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Application of DNA Based Molecular Marker for the Assessment of Genetic Transformation in *Citrus Sinensis*

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Abstract: ***In the present study, Random amplified polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) markers were used to study the occurrence of graft transformation in Citrus sinensis plants. Elite Citrus sinensis scion plants were grafted to wild Citrus sinensis plants collected from Manipur region. Ten decamer oligonucleotide primers along with two SSR primers were used to amplify genomic DNA extracted from leaf samples. A total of 17 strong, unambiguous amplicons were generated. Primer OPAT04 detected one amplicon (1350 bp) in grafts which is present in rootstock but not in scions. This is an instance of transmission of genetic material from stock to scion. OPAD10 primer amplified one excess amplicon of 1000 bp, not found in scion or stock but only noticed in grafts. SSR primer CCSM147 with AG repeat revealed the absence of a 100 bp amplicon in two graft plants which was present in scions. The result indicated that after grafting the genetic fidelity is not maintained in the grafts. The absence of amplicons may be explained as gene silencing by transcriptional down regulation. Genetic transformation may be used for improvement of horticultural and agronomic traits of agriculturally important fruit crops by correlating the molecular marker and phenotypic manifestations.***

Keywords: Citrus sinensis, RAPD, SSR, Genetic transformation, scion, root stock

1. Introduction

Citrus is a medicinally important fruit crop and a huge resource of vitamin C. The consumption of this particular fruit provides ample vitamin and antioxidants. The Citrus orchards of North Eastern India suffer from decline problem due to insufficiency of proper planting material. In some states of North Eastern region Citrus plants are solely developed by grafting. For the improvement of the orange orchards molecular analysis of the grafts are very important. The molecular analysis will provide a clear picture about the genetic architecture of the grafts and their synonymy with scion and stocks.

2. Literature Survey

Most grafted Citrus trees consist of two distinct parts, rootstock and scion. The **rootstock** comprises the root system and lower stem or trunk. It is usually grown from seed. The upper part of the tree, consisting of the limbs, leaves and fruit, is known as the **scion**. The scion is derived by inserting tissue of the desired cultivar into the rootstock in such a way that it unites with the rootstock and develops the fruiting portion. By changing different plants for the rootstock and scion, horticulturists may able to incorporate more desirable characteristics into a single tree. These attributes include tolerance to unfavorable soils, pests, diseases or cold, and greater bearing with high-quality fruit.

From a genetic perspective, grafting involves the creation of a compound genetic system by uniting two (or more) distinct genotypes, each of which maintains its own genetic identity throughout the life of the grafted plant. Mostly it is expected that the scion part will retain its elite characters and no genetic transmission from the stock part will affect the plant. However, controversial claims of graft ''hybridization'' have persisted, and new information on gene silencing cased by the transmission of RNA across the graft union suggests that grafting could have genetic consequences [1]. Another important genetic consideration related to grafting concerns the limits of compatibility. That taxonomic affinity is the arbiter of which species can be grafted successfully onto any other has often been misunderstood through the history of grafting [2]. Broadly speaking, interclonal/intraspecific grafts are nearly always compatible, interspecific/intrageneric grafts are usually compatible, intrageneric/intrafamilial grafts are rarely compatible, and interfamilial grafts are essentially always incompatible. These generalizations are complicated by the observation that the degree of taxonomic affinity necessary for compatibility varies widely across different taxa. Stock like phenotypes reported to be inherited in the scion progeny altering of fruiting direction, fruiting habit, and pericarp color [3]. Ohta states that genetic analysis indicates that these three traits are due to independently inherited Mendelian genes that are highly stable in the cultivars used in the grafting studies. The frequency of transmission of stock like traits in the progeny of the scion was reported to be highly variable across different experiments, but with an average rate of 0.84%.

3. Problem Definition

In West Bengal some sweet orange plants are developed by grafting desired scion plant on hardy sweet orange wild stocks. The stocks were mainly brought from Manipur and are seed propagated. In this paper an attempt is taken to study the molecular profile of the mother scion plant, graft

plants and root stocks to identify the occurrence of graft transmission.

4. Materials & Methods

Investigation presented in the paper was carried out in the laboratory for Plant and Microbial Biotechnology, Research Complex Building, Bidhan Chandra Krishi Viswavidyalaya, Kalyani, Nadia, West Bengal. Leaf sample of root stock plant from Manipur along with three Scion mother plant and their respective grafted plants were brought to laboratory and kept in -20 degree centigrade in Deep freezer.

a. DNA Extraction and RAPD Analysis
Genomic DNA was extracted from the soft leaves of the seedlings using the Plant DNA CTAB Extraction Method with minor modifications for *Citrus sinensis* [4]. The quantity and amount of DNA were determined as described by [5] and spectrophotometer based study.

b. PCR Procedure
Amplification was achieved by the protocol outlined by [6], with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1.5 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10´ buffer, and 15 ng of decamer primers (Bangalore Genei) 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.

c. Electrophoresis of PCR product
Amplification fragments were separated on 1.5% 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).

d. RAPD & SSR band scoring and cluster analysis
Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra.Lum Inc., USA). The size of the fragments (molecular weight in base pairs) 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 patterns of the selected plants were noted.

e. Selection of suitable primers
DNA isolated from sweet orange leaves and other citrus plants were used for primer screening. Arbitrary decamer primers used for Citrus cultivar identification and polyembryony studies were applied to study graft transformation. After preliminary screening primers of Operon series yielding more than one band and strong, intense, unambiguous and reproducible DNA fragment were selected for RAPD analysis. Two SSR primers were also included in the experiment. The selected primers were used for PCR analysis of all the plants including scion, grafts and rootstocks.

f. Details of the Software used for analysis
TOTAL LAB software was used for calculating the fragment size of the generated amplicons along with Excel for PIC calculation.

5. Results & Discussion

DNA isolated from sweet orange leaves was used for primer screening. In total 10 primers belong to the Operon series were used for RAPD analysis and two SSR primer pairs were also there. Arbitrary primers yielding strong, intense, unambiguous and reproducible DNA fragments were selected. The list of the selected primers efficient in discovering the objective of this research was tabulated. Their sequences, maximum number of fragments obtained and range of the size of the amplicons were as shown in Table 1. The amplified fragments varied from 2 to 8 (Table1).

Table 1: Details of RAPD and SSR primer used for study of Graft Transformation

Primer name	Total no. of Amplicons	Polymorphic Bands	Polymorphism (%)	Band Range (bp)
OPAT 04	6	3	50	300 - 1750
OPAD 10	8	6	75	100 - 1000
CCSM 147	3	2	66.67	50 - 400

The size of the fragments ranged from 50 bp to 1350 bp and a total of 17 amplicons were generated by 3 primers. The citrus genome was about 563 mbp [7]. Each set of PCR reaction accompanied positive and negative control. In every PCR reaction no DNA fragments were found in the negative control while similar banding patterns were found in the positive control indicating contamination-free PCR ingredients and the consistency of the protocol. Each experiment was repeated another time for confirmation of data.

Comparison of *Citrus Sinensis* Scion (Mother), Clonal Plants, Rootstocks by Rapd And Ssr

Three scion mother, three grafts and the wild root stock plants were compared to assess the graft fidelity after grafting. Scion and stock both were from sweet orange. The hardy root stocks were collected from Manipur. Two RAPD primers OPAT04 and OPAD10 along with one SSR primer CCSM147 were found unique in identifying some peculiarities in the experiment. OPAT04 identified one DNA amplicon (1350 bp) that is present in root stock and all the grafted plants but not present in mother scion plants, showing the possibility of graft transformation.

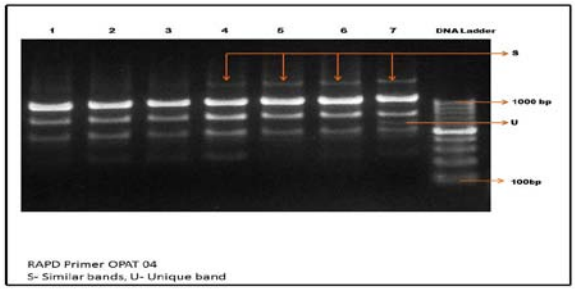

Figure 1: Showing RAPD profile of mother scion plants, their respective clones and root stock

Another important observation was the presence of an extra amplicon (1000 bp) in all the grafted plant which is not present in either scion or root stock. This observation is revealed by decamer primer OPAD10.

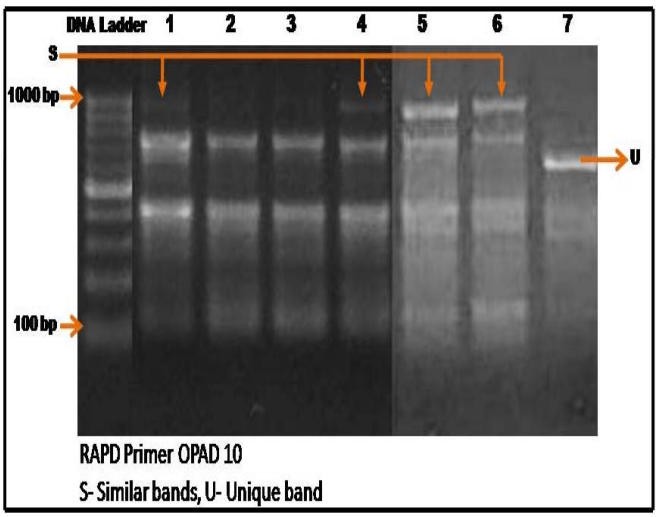

Figure 2: Showing RAPD profile of mother scion plants, their respective clones and root stock

SSR primer CCSM147 with AG repeat motif recorded the absence of a 80 bp fragment in two of the grafted plants unlike their scion mother plant while another graft plant showed smeared SSR profile. Dinucleotide repeats are more abundant and variable and generate stutter bands (*i.e.* smeared bands) during amplification [8]).

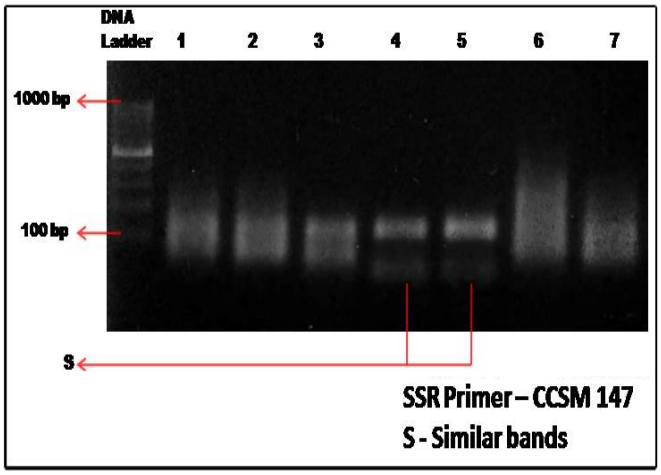

Figure 3: Showing SSR profile of mother scion plants, their respective clones and root stock

All the amplicons generated by root stock are not present in grafted plants, only some fragments are noticed. This observation provides evidence regarding graft transformation which is a controversial and debated phenomenon. The generation of excess bands questions about the genetic fidelity of the grafts with mother scion. [9] reported similar finding with random amplified polymorphic DNA (RAPD) analyses indicating that a stock-specific DNA marker could be detected in graft-induced variants. Various kinds of variations have been found with irregular genetic behaviours in the progenies derived from repeated grafting [10]. Haroldsen et. al, 2012 reported the movement of organellar DNA, RNA and protein across graft unions [11]. Based on our experiments, we especially suggest that transformation is a probable mechanism for graft-induced genetic changes.

While grafted scions and rootstocks are generally assumed to conserve their own genetic identity, it is becoming evident that certain transcription factors, mRNAs, regulatory micro RNAs (miRNAs), small interfering RNAs (siRNAs), peptides, and proteins are mobile in the plant vascular system and thus, may cross the graft union. Potentially, delivery of any of these products from a genetically engineered rootstock is advantageous for the scion, and the grafted plant experiences enhancement of pathogen and pest resistance [12].

To verify the hypothesis of graft transformation, we surveyed DNA transferred from stock to scion by using molecular techniques. We found some extra DNA sequences among stock and graft-induced variant. It is likely that gene transfer and integrated mechanism in the grafted plants might be mediated via special system. By using differential display, further analysis of some genes responsible for some new traits is possible. It is also possible to correlate occurrence of new phenotypic traits with new amplicons. Although it is reported that epigenetic modifications could revert back in the next generation, it presents an opportunity to endow progeny with transcriptional modifications without introduction of heritable transgenic DNA.

6. Conclusion

From this study if is concluded that molecular techniques can detection graft transformation by differential genetic profile of grafts. The presence and absence of new amplicons and matching with scion and root stock may reveal the operation of genetic transmission and up-regulation or silencing of genes.

7. Future of Research

In our study Random amplified polymorphic DNA marker (RAPD) and Simple Sequence Repeat marking (SSR) proved that graft transformation takes place in *Citrus sinensis.* Three primers proved efficiency in detecting the presence of molecular anomaly like presence of amplicon similar to root stock plant or absence of amplicons present in scion mother or presence of altogether new amplicon in grafted plant. This kind of studies is very sparse and requires more elaborate research for horticultural and agronomic

improvement of agriculturally important crops by transcriptional regulation.

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References

[1] Tournier. B, Tabler. M, Kalantidis K. "Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants." Plant Journal, 47, pp.383–394,2006.10.1111/j.1365-313X.2006.02796.x [PubMed] [Cross Ref]
[2] Pease. AS. "Notes on ancient grafting." Trans. Proc. Am. Philological Assoc,. 64, pp. 66-76, 1933.
[3] Ohta. Y, "Graft-transformation, the mechanism for graft-induced genetic changes inhigher plants". Euphytica, 55, pp. 91–99, 1991.
[4] Doyle. JJ, Doyle. JLA, "Rapid DNA isolation method for small quantities of fresh tissues". Phytochemical Bulletin, 19, pp. 11‑15, 1987.
[5] Kahangi. EM, Lawton. MA, Kumar. CACY, "RAPD profiling of some banana varieties selected by small-scale farmers in Kenya." Journal of Horticultural Science and Biotechnology, 77, pp. 393–398, 2002.
[6] Williams. JGK, Kubelik. AR, Livak. KJ, Rafalski. JA, Tingey. SV, "DNA polymorphism amplified by arbitrary primers are useful as genetic markers." Nucleic Acids Research, 18. Pp. 6531–6535, 1990.
[7] Guerra. MDS, "New chromosome numbers in Rutaceae." *Pl. Syst. Evol*, 146, pp. 13–30, 1984.
[8] Schlotterer. C, " Microsatellites. In: Hoelzel AR (ed) Molecular Genetic Analysis of Populations: A Practical Approach." Oxford University Press, New York, pp. 237-261 1998.
[9] Taller. J, Y. Hirata, N. Yagishita, M. Kita, S. Ogata, "Graft-induced genetic changes and the inheritance of several characteristics in pepper (Capsicum annuum L.)." Theor. Appl. Genet, 97, pp. 705–713, 1998.
[10] Hirata. Y. "Graft-induced changes in eggplant (S. melongena L.) I. Appearance of the changes." Euphytica, 35, pp. 395–401, 1986.
[11] Haroldsen. VM, Szczerba. MW, Aktas H, Lopez-Baltazar. J,Odias MJ, Chi-Ham. CL, Labavitch JM, Alan.B, "Mobility of Transgenic Nucleic Acids and Proteins within Grafted Rootstocks for Agricultural Improvement". Front Plant Sci., 3, p. 39, 2012. **doi: 10.3389/fpls.2012.00039**PMCID: PMC3355758.
[12] Gaffney. T, Friedrich. L, Vernooij. B, Negmtto. D, Nye. G, Uknes. S, Ward. E, Kessmann. H, Ryals. J, "Requirement of salicylic acid for the induction of systemic acquired resistance." Science, 261, pp. 754-756, 1993.

Author Profile

Dr. Bidisha Mondal was graduated from University of Calcutta with Botany Honours and was a gold medallist in Genetics & Plant Breeding (Ag.) from University of Calcutta. She was selected for training in Plant Biotechnology: Molecular Marker Technology by DST, Govt. of India among the 10 researcher from all over India. She has worked as guest research worker in Bose Institute after completing her Master's degree. She obtained doctorate degree from Bidhan Chandra Krishi Viswavidyalaya in 2005 with funding by a ICAR-NATP-CGP project of Govt. of India. She has 17 publication in indexed journal out of which 4 are internationally highly reputed one. She has 6 full papers in national &international level proceedings. Adding to this she has written 5 chapters of Subsidiary Botany Book of Netaji Subhas Open University and edited two books of Genetics and Evolution of the same University. Other than that she has written 5 popular articles in Bengali magazines. She has a research career of more than 13 years and a teaching career of 5 years. She served Netaji Subhas Open University as Assistant Professor in Botany from 2009 to 2012 and also taken classes of Post Graduate Diploma in Medicinal & Aromatic Plants of the same University. As Guest Lecturer she took classes of Molecular Biology, Biotechnology & Microbiology. At present she is working as Scientist in Bidhan Chandra Krishi Viswavidyalaya. She has earned the prestigious Bio-CARe Women Scientist award of Department of Biotechnology, Govt. of India in 2012. She has delivered several radio lectures as anchor and distance teacher on several socio-scientific issues. Her research interest involves biotechnology, molecular biology, genetics, plant physiology, plant nutrition and environment.

Reeya Dutta, a 4th year student, from St. Xavier's College, Kolkata, pursuing a course on Int. M.Sc. on Biotechnology. I have recently been awarded a fellowship by the Indian Academy of Sciences Summer Fellowship 2014 (IASc-INSA-NASI). My research experience in Bidhan Chandra Krishi Viswavidyalaya, Kalyani pertained to understanding molecular breeding techniques in *Citrus* plants. My interest lies in plant genetics and transformation techniques. Plant pathology and defense methods adapted by plants also fascinate me. I would like to proceed further with this work on breeding improvement for better yield, using plant molecular biology as my core knowledge.

Dr. Ramkrishna Saha have awarded Ph. D degree in Plant Pathology from Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, W.B. in the year 2004 preceded by M.Sc.(Ag) Hons. in Plant Pathology in 1998 and B.Sc.(Ag) Hons. in 1996 from the same University. He also qualified ICAR NET in 2002 and presently working as Assistant Director of Agriculture at Department of Agriculture, Govt. of W.B. He is engaged in his current employment since 12 years where doing the Agriculture Extension work and implementations of different Agriculture related schemes of Govt. of India and State Govt. The author at the same time continued his laboratory research. He possesses six numbers of other research publications one book chapters in peer reviewed journals. The author is the life member of Indian Science Congress. The area of research interest is in the field of biotechnology and nutritional management in disease development.