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STUDY ON THE RELATED CRACKING-RESISTANT GENES IN CHINESE JUJUBE

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Abstract

The problem of fruit cracking for Chinese jujube (Zizyphus jujuba Mill.) causes serious yield losses in China, however, the existing prevention and control measures are hard to solve it. Two groups of high resistant cracking type and easy cracking type for the filial generation of Dong Zao × Linyili Zao were divided in the study. Two allelic genetic pool and the related genes cDNA libraries for the cracking-resistant characters to be specifically expressed were built to conduct the preliminary feasibility study on the genes related to the resistance of fruit-cracking through the differently expressed genes. The sequencing production of the two group transcriptomes were received more than 4.5 Gb and 4.6 Gb data after removing the low quality segments, and conducted 45,401,606 and 46,468,222 times reading, the Q20 (the base ratio no less than 20) of the two libraries were both greater than 96%. After comparing the genetic expression within two groups, 391 items of differently expressed genes (DEGs) were filtered. Genome function annotation on the 391 items DEGs were conducted and the results showed that there were 92 items genes added, 45 items defined, and 299 items gene without annotation. The annotated genes probably involving in jujube cracking phenomenon were Aquaporin PIP, Tubulin, Calreticulin and Calmodulin.

Key words: *Chinese jujube, fruit cracking, cracking-resistant genes, RNA-Seq.*

INTRODUCTION

The phenomenon of fruit cracking on jujube is widespread, which causes serious yield losses, however, the existing prevention and control measures cannot fundamentally solve the problems. The researches showed that there were great differences between different varieties of jujubes on fruit cracking resistance (Mao et al., 1998 ; Wang et al., 2011 ; Yuan et al., 2013). So, it is effective means to solve the fruit cracking by breeding new varieties with high resistant cracking in Chinese jujube, the molecular marker assisted selection is a important methods in plant breeding.

Researches on the genes related to fruit cracking characteristics have been reported, such as MdExp3 gene in apples (Wakasa et al., 2003; Kasai et al., 2008), β-galactosidase gene (TBG6) in tomatoes (Moctezuma et al., 2003), Expansin gene - LcExp1gene and LcExp2 - in litchi pericarp (Wang et al., 2006), which closely related to cell wall loosening gene and fruit cracking gene. With the rapid development of high throughput sequencing, the function of transcriptome sequencing analysis has opened new avenues for the study of fruit cracking gene. Li used the high throughput sequencing technology to do the transcriptome sequencing analysis on crack resistance and easy to crack pericarps, through the analysis of the differences in gene expression, there were 67 candidate genes about fruit cracking screened out (Li et al., 2014), including 4 water transportation related genes (LcAQP, 1; LcPIP, 1; LcNIP, 1; LcSIP, 1), 5 genes related to Gibberellic Acid (Gibberellic Acid, GA) metabolism (LcKS, 2; LcGA2ox, 2; LcGID1, 1), 21 Abscisic Acid (Abscisic Acid , ABA) metabolism related genes (LcCYP707A, 2; LcGT, 9; Lcβ-Glu, 6; LcPP2C, 2; LcABI1, 1; LcABI5, 1), 13 genes related to calcium transportation (LcTPC, 1; Ca2+/H+ exchanger, 3; Ca2+-ATPase, 4;

LcCDPK, 2; LcCBL, 3), and 24 cell wall metabolism related genes (LcPG, 5; LcEG, 1; LcPE, 3; LcEXP, 5; Lcβ-Gal, 9; LcXET, 1).

The main objectives of this study are (1) building two near-isogenic pools with the hybrid offspring of Dong Zao × Linyili Zao, which have the same genetic background; (2) establishing jujube resistance to cracking fruit traits specific related genes expression cDNA library, and to do the sequencing analysis generating the relevant ESTs sequences; (3) sequencing the differential expression genes, and to do gene function analysis and prediction on the ESTs sequences. This will provide references for the study of new genes and new germplasm about jujube and related plants, and it will have important significance to breed new cracking-resistance varieties through molecular techniques.

MATERIALS AND METHODS

Plant material

The supplied samples were 12 year-old hybrid offspring of Dong Zao × Linyili Zao in Jujube breeding base located in Wangcun town, Daming County, Hebei Province).

Two group of 12 cracking-resistance (cracking fruit rate < 5%, group A) and 12 sensitive-cracking (cracking fruit rate > 80%, group B) hybrid offspring were divided, according to the survey results of consecutive years cracking fruit rate on the hybrid offspring.

During young fruit stage (at the end of the 20 days after flowering), 10 young fruits of each type were picked and covered with silver paper immediately, then put into liquid nitrogen to quick-freeze. The samples were taken back to laboratory and preserved in an icebox (T=-80°C) for subsequent analyses.

RNA extraction

The fruit RNA of the offspring was extracted using the RNeasy plant mini kit (Qiagen); On-column DNase digestion with the RNase-Free DNase set (Qiagen) was performed to remove contaminated DNA.

Twelve mixed Chinese jujube cDNA samples were prepared for each group, and then the RNA samples were sent to prepare library with NEB RNA library prep kit and sequence with Illumina HiSeq2000. The RNA-Seq data were subjected to bioinformatic analysis.

Sequence bioinformatics

The raw reads were filtered with FASTQ_Quality_Filter tool from the FASTX-toolkit. Reads both with more than 35bp and having a quality score higher than 20 were kept. Then all the valid reads of A and B samples were combined to perform de novo splicing by paired-end method with Trinity software (Grabherr et al., 2011). The longest transcript per locus was used as a unigene, and as a result we got 94,984 unigenes.

Several complementary approaches were utilized to annotate the unigenes, which were conducted using the Basic Local Alignment Search Tool (BLAST). The unigenes were compared against the NCBI NR, SWISS-PROT, TrEMBL, Cdd, pfam and KOG databases with an E-value of 1e-5 and Identity of 30%. Functional annotation were operated using gene ontology terms method by Blast2GO software (GO, http://www.geneontology.org) (Ashburner et al., 2000). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were assigned to the sequences using the online KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/kegg/kaas/). This method was used to obtain KEGG Orthology (KO) assignment. The output of KEGG analysis includes KO assignments and KEGG pathways (Kanehisa, 1997; Kanehisa and Goto, 1999).

Gene expression analysis

Researchers compared the reads with unigenes using single-end mapping method by bowtie2-2.2.2 software. To compare the unigene expression level in the A and B libraries, the transcript level of each expressed unigene was calculated and normalized to the reads per kilobase of exon model per million mapped

reads (RPKM) (Mortazavi et al., 2008). Significance of differential unigene expression was determined by using General Chi-squared test and assigned P-values (<0.01). The P-values were adjusted to account for multiple testing by using the false discovery rate (FDR) and assigned error ratio Q-value (<0.05).

The unigenes with an adjusted P-value <0.01 and the absolute value of log2 >1 (expression fold change) were deemed to differently expressed, while the unigenes with an FDR-adjusted P-value <0.01 was considered statistically significant (Audic and Claverie, 1997; Reiner et al., 2003; Simonsen and Mcintyre, 2005).

RESULTS AND DISCUSSIONS

Sequence analysis and assembly

To understand the molecular bases of Zizyphus jujuba and identify the new valid genes, six libraries representing two groups of high resistant cracking fruit and sensitive cracking fruit were constructed. RNA from the two groups was used for Illumina RNA-Seq.

Each sequenced sample yielded 100bp reads from paired-end sequencing of cDNA fragments. After quality assessment and data clearance, 4.5~4.6 billion (G) reads with more than 96% Q20 bases (those with an average base quality greater than 20) were kept as high quality reads for each library and used in the later analysis (Table 1). All the valid reads above were combined to perform de novo splicing by paired-end method with Trinity software (trinityrnaseq_r20131110 version). A total of 94,984 unigenes were obtained, among which 25,287 unigenes were longer than 1kb. An overview of the assembled transcripts and unigenes was presented in Table 2. The length distributions of unigenes were shown in Figure 1. The results demonstrated the effectiveness of Illumina pyrosequencing in rapidly capturing a large portion of the transcriptome.

The distribution of gene expression levels was used to evaluate the normality of the library data. The level of gene expression was determined by calculating the number of unigenes and then normalizes to the RPKM (Mortazavi et al., 2008). As shown in Figure 2, the majority of mRNA was expressed at low levels, whereas a small proportion of mRNA was highly expressed.

The gene expression variations were analysed by compared the two libraries of resistant cracking fruits (group A) and sensitive cracking (group B). A total of 391 differently expressed genes (DEGs) including 218 up-regulated and 173 down-regulated genes were detected.

Table 1 Description of two groups of *Zizyphus jujuba* RNA-Seq libraries

Library name	Total reads	Total bases	Average reads length (bp)	Cycle Q20 (%)
A[a]	45,401,606	4,507,948,150	99.29	96.37
B[b]	46,468,222	4,606,988,891	99.14	96.04
All	91,869,828	9,114,937,041	99.22	96.20

A [a]: cracking-resistant fruit; B [b]: sensitive-cracking fruit;

Table 2 Summary of Illumina transcriptome assembly for *Zizyphus jujuba*

Library name	Total reads	N50[a]	N90[b]	Total Length	Max length	Min length	Average length
Transcript	198,993	2010	589	252,481,032	28,719	201	1268.79
Unigene	94,984	1584	302	79,036,677	28,719	201	832.11

N50 a, sorted the transcripts from long to short, then accumulated bases of transcripts in turn, when the total bases number reached half of total number of bases, the length of transcript, as well as unigenes; N90 b, counted in a similar way.

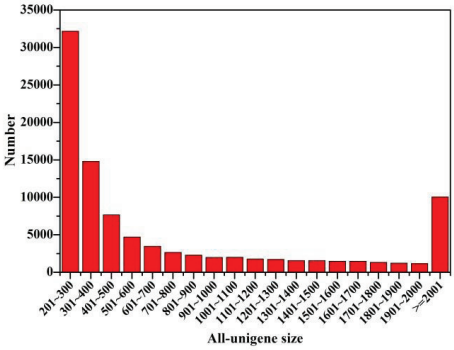

Figure 1. Length distributions of All-Unigenes

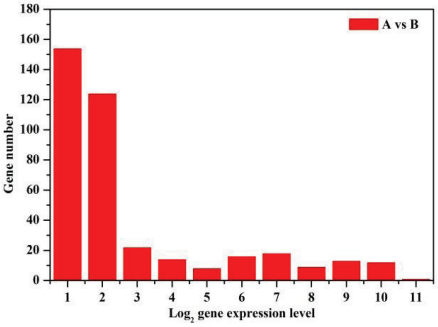

Figure 2. Distribution of gene expression levels

GO enrichment analysis of DEGs

GO enrichment analysis was the function annotations of DEGs. GO term with P-value< 0.05 were defined significantly enriched, and there were 1515 items DEGs involved in it. We mapped the sensitive-cracking higher than cracking-resistant fruit gene expression (UP.A.B) and sensitive-cracking less than cracking-resistant fruit gene expression (DOWN.A.B) DEGs to terms in GO database respectively.

The DEGs up of GO enrichment analysis are shown in Figure 3. There were 467 items UP.A.B DEGs involved in biological metabolism, mainly including metabolic processes, accounting for 30%; cellular processes, 26%; single-organism processes, 15%, and multi-organism processes, 10%; 257 items involved in cellular components, the cell accounted for 17%; cell part, 17%; other organism, 16%, and other organism part, 16%; 255 items involved in molecular function, the catalytic activity took up 60%, and binding was 27%.

The DEGs down of GO enrichment analysis are shown in Figure 4. There were 233 items DOWN.A.B DEGs involved in biological metabolism, mainly including cellular process, taking up 30%; cellular component organization or biogenesis, 15%, and metabolic process, 14%; 256 items involved in cellular component, The cell accounted for 20%; cell part, 20%; macromolecular complex, 14%; organelle, 14% and organelle part, 14%; 73 items involved in molecular function, including binding accounting for 55% and catalytic activity taking up 42%.

We compared GO enrichment analysis between UP.A.B and DOWN.A.B DEGs, there were 11 processes fully upward trend, multicellular organismal process, developmental process, reproductive process, biological adhesion, membrane, membrane part, extracellular matrix, extracellular region part, nucleic acid binding transcription factor activity, molecular function regulator and antioxidant activity. The membrane belongs to cellular component including 24 genes were all up-regulated, membrane component may have important relationship with fruit sensitive cracking.

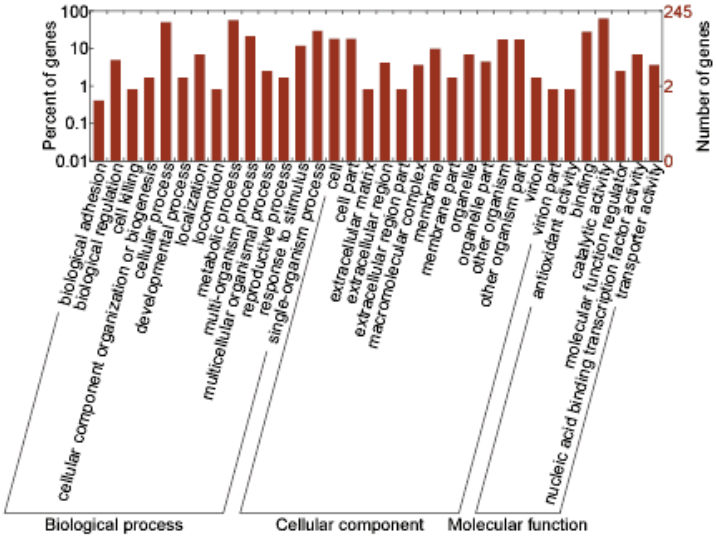

Figure 3 DEGs up of GO enrichment analysis

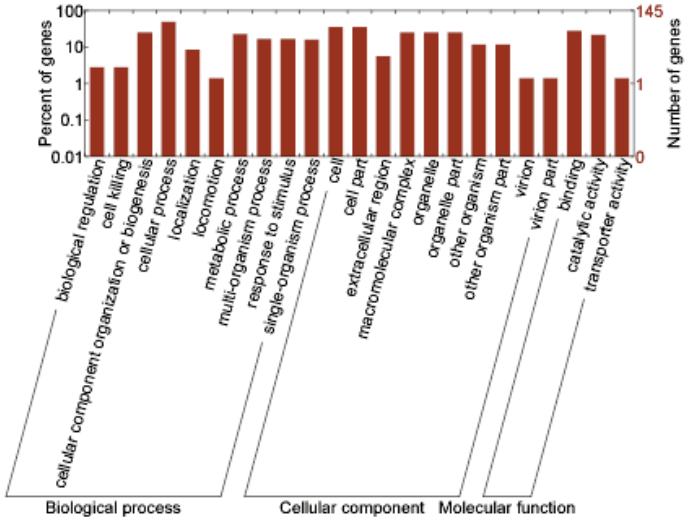

Figure 4. DEGs down of GO enrichment analysis

Pathway enrichment analysis of DEGs

KEGG pathway enrichment analysis was performed to categorize the biological functions of DEGs. (Kanehisa, 1997; Kanehisa and Goto, 1999) We mapped all the genes to terms in KEGG database.

Enrichment analysis of differentially expressed genes by KEGG is shown in Table 3. KEGG with P-value < 0.05 were defined significantly enriched, and there were 246 items DEGs involved in it. Among all the KEGG, there were 10 items significantly enriched KEGG; The DEGs participated in amino acid

metabolism and lipid metabolism were more, there were 15 items and 12 items, respectively. The amino acid metabolism primarily referred to cysteine and methionine metabolism, and valine, leucine and isoleucine biosynthesis. The Lipid metabolism mainly pointed to alpha linolenic acid metabolism and steroid biosynthesis.

The experiment were expected to get the related genes of jujube easy cracking fruit or resistance cracking fruit by the KEGG enrichment analysis of DEGs, the α-linolenic acid is the basic substances of cell membrane and enzymes (SanGiovanni et al., 2005). The result of down-regulated differences in gene enrichment of metabolic pathway suggested that α-linolenic acid metabolism in cracking fruit types were weaker than in resistance cracking fruit types; therefore, α-linolenic acid metabolic pathway can be one of the research targets for cracking fruit study.

The number Ko (ko00592) in the α-linolenic acid metabolic pathway corresponding to KEGG pathway can analyse the relationship of genes and KEGG, and map the information into the pathway. The α-linolenic acid metabolism process with KEGG pathway was shown in Figure 5.

Table 3 Enrichment analysis of differentially expressed genes by KEGG

#	KEGG name term	Classification	P-value	Q-value	Number
1	Cysteine and methionine metabolism [PATH:ko00270]	Amino acid metabolism	2.89E-06	0.000890265	11
2	Flavonoid biosynthesis [PATH:ko00941]	Biosynthesis of other secondary metabolites	4.59E-11	1.41E-08	11
3	Alpha linolenic acid metabolism [PATH:ko00592]	Lipid metabolism	8.11E-07	0.000249766	8
4	Glutathione metabolism [PATH:ko00480]	Metabolism of other amino acids	0.00014206	0.043753378	8
5	Circadian rhythm - plant [PATH:ko04712]	Environmental adaptation	5.29E-06	0.001628153	4
6	ko01220	-	2.84E-05	0.008752995	4
7	Stilbenoid, diarylheptanoid and gingerol biosynthesis [PATH:ko00945]	Biosynthesis of other secondary metabolites	6.33E-05	0.019485288	4
8	Steroid biosynthesis [PATH:ko00100]	Lipid metabolism	0.00015861	0.048852201	4
9	Valine, leucine and isoleucine biosynthesis [PATH:ko00290]	Amino acid metabolism	7.25E-05	0.022328	4
10	Valine, leucine and isoleucine biosynthesis [PATH:ko00290]	Carbohydrate metabolism	0.00014	0.043056	2

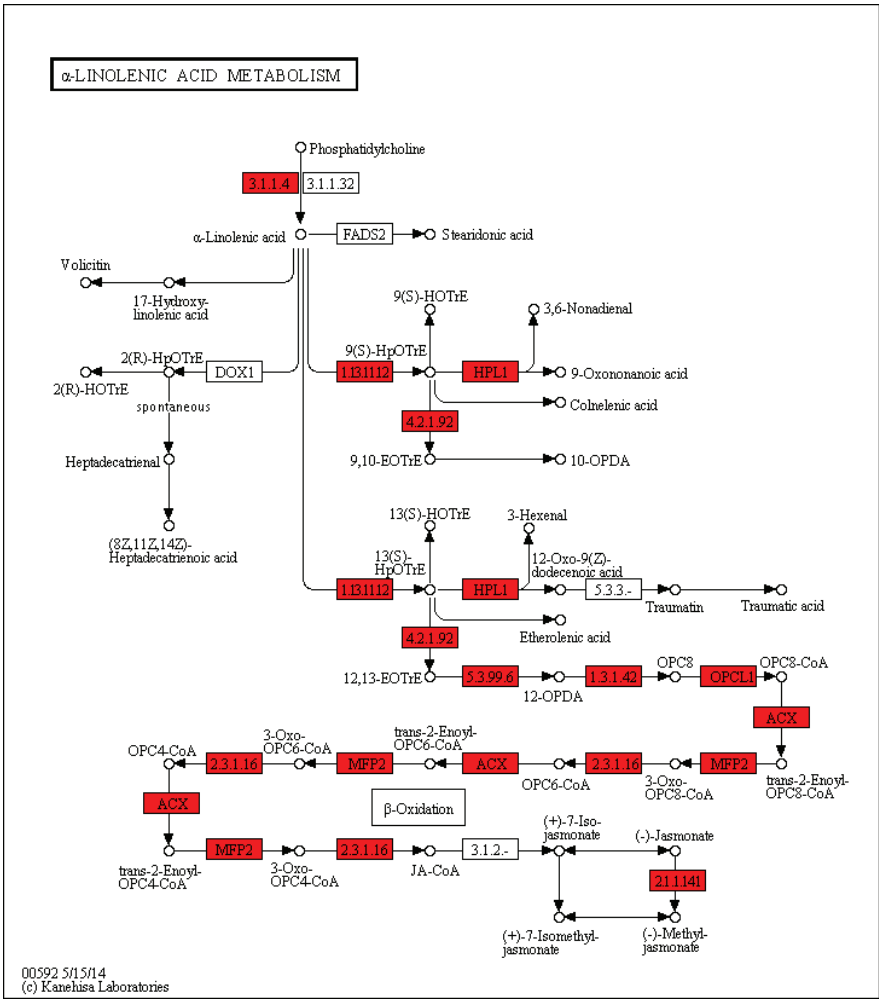

Figure 5. Alpha linolenic acid metabolism with KEGG pathway

Functional annotation of differentially expressed genes

The main gene annotations of DEGs are given in Table 4. The screened 391 items DEGs were functionally annotated, 92 items genes annotated, 45 items genes defined, and 299 items genes still not annotated. The annotated genes related to jujube cracking fruit were Aquaporin PIP, Tubulin, Calreticulin, and Calmodulin; there were 5, 6, 2 and 2 in DEGs, respectively.

Aquaporin PIP is a protein channel in the plasma membrane that facilitates water movement across the membrane, regulating the water balance inside and outside the cell, which are the down-regulated DEGs, suggesting the expression of Aquaporin PIP in resistance to cracking fruit types are higher than that in easy to cracking fruit types. Previous studies showed that

With the development of the fruit, the water in fruit was reduced, and water absorbing capacity was increased. Then when there was water in the fruit surface and dived in water potential gradient, the water would go into the fruits, thus when the turgor pressure within the fruit exceed the skin strength, it would be the cracking fruits (Wang et al., 2013).

And in the resistance to cracking fruit types, the Aquaporin PIP could promote the water transportation inside and outside cells, which reduced the turgor pressure within the fruit to a certain extent, thus reduce the risk of cracking fruits.

Tubulin is the important component of the cytoskeleton, it acts to keep the cells' shape, involved in cell division, cell movement, intracellular material transportation, and assists all kinds of organelles to complete their respective functions (Rao and Zhang, 2013). In our study, we annotated α-tubulin and β-tubulin to down-regulated DEGs, which could promote the high expression of microtubule protein gene, thus reducing the happening of cracking fruit types.

Calreticulin is one of the calcium binding proteins on the endoplasmic reticulum, with the biological function of chaperone, adjusting the steady state of Ca2+, cell adhesion and gene expression regulation (Liu, 2013), Calmodulin is one of the conservative strong regulatory proteins in the structure of biological cell by adjusting a series of enzymes to affect the plant growth, development and stress (Hoeflich and Ikura, 2002). Li (2014) screened 13 genes associated with calcium transportation from the candidate genes related to cracking fruit by DEGs. In our study, the Calreticulin and Calmodulin showed high expression in resistance to cracking fruit types, suggesting the cracking fruit had important relationship with Ca transportation and regulation.

Table 4 The main gene annotation of DEGs

Unigene	Name	Definition	Length	up/down	P-value	Q-value
comp54052_c0_seq1	PIP	AQUAPORIN PIP	1416	down	1.79E-12	2.21E-09
comp40997_c0_seq1	PIP	AQUAPORIN PIP	1508	down	6.84E-13	9.69E-10
comp16213_c0_seq1	PIP	AQUAPORIN PIP	315	down	3.99E-05	1.20E-02
comp16225_c0_seq1	PIP	AQUAPORIN PIP	315	down	3.99E-05	1.20E-02
comp45258_c1_seq1	PIP	AQUAPORIN PIP	1231	up	0.00012	0.03147
comp22094_c0_seq1	TUBA	TUBULIN ALPHA	984	down	0.00011	0.02806
comp10051_c0_seq1	TUBA	TUBULIN ALPHA	704	down	2.65E-06	1.16E-03
comp15927_c0_seq1	TUBA	TUBULIN ALPHA	984	down	4.83E-05	1.41E-02
comp49817_c0_seq1	TUBA	TUBULIN ALPHA	2035	down	4.10E-11	4.33E-08
comp49059_c0_seq1	TUBA	TUBULIN ALPHA	1978	down	2.23E-11	2.43E-08
comp36379_c0_seq1	TUBB	TUBULIN BETA	853	down	1.60E-06	7.43E-04
comp45120_c0_seq1	CALM	CALMODULIN	1210	down	0.000171	0.04298
comp45967_c0_seq1	CALM	CALMODULIN	1150	down	0.000169	0.04249
comp41077_c0_seq1	CALR	CALRETICULIN	1584	down	1.23E-06	0.00059
comp42453_c0_seq1	CALR	CALRETICULIN	1687	down	2.65E-06	0.00116

CONCLUSIONS

In summary, 5 gene fragments annotated Aquaporin PIP, 6 gene fragments annotated Tubulin, 2 gene fragments annotated Calreticulin and Calmodulin have been obtained by the differential gene analysis of two near-isogenic gene pools. All the gene fragments above were down-gene of differential genes. The result suggested that the express level of Aquaporin PIP, Tubulin, Calreticulin and Calmodulin in cracking-resistant fruit were higher than that in sensitive cracking fruit.

It was found that α-linolenic acid metabolism was related to fruit cracking of Chinese jujube according to the GO functional enrichment and KEGG enrichment analysis by building two near-isogenic pools with the same genetic background and different cracking fruit traits. It was concluded that cracking fruit process of Chinese jujube is closely linked to the cellular structure because α-linolenic acid is the basis substance of cell membrane and enzyme (SanGiovanni et al., 2005).The previous researches obtained similar results (Zhou et al., 1999; Xin et al., 2006; Liu et al., 2015).

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