GENOME SIZE AND GENETIC HOMOGENEITY OF REGENERATED PLANTS: METHODS AND APPLICATIONS

Editor:
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Genome Size and Genetic Homogeneity of Regenerated Plants: Methods and Applications

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Preface

The Flow cytometry technique is currently employed in various fields of research. The technique is also used in modern biosystematics, speciation, evolutionary studies and in molecular breeding. Various factors influence the quality of active nuclei isolation, which determines the success of accurate DNA estimation. The importance of extraction buffer, reference standards, fluorochrome dyes, and the process of gating is highlighted in order to understand the various steps of flow cytometry in measuring DNA. An array of compounds act as inhibitors, disrupts fluorochrome binding to DNA, and cause errors in estimating nuclear DNA content; all the above important factors are described in one chapter. Micropropagation using shoot tips and nodal stems produces true-to type plants, while callus regenerated plants show somaclonal variations - a process showing altered DNA. The role of flow cytometry in investigating the genetic homogeneity of tissue cultured populations is reviewed in different chapters. The intra-specific and inter-specific genome and chromosome number variation with reference to gene duplication and DNA sequence loss have been described. To establish the genetic homogeneity / fidelity of regenerated plants, several DNA marker based techniques have been used. Start codon targeted polymorphism (SCoT) has emerged as one of the recently used DNA fingerprinting techniques to assess the genetic stability of tissue cultured plants and for revealingreveal cultivar differentiation and genetic diversity between wild and domesticated plants. A few chapters described the importance, applications and limitations of various molecular markers in studying genetic homogeneity, somaclonal variants and polyploidy in different groups of plants.

Much of the information is available on the website; the need to accumulate cutting-edge knowledge and flow cytometry techniques in a structured book format is still essential. I sincerely hope that this updated literature of the above research will be very useful resource material to a wide range of people, especially researchers, graduate students, teachers and other professionals in various disciplines like Botany, Biotechnology, Agriculture, Horticulture, Pharmacology and other research fields.

I am grateful to all the authors for their valuable contributions. I am indebted to publishers for agreeing to publish this effort. Last but not least, I am thankful to current Ph. D students for their help and support.

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CHAPTER 1

Recent Advancements in CRISPR/Cas-based Genome Editing in Plants

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Abstract: The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated protein (Cas)-mediated genome editing is a recently developed gene editing technology, which has transformed functional and applied genomics. This technology is precise, cost-efficient, and rapid than other previously developed genome editing tools such as Meganucleases (MNs), Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs). The CRISPR-Cas9 system is widely exploited for developing plants with enhanced tolerance towards various environmental stresses, resistance against pathogens, improved yield and nutritional superiority. The method is robustly applied to alter both DNA and RNA at specific target regions. The availability of well annotated genome sequence and an efficient genetic transformation system may open numerous possibilities to gain desirable traits in crop plants employing CRISPR-Cas-mediated genome editing technology. In this chapter, we summarized the basics of CRISPR-Cas technology, various kinds of CRISPR systems and their associated Cas proteins, application in generating abiotic and biotic stress tolerant crops, and bottlenecks of CRISPR-Cas systems.

Keywords: Genome editing, CRISPR-Cas, Abiotic stress, Biotic stress, Crop improvement.

INTRODUCTION

The swiftly rising human population is projected to reach ~10 billion by the end of 2050. To meet the demand of food supply for all, the productivity of the existing crop system needs to be extended further. In addition, crop loss due to various biotic and environmental constraints is needed to be restricted. Due to the continuous global climate change, a significant reduction in cereal grain productivity has been reported, which is a major threat to food security [1].

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Several crop improvement programmes, including molecular breeding and biotechnological approaches, have resulted in the identification of genetic determinants underlying superior agronomical traits. The information has been employed to generate genetically improved varieties conferring tolerance /resistance to abiotic/pathogen stress or nearing biofortification for nutritional traits. Knockout or knockdown of a gene through RNAi or VIGS as per conventional functional genomic approach is largely being replaced by the recently developed Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-CRISPR-associated protein (CRISPR-Cas)-mediated targeted genome editing.

In the last decade, the CRISPR-Cas system has gained much attention due to its precise targeting, versatility, high efficiency, and minimal or negligible effects on non-target DNA regions. The CRISPR-Cas system relies on RNA-DNA recognition to induce a double-strand break, unlike Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), which require protein motifs for target recognition. CRISPR-Cas acts as an adaptive immune system in prokaryotic organisms, which provides resistance to them against foreign DNA [2]. The CRISPR-Cas system consists of two components: a customizable single stranded guide RNA (gRNA) and a Cas endonuclease. The gRNA is created by fusing a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA), and is responsible to locate the target nucleic acid by sequence complementarity. A 2-5 bp protospacer adjacent motif (PAM) is required by the Cas protein to recognize, bind, and cleave the target site. The double stranded break on DNA is created by the Cas-gRNA complex followed by subsequent repair either through homologous recombination (HR) or non-homologous end joining (NHEJ) method. NHEJ-based DNA repair is an error prone pathway that creates random changes, either insertions or deletions of nucleotide base, causing point mutations which usually lead to the knockout of genes [3] (Fig. **1**). The target sequences could be within the coding region of a gene or in the non-coding parts such as promoter, untranslated regions, or regulatory sequences. However, to get the best result for the knockout of a functional gene, the gRNA target sequence should be selected within the coding region, especially towards the 5' end of the sequence. Untranslated regions, intron-exon junction or intergenic regions should be avoided. Multiple sites can also be targeted simultaneously within the same tissue as guided by different gRNAs specific to their target sites. The CRISPR platform is being widely deployed for introducing new traits by disrupting the function of a protein that negatively correlates with stress tolerance, disease infestation, yield, and nutritional quality by creating small insertions or deletions of less than 100 bp.

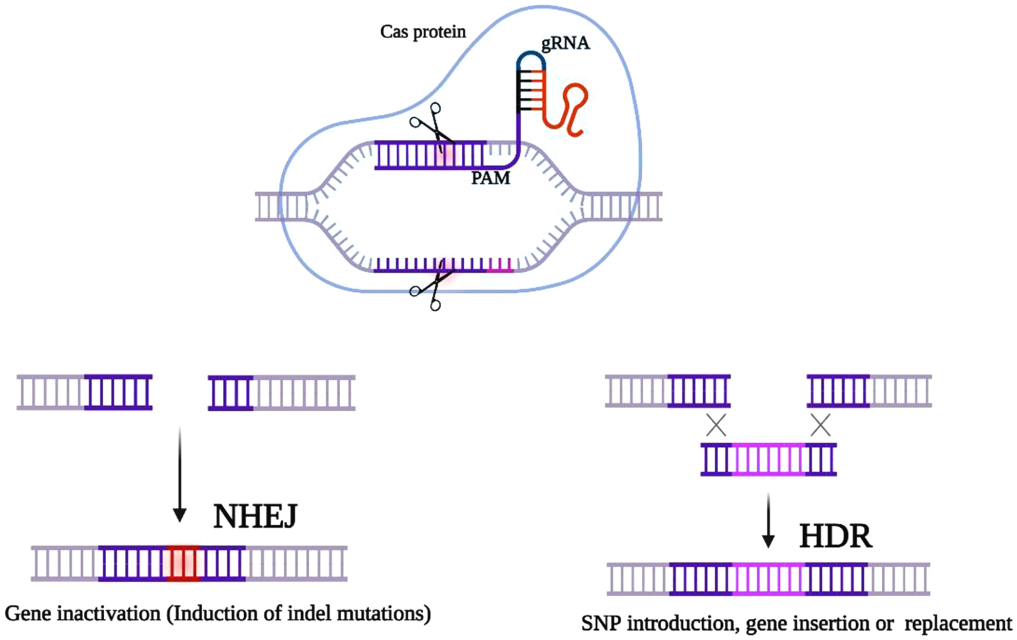

Fig. (1). Mechanism of CRISPR-Cas-mediated gene editing system. Guide RNA (gRNA) directs the Cas protein to the target DNA region to induce double stranded nick near the PAM sequence. The breaks on DNA are repaired either by nonhomologous end joining (NHEJ) or homology directed repair (HDR). The NHEJ repair pathway usually results in InDel mutations, whereas the HDR pathway employs an identical DNA template which can be used to insert, replace, and mutate the target gene or DNA region. Image created using Biorender.com.

CRISPR-CAS SYSTEM AND THEIR NUCLEASES

The CRISPR-Cas system has been broadly categorized into two classes: class 1 that utilizes multiple Cas protein complexes to degrade foreign genetic materials and class 2 that employs single Cas protein. Class 1 is further sub-divided into Type I, III, and IV, whereas class 2 into II, V, and VI [4]. The most commonly used Cas9 nucleases are the component of the type II CRISPR-Cas system. They are RNA-directed DNA endonucleases consisting of HNH and RuvC nuclease domain which makes double stranded breaks (DSBs) of target and non-target DNA strands, respectively. Inactivation of any of these two nuclease domains generates a Cas9 nickase (nCas9) which makes nicks only on one strand. nCas9 is mostly used in precise genome editing through base editors and primer editors, which do not require DSBs [5]. Several orthologs and variants of Cas9 with different PAM sequence preferences have been isolated and used in the editing of plant genomes [6] Table **1**.

CHAPTER 2

Molecular Markers Used in the Analysis of Somaclonal Variation During Plant Tissue Culture

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Abstract: The study of somaclonal variation is a growing research area that has allowed to identify several biological processes involved in genetic instability during plant tissue culture. These changes may be undesirable during the micropropagation of elite plants or desirable during plant breeding programs. There are different molecular techniques that allow to analyze this somaclonal variation. Due to the progress that has been made in the manipulation and analysis of DNA, the number of molecular markers has increased to achieve this objective. These methods have been increasing in number, while some of them have been widely used since their development [simple sequence repeat (SSR), inter simple sequence repeats (ISSRs), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD)], others, such as retrotransposon amplification, SSR-markers derived from expressed sequence tags, targeted region amplification polymorphism (TRAP), transcribed sequences (RNAseq). Whole genome sequencing is increasing their use and they complement each other by providing more information, allowing to link genetic markers with specific phenotypes in somaclonal variants. The aim of this chapter is to highlight the methodology of the most commonly used molecular markers to assess somaclonal variation during plant tissue culture.

Keywords: Micropropagation, Somaclonal variation, Molecular marker.

INTRODUCTION

Molecular markers are a set of powerful tools used to assess somaclonal variation during plant tissue culture. During plant micropropagation, it is desired to obtain

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true-to type regenerants with respect to the mother plant, selected for its important agronomic attributes, as well as during germplasm conservation, where it is desired to selectively store specific genotypes [1]. While other approaches may aim to induce somaclonal variation to select genotypes with some superior agronomic traits [2, 3]. In both cases, confirming genetic stability and genetic changes is of importance. The use of a particular molecular marker may depend on the genetic information available for the plant species of interest, the methodology can be simple or laborious, and it can require more sophisticated equipment. In addition, the objective may be to evaluate genetic variations throughout the genome, in non-coding sequences or in coding sequences, the research may also be more directed towards evaluating the variation of specific genes. In recent years there has been a great advance in the design of new molecular markers that allow the study of these genetic variations, due to the availability of complete genome sequences in various plant species, together with a better understanding of the biological processes involved in somaclonal variation [4]. Although there are several strategies to analyze somaclonal variation (phenotypic characterization, chromosome number and structure, flow cytometry and protein and isoenzyme analysis) [5], the focus of this chapter book is on DNA-based analyses.

SOMACLONAL VARIATION

The term somaclonal variation was first coined by Larkin and Scowcroft [6] and refers to all the changes that can occur in DNA during *in vitro* tissue culture, especially when differentiated somatic cells are directed towards a pluripotent state by organogenic processes [7]. These changes may be reflected in DNA-sequence or epigenetic-modifications, where heterochromatin is modified, either by methylation of cytosine or in a smaller proportion of adenine [8 - 10] by methylation or acetylation of histone proteins [11] or by regulation of heterochromatin by small RNA [12]; some authors have proposed that an erroneous regulation of microRNAs and small RNA pathways can cause somaclonal variation [13]. In addition, cytogenetic analyses have shown a loss or increase in chromosome number within somaclonal variation in plant tissue culture [14]. All these processes can lead to gene activation or silencing, which can cause cellular abnormalities and phenotypic changes in plant height, number of shoots, fruit size, number of stomata, the diameter of vascular tissue, leaf pigmentation, among others (Fig. **1**) [15 - 18] nonetheless, the variation has also been related as an adaptive response of plants to environmental changes and as part of the evolutionary process [19]. The tools used for these purposes are many and have increased in recent years. In addition, next generation sequencing of genomes has provided a broader picture of the genetic changes that occur during PTC.

Fig. (1). Phenotypic changes in somaclonal variants of *V. planifolia* and *Stevia* rebaudiana. A and B) Variegated vanilla plants obtained by indirect organogenesis, **A**) In the right regenerated shoot without variegation, in the left regenerated shoot with variegation, **B**) Right shoots without variegated leaves, left shoots with variegated leaves. C and D) Ste*via* plants showing somaclonal variation, **C**) Plants with abnormal brown pigmentation obtained by direct organogénesis, **D**) Plants with morpho-physiological anomalies micropagated in RITA® bioreactors. Photos taken from Ramírez-Mosqueda *et al.* [17] and Ramírez-Mosqueda *et al.* [18].

Factors Contributing to the Development of Somaclonal Variants Subcultures

During PTC, it is well known that the number of subcultures can influence the genetic stability of the plant material; as subcultures increase, so does the probability of generating somaclonal variants. However, it has been observed that the genotype and the use of different plant growth regulators also have a strong influence [20, 21] In pineapple (*Ananas comosus*), it was observed that maintaining more than 10 subcultures for four years was a factor that favored the increase in somaclonal variation, as well as the regeneration system (indirect regeneration and indirect somatic embryogenesis), this somaclonal variation was

CHAPTER 3

Application of Molecular Markers in Revealing Genetic Stability Among *In Vitro* Regenerants of Different *Valeriana* Species- a Pharmaceutically Valued Plant

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Abstract: *Valeriana* is an important genus due to its immense medicinal properties. This plant contains over 150-200 chemical constituents, which make it useful as a herbal remedy for various ailments. Conventionally, these plant species are cultivated through seeds; however, poor seed setting coupled with low germination rate restricts its cultivation in the wild as well as poses a problem for its cultivation. Due to irregular grazing and excessive harvesting by local people for herbal drugs, the wild population of *Valeriana* species are at a high risk of rapid elimination and extinction. Plant tissue culture is one of the most important methods used for the effective conservation of many rare, endangered and exploited plant species. However, the induction of genetic variability in regenerants may limit the purpose of micropropagation. Assessing the clonal fidelity of *in vitro* derived regenerants is highly essential to know whether plants are true to type or not. The development and utilization of molecular markers for the identification of plant genetic diversity is one of the most important progresses in the field of molecular genetics studies. Molecular markers are a prevalent tool, due to their stability, cost-effectiveness and ease of use for a variety of applications in the field of molecular genetics. Several molecular markers have been efficaciously employed to evaluate the clonal fidelity of the *Valeriana* clones so that only the elite, genetically identical plants are propagated. This chapter highlights the biology, pharmacology, need for micropropagation and application of DNA molecular markers in clonal fidelity assessment of the *in vitro* propagated *Valeriana* species.

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Keywords: Molecular markers, Genetic stability, Chemical constituents, Genetic variability, Clonal fidelity, Molecular genetics, Micropropagation.

INTRODUCTION TO VALERIAN SPECIES

Classification

Kingdom- Plantae

Phylum-Tracheophyta

Class-Magnoliopsida

Order-Dipsacales

Family- Caprifoliaceae

Genus-*Valeriana*

Botanical Description

Valeriana, a major genus, belongs to the Valerianaceae, a family present in all the temperate and sub-tropical regions of the world. Name '*Valeriana'* might be acquired from the Latin word 'valeo', which referred 'to be strong'. Members of this family are mainly herbs, rarely shrubs, having opposite and exstipulate leaves. Flowers are small, arranged in cyme units consisting of 1-4 epipetalous stamens, while gynoecium is represented by three carpels. Ovary is inferior and unilocular with one apical ovule. The fruit is an achene, with a pappus like calyx in some plants. The plant has a distinctive odour which has been described as that of dirty socks or an unpleasant camphorous smell. Others say it is nauseous, and for this reason, it used to be called 'Phu', which meanings the strong feeling of disgust due to the smell arising from long-dried valerian root.

DIVERSITY IN VALERIANA

The genus *Valeriana* consists of about 350 annual and perennial herbs species, which are widely distributed worldwide, usually at high altitudes. Genus *Valeriana* is best known for *Valeriana officinalis,* commonly called valerian. *V. officinalis* originated in Eurasia and naturalised in North America. Traditionally, its roots have been utilized in promoting sleep and possess sedative, anxiolytic and antispasmodic activities [1, 2]. Apart from *V. officinalis*, other commonly studied species include *Valeriana fauriei*, *V. edulis*, *V. wallichii* and *V. hardwickii*. Strachey [3] reported five *Valeriana* species in India, *viz. V. jatamansi* DC., *V. dioica* L., *V. stracheyi*, *V. pyrolaefolia*, and *V. hardwickii*, growing at an

altitude str*etc*hing from 1500-4300m. The most widely used species in India is *V. jatamansi* (synonym *V. wallichii*), commonly called Indian valerian.

CHEMISTRY OF VALERIANA

This plant comprises over 150-200 chemical components (some of which are listed in Table **1**) together with essential oils and their sesquiterpenoid derivatives (valerenic acids), epoxy iridoid esters (valepotriates), alkaloids, flavonoids and lignans which are mostly obtained from root and rhizome [4]. Valerenic acids and valepotriates are the main active ingredients in the commercial and medicinal preparation of valerian, and in many countries, its crude extract is used on a larger scale [5].

Table 1. Chemical constituents found in different *Valeriana species*.

S. No.	Chemical Constituents	References
1.	Sesquiterpenoid derivatives (valerenic acids), epoxy iridoid esters (valepotriates), alkaloids, flavonoids and lignans	[4]
2.	Hydroxypinoresinol	[5]
3.	Bornyl acetate and bornyl, isovalerate, camphene, valerenal, Valerianol	[6]
4.	Bornyl acetate and bornyl, isovalerate	[7]
5.	Camphene, pacifigorgiol, ledol, chlorogenic acids, sitosterol, tannins, choline	[8]
6.	Camphene, valerenol	[8, 9]
7.	Valerianol, bisabolene, caryophyllene,	[9]
8.	Valeranone, valerenal	[9, 10]
9.	Isovaleric and hydroxyisovaleric acids	[10, 11]
10.	Linarin	[17]
11.	Methylapigenin, hesperidin	[17, 18]
12.	Chantinine, valerine, valerianine, actinidine, methyl-2-pyrrole ketone	[19 - 22]

The essential oil of *Valeriana* consists of 150 constituents, including mono-terpenes, sesquiterpenes, and less volatile sesquiterpenic carboxylic acids. Borneol [6, 7], mainly in the form of ester viz. bornyl acetate and bornylisovalerate, camphene, á and â-pinene are the major monoterpenes found in essential oils of *V. officinalis* [6 - 9]. Sesquiterpenes include valeranone [9, 10],valerenal [6, 9, 10], valerenol [8, 9], valerianol [6, 9] *etc*. Isovaleric and hydroxyisovaleric acids are produced while drying and storing the plant which gives the valerian its characteristic unpleasant aroma, that is not present in the fresh plant [10, 11].

CHAPTER 4

Genetic Variation and Genetical Control of Growth and Flowering in Woody Plants: Molecular Techniques

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Abstract: A genetically modified (GM) poplar (*Populusnigra*) that overexpresses a key enzyme in the plant hormone gibberellin (GA) synthesis system was produced by the *Agrobacterium* method. Gibberellin is known to control the elongation and growth of higher plants. GA20-oxidase is a key enzyme in the biosynthesis of active GAs. The major gibberellin biosynthetic pathway involving the participation of GA20-oxidase is shown and described along with other genes and enzymes like GA20ox, GA3ox, GA 3beta-hydroxylase, GA2ox, and GA 2beta-hydroxylase. There are six clades in the GA oxidase gene subfamily. In this chapter, the production of genetically modified (GM) poplar overexpressing a known tobacco GA 20-oxidase gene, *NtGA20ox* and a poplar gene *PnGA20ox,* classified under the same GA 20-oxidase gene, is described. This genetic variant and recombinant poplar showed enhanced above-ground growth and stem biomass production. In addition, GM poplars with altered expression of genes controlling flower bud formation. This successfully reduced the long juvenile phase period to less than one year. Genetic modification has made it possible to control tree growth and reproduction.

Keywords: Biomass production, Floral initiation, Flowering, GA 20-oxidase, Genetic transformation, Genetically-modified (GM), Gibberellin, Growth, Poplar, TERMINAL FLOWER 1 (TFL1), Woody plant.

INTRODUCTION

Following the release of the Climate Change Report [1] and the conclusions of the 26th UN Climate Change Conference of the Parties (COP26) [2], we are required to take more actions to combat climate change and reduce CO_2 emissions than ever before. And Goal 13 of the SDGs also includes the promotion of afforestation, forest protection, and a shift to clean energy to achieve a carbon-

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neutral, decarbonized society [3]. The achievement of BECCS (Bioenergy with Carbon Capture and Storage) [4], in which bioenergy is extracted from biomass and its carbon is captured and stored, is one of the objectives of trait modification of woody plants. Furthermore, efficient control of flower bud formation is also necessary to prevent the spread of recombinant genes and to shorten the breeding period of woody plants.

In this chapter, the increase of biomass production of woody plants using molecular techniques [5] and techniques to control the flowering of woody plants [6], incorporating actual examples, are introduced.

GENETICALLY-MODIFIED POPLAR OVEREXPRESSING THE GENE FOR GIBBERELLIN 20-OXIDASE

To increase the biomass production of woody plants, we have focused on the plant hormone gibberellin (GA), which is known to control the elongation and growth of higher plants [7, 8]. The biosynthetic pathways of the major active forms of GA have already been elucidated, and enzyme genes on these pathways have been isolated from many plant species [7, 9, 10]. GA is a tricyclic diterpenoic acid synthesized from geranylgeranyl diphosphate, a 20-carbon linear isoprenoid. More than 130 natural products with this structure have been identified [8]. Although a detailed description is omitted, a 19-carbon GA is synthesized by oxidase from a 20-carbon GA, and some of them have physiological activity in higher plants Fig. (**1**). The major active GAs, GA_1 and GA_4, are produced by the hydroxylation of the 3-position of GA_{20} and GA_9, precursors formed by GA20-oxidase [7, 11] Fig. (**1**). GA20-oxidase is a key enzyme in the biosynthesis of active GAs, since the amounts of GA_{20} and GA_1 were significantly reduced in recombinant tobacco (*Nicotianatobacum*) in which the expression of the GA20-oxidase gene has been suppressed [12].

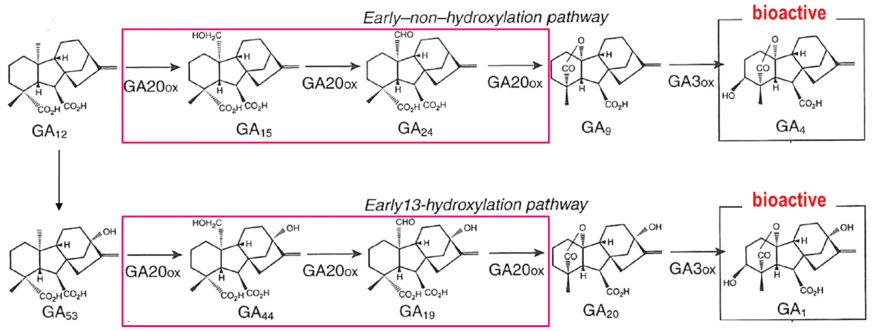

Fig. (1). The major active gibberellin (GA) biosynthetic pathway in higher plants. In this study, we used GA20ox genes from tobacco and poplar. The reaction catalyzed by GA20-oxidase is shown in the red box.GA20ox, GA 20-oxidase; GA3ox, GA 3beta-hydroxylase; GA2ox, GA 2beta-hydroxylase.

The 2-oxoglutarate-dependent dioxygenase gene family, into which the GA20-oxidase gene enters, consists of a number of genes, including the 1-aminocyclopropane-1-carboxylate oxidase (ACCox) gene involved in the synthesis of ethylene, another plant hormone [13] (Fig. **2**). There are six clades in the GA oxidase gene subfamily. A group of enzymes that oxidize the 20th carbon of a 20-carbon GA to a 19-carbon GA; a group of enzymes that oxidize the 20th carbon of a 20-carbon GA but do not reduce the carbon number; a group of enzymes that are considered GA oxidases but whose function is unknown; a group of enzymes that oxidize the 2nd carbon of a 20-carbon GA; a group of enzymes that oxidize the 3rd carbon of a GA, and a group of enzymes that oxidize the 2nd carbon of a 19-carbon GA [13] (Fig. **2**).

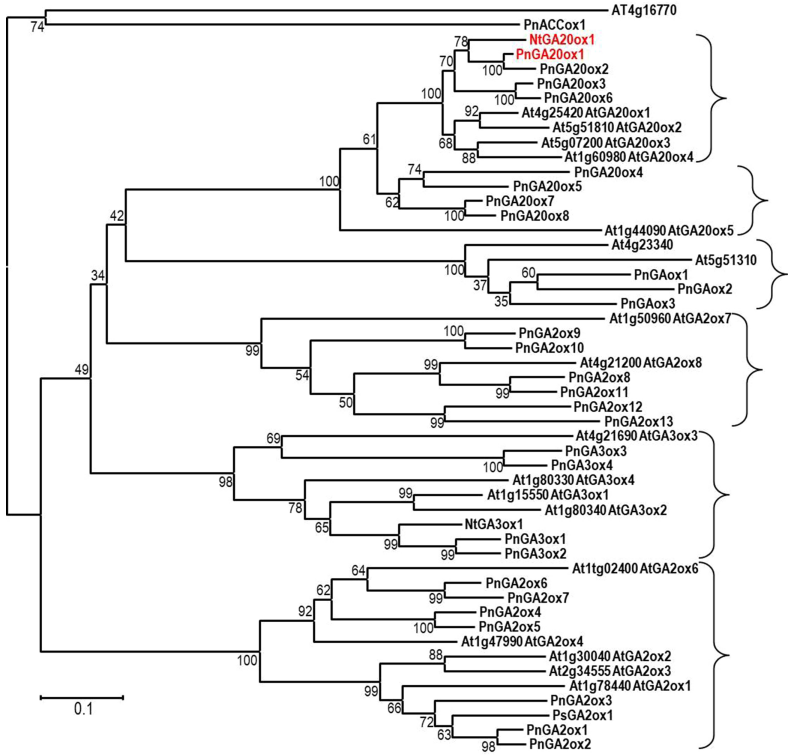

Fig. (2). Phylogenetic analysis of the members in GA oxidase. The tree was constructed by the Neighbor-Joining (N-J) method for members of this family in Lombardy poplar, Arabidopsis (*Arabidopsis thaliana*), tobacco and pea (*Pisumsativum*). The N-J unrooted dendrograms were generated from the alignment of deduced amino acids with the ClustalW program, and the phylogenetic tree was displayed by the MEGA4 software package. The 1-aminocyclopropane-1-carboxylate oxidase (ACCox) gene of poplar and *Arabidopsis thaliana* was used as a gene outside the GA oxidase subfamily of 2-oxoglutarate-dependent dioxygenase family genes. Bootstrap values for 100 resamplings are shown on each branch. The scale indicates the average number of substitutions per site. The red figures indicate the GA 20-oxidase genes of tobacco and poplar, which are the materials used in this study.

CHAPTER 5

Somatic Embryogenesis and Genetic Homogeneity Assessment in *Coffea* - Recent Approaches

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Abstract: Coffee is one of the most preferred beverages consumed by millions of people throughout the world. It is cultivated in more than 80 countries in tropical and subtropical zones of Asia, Africa, and Latin America and provides livelihood to 125 million people worldwide. Among 125 coffee species known so far, only two coffee species, *Coffea arabica* (arabica coffee) and *Coffea canephora* (robusta coffee), are commercially cultivated for beverage production. Coffee is a perennial plant and therefore subjected to constant environmental stress. However, during the last few decades, sustainable coffee cultivation has been threatened by unprecedented climate change. This calls for unified efforts, including cutting-edge research and modified management practices. Although conventional breeding efforts have been developed to address some issues, emerging biotechnology research, especially *in-vitro* propagation technology, could augment the coffee cultivation landscape. Despite the tangible progress made in coffee tissue culture, there were some grey areas, such as the level of somaclonal variation and the genomic changes associated with somatic embryogenesis in coffee, which needs to be addressed imminently. This chapter provides detailed progress on coffee tissue culture and addresses some of the critical issues associated with the genetic homogeneity of tissue culture plants.

Keywords: Coffee, Micropropagation, Somatic embryogenesis, Field evaluation, Genetic fidelity, Molecular markers, Functional genes sequencing.

INTRODUCTION

Coffee is the most preferred beverage consumed by millions of people throughout the world. It is estimated that over 2.25 billion cups of coffee are consumed by people every day, thus making it one of the most essential commodities in world trade. Coffee is a perennial tropical crop belonging to the genus *Coffea* of the

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family Rubiaceae. The genus *Coffea* comprises more than 125 species, of which only two species, *Coffea arabica* (*arabica coffee*) and *C. canephora* (*robusta coffee*), are commercially cultivated for beverage production [1, 2]. More than 80 countries predominantly situated in the tropical and sub-tropical climatic zones in Asia, Africa, and Latin America are engaged in the cultivation and export of coffee [3]. India is one of the world's top 10 coffee-producing countries producing 369000 MT of green coffee during the year 2021-22. Further, the economy of several coffee-growing countries depends heavily on coffee production and export earnings; therefore, many people are directly and indirectly involved in the coffee supply chain for their livelihood.

A majority of the coffee species discovered to date have originated in tropical forests of Africa, Madagascar, and Mascarene islands, while some of the species are found in the Indian subcontinent, Southeast Asia, and tropical Australiasia [1, 4, 5]. Among all the coffee species known, *C. arabica* is the only self-fertile allotetraploid (2n=4x=44) cultivated species. Arabica coffee is thought to be originated in the highlands of southwestern Ethiopia and the Boma Plateau of South Sudan. Further, a wild population of Arabica were also discovered on Mount Marsabit forest in Kenya [6]. The first record of dissemination of *C. arabica* dates back to the 8th century when some seeds were taken from Southern Ethiopia to Yemen and cultivated there by the Arab people till the end of the 14th century [7]. Arabica coffee was introduced to India, Java, Sri Lanka, and Indonesia during the 16th century, and during the early seventeen century, it spread to Europe by the Dutch trader who planted it in the Amsterdam Botanical garden. Currently, all the arabica coffee cultivars grown worldwide are derived from two genetic variants, *i.e.,* Typica and Bourbon and as a consequence of this, most of the cultivated Arabica cultivars have a narrow genetic base [8, 9]. Due to low genetic diversity, arabica coffee is most vulnerable to climate change.

Besides *C. arabica*, *C. canephora* is another cultivated species that contributes about 40% of world coffee production. *C. canephora* is a diploid (2n=2x=22) self-incompatible species believed to be originated from the low land tropical forest of the Democratic Republic of Congo in Africa and further stretched till West African Cameroon on one side and extends up to Northern Angola, passing through Uganda and Northern Tanzania [10]. Robusta coffee was introduced to India from Java during 1903-06 [2]. Currently, India produces 348500 MT of green coffee, of which Robusta accounts for 249500 MT (71.6%). In 2021, India earned more than 949.84 million US dollars by exporting 395655 tonnes of coffee to different countries.

TRADITIONAL BREEDING AND LIMITATIONS

Genetic improvement of coffee has a long history, being initiated about 100 years ago and centres around both *C. arabica* and *C. canephora*, which dominate world coffee production. The main objectives of arabica coffee breeding programs are to enhance yield per unit area and leaf rust resistance. However, for the last 30 years, other objectives have been pursued, such as imparting tolerance to drought and pests, improving organoleptic quality and developing climate resilient coffee varieties. Unlike arabica, the genetic improvement of robusta coffee has been largely neglected until the 1950s, but recently genetic improvement of robusta has gained acceleration in many countries due to the impact of climate change.

For several decades, traditional breeding techniques have been used for the genetic improvement of coffee all over the world. Due to these efforts, several improved coffee cultivars have been developed for commercial cultivation. However, coffee, a perennial crop, is continuously subjected to biotic and abiotic stress, especially the environmental degradation and the co-evolution of plant pathogens due to unprecedented climate change. The emergence of new variant of plant pathogens due to climate change quickly breakdown the defence mechanism in the tolerant coffee cultivars. Because of this, there is a continuous demand for improved cultivars at regular intervals for commercial cultivation. However, coffee breeding using the traditional method is an arduous, time-consuming process involving several critical steps, including selecting parental material, hybridization and progeny evaluation [2]. It is estimated that a minimum of 20-25 years is necessary to develop a new cultivar using conventional breeding methods. Besides the long evaluation period, ploidy barriers between arabica and robusta and other diploid species, incompatibility, constraints of field trial involving high cost and labour and unbridled transfer of genetic traits to hybrids are the major bottlenecks associated with conventional coffee breeding. Further, some genetic traits such as resistance to coffee berry borer (*Hypothenemous hampei*), tolerance to frost, cold, and drought *etc.,* are either unavailable in the coffee gene pool or difficult to transfer using traditional breeding techniques. All these limitations call for the application of biotechnology and traditional breeding for accomplishing the tasks of coffee improvement that is impossible using conventional breeding alone. Among several biotechnological methods used for crop improvement, *in-vitro* multiplication/ tissue culture technique is most important as several techniques like mass production of elite plants, transfer of heterologous genes from various sources, and evaluation of promoters and genes can only be achieved by successful development of this method.

CHAPTER 6

Cellular and Molecular Tools for the Investigation of Somatic Embryogenesis in Medicago Species

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Abstract: The chapter presents the knowledge accumulated on the recent investigation of somatic embryogenesis (SE) in genera *Medicago*. The role of 2,4-D in the process of induction of embryogenic potential in diploid *Medicago* and its transport by the combined action of auxin transporters or diffusion of dissociated molecules is discussed. Among the many methods for studying the process, this chapter is focused on cellular and molecular tools – flow cytometry, assessment of expression level of SE related transcripts of key genes of auxin inducible process and different PCR techniques. Our recent studies on the process of SE in *M. truncatula* are focused on the role of the two genes *MtLAX3* (an auxin transmembrane transporter) and a transcriptional factor *MtARF-B3* (an auxin response factor, containing a B3-binding domain). The transcription profiles of these genes are evaluated and their expression patterns are assessed during indirect somatic embryogenesis – steps of callus formation, embryogenic zone formation and the stages of globular, torpedo and cotyledonary embryos. The localization of expression during the process of SE is traced by the β-glucuronidase reporter gene (*GUS*) under the control of the promoters of these genes. Inverse PCR (IPCR) and Transposon display (TD) are techniques which evaluate transposition and new retrotransposon copies in the investigated mutant lines, and we used these methods as markers for the efficiency of the induction phase of the process of SE. The use of all these methods turns light on a better understanding of the process of somatic embryogenesis in the model species *Medicago truncatula* and other annual medics.

Keywords: Somatic embryogenesis, genus Medicago, Flowcytometry, Transcript profiles, PCR-techniques.

INTRODUCTION

Plant cells possess the unique ability to divide and give rise to a new individual. This remarkable ability is totipotency and is the base of plant cell and tissue cultures. *In vitro* regeneration takes place in two main ways – organogenesis and

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somatic embryogenesis (SE). Organogenesis is characterized by the development of lateral buds, which are connected with explant tissue and their further development to vegetative shoots. The induced somatic embryos possess a bipolar structure with well defined-root and shoot meristem, they do not have a vascular connection with the mother tissue and, in most cases, develop from a single somatic cell. Somatic embryos follow the stages of development of zygotic embryo- globular, heart, torpedo, and cotyledonary [1, 2]. Two main types of *in vitro* SE are established among plant species: direct and indirect (ISE).In the case of direct SE- the somatic embryos are induced from the plant tissue without the formation of a callus, while in indirect SE, the formation of an embryogenic callus precedes the appearance of somatic embryos. One of the pioneer researchers of SE, Stuart (1964) [3], determines three main stages in the process of induction of SE: induction of cell division; acquisition of embryogenic potential; expression of embryogenic program. The last 25 years of work on the regeneration of annual species from genera *Medicago* led to the establishment of a number of protocols for direct and indirect somatic embryogenesis [4 - 8].Together with the development of protocols for direct and indirect somatic embryogenesis of diploid medics, our research also is focused on the selection of tools for understanding the process, as well as for evaluating its effectiveness. Over the years, different tools have been used in order to study first cell division, the efficiency of induction of somatic embryos and their development and conversion. These tools are based on cellular and molecular techniques. Among cellular techniques, confocal microscopy observation on single cell suspension culture and flow cytometry which estimates DNA nuclear content and genome size, are two elements that could be used for evaluation of the efficiency of induction of the SE process. Among molecular techniques, the transcript level of SE related transcripts of key components of auxin inducible process is the tool which turns light on the inside process. Inverse PCR (IPCR) and Transposon display (TD) techniques which evaluate transposition and new retrotransposon copies in the investigated plant could also serve as an estimate for the efficiency of the induction phase of the process of SE.

ROLE OF AUXIN 2,4D IN THE PROCESSES OF INDUCTION AND DEVELOPMENT OF THE SOMATIC EMBRYO

Numerous studies in different species are focused on the involvement of plant hormones in the induction of SE. Among diploid *Medicago*, the external stimuli that allow the induction of an embryogenic pathway are the plant growth regulators - auxins and cytokinins. They are able to reactivate the cell cycle and trigger a series of cell divisions. In most of the cases, the use of high-concentration auxin and low-concentration cytokinin is a key combination able to induce embryogenic potential of plant cells [9 - 14, 8]. The synthetic auxin 2,4-D,

which possesses an embryogenic effect, has been investigated in genera *Medicago* for more than 40 years [15 - 19]. In plants, the auxin is accumulated in the intercellular space in high concentration, which causes embryo induction and formation. This plant hormone act as a signaling molecule and is involved in many processes - cell division, differentiation, cell and root elongation, apical dominance and response to various types of tropism related to plant development [20 - 22]. The auxin is also essential for the process of zygote embryogenesis [23]. The data accumulated from research on auxin biosynthesis, metabolism, transport and development of somatic embryos confirm its major role during induction, formation and subsequent development of somatic embryos [24, 25]. Endogenous auxin is synthesized mainly in young leaves and apical meristems of shoots together with exogenously added PGRs [26, 27] entering the cells by the combined action of auxin transporters or diffusion of dissociated molecule and distributed from the top to the roots [26, 28, 29]. From young leaves and flowers to the roots, auxin is transported *via* long-distance unregulated flow through the phloem [30, 31]. From cell to cell, auxin moves by forming an auxin gradient. Its distribution is due to the combined action coordinated by importing and exporting auxin transporters in the plant cell and this mechanism is known as polar auxin transport-PAT [32 - 35]. This PAT in plant tissues is unique only for auxin and has never been found for other signaling molecules [36]. The transporters for auxin import and export are asymmetrically located on the cell membrane and their interaction is a main requirement for maintaining the PAT [37, 38]. In proto-phloem cells, the auxin transporters, *AUX1/LAX3* importers and *PIN1* exporters are located at opposite ends of each cell. In the model plant *Medicago truncatula*, the asymmetry of the first cell division is an early event of somatic embryogenesis and is caused by exogenously applied auxin and is confirmed in various studies [18, 39]. The auxin enters the plant cell by transmembrane auxin transporter (*LAX3*). 2,4-D is a synthetic auxin analog, mainly used as a herbicide, which is absorbed through the leaves and is translocated into the plants [40, 41]. About 75% of 2,4-D requires an influx carrier *LAX* transporter to enter the cell when its concentration outside the cell is low [42].

Transcript Level of Key Genes of Auxin Inducible Process

Our recent studies on the process of SE in the model legume species *M. truncatula* are focused on the role of the two genes: one encoding an Auxin influx carrier transmembrane transporter (*MT3G072870*, Plaza 2.5, *MtLAX3*) and one transcriptional factor an auxin response factor, containing B3-binding domains (*Mt5g040880*, PLAZA 3.0 Dicots, *MtARF-B3*) [43, 44]. These genes are initially identified by a reverse genetic approach in a population of *Tnt*1 retrotransposon-tagged mutants of *M. truncatula* [45]. The promoters are cloned, and transcriptional reporters and transgenic plants are used to assess the expression of

CHAPTER 7

Flow Cytometry Analysis of *In Vitro* Induced Polyploidy in Plants

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Abstract: Polyploidy is the condition of having more than two sets of chromosomes. The mechanism of polyploidy helps in deriving special traits like an increase in biomass, an increase in the size of various organ systems, and secondary metabolite content for the progeny. Various chemical compounds (colchicine, trifluralin, and oryzalin) that have the capacity to alter the mitotic cycle were used for the purpose of inducing polyploidy. Various techniques, such as counting of chromosome number, chloroplast number, determination of pollen diameter, and estimation of leaf stomatal density and size, were developed to analyze the polyploidy of the plants. However, these methods are not reliable for their regular use. Thus, of all the above-mentioned approaches, the estimation of ploidy level by flow cytometry (FCM) has been the most popular over the last few decades. Flow cytometry is now extensively used for the verification of haploidy, aneuploidy, and polyploidy. The ease of sample preparation, fast acquisition, and accurate measurements have made the method popular in the domains of plant cell biology, systematics, evolution, genetics, and biotechnology. The current chapter discusses the induction of polyploidy and its importance in plant breeding. It also emphasizes the importance of FCM in the analysis of polyploidy and enumerates the various polyploidy studies involving the application of FCM.

Keywords: Ploidy analysis, *In vitro* polyploidy, Colchicine, Flow cytometry, Fluorescence.

INTRODUCTION

Science has been crucial in helping people to unravel the reasons for the changes happening in nature since the dawn of time. From the evolutionary point of view,

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living organisms have undergone loads of changes phenotypically and genotypically and have adapted to the harsh environment to become successful species. The effective establishment of species has been made possible by a variety of mechanisms happening at the genetic level.

POLYPLOIDY

The heritable state of having more than two full sets of chromosomes is known as polyploidy. Polyploidy can be seen in all living organisms but is very much prevalent in plants and helps in creating new species as an evolutionary event. The anatomy and flexibility of plants help in accommodating any morphological changes happening because of polyploidy. The mutation theory of Hugo de Vries gave the initial idea of polyploidy, which was investigated in *Oenothera lamarckiana*. Later studies by Stebbins [1] and Grant provided support for the concept of polyploidization, and it is seen that more than 40% of angiospermic plants are polyploid in nature, which ultimately helped in speciation [2]. Masterson compared the leaf guard cells from fossil flora and the present from the families of Lauraceae. He studied Magnoliaceae and Platanaceae and found that the size of guard cells had been considerably increased, and he estimated that 70% of angiosperms had undergone the phenomenon of polyploidy [3].

When there is damage or unplanned meiotic or mitotic divisions occur, gametes with a greater number of sets of chromosomes emerge, which fuse and eventually aid in the production of polyploids. When a rare diploid gamete fuses with a conventional haploid gamete, triploid zygotes will emerge, which will usually be sterile or try to form polyploid gametes. In another instance, if two diploid gametes fuse, they will also lead to the formation of polyploid individuals [4]. So, studies reveal that, on a broader aspect, there are two types of polyploidy, *i.e.,* autopolyploidy and allopolyploidy. Stebbins noted that autopolyploid individuals arise within or across populations of a single species, whereas allopolyploid individuals are hybrids [1].

There are multiple advantages for a plant if it is becoming a polyploid in nature. The mechanism of heterosis (hybrid vigor) makes polyploid plants into elite hybrids with an exceptional phenotype. In allopolyploidy individuals, the forced pairing of homologous chromosomes precludes intergenomic recombination, successfully preserving the same degree of heterozygosity throughout the generations [4]. The masking of recessive traits by dominant wild-type genes is one advantage offered by gene redundancy, which ultimately comes from polyploidy. Polyploidy has also been found to aid in self fertilization or asexual reproduction, eventually disrupting incompatibility [5].

benefits plants in a variety of ways, induction at various stages enables us to produce plants with specialized features.

INDUCTION OF POLYPLOIDY IN PLANTS

Artificial modes of inducing polyploidy in plants make them able to overcome natural selection, and, usually, they will have exceptional traits that will ultimately help the breeders economically. There are various types of polyploidy inducing agents like colchicine ($C_{22}H_{25}NO_6$), trifluralin ($C_{13}H_{16}F_3N_3O_4$), and oryzalin ($C_{12}H_{18}N_4O_6S$). These chemical substances act as anti-mitotic factors by playing a major role in the depolymerization of microtubules and spindle inhibition in cell division [6].

The induction of polyploidy usually depends on various parameters:

- Characteristics of the explant and their genotype conditions.
- Nature of the polyploidy inducing chemical agent.
- Minimum effective concentration of polyploidy inducing chemical agent.
- Time and method of exposure.

The efficacy of polyploidy induction varies depending on the chemical agent. Among all the chemical agents used, colchicine seems to be very efficient in inducing polyploidy. Various studies have proved their efficiency in improving the traits of exposed individuals when compared to conventional diploid individuals (Fig. **1**). Initially, scientists tried using chemicals like phenylurethane and acenaphthene for inducing polyploids. It is seen that acenaphthene showed no response in polyploidy induction, whereas phenylurethane showed some effect on datura seeds [7]. These chemical agents, depending on the amount of treatment, might be harmful to the cells, causing polyploidy. The chemical combination of amiprophos methyl, pronamide, dimethyl sulfoxide, and nitrous oxide also seems to be playing a similar role to that of colchicine [8]. Colchicine is one of the most accepted and successful antimitotic agents, which is used for inducing polyploidy. In medicinal plants, colchicine at optimal concentrations helped in deriving the tetraploid and mixoploids, and it was also observed that the secondary metabolite yield with respect to biomass also improved [9]; [10]. Colchicine also showed a positive response in deriving tetraploids from ornamental plants and ultimately helped in deriving improved traits with respect to leaf and flower size [11]. The optimal concentration of chemicals for inducing polyploidy is mentioned in Table **1**.

CHAPTER 8

Genetic Fidelity Assessment of Micropropagated Woody Plants Through Molecular Analysis

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Abstract: Somaclonal variation is generally undesirable in woody plant tissue cultures when the main aim is *In Vitro* micropropagation or transformation of selected material, however, it could sometimes be useful for the production of new and valuable varieties. Thus, the determination of somaclonal variation is very important for the genetic fidelity of the micropropagated woody plant species. Molecular markers are generally used in the identification of plant species, analysis of qualitative and quantitative trait loci, determination of the genetic distance between genotypes, detection of stable, high yielding and qualified varieties for variety registration and certification. Moreover, molecular markers are also very useful for the evaluation of the genetic fidelity of micropropagated cultures. Among many markers, ISSRs, SSRs, AFLPs and MSAPs are found to be very efficient for the assessment of genetic stability of micropropagated different woody plants since they are easy to apply, quick to use, and more reliable due to their efficiency and repeatability. In this context, the aim of the present book chapter is to review the advantages of molecular markers together with the summarization of the studies on the determination of genetic stability of micropropagated woody species using this technique in the last decade and causes of somaclonal variation.

Keywords: *In Vitro* propagation, Molecular marker, PCR, Somaclonal variation, Woody plants.

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INTRODUCTION

In Vitro regeneration of somatic cells occurs by the mitotic division that refers to asexual reproduction that enables clonal propagation. However, unexpected and uncontrollable variations also occur in these techniques. Such variations can occur naturally or artificially. These are expressed as either stable or temporary changes in cells and tissues [1]. Variations are not desired in studies conducted with the aim of micropropagation. Therefore, it is of great importance to use the most reliable tissue culture techniques in micropropagation. Nevertheless, whatever technique is used, there is always the possibility of the occurrence of genetic change during micropropagation. On the contrary, these phenotypic and genotypic variations, which are undesirable in micropropagation, are of great importance in plant breeding since they could generate valuable variants where the natural variation is narrowed or difficult to create. Thus, these variations provide new sources in plant breeding and all of these hereditary changes that occur in tissue cultures are defined as 'somaclonal variation' [2 - 4].

Somaclonal variations both contain genetic and epigenetic changes. The variation of DNA sequences due to insertions, deletions, *etc.* is regarded as genetic variation, while epigenetic changes cause the alteration of gene expression in cells. Epigenetic changes occur in nature due to environmental stress, and they are not always inherited [5, 6]. When somaclonal and epigenetic mutations are compared, it is concluded that somaclonal alteration is hereditary and epigenetic variation cannot be transmitted to offspring. Also, epigenetic variation is reversible throughout plant life, while somaclonal variation is not. Another difference is that somaclonal alteration emerges mostly in species consisting of extrinsic meristems, whereas epigenetic alteration can be seen in these species and plants consisting of apical/axillary meristems [7, 8].

The most reliable methods of avoiding the genetic changes that occur in micropropagation are direct shoot formation, direct embryogenesis, and node and shoot cultures. The variations seen in exile cultures are probably due to sudden mutations. Whether they are genotype or epigenetic, those variations cannot be determined without careful analysis [9, 10]. Genetically different plants obtained from shoot cultures depend on shoot formation from buds that occurred from basal callus rather than axillary bud. Plant regeneration occurs from somatic cells because the cells are totipotent. A cell with this ability produces an undifferentiated mass of cells and then differentiates again to form fully organized individuals. However, variations could occur in such a method due to generated stress conditions of *In Vitro* cultures, and the degree of variation thus varies

according to the utilized tissue culture technique. For instance, variations are less common in direct organogenesis or somatic embryogenesis and more common in plants obtained from callus cultures [11, 12].

Somaclonal variation can be determined by phenotypic, cytological and molecular studies. The magnitude of somaclonal alteration is generally identified as the proportion of species showing one or more qualitative deviations [13, 14]. The value of somaclonal alteration is identified by comparing the data of the quantitative traits with the control and the standard deviation values for particular properties in the somaclonal population. Phenotypic variations could be observed with the determination of morphological differences in regenerated plants (Fig. **1a-c**), while cytological differences could be detected by counting the chromosome numbers in mitotic cells, measuring the DNA content of each cell, determining the number of chloroplasts in stomatic protect cells or measuring the stomatic length [15]. Chromosome number changes are the earliest genetic variations seen in *In Vitro* cultures and in species obtained from *In Vitro* cultures. There are studies reported in the literature on the genetic variation degree in callus tissues and regenerating plants species with the determination of chromosome number due to genotype, medium composition, tissue type, and culture length [16, 17].

Fig. (1). Healty micro-shoots of sweetgum (*Liquidamber orientalis* L.) after the first subculture after *In Vitro* culture initiation (**a**); Phenotypic variation in the microshoots after the sixth subculture (**b**). Phenotypic variation in the microshoots after the twelfth subculture (**c**); bars 1cm.

CHAPTER 9

Genetic Stability in Micropropagated Orchids: Assessment by Molecular Markers and Flow Cytometry

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Abstract: Orchids occupy a significant position in the international floricultural market because of their spectacularly beautiful flowers with varied sizes, forms, patterns, and colorations. Apart from their high ornamental values, they are known for therapeutic application in the traditional medicinal system. However, natural orchid resources are quickly depleting because of excessive unregulated commercial collection and mass habitat destruction. Orchid production through conventional propagation methods cannot meet the present demands for these ornamental plants. Micropropagation of orchids through plant tissue culture provides an excellent opportunity to propagate true-to-type quality plants on a large scale rapidly. However, somaclonal variation may appear in the *in vitro* clones producing undesired plants with phenotypic and molecular defects. It is obligatory to test the genetic integrity of the propagated plants to ensure the production of identical quality orchids. Genetic stable orchids are produced by evaluating the fidelity of the regenerants using molecular markers. The present chapter highlights the genetic stability assessment of several micropropagated orchids using molecular markers and the flow cytometry method.

Keywords: Explants, Flow cytometry, Genetic stability, Molecular markers, Monomorphic, Micropropagation, Orchids, Polyploidy, Polymorphism, Soma-clonal variation.

INTRODUCTION

Orchids are incredibly stunning ornamental plants belonging to Orchidaceae, having more than 25,000 species, innumerable hybrids, and varieties [1, 2]. The

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floricultural appeal of the plants is remarkably high as they are besotted with spectacular flowers with an extensive range of floral shapes, sizes, fragrances, and colorations [3, 4]. The orchids, with their variedly colored attractive flowers, are considered highly expensive ornamentals and fetch a great price in the international floriculture market. Apart from their ornamental values, they also possess therapeutic properties due to the rich contents of beneficial phytochemicals [5]. The therapeutic application of orchids in the traditional medicinal system to treat local ailments has been extensively found in many Asian countries, some parts of Europe, America, Australia, and Africa [6]. The population of orchids, despite its rich natural resources, has been diminishing at an astounding rate because of unregulated commercial collection, deforestation, and massive habitat destruction. The situation of population depletion is so alarming that the entire family is now enlisted in Appendix-II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [7]. Orchid production through vegetative and seed culture methods cannot suffice the current requirement. Vegetative propagation through cutting, shoot, and clump division has several limitations as they are slow, time-consuming, and labor-intensive processes. The natural seed culture method has a significant setback as the tiny exalbuminous light orchid seeds have a very low germination rate (0.2 to 0.3%) in nature because of the lack of endosperms and the requirement of appropriate mycorrhizal fungal association [8, 9]. Orchid propagation through plant tissue culture can overcome the drawbacks of conventional methods by rapidly mass-producing quality plants. However, the *in vitro* propagated orchids should be adequately evaluated for genetic stability so that they are not associated with molecular and phenotypic defects. Somaclonal variation may appear in the regenerants as they are constantly exposed to different pressures during *in vitro* culture [10]. The genetic integrity of the clones can be effectively assessed by employing molecular markers and the flow cytometry method. RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeat), SCoT (start codon targeted polymorphism), and IRAP (inter-retrotransposon amplified polymorphism) markers are some prominent markers employed to ascertain the genetic fidelity of many micropropagated orchids [11 - 14]. Flow cytometry, used to analyze cell DNA content, has also been applied to detect somaclonal variation among *in vitro* clones [15, 16]. The present chapter focuses on the aspects of genetic stability assessment of *in vitro* regenerated orchids using molecular markers and the flow cytometry method.

ORCHID MICROPROPAGATION

Many important factors are considered while performing micropropagation of orchids successfully. The culture media type, hormone concentration, and combinations significantly influence *in vitro* growth response of orchids.

Murshige and Skoog (MS) [17], Mitra (M) [18], Knudson C (KC) [19], Vacin and Went (VW) [20], and Gamborg (B5) media [21] are important media usually employed for orchid culture. Primary media can be modified through variations in the concentration of nutrient components and the incorporation of organic additives to suit the requirements of a particular species. Plant growth regulators may accelerate culture growth with auxins promoting root induction, expansion, and multiplication, while cytokinins increase shoot initiation, proliferation, and plant regeneration [22, 23]. Growth hormones act primarily as enhancers of tissue development, but the adverse effect may be seen at a certain concentration. Chen and Chang [24] reported retardation of embryo formation from leaf explants of *Oncidium* 'Gower Ramsey' in elevated auxin concentration. Tikendra and co-workers [25] also showed induction of somaclonal variation in *Dendrobium fimbriatum* under high cytokinin concentration. The selection of an appropriate explant is crucial for successful orchid micropropagation. Plant parts (seeds, shoot and root tips, leaf, nodal segments, rhizome, pseudo bulb, and inflorescences) may be used as explants for *in vitro* culture initiation. Explant responses may vary depending on their juvenility, position, size, and orientation [26 - 28]. Young tissues are more favored for explant preparation as they have more regeneration ability than differentiated ones [26].The surface sterilization of orchid explants is performed using different sterilants to provide contamination-free cultures. Sodium hypochlorite, mercuric chloride, calcium hypochlorite, and bromine water are commonly used for surface sterilization at varying concentrations and treatment periods. However, selecting proper sterilant concentration and treatment duration is vital as they are toxic to the plant tissues. There must be a balance between reducing infection and survivability of the explant [29, 30].

Orchid Micropropagation from Different Explants

Orchid micropropagation has been performed using different explants. Small, light, and non-endospermic orchid seeds germinate very poorly in nature due to many factors. However, with the successful *in vitro* asymbiotic seed germination of orchids in the KC medium, there are many reports of orchid propagation through seed culture [9, 31 - 34]. The seeds start their germination by swelling, eventually developing into spherical-shaped protocorms with the inherent property of developing into complete seedlings with well-formed leaves and roots (Fig. **1**). *in vitro* seed germination is significantly influenced by media and growth hormone combinations and concentrations [35]. David and coworkers [36] tested 3 basal media of KC, MS, and VW, for their efficiency in seed germination of *Vanda helvola.* KC medium produced the best seed germination, but incorporating organic additives like tomato juice, coconut water, peptone, and yeast extract at different concentrations affected the germination percentage. Supplementation of 15% tomato juice in the medium increased germination, while

CHAPTER 10

Application of Flow Cytometry in Biological Sciences

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Abstract: Flow cytometry is one of the sophisticated tools with its applications in different biological disciplines. It is potentially efficient in the characterization of mixed populations of cells present in biological samples, including blood cells, lymphocytes, microorganisms, sperms, cancer cells, metabolites, antibodies, DNA/RNA content, proteins, toxins, plant spores, *etc*. Flow cytometry is widely applied in the determination of cellular characteristics and cellular components profiling like cell size, intracellular pH, DNA, RNA, proteins, surface receptors, membrane potential, calcium, and others. Currently, flow cytometry is pragmatic in basic as well as applied plant research and plant industrial applications like plant breeding. Flow cytometry has been considered a reliable, rapid, efficient, and accurate tool for analysis of ploidy level and nuclear genome size estimation. It is also subjected to taxonomy to study population/subpopulation dynamics. Gender determination from pollen grain is also possible due to flow cytometry.

Keywords: Apoptosis, Bioprocesses, Cell sorting, Cell viability, Caspase-3, Cytomegalovirus, Cell Cycle, Endoreduplication, Flow cytometry, Genome size estimation, Hematologic Malignancy, *HLA* antibody profiling, Intraspecific variation, Immunophenotyping, *IPEX*, *In vivo* compartmentalization, *LRBA*, Ploidy determination, Primary immunodeficiency, Plant breeding, Sorting pollen, Taxonomy, X- linked agammaglobulinemia, Uncultivated diversity, Wiskott-Aldrich syndrome.

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INTRODUCTION

Flow cytometry is used to measure the properties of cells as they flow in a fluid suspension across an illuminated light path. In this process, a biological sample labelled with fluorescent marker cells that move in a linear stream through a focused light source (laser beam) fluorescent molecule gets activated and emits light that is filtered and detected by sensitive light detectors (usually a photomultiplier tube) conversion of analogue fluorescent signals to digital signals.

Flow cytometry is one of the sophisticated tools with its applications in different biological disciplines, including molecular biology, microbiology, cancer biology, hematology, immunology, biotechnology, genetics, plant sciences, cytogenetics, toxicology, pharmacology, and pathology. Cell size, cell count, cell cycle, and other parameters can all be measured using cytometry. Researchers can obtain incredibly detailed information on individual cells using this method. This makes sure that each cell is examined separately.

FLOW CYTOMETRY

Flow cytometry (FCM) is a sensitive device that simultaneously measures multiple physical characteristics like the shape, size, and granularity of the cell within a suspension. Characteristics are measured while cell suspension flows through the measuring device. Its effectiveness and accuracy depend upon the light scattering mechanism (forward scattering & side scattering), which is obtained by different specific dyes or monoclonal antibodies targeting intracellular components or extracellular antigens present on the surface of the cell. With this method, flow cytometry has emerged as a potent instrument for thorough analysis/investigation of the complex population within a short period of time.

Flow cytometry is one the sophisticated tools with its applications in different biological disciplines, including molecular biology, virology, microbiology, cancer biology, hematology, immunology, biotechnology, genetics, ecology, plant sciences, cytology, cytogenetics, toxicology, pharmacology, embryology and pathology. It is potentially efficient in the characterization of mixed populations of cells present in biological samples, including blood cells, lymphocytes, microorganisms, sperms, cancer cells, metabolites, antibodies, DNA/RNA content, proteins, toxins, plants spores *etc* [1].

This chapter discusses the fundamentals of flow cytometry and a few of its applications in the fields of medicine/medical sciences, immunology, diagnosis, microbiology, biotechnology, food science, and plant science. This chapter offers an introduction to flow cytometry technology that is necessary for all end users,

researchers, and scientists working in biological sciences. It includes applications in relative biological fields that will help in the advanced analysis of biological samples. Additionally, current developments in flow cytometry have been reviewed to provide insight into the potential significance of this technology.

Principle of Flow Cytometry

The basic principle of flow cytometry is the passage of cells in a single file in front of a laser so they can be detected, counted and sorted. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths.

The quantity and kind of cells in a sample can then be determined by measuring the fluorescence. It is possible to analyse up to a thousand particles per second as they move through the liquid stream. A hydrodynamically concentrated stream of fluid carrying the cells is the target of a laser beam. The stream is carefully surrounded by a number of detectors at the moment where the fluid enters the light beam. Forward Scatter, or FSC, is measured using one of these detectors that is positioned directly in front of the light beam. Measurement of side scatter (SSC) is done using a different detector that is positioned perpendicular to the stream. Fluorescent detectors are present because fluorescent labels are employed to identify the various cells or components. The 0.2 to 150m in diameter suspended particles or cells pass across the light beam and scatter the light beams. The laser stimulates the fluorescently marked cell parts, which then emit light with a greater wavelength than the light source. The detectors afterwards pick this up. As a result, the detectors detect both fluorescent and dispersed light. A computer connected to the flow cytometer is then used to examine this data using specialised software. For this detection, the brightness of each detector (one for each fluorescence emission peak) is altered. Different details regarding the physicochemical makeup of the cells can be learned by measuring the light. The SSC typically reflects the inner complexity of the particle, such as its cytoplasmic granule content or nuclear structure, whereas the FSC typically detects the cell volume [2]. Working principle of flow cytometry is demonstrated in Figs. (**1a** & **1b**).

Components of a Flow Cytometry

The three main components of a flow cytometer are the fluidics, optics, and electronics (Fig. **2**) [3].

CHAPTER 11

Flow Cytometry-Based Analysis of Tissue Culture-Derived Plants

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Abstract: In tissue culture, plants are genetically identical to native plants. Using methods such as flow cytometry, cytogenetic analysis, and molecular markers such as AFLP, ISSR, RAPD, RFLP, and SSR, we can detect the genetic uniformity of plants. Among these techniques, flow cytometry (FCM) is a fast, easy, cost-effective, and accurate method for screening the genetic stability of propagated plants. FCM involves measuring the fluorescence light of cell nuclei with a flow cytometer after separation and staining with a chemical with fluorescence properties related to DNA. There is a computer with software for receiving, storing, further processing, and displaying result information. The information is presented in an uncomplicated diagram. FCM is used to determine the genome size and ploidy levels of plants produced *In Vitro*. FCM also stimulates cell cycle function and replication rate in various plant organs and tissues. It was used to study plant organs in greenhouse/field conditions and laboratory conditions (anther culture, eggs, and protoplasts). Plant materials grown in tissue culture are unstable due to somaclonal diversity, especially in their DNA content, and therefore, the use of the FCM method is very effective.

Keywords: Haploid, *In Vitro*, Protoplast,Somaclonal diversity, Somatic hybrid.

INTRODUCTION

Plant tissue culture is used *In Vitro*, including cell, tissue, and organ culture. The cultivation of plant materials free of germs and fungi, including seeds, embryos, tissues, cells, and protoplasts, in a disinfected environment, in sterile containers such as test tubes, is called plant tissue culture [1]. Techniques involved in these areas include *In Vitro* pollination and fertility, embryo rescue, embryo culture,

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production of haploid plants through the anther culture, microspores and fertilization, use of cell culture in the presence or absence of selective or mutagenic agents, and exploitation. Among the somaclonal variants are protoplast culture and protoplast fusion [2]. Plants propagated through tissue culture are expected to be genetically uniform. However, many factors that cause environmental stress in cultivation, such as several subcultures, explant source, callus stage, plant growth regulators, or chemicals, lead to somaclonal variation [3, 4].

Genetically propagated plants must therefore be genetically identical to donor plants. The genetic uniformity of plants can be analyzed using many techniques, such as cytogenetic analysis and flow cytometry, or molecular markers, such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD), isozymes, and simple sequence repeats (SSR), were detected [5, 6]. Flow cytometry is a rapid, economical, and easy method for screening the genetic stability of propagated plants [7, 8]. Flow cytometry can be supplemented by traditional cytological studies, such as chromosome counting [9, 10].

Flow cytometry (FCM) involves measuring the fluorescence light of cell nuclei with a flow cytometer after separation and staining with a chemical with fluorescence properties related to DNA. There is a computer with software for receiving, storing, further processing, and displaying result information. The information is presented in an uncomplicated diagram [11]. Fluorescence light intensity accurately estimates the amount of DNA in a sample by comparing it to another piece whose DNA value is known [12]. There are many differences in the DNA content of the plant genome [13, 14]. These changes result from high values of the presence of microsatellites, sequences in individual DNAs, and ploidy enhancement [15, 16]. Due to its high accuracy and speed in FCM, this method is now widely used to assess ploidy levels *In Vitro* instead of the laborious cytogenetic technique [17, 18]. We studied plants derived from tissue culture using flow cytometric analysis in this study.

PLOIDY LEVEL

Ploidy level refers to the number of chromosomes in diploid phase cells (2n) or haploid phase gametophyte cells (1n). The number is indicated by a number followed by the letter X. Diploid cells have two chromosome series and are denoted by 2x. Cells with a chromosome series of more than two are called polyploids and denoted by (n> 2X) [19].

GENOME SIZE

Many DNA flow cytometry applications are simple and conceptually have no complex application and can be performed. Conversely, there are problems in estimating the net amount of DNA or the size of the nuclear genome. FCM analysis is dependent on the relative fluorescence intensity. Hence, it depends on the relative content of DNA. A new specimen's genome size can be determined by comparing it to a specified genome. There are several ways to do this. The external standard includes separate analyzes of unknown and selected samples' cores. Although the device settings remain intact, there is a possibility of error due to accidentally pushing the device by changing the sample preparation and coloring. To prevent this, it is very effective to use an internal standard in which standard cores and samples are isolated, stained, and analyzed simultaneously [20]. Some researchers do this by combining the two methods. The nuclei of the unknown sample and the standard are extracted and stained separately and then analyzed together [13, 21, 22].

The error is not eliminated due to the core extraction and staining variety. The relative fluorescence intensity of the stained nucleus is measured on a linear scale, and typically, 5,000-20,000 nuclei per sample [23] are analyzed. The absolute DNA value of the samples is calculated based on the mean values of the G1 peak based on this formula.

Sample 2C nuclear DNA content (pg)

$$= \frac{\text{Sample } {}^{G1}/_{G2}}{\text{Standard } {}^{G0}/_{G1}} \times \text{standard2C nuclear DNA content (pg)}$$

There are also some minor problems in converting picograms of DNA to base pairs and *vice versa,* which can be solved by calculating the 1:1 ratio of GC:AT pairs and ignoring the presence of variable nucleotides in the DNA molecule.

However, errors should be less than 1% [15]. The researchers also differ in estimating the average relative weight of a pair of nucleotides, resulting in a conversion factor ranging from 109/0965 bp to 109/0980 bp for one picogram of DNA and considering the 1:1 ratio of GC:AT pairs and ignoring the presence of altered nucleotides [24] showed that one picogram of DNA is equal to 109/0978 bp.

Many other conditions are also necessary to estimate reliable genome size:

(1) The nucleus should be sufficiently extracted and remain intact, and its DNA should not be damaged or altered; (2) DNA staining should be specific and

CHAPTER 12

Molecular Techniques for the Detection of Ploidy Level and Genetic Fidelity of Regenerated Plantlets

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Abstract: Most of the medicinal, aromatic and other commercially important crops are poor rooters, and some of them are sessile in terms of seed production; hence these plants are very difficult to propagate either through stem cuttings based vegetative propagation or through seedlings based sexual propagation. During the last two decades, plant tissue culture has emerged as an alternative technique for the propagation of plants with commercial importance. Majorly, the somatic tissues, *viz*., leaf, node and shoot tip, are being used as explants for the production of genetically similar plantlets through tissue culture studies. Recently, abnormalities with respect to ploidy level and genetic fidelity have been reported in *In Vitro* regenerated plantlets. This is mainly due to the usage of synthetic chemicals or artificial plant growth regulators in *In Vitro* culture studies, the fragile nature of callus and exposure of cultures to artificial light sources. In order to ensure the commercial production of genetically true clones of commercial plants, nowadays it has become an obligatory step to assess the ploidy level and genetic fidelity of regenerated plantlets with that of mother plants. This book chapter focussed on different molecular techniques which are in use for the detection of ploidy level and genetic fidelity of *In Vitro* micro propagated plantlets.

Keywords: Medicinal plants, Tissue culture, Molecular techniques, Ploidy level, Genetic fidelity.

INTRODUCTION

Plants are considered vital resources for human beings, animals and microbial organisms, as they provide food and other essential needs [1]. Apart from the crop species of cereals, pulses and oil yielding plants, humans also depend on the tree species with medicinal and other commercial importance [2]. Several plant

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species of commercial importance were enlisted in the category of threatened, vulnerable, endangered and critically endangered list and may become extinct in the near future if proper conservation measures are not taken [3]. And also, in recent times, due to massive urbanization, a concern has been raised to protect and conserve the medicinal, aromatic and other commercially important plants in order to have a sustainable life for the present and future generations [4]. Among the various conservation strategies, the most advanced and reliable type in multiplying the genetically true clones of plant species is *In Vitro* culture based micro propagation system [5].

In Vitro culture based micro propagation technique as other advantages, such as utilizing less space, culturing under pest and pathogen free environment, and culturing under controlled conditions of temperature, nutrient availability, pH, humidity and other factors [6]. There are also certain disadvantages in *In Vitro* culture based system such as in certain plant species, the explants (leaf, petiole, nodal, shoot tip) based callus may not respond for growth (vitrification) and organogenesis (recalcitrance), the *In Vitro* regenerated plants may have an altered genotype with some chromosomal variations (somaclonal variation) and sometimes the *In Vitro* propagated plants may have induced mutations and change in ploidy level [7]. The usage of synthetic chemical based media components, artificial plant hormones, artificial light source and the fragile nature of callus cells are considered the main inducing factors of genetic alterations in micro propagated plants [8].

Hence, in order to ensure the commercial production of genetically true clones of commercial plants, nowadays it has become an obligatory step to assess the genetic integrity in terms of ploidy level and genetic fidelity of regenerated plantlets with that of mother plants [9 - 11]. Several molecular techniques were developed in the recent past and have been utilized in *In Vitro* culture based studies for the assessment of ploidy level and identification of genetically true clones of propagated plantlets [12 - 14]. The present book chapter focuses on flow cytometric technique and cytological measurement/chromosomal counting method for the detection of the ploidy level of regenerated plants and molecular markers (RAPD, RFLP, AFLP, ISSR, SCoT, DAMD, SRAP and DNA Barcoding markers) based detection of genetic fidelity of regenerated plantlets.

FLOW CYTOMETRY TECHNIQUE FOR THE DETECTION OF THE PLOIDY LEVEL OF REGENERATED PLANTS

The chromosomal content along with the other cellular content gets doubled and equally distributed in the daughter somatic cells during mitosis; thus, somatic cell division is considered an equational division. However, during plant tissue culture

in certain plant species, it was reported that the somatic cells present in the leaf, nodal, petiole and shoot tip explants get exposed to artificial chemical components, which might arrest the spindle fibres resulting in a doubling of chromosome number and production of uneven ploidy levels in regenerated plantlets as compared to mother plant [15 - 17]. Hence, confirmation of ploidy levels and somaclonal variations among the regenerated plantlets through molecular techniques is highly essential to confirm the genetic trueness of plantlets raised through *In Vitro* culture techniques.

Flow cytometry is a molecular technique which can be used for the estimation of DNA content and detection of ploidy levels of plants by using a small amount of tissues, mostly preferably leaf and callus cells and by using fluorochrome stains of either 4,6 diamidino-2-phenyl indole (DAPI) or propidium iodide (PI) [18]. Among these stains, propidium iodide has been widely used as a preferred fluorochrome in estimating the ploidy levels by flow cytometer, as it has no base preferences. Whereas the DAPI fluorochrome stain has the base preference, which specifically stains the AT bases, hence the accurate measurement of DNA content by DAPI is not suitable [19].

In flow cytometry, the fluorescent labelled cells of a leaf or any other somatic tissue were passed or flown through a laser beam of a particular wavelength, and based on the light scattering and fluorescence emission by the labelled cells, their ploidy level gets detected in the form of a peak. This technique is relatively faster and can accurately estimate the DNA contents. Flow cytometry is mostly used in laboratories for the cell sorting and multiparametric analysis of cells and tissues. In recent times, there have been several reports about the usage of flow cytometry techniques for the detection and confirmation of ploidy levels of regenerated plants (Table **1**). In plant biotechnological applications, along with the ploidy level, the flow cytometry technique is also used for the detection of somaclonal variations/genetic variations and genetic uniformity/genetic homogeneity of the tissue cultured plantlets [20 - 22] (Table **1**).

CYTOLOGICAL MEASUREMENT/CHROMOSOMAL COUNTING METHOD FOR THE DETECTION OF THE PLOIDY LEVEL OF REGENERATED PLANTS

Chromosome counting is a traditional method for assessing the ploidy level of plants. This method has been used for karyotyping and chromosome banding techniques [68]. For this method, the root tips of actively dividing cells of young seedlings are most suitable. Metaphase is the best stage for chromosome counting, as the entire chromosome lies at the equatorial plate [69]. Beside the root tips, seeds and endosperms have also been used for chromosome counting in maize

CHAPTER 13

Interspecific Genome Size (2C DNA) Variation in Some Ornamental and Medicinal Plants: Is It a Phenomenon of Partial Sequence Amplification or Loss?

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Abstract: The flow cytometry technique has currently been employed in various fields of research, especially in measuring the 2C DNA of plants. The technique is also used in modern biosystematics, speciation, evolutionary studies and in molecular breeding. A large number of tissue culture raised ornamental and medicinal plants' DNAs are currently made and compared with field grown plants. Various factors influence the quality of active nuclei isolation, which determines the success of accurate DNA estimation. The importance of extraction buffer, reference standards, fluorochrome dyes, and the process of gating is highlighted in order to understand various steps of flow cytometry in measuring DNA. An array of compounds act as inhibitors to disrupt fluorochrome binding to DNA, causing errors in estimating nuclear DNA content; these compounds with their families are presented and summarized. Micropropagation using shoot tips and nodal stems produces true-to type plants, while callus regenerated plants show somaclonal variations – a process showing altered DNA. The role of flow cytometry in investigating the genetic homogeneity of tissue cultured plant population is therefore reviewed. The 2C DNA and genome size of a few medicinal and ornamental plants such as *Catharanthus, Allium, Rawolfia, Gladiolus, Caladium, Zephyranthes* from authors' laboratory were measured and described. The intra-specific and inter-specific genome size and chromosome number variation with reference to gene duplication and DNA sequence loss are discussed. The present chapter, in general, discusses the applications of flow cytometry in field and tissue culture grown ornamentals and medicinal plants.

Keywords: 2C DNA, Chromosome number, Flow cytometry, Genome size, Karyotype, Medicinals, Inter and intra-specific genome variation, Ornamentals.

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INTRODUCTION

The term genome is coined to describe the complete set of chromosomes and their genetic material, and it contains all the genetic information needed to develop and grow an organism [1]. Although the chromosome size and the amount of DNA are not always directly linked, and there is a C-value paradox, the information of 2C DNA or the genome size is very important for many reasons. The information helps in identifying species and taxonomic positions in investigating evolutionary relationships by observing the similarity and differences in the diverse flora of plant kingdom [2]. It can minimize taxonomic disputes at intra, inter genera and family levels and can complement in speciation [3, 4]. The genome size is commonly represented in picogram (pg) and mega billion base pairs (Mbp), in which 1 pg corresponds to 978 Mpb [5]. Because of several advantages, the flow cytometry protocol is utilised widely for estimating genome size, ploidy status and genetic fidelity of tissue cultured clones [6, 7]. The technique is reported to be precise, fast, non-laborious and economic [8].

The last few years' information indicates that flow cytometry has a growing impact on areas like genome evolution [9 - 12] and on establishing a correlation of genome size with non-genetic, environmental components [13, 14]. Although immensely valuable, the progress of genome size study is still lagging, and only a mere 3.1% of angiospermic taxa have so far been completed for 2C DNA or genome; the investigated population size is relatively good in gymnosperm, *i.e.*, 41% [9]. As the angiosperm coverage data is low, a special drive on genome size estimation study is the need of the hour to realize its full potential. In a recent effort, Siljak-Yakovlev *et al.* [15] prepared a C-value database after studying 51 Balkan flora (43 genera distributed in 25 families) and categorized into very small ($2C < 2.8$ pg; 50%), small ($2.8 \leq 2C < 7$; 33.3%), intermediate ($7 \leq 2C < 28$;14.8%) and very large ($2C > 75$, 1.85%) 2C DNA class as per Leitch *et al.* [16] guidelines. The study also indicated that the nuclear 2C DNA is low, *i.e.*, 0.28 pg in *Selaginella helvetica*, a lycophyte and very high in *Fritillaria montana* (109.40 pg). In some studied taxa, more than one ploidy was noted; diploid and tetraploidy are more common along with other ploids (2*x* -16x). These ploidy developments may be induced by endoreplication, as partial duplication was observed in plants like *Dactylorhiza cordigera* subsp. *Bosniaca,* - very common in families like Orchidaceae and Brassicaceae. Palomino *et al.* [17] recently investigated the ploidy, chromosome number and DNA content of twenty three different species of *Echeveria* of the family Crassulaceae in which interspecific variation of DNA was noticed; the 2C DNA content lies between 1.26 pg *in E. catorce* to 7.70 pg in *E. roseiflor.* Many of these *Echeveria* species are native to Mexico and are considered to be ornamentals with immense medicinal value.

Although there are limitations and inadequate access, the flow cytometry technique is practised in various fields of research. It is used in modern biosystematics, evolutionary studies and molecular breeding. In recent times, the technique has been used in plant systematics, in investigating similarities and differences between two or more plant groups and their right position on an evolutionary scale [2, 18]. The inter- and intraspecific genome size, complemented with somatic chromosome number count, can shed some light on taxonomic plant diversity, and it may strengthen the concept of nascent speciation [3]. The use of genome size estimation, in particular, raises interest to researchers globally and is used in a variety of taxa [19]. The present chapter discusses the development and applications of flow cytometry in select groups of field and tissue culture derived ornamentals and medicinal plants.

OPTIMIZATION OF FACTORS INFLUENCING QUALITY NUCLEI ISOLATION

The successful isolation of nuclei is primarily dependent upon many internal and external factors, which in turn are responsible for the precise estimation of DNA through flow cytometry. Some of the factors are extraction buffer, reference standard, the used dye or fluorochrome, right tissue source, the instrumentation conditions like voltage and gating *etc.* These controlling factors are highly variable and need optimization for each investigated plant species/genus [20]. The involvement of some considerations is summarized below:

Extraction Buffer for Nuclei Isolation

The use of a proper extraction buffer is very crucial in the flow cytometric nuclei isolation step. In this process, a large number of active functional nuclei are released in the buffer solution. The used buffers fix, preserve the cells/nuclei, facilitate fluorochrome staining and prevent nucleases degradation [21]. Most of the used buffers contain organic compounds, non-ionic detergent solutions and stabilizers. The different buffers contain substances like MOPS, HEPES and TRIS with 7-8 pH. The addition of TRITON X-100 and Tween – 20 in the buffer constitutes the nonionic ions. The NaCl and KCl in the buffer improve ionic balance in maintaining pH and other physiological activities. Various compounds/salts like $MgCl_2$, $MgSO_4$, spermine, EDTA and sodium citrate (as chelating agents) are used as stabilizers in buffer solution. The Gif nuclear isolation buffer (GNB) is tried in several dry samples of angiosperm and gymnospermic plants [11, 12], although there is a risk of increased coefficient of variation (CV) in dry samples compared to living tissue [22].

The use of appropriate buffer has an important say in isolating nuclei, for example, Loureiro *et al.* [3] used two buffers; polyvinylpyrrolidone (PVP-10)

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