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ORIGINAL RESEARCH

Global Transcriptome Analysis Reveals Differences in Gene Expression Patterns Between Nonhyperhydric and Hyperhydric Peach Leaves

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Abstract

Hyperhydricity is a morphophysiological disorder of plants in tissue culture characterized morphologically by the presence of translucent, thick, curled, and fragile leaves as a result of excessive water intake. Since clonal propagation is a major in vitro technique for multiplying plants vegetatively, the emergence of hyperhydricity-related symptoms causes significant economic losses to agriculture and horticulture. Although numerous efforts have been hitherto devoted to the morphological and anatomical responses of plants to hyperhydricity, the underlying molecular mechanism remains largely unknown. Here, a genome-wide transcriptome analysis was performed to identify differentially expressed genes in hyperhydric and nonhyperhydric leaves of peach [*Prunus persica* (L.) Batsch]. The RNA sequencing (RNA-Seq) analysis showed that the expression of >300 transcripts was altered between control and hyperhydric leaf cells. The top 30 differentially expressed transcripts (DETs) were related to the posttranscriptional regulators of organelle gene expression and photosynthesis, cellular elimination, plant cuticle development, and abiotic stress response processes. The expression of 10 DETs was also conformed by quantitative real-time polymerase chain reaction (RT-qPCR) in hyperhydric and nonhyperhydric leaves. As a complex biological process, hyperhydricity alters the expression of various transcripts including transcription factor (*Myb2*), RNA binding protein (pentatricopeptide, *PPR*), transporter protein (*ABC*), and *Laccase3*. Thus, this genome-wide transcriptome profiling study may help elucidate the molecular mechanism of hyperhydricity.

Core Ideas

- Hyperhydricity is a morphophysiological disorder of plants in tissue culture
- RNA-seq showed more than 300 transcripts notably responsive to hyperhydricity
- Expression of Laccase 3 involved in lignin polymerization related to hyperhydricity

RAPID CLONAL MULTIPLICATION of vegetatively propagated plants by tissue culture is a widely used technique in agricultural and horticulture for large-scale propagation of plants. Despite the advantages of tissue culture technique, culturing plants on an artificial medium may cause severe physiological effects on them such as hyperhydricity. Hyperhydricity syndrome, also known as vitrification or glassiness, is generally described as morphological, anatomical, and physiological abnormalities occurring in plantlets as a result of waterlogging stress (Kevers, et al., 2004; Van de Dries et al., 2013). The hyperhydric plantlets tend to exhibit a series of profound morphological alterations including the formation of translucent (less green in color), thick, curled, and fragile leaves (Apostolo and Llorente,

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Abbreviations: cDNA, complementary DNA; DET, differentially expressed transcript; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; PCR, polymerase chain reaction; RNA-Seq, RNA sequencing; RT-qPCR, quantitative real-time polymerase chain reaction.

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2000; Saher et al., 2005). The hyperhydricity can also be anatomically characterized by discontinuous development of epidermis and cuticle, malformations in stomata morphology, the presence of large intercellular spaces in the mesophyll (aerenchyma formation), a sharp decrease in the number of chloroplasts per cell, and the accumulation of large starch grains in plastids (Van den Dries et al., 2013; Olmos and Hellin, 1998; Chakrabarty et al., 2005). In the physiological aspect, the occurrence of excessive water in apoplast, thereby replacing air with water, leads to gas exchange impairment within plant tissues, resulting in oxidative stress. Excessive water uptake can also promote intracellular formation of reactive oxygen species that can attack unsaturated membrane lipids, nucleic acids, enzymes, and other cellular structures. Therefore, the expression levels of antioxidant enzymes—catalase, superoxide dismutase, peroxidase, glutathione peroxidase, and S-transferase—were found to be higher in hyperhydric leaves than in normal leaves (Van den Dries et al., 2013; Chakrabarty et al., 2005; Dewir et al., 2006). Recently, it was also reported that hyperhydricity causes a decreasing amount of the reduced and oxidized pyridine nucleotides showing low metabolic activity in photosynthetic apparatus of leaves (Chakrabarty et al., 2005). Taken together, all these findings demonstrate that hyperhydricity has a serious detrimental effect on plant propagation through tissue cultures.

Despite the availability of a number of treatments to ameliorate adverse effects of hyperhydricity, such as improving the natural ventilation of the culture vessels (Lai et al., 2005; Ivanova and Van Staden, 2010), altering the physical properties of the gel, and optimizing type and concentration of cytokinin (Kadota and Niimi, 2003; Ivanova and Van Staden, 2011), hyperhydricity is still leading to significant economical loss in agriculture. Previous studies are concentrating particularly on the several morphological, anatomical, and physiological responses of plantlets to hyperhydricity. To our knowledge, there have apparently been no studies dealing with genome-wide transcriptional response to hyperhydricity. Therefore, using RNA-Seq-based transcriptome analysis, we set up an experiment to gain a better understanding of the mechanism of hyperhydricity at a genome-wide scale.

Methods

Peach Shoot Micropropagation and Treatments

Shoots of *P. persica* var. *nectarina* (Aiton) Maxim. obtained from explants in culture were grown on standard Murashige and Skoog medium (Duchefa Biochemie B.V.) for 4 wk. By transferring normal shoots to the same medium in which plant agar (Duchefa) was replaced by Gelrite (Carl Roth GmbH) at temperatures above 35°C, hyperhydricity was induced and the plantlets begin to show hyperhydricity characteristic symptoms such as translucent stems and leaves, as well as wrinkled, curled, and fragile leaves. After 30 d of culture, all shoots were found to be hyperhydric. Freshly harvested hyperhydric and nonhyperhydric leaves were immediately subjected to the total RNA isolation.

RNA Extraction, Complementary DNA Library Preparation, and RNA Sequencing

Total RNAs from nonhyperhydric and hyperhydric leaves of peach plantlets were isolated using TRIzol Reagent (Invitrogen) according to the protocol provided by the manufacturer. Residual genomic DNA was eliminated from each RNA samples using DNase I (Thermo Scientific) digestion. The concentration and quality was initially assessed using a Nanodrop ND-2000c spectrophotometer (Nanodrop Technologies) at an absorbance ratio of A260/230 and A260/280 nm. Then, RNA integrity was evaluated by analysis on the Agilent 2100 bioanalyzer (Agilent Technologies, Inc.) and only the samples with an RNA integrity number >8.0 were used for subsequent experiments. An equal amount of total RNA was pooled from three biological replicates for each experimental group (two control and three hyperhydric libraries). The RNA-Seq library preparation and sequencing reactions were conducted in Genetic Engineering and Biotechnology Institute (GEBI) of TUBITAK according to the TruSeq RNA Sample Preparation Guide v2 (Illumina Inc.). Briefly, poly-A messenger RNAs (mRNAs) were purified from 2 μg total RNA using oligo (dT) attached magnetic beads and chemically sheared into short fragments via fragmentation buffer. Sheared fragments were converted to double-stranded complementary DNA (cDNA) using random hexamer primers and a reverse transcriptase Superscript II (Invitrogen). Second strand cDNA was synthesized using DNase I and RnaseH. Subsequently, end repair and single A-base tailing were performed before Illumina adapters were ligated to the cDNA fragments. After an agarose gel size fractionation step to extract fragments of 200 bp, cDNA libraries were enriched by polymerase chain reaction (PCR) amplification. Following this step, the prepared libraries sequenced using the Illumina HiSeq 2000 platform with paired-end 101-bp reads. Base calling was performed using Illumina instrument software.

Bioinformatic Analysis

FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of raw sequence reads. Based on the quality assessment results, low-quality reads and bases from both ends of raw Illumina reads were removed and trimmed using the FASTQ Quality Filter (fastq_quality_filter-q 20-p 80) and FASTQ Trimmer (fastx_trimmer-f 10-l 90) of FASTX-toolkit, respectively. After the high-quality reads were aligned against the peach reference genome (International Peach Genome Initiative et al., 2013) using TopHat 2 (Kim et al., 2013), the mapping results were sorted and indexed using SAMtools (Li et al., 2009). Raw read counts from BAM files were obtained using the multicov option of BEDTools (Quinlan and Hall, 2010). Three differential expression approaches were performed to

identify significantly differentially expressed genes among vitrification conditions. The DESeq method (Anders and Huber, 2010) was used with DESeq normalization and variance stabilizing transformation. Second, edgeR (Robinson et al., 2010) was used with trimmed mean of *M*-values normalization. Finally, limma (Smyth, 2004) was performed using trimmed mean of *M*-values normalization followed by voom transformation (Charity et al., 2014). The *p*-values obtained from each method are adjusted and false discovery rates are computed using Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995). Genes having false discovery rates less than 10% were considered as statistically significant and a Venn diagram was generated to aggregate the results of each method and find the most reliably differentially expressed genes. A plot of the intensity ration vs. the average intensity (MA plot) was constructed by scattering fold change (logarithmic transformed with base 2) vs. mean values of each gene and a heatmap was also generated to cluster the coexpressed genes. To achieve this, hierarchical clustering method was performed using euclidean distance and average method. Additionally, for each method, genes were ranked from most to least significant based on the adjusted *p*-values obtained from differential expression analysis, and Pihur's rank aggregation approach (Pihur et al., 2009) was conducted to obtain a consensus list including most significant genes evaluated from each method. Monte-Carlo cross-entropy optimization approach and Spearman's footrule distance was also performed, and the top 250 genes were selected. To investigate the functions of hyperhydricity-responsive transcripts, gene ontology (GO) and InterProScan analyses were performed with the TRAPID (Van Bel et al., 2013) using GO term (biological process, molecular function, and cellular component) and peptide informations deposited in PLAZA database (Van Bel et al., 2012). We also performed GO analysis by using the existing annotations of these stress-responsive genes in the Phytozome v9.1 plant genome database. Additionally, GO classification of differentially expressed genes based on GO term was performed using agriGO webtool (Du et al., 2010). To see which metabolic pathways hyperhydricity-responsive transcripts from leaf transcriptome, KEGG analysis was performed with KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/tools/kaas/) with bidirectional best-hit (BBH) assignment mode.

Real-Time Qualitative Polymerase Chain Reaction

Total RNA for the RT-qPCR experiment was isolated from nonhyperhydric, hyperhydric, and recurred leaves (turning hyperhydric conditions into normal condition by treatment) of plantlets using TRIzol Reagent (Invitrogen), and isolated RNAs were treated with DNase I to remove contaminating DNA. After DNase I treatment, 2 μg of total RNA was used for first strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) and RT-qPCR experiments were performed on a LightCycler 480 Instrument II (Roche Applied Science) using SYBR Green I Master kit (Roche). All RT-qPCR primers designed for 24 genes using Primer-Blast (Ye et al., 2012) and PCR conditions are listed in Supplemental Table S3. Peach translation elongation factor 2 (*TEF2*) was used as reference gene (Tong et al., 2009). Technical triplicates (in RT-qPCR well plates) were always run for each of the three biological replicates. The $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) was used to normalize and calculate the fold change of each transcript. Finally, an unpaired *t*-test was used to compare transcript abundance between control and hyperhydricity conditions.

Results

Overview of the RNA Sequencing Data

To elucidate genome-level responses to hyperhydricity sendrome, we performed transcriptome profiling using an Illumina HiSeq 2000 instrument to identify genes that are differentially expressed in normal and hyperhydric leaves of peach plantlets regenerated from leaves. A total of 60,994,078 raw reads were obtained from the five-leaf libraries (two control and three hyperhydric leaves), encompassing ~6 Gb of sequencing data. After removing low-quality and short-length reads, 45,491,774 high-quality reads (74.5% of total sequences) were retained for further downstream analysis. The majority of high-quality sequences (>90.30%) were successfully mapped to peach v1.0 reference genome regardless of library type. So far, a total of 27,852 protein-coding genes and 28,701 protein-coding transcripts were predicted in various tissue of peach (International Peach Genome Initiative et al., 2013). Our analysis showed that 23,519 protein-coding transcripts were found to be expressed in leaf tissues (Supplemental Table S1). Of these, 18,365 transcripts (78.09%) were expressed in control leaves, while 18,678 transcripts (79.42%) appeared to be expressed in hyperhydric leaves.

Identification of Differentially Expressed Genes Between the Nonhyperhydric and Hyperhydric Leaves

For comprehensiveness, we used three differential expression (DE) detection methods (DESeq, edgeR, and limma) to obtain a robust picture of the effect of hyperhydricity on the leaf transcriptome dynamics. After applying differential expression analysis between normal vs. hyperhydric leaves, 304, 782, and 1583 transcripts were found to be as statistically significant for DESeq, edgeR, and limma methods, respectively ($p < 0.1$; Supplemental Table S1). DESeq gave the most conservative results among the three algorithms, which is concordant with the findings of previous studies (Charity et al., 2014; Soneson and Delorenzi, 2013; Kvam et al., 2012). For each method, number of statistically significant genes with different α-values and the agreement of results can be found in Fig. 1. To obtain more reliable results, a rank aggregation method was used to rank DETs identified by all these methods according to their significance of differential expression. The transcripts were ranked from

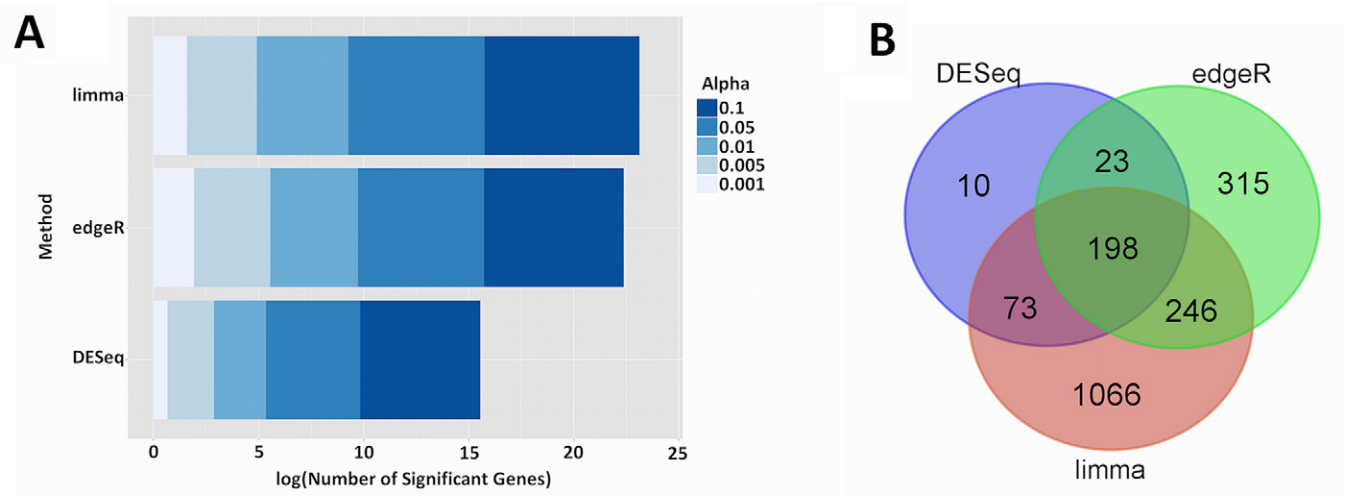


Fig. 1. The number of transcripts with significantly changed expression under hyperhydricity. (A) The number of significant transcripts determined by three statistical methods; DESeq, edgeR, and limma based on their α-value. (B) Venn diagram showing the overlap of differentially expressed transcripts (DETs). In total, 198 DETs were shared among the three software using different statistical approaches.

most significant to less and Pihur's rank aggregation method was performed (Supplemental Table S1). A total of 276 DETs were determined based on analysis results of rank aggregation method. Of these, 176 transcripts (64%) were downregulated, whereas 100 transcripts (36%) were upregulated in response to hyperhydricity. These genes exhibited a clearly different expression pattern in hyperhydricity and control leaves tissues. A heatmap plot displaying the coregulated genes in both vitrificated and control groups is shown in Supplemental Fig. S1. As seen from the plot, both vitrificated and control samples are clustered within their groups, and the groups are well separated from each other.

Moreover, RT-qPCR was applied to validate DETs identified by RNA-Seq. Ten DETs were selected based on their possible involvement in hyperhydricity. Results of RT-qPCR demonstrated a reduction in the mRNA levels of eight transcripts (*ppa010550m*, ABC transporter; *ppa023907m*, FAD/NAD reductase; *ppa026071m*, FAD/NAD(P) oxidoreductase; *ppa003249m*, phospholipase-C; *ppa003408m*, laccase 3; *ppa025096m*, PIP2 aquaporin; *ppa1027224m*, Ara12; and *ppa010269m*, haloacid dehydrogenase), whereas the expression of two transcripts (*ppa007605m*, alchol dehydrogenase and *ppa015973m*, Myb2) were induced in hyperhydric leaf tissue of peach compared with control. Statistical analysis revealed that among 10 hyperhydricity-inducible transcripts, four were (alchol dehydrogenase, MYB2, laccase 3, and phospholipase C) were found to be significant at the $p < 0.05$ level (Fig. 2). Although the remaining six transcripts did not show statistical significance at $p < 0.05$, p-values were obtained between 0.063 and 0.118. We believe that this limitation exists as a result of the small sample size, and increasing the sample size will lead to more significant results. Among the selected transcripts, the RT-qPCR results of nine transcripts was concordant with the results obtained by RNA-Seq except for one transcript encoding a halo dehydrogenase, suggesting that RNA-Seq and RT-qPCR generated similar profiles of transcript abundance.

Intriguingly, 50% of top 20-most differentially expressed transcripts in response to hyperhydricity were annotated as unknown function. In a further attempt to annotate these unknown transcripts, one of the most differentially expressed transcript, *ppa013541m* (the most differentially expressed according to the results by DeSeq and edgeR) was compared against de novo-assembled peach unigenes (obtained from Trinity de novo assembly, data not shown) using BlastN (E-value $= 1 \times 10^{-30}$). The unigenes with the best Blast hits were searched against NCBI nonredundant protein databases using BlastX, but we failed to obtain positive findings. We believe that these transcripts could be currently unannotated protein-coding transcripts or functional RNAs such as long noncoding RNAs.

Functional Annotation of Differentially Expressed Genes

Metainformation analysis of 276 DETs showed that 174 (63%) transcripts were identified to be potential full-length genes, while 10 (3.6%) of them yielded no information. Sequence homology analysis based on BlastP (E-value $\leq 1 \times 10^{-10}$) revealed that these transcripts are 62.5% (168) identical to apple (*Malus domestica* Borkh.), 25.3% (68) identical to wild strawberry (*Fragaria vesca* L.), and 3.7% (10) similar to cacao (*Theobroma cacao* L.) and black cottonwood (*Populus trichocarpa* Torr. & A. Gray). An InterProScan analysis performed on these DETs revealed that 75% (207 transcripts) were characterized by at least one functional annotation, resulting in identification of 463 conserved protein families. Further, a total of 3221 GO terms were assigned to 164 (59.40%) differential transcripts based on similarity to annotated coding sequences (Supplemental Table S2).

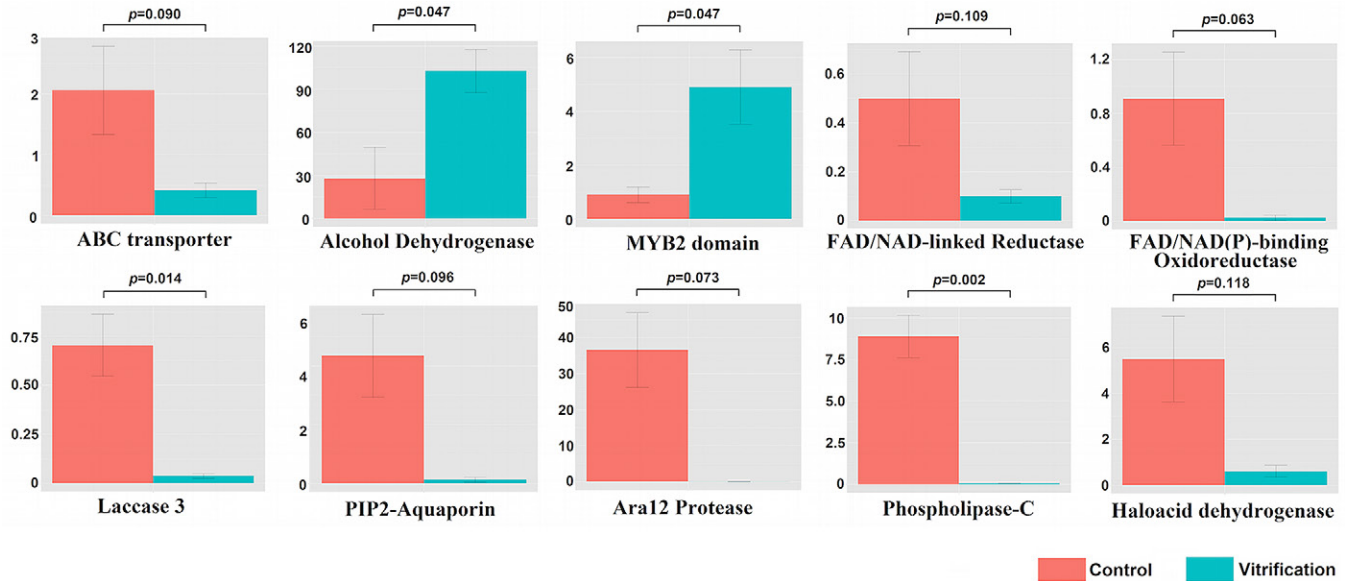


Fig. 2. Validation of 10 differentially expressed transcripts in response to hyperhydricity by qualitative polymerase chain reaction (qPCR). The results of qPCR showed that a reduction in the messenger RNA levels of eight transcripts (ABC transporter, FAD/NAD reductase, FAD/NAD(P) oxireductase, Laccase 3, PIP-aquaporin, Ara12, Phospholipase-C, and Haloicacid dehydrogenase) and an upregulation of two transcripts (Alcohol dehydrogenase, MYB2) in hyperhydric leaves compared with control. Peach translation elongation factor 2 (*TEF2*) was used as reference transcript. The error bars represent the standard deviation (n = 3). All vertical axis show the relative expression of transcripts.

The majority of GO terms assigned from the molecular-function category belong to binding (GO:0005488; 27%), catalytic activity (GO:0003824; 19%), nucleotide binding (GO:0000166; 9%), transferase (GO:0016740; 8%), nucleic acid binding (GO:0003676; 6%), hydrolase (GO:0016787; 6%), and protein binding activity (GO:0005515; 5%). For biological processes, metabolic process made up the majority of the GO annotation (GO:0008152; 21%) followed by biological process (GO:0008150; 18%), cellular process (GO:0009987; 18%), biosynthetic process (GO:0009058; %8), and nucleobase-containing compound metabolic process (GO:0006139; %6). In the cellular component category, GO terms related to cellular component (GO:0005575; 27%), cell (GO:0005623; 27%), intracellular (GO:0005622; 16%), membrane (GO:0016020; 11%), and cytoplasm (GO:0005737; 7%) (Fig. 3). The pathway-based analysis (Kyoto Encyclopedia of Genes and Genomes [KEGG]) was used to further identify metabolic pathways in which DETs were involved. A total of 38 unique differential transcripts were predicted to be involved in a total of 49 metabolic pathways and assigned to 40 KEGG orthology terms. The DETs were heterogeneously distributed in metabolic pathways and the majority of genes with altered expression encode proteins involved in metabolic pathways (21%), biosynthesis of secondary metabolites (9%), sugar metabolism (6%, pentose, fructose and sucrose metabolism), RNA metabolism (4%, ribosome and RNA transport), phenylpropanoid biosynthesis (4%), and so on (Supplemental Table S2).

Discussion

As well established in recent literature, hyperhydricity-induced morphological and physiological changes on the whole plantlets result in significant economic loss fruit production. However, the mechanism of hyperhydricity still remains poorly understood at the molecular level. Our RNA-Seq results show that a relatively high number of genes were involved in this physiological malformation. Since the hyperhydricity leads to a general reduction in photosynthesis, oxidative phosphorylation, and pyridine nucleotide synthesis (Van den Dries et al., 2013; Chakrabarty et al., 2005), the KEGG pathway analysis revealed that most of DETs participated in metabolic pathways, carbon and sugar metabolism, as well as RNA metabolism. Two downregulated transcripts (*ppa019520m* and *ppa020876m*) among the top 30 DETs were identified to be a gene encoding pentatricopeptide (PPR) protein whose function is the postranscriptional regulation of organelle gene expression and photosynthesis. Kotera et al. (2005) and Okuda et al. (2007) reported that a PPR protein, chlororespiratory reduction 4 (*CRR4*), functions as site-specificity factor for RNA editing site of plastid *ndhD-1* transcripts, encoding a subunit of NADPH dehydrogenase complex. Therefore, this RNA-binding protein involved in the regulation of NADPH protein, which is essentially required for photosystem and thereby energy metabolism (Kotera et al., 2005). This result is consistent with the conclusion reached by Chakrabarty et al. (2005) that pyridine nucleotide production (NADPH and NADP+) were significantly decreased in hyperhydric apple leaves compared with nonhyperhydric leaves. These findings are concordant

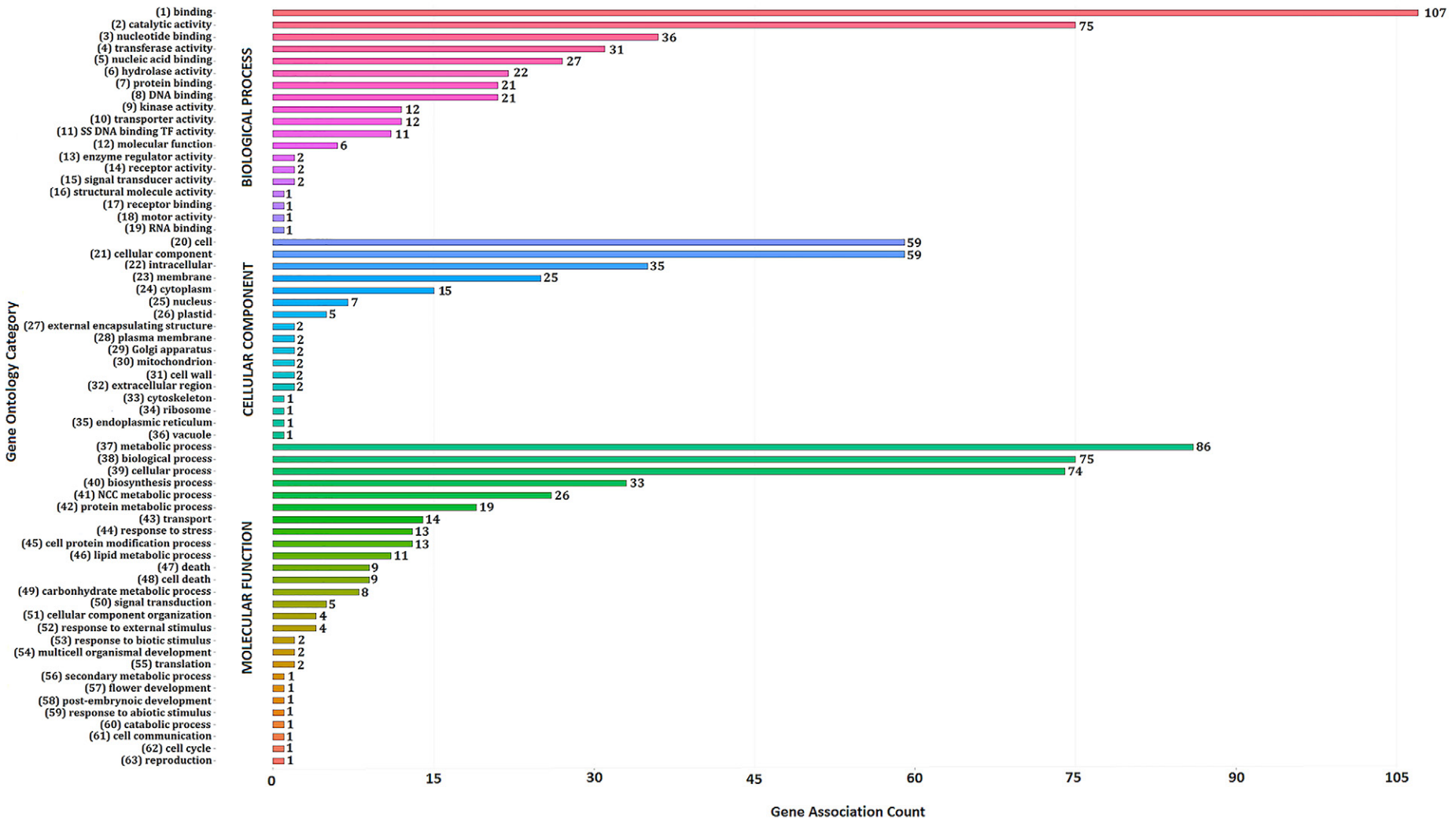


Fig. 3. Gene ontology (GO) analysis of differentially expressed transcripts. The bar indicates the number of GO terms summarized into three main categories: biological process, cellular component, and molecular functions.

with our results showing the downregulation of both transcripts can explain the low photosynthetic rates of hyperhydric leaves.

Plant cuticle, essentially consisting of wax and cutin, covers the epidermis of all aerial parts of plant tissue and act as a hydrophobic barrier for protection of inner tissues. Microscopic observations have revealed that there appeared to be a discontinuous development of cuticle with less or poorly developed epicuticular wax layer in leaves of hyperhydric plants compared with normal leaves (Chakrabarty et al., 2005; Olmos and Hellin, 1998; Gribble et al., 2003; Jausoro et al., 2010). The same observation was also reported in another study where *Arabidopsis thaliana* (L.) Heynh. plants develop cuticleless hyperhydric leaves in submerged culture (Ohyama et al., 2008). On the other hand, reverse genetic studies (insertional knock-out mutants) showed that *Arabidopsis* ATP-binding cassette (ABC) transporter CER5 (*AtWBC12*) and *AtWBC11* were required for plant cuticle development via exporting and accumulation of both cutin and wax (Bird et al., 2007; Luo et al., 2007). Accordingly, in our study, both RT-qPCR and RNA-Seq analysis indicated that the levels of ABC transporter were reduced in hyperhydric leaves compared with normal leaves. The RT-qPCR displayed a 2.3-fold (log2FC, $p = 0.09$) lower expression in the hyperhydric leaves as compared with control (Fig. 2). Therefore, we can conclude that the downregulation of ABC transporters in response to hyperhydricity can be strongly related to discontinuous development of cuticle.

Several studies showed that transcription factors have a variety of important functions in abiotic stress responses (Nakashima et al, 2012; Todaka et al., 2012). Research by Hoeren et al. (1998) attempted to investigate the role of transcription factor Myb2 under hypoxic conditions. In this study, *Arabidopsis* Myb2 transcription factor (*AtMyb2*) was upregulated in plantlets by hypoxia and transactivates the ADH1 (alcohol dehydrogenase) gene promotor via specially binding to the GT-motif. This experiment further validated the transient assay by showing that mutations in the GT-motif disrupted the induction of ADH1. These results are in line with our findings because RT-qPCR and transcriptome analysis showed that both Myb2 (*ppa015973m*) and alcohol dehydrogenase (*ppa007605m*) transcripts were induced in hyperhydric leaves compared with control (Supplemental Table S2; Fig. 2). This possibly can be explained by the fact that ADH1 involved in the final stage of anaerobic glycolysis and regenerates NAD+ by reducing acetaldehyde to ethanol to produce a limited amount of ATP in the absence of oxygen (Strommer, 2011). Therefore, when normal respiration is disrupted, plants are forced to use alternative biochemical pathways for energy production.

As an aromatic biopolymer, lignin an integral cell-wall constituent in vascular plants, providing structural support to the stem and leaves as well as constituting a barrier to environmental abiotic and biotic stress factors. Previous cytological and histochemical studies indicated that there was a significant reduction in lignin content and composition in hyperhydric shoots (Rasco and Pateña, 1997; Kevers et al., 1987) and this physical weakness is attributed to low lignification (Piqueras et al., 2002). More recent studies showed that two types of laccases, LAC4 and LAC17, have been shown to play crucial role in lignin polymerization and double knock out of these genes in *Arabidopsis* resulted in small reduction of lignin content (Berthet et al., 2011). However, simultaneous disruption of LAC11 along with LAC4 and LAC17 leads to severe plant growth and vascular development arrest from lack of lignification (Zhao et al., 2013). Another study with maize (*Zea mays* L.) roots found that laccase 3 (*ZmLac3*) was involved in the polymerization of phenolic compounds such as lignin (lignification process) (Caparrós-Ruiz et al., 2006). Our RNA-Seq analysis showed that the expression of laccase 3 (*ppa003408m*) was reduced compared with control leaf tissue. In particular, the data obtained by RT-qPCR suggested that lac3 expression was 4.2-fold (log2FC, $p = 0.014$) downregulation compared with control (Fig. 2). Taken together, we suppose that the fragility and weakness of hyperhydric leaves can be attributed to inadequate lignin content in which synthesis is dependent on laccase enzyme.

In summary, as a physiological and morphological malformation, hyperhydricity is a complex biological process, which is reflected at the molecular level by alternations in expression of many transcripts (~300) revealed by RNA-Seq coupled with robust statistical analysis. As the first genome-wide study, our results provide a valuable resource for understanding the molecular mechanism underlying hyperhydricity, which causes significant economic loss in fruit production.

Supplemental Information Available

Supplemental Table S1. The raw and normalized count of differentially expressed transcripts.

Supplemental Table S2. A summary of functional annotations (InterProScan, GO, KEGG).

Supplemental Table S3. RT-qPCR primer sequences and other pertinent information regarding the use of RT-qPCR in this study.

Supplemental Figure S1. This heat map shows the clear separation between hyperhydric and non-hyperhydric leaves tissues.

Acknowledgments

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Author Contributions

YB, TU, VE, and GZ conceived and designed the study. YB generated hyperhydric plantlets using tissue culture and did RT-qPCR experiment. VE performed RNA extraction, performed bioinformatics analysis and sequence annotation as well as helped to draft the manuscript. GZ performed bioinformatics and statistical analysis of sequencing data and did statistical analyses of RT-qPCR data as well as drafted the manuscript. TU and YB coordinated the study, participated in the analysis of the results, and helped to accomplish the final form of the manuscript. All authors read and approved the final manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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