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Training Manual

on

Genetic Fidelity Testing of Tissue Culture-Raised Plants

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Preface

Micropropagation, a tissue culture based approach for clonal multiplication of superior genotypes has been practised in our country for the past three decades across a variety of species. This technology is being successfully employed in vegetatively propagated crops which have complex genomes and higher ploidy. Of these, banana, sugarcane, potato followed by orchids and other ornamentals are the major ones. Horticultural crops with longer growth cycles such as apple, oilpalm, datepalm and pomegranate are also being clonally multiplied in the country. With more and more farmers planting tissue culture-raised plants, the quality of the planting material needs to be ensured to augment farmer's income. To identify somaclonal variations in tissue culture-raised plants, molecular marker systems could be a better choice.

NRCPB, as a referral centre for National Certification System for Tissue Culture Raised Plants (NCS-TCP) has the responsibility to identify a set of markers as well as marker systems for unequivocal DNA profiling of the mother plant and its tissue culture multiplied progeny. It also has the obligation of developing trained and skilled human resource in this area of agricultural biotechnology esp., for the Accredited Testing Laboratories (ATLs) established across the country. In this regard, a training to strengthen the capability of ATLs was organized in 2015 at NRCPB by providing basic principles behind the DNA marker techniques and the most common trouble shooting strategies. In the last one year, most informative set of molecular markers have been developed at NRCPB for genetic fidelity testing of banana, potato and sugarcane. The procedure for genetic fidelity testing in date palm has also been streamlined. The current training programme and the manual intend to disseminate these latest developments to the ATLs. The ultimate aim is to equip the ATLs to handle the techniques with knowledge and confidence.

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1. Plant genetic fidelity and its significance

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Unlike animals which follow sexual mode reproduction for the multiplication and continuation of their genetic content to the subsequent generation, plants either follow sexual or asexual or both the methods of propagation. The nature of multiplication i.e., sexual or asexual has significant bearing on the genetic constitution of its progeny and progenies thereafter. Sexual mode of reproduction enhances the genetic variability of offspring produced from genetically divergent parents, and maintains diversity of the population. It is this genetic variation which plays key role in evolution of a species. On the contrary the asexual or vegetative reproduction ensures preservation of genome from parents to offspring, thereby ensuring the conservation of novelty of the elite germplasm of an organism. Preservation of genetic uniformity through generations (true to type) of the superior parental genotype is more important in tissue culture regenerated plants so as to preserve the specific agronomical traits associated with those plants. Therefore, genetic fidelity is defined as faithful transfer of genetic component from one generation to the next generation without modifications of any sort. Naturally plants do produce true to type plants to preserve their genetic content through vegetative propagation; on the other hand, modern day biotechnology tools, such as tissue culture based micro propagation, are being exploited to conserve valuable plant genetic resources and elite genotypes.

Plant tissue culture technique has wide spread application in agriculture and allied fields. The important applications are:

1. Micro propagation of agriculturally important plants like banana, potato, papaya, sugarcane, date palm, cashew, coconut etc.
2. Clonal multiplication of forest trees like eucalyptus and teak, which never breed true to type.
3. Meristem culture of banana, potato, citrus and other crop plants to eliminate spread of viral diseases.
4. Generation of double haploid plants
5. Shortening the breeding cycle of tree species, which otherwise take many years to complete life cycle.
6. It is the important component of transgenic development

7. Production and extraction of plant secondary metabolites

Though micro-propagation through tissue culture guarantees the maintenance of high genetic fidelity, the scaling up of any micro-propagation methodology and continuous subculturing carries the associated risk of induced genetic variability, mainly somaclonal variations (Larkin and Scowcroft, 1981). Somaclonal variations are induced due to the stresses imposed on the plant under *in vitro* culture conditions and are manifested in the form of DNA methylation, chromosome rearrangements, point mutations and retrotransposon activation leading somaclonal variations. The primary factors that induce somaclonal variation are culture method and conditions, source of explant, stage of explant, ploidy level and culture age i.e. frequency of subculture. High concentration of growth regulators usually used in the culture media for enhancing the rate of shoot multiplication has been reported to induce somaclonal variations in the micro-propagated plantlets**.** The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerated plants can seriously limit the broader utility of the micro-propagation system (Salvi et al., 2001). Therefore, it is imperative to ascertain the suitability of a particular micro-propagation protocol developed for a particular species to establish genetic uniformity of micro-propagated plants where commercial success depends solely on the maintenance of clonal uniformity. In this regard, it should be mandatory for commercialisation of such crop plants to undergo quality checks implying identification of off types and genetically true-to-type progenies of the mother plant.

Several techniques have been developed to assess the genetic purity of tissue culture raised plants such as morphological descriptions, physiological supervisions, cytological studies, field assessments, isozyme analysis and molecular studies. Among other approaches, molecular markers assisted approach is one of the important methods to preserve the genetic fidelity of clonally multiplied plants, since this can be accomplished well before the distribution of planting material to the farmers. Most commonly used molecular markers for genetic fidelity testing include, Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or Sequence Tagged Microsatellite Sites (STMS), Sequence Tagged Sites (STS), DNA Amplification Fingerprinting (DAF) and Microsatellite Primed-PCR (MP-PCR) (Mohan et al*.,* 1997) and Inter simple sequence repeats (ISSR) markers (Williams et al*.,* 1990; Godwin et al*.,* 1997). In India, use of molecular markers for genetic fidelity was reported way back in 1995 (Rani, 1995). Among the available molecular markers, RAPDs and ISSR are most commonly used

as these markers are very simple, fast, cost-effective and highly discriminative. They have higher effective multiplexing ratio since they can profile multiple loci simultaneously. Besides, they need only a small quantity of DNA sample and there is no need of prior sequence information to design the PCR primer.

Among the available molecular markers used for genetic fidelity testing, ISSR markers are most commonly used as. These are simple sequence repeat motifs used to prime the polymerase chain reaction and thereby amplify regions between adjacent, but inversely orientated, microsatellites. Compared to RAPD primer, ISSR primers are longer, so they offer advantages in the detection of somaclonal variation notably with a higher degree of sensitivity, increased stringency, higher reproducibility and the dominant representation of polymorphic genetic alleles. ISSR markers have been successfully applied in the analysis of genetic fidelity in cauliflower (Leroy et al., 2000), almond (Martins et al., 2004), banana (Lakshman et al., 2007; Rout et al., 2009), *Cymbopogon martinii* var motia (Bhattacharya et al., 2010), gerbera (Bhatia et al., 2009), *Swertia chirayita* (Joshi and Dhawan 2007) and bamboo (Goyal et al., 2015).

2. History and significance of plant tissue culture

Solanke AU, Shingote P, Devanna BN, and Sharma P, Arora K and Amitha Mithra SV

Plants are propagated through sexual as well as asexual (vegetative) mode of propagation. Vegetative propagation using *in vitro* approach is called as micro-propagation, where in plant tissue/ cells give rise to complete plants under controlled aseptic culture conditions. Plant tissue culture (PTC) exploits the totipotent nature of plant cells and the phenomenon of regenerating entire plants from any single cell is known as totipotency of cells. Micropropagation comprises of different stages viz. creation of a dedifferentiated cell from well differentiated tissue under specific culture conditions; proliferation of dedifferentiated cells for a number of generations and; its subsequent redifferentiation and regeneration in to whole plant. In other words, one imposes a period of essentially dedifferentiated cell proliferation between an explant and the next plant generation. The source of explants may be different plant parts including leaves, stems, petiole, embryos, roots, microspores and protoplasts. The ease and efficiency with which such manipulations can be made varies enormously from species to species.

Haberlandt in 1902 for the first time gave the concept of plant tissue culture where he worked on the culture of single cells. The actual tissue though was reported by Gautheret (1939) where he developed a method for *in vitro* growth of carrot tissues. Tissue-culture techniques have subsequently been developed and established for a lot of plant species. The major breakthrough in plant tissue culture leading to commercial scale tissue culture however begun after the discovery of artificial medium by Murashige and Skoog in 1962. Micropropagation is an important tool in both basic and applied studies, as well as in commercial application (Thorpe 1990).

Propagation of plants through micropropagation has become an established and popular technique to reproduce crops that are otherwise difficult to propagate conventionally by seed and/or vegetative means. Micropropagation offers many unique advantages over conventional propagation methods of propagation, such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free plants, non-seasonal production (round the year), germplasm conservation and international exchange. Micropropagation is also one of the key steps in development of transgenic plants and doubled haploids. Through the use of micropropagation the production of secondary

metabolites is considerably accelerated. In spite of its widespread applications, plant tissue culture still has limitations such as cost of production, choice of crops which is restricted to species with well-established and reproducible micropropagation protocols etc. As tissue culture raised plants are grown in aseptic, highly protected environment, they need to be hardened or acclimatized in nurseries before transfer to the field. Advances in commercialization of plant tissue culture and acceptance of tissue cultured plantlets by the commercial sector have led to continued exponential growth within the industry in terms of number of new units as well as number of plants produced by these units.

Different techniques used for micropropagation of plant material are summarised briefly in the following sections. *In vitro* propagation can be achieved either through organized and unorganized culture approaches.

Organogenesis /Direct regeneration/

It is a method of development of embryo (embryogenesis) or formation of organs (organogenesis) directly leading to the generation of plants from the explant, it is called as direct regeneration or direct organogenesis, respectively. Embryos that form directly from explants is called somatic embryos and they are bipolar in nature possessing both root and shoot meristems. This process can also be called as somatic embryogenesis. On the other hand, in direct organogenesis, either shoots or roots develop directly from the explants which are later cultured to develop into entire plants. Direct organogenesis/regeneration is adventitious in nature. Adventitious refers to the development of organs/embryos from organized explants where preformed meristem is lacking.

Regeneration through callus/Indirect regeneration

A method in which plant regeneration is achieved through intervening callus culture and subsequent regeneration of callus to give rise to organs (organogenesis) or entire embryos (somatic embryogenesis) de novo, and it is called as indirect regeneration or callogenesis, respectively. Under *in vitro* cultures, the meristematic cells undergo dedifferentiation to become parenchyma cells and which further divides and re-divides to form unorganized mass of cells called callus. These callus cells later undergo redifferentiation thereby regenerating into entire organs or plants.

Naturally the unorganized growth is observed very rarely, but it is frequently observed under *in vitro* culture conditions. The resulting unorganized cell typically lacks any recognizable structure and contains only a limited number of the many kinds of specialized and differentiated cells of plant parts. A differentiated cell is one that has developed a

specialized morphology and/or function. The formation of differentiated cell types can only be partially controlled in culture medium. It is not possible, for example, to maintain and multiply a culture composed entirely of epidermal cells. By contrast, unorganized tissues can be increased in volume by subculture and can be maintained on semisolid or liquid media (suspension cultures) for long periods.

Organ cultures

Differentiated plant organs can usually be grown in culture without loss of integrity.

I. Meristem and shoot culture

The growing points of shoots can be cultured in such a way that they continue uninterrupted and organized growth. As these shoot initials ultimately give rise to small organized shoots which can then be rooted, their culture has great practical significance for plant propagation. Two important applications are:

a. Meristem culture

Culture of the extreme tip of the shoot is used as a technique to free plants from virus infections. Explants are dissected from either apical or lateral buds. They comprise a very small stem apex (0.2-1.0 mm in length) consisting of just the apical meristem and one or two leaf primordia. This technique is very popular in virus-free citrus propagation.

b. Shoot culture or shoot tip culture

Culture of larger stem apices or lateral buds (ranging from 5-10 mm in length to undissected buds) is a very successful method of propagating plants.

c. Node cultures

Node culture is an adaptation of shoot culture; stem pieces carrying either single or multiple nodes may be cultured.

II. Isolated root cultures

The growth of roots, unconnected to shoots: a branched root system may be obtained.

III. Embryo culture

Zygotic or seed embryos are often used advantageously as explants in plant tissue culture, for example, to initiate callus cultures. In embryo culture, however, embryos are dissected from seeds, individually isolated, and 'germinated' *in vitro* to provide one plant per explant. Isolated embryo culture can assist in the rapid production of seedlings from seeds

that have a protracted dormancy period or low viability (e.g. those resulting from distant or interspecific or incompatible crosses). This technique is also known as embryo rescue.

a. Immature embryo culture

Culturing of embryo prior to the formation of zygote to circumvent pre-zygotic incompatibility which prevents pollen germination and/or pollen tube growth so that a zygote is never formed is called as immature embryo culture.

b. Mature Embryo Culture

Culturing of mature zygotic embryo on artificial medium to circumvent post-zygotic incompatibility which rejects the acceptance of zygote by the endosperm is called as mature embryo culture. When there is embryo-endosperm incompatibility, the embryo, not receiving sufficient nutrition, disintegrates or aborts.

Stages of micropropagation

Vegetative propagation *in vitro* is called as Micropropagation. Murashige (1974) defined three steps or stages (I-III) in the *in vitro* multiplication of plants. These have been widely adopted by both research and commercial tissue culture laboratories. The treatment and preparation of stock plants is regarded as a stage '0' while hardening is used as fourth stage (IV). A general description of these 0-IV stages is as follows:

Stage 0: Mother plant selection and preparation

Before micropropagation commences, careful attention should be given to the selection of the stock or parent plants. They must be typical of the variety or species, and free from any symptoms of disease. It may be advantageous to treat the chosen plant (or parts of it) in some way to make *in vitro* culture successful. Chemical sterilization is the most common procedure.

Stage I: Establishing an aseptic culture

The customary second step in the micropropagation process is to obtain an aseptic (sterile) culture of the selected plant material. Success at this stage primarily requires that explants should be transferred to the cultural environment, free from microbial contaminants; and this should be followed by some kind of growth (e.g. growth of a shoot tip, or formation of callus). Stage I would be regarded as satisfactorily completed if an adequate number of explants had survived without contamination, and kept growing.

Stage II: The production of suitable propagules

The object of Stage II is to bring about the production of new plant outgrowths or propagules which, when separated from the culture are capable of giving rise to complete plants. According to the *in vitro* procedure that is being followed, multiplication can be brought about from newly derived axillary or adventitious shoots, somatic embryos, or miniature storage or propagative organs. In some micropropagation methods, Stage II will include the prior induction of meristematic centers from which adventitious organs may develop. Some of the propagules produced at Stage II (especially shoots) can also be used as the basis for further cycles of multiplication in that they can usually be sub-cultured to increase their number.

Stage III: Preparation for growth in the natural environment

Shoots or plantlets derived from Stage II are small, and not yet capable of self-sustaining growth in soil or compost. At Stage III, steps are taken to grow individual or clusters of plantlets, capable of carrying out photosynthesis, and survive without an artificial supply of carbohydrates. Some plantlets need to be specially treated at this stage so that they do not become stunted or dormant when taken out of the cultural environment. As originally proposed by Murashige, Stage III includes the *in vitro* rooting of shoots prior to their transfer to soil.

Stage IV: Transfer to the natural environment

Although not given a special numerical stage by Murashige, the methods whereby plantlets are transferred from the *in vitro* to the external environment are extremely important. If not carried out carefully, transfer can result in significant loss of propagated material. In practice, plantlets are removed from their Stage III containment, and if they have been grown on agar medium, the gel is carefully washed from the roots. The application of an anti-transpirant film to the leaves has been recommended at this stage, but in practice, seems to be seldom used. Plantlets are then transplanted into an adequate rooting medium (such as a peat: sand compost) and kept for several days in high humidity and reduced light intensity. A fog of water vapour is very effective for maintaining humidity. Alternatively, intermittent water misting may be applied automatically, or the plants may be placed inside a clear plastic enclosure and misted by hand. With some plants, an *in vitro* Stage III can be omitted; shoots from Stage II are rooted directly in high humidity, and, at the same time, gradually hardened to the exterior environment.

Advantages

i. Economy of space is the major advantage of micropropagation. Explant requirement is also minimal.
ii. The rate of propagation is much greater than in macropropagation and many more plants can be produced in a given time. This may enable newly selected varieties to be made available quickly and widely, and numerous plants to be produced in a short while.
iii. Production of plants free from specific virus diseases thereby assuring productivity is a major commercial application of PTC.
iv. It is possible to produce clones of rare species/genotypes, and also plants that are otherwise slow and difficult (or even impossible) to propagate vegetatively.
v. Plants may acquire a new temporary characteristic through micropropagation which makes them more desirable to the grower than conventionally-raised stock.
vi. Production can be continued all the year round and is more independent of seasonal changes.
vii. Vegetatively-reproduced material can often be stored over long periods.
viii. Plant material needs little attention between subcultures and there is no labour or material requirement for watering, weeding, spraying etc.;

Limitation

i. The cost of propagules is usually relatively high due to the requirement of sophisticated tools and techniques required to maintain stringent aseptic environment and also cost medium requirements is also high.
ii. In order to survive *in vitro*, explants and cultures have to be grown on a medium containing sucrose or some other carbon source.
iii. As they are raised within glass or plastic vessels in a high relative humidity, and are not usually photosynthetically self-sufficient, the young plantlets are more susceptible to water loss in an external environment.
iv. Further undesirable consequence of using *in vitro* adaptations is that the plantlets obtained are initially small and sometimes have undesirable characteristics caused by somaclonal variation.

3. Somaclonal variations: Causes and importance

Amitha Mithra SV, Shingote P, Devanna BN, Sharma P, Arora K and Solanke AU

Somaclonal variation is the genetic variations produced in plants in response to plant tissue culture or any form of cell culture and the term somaclone was coined by Larkin and Scowcroft (1981). Somaclonal variation can be a serious limitation in commercial tissue culture practices, and it can be occurring both during *in vitro* propagation and in the field conditions. The main objective of the clonal propagation is regeneration of phenotypically identical individuals, but in contrast it is often not the case in practice. One of the popular examples of adverse effect of somaclonal variation is the mantled floral phenotype in oil palm which affects ~5% of regenerated palms. Causes of somaclonal variations are genetic and epigenetic changes induced in response to micropropagation. Somaclonal variation can also be a useful tool for crop improvement through selection and characterization of novel variations. The successful example of such variations is a Fiji disease resistant variety 'ono' developed through somaclonal variation in sugarcane from variety Pindar; and a bold seeded and high yielding variety Pusa Jai Kisan developed in Indian mustard from variety Varuna. Another example is a somaclone scarlet in sweet potato which has darker and more stable skin colour.

Somaclonal Variations may show either heritable or epigenetic. Heritable mutation(s) persisting in plant population even after plantation into the field could be detected by variations in karyotype, isozyme characteristics, DNA marker profile and morphology.

Mechanism of Somaclonal Variations

1. **Genetic (Heritable Variations)**

- Pre-existing variations in the somatic cells of explant
- Caused by point mutations or gross chromosomal rearrangements or ploidy changes or activated transposons and other DNA changes/aberrations

2. **Epigenetic (Non-heritable Variations)**

- Variations generated during tissue culture
- Caused by temporary phenotypic changes

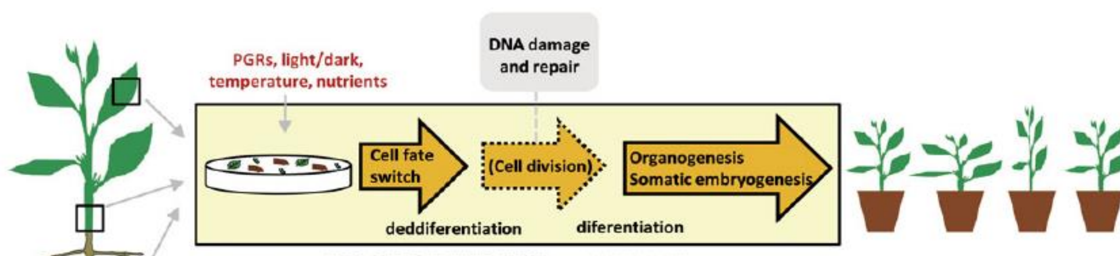

Figure 3.1: Illustartion of somaclonal variation induced by tissue culture (Source, Miguel and Marum 2011)

Causes of Somaclonal Variations

1. **Physiological**
    - Exposure of culture to plant growth regulators
    - Culture conditions
2. **Biochemical**
    - Lack of photosynthetic ability due to alteration in carbon metabolism
    - Biosynthesis of starch via carotenoid pathway
    - Nitrogen metabolism and Antibiotic resistance

Detection and Isolation of Somaclonal Variants

a. **Analysis of morphological characters**
- Qualitative characters: Plant height, maturity date, flowering date and leaf size
- Quantitative characters: yield of flower, seeds and wax contents in different plant parts

b. **Variant detection by cytological Studies**
- Staining of meristematic tissues like root tip, leaf tip with feulgen and acetocarmine provide the number and morphology of chromosomes

c. **Variant detection by DNA content**
- Cytophotometer detection of feulgen stained nuclei can be used to measure the DNA content

d. **Variant detection by gel electrophoresis**
- Change in concentration of enzymes, proteins and chemical products like pigments, alkaloids and amino acids can be detected by their electrophoretic pattern

e. **Detection of disease resistance variant**
- Pathogen or toxin responsible for disease resistance can be used as selection agent during culture

f. **Detection of herbicide resistance variant**

- Plantlets generated by the addition of herbicide to the cell culture system can be used as herbicide resistance plant

g. Detection of environmental stress tolerant variant

- Selection of high salt tolerant cell lines in tobacco
- Selection of water-logging and drought resistance cell lines in tomato
- Selection of temperature stress tolerant in cell lines in pear
- Selection of mineral toxicities tolerant in sorghum plant (mainly for aluminium toxicity)

Advantages of Somaclonal Variations

- Help in crop improvement
- Creation of additional genetic variations
- Increased and improved production of secondary metabolites
- Selection of plants resistant to various toxins, herbicides, high salt concentration and mineral toxicity
- Suitable for breeding of tree species

Disadvantages of Somaclonal Variations

- A serious disadvantage occurs in operations which require clonal uniformity, as in the horticulture and forestry industries where tissue culture is employed for rapid propagation of elite genotypes
- Sometime leads to undesirable results
- Selected variants are random and genetically unstable
- Require extensive and extended field trials
- Not suitable for complex agronomic traits like yield, quality etc.
- May develop variants with pleiotropic effects which are not true

4. Molecular genetic markers for ensuring plant genetic fidelity

Amitha Mithra SV, Devanna BN, Sharma P, Shingote P, Arora K and Solanke AU

A molecular genetic marker is the differences in the nucleotide sequence at the corresponding position of the homologous chromosomes (physical position) and it shows Mendelian pattern of inheritance. Molecular markers, including DNA based genetic markers have found their applications in almost all areas of biology, more so in evolutionary biology, genetics and breeding in order to identify a particular sequence of DNA in a pool of unknown DNA. For many applications, DNA markers are preferred over other molecular markers since DNA is stable within an organism over time and among stages of development whereas other molecules are dynamic.

Variations in DNA across genotypes are substantial in any species and all DNA variations are essentially due to single nucleotide polymorphisms (and additions and deletions), chromosomal aberrations and copy number variations. Diversity within a species is a result of these sequence variations and interaction between them and the environment. Since the former is inherited across generations, it is worthwhile to identify and employ these variations in DNA for both basic (e.g. phylogeny and diversity analysis, molecular tagging of traits of interest) and applied studies (e.g. marker assisted selection, fingerprinting, genetic fidelity testing). There is array of molecular markers developed to identify these variations all of which rely either on hybridization or PCR based approaches. Most commonly used molecular markers in biological sciences include Random Amplified Polymorphic DNAs (RAPDs), Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Cleaved Amplified Polymorphic Sequences (CAPS), DNA Amplification Fingerprinting (DAF), Simple Sequence Repeats (SSRs) or Sequence Tagged Microsatellite Sites (STMS), Sequence Tagged Sites (STS), and Microsatellite Primed-PCR (MP-PCR), Inter simple Sequence Repeats (ISSR), Inter-Retrotransposon Amplified Polymorphism (IRAP) and Retrotransposon-Microsatellite Amplified Polymorphism (REMAP).

A. RFLP Restriction Fragment Length Polymorphism (RFLP)

RFLP is the first molecular marker technique which was developed by Botstein et al., (1980) and was based on restriction enzyme digestion of genomic DNA. This is a landmark invention in molecular biology which revolutionized the field of molecular genetics.

Polymorphisms are detected based on the restriction enzyme digestion and in combination with probe hybridization (a labelled DNA fragment which is used for hybridization with the restriction digested genomic DNA). The Southern blots of restricted genomic DNA from different strains of the same species are denatured and hybridized to a probe. This results in the visualisation of variation in the size and/or number of detected restriction fragments generated from the different strains. The detected length variation is because of the differences in restriction sites caused by insertions or deletions. RFLP is largely codominant. It could be multi or single locus depending upon the source and sequence of the probe used (Figure 4.1).

B. Random Amplified Polymorphic DNA (RAPD)

RAPD makers were initially described by William (William et al., 1990). It is a PCR based marker which uses a single random arbitrary primer of 10 bases for amplification of DNA fragments. Principle is when nanogram quantity of genomic DNA is subjected to PCR, the probability of finding priming sites for random decamers of inverted orientation and close enough to be amplified by PCR, in the whole genomes, is very high. This technique amplifies the intervening sequences of variable length between such priming sites giving rise to a multitude of bands for any primer which are resolved by agarose gel electrophoresis (Figure 4.2). Therefore, RAPD is a multi-locus dominant marker that cannot differentiate between homozygous and heterozygous lines. However, different PCR conditions may give rise to a different profile of amplicons and hence the same set of PCR conditions should be used for comparable and reliable results. The main advantage of this technique is that no prior genomic information is necessary and can be employed in any species.

These polymorphisms, simply detected as DNA segments which are amplified in one genotype but not the other, and inherited in a Mendelian fashion can be used for genetic analysis and unequivocal identification of the genotype. RAPD primers normally use low annealing temperatures and generates ~10 fragments for each decamer primer. Due to low Tm, RAPDs are largely not reproducible. To overcome this problem, SCAR markers were developed.

C. DNA Amplification Fingerprinting (DAF)

Caetano-Anolles (1991) developed DAF molecular marker and it refers to DNA amplification using a single arbitrary primer of 7-8 GC rich primers. The principle of DAF is similar to that of RAPD. However, they differ with each other in two aspects; 1. DAF uses primers of 7-8 bases unlike 10 bases in RAPDs and 2. DAFs markers are resolved on

polyacrylamide gels and detected by silver staining rather than normal agarose gel used for RAPDs. Similar to the RAPDs, DAF is also dominant giving multiple anonymous amplification products, and there are problems with reproducibility and problems of co-migration of bands (homoplasy) from different loci. But the strength is that there is no need of sequence information and they also work across the species or genera.

D. Sequence Characterized Amplified Regions (SCAR)

Paran and Michelmore (1993) developed SCAR markers to overcome the relative inconsistency of RAPDs. SCAR markers were developed from polymorphic RAPD marker. To develop a SCAR marker, polymorphic marker band differentiating two genotypes is eluted and sequenced, based on which longer and more specific primers are designed. Since the primer sequence has been made more specific and higher annealing temperatures are used, the amplifications products are specific and reproducible. Thus, SCAR is largely a codominant marker, with single locus profiling (Figure 4.3). Therefore, SCAR marker can differentiate homozygous and heterozygous lines.

E. Cleaved Amplified Polymorphic Sequences (CAPS)

PCR amplified DNA (STS-, EST- or SCAR-product) which is monomorphic (i.e. does not show any length variation) is digested with restriction endonucleases to detect polymorphisms, if any, in restriction sites. Principle is sequence variation namely Single Nucleotide Polymorphisms (SNPs) or small Insertions/Deletions (InDels) present in a monomorphic sequence, could be detected by amplifying the sequence of interest, digesting with appropriate restriction enzyme and subsequent electrophoresis. Koneiczny and Ausubel (1993) named these markers as CAPS for cleaved amplified polymorphic sequences and employed them for the first time in Arabidopsis to detect polymorphisms between two parents of a F_2 population, the popular ecotypes, Columbia (Col) and Landsberg erecta (Ler) and later in the population. Since monomorphic PCR products of known gene sequences or sequence tagged sites (STS) or sequence characterized amplified regions (SCAR) are restriction digested in this method, it always remains a **locus specific, codominant marker** (Figure 4.4). The only drawback is that it cannot be automated or multiplexed unlike other marker types since it involves restriction digestion. The original procedure of developing CAPS, did not involve sequencing, but testing with many restriction enzymes till polymorphism is obtained. Nowadays, with the advent of automated Sanger sequencing and next generation sequencing technologies, sequence data generation has become routine and a lot of such data are available in the public domain. Thus, wherever locus specific sequence

information from two or more genotypes are available, CAPS markers can be developed, based on the sequence variations and restriction sites, easily using appropriate tools. SNP2CAPS is one such tool which is freely available and can be downloaded from http://pgrc.ipk-gatersleben.de/snp2caps/ (Thiel et al. 2004).

F. Amplified Fragment Length Polymorphism (AFLP)

Zabeau and Vos (1992) developed a marker system which combines restriction digestion and PCR amplification to identify polymorphism. Genomic DNA of any organism and any complexity can be subjected to AFLP. Similar to RAPD, this marker system also does not require any prior sequence information of the species to design primers. However, the priming of oligos is not arbitrary; rather amplification is from specifically selected restriction fragments. Therefore, AFLP is defined as PCR based amplification of a set of fragments generated by restriction digestion. In brief, AFLP has three important steps: 1. Restriction digestion of genomic DNA with two restriction enzymes, one a frequent cutter and a rare cutter, and ligation of adaptor (compatible with the restriction site) sequences – adaptors have sequences complimentary to the restriction cleavage site and arbitrary core sequences; 2. Selective PCR amplification (of only those fragments which have been cleaved by both the restriction enzymes) using adaptor specific primers – to understand AFLP, it is important to understand the structure of the primers – primer has three important parts namely the core sequence (which is complementary to the arbitrary portion of the adaptor sequence), a restriction enzyme specific sequence and finally a set of three nucleotides for selective amplification (this is to make the priming sites more specific) 3. Gel electrophoresis to resolve the amplified fragments. A typical AFLP reaction amplifies 50-100 fragments. AFLP polymorphism is based on variations in the sites of restriction enzymes used and the priming sites of the oligos. AFLP therefore produces a multilocus profile which is largely dominant (Figure 4.5).

G. Sequence-Tagged Site (STS)

STS is a relatively short (200 to 500 bp), easily PCR-amplified sequence whose location in the genome is mapped. PCR amplification of STS marker is very specific and hence can be detected in the presence of all other genomic sequences. The STS concept was introduced by Olson et al., (1989). One of the major reasons for acceptance of hybridization based RFLP marker was that it could indicate map locations and is a valuable tool in developing genetic and physical maps of any organism. However, the PCR based markers such as RAPD and DAF were not very useful for this purpose. In this context, Olson and co-

workers realized that single-copy DNA sequences of known map location which could be uniquely amplified by PCR could serve as markers for genetic and physical mapping of genes. The advantage of STSs over other mapping landmarks is its universality: the means of testing for the presence of a particular STS in genomic DNA can be completely described as specific information in a database: anyone who wishes to make copies of the marker would simply look up the STS in the database, synthesize the specified primers, and run the PCR under specified conditions so as to genotype the STS. STS-based PCR produces a simple and reproducible pattern on agarose or polyacrylamide gel. In most cases STS markers are codominant, i.e., allow hetorozygotes to be distinguished from the two homozygotes. The DNA sequence of an STS may contain repetitive elements, sequences that appear elsewhere in the genome, but as long as the sequences at both ends of the site are unique and conserved, researches can uniquely identify this portion of genome using tools usually present in any laboratory. Thus, in broad sense STS include such markers as microsatellites (SSRs, STMS or SSRPs), SCARs, CAPS, and ISSRs.

H. Microsatellites based markers or simple sequence repeats

Microsatellites are simple sequence tandem repeats which are 1 to 6 nucleotides long and repeated n number of times. CA dinucleotides are the most abundant microsatellites in mammals whereas TA and GA are more common in plants. In fact, in plants trinucletides are the most abundant. Microsatellite based markers were initially developed by Zietkiewicz et al. (1989). They are highly polymorphic and distributed in both genic and non-genic regions and serve a very useful class of markers. Being locus specific and codominant markers, they are the markers of choice even today despite the increasing popularity of single nucleotide polymorphisms (SNPs). This is because the polymorphic content of SSRs is very high compared to SNPs. The higher PIC comes from the multiple allelic nature of the microsatellites. The only problem with SSRs is that developing them is a long-drawn process. Once discovered, they are known to work across species within a genus. They are quite suitable for all genetic applications such as construction of genetic maps, molecular tagging, marker assisted selection and diversity studies within a species. Microsatellites are flanked by unique regions and primers are developed from these regions enabling the amplification of intervening repeat sequences. Since the repeats evolve faster than other nucleotides, due to slippage and unequal crossing over, they are highly polymorphic. It is also possible to multiplex their detection using fluorescent primers and automated fragment analyzers.

Microsatellite markers are also known in literature by the name Simple Sequence Repeats (SSR) (Figure 4.6) and Sequence Tagged Microsatellite Site (STMS).

I. Inter-Simple Sequence Repeat Amplification (ISSR)

It is a PCR-based multi-locus marker system which used single primers of SSR sequences. Kantety et al., (1995) developed these markers to assess genetic diversity in dent and popcorn. To avoid stuttering, these primers can be anchored to unique genomic sequences flanking the repeat either in the 5' or 3'. 3'-anchoring give better results than 5'-anchoring. Amplification products are only obtained in case SSRs in opposite orientation are found within a PCR-able distance, with flanking sequences matching the oligo's. Repeat polymorphisms within the SSR do not influence the chance for ISSR polymorphisms. ISSR are dominant, easy-to-use and reliable markers with high polymorphic information content. They are multi-locus which amplify 3-7 bands on an average.

J. Inter-Retrotransposon Amplified Polymorphism (IRAP)

IRAP is a retrotransposon-based dominant marker developed by Kalendar. Transposable elements are abundant in genomes of eukaryotes. Among them, SINES are found in large numbers and the pattern of their insertion varies in genomes of different species and varieties. Transposons are characterized by the sequences found in their ends which are known popularly as long terminal repeats (LTR). Thus, LTR specific primers can amplify the regions between two LTR regions of neighbouring transposons. For this, the primers face outwards from terminal retrotransposon regions. As retrotransposon integration patterns vary between genotypes, the number and size of the resulting amplicons can be used for differentiation of genotypes or cultivars, to measure genetic diversity or to reconstruct phylogenies. IRAP generates multilocus dominant marker profile. Since prolonged plant tissue culture activates transposable elements resulting in somaclonal variation, IRAP could be one of the best marker systems for genetic fidelity testing of tissue culture raised plants.

K. REtrotransposon-Microsatellite Amplified Polymorphism (REMAP)

The REMAP method is similar to IRAP, but one of the two primers (forward) matches an SSR motif. Besides SSR motif, one or more non-SSR anchor nucleotides are present at the 3' end of the primer. Anchor nucleotides serve the same purpose as in the case of ISSRs: it avoids slippage while annealing with the template thereby avoiding stuttering effect. Differences in the number of SSR units in a microsatellite are generally detected using primers designed to unique sequences flanking microsatellites. Alternatively, the stretches of the genome present between two microsatellites may be amplified by ISSR, in a way akin to

IRAP. REMAP uses primer types that are shared by IRAP and ISSR: one primer is specific to LTR region and the other to SSR motif. Thus, PCR amplifies the region between a SSR motif and adjoining retrotransposon. The polymorphism is therefore generated by both the location of retrotransposon element and SSR motif. Similar to IRAP and ISSR, REMAP also generates multilocus dominant markers. Since REMAP also works on the principle akin to IRAP, they can also be used for detecting somaclonal variation.

L. Single Nucleotide Polymorphisms (SNPs)

A single nucleotide polymorphism or SNP (pronounced as *snip*) is an individual nucleotide base difference (could be insertion/deletion/substitution) between two homologous DNA sequences. They are hailed as the newest markers of choice since they make up about 90% of all genetic variation. As a nucleotide base is the smallest unit of inheritance, SNPs provide the ultimate form of molecular marker. SNPs are meaningful only when their position is clearly defined and also with respect to a reference genome. Reference genome can either be the standard ones, such as B73 in maize or can be fixed by the researcher with respect to the phenotype the gene is responsible for. For example, if the trait is low phytate soybean, then the candidate genes between low and high phytate genotypes are compared using the best low phytate genotype as reference. Jordan and Humphries first time used SNP based diagnostic marker for a specific trait. SNPs are often mentioned in connection with a technique which allows the specific recognition of the SNP. SNP are detected by techniques like resequencing, comparison of genomic sequences, denaturing gradient gel electrophoresis (DGGE), Field-Inversion Gel Electrophoresis (FIGE), Pulsed-Field Gel Electrophoresis (PFGE), Single Cell Gel Electrophoresis (SCGE), Single-Strand Conformation Polymorphism (SSCP), Temperature Gradient Gel Electrophoresis (TGGE), RFLP, CAPS etc. SNPs are locus-specific, codominant and more often than not bi-allelic.

Prospects of molecular markers in plants are limited by the exploitation of variance analysis, which is being explored in the case of animals, especially in humans. Therefore, there is greater scope for developments in the DNA markers in case of plants.

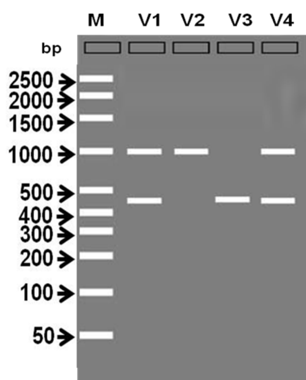

Fig. 4.1: The polymorphic RFLP marker reveals size differences for the marker alleles in four genotypes. M: 100 bp DNA ladder, V1-V4: Varieties.

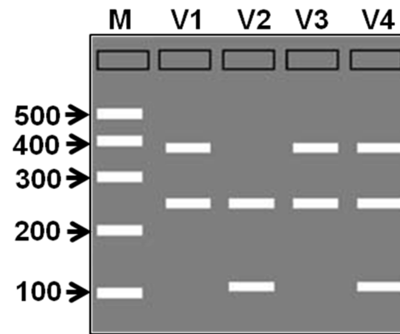

Fig. 4.2: The polymorphic RAPD marker reveals size differences for the marker alleles of the four genotypes. M: 100 bp DNA ladder, V1-V4: Varieties.

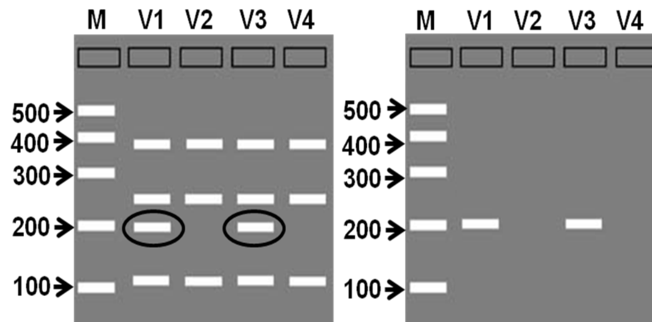

Fig. 4.3: Development of polymorphic SCAR marker (right panel) from a RAPD profile (left panel) M: 100 bp DNA ladder, V1-V4: Varieties. Here SCAR is shown as a dominant marker. However, it could also be a codominant marker.

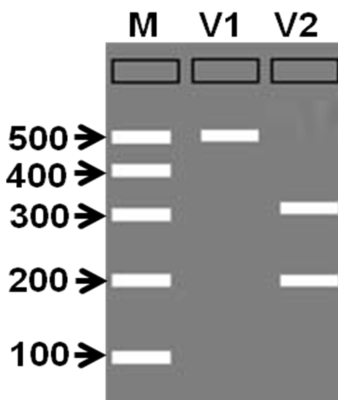

Fig.4.4: The polymorphic CAPS marker reveals size differences for the marker alleles of the two genotypes. M: 100 bp DNA ladder, V1 and V2: Varieties. Note that the total length of the amplicons is same in both the varieties.

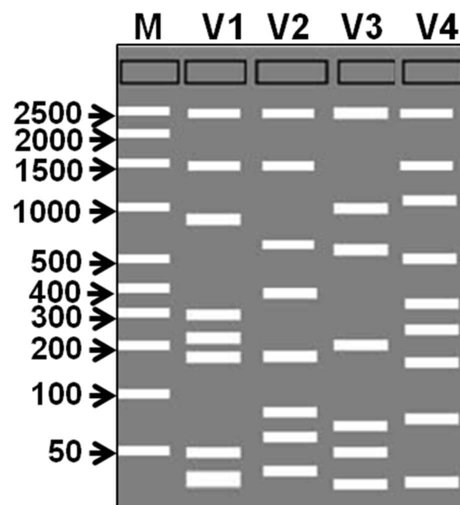

Fig. 4.5: The polymorphic AFLP marker reveals size differences for the marker alleles of the four genotypes. M: 100 bp DNA ladder, V1-V4: Varieties.

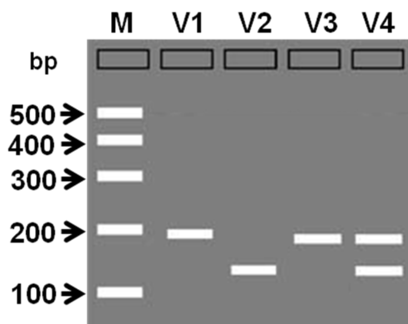

Fig. 4.6: The polymorphic SSR marker reveals size differences for the marker alleles of the four genotypes. M: 100 bp DNA ladder, V1-V4: Varieties. Note their multiple allelic nature.

5. Plant genomic DNA isolation, an overview

Arora K, Devanna BN, Sharma P, Shingote P, Amitha Mithra SV and Solanke AU

Many protocols have been reported for extraction of DNA and the primary objectives of them is development of relatively quick, inexpensive and consistent protocol to extract high quality DNA with better yield from expanded leaf material containing large quantities of polyphenols, tannins and polysaccharides. However, the basic principle of all these DNA extraction protocols remains the same. The general principle is disruption of the cell wall, cell membrane and nuclear membrane to release the highly intact DNA into solution followed by precipitation of DNA while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites by enzymatic or chemical methods.

The most commonly used detergents such as cetyl trimethyl ammonium bromide (CTAB) or SDS (sodium dodecyl sulfate) disrupts the membranes whereas β-mercaptoethanol, a reducing agent, helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and removes tannins and polyphenols present in the crude extract. EDTA (Ethylenediaminetetraacetic acid) chelates the Mg^{+2} ions required for DNase activity, thereby keeping DNA intact. Buffer such as Tris is used to maintain the pH at 8 and a salt such as NaCl aid in precipitation of DNA by neutralizing the negative charges and aggregating the DNA molecules. Further, nucleic acid solutions are commonly contaminated with molecules made of proteins. These contaminants are removed by successively washing with phenol: chloroform: isoamyl alcohol (25: 24: 1) and chloroform: isoamyl alcohol (24:1). Phenol denatures proteins and dissolves denatured proteins. Chloroform helps in binding to the complexed proteins and polysaccharides whereas isoamyl alcohol acts as an antifoaming agent. Finally alcohol is used for nucleic acid precipitation. This requires diluting the nucleic acid with monovalent salts, such as sodium acetate ($C_2H_3NaO_2$), sodium chloride (NaCl), ammonium acetate ($C_2H_7NO_2$), lithium chloride (LiCl) and potassium chloride (KCl). In the presence of cations, ethanol induces structural change in DNA molecules that causes them to aggregate and precipitate out of solution.

The nucleic acid precipitates spontaneously and can be pelleted by centrifugation. The remaining salts and alcohol are removed by washing with 70% alcohol. The clean DNA is lastly resuspended in a buffer to ensure stability and long term storage. The most commonly

used buffer for resuspension is 1X TE. An overview of DNA isolation from plant sample is given in Figure 5.1.

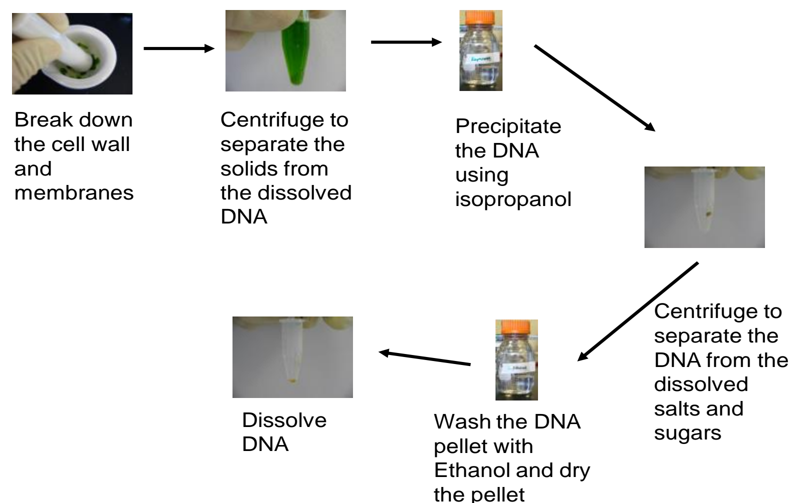

Fig. 5.1. Overview of DNA extraction procedure

1.1 Genomic DNA isolation protocol for Tissue culture raised plants (Sugarcane, Potato and Date palm)

Plant samples were harvested and stored in aluminium foil at -20 °C. For crops like Sugarcane (isolation from leaf tissue), Potato (isolation from sprouts), Datepalm (isolation from leaf tissue), following protocol; a modification of the Doyle and Doyle (1990) CTAB extraction procedure was adopted. This protocol uses a higher CTAB and NaCl concentration to remove polysaccharides (Lodhe, et al., 1995), thereby preventing their interaction with DNA. We also used PVP and sodium sulphite (Na_2SO_3) to prevent the oxidation of phenolic compounds that results in brown coloured DNA (Loomis, 1974).

Solutions

1. Homogenization buffer:
200 mM Tris-HCl
50 mM EDTA
2.2 M NaCl
2% CTAB
0.06% sodium sulphite
pH8.0

2. Phenol: Chloroform: Isoamyl alcohol (25:24:1)

3. 6 M NaCl

4. 10% Polyvinylpyrrolidone (PVP)

5. 5% N-lauroyl-sarcosine

6. 20% CTAB

7. TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

Methodology:

- Weigh about 2 g of leaf sample (after removing midrib) in case of sugarcane and datepalm, and sprouts in case of potato, grind to fine powder in pre-chilled pestle and mortar using liquid nitrogen.
- Transfer the ground tissue powder into 50 ml Falcon tubes containing homogenization buffer (4 ml/g fresh tissue) using a clean sterilized spatula and gently invert the tubes (around 5 times) for proper mixing.
- Add 2 ml of 5% N-lauroyl-sarcosine, 2 ml of 10% PVP and 2 ml of 20% CTAB and mix well by gentle inversion.
- Incubate the Falcon tubes for 60 min at 65 °C in a water bath with intermittent mixing (by inverting 3–4 times at every 15 minutes interval) during incubation.
- Take samples from the water bath and cool down to room temperature. Then add an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mix by inversion.
- Then centrifuge the tubes at 6000 rpm for 15 min at room temperature (25 °C).
- Recover aqueous phase (top layer) from the three layers obtained after centrifugation, and transfer the supernatant to a fresh tube.
- To the supernatant add an equal volume of isopropanol (pre-chilled) followed by 2 ml of 6 M NaCl.
- Incubate the tubes at -20 °C for at least 1 h or overnight.
- Fish out the DNA with a glass pipette and transfer to a fresh tube containing 10 ml of 70% ethanol. Alternatively, spin down the DNA pellet at 6000 rpm for 10 min, wash with 10 ml of 70% ethanol, air dry the pellet, and resuspend in 2 ml of TE buffer

Notes

- Our observation reveals that initial centrifugation at room temperature leads to better separation of three phases.
- Use cut-tips to transfer supernatant after initial centrifugation to minimise shearing of DNA.
- Avoid disturbing the interface (white colour) while pipetting the supernatant phase.

- There is no need of RNAse treatment as RNA is removed/ degraded during the process.

1.2 Genomic DNA isolation protocol for banana

DNA from banana samples was isolated using Sigma's GenElute™ Plant Genomic DNA Miniprep Kit using following procedure:

1. Grind leaf tissue sample of banana into fine powder in liquid nitrogen using a mortar and pestle. Transfer up to 100 mg of the powder to a microcentrifuge tube and keep the sample on ice for immediate use or freeze at –70 °C for later use.
2. Add 350 µl of lysis solution [Part A] and 50 µl of Lysis Solution [Part B] to the tube; thoroughly mix by vortexing and inverting. A white precipitate will form upon the addition of lysis Solution [Part B]. Incubate the mixture at 65 °C for 10 minutes with occasional inversion to dissolve the precipitate. RNase digestion is optional (50 units of RNaseA is added prior to incubation).
3. To precipitate cellular debris, proteins, and polysaccharides debris, 130 µl of precipitation solution is added to the mixture; mix completely by inversion and the sample is then placed on ice for 5 minutes. Centrifuge the sample at maximum speed (12,000–16,000g) for 5 minutes.
4. Pipette supernatant from step 3 onto a GenElute filtration column (blue insert with a 2 ml collection tube). Centrifuge at maximum speed for 1 minute. This removes any cellular debris not removed in step 3. Discard the filtration column, but retain the collection tube.
5. Add 700 µl of binding solution directly to the flowthrough liquid from step 4. Mix thoroughly by inversion.
6. Insert a GenElute miniprep binding column (with a red o-ring) into a provided microcentrifuge tube. Add 500 µl of the column preparation solution to each miniprep column and centrifuge at 12,000g for 30 seconds to 1 minute. Discard the flow-through liquid.
7. Pipette 700 µl of the mixture from step 5 onto the column prepared in step 6 and centrifuge at maximum speed for 1 minute. Discard the flow-through liquid and retain the collection tube. Return the column to the collection tube and apply the remaining lysate from step 5 onto the column. Repeat the centrifugation as above and discard the flow-through liquid and collection tube.

8. Place the binding column into a fresh 2 ml collection tube and apply 500 ml of the diluted wash Solution to the column. Centrifuge at maximum speed for 1 minute. Discard the flow-through liquid, but retain the collection tube.
9. Apply another 500 µl of diluted Wash Solution to the column and centrifuge at maximum speed for 3 minutes to dry the column. Do not allow the flow-through liquid to contact the column; wipe off any fluid that adheres to the outside of the column.
10. Transfer the binding column to a fresh 2 ml collection tube. Apply 100 µl of pre-warmed (65 °C) elution solution to the column and centrifuge at maximum speed for 1 minute. Repeat the elution. Do not allow the flow-through liquid to contact the column. Eluates may be collected in the same collection tube. Alternatively, a second collection tube may be used for the second elution to prevent dilution of the first eluate.

6. Quantitative and qualitative analysis of genomic DNA

Arora K, Devanna BN, Sharma P, Shingote P, Amitha Mithra SV and Solanke AU

All downstream applications of genomic DNA isolated from plants as well as other organisms are largely affected by the quality and quantity of the DNA used in the respective experiments. Therefore, it is imperative to ensure the quality and quantity of DNA isolated before embarking on any further uses. The different approaches used for this purpose are discussed as follows.

A. Agarose gel electrophoresis and DNA quantification:

Reagents needed

50X TAE buffer

Tris base	242 g
Disodium EDTA	18.61 g
Glacial acetic acid	57.1 ml

Volume to 1 litre with distilled water

DNA loading dye 6X

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol

Store at 4 °C

Ethidium bromide (10 mg/ml)

Store EtBr solution in dark

Protocol for agarose gel electrophoresis:

1. Select appropriate gel casting tray and matching comb; fix the comb into slots
2. Weigh 0.8 g agarose into 250 ml flask containing 100 ml 1X TAE and boil to dissolve agar completely
3. After cooling agarose to around 50 °C, add 5 µl EtBr and mix gently so that no bubbles are formed
4. Pour agarose into sealed casting tray slowly in order to avoid bubble formation and let it solidify for 30-45 min

5. Remove the sealing and comb and keep the tray in electrophoresis tank containing 1X TAE buffer sufficient enough to dip the gel
6. Mix 1 µl DNA sample with 1 µl of 6X loading dye and pipette the mix into the wells carefully to avoid floating out of sample. Also load 4 µl DNA ladder of known base pairs, preferably 1 kb
7. Place the lid back on the running tank and connect the power supply. Run the apparatus at 60-80 volts for 45 min to 1h or till the bromophenol blue dye front migrates around 2/3 of the gel
8. At the end take put gel along with tray and capture the image under UV light using gel documentation unit

Note

- To quantify DNA concentration, uncut λ genomic DNA of known concentration (25, 50, 100, 150, 200) should be used during electrophoresis.
- The gel image should be checked for the presence of any kind of RNA contamination or DNA degradation in the samples

B. Spectrophotometer Method:

- Take 1ml of TE buffer in a cuvette and calibrate the spectrophotometer at wavelength of 260 nm and 280 nm.
- Add 2 to 5µl of DNA mix properly and record the optical density (OD) both at 260 nm and 280 nm.
- Estimate the DNA concentration employing the following formula:
- Amount of DNA (µg / µl) = $\frac{\text{(OD) 260 x 50 x dilution factor}}{1000}$
- This is based on the fact that an OD of 1 at 260 nm corresponded to approximately 50µg /ml for double stranded DNA.
- The quality of DNA can be analysed from the ratio of OD values recorded at 260 and 280 nm for each sample. The ratio between the readings (OD_{260}/OD_{280}) provides an estimate of sample purity. Pure preparations have values close to 1.8; whereas protein contaminated samples have significantly lower ratio. On the contrary RNA contamination shoots up the ratio much over 1.8

C. Nanodrop analysis of DNA samples:

The working principle of Nanodrop is same as that of previous method, with the added advantage that Nanodrop provides direct readings after software based internal analysis. Therefore, it is more user-friendly. The parameters to check the quality do remain the same as in the previous method.

Formula to calculate DNA quantity

Concentration (µg/ml) = (O.D at A_{260} – O.D at A_{320}) × dilution factor × 50 µg/ml

Total yield of DNA is calculated using the formula

Total DNA= Concentration/µl x total volume

7. PCR: principle and procedure

Sharma P, Shingote P, Devanna BN, Arora K, Amitha Mithra SV and Solanke AU

The polymerase chain reaction (PCR) is basically defined as a scientific technique in molecular biology which is used to amplify a single or a few copies of a DNA fragment to several orders of magnitude thereby generating millions of copies of a particular DNA sequence. Kary B. Mullis invented PCR technique in 1983 which is hailed as one of the greatest innovations that revolutionized the modern filed of molecular biology. PCR is now a common and often indispensable technique used in biological research labs for a variety of applications. The applications range from the daily practicalities of medical diagnosis to the theoretical framework of systematics, from courts of law to field studies of animal behavior, and PCR takes analysis of tiny amounts of genetic material; even damaged one to a new level of precision and reliability. A brief history of 'development of PCR' is given.

PCR History:

- **1966:** *Thermus aquaticus*, a thermostable bacteria discovered by Thomas Brock. This bacterium was found in the hot springs of Yellowstone National Park.
- **1983:** The concept of PCR was postulated by Kary B Mullis.
- **1985:** The first application of PCR was published in 'Science' Journal by Saiki et al.
- **1985:** Isolation of thermostable Taq Polymerase from *Thermus aquaticus* by Cetus Corp. researchers
- **1988:** PerkinElmer introduced the automated thermal cycler
- **1991:** RT-PCR was developed using a single thermostable polymerase, facilitating diagnostic tests for RNA viruses.
- **1993:** Dr. Kary Mullis shared the Nobel Prize in Chemistry for conceiving PCR technology.

Principle of PCR:

The basic principle of PCR involves the exploiting the ability of DNA polymerase to add nucleotides to free 3' end in a template dependent manner under standard condition. In brief, the DNA sample is denatured to produce single stranded template DNA, to which the oligonucleotide primers can bind. The enzyme DNA polymerase then add nucleotide bases to the 3' end of each primer, using the template DNA as a guide to extend the primer thereby

producing new double stranded DNA. This process is repeated for a number of cycles to enrich the DNA sample for the desired genes targeted by the oligonucleotide primers. Since each cycle of PCR involves creating two new double stranded DNAs from each DNA molecule, the amount of DNA theoretically doubles with every cycle of PCR. After N cycles, PCR generates a 2N-fold increase in the target DNA. The following are the three cycling conditions in PCR.

1. **Denaturation of double stranded (ds) DNA template:**
   At a temperature ranging from 94 – 98 °C, double stranded DNA melts to form two single strands of DNA. It causes DNA separation by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA.

2. **Annealing of primers:**
   After denaturation, the temperature is then further lowered to allow the oligonucleotides or primers to bind to the sequence specific target DNA segments. This process is known as annealing. For the primers to anneal with the target DNA, complementarity in base sequences is mandatory. Depending on the melting temperature of the expected duplex, primers bind to their complementary sequences at a range of temperatures.

3. **Extension of dsDNA molecules:**
   The temperature is raised again at which DNA polymerase binds and extends DNA from the 3' end of the primer by adding nucleotides to the developing DNA strand. This requires a temperature of nearly 72 °C for a time period of 30 s to7 min, depending on the enzyme used. Generally the extension time varies with the type of DNA polymerase used and also on the length of the DNA fragment to be amplified. Under optimum conditions and adequate substrate or reagent concentration, the amount of DNA target is doubled after each cycle, leading to exponential amplification of the specific DNA fragment.

The pictorial depiction of the protocol is given in Figure. 7.1. and the different components involved in PCR reaction is given Figure. 7.2.

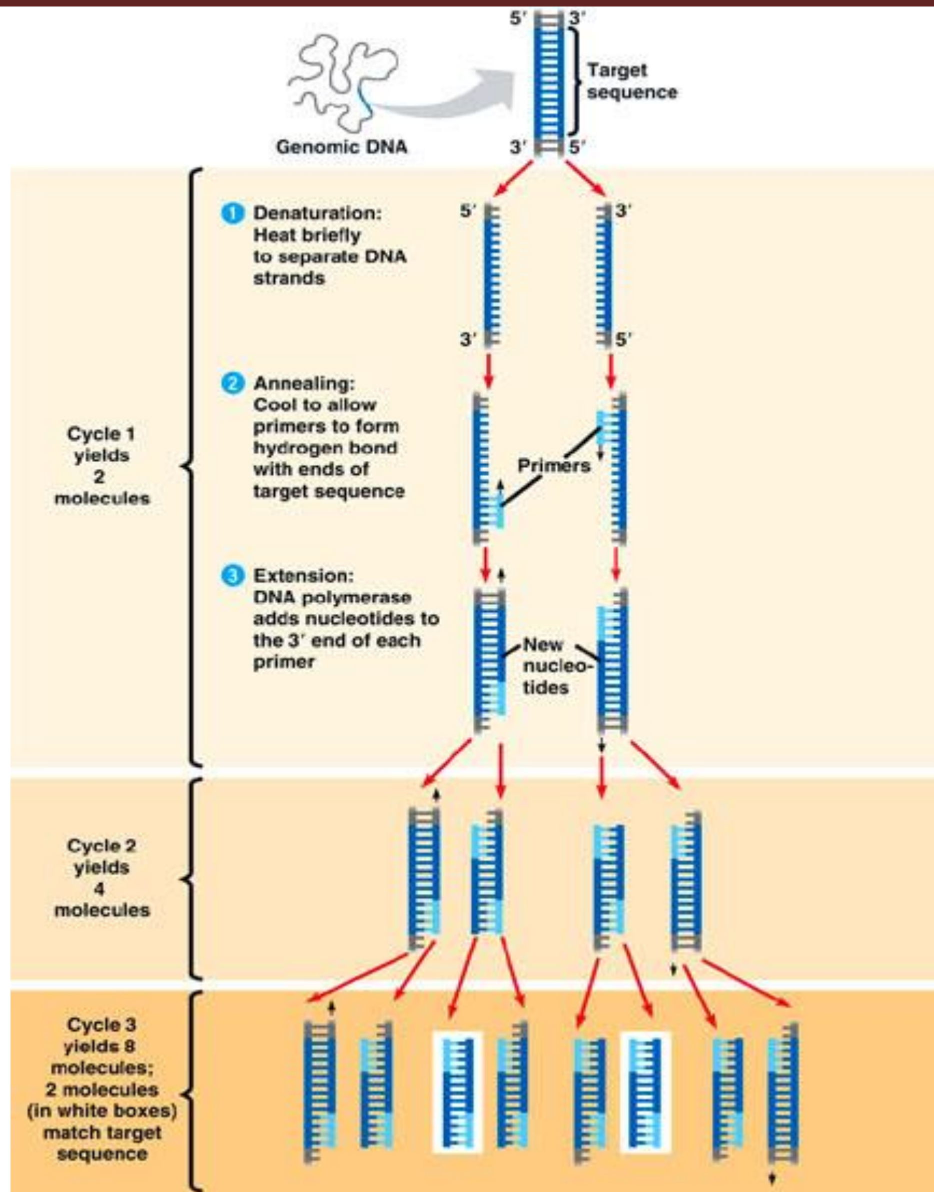

Fig. 7.1: Illustration of PCR Source: "PCR: Uses, Steps, Purpose. http://schoolworkhelper.net/pcr-uses-steps-purpose/. St. Rosemary Educational Institution

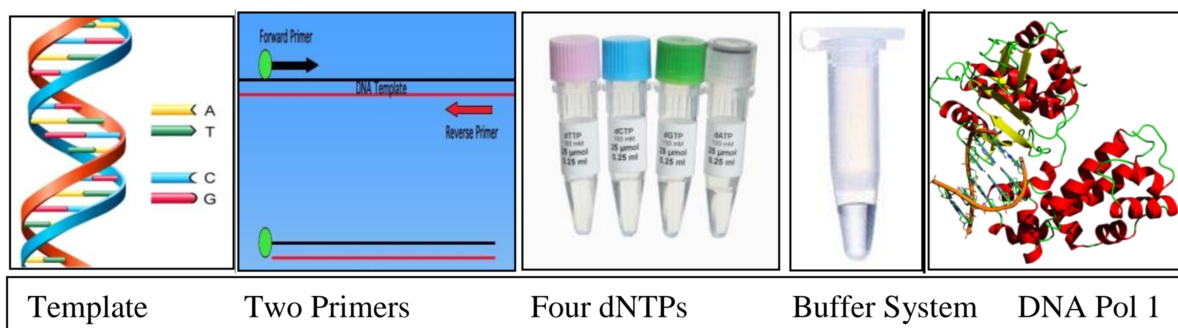

Fig. 7.2: Different components used in PCR reaction.

A. DNA Template:

In PCR, usually, the template intended for amplification is a double-stranded DNA. Any damage caused to the DNA prior to or during the PCR reaction may affect the precision, resulting in decreased efficiency and yield basically caused due to base misincorporation, formation of partial products or many other artifacts. Foreign DNA or some other intrusive DNA from unwanted sources can contaminate the PCR and confound the results. Depending on the nature or source of the template, the quantity of DNA required for PCR varies. The basic thing to keep in mind is that the quality of DNA to be used must be sufficiently pure. Even if a sample does not need to be highly purified, some contaminants such as heparin, heme, formalin, Mg^{2+}-chelating agents, as well as detergents should be eliminated to avoid inhibition of the amplification process. In general various concentrations of DNA required are listed below:

- ❖ Human genomic DNA should be up to 100 ng
- ❖ Plant DNA should range from 20-60 ng
- ❖ Bacterial DNA 1-10 ng
- ❖ Plasmid DNA 0.1-1 ng

B. A pair of synthetic oligonucleotides to prime DNA synthesis:

Short fragments of ssDNA usually 18-30 nucleotides in length, which are complementary to target DNA sequences, flanking the target region of interest are defined as PCR primers. The purpose of PCR primers is to provide a “free” 3’-OH group to which the DNA polymerase can add dNTPs. Of the various factors influencing the specificity of amplification reaction, primer designing is the most crucial factor that determines the success of PCR. The important points to be considered while designing primers for PCR amplification are as follows:

- ❖ Typically length of primers should be 18-30 bases.
- ❖ Primers should have 40- 60% GC content. Stretches of polybase sequences or repeating motifs should be avoided as these can hybridise inappropriately on the template.
- ❖ Also, Inverted repeat sequences should be avoided so as to prevent formation of secondary structure in the primer, which would prevent hybridisation to template.
- ❖ If possible, the 3' end of the primer should be rich in G, C bases to enhance annealing of the end that will be extended.

- The distance between primers should be less than 10 Kb in length. Typically, substantial reduction in yield is observed when the primers extend from each other beyond ~3 Kb.
- Calculated melting temperatures (T_m) should be in the range of 42-65 °C
- If the primer is shorter than 25 nucleotides, the approx. melting temperature (Tm) is calculated using the following formula:

  Tm = 4(G + C) + 2(A +T),

  Where G, C, A, and T, are the number of respective nucleotides in the primer.
- Optimal primer concentration should range between 0.1 and 0.6 µM as higher concentrations of primers favor mispriming leading to non specific amplification.
- Primer pairs should have T_m within 5°C of each other
- Primers should not contain internal secondary structure
- Primers must not be complementary to each other at the 3' ends and also should not be used at very high concentrations to avoid primer- dimer forming artifacts.

C. Deoxynucleosides triphosphates (dNTPs)

Standard PCR contains equimolar amounts of dATP, dTTP, dCTP and dGTP. Typical concentration used is 200 µM - 250 µM of each dNTP. Lesser concentration (50-100µM) although, enhances fidelity of polymerization, reduces yield. Similarly, yield increases at higher concentrations especially in long PCR, but fidelity is reduced. High concentrations of dNTPs (>4 mM) are inhibitory because of sequestering of Mg^{2+}.

D. Divalent cations:

Thermostable DNA Polymerases require free divalent cations usually Mg^{2+} for its activity. Since dNTPs and oligonucleotides bind Mg^{2+}, the molar concentration of the cation must exceed the molar concentration of phosphate groups (contributed by dNTPs & Primers). Therefore, it is difficult to recommend some optimal value, which works in all circumstances. Although, $MgCl_2$ concentration ranging between 1.5-2.0 mM is optimal for *Taq* DNA polymerase to function, still standard PCR buffer has 1.5 mM of $MgCl_2$. No PCR product will be seen if magnesium ion concentration is low and undesired PCR products may be seen if too high concentrations are used. At higher concentration, the enzyme loses fidelity. Alternatively, optimization can be achieved by comparing yield obtained from a series of PCRs containing concentrations of Mg^{2+} ranging from 0.5 mM to 5.0 mM.

E. Buffer system (Generally supplied as 10X):

- Tris-HCl 100 mM
- KCl 500 mM: Use of these monovalent cations in PCR Buffer helps in amplification of segments of DNA > 500 bp in length. Raising the concentration often improves the yield of shorter DNA segments.
- 0.1% Triton X-100
- $MgCl_2$ 15mM (If containing)

F. A thermostable DNA polymerase to catalyze template dependent DNA synthesis:

Enormous range of enzymes varying in their fidelity and efficiency are available for synthesizing large DNA products. For routine PCR, use of 0.5–2.5 units of enzyme per standard 25-50 µl reaction commonly practiced. The specific activity of most commercial preparations of Taq is nearly 80,000 units/mg of protein.

***Points to remember*:**

While handling the sample:

- Always wear gloves.
- Use autoclaved and sterilized nuclease free water.
- Glassware, plasticware and pipettes used to prepare the PCR reagents and template DNA should be either new or sterilized/autoclaved.
- PCR reagents and solutions should be stored in small aliquots to avoid frequent use of main stock. This is to save it from contaminants and frequent freezing and thawing.
- Always use positive and negative controls in every PCR reaction.

While preparing reaction mix:

- Assemble all reaction components on ice
- Add Taq polymerase at last after adding all other reagents.
- Give a short spin to PCR tube/plate to settle down all PCR reaction components before transferring to thermocycler.

Thermal Cycling Profile (for Standard PCR):

Initial denaturation at 95 °C for 2-5 minutes is recommended prior to PCR cycling to fully denature the DNA.

- Avoid longer or higher temperature incubations (unless required due to high-GC content of template)

- ❖ As a general rule, use **extension times** of one minute per 1000 base pairs (e.g. 3 minutes for a 3 kb product), for products less than 1 kb, use 45-60 seconds
- ❖ Most PCR should include only 25 – 35 cycles because as the number of cycles increase, nonspecific products accumulate.

Post extension and holding:

- ❖ Cycling should conclude with a **final extension** at 72 °C for 5 – 12 minutes to promote completion of partial extension products and then **holding** at 4 °C.

Trouble shootings:

A) Checkpoints if the desired product of correct size is not obtained:

- Check DNA quality and quantity by running on gel and/or nanodrop reading.
- Reduce annealing temperature
- Increase $MgCl_2$ concentration
- Add dimethylsulphoxide (DMSO) to assay (at around 10%)
- Use different thermostable enzyme
- Check primers for degradation, throw out degraded primers & make new stocks

B) Checkpoints if extra or non-specific product/bands are present

- Increase annealing temperature
- Reduce $MgCl_2$ concentration
- Reduce the number of cycle

C) **Checkpoints if amplification is weak or non detectable**

- Optimize concentrations of $MgCl_2$, template DNA and dNTPs
- Repurify template DNA to remove inhibitors

8. Variants of PCR and their applications

Sharma P, Shingote P, Devanna BN, Arora K, Amitha Mithra SV and Solanke AU

Besides the standard PCR technique, many variants of PCR for defferent applications have been developed over the time. Some of the important ones are briefly described below for your ready reference.

A. Gradient PCR

The sequence and length of PCR primers generally determine the annealing temperature of the thermal cycling reaction for a specific assay. Primers are usually supplied with theoretical melting temperatures (T_m), which can be calculated in different ways giving widely varying values. Using too low annealing temperature can produce non-specific products whereas too high annealing temperature may compromise on PCR yield. Moreover, there can be difference in block tolerance of different makes of thermal cyclers leading to differences in PCR amplification. Such issues significantly affect results, especially, while using multiple locus based marker such ISSR or AFLP. An annealing temperature optimisation step is mandatory to avoid such problems and is especially important when changing a sensitive assay from one thermal cycler to another. By using the gradient feature of a thermal cycler, the PCR can be optimised for each particular instrument and primer. The basic principle is that under gradient option, different columns of the thermal cycler block sense different temperature for annealing and a primer is tested in several annealing temperatures across the block. Generally, a gradient of $\pm$10 °C is tested around the optimum theoretical Tm calculated. Depending on the make and the model, one may test up to $\pm$30 °C. Based on the specific product profile and the clarity and brightness of bands produced, the optimum annealing temperature is chosen.

B. Hot Start PCR:

The method used to optimize the yield of desired amplified product in PCR simultaneously suppressing nonspecific amplifications, is the Hot Start PCR. This is achieved by withholding an essential component of the PCR i.e. DNA polymerase or Mg^{2+}, until the reaction mixture has been heated to a temperature that inhibits nonspecific priming. It can be performed manually by heating the reaction components (Buffer, dNTPs, template DNA and primers) held at a temperature above the threshold of nonspecific binding of primer

to template i.e denaturation temperature (95^0C). Thermal cycling is initiated after the final addition of the DNA polymerase to the preheated reaction mixture. The elimination of the warm-up phase preceding the first cycle of PCR reduced the opportunities for nonspecific annealing of oligonucleotide primers, whereas the absence of DNA polymerase activity prevented extension of mismatched primers.

C. Quantitative PCR

Real-Time PCR or quantitative real-time PCR (qRT-PCR) is a PCR-based technique that is able to simultaneously amplify and detect changes in the amplicon concentration in a real-time manner. Real-time PCR collects data during PCR amplification by utilizing fluorescence signal emitted by either special probes or DNA binding dyes. qRT-PCR uses either fluorescent labelled probes like TaqMan or molecular beacon or SYBR green labelled nucleotides (Figure 8.1).

D. Multiplex PCR:

For the simultaneous amplification of two or more loci (multiple targets) in a single PCR experiment, a variant of PCR is employed which is Multiplex PCR (Figure 8.2). This has been made possible by using multiple primer pairs in a reaction mixture. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands when visualized by gel electrophoresis. There should not be complementarity among primer sequences for multiplexing. This technique potentially saves time and effort without hampering the utility of the experiment. This method can be applied for analysing deletions and mutations in DNA, polymorphisms, quantitative assays and RT – PCR.

E. Reverse Transcriptase – PCR (RT– PCR)

This is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA. In this type of PCR variant, gene expression is qualitatively detected by cDNA transcripts synthesized from RNA (Figure 8.3). Uses of RT-PCR are mentioned below:

- Expression profiling
- To identify the sequence of an RNA transcript
- To map the location of exons and introns in the gene.
- The 5' end of a gene (transcription start site) identified by a RT-PCR method, named RACE-PCR (*Rapid Amplification of cDNA Ends*)

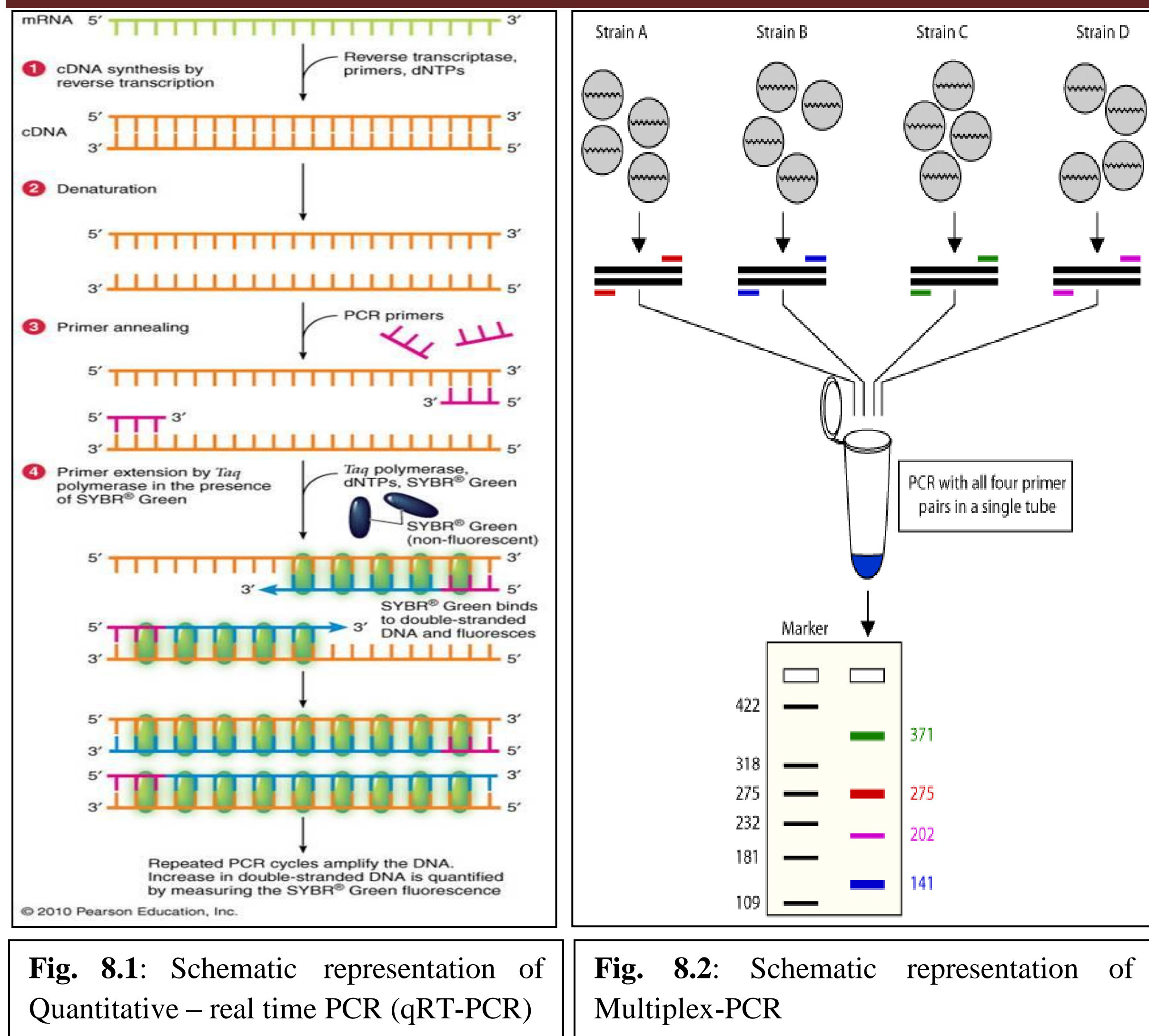

Fig. 8.1: Schematic representation of Quantitative – real time PCR (qRT-PCR)	**Fig. 8.2**: Schematic representation of Multiplex-PCR

F. Inverse PCR:

In general, standard PCR is used to amplify a segment of DNA that lies between two inward pointing primers (Figure 8.4). However, inverse PCR is used to amplify unknown DNA sequences that flank the known DNA sequence and for which no primers are available. The technique involves restriction digestion of DNA using enzyme that does not have restriction site in the target sequence followed by circularization of the individual restriction fragments (linear DNA fragments) through intramolecular ligation. The circularized DNA is then used as a template in the PCR. The unknown sequence is amplified by two primers that bind specifically to the known sequence but in opposite orientation. The product of the amplification reaction is a linear DNA fragment containing a single site for the restriction enzyme originally used to digest the DNA. This site marks the junction between the previously cloned sequence and the flanking sequences. The size of the amplified fragment depends on the distribution of restriction sites within known and flanking DNA sequences.

Inverse PCR (iPCR) is used extensively for rapid allelotyping and to determine the genomic locations at which retroviruses, transgenes, and transposons are integrated into genomes.

G. Nested PCR:

In certain cases where the primers which are closely flanking the region of interest hybridize/mishybridize elsewhere on substrate DNA leading to amplification of unwanted targets, a new modification of Standard PCR known as nested PCR is used , which increases the specificity of DNA amplification by reducing background (product contamination) due to the amplification of unintended primer binding sites. Nested PCR uses two sequential sets of primers in two successive PCR reactions which are performed for 15-30 cycles with one primer set and then additional similar number of cycles with second primer set. In the first reaction, one pair of primers binds to sequences outside the target DNA, as expected in standard PCR, but it may also bind to other areas of the template producing a larger fragment. The second primer set binds to sequences in the target DNA that are within the portion amplified by the first set (that is, the primers are nested) (Figure 8.5). Thus, the second set of primers will bind and amplify target DNA within the products of the first reaction. The primary advantage of nested PCR is that if the first primers bind to and amplifies an unwanted DNA sequence; it is very unlikely that the second set of primers will also bind within the unwanted region. Nested PCR has been used in highly sensitive method of preimplantation genetic diagnosis. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

H. Touchdown PCR (Step-down PCR):

It is a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. This method is usually used to optimize yields of amplified DNA when the melting temperature of hybrids between the oligonucleotide primers and their target sequences is not known with certainty, for example:

- Using primers designed from amino acid sequences
- Amplifying members of a multigene family
- Using extremely complex template DNA occupying large sequence space.
- Amplifying DNA from different species than that for which the primers were designed
- While using, primers containing "universal" bases such as inosine.

In all the above-mentioned circumstances, mismatches between the oligonucleotide primers and the template DNA can be predicted, as undesirable nonspecific amplification products will be generated. For proper optimization, the best alternative is touchdown PCR, in which a range of annealing temperatures is utilized in a single PCR.

The annealing temperature at the initial cycles is usually a few degrees (3-5°C) above the Tm of the primers used, while at the later cycles, it is a few degrees (3-5°C) below the primer Tm (Figure 8.6). The higher temperatures give greater specificity for primer binding, reduces the likelihood of misannealing, and ensures that PCR amplicons generated in the first cycles are more likely to be accurate copies of the target sequence and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles

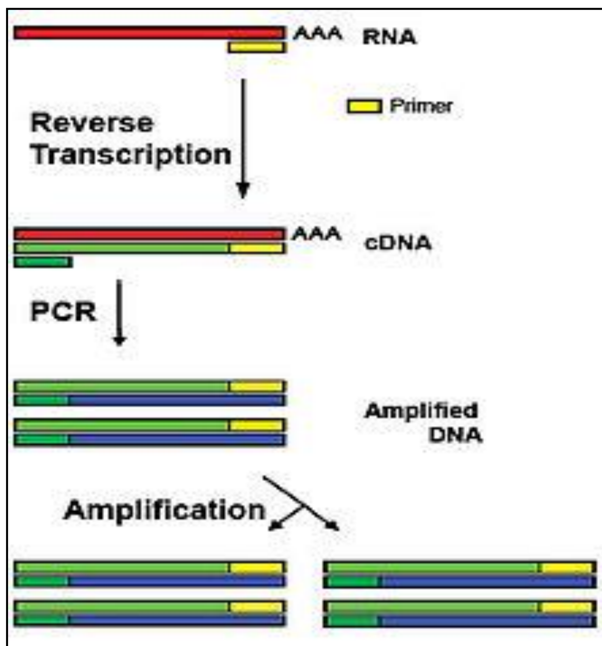

Fig. 8.3: Schematic representation of Reverse Transcriptase PCR

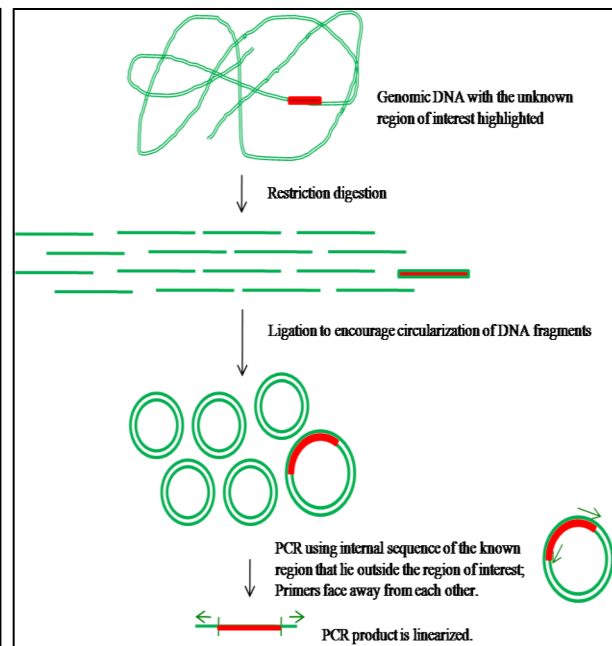

Fig. 8.4: Schematic representation of Inverse PCR

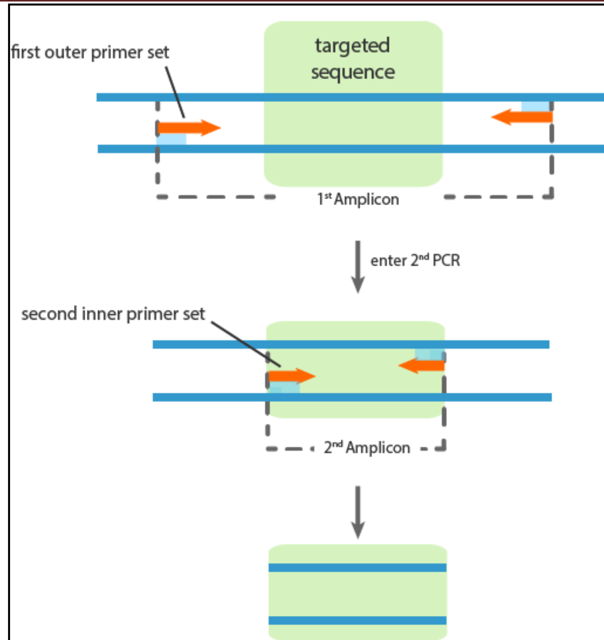

Fig. 8.5: Schematic representation of Nested PCR

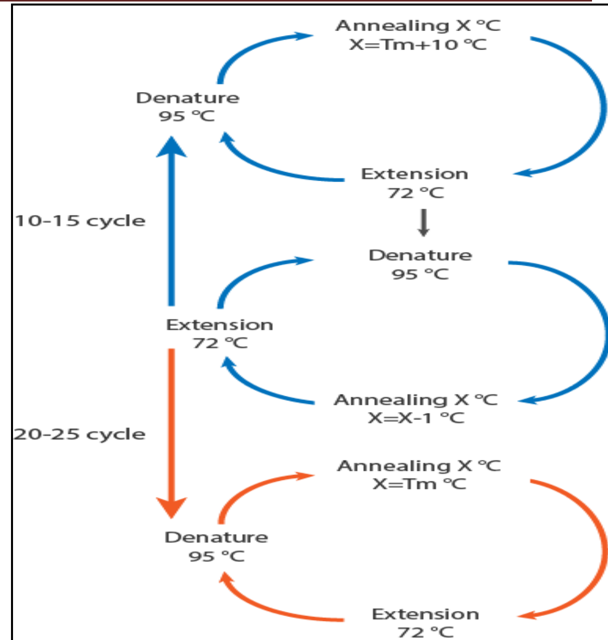

Fig. 8.6: Schematic representation of Touch-down PCR

9. ISSR markers mediated analysis of genetic fidelity of sugarcane

Shingote P, Sharma P, Devanna BN, Arora K, Amitha Mithra SV and Solanke AU

Sugarcane (*Saccharum spp*.) is the most important cash crop and the third largest crop in terms of value next only to rice and wheat. Sugarcane hybrids display varying ploidy level and in particular aneuploids with an average chromosome number of 100-120 are commonly found and the estimated genome size 10,000 Mb (D'Hont and Glaszman, 2001, Grivet et al., 2001). Genetic studies and genome mapping of sugarcane is difficult due to polyploidy nature in different varieties. Therefore, it is more important to ensure the genetic fidelity of the micropropagated sugarcane planting materials and in the present study we used a set of ISSR markers available from the University of British Columbia (UBC) for genetic fidelity analysis of sugarcane. The principle of ISSR marker system is PCR based amplification using repeat sequences as primer to amplify varying number of DNA fragments flanked by ISSR sequences, among the crop lines. Leaf samples of Sugarcane varieties received from Indian Institute of Sugarcane Research (IISR), Lucknow were subjected to DNA isolation ((Figure 9.1)). The quality of isolated DNA was determined by both nanodrop and gel based methods. The readings from the nanodrop are shown in Table 9.1

Table 9.1: Nanodrop analysis of sugarcane DNA isolated from leaf samples received from Indian Institute of Sugarcane Research, Lucknow

Code No	Sample name	Concn in ng/µl	Concn in ng/µl	Final concn	Ratio
1	CoLK94184	198	193	195.5	1.91
2	CoPK05191	178	183	180.5	1.92
3	Co0238	140	138	139	1.98
4	CoLK9709	210	209	209.5	1.92
5	Co05011	94	93	93.5	1.96
6	BO153	182	183	182.5	1.86

Requirements for 25 µl PCR reaction

1. ISSR markers
2. High quality DNA of working standard: 50 ng/µl
3. Taq DNA polymerase (1 unit/reaction)

4. dNTPs

5. Components of gel electrophoresis

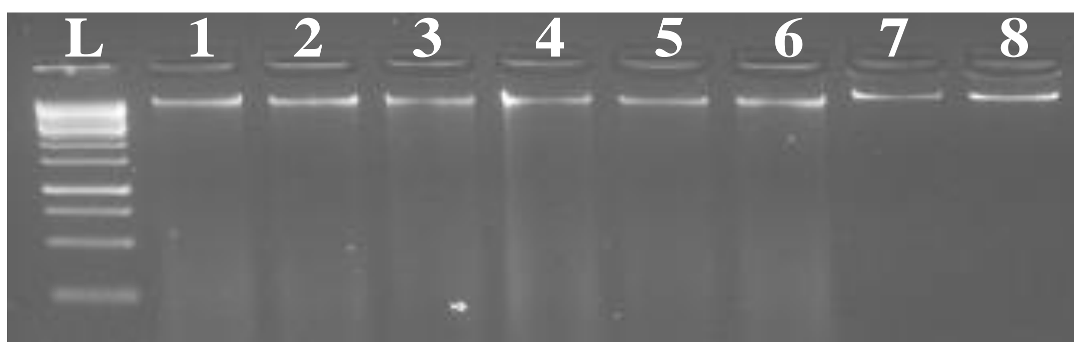

Fig.9.1: Gel picture showing genomic DNA of diluted Sugarcane checked on 0.8% Agarose L: 1Kb DNA ladder; 1: CoLK94184; 2: CoPK05191; 3: Co0238; 4: CoLK9709; 5: Co05011; 6: BO153; 7: 50 ng λ DNA; 8: 100 ng λ DNA

PCR reaction mix

- Buffer 2.5 µl
- dNTPs (2.5 mM each) 2.0 µl
- Primer (200-500 pg) 1.0 µl
- DNA template (~50 ng) 1.0 µl
- Taq DNA polymerase (1U) 0.2 µl
- Nuclease free water 18.3 µl

Total: 25.0 µl

PCR conditions

- 95^0C 5 min
- 95^0C 30 s
- 45-50 ^{0}C 30 s
- 72 ^{0}C 2 min
- 72 ^{0}C 10 min
- 4 ^{0}C ∞

(Steps 2–4: 35 cycles)

PCR based analysis of ISSR primers was performed. We used ISSR primers UBC_810, UBC_813, UBC_815, UBC_834, UBC_835, UBC_840 and UBC_847 for PCR with annealing temperature of 45 °C (Fig. 9.2 and Fig.9.3). The annealing temperature used for primers UBC_826, UBC_827, UBC_855 and UBC_856 was 50 °C (Fig. 9.4).

Gel electrophoresis of PCR products

1. Select appropriate gel casting tray and matching comb, fix into slots
2. Weigh 2.0 g agarose into 500 ml flask containing 200 ml 1X TAE and boil to dissolve agar completely

3. After cooling agarose gel to around 50 °C add 10 µl EtBr and mix gently so that no bubbles would form
4. Pour agarose into sealed casting tray slowly in order to bubble formation and let it solidify for 30-45 min
5. Remove the sealing and keep the tray in electrophoresis tank containing 1X TAE buffer sufficient enough to dip the gel
6. Mix 25 µl DNA sample with loading dye (5 µl) and pipette the mix into the wells carefully to avoid floating out of sample. Also load 4 µl DNA ladder of known base pairs, preferably 1 kb
7. Place the lid back on the tank and connect the power supply. Run the apparatus at 60-80 volts for 45 min to 1h or till the front bromophenol blue dye travel around 2/3 of the gel
8. At the end take put the tray along with gel and capture the image under UV light using gel documentation unit.

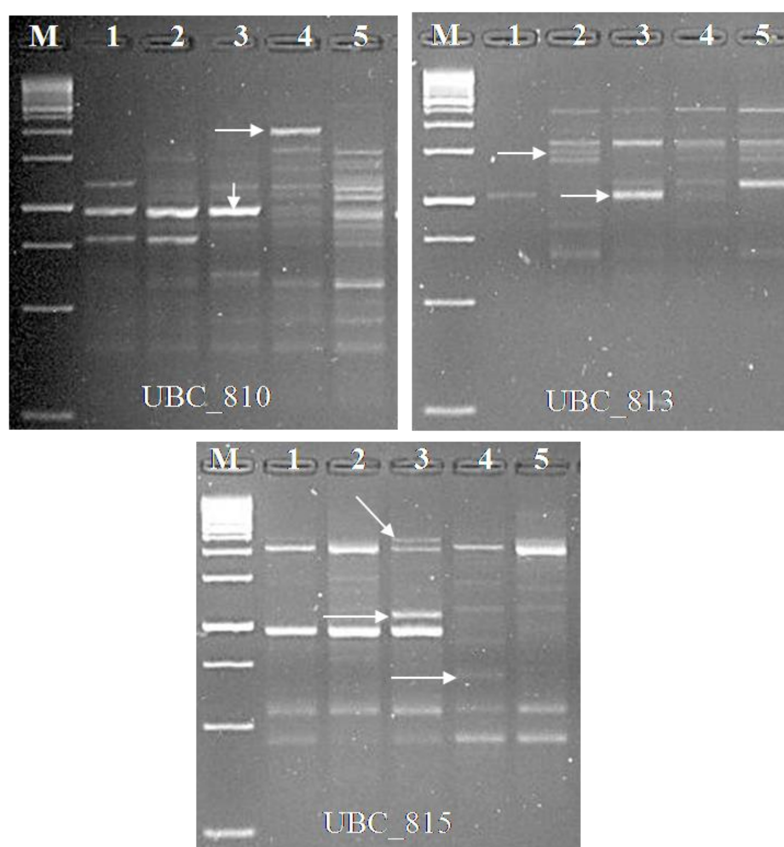

Fig. 9.2. ISSR profiling of five sugarcane varieties with UBC_810, UBC_813, UBC_815. M: 1Kb DNA ladder, 1: CoLK94148, 2: CoPK05191, 3: Co0238, 4: CoLK9709, 5: Co05011.

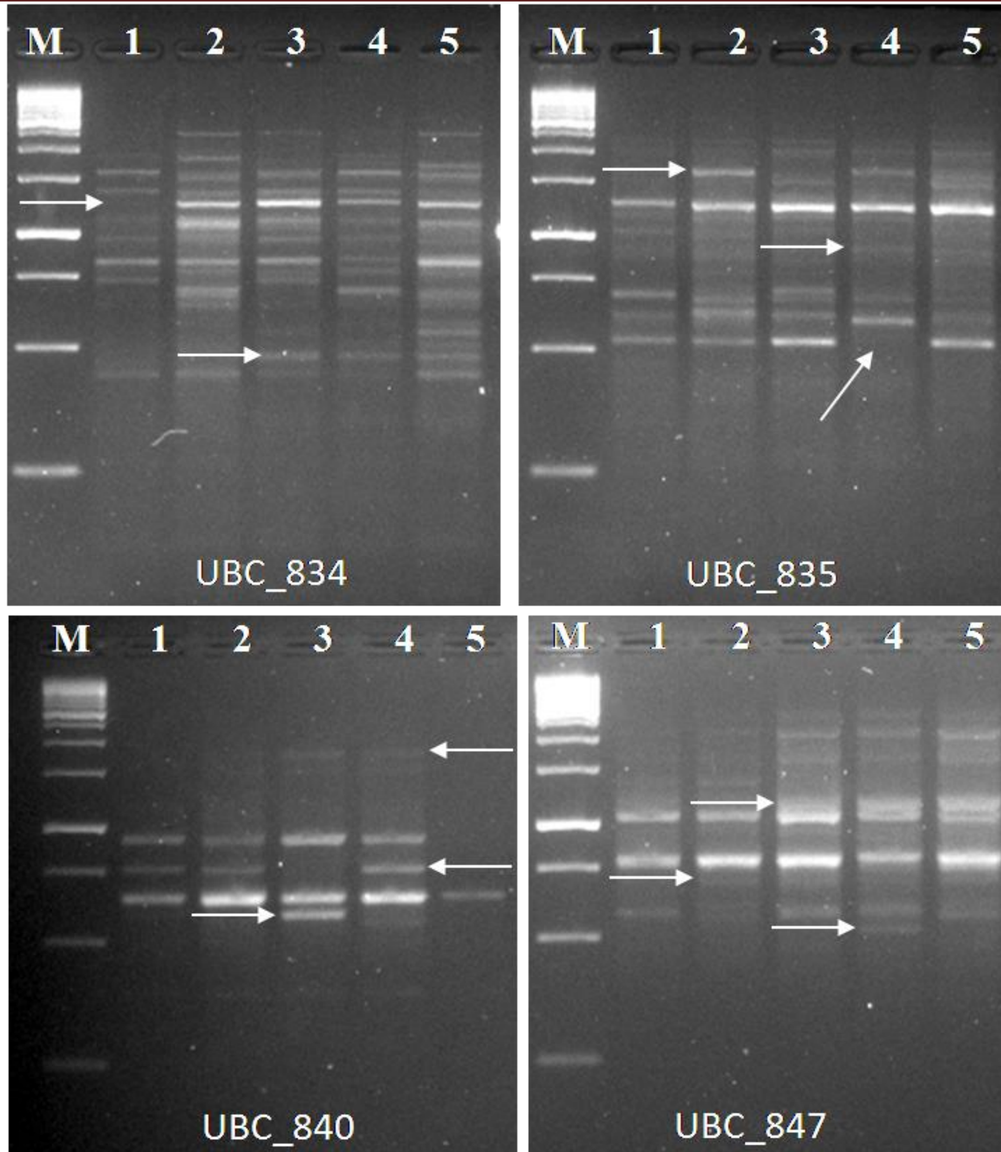

Fig. 9.3. ISSR profiling of five sugarcane varieties with UBC_834, UBC_835, UBC_840 and UBC_847. M: 1Kb DNA ladder, 1: CoLK94148, 2: CoPK05191, 3: Co0238, 4: CoLK9709, 5: Co05011.

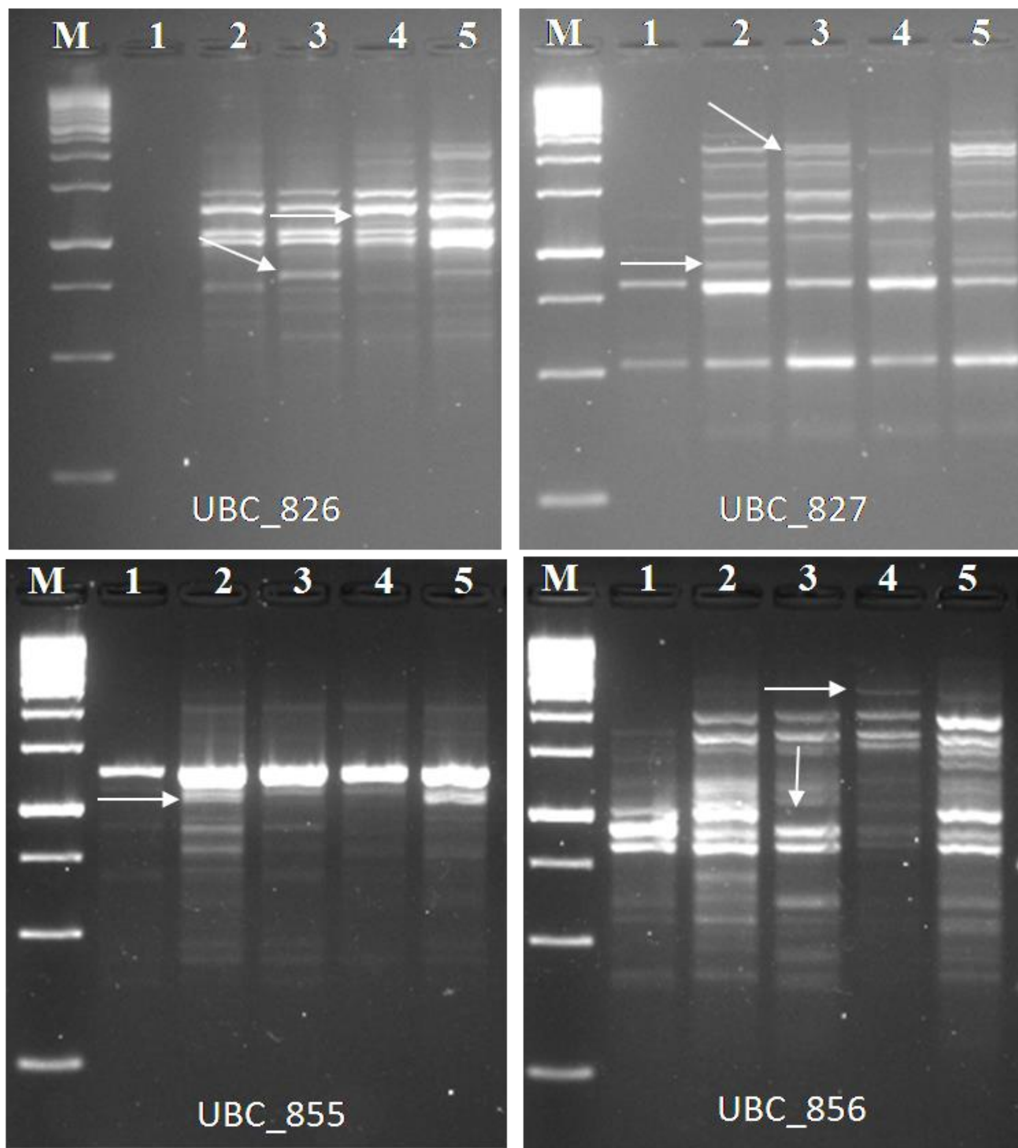

Fig. 9.4. ISSR profiling of five sugarcane varieties with UBC_826, UBC_827, UBC_855 and UBC_856. M: 1Kb DNA ladder, 1: CoLK94148, 2: CoPK05191, 3: Co0238, 4: CoLK9709, 5: Co05011.

10. IRAP markers mediated analysis of genetic fidelity of sugarcane

Shingote P, Sharma P, Devanna BN, Arora K, Amitha Mithra SV and Solanke AU

Inter Retrotransposon Amplified Polymorphism (IRAP) is a PCR based molecular genetic marker which uses the primers derived from a retrotransposon elements. The advantage of IRAP is its experimental simplicity, which requires simple PCR followed by electrophoresis to resolve the PCR products. IRAP can be carried out with a single primer matching either the 5' or 3' end of the LTR but oriented away from the LTR itself, or with two primers. Transposable elements (TEs) which are found randomly in the genomes may be found in different orientations and different orders (head-to-head, tail-to-tail or head-to-tail) enabling the range of tools available to detect polymorphism depending on the method and primer combinations. If two primers are used, they may be from the same retrotransposon family or from different families. The PCR products, and therefore the fingerprint patterns, result from amplification of hundreds to thousands of target sites in the genome. The pattern obtained will be related to the TE copy number, insertion pattern and size of the TE family.

Current study was based on screening of sugarcane lines using of IRAP primers to select the best combinations which show good polymorphism with reproducible amplification. The selected polymorphic primers were then used to screen DNA isolated from different sugarcane lines (Figure.10.1) and to test clonal fidelity/true-to-typeness in micropropagated somaclones.

Table 10.1: Nanodrop analysis of sugarcane DNA isolated from leaf samples received from Indian Institute of Sugarcane Research, Lucknow

Code No	Sample name	Concn in ng/µl	Concn in ng/µl	Final concn	Ratio
1	CoLK94184	198	193	195.5	1.91
2	CoPK05191	178	183	180.5	1.92
3	Co0238	140	138	139	1.98
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6	BO153	182	183	182.5	1.86

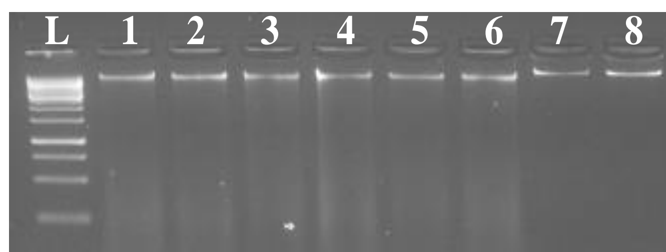

Fig. 10.1: Gel picture showing genomic DNA of diluted Sugarcane checked on 0.8% Agarose L: 1Kb DNA ladder; 1: CoLK94184; 2: CoPK05191; 3: Co0238; 4: CoLK9709; 5: Co05011; 6: BO153; 7: 50 ng λ DNA; 8: 100 ng λ DNA

PCR Requirements:

1. IRAP primers
2. High quality DNA of working standard: 20 - 30 ng/µl
3. Taq DNA polymerase (1 unit/reaction)
4. dNTPs (200 µM each)
5. Buffer
6. Nuclease free autoclaved distilled water
7. $MgCl_2$

PCR reaction mix

Component	Volume
• Buffer (Containing 1.5mM $MgCl_2$)	2.5 µl
• dNTPs (2.5mM each)	2.0 µl
• Primer (200-500 pg)	1.0 µl
• DNA template (~50 ng)	1.0 µl
• Taq DNA polymerase (5U/ µl)	0.2 µl
• $MgCl_2$	0.5 µl
• Nuclease free water	17.8 µl
Total Volume/reaction	25.0 µl

PCR conditions:

Temperature		Time	Cycles	Step
• 95^{0}C	:	5 min		(Initial Denaturation)
• 95^{0}C	:	40 s	35 cycles	(Denaturation)
• 50 ^{0}C	:	40 s		(Annealing)
• 72 ^{0}C*	:	2 min		(Extension)
• 72 ^{0}C	:	12 min		(Final Extension)
• 4 ^{0}C	:	∞		(Hold)

Where, * is Extension carried out at 72^{0}C for 2 min +3 s extension/cycle

Sixteen IRAP primers alone and sixteen different primer combinations were used for current study to study genetic variability in 6 sugarcane varieties. The PCR conditions for all these IRAP primers were standardized for obtaining reproducible and scorable banding patterns with different number of bands. Out of 32 IRAP primers only 8 primers showed reproducible polymorphism thus only these polymorphic primers were used for further testing (Table 10.2).

Table 10.2. List of primer combinations used for IRAP PCR assays

S.NO.	IRAP Primers	$T_m(^0C)$
1	Gypsy	45
2	Tst-2	45
3	LTR6150	45
4	3'LTR	45
5	LTR6149+3'LTR	45
6	LTR6150+3'LTR	45
7	LTR6149+Sukkula	45
8	LTR6150+Sukkula	45

Gel electrophoresis of PCR products:

The methodology used for agarose gel electrophoresis was followed similarly as mentioned in Chapter 6 of this manual and results are given in (Figure 10.2 and Figure 10.3).

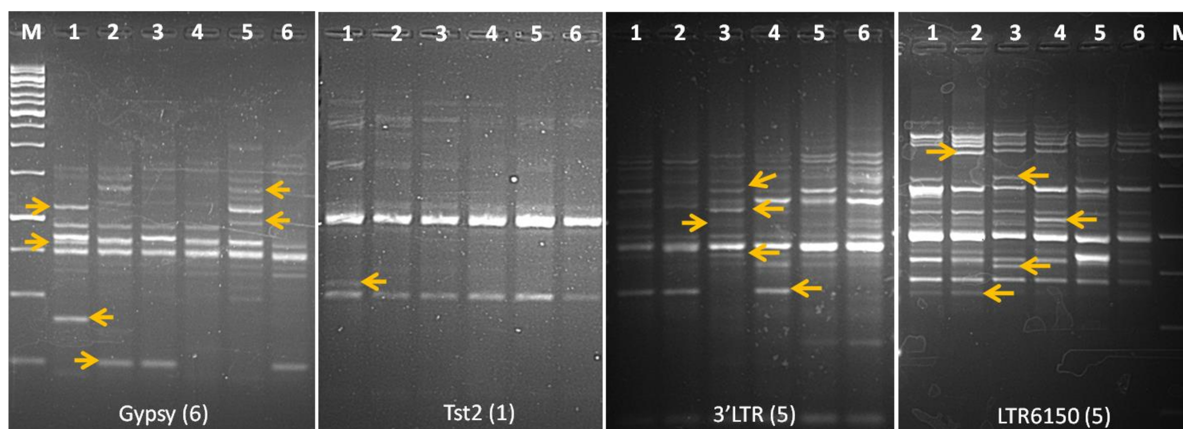

Fig. 10.2: IRAP profiling of six sugarcane varieties by using primers Gypsy, Tst-2, 3'LTR and LTR6150. M: 1Kb DNA ladder; 1: CoLK94184; 2: CoPK05191; 3: Co0238; 4: CoLK9709; 5: Co05011 & 6: BO153.

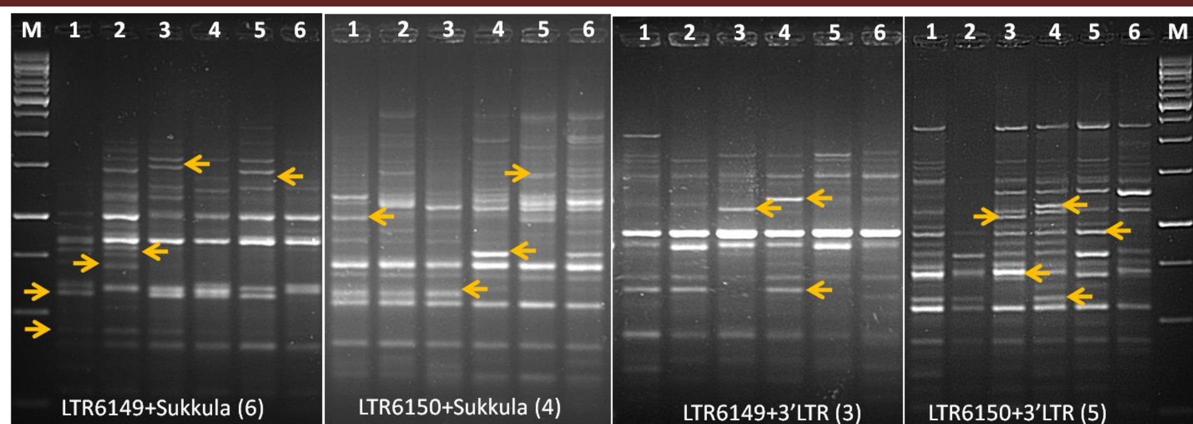

Fig. 10.3: IRAP profiling of six sugarcane varieties by using primers LTR6149+Sukkula, LTR6150+Sukkula, LRT6149+3'LTR & LTR6150+3'LTR. M: 1Kb DNA ladder; 1: CoLK94184; 2: CoPK05191; 3: Co0238; 4: CoLK9709; 5: Co05011 & 6: BO153.

Results inferred:

- Eight sets of IRAP primer combinations (Gypsy, Tst-2, LTR6150, 3'LTR, LTR6149+3'LTR, LTR6150+3'LTR, LTR6149+Sukkula & LTR6150+Sukkula) were screened against six potato varieties to test polymorphism.
- A total of 36 polymorphic bands were obtained.
- The range of polymorphic bands amplified by each primer varied from 1-6 bands.
- One constant temperature of 45^0C was tried out.

11. ISSR markers mediated analysis of genetic fidelity of potato

Sharma P, Shingote P, Devanna BN, Arora K, Amitha Mithra SV and Solanke AU

Potato (*Solanum tuberosum* L.), a tuber crop of the family solanaceae is the fourth most important food crop which contributes to nutritional security after wheat, maize and rice. It is a highly heterogeneous annual, herbaceous, dicotyledonous and vegetatively propagated plant and a wholesome food containing carbohydrates, proteins, minerals, vitamins and high quality dietary fibre. The tetraploid genome of potato has 12 chromosomes (4n=48; x=12) and the genome size is 840 Mb. Plant tissue culture methodology has immense potential to rapidly multiply "true-to-type" genotypes leading to commercial production of planting material. Potato being a vegetatively propagated crop, this technology has proven to be of utmost importance in case of potato for maintenance of healthy stocks and rapid propagation of planting materials.

In vitro clonal propagation is used to produce micro or mini tubers for healthy seed stocks identical to mother plant after repeated sub-culturing. However, problem of genetic stability mediated by genetic (somaclonal variation) and epigenetic variations has been observed in tissue culture regenerated plants, leading to altered morphological and biological parameters and affecting agronomic performance. Hence a quality check to validate the true to type planting material at an early stage of development is considered to be very useful in plant tissue culture.

Among the various approaches used, molecular markers mediated genetic fidelity testing is most commonly followed method. Molecular markers used in potatoes include, RAPD, AFLP, SSRs, ISSRs and other retrotransposon based markers like IRAP and REMAP. Among the various DNA-based markers used, ISSR markers are simple, fast, cost effective and a highly reliable. Also, retrotransposons are known to be activated under tissue culture conditions. These are highly abundant, ubiquitous in distribution, high copy number and provide excellent potential for developing DNA based marker systems. Retrotransposon insertional polymorphism can be detected by PCR in which outward facing primers are designed for conserved domains such as LTRs within the elements.

A. Inter Simple Sequence Repeat (ISSR) PCR Assay:

ISSR is a PCR based technology which employs the use of universal primers which were used to screen for polymorphism between different varieties of the crop. In this study, hundred ISSR markers available from the University of British Columbia (UBC) were used in two lots (UBC – 801 to UBC - 860) & (UBC – 861 to UBC - 900) for genetic fidelity analysis of DNA from two different sets of five potato samples; each obtained from CPRI (Central Potato Research Institute, Shimla) and most polymorphic markers were identified.

DNA was isolated using the protocol as mentioned in chapter 5 and its quality was determined by both nanodrop and gel based methods (Figure 11.1). The readings from the nanodrop are shown in Table 11.1.

Table 11.1: Nanodrop analysis of DNA concentration of potato samples received from CPRI, Shimla

Potato Cultivar	I.D.	Conc (ng/µl)	260/280
Kufri Bahar	1	65.8	1.84
Kufri Gaurav	2	68.1	1.86
Kufri Surya	3	95.1	1.83
Kufri Chipsonal	4	67.5	1.82
Kufri Girdhari	5	52.6	1.85
Kufri megha	P1	130	1.81
Kufri satluj	P2	119.8	1.79
Kufri shailja	P3	135	1.75
Kufri chipsona-II	P4	198	1.82
Kufri naveen	P5	157	1.78

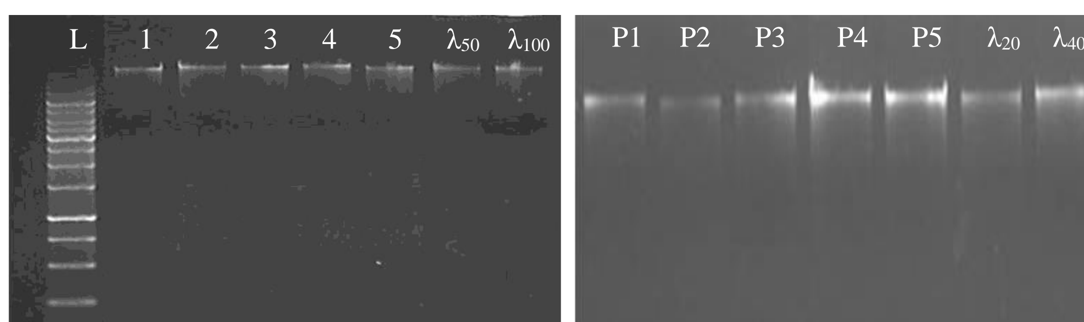

Fig. **11.1**: Gel image showing genomic DNA of potato samples run on 0.8% agarose L: Ladder; 1: Kufri Bahar; 2: Kufri Gaurav; 3: Kufri Surya; 4: Kufri Chipsona 1; 5: Kufri Girdhari; P1: Kufri megha; P2: Kufri satluj; P3: Kufri shailja; P4: Kufri chipsona-II; P5: Kufri naveen.

For identification of most polymorphic ISSR primers PCR was carried out with five genotypes of potato at a constant annealing temperature of 50 ^{0}C.

PCR Requirements:

1. ISSR primers
2. High quality DNA of working standard: 50 ng/µl
3. Taq DNA polymerase (1 unit/reaction)
4. dNTPs (200 µM each)
5. Buffer
6. Nuclease free autoclaved distilled water

PCR reaction mix

Component	Volume
• Buffer	2.5 µl
• dNTPs (2.5mM each)	2.0 µl
• Primer (200-500 pg)	1.0 µl
• DNA template (~50 ng)	1.0 µl
• Taq DNA polymerase (5U/ µl)	0.2 µl
• Nuclease free water	18.3 µl
Total Volume/reaction	25.0 µl

PCR conditions:

Temperature		Time	Cycles	Step
• 95^0C	:	5 min		(Initial Denaturation)
• 95^0C	:	2 min	35 cycles	(Denaturation)
• 50 ^{0}C	:	30 s		(Annealing)
• 72 ^{0}C	:	2 min		(Extension)
• 72 ^{0}C	:	10 min		(Final Extension)
• 4 ^{0}C	:	∞		(Hold)

Out of all the hundred screened primers fifty-five primers showed amplification at 50 $^{\circ}$C, whereas only thirty-five primers were found to be polymorphic exhibiting 98 polymorphic bands. Of these thirty-five primers two sets of six primers each were selected for screening potato samples for genetic fidelity testing. First set comprises of ISSR primers UBC_807, UBC_817, UBC_822, UBC_826, UBC_880 & UBC_890 (Fig. 11.2). If during screening any variation is observed exhibiting 3 or more polymorphic bands then further second set need not to be screened. But if only 1-2 polymorphic bands are seen with first set then to reconfirm any type of variation the second set which comprises of UBC_808, UBC_812, UBC_864, UBC_886, UBC_887 & UBC_891 primers are to be used (Fig....).

Gel electrophoresis of PCR products:

The methodology used for agarose gel electrophoresis was followed similarly as mentioned in Chapter 6 of the Manual. The results obtained is given in (Figure 11.2 and Figure. 11.3)

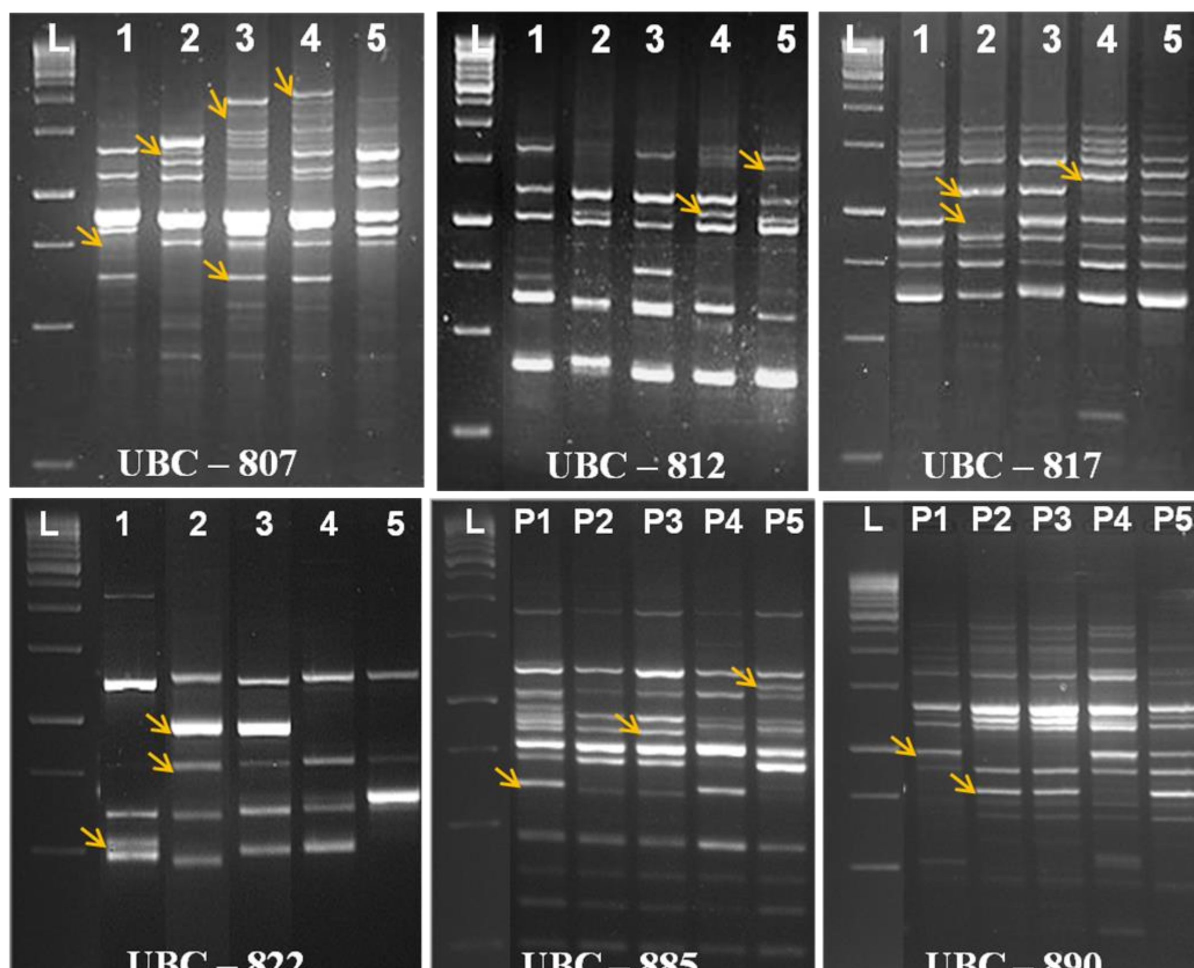

Fig. 11.2: Gel images of amplified PCR product showing polymorphism using second set of ISSR (UBC) Primers for Potato ; L: Ladder; 1:Kufri Bahar; 2:Kufri Gaurav; 3:Kufri Surya; 4:Kufri Chipsona 1; 5: Kufri Girdhari; UBC: University of British Columbia & P1: Kufri megha; P2: Kufri satluj; P3: Kufri shailja; P4: Kufri chipsona-II; P5: Kufri naveen.

S.NO.	DETAIL	AT 50^0C	AT 45^0C
1	No. of primers screened	100	9
2	No. of primers showing amplification	55	4
3	No. of primers showing monomorphism	20	3
4	No. of primers showing polymorphism	35	1
5	No of polymorphic bands	98	4
6	Total polymorphic bands	102	

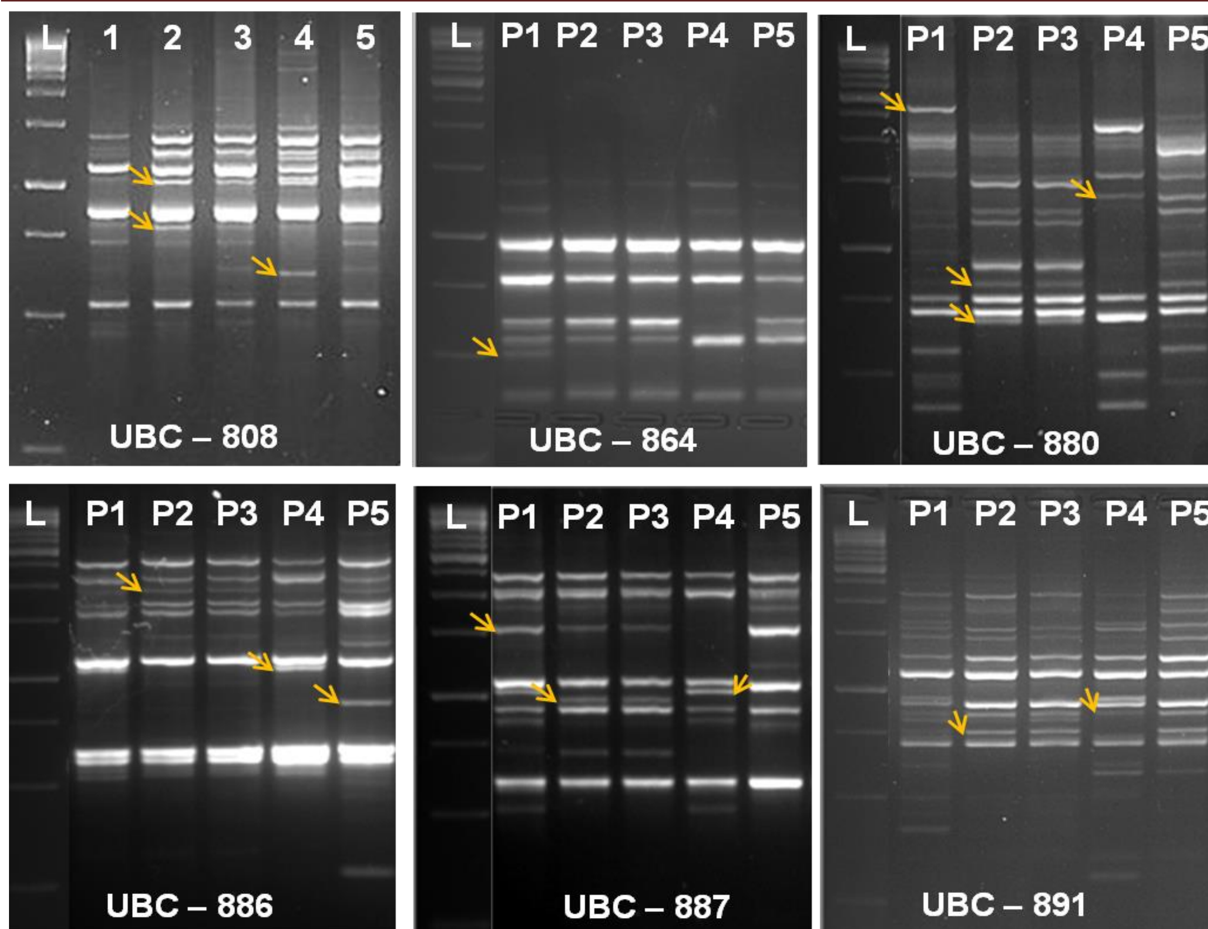

Fig. 11.3: Gel images of amplified PCR product showing polymorphism using second set of ISSR (UBC) Primers for Potato ; L: Ladder; 1:Kufri Bahar; 2:Kufri Gaurav; 3:Kufri Surya; 4:Kufri Chipsona 1; 5: Kufri Girdhari; UBC: University of British Columbia & P1: Kufri megha; P2: Kufri satluj; P3: Kufri shailja; P4: Kufri chipsona-II; P5: Kufri naveen.

Results inferred:

- 100 ISSR primers (UBC-801to UBC-900) were screened against five potato varieties, out of which 36 primers were found to be polymorphic.
- A total of 102 polymorphic bands were obtained.
- Number of polymorphic bands amplified by each primer varied from one - six bands.
- Two constant temperatures 50°C and 45°C were tried out of which 35 ISSR primers showed polymorphism at 50^0C whereas 1 primer showed polymorphism at 45°C.

12. IRAP markers mediated analysis of genetic fidelity of potato

Sharma P, Shingote P, Devanna BN, Arora K, Amitha Mithra SV and Solanke AU

IRAP is a PCR based technique which includes the use of primers derived from a retrotransposon source. In IRAP, combinations of primers pointing outward from the LTR of retrotransposons are used to amplify the region between two retroelements. In the present study IRAP primers were screened to select the best combinations which show good amplification and reproducibility. The selected primers were then used to screen genomic DNA isolated from potato varieties (Table 12.1) and to test clonal fidelity/true-to-type in micropropagated plants.

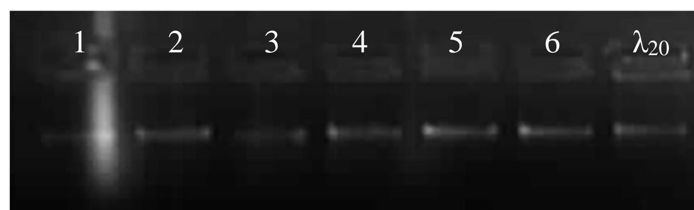

Fig. 12.1: Genomic DNA of potato samples run on 0.8% agarose;1: Kufri alankar; 2: Kufri khyati; 3: Kufri sindhuri; 4: Kufri sharpa; 5: Kufri muthu; 6: Kufri laykar

Table 12.1: List of varieties of potato crop used:

S.No.	VARIETY NAME	I.D.
1	Kufri Alankar	R1
2	Kufri Khyati	R2
3	Kufri Sindhuri	R3
4	Kufri Sharpa	R4
5	Kufri Muthu	R5
6	Kufri Laykar	R6

PCR Requirements:

1. ISSR primers
2. High quality DNA of working standard: 20 - 30 ng/µl
3. Taq DNA polymerase (1 unit/reaction)
4. dNTPs (200 µM each)
5. Buffer
6. Nuclease free autoclaved distilled water

7. $MgCl_2$

PCR reaction mix

•	Buffer (Containing 1.5mM $MgCl_2$)	2.5 µl
•	dNTPs (2.5mM each)	2.0 µl
•	Primer (200-500 pg)	1.0 µl
•	DNA template (~50 ng)	1.0 µl
•	Taq DNA polymerase (5U/ µl)	0.2 µl
•	$MgCl_2$	0.5 µl
•	Nuclease free water	17.8 µl
	Total Volume/reaction	25.0 µl

PCR conditions:

•	95^0C	:	5 min		(Initial Denaturation)
•	95^0C	:	40 s	35 cycles	(Denaturation)
•	50 ^{0}C	:	40 s	35 cycles	(Annealing)
•	72 ^{0}C*	:	2 min	35 cycles	(Extension)
•	72 ^{0}C	:	12 min		(Final Extension)
•	4 ^{0}C	:	∞		(Hold)

Where, * is Extension carried out at 72 ^{o}C for 2 min +3 s extension/cycle

Out of all the screened sets of primer combinations, eight primer pairs showed amplification and polymorphism at 50^0C eliciting 33 polymorphic bands when screened against six potato varieties. Various IRAP primers used are listed below (Table 12.2):

Table 12.2. List of primer combinations used for IRAP PCR assays

S.NO.	Primers	T_a(^{0}C)
1	Copia F + 3’ LTR	50
2	Copia F + 5’ LTR2	50
3	Copia F + LTR 6150	50
4	Tst 6 + 3’ LTR	50
5	LTR 6149 + Sukkula	50
6	Sukkula + 5’ LTR 1	50
7	Nikita + Sukkula	50
8	5’ LTR 1 + 3’ LTR	50

Gel electrophoresis of PCR products:

The methodology used for agarose gel electrophoresis was followed similarly as mentioned in Chapter 6 of the Manual. The PCR results are given in Figure 12.2 and Figure 12.3.

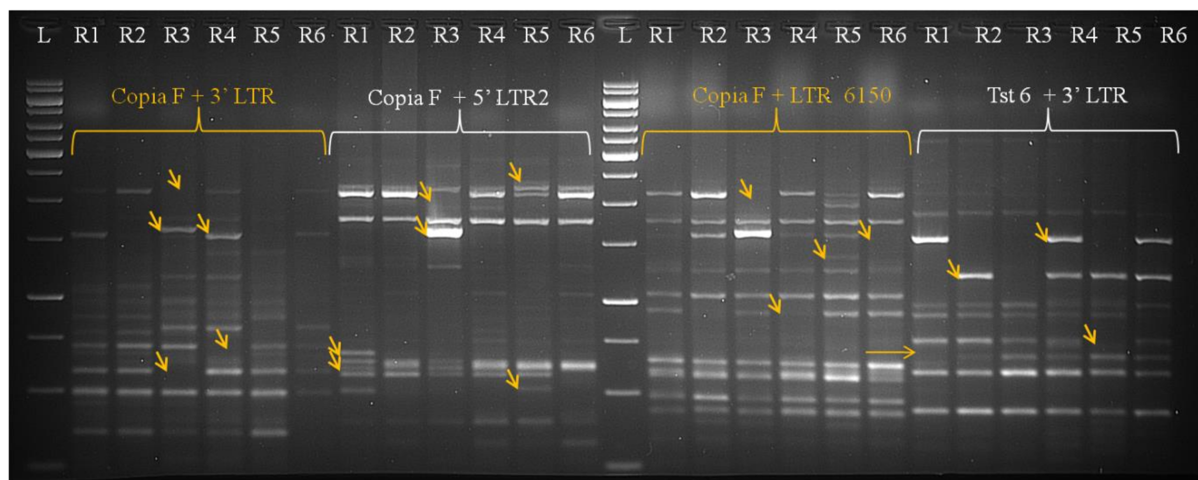

Fig. 12.2: Gel images of amplified PCR product showing polymorphism using IRAP Primers sets for Potato Fig. L: 1 Kb Ladder; R1: Kufri alankar; R2: Kufri khyati; R3: Kufri sindhuri; R4: Kufri sharpa; R5: Kufri muthu; R6: Kufri laykar. IRAP Primer combinations: Copia F + 3' LTR, Copia F + 5' LTR2, Copia F + LTR 6150, Tst 6 + 3'

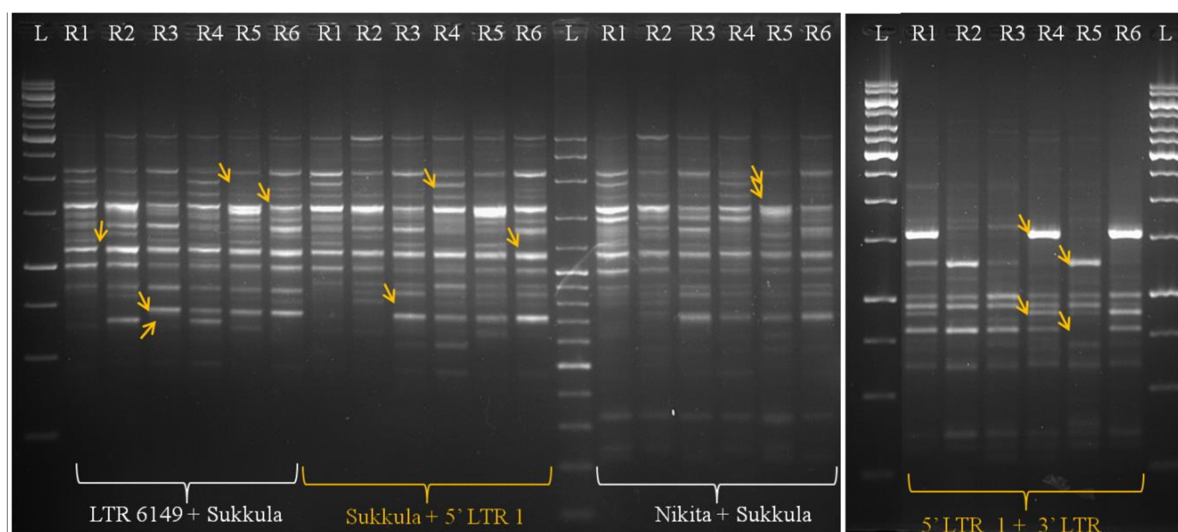

Fig. 12.3: Gel images of amplified PCR product showing polymorphism using IRAP Primers sets for Potato Fig. L: 1 Kb Ladder; R1: Kufri alankar; R2: Kufri khyati; R3: Kufri sindhuri; R4: Kufri sharpa; R5: Kufri muthu; R6: Kufri laykar, IRAP Primer combinations: LTR 6149 + Sukkula, Sukkula + 5' LTR 1, Nikita + Sukkula, 5' LTR 1 +

Results obtained:

- Eight sets of IRAP primer combinations (Copia F + 3' LTR, Copia F + 5' LTR2, Copia F + LTR 6150, Tst 6 + 3' LTR, LTR 6149 + Sukkula, Sukkula + 5' LTR 1,

Nikita + Sukkula, 5' LTR 1 + 3' LTR) were screened against six potato varieties to test polymorphism.

- A total of 33 polymorphic bands were obtained.
- Number of polymorphic bands amplified by each primer varied from two - six bands.
- One constant temperature of 50^0C was tried out.

13. ISSR markers mediated analysis of genetic fidelity of banana

Arora K, Sharma P, Devanna BN, Shingote P, Amitha Mithra SV and Solanke AU

Banana (Musa) is a good model system for plant genomic studies and is also one of the most important food security crops for developing countries. Banana (*Musa acuminata*) belongs to the family Musaceae. Banana has a genome size of 600 million base (Mb) pairs and has a haploid chromosome number of 11. The cultivated species of banana are usually diploid or triploid with 3n=33. Banana is the second largest produced fruit after citrus, contributing about 16% of the world's total fruit production. India is the largest producer of banana, contributing to 27% of world's banana production (Mohapatra et al., 2010). Maharashtra leads banana production in India with its higher productivity and area coverage of 60,000 hectares. To increase the production of banana, it is highly desirable to adopt new production technologies to ensure better quality production. Tissue culture could be one of the promising alternatives for fast replacement of planting material. Therefore, a qualitative check for the true to type planting material at an early stage of banana development is considered to be desired in its tissue culture.

Molecular markers serve as an important tool to check the genetic uniformity and true to type nature of micropropagated plants. Among the various DNA-based markers used, ISSR markers are simple, fast, cost effective and a highly reliable. A set of hundred ISSR markers available from the University of British Columbia (UBC) were used for genetic fidelity analysis of DNA (Figure 13.1) from three varieties of Banana procured from UAS, GKVK (University of Agricultural Sciences, Gandhi Krishi Vigyan Kendra), Bangalore and most polymorphic markers were identified.

Quality of DNA was determined by both Nanodrop and gel based methods. The readings from the nanodrop are shown in Table 13.1.

Table 13.1: Nanodrop analysis of DNA concentration of banana leaf tissues

Banana Cultivar	Volume (µl)	Conc. (ng/µl)	$A_{260/280}$
Grand Naine	100	11.82	1.68
Elakki	100	11.24	1.74
Poovan	100	9.72	1.75

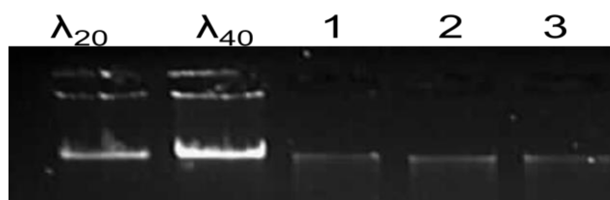

Fig. 13.1: Genomic DNA of banana samples run on 0.8% agarose gel; Lane 1, 2: Uncut λ ; 1: Grand Naine; 2: Elakki; 3: Poovan

For identification of most polymorphic ISSR primers PCR was carried out with three genotypes of banana at a constant annealing temperature of 50 °C.

PCR Requirements:

1. ISSR primers
2. High quality DNA of working standard: 50 ng/µl
3. Taq DNA polymerase (1 unit/reaction)
4. dNTPs (200 µM each)
5. Buffer
6. Nuclease free autoclaved distilled water

PCR reaction mix

Component	Volume
• Buffer	2.5 µl
• dNTPs (2.5mM each)	2.0 µl
• Primer (200-500 pg)	1.0 µl
• DNA template (~50 ng)	1.0 µl
• Taq DNA polymerase (5U/ µl)	0.2 µl
• Nuclease free water	18.3 µl
Total Volume/reaction	25.0 µl

Out of thirty-two polymorphic primers, two sets of primers with six each were selected for screening banana samples for genetic fidelity testing. First set comprises of ISSR primers UBC_810, UBC_818, UBC_826, UBC_834, UBC_840 and UBC_848 (Fig. X). For screening, if any variation consisting of 3 or more polymorphic bands is observed in the first set, then further screening with second set of primers is required to validate the variation. If the number of polymorphic bands found with the first set of primers is less than 3, then we

need to further confirm the variation by screening with the second set of primers which comprises of UBC_855, UBC_857, UBC_864, UBC_873, UBC_880 and UBC_881 primers (Fig. 13.2 and Fig. 13.3). However, there is no need to screen with the second set of primers if we could either get the polymorphism for more than 3 bands or if no polymorphism is found with the first set of primers.

PCR conditions:

- 95 °C : 5 min (Initial Denatiuration)
- 95 °C : 2 min (Denaturation)
- 50 °C : 30 s (Annealing)
- 72 °C : 2 min (Extension)
- 72 °C : 12 min (Final Extension)
- 4 °C : ∞ (Hold)

35 cycles (steps 2–4)

Gel electrophoresis of PCR products:

The methodology used for agarose gel electrophoresis was followed similarly as mentioned in Chapter 6 of the Manual.

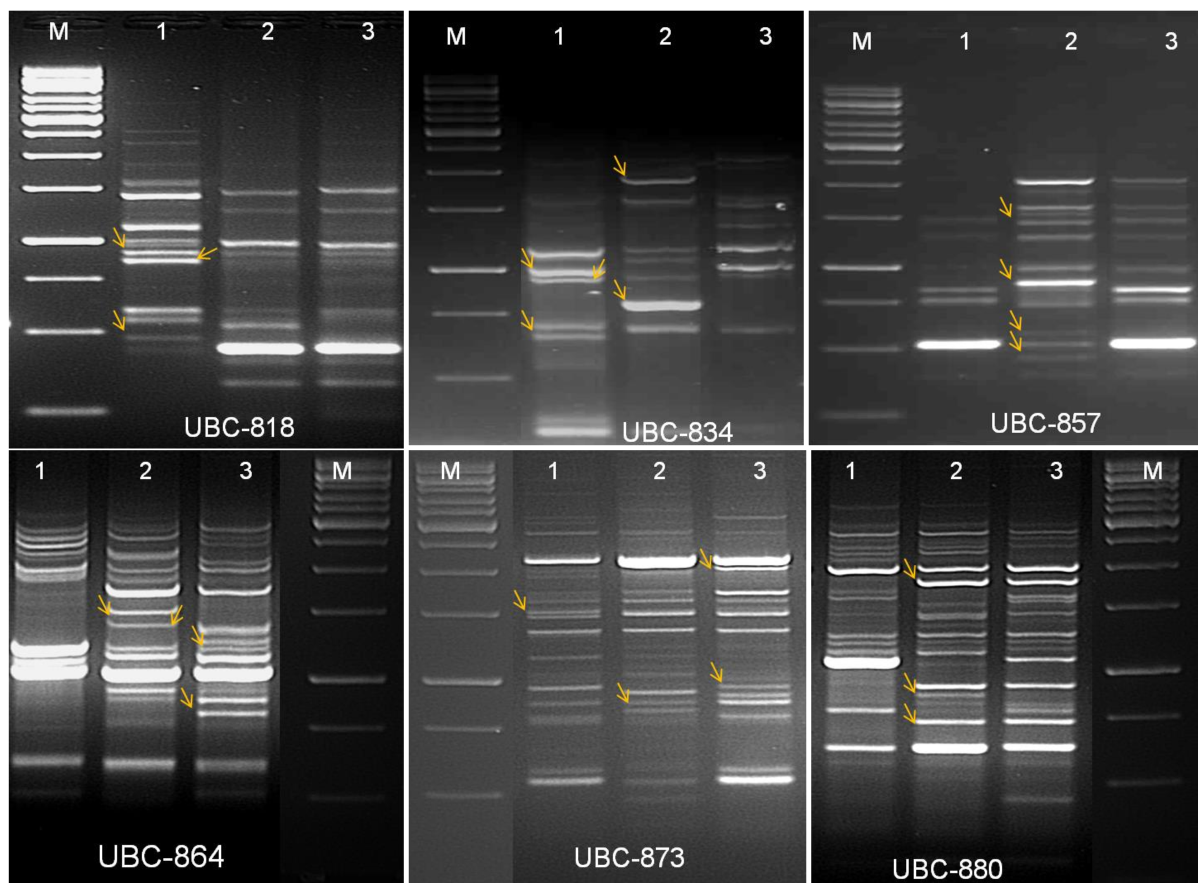

Fig. 13.2: ISSR profiling of three banana varieties with primers UBC-818, 834, 857, 864, 873, and 880. Lane M: 1kb DNA ladder, 1: Grand Naine, 2: Elakki, 3: Poovan.

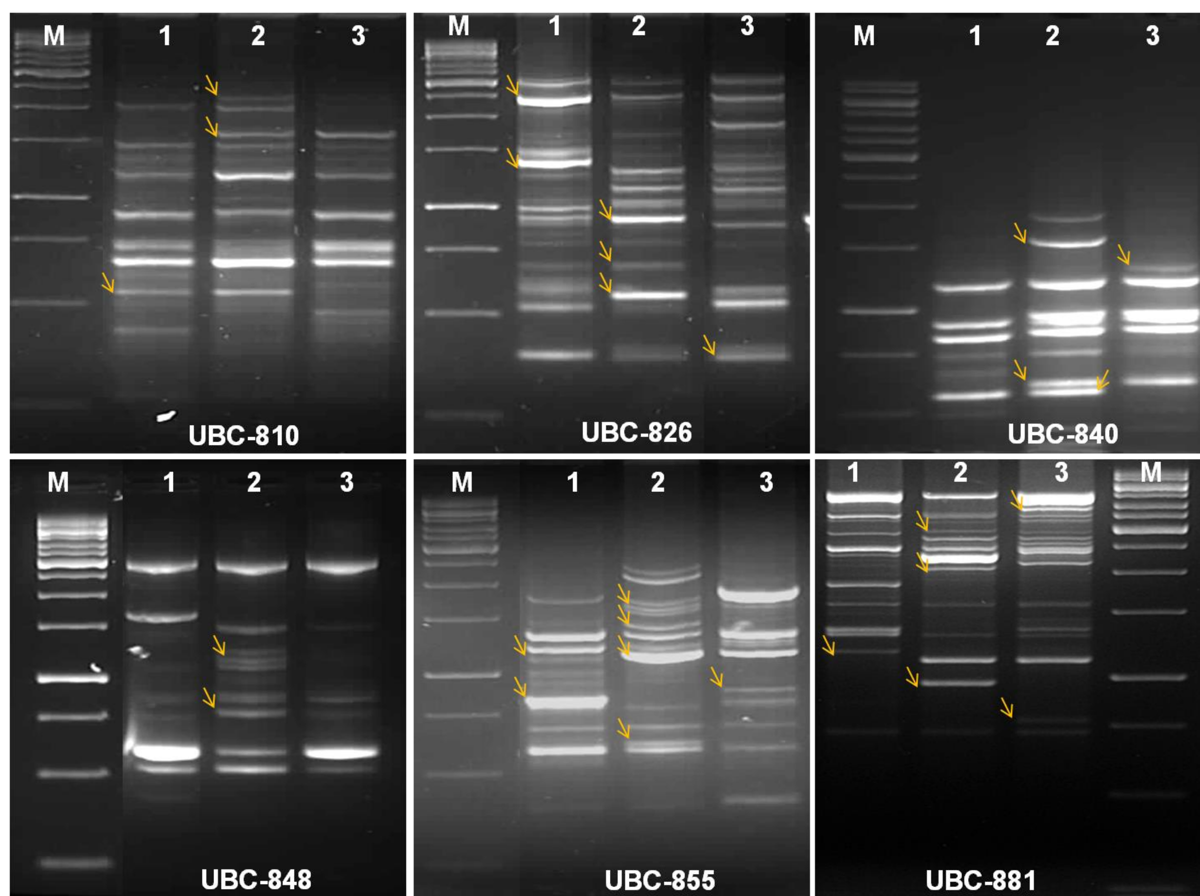

Fig. 13.3: ISSR profiling of three banana varieties with primers UBC-810, 826, 840, 848, 855, and 881. Lane M: 1kb DNA ladder, 1: Grand Naine, 2: Elakki, 3: Poovan.

Results:

- 100 ISSR primers (UBC-801 to UBC-900) were screened against three banana varieties, out of which 51 primers were amplified and 32 of these primers were found to be polymorphic and 19 were found to be monomorphic.
- A total of 103 polymorphic bands were obtained.
- Number of polymorphic bands amplified by each primer varied from two-six bands.
- Constant temperature of 50°C was standardized and used.

14. ISSR markers mediated analysis of genetic fidelity of date palm

Devanna BN, Sharma P, Arora K, Shingote P, Amitha Mithra SV and Solanke AU

Date palm (*Phoenix dactylifera* L. Arecaceae) (2n=2x=36) is a dioecious, perennial, monocotyledonary fruit bearing tree, with a very slow growth rate and a late reproductive phase. Palm trees are an excellent candidate for cultivation in harsh climatic conditions of arid and semi-arid regions of the world due to their high tolerance to environmental stresses where no other crops give economic returns. This fruit crop also has the potential of generating good income and foreign exchange from unproductive tracts. Date palms are commonly propagated by offshoots as this maintains the genetic integrity of the cultivars but offshoots are produced in limited numbers during a date palm's life span. Therefore the developments in the field of agro-biotechnology have increased the importance of developing *in vitro* methods of propagation in date palm. However, scaling up of any micropropagation protocol is severely hindered due to incidences of somaclonal variations; hence a stringent quality check in terms of genetic similarity of the tissue culture raised plants becomes mandatory. Therefore, it is necessary to check the genetic uniformity of micropropagated plants by molecular techniques.

We have started standardization for ISSR primers in date palm and used a set of six date palm genotypes received from Atul Ltd. Kuch, Gujarat for ISSR analysis. Genomic DNA was isolated using modified CTAB method from all the six samples before further analysis. Initially we used a uniform annealing temperature of 50 ^{0}C for all the 100 ISSR primers. Genomic DNA was isolated from 6 date palm varieties and used for primer screening.

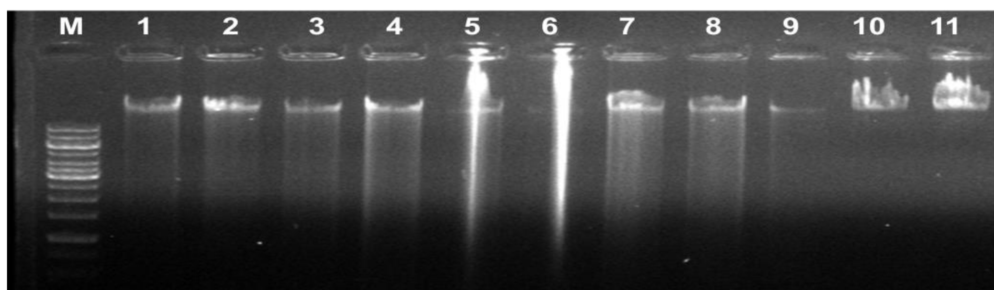

Fig. 14.1: Gel analysis of genomic DNA isolated from date palm samples. 1-9 DNA from date palm samples; 10- 75ng and 11- 150 ng λ DNA.

Nanodrop analysis of DNA

Sample No.	Volume (µl)	Conc. (ng/µl)	$A_{260/280}$
1	100	699	1.74
2	100	355	1.84
3	100	692	1.74
4	100	897	1.72
5	100	589	2.01
6	100	569	1.81

PCR reaction mix:

Component	Volume
• Buffer	2.5 µl
• dNTPs (2.5mM each)	2.0 µl
• Primer (200-500 pg)	1.0 µl
• DNA template (~50 ng)	1.0 µl
• Taq DNA polymerase (5U/ µl)	0.2 µl
• Nuclease free water	18.3 µl
Total Volume/reaction	25.0 µl

PCR conditions:

Temperature	Time	Cycles	Step
• 95^0C	5 min		(Initial Denaturation)
• 95^0C	30 s	35 cycles	(Denaturation)
• 45	30 s	35 cycles	(Annealing)
• 72 ^{0}C	2 min	35 cycles	(Extension)
• 72 ^{0}C	12 min		(Final Extension)
• 4 ^{0}C	∞		(Hold)

Results:

- Out of 100 ISSR primers screened, 32 primers could show PCR amplification at given PCR conditions.
- Among the 32 PCR amplified primers, 9 primers (Fig. 14.2) were polymorphic among the six date palm genotypes
- Totally 16 polymorphic bands were identified from 9 ISSR markers (UBC_818, 825, 826, 827, 836, 845, 850, 851 and 855)

Conclusions:

- We found 16 polymorphic bands from 9 primers at 45 ^{o}C
- The polymorphism analysis indicates that these six date palm lines are more closely related or derivatives of the same parental plant

Gel electrophoresis of PCR amplified products:

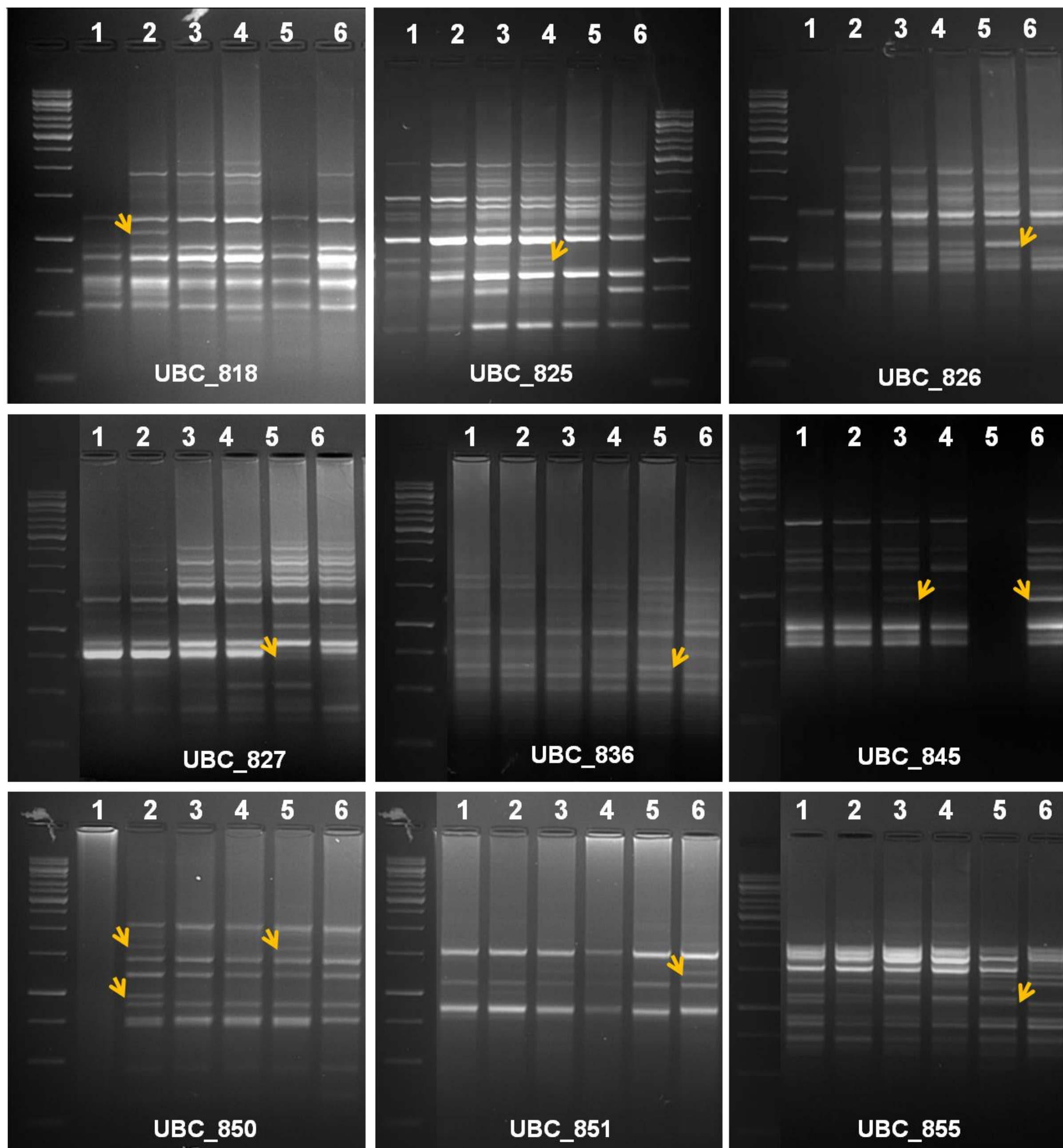

Fig. 14.2: ISSR profiling of six date palm genotypes using UBC primers. M: 1 Kb Ladder, 1-6 date palm genotypes

15. Increasing the genetic fidelity testing efficiency through pooled DNA sample analysis

Sharma P, Shingote P, Arora K, Devanna BN, Amitha Mithra SV and Solanke AU

The idea of using pooling DNA samples is to reduce the burden of screening large number of plant samples for genetic fidelity testing to save time and resources without compromising the authenticity of the test. Thus, in the present study an experiment was conducted to determine the number of plant samples whose DNA could be pooled without disturbing the banding pattern obtained for that specific marker. Two differentiating/ contrasting plant samples of potato exhibiting explicit band pattern were selected for the study. For example; sample A shows presence of a prominent band which is absent in sample B. When the DNA from these two species was pooled in various ratios (A:B - 1:1, 4:1, 9:1, 1:4 & 1:9) and used for PCR using a specific ISSR marker, the results thus obtained clearly showed that 1:1, 1:4/4:1 and 1:9/9:1 pooling results exhibited no significant difference in band pattern (Figure 15.1). However, selection of marker needs a conscious one as the marker with faint band disappears easily in pooled samples than the one with a major intense band at higher ratio of pooling.

Inference:

- If the number of mother plant tissue/stock is large, the samples from batches consisting of a maximum of 10 mother plants/stock cultures may be pooled for testing.
- In cases where the pooled samples show variation, it is necessary to further analyze individual samples from the pool so as to identify the actual variant.

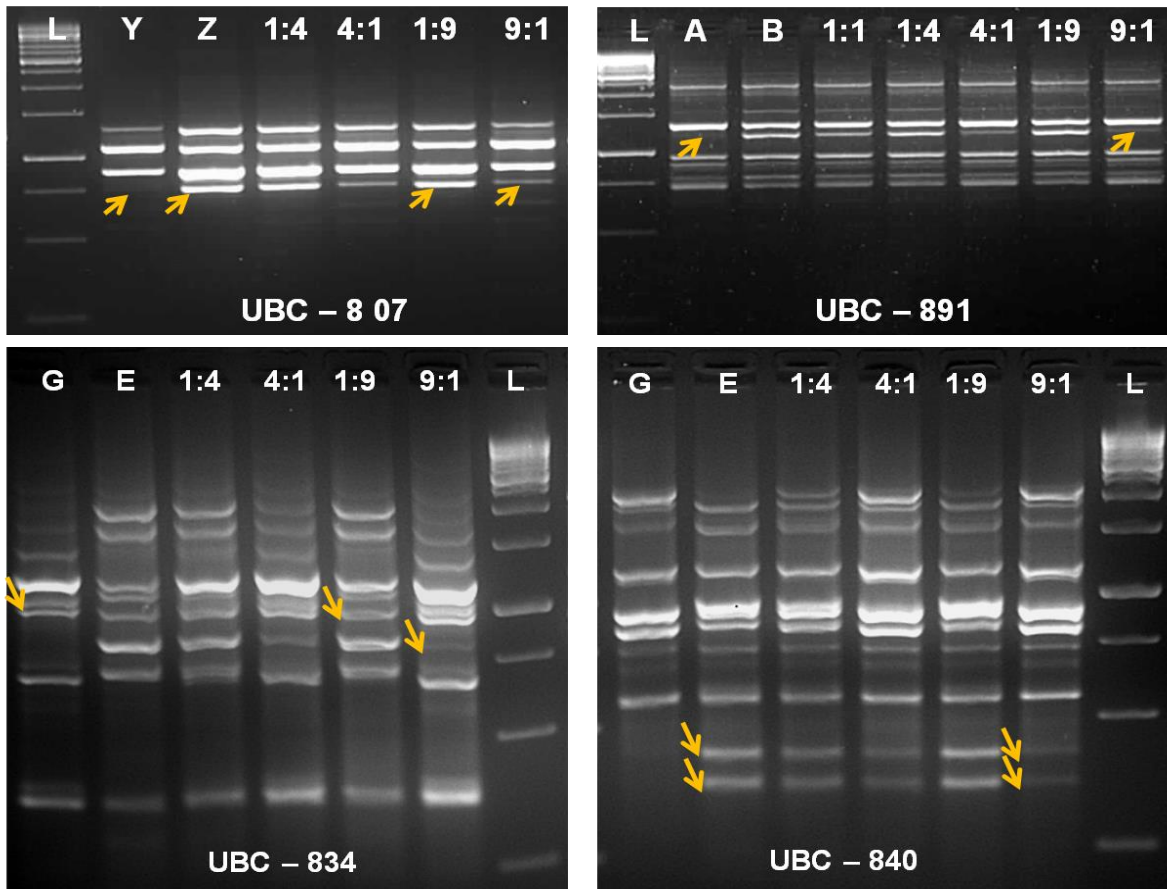

Fig. 15.1: Pooling of potato (upper panel) and banana (lower panel) samples in different ratios. Band pattern and ratios of potato samples in upper panel, where; Y: Kufri alankar; Z: Kufri sharpa; A: Kufri giriraj; B: Kufri jyoti. Band pattern and ratios of banana samples in lower panel: where G: Grand Naine; E: Elakki.