De Novo Transcriptome Sequencing Reveals Important Molecular Networks and Metabolic Pathways of the Plant, Chlorophytum borivilianum

Shikha Kalra1, Bhanwar Lal Puniya2, Deepika Kulshreshtha2, Sunil Kumar1, Jagdeep Kaur1, Srinivasan Ramachandran2, Kashmir Singh1*

1Department of Biotechnology, Panjab University, Chandigarh, India, 2G N Ramachandran Knowledge Center, Institute of Genomics and Integrative Biology (Council of Scientific and Industrial Research), New Delhi, India

Abstract

Chlorophytum borivilianum, an endangered medicinal plant species is highly recognized for its aphrodisiac properties provided by saponins present in the plant. The transcriptome information of this species is limited and only few hundred expressed sequence tags (ESTs) are available in the public databases. To gain molecular insight of this plant, high throughput transcriptome sequencing of leaf RNA was carried out using Illumina's HiSeq 2000 sequencing platform. A total of 22,161,444 single end reads were retrieved after quality filtering. Available (e.g., De-Bruijn/Eulerian graph) and in-house developed bioinformatics tools were used for assembly and annotation of transcriptome. A total of 101,141 assembled transcripts were obtained, with coverage size of 22.42 Mb and average length of 221 bp. Guanine-cytosine (GC) content was found to be 44%. Bioinformatics analysis, using non-redundant proteins, gene ontology (GO), enzyme commission (EC) and kyoto encyclopedia of genes and genomes (KEGG) databases, extracted all the known enzymes involved in saponin and flavonoid biosynthesis. Few genes of the alkaloid biosynthesis, along with anticancer and plant defense genes, were also discovered. Additionally, several cytochrome P450 (CYP450) and glycosyltransferase unique sequences were also found. We identified simple sequence repeat motifs in transcripts with an abundance of di-nucleotide simple sequence repeat (SSR; 43.1%) markers. Large scale expression profiling through Reads per KiloBase per Million mapped reads (RPKM) showed major genes involved in different metabolic pathways of the plant. Genes, expressed sequence tags (ESTs) and unique sequences from this study provide an important resource for the scientific community, interested in the molecular genetics and functional genomics of C. borivilianum.

Introduction

Chlorophytum borivilianum is an important species of liliaceae family due to its exceptional medicinal properties. Overexploitation and extensive harvesting of the wild strands has threatened its status as ‘endangered’ species by International Union for Conservation of Nature and Natural Resources (IUCN) [1]. Due to its high medicinal properties, the species has been recognized as 26th among the top priority medicinal plants to be protected and promoted by the Medicinal Plant Board, Government of India. Chlorophytum borivilianum brags the exuberant references in many Ayurvedic classics like Charka Samhita (2nd century B.C.), Sushrut Samhita (2nd century A.D.), Raja Nighantu (17th century A.D.) etc. (http://www.safedmusli.net). Its tubers are used for aphrodisiac, adaptogen, antiaging, health restorative and health promoting purposes. The amalgamation of C. borivilianum leaves with other herbs such as Withania somnifera, Emblica officinalis etc. makes the body resistant against sex related diseases and also delays menopause [2]. The above attributes have made C. borivilianum an essential ingredient in Ayurvedic, Unani and Allopathic formulations. Major phytochemical components reported from the roots of C. borivilianum include steroidal saponins, fructans and fructoligosaccharides (FOS), acetylated mannans, phenolic compounds and proteins [3,4]. Steroidal saponins, are considered to be the principal bioactive components responsible for the pharmacological properties [5] and borivilianosides, furostane type steroidal saponins, have been isolated and characterized from this plant [6,7].

Steroidal saponins are synthesized via the mevalonic acid (MVA) pathway, pervasively operating in cytoplasm [8], or through the newly discovered non-mevalonate pathway (MEP) located in plastids [9,10]. Cyclization of precursor compound, 2, 3-oxidosqualene, involving oxidosqualene cyclase (OSC) combined with modifications on steroid skeletons like hydroxylations and glycosylations lead to the formation of various saponins. Several OSC genes like cycloartenol synthase (CAS), farnesyl synthase (LS), β-amyrin synthase (β-AS) have been cloned from various plant systems [11,12]. According to the proposed pathway [13], some specific CYP450s and UDP-glycosyltransferases (UGTs) may catalyze the conversion of cycloartenol to various steroidal
saponins. Little is known about the molecular mechanism of the biosynthetic pathway downstream of cyclization process involved in saponin biosynthesis. The functional genomics studies in *C. borivilianum* is still in its infancy and few expressed sequence tags (ESTs) have been generated by our group [13], with an aim to identify the differentially expressed genes in the leaf and root tissues of *C. borivilianum*. Also, efforts are being made to clone the genes of interest via the candidate gene approach [14] for this plant.

ESTs play significant role in accelerating gene discovery, especially in non model organisms where reference genome sequence is unavailable, as it is a rapid and relatively economical method for analyzing the transcribed region of the genome [15]. Besides this, ESTs are also helpful in large scale expression analysis, improving genome annotation, identifying splice variants, molecular markers identification and physical mapping [16]. For large scale transcriptome analysis, next generation sequencing has evolved to be a very useful technique for providing large expression data in much shorter time period, depth and coverage to expedite understanding of metabolic pathway as well as contribute to comparative transcriptomics, evolutionary genomics and gene discovery [17,18]. Illumina high throughput sequencing technology yields huge amount of parallel sequence short reads with larger coverage. Assembling these short read sequences is a challenging task in the absence of any reference sequence. However, many *de novo* assembly tools have been developed that can be used to analyze the short read sequences [19,20].

We have undertaken the first global analysis of *C. borivilianum* transcriptome. Strategy has been developed for *de novo* assembly of transcriptome using short-read sequence data generated by Illumina RNA-Seq method in lieu to identify candidate genes involved in saponin biosynthetic pathway. Furthermore, GC content analysis, identification of EST-SSRs and gene expression analysis has also been done. Transcriptome coverage, at 22.42 megabase pairs, was comprehensive enough to discover all known genes of several major metabolic pathways. The data has been submitted to the Sequence Read Archive (SRA) of NCBI database under the accession ID PRJNA 196968 and will serve as a public information platform for further studies in *C. borivilianum*.

**Materials and Methods**

2.1 Plant Material, growth conditions and saponins extraction

Plants of *C. borivilianum* were grown under controlled conditions in the growth chamber with a day/night temperature of 27±1°C, for 16 h photoperiod (flux density of 200 μmol m$^{-2}$ s$^{-1}$) with 30% relative humidity at Panjab University, Chandigarh (India). Plants were regenerated from the previous year's roots in the month of April. Young leaves and roots of a two month old plant were harvested during the period of active growth (June, average outside temperature 38°C), snap frozen in liquid nitrogen and later transferred to a −80°C freezer until further processing.

Total saponins were isolated from leaf and root tissues by soxhletation method and analyzed by thin layer chromatography (TLC) [21]. Briefly, powdered tissues were defatted with petroleum ether (60–80°C). Ethanolic extracts were prepared by extracting defatted tissues with 95% ethanol in a soxhlet extractor. Ethanolic extract was suspended in water (100 ml) and then extracted with n-butanol (300 ml). The volume of n-butanol soluble portion was reduced to half under reduced pressure and finally saponins were precipitated by addition of diethyl ether. Chromatographic analysis of ethanolic extract was performed on silica gel-GF254 precoated plates using chloroform: glacial acetic acid: methanol: water (16:8:3:2, v/v) as mobile phase. Anisaldehyde (0.5 ml) mixed with glacial acetic acid (10 ml), methanol (85 ml) and concentrated sulphuric acid (5 ml) was used as spraying reagent. TLC plates, after spraying the reagent, were heated at 100°C and saponin spots were visualized.

2.2 RNA isolation, cDNA library construction and Illumina sequencing

Total RNA was extracted by combining the methods as described by Ghawana et al [2011], [22] followed by RNA purification and on column DNaseI digestion using miRNA Easy kit (Qiagen, Germany). The integrity of RNA samples was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Sample with RNA integrity number (RIN) value more than 8.0 was selected for further use.

Library construction and sequencing was performed at Microarray core facility, Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah, USA. cDNA library was generated using TruSeq™ RNA Sample preparation kit (Illumina, USA) according to the manufacturer's instructions. Briefly, oligo(dT) beads were used to purify poly(A) mRNA from total RNA. mRNA was fragmented using RNA fragmentation kit (Ambion, USA). First strand cDNA was synthesized from the fragmented mRNA using random hexamer primer and reverse transcriptase (Invitrogen, USA). Single-end cDNA library was prepared in accordance with Illumina’s protocol with an insert size of 150 bp. 50-cycled single end library sequencing was performed using Illumina Hiseq2000 platform. Raw reads were filtered to obtain high-quality clean reads by removing adaptor sequences, duplication sequences using FastQC software and bases with Phred score <20 were trimmed. Based on the quality check for each base pair in the reads, last two base pairs from each read were removed in order to minimize the sequencing error, which is usually higher in the 3' end of reads.

2.3 *De novo* assembly of the sequences and clustering

All the assemblies were performed on Red Hat based SGI workstation with 48 cores, 2.27 GHz Intel Xeon processor and 50 Gb random access memory. We used SOAPdenovo (version 1.04; http://soap.genomics.org.cn/soapdenovo.html) which applies de Bruijn graph algorithm for *de novo* assembly of high quality (HQ) sequence reads to generate a non-redundant set of transcripts. Clean reads were first split into different ‘k-mers’ for assembly, in order to produce contigs, using the de Bruijn graph. K-mer size of 23 achieved best balance between the number of contigs produced, coverage and average sequence length obtained. In order to reduce sequence redundancy, clustering process was supplemented with TIGR gene indices clustering tools (TGICL), which is based on pairwise sequence similarity and then assembly by individual clusters in order to retrieve better results [23]. Clusters were then passed to contig assembly program (CAP3) assembler for multiple alignments and consensus building. TGICL and CAP3 assembly were run under default parameters. Resulting singletons and consensus contigs were merged for final assembled transcripts.

2.4 Functional annotation and Classification

All the assembled unigenes (consensus and singletons) longer than 100 bp were annotated by assigning putative gene descriptions and Gene Ontology (GO) terms on the basis of sequence similarity with previously identified genes annotated with similar details. Unigenes were subjected to BLASTX search against non-redundant protein database and significant hits with e-value ≤1.0e−05 were extracted. Transcripts that did not show any
significant hit were searched using BLASTN tool against the non-redundant database. Functional categorization by GO terms (http://www.geneontology.org) [24] and Enzyme Commission (EC) database was carried out based on AnnoMR tool [http://www.nematodes.org/bioinformatics/annoMR/] with E-value threshold of 1.0e-05. GOslim terms for molecular function, biological process, and cellular component categories associated with the best BLASTX hit with non-redundant database were assigned to the corresponding *C. borivilianum* transcripts. KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/home.do) software was used for annotation of transcripts with KEGG pathways. For this, we first used KEGG orthology data provided at website and locally BLAST all of transcripts with KEGG pathways. Identification of Transcription Factor families represented in *C. borivilianum* transcriptome, transcripts were probed against all *C. borivilianum* transcripts. The 'Identify' program in KOBAS 2.0 uses transcript ID's to KO terms which finally annotate the transcripts with KEGG pathways. The 'Identify' program in KOBAS 2.0 uses annotation to identify enriched pathways. Arabidopsis thaliana, being a monocot, was used as background species. Hyper geometric distribution was used for p-value calculation. The false discovery rate (FDR) was calculated by using q-value and being a model organism was also used as background species.

2.5 Read mapping onto *C. borivilianum* transcripts

The expression level of each assembled transcript was measured through reads per kilobase per million mapped reads (RPKM) values. All reads were mapped onto the non-redundant set of transcripts to quantify the abundance of assembled transcripts. SeqMap [25] was used for read mapping and rSeq [26] was then applied for RPKM based expression measurement. Assembled sequences were used as reference sequence to map back short reads and to measure RPKM for all assembled transcripts as suggested by Mortazavi et al. [26] and Jiang and Wong [25]. For RPKM measurement, filtered reads were first mapped back to various assembled transcripts and total mapped reads were estimated. Unique mapped reads were assigned to each assembled transcript allowing maximum two mismatches. For each such cluster having similar sequences in database, longest sequence was considered as the representative sequence for the unique gene it represented. The associated GO terms and Id's were parsed for each of such sequence and their corresponding RPKM values for different assembled genes were calculated.

2.6 GC content analysis and Simple Sequence Repeats (SSRs) identification

GC content analysis was done using in-house developed R script. A Perl script known as MiCroSATellite (MISA, http://pgrc.ipk-gatersleben.de/misa/) was used to identify SSRs in the unigenes. Repeats of mono-nucleotide more than 10 times, di-nucleotide repeats more than 6 times, tri-, tetra-, penta- and hexa-nucleotide repeats more than 5 times were considered as search criteria in MISA script.

2.7 Identification of Transcription Factor families

For identification of transcription factor families represented in *C. borivilianum* transcriptome, transcripts were probed against all the transcription factor protein sequences at Plant transcription factor database (PhTTFDB; http://phl.tfdb.bio.uni-potsdam.de/v3.0/downloads.php) using BLASTX with an e-value cut-off of 1.0e-05.

### Results and Discussion

#### 3.1 Transcriptome sequencing and *de novo* assembly

Illumina Hiseq 2000 (Illumina, USA) run representing the cDNA library from leaf tissues produced 22,595,634 single end (SE) reads. Each read was 50 bp in length encompassing nearly 4.0 Gb of sequencing data in fastq format. Since 3rd ends of reads are more prone to sequencing error, so after quality check for every 50 bp read, only 48 bases (excluding 2 bases at 3rd end) were considered for further analysis. Sequence data was filtered for low-quality reads and reads containing primer/adaptor sequences. Reads having the Phred quality score ≥20 (an error probability of 0.01) were selected for further use. After quality filtering, a total of 22,161,444 high-quality SE reads (3.8 Gb, 95% of the raw data) remained. Quality of the clean reads data was assessed using FASTX Toolkit (www.hannonlab.cshl.edu/fastxtoolkit).

*De novo* assembly of *C. borivilianum* transcriptome was optimized after assessing effect of various k-mer lengths. The high quality trimmed reads (48 bp) were assembled using SOAPdenovo program at k-mer length of 17, 21, 23, 25, 27, 31, and 35 (Table 1). Total number of transcripts decreased linearly with the increment of k-mer size suggesting over-representation at lower k-mer and under-representation at higher k-mers. It was observed that sequences assembled at higher k-mer were enriched for transcripts with higher coverage/higher expression. For assembly process, only those reads were considered that produced high frequency k-mer. Several output parameters were analyzed that included total number of contigs, contigs with length 100 bp and above, N50 length, longest contig length, and average contig length as a function of k-mer. For the above mentioned data set, k-mer size of 23 emerged as the best for assembly with N50 length of 245 bp, largest contig length 3,168 bp and average contig length of 221 bp (Table 1). A total of 101,589 contigs having length of at least 100 bp were generated. These contigs made the final representatives for assembled sequences for this study. Total bases covered by contigs with length greater than 100 bp and above came out to be 22.47 Mb (Table 2). Although the majority of the contigs lies between 1 bp to 100 bp, a total of 6568 contigs with a size of 500 bp and above were generated (Fig 1a).

#### 3.2 Sequence clustering and similarity search

To reduce any redundancy, assembled sequences from the above analysis were clustered by hierarchical clustering with TGICL. [27], which generated 1273 clusters. These clusters were further assembled using CAP3 program [28] and using 896 sequences, 448 consensus sequences were generated. This resulted in reduction of uniquely assembled contigs from 101,589 to 101,141. Contigs shorter than 100 bp were removed and total coverage with contig length ≥100 bp came out to be 22.42 Mb. Although majority of the contigs lie between 150 bp to 500 bp, we obtained a total of 6,290 contigs with a size of >500 bp (Fig 1b).

For contig annotation, similarity search was performed using BLASTX against the non-redundant (NR) protein database with an E-value cut-off 1.0e-05. For read assembled transcript sequences, significant BLASTX hits were found for a total of 50,826 sequences (50.4% of the total unigenes obtained; Table S1). The E-value distribution of the top hits in the NR database showed that 15.9% of the mapped sequences have high similarity (<1.0e-30), while the other 84.1% of sequences, similarity ranged between 1.0e-05 to 1.0e-30 (Fig 2a). The similarity distribution revealed 67.5% of the query sequences have a similarity higher than 80%, while 32.4% of the unigenes have a similarity ranging from 20% to 80% (Fig 2b). Homologous genes come from different species, with 23.5% of the unigenes having the highest homology
to genes from *Vitis vinifera* followed by *Oryza sativa* (16.3%), *Populus trichocarpa* (10.8%), *Ricinus communis* (9.0%), *Glycine max* (8.1%) and *Sorghum bicolor* (7.2%) (Fig 2c).

Due to lack of *C. borivilianum* genome information, nearly half of the unigenes did not show a match in the plant protein dataset of NR database. Moreover, 63.4% of the above mentioned 50,826 annotation descriptions were uninformative (e.g., ‘unknown’, ‘unnamed’, ‘putative’, or ‘hypothetical’ protein).

3.3 Validation of assembled sequences against the available ESTs of *C. borivilianum*

The assembled sequences were validated by sequence alignments against ESTs of *C. borivilianum* submitted at NCBI dbEST by our group [13]. BLASTN analysis of assembled transcripts was performed with 459 ESTs with an E-value threshold of 1.0e-05 (Table S2). Alignment parameters were manually checked and also analyzed for mis- assemblies. Significant hits were observed for 394 sequences (85.8%), while no hit could be obtained for 65 ESTs from the assembled transcript set. It was observed that most of the assembled transcript sequences aligned correctly and in continuous form, with average identity of 95.67%, showing good assembly quality. The possible reason for unmapped unigenes of *C. borivilianum* could be due the presence of relatively short and low quality singletons, UTR sequences far from the translation start or stop sites (>1,000 bp), fusion transcripts and those having incomplete coverage by the genome. Similar results were shown in *A. thaliana* where around 13% of the ESTs could not be aligned to the predicted genes [29] and in human only 64% of the reads could be mapped to the RefSeq database of well annotated human genes [30]. *Picorrhiza kurrooa* had 14% of the ESTs that could not be aligned to its predicted genes [31].

3.4 Functional annotation and classification of *C. borivilianum* transcriptome

*Chlorophytum borivilianum* transcripts were assigned GO terms to describe functions of genes and associated gene products into three major categories namely, biological process, molecular function, and cellular component, and their sub-categories [24]. A total of 30,625 out of 50,826 assembled sequences yielded significant annotation against GO database. These genes were further classified into three major categories namely, biological process, molecular function, and cellular component using plant specific GO slim that broadly provides an overview of the ontology content. Functional classification of *C. borivilianum* transcripts in
Table 1. Effect of k-mer size on assembly of transcriptome data.

| k-mer | Total contigs | Contigs >100 bp | Longest contig length (bp) | Average length (bp) | Total bases covered by 100 bp length | N50 |
|-------|---------------|----------------|---------------------------|--------------------|--------------------------------------|-----|
| 17    | 1079131       | 99031          | 1862                      | 178                | 17930159                             | 180 |
| 21    | 516724        | 101445         | 3216                      | 220                | 22401122                             | 243 |
| 23    | 408567        | 101589         | 3168                      | 221                | 2247281                              | 245 |
| 25    | 337186        | 101765         | 3179                      | 216                | 2207354                              | 240 |
| 27    | 276611        | 101491         | 5256                      | 211                | 2240112                              | 232 |
| 31    | 183200        | 95717          | 5256                      | 199                | 1911429                              | 211 |

doi:10.1371/journal.pone.0083336.t001

biological process category (Fig 3) showed that metabolic process (GO: 0008152), response to stimulus (GO: 0050896), nucleobase containing compound metabolic process (GO: 0006139) and extracellular structural organization (GO: 0009967) were among the highly represented groups indicating that the plant is undergoing rapid growth and extensive metabolic activity. In cellular component group, sequences related to the intracellular regions (GO: 0005622) and membrane regions were well-represented categories (GO: 0016020) (Fig 3). Transcripts belonging to major subgroups of molecular function category included binding functions (GO: 0005488), transferase activity (GO: 0016740), catalytic activity (GO: 0003824) and oxidoreductase activity (GO: 0016149) (Fig 3). These GO annotations provide a comprehensive information on C. borivilianum expressed genes that are encoding diverse proteins.

Best EC classification was obtained for 14,998 assembled sequences, which were annotated with 15,866 enzyme codes. Fig 4 lists major 20 abundant enzyme classes observed for C. borivilianum transcriptome. Interestingly, a large amount of assembled transcripts belonged to non-specific protein tyrosine kinases enzyme class alone (14.7%), in arabidopsis, this enzyme controls shoot and floral meristem size [32] and also contributes to signal transduction [33]. Presence of this enzyme in such abundance may suggest that the plant is in active metabolic phase.

We also compared KEGG pathways mapping of C. borivilianum with KEGG mapping of O. sativa and A. thaliana. Sample score corresponds to the transcripts of C. borivilianum mapped on KEGG pathways and background score is the total genes in the genome of the background species mapped on KEGG pathways. We found 50,034 transcripts showing similarity with GO database at e-value cut-off of 1e-05. These transcripts were used for annotation of KEGG pathways. These transcripts were annotated with 257 KEGG pathways which were present in O. sativa and A. thaliana. A total of 236 pathways were common in both of background species. 10 unique pathways of O. sativa and 11 of A. thaliana were also mapped. Total 3,805 transcripts were mapped on these KEGG pathways common with O. sativa and 3,806 transcripts were mapped on KEGG pathways common with A. thaliana. Out of these 43 KEGG pathways were significantly enriched (p-Value <0.05) with the genes present in O. sativa. Non-homologous end joining, Lipopolysaccharide biosynthesis, Spliceosome, RIG-I like receptor signaling pathway, mineral absorption, GPI-anchor biosynthesis, RNA transport, Basal transcription factors, N-Glycan biosynthesis and RNA degradation are top enriched pathways. In comparison to A. thaliana, 42 pathways were identified as enriched (p-value <0.05). Spliceosome, RNA degradation, RNA transport, Pyrimidine metabolism and GPI anchor biosynthesis were found to be most enriched pathways in comparison with A. thaliana. A total of 100 transcripts in C. borivilianum were mapped on spliceosome KEGG pathway compared to 106 genes of O. sativa and 117 genes of A. thaliana. Highest number transcripts (106) were mapped to Ribosome pathways in C. borivilianum compared to 257 genes of O. sativa and 311 genes of A. thaliana. Other pathways with highest transcripts mapping include RNA transport (96), Purine metabolism (79) and protein processing in endoplasm (73), thus suggesting that the plant was in actively growing stage at the time of harvesting. Besides all these pathways, flavonoid biosynthesis and steroid biosynthesis were the most enriched pathways in C. borivilianum. The enrichment of these pathways clearly suggested that the plant possess antioxidant properties, anti-arthritic properties, and anticancer properties. Pathways comparison (Fisher’s Exact P-value <0.05) with respect to the background species O. sativa and A. thaliana is shown in Fig 5a and 5b. All the data of pathways mapping is provided in Table S3A and Table S3B for O. sativa and A. thaliana respectively.

3.5 Discovery of the transcripts encoding putative transcription factors

Transcription factors interact with the promoter regions of a gene and modulate its expression. Associative modulation of several plant processes suggests involvement of transcription factors (TFs) for coordinated regulation of gene expression. By sequence comparison with known transcription factor gene families, 8,369 putative C. borivilianum transcription factor genes, distributed in at least 62 families, were identified representing 8.3% of C. borivilianum transcripts (Fig 6). These genes covered transcription factor gene families like MYB, MADS, Orphans, basic Helix-Loop-Helix (bHLH), bZIP, WRKY and many more. Among all these TF gene families, C3H, PHD and MIB were most abundant families (Table S4). MYB family proteins are characterized by DNA-binding domains and it is the largest transcription factor family in Arabidopsis thaliana as well, where it comprises of 163
genes [34]. These TFs have been associated with varied processes. It has been observed that many protein members of the MYB, bZIP and WRKY transcription factor families insinuates the regulation of stress responses [35]. Members of C3H family are involved in embryogenesis [36] and members of PHD family TFs are involved in vernalization processes [37]. bHLH members are involved in controlling cell proliferation and development of specific cell lineages [38] and bZIP regulates processes including pathogen defense light and stress signaling seed maturation and flower development. A complete picture about evolution of various transcription factor families will emerge once the complete genome sequence of \textit{C. borivilianum} will be available.

3.6 GC content and SSRs analysis from the transcriptome of \textit{C. borivilianum}

Deep sequencing of \textit{C. borivilianum} transcriptome rendered us with an opportunity to calculate the ratio of GC contents. Knowledge of GC content helps in understanding ecology and evolution of particular taxa. It also plays a vital role in gene and genome regulation and also helps in determining the physical properties of the genome. It is an important indicator of stability of DNA [39]. Average GC content of \textit{C. borivilianum} transcripts was 44\% (Figure S1). This is in range with the GC levels of coding sequences of \textit{O. sativa} (43.6\%) which also belong to monocot family.

Assembly of \textit{C. borivilianum} was further assessed for the molecular markers. Morphological as well as biochemical markers are used in the authentication of herbal drugs. SSRs or microsatellite markers are polymorphic stretches of 1 to 6 nucleotide units repeated in tandem and randomly spread in eukaryotic genomes. SSRs are generally associated with functional and phenotypic variations. Moreover, SSRs allow identification of many alleles at a single locus, are easy to develop, distributed evenly all over the genome and are co-dominant [40]. SSRs are particularly useful when genome information of the crop is lacking. Since \textit{C. borivilianum} is a cross pollinated species [41], and hence seed raised population will have variability.

For identification of SSRs, transcripts of \textit{C. borivilianum} were analyzed with perl script MISA. 3,487 SSRs were identified in 3,321 (3.28\%) transcripts comprehensively out of which, 160 sequences contained more than 1 SSR. With a frequency of over 43.1\% (1,503/3,321), di-nucleotides were most abundant of all the SSRs obtained. Tri-nucleotides were more than 32.89\% (1,147/...
followed by tetra-nucleotides (0.4%, 15/3,487) and penta-nucleotides (0.05%, 2/3,487). Most prevalent of mononucleotide was poly-A. The edge of poly-A mono-nucleotide SSRs over others may be due to the presence of poly-A tails in the RNA sequences (Table S5). Di-nucleotide SSRs were the most abundant in the transcripts, which is similar to results obtained from other plants [42]. Among the di-nucleotide repeat classes, AG/GA/CT/TC (90.7%) was the most frequent dimer motif. Other dimer motifs included TG/CA and AT/TA (Table S5). CG repeats were infrequent in the plant (0.6%), which is consistent with previous observations [42,43]. Among tri-nucleotide repeats, CTC/CCT/GAG/GAA was the largest repeat class followed by CTT/AAG/GGA/AGA (Table 3). These findings indicated that unique sequences containing SSR markers were indeed abundant in C. borivilianum. Di- and tri-nucleotide SSR motifs and the number of repeats are presented in Table 3. In particular, several SSR motifs were linked with the unique sequences encoding enzymes (e.g. MVK, DXS, SqS) involved in saponin biosynthesis (Table 4). These unique sequence-derived markers generated in this study represent a valuable genetic resource for future studies in this and related species.

### 3.7 Analysis of metabolic pathway genes

Saponins present in C. borivilianum are the primary source of its significant medicinal properties and are synthesized by mevalonate and non-mevalonate pathways in plants. TLC analysis indicated that significant levels of saponins were present in C. borivilianum tissues (Figure S2). Nearly 10 spots were visualized on TLC plate upon derivatization with anisaldehyde - sulfuric acid reagent. Previous studies reported that initial reactions of isoprenoid biosynthetic pathway occur in leaves, while later step modifications and storage of saponins occurs in roots [13,14], thus the amount of saponins is high in roots. There are also few reports on the presence of flavonoid compounds in C. borivilianum [44]. These reports very well validate the presence of saponins and flavonoids in C. borivilianum, thus, our interest was to identify the genes responsible for biosynthesis of saponins and other metabolites in our dataset. The genes related to following pathways/compounds were identified:

#### 3.7.1. Saponin biosynthesis pathway

Saponins are derived from geranyl pyrophosphate (GPP). GPP is synthesized by sequential head to tail addition of isopentenyl pyrophosphate (IPP) and its allelic isomer dimethylallyl pyrophosphate (DMAPP) [45]. IPP and DMAPP are synthesized via the cross talks between the cytosolic mevalonate (MVA) pathway and plastidial 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [46]. MVA pathway starts from the condensation of acetyl-CoA [47], whereas MEP pathway needs pyruvate and 3-phospho-D-glycerate. Both biosynthetic routes yield the activated isoprene units, dimethylallyl diphosphate (DMAPP) and IPP [49]. Genes involved in MVA pathway that were found in our transcriptome included acetyl CoA- acetyltransferase (EC 2.3.1.9, 5 unigenes), HMG-CoA reductase (EC 1.1.3.34, 13 unigenes), HMG-CoA synthase (EC 2.3.3.10, 15 unigenes), mevalonate kinase (EC 2.7.1.36, 3 unigenes), phosphomevalonate kinase (EC 2.7.4.2, 2 unigenes), mevalonate diphosphate decarboxylase (EC 4.1.1.33, 2 unigenes). MEP pathway genes are also converted to IPP by reaction catalyzed by series of enzymes. These included 1-deoxy-D-xylulose-5-phosphate synthase (EC 2.2.1.7, 18 unigenes), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (EC 1.1.1.267, 5 unigenes), 2-C-methyl-D-erythritol 4-phosphate
cytidyl transferase (EC 2.7.7.60, 2 unigenes), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (EC 2.7.1.148, 1 unigene), 2-C-methyl-D-erythritol, 2,4-cyclodiphosphate synthase (EC 4.6.1.12, 1 unigene), 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (EC 1.17.7.1, 3 unigenes), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC 1.17.1.2, 13 unigenes). IPP was then converted to squalene by a series of enzymes which included isopentenyl diphosphate isomerase (EC 5.3.3.2, 6 unigenes), geranylgeranyl diphosphate synthase (EC 2.5.1.29, 5 unigenes), farnesyl diphosphate synthase (EC 2.5.1.10, 14 unigenes), squalene synthase (EC 2.5.1.21, 9 unigenes). Subsequently, squalene monooxygenase (EC 1.14.13.132, 14 unigenes) catalyzes the conversion of squalene to 2,3-oxidosqualene. The cyclization event of 2,3-oxidosqualene is catalyzed by a class of enzymes, OSCs. Cyclization of 2,3-oxidosqualene is rate limiting step and this event is also the branch point for sterol and triterpenoid biosynthesis in many plants [50]. Three OSC genes of C. borivilianum, cycloartenol synthase (E.C. 5.4.99.8, 16 unigenes) [51], β-amyrin synthase (E.C. 5.4.99.39, 1 unigene) [52] and Dammarenediol II synthase (E.C. 4.2.1.125, 1 unigene) [53] exist in our dataset. Steroidal saponins, borivilianosides, are the major type of saponins present in C. borivilianum [6]. As yet, no reports reveal the presence of triterpenoid type of saponins in C. borivilianum. However, two singleton sequences (Transcript ID: 664097 and 798016) matched with β-amyrin synthase and Dammarenediol II synthase of V. vinifera and R. communis respectively.

3.7.2 Flavonoid biosynthesis pathway. Genes associated with flavonoid biosynthesis pathway were also detected in the dataset (Fig 7b). Flavonoids are synthesized via the phenylpropanoid pathway and are converted from phenylalanine to chalcone by the enzymes phenylalanine ammonia lyase (EC 4.3.1.24, 25
unigenes), 4-coumarate CoA ligase (EC 6.2.1.12, 23 unigenes), and chalcone synthase (EC 2.5.1.74, 16 unigenes). Chalcone isomerase (EC 5.5.1.6, 12 unigenes) then catalyzes the isomerization of chalcones into naringenin. Naringenin can be converted by flavonoid 3′-hydroxylase (EC 1.14.13.21, 18 unigenes, 421.18) and flavonoid 3′, 5′-hydroxylase (EC 1.14.13.88, 3 unigenes) to produce eriodictyol and dihydrotricetin, respectively. Flavone synthase (EC 1.14.11.22, 1 unigene) catalyzes the conversion of flavanones to flavones, and flavone 3′-hydroxylase (EC 1.14.11.9, 24 unigenes) can convert these flavanones to dihydroflavonols. Dihydroflavonols can then lead to production of flavonols and flavan-3, 4-diols (leucoanthocyanidin), reactions being catalyzed by flavonol synthase (EC 1.14.11.23, 9 unigenes) and by dihydroflavonol 4-reductase (EC 1.1.1.219, 6 unigenes) respectively. Leucoanthocyanidins can be converted either to anthocyanidins and subsequently anthocyanins through the subsequent action of anthocyanidin synthase (EC 1.14.11.19, 6 unigenes) or reduced to catechins through the action of the enzyme and anthocyanidin reductase (EC 1.3.1.77, 8 unigenes). Genes of the phenylpropanoid pathway have already been reported in several plant species such as *Camellia sinensis* [54,55], *Petroselinum hortense* (parsley) [56], *Zea mays* [57], *Arabidopsis thaliana* [58], *Vitis vinifera* [59], *Citrus unshiu* (Marc.) [60] and *Fragaria* spp. [61]. Above descriptions showed vertical pathway responsible for the formation and conversion of the sub-categories of flavonoids.

Another revelation came out with the genes involved in isoflavonoid metabolism. These genes included 2-hydroxyisoflavone synthase (EC 1.14.13.86, 3 unigenes), isoflavone 4′-O-methyltransferase (EC 2.1.1.46, 1 unigene), isoflavone 7-O-methyltransferase (EC 2.1.1.150, 2 unigenes), isoflavone 2′-hydroxylase (EC 1.14.13.89, 9 unigenes) and isoflavone reductase (EC 1.3.1.45, 12 unigenes). *C. borivilianum* is not known to produce isoflavones. Similar kinds of findings have been reported in other non-isoflavone accumulating plants [62], suggesting homologs of isoflavone reductase and isoflavone O-methyltransferase may have general metabolic functions in many plant species.

### 3.7.3. Discovery of transcripts encoding CYPs and UGTs involved putatively in saponin and flavonoid metabolism

**Table 3.** Simple sequence repeats (SSRs) identified in transcripts of *C. borivilianum*.

| SSR mining | Number |
|------------|--------|
| Total number of sequences examined: | 10,11,41 |
| Total size of examined sequences (Mb): | 22.42 |
| Total number of identified SSRs: | 3,487 |
| Number of SSR containing sequences: | 3,321 (3.28%) |
| Number of sequences containing more than one SSR: | 160 |
| Number of SSRs present in compound formation: | 109 |

| Distribution of SSRs in different repeat types Unit size | Number of SSRs (%) |
|--------------------------------------------------------|---------------------|
| Mononucleotide | 820 (23.42%) |
| Dinucleotide | 1,503 (43.1%) |
| Trinucleotide | 1,147 (32.89%) |
| Tetrancleotide | 15 (0.4%) |
| Pentanucleotide | 2 (0.05%) |

To put it in a table for easy reading:

| Table 3. Simple sequence repeats (SSRs) identified in transcripts of *C. borivilianum*. |
|----------|
| **SSR mining** | **Number** |
| Total number of sequences examined: | 10,11,41 |
| Total size of examined sequences (Mb): | 22.42 |
| Total number of identified SSRs: | 3,487 |
| Number of SSR containing sequences: | 3,321 (3.28%) |
| Number of sequences containing more than one SSR: | 160 |
| Number of SSRs present in compound formation: | 109 |

| Distribution of SSRs in different repeat types Unit size | Number of SSRs (%) |
|--------------------------------------------------------|---------------------|
| Mononucleotide | 820 (23.42%) |
| Dinucleotide | 1,503 (43.1%) |
| Trinucleotide | 1,147 (32.89%) |
| Tetrancleotide | 15 (0.4%) |
| Pentanucleotide | 2 (0.05%) |
biosynthesis. Cytochrome P450 (CYP 450) are versatile biocatalysts. These enzymes constitute largest family of plant proteins (http://drnelson.utmem.edu/CytochromeP450.html). CYP450s are involved in the NADPH-dependent regio- and stereo-specific oxygenations during the biosynthesis of terpenoids, sterols, lignins, hormones, fatty acids, pigments, and phytoalexins in plants [63]. In biosynthesis of steroidal-type of saponins, CYP450 catalyzes the conversion of obtusifoliol to $D_8, 14-$ sterol. A total of 490 unique transcripts, were annotated as CYP450s (Table S6). Previous studies reported characterization of obtusifoliol 14-alpha-demethylase (EC 1.14.13.70, 7 unigenes) of CYP51 family from *Sorghum bicolor* [64] and *Triticum aestivum* [65] and CYP 710 [66] from *Arabidopsis*, both of which are involved in steroid saponin biosynthesis. Therefore, the CYP450s belonging to CYP51 and CYP710 family might be involved in borivilianoside biosynthesis in *Chlorophytum* genus.

Amongst reactions for the modification of secondary metabolites, glycosylation plays an important role in plants, contributing to biosynthesis and storage of secondary metabolites [67]. UDP-

| Gene name                | Unique sequence | SSR motif | Number of repeats | SSR start (bp) | SSR end (bp) | Sequence length (bp) |
|--------------------------|-----------------|-----------|-------------------|----------------|--------------|----------------------|
| Mevalonate kinase (MVK)  | contig 780100    | ag        | 7                 | 6              | 19           | 1326                 |
| 1-deoxy-D-xylulose 5-     | contig 801760    | ac        | 20                | 1              | 20           | 787                  |
| phosphate synthase (DXS) | contig 809736    | ta        | 16                | 64             | 79           | 1647                 |
| Squalene synthase (Sq)   | contig 676689    | cat       | 16                | 1              | 16           | 355                  |
|                          |                 |           |                   |                |              |                      |

Table 4. The discovery of SSR motifs in putative genes involved in saponin biosynthesis.

doi:10.1371/journal.pone.0083336.t004

Figure 7. *Chlorophytum borivilianum* unigenes involved in two secondary metabolic pathways. *C. borivilianum* unigenes involved in; (A) saponin biosynthesis, (B) flavonoid biosynthesis and (C) alkaloid biosynthesis. Red number in the bracket following each gene name indicates the number of corresponding unigenes.

doi:10.1371/journal.pone.0083336.g007
and flavonol-3-O-glucosyltransferase (EC 2.4.1.91, 46 unigenes). Presence of such glycosylation enzymes and cytochrome P450 genes might contribute to extensive modifications of various secondary metabolites in *C. borivilianum*. 

### 3.7.4. Alkaloid biosynthesis pathway.

Alkaloids are heterocyclic nitrogen compounds synthesized from amino acids and are the largest groups of natural plant products [69]. Potent biological activity of some alkaloids has led to their exploitation as pharmaceuticals, stimulants, narcotics and poisons. Plant-derived alkaloids currently in clinical use include, but not limited to, analgesics morphine and codeine, anticancer agents vinblastine and taxol, gout suppressant colchicine, muscle relaxant (*C.* tubocurarine, antiarrhythmic ajmaline, antibiotic sanguinarine, and sedative scopoamine. Other important alkaloids of plant origin include caffeine, nicotine, cocaine, and synthetic O, O-acetylated morphine derivative heroin.

In *C. borivilianum* transcriptome dataset, in addition to saponins and flavonoids, we also discovered genes involved in the biosynthesis of benzylisoquinoline alkaloids (BIA). Amino acid tyrosine is the precursor of benzylisoquinoline alkaloids. BIA biosynthesis begins with a metabolic lattice of decarboxylations, ortho-hydroxylations and deaminations that convert tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde [70]. This reaction is catalyzed by tyrosine decarboxylase (TYDC; EC 4.1.1.25, 8 unigenes), the only enzyme involved in these early steps that has been purified [71], and for which the corresponding cDNA has been cloned [72,73] (Fig 7c). TYDC cDNAs have also been reported from parsley [74] and *Arabidopsis thaliana* [75], which do not accumulate tyrosine-derived alkaloids. TYDC mRNAs were shown to be rapidly induced in response to elicitor treatment [74,75] and pathogen challenge [76] in various plants. Induction of TYDC mRNAs in parsley and *Arabidopsis* suggests that, in addition to BIAs, tyramine serves as precursor to a ubiquitous class.

### Table 5. Identification of genes involved in saponin biosynthesis along with their RPKM values.

| Gene Name | EC number | Transcript ID | Total ESTs | RPKM value | Number of reads |
|-----------|-----------|---------------|------------|------------|----------------|
| acetyl Co-acetyl transferase | 2.3.1.9 | contig 662055, 789282, 807132, 807650, 810900 | 5 | 420.22 | 2162 |
| HMG-CoA synthase | 2.3.3.10 | contig 617027, 642120, 642122, 642652, 643560, 648291, 662091, 685089, 714060, 719432, 730642, 750334, 752180, 787594, 791328 | 15 | 588.8 | 399 |
| HMG-CoA reductase | 1.1.1.34 | contig 616693, 642352, 726822, 731828, 739034, 750204, 751864, 757982, 761916, 765000, 777172, 789074, 805890 | 13 | 684 | 1144 |
| mevalonate kinase | 2.7.1.36 | contig 780100, 790236, 808328 | 3 | 116.9 | 337 |
| phosphomevalonate kinase | 2.7.4.2 | contig 796764, 802836 | 2 | 83.9 | 351 |
| mevalonate diphosphate decarboxylase | 4.1.1.33 | contig 731872, 807394 | 2 | 183.9 | 932 |
| 1-deoxy-D-xylulose-5-phosphate synthase | 2.2.1.7 | contig 630630, 639174, 659061, 672215, 692780, 701402, 716282, 758550, 770832, 776522, 785368, 788176, 796714, 799960, 801760, 805736, 809736, 812228 | 18 | 33.8 | 31 |
| 1-deoxy-D-xylulose-5-phosphate reductoisomerase | 2.2.1.267 | contig 767146, 807128, 811584 | 3 | 49.4 | 325 |
| 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | 2.7.7.60 | contig 697584, 815196 | 2 | 35.05 | 126 |
| 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase | 2.7.1.148 | contig 815196 | 1 | 30.19 | 302 |
| 2-C-methyl-D-erythritol, 2,4-cyclodiphosphate synthase | 4.6.1.12 | contig 809428 | 1 | 35.16 | 200 |
| 4-hydroxymethylbut-2-enyl diphosphate reductase | 1.7.1.71 | contig 768924, 813016, 815362 | 3 | 91.9 | 179 |
| 4-hydroxymethylbut-2-enyl diphosphate synthase | 1.7.1.2 | contig 663803, 674257, 674259, 697268, 713070, 757110, 770076, 775264, 776012, 780586, 780710, 813560 | 13 | 91.6 | 217 |
| isopentenyl diphosphate isomerase | 5.3.3.2 | contig 622716, 640576, 677655, 684005, 771148, 793800 | 6 | 239.49 | 128 |
| geranylgeranyl diphosphate synthase | 2.5.1.29 | contig 669873, 709788, 771708, 791512, 804002 | 5 | 37.97 | 166 |
| farnesyl diphosphate synthase | 2.5.1.10 | contig 622530, 633119, 669873, 694410, 700520, 709788, 759242, 764802, 771708, 774914, 782044, 785872, 791512, 804002 | 14 | 337.61 | 174 |
| squalene synthase | 2.5.1.21 | contig 660543, 669241, 676689, 684225, 720378, 762052, 771110, 777187, 779602, 805830 | 9 | 178.12 | 119 |
| squalene monoxygenase | 1.14.13.132 | contig 625956, 650031, 684205, 693988, 718888, 724460, 735738, 749834, 752160, 753330, 759096, 772472, 773000, 880304 | 14 | 789.79 | 897 |
| cycloartenol synthase | 5.4.99.8 | contig 634716, 641806, 644535, 660567, 669397, 737170, 761218, 762990, 777848, 809202, 813566, 814320, 815892 | 16 | 177.58 | 100 |
| *β*-amyrin synthase | 5.4.99.39 | contig 664097 | 1 | 30.56 | 21 |
| dammaradienol II synthase | 4.2.1.125 | contig 663453 | 1 | 125.43 | 85 |

doi:10.1371/journal.pone.0083336.t005
methyltransferases (6-O-MT and 4-N-methyltransferase) are condensed by norcoclaurine synthase (EC 2.1.1.128, 6 unigenes), an N-methyltransferase (EC 2.1.1.129, 1 unigene) that catalyses the S-N-debenzoyl-2-deoxytaxol N-benzoyltransferase, taxane 10-beta-hydroxylase (5a-taxadienol-10-beta-hydroxylase) and deacetyl baccatin genes were discovered that are involved in taxol biosynthetic pathway. Few genes namely pulegone reductase, and pulegone reductase, involved in menthol biosynthesis pathway, were also discovered. Menthol biosynthetic genes are primarily involved in plant defense, although there are evidences that menthol has a potent anticancer property, effecting cell death through TRPM8 receptor [82]. Further studies on this pathway could be beneficial as the combination of C. borivilianum with other herbs could be beneficial for cancer treatment.

3.7.5. Discovery of anticancer and plant defense genes. C. borivilianum possesses antitumor and anticancer properties [80]. It contains cytotoxic steroidal glycoside saponinichloromaloside-A and spirostanopentaglycosides embracing beta-D-apiofuranose which are used for their anticancer properties [81]. In the present study, for the first time, we reported the presence of genes involved in taxol biosynthesis. 3’-N-debenzyl-2’-deoxytaxol N-benzoyltransferase, taxane 10-beta-hydroxylase (5a-taxadienol-10-beta-hydroxylase) and deacetyl baccatin genes were discovered that are involved in taxol biosynthetic pathway. Few genes namely 2-deoxytaxol N-benzoyltransferase, taxane 10-beta-hydroxylase (5a-taxadienol-10-beta-hydroxylase) and deacetyl baccatin genes were discovered that are involved in taxol biosynthetic pathway. Few genes namely 2-deoxytaxol N-benzoyltransferase, taxane 10-beta-hydroxylase (5a-taxadienol-10-beta-hydroxylase) and deacetyl baccatin genes were discovered that are involved in taxol biosynthetic pathway. Few genes namely 2-deoxytaxol N-benzoyltransferase, taxane 10-beta-hydroxylase (5a-taxadienol-10-beta-hydroxylase) and deacetyl baccatin genes were discovered that are involved in taxol biosynthetic pathway. Few genes namely 2-deoxytaxol N-benzoyltransferase, taxane 10-beta-hydroxylase (5a-taxadienol-10-beta-hydroxylase) and deacetyl baccatin genes were discovered that are involved in taxol biosynthetic pathway. Few genes namely

| Gene Name                    | EC number | Transcript ID | Total ESTs | RPKM value | Number of read |
|------------------------------|-----------|---------------|------------|------------|---------------|
| phenylalanine ammonia lyase  | 4.3.1.24  | contig 634698, 642066, 657575, 668909, 672281, 691416, 702626, 703366, 703960, 715686, 719064, 735030, 734228, 748710, 753296, 754648, 755468, 760124, 760936, 769576, 777484, 783272, 788484, 790736, 799910 | 25 | 241.09 | 474 |
| cinnamate 4-hydroxylase      | 1.14.13.11| contig 626042, 673051, 688226, 704750, 755308, 758396, 791376, 794622 | 8 | 207.68 | 111 |
| 4-coumarate CoA ligase       | 6.2.1.12  | contig 658459, 673103, 685642, 712208, 722686, 734074, 752094, 752608, 757946, 758914, 760860, 761416, 773542, 776318, 790346, 790364, 792818, 793014, 793670, 803146, 811102, 815080 | 23 | 1206.79 | 979 |
| chalcone synthase            | 2.3.1.74  | contig 619401, 623782, 642216, 644333, 694872, 703744, 711022, 729452, 742538, 750790, 752302, 775504, 776662, 790158, 803096, 804748 | 16 | 158.11 | 83 |
| chalcone isomerase           | 5.5.1.6   | contig 618735, 654743, 666277, 716164, 716334, 761068, 76552, 772452, 776068, 783048, 783534, 790738 | 12 | 343.98 | 174 |
| flavonoid 3’-hydroxylase     | 1.14.13.21| contig 645173, 688012, 697330, 716994, 717416, 719790, 732470, 733296, 748620, 753294, 765426, 780410, 781518, 796884, 799424, 803552, 813496 | 18 | 421.18 | 1013 |
| flavonoid 3’, 5’-hydroxylase | 1.14.13.88| contig 638078, 679673, 705566 | 3 | 31.43 | 18 |
| flavone synthase             | 1.14.11.22| contig 690218 | 1 | 30.66 | 24 |
| flavone 3-hydroxylase        | 1.14.11.9  | contig 617797, 623238, 628840, 631844, 649435, 649459, 655551, 665101, 712318, 715714, 721514, 724718, 728206, 741180, 760132, 763024, 767854, 789424, 790626, 795272, 800076, 801784, 803294, 812892 | 24 | 475.11 | 263 |
| flavonol synthase            | 1.14.11.23 | contig 631844, 656551, 721514, 741180, 763024, 767854, 800076, 801784, 812892 | 9 | 475.11 | 263 |
| dihydrolavonol 4-reductase   | 1.1.1.219  | contig 701984, 702106, 781542, 789910, 789920, 796570 | 6 | 372.12 | 679 |
| anthocyanidin synthase       | 1.14.11.19 | contig 698156, 706394, 733490, 752474, 778182, 794658 | 6 | 18.9 | 61 |
| anthocyanidin reductase      | 1.3.1.77   | contig 660955, 667861, 675719, 718414, 719766, 730968, 756916, 794760 | 8 | 22.83 | 17 |

doi:10.1371/journal.pone.0083336.t006

Table 6. Genes involved in flavonoid biosynthesis along with their RPKM values.
saponin biosynthesis and in ketogenesis. This may be the reason of its high abundance in *C. borivilianum* transcriptome dataset. The expression of cycloartenol synthase (EC 5.4.99.8) (100 reads) was 5 fold higher than that of α-amyrin synthase (21 reads) (EC 5.4.99.39) (Table 5) thus, further confirming the diversion of the pathway towards steroidal type of saponins in this plant. Most of the genes of mevalonate pathway had expression greater than the genes of non-mevalonate pathway (Table 5). It might be due to the fact that mevalonate pathway is more active in the production of saponins in *C. borivilianum* as compared to non-mevalonate pathway as is the case commonly seen in monocot plants. In case of flavonoid biosynthesis, expression of mevalonate pathway as is the case commonly seen in monocot plants or in microbial hosts by metabolic engineering. In our data set, we also identified transcripts that encode for enzymes involved in anticancer and plant defense properties. Further, we annotated a large number of genes involved in the various pathways. Dataset of assembled *C. borivilianum* unigenes presented here will provide the foundation for other functional and comparative genomic studies.

**Conclusion**

In conclusion, identification of genes involved in saponin and flavonoid biosynthesis will contribute to future functional studies in the plant and provide a basis for improving production levels in plants or in microbial hosts by metabolic engineering. In our data set, we also identified transcripts that encode for enzymes involved in anticancer and plant defense properties. Further, we annotated a large number of genes involved in the various pathways. Dataset of assembled *C. borivilianum* unigenes presented here will provide the foundation for other functional and comparative genomic studies.

**Supporting Information**

**Figure S1** Guanine-cytosine (GC) content analysis of *C. borivilianum* transcripts. (TIF)

**Figure S2** Analysis of total saponins contents in root and leaf tissue of *C. borivilianum* by soxhlet method and TLC. (TIF)

**Figure S3** Distribution of RPKM values for different transcripts in *C. borivilianum* transcriptome. (TIF)

**Table S1** Assembled unigenes after CAP3 assembly. (XLS)

**Table S2** Assembly validation with known *C. borivilianum* leaf nucleotide sequences. (XLS)

**Table S3** *Chlorophytum borivilianum* metabolic pathway analysis using KEGG with; (A) *O. sativa* and (B) *A. thaliana* background databases. (XLS)

**Table S4** Details on transcription factor (TF) families identified in *C. borivilianum*. (XLS)

**Table S5** Simple sequence repeats (SSRs) identified in transcripts of *C. borivilianum*. (XLS)

**Table S6** Annotated cytochrome P450s (CYPs) in *C. borivilianum* transcriptome. (XLS)

**Table S7** Annotated glycosyltransferases (GTs) in *C. borivilianum* transcriptome. (XLS)

**Table S8** Reads per exon kilobase per million (RPKM) based expression of annotated genes of *C. borivilianum* transcriptome. (XLS)

**Acknowledgments**

We are grateful to Dr. Brian Dalley, Director, Microarray core facility, Huntsman Cancer Institute, University of Utah, Salt Lake City, USA for their valuable services for mRNA library preparation and high-throughput sequencing. S. Kalra acknowledges the senior research fellowship from ICMR, India. Guidance of Mr. Gurpreet Singh Sekhon for data analysis is duly acknowledged. Authors are thankful to Dr Ravneet Kaur for critically reading the manuscript.

**Author Contributions**

Conceived and designed the experiments: KS JK. Performed the experiments: S. Kalra, S. Kumar. Analyzed the data: S. Kalra BLP DK. Contributed reagents/materials/analysis tools: KS SR. Wrote the paper: S. Kalra S. Kumar.

**References**

1. Bordia PC, Joshi A, and Simlot MM (1995) Safed musli In: K.L. Chadha and R. Gupta ed. Advances in Horticulture Vol. 11 - Medicinal and Aromatic Plants. (Malhotra Publishing House, New Delhi) pp–429–449.

2. Kaushik N (2005) Saponins of *Chlorophytum* species. Phytochem Rev 4: 191–196.

3. Narasimhan S, Govindarajan R, Madhavan V, Thakur M, Dixit VK, et al. (2006) Action of (2→1)fructo-oligosaccharides on yeast growth and expression of genes involved in the production of saponins in *C. borivilianum*. (TIF)

4. Thakur M, Loepert R, Praznik W, Dixit VK (2008) Effect of some ayurvedic vajikarana rasayana herbs on heat induced testicular damage in male rats. *Inter J Comp Med* 3: 1–14.

5. Mainjumaha G, Tyagi S, Srivivasan K (2004) Safed musli: A White Gold. (Aydogus, India).

6. Acharya D, Mitaine-Offer AC, Kaushik N (2009). Cytotoxic spirostanol-type saponins from the roots of *Chlorophytum borivilianum*. *J Nat Prod* 72: 177–181.

7. Deore SL and Khadabadi SS (2010) Effect of *C. borivilianum* on streptozotocin-induced oxidative stress. *Planta Medica* 72: 1421–1424.

8. Chavan VR, Thakur M, Dixit VK (2006) Action of (2→1)fructo-oligosaccharides on yeast growth and expression of genes involved in the production of saponins in *C. borivilianum*. (TIF)

9. Rohmer M (2003) Mevalonate-independent methylerythritol phosphate pathway for isoprenoid biosynthesis. *Eur J Biochem*. 270: 375–387.

10. Corey EJ, Matsuda SPT, Bartel B (1993) Isolation of an *Arabidopsis thaliana* gene encoding cycloartenol synthase by functional expression in a yeast mutant lacking lanosterol synthase by the use of a chromotographic screen. *Proc Natl Acad Sci USA* 90: 11620–11632.

11. Parkman J (2009) Expressed Sequence Tags (ESTs) Generation and Analysis, Methods in Molecular Biology. Vol 533. Humana Press, New York.

12. Rovd S (2003) Expressed sequence tags: alternative or complement to whole genome sequencing. *Trends Plant Sci* 8: 321–329.

13. Morozova O, Hirst M, Marra MA (2009): Applications of new sequencing technologies for transcriptome analysis. *Annu Rev Genomics Hum Genet* 10:135–151.
41. Geetha KA, Maiti S (2001). Reproductive biology of Safed musli (A. C. P. 1998) Compositional properties of the vernalization and photoperiod pathways in Arabidopsis. Genes Dev 20(23): 1135–1145.

42. Senthilvel S, Jayashree B, Mahalakshmi V, Kumar PS, Nakka S, et al (2008) Flavonol synthase gene expression during citrus fruit development. Physiologia Plantarum 114: 231–239.

43. Mourigou T, Kita M, Ogawa K, Tomono Y, Endo T, et al (2002) An RNA isolation system for plant tissues rich in secondary metabolites. BMC Res Notes 144: 32–42.

44. Jiang H, Wong WH (2008) SeqMap: mapping massive amount of oligonucleotide to the genome. Bioinformatics 19: 651–652.

45. Li Z, Thomas TL (1998) PEI1, an embryo-specific zinc finger protein gene that phosphorylates tyrosine, serine and threonine. Plant Mol Biol 20(4): 653–62.

46. Schuhr CA, Radykewicz T, Sagner S, Latzel C, Zenk MH, et al (2003) oxidosqualene cyclase that catalyzes the formation of the most popular triterpene D- erythritol, a putative C-5 intermediate in the mevalonate independent pathway for isoprenoid biosynthesis. Tetrahedron Lett 38: 4769–4772.

47. Schmid A, Rass S, Rajapaksa J, Schmutz J, MacDonald M, et al (2010) Expression of the genes. Plant Cell Physiol 35: 1011–1018.

48. Dourdel T, Bravo JM, Pale GC, Rhoemer M (1997) Biosynthesis of 2-C-methyl-D- erythritol (C5) intermediate in the mevalonate independent pathway for isoprenoid biosynthesis. Tetrahedron Lett 38: 4769–4772.

49. Park J, Rhee D, Lee Y (2005) Biological activities and chemistry of saponins from Panax ginseng C. A. Meyer. Phytochemistry 49(1): 159–173.

50. Ohyama K, Suzuki M, Kikutani J, Saito K, Muranaka T (2009) Dual biosynthetic pathways to phytosterols via cycloartenol and lanosterol in Arabidopsis. Proc Natl Acad Sci USA 106(3): 723–730.

51. Kusunori T, Shibuya M, Ebizuka Y (1998) Cloning of the chalcone synthase from Camellia sinensis. FEBS Lett 584: 2258–2264.

52. Tansakul P, Shibuya M, Kusunori T, Ebizuka Y (2006): Dammarenediol-ll synthase, the first dedicated enzyme for ginsenoside biosynthesis, in Panax ginseng. FEBS Lett 584(21): 1413–1417.

53. Takeuchi A, Tatsunori M (1994) Chalcone synthase from Camellia sinensis isoenzymes and the organ-specific and sugar responsive expression of the genes. Plant Cell Physiol 35:1011–1018.

54. Lin GF, Lian YJ, Ryu JH, Sung MK, Park JS, et al (2007) Expression and puriﬁcation of His-tagged favonol synthase of Camellia sinensis from Escherichia coli. Protein Expr Purif 53:287–292.

55. Kreuzer F, Ragg H, Fautez E, Kuhn DN, Halbritter K (1983) UV-induction of flavonol synthase mRNA in cell suspension cultures of Petunia hybrida. Proc Natl Acad Sci USA 80: 2591–2593.

56. Goff SA, Klein TM, Roth BA, Fromm ME, Cone KC, et al (1990) Transactivation of anthocyanin biosynthetic genes following transfer of regulatory genes into maize tissues. EMBO J 9: 2517–2522.

57. Shirley BW, Hanley S, Goodman H (1992) Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis transparent testa mutations. Plant Cell 4: 333–347.

58. Boss PK, Davies C, Robinson S (1996a) Analysis of the expression of anthocyanin pathway genes in developing Microtus agrestis leaf shoots. Biochemical and Biophysical Research Communications 230: 381–385.

59. Moriguchi T, Mizutani M, Aoki N, Watanebe B, Saga H, et al (2006) Genome sequence analysis of two Arabidopsis reference samples using next generation sequencing. BMC Genomics 10: 264.

60. Huang XQ, Madan A (1999) CAP3: a DNA sequence assembly program. Biotechniques 26: 97–105.

61. Pertea G, Huang XQ, Liang F, Antonescu V, Sultana R, et al. (2003) TIGR plant genome: analysis of two Arabidopsis plant transcriptomes by RNA-Seq. Nucleic Acids Res 31: 62–62.
77. Stadler R, Kutchan TM, Loeffler S, Nagakura N, Cassels B, et al (1987) Revision of the early steps of reticuline biosynthesis. Tetrahedron Lett 28: 1251–54.

78. Sato F, Tsujita T, Katagiri Y, Yoshida S, Yamada Y (1994) Purification and characterization of S-adenosyl-L-methionine: noncoelaurine 6-O-methyltransferase from cultured Coptis japonica cells. Eur J Biochem 225:125–31.

79. Morishige T, Tsujita T, Yamada Y, Sato F (2000) Molecular characterization of the S-adenosyl-L-methionine: 30-hydroxy-N-methylcoclaurine-40-O-methyltransferase of isoquinoline alkaloid biosynthesis in Coptis japonica. J Biol Chem 275: 23398–405.

80. Kumar M, Meena P, Verma S, Kumar M, Kumar A (2010) Anti-tumour, Anti-mutagenic and Chemomodulatory Potential of Chlorophytum borivilianum. A Pac Jour Canc Preven 11: 327–334.

81. Qiu SX, Li XC, Xiong Y, Dong Y, Chai H, et al (2000) Isolation and characterization of cytotoxic saponin Chloromaloside-A from Chlorophytum. Planta Med 66: 587–90.

82. Li Q, Wang X, Yang Z, Wang B, Li S (2009) Menthol induces cell death via the TRPM8 channel in the human bladder cancer cell line T24. Oncology 77(6): 335–41.