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Molecular Genetic Analysis of Nucellar Embryony (Apomixis) in *Citrus maxima* x *Poncirus trifoliata*

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by

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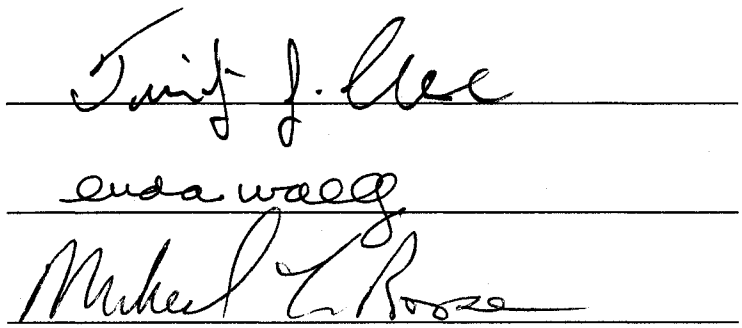

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ABSTRACT OF THE DISSERTATION

Molecular Genetic Analysis of Nucellar Embryony (Apomixis) in
Citrus maxima x *Poncirus trifoliata*

by

Joseph Lee Kepiro

Doctor of Philosophy, Graduate Program in Plant Biology (Plant Genetics)
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Dr. Mikeal L. Roose, Chairman

Some citrus varieties express the trait of nucellar embryony (NE). The nucellus tissue surrounding the embryo sac gives rise to embryos genetically identical to the seed-producing tree. The phenotype of the trait is commonly called polyembryony because when NE is expressed, multiple embryos can develop within one seed. The cross *Citrus maxima* 'Chandler' (monoembryonic) X *Poncirus trifoliata* (polyembryonic) provided a population of 88 siblings segregating for NE. Progeny were classified for polyembryony. Molecular marker data were generated for the parents and progeny using Amplified Fragment Length Polymorphism (AFLP) System II with multiplexed PCR. The extreme monoembryonic and polyembryonic phenotypes of this population were used in a bulked segregant analysis screen of 256 primer combinations that identified AFLP markers linked to a major gene (*N*) conferring nucellar embryony in *P. trifoliata*. No locus in 'Chandler' was linked to NE. Three of the markers most closely linked to NE in *Poncirus* were cloned, sequenced, and used to probe a 9-fold coverage BAC library. Each of thirteen positive BAC clones was confirmed to carry one DNA fragment matching the

exact mobility of the original genomic AFLP polymorphism when amplified with the appropriate primer combination.

Scoring 42 AFLP primer combinations in the 88 progeny identified over 600 segregating AFLP markers. Molecular marker linkage maps were constructed for *C. maxima* and *P. trifoliata* with JoinMap 3.0® using a pseudo-testcross analysis. The map of *C. maxima* 'Chandler' (Ch) had 257 markers distributed across nine linkage groups. The map of *P. trifoliata* constructed from *P. trifoliata* 'Rubidoux' and 'Webber Fawcett' (RW) had 211 markers distributed across 11 linkage groups.

Quantitative trait values for the percentage of polyembryonic seed produced by each hybrid in the population was used in conjunction with the Ch and RW maps for Quantitative Trait Loci (QTL) mapping. The QTL analysis confirmed the major gene *N* on RW-10 that confers nucellar embryony. Multiple QTL method mapping provided strong evidence that a second independent gene had a large effect on the percentage of polyembryonic seed that a hybrid produced. That locus, named *P1* (polyembryony one), explained ~ 63 % of the variance in the percentage of polyembryonic seed produced by hybrids with NE.

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GENERAL INTRODUCTION

Sexual reproduction in Citrus

Sexual reproduction results in a recombination of parental genetic material. The offspring have a new and unique genetic composition, not identical to either parent. Sexual reproduction involves a well-defined series of events that occur in the ovule of angiosperms: megaspore mother cell differentiation from the nucellus, megaspore production by meiosis, megaspore selection, embryo sac development by mitotic processes, embryo sac maturation, double fertilization, and endosperm and embryo development (Koltunow, 1993).

In a normal sexual reproductive process, one cell near the center of the nucellus differentiates to become the megaspore mother cell, while the surrounding cells maintain their identity and function as nucellar cells. The megaspore mother cell is initialized to enter the meiotic pathway during which homologous chromosomes pair and disjoin, and non-homologous chromosomes segregate independently. The chromosome number in each cell is reduced from the diploid (2n) number to the haploid number (1n). The result is four haploid cells. Three of the cells degenerate, leaving one viable haploid megaspore that divides mitotically to produce an eight-nucleated gametophytic structure called the polygonum embryo sac. One nucleus is

contained in the egg. Two other key nuclei, the polar nuclei, are centrally located in the embryo sac.

The male gametes, sperm, are produced in the anther through a related meiotic process and are disseminated within pollen grains. Pollination occurs at or shortly after anthesis, when the flower petals open and anthers dehisce, allowing pollen to be carried to a receptive stigma by insects or wind. Pollen germinates on the stigma, and a pollen tube carrying the two sperm grows along the transmitting track in the style towards the ovule. The sperm are released into the embryo sac after the pollen tube grows into the micropylar end of the ovule. Pollination to fertilization takes about 10 days.

At fertilization, one sperm nucleus fuses with the egg nucleus to restore the diploid condition of a single cell within the embryo sac forming the zygote. The fusion of nuclei containing one set of chromosomes from each parent results in a zygote of new genetic composition, with one-half of its chromosomes from the male parent and one-half from the female parent. The second sperm nucleus undergoes triple fusion with the two polar nuclei forming the triploid (3n) endosperm. The endosperm begins rapid cell division and proliferates in a free-nucleated state to become a un-cellularized nutritive mass. When the zygote/embryo begins to divide, it is pushed into the endosperm by a structure resembling a stalk called the suspensor. The endosperm begins to partition nuclei by forming cell walls. As the embryo develops, cotyledons

are formed. The endosperm is consumed as its energy supplies are transferred to the zygote with excess energy stored in the cotyledons of the maturing embryo. The mature seed contains an embryo surrounded by its two large cotyledons and is capable of germinating to become a seedling.

Nucellar embryony (apomixis) in citrus

Apomixis refers to those asexual reproductive processes that occur in the ovules of certain species of flowering plants (Nogler, 1984; Asker and Jerling, 1992). Three major types of apomixis have been described: diplospory, apospory and adventitious embryony. Each type of apomixis differs in particular aspects of development and timing. In citrus varieties and their relatives, apomixis can occur as adventitious embryony, specifically called "nucellar embryony", in which adventitious embryos arise from the nucellus. When this occurs, multiple embryos can develop within one seed; therefore nucellar embryony also causes polyembryony.

The nucellus tissue surrounding the embryo sac gives rise to adventitious embryos genetically identical to the tree bearing the fruit. Cells within the diploid sporophytic nucellus tissue are initialized to follow an embryonic developmental pathway. These particular cells are nucellar embryo initial cells (NEICs). NEICs do not go through meiosis and there is no reduction and subsequent restoration of diploid number and, therefore, no recombination of genetic material. The progeny originating from NEICs

are genetically identical to the plant that bears the flower structure in which they arise. Embryos that develop from these NEICs are called nucellar embryos.

The term monoembryony (one embryo) is used to refer to the result of normal sexual reproduction. However, multiple embryos can arise by twinning, which is an aberration of the normal sexual process. The origins of twins are generally of two types. Monozygotic (identical) twinning occurs after a zygote has formed and the zygote/embryo experiences an abnormal cell division that gives rise to a second embryo. Dizygotic (fraternal) twinning occurs if two eggs are present in a single embryo sac or if two embryo sacs have developed within a single ovule and are fertilized. When used in reference to nucellar embryony, the term monoembryony means the genetic capacity to produce offspring only by sexual reproduction (sexual obligate reproduction).

The presence of multiple embryos within one seed is polyembryony. The term polyembryony is commonly used as an alternate term to describe the trait of nucellar embryony. The term polyembryony implies that the multiple embryos arise from NEICs, not twinning. The term "multiple embryos" does not distinguish the embryos origin as zygotic or nucellar.

Initialization of NEICs

A detailed morphological description of the nucellar embryo initial cell revealed that the first evidence of differentiation can be distinguished by an angular cell shape, a large, deeply staining nucleus and granular, deeply staining cytoplasm (Kobayashi et al., 1979; Naumova, 1993). A thickening of the cell wall occurs by deposition of an electron dense material that is or resembles callose (Wilms et al., 1983; Wakana and Uemoto, 1988). These distinguishing characteristics have been observed in a few cells (2-3) of the cv. 'Valencia' orange nucellus at anthesis, when the embryo sac contains four nuclei (Koltunow et al., 1995). The mechanism of adventive embryogenesis was studied at the ultrastructural level with electron microscopy by Wilms et al. (1983). He described the appearance and distinguishing characteristics of NEICs. One of his most relevant observations was that NEICs are first detected between the anthesis and fertilization stages of development.

Development of nucellar embryos

By the time of style abscission, approximately 10 days after anthesis (10 DAA), the number of detectable NEICs increases to at least 50 (Koltunow et al., 1995). After double fertilization, the ovules enlarge rapidly by cell division of the chalazal end and the embryo sac expands as endosperm develops. The nucellus begins to degenerate and NEICs are found predominantly clustered in the micropylar end of the ovule with few

NEICs in the chalazal end (Wakana and Uemoto, 1988). The cell walls of the NEICs become thinner, the cells become rounder, larger, contain more vacuoles, and the nuclei are very prominent. Cell division of the NEICs appears to be nearly synchronous with zygotic division but varies slightly between cultivars. Esan and Soost (1977) reported the presence of globular nucellar embryos before the zygote had divided in cv. 'Ponkan' mandarin. Wakana and Uemoto (1988) reported the first cell division of NEICs at 44 days after pollination and the first zygotic cell division at 45 days after pollination in cv. 'Satsuma' mandarin. Once the NEICs begin to divide, their development is similar to a zygotic embryo.

In citrus, one or more nucellar embryos can mature and germinate along with a zygotic embryo, but maturation of nucellar embryos is contingent upon endosperm having been formed. Without endosperm formation, NEIC development is arrested at or before the early heart stage. The 3n endosperm is required as a source of nutrition for the developing embryos. Johri and Ahuja (1956) reported that adventive embryos developed in a related genus *Aegle marmelos* (Ruteaceae) in which the egg disintegrated before the pollen tube entered the embryo sac, however triple fusion had occurred to create endosperm. If these series of events should occur in citrus, a nucellar embryo could mature without the presence of a zygote. The result would be a viable seed with only one mature embryo that is genetically identical to the seed progenitor (female parent).

Ueno et al. (1967) undertook a comprehensive study of the mean embryo numbers in *Citrus*, *Poncirus* and *Fortunella*. Out of 116 varieties, 85 were defined as polyembryonic. The mean embryo number per seed of polyembryonic varieties shows continuous variation, ranging from 1.10 through 49.96. When examined by dissection, the varieties with a high degree of polyembryony had many small spherical embryos along with thickly developed nucellus at the micropylar end of the inner seed coat. This suggests that the nucellus persists and NEICs continue to be initiated over a long period of time. In varieties with lower degrees of polyembryony the cells of the nucellus seem to disappear in an earlier stage.

There are several developmental characteristics that can influence the number of nucellar embryos in seeds, such as persistence of the nucellus versus early degradation. The stage at which the endosperm begins to form cell walls may play a role in the final maturity of NEIC-derived embryos. If the nucellus tissue does not begin to degrade prior to endosperm cell wall formation, the NEICs will remain outside the endosperm. If the endosperm begins cellularization precociously, the NEICs will again be excluded from the endosperm. Therefore, some variation in the mean number of embryos per seed can be expected based on developmental characteristics not directly related to the initiation of NEICs.

Single gene inheritance is difficult to establish using Mendelian ratios of inheritance

The inheritance of nucellar embryony in common citrus species appears to be controlled by one or a few genes. The trait appears dominant with strictly sexual varieties homozygous recessive for the gene. Assuming a single dominant gene model, crosses between monoembryonic x monoembryonic parents would be represented (nn) x (nn) and all offspring would be (nn), not capable of expressing nucellar embryony. In crosses between monoembryonic and polyembryonic parents, the ratio of offspring in a population expressing the trait depends on the zygosity of the polyembryonic parent at the locus for a major dominant gene (N).

When a variety with the trait is crossed with a monoembryonic variety, two types of populations can arise: 1) segregating with monoembryonic and polyembryonic progeny, or 2) all progeny polyembryonic. In the first population type, the polyembryonic parent is heterozygous (Nn) and the monoembryonic parents homozygous (nn). The testcross of the nucellar embryony locus (Nn) x (nn) should produce a population of genotypes (Nn) and (nn) and the offspring should segregate with a ratio of 1:1 for the presence or absence of the trait of nucellar embryony. In the second population type, the polyembryonic parent would be homozygous dominant (NN) and the cross (NN) x (nn) should yield all (Nn) genotypes expressing the trait. However, crosses in citrus have not always produced progeny with the segregation ratios expected (Parlevliet and Cameron, 1959; Cameron and Soost, 1979).

In 1945, under the direction of Dr. H. B. Frost at the University of California Riverside, crosses were made to test the single major gene model. Six crosses of monoembryonic x monoembryonic parents were made. In 1958, F_1 progeny from those crosses were evaluated for polyembryony by seed dissection (Parlevliet and Cameron, 1959). Twelve trees were evaluated from each of the first five crosses and three trees of the sixth cross. All 63 trees were determined to be monoembryonic based on 3150 seeds examined and detection of multiple embryos in only 0.3 % of the seeds, with those presumed to have arisen by twinning.

Frost made fourteen crosses between polyembryonic parents (with evidence of heterozygosity) and monoembryonic parents. Monoembryonic pummelo and mandarin parents were used for these crosses and progeny evaluated by group based on parentage. Seven crosses were made between presumed (Nn) polyembryonic parents and monoembryonic pummelo parents. The ratios of progeny that were polyembryonic to monoembryonic (P:M) in the pummelo group were: 3:6, 4:8, 13:14, 6:6, 8:4, 9:4, and 19:3. Seven other crosses were made between presumed (Nn) polyembryonic parents and monoembryonic mandarin-type parents. In the mandarin-type group, the segregation ratios of progeny (P:M) were: 8:19, 16:22, 34:32, 8:5, 8:3, 4:1, and 10:2 (Parlevliet and Cameron, 1959).

Some polyembryonic varieties were used as parents in both groups and a few polyembryonic parents were used only in one group or the other. For each

monoembryonic parent group, the number of embryos per seed was evaluated by the mean of hybrid tree means derived from the crosses. The mean number of embryos per seed ranged from 1.2-3.8 among the seven pummelo crosses and 2.8-24.4 among the seven mandarin populations. The lower maximum mean and overall lower mean of embryos per seed for pummelo hybrids versus mandarin hybrids suggests the presence of a minor gene reducing embryo number per seed in pummelo (Parlevliet and Cameron, 1959).

One polyembryonic parent ('Frua' tangerine) was used as a polyembryonic parent in both groups for a total of three crosses, producing only polyembryonic offspring with (P:M) ratios of: 8:0, 10:0, 8:0 and was presumed to be homozygous for (N) (Parlevliet and Cameron, 1959). Other crosses between polyembryonic x polyembryonic parents have produced offspring consistent with single gene inheritance of nucellar embryony dominant to monoembryony, with (Nn) x (Nn) producing offspring in the polyembryonic classes of the (NN), (Nn), and monoembryonic class (nn) but with skewed segregation ratios (Cameron and Soost, 1979).

Many of the segregation ratios reported by Parlevliet and Cameron (1959) and Cameron and Soost (1979) are significantly skewed from the expected ratios with too few polyembryonic progeny observed. Three probable causes exist to explain this: epistasis, mis-classification and lethal alleles that cause segregation distortion. Epistasis is highly probable. Quantitative models of trait expression provides for both positive and negative

effects. During epistatic interactions the presence or absence of one gene product might increase or reduce the expression or effect of another gene product. These epistatic alleles can be called modifier genes because they modify the expression of a trait under investigation. They have also been termed minor genes to distinguish them from the gene(s) that cause major effects such as conferring a trait, i.e., nucellar embryony. With strong negative epistatic effects, it is possible for an individual to have the major gene for a trait but not exhibit the phenotype of the trait or to express the trait at such a low level that the phenotype is not recognizable.

The skewed ratios of Cameron and Soost (1979) are likely due in part to mis-classification because individuals producing 7 % or less seed with multiple embryos were classified as monoembryonic and progeny producing 8-10 % seed with multiple embryos were considered near-monoembryonic. Only those progeny with greater than 10 % polyembryonic seed were classified as having nucellar embryony, although models in which the near-monoembryonic class was considered to have nucellar embryony were also tested. In addition, only 50-150 seeds per individual were scored for multiple embryos. Parlevliet and Cameron (1959) used the number of embryos per seed as the criterion for classification and arbitrarily defined individuals producing seed with multiple embryos at 1.01 to 1.04 embryos per seed as monoembryonic. They also dissected a very small sample of 50 seeds per tree and their study collected data for only one year. The work of Parlevliet and Cameron (1959) supported the findings of Nasharty (1945) who reported much higher mean embryo numbers in Los Angeles CA, than in the same

varieties evaluated in Riverside (60 miles inland), during the same season. Furusato (1954) also found the mean embryo number from the north side of the tree was significantly higher than the south side. This suggests that temperature during the flowering and growing season can affect mean embryo numbers.

In a study following the experimental designs above, using the same classification techniques, limiting data collection to one season, or scoring only 50-150 seeds per tree, a tree might be mis-classified as monoembryonic. This might occur if the tree had the major gene for nucellar embryony but lacked a modifier gene with a positive effect or had a modifier gene with a strong negative effect. In a breeding program such an individual might unexpectedly produce offspring with significant polyembryony if the combination of alleles in the offspring relaxed epistatic constraints. Therefore, multiple year sampling with fruit collected from the cooler side of trees should increase the probability that trees with low levels of nucellar embryony can be identified. Increasing the seed sample size per tree would also reduce the risk of mis-classification from twinning. But ultimately, molecular techniques are required for unambiguous classification of individuals with low levels of multiple embryony by verifying that at least some offspring are genetically identical to the maternal parent.

The advent of molecular markers allows a researcher to determining the genetic composition of the parent and create molecular marker maps. The progeny can be tested for inheritance of the molecular markers, and when the ratios are calculated, segregation