苹果苦痘病对果实类黄酮总含量的影响及其分子生物学机制

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摘要 类黄酮在多种植物生物学功能和人类慢性病的治疗上发挥了重要的作用. 苹果苦痘病是苹果生产上的一种重要生理性病害, 严重影响了果品的品质, 造成了巨大经济损失. 研究苹果苦痘病对苹果果实类黄酮的影响及其分子生物学机制, 可为医用类黄酮的获得挖掘新的资源. 采用氯化铝比色法测定了健康果实和苦痘病果的类黄酮总含量, 结果显示, 苦痘病果的类黄酮总含量为健康果实的4.28倍. 通过转录组测序和qRT-PCR对类黄酮总含量变化的分子生物学机制进行了研究. 经测序, 从苹果健康果实和苦痘病果2组样品中, 分别获得226.702和202.951 M Clean reads, 包含32.807和29.626 Gb测序数据, 其碱基百分比(Q30)大于95.0%. 以健康果实为参考, 苦痘病果共获得2632个差异表达基因, 其中上调基因2080个, 下调基因552个. 经GO基因功能注释和KEGG代谢通路富集分析, 获得与类黄酮合成相关的差异表达基因26个(24个上调基因和2个下调基因), 选取其中8个差异表达基因: *CYP98A3* (1), *CYP98A3* (1), *BADH*, *DAT*, *MdHCT* (1), *MdHCT* (2), *CHI* (1)和*CHI* (2)进行qRT-PCR分析验证, 结果显示, 该8个基因均表现为上调表达, 上调倍数为1.05~7.17倍. 研究表明, 苹果苦痘病的发生刺激了苹果果实中类黄酮的大量表达和积累, 可为医学研究中类黄酮的获得, 提供更广泛的资源.

关键词 全转录组测序分析, 苹果苦痘病, qRT-PCR分析, 类黄酮生物合成与积累, 类黄酮总含量

类黄酮是一类广泛存在于植物界, 尤其是彩色水果和花卉中的重要多酚类次生代谢产物^[1,2]. 类黄酮在植物生长发育、色素沉淀、植物抗毒素的合成等多种植物生物学功能上发挥了重要的作用, 能够保护植物免受紫外线、植物病原等生物与非生物因素的危害^[3,4]. 类黄酮因具有抗氧化、消炎、抗真菌、抗病毒及抗肿瘤等作用, 在医学上常用于心脑血管病、老化病、糖尿病、肝脏疾病、白血病、癌症等慢性病的治疗^[1,5-11]. 苹果果实因含有较高比例、人体容易吸收利用的类黄酮等游离多酚, 具有较高的营养保健价值, 在

预防心脑血管病、抗肿瘤及抗氧化等人类慢性疾病方面有较好的作用^[12,13].

苹果产业已成为世界最大水果产业, 中国是世界苹果生产大国和消费大国^[14,15], 而苹果苦痘病在整个发育期直至采后均可发生, 常造成严重的经济损失, 迄今未找到有效的解决办法^[16-18]. 研究苹果苦痘病对果实中类黄酮含量的影响及其分子生物学机制, 可为医学研究中类黄酮物质的获得, 挖掘和开发更多的资源来源. 苹果果实中类黄酮的含量受品种、株系、授粉组合、温度、光、压力以及木质素等生物或非生物因

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素的影响^[13,19-25]。苹果苦痘病对果实中类黄酮含量的影响及其机制研究尚未见报道。本研究采用氯化铝比色法测定了健康果实和苦痘病果的类黄酮总含量,通过基于高通量测序技术的全转录组测序分析^[26,27],从整体RNA水平研究苹果健康果实和苦痘病果的差异,获得与类黄酮生物合成相关的差异表达基因,并通过qRT-PCR分析验证其差异表达,以期为苹果苦痘病果的回收利用提供新途径,为医学研究中类黄酮的获得提供更广泛的资源来源。

1 材料与方法

1.1 试材

商业成熟期的“烟富2号”苹果(*Malus domestica* Borkh. cv. Yanfu2)采自山东蒙阴苹果园,从同一棵苹果树内膛选取成熟期和大小一致的苹果健康果实(JKG)和五级苦痘病果(BG)^[28]。

1.2 类黄酮总含量测定

从健康果实和苦痘病果中分别取5.0 g薄片样品,采用氯化铝比色法^[29]测定苹果果实中的类黄酮总含量,以mg g⁻¹ FW(鲜重)表示。

1.3 转录组测序分析

(i) 样品处理。从健康果实和苦痘病果中分别取20 g薄片作为样品,快速取样后用锡纸包好并迅速放入液氮中冷冻保存备用,每个处理重复3次。

(ii) RNA提取和质量检测。采用TRNzol总RNA提取试剂盒(北京天根生物技术有限公司)提取样品总RNA,通过分光光度计NanoDrop® ND-1000 (Thermo Scientific, DE, USA)检测RNA浓度和纯度,OD吸收峰比值合格标准为 A_{260}/A_{280} , $A_{260}/A_{280} > 1.8$, $A_{260}/A_{230} > 1.6$;通过安捷伦生物分析仪Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA)和RNA 6000 Nano LabChip Kit (Agilent, CA, USA)检测RNA总量和完整性,合格标准为RIN分值>7.0。

(iii) RNA文库制备、测序与数据分析。RNA文库制备,采用去核糖体试剂盒Ribo-Zero® rRNA Removal Kit (Plant) (Illumina, San Diego, CA, USA)和NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs)试剂盒构建全转录组文库,包括mRNA (messenger RNAs)、lncRNA (long non-coding RNAs)和

CircRNA (circular RNAs)文库,严格按照试剂盒的标准步骤执行。

构建好的文库用测序仪Illumina Hiseq™ 2500 system进行高通量测序,测序读长为双端2×100/150 bp。通过FastQC进行数据质量评估,获得Q30数据后,去除接头序列、含有模糊碱基N的序列、长度小于60 bp的序列,利用TopHat 2.0程序与苹果参考基因组(<https://www.rosaceae.org/>)进行序列比对。

mRNA与lncRNA的分析选择Ensembl 数据库的参考基因组作为注释文件对转录本与基因进行注释。利用CPC 程序^[30]对转录本编码潜能进行预测,得到CircRNA。

通过软件edgeR software (<http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>),采用FPKM (Fragments Per Kilobase of exon model per Million mapped reads)值来度量基因表达的丰度值,消除基因长度和测序量差异对计算基因表达的影响^[31],计算得到的基因表达量可直接用于比较不同样品间的基因表达差异。筛选差异表达基因的标准是 $|\log_2(\text{fold-change})| \geq 1$, $P \leq 0.05$ 和 $Q \leq 0.05$ 。

(iv) miRNA文库制备、测序与数据分析。miRNA (microRNAs)文库制备采用NEBNext® Small RNA Library Prep Set for Illumina (New England Biolabs)试剂盒,严格按照试剂盒的标准步骤执行。采用安捷伦2100 Bioanalyzer系统和qPCR对文库进行质检和定量。

构建好的文库用测序仪Illumina Hiseq™ 2500 system进行高通量测序,测序读长为单端1×150 bp。通过FastQC进行数据质量评估,获得Q30数据后,去除接头序列、含有模糊碱基N的序列、长度范围在15~34 nt之外的reads,通过BLAST与Rfam数据库进行序列比对和注释分类,对于未鉴定为已知miRNA的序列,先用bowtie2将其定位到参考基因组上,再用MIREAP程序(<http://sourceforge.net/projects/mireap/>)根据定位结果进行novel miRNA预测。对各miRNA在各样本中的reads数量进行统计,用RPM (reads per million)进行表达量归一化处理。

对于单重复、reads数较多的(两样本都大于20) miRNA采用卡方检验,较少的采用Fisher精确检验;对于多重复,在不事先假设其分布的情况下采用置换检验。差异miRNA 筛选标准: $|\log_2(\text{fold-change})| \geq 1$, P 值 ≤ 0.05 。采用软件TargetFinder,对差异miRNA 靶基因进行预测。

(v) 差异表达基因的GO分类和KEGG富集分析。

利用软件Goseq R package和KOBAS software^[32,33]对差异表达基因进行GO (gene ontology)分类和基因功能注释, 及KEGG (Kyoto encyclopedia of genes and genomes)通路富集分析, 检验 $P \leq 0.05$ ^[34].

1.4 差异表达基因qRT-PCR验证

选取转录组测序中差异倍数大于1.0的8个差异表达基因进行qRT-PCR验证, 根据测序获得的序列, 采用软件Primer Premier 6.0 (PREMIER Biosoft International, Palo Alto, CA, USA) 设计引物, 以苹果*Actin* 为内参, 每个基因扩增均伴有内参基因同时扩增, 基因及其引物序列见表1.

表 1 用于qRT-PCR验证的8个基因及其引物序列

Table 1 The qRT-PCR primers and their sequences for the 8 selected genes

基因ID	基因名称	预测功能	引物序列(5'→3')
MD08G1242900	<i>CYP98A2</i> (1)	细胞色素p450 (LOC103441965)	F: AACCACTGCACCAAACCTGA R: AGCGATCCGCCTATCTTCAC
MD15G1436500	<i>CYP98A2</i> (2)	细胞色素p450 (LOC103441965)	F: CGGTATCAACTTGGTGGCCT R: CGTCCCTGGATTTTCGGACA
MD05G1219000	<i>BAHD</i>	BAHD酰基转移酶BAHD	F: GGCGGATGTTTCACACTCA R: CAGCCCCAAATGCTACGAGA
MD16G1110600	<i>DAT</i>	去乙酰乙烯醇4-O-乙酰转移酶	F: CAGTGAACCTGCGCACTAGA R: GCAACTCAATCACGCTGTCC
MD17G1224900	<i>MdHCT</i> (1)	羟肉桂酰辅酶A羟肉桂酰转移酶	F: AATAAGACCGCACAGACGGA R: GTCGAAGAAGGAGTTGCCCT
MD17G1225100	<i>MdHCT</i> (2)	羟肉桂酰辅酶A羟肉桂酰转移酶	F: GGTGATTCCGAGCATCCACA R: GCCGCAGTCAATCTCGATAC
MD01G1118000	<i>CHI</i> (1)	查耳酮-二氢黄酮异构酶	F: CAGAGAAGCGGGTCTCTCCC R: GCGTTGGAGCCATTTGACAAT
MD01G1118100	<i>CHI</i> (2)	查耳酮-二氢黄酮异构酶	F: ATTTCCCACCCGGTGCTT R: CCGGCTTCAGCACCATTAGA
	<i>MdActin</i>	内参基因	F: TGACCGAATGAGCAAGGAAATTACT R: CTCAGCTTTGGCAATCCACATC

用试剂盒FastQuant RT Kit (with gDNAase)(北京天根生物技术有限公司)对1.2中提取的RNA进行反转录; 用2×RealStar Green Mixture (GenStar Technologies Company Inc., Chino, CA, USA)进行qRT-PCR分析, 以 $2^{-\Delta\Delta C_t}$ 法计算基因的差异表达倍数^[35]. 每个样品3个重复.

2 结果

2.1 类黄酮总含量的测定

采用氯化铝比色法对苹果健康果实和苦痘病果(图1)中的类黄酮总含量进行测定, 结果显示, 苦痘病果中

图 1 健康果实(a)和五级苦痘病果(b)
Figure 1 The healthy apple fruit (a) and bitter-pit apple fruit (b)

的类黄酮总含量明显高于健康果实(图2), 其含量差异倍数为4.28倍。

2.2 测序数据及质量分析

经RNA文库构建和测序, 从健康果实和苦痘病果2组样品中分别获得257.002和225.713 M raw reads, 包含38.55和33.857 Gb raw bases, 经净化和质量过滤, 获得226.702和202.951 M Clean reads, 包含32.807和29.626 Gb Clean bases, 其碱基百分比Q20和Q30分别大于98.3%和95.0%(表2); 同时, 样品间测序数据聚类结果良好(图3), 说明RNA-Seq测序数据质量较高, 可用于后续分析。测序数据与参考基因组的mapping率为63.32%~72.27%(Total Mapped为63.32%~69.91%, Uniquely Mapped为65.65%~72.27%)(表3), 均低于70%, 但无显著差异, 说明本研究所用试材“烟富2号”与数据库中选用的参考基因组*Malus domestica* Borkh的亲缘关系较远。miRNA文库构建与测序结果显示, 总共获得4331 Clean reads, 与3753个靶标基因相关联, 这些靶标基因中, 2571个基因上调, 1181个基因下调。序列分析和注释结果显示, 总共获得41894个RNA, 已知RNA和新RNA分别为15852个和26042个(表4)。

2.3 序列长度分布及差异表达基因筛选

通过对苹果果实转录组FPKM值的测定, 发现健康果实和苦痘病果的转录本和基因数量差异不显著(表5)。健康果实转录组FPKM值<5.0的转录本为17086, 占48.17%, >50.0的转录本为3874, 占10.92%; 苦痘病果

图2 苹果样品中的类黄酮总含量

Figure 2 The total flavonoid content from different apple fruit samples

转录组FPKM值<5.0的转录本为16396, 占45.80%, >50.0的转录本为4044, 占11.30%。相对应地, 健康果实转录组FPKM值<5.0的基因为9382, 占35.64%, >50.0的基因为3963, 占15.06%; 苦痘病果转录组FPKM值<5.0的基因为8670, 占32.62%, >50.0的基因为4099, 占15.42%。

将差异倍数(fold change)≥1, 显著水平 $P \leq 0.05$ 筛选为显著差异的表达基因。从JKG vs. BG 样品中获得35920个转录本, 其中, 差异表达转录本(differentially expressed transcripts, DETs)为2577个, 上调转录本和下调转录本分别为2085和492个; 26600个基因中, 共获得2632个差异表达基因(differentially expressed genes, DEGs), 其中, 上调基因2080个, 下调基因552个。

表2 全转录组测序数据质量控制

Table 2 The quality control of whole-transcriptome RNA-Seq stats

样本	Raw reads (M)	Raw bases (G)	Raw Q20 (G)	Raw Q30 (G)	Clean reads (M)	Clean bases (G)	Clean Q20 (G)	Clean Q30 (G)	平均长度 (bp)
JKG_1	93.383	14.007	13.308 (95.0%)	12.528 (89.4%)	82.488 (88.3%)	11.988 (85.6%)	11.787 (98.3%)	11.388 (95.0%)	145.3
JKG_2	75.388	11.308	10.721 (94.8%)	10.095 (89.3%)	66.332 (88.0%)	9.559 (84.5%)	9.412 (98.5%)	9.108 (95.3%)	144.1
JKG_3	88.231	13.235	12.573 (95.0%)	11.856 (89.6%)	77.882 (88.3%)	11.260 (85.1%)	11.084 (98.4%)	10.730 (95.3%)	144.6
BG_1	81.606	12.241	11.703 (95.6%)	11.063 (90.4%)	73.073 (89.5%)	10.652 (87.0%)	10.481 (98.4%)	10.140 (95.2%)	145.8
BG_2	63.588	9.538	9.133 (95.8%)	8.649 (90.7%)	57.173 (89.9%)	8.378 (87.8%)	8.250 (98.5%)	7.991 (95.4%)	146.5
BG_3	80.519	12.078	11.575 (95.8%)	10.966 (90.8%)	72.705 (90.3%)	10.596 (87.7%)	10.433 (98.5%)	10.103 (95.3%)	145.7

图3 RNA-seq测序数据聚类结果
Figure 3 Clustering result of RNA-Seq stats

2.4 差异表达基因GO富集分析

利用软件Goseq R package和KOBAS software对差异表达基因进行GO分类和功能注释。结果显示, 4643

个基因(3922个上调基因和721个下调基因), 共分为3大类(生物过程、细胞组分和分子功能), 34个小类(图4)。其中, 2632个基因分类到生物过程的14个类别, 946个基因分类到细胞组分的12个类别, 1065个基因分类到

表 3 测序数据与参考基因组比对情况^{a)}

Table 3 Mapping of sequencing data with reference genome^{a)}

样本	Total reads (M)	Total mapped (M)	Multiple mapped (M)	Uniquely mapped (M)
JKG_1	82.488	57.352 (69.53%)	2.058 (2.50%)	55.293 (67.03%)
JKG_2	66.332	44.818 (67.57%)	1.757 (2.65%)	43.061 (64.92%)
JKG_3	77.882	52.132 (66.94%)	1.908 (2.45%)	50.225 (64.49%)
BG_1	73.073	47.973 (65.65%)	1.704 (2.33%)	46.269 (63.32%)
BG_2	57.173	41.321 (72.27%)	1.354 (2.37%)	39.967 (69.91%)
BG_3	72.705	49.360 (67.89%)	1.802 (2.48%)	47.557 (65.41%)

a) Total reads: 用于比对的总reads数; Total mapped: 各样本中, 能比对上的reads数及比例; Multiple mapped: 比对上的reads中, 存在多个比对位置的reads; Uniquely mapped: 比对上的reads中, 只能比对到唯一一个位置的reads

表 4 RNA-seq测序数据中转录本类型

Table 4 Transcript types of RNA-seq data

分类	mRNA(条)	lncRNA(条)	circRNA(条)	miRNA(条)	其他(条)	未知(条)	总计
已知	12645	0	52	3017	0	138	15852
新发现	20937	1860	46	2877	322	0	26042
总计	33582	1860	98	5894	322	138	41894

表 5 测序数据中转录本和基因序列长度分布

Table 5 Sequence-length distribution of transcripts and genes assembled from illumina reads

FPKM值(%)	0~0.5	0.5~1.0	1.0~5.0	5.0~10.0	10.0~50.0	>50.0	总计
转录本	JKG 3402(9.59%)	2866(8.08%)	10818(30.50%)	5356(15.10%)	9156(25.81%)	3874(10.92%)	35472
	BG 2354(6.58%)	2801(7.82%)	11241(31.40%)	5664(15.82%)	9694(27.08%)	4044(11.30%)	35798
基因	JKG 1580(6.00%)	1348(5.12%)	6454(24.52%)	4282(16.27%)	8694(33.03%)	3963(15.06%)	26321
	BG 816(3.07%)	1208(4.55%)	6646(25.01%)	4554(17.14%)	9254(34.82%)	4099(15.42%)	26577

分子功能的8个类别. 在生物过程分类中, 有763个基因涉及代谢过程(metabolic process), 666个为上调基因, 97个为下调基因.

2.5 差异表达基因KEGG通路富集分析

KEGG通路富集分析结果显示, 共有646个差异表达基因被注释到60个通路中, 其中, 26个差异表达基因(24个上调基因和2个下调基因)与苹果果实的类黄酮生物合成有关(图5). 这26个差异表达基因均为mRNA, 未发现lncRNAs, circRNAs和miRNA中与类黄酮生物合成有关的差异表达基因. 类黄酮生物合成KEGG图谱(图6)显示, 差异表达的基因总体表现上调(红色), 说明KEGG通路富集分析获得的2个下调基因, 对类黄酮总

体表达量的影响极小, 因而未在KEGG图谱中体现下调趋势.

2.6 差异表达基因的qRT-PCR验证

通过qRT-PCR分析, 对涉及苹果果实类黄酮生物合成的8个差异表达基因进行荧光定量分析, 发现与健康果实相比, 苹果苦痘病果各基因的表达均表现上调趋势(表6), 仅在差异倍数(1.05~7.17)上, 与转录组数据中FPKM值差异倍数(1.43~29.42)略有不同, 除基因*DAT*的差异倍数高于转录组数据, 其他7个基因的差异倍数均低于转录组数据. 8个基因的qRT-PCR分析结果与转录组测序结果相一致, 二者呈正相关, 相关系数 $R^2=0.3994$ (图7(a)), 去除基因*DAT*之后, 相关系数

图4 苹果健康果实和苦痘病果差异表达基因GO富集分析. B1, 生物调节; B2, 组织或生物起源细胞组件; B3, 细胞过程; B4, 发育过程; B5, 生长; B6, 定位; B7, 代谢过程; B8, 多生物体过程; B9, 多细胞生物过程; B10, 繁殖; B11, 繁殖过程; B12, 刺激反应; B13, 信号; B14, 信号组织过程; C1, 细胞; C2, 细胞部分; C3, 胞外区; C4, 细胞外区域部分; C5, 高分子复合物; C6, 膜; C7, 膜部分; C8, 膜包围腔; C9, 细胞器; C10, 细胞器部分; C11, 突触; C12, 突触部分; M1, 抗氧化活性; M2, 结合; M3, 催化活性; M4, 电子载体活性; M5, 分子功能调节因子; M6, 核酸结合转录因子活性; M7, 蛋白结合转录因子活性; M8, 运输活性

Figure 4 The GO enrichment analysis of DEGs from JKG vs. BG. B1, Biological regulation; B2, cellular component organization or biogenesis; B3, cellular process; B4, developmental process; B5, growth; B6, localization; B7, metabolic process; B8, multi-organism process; B9, multicellular organismal process; B10, reproduction; B11, reproductive process; B12, response to stimulus; B13, signaling; B14, single-organism process; C1, cell; C2, cell part; C3, extracellular region; C4, extracellular region part; C5, macromolecular complex; C6, membrane; C7, membrane part; C8, membrane-enclosed lumen; C9, organelle; C10, organelle part; C11, synapse; C12, synapse part; M1, antioxidant activity; M2, binding; M3, catalytic activity; M4, electron carrier activity; M5, molecular function regulator; M6, nucleic acid binding transcription factor activity; M7, transcription factor activity, protein binding; M8, transporter activity

$R^2=0.8551$ (图7(b)).

3 讨论

类黄酮广泛分布于植物中, 易被人体吸收利用, 常用于人类多种慢性疾病的治疗^[7,10]. 已鉴定的类黄酮物质超过10000个, 分属于黄酮、黄烷、黄烷酮、黄酮醇、二氢黄酮醇、花青素、橙酮、生物类黄酮、查尔酮、二氢查尔酮、异黄酮等亚组^[36]. 苹果果实富含类黄酮等多酚类物质, 具有较高的营养保健价值, 苹果果实中类黄酮的生物合成和积累受温度、光照、品种、栽培措施等生物和非生物因素的影响^[12,13,19~25].

Abid等人^[20]发现, 高压(450 MPa/25°C/10 min)处理显著增加了苹果果汁中的类黄酮总含量. 在苹果成熟期和采收期, 适度的低温和良好的光照能增加苹果果皮中类黄酮的总含量^[19,23]. Mahunu等人^[4]研究发现, 不同品种的苹果, 其类黄酮总含量差异较大, 新疆红肉苹

果3个品系的果皮和果肉中类黄酮总含量分别为对照富士系苹果“长富2号”的1.23~1.61和1.43~3.49倍. Shafiq和Singh^[24]发现, 在果实商业成熟期前3~4周喷施100 mg/L的L-苯丙氨酸, 能够在不影响果实品质的情况下增加苹果果皮中类黄酮的积累. 张曼曼等^[25]研究发现, 在果实发育的整个生长期, 不同授粉品种处理的“富士”和“新红星”苹果中类黄酮总含量均高于对照, 其含量与对照相比提高了4.24%~19.63%, 不同品种间存在显著差异.

本研究采用氯化铝显色法测定了苹果健康果实和苦痘病果中的类黄酮总含量, 结果显示, 苦痘病果中的类黄酮总含量显著高于健康果实, 其含量为健康果实中的4.28倍, 说明苦痘病的发生, 显著提高了苹果果实中的类黄酮总含量.

类黄酮含量的变化, 与类黄酮生物合成通路中各相关基因的表达有关. 许海峰等人^[21]研究了新疆红肉

Figure 5 The KEGG-pathway enrichment analysis of DEGs from JKG vs. BG. C. Cellular process; C1, cell growth and death; C1-1, apoptosis; C2, cellular community; C2-1, gap junction; C3, transport and C3-1, peroxisome; C3-2, phagosome; C3-3, regulation of autophagy; E, environmental information processing; E1, signal transduction; E1-1, calcium signaling pathway; G, genetic information processing; G1, folding, sorting and degradation; G1-1, ubiquitin mediated proteolysis; M, metabolism; M1, amino acid metabolism; M1-1, cysteine and methionine metabolism; M1-2, valine, leucine and isoleucine degradation; M1-3, glycine, serine and threonine metabolism; M1-4, phenylalanine metabolism; M1-5, phenylalanine, tyrosine and tryptophan biosynthesis; M1-6, lysine degradation; M1-7, valine, leucine and isoleucine biosynthesis; M1-8, tyrosine metabolism; M1-9, arginine and proline metabolism; M1-10, glutathione metabolism; M1-11, selenocompound metabolism; M1-12, taurine and hypotaurine metabolism; M1-13, beta-Alanine metabolism; M2, Biosynthesis of other secondary metabolites; M2-1, Flavonoid biosynthesis; M2-2, phenylpropanoid biosynthesis; M2-3, stilbenoid, diarylheptanoid and gingerol biosynthesis; M2-4, tropene, piperidine and pyridine alkaloid biosynthesis; M2-5, flavone and flavanol biosynthesis; M2-6, monobactam biosynthesis; M3, carbohydrate metabolism; M3-1, amino sugar and nucleotide sugar metabolism; M3-2, glycolysis/Gluconeogenesis; M3-3, butanoate metabolism; M3-4, glyoxylate and dicarboxylate metabolism; M3-5, c5-Branched dibasic acid metabolism; M3-6, fructose and mannose metabolism; M3-7, starch and sucrose metabolism; M4, energy metabolism; M4-1, photosynthesis; M4-2, sulfur metabolism; M4-3, carbon fixation in photosynthetic organisms; M4-4, methane metabolism; M4-51, nitrogen metabolism; M5, lipid metabolism; M5-1, alpha-Linolenic acid metabolism; M5-2, linoleic acid metabolism; M5-3, fatty acid degradation; M5-4, steroid biosynthesis; M5-5, arachidonic acid metabolism; M5-6, synthesis and degradation of ketone bodies; M5-7, ether lipid metabolism; M6, metabolism of terpenoids and polyketides; M6-1, sesquiterpenoid and triterpenoid biosynthesis; M6-2, monoterpene biosynthesis; M6-3, terpenoid backbone biosynthesis; M6-4, limonene and pinene degradation; M7, overview; M7-1, degradation of aromatic compounds; M7-2, biosynthesis of amino acids; M7-3, carbon metabolism; M7-4, 2-oxocarboxylic acid metabolism; M8, xenobiotics biodegradation and metabolism; M8-1, P450 drug metabolism-cytochrome P450; M8-2, metabolism of xenobiotics by cytochrome P450; M8-3, chloroalkane and chloroalkene degradation; M8-4, caprolactam degradation; M8-5, benzoate degradation; O, organismal Systems; O1, aging; O1-1, longevity regulating pathway-multiple species; O2, environmental adaptation; O2-1, circadian rhythm-plant species.

图6 苹果健康果实和苦痘病果实黄酮生物合成通路KEGG图谱。■, 上调; ■, 正常表达
Figure 6 Flavonoid biosynthesis pathway in KEGG from JKG vs. BG. ■, upregulated; ■, normal expression

表 6 8个差异表达基因的RNA-seq FPKM值、qRT-PCR相对表达水平及其比率

Table 6 RNA-seq FPKM value, qRT-PCR relative expression level and ratios of 8 candidate DEGs

方法	样品	<i>CYP98A2</i> (1)	<i>CYP98A2</i> (2)	<i>BADH</i>	<i>DAT</i>	<i>MdHCT</i> (1)	<i>MdHCT</i> (2)	<i>CHI</i> (1)	<i>CHI</i> (2)
RNA-seq	JKB	61.80	2.32	0.37	5.67	60.45	4.04	10.29	0.71
	BG	88.61	25.19	4.25	9.66	405.70	9.13	69.70	20.83
	BG/JKG	1.43	10.86	11.48	1.70	6.71	2.26	6.77	29.42
qRT-PCR	JKB	1.58	1.71	5.33	1.27	5.29	2.08	4.76	1.83
	BG	1.80	6.41	35.60	7.61	17.97	2.18	17.26	13.14
	BG/JKG	1.14	3.74	6.69	6.01	3.40	1.05	3.62	7.17

图 7 (网络版彩色)8个差异表达基因转录组测序与qRT-PCR结果的相关性分析

Figure 7 (Color online) Correlation analysis of the RNA-seq results and qRT-PCR results for the 8 DEGs

苹果与富士等苹果品种杂交后代株系间果实类黄酮合成差异的分子机理,发现*MYB12*, *MYB16*和*MYB111*等转录因子及*DFR*, *FLS*, *LAR* 和*ANR* 类黄酮生物合成相关结构基因的差异表达,可能是导致红脆1号、2号和4号3个株系类黄酮组分及含量差异的主要原因。

CYP, BADH, DAT, HCT和CHI是植物类黄酮生物合成中重要的酶。CYP (cytochrome P450, 细胞色素P450)在高附加值天然产品的生物合成方面,发挥非常重要的作用^[37]。CYPs的主要功能是在激素和类黄酮等各种内源性化合物生物合成的最后几步反应中起催化作用,并与大量外源性化合物发生氧化代谢反应,进而发挥其抗氧化功能^[38]。植物BAHD酰基转移酶是脂酰辅酶A应用酶类的一个大家族,能产生小分子挥发性酯类、花青素、组成型防御化合物和植物抗毒素等产物^[39]。DAT (deacetylindoline 4-O-acetyltransferase, 去乙酰乙烯醇4-O-乙酰转移酶)是长春花生物碱合成中的终端酶^[40]。HCTs (hydroxycinnamoyl-coenzyme A (CoA) hydroxycinnamoyl transferases, 羟肉桂酰辅酶A羟肉桂酰转移酶)作为BAHD酰基转移酶的家族成员,在生物合成中间体和特殊代谢物的生产等专性代谢方面发挥

重要作用^[41]。CHI (chalcone isomerase, 查耳酮异构酶)是植物类黄酮生物合成中的一个关键酶,主要催化二查耳酮分子内环化,生成三查耳酮,以保证类黄酮生物合成通路中足够的底物供给^[42]。

本研究通过全转录组测序分析,分析了苹果健康果实和苦痘病果总RNA (包括mRNAs, lncRNAs, circRNAs和miRNA)^[43~46]转录水平上的差异。其中涉及类黄酮生物合成的差异表达基因26个。通过qRT-PCR分析验证了其中8个差异表达基因[*CYP98A2* (1), *CYP98A2* (2), *BADH*, *DAT*, *MdHCT* (1), *MdHCT* (2), *CHI* (1)和*CHI* (2)]的表达情况。结果显示,8个差异表达基因的表达趋势与全转录组测序结果一致,二者呈正相关,仅差异倍数不同。测序数据FPKM值和qRT-PCR相对表达量的差异倍数分别为1.43~29.42和1.05~7.17倍,说明苹果苦痘病的发生显著促进了苹果果实中类黄酮的生物合成。

本研究发现,GO富集分析和KEGG通路富集分析获得的与类黄酮生物合成相关的差异表达基因均为mRNA,未发现lncRNAs, circRNAs和miRNA中与类黄酮生物合成有关的差异表达基因。因此,无法进行基因

关联分析. 对于苦痘病的发生是否会引起果实中与类黄酮生物合成相关的lncRNAs, circRNAs和miRNA的差异表达, 有待于进一步研究.

另外, 由于同一棵苹果树中, 内膛的苦痘病发病率及严重程度高于其他部位, 因此本研究仅从苹果树内膛采集病果和健康果实作为样品, 研究了五级病果与健康果实中类黄酮含量及表达的差异. 对于不同发育时期或同一棵树不同部位的苹果中, 苹果苦痘病的发生和严重程度对果实类黄酮含量和表达的影响, 以及苹果苦痘病对果实中不同类别类黄酮物质含量和表达

的影响, 有待于进一步的研究探讨.

4 结论

本研究通过氯化铝显色法、全转录组测序和qRT-PCR分析, 研究了苹果健康果实和苦痘病果中类黄酮的含量及表达差异. 发现苹果苦痘病果中的类黄酮总含量及其生物合成显著高于健康果实, 说明苹果苦痘病的发生极大地促进了苹果果实中类黄酮的生物合成和积累, 表明苹果苦痘病果可作为提取用于治疗人类慢性疾病的类黄酮物质的潜在资源.

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Summary for “苹果苦痘病对果实类黄酮总含量的影响及其分子生物学机制”

Influence of bitter pit on the flavonoid contents in apple fruits and its molecular biological mechanism revealed by the whole-transcriptome RNA-Seq analysis

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Flavonoids are an important group of polyphenolic compounds which are widely distributed throughout the plant kingdom especially in colored fruits and flowers. They play important roles in many plant biological functions and have therapeutic effect on many chronic diseases in humans. The flavonoid biosynthesis and productivity in plants could be stimulated by many biotic and abiotic factors. Bitter pit in apple is an important physiological disorder in apple production, which consequently affects the quality of apple fruits and causes significant economic losses. This study was aimed to investigate the influence of bitter pit on the flavonoid contents in apple fruits and its molecular biological mechanism, so as to explore the alternative bioresource of flavonoids for medical application in human chronic diseases. The total flavonoid content was determined using the aluminum chloride colorimetric method. The result showed that the total flavonoid content in bitter-pit apple fruits (BG) were 4.28-fold as that in healthy apple fruits (JG). By RNA sequencing, 257.002 and 225.713 M raw reads containing 38.55 and 33.857 Gb raw bases were obtained from JG and BG respectively, after trimming the adapter sequences, primers, Ns, and reads with quality scores below 30, 226.702 and 202.951 M clean reads containing 32.807 G and 29.626 nucleotides were obtained respectively, their base ratios with quality values higher than 20 and 30 in reads (Q20 and Q30) were more than 98.3% and 95.0%, respectively. By miRNA sequencing, 4331 clean reads associated with 3753 target genes (2571 upregulated and 1181 downregulated) were obtained. After annotation, a total of 41894 RNAs were obtained, of which, 15852 were known transcripts and 26042 were novel transcripts. Among the obtained transcripts, there were 33582 mRNAs (12645 known and 20937 novel), 1860 lncRNAs (0 known and 1860 novel), 98 circRNAs (52 known and 46 novel), 5894 miRNAs (3017 known and 2877 novel), 322 other type of RNAs (0 known and 322 novel) and 138 unknown type of RNAs (138 known and 0 novel). Based on Gene Ontology (GO) gene functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, 2632 differentially expressed genes (DEGs) were obtained from JG vs. BG comparison, in which, 2080 DEGs were upregulated and 552 were downregulated. Among the obtained DEGs, 26 DEGs (2 downregulated and 24 upregulated) were involved in flavonoid biosynthesis [ko00941], all of them are mRNAs, there were no lncRNAs, circRNAs and miRNAs related to flavonoid biosynthesis. Then, 8 of the 26 DEGs [*CYP98A3*(1), *CYP98A3*(2), *BADH*, *DAT*, *MdHCT*(1), *MdHCT*(2), *CHI*(1) and *CHI*(2)] with fold change > 1.0 (1.43–29.42) were selected for qRT-PCR validation, the result exhibited the consistent expression patterns with that of RNA-seq analysis ($R^2=0.8551$), that is, the expression of the 8 DEGs were upregulated with the BG/JG ratio of 1.05–7.17 in JG vs BG comparison, and the ratios of qRT-PCR relative expression level were less than the fold change of RNA-seq FPKM value except *DAT*. The results of this study showed that the flavonoid biosynthesis and accumulation in apple fruits was significantly stimulated by bitter pit disease, which provided new insights into the influence of bitter pit on the flavonoid biosynthesis and accumulation in apples and indicated that the bitter-pit apples could be use as the potential bioresource of flavonoids for therapeutic utilization in human chronic diseases.

transcriptome sequencing analysis, bitter pit in apples, qRT-PCR analysis, flavonoid biosynthesis and accumulation, the total flavonoid content

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