

High-throughput identification of chrysanthemum gene function and expression: An overview and an effective proposition

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Abstract Since whole-genome duplication (WGD) of diploid *Chrysanthemum nankingense* and de novo assembly whole-genome of *C. seticuspe* have been obtained, they have afforded to perceive the diversity evolution and gene discovery in the improved investigation of chrysanthemum breeding. The robust tools of high-throughput identification and analysis of gene function and expression produce their vast importance in chrysanthemum genomics. However, the gigantic genome size and heterozygosity are also mentioned as the major obstacles preventing the chrysanthemum breeding practices and functional genomics analysis. Nonetheless, some of technological contemporaries provide scientific efficient and promising solutions to diminish the drawbacks and investigate the high proficient methods for generous phenotyping data obtaining and system progress in future perspectives. This review provides valuable strategies for a broad overview about the high-throughput identification, and molecular analysis of gene function and expression in chrysanthemum. We also contribute the efficient proposition about specific protocols for considering chrysanthemum genes. In further perspective, the proper high-throughput identification will continue to advance rapidly and advertise the next generation in chrysanthemum breeding.

Keywords Chrysanthemum breeding, Gene expression, Gene function, Genomics analysis, High-throughput sequencing, Phenotyping data

Introduction

High-throughput sequencing methods are recognized as

essential innovation tools for quantitative sequencing nucleic acid molecules. High-throughput sequencing techniques (HST) are now reducing price, replacing the analysis of traditional cloning library, and probably identifying techniques in the adjacent future (Huse et al. 2008). Based on the read-length achievement, HST accesses an increase in coverage read-depth to identify phylotypes, to estimate diversity, and discover metagenomic characterization of genetic complex traits.

Chrysanthemum, which is an important crop of the Asteraceae family, is high-value in floricultural crops and is stood the second in the florist market trade throughout the world (Nguyen and Lim 2019; Nguyen et al. 2020). Chrysanthemums were used for medicinal purposes as the birth-place in China where they were first cultivated for traditional medicine treatments (Nguyen and Lim 2019). They are characterized by types, flower shapes, and their colors are made as high value in floral crop (Nguyen and Lim 2019; Nguyen et al. 2020).

Despite the challenges conferred by global climate change and the immediately burgeoning human population, techniques to achieve the giant production and high quality chrysanthemum with reducing input are critically required (Tester and Langridge 2010). Therefore, relevant approaches for progressing the best chrysanthemum production that would obtain disease and insect resistances, various petal colors, shapes, and many type of flowers, has been recommended (Teixeira da Silva et al. 2013).

Additionally, the lack of genomic chrysanthemum data has been solved by various achievements to study deeply in chrysanthemum genomic functional with high-throughput sequencing tools (Su et al. 2019). The improvement of functional genomic and genetic techniques is over three decades, principally technological sequencing tool. In *C. nankingense*, the WGD of diploid level have been sequenced (Song et al. 2018), and the functional investigation of the chrysanthemum genome has accessed the high-throughput

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Table 1 Chrysanthemum key genes and their functions

Name	Function	References
<i>CmHSFA4</i>	<i>CmHSFA4</i> (in overexpression) improved the salt tolerance of chrysanthemum	(Li et al. 2018)
WRKY transcription factor	correlated in stress responses including salt stress, drought, disease, aphid resistance	(Fan et al. 2015; Li et al. 2015a; Li et al. 2015b; Fan et al. 2016; Jaffar et al. 2016; Liang et al. 2017; Wang et al. 2017)
<i>CmDREB6</i>	<i>CmDREB6</i> (in overexpression) improved the heat tolerance	(Du et al. 2018)
<i>CmCPL1</i>	<i>CmCPL1</i> increased (in overexpression) or decreased (in knockdown) the heat tolerance	(Qi et al. 2018)
<i>CmCBF1</i>	<i>CmCBF1</i> (in overexpression) improved its drought tolerance and salt stress in <i>C. lavandulifolium</i> ‘White Snow’	(Gao et al. 2018b)
<i>CmCYC2</i>	<i>CmCYC2c</i> increased (in overexpression) flower number and ray floret length, deficient to completely modify flower shape	(Huang et al. 2016)
<i>CmTCP20</i>	obligation for petal elongation growth	(Wang et al. 2019a)
<i>CmANR1</i>	encouragement nonessential root and lateral root development	(Sun et al. 2018)
<i>CmERF053</i>	maintaining the new regulating shoot function and developing lateral root, affecting drought resistance	(Nie et al. 2018)
<i>Cm-BBX24</i>	transgenic RNA interference (<i>Cm-BBX24-RNAi</i>) suppressed flowering earlier ~ 20 days than wild type	(Yang et al. 2014)
<i>CmNF-YB8</i>	<i>CmNF-YB8</i> expression with <i>cmo-MIR156</i> influences flowering time	(Wei et al. 2017)
<i>CtA3 5'GT</i>	<i>CtA3 5'GT</i> (true blue color), encoding UDP-glucose: <i>Clitoria ternatea</i> with anthocyanin 3',5'-O-glucosyltransferase, and <i>Campanula medium</i> with <i>CamF3 5'H</i> , encoding F3'5'H	(Noda et al. 2017)

platform (Yagi 2018). In *C. seticuspe*, de novo assembly was studied in whole genome (Hirakawa et al. 2019). Some of chrysanthemum key genes were characterized their functions such as stress tolerance (abiotic and biotic), plant growth and development, flowering time, and flower color (Table 1).

High-throughput sequencing techniques have been enabled by increased the new knowledge of modern plant genomes (Furbank 2009). The applicable genomic data have been obtained with accommodate technologies to be enable to identify the functional of plant genes such as photonics applications (Yeong et al. 2019), functional plant biology (Poorter et al. 2012), phenotyping computers vision-based (Mochida et al. 2018), and robotics (Coppens et al. 2017). Thus, they should be expanded to allow chrysanthemum physiology and phenomics to improve correlation of chrysanthemum genomics.

Here, we summarize the emerging methods in chrysanthemum species and highlight the technological biases of high-throughput sequencing technologies. Next, we focus on the ongoing applications in chrysanthemum phenomics, and note the efficient proposition in high-throughput sequencing based on research design. We conceive the major challenges in chrysanthemum phenomics.

High-throughput sequencing methods for Chrysanthemum species identification

High-throughput techniques could be carried quantification PCR, hybridization-based (microarrays), second-generation fingerprinting (RADseq), and sequence-based (meta-barcoding, meta-transcriptomics, meta-genomics).

In 2005, PCR quantification of phytoplasma DNA in *C. carinatum* and insect (*Macrosteles quadripunctulatus*) host was described. Primers and a TaqMan probe were designed on a target cloned specific ribosomal chrysanthemum fragment. They were also applied on ITS1 (internal transcribed sequences) and 18S rDNA (plant) for amplification from *C. carinatum* and *M. quadripunctulatus* (Marzachi and Bosco 2005). Chrysanthemum yellows phytoplasma (CYP) were found a few hundred thousand in the highest amounts in leaves and roots (Saracco et al. 2005). CYP concerning young developing leaves and roots had been indicated (Saracco et al. 2005).

The Asteraceae introduces various polyploid species, and broad crossing in the proceeding of hybridization by genetic and epigenetic alterations. In Asteraceae, the consequences of hybridization were characterized in the large genomic, transcriptomic and epigenomic. These adjustments conducted

the processing hybridization in the cross-sections *C. nankingense* × *Tanacetum vulgare* and *C. crassum* × *Crossostephium chinense*. These cytosine methylation changes could be introduced the evolutionary processes of the mechanisms newly formed intergeneric hybrids formation (Wang et al. 2013a). The paired-end sequencing presented over 53 million reads among 81 amplicon markers, 20 polymorphic markers, and 100 random primer pairs from *C. nankingense* mRNA in Illumina platform (Wang et al. 2013b).

RNA-Seq technology was applied to chrysanthemum transcriptomic which was responded to *Alternaria tenuissima* inoculation by the Illumina HiSeq™ 2000 platform. Twenty three gene selections were identified on the profiled transcription by validating the RNA-Seq output using qRT-PCR (Li et al. 2014). The chrysanthemum embryo including 1037 miRNA target genes was characterized by transcriptome data and 228 miRNAs were validated with symbolic changes in frequency expression through embryotic development (Zhang et al. 2015). The miRNAs response was discovered to low-nitrogen stress in *C. nankingense* which the results presented overlapping or effecting unique response to nitrate stress in leaves and roots, including miR156, miR169, and miR393 (Song et al. 2015).

Quantitative real-time PCR (qPCR) uses to identify the stage of flower development, which is an identified 9 candidate reference genes for their expression through *C. morifolium* and *C. lavandulifolium* samples. *SAND* (*SAND* family protein) was most thoroughly expressed in all or different *C. lavandulifolium* tissues. Both *SAND* and *PGK* (phosphoglycerate kinase) achieved the most stable in large flower, and the best *PGK* was presented in pot chrysanthemums (Qi et al. 2016). Based on the use of 454 sequencing platform, the *C. indicum* completed chloroplast genome was achieved with 150,972 bp in length including inverted repeats (IR; 24,956 bp), large single copy (LSC; 82,741 bp), and small single (SSC; 18,319 bp) regions (Xia et al. 2016). miRNAs target genes were studied on the normal and abnormal embryos within three sRNA libraries by RNA-Seq to identify 170 miRNAs with 41 special miRNAs in the paternal chromosome doubling cross including miR169b, miR440, and miR528-5p (Zhang et al. 2017). For floral traits of chrysanthemum, the 454-pyrosequencing technology was used to construct normalized cDNA libraries in chrysanthemum. Chrysanthemum libraries presented ~3,77 million high-quality reads through assembling into ~213,000 contigs (Sasaki et al. 2017).

Oxford Nanopore long-read-technology was used to sequencing *C. nankingense* genome, its genome evolution was consumed by repetitive element expansion bursts, and the

WGD chrysanthemum classified near WGD sunflower, which deviated from chrysanthemum ~38.8 million years ago (Song et al. 2018). The diploid *C. seticuspe* was studied as a model plant in the cultivated chrysanthemum and analyzed de novo whole-genome assembly by Illumina platform. The results showed that the assembled sequences were 2.72 Gb; 354,212 consisting scaffolds; the 3.06 Gb of *C. seticuspe* genome was covered to 89.0% estimated by k-mer analysis (Hirakawa et al. 2019).

Chrysanthemum gene functions and expressions

MADS-box transcription factors were designed in functionally characterized in *C. grandiflorum* such as *MADS (CDM) 8*, *CDM41*, *CDM111*, and *CDM44*. *CDM44* protein combines to *CDM* proteins which are constructed to the *API/FUL* and *AG* subfamilies, and made the expression in larger than order heterodimer complex with B-type *CDM* proteins in *C. grandiflorum* (Shchennikova et al. 2004).

CsFTL3 had found early induced flowering time in non-inductive conditions in *C. seticuspe* accessions. The expression levels of *CsFTL3* were higher in its leaves, therefore, the *CsFTL3* gene product is one of the key regulators in photoperiodic flowering time (Oda et al. 2012). In the study of cultivated chrysanthemum breeding, the salt stress can be arranged valuable information for describing the salt tolerance mechanism in plant. In *C. lavandulifolium*, cDNA amplified fragment length polymorphism (cDNA-AFLP) was analyzed to identify the differential gene expression in chrysanthemum leaves in NaCl response. By using cDNA clone and BLASTx program, 25 expressed sequence tags (ESTs) were identical to known functional genes (He et al. 2012).

The systemic flowering inducers (florigens) and inhibitors (antiflorigens) can be changed in the day length in leaves that regulated to floral initiation in shoot. The *Anti-florigenic FT/TFL1 family protein (AFT)* was studied in *C. seticuspe*. *CsAFT* induction was produced by phytochrome signals; and flowering step was developed in the photosensitive phase (Higuchi et al. 2013). In *C. morifolium* ‘Fall Color’, the study of regulated hormone biosynthesis and signaling was performed in two cDNA libraries to identify 8,558 unique transcripts in responsive dehydration, containing 307 transcription factors, and 229 protein kinases by Illumina technology (Xu et al. 2013).

The *C. lavandulifolium* transcriptome was shown 108,737 unigenes high-quality (~4 GB data), 58,093 proteins sequencing, and 211 flowering-related unigenes by using

Illumina HiSeq™ 2000 sequencing platform. Moreover, the genes expression analysis identified 14,406 unigenes related to the visible bud form and seedling stage. In the transcriptome of *C. lavandulifolium*, many unigenes with high correspondence to important genes that are complicated in the gibberellin, photoperiod, autonomous pathway, and vernalization were analyzed, including *FLOWERING LOCUS C (FLC)*, *CONSTANS (CO)*, *FLOWERING LOCUS T (FT)*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* (Wang et al. 2014).

The function of an MYB transcription factor (especially focuses on *RsMYB1* ectopic expression isolated from radish *Raphanus sativa*) was identified *CmF3H*, *CmDFR*, and *CmANS* biosynthetic key genes which are responsible for anthocyanin products in transgenic chrysanthemum and are in possession a negative feedback to non-transgenic line. Especially, *RsMYB1* was not found any change in morphological characteristics, such as number of flower, leaf size and shape, and plant height in chrysanthemum cv. ‘Shinma’ (Naing et al. 2015). The Illumina platforms were performed to sequence chrysanthemum buds (from vegetative buds to floral buds) in *C. morifolium*. 91,367 unigenes were identified in 15.4 Gb of reads, including 43,137 unigenes showed similarity protein. DEGs expressed genes (1,876 DEGs including 1,516 up- and 360 down-regulated) were analyzed between floral buds and vegetative buds, and 3,300 DEGs (1,277 up- and 1,706 down-regulated) were scanned between floral buds and buds (Liu et al. 2015).

qPCR was identified reference genes in the developmental flower stage in *C. morifolium* and *C. lavandulifolium*. The suggestion of good candidate genes was appraised and validated by four algorithms such as Bestkeeper, Norm-Finder, GeNorm, and Ref-finder. In *C. lavandulifolium*, all samples were found the most strongly expressed genes in *SAND* family protein. The study was screened on both *SAND* and *PGK* (phosphoglycerate kinase) to achieve the most constant in Chinese chrysanthemum cultivars with large flower, and the potted chrysanthemums was the best in *PGK* (Qi et al. 2016). The *SQUAMOSA promoter binding protein-like (SBP or SPL)* family is defined to know as functional transcription factors by a plant-specific DNA-binding domain. Twelve *SBP-like (SPL)* genes were shown six of these genes included a miR156 target site, and five *CmSPLs* were targeted by miR157 in *C. morifolium*. Furthermore, 50 *RLM-RACE* were mapped to the cleavage sites in *CmSPL2* and *CmSPL3* (Song et al. 2016b). The analyses of 20 DNA binding with one finger (*DOF*) genes were shown that 13 *CmDOFs* could be focused by 16 miRNA families in *C. morifolium* based on transcriptomic

sequences. These genes were expressed patterns of 6 pairs of paralogous *CmDOF* genes which were discovered to effectively differ from one another, except for *CmDOF6* and *CmDOF7* (Song et al. 2016a). The screening of MADS-box gene was performed on short vegetative phase (*SVP*) like genes from *C. morifolium* ‘Jinbudiao’ and constructed as *CmSVP* to consist 672 bp encoding 223 amino acids. *CmSVP* is conversely expressed in the shoot apex and leaves, and it is expressed in the decreasing concentration in both flower buds and flowers (Gao et al. 2017).

The isolated stem segments was performed to analyze bud outgrowth in auxin (IAA), strigolactone (GR24), and auxin transport inhibitor (NPA) to identify expression levels in auxin transport (*CmPIN1*) and signaling (*CmAXR2*), bud dormancy (*CmBRC1*, *CmDRM1*) and strigolactone biosynthesis (*CmMAX1*, *CmMAX3*). The expression of *CmBRC1* and *CmDRM1* was decreased and the expression of *CmPIN1* was increased in the control of high dividing cell percentage in bud outgrowth. The inhibition by IAA and IAA and GR24 concurred with low dividing cell percentage and unaltered or increased expressions of *CmBRC1*, *CmDRM1*, and *CmPIN1*. Notwithstanding, GR24 treatment indicated reduced bud outgrowth that was prevented by NPA (Dierck et al. 2018). The study of anthocyanin biosynthesis-related genes *CmplCHI*, *CmplANS*, *CmplCHS*, *CmplF3H*, and *CmplDFR* was performed in *C. morifolium* ‘Pelican’. Two genes, *CmplCHSs* and *CmplCHI*, indicated that a high gene expression level from stage 1 to show un-pigmented petals. Two genes (*CmDFRs* and *CmANS*) performed in a high gene expression level with pale pink petals from stage 2. Notwithstanding, *CmplDFR2* displayed constant gene expression from stage 2 to 6, dissimilar the other isolated genes (Puangkrit et al. 2018). The analyses of 23 *CmGRAS* genes in the *GRAS* gene family were performed to *C. morifolium* transcriptome. The *CmGRAS4* and *CmGRAS10* were powerfully transcribed in flowers and roots. In the other hand, the *CmGRAS19* and *CmGRAS20* was immersed in the reproductive tissues to be reactive to phytohormones and stresses (Gao et al. 2018a). A transcriptional regulator response to many abiotic stresses, such as *RNAPII CTD phosphatase-like 1 (CmCPL1)*, was isolated in *C. morifolium*. This analysis was performed 955 residue gene product containing the FCPH catalytic domain, one local nuclear signal, and two binding motifs in double-stranded RNA. The overexpression and knockdown of *CmCPL1* was over-expressed and knock-downed to come out the result to increase and diminish the tolerance heat stress, which can be dependent on the regulation of *CmCPL1* and on the expression of downstream responsive genes in chrysanthemum

(Qi et al. 2018). RNA-Seq platform achieved a de novo transcriptome assembly to determine chrysanthemum (*Chrysanthemum grandiflorum* var. ‘Jinba’) transcription response to low temperature. By using Illumina technology, total 7583 differentially expressed genes (DEGs) of 36,462 annotated unigenes were discovered (Wang et al. 2018a). The high-throughput sequencing was used to analyze the genome-wide transcriptome of *C. morifolium* ‘Chuju’, which was well-known as a famous traditional Chinese medicine species. Total 63,854 unigenes were obtained with an average length of 741 bp, and 12 unigenes complicated in flavonoid biosynthesis were commonly verified by using qPCR (Yue et al. 2018).

The functions of *R2R3-MYB* transcription factor (TF) genes are mainly associated to the accumulation of anthocyanins that have not been study well in chrysanthemum. By the use of phylogenetic and alignment analyses, some genes such as phenylpropanoid (*CmMYB4* and *CmMYB5*), flavonoid repressor (*R2R3-MYB*), anthocyanin biosynthesis activator (*CmMYB6*), flavonol biosynthesis regulator (*CmMYB7*), and negatively regulate anthocyanin biosynthesis (*CmMYB4* and *CmMYB5*) were identified, while *CmMYB6* and *CmMYB7* were played a positive role (Hong et al. 2019).

A moderate domain spliced hairpin RNA (ihpRNA) is known as expression vector to intent *DNA METHYLTRANSFERASE1* (*CmMET1*). Transgenic chrysanthemum materials (Zijingling) were consumed as root stock, or grafted onto Guoqinghong (GQH), and Huanshuijingju (HSJQ) non-transgenic the scions which were no signal in *CmMET1* gene, and presented phenotypes with early flowering. Methyltransferase gene expression was up-regulated to decreased expression (*CmMET1*), *METHYLTRANSFERASE2* (*CmDRM2*), however, demethylating enzyme gene expression was down-regulated, *DEMETER* (*CmDME*), while the *CHROMOMETHYLASEA* (*CmCMT3*) expression level continued low and could be almost imponderable. Among the *CmFT-like*s genes that involve flowering time, *CmFTL1* expression was down-regulated, as well as *CmFTL2* and *CmFTL3* were up-regulated. The use of silencing *CmMET1* could make less height, adjust phenotype, and improve earlier flowering (Li et al. 2019). Four genes encoding pseudo-response regulator (PRR) related to flowering time were isolated from *C. morifolium*. The *CmPRR2*, *CmPRR7*, *CmPRR37*, and *CmPRR73* genes are decidedly homologous to the counterparts of PRRs of *Helianthus annuus* (Wang et al. 2019b). The expression levels of six Chrysanthemum stunt viroid (CSVd) *CSVd-siRNAs* genes performed in cytokinin (CK), gibberellic acid (GA), and indole-3-acetic acid (IAA) biosynthesis transport. The growth of cell walls was down-regulated in infected plants,

but mRNAs of those genes were not detected as possible target sequences of *CSVd-siRNAs*. On the different way, endogenous levels of abscisic acid, IAA, GA, CK, and the rate of IAA movement were not different between healthy and infected plants (Takino et al. 2019). A reference based on *C. lavandulifolium* transcriptome database was concerned the global changes in gene expression in *C. lavandulifolium* seedlings treated with 200 mM NaCl for 12 hours to compare with normal conditions. Total of 2254 DEGs (1418 up-regulated and 836 down-regulated genes) were discovered (He et al. 2019).

Efficient propositions in high-throughput sequencing

The Asteraceae family (~24,000 to ~35,000 species) with gigantic genome sizes (~3 Gbp) (Luo et al. 2017; Vallès et al. 2013) and 208 kb mitogenome sizes (Wang et al. 2018b). Based on your objective research goal for Chrysanthemum species, short-reads will not be respected for whole genome sequencing, but long-reads for the short-time sequencing is not an opportunity for the gigantic genome size such as Asteraceae (Nguyen and Lim 2019). Thus, chrysanthemum researchers should design their experiments based on their objective research. In chrysanthemum, miRNA genes play as gene expression in demanding regulator at infrequently transcriptional and post-transcriptional levels such as monitoring the transcriptional procedures, processing and maturation, control of accumulation levels, and verification of miRNA-target interactions based on the great achieved sequencing progresses. The finds on the great progresses must be achieved for high-throughput sequencing technology. These finds have been provided some original ways for the studies on genome-wide or transcriptome-wide.

Several HTS platform-based methods are considered to use for plant miRNA studies, such as ssRNA-seq (single-stranded RNA sequencing), dsRNA-seq (doublestranded RNA sequencing), degradome-seq (degradome sequencing), RNA-seq (RNA sequencing), sRNA-seq (small RNA sequencing), and RNA-PET-seq (paired end tag sequencing of RNAs). The innovative use of HST methods for inspected processing approaches of the miRNA precursors, recognition of the RNA rearranging sites on miRNA precursors, and discovery on the target interactions of novel miRNA species. The obliteration of the independent biological replicates seems to present in multiple spatially autocorrelated subsamples and vary recovered replicates. The origin of sample collection must be particularly consisted with essential hierarchical design and various level spatial autocorrelation. Pooling

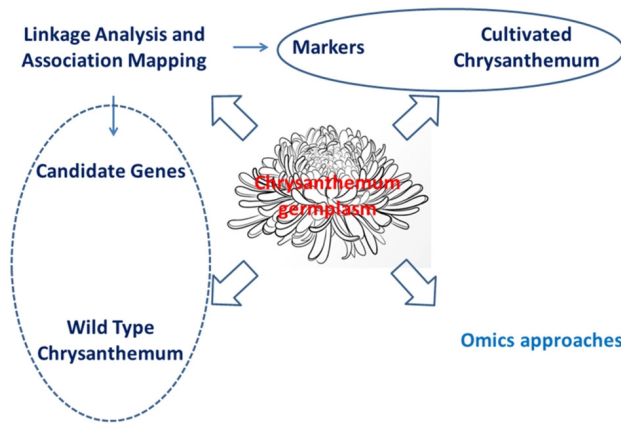


Fig. 1 Chrysanthemum germplasm studies. The circle shows the present study and the dashed circle shows the further studies in Chrysanthemum species

can be allowed vigorously examination cost, however, it is also diminished the small-scale resolution. However, the multiple testing independent samples are favored to the performance of approximate calculation in sampling error and number of spatiotemporal variability. Researchers should consider an extra replicate because it includes DNA in low-quality or restricted sequence reads from some samples (commonly 1%~10%). In the diversity study, the researchers must load more sample for univariate tests in the lower statistical power.

Based on the sensitive techniques to demolishing, external contamination and cross contamination; thus, HTS techniques will be required carefully collected samples, pre-treatment and handling inspect to avoid contamination and overrun by quick growing mildews or degradation of DNA or RNA. The pre-treatment steps, such as storage, drying with air or freeze, deep freezing, and preservation buffer fixing, were managed evenly fine for DNA. Dried, frozen, and deep-freezing DNA/RNA samples (-80°C) would be considered for preservative potential analysis of DNA/RNA, fatty acids, and protein for further analyses. For considering contamination during all various analysis steps, it can be approved to set up the laboratory working space into moisten laboratory, PCR laboratory, and HTS preparation laboratory. The main authority of the contaminated HTS analyses is the different previous source of PCR steps, because of single strain of DNA molecule may be increasingly automatic amplified and sequenced. All the exteriors of RNA/DNA molecule can be control by efficient and economic cleaning of laboratory using DNase/RNase-containing solutions and UV-light. Negative controls should be considered in all stages of analyses because it can be detected mistakes and confirmed the track contamination.

Based on the work of HTS-based diversity analyses, the DNA/RNA markers should be clearly considered in the decision of taxonomic resolution. It is important to select the guiding methods for primers and choices of marker genes. For targeting specific host molecular, researchers should consider the primers that removing DNA host or adding blocking primers. Providing with 3' nucleotide-terminal modifications and oversupplied concentration, blocking primers inhibit annealing and make elongation of DNA host marker by exactly binding to DNA host downstream of regular primers. Based on sample molecular identification, forward and reverse primers should be tagged to enable multiplexing sequences. For Illumina sequence technique, 96 wells of DNA ligation can be combined by PCR. Identifiers *tags* are only cheaper and useful for sequencing analysis in their platform. In the ligation step, it is recommendable to reduce the *tag* amplicons because of starting with the use of two-base linker sequence in the same nucleotide with no match to any templates. To reduce the *tag* switching to minimize, the researchers have to consider strongly to identifier tags through both the reverse and forward primers.

For HTS analysis, the researchers should control the primer annealing temperature to benefit the amplification of sample templates which is with one or two mismatches to primers. On the other hands, the low input DNA template should be affected to the result of lower inhibitors and reduced chimeric sequences. Based on the stochastic variation, the researchers should be used at least two PCR replications that can be used for the next sequencing step; and it is defended on the use of sequencing platform and further analyses. It is recommend that the use of specific adapter ligation and the choice of platform sequencing should be linked together to reduce the risk and the failure of service provider. To reduce the contamination and sequencing errors, the researchers should run three types of control sample in the same direction. If the experience has a few samples, the limited technical replication may be appropriate to estimate the method reproducibility and performance for upgrading protocols.

Conclusion

This study provides the overview in chrysanthemum molecular breeding and gene expression. To further investigation, the higher techniques should be upgraded for phenotyping and sequencing in chrysanthemum genome, especially in wild chrysanthemum (Fig. 1). On the other hand, the proper protocol for identifying chrysanthemum gene function should be deeply studied.

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