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(REVIEW ARTICLE)

Curry tree, *Murraya koenigii* (L.) (Spreng): A review and future prospects using applications of next generation sequencing technology

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Abstract

Murraya koenigii is a multipotential medicinal plant. Almost each and every part of the plant has numerous medical applications. Thus, it can be considered as most suitable candidate for new drug discovery. The present review proposes very useful idea for developing significant amount of both genetic and genomic resource for *M. koenigii.* The transcript data which can be developed using these approaches can be used for both basic and applied aspects in *M. koenigii* genetics and breeding. Further, this will be helpful for the discovery and characterization of genes responsive to various medicinal values of the plants and can be further studied and validated. The present review is an attempt which will help to isolate the active principles, as well as to elucidate the role of various interacting phytocompounds which influence therapeutic potential and efficacy *in vivo*.

Keywords: Next generation sequencing; Transcriptome assembly; Gene discovery; Molecular marker

1. Introduction

India is rich in the medicinal herbs we have been getting a huge number of medicinal agents since a long time from nature and we can produce multitude of modern drugs with the help of these agents. The medicinal plants are almost the exclusive source of drugs for majority of world population today. *Murraya koenigii* (L.) Spreng., Popularly known as curry leaf in many Indian languages is basically known for its' aroma and medicinal property. Almost each part of this plant have distinctive order. Fresh leaves, dried leave and essential oils are generally used for flavoring soups, curries, fish and meat dishes etc. essential oil from the plant is used by aroma therapy industries for soaps and costmetics[1]. Properties like blood purification, antifungal, anti-inflammatory, against body ache and antibacterial etc. are very well known[2-5]. It is very effective against diabetics Mellitus, kidney pains and volmiting[6-8]. Fresh leaves paste is also shown to be an effective against bites of some poisonous animals [9].

2. Morphology

A small spreading shrub, about 13-20feets high; the main stem, dark green to brownish, with numerous dots on it; its bark can be peeled off longitudinally, exposing the white wood underneath; the girth of the main stem is 16 cm. Leaves, exstipulate, bipinnately compound, 30 cm long, each bearing -1224 leaflets, having reticulate venation; leaflets, lanceolate, -2 4 cm long, 1.8 cm broad, having 0.5-cm-long petiole. Flowers, bisexual, white, funnel-shaped, sweetly scented, stalked, complete, ebracteate, regular, actinomorphic, pentamerous, hypogynous, the average diameter of a fully opened flower being 1.12 cm; inflorescence, a terminal cyme, each bearing 60 to 90 flowers; calyx, 5-lobed, persistent, inferior, green; corolla, white, polypetalous, inferior, with 5 petals, lanceolate; length, 5 mm; androecium, polyandrous, inferior, with 10 stamens, dorsifixed, arranged into circles of five each; smaller stamens, 4 mm. long whereas the longer ones, 5 to 6 mm; gynoecium, 5 to 6 mm long; stigma, bright, sticky; style, short; ovary, superior.

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Fruits, round to oblong, 1.4 to 1.6 cm long, 1 to 1.2 cm in diameter; weight, 880 mg; volume, 895 microliters; fully ripe fruits, black with a very shining surface; pulp, Wistaria blue 640/2; the number of fruits per cluster varying from 32 to 80. Seed, one in each fruit, 11 mm long, 8 mm in diameter, color spinach green 0960/3; weight, 445 mg; volume, 460 microliters.

2.1. The flowering and fruiting season

Flowering starts from the middle of April and ends in the middle of May. The peak flowering season was observed to be the last week of April. The fruiting season was observed to continue from the middle of July to the end of August. The peak fruiting season, however, was found to continue from the last week of July to the 1st week of August.

2.1.1. Yield

The average yield of a medium-sized bush was found to be 480 g in three to four pickings.

2.2. Chemical composition of the fruit

The pulp of the fruit contains 64.9 per cent moisture. The content of total soluble solids of the fruit juice is 16.8 per cent. The pulp contains 9.76 per cent total sugars, 9.58 per cent reducing sugars, 0.17 percent non-reducing sugars and almost a negligible quantity of tannins and acidity. The vitamin C content of the fruit, which is 13.35 mg per 100 g of the pulp, is better than that of many fruits studied during the course of the present investigation. The mineral content of the edible portion of the fruit, as represented by its ash, is 2.162 per cent. Similarly, 100 g of the edible portion of the fruit contains, protein, 1.97 g; phosphorus, 0.082 g, potassium, 0.811 g, calcium, 0.166 g; magnesium, 0.216 g; and iron, 0.007 g.

2.3. Medicinal importance of plant

Fresh leaves, dried leaves and essential oils of the plant are used in food. It is also used as anthelmintics, febrifuge, blood purifier, antifungal depressant, anti-inflammatory, body aches, for kidney pain, bronchial disorders, piles and vomiting. Furthermore, antimutagenic properties of this plant have been reported in different studies. In spite of its medicinal importance no efforts have been done in developing genomic resources of this very important herb. Presence of important photochemical in *M. koenigii* make it a medicinally as well as economically important herb. Many studies have shown that almost all parts of this plant are useful in one or the other way. The leaves, bark and roots of *M. koenigii* can be used as a tonic and a stomachic. The bark and the roots are used as a stimulant by the physicians. They are also used externally to cure eruptions and the bites of poisonous animals. The green leaves are stated to be eaten raw for curing dysentery, and the infusion of the washed leaves stops vomiting[8].

Phytochemical screening of leaf like qualitative chemical analysis revealed presence carbohydrates, alkaloids, sterols tannins, volatile oil, saponins, anthraquinone glycosides and flavonoids. Various extracts from different parts of the plant have been shown to have different medicinal properties. Ethanol extracts of leaf show anti-inflammatory, hypocholesterolemic and wound healing activity. Petroleum ether extract from leaf show anti-amnesic memory enhancer anti-bacterial, anti-fungal, and anti-tumor properties. Methanol extract of leaf show radioprotective, chemoprotective analgesic, antinociceptive and phagocytic activity. n-hexane extract of seed shows antidiarrheal properties. Aqueous and methanol extract of whole plant, fresh leaf and fruit show anti-diabetic properties. Aqueous extract from roots and stem show cytotoxicity[10].

2.4. Ani-Mutagenic properties

An investigation on *M. koenigii* ethanol extract showed maximum activity against *M. bovis* BCG in combination with a first line anti-TB drug rifampicin. *M. koenigii* leaf extract also exerted more cytotoxic (IC50 20 µg/ml), genotoxic and apoptosis in mouse macrophage RAW 264.7 cell line[11]. The anticancer effects of girinimbine, a carbazole alkaloid isolated from *M. koenigii*, on A549 lung cancer cells indicated that girinimbine may be a potential agent for anticancer drug development[12]. Hydro-methanolic extract of curry leaves (CLE) decreased cell viability and altered the growth kinetics in both the breast cancer cell lines in a dose-dependent manner and leads to cancer cell death. Therefore, identification of active component(s) from the leaf extract could lead to the development of anti-cancer agents which could be useful in the treatment of different types of cancers[13].

A study indicated that the pharmaceutical quality of curry leaves could be improved significantly by optimizing the extraction process of Malaysian curry leaf using response surface methodology[14]. Yet another study, concluded that Malaysian curry leaf collected from the North (Kelantan) might be potential source of potent natural antioxidant and beneficial chemo preventive agents[14].

3. NGS platforms

Genome sequencing is a robust method for gene discovery and for identifying transcripts involved in specific biological process. Over the past decade genome sequencing technology has become more efficient for complex genomes. Sequencing projects have provided not only the first insight into the gene complement for these tissue regions but also sets of genes involved in a number of biological processes. Several approaches were explored as a replacement to conventional Sanger sequencing technology these include sequencing by hybridization[15], mass spectrometry resolution[16], direct imaging of DNA sequence by atomic force microscopy[17], other approaches include techniques-based sequencing by synthesis[18,19] and microfluidics to sequencing[20]. With the advent of reduced costs and higher throughput sequencing methods, expressed sequence tags (ESTs) can be economically generated for a wider range of organisms, thereby providing a more comprehensive assessment of an organism's transcriptome. In recent years, high-through expression profiling technologies like pyrosequencing have transformed molecular genetics approaches in legumes significantly[21]. The advent of high throughput next generation sequencing technologies such as Roche FLX/454 sequencing developed by 454 Life Sciences (acquired by Roche), Solexa by Illumina Genome Analyser (Hayward, CA, USA) and SOLiD from ABI has created the potential for generating considerably increased amounts of information for many organisms including orphan legume crop like pigeon pea.

However, the availability of various *denovo* assembly software programs such as CAP3[22], PCAP[23], RePS[24], and Phusion[25], MAQ, SOAP, ELAND, MOSAIK, VALVET, EULER, SSAKE, SHARCGS can effectively assemble the shorter reads. Previously, combinatorial strategy involving cDNA normalization and FLX-454 deep sequencing platform has been employed in transcriptome characterization studies in *Medicago*[26], Coral[27], Melitaea cinxia (Glanville fritillary butterfly) [28] and many other non-model organisms.

3.1. Roche FLX/454 sequencing

Development of next generation sequencing technologies has significantly increased the volume of sequencing projects conducted by scientific community. Three main evolutionary improvements enabled genome sequencing projects in many species. These include, i) use of fluorescent tags instead of radioactive labels to detect the terminated ladders; ii) use of capillary electrophoresis in place of slab gels; and iii) development of paired-end sequencing protocols incorporating hierarchical template sizes (plasmids, cosmids and bacterial artificial chromosomes (BACs) to provide sequence context and orientation beyond the constraints of the actual sequence read-length in the conventional sequencing techniques[29]. The 454 Life Sciences sequencing platform initiated the next generation sequencing by providing solution to three main bottlenecks of conventional sequencing faced by scientific community i.e. library preparation, template preparation and sequencing[30]. As direct incorporation of natural nucleotides seemed more efficient than repeated cycles of incorporation, detection and cleavage, technology based on pyrophosphate release with an enzymatic cascade ending in luciferase and is detection by emitting light was used for Roche FLX/454 platform. Roche FLX/454 sequencing was based on moving both the template preparation step and the pyrosequencing chemistry to the solid phase[31, 32]. Template DNA is nebulized and size-selected to produce a population of double-stranded fragments ranging from 400 to 600 bases.

Two distinct oligonucleotide adapters are ligated onto the fragments, providing priming sites for subsequent amplification and sequencing. One of the adapters is biotinylated, permitting collection of single-stranded templates. The templates are amplified and immobilized by compartmentalizing individual template molecules and 28 μm DNA capture beads within droplets of an emulsion. PCR reactions conducted inside the droplets amplify the template molecules and complementary primers covalently attached to the DNA capture immobilize the product on the bead surface. Template-covered DNA capture beads are loaded into individual wells etched into the surface of a fiber-optic slide. The sequencing process uses an enzymatic cascade to generate light from inorganic pyrophosphate (PPi) molecules released by the incorporation of nucleotides as a polymerase replicates the template DNA[21]. Individual nucleotides are provided to the open wells by flowing them over the fiber-optic slide. The number of photons generated by the cascade is proportional to the number of nucleotides incorporated by the polymerase and the release of the PPi generated by the individual sequencing reactions. Initially the system generated ~ 20 Mb of 110 base-read per 8 hrs run, subsequent released product generated an average of 100 Mbs of 250 base-reads. Using high density fiber-optic 400-600 Mbs of data is generated per run with an average size of 450 bps. Assembly of 148 Mbp of Roche/454 ESTs obtained for multiple genotypes was aligned and 23,742 SNPs were found in Eucalyptus[33]. Roche FLX/454 sequencing of shoot apical meristem generated 261 000 ESTs of which 30% were novel; ~400 unique ESTs were also identified, for which 27 genes were validated using RT-PCR[34]. A total of 292,465 ESTs were generated using Roche FLX/454 sequencing in *Medicago*, 184,599 unique sequences were identified. This study also include identification of 400 EST SSRs in *Medicago*[26].

3.2. Illumina/Solexa 1G sequencing

In contrast to the 454 and ABI methods which use a bead-based emulsion PCR to generate "polonies", Illumina utilizes a unique "bridged" amplification reaction that occurs on the surface of the flow cell. The flow cell surface is coated with single stranded oligonucleotides that correspond to the sequences of the adapters ligated during the sample preparation stage. Single-stranded, adapter-ligated fragments are bound to the surface of the flow cell exposed to reagents for polymerase-based extension. Priming occurs as the free/distal end of a ligated fragment "bridges" to a complementary oligo on the surface. Repeated denaturation and extension results in localized amplification of single molecules in millions of unique locations across the flow cell surface. This process is referred to as Illumina's "cluster station", an automated flow cell processor. A flow cell containing millions of unique clusters is now loaded into the 1G sequencer for automated cycles of extension and imaging. The first cycle of sequencing consists first of the incorporation of a single fluorescent nucleotide, followed by high resolution imaging of the entire flow cell. These images represent the data collected for the first base. Any signal above background identifies the physical location of a cluster (or polony), and the fluorescent emission identifies which of the four bases was incorporated at that position. This cycle is repeated, one base at a time, generating a series of images each representing a single base extension at a specific cluster. Base calls are derived with an algorithm that identifies the emission color over time. At this time reports of useful Illumina reads range from 26-50 bases. Illumina/Solexa 1G sequencing was used for identification of 8, 23,325 unique SNPs in Arabidopsis[35]. Illumina/Solexa 1G sequencing generated 574 Mbp data which was used to identify and mark repetitive regions and define putative gene space in barley[36].

3.3. Transcriptome analysis

Next generation sequencing was used to generate the transcriptome of the curry leaf to detect changes in gene expression during leaf development transcriptome when annotated with BLASTx using the non-redundant (nr) protein database, and Gene Ontology (GO). A transcriptome for the curry leaf was assembled using ~76 million single-end sequence reads, pooled from both immature and mature leaf libraries such that the final assembly shows a more accurate estimation of genes expressed throughout leaf ontogeny.

The transcriptome pattern of the curry leaf was a useful tool in the analysis of the differential expression of genes and pathways in immature and mature tissue. Until this study, only a few nuclear genes, complete chloroplast genome[37], and a transcriptome[38] in mature leaves had been sequenced for the curry tree; this study adds 57,383 newly sequenced transcripts (including multiple transcriptional isoforms of the same gene), contributing to a new understanding of the molecular genetics of the curry leaf development. [39].

4. Applications of NGS technology

NGS technologies have already been used for variety of applications, such as development of SSR and SNP- based molecular markers. Applications of NGS technology resequencing of well-characterized sp.[35], *de novo* sequencing of crop sp. without reference sequence likepigeonpea[40], association mapping using natural population, expression and nucleotide polymorphism in transcriptome, wide crosses and alien introgression, population genetics and evolutionary biology, organellar and genome-wide assembly[38].

4.1. Application of NGS technologies in *M. koenigii* genomics

Although crude extract from various parts of *M. koenigii* have numerous medical applications, modern drugs can be developed after extensive investigation of genome through sequence data analysis of this plant. Till date only 55 genes of *M. koenigii* have been sequenced. Furthermore, all the studies done majorly involved leave extract from the plant were as not much research was done using other parts of plant which are equally medicinally important. Hence there is a need to involve transcriptomic to have a better idea of expression profile of the genome. Furthermore, generation of sequence data is the key question for the same and this can be done using next generation sequencing technologies[42]. Next generation sequencing technologies are being extensively utilized not only for cDNA sequencing projects but also whole genome sequencing both in plants and animal species like humans, nematodes and Arabidopsis etc. For example, researchers are using 454 sequencing to publish the complete genome of an individual human, achieving a key milestone on the path to personalized genome sequencing. This can be done using following approaches:

4.1.1. RNA extraction

Tissues from different developmental stages can be targeted for collection and construction of cDNA libraries. Total RNA can be extracted from all the harvested tissues using modified hot-acid phenol method[41].

4.1.2. Quantification of RNA and Sample preparation

The Quantification of RNA can be performed by UV spectrophotometer.

4.1.3. Roche FLX/454 sequencing, Sequence data assembly and clustering

RNA samples can be out source for sequencing. All the sequence analyses can be conducted using publicly available software and custom Perl scripts.

4.2. Characterization of 454 transcriptome assembly

4.2.1. Functional annotation and gene ontology (GO) categorization

Homology searches can be performed against non-redundant (nr) nucleotide sequences available at the **TIGR Plant** Transcript A**ssemblies database** using BLASTN algorithm at a significance threshold of ≤ 1E-30. TUSs which show a significant BLASTX hit can be used for functional annotation based on Gene Ontology categories from UniProt database.

4.2.2. Identification of paralogues

Identification of paralogous genes was conducted using both the contig consensus sequences and the singletons following assembly.

4.2.3. Development of gene-based molecular markers

Mining and development of gene-based marker can be done using custom Perl scripts.

M. koenigii contains many important properties, still there is need of extensive genomic investigation of the same to exploit these important properties of the plant. With the advent of new approaches NGS it will be very easy still remarkable to explore this genome and get an insight of various pathways owing to its medicinal properties. Anti-mutagenic properties shown by various extracts of the plant can be an important source for new drug delivery and technology development using various advanced biotechnological approaches.

5. Conclusion

Using all these above approaches huge genomic data for *M. Koenigii* can be developed. Medicinal plant like *M. Koenigii* can be an exclusive source of herbal drug discovery. Majority of world population today is interested in herbal as they are considered safe, inexpensive and have no adverse effect. The upregulated pathways identified in immature leaves can now be studied in this species, as well as other molecular resources for studying the antiherbivore mechanisms of vulnerable meristematic tissues in the absence of mature oil glands. This will enable future studies to investigate the role of these enzymes and metabolites in the ecology and physiology of this species as well as its application in food and medicine. Further plants have advantages like they are self-generated and produce range of beneficial bioactive product which have potential to develop medicines for various ailments.

Future prospect

The data which can be generated using these approaches can have significant impact on *M. Koenigii* genomics. Huge genomic resources which include development of transcriptome assembly of *M. Koenigii* containing contigs and unigene. Further the characterization of this assembly will enable coding DNA sequences (CDS) prediction and functional annotation of the predicted CDS with non-redundant database. GO functional classification of CDS/unigenes in biological processes, cellular components and molecular function will be helpful in identifying important genes. Paralogous and orthologous genes will be identified using COG/KOG functional classification of unigenes/CDS. Genes involved in various pathways will be identified using KEGG. Further the study will also result in identification of transcription factor, simple sequence repeats (SSRs) discovery. Lastly, the classes of enzymes involved in the synthesis of terpenes were to be characterized in curry leaves, and the differential expression of these terpene synthase genes can be studied, it was reported that significant increase in terpene biosynthesis after leaf maturation. In summary the study will give vast information about the gene and genic factors owing to its medicinal property such as antimutagenic, anthelmintic, anti-inflammatory and antifungal depressant etc. This kind of information can play a vital role in the development of safe formulation for treatment of various diseases.

Compliance with ethical standards

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Disclosure of conflict of interest

We have no conflicts of interest to disclose.

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