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Technology development for identification of citrus *(Citrus* spp*)* rootstocks based on Sequence Tagged Microsatellite marker

AMBIKA BALDEV GAIKWAD[1], SUNIL ARCHAK[2] and SWATI SAXENA[3]

National Research Centre on DNA Fingerprinting,National Bureau of Plant Genetic Resources,Pusa Campus, New Delhi 110 012

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Citrus is an important fruit producing genus of the world.China and Nigeria are the major citrus producers with India ranking fourth,contributing a 4.48% in the global production (FAO Statistics 2011). In India, citrus fruits rank third in importance after mango and banana. Andhra Pradesh, Maharashtra and Punjab produced 24.2, 18.9 and 12.1% respectively of the total produce of 74.64 lakh tonnes from 8.46 lakh ha area in the year 2010-2011(Kumar 2011). The major citrus fruits grown in India are lime, lemon, mosambi and orange. The average productivity from India is approximately 9 mt/ha.This is contrast to an average of 30-40 mt/ha in countries like Brazil and USA.

Limiting growing conditions, water resources and high incidence of pests and diseases are some problems that affect productivity (Radha and Matthew 2007). Another major cause for concern for citrus growers is the availability of appropriate rootstocks. For centuries commercial citrus cultivation has been done by grafting and budding. The rootstock is a very important part of a citrus orchard and unlike a cultural practice, a fertilizer dosage or an irrigation schedule, it cannot be changed overnight. It has varied effects on scion vigour and size, fruit yield, quality, tolerance to various biotic and abiotic stresses,adaptability to various soils and mycorrhizal dependency (Sonkar *et al*. 2002). In addition,the effect of the stock- scion interaction is reflected in various physiologically and agronomically important traits,so much so that breeding for rootstocks has become an important aspect for the success of any citrus breeding programme.

The performance of a rootstock varies greatly with the scion variety and the agroclimatic conditions. It is therefore of utmost importance to select the best performing rootstock for a given variety in a given region to attain maximum productivity and quality. Rough lemon and Rangpur lime are the most time tested and widely used rootstocks in India (Sonkar *et al*. 2002). The fact that many species of Citrus, including the desired rootstocks are highly polyembryonic and produce true to type seedlings from nucellar seeds is a boon to the citrus grower.However, there can be 1-40% zygotic seedlings in a seedbed (Wutscher 1979).Matters are further complicated by inadvertent seed mixtures of related species. In India, the largest area under citrus cultivation is in the Vidarbha region of Maharashtra where farmers totally rely on the public and private nurseries for supply of planting material. Many nurseries unfortunately do not maintain the mother plants of the Rangpur lime (*Citrus limonia*) and Rough lemon (*Citrus jambhiri*) rootstocks and import seeds from the Himalayan foothill states (Hom *et al.* 2012). These rootstock seeds are randomly collected from different citrus species, particularly Galgal (*Citrus pseudolimon*). Although scions grafted on Galgal are vigorous and healthy, they are susceptible to *Phytophthora* and have a lesser life span thus requiring replanting of the orchard after six or seven years. A comparison of the citrus cost and returns (www.nabard.org) shows that a farmer cuts even with the input cost at the end of the seventh year of starting an orchard. Replanting of an orchard would thus lead to huge economic losses for a farmer with no recovery whatsoever of the input cost.

DNA based markers provide ideal nondestructive assays for identification of cultivars at a juvenile stage (Karp 1997). In citrus, molecular markers such as RAPD,ISSR, and RFLP have been used for fingerprinting (Fang and Roose 1997), genetic diversity analysis (Filho *et al*. 1998,Luro *et al*. 1995),phylogenetic studies (Nicolosi *et al*. 2000) and discrimination of cultivars (Bernet *et al*. 2004). Sequence tagged microsatellite markers (STMS) are codominant, highly reproducible markers,easily automated with good analytical resolution making them the preferred choice of markers (Matsuoka *et al.* 2002).The development of these in citrus (Kijas *et. al*.1995) and their utilization for discriminating species (Ahmad *et al*. 2003) has opened avenues for genomic

[1]Senior Scienctist (email: ambikabg@yahoo.co.in), [2]Senior scientist (email: sarchak@nbpgr.ernet.in), [3]email: swati.saxena2003@yahoo.in

evaluation. The present study describes the application of STMS markers to differentiate the undesirous Galgal seedlings from the preferred rootstocks of citrus, i e Rangpur lime and Rough lemon. This protocol has been developed into a technology that is successfully transferred to two public sector organizations for identification of quality rootstocks in citrus nurseries.

Reference material comprising fifteen seedlings each of Rough lemon, Rangpur lime and Galgal were obtained from the Director of Horticulture,Commissionerate of Agriculture, Maharashtra State, Pune. DNA was extracted from 2 g of leaf material using CTAB method with minor modifications (Saghai Maroof *et al.* 1984). The homogenization buffer contained 100mM Tris, 20mM EDTA, 1.4M NaCl, 2% CTAB, and 0.2% 2-Mercaptoethanol. The DNA was treated with bovine pancreatic RNase,extracted once with phenol: chloroform(1:1) and twice with chloroform:isoamyl alcohol (24:1). It was precipitated with 0.6 volumes of isopropanol, washed with 70% ethanol,dissolved in TE buffer and quantified using a fluorometer (DyNA Quant 200, Hoefer).Twenty five STMS primer-pairs were screened against the bulked DNA of each of the three genotypes. Based on the results two STMS primer-pairs were identified for amplification of each of the forty five individuals. Most STMS primer pair amplified markers that identified loci shared between individuals of Galgal and either Rangpur lime or Rough lemon and thus could not be used for differentiating these from each other. One STMS primer pair (CIT2), flanking the core repeat TAA however amplified distinct alleles that were specific only to Rangpur lime and Rough lemon. The conditions for amplification were optimized at 25ng of DNA, 3.0 mM magnesium chloride, 0.2 mM deoxyribonucleotide triphosphates(dNTP mix), 1 microlitre of each of the forward and reverse primer, 1.0 unit *Taq* DNA polymerase and 1X of reaction buffer(as provided by the manufacturer with *Taq* DNA polymerase) amplified at 95ºC for 5 minutes; followed by 35 cycles of 98ºC for 1 min, 55ºC for 30 seconds and 72ºC for 1 minute; and a final extension of 72ºC for 7 minutes.The amplified fragments were separated by electrophoresis in 2.5% routine agarose and through 1X Tris Borate EDTA buffer at 4-7volts/cm along with a standard DNA marker of 50 base pair interval. An allele of 160 base pairs was amplified in all the three genotypes. However in Rough lemon (Jambhiri) and Rangpur lime, an additional allele of 200 base pairs and 180 base pairs respectively was present (Fig 1). The presence of this allele (200bp or 180bp) confirms the material to be tested as the preferred rootstock of Rough lemon and Rangpur lime. Thus on the basis of presence/ absence of a fragment, Rough lemon and Rangpur lime can be differentiated from Galgal.

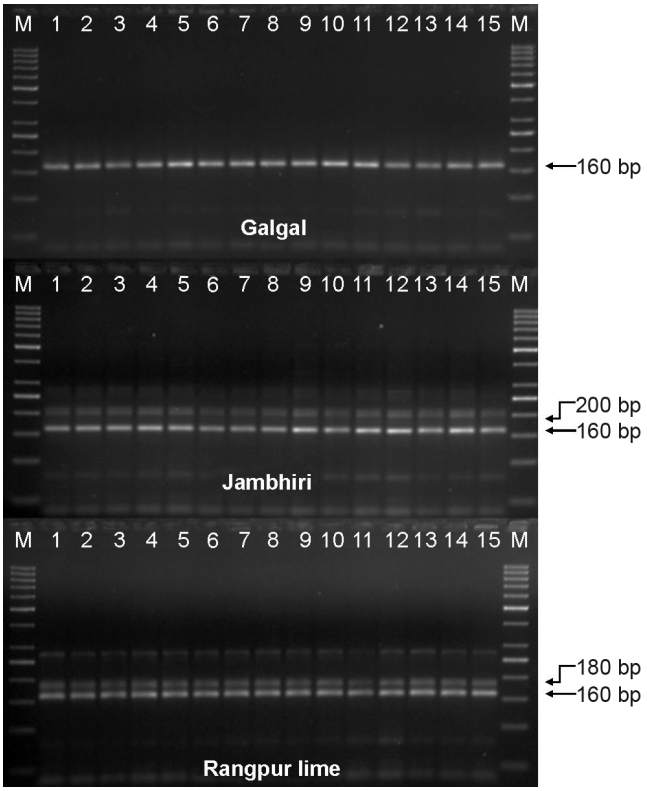

Fig 1 Allelic differences between individuals of Galgal, Jambhiri, and Rangpur lime based on STMS markers.M is the 50 bp DNA standard.

SUMMARY

Grafting and microbudding in citrus requires excellent quality root stock material. Traditionally Rough lemon (Jambhiri) and Rangpur lime provide quality rootstock seedlings. Galgal is avoided as a rootstock particularly for its susceptibility to diseases. It is difficult to discriminate between Galgal, Rough lemon and Rangpur lime at seedling stage. This results in inadvertent as well as deliberate mixture of Galgal plants in the nursery. This impediment may eventually lead to great economic loss in the citrus orchards.A DNA marker based technology for citrus rootstock identification at seedling stage has been developed at the National Research Centre on DNA Fingerprinting, NBPGR New Delhi. The PCR based protocol uses microsatellite markers, is easy and quick to be adopted by any laboratory with basic molecular biology facility.The technology has been transferred to Krishi Vigyan Kendra, Durgapur (Badnera), Amravati and Maharashtra State Seed Certification Limited, Akola.

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