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基于高通量测序发掘番木瓜果实成熟相关miRNA

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摘 要:【目的】番木瓜是典型的呼吸跃变型果实,外源乙烯处理使番木瓜呼吸跃变提前,促进果实成熟。分离番木瓜果实成熟相关miRNA,为深入了解呼吸跃变型果实的成熟分子机制奠定基础。**【方法】**利用高通量测序技术对乙烯(ETH)、1-MCP和清水对照(CG)处理的番木瓜果实进行miRNA和转录组高通量测序,然后对测序获得数据进行生物信息学分析,进行miRNA鉴定和靶基因预测,并与转录组测序结果进行关联分析。**【结果】**乙烯、1-MCP和对照处理分别获得10 734 196、16 486 803和16 067 290条纯净序列,共鉴定出523个miRNA。其中,已知miRNA个数为1-MCP(303)、CG(214)和ETH(239),新miRNA个数为1-MCP(184)、CG(188)和ETH(114)。与对照相比,在乙烯处理中上调和下调表达的miRNA分别是123和72条。靶基因预测共获得5 053个靶基因,KEGG功能富集分析显示它们参与了戊糖、葡萄糖醛酸转换、淀粉和蔗糖代谢、卟啉和叶绿素代谢、类胡萝卜素合成等代谢途径。筛选出的番木瓜果实成熟衰老相关候选miRNA,包含4个果实软化调控相关miRNA(miR167-y、miR4993-x、miR3946-x和miR5059-x)、3个果实颜色调控相关miRNA(miR4993-x、miR815-y和miR7810-x)、3个激素调控相关miRNA(miR4993-x、miR8004-x和miR9722-x)和4个转录因子调控相关miRNA(miR5641-y、miR9722-x、miR838-y和miR319-y)。**【结论】**筛选的番木瓜果实成熟衰老相关miRNA为今后果实成熟衰老调控网络研究提供了可能的线索。

关键词:番木瓜;乙烯;小RNA;高通量测序;果实

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Isolation of ripening-related miRNAs from *Carica papaya* fruit based on high-throughput sequencing

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Abstract:【Objective**】***Carica papaya* is a typical climacteric fruit, and exogenous ethylene (ETH) applications can induce premature and quicker ripening. 1-methylcyclopropene (1-MCP) is one of ethylene perception inhibitors, which is used commercially to slow down the ripening of fruits. In previous studies, a lot of genes were found playing important roles in the process of papaya fruit ripening. However, the regulation mechanism of these genes is not clear. MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate the expression of target messenger RNAs (mRNAs), which are widely involved in the regulation of a variety of biological processes in plant, such as cell development and differentiation, biological and abiotic stress, maturation and senescence, etc. Isolation of fruit ripening-related miRNAs will lay a foundation for further understanding the fruit ripening regulation mechanism. **【**Methods**】**Papaya fruits (*C. papaya* L. 'Daqing No.7') at the green-mature stage were harvested from a local commercial plantation in Zhangzhou, China. Thirty-six fruits were incubated with 1 $\mu L \cdot L^{-1}$ of 1-methylcyclopropene (1-MCP) gas for 18 h in a sealed box; thirty-six fruits were dipped into 0.5 $g \cdot L^{-1}$ of ethephon solution for 3 min, then dried and put in a sealed box for 2 h; thirty-six fruits (Control Group, CG) were dipped into water for 3 min, then dried and put in a sealed box for 2 h. After treatments, all

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fruits were stored at 25 ℃ and allowed to ripen. Fruits were taken randomly at 24 h after treatments. Three fruits were peeled, seeds were removed, and the flesh was cut into some pieces. The pieces of papaya flesh were mixed, frozen in liquid nitrogen, and stored at −80 ℃. The flesh samples of different treatments (ETH, 1-MCP and CG) were sent to Genedenovo Biotechnology Co., Ltd (Guangzhou, China) for miRNA sequencing and RNA-seq using Illumina Hiseq2500. The raw reads were filtered to remove the low quality reads, including the adaptor sequences, the sequences of shorter than 18 nt or longer than 30 nt. Then, the sequences of rRNA, scRNA, snoRNA, snRNA, tRNA, and degradation fragments were also removed. Through miRNA sequences searching, the structure prediction was undertaken to identify the known miRNA and new miRNA. Patmatch was used to conduct the target gene prediction. Then all of the target genes were annotated using the papaya reference genome database, NCBI non-redundant (Nr) database, Gene Ontology (GO) database, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Based on the differential miRNAs expression and the KEGG pathway enrichment of their target genes, fruit ripening-related miRNAs were selected.【Results】A total of 18 421 155(1-MCP), 17 669 301(CG) and 13178734(ETH) raw reads, and 16 486 803(1-MCP), 16 067 290(CG) and 10 734 196(ETH) clean reads were obtained, respectively. Bioinformatics analysis identified 523 conserved miRNAs. Among them, the numbers of known miRNAs were 1-MCP (303), CG (214) and ETH (239), and the numbers of new miRNAs were 1-MCP (184), CG (188) and ETH (114). Compared with the untreated papaya, 123 miRNAs were up-regulated and 72 miRNAs were down-regulated with ETH-treatment; however, 29 miRNAs were up-regulated and 15 miRNAs were down-regulated with 1-MCP-treatment. Target gene prediction showed that a total of 5 053 target genes were predicted. The target genes were annotated using the GO database and genes were classed into three categories: molecular function, cellular component and biological process. The terms of binding and catalytic activity were observed to occur most frequently in the ontology of molecular function; while the terms of cell and cell part were observed to occur most frequently in the ontology of cellular component; and the terms of metabolic process and cellular process were observed to occur most frequently in the ontology of biological process. KEGG analysis revealed that these genes may be involved in pentose and glucuronate interconversions, starch and sucrose metabolism, porphyrin and chlorophyll metabolism, carotenoid biosynthesis pathway, etc. A total of eleven fruit ripening-related miRNAs were selected: four fruit softening regulation-related miRNAs including miR167-y, miR4993-x, miR3946-x and miR5059-x, with their corresponding target genes being UDP-glucuronate 4-epimerase (6*GAE6*), cellulose synthase 6 (*CESA6*), cellulose synthase-like protein G3 isoform X1 (*CSLG3*) and expansin-A8-like (*EXP8*); three fruit coloring regulation-related miRNAs containing miR4993-x, miR815-y and miR7810-x, with their corresponding target genes being magnesium-protoporphyrin IX methyltransferase (*CHLM*), protoporphyrinogen oxidase 2 (*HEMG2*) and lycopene ε-cyclase (*LCYE*); three hormone related miRNAs including miR4993-x, miR8004-x and miR9722-x, with their corresponding target genes being ethylene-responsive transcription factor RAP2-3-like(*EBP*), ERF domain protein 12 (*ERF12*), gibberellin 2-*β*-dioxygenase 8 (*GA2OX8*) and growth-regulating factor 7 (*GRF7*); and four transcription factors regulation-related miRNAs including miR5641-y, miR9722-x, miR838-y and miR319-y, with their corresponding target genes being zinc finger (CCCH-type) family protein (*C3H53*), WRKY DNA-binding protein 23 (*WRKY23*) and MYB domain protein 65 (*MYB*). The expression of selected miRNAs was negatively correlated with their target genes' expression, indicating that these miRNAs may negatively regulate the expression of their target genes.【Conclusion】High throughput sequencing is a sound approach to isolating miRNAs. Based on the correlation analysis between the

miRNA sequencing and RNA-seq of ETH/1-MCP-treated papaya, eleven fruit ripening-related miRNAs were selected. These miRNAs provide a useful resource for further elucidation of the regulatory roles of miRNAs that participate in papaya fruit ripening.

Key words: *Carica papaya* L.; Ethylene; microRNA; High-throughput sequencing; Fruit

番木瓜（*Carica papaya* L.）是热带、亚热带地区重要果树之一，具有较高的营养价值和药用价值，是世界第三大热带水果，仅次于杧果和菠萝[1]。番木瓜是典型的呼吸跃变型果实，后熟过程中快速软化，果皮由绿转黄，果肉从白转红。外源乙烯（ethylene，ETH）处理使番木瓜呼吸跃变提前，促进果实软化和转色；而乙烯受体抑制剂1-甲基环丙烯（1-methylcyclopropene，1-MCP）能不可逆地作用于乙烯受体，从而阻断其与乙烯的正常结合，延迟果实后熟[2-3]。乙烯/1-MCP处理番木瓜果实使其硬度和颜色差异显著，是一系列基因差异表达的结果。已有研究证实，多聚半乳糖醛酸酶（Polygalacturonase，PG）、果胶裂解酶（Pectate lyase，PL）、β-半乳糖苷酶（β-galactosidase，GALB）、果胶甲酯酶（Pectin methylesterase，PME）和木葡聚糖内转糖苷酶/水解酶（Xyloglucan endotransglucosylase /hydrolase protein，XTH）等基因在果实软化过程中起重要作用[4-7]。番木瓜果实的主要呈色色素是类胡萝卜素，类胡萝卜素生物合成途径中的番茄红素合成酶（Phytoene synthase，PSY）、八氢番茄红素脱氢酶（Phytoene desaturase，PDS）、ζ-胡萝卜素脱氢酶（ζ-carotene desaturase，ZDS）、番茄红素β-环化酶（Lycopene β-cyclase，LCYB）、β-胡萝卜素羟化酶（β-carotene hydroxylase，CHYB）等基因相继被克隆和分析，在番木瓜果实颜色形成中起关键作用[8-10]。

果实软化和类胡萝卜素合成相关基因的差异表达，是导致乙烯/1-MCP处理番木瓜果实硬度和颜色差异的主要原因，然而调控这些基因差异表达的机制却不清楚。MicroRNA（miRNA）是一类长约17~25个核苷酸的非编码RNA，能够调控真核生物的基因表达。miRNA广泛参与植物生长发育各个生物过程的调控：细胞发育分化、生物和非生物胁迫与成熟衰老等[11]。随着测序技术的快速发展和测序成本的大大降低，高通量测序应用广泛。这项技术也被成功应用于分离鉴定一些低丰度和差异表达的miRNA[12-13]。笔者通过对乙烯、1-MCP和对照处理番木瓜果肉miRNA和RNA进行高通量测序和分析，筛选番木瓜果实成熟衰老相关miRNA，为进一步深入研究其在果实成熟衰老中的功能奠定基础。

1　材料和方法

1.1　试材与取样

从福建省漳州市采摘表面完好、无损伤、大小均匀一致的‘大庆七号’番木瓜果实作为实验材料。按成熟度将果实分为4个时期：绿色期（Green stage，GS），果皮浓绿色，果肉白色，种子也为白色；绿熟期（Mature-Green stage，MG），果皮淡绿色，果肉淡红色，种子黑色；破色期（Color Break stage，CB），果皮出现黄色，果肉红色；半黄期（Half Yellow stage，HY），约一半果皮为黄色，果肉红色。将绿熟期果实分为3组分别进行以下处理：①乙烯处理（ETH），将番木瓜果实用0.5 g·L^{-1}乙烯利浸泡3 min，然后放到密封的箱子中用生成的乙烯气熏2 h；②1-甲基环丙烯（1-MCP）处理：在密闭的箱子中，用1 μL·L^{-1}的1-MCP处理番木瓜果实18 h；③对照（Control Group，CG）：将番木瓜果实用清水浸泡3 min，然后放到密封的箱子中2 h[3]。将处理后的果实放于25 ℃后熟，在贮藏24 h后取3个果实分别去除果皮和种子，将果肉切成小块混匀，用液氮速冻后于−80 ℃贮存备用。将绿色期、破色期和半黄期，每个时期各5个果实分别去除种子，将果肉和果皮分别取样，获得6种类型的样品：F1，绿色期的果肉（the flesh of GS）；F2，破色期的果肉（the flesh of CB）；F3，半黄期的果肉（the flesh of HY）；P1，绿色期的果皮（the peel of GS）；P2，破色期的果皮（the peel of CB）；P3，半黄期的果皮（the peel of HY）[4]。将样品用液氮速冻后于−80 ℃贮存备用。

1.2　RNA文库构建与测序

将不同处理（ETH、1-MCP和CG）的果肉组织样品送到广州基迪奥生物科技有限公司进行miRNA测序和RNA-seq测序，同时将F1、F2、F3、P1、P2、P3样品也进行RNA-seq测序。用Trizol法提取番木瓜果肉总RNA，然后采用赛默飞世尔科技的NanoDrop检测RNA纯度，安捷伦2100生物分析仪检测RNA完整性。然后采用美国纽英伦生物技术有限公司的小RNA建库试剂盒（NEBNext Multiplex

Small RNA Library Prep Set for Illumina）进行miRNA文库构建，Illumina Hiseq2000进行miRNA测序。RNA-seq文库构建则采用Illumina Dynabeads® mRNA DIRECT™ Kit进行，Hiseq2500 PE125双端测序，具体测序和分析方法见Shen等[3-4]。

1.3 miRNA测序序列的基本分析

对测序所获得的原始序列（raw reads）进行如下分析：首先过滤原始序列中低质量reads，即去除质量值低于20、碱基数超过1个或含有N的reads，剩余的reads即为“high_quality”；接着，去除5’端和3’端接头序列、污染序列，以及小于18 nt和大于30 nt的序列，得到纯净序列（clean reads）。根据得到的tag序列，统计长度在16~27 bp的tag序列长度的分布情况；然后，选取Genebank数据库和Rfam（11.0）数据库分别注释tag序列，尽可能的发现并去除样本中的rRNA、scRNA、snoRNA、snRNA与tRNA。最后，通过比对番木瓜基因组，去除重复序列和来自mRNA降解片段的tag序列。

1.4 miRNA的鉴定与差异表达分析

通过比对获得的mirbase中已收录的番木瓜miRNA为已存在miRNA；同mirbase中已知动植物miRNA进行比对，鉴定到的为已知miRNA。结合参考序列进行发卡结构预测，鉴定到的为新miRNA。将分析得到的已存在miRNA、已知miRNA、新miRNA进行合并得到全部miRNA，结合miRNA在各样本中的表达情况，得到全部miRNA的表达谱。将表达量变化2倍以上，并且$p < 0.05$的miRNA，定义为差异表达miRNA。

1.5 miRNA靶基因的预测和功能富集分析

使用patmatch软件进行靶基因预测，然后对靶基因进行番木瓜参考基因组和NCBI Nr（non-redundant）数据库注释，以及GO（Gene Ontology）和KEGG（Kyoto Encyclopedia of Genes and Genomes）分析。

1.6 番木瓜果实成熟衰老相关miRNA的筛选

将不同处理（ETH、1-MCP和CG）番木瓜果实的小RNA和RNA-seq测序结果进行关联分析，通过分析每个miRNA在乙烯/1-MCP处理中的TPM值（Transcripts per Million），比较miRNA的表达差异，并参考其靶基因的KEGG代谢通路分析，以及靶基因在RNA-seq测序中的表达差异，筛选番木瓜果实成熟衰老相关miRNA。

2 结果与分析

2.1 番木瓜果实miRNA测序序列的分析

基于Illumina高通量测序，分别在番木瓜1-MCP、CG和ETH处理番木瓜果实中各获得18 421 155、17 669 301和13 178 734条原始序列（raw reads）。通过去除5’端和3’端接头、污染序列及低质量序列，最后分别保留16 486 803、16 067 290和10 734 196条纯净序列（clean reads，表1）。统计了长度在16~27 bp的tag序列长度的分布情况，在1-MCP与CG处理中以21 nt长度的序列比例最高，而在ETH处理中19 nt长度的序列比例也比较高（图1）。

2.2 番木瓜果实miRNA的鉴定与差异表达分析

把3个处理（1-MCP、CG和ETH）的纯净序列分别与Rfam数据库和番木瓜基因组进行比对，3 248 366、3 344 179、1 171 377条序列匹配上Rfam数据库，1 491 884、1 584 735、488 923条序列比对上番木瓜基因组。在乙烯处理中，snRNA、tRNA、rRNA、snoRNA和总tags的数量都远低于其他两个处理，而1-MCP处理和对照差异不大（表2）。这说明外源乙

表1 不同处理番木瓜果实miRNA测序数据的基本分析

Table 1 Statistics of sequencing reads in the fruit of papaya

类型 Category	1-MCP		对照 CG		乙烯 ETH	
	数量 Reads No.	比例 Percentage/%	数量 Reads No.	比例 Percentage/%	数量 Reads No.	比例 Percentage/%
总序列 Total reads	18 421 155	100.00	17 669 301	100.00	13 178 734	100.00
高质量的序列 High quality reads	17 100 760	92.83	16 556 969	93.70	12 367 188	93.84
无3’接头的序列3’ adapter null	37 362	0.22	18 949	0.11	34 112	0.28
无插入片段的序列 Insert null	305 493	1.79	256 246	1.55	383 892	3.10
5’接头污染的序列 5’ dapter contaminants	24 650	0.14	18 754	0.11	29 033	0.23
小于18 nt的序列 Smaller than 18 nt	244 115	1.43	193 260	1.17	1 184 762	9.58
有多聚A的序列 PolyA	2 337	0.01	2 470	0.01	1 193	0.01
纯净序列 Clean reads	16 486 803	96.41	16 067 290	97.04	10 734 196	86.80

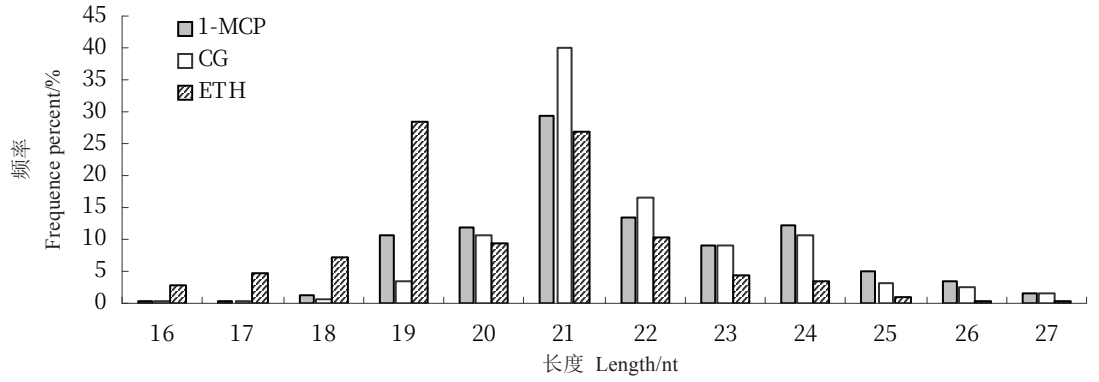


图1　不同长度序列 miRNAs 的读数与数量

Fig. 1　Distribution of small RNAs with different sequence length according to their total reads and unique tags

烯处理番木瓜果实抑制了一些RNA的表达。

表2显示，对总miRNA进行鉴定，共获得了523个miRNA。其中，与mirbase中番木瓜miRNA进行比对，在3个处理（1-MCP、CG、ETH）中分别获得已存在miRNA数量为71、70和68；与已知动植物miRNA比对，鉴定到的已知miRNA个数为303、214和239；通过发卡结构预测，鉴定到的新miRNA个数为184、188和114。

对鉴定到的miRNA进行差异表达分析，将表达量变化2倍以上并且$p < 0.05$的定义为差异表达

表2　番木瓜果实 miRNA 的鉴定

Table 2　Identification of miRNAs in the fruit of papaya fruit

类别 Category	1-MCP		对照 CG		乙烯 ETH	
	数量 No.	比例 Percentage/%	数量 No.	比例 Percentage/%	数量 No.	比例 Percentage/%
比对到Rfam的snRNA Total sRNAs mapping to Rfam	7 035	0.22	7 826	0.23	5 994	0.51
比对到Rfam的tRNA Total tRNAs mapping to Rfam	14 122	0.43	12 465	0.37	9 856	0.84
比对到Rfam的rRNA Total rRNAs mapping to Rfam	87 639	2.70	88 707	2.65	76 945	6.57
比对到Rfam的snoRNA Total snoRNAs mapping to Rfam	2 455	0.08	2 712	0.08	2 185	0.19
比对到Rfam的其他tags Other tags mapping to Rfam	3 137 115	96.58	3 232 469	96.66	1 076 397	91.89
比对到Rfam的总tags Total tags mapping to Rfam	3 248 366	100.00	3 344 179	100.00	1 171 377	100.00
比对到基因组的tags Total tags mapping to genome	1 491 884	45.93	1 584 735	47.39	488 923	41.74
鉴定到的已存在miRNA Exist miRNAs	71	13.58	70	13.38	68	13.00
鉴定到的已知miRNA Known miRNAs	303	57.93	214	40.92	239	45.70
鉴定到的新miRNA New miRNAs	184	35.18	188	35.95	114	21.80

miRNA。由图2可以看出，与对照相比1-MCP处理获得的差异表达miRNA数量较少，上调表达的为29，下调表达的为15；乙烯处理则获得较多的差异表达miRNA，上调表达miRNA数量为123，下调表达的为72。

2.3　miRNA靶基因的功能富集分析

使用patmatch软件进行了miRNA靶基因预测，靶基因数量为5 053。对乙烯处理番木瓜果实的差异miRNA靶基因进行GO注释，将其分为三大类：分子功能（Molecular function）、细胞组分（Cellular component）和生物过程（Biological process）。在分子功能中，靶基因主要集中在结合作用（Binding）和催化活性（Catalytic activity）；在细胞组分中，主要集

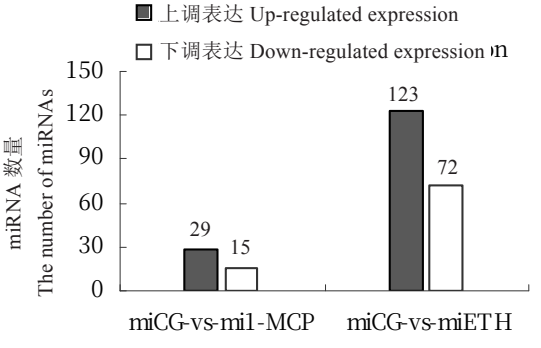


图2　miRNA 差异表达统计图

Fig. 2　Differential expression miRNAs statistics

中在细胞（Cell）、细胞部分（Cell part）等；在细胞过程中，主要集中在代谢过程（Metabolic process）和细

胞过程(Cellular process)(图3)。对这些靶基因进行KEGG分析,富集到的代谢途径有戊糖和葡萄糖醛酸转换(Pentose and glucuronate interconversions)、淀粉和蔗糖代谢(Starch and sucrose metabolism)、卟啉和叶绿素代谢(Porphyrin and chlorophyll metabolism)、类胡萝卜素合成代谢(Carotenoid biosynthesis)等途径。

2.4 番木瓜果实成熟衰老相关miRNA的筛选

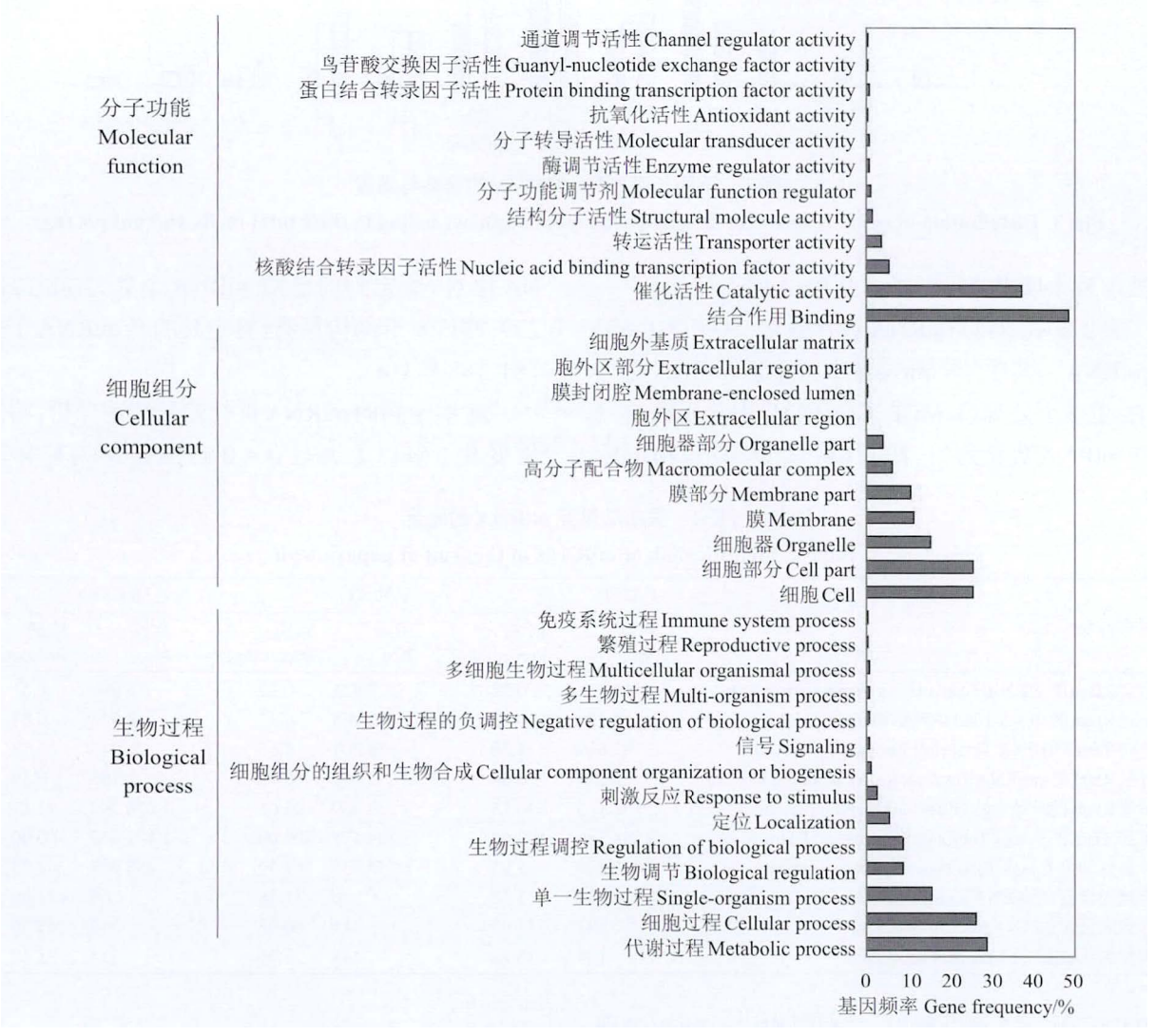


图3 番木瓜不同处理果实中差异表达miRNA靶基因的GO注释(miCG_vs_miETH)

Fig. 3 GO annotation of the predicted target genes of differentially expressed miRNAs in the fruit of papaya (miCG_vs_miETH)

通过分析每个miRNA在乙烯、1-MCP和对照中的TPM值,比较miRNA的表达差异,并将其与靶基因进行关联分析,最终我们获得15个具有相反表达趋势的miRNA-靶基因对,包含4个果实软化相关miRNA、3个果实颜色相关miRNA、3个激素相关miRNA和4个转录因子相关miRNA。从表3可以看出,筛选出的miRNA长度主要集中在18 nt。

筛选的果实软化相关miRNA有miR167-y、miR4993-x、miR3946-x和miR5059-x,其靶基因分别为UDP-葡糖醛酸差向异构酶(UDP-glucuronate 4-epimerase 6,GAE6)、纤维素合成酶6(Cellulose synthase 6,CESA6)、纤维素合酶样G3蛋白同工型X1(Cellulose synthase- like protein G3 isoform X1,CSLG3)和扩展蛋白A8(Expansin-A8-like,EXP8)基因。筛选出3个miRNA可能参与调控叶绿素和类胡萝卜素代谢,它们是miR4993-x、miR815-y和miR7810-x,其靶基因分别为镁原卟啉IX甲基转移酶(Magnesium-protoporphyrin IX methyltransferase,

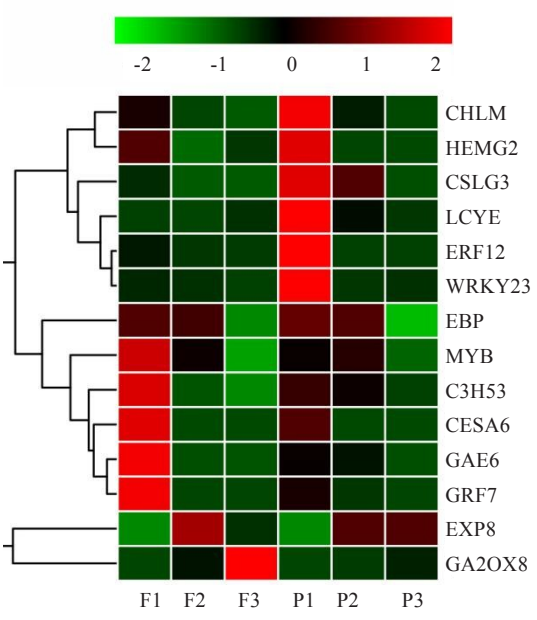
表3　筛选的番木瓜果实成熟相关 miRNA

Table 3　Selected fruit ripening-related miRNAs in *C. papaya*

类别 Category	序号 No.	miRNA ID	长度 Length/nt	序列 Sequence (5'-3')	基因ID Gene ID	基因名称 Symbol	靶基因注释 Description
果实软化调控相关miRNA Fruit softening regulation- related miRNAs	1	miR167-y	18	CTTGCTG - CAGCTTCCTTT	evm.TU.supercontig_157.50	GAE6	UDP-葡糖醛酸差向异构酶 UDP-D-glucuronate 4-epimerase 6 (XM_022033270.1)
	2	miR4993-x	18	GGCGGCGGTG - GAGGCGGA	evm.TU.supercontig_36.82	CESA6	纤维素合成酶 Cellulose synthase 66 (XM_022044953.1)
	3	miR3946-x	18	AGAGAGAGAGA - ACAGAGC	evm.TU.supercontig_15.5	CSLG3	纤维素合酶样蛋白3同工型X1 Cellulose synthase - like protein G3 isoform X1 (XM_022049442.1)
	4	miR5059-x	18	TCCTGGGCAG - CAACACCA	evm.TU.supercontig_2.303	EXP8	扩展蛋白A8 Expansin-A8-like (XM_022054114.1)
果实颜色调控相关miRNA Fruit coloring regulation- related miRNAs	1	miR4993-x	18	GGCGGCGGTG - GAGGCGGA	evm.TU.supercontig_13.164	CHLM	镁原卟啉IX甲基转移酶 Magnesium-protoporphyrin IX methyltransferase (XM_022049998.1)
	2	miR815-y	18	AGAATGGGATT - GAGGAGA	evm.TU.supercontig_130.4	HEMG2	原卟啉原氧化酶2 Protoporphyrinogen oxidase 2 (XM_022034724.1)
	3	miR7810-x	18	GAGAG - GAAGAATTTTCTC	evm.TU.supercontig_28.134	LCYE	番茄红素ε-环化酶 Lycopene ε-cyclase (XM_022046323.1)
激素调控相关miRNA Hormone regulation-related miRNAs	1	miR4993-x	18	GGCGGCGGTG - GAGGCGGA	evm.TU.supercontig_2304.1	EBP	乙烯应答转录因子RAP2-3 Ethylene-responsive transcription factor RAP2-3-like (XM_022032616.1)
	2	miR4993-x	18	GGCGGCGGTG - GAGGCGGA	evm.TU.supercontig_27.18	ERF12	ERF功能域蛋白12 ERF domain protein 12(XM_022046585.1)
	3	miR8004-x	18	GTGTCTGTGTGT - GGCCTT	evm.TU.supercontig_198.6	GA2OX8	赤霉素2-β-双加氧酶8 Gibberellin 2-β-dioxygenase 8 (XM_022031678.1)
	4	miR9722-x	18	GAAGGAAGAA - GATGAAGA	evm.TU.supercontig_3.145	GRF7	生长调节因子7 Growth-regulating factor 7(XM_022053960.1)
其他转录因子调控相关miRNA Other transcription factors regulation-related miRNAs	1	miR9722-x	18	GAAGGAAGAA - GATGAAGA	evm.TU.supercontig_197.24	C3H53	CCCH型锌指蛋白 Zinc finger (CCCH-type) family protein (XM_022031723.1)
	2	miR5641-y	18	GAAGAAGAA - GATGGAATT	evm.TU.supercontig_197.24	C3H53	CCCH型锌指蛋白 Zinc finger (CCCH-type) family protein (XM_022031723.1)
	3	miR838-y	18	TCTTCTCTTCTTC TTCTT	evm.TU.supercontig_18.82	WRKY23	WRKY转录因子 WRKY DNA-binding protein 23 (XM_022048576.1)
	4	miR319-y	20	ATTGGACT - GAAGGGAGCTCC	evm.TU.supercontig_34.210	MYB	MYB转录因子 MYB domain protein 65(XM_022045295.1)

CHLM）、原卟啉原氧化酶2（Protoporphyrinogen oxidase 2，HEMG2）和番茄红素ε-环化酶（Lycopene ε-cyclase，LCYE）基因。筛选出3个miRNA可能参与调控激素水平，miR4993-x、miR8004-x和miR9722-x，其靶基因为乙烯应答转录因子RAP2-3（Ethylene-responsive transcription factor RAP2-3-like，EBP）、ERF功能域蛋白12（ERF domain protein 12，ERF12）、赤霉素2-β-双加氧酶8（Gibberellin 2-β-dioxygenase 8，GA2OX8）和生长调节因子7（Growth-regulating factor 7，GRF7）。另外，我们还筛选到4个miRNA：miR9722-x、miR5641-y、miR838-y和miR319-y，可能参与调控转录因子表达，其靶基因为*C3H53*、*WRKY23*和*MYB*。表3还显示，miR4993-x具有4个靶基因：*CESA6*、*CHLM*、*EBP*和*ERF12*。miR9722-x具有2个靶基因：*GRF7*和*C3H53*。同时，转录因子*C3H53*受到miR5641-y、miR9722-x两个miRNA的调控。

利用番木瓜果肉和果皮的转录组测序结果分析miRNA靶基因的表达趋势，了解靶基因在果实成熟过程中的功能。从图4可以看出，根据靶基因的表达模式聚为三大类，*CHLM*、*HEMG2*、*CSLG3*、*LCYE*、*ERF12*和*WRKY23*聚到一起，它们在果皮中的表达量高于果肉，而且随着果实成熟表达量逐渐降低；*EBP*、*MYB*、*C3H53*、*CESA6*、*GAE6*和*GRF7*也是随着果实成熟表达量逐渐降低，但是它们在果肉中的表达量高于果皮；*EXP8*和*GA2OX8*的表达量则随着果实成熟，表达量逐渐升高，*EXP8*是在破色期果肉中表达量最高，而*GA2OX8*在半黄期果肉中的表达量



根据 FPKM 值绘制靶基因热图，图中的列和行分别代表样本和基因。颜色标度表示基因表达量变化。

The heat map was drawn according to FPKM values. Columns and rows represent samples and genes respectively. Color scale indicates fold changes of gene expression.

图 4 miRNA 的靶基因在不同成熟期果肉和果皮中表达差异

Fig. 4 Heat map diagram of expression levels for targeted genes in the flesh and peel of different ripening papaya fruits

最高。

利用不同处理（ETH、1-MCP 和 CG）番木瓜果实 miRNA 和 RNA-seq 测序获得的 TPM 值和 FPKM 值分别做表达趋势图，从图 5 可以看出，筛选出的 miRNA 与其靶基因表达模式具有互补关系，呈负相关，表明这些 miRNA 通过负向调控它们的靶标，实现对番木瓜果实成熟的调控作用。

3 讨 论

果实软化和颜色转变是番木瓜果实成熟过程中最重要的两个特征。外源乙烯处理大大促进果实成熟衰老进程，在处理后 24 h 果实硬度迅速下降[3]。在果实成熟过程中，检测到多聚半乳糖醛酸酶（PG）、果胶裂解酶（PL）、半乳糖苷酶（GAL）和木聚糖酶（XTH）的表达，伴随着果胶、纤维素和半纤维素解聚增加[14-16]。经 RNA-Seq 分析发现，乙烯处理番木瓜果实诱导 *PG1*、*XTH30*、*PL* 和 *EXP* 等基因表达量升高，从而加速了果胶、纤维素和半纤维素的降解，促进了果实软化[3]。作者通过分析乙烯、1-MCP 和清水对照处理番木瓜果实的 miRNA 差异表达，筛选了 4 个果实软化相关 miRNA，他们分别是 miR167-y、miR4993-x、miR3946-x 和 miR5059-x，其靶基因分别是 *GAE6*、*CESA6*、*CSLG3* 和 *EXP8*。GAE6 是果胶合成关键酶[17]，CESA6 和 CSLG3 是纤维素合成酶，这 3 个酶基因的表达量在绿色期果肉中最高，随着果实成熟逐渐降低，果胶和纤维素合成量减少，与果实软化进程一致。扩展蛋白 A 参与调节细胞壁伸展和结构松弛，研究表明它在果实成熟过程中发挥着重要作用[18-19]。*EXP8* 在果实成熟过程中表达量先升高后降低，但是在乙烯和 1-MCP 中表达量都降低，这个基因在果实成熟中的功能还需进一步研究确定。

色泽是果实品质的一个重要组成部分，它既是果实成熟程度的标志，又与果实营养成分变化相关。类胡萝卜素是番木瓜果实的主要呈色色素，其含量和组成对果实外观和营养价值具有重要作用。番木瓜按果肉颜色分为红肉和黄肉两种。成熟的黄肉番木瓜主要含有 *β*-隐黄质和 *β*-胡萝卜素，红肉番木瓜则含有大量番茄红素，果肉为橙红色[20-21]。番木瓜果实成熟过程中，叶绿素降解导致叶黄素、*β*-胡萝卜素等物质显现是果皮从绿色向黄色转变的原因。因此，叶绿素与类胡萝卜素代谢是番木瓜果实颜色相关的两个重要代谢途径。本文中我们筛选出 2 个 miRNA，分别是 miR4993-x 和 miR815-y，其靶基因是叶绿素合成途径关键酶基因 *CHLM* 和 *HEMG2*，在果皮中的表达量高于果肉，乙烯处理抑制其表达，且随着果实成熟表达量显著下降，与果实成熟褪绿一致。筛选出 1 个 miRNA（miR7810-x）可能参与类胡萝卜素代谢，其靶基因为 *LCYE*。番茄红素环化酶是类胡萝卜素合成途径的关键酶，在番木瓜果实转录组中检测到 4 个番茄红素环化酶基因——*CYCB*、*LCYB1*、*LCYB2* 和 *LCYE*[4,22]。LCYE 是叶绿体特异性番茄红素环化酶，催化番茄红素生成叶黄素，在绿色组织果皮中表达量高，且随着果实成熟表达量降低[4]。*CHLM*、*HEMG2* 和 *LCYE* 是番木瓜果实从绿色向黄色转变的关键酶基因，本文获得了调控它们表达的 miRNA，为今后深入研究这些基因的表达调控机制奠定了基础。

乙烯是一种植物催熟激素，目前已经被广泛应用于采后果实的催熟。尤其是对于呼吸跃变型果实番木瓜，少量外源乙烯处理促进果实快速软化变黄，

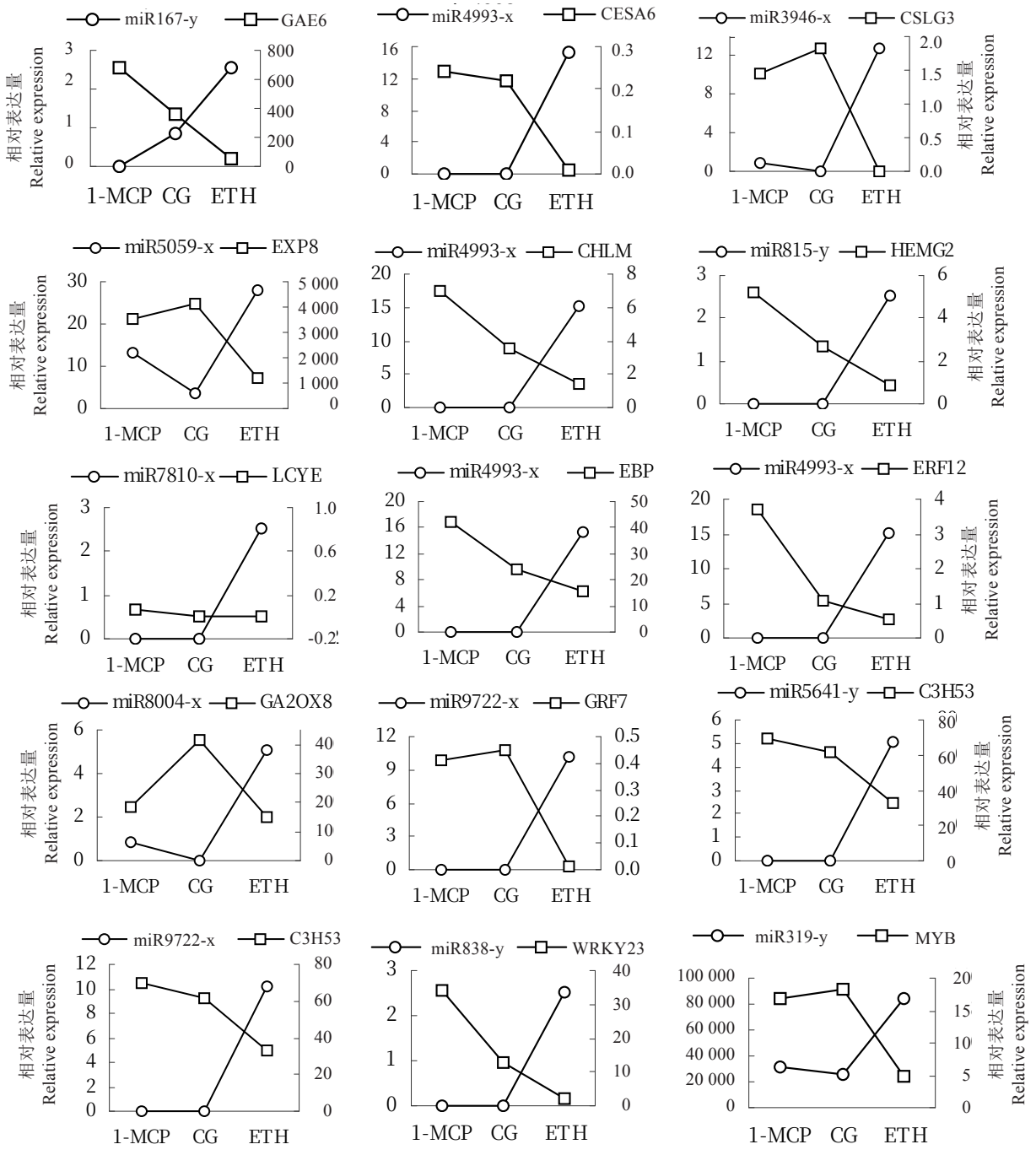


图 5　miRNA 及其靶基因在不同处理番木瓜果实中的表达分析

Fig. 5　Expression analysis of miRNAs and their target genes in different treated papaya fruits

大大加快成熟进程。miR4993-x 的靶基因是乙烯响应转录因子 *EBP* 和 *ERF12*，在乙烯信号转导中起重要调控作用。有研究报道，脱落酸（abscisic acid，ABA）通过促进乙烯合成来达到对跃变型果实成熟的调控[23]。在本文中，miR9722-x 的靶基因为 *GRF7*，*GRF7* 是脱落酸相关基因的转录抑制因子[24]。本文也筛选到一个参与调控赤霉素代谢的 miR8004-x，其靶基因是 *GA2OX8*，它可将 GA 转变为无生物活性的分解代谢物，使植物体内 GA 的活性降低[25]，*GA2OX8* 在果实衰老期明显高于绿色期。作者还筛选了 4 个 miRNA 参与调控 *C3H53*、*WRKY23* 和 *MYB* 的表达。3 个转录因子都随着果实成熟表达量逐渐降低，但是 *WRKY23* 在果皮中的表达量大大高于果肉，而 *C3H53* 和 *MYB* 在果肉中的表达量高于果皮。这 3 个转录因子的表达趋势与番木瓜果实成熟具有相关性，可能参与了果实的成熟调

控，而这些转录因子及其对应miRNA在果实成熟中的具体功能有待于进一步研究。

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