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Production and characterization of new triploid seedless progenies for mandarin improvement

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Abstract

Eighty-six triploid *Citrus* plants were recovered from interploid crosses between a natural tetraploid selection of the tangerine 'Dancy' (*Citrus reticulata* Blanco, cultigroup 'Tangerine'), used as the pollen parent, and two seedy selections of diploid mandarins (*C. reticulata* Blanco) as well as one clementine (*Citrus clementina* Hort. ex Tan.): 'Fortune' mandarin, 'Wilking' mandarin and 'Monreal' clementine. Flow cytometric analysis was used for screening the triploid plantlets and the ISSR-PCR technique was used to characterize the obtained triploids through a double approach, confirming the hybrid nature of the offspring and allowing the analysis of the genetic pool obtained. Selection among triploid genotypes generated from elite *seedy* parents will give us a better chance to obtain superior mandarin cultivars characterized by true seedlessness.

Keywords: *Citrus reticulata*; Triploidy; Marker-assisted selection; Flow cytometry; PCR-ISSR

1. Introduction

Seedlessness is a major objective in *Citrus* improvement programmes. Seedy *Citrus* fruits are, indeed, considered unacceptable on the international fresh market and it is a priority that any new hybrid developed has a high degree of seedlessness. To make it competitive, the primary goal of the Italian *Citrus* breeding programme is the production of new, seedless, easy-peel mandarin cultivars suitable to take the place of the 'Avana' and 'Tardivo di Ciaculli' mandarins (*Citrus reticulata* Blanco), both very seedy varieties.

Triploid *Citrus* cultivars ($2n = 3x = 27$) have great commercial potential because of their high degree of seedlessness. Worldwide, the selection of promising triploid genotypes is, therefore, considered the ultimate task of *Citrus* breeders, but the frequency of naturally occurring triploids in *Citrus* is extremely low, with the 'Tahiti' lime (*Citrus aurantifolia* Swing.) being the only example of a spontaneous triploid *Citrus* cultivar to be commercialized. The production of triploids by crossing tetraploids with diploids has proven to be a useful and efficient method for producing seedless and fruitful selections in *Citrus* (Soost and Cameron, 1969, 1980, 1985; Reforgiato Recupero et al., 2005). Following this approach, we have crossed a natural tetraploid selection of tangerine 'Dancy' (*C. reticulata* Blanco, cultigroup 'Tangerine'), used as male parent, with the diploid seedy selections of 'Fortune' and 'Wilking' mandarin (*C. reticulata* Blanco), and the 'Monreal' clementine (*Citrus clementina* Hort. ex Tan.).

'Dancy' tangerine and 'Fortune' and 'Wilking' mandarins all produce juicy, sprightly, aromatic, richly flavoured and easy-to-peel fruits, and particularly large for the case of 'Dancy'. The ripening occurs late for 'Fortune' and during the midseason (January–February) for 'Wilking' and 'Dancy', with a strong tendency to alternate bearings for the latter two. On the contrary, 'Monreal' clementines produce early-ripening (October) small fruits. Unfortunately, all of these cultivars are characterized by the presence of a high number of seeds per fruit (up to 15 in 'Monreal').

The parent plants were chosen to obtain progenies of a triploid genotype for the selection of high-quality, seedless mandarin cultivars, marketable for a long period of time (from November to March).

In order to analyze the relative nuclear DNA content of the cells of the obtained plants and to confirm the triploidy, flow cytometric analysis (FCM) was used.

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Furthermore, to determine the hybrid origin of the triploid offspring obtained, an intersimple sequence repeat-polymerase chain reaction (ISSR-PCR) analysis has been carried out. The ISSR-PCR technique has already used successfully to characterize allotetraploid somatic hybrids of grapefruit (*Citrus paradisi* Macfadyen) and mandarin (Scarano et al., 2002; Tusa et al., 2002) and allohexaploid hybrids of cotton (*Gossypium hirsutum* and *Gossypium anomalum*) (Liu and Wendel, 2001). Six ISSR primers have been selected to give polymorphic patterns between the parents of each cross and were then used to analyze the relative offspring. The ISSR analysis showed specific markers from both parents to segregate into all components of the progeny and new bands were not detected.

Fingerprinting data were also used to calculate the genetic diversity and genetic distances within the progenies obtained. Genetic diversity values and the percentage of polymorphic loci can be used to identify the parental lines to be crossed for future hybridization programmes. The genetic distances of the offspring can be used to predict the yields of future crosses between lines from the same germplasm group or to select individuals more similar to a specific parent, in order to maintain a favourable gene combination. In our study, ISSR analysis was demonstrated to be a useful and simple method to recognize the hybrid nature of the offspring arising from interploid hybridization and to study the genetic pool obtained.

2. Materials and methods

2.1. Plant material

In all of the crosses, a natural tetraploid selection of 'Dancy' tangerines (*C. reticulata* Blanco, cultigroup 'Tangerine') was used as the pollen parent. Two selections of diploid mandarin and one of clementine were used as the mother plants: 'Fortune' mandarin (*C. reticulata* Blanco), 'Wilking' mandarin (*C. reticulata* Blanco) and 'Monreal' clementine (*C. clementina* Hort. ex Tan). All parent plants were grown in the germplasm collection of the Lascari field station (38°N, 14°E).

'Dancy' tangerines are characterized by a rich, sweet and intense juice flavour and by a thin and deep reddish-orange, easily peeled rind. It ripens in January–February and, despite its tendency to alternate bearings, it produces large and attractive fruits.

'Fortune' mandarin is a monoembryonic, late-ripening variety characterized by high-quality, large fruit possessing rich and sprightly (subacid) flavour and a reddish-orange and peelable skin. The fault associated with this cultivar is the high presence of seeds. The 'Wilking' mandarin produces juicy, richly flavoured and easy-to-peel fruits. The problems associated with this midseason variety are the strong tendency to alternate bearings and, again, the high presence of seeds per fruit. 'Monreal' clementine is one of the most important mandarin varieties in the Mediterranean basin. Its relevance is mainly due to its early-ripening (October). However, fruits are small in size and contain an excessive number of seeds.

2.2. Interploid hybridization

Flowers from the seed parent were emasculated before anthesis and immediately pollinated with pollen collected, dried and stored 2–3 days at 4 °C in the dark. At maturity, the fruits were harvested and stored temporarily at 4 °C. Fruits were surface sterilized for 30 min with a solution of 20% bleach (NaOCl 5.25%). After rinsing with sterilized water, the fruits were carefully cut at the equatorial region, seeds were extracted and the teguments removed under a stereo microscope. Seeds were cultured on MS medium under continuous light at 25 °C in magenta vessels. Resulting plantlets were transplanted in a commercial soil mixture in plastic vessels for acclimatization at 80% RH, 28 °C, then moved into a greenhouse under normal conditions.

2.3. Flow cytometric analysis

Flow cytometry (FCM) was used to analyze the relative nuclear DNA content of the leaf cells according to method of reference (Lucretti et al., 1997). The intercalating DNA specific fluorescent dye propidium iodide was used for staining leaf nuclei in suspension, and they were then analyzed with a FACStarPlus flow cytometer and sorter (Becton Dickinson, San José, CA). As an internal standard, nuclei from diploid *C. reticulata* 'Fortune' were used through all FCM analysis.

2.4. DNA extraction

DNA was extracted from the parent plants of each cross and from the relative hybrid progenies. Young leaves were harvested and carefully washed, as advised, to avoid insect and fungal contamination (Fang and Roose, 1997), then they were frozen in liquid nitrogen and stored at −80 °C. They were ground in a mortar with liquid nitrogen and genomic DNA was extracted using the procedure described by Doyle and Doyle (1987). DNA was quantified by measuring OD_{260} as described by Sambrook et al. (1989).

2.5. ISSR analysis

A total of six primers – i.e. $(AC)_8YG$, $(AG)_8YC$, $(AC)_8YA$, $(AC)_8YT$, $(AG)_8YT$ and $(CA)_8RG$ (Fang and Roose, 1997) – were used to amplify the DNA. The primers were purchased from MWG Biotech AG.

Each 25-μL amplification reaction consisted of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 800 μM dNTP, 0.5 μM of each primer, 1 U of Platinum *Taq* polymerase (Invitrogen, Life Technologies) and 30 ng of template DNA. The amplification was performed in a 96-well GeneAmp® PCR System 9700 (Applied Biosystem) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 4 min at 94 °C, followed by 36 cycles at 94 °C for 30 s (denaturation), 48–50 °C for 45 s (annealing) and 72 °C for 120 s (extension), followed by a final extension step at 72 °C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1× TBE (45 mM Tris-borate,