Origin and frequency of 2n gametes in *Citrus sinensis* × *Poncirus trifoliata* and their reciprocal crosses

Chunxian Chen a, Matthew T. Lyon b, David O’Malley c, Claire T. Federici b, Julie Gmitter a, Jude W. Grosser a, Jose X. Chaparro c, Mikeal L. Roose b, Fred G. Gmitter Jr. a,*

a University of Florida, Citrus Research and Education Center, Lake Alfred, FL 33850, USA
b University of California, Department of Botany and Plant Sciences, Riverside, CA 92521, USA
c University of Florida, Horticultural Sciences Department, Gainesville, FL 32611, USA

Received 2 May 2007; received in revised form 13 August 2007; accepted 20 August 2007
Available online 25 August 2007

Abstract

A total of 293 putative citrange F1 seedlings, derived from 13 crosses and visually identified by their trifoliate leaf morphology, were investigated using six characterized expressed sequence tag (EST) microsatellite primers. Their origins and ploidy levels were simultaneously and unambiguously determined by these markers. Besides a majority of diploid zygotic hybrids, as high as 24% triploid hybrids were produced, only by *Citrus sinensis* (sweet orange) × *Poncirus trifoliata* (trifoliate orange), but not the reciprocal crosses. The results indicated that 2n eggs originated only from the maternal sweet orange parent, contributing the extra haploid genome in the triploids. It was further inferred that the 2n eggs in sweet orange result from first division restitution (FDR), not second division restitution (SDR), according to the marker genotypes of these triploids. High percentages of self-cross and nucellar origin seedlings were found only from *P. trifoliata* × *C. sinensis*, but not from the reciprocals, simply due to their different reliabilities in the visual leaf identification of the seedlings. Survey of leaves indicated that adult triploid seedlings had predominantly monofoliate leaves, compared to diploid hybrids with mostly trifoliate leaves. The possible genetic mechanism and potential applications were discussed.

#2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: Simple sequence repeat (SSR); Allele dose effect; Meiosis; Seedless

1. Introduction

*Citrus* is the origin of commercial citrus cultivars. Sweet orange (*C. sinensis* [L.] Osbeck) ranked first among diverse types of citrus fruits in terms of total production and market value worldwide. *Poncirus* is a sexually compatible relative of *Citrus* in the family Rutaceae. Trifoliate orange (*P. trifoliata* [L.] Raf.), the only species in the genus, is an important rootstock in certain countries and a widely used rootstock breeding material [1]. *Poncirus* possesses several disease resistance or stress tolerance genes not found in *Citrus*. Citrus cultivars and wild species mostly are diploids of 18 chromosomes [2,3]. Complete or partial sexual compatibility is one common feature in citrus. Fertile hybrids among *Citrus*, *Poncirus*, and *Fortunella* can be rather easily acquired, for the selection of compatible rootstocks or desired scions and/or the provision of genetic mapping populations [4–6]. For example, two widely used rootstocks, Carrizo citrange and Swingle citrumelo, were selected from the hybrids of *C. sinensis* cv. Washington Navel × *P. trifoliata*, and of *C. paradisi* cv. Duncan × *P. trifoliata*, respectively [5].

Despite ease of hybridization, acquisition of normal zygotic diploids has been impeded by two reproductive phenomena found in citrus, polyembryony (a form of apomixis) and 2n gametes, causing the uncertainties of the origin (zygotic versus nucellar) and ploidy (diploid versus polyploids) of seedlings [7,8]. A monoembryonic seed parent is preferred for hybridization because generally only zygotic embryos can be produced. Most citrus varieties are polyembryonic, and when
3.3. Some phenotypes of adult triploids

A tree survey was done for a preliminary evaluation on how the extra sweet orange haploid genome affects the phenotype. Descriptively, the color of the tree canopy of triploids looked like that of sweet orange (green or dark green) with denser branches, compared to that of diploids like that of *Poncirus* (lighter green). The major difference was the composition of leaf types. The averages of uni-, bi-, and trifoliates on triploids were 6.07 (73%), 1.27 (15%), and 0.95 (12%), while those on diploids were 0.72 (8%), 1.58 (18%), and 6.54 (74%). There were statistically significant differences between two pairs of the averages of uni- and trifoliates, but no difference between that of bifoliates on triploids and diploids (*p* < 0.001). Unlike most trifoliate leaves on diploid hybrids, a majority of unifoliate leaves on triploids may be the consequence of expressing double sweet orange gene sets. Some phenotypically triploid trees looked substantially more vigorous than most diploid citranges. In addition, most triploids had just a few fruits, always seedless, but diploid trees of the same age had fruit numbers ranging from 9 up to nearly 150. Further evaluation of the fertility may need continuous full-season observations for multiple years.

Formation of 2n gametes in plants has been considered primarily occurring in either phase of abnormal meiosis, first division restitution (FDR) or second division restitution [19,20,38,39]. FDR occurs if failure of spindle formation results in the nuclear membrane reforming around the chromosomes without movement to two opposite poles during meiosis I, the reduction process. SDR occurs when no cell plate is formed at the conclusion of separation of sister chromatids during meiosis II, the mitosis-like division [19,20]. SDR was thought to be the mechanism forming 2n gametes in *C. clementina*, based on comparison of the allele number and intensity profiles theoretically deduced from monomeric or dimeric isozyme patterns [36]; however, isozyme-based profiles are usually far less precise than DNA markers. In this study, we found all triploids were derived from three genotypes of sweet orange 2n eggs, ab, aa, and bb, at a certain ratio at all the heterozygous loci. We inferred that FDR, not SDR, is the primary mechanism to form 2n eggs in sweet orange, illustrated in Fig. 2. The scenario of three-genotype 2n eggs only occurred under FDR and with chiasma, shown at the heterozygous locus “ab” that could produce the three genotypes, i.e., ab (50%), aa (25%), and bb (25%), at a ratio of 2:1:1. None of the other cases could result in this scenario.
Under FDR and without chiasma, shown at the locus "cd", only one heterozygous genotype of 2n eggs, cd (100%), would be formed. Under SDR, either one heterozygous genotype of eggs, ab (100%), at chiasmatic locus "ab", or two different homozygous genotypes of eggs at a ratio of 1:1, cc (50%) and dd (50%) at locus "cd" without chiasma, only could be produced. So it could be concluded that the 2n eggs were formed via FDR, not SDR, only in the maternal sweet orange parent.

4.2. Genetic control and potential application of 2n gametes in citrus breeding

Nuclear restitution, failure in reduction or division likely occurring at various stages of meiosis, has been considered a major mechanism resulting in 2n gametes [38–40]. Other possible mechanisms could be involved in formation of 2n gametes. About a dozen genes related to alteration in meiotic reduction and nuclear restitution were found or isolated in Arabidopsis and other plant mutants [41,42]. Combining the fact that almost all seed parents capable of producing triploids were from Citrus [3,26–31] with our results from these reciprocal crosses, we should have a strong inference that the genes controlling this genome-dependent phenomenon may exist in only Citrus but not in Poncirus. Moreover, the genes are particularly expressed during only megagametogenesis that is in consistency with an early cytogenetics-based speculation [30]. Further genomic exploration, either using known genes or profiling gene expression, may isolate or differentiate the gene(s) in Citrus.

Triploidy has always been attractive to fruit breeders because it can likely yield seedless fruits that are highly favored by growers and consumers [31]. More crosses among Citrus varieties, including self-crossing, needs to be made to confirm if the gene(s) related to the formation of 2n eggs indeed exist in Citrus genome. If this mechanism of generating a high frequency of 2n eggs exists in all Citrus varieties, it can be of huge potential for citrus breeder to select desired seedless triploid varieties [28,29,31,35,36]. Utilization of crosses among good-quality Citrus cultivars to produce triploids can likely maximize the chance to acquire good-quality seedless selections. Unlike other crosses with citrus wild species, those unpalatable genes are avoided.

4.3. Approaches to determine the origin and ploidy of citrus seedlings

In practice, polyembryony resulting in nucellar seedlings can maximize the uniformity of citrus rootstocks [7]. 2n gametes may potentially be used for the selection of seedless varieties [29,31]. On the other hand, the ubiquity of polyembryony and high frequency of 2n eggs in Citrus have really been a genetic barrier and extra burden for conventional breeding; mixed seedlings of different origins and ploidy levels from sexual hybridization must be distinguished prior to further use [7,26], as indicated in this study. Zygotic and nucellar
seedlings were usually distinguished by visual leaf traits, and then genetic markers followed to confirm in most cases [13–17]. Ploidy was determined either by laborious and time-consuming chromosome counting methods [2,3] or more recently by fairly rapid and efficient flow cytometry [32,33]. Reliable, high-throughput approaches are demanded in order for a citrus breeder to be able to simultaneously determine the origins and ploidy levels of mixed seedlings to facilitate further applications. In this study, we have demonstrated the advantages of selected EST-SSR markers and the capabilities to utilize these markers to determine the origins and ploidy levels in a large scale. The basic criteria are that co-dominant PCR markers must have polymorphic alleles different between two parents and heterozygous in at least one parent. But the ideal are those different and heterozygous in both parents, like CX5F56 in this study, from which the inheritance and/or segregation of the alleles can be unambiguously tracked in progeny from both parents. We may use such primers to track and count chromosomes if nine or more chromosomes-associated primers are used [11]. Allele dose effect of DNA markers has been used to determine the donor genome in polyploids and extra chromosomes in aneuploids. In order to avoid misjudging the band intensities, representing the dose effects in RFLP probing assays, the same DNA amount of an external single-dose control was required to use as accurately as possible [34]. However, in this study, the allele dose effects, exhibited as fluorescent peak heights in the ABI chromatographs, were easy to visualize and measure in the “aap” or “bbp” triploids. No external control was needed because the internal single allele “p” or “q” peak was used as the single dose control.

Acknowledgments

The authors would like to thank Mrs. Margie Wendell and Misty Holt for their excellent technical support. This research was supported in part Project Number 0110-031 of the Florida Citrus Production Research Advisory Council, Peter McClure, Chairman, and 5200-125 from the California Citrus Research Board.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2007.08.005.

References

[29] N. Handaji, D. Hnoumi, N. Arsalane, New Citrus triploids from diploid crosses between clementine and eight mandarins, in: Proceedings of the...


