International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(3): 426-432

Received: 22-03-2019
Accepted: 24-04-2019

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Development and validation of simple sequence repeat markers from genome of ajwain (*Trachyspermum ammi* L.)

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Abstract
Ajwain (*Trachyspermum ammi* L.) is an annual herbaceous plant belonging to the highly valued medicinally important family, Apiaceae. In present study, Ion Torrent Genome Sequencing (Ion S5) technology was used to generate ajwain draft genome using genotype Gujarat Ajwain 1. Gujarat Ajwain 1 genotype was sequenced using next generation sequencing platform Ion S5 which yielded 8.4 Gb of raw data. After trimming of reads, the quality data were of 28,450,128 bp with average read length of 193.8 bp. The average percentage of quality filtered read was 99.43%. The *de novo* assembly yielded assembled reads of 499,199,313 bp and number of contigs were 788,836. In the assembly the N25, N50, N75 contig size were 1151 bp, 644 bp and 416 bp respectively. Based on the contig length more than 5000 sequences were generated from which 425 sequences were selected for further analysis. In Blast 2GO analysis, 425 sequence were functionally annotated out of which 22 showed positive interpro. While 414 got Blast hits, of which 408 and 339 sequence were mapped and annotated, respectively. During the gene ontology, total 33594 GO IDs were found, which were grouped in to biological process, cellular components and molecular function, respectively. Out of 425 sequences scanned 17 showed positive InterPro result while 408 did not showed any InterPro results. KEGG pathway rewarded that among all sequences the maximum sequence was in Purine metabolism (63). KEGG analysis created the enzymes ID of which maximum enzymes count ID were found in the pathway of Diterpenoid biosynthesis, which was 12 while for other pathway few enzyme ID count were found. From the sequenced genome a total of 228 SSRs were identified with the Tm range of 56-62 °C and GC% of 40-70%. The length of SSR primer was between 18-23 bp and the product size in the range of 100-300 bp. The percentage of dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, hexanucleotide repeats comprised 26.31%, 27.9%, 35.52%, 7.89% and 3.07%, respectively. Validation of selected 25 SSR primers was carried out in 5 different genotypes of ajwain namely Ajmer ajwain-1, Ajmer Aawain-2, Ajmer ajwain-93, Gujarat ajwain-1, Gujarat ajwain-2. Out of 25 primers 19 primers were amplified to produce a total 24 band. The largest amplicon of 734 bp was amplified by SSR primer AJ 12 and smallest fragment of 58 bp was applied by SSR primer AJ16. The polymorphic primer can be used in genetic diversity analysis of Ajwain and its relatives.

Keywords: *de novo*, IPS, SSR, putative

Introduction
Ajwain (*Trachyspermum ammi* L.) is an annual herbaceous plant belonging to the highly valued medicinally important family, Apiaceae (Gersbach and Reddy, 2002) [8]. It is a cross pollinated crop and has a somatic chromosome number of 2n=18. Flowers are self-fertile, but cross pollination occurs through insects (Mostafavi and Pezhannfar, 2015) [18]. It is said that the herb is widely grown in arid and semi-arid regions where the soil involve high amount of salts (Joshi, 2000) [13]. Ajwain has an erect and striate stem involving glabrous or minutely pubescent properties which may grow up to 90 cm tall (Chatterjee, 1995) [1]. Ajwain is widely distributed and cultivated in various regions such as Iran, Pakistan, Afghanistan, and India as well as Europe while it is indigenous to Egypt (Shojaaddini *et al.*, 2008) [26]. The herb is generally grown in October–November and should be harvested in May–June (Ranjan *et al.*, 2012) [23]. Usually grayish brown seeds or fruits of Ajwain are considered for medical and nutritional purposes (Chauhan *et al.*, 2012) [2]. The seeds contain 2 - 4.4% brown color-red oil known as ajwain oil. The main component of this oil is thymol, which is used in the treatment of gastro-intestinal ailments, lack of appetite and bronchial problems (Choudhary *et al.*, 1998)

[3]. Ajwain seed analysis has revealed it to contain fiber (11.9%), carbohydrates (24.6%), tannins, glycosides, moisture (8.9%), protein (17.1%), fat (21.1%), saponins, flavones and other components (7.1%) involving calcium, phosphorous, iron, cobalt, copper, iodine, manganese, thiamine, riboflavin and nicotinic acid are of reported phytochemical constituents of Ajwain (Qureshi and Kumar, 2010) [22].The non-thymol fraction (Thymene) contains Paracymene, Gamma-terpinene, Alpha-pinene, Betapinene, α-terpinene, Styrene, Delta-3-carene, Betaphyllanderene, terpinene-4-ol and Carvacrol [6, 13]. On the other hand, in an investigation, carvone (46.2%), limonene (38.1%) and dillapiole (8.9%) were introduced as principal oil constituents (Mohagheghzadeh *et al.,* 2005) [17].

In the alcoholic extraction process, a large amount of saponin has been derived (Duke, 1992) [5]. Ajwain has been shown to possess antimicrobial, hypolipidemic, digestivestimulant, antihypertensive, hepatoprotective, antispasmodic, broncho dilating, antilithiasis, diuretic, abortifacient, galactogogic, antiplatelet-aggregatory, anti-inflammatory, antitussive, antifilarial, gestroprotective, nematicidal, anthelmintic, detoxification of aflatoxins, and ameliorative effects (Gilani *et al.,* 2005) [9].

Genome sequencing refers to methods for determining the order of the nucleotides bases adenine, guanine, cytosine and thymine of an organism's genome at a single time. Numerous technologies have been developed to analyze and quantify the sequencing. Any high throughput sequencing technology can be used for genome sequencing such as the Illumina Genome Analyzer, Applied Biosystems SOLiD TM, Roche 454 Life Sciences system, Ion Torrent. Genome sequencing work will be helpful to decipher information from coding and noncoding part of Ajwain. It also provides facts of genome construct its constituent and genic variation.

Simple sequence repeats (SSR) developed from genome data will be helpful to develop a breeding program for mapping and marker assisted selection. The main genetic tools used for the identification and breeding of cultivars of domesticated species are morphological and molecular marker (Tanksley *et al.,* 1993) [28]. SSRs have been the most widely employed class of molecular markers used in genetic studies with applications in many fields of genetics including genetic resources conservation, population genetics, molecular breeding and paternity testing (Ellegren, 2004) [7]. This range of applications is due to the fact that SSR markers are codominant, multiallelic, and highly reproducible, have high resolution, are amenable to high throughput and are based on polymerase chain reaction (PCR) (Oliveira *et al.,* 2006) [19]. As a convention, SSRs are regions in the genome where a group of bases (1–8 bp long) are repeated in tandem (Richard *et al.,* 2008) [24]. These regions can be isolated either by data mining of existing sequences or by generating SSR-enriched libraries. Microsatellites or simple sequence repeats (SSRs) are found throughout the eukaryotic genomes and occur in both coding and noncoding regions. Microsatellites are stretches of DNA consisting of tandemly arranged units in 1-6 bp (Gupta *et al.,* 1996; Thiel *et al.,* 2003) [11, 29], characterized by their co-dominant inheritance, wide genomic distribution, hyper variable and multiallelic nature (Powell *et al.,* 1996; Parida *et al.,* 2009) [21, 20]. They are also termed as simple sequences (Tautz, 1989) [28], Short Tandem Repeats (STRs) (Edwards *et al.,* 1991) [6] and SSRs (Jacob *et al.,* 1991) [12]. Microsatellites are ubiquitous in the coding and noncoding regions with a higher density of simple sequence motifs in the noncoding regions of eukaryotes.

In plants, SSRs are much more abundant and preferentially associated within untranslated regions (UTRs) of the transcribed regions. With the importance of dill as a medicinal and spices crop, this research has been challenge to discover the genes responsible for different cellular, biological and molecular aspect through genome sequencing. Genome sequencing work will be helpful to decipher information from coding and noncoding part of an organism. It also provides facts of genome construct its constituent and genic variation. Polymorphic SSR markers development would be useful for the population genetic studies and germplasm management of ajwain. Sequencing of *Trachyspermum ammi* genome will provide a model for characterization of metabolic pathway, involved in synthesis of bioactive compound, comparative evolutionary studied among various Apiaceae family members and help annotate their genome (Richard *et al.,* 2008) [24].

Material and Methods

Sample collection and DNA isolation

For this study five genotypes of ajwain were used namely, Gujarat Ajwain 1, Gujarat Ajwain 2, AjmerAjwain 1, AjmerAjwain 2 and AjmerAjwain 93. The seeds of ajwain genotypes Gujarat Ajwain 1 and Gujarat Ajwain 2 were obtained from Seed Spices Research Station, Sardarkrushinagar Dantiwada Agricultural University, Jagudan and seeds of Ajwain 1, AjmerAjwain 2 and Ajmer Ajwain 93 collected from National Research Centre on Seed Spices, Ajmer. Genomic DNA isolated from fresh seedling tissue by following modified CTAB method as described by Doyle and Doyle (1990) [4]. After DNA isolation the concentration was determined by using Picodrop PET01 with software v2.08 (Picodrop Ltd., Cambridge U.K). The ratio at A260/A280 was 1.85. For the good quality and purity of genomic DNA, plant DNA extraction kit (Nucleopore DNA extraction kit) also was used in the study.

Sequencing of sample by ION Torrent S5

gDNA fragment library were prepared using Ion Shear™plus enzyme mix II (as per the Ion S5 library preparation protocol), then fragments were purified by Agencourt® AMPure® XP reagent (1.8X sample volume) followed by adapter ligation and nick repair. Adapter ligated fragments were purified. Size selection of library performed on 2% EGel. Stop the run when the 500-bp ladder band is at the top edge of the collection well. Fragments of the desired size are enriched by amplifying library. Emulsion PCR was carried out to generate multiple copies of fragments on the Ion Sphere (IPS) beads using the Ion torrent OT2 machine. Sequencing of each IPS was carried out in Ion Torrent S5 sequencer system using Ion 530/540 chip.

Data Analysis

Raw data quality analysis using CLC Version 9.5.4. Quality control check of raw sequence data coming from high throughput sequencing pipelines provides a modular set of analyses which you can use to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis. These tools analyze FASTA format file to calculate different sequence statistics and calculates the average quality score for each read and overall average quality score for all the reads. *de novo* genome assembly was also carried by using CLC Version 9.5.4 *de novo* Assembler (Merida *et al.,* 2013) [16]. Functional annotation was carried out by using Blast2Go software to

align the consensus sequences from the assembled contigs and the singleton sequences (Liu *et al.,* 2013) [7]. Identification of putative genes was carried out using CLC software Genomics Workbench (CLC Genomics Workbench v9.5.4 or according to the manufacturer's instructions (User manual according respective company) by company (Zhou *et al.*, 2012) [30].

Identification of genomic SSR marker, primer designing and validation

Genomic SSR markers were designed from those contigs which were involved in biological, molecular and cellular function. SSR markers were designed using a high through put web tool Batch Primer3 v1.0 (Guimaraes *et al.,* 2012) [10]. For validation of designed SSR markers in five genotype of dill, the mastermix was prepared in a microfuge tube in which the buffer was added first followed by sterile water, Primer, dNTPs mix followed by Taq DNA polymerase (Table 1). At the last DNA was added in each tube separately. The reagents were mixed gently by tapping against the tube. The tubes were then placed in the Thermal Cycler for amplification. The PCR condition for thermal cycler is given in table 2. PCR products were subjected to electrophoresis with marker DNA of known molecular weight in 1.8% agarose gel. After electrophoresis, the gel was carefully taken out of the casting tray and photographed in GeneSys gel documentation system.

Table 1: Preparation of reaction mixture for SSR

Sr. No.	Reagent	Quantity
1	PCR buffer (10X)	2µl
2	Taq polymerase (3 U.µl^{-1})	0.3µl
3	dNTPs mix (2.5 mM each)	0.06µl
4	Primer-F (25 pmoles.µl^{-1})	1µl
5	Primer-R (25 pmoles.µl^{-1})	1µl
6	Template DNA (50 ng.µl^{-1})	1µl
7	Millipore sterile distilled water	14.74µl
Total		20µl

Table 2: PCR conditions for SSR

Sr. No	Steps	Temperature (ºC)	Duration
1	Initial Denaturation	94	3.0 min
2	Denaturation	94	30 sec
3	Annealing	(52 -57)	1min
4	Extension	72	1 min
Repeat the steps 2 to 4 for 40 times			
5	Final extension	72	5.0 min
6	Hold	4	--

Result and Discussion

Genome sequencing and *de novo* assembly of the raw data

The study was initiated with the objective of genome analysis of Gujarat Ajwain 1. A total of three run were performed on Ion Torrent S5 next generation system. In first run of sample, total data generated was 1.9 Gb. ISPs loading was 75% on the Ion 530^{TM} chip and 28,172,861 total numbers of bases were generated after removing of polyclonal (39%) and low quality data (59%), and adaptor dimer with mean length of 281 bp. The loading of second run sample's ISPs was 73% on the chip and total number of reads of 1,180,588,577 was obtained after removing of polyclonal (19%) and low quality data (73%) and adapter dimer, with mean read length of 308 bp. Total data generated in second run was 1.1 Gb. Second run was carried out by using bar codes for different samples, out of which Ajwain was barcoded with IonXpress_002 barcode name. The third run of sample was carried out in Ion 540^{TM} chip and total data was generated 5.4 Gb. ISPs loading was 90% on the chip. Third run was also carried out by using barcodes for different samples, out of which Ajwain was barcoded with IonXpress_003 barcode name. For Ajwain, 5,412,056,746 bp total numbers of bases were generated after removing of polyclonal (18%), low quality data (7%) and adapter dimer, with mean length 198 bp. The statistical data of all three runs is shown in Table 3.

Total of 8.4 Gb raw data was generated through sequencing. Raw sequence data were processed for quality assessment using CLC workbench v9.5.4, in which the adapter sequences were removed and trimmed on quality bases. Total nucleotides in data sets were 449,199,313 respectively. In order to get quality reads the data was trimmed. The *de novo* assembly of assembled reads has yielded data of 225,277,958 base pairs (225 Mb). Total numbers of contigs were 788,836 in number with 200 bp of minimum, 17,216 bp of maximum contig length. N25, N50 and N75 measurement of contigs were found 1151 bp, 644 bp, and 416 bp respectively *viade novo* assembly. In order to maintain the quality of assembled data contigs were filtered, in which only those contigs who have sequence above and equal to 5000 bp were selected for the annotation. This whole process was carried out in CLC Genome Workbench. After filter 425 contigs were remain with 5011 bp of minimum, 17,296 bp of maximum and 7299 bp of average contig length.

Table 3: Raw data of run on Ion S5

Sr. no.	Parameter	1st run	2nd run	3rd run
1	Total number of bases	28,172,861	1,180,588,577	5,412,056,746
2	Total reads (Gb)	1.9	1.1	5.4
3	Mean length (bp)	281	308	198
4	ISP Loading (%)	75	73	90

Blast2go analysis of genome sequencing data

Sequence from Ajwain (Gujarat Ajwain 1) cultivar was assembled using CLC workbench. Validation and functional annotation of these sequences were done using Blast2Go tool. Data analysis progress of functional annotation is given in (Figure 2). Total 425 sequences were functionally annotated out of which 22 (5.17%) were showed positive interpro, while 414 (97.41%) were got Blast hits. From the total 408 (96%) and 339 (79.96%) sequence were mapped and annotated respectively.

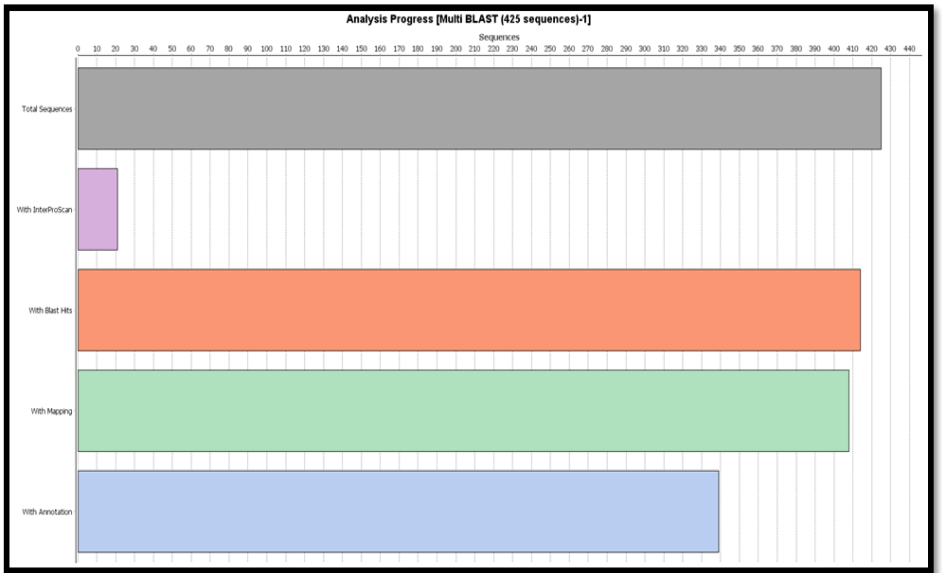

Fig 1: Analysis progress of Blast2Go result

Gene ontology (GO) IDS and sequence distribution

Gene ontology mainly divided in to three groups, biological process, cellular components and molecular function. Collectively 33,594 numbers of GO IDs were found which were grouped into biological process, cellular components and molecular function, respectively responsible to cellular process and metabolic process. During gene ontology direct Go count were generated. In molecular function maximum sequences from total sequences respond to ubiquinone activity and protein binding showing maximum Go count followed by Magnesium ion binding, iron-sulfur cluster binding. In biological processes maximum sequences were showing maximum Go count to ATP synthesis followed by protein – chromophore linkage and aerobic respiration. In cellular component maximum sequences having maximum Go count which respond to integral component of the membrane followed by integral component of the plasma membrane.

Biological process

Ajwain genotype differs from the model plant on genetic basis, so, limited GO term could be identified. For Biological Process (BP), 45 GO terms were identified. Common GO term were response to biosynthetic process, response to stress, cellular component organization, response to abiotic stimulus, response to endogenous stimulus and nucleobase containing compound metabolic process. Out of 45 biological process GO terms,response to biosynthetic process, response to stress and cellular component organization maximum GO count were found between 145 to 150 sequence, while remaining other GO term were found below 115 sequence which is shown in figure 2.

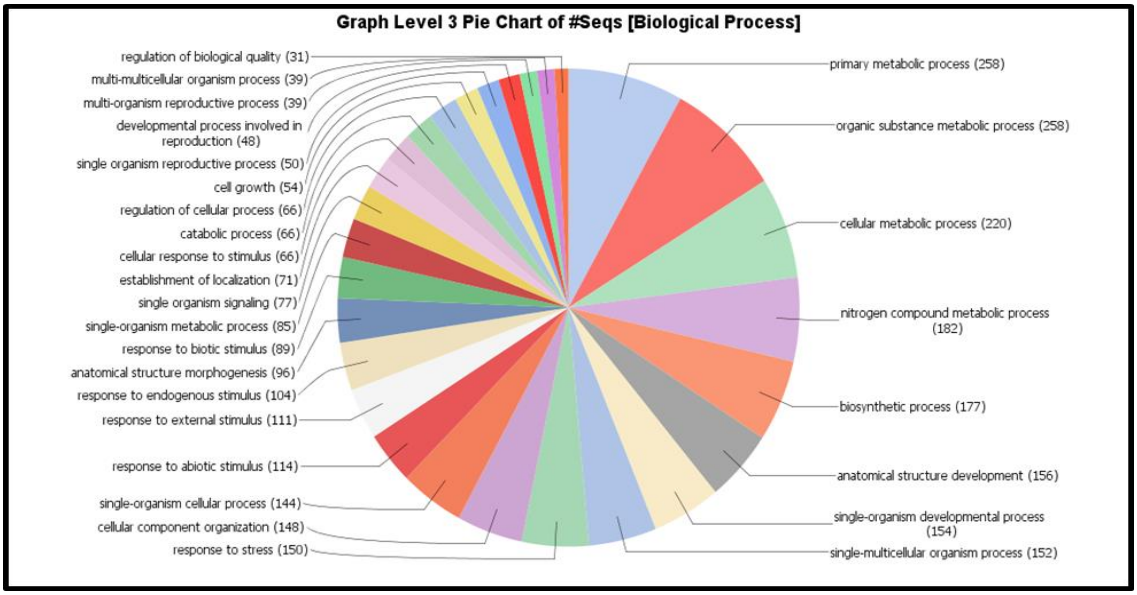

Fig 2: Pie chart at graph level 3 of biological process.

Molecular function

For Molecular Function, 25 GO terms were identified, which included protein binding, enzyme regulator, molecular transducer, structural molecule, translation regulator, antioxidant activity and catalytic activity. Out of 25 molecular function GO terms, response to binding, enzyme regulator, molecular transducer, structural molecule, translation regulator and antioxidant activity maximum GO count were found between 125 to 130 sequence, while remaining other GO term were found below 90 sequence which is shown in figure 3.

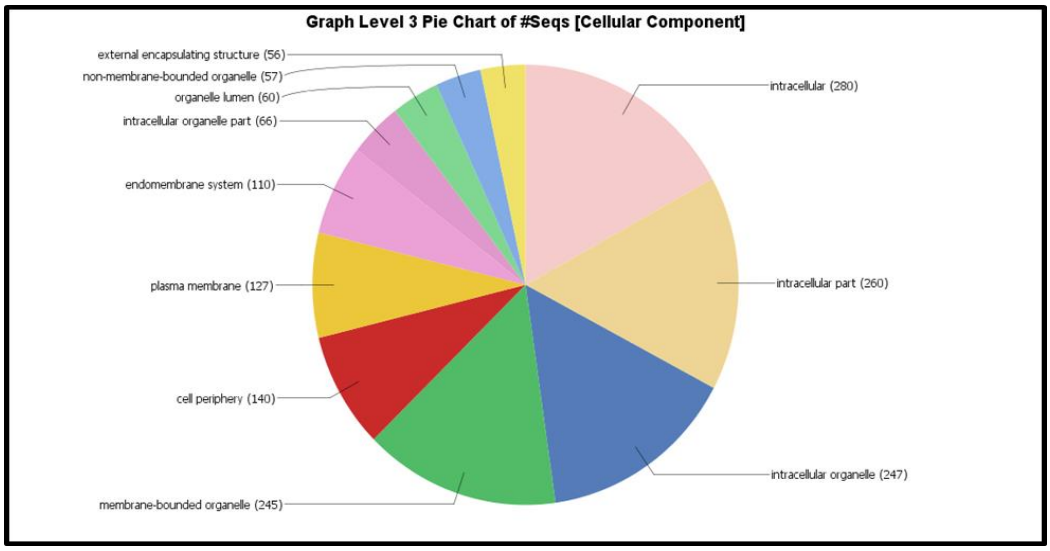

Fig 3: Pie chart at graph level 4 of molecular function

Cellular component

For Cellular Component, 26 GO terms were identified, which included plasma membrane, plastid, mitochondrion, cytosol, nucleus, extracellular region, Golgi apparatus, vacuole, membrane, endoplasmic reticulum, cell wall, nucleoplasm and endosome. Out of 26 cellular component GO terms,response to plasma membrane, plastid, mitochondrion, cytosol, nucleus, extracellular region, Golgi apparatus, vacuole and membrane maximum GO count were found between 115 to 130 sequence, while remaining other GO term were found below 100 sequence which is shown in figure 4.

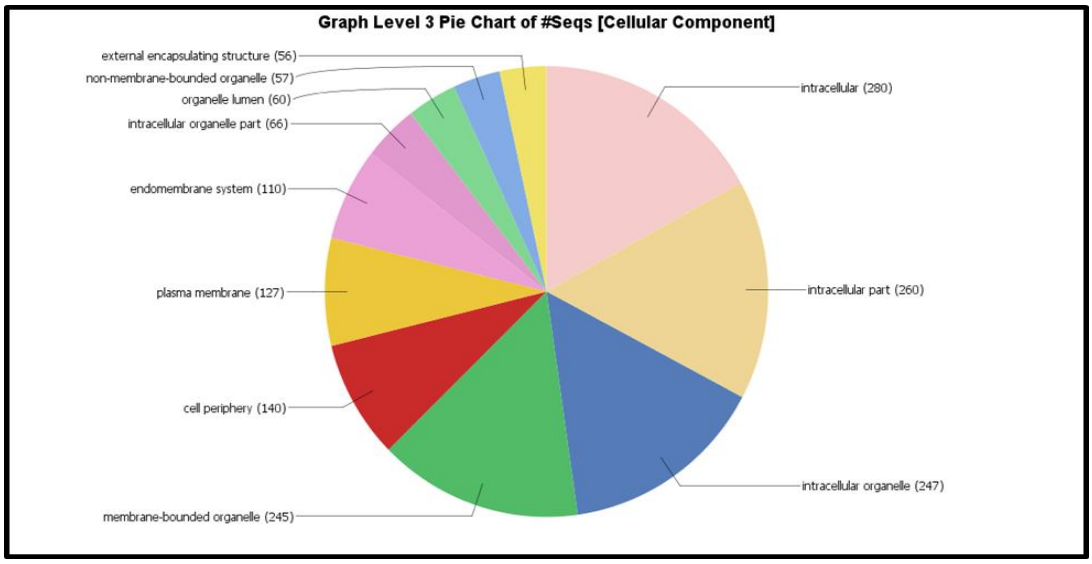

Fig 4: Pie chart at graph level 3 of cellular components

Putative/hypothetical genes identification

Putative genes identification is a requirement for gene investigation in the era of genomics. The strategy for discovery of potential ORFs at a large-scale in ajwain genomes described here will contribute to their annotation and identifies new potential regulators of diverse biological processes in plants and that improve understanding of plant biology. The putative function was bifurcate into biological process, cellular process and molecular process. The average contig length was 5314 bp, however only those contigs with the length of 9000 bp and above were used for identification of putative function.

In biological process, total 44 contig were found which were responsible for putative function Among the 44 contigs, contig no 41 had the maximum length of 15,554 bp and also had maximum number of GO function allotted. this contig was followed by contig no 48 which has length of 10,664 bp and had function like embryo development, catabolic process, cellular protein modification process, response to biotic stimulus etc. Among 44 contigs, three contigs were those which showed similarity above 75% with available database. Contig no 24 showed highest similarity by 90.17% followed by contig no 102 (80.86%) and contig no 421 (78%) similarity.

In cellular process, total 42 contig were found which were responsible for putative function. Among the 42 contigs, contig no 42 had the maximum length of 15,429 bp. This contig was followed by contig no 48 which has length of 10,664 bp and also had maximum number of GO function allotted. Which had function like thylakoid, plastid, nucleuolus etc. Among 42 contigs, three contigs were those which showed similarity above 75% with available database. Contig no 24 showed highest similarity by 90.17% followed by contig no 102 (80.86%) and contig no 421 (78%) similarity.

In molecular process, total 45 contig were found which were responsible for putative function. Among the 45 contigs, contig no 41 had the maximum length of 15,554 bp. This contig was followed by contig no 230 which has length of 9150 bp and also had maximum number of GO function allotted. Which had function like Double-stranded methylated DNA binding, transcription regulatory region sequence-specific DNA binding, Zinc ion binding, Aspartic-type endopeptidase activity etc. Among 45 contigs, two contigs were those which showed similarity above 75% with available database. Contig no 24 showed highest similarity by 90.17% followed by contig no 102 (80.86%) similarities.

SSR marker identification and primer design using BatchPrimer3, V 1.0

Genomic SSRs of Ajwain were identified and developed from the draft genome of Ajwain genotype, Gujarat Ajwain-1 by using online tool BatchPrimer3 v1.0. For the identification of SSRs FASTA files of four contigs were used. Using the filtered assembly report, 228 SSR markers were developed. . Total selected 25 primers met the following parameters: 100–300 final product length (optimal 200 bp), primer size from 18 to 23 bp (optimal 20 bp) and GC content 40%–70% (optimal 50%); the annealing temperature was set at 56–62 °C (optimal 60 °C). Tm and GC % was found between 61-59 ºC and 50% respectively. Length of SSR primers was between 18-22 bp with 100-300 bp product size.

Validation of SSR markers

In order to validate the SSR primers in five different genotypes of Ajwain (Ajmer Ajwain-1, Ajmer Ajwain-2, Ajmer Ajwain-93, Gujarat Ajwain-1, Gujarat Ajwain-2), 25 SSRs were selected manually out of 228 primers. Criteria used to select the SSR primers for validation purpose was based on the melting temperature (Tm) and GC content (GC%) of the primers. Those primers were selected which were having melting temperature above 54 ºC and GC% above 40% and primers were chosen from those contigs which were involved in putative function. Firstly, all the 25 primers were screened on different annealing temperature and total of 19 primers amplified the DNA.

Polymorphism pattern of SSR

All the 19 SSRs primers were amplified a total of 24 bands. Among 19 amplified primers, only three primers (AJ4, AJ5 and AJ21) showed polymorphism (Figure 5), rest were monomorphic. The SSR primer AJ16 produced maximum number of three bands, while AJ12, AJ14 and AJ18 produced two bands and others were produced one band. Out of 24 bands, 8 bands were polymorphic and 16 bands were monomorphic. Among the 8 polymorphic bands, 6 bands were shared polymorphic within two or more varieties, while 2 bands were unique-polymorphic. The amplified fragments ranged from 58-734 bp. The largest amplicon of 734 bp was amplified by SSR primer AJ12 and smallest fragment of 58 bp was found with SSR primer AJ16. The percent polymorphism obtained for SSR primers were ranged from 0% to 100%. The polymorphic information content (PIC) was calculated for each primer was ranged from 0.0 to 0.59 respectively. The SSR primer index (SPI) was ranged from 0.55 (AJ14 and AJ18) and 1.77 (AJ16).

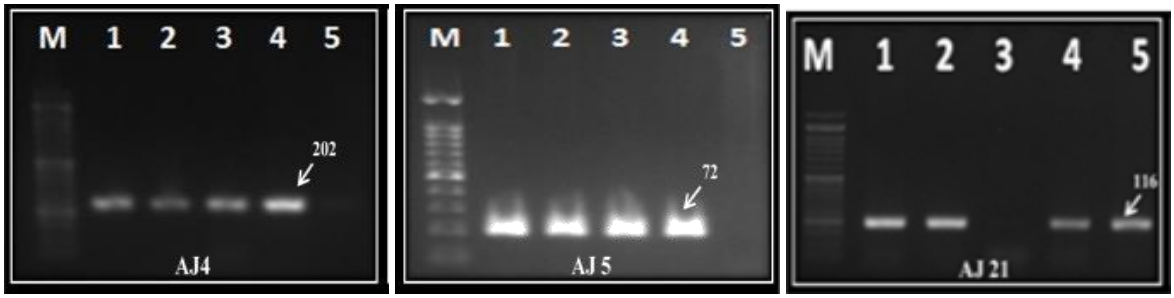

Fig 5: Primer amplification 1: Gujarat Ajwain 1, 2: Gujarat Ajwain 2, 3: AjmerAjwain 1, 4: Ajmer Ajwain 2 and 5: AjmerAjwain 93

Conclusion

The whole study provides important information about the biological functions, molecular functions and cellular components occurring in ajwain. This study can be of great importance in the field of research to understand metabolic pathways and genetic basis of all the physiological processes occurring in ajwain plant. It also provides data through which we can identify gene and functional elements of genome and give basis for annotation of complete plant genome. The designed primers having putative function can be used in genetic diversity studies.

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