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Comparative analysis of bioinformatic tools to predict and quantify active circular RNAs during grape cluster development

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Abstract

Circular RNAs (circRNAs) are relatively new members of the RNA world and can contribute to crucial biological functions. CircRNAs have tissue-specific expression profiles depending on cell type and developmental stage. In Sistan region cultivated grapes are seedless but have small berries. The compact clusters are another notable characteristic of these grape cultivars, which negatively impacts their marketability. In this study, we aimed to identify the circRNAs that are active in cluster formation and investigated the effects of gibberellin treatment on their expression. Eight detection tools were used to predict the expressed circRNAs. Reliable circRNAs were used to identify potential functions of differentially expressed circRNAs by gene ontology (*GO*) analysis and prediction of target microRNAs (miRNAs). Of the 28 157 circRNAs detected, 3 715 were reliable. 900 differently expressed circRNAs were identified in the three developmental stages of the cluster under gibberellin treatment. Among the 503 target miRNAs found, 12 miRNAs were selected based on the number and expression of their circRNA sponges. Of the 29 circRNAs in the circRNAs-miRNAs-mRNAs interaction network, 12 circRNAs are highly conserved. Our results suggest that circRNAs in grape may play a key role in developmental and environmental adaptation in perennial plants.

Keywords: circular RNAs, gene ontology, gibberellin, micro RNAs.

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Abbreviations: AP2 - APETALA2; BC - betweenness centrality; BSJ - back-splice junction; CC - centrality parameter; circRNAs - circular RNAs; DE - differentially expressed; FDR - false discovery rate; *GO* - gene ontology; miRNAs - microRNAs; RAP2.7 - related to APETALA2; RPM - reads per million mapped; SPL - squamosa promoter binding protein-like.
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Introduction

CircRNAs are among the relatively new members of the RNA world and they are a significant class of RNAs. CircRNAs arise from pre-mRNAs *via* a non-canonical back-splicing process and have a circular structure in which the 5'- and 3'-ends are joined by a covalent bond (Chen *et al.* 2021). The specific expression profiles of circRNAs depending on cell type and developmental stage suggest that they are intentionally produced by the cell (Jakobi and Dieterich 2019). Overall, circRNAs are a group of regulatory molecules with diverse biological functions. They can regulate gene expression at transcriptional and post-transcriptional levels, act as miRNA sponges, prevent binding of miRNAs to target transcripts, and bind to protein factors (Kalwan *et al.* 2023).

Biotic and abiotic stresses are among the most serious limiting factors in crop production and harvest worldwide. Studies suggest that circRNAs play a critical role in plant resistance to biotic and abiotic stresses, at different developmental stages (Chen *et al.* 2022), and in specific tissues (Li *et al.* 2020). In the case of circRNA expression profile of maize infected with maize Iranian mosaic virus, 155 and 55 up- and down-regulated circRNAs were detected in infected plants, respectively. Inhibition of several miRNAs under stress revealed the role of circRNAs as miRNA sponges and the biological functions of these circular molecules (Ghorbani *et al.* 2018, 2022). CircRNAs also show differential expression in response to abiotic stresses such as nutrient deficiency (Ma *et al.* 2021), heat (He *et al.* 2020), chilling (Zuo *et al.* 2018), drought (Zhang *et al.* 2019), or salt (Zhu *et al.* 2019). Samples from the roots of *Oryza sativa* under phosphate deficiency and leaves of *Arabidopsis thaliana* under excessive radiation treatment were the first circRNAs identified in plants (Ye *et al.* 2015). In *Solanum lycopersicum*, target gene analysis was performed to determine circRNA function under chilling exposure. The results showed that target genes regulate the major processes in the chilling response (Zuo *et al.* 2016). In another study, *GO* enrichment analysis of circRNA parental genes showed cold response in grapevine (Gao *et al.* 2019).

The *Vitis vinifera* cv. Yaghooti is cultivated to a limited extent in south-eastern Iran, western Pakistan, and southern Afghanistan and it is one of the few horticultural crops adapted to heat and drought in this region. A study of the transcriptome of this grape cultivar shows that it accelerates its growth before 120-day storms. By mid-June, it was fully mature and ready for harvest. This means that the Yaghooti grape has one of the shortest growth period from flowering to full fruit ripeness. This grapevine cultivar is a seedless type with small berries. The compactness of the clusters is another striking characteristic of this plant. This impairs marketability and, in some cases, leads to split berries, making them susceptible to fungal diseases. Evolution has developed the seeded grapes into seedless grapes. Since gibberellin is synthesized in the seed, seedless grape cultivars produce more compact grapes than seeded cultivars (Shiri *et al.* 2020). Studies have shown that spraying with 40 mg l^{-1} gibberellin at the time of cluster formation and flowering increases the quality and quantity of the harvest. This is achieved by reducing the compactness (Shiri *et al.* 2020).

Given the importance of gibberellin in grape tissue growth and development and its role in grape morphology, size, and ripening, our study investigated the expression profile of circRNAs associated with grape cluster formation using multiple detection tools. The identification and prediction of the functional role of differentially expressed circRNAs may clarify the crucial role of circRNAs in the response to developmental stages and phytohormones in grapevines.

Materials and methods

Plant materials: Yaghooti grape from Sistan region was obtained from cloned grapevines (*Vitis vinifera* L.) in the gardens of the Agricultural Research Institute of Zabol University. Three stages of cluster formation were sprayed with gibberellin (*Merck*, Gernsheim, Germany) at a concentration of 40 mg l^{-1} 24 h before sampling. Gibberellin concentrations were determined according to previous studies (Casanova *et al.* 2009, Amkha *et al.* 2017). Control samples were sprayed with distilled water simultaneously. The first (2 April 2016), second (16 April 2016), and third (29 April 2016) sampling coincided with the formation of the first clusters, the time of berry formation, and the final size of the clusters, respectively. Control samples were also taken at the same intervals. A sample was taken from each cluster and berries were removed from the peduncles and pedicels for RNA extraction.

RNA extraction, library construction, and sequencing: In the control and treatment groups, six pools were prepared for each time point based on three healthy clusters from each plant. At the end, we selected only one sample from each pool. RNA was extracted from six samples, including three untreated (control group) and three treated with gibberellin (treatment group), based on the Japelaghi technique (Japelaghi *et al.* 2011). RNA quality and quantity was assessed using *Bioanalyzer 2100* (*Agilent Technologies*, Santa Clara, California, USA) and *2200 TapeStation* (*Agilent Technologies*, Santa Clara, California, USA). Ultimately, samples with high RNA quality and quantity were selected for library construction. Total RNA from six samples was sequenced using the *Illumina HiSeq 2500* method (*Macrogen*, Seoul, South Korea) with a commercial kit *TruSeq Stranded* total RNA sample preparation kits with *Ribo-Zero Plant* (*Illumina*, San Diego, California, USA) along with 101 bp paired-end reads.

Identification of circRNAs in total RNA-seq data: In this study, the *CirComPara* (University of Padua, Padua, Italy) integrated pipeline (Gaffo *et al.* 2017) with eight detection tools was used to identify and quantify circRNAs from grape rRNA-depleted RNA-Seq (*Ribominus-Seq*). Quality control of raw reads and read statistics was

performed with *FastQC* (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter sequences and low-quality raw reads were removed from the pipeline using *Trimmomatic* (Bolger *et al.* 2014). The circRNA identification tools based on default parameters were: *Find-circ* (Memczak *et al.* 2013) uses *Bowtie2* (Langmead and Salzberg 2012) read mapper, *DCC* (Cheng *et al.* 2016) with *STAR* (Dobin *et al.* 2013) aligner, *Ciri* (Gao *et al.* 2015) has *BWA-MEM* (Houtgast *et al.* 2018) read mapper, *CircRNA_finder* (Westholm *et al.* 2014) utilizes the *STAR* aligner, *CircExplorer2* (Zhang *et al.* 2014) links to *STAR*, *Segemehl* (Hoffmann *et al.* 2009) and *TopHat* (Trapnell *et al.* 2009) aligners, and *Testrealign* (Hoffmann *et al.* 2014), which uses the *Segemehl* aligner. The *V. vinifera* genome and annotation files (GTF) were downloaded from the *Ensemble Plant* database (Bolser *et al.* 2016). Only circRNAs expressed with at least two back-splice reads and identified together by at least two methods were considered reliable circRNAs for downstream analyses (Gaffo *et al.* 2022). Expression levels of identified circRNAs were normalized by reads per million mapped (RPM). *ANOVA* was used to assess significant differences between stages and conditions.

Bioinformatic approach and functional prediction of circRNAs: The differentially expressed (DE) circRNAs were determined using *Microsoft Excel 2016*, with a fold-change < 2 thresholds between each stage and condition. For each sample, 150 circRNAs that were most up-regulated and least down-regulated were selected. The major miRNAs that showed interactions with up-regulated circRNAs were finally identified by *miRBase v. 21.0* (Kozomara and Griffiths-Jones 2014) and *psRNATarget* (Dai and Zhao 2011). Only miRNAs with an expectation coefficient of ≤ 5 in *psRNATarget* were selected for subsequent analysis. Target genes for miRNAs were identified using *miRNEST* (Szcześniak and Makałowska 2014), *TarDB* (Liu *et al.* 2021), *PsRobot* (Wu *et al.* 2012), and articles (Ding *et al.* 2012, Meng *et al.* 2014). The circRNAs-miRNAs-mRNAs network and the network of target genes for miRNAs were reconstructed by *Cytoscape 3.7.2* (Shannon *et al.* 2003). The network topology was also identified using the *NetworkAnalyzer 2.7* plugin (Assenov *et al.* 2008). The interaction of genes in the co-expression network was calculated based on co-expression, co-occurrence, and text mining parameters using the *STRING* database (Szklarczyk *et al.* 2021). The *GO* was analyzed using the *BiNGO 3.0.3* plugin (Maere *et al.* 2005) in *Cytoscape* software. *GO* terms were selected based on *Hypergeometric test* (Rivals *et al.* 2007), *Benjamini-Hochberg* false discovery rate (FDR) correction (Benjamini and Hochberg 1995), and *P*-value < 0.05.

Conservation analysis of circRNAs: The robust circRNAs identified with 4 ≥ 8 detection tools were selected for the interaction network. Twenty-nine circRNAs that could function as miRNA sponges were aligned to *PlantcircBase v. 7* (E value < $1e^{-5}$) to analyze conservation and confirm authenticity (Xu *et al.* 2022).

Results

Identification of differentially expressed circRNAs: The entire RNA-Seq library of Yagooti grape was used for circRNA detection and quantification. Back-splice junction (BSJ) reads provide a molecular signature for circRNA identification. Most circRNA recognition tools identify circRNA by scanning BSJ reads. For each detection tool, only circRNAs with at least two BSJ reads were considered detected circRNAs. The total number of detected circRNAs in six samples with eight detection tools was 28 157. Among the eight tools, *Testrealign* had the highest number of detected circRNAs with 24 147 BSJ events (64.8% of the total detected). Next, *CircRNA_finder* (13%) and *DCC* (5.3%) accounted for the total number of circRNAs detected. The number of circRNAs detected by *Find-circ* and *Ciri* (4.4 and 4%, respectively), was almost identical to that of *Circexplorer2segemehl* (3.1%), *Circexplorer2star* and *Circexplorer2tophate* (2.5%) (Fig. 1*A*). CircRNAs predicted by a single tool are not always accurate; in particular, circRNAs detected by a single method are generally less precise. Therefore, combining at least two detection tools is an appropriate strategy to increase sensitivity and decrease false-positive rates. For each method, at least 2 reads were required for a circRNA to be considered detected. In addition, circRNAs detected by at least 2 methods are considered reliable. Of the 28 157 circRNAs detected, 3 715 were reliable circRNAs (Fig. 1*B*). Considering all eight methods, 430 circRNAs were identified in the most restrictive setting. The number of circRNAs shared by different combinations of tools was determined (Fig. 2). To continue the analysis, in this study, we only used circRNAs that were independently determined by two or more tools (reliable circRNAs) to check further details, including the annotation of circRNAs according to the reverse splice position with respect to exons or introns.

Of the total 3 715 reliable circRNAs expressed in the present study, 380 and 3 335 cases originated from intergenic and genic regions, respectively. Exon fragments accounted for over 82% of circRNAs from genic regions (Fig. 3*B*). Expression values represent the median of raw counts predicted by reliable circRNAs. The expression values of circulars in all stages and conditions were not significantly different (*P*-value ≤ 0.05), but total circRNA expression was enhanced with treatment (Fig. 3*C*).

The gene ontology of host genes for circRNAs: Based on previous studies, a host gene ontology is used to determine circRNA functions (Gao *et al.* 2019). For Yaghooti grape clustering, *GO* analyses were performed on approximately 900 host genes whose circRNAs showed the most up-regulated and down-regulated expression (Fig. 4).

CircRNAs-miRNAs-mRNAs interaction network: One possible function of circRNA is to repress miRNA function by binding directly or indirectly to the target miRNA through a process called miRNA sponging. A single circRNA can bind to one or more miRNAs in one or more regions (Li *et al.* 2018). Based on circRNAs-

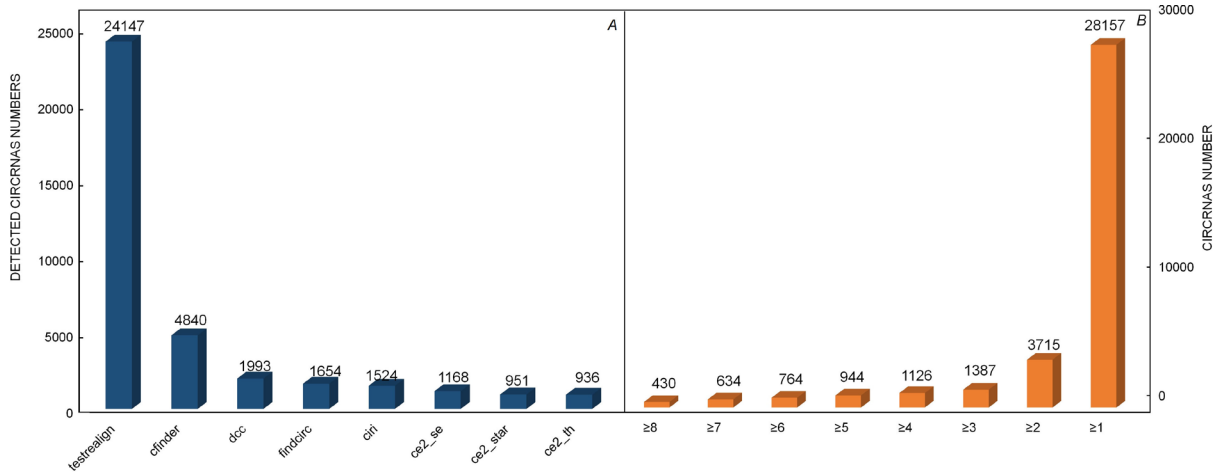

Fig. 1. *A*. Detected circRNAs; circRNAs detected with ≥ 2 reads by each method. *B*. Cumulative detections; the number of circRNAs identified jointly through one and more than one method. The use of two detection methods reduces the false positive rate. *cfinder*: *circRNA_finder*; *ce2_se*: *circexplorer2segemehl*; *ce2_star*: *circexplorer2star*; *ce2_th*: *circexplorer2tophate.*

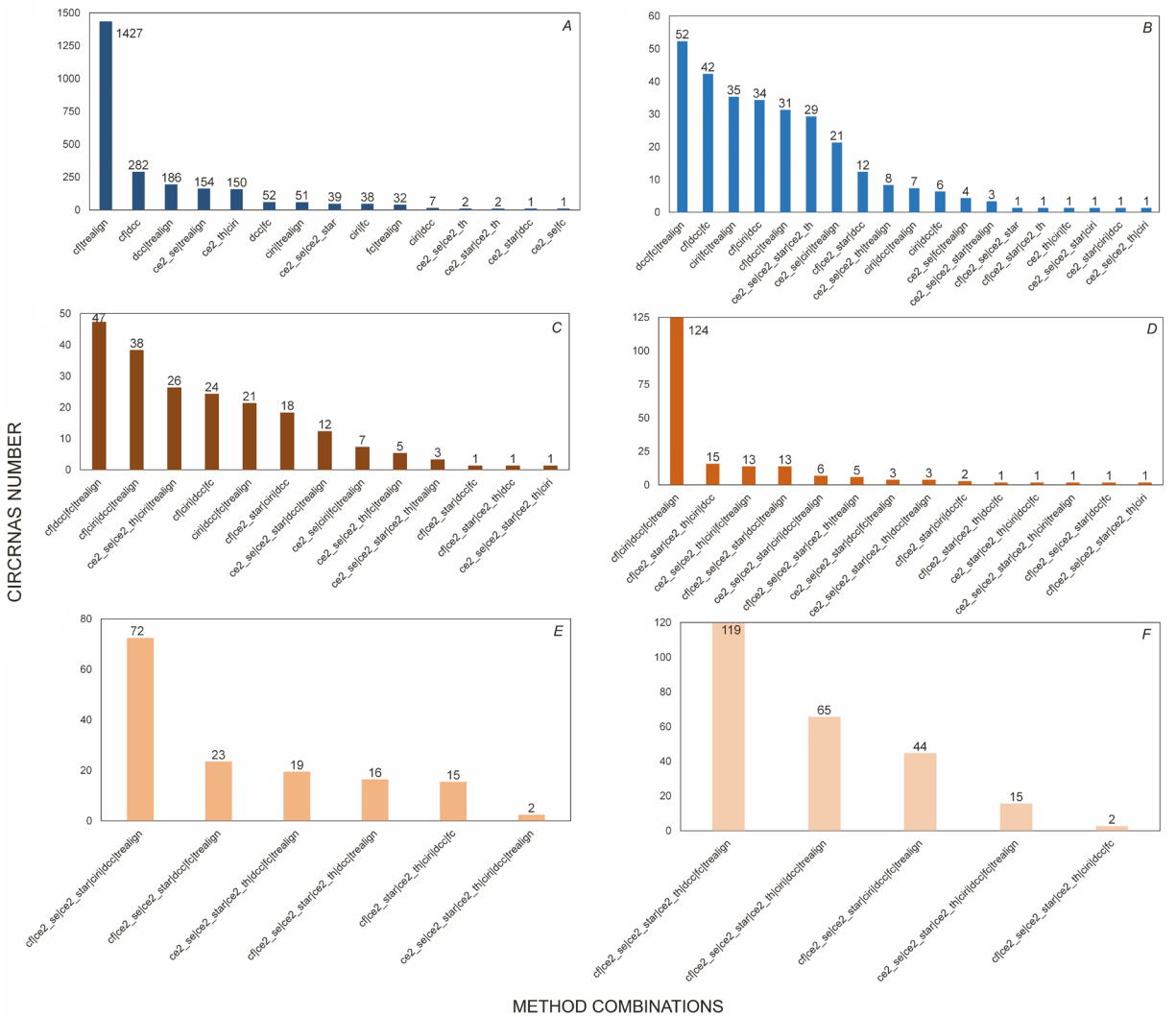

Fig. 2. CircRNAs shared by the methods. *A*. Two methods. *B*. Three methods. *C*. Four methods. *D*. Five methods. *E*. Six methods. *F*. Seven methods. *cf*: *circRNA_finder*; *fc*: *find-circ*; *trealign*: *testrealign*; *ce2_se*: *circexplorer2segemehl*; *ce2_star*: *circexplorer2star*; *ce2_th*: *circexplorer2tophate*.

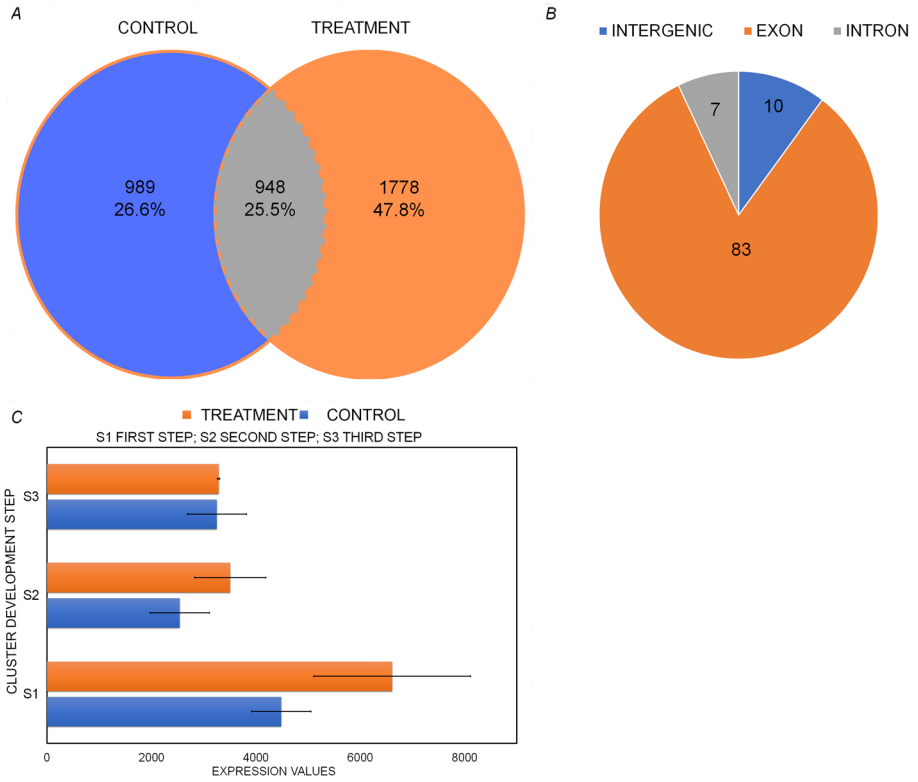

Fig. 3. The distribution of circRNAs expressed in Yaghooti grapes. *A*. The Venn diagram illustrates the number of circRNAs identified as a function of the number of samples. The higher number of circRNAs in gibberellin-treated samples compared to control samples shows the extensive changes in the biological pathways active in the two groups. *B*. Of the estimated 3 715 reliable circRNAs identified, the majority (83%) originated from exon regions within the gene. *C*. Expression values of total circRNAs identified in control and treatment samples at the three developmental stages show that total circRNA expression increased with treatment. *Bars* are based on standard deviations (SDs) and are not significantly different.

miRNAs interaction, reliable circRNAs could bind 502 miRNAs. To construct a circRNAs-miRNAs-mRNAs interaction network, we used circRNAs identified by four or more detection tools. 12 miRNAs were sponged by up-regulated circRNAs in interaction network (Fig. 5). Subsequently, the target gene co-expression network was reconstructed (Fig. 6). The color of each node in the reconstructed network varied from red for larger values to blue for smaller values, depending on the betweenness centrality (BC) parameter. In addition, the size of the nodes varied from small for low values to large for larger values, based on the closeness centrality parameter (CC). The BC component refers to a node's centrality in a complex network. It is calculated based on each node's communication lines. On the other hand, CC refers to the shortest distance between a node and others. In other words, large BC and CC values for a node indicate the high importance of that node in the network (Shiri *et al.* 2020).

Conservation analysis of circRNAs: A comparison was made between our reliable circRNAs and those in other plants. Of the 29 robust circRNAs in the interaction network (Fig. 5), 12 circRNAs showed more than 70% similarity with 610 circRNAs belonging to seven plants in *PlantcircBase*. Based on the *BLAST* results, the circRNAs of Yaghooti grapevine are homologous and conserved with circRNAs from *Arabidopsis thaliana* (78%), *Glycine max* (2.92%), *Gossypium hirsutum* (0.16%), *Solanum lycopersicum* (0.16%), *Solanum tuberosum* (8.81%), *Oryza sativa* ssp. *japonica* (1.16%), and *Poncirus trifoliata* (0.99%) (Table 1).

Discussion

Our study examined the different expression of circRNAs in two groups, the control and gibberellin treated, during three different stages of cluster development. The expressed circRNAs were identified using eight circRNA detection tools. For downstream analysis, we considered circRNAs expressed independently by two or more methods as reliable circRNAs (Fig. 1). The number of reliable circRNAs decreased when the number of recognition tools increased. Each algorithm's performance is evaluated based on its specificity and sensitivity. The algorithm's specificity is determined by the false positive rate. The reason is that each method has specific circRNA prediction criteria. This may lead to the selection of circRNAs with high confidence and lower FDR in circRNA prediction (Gaffo *et al.* 2017, Kalwan *et al.* 2023). Previously, *Circexplorer*, *Find-circ*, and *Ciri* tools

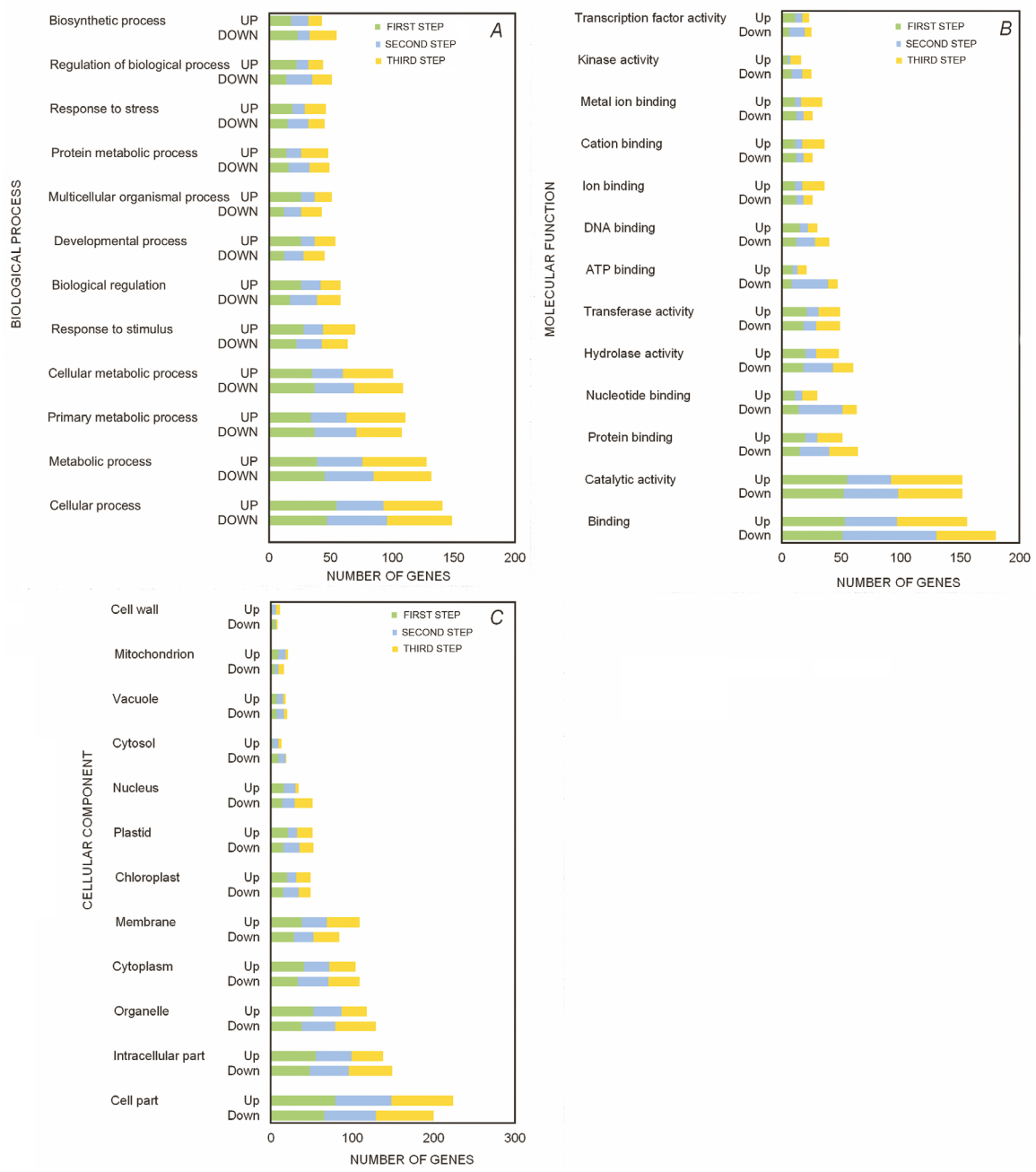

Fig. 4. *GO* analysis plots of host genes for circRNAs show the most significant expression variation at the three developmental stages. This is under gibberellin treatment. In this analysis, 900 host genes were examined. The word "Up" indicates the number of circRNAs with more expression, and the word "Down" indicates the number of circRNAs with less expression in *GO* under gibberellin treatment. The color pattern of the graphs is based on the three developmental stages of the clusters. *A*. Biological process. *B*. Molecular functions. *C*. Cellular component.

were tested to identify circRNAs in grape in response to cold stress. A total of 8 354 circRNAs were detected in five grape tissues, with most detections made using the *Circexplorer* tool. This was followed by *Find-circ* and *Ciri* with 3 509 and 3 181 discoveries, respectively. Considering all three tools, the number of reported putative circRNAs was 1 432 (17.1% of the total circRNAs discovered) (Gao *et al.* 2019).

A comparison of the results of *GO* based on the biological process shows that the number of up-regulated circRNAs was larger than that of down-regulated circRNAs under gibberellin treatment at the first stage of cluster development. This includes the concepts of the multicellular organism process (*GO*: 0032501) and developmental process (*GO*: 0032502). However, this process reversed at the second developmental stage of

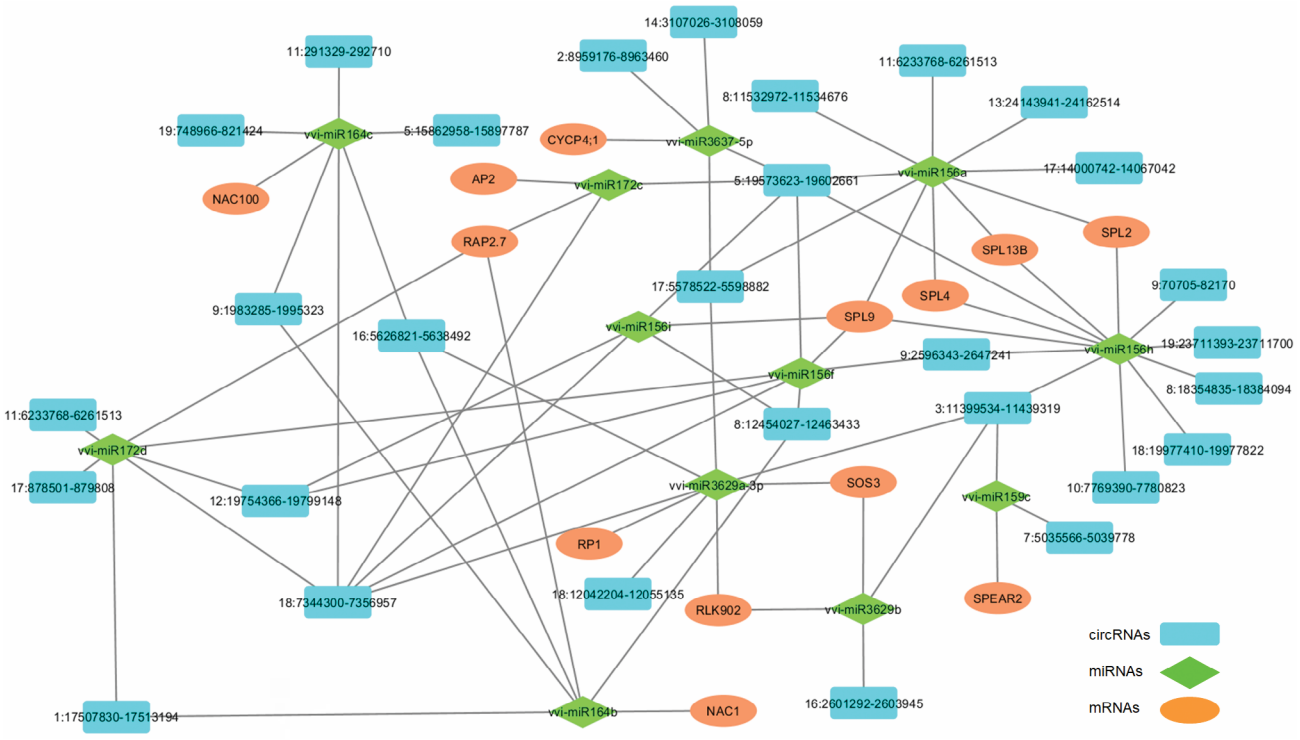

Fig. 5. CircRNAs-miRNAs-mRNAs interaction network.

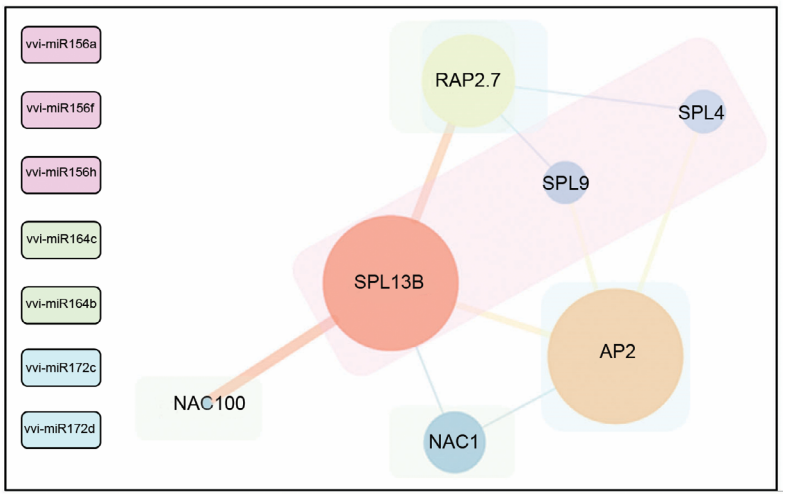

Fig. 6. The co-expression network of target genes for miRNAs is repressed by up-regulated circRNAs. Among the 12 index miRNAs, the target genes for eight miRNAs formed co-expression networks. The size of the nodes varied according to BC (smaller sizes for lower levels), and the color of the nodes ranged between *blue* (low CC) and *red* (high CC). The color and thickness of the communication lines varied from *red* and *thick* for high values of edge linkage to *blue* and *thin* for low values. The miRNAs in the left figure control the genes in the same color frames.

the cluster, when the expression of circRNAs in these terms declined compared to control samples. This expression pattern was repeated in most biological processes. This means that circRNA were up-regulated in the first developmental stage of the cluster and down-regulated in the second and third developmental stages under gibberellin treatment. However, some cases contradicting these expression patterns were also observed. For example, the expression of circRNAs derived from host genes involved in the biosynthetic process (*GO*: 0009058) decreased under gibberellin treatment. The molecular function of host genes can be illustrated by a strong down-regulation of host genes with ATP binding (*GO*: 0005524) molecular function. This significant difference was observed only at the second cluster developmental stage of grape, and only four of the 31 genes with ATP

Table 1. Preserved grape circRNAs also identified in other plants.

Predicted grape circRNAs	Crops with conserved circRNAs
5:19573623:19602661	*Poncirus trifoliata* (1) *Arabidopsis thaliana* (4) *Oryza sativa* ssp. *japonica* (3)
3:11399534:11439319	*Arabidopsis thaliana* (515)
12:19754366:19799148	*Gossypium hirsutum* (1) *Solanum tuberosum* (1)
7:5035566:5039778	*Poncirus trifoliata* (1)
19:748966:821424	*Solanum tuberosum* (1) *Glycine max* (1)
16:2601292:2603945	*Oryza sativa* ssp. *japonica* (4)
17:14000742:14067042	*Glycine max* (6) *Solanum lycopersicum* (1) *Solanum tuberosum* (41) *Poncirus trifoliata* (2)
10:7769390:7780823	*Glycine max* (5) *Solanum tuberosum* (5)
18:7344300:7356957	*Poncirus trifoliata* (2)
9:1983285:1995323	*Glycine max* (1)
5:15862958:15897787	*Solanum tuberosum* (5) *Glycine max* (5)
9:2596343:2647241	*Arabidopsis thaliana* (4)

binding molecular function showed up-regulated levels in gibberellin-treated samples compared with control samples. A clear reason for this observation is that Yaghooti grapes require free energy in the form of ATP to drive their biochemical processes and to tolerate the hot and dry climate of the Sistan region, which intensifies during the second stage of cluster development (Shiri *et al.* 2020). However, as mentioned in the study of the biological process diagram, gibberellin treatment promoted biological processes in the first developmental stage of the cluster, which coincides with the beginning of April, when the climate in the Sistan region is still temperate. Many developmental processes in grape treated with gibberellin are completed at the first stage of cluster development compared to the control sample. Compared to the control sample, the gibberellin-treated sample reduces its biological interaction in the second stage to reduce environmental damage and conserve energy, by reducing the need for energy molecules in the form of ATP. Gibberellin synthesis in *Arabidopsis thaliana* was increased in the presence of elevated ATP. Therefore, an increase in the external use of gibberellin could inhibit ATP synthesis (Zhu *et al.* 2012). Similarly, GO analysis of circRNA host genes obtained from tissue stages in developing leaves of *Camellia sinensis* reveals the enrichment of the molecular function domain of ATPase activity (Tong *et al.* 2018). An examination of the cellular components of the host genes revealed that the activity of the cellular components increased similarly to the biological processes and molecular functions, during treatment with gibberellin in the first developmental stage of the cluster. The activity of host genes with a cellular component of the nucleus (*GO*: 0005634) decreased sharply in the third developmental stage of the cluster under gibberellin. As mentioned above, the activity is increased in the first stage of development of the cluster under the influence of gibberellin, while it flattens in the second stage and further decreases in the third stage. The study of transcriptional changes in Yaghooti grape from Sistan under gibberellin treatment revealed the process of cell activity in grape during the three stages of cluster development. According to this, the nucleus and cytoplasm are more active than the other components in the first and second stages, respectively. In the third stage, the cell wall and membrane are involved in the developmental cycle of the grape cluster. However, this cellular order changes under the influence of gibberellin treatment, so that both the nucleus and cytoplasm activity is increased in the first stage. In contrast, the activity of the cytoplasm and cell wall was observed in the second stage, and the cell wall was more active in the third stage of cluster development (Shiri *et al.* 2018, 2020). These results demonstrate the function of circRNAs during cluster development and berry ripening in grape. Identification of DE circRNAs and the function of host genes in other plants revealed the key role of circRNAs in different developmental states. *GO* enrichment analysis of parent genes of DE circRNAs revealed the cell wall organization or biogenesis, structural molecule activity, structure-specific DNA binding, and signal transducer activity between green tomato and mature red tomato (Yin *et al.* 2018).

Of the 503 miRNAs identified, 12 index miRNAs were selected based on their sponge circRNAs and expression level (Table 1 Suppl.). As mentioned previously, circRNAs were up-regulated in gibberellin-treated samples compared with control samples (Fig. 3). This suggests that circRNAs play an important role in the biological changes of gibberellin-treated plants. CircRNAs can act as miRNA sponges and prevent miRNAs from binding to their target transcript, which means that the products of the target genes of these miRNAs can play a key role in the plant.

Interaction networks were dominated by the miR156, miR172, and miR164 families (Fig. 5). They have been implicated in a variety of biological and developmental processes, including vegetative growth, fertility, and fruit ripening (Waititu *et al.* 2020). They regulate flower development in *Arabidopsis* (Jung *et al.* 2014), *Brassica napus* (Wang *et al.* 2019), and strawberry (Zheng *et al.* 2019). Developmental phase transition in plants is regulated by miR156 and miR172 expressions. During plant growth, miR156 is highly expressed, whereas miR172 is low expressed. As plants are ageing, miR156 expression decreases and miR172 expression increases, which results in a transition from vegetative to reproductive growth (Babaei *et al.* 2023). The miR156 family controls the expression of genes of the *squamosa promoter binding protein-like* (*SPL*) genes related to stage transition and flowering time. The regulatory target of miR172 is a subfamily of *APETALA2* (*AP2*) transcription factor genes. Both *AP2* and *related to AP2* (*RAP2.7*) target genes are critical for the onset of the flowering process, and miR172 regulates flowering time by reducing their activity (Chung *et al.* 2020). The miRNA164 family restrict the

NAC gene expression in plant. The NAC proteins form one of the largest families of plant-specific transcription factors that play a crucial role in plant development and stress response (Hernández and Sanan-Mishra 2017).

In co-expression network (Fig. 6), the *SPL13B* target genes were the most critical genes among the DE circRNAs and had the largest BC and CC-values based on topology analysis. The greatest number of circRNAs as miR156 sponges was observed at the first and third cluster developmental stages in control and gibberellin treated samples (Table 2 Suppl.). A decrease in the number of circRNAs as miR156 sponges is observed at the second stage. This is due to the role of *SPL*-box family genes in the flowering process at the first cluster developmental stage and in fruit ripening at the third cluster developmental stage.

The constructed circRNA-miRNA-mRNA network identified four circRNAs that had predicted binding sites for miR156 and miR172 involved in developmental phase transition in soybean (Babaei *et al.* 2021). DE circRNAs during pollen development in *Brassica rapa* have the ability to sponge the significant microRNA regulatory module for flower development, miR156, miR172, and miR164 (Babaei *et al.* 2021).

Our examination of the up-regulated circRNAs and their expressions revealed that the expression of sponge circRNAs was more pronounced in the index miRNAs under gibberellin treatment (Fig. 5). This would mean that the target genes (Table 2 Suppl.) had lower post-transcriptional control after gibberellin treatment and were more active than in the control sample. Thus, our results suggest, gibberellin treatment could reduce compact clusters in the Yagooti grape through circRNA expression levels at developmental stages.

Reliable circRNAs identified with 4 or more detection tools, revealed high conservation (Table 1). 18:7344300:7356957 circRNA could interact with miR172c and miR172d, miR164c, miR156i, and miR156f simultaneously in the network. It had e-value 2^{-87} and identities 74% with ptr_circ_000242. Interestingly, ptr_circ_000242 expressed in DE circRNAs when the early flowering process was studied in trifoliate orange. Functional analysis showed that circRNAs influence the flowering process by regulating conserved miRNA families such as miR172 (Zhang *et al.* 2012, Zeng *et al.* 2018). 12:19754366:19799148 circRNA had 96 and 75% similarity to stu_circ_000772 and ghi_circ_000090, respectively. Three miRNAs of miR172, miR156, and miR164 family are targeted by this circRNA in the grape interaction network. It is possible that these results indicate the importance of conserved circRNAs during crucial processes in plants.

Conclusions

CircRNA expression profiles were compared to determine their functions in clustering of gibberellin-treated Yaghooti grape from Sistan region in three developmental stages. The total number of reliable circRNAs identified in the present study was 3 715, with 380 and 3 335 cases belonging to the intergenic and corresponding genic regions, respectively. More than 82% of the circRNAs from the genic regions belonged to exon fragments. Our examination of the expression profiles of circRNAs up- and down-regulated under gibberellin treatment revealed 503 miRNAs with binding components to target genes. Starting from the index miRNAs, vvi-miR156, vvi-miR164, and vvi-miR172 controlled the flowering process in Yaghooti grape. The regulatory target of miR172 is a subfamily of *AP2* transcription factor genes. Both *AP2* and *RAP2.7* target genes are critical for the onset of the flowering process, and miR172 regulates time of flowering by reducing their activity. In addition, miRNAs from the miR164 family affect the lateral root development by controlling the expression of *Nac1* and *NAC100* genes. Although the results indicate the key role of circRNAs in the process of development in Yaghooti grapevines, the functional and regulatory mechanisms of circRNAs need to be further confirmed in the laboratory.

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