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MOLECULAR MARKERS FOR THE DETETECTION OF PHARMACEUTICAL ALKALOIDS OF *CATHARANTHUS ROSEUS*: A REVIEW

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ABSTRACT

The periwinkle [*Catharanthus roseus* (L.) G. Don, 2n=16], is a seasonal to perennial garden plant and the exclusive source of the anticancer drugs vincristine and vinblastine. Apart from these, its leaves and roots hyper-accumulate the other terpenoid indole alkaloids, which are required for the economic production of pharmaceutical molecules vindoline, catharanthine, ajmalicine and serpentine. Morphological, molecular and biochemical markers have been significantly used to detect these traits. In the present article several such marker have been discussed.

Keywords: *Catharanthus roseus*, RFLP, RAD, SSCP, EST markers, Sequence-tagged sites, DNA Amplification Fingerprinting, SCAR, CAZPS

INTRODUCTION

The periwinkle *Catharanthus roseus*, is a plant that is displayed indoors in all kinds of geographical locations, especially the temperate locations and grown in gardens in semi- temperate to tropical locations on account of its ability to produce flowers all round the year, small size and perenniality. It occurs in almost every tropical and subtropical region of the world, occurring on every continent except Antarctica and on many islands. It is rarely encountered in temperate environments as low winter temperatures inhibit its growth. The characteristic of wide adaptability to all types of soils facilitates its geographically spread distribution in India. Water logged and highly alkaline soils are not suitable for this plant species. The name *Catharanthus* (L.) G. Don is derived from the Latin words *Katharos* (pure) and

anthos (flower). Several names such as *Ammocallis rosea, Lochnera rose*a and most commonly *Vinca rosea* were used. The genus *Catharanthus* is comprised of eight species of small shrubs and herbs, six of which are predominantly indigenous to Madagascar. The information about the designations and origins of the 8 species has been shown in **Table 1.**

Taxonomical hierarchy

Kingdom : Plantae
Subkingdom : Tracheobionta (Vascular Plants)
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Asteridae
Order : Gentianales
Family : Apocynacea (dog-bane family)
Sub family : Plumeroideae
Tribe : Plumerieae
Sub Tribe : Alstonrieae
Genus : *Catharanthus* G. Don
Species : *Catharanthus roseus* (L.) G. Don

Table 1: Designations and origins of the *Catharanthus* species

S.No.	Name of the *Catharanthus* species	Origin
1	*C. roseus* L.G. Don	Madagascar, now naturalized throughout the tropics
2	*C. ovalis* Markgraf	Madagascar
3	*C. trichophyllous* Baker	Madagascar
4	*C. longifolius* Pichon	Madagascar
5	*C. coriaceous* Markgraf	Madagascar
6	*C. lanceous* Bojer ex ADC	Madagascar
7	*C. saitulus* Pichon	Madagascar
8	*C. pusillus* Murray G. Don	India and Sri Lanka

C. roseus is a diploid plant species with estimated haploid genome size between 696Mbp and 2377 Mbp (Galbraith et al. 1983; Zonneveld *et al.* 2005) comprising of 16 chromosomes. The floral morphology of *C. roseus* is conducive both for self and insect pollination. The natural populations of *C. roseus* have been observed to harbour considerable genetic variability; natural variability has been used to develop horticulture and drug types. Variation resulting from cross breeding, induced mutagenesis and polyploidy has been variously employed in *C. roseus*. Initial

crossing studies have shown that although the plant is self pollinated, frequent out crossing has been observed resulting into different intermediate types. Artificial tetraploids have been produced by a number of workers. Although some workers have reported increase in alkaloid content in tetraploids. Tetraploid plants have shorter, thicker stem, larger and thicker leaves. Tetraploids have also been reported to be less susceptible to die back disease.

The plant contains about 130 alkaloids of the indole group out of which 25 are dimeric in nature. Two of the dimeric alkaloids VB and VC mainly present in the aerial parts, have found extensive application in the treatment of human neoplasma. Among the monomeric alkaloids A (raubacine) found in the roots has been confirmed to have a broad application in the treatment of circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow. Vinblastine sulphate (sold as Velban) is used particularly to treat Hodgkin's disease besides lymphosarcoma, choriocarcinoma, neuroblastoma, carcinoma of breast, lungs and other organs in acute and chronic leukemia. Vincristine sulphate (sold as Oncovin) arrests mitosis in metaphase and is very effective for treating acute leukaemia in children and lymphocytic leukemia. It is also used against Hodgkin's disease, Wilkins's tumor, neuroblastoma and reticulum cell sarcoma. High demand and low yield of these alkaloids in the plant has led to research for alternative means for their production. VB is also modified structurally to yield deacetyl vinblastine amide (vindesine) introduced recently as Eldisine for use in the treatment of acute lymphoid leukemia in children. The periwinkle tea prepared from leaf decoction of *C. roseus* is used for curing diabetes. Hypoglycemic effects of a number of other indole alkaloids such as lochnerine, tetrahydroalstonine, and vindolidine have also been revealed. The plant has been reported to possess vermifungal properties. Dried leaves, incorporated into the soil produce nematicidal and ovistatic effects.

Alkaloid content

Table 2: Percent total alkaloid content in different parts of *C. roseus*

Plant part	% Total alkaloid content on dry weight basis
Root	**0.12 - 9.00**
Stem	**0.07 - 0.46**
Leaf	**0.10-1.16**
Flower	**~ 0.005**
Fruit	**~ 0.40**
Seed	**~ 0.18**
Pericarp	**~ 0.14**

Markers

Morphological markers

These are the traditional markers. Morphological mutant traits in a population are mapped and linkage to a desirable or undesirable trait is determined and indirect selection is carried out using the physically identifiable mutant for the trait. There are several undesirable factors that are associated with morphological markers. The first is the high dependency on environmental factors. Often the conditions that a plant is grown in can influence the expression of these markers and lead to false determination. And secondly, performing breeding experiments with these markers is time consuming, labor intensive and the large populations of plants required need large plots of land and/or greenhouse space in which to be grown various morphological markers used by Mendel.

Biochemical markers

Biochemical markers are superior to morphological markers as they are generally independent of environmental growth conditions. The only problem with isozymes in MAS is that most cultivars (commercial breeds of plants) are genetically very similar and isozymes do not produce a great amount of polymorphism and polymorphism in the protein primary structure may still cause an alteration in protein function or expression. Proteins and enzymes also serve as markers, since it is possible to visualize them on gel by or on account of their activity. The weakness of isozyme markers is that each of the proteins that are being scored may not be expressed in the same tissue and at the same time in development. Therefore several samplings of the genetic population need to be made occurring in isozymes analysis of plant samples. Although useful in some plant varieties, isozymes provide little variation in highly bred cultivars.

Molecular or DNA markers

A molecular marker is a DNA sequence which is readily detected and whose inheritance can easily be monitored. The use of molecular markers is based on naturally occurring DNA polymorphism. A marker must be polymorphic, that is, it must exist in different forms so that chromosome carrying mutant gene can be distinguished from the chromosome with the normal gene by a marker it carries. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Polymorphism in the nucleotide sequence usually is sufficient for it to function as a molecular marker in mapping. The molecular markers are of three kinds based on their detection. (1) Non – PCR based

detection: Restriction fragment length polymorphism (RFLP), (2) PCR – based detection: Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), Arbitrarily Primed PCR (AP – PCR), Single nucleotide polymorphism (SNP), (3) Targeted PCR and sequencing: Sequence Tagged Sites (STS), Sequence Characterized Amplified Region (SCARS) etc.

Restriction fragment length polymorphism (RFLP)

Among the various molecular marker techniques developed, restriction fragment length polymorphisms (RFLPs) was used to construct the first molecular map of the human genome (Botstein et al. 1980). This technique uses cDNA or other cloned single-copy DNA elements as radioactively labeled probes in hybridization with restricted genomic DNA. Usually, several endonucleases and different genotypes are screened. The combination of DNA probe and genotype-specific restriction enzyme pattern reveal a restriction fragment length polymorphism. RFLP is a reliable polymorphism which can be used for accurate scoring of genotypes. RFLPs are co-dominant and identify a unique locus and, therefore, are very informative. When cDNAs with known gene function are used as markers, the chromosomal position of the specific gene or genes can be identified. RFLP mapping together with molecular cloning of genes set the stage for establishing syntenic relationships for a number of plant and animal species. Most comparative maps made to date have relied on RFLP analysis using cDNAs as a probe (Kowalski et al. 1994; Livingstone et al. 1999). In plants, RFLP remains the most widely used DNA marker assay, and is the basis for detailed genetic maps of major crops. RFLP markers are converted in to PCR based-markers as:

Sequence-tagged sites (STS)

RFLP probes specifically linked to a desired trait can be converted into PCR-based STS markers based on nucleotide sequence of the probe giving polymorphic band pattern, to obtain specific amplicon. Using this technique, tedious hybridization procedures involved in RFLP analysis can be overcome.

Allele-specific associated primers (ASAPs)

To obtain an allele-specific marker, specific allele (either in homozygous or heterozygous state) is sequenced and specific primers are designed for amplification of DNA template to generate a single fragment at stringent annealing temperatures.

Expressed sequence tag markers (EST)

Such markers are obtained by partial

sequencing of random cDNA clones. Once generated, they are useful in cloning specific genes of interests. ESTs are popularly used in full genome sequencing and mapping EST markers are identified to a large extent for rice, *Arabidopsis* etc. wherein thousands of functional cDNA clones are being converted in to EST markers.

Single strand conformation polymorphism (SSCP)

This is a powerful and rapid technique for gene analysis particularly for detection of point mutations and typing of DNA polymorphism. SSCP can identify heterozygosity of DNA fragments of the same molecular weight and can even detect changes of a few nucleotide bases as the mobility of the single-stranded DNA changes with change in its GC content due to its conformational change. To overcome problems of reannealing and complex banding patterns, an improved technique called asymmetric-PCR SSCP was developed, wherein the denaturation step was eliminated and a large-sized sample could be loaded for gel electrophoresis, making it a potential tool for high throughput DNA polymorphism.

RAPDs (Randomly Amplified Polymorphic DNA)

PCR-based techniques for detecting DNA markers require the development of specific DNA primers as a start site for amplification. The widely used RAPD analysis (Williams et al. 1990) relies on a single 10-base primer of largely arbitrary sequence, except that primers are selected to have 60% or more G+C content, to obtain stronger binding to the template. PCR amplification would only be expected when the priming site occur twice in opposite orientation within approximately 2000 bases. RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with previous methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications.

Despite the reproducibility problem, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time and labor. It requires small amounts of DNA (15-25 ng), is a non-radioactive assay and can be performed in several hours. RAPD fragments linked to a trait of interest could easily be identified by using two pooled DNA samples; one from individuals that express the trait, the other from individuals that do not. Any

polymorphism between the two pools should be linked to the trait. Identified markers are subsequently confirmed by mapping in a segregating population (Caetano-Anolles et al. 1991). Once a marker has been linked to a trait of interest, it is relatively easy to convert the RAPD assay into a more reproducible PCR- type assay based on secondary DNA sequence information, by the use of allele-specific PCR (AS-PCR) or a sequence-characterized amplified region (SCAR) assay (Paran and Caetano-Anolles 1991). Some variations in the RAPD technique include:

DNA Amplification Fingerprinting (DAF)

Caetano-Anolles et al. (1991) employed single arbitrary primers as short as 5 bases to amplify DNA using polymerase chain reaction. In a spectrum of products obtained, simple patterns are useful as genetic markers for mapping, while more complex patterns are useful for DNA fingerprinting. Band patterns are reproducible and can be analysed using polyacrylamide gel electrophoresis and silver staining. DAF is extremely amenable to automation and fluorescent tagging of primers for early and easy determination of amplified products.

Arbitrary Primed Polymerase Chain Reaction (AP-PCR)

This is a special case of RAPD, wherein discrete amplification patterns are generated by employing single primers of 10–50 bases in length in PCR of genomic DNA. In the first two cycles, annealing is under non-stringent conditions. The final products are structurally similar to RAPD products. Compared to DAF, this variant of RAPD is not very popular as it involves autoradiography. Recently, however, separating the fragments on agarose gels and using ethidium bromides staining for visualization have simplified it.

Sequence Characterized amplified regions for amplification of specific band (SCAR)

In this technique the RAPD marker termini are sequenced and longer primers are designed (22–24 nucleotide bases long) for specific amplification of a particular locus. These are similar to STS markers in construction and application. The presence or absence of the band indicates variation in sequence. These are better reproducible than RAPDs. SCARs are usually dominant markers, however, some of them can be converted into codominant markers by digesting them with tetra cutting restriction enzymes and polymorphism can be deduced by denaturing gel electrophoresis. Compared to arbitrary primers, SCARs exhibit several advantages in mapping studies. SCAR are identified as distinct single bands in agarose gel, there amplification is less sensitive to reaction

conditions. Co-dominant SCARs are informative for genetic mapping than dominant RAPDs), map-based cloning as they can be used to screen pooled genomic libraries by PCR, physical mapping, locus specificity, etc. SCARs also allow comparative mapping or homology studies among related species, thus making it an extremely adaptable concept in the near future. Scars linked to the trait of interest can be readily applied to commercial breeding programmes such as cultivar identification and marker assisted selection (Naqvi and Chattoo 1996)

Cleaved amplified polymorphic sequences (CAPS)

These polymorphic patterns are generated by restriction enzyme digestion of PCR products. Such digests are compared for their differential migration during electrophoresis (Jarvis et al. 1994). PCR primer for this process can be synthesized based on the sequence information available in databank of genomic or cDNA sequences or cloned RAPD bands. These markers are co-dominant in nature. This is also sometimes known as PCR-RFLP. Konieczny and Asubel (1993) first adapted the CAPS procedure for genetic mapping by developing a set of CAPS markers for use with Arabidopsis. They designed 18 sets of primers, which amplified products ranging in size from 0.316 to 1.728 kb from Columbia and Landsberg ecotypes. These amplified products were digested with a panel of restriction endonuclease to identify restriction enzymes that generated ecotype specific patterns.

Simple Sequence Repeats (SSRs)

Microsatellites, or Simple Sequence Repeats (SSRs), is a class of repetitive sequences which are widely-distributed in all eukaryotic genomes. They consist of arrays of tandemly repeated short nucleotide motifs of 1-4 bases, and are called mono-, di-, tri- or tetranucleotide repeats respectively that generate repeating regions 100-250 bp. These regions are highly interspersed throughout the eukaryotic genome suggesting having a microsatellite sequence distributed once in every 10 Kb. It had been known that such arrays of short DNA elements repeated in tandem tend to be imprecisely replicated during DNA synthesis, and generate new alleles with different numbers of repeating units. Variable number of repeats between individuals or array length is a result of slippage of the DNA polymerase during DNA replication (Tautz et al. 1986). This length variation is a source of polymorphisms even between closely related individuals. Such microsatellite sequences can be easily amplified by PCR using a pair of flanking locus- specific oligonucleotides as primers

and detect DNA length polymorphisms (Litt and Luty 1989; Weber and May 1989). Further, these microsatellite markers use long PCR primers which are specific to a single genetic locus, they are co dominant and, most importantly, they are multiallelic and detect a much higher level of DNA polymorphism than any other marker system. There has been recent interest in the development of high- density linkage maps based on biallelic markers that can be assayed by PCR. With only two alleles, SNP markers are generally less informative than SSRs. Human genetic maps consisting of SNPs used in linkage studies need at least three times more markers than those containing SSRs at comparable resolution (Kruglyak 1997).

Inter Simple Sequence Repeat (ISSR)

Inter simple sequence repeat (ISSR) is a PCR based technique, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multilocus markers. It is a simple and quick method that combines most of the advantages of SSRs and AFLP to the universality of RAPD. The primers used can be either unanchored (Meyer et al. 1993; Wu et al. 1994) or more usually anchored at 3'or 5' end with 1 to 4 degenerate bases extended into the flanking sequences. Various microsatellites anchored at the 3' end are used for amplifying genomic DNA, which increases their specificity. These are mostly dominant markers, though occasionally a few of them exhibit co - dominance. An unlimited number of primers can be synthesized for various combinations of di, tri, tetra and penta-nucleotides [(4) 3 = 64, (4) 4 = 256] etc. with an anchor made up of a few bases and can be exploited for a broad range of applications in plant species.

Amplified fragment length polymorphism (AFLP)

This technique is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity. The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. AFLP technique is reliable since stringent reaction conditions are used for primer annealing. This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism between closely related genotypes.

AFLP is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of a few nucleotide bases (Vos et al. 1995). This

method generates a large number of restriction fragments (50-100) facilitating the detection of polymorphisms. By choosing different base numbers and composition of nucleotides in adapters the number of DNA fragments which are amplified can be controlled. Although not many maps have been developed so far using AFLPs, this method is now widely used for developing polymorphic markers. The approach is very useful in saturation mapping and for discrimination between varieties.

High reproducibility, rapid generation and high frequency of identifiable polymorphisms make AFLP analysis an attractive technique for determining linkages by analyzing individuals from segregating populations. However, AFLPs are predominantly not codominant and still expensive to generate because silver staining, fluorescent dye or radioactivity detects the fragments.

Single nucleotide polymorphism (SNP)

A single-nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide- A, T, C or G- in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes in an individual. SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions (regions between genes). These genetic variations between the individuals (particularly in the non-coding parts of genome) are exploited in DNA fingerprinting.

MicroRNAs

MicroRNAs were discovered in 1993 during a study of the gene *lin-14* in *C. elegans* development (Lee et al. 1993). A microRNA (abbreviated as miRNA) is a short ribonucleic acid (RNA) molecule found in eukaryotic cells. A microRNA molecule has very few nucleotides (an average of 22) compared with other RNAs. miRNAs are post- transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing. In plants the miRNA complementarity to its mRNA target is nearly perfect, with no or few mismatched bases.

Use of markers for the construction of genetic linkage map

Genetic markers allow tracking of characteristics of interest, to which markers are linked, in the segregating generation. The detection and exploitation of naturally occurring DNA sequence polymorphism represents one of the most significant developments in molecular biology. The preparation of molecular maps implants has been a high priority area of research. The

utility for plant improvement has been emphasized since 1980s. In plants three different genomes exist within most cells: nuclear, chloroplast and mitochondria .The presence of these genomes does not interfere with the genetic analysis of the most molecular loci. In deriving a linkage map for particular species, a mapping population, which will produce segregation at the loci, is produced.

The choice of an appropriate mapping population depends on the type of marker systems employed.

For the preparation of molecular genetic maps the following steps are involved

1. Development of mapping population
2. Selection of one or more molecular marker systems
3. Identification of adequate no of molecular markers showing polymorphism in mapping population and genotyping of individual plants/lines of mapping population using each polymorphic molecular marker, and
4. Construction of molecular map using suitable computer software

Several molecular marker systems including RFLP, RAPD, SSR, ISSR, AFLP, SNP have been utilized for preparation of molecular maps. Before molecular markers are mapped they are to be tested for detection of polymorphism in the parents of mapping population. Only those markers are used in population, which exhibit polymorphism during parent testing, because markers showing no polymorphism cannot be mapped genetically although they can be mapped physically.

The two polymorphic states of the marker observed in parents are given allelic symbols A and B and all the lines of the mapping population are genotyped using this symbolic assignment. The next step is to determine if the segregation at each locus is Mendelian. According to the prediction of Mendels first law, alleles have no permanent effect on one another when present in the same plant, but segregate unchanged by passing randomly into different gametes.

In order to determine if the locus is segregating as expected, the observed segregation is compared with the expected. The statistical test use for this is the chi-square test and iscalculated below.

$$\chi^2 = \Sigma \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

After one or more markers have been checked and the genotypes determined, the next step is to determine if any of the markers are linked. Mendel's second law concerns the independent assortment of genes. When two homozygous parents are crossed, two classes of individuals will be obtained in the next generation: parental and recombinant. This data is subsequently fed and worked out by softwares, which

prepare the genetic maps and determine genetic distances based on recombination frequencies. Genetic map construction requires calculation of pair wise recombination frequencies, establishment of linkage groups and estimation of map distances and determination of map order. Common software packages used for map construction are Linkage1, G Mendel, Mapmaker, Map manager, and Join map.

CONCLUSION

It can been concluded that morphological, biological and molecular markers are equally important for *Catharanthus roseus*. It become more reliable when the several markers are used and the consensus data are analyzed.

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