these regenerants were derived were induced from adjacent explants or explants excised from the same spear.

It is also important to consider the results obtained using the different approaches for characterizing somaclonal variation as related to the callus from which each regenerant was obtained. Thirteen out of the sixteen calluses yielded more than one regenerant but in only two of these (calluses 45 and 56 of clone 265) were all regenerants diploid, had similar morphology and fruit and seed set as the donor clone and exhibited normal chromosome behavior. The regenerants obtained from the remaining 11 calluses showed variations in one to all of the factors analyzed (plant phenotype, ploidy, meiotic behavior, reproductive parameters and AFLP profile) as compared with other regenerants derived from the same callus. It is particularly important to consider this variation when regenerating plants from callus after *in vitro* screening for a certain trait because, according to our results, many calluses seem to exhibit a heterogeneous (epi)genetic and/or chromosomal constitution after long-term culture that can eventually be manifested in the regenerants.

The different approaches used by us in this study appear to be reliable and complementary tools for assessing somaclonal variation in asparagus. The conspicuous variability detected appears to be exploitable for *in vitro* selection programs.

Acknowledgments

This study was partially financed by the Consejo Nacional de Investigaciones Científicas y Técnicas

(CONICET) and the Universidad Nacional de Mar del Plata (UNMdP).

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Associate Editor: Márcio de Castro Silva Filho