

Microsporogenesis in a *Citrus* interspecific tetraploid somatic hybrid and its fusion parents

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Microsporogenesis was analysed in a tetraploid somatic hybrid (SH) ($2n = 4x = 36$) of *Citrus* and its diploid fusion parents ($2n = 2x = 18$), Valencia sweet orange (*C. sinensis* L. Osbeck) and Femminello lemon (*C. limon* L. Burm. f.). Intergenomic pairing between lemon and orange occurred in the somatic hybrid which showed multivalent chromosome associations in diakinesis, although one quadrivalent was definitely because of a reciprocal translocation present in Valencia. The behaviour of univalents was variable in the somatic hybrid and its parents. In the somatic hybrid and Valencia, the univalents preferentially formed micronuclei and polyads whereas, in Femminello, they were generally enclosed in a nucleus although distributed randomly. The somatic hybrid showed a rate of pollen stainability of 64% and germinability of 41%. The chromosomally unbalanced pollen from the tetraploid SH was presumed viable and able to fertilize because different nuclear DNA contents were found in the back-cross progeny. Moreover, meiotic nuclear restitution mechanisms, which could be mainly dependent on the abnormal orientation of the spindles in meiosis II, are described.

Keywords: lemon, meiosis, orange, somatic hybridization, unreduced pollen.

Introduction

Cultivar development in *Citrus* by conventional breeding methods has been limited by its complex reproductive biology (Swingle & Reece, 1967; Grosser & Gmitter, 1990a). Somatic hybridization was applied successfully in *Citrus* for combining complementary diploid elite scion varieties (Grosser & Gmitter, 1990a, 1990b). Allotetraploid somatic hybrids of *Citrus* do not have the potential for direct use as scions but, if fertile, can be used as pollen parents in interploid sexual hybridization to generate new and improved seedless triploid *Citrus* varieties (Grosser & Gmitter, 1990a). However, the relationship between the genomes of the parental species has great influence on determination of the process of chromosome pairing and recombination and thus the extent of meiotic irregularities and viability of the gametes (De Jong *et al.*, 1993). Indeed, somatic hybrids generally have a lower fertility, which is a major problem in sexual reproduction (Pijnacker *et al.*, 1992).

The present study was conducted in an interspecific tetraploid *Citrus* somatic hybrid between Valencia sweet orange (*C. sinensis* L. Osbeck) and Femminello lemon (*C. limon* L. Burm. f.) that was produced in an effort to combine the good quality and performance of Femminello lemon with the cold hardness and tolerance to 'mal secco' [a systemic fungal disease caused by *Phoma tracheiphila* (Petri) Kantsh et Ghik] from Valencia orange (Tusa *et al.*, 1990). The somatic hybrid Valencia (+) Femminello has already been used in interploid sexual hybridization with diploid lemon. Cytofluorimetric analysis of nuclear DNA content revealed variation in the ploidy level among the seedlings of the back-cross progeny which was expected to be uniformly triploid (Tusa *et al.*, 1996). However, successful introgression of useful Valencia traits into the back-cross lemon offspring can be guaranteed by meiotic recombination between homologous chromosomes in the somatic hybrid. Therefore, in this work, meiotic behaviour was investigated in the tetraploid somatic hybrid to determine the level of intergenomic recombination and to identify the cytological mechanisms underlying the ploidy variation in the back-cross progeny.

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Materials and methods

Two diploid fusion parents ($2n = 2x = 18$), Femminello lemon and Valencia sweet orange and a tetraploid somatic hybrid (SH) Valencia (+) Femminello ($2n = 4x = 36$), were used. The somatic hybrid was produced by polyethylene glycol (PEG)-mediated protoplast fusion of nucellus-derived embryogenic protoplast with nucellar seedling leaf-derived protoplast (Tusa *et al.*, 1990). All the genotypes were grafted onto sour orange (*C. aurantium* L.) and grown at the same location in an experimental field station (Lascari, Palermo, Italy).

For microsporogenesis, floral buds were harvested at various stages of development from early (2 mm) to nearly full-grown. After removal of petals and calyx lobes, the buds were fixed in ethanol-propionic acid (3:1) with a small amount of ferric chloride at room temperature for at least 48 h and squashed in acetocarmine 2% onto slides after gentle heating. After coverslip removal by freezing with liquid N₂, the slides were made permanent by dipping them twice in butanol-acetic acid (1:1), once in absolute butanol, and finally by mounting the coverslip with Entellan. Pollen stainability and viability were tested by acetocarmine (2%) and fluorescein diacetate (FDA), respectively. Pollen germinability was determined on agar medium (2% agar and 20% sucrose) at 25°C after 36 h.

Results

Chromosome pairing was examined at diakinesis during microsporogenesis in the tetraploid SH and its diploid fusion parents (Table 1). SH exhibited multivalent chromosome configurations with, on average, 1.6 quadrivalents per pollen mother cell (PMC) (Fig. 1a). One quadrivalent was found in the donor parent Valencia (Fig. 1b). In the recipient parent Femminello, the mean number of bivalents per PMC was 6.4, but pairing failure often occurred, giving a high number of

univalents (Fig. 1c). In contrast, the occurrence of univalents (mean of 1.1 per PMC) was very low in Valencia. The somatic hybrids (SH) exhibited a range and number of univalents that were intermediate between the parents.

Pollen stainability and viability were higher in SH than in its parents (Table 2); pollen germinability was 41% in SH, whereas in the parental lines it varied from 40% in Femminello to 46% in Valencia. Variable size characterized pollen grains, fertile and sterile, in both SH and parents. In SH, the sterile pollen corresponded largely to micropollen grains which were produced by the polyads (Table 2). They predominated among sporads, with a frequency of 66% in SH, depending on the occurrence of univalents. The unpaired chromosomes showed variable behaviour during microsporogenesis. In SH, they divided at meiosis I (Fig. 1d) or were scattered off the equatorial plate. In the latter case, the lagging chromosomes did not migrate towards the two poles at anaphase I and they were enclosed in an extra nucleus at interkinesis (Fig. 1e). Subsequently, they passed through the second division (Fig. 1f). When the univalents divided during the first meiotic division they formed lagging chromosomes at anaphase/telophase II. At the end of meiosis, the laggards formed two or more micronuclei per PMC in the polyads. Similar behaviour of the univalents was also found in the fusion parent Valencia. In contrast, the univalents in Femminello, which occurred at higher frequencies than in SH, rarely behaved as laggards and generally went to the poles at anaphase I, although the distribution was sometimes asymmetric. However, in both fusion parents, the tetrads were the most frequent type of sporads (Table 2), occasionally containing microspores of different sizes. Moreover, the triads occurred equally in SH and its parents because of abnormalities in spindle orientation in metaphase/anaphase II (Fig. 1g,h). Meiotic nuclear restitution mechanisms which gave rise to dyads or monads were seen as a consequence of the abnormalities in spindle orientation (Fig. 1i,j).

Table 1 Chromosome associations at diakinesis in meiocytes of *Citrus sinensis* (+) *C. limon* somatic hybrids and its fusion parents (Valencia sweet orange and Femminello lemon). Four plants of each genotype were analysed

Genotype	Chromosome number ($2n$)	Number of diakineses	Mean number (range) per diakinesis of		
			Univalents	Bivalents	Quadrivalents
Valencia	18	25	1.1 (0–2)	7.1 (6–8)	0.8 (0–1)
Femminello	18	21	5.2 (0–18)	6.4 (0–9)	0
Somatic hybrid	36	24	2.0 (0–10)	13.8 (9–17)	1.6 (1–3)

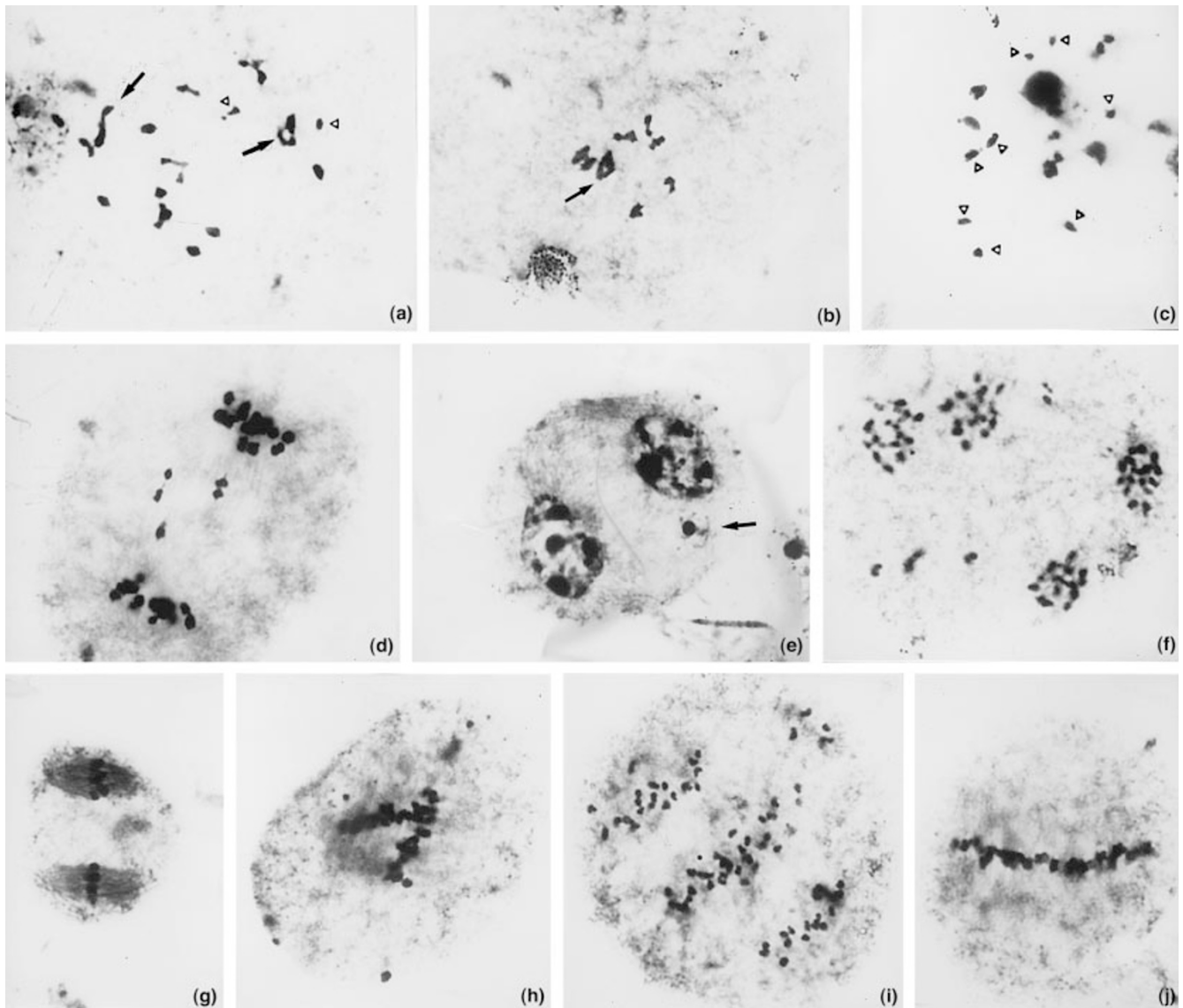


Fig. 1 Microsporogenesis in the *Citrus sinensis* (+) *C. limon* somatic hybrid (SH) and its fusion parents. Chromosome pairing at diakinesis in pollen mother cells (PMCs) showing: (a) 2 I + 13 II + 2 IV in SH; (b) 7 II + 1 IV in *C. sinensis* Valencia; (c) 8 I + 5 II in *C. limon* Femminello; (d) equational division of univalents at anaphase I in SH; (e) micronucleus (indicated by an arrow) at interkinesis; (f) lagging chromosomes scattered in the cytoplasm at telophase II. Abnormalities in meta-anaphase II and spindle orientation: (g) parallel spindles in *C. sinensis* Valencia; (h) V-shaped metaphase plates in SH; (i, j) meiotic nuclear restitution events in SH ($\times 4800$).

Table 2 Frequency of sporads and pollen stainability in *Citrus sinensis* (+) *C. limon* somatic hybrids and its fusion parents (Valencia sweet orange and Femminello lemon). Four plants of each genotype were analysed

Genotype	Number of sporads	Sporad types (%)					Pollen stainability (%)
		Monad	Dyad	Triad	Tetrad	Polyad	
Valencia	101	1	0	10	62	27	58
Femminello	157	1	3	8	76	12	51
Somatic hybrid	110	1	1	10	22	66	64

Discussion

Because of the formation of quadrivalents, gene exchange between Femminello and Valencia occurs in SH. One quadrivalent results from a reciprocal translocation which is present in the Valencia parent and which was also found in different selections by Iwamasa (1969). However, the additional quadrivalents can be considered a consequence of intergenomic pairing. Indeed, good pairing is generally reported between the chromosomes of different *Citrus* species and between *Citrus* and its allied genera like *Poncirus* and *Fortunella* (Iwamasa, 1969; Raghuvanshi, 1969; Mohammad-Anis & Raghuvanshi, 1980; Agarwal, 1987).

The behaviour of univalents was variable in SH and its parents and was similar to that described by Raghuvanshi (1969) in most *Citrus* species. In SH and Valencia the univalents preferentially formed micronuclei and polyads, whereas in Femminello they were generally enclosed in a nucleus although distributed randomly. In the latter case, the tetrad is the final meiotic product but it contains microspores with an unbalanced chromosome number. The presence of univalents, which is a common feature of *Citrus* meiosis, is explained by heterozygosity for structural change in chromosomes as well as genetic mutations in synapsis control (Iwamasa, 1969; Raghuvanshi, 1969; Gmitter *et al.*, 1992). The frequency of univalents is negatively correlated with pollen stainability in diploid genotypes of *Citrus* (Agarwal, 1987; Ibrahim *et al.*, 1994). However, in the tetraploid SH analysed in this work the pollen stainability and germinability are not low (64% and 41%, respectively), even though as many as 10 univalents per cell and a high frequency of polyads occur. A possible explanation for this behaviour is that the chromosomally unbalanced pollen from the tetraploid SH is viable and able to fertilize. Indeed, different DNA contents were found in the back-cross progeny obtained from diploid Femminello lemon crossed with SH as the pollen parent (Tusa *et al.*, 1996). The nuclear DNA amounts, estimated by laser flow cytometry, ranged from 1.1 to 1.7 pg of DNA, whereas the *C. limon* nuclear DNA content was 0.8 pg. Thus, based on these values, aneuploids, triploids, tetraploids and maybe pentaploids occurred. The presence of tetraploids and pentaploids could be explained through the formation of unreduced eggs in Femminello and unreduced pollen in SH. In the latter, large-sized pollen grains and meiotic abnormalities affecting spindle orientation at meiosis-II and sporad formation are found. Similar cytological features, the most common being parallel spindles, are well known in *Solanum* where they cause the formation of $2n$ pollen (Mok & Peloquin,

1975). In *Citrus*, $2n$ egg occurrence is reported in unilateral sexual polyploidization: triploids and tetraploids are obtained from $2x \times 2x$ and $2x \times 4x$ crosses, respectively (Esen & Soost, 1972; Esen *et al.*, 1979). Additionally, tetraploids are obtained from $2x \times 2x$ crosses by bilateral sexual polyploidization (Russo & Torrisi, 1951) which supports the formation of $2n$ pollen in *Citrus*.

Because the somatic hybrid described in this study is characterized by fertile pollen with different ploidy levels and because it showed intergenomic recombination, it offers interesting possibilities for *Citrus* breeding programmes for the introgression of useful traits from orange into lemon and for ploidy manipulation.

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