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Research article

Transcriptional changes during tomato ripening and influence of brackish water irrigation on fruit transcriptome and sugar content

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ABSTRACT

Efficient management and utilization of brackish water irrigation help to minimize yield losses and promote fruit quality and sugar content in tomato fruit. However, the functional genes involved in sugar metabolic pathways and potential molecular pathways responsive to brackish water irrigation remain unknown. To this end, physiological responses and comparative transcriptional profiling was used to analyze the tomato fruit during the white-ripe period (CK1) and mature period (CK2) in plants grown under four water management strategies (rotating irrigation with brackish and fresh water during fruit development, T1; fresh water irrigation, T2; mixed brackish and fresh water irrigation, T3; mixed water and fresh water irrigation in sequence, T4). Comparative analysis revealed that during fruit development (CK2 cv CK1) differentially expressed genes (DEGs) involved in photosynthetic pathways and sucrose-starch metabolism were downregulated. However, two DEGs encoding putative beta-fructofuranosidases were significantly upregulated at the mature stage, which promoted the accumulation of glucose and fructose in CK2. Comparing four types of management strategies, rotating irrigation with brackish water and fresh water (T1) led to reprograming of global gene expression. Moreover, the upregulated DEGs in T1 were significantly enriched for signaling, hormone metabolism, and stress tolerance, suggesting the coordination of both stresses signaling as well as the plant hormone. These results provide a valuable reference for rational use of brackish water in the production of high-quality tomato in arid and semi-arid regions.

1. Introduction

Irrigation is one of the most important factors for the production of horticultural crops in many areas. With the increasing shortage of fresh water resources in the past decade, the utilization of brackish water has received more and more attention (Wang and Shan, 2015). The rational utilization of brackish water has become one of the most important ways to alleviate water shortage (Wang and Shan, 2015). However, the use of brackish water inevitably increases soil salinity (Pang et al., 2010), which influences growth, yield and quality of the crop, especially sugar content in fruit (Wang and Shan, 2015; Takahata and Miura, 2014) through changes in osmosis, ion content and soil physical and chemical properties (Feleafel and Mirdad, 2014; Davies and Hobson, 1981; Beckles, 2012). Therefore, effective measures to regulate soil water and salinity have become the basis for the safe use of brackish water. There are three main approaches to irrigation with brackish water: direct irrigation, mixed irrigation of saline water and fresh water, and rotating irrigation between saline water and fresh water. For irrigation water of the same salinity, different irrigation methods can have different effects (Cetin and Kirda, 2003; Al-Sulaimi et al., 1996). Specific irrigation approaches with brackish water can affect flavor, color and the soluble acid content of tomatoes (Leuschner et al., 1985; Pasternak et al., 1986; Mitchell et al., 1991; Pascale et al., 2001).

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetables worldwide (Causse et al., 2010; Thakur et al., 1996), and is rich in many essential vitamins and minerals. In China, tomato is one of the most important and valuable horticultural crops. However, tomato production in China increasingly relies on controlled environment facilities and intensive use of fertilizer and irrigation (Lin and Huang, 2000; Adams et al., 2001; Qiang et al., 2012), often leading to significantly reduced fruit quality and environmental pollution. For tomato, fruit quality is largely determined by the types and quantities of accumulated sugars (Davies and Kempton, 2010; Kanayama, 2017). Therefore, sugar accumulation is one of the most important traits for

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quality (Yamaguchi and Okamoto, 1977). It has also been documented that sugars are at the core of primary metabolism in plants (Patrick et al., 2013). As signaling molecules, sugars play critical roles in fruit set, growth, ripening, and composition (Kanayama, 2017).

The sugar content in mature tomato fruit comprises mainly sucrose, fructose and glucose (Davies and Hobson, 1981; Stevens, 1972), and sugar composition is also a major determinant of fruit quality (Dorais et al., 2001; Malundo et al., 1995). Enzymes involved in sucrose metabolism include acid invertase, neutral invertase, sucrose synthase and sucrose phosphate synthase. These four enzymes play important regulatory roles in sugar accumulation and consumption (Lu et al., 2010). Changes in sugar contents and enzyme activities related to sucrose metabolism in tomato fruit have been intensively studied (Demnitzking et al., 1997; Jiang et al., 2007; Yuan, 2009; Lu et al., 2010). The enzymes involved are sensitive to environmental conditions and various means can be used by growers to influence their activities by controlling the environment (Koch, 2004), thereby improving fruit quality. There have been several reports describing the effects of brackish water irrigation patterns on growth and quality of tomato (Pasternak and Malach, 1995; Pasternak et al., 1986; Shalhevet and Yaron, 1973). However, the mechanisms of influence of different brackish water irrigation patterns on tomato fruit sugar metabolism, especially key enzymes of sucrose metabolism, related genes, sugar transporter genes and the expression of major regulatory transcription factors are still unclear.

Understanding the mechanism(s) by which different brackish irrigation patterns influence sugar accumulation in tomato is thus the key to establish an optimized irrigation method that comprehensively considers yield and fruit quality. In this study, we investigated the influence of different brackish water irrigation patterns on sugar content, enzymes activity, and gene expression in the mature fruit. Moreover, differentially expressed genes (DEGs) associated with sugar accumulation and sucrose metabolism in tomato fruit under different brackish water irrigation patterns were identified using transcriptome sequencing. Our results reveal the biochemical mechanisms of the effects of different irrigation methods of brackish water on sugar accumulation and sucrose metabolism of tomato fruit, which provide a scientific basis for efficient and sustainable production of tomato using brackish water irrigation.

2. Material and methods

2.1. Plant material, growth conditions and irrigation treatments

Tomato cv. ‘Jingfan 301’, a medium-maturing variety with pink fruit and an indeterminant growth habit, was obtained from the Chinese Academy of Agricultural Sciences, Beijing, China. Experiments were conducted in a polyethylene greenhouse with natural sunlight in research plots at Ningxia University, Yinchuan, Ningxia Province, China (38° 30′13.44″N; 106°08′19.67″E) from July to December 2016. Plants were cultivated according to the production standard for high-quality tomato fruit routinely used in Ningxia Province. For physiological analyses and transcriptome sequencing, white-ripe and mature fruit from the middle parts of tomato plants (about 65 cm in height) from three individual plants were harvested either 42 days (white-ripe fruit with brackish water irrigation; CK1) or 52 days (mature fruit with brackish water irrigation; CK2, or different irrigation patterns; T1-T4) after the cultivated plants were transplanted. All samples were immediately frozen in liquid nitrogen until analysis. For irrigation treatments, water from two sources was used: fresh water (EC = 1.07 mS/cm)from local groundwater, and brackish water (EC = 3.0 mS/cm)from local groundwater with the addition of four industrial salts ($NaHCO_3$, $MgSO_4.7H_2O$, $CaCl_2$, and K_2SO_4) according to the ionic components of brackish water in the Yinchuan area of Ningxia. The soil was sand with 0.22 g kg^{-1} total salt content, with a pH of 8.9 and electrical conductivity (EC) of 0.08 mS/cm. The experiment consisted of a control treatment and four different types of irrigation treatments: rotating irrigation with brackish water and fresh water during fruit development (T1), fresh water irrigation (T2), a mixture of brackish and fresh water at a 1:1 ratio (T3), and mixed water irrigation of saline water and fresh water in sequence (T4). The control treatment (CK2) was brackish water. The experimental layout in all trials was a split plot-block design with three replications per treatment. Tomato fruits were sampled either in the white-ripe stage (CK1) or mature stage (CK2) for brackish water treatment.

2.2. Determination of sugar contents and enzyme activity in pericarp and pulp

Tomato fruit were collected at either the white-ripe or mature stage and six to eight fruits of the similar size were dissected into pericarp and pulp. The total amounts of soluble sugar and starch were determined using the anthrone method as described previously (Srivastava and Dwivedi, 2000). Three biological replicates were used in each control or treatment. The activity of acid invertase, neutral invertase, sucrose synthase, and sucrose phosphate synthase was determined using methods described by Samac et al. (2015). The enzymatic activities were measured in three independent samples from three tomato plants for each treatment.

2.3. Library construction and sequencing

Total RNA from fruit pericarp/pulp was extracted with Trizol reagent (Invitrogen, Shanghai, China). RNA quality and quantity were assessed using the ND2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and an Agilent 2100 spectrophotometer (Agilent Technologies, Cheadle, UK). Libraries were constructed using the TruSeq Kit (Illumina) and sequencing was performed using Illumina HiSeqTM 4000 system by Guangzhou Sagene Biotech Co., Ltd (Guangzhou, China).

2.4. Data analysis

Sequence data were filtered to remove reads containing adapters, > 10% unknown nucleotides, and > 50% low-quality bases (base quality ≤ 20) using RobiNA (Lohse et al., 2012). The resulting high-quality, clean reads were aligned with a reference genome of tomato (The Tomato Genome Consortium, 2012) using TopHat2 (Kim et al., 2013) with default parameters. Gene expression was calculated and normalized by FPKM (Fragments Per Kilobase of transcript per Million mapped reads) (Mortazavi et al., 2008). Pearson correlation analysis was performed with R package models (http://www.r-project.org/). To identify differentially expressed genes across samples or groups, the DEseq2 package (http://www.r-project.org/) was used (Love et al., 2014). Significant DEGs in comparisons either between CK2 and CK1 or T1-T4 and CK2 were defined as those with a fold change (FC) ≥ 2 for up- and downregulation and a false discovery rate (FDR) < 0.05 (Xu et al., 2014). All DEGs were mapped to GO terms in the Gene Ontology database (http://www.geneontology.org/), gene numbers were calculated for every term and significantly enriched GO terms in DEGs compared to the genome background were defined using R-based GO function software packages by hypergeometric test (Lomax, 2005; Noble, 2009). The calculated p-value was adjusted for FDR Correction, taking FDR ≤ 0.05 as a threshold. GO terms meeting this condition were defined as significantly enriched in DEGs. GO enrichment was subjected to agriGO v2.0 with default parameters (Tian et al., 2017). This analysis was able to recognize the main biological functions that DEGs represent. In addition, KEGG enrichment was conducted to generated significant KEGG pathways of DEGs (Ogata et al., 2000). DEGs were loaded into MapMan to display individual genes mapped on their pathways (Thimm et al., 2004). All sequence reads are available at GenBank SRX6801253 ~ SRX6801270.

2.5. Validation of differentially expressed genes by qRT-PCR

Total RNA was extracted from fruit pericarp/pulp samples of CK1, CK2, T1, T2, T3, and T4 with Trizol reagent (Invitrogen). The residual genomic DNAs were treated with RNase-free DNase I (Invitrogen), and the purified RNA samples were further used for cDNA synthesis with PrimeScript RT reagent Kit with gDNA eraser (TaKaRa). Reaction mixtures were diluted 1:40 with distilled water and used as templates for qRT-PCR. The primer sequences are listed in Supplementary Table S1. Quantitative assays were carried on a qTOWER2.2 system (Analytik Jena, Jena, Germany) and a SYBR Premix Ex Taq™ II kit (Takara) in triplicate on each cDNA sample and transcript levels were calculated relative to the level of tomato *actin (SlACT,* FJ532351) using the $2^{-\Delta\Delta Ct}$ method (Livaka and Schmitten 2001). All data are presented as mean ± SD (n = 3).

Transcriptomic data of T1 and T2 were normalized to transcriptomic data of CK2, and qRT-PCR data of T1 and T2 were normalized to CK2 using z-score transformation as the normalization process. Hierarchical clustering analysis of the expression data was performed on OmicShare tools (http://www.omicshare.com/tools/Home/Soft/heatmap). The color scale represents log2 expression values and the expression levels in heatmap were log2-based.

2.6. Statistical analyses

Significant differences among the data were determined using an analysis of variance with SPSS version 19.0 (SPSS, Inc, an IBM Company, Chicago, IL). Data of continuous variables were tested for normality using a Shapiro–Wilk's test and for homogeneity of variance using a Levene's test. Where data conformed to the above assumptions for ANOVA, this method of analyses was used. Where data were non-parametric, comparisons were made using a Kruskall-Wallis test at $P \leq 0.05$ between treatments and control. All of the data are presented as the means ± standard error (SE) of at least three biological replicates. Pearson's correlation coefficients between contents of sugar/starch and enzymes activities were calculated in R using function cor () from package stats.

3. Results

3.1. Sugar and starch content of tomato fruit treated with different irrigation patterns

Prior to transcriptome analysis, we determined the contents of sugar and starch in tomato fruit collected at either the white-ripe stage (CK1) with brackish water irrigation or mature stage, with brackish water irrigation (CK2) or four different water irrigation patterns (T1-T4). Since the data does not conform to Gaussian distribution and the homogeneity of the variance, non-parametric test (Kruskall-Wallis test) was further used for the significance analysis. Compared to fruit harvested at the white-ripe stage with brackish water irrigation (CK1), mature fruit from plants irrigated with brackish water or from the four irrigation pattern treatments showed significantly increased glucose and fructose content in pericarp and pulp except for the content of glucose in pericarp of T2 treatment (Fig. 1B and D), and significantly decreased sucrose content (Fig. 1C). Similarly, starch content showed a substantial decrease at the mature period in all treatments (Fig. 1E), although there were differences among the different irrigation patterns. At the mature period, there was no significant difference in sugar content in pericarp between the three irrigation patterns (T1, T3 and T4) relative to brackish water irrigation (CK2), but T2 treatment causes significantly difference in sucrose content either in pericarp or pulp (Fig. 1C and E).

3.2. Activities of carbohydrate metabolism-related enzymes in different tissues of tomato fruit

The activities of AI and NI were increased (Fig. 2A and B), whereas the activity of sucrose synthase (SS)decreased in the pericarp and pulp from the white-ripe stage to mature stage (Fig. 2C). However, the activity of sucrose phosphate synthase (SPS) decreased in the pericarp but did not change significantly in pulp from the green-ripe to mature stage (Fig. 2D).

3.3. Correlation analysis between sugar/starch contents and enzymes activities

To further explore the correlation between sugar/starch accumulation and their corresponding enzymes activities, a correlation analysis was performed on both pericarp and pulp either at white-ripe stage (CK1) with brackish water irrigation, or at the mature stage, with brackish water irrigation (CK2) or four different water irrigation patterns (T1-T4). In pericarp, soluble starch content was positively correlated to total sugar content ($r = 0.98$, $P \leq 0.05$). The activity of SS and SPS positively correlated to the content of sucrose and starch with the correlation coefficient of 0.89–0.99. The content of glucose positively correlated to the content of fructose with the correlation coefficient of 0.90, whereas the activity of AI and NI positive correlated to glucose and fructose with the correlation coefficient of 0.71–0.85 (Fig. 3A). A similar correlation pattern either between the content of starch and sugar and SS activity or between the content of glucose and fructose and the activities of AI and NI in pericarp was observed in the pulp, however, there was a negative correlation between SPS activity and the content of sucrose and starch (Fig. 3B).

3.4. Analysis of differentially expressed genes in different tissues of tomato fruit

Following the biochemical analyses, we conducted transcriptional analyses to discover which genes or gene networks responded to different water irrigation patterns. We generated transcriptome data by RNA-based sequencing (RNA-Seq) of pericarp from different irrigation patterns at either the white-ripe stage (CK1) or mature stage (CK2, T1-T4). After removing reads with poor quality, around 5.6 billion clean reads were generated. A summary of RNA-Seq data from a total of 18 samples is shown in Supplementary Table S2. On average, the mapping rate for each sample was ~90.3%. Pearson correlation of 18 samples were obtained to evaluate the repeatability of RNA-Seq data using R software. White-ripe fruits transcriptome was clearly distinct from mature fruits one, regardless of the irrigation treatment. Moreover, the sequencing results were highly reproducible (Fig. S1).

The expression for each gene in each sample was then quantified based on FPKM value. Our RNA-seq analysis suggested that, of the putative 34,727 genes in the tomato genome, 20,148 were expressed in CK1 (white-ripe stage, with brackish water irrigation) and 18,786 were expressed in CK2 (mature stage, with brackish water irrigation) (FPKM ≥ 0.1). There were 18,080 genes expressed in both CK1 and CK2, whereas 2068 and 706 were exclusively expressed in either CK1 or CK2, respectively (Fig. 4A). We identified 6235 differentially expressed genes (DEGs) between CK1 and CK2 (1750 genes up-regulated and 4485 genes down-regulated) (Fig. 4B, Supplementary Table S3). A Venn diagram of DEGs between CK2 and T1 (rotating irrigation with brackish water and fresh water during fruit development), T2 (fresh water irrigation), T3 (a mixture of brackish and fresh water at a 1:1 ratio) or T4 (mixed water irrigation of saline water and fresh water in sequence) (Fig. 4C) showed that there were few shared genes among the four DEG datasets and the numbers of novel DEGs specific to each dataset were as follows: 170 for CK2/T1, 80 for CK2/T2, 31 for CK2/T3, and 13 for CK2/T4 (Fig. 4C). The numbers of down- or up-regulated genes for CK2/T1, CK2/T2, CK2/T3, and CK3/T4 were 187 and 59, 104 and 55,

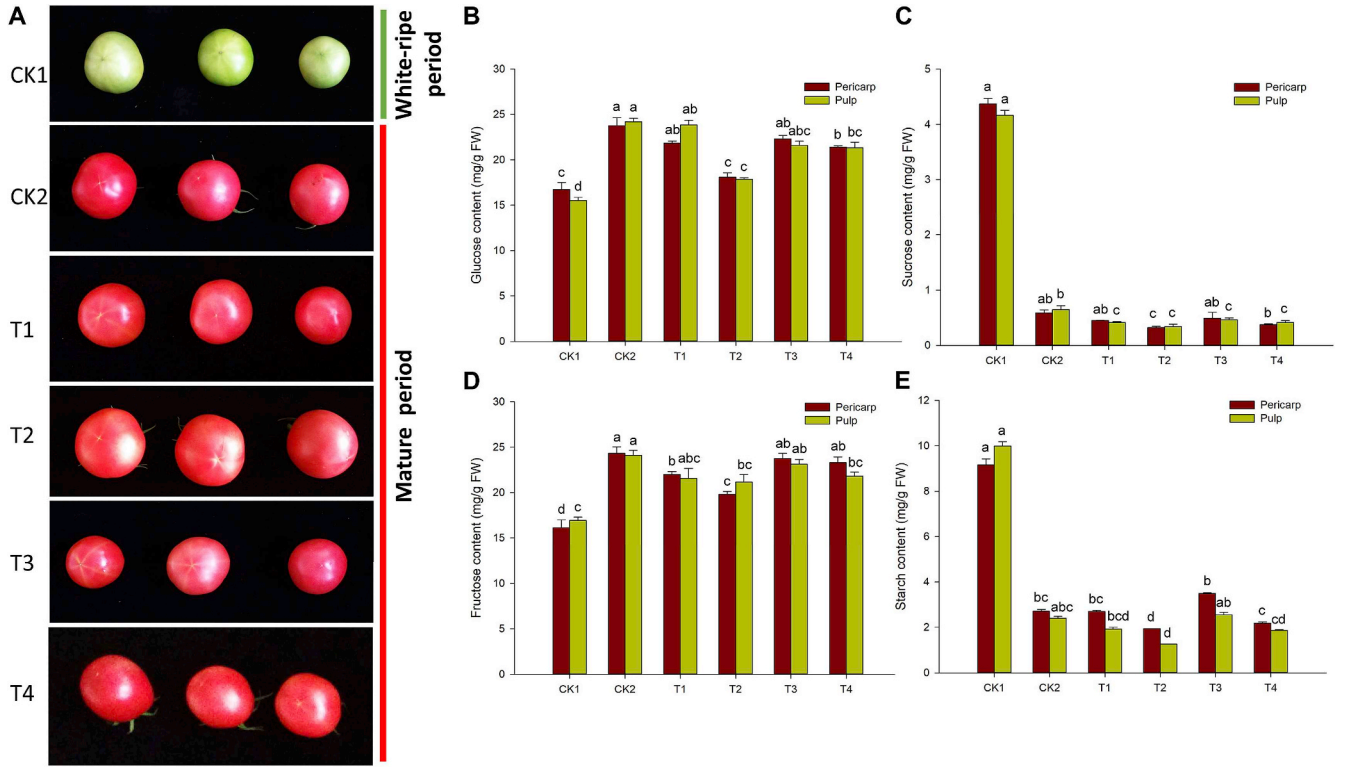

Fig. 1. Determination of carbohydrate content in different organizations of tomato fruits. (A) Representative pictures of harvested fruit at white-ripe and mature period with different water irrigation patterns. Brackish water irrigation at the white-ripe period (CK1) and mature period (CK2); rotating irrigation with brackish water and fresh water at the fruit development (T1), fresh water irrigation (T2), mixing water irrigation with 1:1 of ration of brackish and fresh water (T3), mixed water irrigation of saline water and fresh water irrigation in sequence (T4).(B) glucose, (C) sucrose, (D) fructose, and (E) starch. Different letters above columns indicate significant difference between means at least at the $P \leq 0.05$ level of probability. Values with the same letter were not significantly different according to Kruskall-Wallis test.

17 and 30, and 19 and 7, respectively (Fig. 4D, Supplementary Tables S4, S5, S6, and S7).

3.5. Functional classification of differentially expressed genes in different tissues of tomato fruit

To generate a comprehensive overview of the potential function of DEGs from the white ripe to mature stage, we used MapMan to reveal the associated pathways (Table 1, Supplementary Tables S8, S9, S10, S11, and S12). Major bin codes exhibiting changes during fruit ripening were 'signaling' (BIN 30), 'stress' (BIN 20), 'development' (BIN 33) and hormone metabolism (BIN 17). Additional bin codes included 'PS' (BIN 1), secondary metabolism (BIN16) and 'redox' (BIN21) (Table 1, Supplementary Tables S8–12). A similar grouping of functional categories was found in DEGs for CK2/T1, CK2/T2, CK2/T3 and CK2/T4 but with the fewer number of DEGs (Table 1).

3.6. Functional classification of DEGs from white ripe to mature by GO and KEGG analysis

To detect molecular and cellular functions impacted by developmental stage, DEGs (CK2/CK1) with expression change FDR < 0.2 were subjected to agriGO v2.0 (Tian et al., 2017). The GO terms for down-regulated DEGs were enriched within the GO category 'photosystem I reaction center' (Fig. 5A), which suggested photosynthesis intensity was inhibited from the green-ripe to mature stage. In addition, we found that one GO category, 'catalytic activity' was enriched for up-regulated DEGs (Fig. 5B). The KEGG database was used to determine the biological processes of the DEGs. The DEGs were mainly associated with photosynthesis–antenna proteins, photosynthesis, and carbon fixation in photosynthetic organisms (Fig. 5C). Moreover, the DEGs were also associated with starch and sucrose metabolism, other glycan degradation and fructose and mannose metabolism (Fig. 5C).

3.7. Candidate regulatory and functional genes involved in enriched metabolic pathways

To thoroughly characterize DEGs involved in the enriched pathways depicted in Fig. 5C, and in view of the biochemical indicators described above (Figs. 1 and 2), we focused further analysis on photosynthesis metabolism, sucrose-starch metabolism, and glycolysis-TCA metabolism. Also, we used MapMan to map all the DEGs to depict overall metabolism (Fig. S2) and regulation (Fig. S3) in the development from white ripe to mature fruit. Among the 6114 DEGs, 810 were assigned to 28 known functional categories (Fig. S2, Supplemental Tables S3 and S8). Genes involved in "signaling", "amino acid metabolism", "secondary metabolism", "cell wall synthesis" and "stress response" were significantly enriched in the up-regulated DEGs (Supplemental Table S8), suggesting that tomato fruit at the mature period (CK2) went through a transcriptional reprogramming, both in signaling and metabolism relative to the white-ripe period (CK1). Consistent with GO analysis results, the functional category "Transcription factor", "Protein modification", "Protein degradation" and "Receptor kinases" were significantly enriched. In addition, hormone-related genes were enriched in ethylene category (Fig. S3, Supplemental Table S8).

3.8. Genes regulated in photosynthesis metabolism

In the present study, most DEGs related to photosynthesis metabolism were down-regulated from the white ripe to mature stage. As shown in Fig. 6, photosynthesis metabolism could be divided into three parts including Light reactions (Fig. 6A), Calvin cycle (Fig. 6B) and

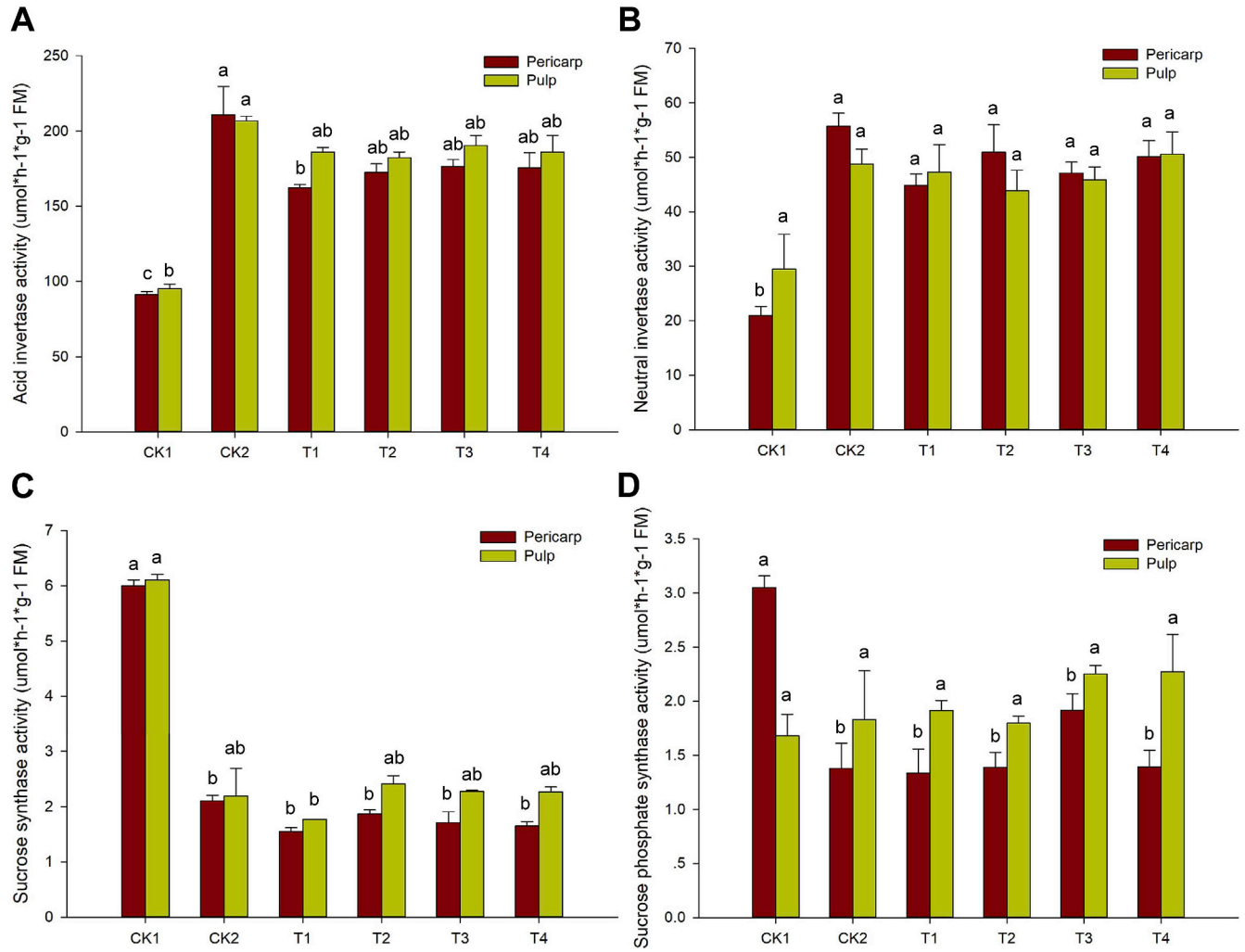

Fig. 2. Activities of carbohydrate metabolism-related enzymes in different organizations of tomato fruits. (A) Acid invertase (AI), (B) Neutral invertase (NI), (C) Sucrose synthase (SS), and (D) Sucrose phosphate synthase (SPS). Different letters above columns indicate significant difference between means at least at the $P \leq 0.05$ level of probability. Values with the same letter were not significantly different according to Kruskall-Wallis test.

Photorespiration (Fig. 6C). In light reactions, all 14 photosystem I subunit (BIN 1.1.2.2) DEGs were down-regulated including several photosystem I reaction center protein subunits, e.g. PSAD (Solyc06g054260), PSAE (Solyc06g083680, Solyc09g063130), PSAF (Solyc02g069450, Solyc02g069460), PSAG (Solyc07g066150), PSAH (Solyc03g120640, Solyc06g066640, Solyc12g044280), PSAL (Solyc06g082940, Solyc06g082950), PSAN (Solyc08g013670), PSAO (Solyc06g074200) and PSAP (Solyc10g005050). In addition, 19 out of 21 PHOTOSYSTEM II REACTION CENTER (BIN 1.1.1.2) related DEGs and all 26 LIGHT-HARVESTING associated DEGs were also down-regulated (Fig. 6A). In the Calvin cycle, there are at least one DEGs at each node and all the genes showed a tendency for down-regulation, including ribulose-bisphosphate carboxylase (BIN 1.3.2), phosphoglycerate kinase (BIN 1.3.3), glyceraldehyde-3-phosphate dehydrogenase (BIN 1.3.4), triose-phosphate isomerase (BIN 1.3.5), fructose-bisphosphate aldolase (BIN 1.3.6), fructose-1,6-bisphosphatase (BIN 1.3.7), transketolase (BIN 1.3.8), sedoheptulose-bisphosphatase (BIN 1.3.9), phosphoribulokinase (BIN 1.3.12) (Fig. 6B). Furthermore, in Photorespiration, ribulose-1,5-bisphosphate carboxylase (BIN 1.3.13), phosphoglycolate phosphatase (BIN 1.2.1), (S)-2-hydroxy-acid oxidase (BIN 1.2.2), alanine-glyoxylate transaminase (BIN 1.2.3), glycine cleavage system H protein (BIN 1.2.4.4), glycine hydroxymethyltransferase (BIN 1.2.5), glycerate dehydrogenase (BIN 1.2.6) were also down-regulated from the white ripe to mature stage (Fig. 6C). In summary, our results suggest that photosynthesis rate was decreased as most related genes were down-regulated during the ripening of tomato fruit.

3.9. Sucrose-starch metabolism

We analyzed the soluble sugar content within the pericarp and pulp of the fruit (Fig. 1). To understand dynamic expression changes of genes involved in sucrose-starch metabolism, we selected DEGs associated with sucrose-starch metabolism (Fig. 7). Sucrose-phosphate synthase (BIN 2.1.1.1) and sucrose-phosphatase 1 (BIN 2.1.1.2) were down-regulated, suggesting that synthesis of sucrose was inhibited during ripening (Fig. 7A). Similarly, glucose-1-phosphate adenylyltransferase, a key enzyme for starch synthesis, was also down-regulated (Fig. 7B). However, several beta-amylase genes, which are involved in starch degradation, were up-regulated, indicating that starch was processed into small sugar molecules during tomato ripening (Fig. 7B).

3.10. Glycolysis-TCA metabolism

As reported previously, energy metabolism plays an important role in fruit ripening (Saquet et al., 2003; Carrari et al., 2006). Therefore, we monitored all of the DEGs involved in glycolysis and found that most were down-regulated (Fig. 8). These DEGs included phosphopyruvate hydratase (BIN 4.1.13), 6-phosphofructokinase (BIN 4.2.4),

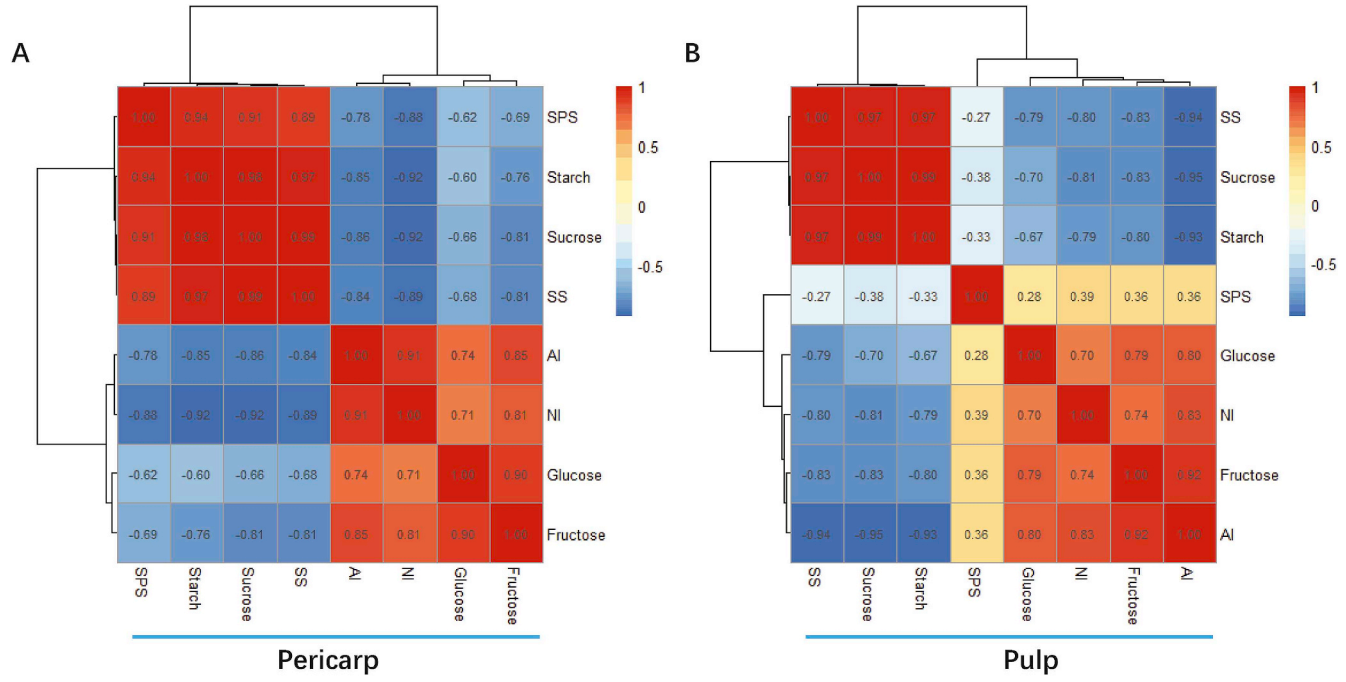

Fig. 3. Heatmap of correlations between the content of sucrose, starch, glucose, fructose and enzymes activities either in the pericarp (A) or pulp (B). Pearson's correlation coefficients between sugar contents and enzymes activities were calculated in R using function cor () from package stats.

phosphoglycerate (BIN 4.3.12), and hexokinase (BIN 2.2.1.4). However, DEGs involved in TCA metabolism showed various transcriptional responses. Cytochrome *c* oxidase (BIN 9.7), NADH-ubiquinone oxidoreductase (BIN 9.1.2), malate dehydrogenase (BIN 8.2.9), malate synthase (BIN 6.2) and malate dehydrogenase (BIN 8.1.9) were down-regulated, whereas NADH dehydrogenase (BIN 9.2.3), succinate dehydrogenase (BIN 8.1.7), fumarate hydratase (BIN 8.1.8), L-malate dehydrogenase (BIN 6.3) and isocitrate dehydrogenase (BIN 8.1.4) were up-regulated (Fig. 8).

3.11. Functional categorization of differentially expressed genes between different irrigation methods

To further identify the possible molecular pathways responsive to different water management strategies, we identified genes that were differentially expressed in mature fruit between the strategies (Fig. 4, Table 1). We identified 246 and 159 DEGs in the T1 and T2 treatments, respectively, relative to CK2 (Table 1). However, there were only 47 and 26 DEGs found in T3 and T4, respectively (Table 1). These results

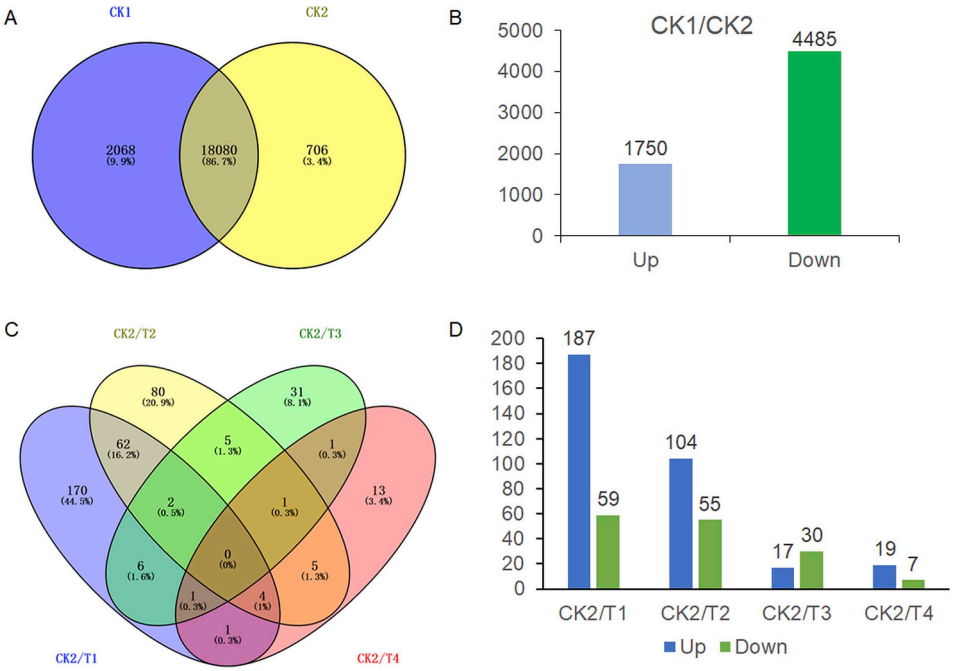

Fig. 4. Analysis of differentially expressed genes in different organizations of tomato fruits. (A) A Venn diagram showing expressed genes that are unique to CK1. There were 18080 (overlapped area) shared by CK1 and CK2 and 706 genes were specifically expressed in CK2, (B) Numbers of differentially expressed genes between CK1 and CK2. There were 1750 up-regulated genes and 4485 down-regulated genes in CK2, (C) A Venn diagram showing expressed genes of CK2 compared to T1, T2, T3 and T4, and (D) Numbers of differentially expressed genes of CK2 compared to T1, T2, T3 and T4. Numbers of down- or up-regulated expressed genes are listed at the histogram of the figure.

Table 1
Functional classification of differentially expressed genes in different organizations of tomato fruits.

Bin	Name	CK1/CK2		CK2/T1		CK2/T2		CK2/T3		CK2/T4	
		No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
30	signaling	356	5.71%	12	4.88%	4	2.52%	2	4.26%	–	–
20	stress	246	3.95%	14	5.69%	14	8.81%	2	4.26%	3	11.54%
31	cell	232	3.72%	9	3.66%	5	3.14%	2	4.26%	1	3.85%
17	hormone metabolism	188	3.02%	10	4.07%	7	4.40%	5	10.64%	2	7.69%
10	cell wall	175	2.81%	9	3.66%	6	3.77%	2	4.26%	4	15.38%
33	development	168	2.69%	6	2.44%	5	3.14%	5	10.64%	1	3.85%
28	DNA	151	2.42%	5	2.03%	1	0.63%	1	2.13%	–	–
11	lipid metabolism	138	2.21%	11	4.47%	7	4.40%	–	–	–	–
1	PS	126	2.02%	9	3.66%	3	1.89%	1	2.13%	–	–
16	secondary metabolism	120	1.92%	3	1.22%	2	1.26%	1	2.13%	1	3.85%
13	amino acid metabolism	108	1.73%	4	1.63%	2	1.26%	1	2.13%	–	–
23	nucleotide metabolism	55	0.88%	1	0.41%	–	–	–	–	–	–
21	redox	55	0.88%	–	–	–	–	–	–	–	–
2	major CHO metabolism	43	0.69%	1	0.41%	1	0.63%	1	2.13%	–	–
3	minor CHO metabolism	41	0.66%	–	–	2	1.26%	2	4.26%	–	–
8	TCA/org. transformation	25	0.40%	–	–	1	0.63%	–	–	–	–
Others and Unknown		1643	26.35%	55	22.36%	38	23.90%	11	23.40%	3	11.54%
Total		6235	100%	246	100.00%	159	100.00%	47	100.00%	26	100.00%

Differentially expressed genes were ascribed to 36 bins in MAPMAN. Only the top 17 groups were listed. The order was made based on the number of differentially expressed genes in a given functional category (BIN), excluding the categories of 'Others' and 'Unknown'.

revealed that brackish water irrigation had a larger influence on gene expression during fruit development. A functional categorization analysis showed that these DEGs were enriched in signaling, hormone metabolism, regulation of transcription, photosystem, carbohydrate metabolism and stress (Table 2). Considering the number of DEGs and functional categorization, we focused our subsequent analyses on the T1 and T2 treatment in order to gain further insights into the molecular pathways regulated by brackish water irrigation.

We detected 8 DEGs in T1 compared with CK2 involved in signaling (Table 2). Seven DEGs, encoding four serine/threonine-protein kinases, a Calcium-transporting ATPase, a COP1-like protein, an early light-inducible protein and a Hcr2-0B protein were up-regulated, while one DEG, encoding the GTP-binding nuclear protein Ran2, was down-regulated. However, none of these genes was changed in T1 compared with CK2. We also analyzed the expression of DEGs involved in the regulation of transcription (Table 2). One gene encoding a CONSTANS interacting protein 1 (CIP1, Solyc06g073180) which was involved in positive regulation of shade avoidance, photomorphogenesis and salt stress (Ben-Naim et al., 2006), was up-regulated in T1 and T2 compared with CK2. Four transcription factor genes were significantly down-regulated in T1, but not in other treatments. One of four genes encoding a C2H2-type zinc finger protein (CZFP1, Solyc04g077980) was involved in photosynthesis, salt stress, wounding, water deprivation, cold and oxidative stress (Aoki et al., 2010). The other three TFs belong to the *MYB* (Solyc06g076350), *GRAS* (Solyc02g085600) and *WRKY* (Solyc09g066010) families, respectively. These results revealed that brackish water irrigation changed the expression of TFs. These TFs might be play positive or negative roles in regulating the transcriptional level of target genes.

An interesting observation was that stress-related genes were generally enriched and up-regulated in response to brackish water irrigation (Table 2). Three out of six DEGs encoding an endochitinase (Solyc02g082920), a wound-induced protein (Solyc01g097270) and an *MLO*-like protein (Solyc06g010030) were significantly up-regulated in T1 relative to CK2. Furthermore, another two DEGs encoding a *PR1* protein (Solyc06g071280) and a heat shock protein (Solyc11g066060)

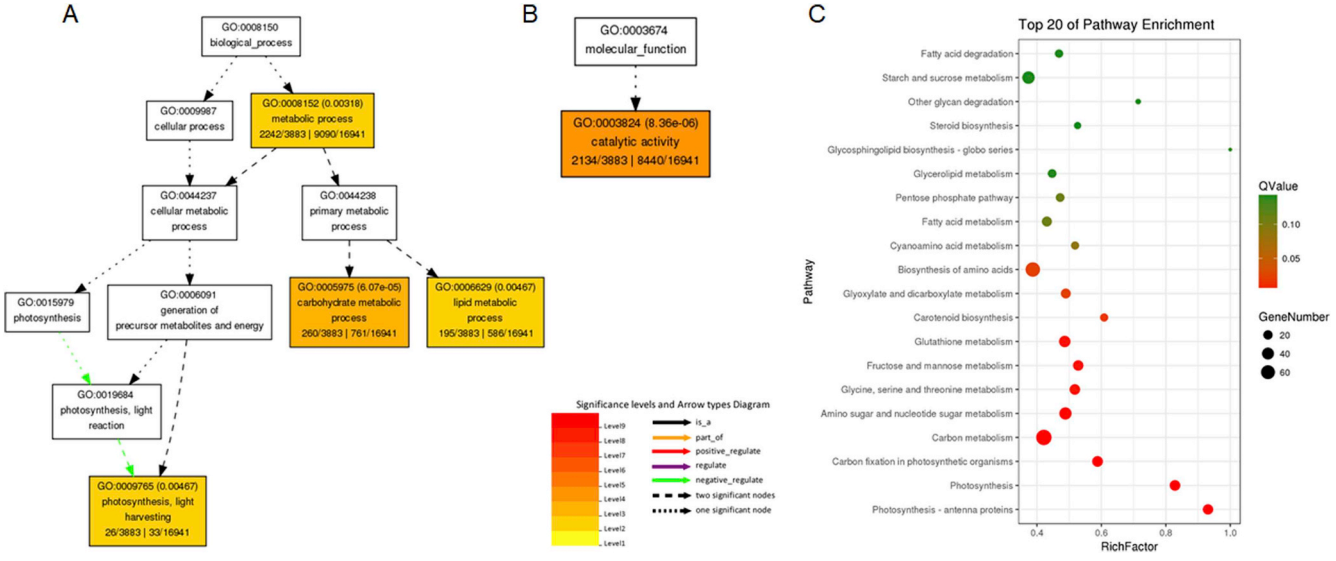

Fig. 5. Functional analysis of differential expressed genes between CK1 and CK2. (A) Biological process of GO enrichment analysis for down-regulated genes. (B) Molecular function of GO enrichment analysis for up-regulated genes. (C) KEGG enrichment analysis of differentially expressed genes between CK1 and CK2.

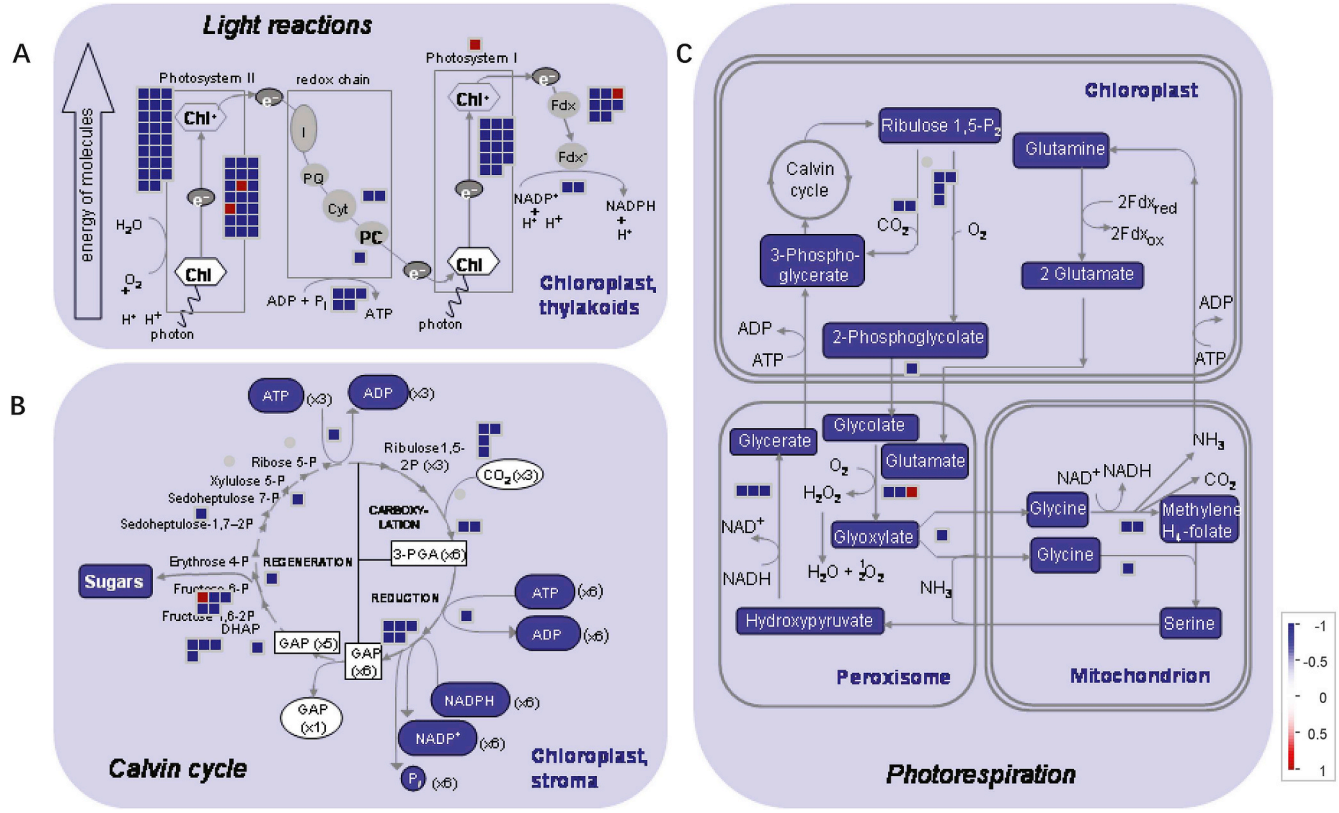

Fig. 6. MapMan analysis of differential expressed genes between CK1 and CK2 in photosynthesis metabolism. (A) Light reactions. (B) Calvin cycle. (C) Photorespiration. Values are log2 fold changes. Blue indicates up-regulation in gene expression, and red indicates down-regulation in gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

also increased in both the T1 and T2 treatments. These results showed that biotic and abiotic stress-related genes were activated by the brackish water irrigation during fruit development.

3.12. DEGs related to hormonal regulation

It has been reported that hormones play important roles in fruit development and sugar-hormone interactions (León and Sheen, 2003;

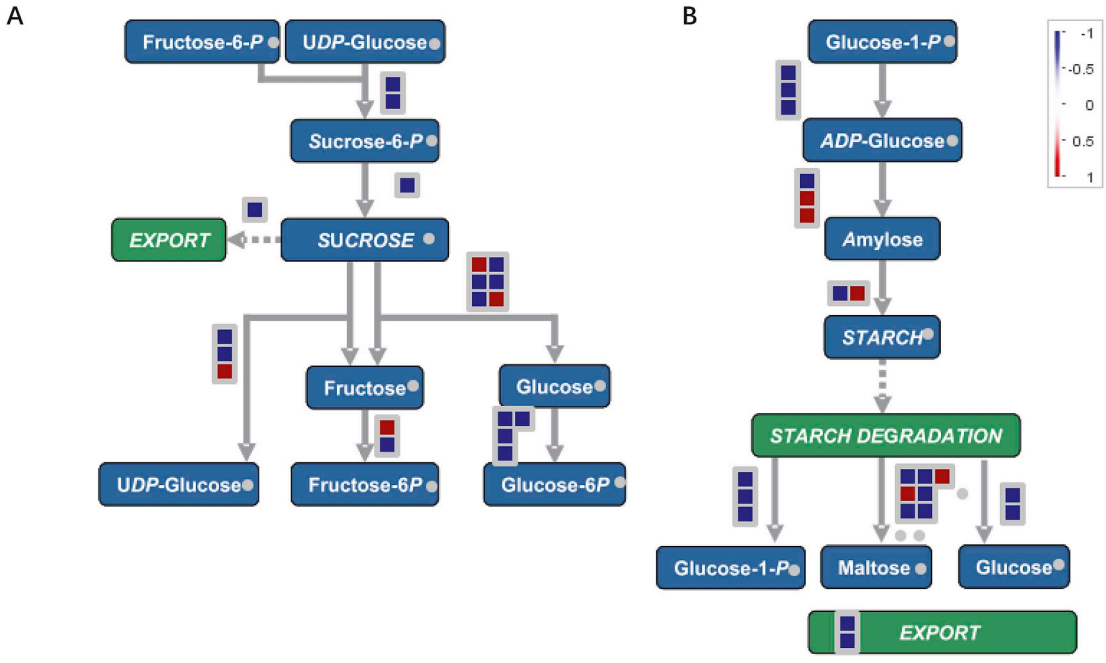

Fig. 7. MapMan analysis of differential expressed genes between CK1 and CK2 in Sucrose-starch metabolism. (A) Sucrose metabolism. (B) Starch metabolism. Values are log2 fold changes. Blue indicates up-regulation in gene expression, and red indicates down-regulation in gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

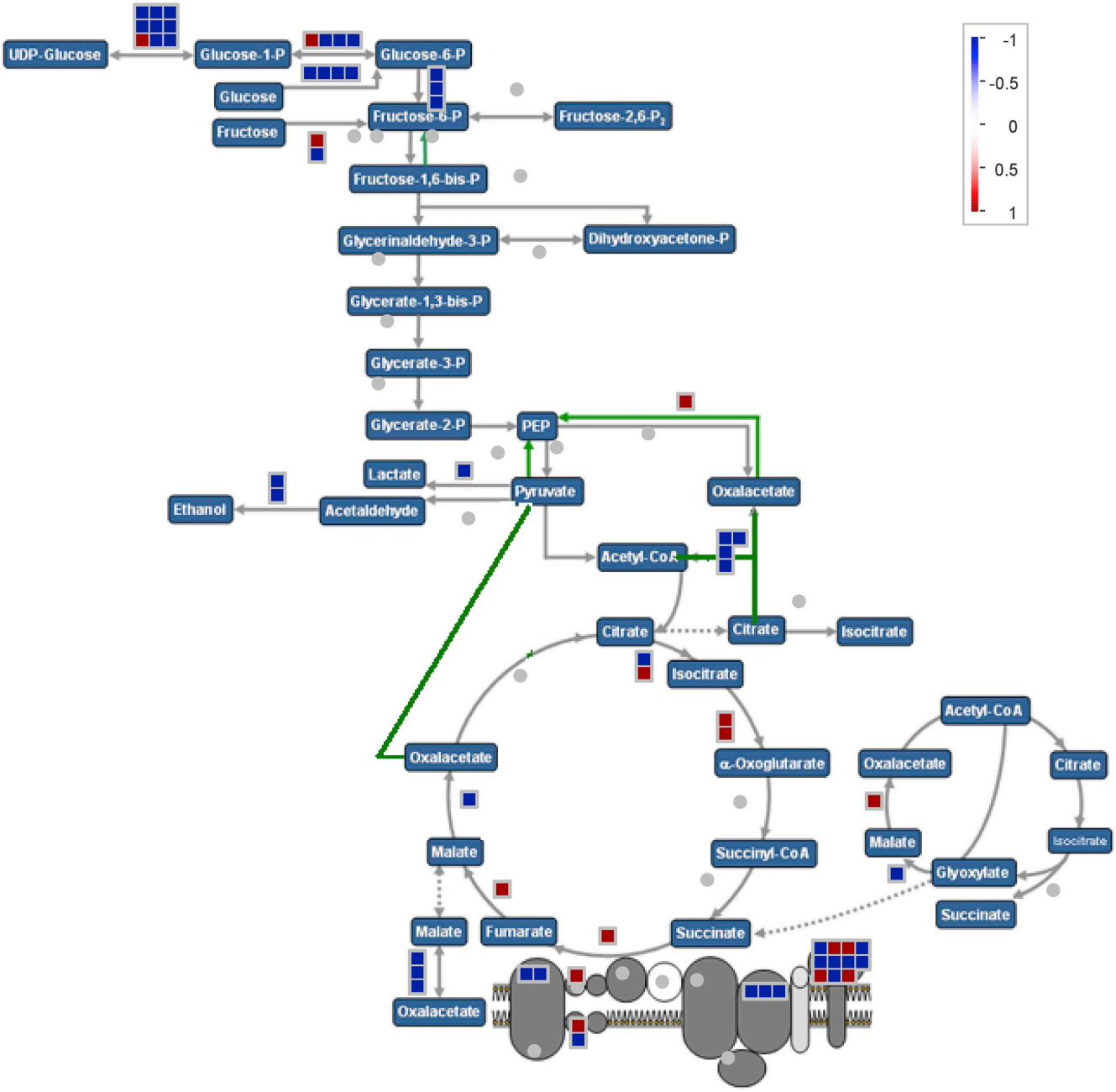

Fig. 8. MapMan analysis of differential expressed genes between CK1 and CK2 in Glycolysis-TCA metabolism. Values are log2 fold changes. Blue indicates up-regulation in gene expression, and red indicates down-regulation in gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Kumar et al., 2014; Mao et al., 2018). For example, ethylene plays a critical role in fruit ripening (Noushina et al., 2017), which is tightly correlated with the expression of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and ACC oxidase (ACO) genes (Smith et al., 1988). In our transcriptomic data, one DEG, encoding 1-aminocyclopropane-1-carboxylate oxidase 4 (ACO4), was found to be increased in T1 and T2 treatments. Allene oxide synthase (AOS) is reported to be involved in JA biosynthesis (Vick and Zimmerman, 1984), which was increased in T1. GH3-1 is encoded by a probable indole-3-acetic acid-amido synthetase and its expression increased at the onset of ripening (veraison), suggesting that it might be involved in the establishment and maintenance of low IAA concentrations in ripening berries (Bottcher et al., 2010). The expression level of one DEG, GH3.1, was highly expressed in T1 treatment (Table 2). These results showed that auxin, ethylene and jasmonic acid biosynthetic pathways were responsive to the brackish water irrigation.

3.13. Confirmation of RNA-seq data using qRT-PCR

To verify the transcriptomic results, 23 DEGs related to sugar metabolism and abiotic stresses in CK2, T1 and T2 were selected and further analyzed using qRT-PCR. The transcriptomic and qRT-PCR

Table 2
Functional cluster of differentially expressed genes in different organizations of tomato fruits.

Gene locus	Annotation	CK2/T1	CK2/T2	CK2/T3	CK2/T4
Signaling					
Solyc06g008300	Hcr2-0B	**1.1084**	0.6196	−0.159	0.5322
Solyc01g005760	NL0D	−0.35	−0.237	**−1.034**	−0.159
Solyc07g063770	Serine/threonine-protein kinase	**2.269**	1.4752	−1.032	−0.317
Solyc07g053130	Serine/threonine-protein kinase	**1.6279**	0.6066	−1.004	−0.538
Solyc11g005630	Serine/threonine-protein kinase	**1.0953**	0.9792	0.1102	0.5717
Solyc04g016260	Calcium-transporting ATPase	**1.0877**	0.3855	−0.227	0.1069
Solyc01g104680	GTP-binding nuclear protein Ran2	**−1.036**	−0.681	−0.2	−0.326
Solyc10g084880	Putative uncharacterized protein	−1.575	−0.232	**−4.289**	−0.314
Solyc11g005190	COP1-like protein	**3.2359**	1.6621	0.3126	0.4285
Solyc09g082690	Early light inducible protein	**1.0745**	0.0164	0.3362	−0.149
Hormone metabolism					
Solyc02g092820	Probable indole-3-acetic acid-amido synthetase GH3.1	**1.46**	−0.765	−1.239	0.2784
Solyc07g049550	1-aminocyclopropane-1-carboxylate oxidase 4	**1.2334**	**1.2587**	0.5119	0.5687
Solyc01g095080	1-aminocyclopropane-1-carboxylate synthase 2	0.9087	0.7848	**1.0516**	0.0127
Solyc05g052040	Ripening regulated protein DDTFR10/A	−1.727	−0.647	**−2.35**	−0.701
Solyc03g093560	Ethylene response factor 5	**−2.256**	−1.302	**−2.815**	−1.434
Solyc01g104740	Ethylene-responsive transcriptional coactivator	**1.3912**	**1.9258**	0.7639	−0.335
Solyc02g089350	Protein GAST1	0.3304	**1.1039**	−0.039	**1.0924**
Solyc08g014000	Linoleate 9S-lipoxygenase A	−0.68	**−1.677**	−1.12	−0.427
Solyc11g069800	Allene oxide synthase	**1.1143**	0.6859	0.3684	-8E-04
RNA. regulation of transcription					
Solyc06g073180	CONSTANS interacting protein 1	**1.3583**	**1.194**	0.1886	−0.058
Solyc04g077980	C2H2-type zinc finger protein	**−3.231**	−0.832	**−3.947**	−0.275
Solyc06g076350	LePCL1 protein	**−1.697**	−0.889	−0.073	0.2031
Solyc02g085600	BAC19.14	**−1.526**	−0.781	0.2701	0.4976
Solyc01g100510	Knotted protein TKN4	−0.881	**−1.667**	−1.113	−0.57
Solyc04g081000	Floral homeotic protein DEFICIENS	−0.846	−0.014	**−1.585**	−1.154
Solyc09g066010	WRKY transcription factor IId-3	**−1.34**	−0.654	0.2495	0.1307
Solyc08g079700	Stress-associated protein 6	0.9264	**1.4491**	0.4492	−0.86
Photosystem					
Solyc10g006230	Chlorophyll *a-b* binding protein 7	−0.709	−0.755	**−2.382**	−0.937
Solyc02g065400	Oxygen-evolving enhancer protein 1	**1.1766**	**1.2572**	−0.154	0.6101
Solyc09g064500	Photosystem II reaction center Psb28 protein	**1.6361**	−0.901	−1.336	−0.113
Solyc06g060340	Photosystem II 22 kDa protein	**3.9423**	−0.059	−2.04	2.1508
Gene locus	**Annotation**	**CK2/T1**	**CK2/T2**	**CK2/T3**	**CK2/T4**
Solyc10g044520	Ferredoxin-1, chloroplastic	**1.3213**	0.701	−0.366	0.1249
Solyc12g099930	Hop-interacting protein THI032	**1.4273**	**1.2702**	0.1549	−0.22
Solyc02g091560	Serine hydroxymethyltransferase	0.3731	**−1.509**	−1.306	−0.273
Solyc04g009030	Glyceraldehyde-3-phosphate dehydrogenase	**2.0312**	−0.403	−1.618	−0.063
Major CHO metabolism					
Solyc07g052690	Beta-amylase	−0.339	**−1.182**	−0.366	−0.353
Solyc08g007130	Beta-amylase	**2.3208**	−0.502	−0.119	0.3401
Solyc05g012510	Alpha-1,4 glucan phosphorylase	0.1545	−0.426	**−1.244**	−0.224
Minor CHO metabolism					
Solyc05g051850	Putative myo-inositol-1-phosphatase	0.5031	**−1.316**	−1.152	−0.747
Solyc04g008730	Melibiase	−0.993	**−1.34**	**−1.358**	−0.725
Glycolysis					
Solyc08g066100	Phosphohexokinase	−0.228	**−1.671**	−0.338	−0.32
Transport					
Solyc11g012360	Tonoplast dicarboxylate transporter	1.7489	**1.6429**	0.8713	1.3265
Solyc06g060760	Rb7	0.4087	**−3.102**	−0.887	−0.83
Stress					
Solyc02g082920	Acidic 26 kDa endochitinase	**2.4096**	0.7024	0.1547	−0.297
Solyc10g055810	Basic 30 kDa endochitinase	0.5651	**1.3689**	1.0888	**1.7603**
Solyc01g097270	Wound-induced protein	**1.6554**	1.3473	0.6917	0.2526
Solyc01g106620	*PR1* protein	**1.407**	**1.9451**	1.7621	0.3555
Solyc06g071280	*EDS1*	**2.1662**	**1.761**	−0.107	−0.23
Solyc06g010030	*MLO*-like protein	**1.4594**	0.9954	−0.066	1.0633
Solyc06g076540	Class I small heat shock protein 20.1	0.0552	**2.0667**	0.6206	−0.315
Solyc11g066060	H	**1.3526**	**1.1725**	0.4307	0.4202

results are compared in Fig. 9. Nineteen out of 23 (82.6%) selected DEGs were concordant with the trend estimated by RNA-Seq. This high concordant result showed that RNA-Seq do not introduce bias to the results of the gene expression profiling under study.

4. Discussion

Whether it is possible to use brackish water for supplementary irrigation is one of the current hotspots, which has been debated for a

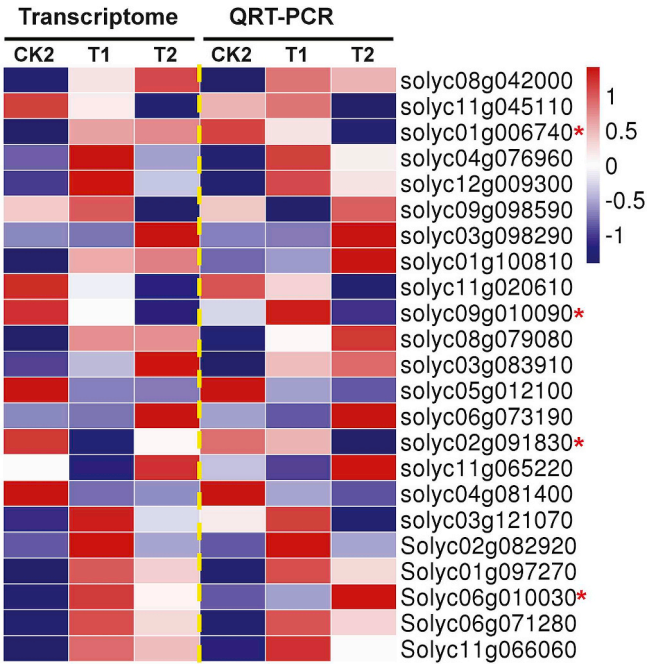

Fig. 9. Validation and expression analysis of 23 genes in response to three different irrigation patterns in fruits of CK2, T1 and T2. Gene expression analysis were examined by qRT-PCR using cDNA from fruits of CK2, T1 and T2. All of the qRT-PCR values were expressed relative to the expression level of CK2. The data of T1 and T2 from transcriptome was expressed relative to the transcript abundance of CK2 from the transcriptome. The color scale represents log2 expression values and the expression levels presented in heatmap were log2-based. All data are shown as the mean ± SD (n = 3). * indicates the different expression patterns of data between transcriptome and qRT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

long time. For instance, irrigation with brackish water could cause salt accumulation in soil to further affect the normal growth of crops (Pang et al., 2010). However, the use of brackish water can significantly improve fruit quality, especially sugar content in fruits (Wang and Shan, 2015; Takahata and Miura, 2014) through changes in osmosis, ion content and soil physical and chemical properties (Feleafel and Mirdad, 2014; Davies and Hobson, 1981; Beckles, 2012). Therefore, reasonable exploitation and utilization of brackish water have become an important way to alleviate the contradiction between water supply and demand. Our result demonstrated that brackish water irrigation increased the contents of glucose and fructose at mature period to improve fruit quality. This may be caused by a change in the activity of acid invertase (AI) and neutral invertase (NI) which hydrolyzes sucrose and determines the types of sugars that accumulate in the storage cells (Fig. 2A and B).

Sucrose metabolism plays crucial roles in plant development (Hu et al., 2016). Both fruit ripening and irrigation patterns are complex regulation processes (Giovannoni, 2007; Stefania et al., 2016). The effect of brackish water treatment on the relationship between increased sugars and the regulation of sugar metabolism in tomato fruit is not yet clear. To identify genes with potential functions in sugar metabolism, the transcriptomes of tomato fruits were analyzed and compared between the white-ripe and mature stages. Compared to the white-ripe stage, there were 1750 up-regulated DEGs and 4485 down-regulated DEGs at the mature stage (Fig. 4B). This result suggested that the metabolism of sugar is developmentally regulated, which is consistent with the previous investigation (Hu et al., 2016). Under brackish water treatment, two beta-fructofuranosidase genes (solyc01g100810 and solyc03g083910) at the mature stage were highly expressed (Fig. 7A), which may promote the hydrolysis of sucrose into glucose and fructose (Rohde et al., 1995). Rice sucrose transporter *OsSUT2* was found to be up-regulated during salinity treatment, thus facilitating the transport of sucrose from photosynthetic cells to cope with salt stress (Ibraheem et al., 2011). One sucrose transporter 4 (solyc04g076960) was found to be significantly down-regulated in our study (Fig. 7A), which may have limited sucrose transport. All these factors likely contributed to the accumulation of glucose and fructose at the mature stage under brackish water treatment.

Suitable management strategies for saline water (e.g. mixed with fresh water in different ratios) is crucial for tomato growth and berry development (Malash et al., 2005). Increasing salinity led to decreases in leaf area index, plant dry weight, fruit total yield, individual fruit weight (Malash et al., 2008) as well as a decrease in seasonal accumulative water use (Wan et al., 2007). To identify potential molecular pathways mediating the response to brackish water irrigation (CK2), four types of water management strategies were performed. Compared to brackish water irrigation (CK2), there were 246 DGEs in T1, 159 DGEs in T2, 47 DGEs in T3, and 26 DGEs in T4 (Fig. 4C and Table 1). Treatment of rotating irrigation with brackish water and freshwater (T1) led to reprograming of global gene expression to a large extent, relative to the other treatments. These results suggested that different irrigation modes influence on the transcriptome in different patterns. An interesting observation is that genes involved in signaling, hormone metabolism, photosystem, and stress responses were significantly up-regulated in T1 (Table 2). It has been well-documented that sugar accumulation is tightly associated with the expression of genes involved in defense responses (Ho, 1996; Price et al., 2004). This is in good accordance with our data, where the 4 genes regulated were most frequently related to abiotic and biotic stress responses. For example, endochitinase (Solyc02g082920), pathogenesis-related protein 1(*PR1*, Solyc01g106620), Enhanced disease susceptibility 1 (*EDS1*,Solyc06g071280) and Mildew resistance locus o (*MLO*)-like protein (Solyc06g010030) were highly expressed, whereas the expression of one wound-induced protein (Solyc01g097270) and two heat shock proteins (*HSP*,Solyc06g076540 and Solyc11g066060) were differentially expressed in response to brackish water treatment during fruit development (T1). It is worth noting that 4 transcription factors (Solyc04g077980, Solyc06g076350, Solyc02g085600 and Solyc09g066010), encoding to *C2H2*-type zinc finger proteins, were down-regulated. CONSTANS interacting protein 1 (Solyc06g073180) participates in positive regulation of shade avoidance, photomorphogenesis, and salt stress (Ben-Naim et al., 2006). Under brackish water treatment, CONSTANS interacting protein 1 was observed to be up-regulated in our transcriptomic analysis. These findings indicated that brackish water irrigation during fruit development (T1) could induce or enhance the defense against pathogen attack and abiotic stress by activating the expression of stress-responsive genes.

Based on the results, we present a general model to highlight how brackish water irrigation during fruit development may contribute to fruit quality and enhanced resistance to biotic and abiotic stress (Fig. 10). When irrigated with brackish water, the influx of ions could lead to activation of the expression of genes involved in signal transduction. The up- or down-regulated expression of transcription factor genes may induce or enhance the expression of R genes (e.g. *EDS1*, *MLO*, *PR1* and endochitinase) and abiotic stress-related genes (such as wound-induced heat shock genes). The influx of ions could also promote photosynthesis to synthesize and accumulate sugars. More efficient management and utilization of irrigation with brackish water will be helpful to improve tomato fruit quality in arid and semi-arid regions.

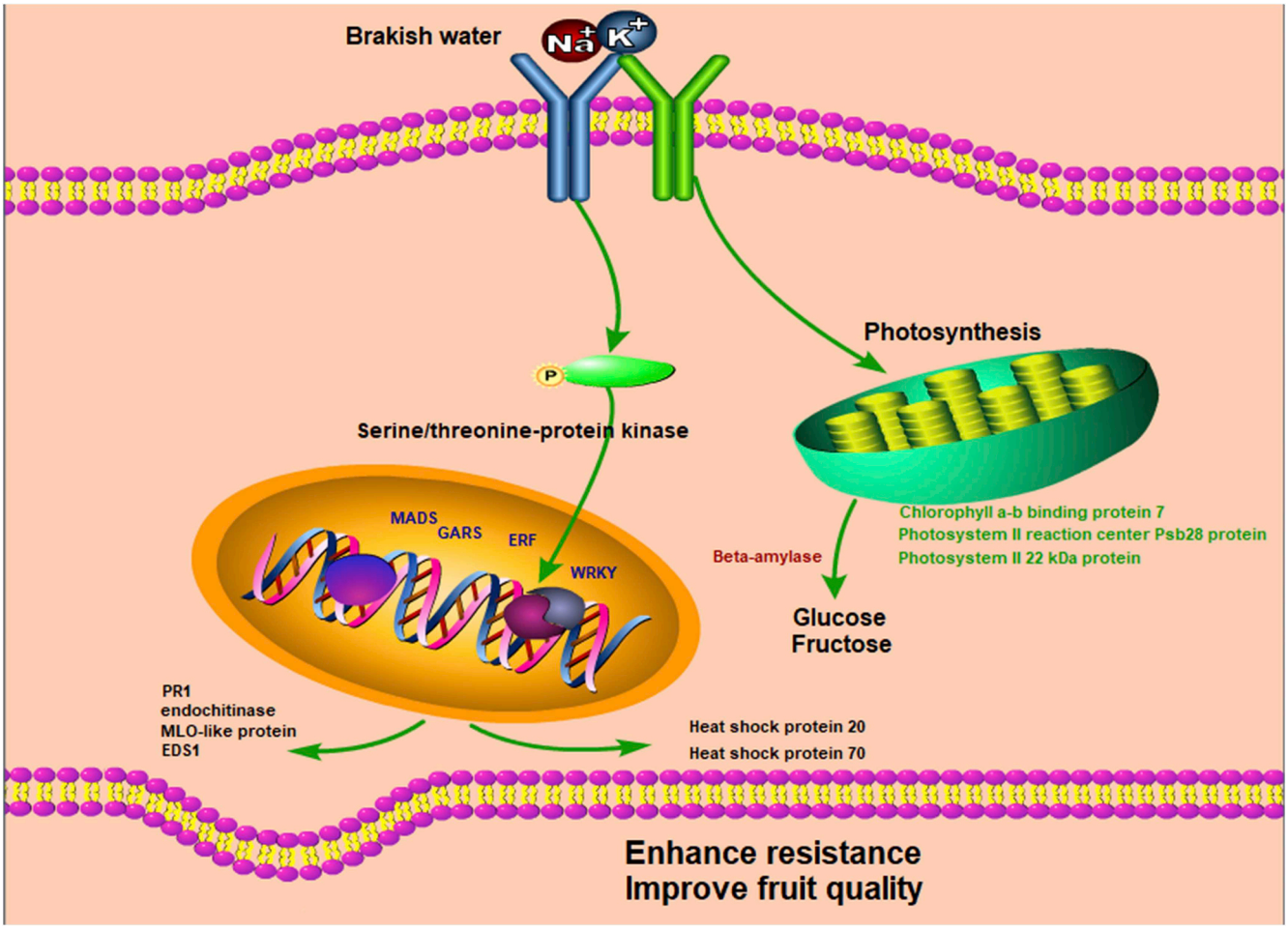

Fig. 10. A model of how different irrigation methods regulate signaling may in tomato fruit. See Discussion for more details. Arrows indicate differential expressed genes involved in the specified.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.025.

Contribution

Jianshe Li and Weirong Xu conceived and designed research, Yanming Gao, Ping Tian, Juan Li and Yune Cao conducted experiments, Yanming Gao and Ping Tian analyzed data, Yanming Gao and Weirong Xu wrote the manuscript. All authors read and approved the final manuscript.

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