褪黑素对葡萄果实成熟及乙烯和ABA含量的影响

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摘要: 褪黑素不仅是一种抗氧化剂, 而且能作为激素调控植物生长和发育。本文主要研究了外源褪黑素处理对葡萄果实成熟的影响, 并测定了其对成熟调控因子乙烯和ABA的影响。结果表明, 外源褪黑素处理能极显著提高果实内源褪黑素含量。褪黑素处理提高了总花青苷和可溶性固形物含量, 降低了可滴定酸含量, 表明褪黑素促进了葡萄果实成熟。RNA-Seq分析表明褪黑素处理显著诱导了乙烯生化合成和信号传导关键基因的表达水平, 包括2个*ACS*、2个*ACO*和10个*ERF*。进一步分析表明褪黑素处理提高了转色期葡萄果实中乙烯前体物质ACC含量和乙烯释放速率。此外, 研究还发现褪黑素处理6 h后显著下调了ABA合成基因*NCED1*, 上调了ABA降解基因*CYP707A1*的表达, 但对ABA含量未产生显著性影响。总之, 本研究表明褪黑素促进了葡萄果实的成熟, 乙烯很可能在此过程中起关键作用。

关键词: 褪黑素; 乙烯; ABA; 成熟; RNA-Seq; 葡萄

褪黑素(*N*-乙酰基-5-甲氧基色胺)为吲哚胺类物质, 存在于多种植物上(Tan等2012)。褪黑素作为抗氧化剂, 能提高植物的各种生物和非生物胁迫抗性(Tan等2012); 作为生长调节剂, 则能调节根系分化、叶片衰老、种子萌发、开花和果实发育等生物学过程(Arnao和Hernández 2014)。褪黑素存在于苹果、樱桃、香蕉和番茄等各种果实及其它组织中(Jemima等2011; Sae-Teaw等2013)。就葡萄果实而言, 褪黑素存在于果皮、果肉和种子中; 在葡萄果皮中, 褪黑素含量随着成熟而下降, 而在果肉和种子中随果实成熟上升(Vitalini等2011)。尽管目前褪黑素在果实中的生理功能知之甚少, 但是已有研究表明了褪黑素可能调控果实发育。比如, 外源褪黑素处理能够增加葡萄果粒大小和一致性(Meng等2015); 在樱桃果实中, 褪黑素可能作为抗氧化剂来防止果实的氧化胁迫(Zhao等2012)。褪黑素处理通过提高桃果实抗氧化能力延长了其衰老(Gao等2016)。50~100 μmol·L^{-1}的褪黑素能够提高番茄的产量、果实品质、增强果香(杜天浩等2016; Liu等2016), 并且蛋白组学分析表明了50 μmol·L^{-1}的褪黑素处理影响了番茄果实成熟相关的几个代谢途径, 包括细胞壁代谢、氧化磷酸化、碳水化合物和脂肪酸代谢(Sun等2016)。

葡萄属于非呼吸跃变型果实, 脱落酸(abscisic acid, ABA)在非跃变型果实成熟过程中起重要调控作用(Zhang等2009)。大量研究表明在葡萄发育的各个阶段、离体或活体外源ABA处理均能促进葡萄果实着色(Gagné等2011)。但是, 一些研究也表明乙烯合成和信号传导途径存在于葡萄果实中, 并且功能完全正常, 乙烯信号对葡萄成熟至关重要(Chervin等2004, 2008)。外源乙烯处理能影响葡萄果实着色等成熟指标(Chervin等2004, 2009)。此外, ABA、乙烯代谢和信号传导相关的基因已研究的比较清楚, 其中9-顺式-环氧类胡萝卜素双加氧酶合成基因(9-*cis*-epoxycarotenoid dioxygenase gene, *NCED*)是ABA合成的关键酶基因(Luchi等2001; Wheeler等2009), 1-氨基环丙烷-1-羧酸合酶合成基因(1-aminocyclopropane-1-carboxylate synthase gene, *ACS*)和1-氨基环丙烷-1-羧酸氧化酶合成基因(1-aminocyclopropane-1-carboxylate oxidase gene, *ACO*)是乙烯合成的关键酶基因(Yang和Hoffman 1984; Merchante等2013)。

最近研究发现褪黑素能通过调节ABA和赤霉素(gibberellin A4, GA_4)来调节种子萌发、抗病性和抗旱性(Zhang等2014; Lee等2015; Li等2015); 尤其是在呼吸跃变型番茄果实上, 褪黑素处理增加了乙烯产生, 加速了跃变峰的出现, 进而促进了番茄果实着色和软化(Sun等2015)。基于以上, 本文主要测定褪黑素对非跃变型葡萄果实中ABA和乙烯以及果实发育的影响, 以期为揭示褪黑素调控葡萄果实的机理奠定基础。

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材料与方法

1 时间、地点

试验基地位于东经117.0°北纬36.7°, 属于暖温带半湿润性季风气候; 年平均气温为12.9°C, 10°C以上积温4 213°C; 年平均降水量约700 mm; 年平均日照数2 627 h。

2 材料与处理

以田间三年生鲜食与酿酒兼用葡萄品种'摩尔多瓦' (*Vitis vinifera* cv. Moldova, Guzal kara× SV.12-375)为试材, 选取5行, 每行20棵葡萄苗, 处理与对照交叉。从新梢发育开始控制留梢量, 每平米50~60个叶片、6个结果枝, 每个结果枝留2个果穗, 豆果期疏去副穗和穗尖。生长期内修剪及水肥管理等技术统一。转色初期(花后68 d)用100 μmol·L^{-1}褪黑素浸果, 每穗浸泡5 min; 对照用水处理, 方法相同。于处理后0、2、4、6和8 d取样, 用于测定基因表达量。处理后大约每隔1周取一次样, 直至果实成熟, 用于测定果实品质和激素。每次取样选取果穗的上中下部果实3~4粒, 共计300~400粒, 对照保持取样一致。于样品中随机选取100粒果实测定果实品质指标、乙烯及1-氨基环丙烷羧酸(1-aminocyclopropanecarboxylic acid, ACC)的含量, 然后快速将剩余果实的果皮果肉分离, 液氮研磨, 冷冻干燥后置–70°C保存。

3 测定指标与方法

3.1 果实可溶性固形物(TSS)、可滴定酸、总花青苷含量的测定

取单粒葡萄果实挤汁, 用数显糖度计测定(0~45, 0.01), 重复50次, 计算得果实可溶性固形物(total soluble solid, TSS)。可滴定酸测定参照国家标准《水果、蔬菜制品可滴定酸度的测定》GB12293-90, 取20粒葡萄果实挤汁, 混匀, 试样浸出液用0.1 mol·L^{-1} NaOH溶液进行电位滴定, 以pH=8.10为滴定终点, 重复5次, 计算结果以mg·g^{-1}的酒石酸表示。取液氮速冻的葡萄果皮, 随机称取0.2 g, 研磨至粉末状, 用10 mL的提取液(盐酸-甲醇1%)萃取12 h, 8 000×*g*离心10 min, 取上清液经稀释后测定其在525 nm波长处的吸光值, 重复3次, 计算果实总花色苷含量。

3.2 激素测定方法

3.2.1 褪黑素及ABA提取及测定

取液氮速冻样品置于冷冻干燥机冻干(≥48 h)后, 在液氮冷却下用冷冻研磨机磨成冻干粉, 加80%甲醇提取12 h, 4°C、10 000×*g*离心15 min, 收集上清液并用0.22 μm有机滤膜进行过滤, 置于新试管中。低温旋转蒸发(不超过40°C, 避光), 将蒸干后的物质用3 mL的甲醇水溶液(5%)分3次重新溶解, 过0.22 μm滤膜到新试管中。SPE固相萃取后, 经高效液相色谱-荧光(TSQ-Quantum-Access-MAX三重四极杆)检测, 重复3次。褪黑素检测参数激发波长λ_{ex}=288 nm, 发射波长λ_{em}=333 nm; 流动相乙腈:水=25:75 (*V*/*V*); 流速: 0.5 mL·min^{-1}; 柱温: 22°C; 上样量: 10 μL (Erland等2016)。ABA检测参数: 激发波长λ_{ex}=288 nm, 发射波长λ_{em}=333 nm; 流动相甲醇:乙酸水溶液(pH 3.5)=4.5:5.5 (*V*/*V*); 流速: 0.8 mL·min^{-1}; 柱温: 40°C; 上样量: 10 μL (Zhai等2017)。

3.2.2 乙烯及ACC的测定

将葡萄果实置于容器后密封, 室温下放置2 h, 抽取2 mL气体, 注入气相色谱仪(日本岛津GC-16A), 测定乙烯含量, 重复3次(Farmer等1986)。检测参数: FID检测器, N2流速为85 mL·min^{-1}, H2压力为0.6·kg·cm^{-2}, AIR压力为0.4 kg·cm^{-2}, 柱温105°C, 进样口温度125°C, 外标法定量。

取葡萄果肉10 g, 加入20 mL 的三氯乙酸(TCA, 0.2 mol·L^{-1})溶液研磨成浆, 12 000×*g*离心15 min, 收集上清液, 取2 mL上清封闭与10 mL青霉素小瓶中, 加入1 mL反应液(5%次氯酸钠:饱和氢氧化钠=2:1), 摇匀后静置2 h, 用注射器抽取上空气体, 待测, 重复3次(Lizada和Yang 1979)。气相色谱测定条件同乙烯。

3.3 RNA-Seq及相关基因表达量的测定方法

RNA-Seq由北京安诺优达基因科技有限公司协助完成。利用NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (#7530L, NEB, USA)试剂盒构建测序文库, 文库RNA浓度利用Qubit® RNA Assay Kit和Qubit® 3.0荧光仪(Life Technologies, CA, USA)检测和定量, 稀释至1 ng·μL^{-1}。插入大小利用Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA)分析评价。利用HiSeq PE Cluster Kit v4-cBot-HS (Illumina)和cBot 聚类产生系统对样品进行聚类, 然后在Illumina Hiseq 4000平台上进行测序, 测序长度为基因两端各150 bp。以葡萄基因组数据库为参考(http://genomes.cribi.unipd.it/grape/), 利用

Cufflinks对clean reads进行组装。单基因表达水平利用reads per fragments per kilobase of transcript per million mapped reads (RFKM)进行定量。差异水平根据*P*值和$\log_2$(差异倍数)来判断显著性, $P<0.05$且$\log_2 \geqslant 1$为显著性差异。选用Takara RNA提取试剂盒、Takara反转录试剂盒进行RNA的提取及反转录。以美国国家生物技术信息中心(https://www.ncbi.nlm.nih.gov/)上报道的各基因序列为参考序列, 利用Primer 5.0设计实时荧光定量引物。采用SYBR Green染色, 实时荧光定量(real-time fluorescence quantitative PCR, RT-qPCR)方法对相关基因相对表达量进行测定。

本文选取ABA合成基因*NCED*与降解基因8′-羟化酶合成基因(8′-hydroxylase synthase gene, *CYP707A1*), 以及乙烯合成基因*ACS*、*ACS1*与降解基因*ACO*做荧光定量分析 。

4 统计分析

所有的统计分析采用DPS软件(2005)。采用单因素方差分析(方差分析), 个体均值之间显著差异($P<0.05$)。

表1 定量PCR所用引物

Table1 Primers for qRT-PCRs

基因	正向引物 (5′→3′)	反向引物 (5′→3′)
NCED1	TGAGGTAATTTGCAATGAAGGG	TTCTTCCGAAAACCGAAACAGT
CYP707A1	ACTGAACCCAAGTTTTCTTTCTCT	TTCACCGGACGATCAGCAAA
ACO1	CCTGTTCTCATCATCCTGGGTC	AGCCTTTGTTTCAGCCCTTT
*ACS*1	ACCGTCTTTTCTCTCTCGCC	TCTTGGACAACAACTGCGGT
ACS	TGTGATGTCCCCTCATTCCCC	GCATCTAACTGCCAACCAA
ACTIN	TCTCAACCCAAAGGCTAATC	GCATAGAGGGAAAGAACAGC

实验结果

1 外源褪黑素处理对葡萄果实中褪黑素含量的影响

花后75 d褪黑素含量开始增加, 在花后89 d达最大值, 然后随着果实成熟逐渐下降(图1)。转色初期(花后68 d)进行褪黑素处理, 处理1周的褪黑素含量为对照果实的3.06倍; 在花后75 d至103 d内, 处理果实的褪黑素含量显著高于对照, 且保持在1~3倍。随着果实发育, 处理和对照果实中褪黑素含量的差异变小。

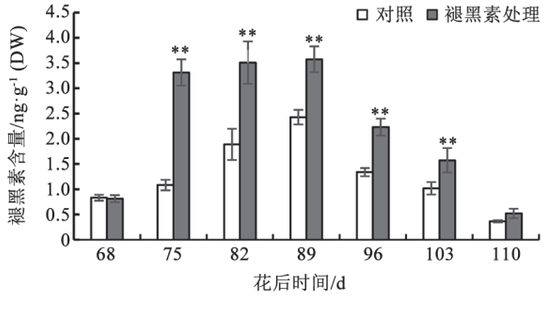

图1 外源褪黑素处理对葡萄果实褪黑素含量的影响

Fig.1 Effect of exogenous melatonin treatment on endogenous melatonin contenst in grape berries

*表示不同处理间在0.05水平上差异显著; **表示不同处理间在0.01水平上差异极显著。下图同此。

2 褪黑素处理对葡萄果实成熟的影响

从花后75 d开始, TSS和花青苷快速积累, 而可滴定酸含量大幅度下降, 表明果实进入转色期(图2)。从花后96 d (处理第4周), 褪黑素处理显著提高了TSS含量; 在花后110 d, 褪黑素处理的果实TSS最高, 为16.25°, 而对照为14.96° (图2-A)。褪黑素处理降低了可滴定酸含量, 在花后96和103 d, 与对照差异达到显著水平(图2-B)。相比之下, 花青苷对褪黑素处理非常敏感, 在花后75 d (处理后7 d), 褪黑素含量为对照的1.47倍, 总体上处理显著提高了花青苷含量(图2-C)。以上结果表明褪黑素处理促进了葡萄果实成熟。

3 褪黑素对乙烯代谢和信号传导相关基因的调控

为了研究褪黑素处理对激素代谢和信号传导相关基因表达的直接影响, 本文检测了褪黑素处理6 h后葡萄果实的转录组变化。结果(表2)表明, 褪黑素处理后, 葡萄果实的14个乙烯合成和信号传导相关基因的表达被显著改变, 且均为上调; 包括4个乙烯合成关键酶基因*ACS*和*ACO*, 其中*ACS1*上调幅度最大, 为对照的3.58倍; 以及10个乙烯响应转录因子*ERF*基因, *ERF115*上调幅度最大, 为对照的2.86倍。此外, 还检测到一个ABA降解相关的

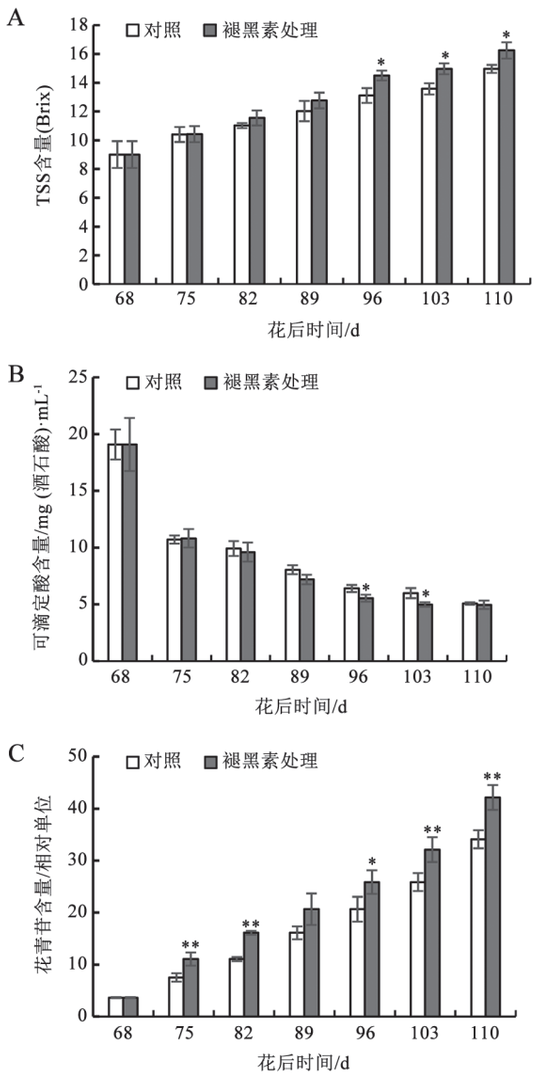

图2 褪黑素处理对葡萄果实TSS、可滴定酸和花青苷含量的影响

Fig.2 Effects of melatonin treatment on TSS, titratable acidity and anthocyanins contents in grape berries

基因*CYP707A1*, 为对照的2.78倍。因此, 乙烯生化合成和信号传导对褪黑素处理较为敏感, 能迅速响应褪黑素处理。

4 褪黑素处理对葡萄果实中乙烯代谢关键基因表达及乙烯含量的影响

为了进一步研究褪黑素处理对乙烯合成的影响, 首先检测了褪黑素处理下乙烯释放速率。在对照和处理果实中, 乙烯释放速率在花后75 d达到最大值, 然后随着果实发育逐渐下降。褪黑素处理极显著提高了转色前果实的乙烯释放速率, 但对发育后期果实影响不大(图3-A)。褪黑素处理显著提高了转色期乙烯合成前体物质ACC的含量(图3-B)。

此外, 乙烯合成关键酶基因*ACS*和*ACO*的表达均受褪黑素诱导(图3-C~E), 这与RNA-Seq的结果一致。在处理后4和6 d, 2个*ACS* (VIT_215s0046g02220-*ACS1*和VIT_202s0025g00360-*ACS2*)基因的表达量达到对照的2倍以上; *ACO1* (VIT_211s0016g02380)为对照的4倍以上, 三者均受到褪黑素的持续诱导。因此, 褪黑素可能通过上调乙烯合成基因的表达提高了乙烯含量。

5 褪黑素处理对葡萄果实ABA含量及相关基因表达的影响

ABA为葡萄果实成熟的主要调控激素, 因此本文检测了褪黑素处理后葡萄果实的ABA含量和ABA合成关键基因*NCED1*和分解关键基因*Abscisic acid 8′-hydroxylase 1* (*CYP707A1*)的相对表达水平。结果表明, 与对照相比, 褪黑素处理略微降低了ABA含量, 其中花后82和89 d的降低幅度较大, 在3.2%左右(图4-A)。基因表达分析表明, 褪黑素在处理6 d显著下调了*NCED1*的表达(图4-B); 总体上上调了*CYP707A1*的表达量, 在处理2和4 d达到显著水平, 增幅分别为46.3%和28.1% (图4-C)。

讨 论

褪黑素作为吲哚胺类物质和IAA具有相似的结构, 二者在促进根系生长和分化方面具有相似的功能; 但是, 在果实中IAA延迟了番茄果实成熟(Su等2015), 而褪黑素处理却促进了番茄成熟(Sun等2015)。本研究结果也表明了褪黑素处理能促进葡萄果实成熟, 这表明褪黑素和IAA作为调节剂共存于果实, 可能具有不同的调节靶基因和途径(Arnao和Hernández 2014)。目前, 褪黑素的感受和信号传导途径还不清楚, 但一些研究表明褪黑素可能通过其它激素来行使调节功能。比如, 褪黑素抑制ABA合成基因*NCED1*、*NCED2*的表达, 提高ABA降解基因ABA 8′-羟化酶(*CYP707A1*和*CYP707A2*)和GA合成基因*GA20*、*GA3*等表达, 进而促进了ABA降解和GA合成, 促进了高盐条件下黄瓜种子萌发(Zhang等2014)。干旱胁迫条件下, 褪黑素处理的苹果选择性下调了*NCED3*, 上调了*CYP707A1*和*CYP707A2*, 进而降低了ABA含量(Li等

表2 褪黑素诱导的激素代谢和信号传导关键基因的表达水平

Table 2 The expression levels of the melatonin-induced genes involved in hormone metabolism and signaling

基因功能	基因ID	基因名称	$\log_2$(倍数变化)	*P*值
乙烯生化合成	VIT_215s0046g02220	*ACS1*	3.58	0.00015
	VIT_211s0016g02380	*ACO1*	1.74	0.00001
	VIT_202s0025g00360	*ACS*	1.43	0.00001
	VIT_212s0059g01380	*ACO1*	1.00	0.00000
乙烯信号转导	VIT_217s0000g00200	*ERF115*	2.86	0.00042
	VIT_205s0049g00510	*ERF1B*	1.87	0.00029
	VIT_201s0011g03070	*AP2/ERF*和*TEM1*	1.72	0
	VIT_201s0150g00120	*ERF114*	1.69	0
	VIT_216s0013g00890	*ERF2*	1.57	0.00187
	VIT_218s0072g00260	*ERF110*	1.50	0
	VIT_202s0234g00130	*ERF1A*	1.07	0
	VIT_207s0005g00820	*ERF071*	1.00	0
	VIT_203s0063g00460	*ERF109*	1.00	0.01367
	VIT_210s0003g00140	*ERF9*	1.00	0
ABA降解	VIT_202s0087g00710	*CYP707A1*	2.78	0

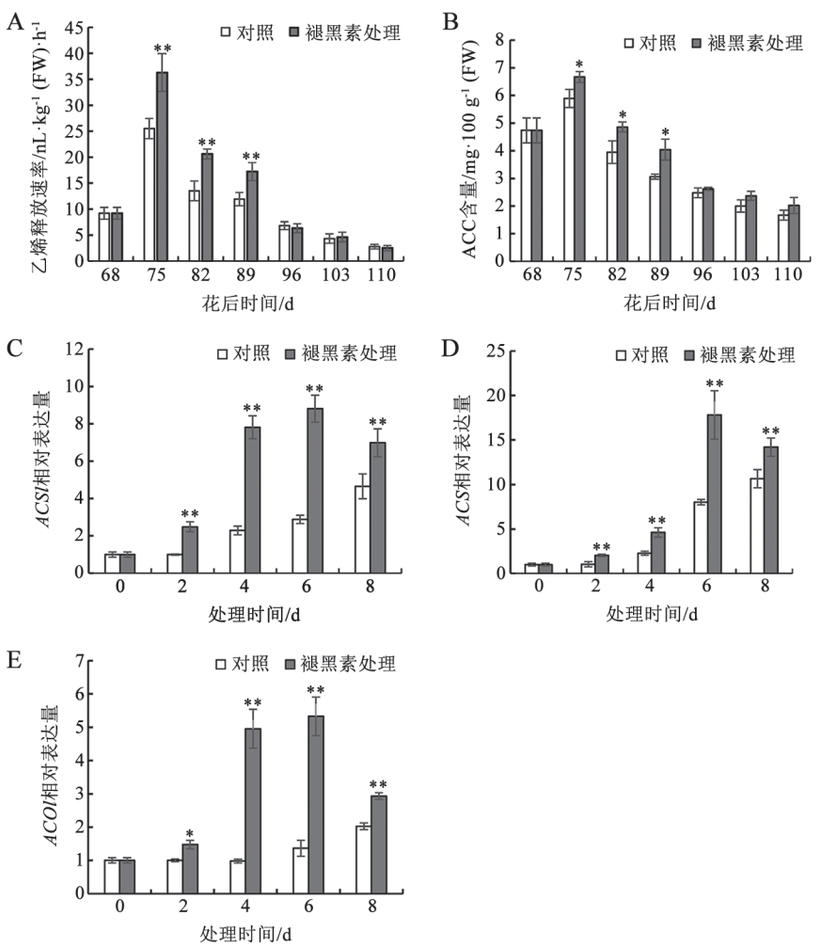

图3 褪黑素处理对乙烯释放速率、ACC含量及乙烯合成关键基因相对表达量的影响

Fig.3 Changes of ethylene release rate, ACC content and the expression levels of the genes involved in ethylene biosynthesis under melatonin treatment

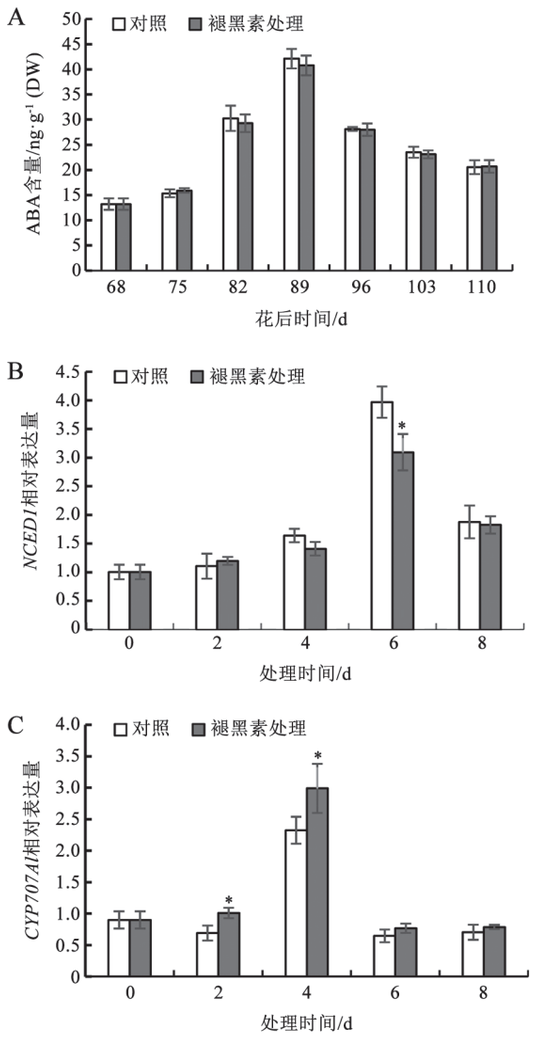

图4 褪黑素处理对ABA含量及ABA代谢关键基因表达的影响

Fig.4 Changes of ABA content and the expression levels of the genes involved in ABA metabolism under melatonin treatment

2015)。褪黑素在水杨酸合成的上游起作用, 褪黑素介导的病菌响应依赖于水杨酸(Lee等2015)。以上表明褪黑素介导的生物学作用一定程度上依赖于其它激素。本文结果中, 在葡萄果实的基因表达和ABA含量分析表明, 褪黑素负调控ABA, 但在正常发育的果实中负调控效应非常弱, 未能显著降低ABA含量。

相比之下, 本研究发现褪黑素处理显著促进了葡萄果实中乙烯生化合成和信号传导。大量研究表明乙烯生化合成和信号传导在调节番茄果实成熟上起重要作用(Li等2010; Klee和Giovannoni等2011; Hayama等2006)。尽管葡萄为非乙烯跃变型果实, 但是一些研究表明乙烯在葡萄果实发育和成熟过程中起重要作用(Coombe 1992; EI-Kereamy等2003)。本研究发现葡萄中褪黑素和ABA含量高峰出现在花后89 d, 而乙烯高峰出现在花后75 d; 而在其它葡萄品种上也发现乙烯峰先于ABA出现在转色前期(Sun等2010; Chervin等2004); 以上暗示了乙烯可能在启动葡萄果实成熟上具有重要作用。在番茄果实上, 褪黑素主要通过诱导*ACS4*上调来促进乙烯合成(Sun等2015), 而2个*ACS*和2个*ACO1*可能参与了褪黑素诱导的葡萄乙烯合成; 以上暗示了在番茄和葡萄两种类型的果实中, 褪黑素诱导乙烯产生的途径可能存在差异。此外, 乙烯生化合成调节主要发生在转录水平上。研究发现RIN、MADS8等转录因子能通过调节*ACS*和*ACO*表达来改变乙烯水平(Ito等2008; Ireland等2013); ERF11、ERF2、ERF3也能通过调节*ACS1*或*ACO1*的表达来调节乙烯水平(Han等2016; Li等2016)。尤其是, 最近研究发现外源MYC2能直接结合到*ACO1*和*ACS1*的启动子上激活其表达, 并且MYC2也能通过ERF3来激活*ACS1*的表达(Li等2017)。在本文结果中2个*ACS*、2个*ACO*和7个*ERF*基因表达受褪黑素诱导, 结合以上前人研究, 我们推测褪黑素处理可能通过调控*ACS*和*ACO*的表达来促进乙烯水平, 并且*ERF*在此过程中可能起重要作用。

总之, 本研究发现了褪黑素处理能促进葡萄果实成熟; 褪黑素显著改变了乙烯合成和信号转导基因的表达及乙烯水平; 褪黑素至少部分通过乙烯信号来调节葡萄果实成熟。

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Effects of melatonin treatment on grape berry ripening and contents of ethylene and ABA

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Abstract: Melatonin acts not only an antioxidant, but also a hormone that regulates plant growth and development. In this paper, the effects of external melatonin treatment on the ripening of grape (*Vitis vinifera*) fruit were studied, and its effects on mature regulatory factors of ethylene and ABA were determined. It was found that the content of endogenous melatonin of grape was significantly improved in the treatment. Melatonin treatment increased the contents of anthocyanins and TSS and decreased the titratable acidity, indicating the accelerated berry ripening. RNA-Seq analysis showed that melatonin treatment significantly induced the expression of the key genes related to ethylene biosynthesis and signaling, including two *ACSs*, two *ACOs* and ten *ERFs*. Further research indicated that melatonin treatment enhanced the content of ethylene biosynthesis substrate ACC and ethylene release rate in grape berries. Besides, it was also found that melatonin treatment significantly decreased the expression levels of *NCED1* (ABA biosynthesis) and enhanced *CYP707A1* (ABA degradation), respectively. In contrast, melatonin did not affect the ABA content of grape berries in a statistically significant manner. Therefore, it was indicated that melatonin promoted berry ripening and ethylene most likely played a key role in this process.

Key words: melatonin; ethylene; ABA; berry ripening; RNA-Seq; grape (*Vitis vinifera*)

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