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Detection of zygotic and apomictic embryonic origin in *Citrus sinensis* Osbeck based on RAPD markers

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ABSTRACT

Purpose: Citrus plant exhibits a unique trait called polyembryony. In open-pollinated plants the pollen source of the plant remains unknown but it is assumed that the apomictic nucellar embryos mimic the genetic architecture of the mother plant. This assumption was exploited in the detection of true-to the type seedlings of polyembryonic *Citrus sinensis* plants for fruit quality retention and smooth maintenance of the orchards. **Research Method:** The randomly amplified polymorphic DNA (RAPD) technique was employed to distinguish nucellar and zygotic seedlings obtained from a selected *Citrus sinensis* plant marked in South 24- Parganas district of state of West Bengal in India. The embryos were extracted from a fruit and seedlings were raised in poly-house. To identify DNA marker for tracing the embryonic origin, ten vigorous seedlings marked in poly-house were used for RAPD analysis. DNA was extracted from three-month-old seedlings along with the mother plant and RAPD analysis was performed with 25 arbitrary decamer primers with a negative control. **Findings:** Four decamer primers OPQ15, OPAH02, OPAA02 and OPA11 were able to differentiate the sexual seedlings from the apomictic nucellar types. The total study took 48 hours for tracing the embryonic origin of the seedlings. **Research limitations:** This study could be extended with inclusion of more primers and screening of more fruits from diverse locations of India. **Originality/Value:** This process could act as a fast technique for preliminary identification of true-to the-type plants for quality control of Citrus fruit industry and sustainable nursery management.

INTRODUCTION

Apomixis is a unique phenomenon of embryo development found in a couple of families of angiosperm. Apomixis is detected in more than 400 species of flowering plants (Carman, 1997) including important horticultural plant families represented by citrus (Wakana & Uemoto, 1987), mango (Aron et al., 1998), walnut (Peng-fei et al., 2007) and pepper (Nowaczyk, 1987). Apomixis is divided into gametophytic and sporophytic type where both display potential usefulness in plant breeding. Sporophytic apomixis also known as adventitious embryony is a process in which the embryo arises directly from the nucellus or the integument part of the ovular tissue. Sporophytic apomixis occurs commonly in citrus species but rarely found in any renowned cereal crop plants. The nucellar apomixis is very promising in permanent fixation of superior genetic architecture of female plants (Xu et al., 2021). The valuable heterotic potential present in highly cross-pollinated crops could be preserved through apomictic embryos.

In Citrus, the apomictic nucellar embryos could be utilized as explants for *ex-vivo* clonal propagation or as superior scion in grafting operation. The sexual reproduction in cross pollinated crops may involve integration of foreign genes from pollen donor. The fertilization event from unknown pollen source often deteriorates the quality of fruits, field performance and affects disease resistant properties of the sexual progeny plants in citrus. Whereas the nucellar apomictic progeny population developed from an elite well performing genotype could ensure uniformity in progeny population with equivalent performance as the mother progenitor (Xu et al., 2022).

The apomictic trait promotes a short-circuited life cycle developing embryos identical to the mother plant. The early screening of nucellar embryos helps in retention of fruit quality along with effective utilization of financial resource, man-power, and farm-input in orchard management programme. Scientists applied several techniques including the low cost *in-vitro* embryo germination, isozyme and DNA marking techniques for discrimination of nucellar apomictic embryos in citrus. The isozyme system displays many limitations such as excessive dependence on tissue age, environmental factors, accuracy of the enzyme system and efficacy of the researcher (Ashari et al., 1988). The constraints in the application of isozyme markers to differentiate the embryos created room for application of strong molecular markers such as direct involvement of DNA based molecular marker.

The DNA based marking is devoid of any direct influence from the tissue age, type and environment. The random amplified polymorphic DNA markers (RAPD) has been widely used in fruit crops for solving diverse problems due to their phenotypic neutrality, high polymorphism, low-cost and fast result. The versatile technique was used for genotype identification, genome analysis, duplicate identification, phylogenetic studies, mapping and mutant identification (Babu et al., 2021; Pillay et al., 2000; Subudhi et al., 2016; Zarei et al., 2017; Li et al., 2019; Abdein et al., 2022; Wahyudi et al., 2020). The RAPD technique does not require previous information about the targeted DNA and may reveal immense polymorphism with easy, simple method of operation suitable to conduct in a moderate laboratory set-up (Bardakci, 2001).

In the present study RAPD marking technique is used for molecular detection of the apomictic seedlings from sexual one with an assumption of early detection of seedlings similar to the mother plant. The results of this preliminary trial will help to develop advance markers for identification of desired apomictic embryos for Citrus propagation. The detection of reproducible RAPD markers in long run could be converted into robust markers for the study of sporophytic polyembryony in other horticultural crop species and tracing of elite plant types for quality control in fruit business.

MATERIALS AND METHODS

Selection of plant and embryo extraction

One open pollinated sweet orange (*Citrus sinensis)* plant from Krishnanagar, Amtala at South 24 Parganas district of West Bengal, India was marked for high productivity, regular bearing and excellent fruit quality. Mature fruits were collected and brought to the laboratory of the Biochemistry and Crop Physiology department of School of Agriculture & Allied Sciences, The Neotia University. Seeds collected from five representative fruits from the plant were surface sterilized with 0.1% mercuric chloride solution, placed between two layers of sterile moist sterile cotton pad in Petri dishes, and incubated for 5 to 7 days at 30-32°C to germinate in laboratory incubator. Upon swelling of the seeds, the germinating nucellar and zygotic embryos were identified following the procedure standardized by Tisserat (Tisserat, 1985). Under aseptic conditions, the integument of the mature seed was carefully rolled away by making a longitudinal incision with a fine scalpel from the micropylar end. The germinating embryo holding the two original cotyledons originating from the micropylar end was considered as the zygotic embryo. All other germinating embryos under the integument, each with two newly differentiating tiny cotyledons or globular shaped embryos were considered as nucellar embryos (Dubey et al., 2020). The number of embryos and seedlings originating from the zygotes and from nucellar tissue were carefully examined.

Establishment of seedling progeny

After observation on polyembryony, the germinating seeds were allowed to grow in aseptic conditions on a cotton bed for another 10 to 12 days, and then put into a sterile soil-sand-organic matter mixture (2:1:1) under controlled conditions with high humidity for further growth of the seedlings, and were marked separately according to their origin. The growth pattern of different seedlings was carefully noted and recorded. The most vigorous seedlings developed from a single fruit were grown for three months in poly-house. As all the embryos were selected from the same fruit, it was expected that the developed seedlings will be genetically similar in majority of the characters except few ones. The mother plant was also included in the experiment for marking of the apomictic nucellar seedlings.

DNA Extraction

Genomic DNA was extracted from the soft leaves of the seedlings and mother plant using the Plant DNA CTAB Extraction procedure (Schenk et al., 2023). The quantity and amount of DNA were determined using intact bacteriophage lambda DNA in 1% agarose gel (Kahangi et al., 2002).

Primer selection and RAPD Analysis

The PCR amplification was achieved by the protocol outlined by Williams 1990 with slight modifications (Williams, 1990). Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1.5 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10X Taq Polymerase buffer´(Bangalore Genei) and 15 ng of decamer primers (Nalbiogen, India) in a total volume of 25 µL. The amplification was performed in a thermocycler (Gene Amp PCR System 9700, Applied BioSystems). Total reaction consisted of 45 cycles, each cycle comprising three steps (denaturation at 92°C for 30 seconds; annealing at 38°C for 30 seconds; extension at 72°C for 1 minute), with an initial denaturation at 94°C for 30 seconds and a final extension at 72°C for 5 minutes, followed by cooling at 4°C. RAPD analysis was carried with 25 Operon decamer primers selected by preliminary screening to give

polymorphism and reproducible fragment patterns in the species using mother plant DNA and analysis of polyembryony in *Citrus reticulata* (Mondal et al., 2015).

Horizontal gel electrophoresis and amplicon detection

Amplified fragments were separated on 2% agarose (Merck-Genei) gels containing ethidium bromide (0.5 µg per mL of agarose) at 60 V for 6 hours in Tris Borate EDTA buffer. The gel was visualized and photographed under UV excitation using an electronic dual wave transilluminator system (Ultra. Lum Inc., USA). Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra. Lum Inc., USA). The size of the amplicons (DNA fragments) in base pairs (molecular weight) was estimated by using a 100-bp ladder marker (Bangalore Genei), which was run along with the amplified products. The primers that could generate differential banding pattern of the seedlings of different origin (apomictic and sexual) of a fruit were noted by comparing with the DNA profile of the mother plant. The experiment randomly included a positive and negative control in 10% of the PCR run. One of the selected apomictic seedlings was used as positive control and pure de-ionised water was used as negative control in PCR reactions.

RESULTS & DISCUSSION

The phenomenon of apomixis is very common in Citrus. Addition to the presence of the single normal sexual embryo, small, plural embryos were also observed. In further course of development these embryos compete for nutrient resource. In *Citrus sinensis* several embryonic anomalies were noticed in this experiment. Figure 1 revealed the presence of several embryos per seed along with presence of zygotic twin, triplets, and anomalies such as fused radical or fused plumule of two seedlings. The number of twin and triplet seedlings was significantly less in comparison to large number of polyembryonic seeds.

For the molecular experiment a single fruit was used for extraction of all the seeds. The embryos extracted from the seeds were screened on the basis of morphological character and utilized for raising the seedlings. The embryos were carefully kept in poly-house with high humidity for seedling development. During the period of seedling growth, DNA was isolated from the leaves of the mother plant and was used for primer screening. In total 50 primers belonging to the Operon series were selected for RAPD analysis. The primer selection was based on previous literature search and ongoing research of the laboratory on citrus. The three-month-old seedlings with 7-8 leaves were used for DNA extraction using CTAB Plant DNA extraction method. After preliminary screening, 25 primers yielding strong, intense, unambiguous and reproducible DNA fragments were selected and utilized for conducting the reactions. Out of which four primers were able to trace the embryonic origin of the seedlings. The details of decamer primer able to differentiate the zygotic and apomictic embryos in sweet orange were as shown in Table 1. Out of 25 primers four primers, OPQ15, OPAH02, OPAA02 and OPA11were able to detect variation in DNA profile among the selected seedlings. The seedlings showing banding pattern dissimilar to the mother plant were marked as the sexual or zygotic types. Out of the ten seedlings two displayed difference in banding pattern from the mother plant (Fig. 2).

In horticulture industry the fruit quality parameter plays an instrumental role in the profitability and growth of the business. The early detection of a high performing plant could assist in meaningful orchard management. Nowadays the scientists are relying on diverse rapid and novel techniques for early diagnosis of internal and external quality of fruits. In majority of the cases the non-destructive sensing methods are regarded as excellent tool for advance quality assessment of the fruits before marketing of the produce (Li et al., 2018). In a

study led by a group of scientists of Portugal, non-destructive near infrared spectroscopy method was used to diagnose the quality of Citrus fruits. The method was able to measure several biochemical parameters using NIR spectra without damaging the fruits. These new-age techniques are regarded as low cost, environment friendly, accurate, easy-to-use measures by the scientist for quality control studies (Magwaza et al., 2012; El Khaled et al., 2017; Santos et al., 2021).

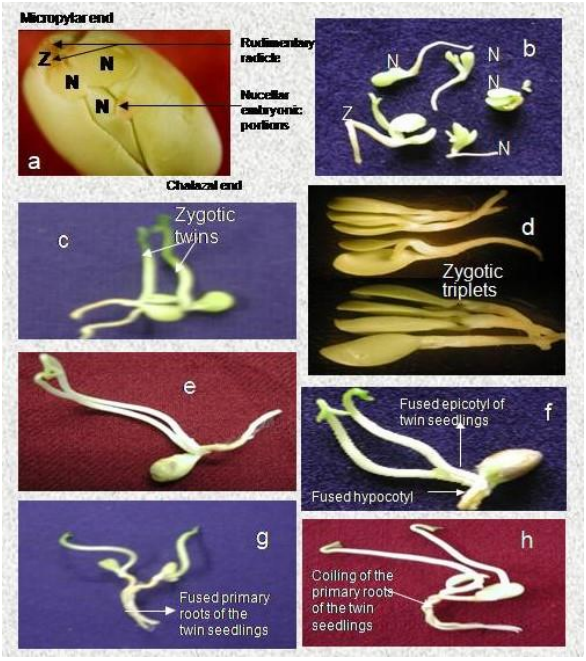

Fig. 1. a. Position of zygotic and nucellar embryo inside a dissected seed, b. extracted embryos, c. zygotic twins, d. zygotic triplets, e., f., g., h – diverse anomalies in embryonic development.

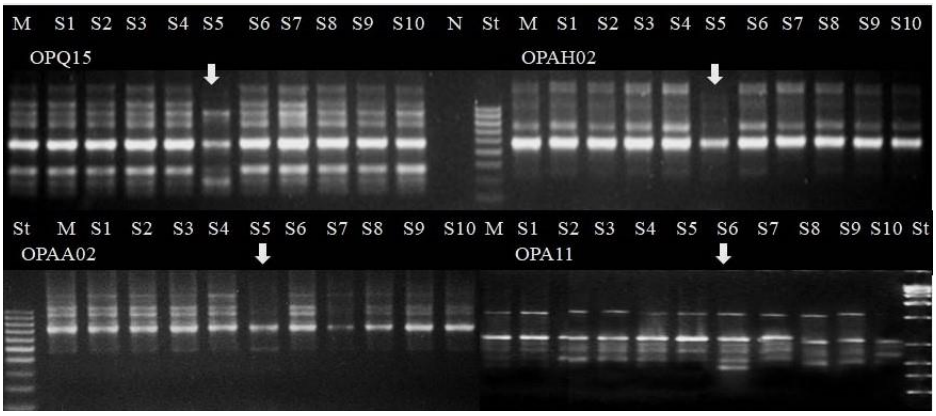

Fig. 2. The RAPD profile of the seedlings developed from a single fruit, M= mother plant, S1-S10= seedlings, N= negative control, St= 100 bp ladder.

Table 1. The details of decamer primers able to differentiate sexual and apomictic seedlings

Primer	Sequence (5'-3')	Total number of amplicon	Polymorphic amplicon	Range of amplicon	Size of unique amplicon (Approximate)
OPQ15	GGGTAACGTC	8	2	175 bp - 1.4 kb	1.3 kb, 1.4 kb
OPAH02	CACTTCCGCT	5	4	350 bp - 1.2 kb	1.2kb,1.1 kb,1.0 kb, 600 bp
OPAA02	GAGACCAGAC	5	3	200 bp – 1.12 kb	1.12 kb, 900 bp, 700bp
OPA11	CAATCGCCGT	6	2	280 bp – 2.1 kb	300 bp, 250 bp

This article emphasizes the scope of using molecular techniques as an efficient tool for quality control of fruits at a very early stage of seedling development. The RAPD based molecular technique utilized in this research experiment could detect quality of plants just after establishment of the seedlings. The Horticultural business sector requires sizable land for rearing and maintenance of the plants. In Citrus usually the flowering and fruiting occurs after 5-6 years due to the prevalence of a long juvenile period. The maintenance of low quality non-performing plant types for five to six year could become a costly venture for orchard owners and growers. The rouging of the plants at the age of 5-6 year will generate a problem of agricultural waste removal. The DNA based molecular technique discussed here could detect the true-to-the-type seedlings within 48 hours without destruction of the plant in net house. The process requires only 400 mg of leaf tissue that could be obtained from a 2-3 month old seedling. In this experiment one to two leaves from the three month old seedlings were used for RAPD analysis. Considering the same by three months of establishment of the seedling the PCR based molecular technique could identify nucellar plants in a sustainable way without destruction of the germplasm. The research described above could be used by the horticulturist for quality control of fruits in lucrative Citrus industry with a nominal input.

CONCLUSION

The unidentified pollen source may significantly influence the performance of an elite genotype. The contribution of an undesirable allele may negatively alter the fruit quality of the extracted embryos. The initial detection of off-type plant in nursery and orchard may assist in quality control of fruits and production of uniform progeny population for citrus industry. The DNA fingerprinting process described in this article could trace the embryonic origin of the seedlings within 48 hours. This technique could be used as a rapid, low cost molecular technique for fruit quality control.

Conflict of interest

The author declares no conflict of interest regarding publication of this work.

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