Differential Expression of Hyperhydricity Responsive Peach MicroRNAs

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Summary

Hyperhydricity is a syndrome that causes morpho-physiological malformations in tissue culture plantlets. Micro-RNAs (miRNA) are small non-coding RNAs that play important regulatory roles in plant development, stress response, and adaptation to environmental conditions. In this study, differential expression analysis indicated that miRNAs play an underlying role in the responses to the hyperhydricity syndrome in peach *Prunus persica* (L.) leaves. 24 known and three novel potential miRNAs were characterized in hyperhydric and non-hyperhydric transcriptome libraries. The miRNA-target transcript analyses indicated that transport, plant cuticle development, intracellular part, and stress response are regulated by miRNAs in hyperhydric leaves. It is also suggested that miR5021 and miRnovel2 might play critical regulatory roles in hyperhydricity regarding miRNA-based response to stress. This study went one step further to advance understanding of molecular miRNA-based regulatory mechanisms regarding responses to hyperhydricity in peach.

1 Introduction

Micro-RNAs (miRNAs), are non-coding, endogenously expressed short RNAs which play critical roles in transcriptional regulation [1]. They post-transcriptionally suppress the expression of targeted genes by interfering or cleaving the targeted transcript (mRNA) in collaboration with RNA-induced silencing complex (RISC) [2]. Primary transcripts, called pri-miRNA, of the miRNA encoding genes, are processed into miRNA precursors (pre-miRNA) formed with their characteristic secondary structures, further digested by Dicer-like endo-nucleases to generate mature miRNAs [3]. It is clear that pre-miRNAs have special structures which are crucial to recognition and cleavage during miRNA biogenesis [4]. The stem-loop secondary structure consisting of highly conserved miRNA:miRNA* complementary arms is one of the main characteristics of a pre-miRNA [5]. Features as the stability of the secondary structure, minimum free energy (MFE), length, and hundreds more [6, 7], are used as parameters to discriminate miRNA precursors from other pseudo-hairpins, mRNAs, genomic background and long-noncoding RNAs (lncRNAs) [8-10].

Plant miRNAs play crucial roles in biological processes such as development, stress response, cell differentiation and hormone signaling [11]. They appear to be evolved to respond environmental stresses and diseases under certain conditions and their expression follows time specific manner to respond environmental and biological changes [12]. A number of reports revealed that miRNAs are differentially expressed under conditions as drought, salinity, temperature, nutrient, metals, and diseases to convey tolerance [13-16].

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Hyperhydricity, formerly known as vitrification, is a type of physiological malformation syndrome seen in plants regenerated in tissue culture containers with glassy and water-soaked appearance in leaves [17]. Excess water uptake to the apoplasts is the primary effect of hyperhydricity. Waterlogging in tissues, especially leaves, causes a reduction in the gas exchange between cells and environment leading to hypoxic stress, and the increase of reactive oxygen species [18, 19]. Hyperhydricity is a serious problem since it can affect generation, survival and quality of plantlets in culture and lead to economic loss during agricultural usage [18, 20]. Although morphological and anatomical changes in the culture regenerated-plants in response to the underlying syndrome were largely studied, only few works have investigated the molecular level [21-23]. In a recent report performed by our group, the genome-wide expression pattern of hyperhydric and non-hyperhydric peach leaves were comprehensively analyzed [24]. In the light of such information, here, we investigated the hyperhydricity syndrome in respect to miRNA-based responses. To reveal the role of miRNAs in such regulatory mechanisms regarding responses to hyperhydricity in peach, we performed miRNA identification and expression measurement as well as target transcript annotation via computational approaches.

2 Materials and methods

In this study, we utilized the peach transcriptome libraries, which were previously generated by our groups. Briefly, the RNA-sequencing (RNA-seq) libraries generated from two groups; two non-hyperhydric (as control) and three biological replicates of hyperhydric peach leaves (Non-hyperhydric: SRR1300787; SRR130078 and hyperhydric biological replicates: SRR1300790; SRR1300834; SRR1300836) [24], were examined in terms of their quality graphs using the Sequence Read Archive (SRA) browser [25]. FASTQ files were transformed from sequence files by using -skip-technical, -clip-files and -split-files options of the fastq-dump alternate of the SRA Tool Kit. Trimmed sequencing reads were analyzed via RNA-seq analysis software tools (Figure 1).

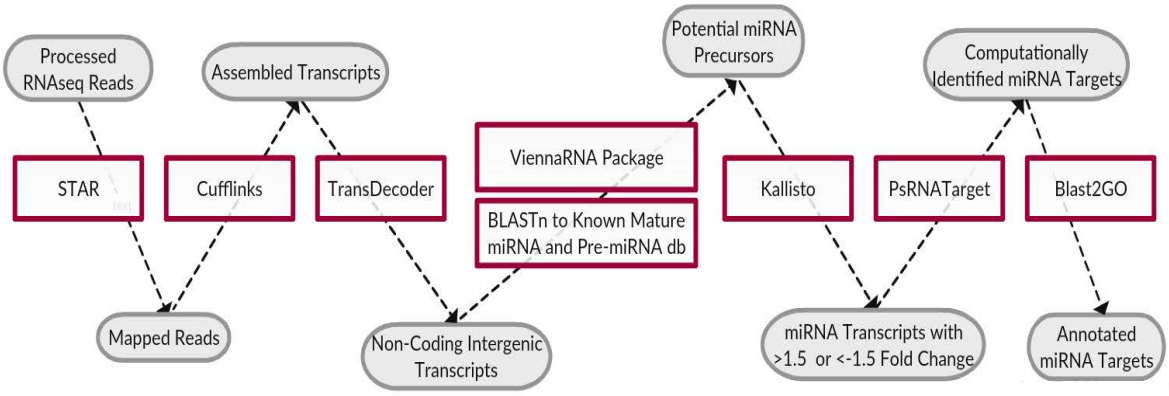

Figure 1: Demonstration of the computational strategy, red boxes indicate the software tool used in this study.

2.1 Identification of putative lncRNAs

High-quality sequencing reads were mapped to the peach reference genome (assembly Prupe1_0) [26] using STAR v2.5.2a [27], a spliced aware-alignment tool. Aligned reads were then assembled to determine novel transcript sequences using the popular *ab initio* assembler, Cufflinks v2.2.1 [28]. The Cuffmerge tool was utilized to merge all gene transfer format (GTF) files generated via *ab initio* assembly. Afterward, previously un-annotated transcript sequences were revealed with the Cuffcompare utility in the Cufflinks suit; transcript features

were utilized as the query against Ensembl Plant database (release33) [29]. Annotated transcript sequences were downloaded from GreeNC database (v1.12) [30] to create a comprehensive lncRNA list. In order to differentiate transcripts into coding and noncoding; coding potentials of each was tested using TransDecoder v2.0.1 with default parameters [31]. Transcripts with no coding potential were filtered based on their length. The ones equal or longer than 200 bp were utilized as input data for downstream approaches as identified lncRNAs.

2.2 Prediction of pre-miRNAs

Blast+ v2.5.0 [32] search database was generated from transcripts which were filtered according to their length and coding potential. Databases containing hairpins (pre-miRNAs) and mature miRNAs were acquired from miRBase v.21 <http://www.mirbase.org/> and were used as queries against our custom Blast database. Blast-search was performed with default parameters. Then, all the filtered transcripts were subjected to the ViennaRNA Package v2.1.9 [33] with default parameters to estimate the secondary structures of the all the filtered transcripts. The MFE indexes of all transcripts were calculated according to the equation described previously [34]. Then Blast results were subjected to the three threshold criteria, transcripts with >0.67 MFEI, e-value <0.001, and only transcripts that have homology with plant miRNAs and pre-miRNAs were selected for downstream analysis. These filtered Blast results were separated into four sub-groups as hits only to hairpin queries, hits only to mature miRNA queries, hits which include both hairpin and mature miRNA queries, and transcripts with no hits. It is suggested that transcripts with hits including both mature and pre-miRNA queries are potentially known miRNA precursors and the transcripts with no hits are potential novel miRNA precursors [35]. These sub-groups were manually investigated to reveal potential known and novel miRNA precursors.

2.3 Quantification of potential miRNA precursors

The abundance of miRNA-precursors was estimated using Kallisto v0.43.0 tool [36]. Bootstrap-samples option was set to 100, and normalized transcripts per million (TPM)

$$\frac{\text{Actual miRNA precursor count}}{\text{Total count of clean reads}} \cdot 10^6$$

values were calculated for each transcript as previously described [37]. Fold changes (log2) of normalized counts between control and hyperhydric libraries were calculated and transcripts with more than 1.5 or less than -1.5 fold changes were selected [38]. It was assumed that these ones which expressions were significantly changed were associated with the hyperhydricity in peach.

2.4 Target transcript identification and gene ontology analysis

MicroRNA targets are often identified using machine learned models [39, 40], but in this case the target transcripts were identified via the web-based psRNATarget server [41]. The program was run with default parameters; user submitted miRNA/ preloaded transcripts option was chosen and differentially expressed known and novel miRNAs were utilized as input. Functional annotation of predicted miRNA-target transcripts was revealed using the Blast2Go software v4.0 [42]. All miRNA target transcript sequences were uploaded as an input fasta formatted text file and BlastX was performed with an e-value threshold of 10^{-10}. Then, BlastX results were utilized to retrieve GO terms associated with each hit. All the transcripts targeted by the miRNAs were clustered by their category: molecular function, cellular component, or biological process.

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3 Results

A total of 4,218 transcripts were assigned to lncRNAs in our computational analyses; 1,789 of them were previously un-annotated (Suppl. Table 1) while the rest of them were downloaded from GreeNC database to create a comprehensive lncRNA list. MFE indexes of all these transcripts were within the range of between 0.05 to 1.42. The MFE indexes of 572 lncRNA transcripts were calculated as greater than 0.67, which were the candidate miRNA precursors. Only 55 of them had sequence similarity to known mature miRNAs and pre-miRNAs according to the Blast-search. From these 55 transcripts; 22 transcripts had homology with only pre-miRNA sequences while 34 transcripts had Blast hits with only mature miRNA sequences, and 19 transcripts had Blast hits with both mature and pre-miRNA sequences (Suppl. Table 2). Remaining 517 transcripts had MFE indexes greater than 0.67 were the potential novel miRNA precursors (Suppl. Table 2). Fold changes of 140 transcripts among the 517 potential novel miRNA precursors were greater than 1.5.

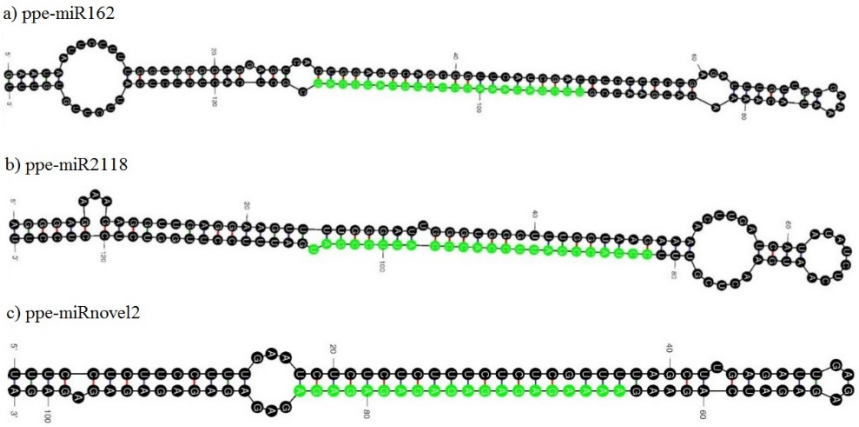

Figure 2: Secondary structure representations of selected known and novel pre-miRNAs.

We predicted 3 novel miRNA-sequences and their precursors among these potentials and annotated them as ppe-miRnovel1, ppe-miRnovel2, and ppe-miRnovel3 (Table 1). We identified 24 known miRNAs associated with the hyperhydricity in peach via Blast-search. All of the known and novel miRNAs identified in this study are shown in Table 1. miRNA:miRNA* complementarity of characterized miRNAs was manually investigated and confirmed. The secondary structures of 3 representative miRNA precursors; ppe-miR162, ppe-miR2118, and ppe-miRnovel2 were predicted and are presented in Figure 2.

3.1 Identification of differentially expressed miRNAs in hyperhydric leaves

Differential expression patterns of miRNA precursors under hyperhydricity, for 572 potential precursor transcripts, demonstrated as a scatter plot in Figure 3. Most of the potential precursors were found to be suppressed in hyperhydric leaves of peach. Expression of the corresponding miRNA precursors were also determined to be down-regulated with the fold changes ranging between 0.5 and 15. The most striking differential expression were observed for miRnovel1 and miR398b with the fold changes of 15 and 9, respectively (Suppl. Table 4, Table 2).

3.2 Identification and functional characterization of miRNA target transcripts

We predicted 405 targets for 17 peach miRNAs which are differentially regulated under hyperhydricity, some of them are presented with their target description and gene ontology (GO) terms in (Table 3). In this study, 1,106 GO terms were assigned to the 405 targets based

on similarity to annotated coding sequences (Suppl. Table 4). The majority of GO terms assigned from the molecular function category associated with the catalytic activity (GO: 0003824; 57.14%) and binding (GO: 0005488; 14.29%), on the other hand for biological process, localization (GO: 0051179; 25%) is the most abundant GO annotation and metabolic process (GO: 0008152; 20%) tag after that. Single-organism process (GO: 0044699; 10%) and response to stimulus (GO: 0050896; 10%) are the other major GO terms which were assigned to the miRNA-target transcripts. Major GO terms associated with the cellular components were cell (GO: 0005623; 59.46%), organelle (GO: 0043226; 32.43%) and macromolecular complex (GO: 0032991; 10.81%). The GO term distributions over the putative miRNA-targets for the top 50 is presented in (Figure 4).

Table 1: Computationally identified novel and known miRNAs in peach and their features.

miRNA Name	miRNA Sequence (5'-3')	Length of Mature miRNA	Length of pre-miRNA
miR156g	TTGACAGAAGATAGAGAGCAC	21	83
miR156h	TTGACAGAAGATAGAGAGCAC	21	110
miR156i	TTGACAGAAGATAGAGAGCAC	21	124
miR156f	TGACAGAAGATAGAGAGCAC	20	146
miR162	TCGATAAACCTCTGCATCCAG	21	145
miR166c	TCGGACCAGGCTTCATTCCCC	21	180
miR166a	TCGGACCAGGCTTCATTCCCC	21	95
miR166d	TCGGACCAGGCTTCATTCCCC	21	168
miR166e	TCGGACCAGGCTTCATTCCCC	21	162
miR398b	CGTGTTCTCAGGTCGCCCCTG	21	127
miR399b	TCTGCCAAAGGAGAATTGCCC	21	120
miR535a	TGACAACGAGAGAGAGCACGC	21	106
miR535b	TGACGACGAGAGAGAGCACGC	21	131
miR169h	TAGCCAAGGATGACTTGCCTGC	22	176
miR169j	TAGCCAAGGATGACTTGCCTGC	22	207
miR482c-5p	GGAATGGGCTGTTTGGGATG	20	123
miR482c-3p	TTCCCAAGCCCGCCCATTCCAA	22	123
miR6295	GAGGACAGAAGATGATTCAGC	21	335
miR8132	TCCAACGATGGGTGACCACAA	21	111
miR167b	TGAAGCTGCCAGCATGATCTA	21	139
miR5021	TGAGAAGAAGAAGAAGAAAA	20	173
miR2118	TTACCGATTCCACCCATTCCTA	22	126
miR845	TGCTCTGATACCAATTGTTGG	21	96
miR8051	TAGTATGGTAGAAAGATTCA	20	43
*miRnovel1	ACTTTGGTGCCAAGGTATA	19	210
*miRnovel2	AAAAGAGAGAGAGAGAGAGA	20	102
*miRnovel3	TCTTCAAATATAGAGAGGCAT	21	210

* indicates the novel miRNAs identified in this study.

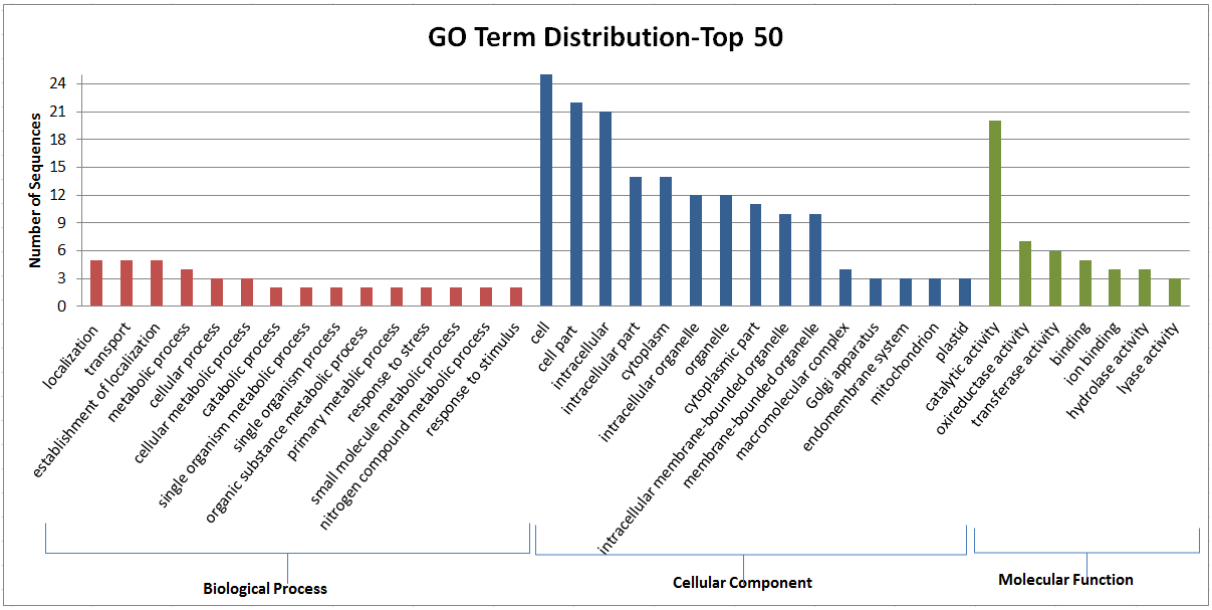

Figure 4: GO term distribution over the target sequences for top 50 GO annotations.

4 Discussion

Hyperhydricity is an important issue to uncover molecular responses of plant tissues to stress. Previous studies revealed that hyperhydricity affects morphology and physiology of plants regenerated in tissue culture. In this study, we consider hyperhydricity with respect to miRNA-mediated regulation.

A great majority of plant miRNAs are conserved among species [43]. These conserved miRNAs share some conserved sequences almost identical, with the help of this homology this study revealed the conserved orthologous miRNA families in peach. Axtell and Bartel's [44] comprehensive data suggested that most of the miRNA families (~%30) are conserved in different species, also these ancient miRNAs in plants may have been regulating the same targets, and this indicates that the regulatory roles are also conserved among the species. miR156, miR399, miR166, and miR162 are the highly conserved miRNAs expressed among plant species and also identified in peach so our data is consistent with the previous reports [37, 45, 46] (Table 1). Beside the known miRNAs, in this study three novel peach miRNA sequences were predicted according to the well-established features of miRNA precursors [34, 47]. Stem-loop structures and MFE indexes of the transcripts were consistent with the established criteria (Table 2, Figure 2).

Small RNA sequencing is the commonly used approach to identify small non-coding RNAs [48]. In this study, we employed transcriptomic data to identify miRNAs [49, 50] which is a convenient way to reveal novel miRNAs. It is clear that miRNA precursors have well-established features, therefore, the primary transcripts can be distinguished from others easily in transcriptome data [34]. In one of the recent studies about miRNA identification from transcriptome data, Ozhuner et al. reported 32 known and four new boron stress responsive barley miRNAs [51]. As compared to that; here less number of hyperhydricity related-miRNAs were identified with 24 known and three novel ones (Table1).

Table 2: Differential expression levels and MFE indexes of miRNA precursors.

MFEI: Minimum Free Energy Indexes of the transcripts; non-hyperhydric Mean; mean of normalized read counts for 2 control (Non-hyperhydric) libraries; Hyperhydric Mean: mean of normalized read counts for 3 hyperhydric biological replicate. Read counts normalized according to TPM method. * indicates the novel miRNAs identified in this study.

Transcript ID	miRNA Name	MFEI	Nonhyperhydric Mean	Hyperhydric Mean	Fold Change
TCONS_00036792	miR8051	0.733	12.071	4.587	3 ↓
TCONS_00036379	miR156g/miR156h	0.768	31.890	5.812	5 ↓
TCONS_00021312	miR156g/miR156h /miR156i/miR156f	0.707	20.278	5.958	3 ↓
TCONS_00024679	miR156g/miR156h /miR156i/miR156f	0.715	8.030	4.212	2 ↓
lcl|Ppersica_Prupe.5G213900.1	miR162	0.718	23.628	7.326	3 ↓
lcl|Ppersica_Prupe.5G213900.2	miR162	0.731	3.662	2.116	0.5 ↓
lcl|Ppersica_Prupe.5G130100.1	miR166c/miR166a/ miR166d/miR166e	0.741	3.455	1.370	2 ↓
lcl|Ppersica_Prupe.4G263200.1	miR398b	0.706	16.941	1.858	9 ↓
lcl|Ppersica_Prupe.4G066200.1	miR399b	0.715	4.456	1.675	3 ↓
lcl|Ppersica_Prupe.8G073800.1	miR535a	0.684	10.663	4.817	2 ↓
lcl|Ppersica_Prupe.8G187600.1	miR535a/miR535b	0.782	21.722	11.183	2 ↓
lcl|Ppersica_Prupe.8G187700.1	miR535a/miR535b	0.739	17.358	10.017	0.5 ↓
TCONS_00037437	miR845	0.697	5.373	15.800	3 ↓
lcl|Ppersica_Prupe.1G219200.1	miR169h/miR169j	0.709	11.404	4.013	3 ↓
lcl|Ppersica_Prupe.3G149400.1	miR482c-5p / miR482c-3p	0.787	3.532	2.209	0.5 ↓
lcl|Ppersica_Prupe.1G318600.1	miR2118	0.715	5.594	2.901	2 ↓
lcl|Ppersica_Prupe.3G233100.1	miR5021	0.694	2.606	1.426	2 ↓
lcl|Ppersica_Prupe.6G304700.1	miR6295	0.856	9.724	6.386	0.5 ↓
lcl|Ppersica_Prupe.6G208000.1	miR8132	0.740	7.217	3.722	2 ↓
TCONS_00030948	miR167b	0.671	2.315	1.186	2 ↓
TCONS_00013697	*miRnovel1	0.822	87.942	5.632	15 ↓
lcl|Ppersica_Prupe.4G006200.1	*miRnovel2	0.713	69.640	7.111	9 ↓
TCONS_00051306	*miRnovel3	0.894	57.790	9.052	6 ↓

Plant miRNAs appear to be involved in response to environmental stresses and biological changes such drought, salinity, temperature, nutrient, metals and diseases [52, 53]. Several differential expression analyses revealed that miRNA expression level changes to show tolerance to such conditions [54, 55]. In this study, differential expression of miRNAs (Table 2) and expression patterns of potential miRNA precursors (Figure 3) clarified that miRNA expressions also changed in response to hyperhydricity in peach. As demonstrated in Figure 3, the majority of miRNA precursor transcripts were down-regulated such as miR398 which targets copper superoxide dismutase and cytochrome C oxidase subunit V [56]. It was supposed to be related with the hyperhydricity tolerance mechanisms. In relation to that, Sunkar et al. confirmed that miR398 down-regulation is important for oxidative stress tolerance in *Arabidopsis thaliana* [57]. It is also known that regulation of miR398 is important to respond to high-concentration of salt and abscisic acid ABA stress [58].

Excessive water uptake caused by the hyperhydric condition impairs the gas exchange mechanism between tissues leading to reactive oxygen species (ROS) formation and oxidative stress [24]. As miR398b plays an important role in the response to stress conditions; its down-regulation upon hyperhydricity in peach may be correlated with abiotic stress response.

Computational target prediction of the miRNAs is the most favorable strategy for discovery and functional annotation. Here, target transcripts were predicted via a homology-based bioinformatics approach, and most of the target transcripts were found as homologous to *A. thaliana.* Their functional characterizations showed that the majority of the target transcripts associated with the transport, plant cuticle development, intracellular part, and stress response. One of the predicted target transcript TC14848 encoding NADP-dependent malic enzyme (NADP-ME) which is localized in bundle sheath chloroplasts was targeted by the miRNAs miR156g, h, and i [59]. The enzyme associated with the breakdown of photosynthesis and its expression is differentially regulated under abiotic stress conditions [60]. Due to hyperhydricity, plant leaves under the absence of proper light chain reactions further altered the NADP-ME activity. Therefore, miRNA-based regulation of the NADP-ME transcript might be correlated with the findings reported by Bakir et al. and Chakrabarty et al. [24, 61]. Their genome-wide studies revealed differentially expressed transcripts which are functionally related to the NADPH dehydrogenase activity and photosynthesis. On the other hand, some reports indicate that miR156 is targeting the squamosa promoter-binding protein-like (SPL) family transcription factor [45, 62, 63]. In this study, we annotated a new putative targeted transcript, which is annotated as TC14848 for miR156 related with the hyperhydricity in peach as described above.

Chakrabarty et al. also observed discontinuous development of cuticle and epicuticular wax layers in hyperhydric plant leaves. In relation to this, some researchers suggested that the ATP binding cassette (ABC) transporters take role in plant cuticle formation [64, 65]. Here, we predicted that the ABC transporter family I as a miR5021 target transcript might be related with the mechanism of the hyperhydricity.

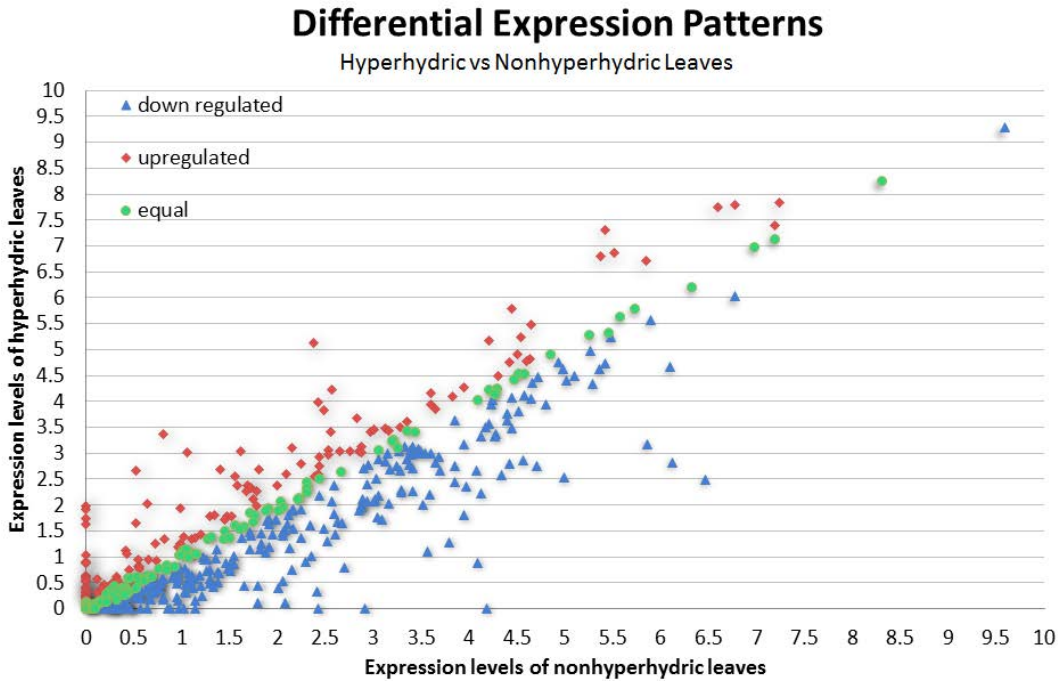

Figure 3: Scatter-plot demonstration of differentially expression patterns of miRNA precursors between hyperhydric and non-hyperhydric peach leaves.

Table 3. Selected miRNA target descriptions and GO terms.

miRNA Name	Target Accession	Target Sequence Description	Go Molecular Function	Inhibition
miR8051-5p	TC12081	Nuclear transcription factor Y subunit B-3-like	Sequence-specific DNA binding	Cleavage
miR156g	TC14848	NADP-dependent malic enzyme	Oxidoreductase activity	Cleavage
miR156h	TC13144	Fatty acid amide hydrolase	Hydrolase activity	Cleavage
miR156i/ miR156f	BU040190	Teosinte glume architecture 1-like	DNA binding	Cleavage
miR162	FC864998	Endoribonuclease Dicer homolog 1-like	Hydrolase activity	Cleavage
miR399b	TC10279	Aspartic ase-like	Peptidase activity	Cleavage
miR535a	AJ827655	Ethylene-responsive transcription factor 1B-like	DNA binding	Cleavage
miR845	DW344875	Probable serine threonine- kinase At1g54610	nucleic acid binding	Cleavage
miR482c-3p	DW341459	Receptor kinase HERK 1	ATP binding	Cleavage
miR2118	TC15344	Uridine kinase chloroplastic	Transferase activity	Cleavage
miR5021	TC12747	ABC transporter I family member chloroplastic	ATPase activity	Cleavage
*miRnovel2	TC13858	Gibberellin 2-beta-dioxygenase 2-like	Oxidoreductase activity	Cleavage
*miRnovel2	TC14464	Auxin-responsive IAA9	Biological process	Cleavage

* indicates the novel miRNAs identified in this study.

Among the predicted novel miRNAs, miRnovel2, which was 9 fold suppressed in hyperhydric leaves (Table 2), targets a diverse range of target transcripts. One of them is the gibberellin 2-beta-dioxygenase 2 which catalyzes the 2-beta-hydroxylation of several biologically active gibberellins which is crucial for plant development [66]. Developmental retardation is one of the significant observations in hyperhydric plants. Thus, miRnovel2 may contribute to the regulation of gibberellin 2-beta-dioxygenase transcript in response to hyperhydricity.

Gene Ontology analysis of the predicted targets of identified miRNAs indicated that the majority of the targets functionally annotated as catalytic activity (GO: 0003824) for molecular function; most of the GO-terms related with cellular component were cell (GO: 0005623) and intracellular organelle (GO: 0043229) (Figure 4). These results were also consistent with the findings reported by Bakir et al. [24]. Due to the fact that hyperhydricity affects the physiology of the plants and most of the miRNA-target transcripts take a role as cellular component and function in the catalytic activities like oxyreductase activity.

Briefly, we revealed miRNA-based regulation patterns of hyperhydricity utilizing RNA-seq libraries by computational approaches. Besides, we presented three novel peach miRNAs which are potentially related to the hyperhydricity response.

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