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薄壳山核桃赤霉素氧化酶基因 *CiGA2ox1* 克隆与功能分析

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摘 要:【目的】从薄壳山核桃(*Carya illinoinensis*)中分离克隆赤霉素氧化酶 *CiGA2ox1* 基因,通过遗传转化及功能验证研究该基因对薄壳山核桃生长发育的影响,为薄壳山核桃新品种选育提供理论参考。【方法】采用PCR扩增技术从薄壳山核桃体胚中克隆出 *CiGA2ox1* 基因全长序列,分析其序列和表达特性。通过亚细胞定位观察其在烟草中发挥功能的场所。构建35S::*CiGA2ox1*::GFP过表达载体,利用农杆菌介导法将35S::*CiGA2ox1*::GFP过表达载体转化到薄壳山核桃体胚中,获得 *CiGA2ox1* 过表达株系。通过qRT-PCR方法分析转基因薄壳山核桃中相关基因的表达水平,采用丙酮乙醇提取方法测定再生植株中叶绿素含量。【结果】薄壳山核桃 *CiGA2ox1* 基因全长1053 bp,编码350个氨基酸。CiGA2ox1蛋白含有1个2OG-FeⅡ-Oxy保守结构域和3个 Fe^{2+} 结合位点。序列系统进化分类结果表明,*CiGA2ox1* 基因与核桃 *JrGA2ox1* 基因亲缘关系最近。烟草叶片亚细胞定位显示,CiGA2ox1蛋白定位于细胞核和细胞膜中。对薄壳山核桃进行遗传转化后经荧光检测及PCR验证表明,35S::*CiGA2ox1*::GFP过表达载体成功转入薄壳山核桃体胚以及再生植株中。与野生型相比,过表达株系 *CiGA2ox1* mRNA相对表达量极显著增加,节间极显著缩短,呈半矮化表型,叶片长宽比降低,叶色深绿,叶绿素含量极显著提高。【结论】薄壳山核桃 *CiGA2ox1* 基因对植株高度具有负调节作用,对叶绿素含量具有正调节作用。研究结果为开发新的半矮化薄壳山核桃突变体提供有价值的参考数据。

关键词:薄壳山核桃;*CiGA2ox1*;亚细胞定位;植株高度;叶绿素含量

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Cloning and functional analysis of gibberellin 2-oxidase1 gene of pecan (*Carya illinoensis*)

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Abstract:【Objective】Changing plant type can effectively improve crop yield, quality and resistance to stresses The research in this field mainly focuses on the creation and application of semi-dwarfing and dwarfing mutants. Different from the breeding objectives of crops, the innovative cultivation of germplasm resources of grafted rootstocks and scions is usually carried out separately for the needs of variegated cultivation of fruit crops. The creation of new rootstock germplasm mainly focuses on important traits such as plant stature, stress resistance and adaptability. Gibberellins (GAs) is one of the five class of hormones that control all aspects of plant growth and development. Semi-dwarfing and dwarfing mutants may shed light on the mode of action of gibberellin. The *GA2ox* is one of the key genes that

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reduce endogenous bioactive GA content and lead to semi-dwarfing and dwarfing plant. So far, few studies have been reported on the cloning of the genes related to gibberellin synthesis in *Carya illinoensis* (pecan). To construct the dwarf pecan plant line for the selection of the rootstock, *CiGA2ox1* was isolated and cloned from pecan, and the effects on the growth and development were studied by overexpression of the *CiGA2ox1*, so as to provide theoretical reference for functional analysis of the gene and breeding. 【Methods】The full length of *CiGA2ox1* gene was isolated from pecan somatic embryos by RT-PCR. The nucleotide sequence and the corresponding amino acid sequence verified by sequencing were analyzed by BLASTn and BLASTp in the database of National Biotechnology Information Center (NCBI). The sequence of the *CiGA2ox1* was analyzed by bioinformatics MEGA 7.0 software to construct the phylogenetic tree. The transient expression was observed by subcellular localization in tobacco. The constructed plant fusion expression vector 35S::*CiGA2ox1*::GFP, empty vector pC1300-GFP and Marker were respectively transferred into *Agrobacterium tumefaciens*, injected into tobacco epidermis and the tobacco plants were cultured under low light for 2 days. The labeled tobacco leaves were made into a pack and observed with a Zeiss LSM710 confocal laser microscope and photographed. Then, 35S::*CiGA2ox1*::GFP overexpression vector was transformed into pecan somatic embryos by *agrobacterium* to obtain *CiGA2ox1* overexpression plant lines. The modified pC1300 plasmid was doubly digested by BamHI and SalI, and the doubly digested products were recovered. The target band and plasmid vector were connected by ClonExpress Ultra One Step Cloning Kit. The somatic embryos of the pecan overexpressing *CiGA2ox1* gene were placed under Carl Zeiss stereo d13covery V12 (Axio cam MRC system) and stimulated by blue light (488 nm). The transformed somatic embryos with green fluorescence excitation were identified by PCR. qRT-PCR was used to analyze the expression levels of the related genes in the transformed pecan lines. At the same time, the terminal buds of the wild-type and positive regenerated plant lines with the same growth conditions were cut, and cultured for 2 weeks for phenotype observation. Chlorophyll was extracted by acetone ethanol (1∶1) from leaves of the transformed and wild-type lines. IBM SPSS Statistics 25 software was used for one-way anova, and Graphpad7.0 software was used to plot the results of the above physiological indicators. 【Results】The length of the *CiGA2ox1* gene was 1053 bp and encoded 350 amino acids. It contained a conserved 2OG-FeⅡ-Oxy domain and three Fe^{2+} binding sites. The relative molecular weight was 39.22 ku, the isoelectric point (pI) was 6.55, and the molecular formula is $C_{1761}H_{2750}N_{464}O_{516}S_{17}$. The transmembrane region value of CiGA2ox1 protein had no transmembrane region. The sequence phylogenetic analysis showed that the *CiGA2ox1* was the most closely related to the *JrGA2ox1* in walnut. The subcellular mapping of the tobacco leaves showed that the *CiGA2ox1* gene was localized in the nucleus and cell membrane. The result suggested that the CiGA2ox1 might have catalytic function on plasma membrane. After gene transformation, fluorescence detection and PCR verification showed that the 35S::*CiGA2ox1*::GFP overexpression vector was successfully transformed into the pecan somatic embryos and the plants were regenerated. The relative expression of the *CiGA2ox1* gene significantly increased in transformed plants compared with the WT. Compared with the wild-type plants, the internode of the transformed plants was shortened and dwarfed. The plant height was about 0.64 times as high as that of wild-type, indicating that the *CiGA2ox1* gene of pecan had a negative effect on plant height. The transformed plant had reduced the leaf length, darkened the green color and increased the chlorophyll content. It showed that GA was an important signal to regulate chlorophyll biosynthesis. Reducing the concentration of endogenous bioactivity GA by overexpression of the *GA2ox1* would increase the content of chlorophyll in plants. 【Conclusion】The *CiGA2ox1* gene mainly played a negative role in regulating plant height and a

positive role in regulating chlorophyll content in pecan.

Key words: Pecan（*Carya illinoinensis*）; *GA2ox1*; Subcellular localization; Plant height; Chlorophyll content

改变植物株型可有效提高作物的抗逆性[1-2]、增加产量[3]以及改善品质[4]等，目前该领域研究多集中于半矮化和矮化突变体的创制和应用。Zhuang等[5]研究表明，与野生型植株相比，过表达*PdC3H17*基因导致杨树（*Populus*）矮化，并显示出更强的光合和活性氧（reactive oxygen species，ROS）清除能力，从而增强了植株的耐旱能力。一般而言，矮化植株高度通常小于正常植株高度的1/2，而半矮化植株高度约为正常植株高度的2/3[6-7]。目前，半矮化和矮化新品种的培育已在农作物中大量开展，包括水稻（*Oryza sativa*）[8-10]、小麦（*Triticum aestivum*）[11-14]、玉米（*Zea mays*）[15-16]、高粱（*Sorghum vulgare*）[17]和大豆（*Glycine max*）[18]等。Zhang等[16]利用CRISPR/Cas9技术编辑了玉米*GA20ox3*基因获得了半矮秆玉米植株，经外源赤霉素（gibberellins，GAs）处理可以恢复植株高度，表明*GA20ox3*基因参与了GAs的生物合成，而缺失*GA20ox3*基因GAs含量显著降低。与农作物的育种目标不同，果树由于品种化栽培的需要，嫁接砧木和接穗的种质资源创新培育往往分别开展。与接穗侧重产量和品质不同，砧木的新种质创制主要集中于株型、抗逆性以及适应性等重要性状[19-22]。目前，关于果树半矮化和矮化基因的相关研究较少，主要集中于苹果（*Malus pumila*）[23-24]、梨（*Pyrus sorotina*）[25]、桃（*Amygdalus persica*）[26-27]、香蕉（*Musa paradisiaca*）[28]和柿树（*Diospyros kaki*）[29]等果树中。其中，植物内源激素GAs[30]、油菜素内酯（brassinosteroids，BRs）[31]、生长素（auxin，IAA）[32]等激素被认为是导致果树矮化的重要生长调节物质，参与果树生长发育的各个阶段。Zheng等[33]对苹果蛋白质组学分析表明，外施BRs处理增加了内源BR、IAA和GAs含量，并通过多种信号蛋白改变激素信号，包括BRs、细胞分裂素（cytokinin，CTK）、脱落酸（abscisic acid，ABA）和GAs信号，表明激素途径可能通过影响细胞生长和木质素相关蛋白加速细胞生长和木质素合成，从而共同控制苹果树的生长。

GAs为5大类激素之一，控制植物生长和发育的各个方面[34-37]。半矮化和矮化突变体可能有助于阐明GAs的作用方式。在一些物种中，如拟南芥[38]、水稻[39-40]和玉米[15]等描述了缺乏或改变内源GAs会表现出矮化突变表型。*GA2ox*基因是降低内源生物活性GA含量并导致植株半矮化和矮化的关键基因之一[27,41]。研究表明，过量表达*GA2ox*基因可导致植株矮化以及活性GAs含量减少[42]。目前，已从香蕉[43]、梨[44]、杧果（*Mangifera indica*）[45]、苹果[46]、葡萄（*Vitis vinifera*）[47]、核桃（*Juglans regia*）[48]等果树中克隆获得*GA2ox*基因。迄今，有关薄壳山核桃GAs合成相关基因克隆的研究鲜有报道，对薄壳山核桃*CiGA2ox1*基因的分离及功能研究尚未见报道。笔者在本研究中采用PCR扩增技术从薄壳山核桃中分离克隆出*CiGA2ox1*基因，对其序列和表达特性进行分析，构建35S::*CiGA2ox1*::GFP过表达载体，通过农杆菌介导方法转化到薄壳山核桃体胚中，分析过表达*CiGA2ox1*基因对薄壳山核桃组培再生植株生长的影响，为果树矮化机制研究提供理论依据。

1 材料和方法

1.1 材料

1.1.1 植物材料 本研究的植物材料源于省部共建亚热带森林培育国家重点实验室培育的薄壳山核桃（*Carya illinoensis*）体细胞胚（简称“体胚”）以及薄壳山核桃组培苗。体胚培养于25 ℃的暗箱中；组培苗培养于温度25 ℃、光照度1500~2000 lx、光周期16 h/8 h、相对湿度80%~90%的组织培养室中。总RNA提取采用生长良好的薄壳山核桃体细胞胚，液氮速冻后于−80 ℃保存。烟草悬浮培养液配置：100 μL 1 mol·L^{-1} $MgCl_2$、200 μL 0.5 mol·L^{-1} MES、15 μL，100 mmol·L^{-1}乙酰丁香酮，ddH_2O定容至10 mL。

1.1.2 克隆菌株与试剂 大肠杆菌菌株DH5α购自于杭州有康生物技术有限公司，农杆菌菌株GV3101购自于上海唯地生物技术有限公司。多糖多酚的总RNA提取试剂盒购自北京天根生化科技有限公司，cDNA反转录试剂盒、SanPrep柱式凝胶回收试剂盒、质粒提取试剂盒、DNA Marker、各种限制性内切酶、PrimeSTAR高保真酶、r*Taq* DNA聚合酶及连接酶均购自TaKaRa公司。过表达载体由

pCAMBIA1300（简称pC1300）改造而来[49]。

1.2 方法

1.2.1 薄壳山核桃*CiGA20ox*基因的克隆及过表达载体的构建 根据薄壳山核桃*CiGA2ox1*基因的全长蛋白质编码区（coding sequence，CDS）序列，采用Primer 5.0设计引物序列（表1），由上海生工生物工程有限公司合成。

以薄壳山核桃体胚为植物材料，提取总RNA，反转录得到的cDNA，引物*CiGA2ox1*-F和*CiGA2ox1*-R用于*CiGA2ox1*基因开放阅读框（open reading frame，ORF）克隆。PCR反应体系：Prime STAR Max（2×）酶12.5 μL，*CiGA2ox1*-F和*CiGA2ox1*-R引物各1.0 μL，cDNA 1.0 μL，用双蒸水补足至25 μL。反应程序：94 ℃ 2 min；94℃ 10 s；55 ℃ 30 s；68 ℃ 2 min；共32个循环；68 ℃延伸7 min；4 ℃保存。产物回收：按照SanPrep柱式胶回收试剂盒说明书的步骤对PCR产物进行回收。

过表达载体的构建：将改造后的pC1300质粒进行*Bam*HⅠ和*Sal*Ⅰ双酶切，回收双酶切产物。利用诺唯赞One step clonging试剂盒连接目的条带和质粒载体。将融合载体35S:*CiGA2ox1*::GFP转化到大肠杆菌DH5α，PCR检测，挑取阳性克隆，测序验证。将成功构建的载体转化农杆菌GV3101菌株，具体步骤参照说明书，将PCR检测阳性的菌液进行保菌用于后续试验。

表1 薄壳山核桃*CiGA2ox1*基因克隆、表达及鉴定分析所用引物

Table 1 Primers used for cloning, expression and testing analysis of *CiGA2ox1*

引物名称Primer name	引物序列Primer sequence（5'—3'）	用途Usage
*CiGA2ox1-Bam*HⅠ	GAGCTCGGTACCCGGGGATCCATGTTGGTCCTTTCCAAAC	扩增Amplification
*CiGA2ox1-Sal*Ⅰ	TCGCCCTTGCTCACCATGTCGACTGAGGCTATGATTCTCTCAAAG	扩增Amplification
QCiGA20ox1-F	CAGGTAGGTGGGCTTCAAGTGT	定量PCR qPCR
QCiGA2ox1-R	CCCGATGCAAGCAACTTTTGTA	定量PCR qPCR
Actin-F	TGCGGGTGCTCGCTTCGGCAGC	内参Action
Actin-R	GGGCAGCCAAGGATGACT	内参Action
CiGA2ox1-F	ATGGCCATTGACTGCATCAC	鉴定Testing
GFP-R	AACCGATGATACGAACGAAAGC	鉴定Testing

注：GGATCC和GTCGAC（下划线）分别为*Bam*HⅠ和*Sal*Ⅰ的酶切位点。

Note: GGATCC and GTCGAC (underlined) are *Bam*HⅠ and *Sal*Ⅰ restriction sites, respectively.

1.2.2 薄壳山核桃*CiGA2ox1*基因的生物信息学分析 将测序验证正确的核苷酸序列和对应的氨基酸序列分别在美国国立生物技术信息中心（National Center for Biotechnology Information，NCBI）数据库中用BLASTn和BLASTp进行序列相似性分析；利用ORF Finder（https://www.ncbi.nlm.nih.gov/orffinder/）在线程序分析*CiGA2ox1*基因的开放阅读框；应用PROSITE Scan软件预测分析蛋白保守区域；利用Expasy（http://web.expasy.org/protparam/）在线软件分析蛋白质的理化性质；TMHMM法（http://www.cbs.dtu.dk/services/TMHMM/）分析蛋白质的跨膜区；利用Wolf psor（https://www.genscript.com/tools/wolf-psort）在线分析软件进行蛋白亚细胞定位预测；系统发育树使用MEGA 7.0软件构建，分析*CiGA2ox1*基因在进化过程中与其他物种的亲缘关系。

1.2.3 薄壳山核桃CiGA2ox1蛋白亚细胞定位 烟草叶片的瞬时转化：将含有35S::*CiGA2ox1*::GFP以及pC1300-GFP空载体的农杆菌扩大培养，8000 r·min^{-1}离心10 min，弃上清液，收集菌液。1∶1比例悬浮液重悬，OD_{600}为0.7~1.0。以pC1300-GFP空载体作为阴性对照，同时以核定位蛋白Marker（由水稻ART1[50]和1个具有RFP信号的载体蛋白连接而来）及膜定位蛋白Marker（AtPIP2A - mCherry）[51]对烟草下表皮细胞中的细胞核和细胞膜进行准确定位。将构建的植物融合表达载体35S::*CiGA2ox1*::GFP，空载体pC1300-GFP和Marker分别转入农杆菌，注射烟草下表皮，弱光培养2 d。取标记的烟草叶片制作成装片，用蔡司LSM710激光共聚焦显微镜观察并拍照。

1.2.4 薄壳山核桃*CiGA2ox1*基因的遗传转化及表达分析 将含有35S::*CiGA2ox1*::GFP的农杆菌菌液在含有卡那霉素（100 mg·L^{-1}）和利福平（100 mg·L^{-1}）LB液体培养基中扩大培养至*OD*值为1.0左右，6000 r·min^{-1}离心10 min，取上清液。加入含有乙酰丁香酮（40 mg·L^{-1}）的DKW液体培养基吹打悬浮菌液。选取生长状态良好的薄壳山核桃体胚进行遗传

转化[52]。将过量表达*CiGA2ox1*基因的薄壳山核桃体胚置于体式荧光显微镜(Carl Zeiss Stereo D13covery V12,Axio Cam MRc system)下,在蓝光(488 nm)激发下,观察体胚荧光激发情况。对具有绿色荧光激发的薄壳山核桃体胚进行PCR验证,引物参照表1。筛选出的阳性体胚培养至子叶胚阶段,干化处理5~7 d后进行植株再生[51]。利用实时荧光定量PCR,通过$2^{-\Delta\Delta CT}$法[53]计算*CiGA2ox1*基因在薄壳山核桃阳性植株中的相对表达量,引物参照表1(Q*CiGA20ox1*-F及Q*CiGA20ox1*-R)。同时分别剪取等长且生长条件较一致的野生型及阳性再生株系的顶芽,培养14 d后观察表型。

1.2.5 薄壳山核桃*CiGA2ox1*转基因株系叶绿素含量测定 选取转基因株系与野生型薄壳山核桃再生株系叶片0.1g,剪碎后置于标号的试管中,向每个试管中加入10 mL 80%的丙酮溶液。放入室温、黑暗处浸提,直至试管内叶片材料全部变白。以80%的丙酮作空白对照,测定663 nm和645 nm下吸光度值。

w(叶绿素)(mg·g^{-1})=[(Ca+8Cb)×Vt]/FW×1000。

Ca=20.3×OD_{645};Cb=8.04×OD_{663}。

其中,Ca、Cb分别为叶绿素a、叶绿素b浓度,Vt为提取液总体积10 mL;FW为叶片鲜质量0.1 g。

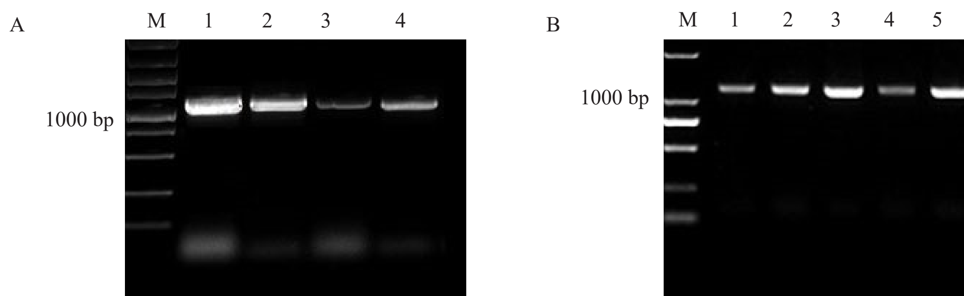
1.3 数据统计与分析

利用IBM SPSS Statistics 25软件进行单因素方差分析,使用Graphpad 7.0软件根据上述生理指标结果绘图。

2 结果与分析

2.1 薄壳山核桃*CiGA2ox1*基因克隆和过表达载体构建

根据设计的特异性引物,通过PCR扩增,琼脂糖凝胶电泳获得1条1000~1500 bp之间的特异条带(图1-A),与预期结果一致。将目的条带胶回收产物与改造后的过表达载体pC1300质粒连接,转化到大肠杆菌中,挑选单克隆,对培养的菌液进行PCR鉴定。将1000~1500 bp电泳条带(图1-B)送出测序。序列长度为1053 bp,测序比对正确的菌液保菌备用。所获过表达载体命名为35S::*CiGA2ox1*::GFP。



A为薄壳山核桃*CiGA2ox1*基因扩增电泳图,M为Maker DL5000,泳道1~4为35S::*CiGA2ox1*::GFP目的基因PCR产物;B为薄壳山核桃*CiGA2ox1*基因大肠杆菌菌液PCR检测,M为Maker DL 2000,泳道1~5为35S::*CiGA2ox1*::GFP基因的单菌培养后的PCR产物。

A. Amplification electrophoresis map of *CiGA2ox1* gene, M. Marker DL5000, Lane 1-4 indicate PCR product of 35S::*CiGA2ox1*::GFP gene; B. Electrophoretogram of *CiGA2ox1 Escherichia coli*. M. Marker DL 2000, Lane 1-5 indicate PCR product of single colony of 35S::*CiGA2ox1*::GFP gene.

图1 薄壳山核桃*CiGA2ox1*基因克隆与大肠杆菌菌液PCR检测

Fig. 1 *CiGA2ox1* gene cloning and PCR detection of *E. coli* bacteria

2.2 薄壳山核桃*CiGA2ox1*基因编码的蛋白氨基酸序列的理化性质分析及系统进化分析

利用ORF Finder在线分析软件得出,薄壳山核桃*CiGA2ox1*基因ORF长度为1053 bp,编码350个氨基酸。ExPASy在线软件预测,薄壳山核桃*CiGA2ox1*基因所编码蛋白的分子质量为39.22 ku,等电点(pI)为6.55,分子式为$C_{1761}H_{2750}N_{464}O_{516}S_{17}$,总平均亲水性-0.158。Wolf psor在线分析软件显示,薄壳山核桃CiGA2ox1蛋白亚细胞定位50%位于细胞核,28.5%位于细胞质中。PROSITE Scan软件预测薄壳山核桃CiGA2ox1蛋白的保守结构域,发现该蛋白含有1个比较保守的2OG-FeⅡ-Oxy(175~281)结构域和3个Fe^{2+}结合位点(205、207、262)。TMHMM在线分析显示,CiGA2ox1蛋白的跨膜区域值为0,该蛋白没有跨膜区域(图2)。在进化关系图中可以发现,薄壳山核桃*CiGA2ox1*基因编码的蛋白氨基酸序列与核桃的相似度最高,达到96.69%,同属1个小分支,亲缘关系最近(图3)。根据亲缘关

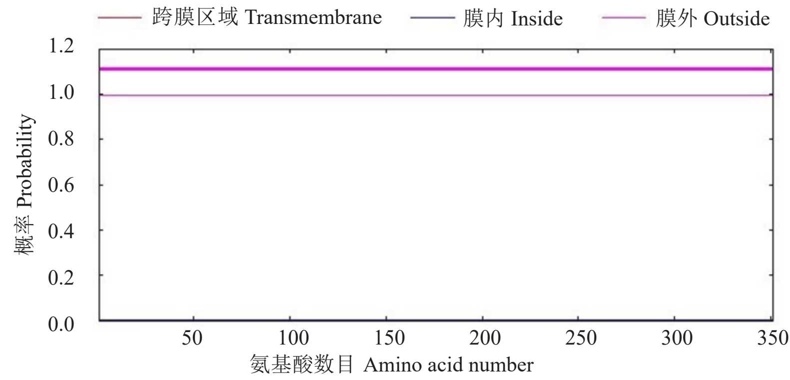
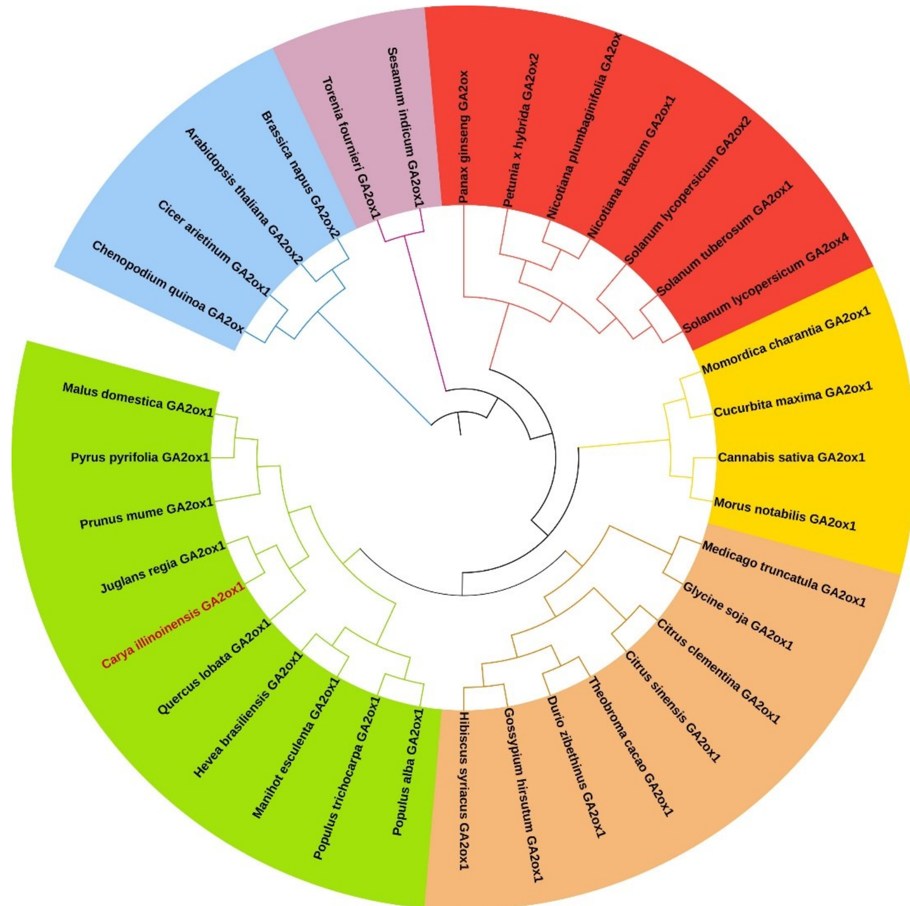


图 2　薄壳山核桃 *CiGA2ox1* 蛋白跨膜区域分析

Fig. 2　Prediction of *CiGA2ox1* transmembrane region of hickory



藜麦 *Chenopodium quinoa*. *CqGA2ox*（XM_021890681.1）；鹰嘴豆 *Cicer arietinum*. *CaGA2ox1*（XM_004485965.3）；拟南芥 *Arabidopsis thaliana*. *AtGA2ox2*（AJ132436.1）；油菜 *Brassica napus*. *BnGA2ox2*（XM_013797003.2）；夏堇 *Torenia fournieri*. *TfGA2ox1*（AB613271.1）；芝麻 *Sesamum indicum*.*SiGA2ox1*（XM_011084568.2）；人参 *Panax ginseng*. *PgGA2ox*（KT692958.1）；矮牵牛 *Petunia x hybrida*. *PhGA2ox2*（JQ323102.1）；皱叶烟草 *Nicotiana plumbaginifolia*. *NpGA2ox*（FM244693.1）；烟草 *Nicotiana tabacum*. *NtGA2ox1*（XM_016644757.1）；番茄 *Solanum lycopersicum*. *SlGA2ox2*（NM_001247409.1）；马铃薯 *Solanum tuberosum*. *StGA2ox1*（XM_006348309.2）；番茄 *Solanum lycopersicum*.*SlGA2ox4*(NP_001234752.1）；苦瓜 *Momordica charantia*.*McGA2ox1*(XP_022142867.1）；笋瓜 *Cucurbita maxima*. *CmGA2ox1*（XM_023675454.1）；大麻 *Cannabis sativa*. *CsGA2ox1*（XM_030634384.1；川桑 *Morus notabilis*. *MnGA2ox1*（XM_010112317.2）；蒺藜苜蓿 *Medicago truncatula*. *MtGA2ox1*（XM_013608940.3）；野大豆 *Glycine soja*. *GsGA2ox1*（XM_028332010.1）；克莱门氏小柑橘 *Citrus clementina* .*CcGA2ox1*(XP_006449692.1)；甜橙 *Citrus sinensis*. *CsGA2ox1*（XM_006477863.3）；可可 *Theobroma cacao*. *TcGA2ox1*（XM_018124899.1）；榴莲 *Durio zibethinus*. *DzGA2ox1*（XM_022877473.1）；陆地棉 *Gossypiumhirsutum*. *GhGA2ox1*（XM_016868828.2）；木槿 *Hibiscus syriacus*. *HsGA2ox1*（XM_039170793.1）；银白杨 *Populus alba*. *PaGA2ox1*（XM_035040541.1）；毛果杨 *Populus trichocarpa*. *PtGA2ox1*（XM_002300394.3）；木薯 *Manihot esculenta* . *MeGA2ox1*（XM_021751000.1）；橡胶树 *Hevea brasiliensis*. *HbGA2ox1*（XM_021813605.1）；大叶栎 *Quercus lobata*. *Ql GA2ox1*（XM_031106239.1）；薄壳山核桃 *Carya illinoinensis*.*CiGA2ox1*（XM_043095786.1）；核桃 *Juglans regia*. *JrGA2ox1*（XP_018824979.1）；梅花 *Prunus mume*. *PmGA2ox1*（XM_008228016.1）；沙梨 *Pyrus pyrifolia*. *PpGA2ox1*（BAU19310.1）；苹果 *Malus domestica*. *MdGA2ox1*（XP_008372291.2）.

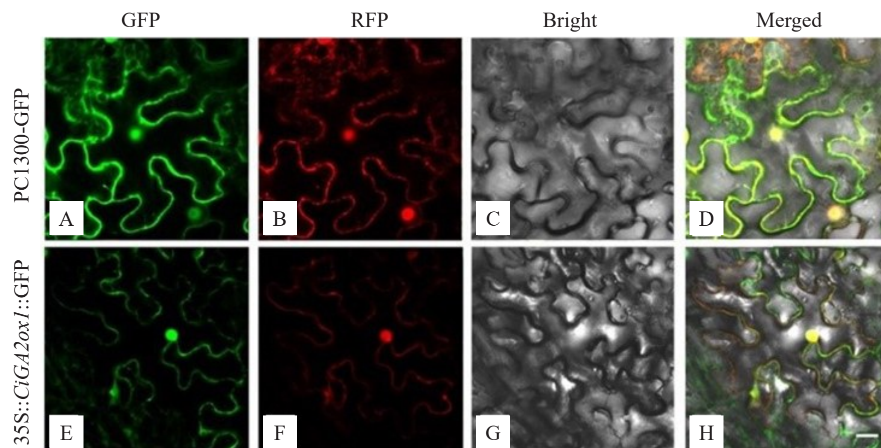
图 3　不同物种 *GA2ox1* 基因的系统发育树分析

Fig. 3　Phylogenetic tree analysis of *GA2ox1* in different species

系远近，可将*CiGA2ox1*基因分为6簇族。其中，薄壳山核桃*CiGA2ox1*基因与核桃、苹果、沙梨、梅花、大叶栎、橡胶树、木薯、毛果杨及银白杨聚为一簇，与薄壳山核桃遗传距离较近；其次木槿、陆地棉、榴莲、可可、甜橙、野大豆和蒺藜苜蓿为第二簇；川桑、大麻、笋瓜及苦瓜聚为第三簇；马铃薯、番茄、烟草、皱叶烟草、矮牵牛和人参聚为第四簇；芝麻和夏堇为第五簇；油菜、拟南芥、鹰嘴豆及藜麦为第六簇。同一基因在同一簇族内，各物种差异性较小；而随着分支不断扩大，各物种间的亲缘关系值逐渐减弱。

2.3 薄壳山核桃*CiGA2ox1*基因编码蛋白的亚细胞定位

为验证*CiGA2ox1*基因编码蛋白的定位，将构建好的35S::CiGA2ox1::GFP融合蛋白、pC1300-GFP空载体及2个定位Marker（核定位蛋白Marker及膜定位蛋白Marker）转化到烟草叶片中，在激光共聚焦显微镜观察35S::*CiGA2ox1*::GFP融合蛋白的定位情况。试验表明，在GFP通道下，pC1300-GFP空载体绿色荧光蛋白信号弥散于细胞核和细胞膜中（图4-A）；在RFP通道下，细胞核和细胞膜呈现红色荧光（图4-B）；在融合通道中细胞核和细胞膜荧光信号呈黄色（图4-D）。这表明pC1300-GFP空载体、核定位蛋白Marker及膜定位蛋白Marker均可正常表达。而在含有35S::*CiGA2ox1*::GFP融合蛋白的烟草叶片中（图4-E~H）观察到绿色荧光信号、红色荧光信号和黄色荧光信号均在细胞核和细胞膜中表达。这表明*CiGA2ox1*蛋白在细胞中主要定位于细胞核和细胞膜中。



A，E. GFP 通道；B，F. RFP 通道；C，G. 白光通道；D，H. 融合图像。A~D. pC1300 空载、膜 marker 和细胞核 marker；E~H. *CiGA2ox1*-pC1300、膜 marker 和细胞核 marker。比例尺 50 μm。

A，E. GFP field；B，F. RFP field；C，G. Bright field；D，H. Merged field. A-D. 35S-GFP indicates the empty vector, cell membrane marker and the nucleus marker；E-H. 35S::*CiGA2ox1*::GFP indicates the fusion protein with *CiGA2ox1*, cell membrane marker and the nucleus marker. Bar: 50 μm.

图4 薄壳山核桃 *CiGA2ox1* 基因编码蛋白在烟草叶片中的亚细胞定位

Fig. 4 Fluorescent localization of *CiGA2ox1* in tobacco leaf epidermal cells

2.4 *CiGA2ox1*基因在薄壳山核桃中的遗传转化及阳性检测

用农杆菌侵染法将35S::*CiGA2ox1*::GFP过表达载体转入到薄壳山核桃体胚中，将侵染后的体胚标记为E0代，筛选培养到E3代，在体式显微镜观察转化体胚可正常生长发育和增殖，形态与野生型体胚相似。挑取15个生长健壮的子叶胚时期的体胚进行侵染，数据统计结果表明，E1代体胚GFP阳性率为34.1%；E2代体胚GFP阳性获得率为32.2%；E3代体胚GFP阳性获得率为65.9%（表2）。将转化体胚放置于体式显微镜下观察，发现转基因阳性体胚在488 nm蓝光激发下呈现绿色荧光，而野生型体胚无荧光信号（图5）。为排除荧光假阳性，进一步对荧光阳性体胚及再生植株进行PCR验证。用外源*GFP*基因（729 bp）及目的基因进行验证（引物序列见表1）。结果发现，凝胶电泳条带大小为1800 bp左右，说明转化成功（图6）。

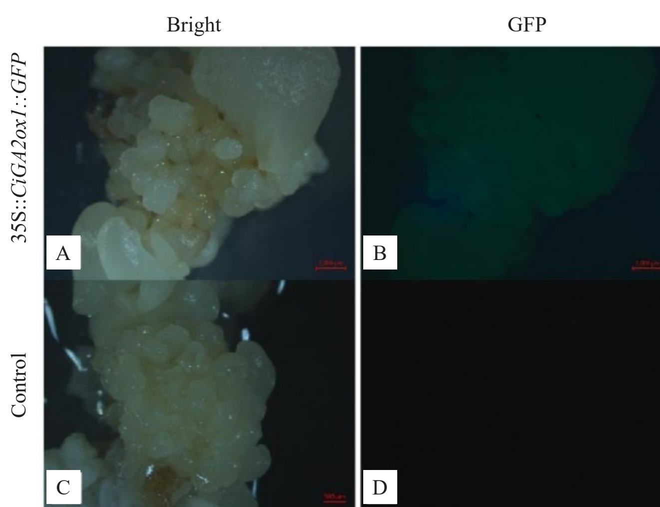
2.5 薄壳山核桃*CiGA2ox1*基因功能验证

2.5.1 薄壳山核桃*CiGA2ox1*基因过表达株系株形鉴定　为了研究过表达*CiGA2ox1*基因与薄壳山核桃植株高度之间的相关性，将野生型体胚和阳性体胚同时萌发。进一步对再生植株高度进行测定，

表2　薄壳山核桃体胚转化再生个数

Table 2　The number of transformation in somatic embryos

E0		E1		E2		E3	
侵染数 Infection number	成活数 Survival number	萌发数 Germination number	阳性数 Positive number	萌发数 Germination number	阳性数 Positive number	萌发数 Germination number	阳性数 Positive number
15	6	92	31	202	65	41	27

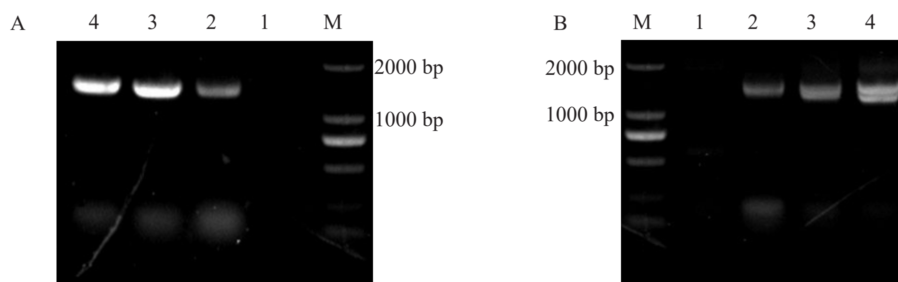


A，B. 白光和蓝光激发下的转化薄壳山核桃体胚；C，D. 白光和蓝光激发下的野生型薄壳山核桃体胚。比例尺：1000 μm。

A，B. Somatic embryos of transformed hickory pecans stimulated by white and blue light；C，D. Somatic embryos of wild-type hickory pecan stimulated by white and blue light. Bar: 1000 μm.

图5　薄壳山核桃35S::*CiGA2ox1*::GFP转化体胚荧光表达

Fig. 5　Fluorescent expression of *Carya illinoinensis* 35S::*CiGA2ox1*::GFP transformed somatic embryos



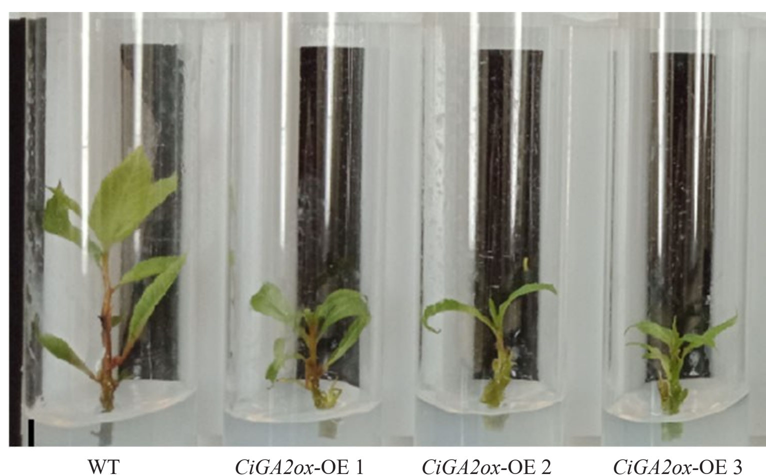
A：1. 野生型薄壳山核桃体胚；2~4. 薄壳山核桃35S::*CiGA2ox1*::GFP 转化体胚；B：1. 野生型薄壳山核桃再生植株；2~4. 薄壳山核桃35S::*CiGA2ox1*::GFP 转化再生植株。M. DNA marker DL2000。

A: 1. Control somatic embryos; 2-4. 35S::*CiGA2ox1*::GFP somatic embryos; B: 1. Control regenerated plants; 2-4. 35S::*CiGA2ox1*::GFP regenerated plants; M. DNA marker DL2000.

图6　薄壳山核桃体细胞胚及再生植株35S::*CiGA2ox1*::GFP的PCR检测

Fig. 6　PCR assay of 35S::*CiGA2ox1*::GFP in overexpression *Carya illinoinensis* somatic embryos and regenerated plants

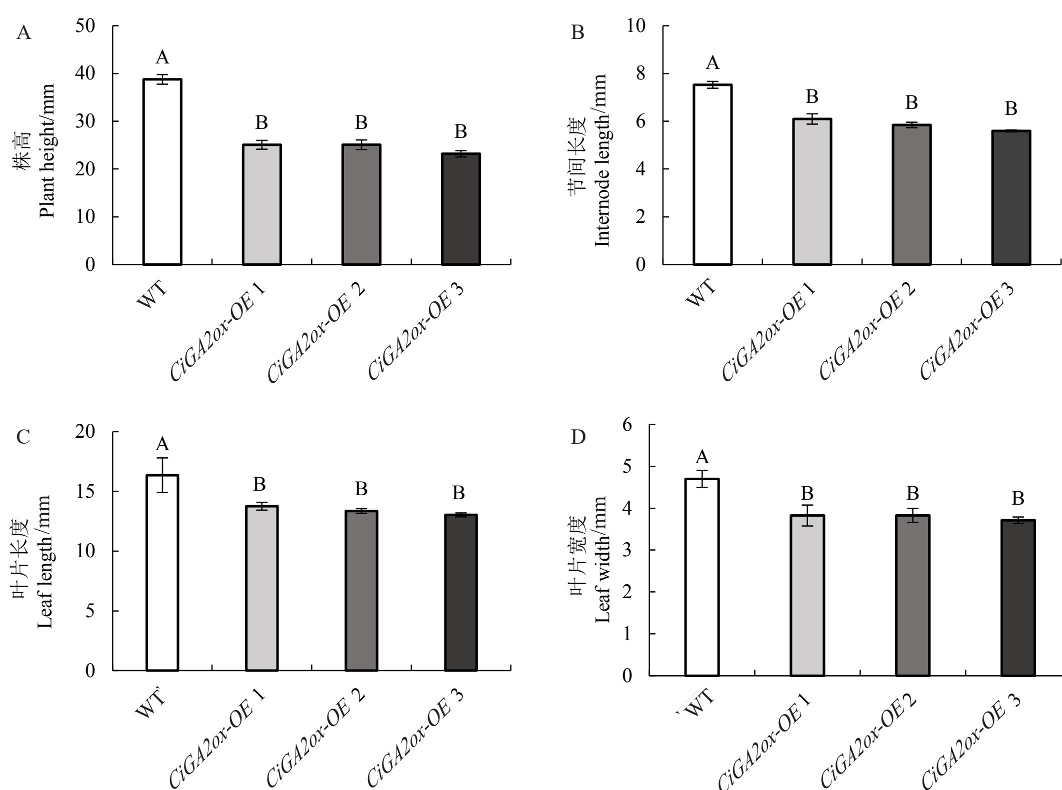
*CiGA2ox1*基因过表达植株选取3个株系，分别命名为*CiGA2ox1*-OE1、*CiGA2ox1*-OE2、*CiGA2ox1*-OE3（图7）。试验结果表明，在体胚萌发后30 d中野生型植株生长较快，而阳性再生植株生长较慢。而后高度基本保持不变。在第30天时*CiGA2ox1*基因过表达植株高度分别为野生型的65.1%、64.7%和59.8%，植株高度极显著减小（图8-A）；第3至第4功能叶的节间长度分别为野生型的81.0%、77.7%和74.4%，节间长度极显著减小（图8-B）；在叶片长度和叶片宽度指标上，叶片长度分别为野生型的72.5%、66.0%和66.1%，叶片宽度为野生型的65.1%、60.2%和55.6%，极显著减小（图8-C~D）。结



比例尺：1 cm。Bar: 1 cm.

图 7　阳性植株与野生型植株的表型

Fig. 7　Positive plants versus wild type B



单因素方差分析显著性，不同大写字母表示差异极显著（$p<0.01$）。下同。

Significance of one-way ANOVA, different capital letters indicated extremely significant difference ($p<0.01$). The same below.

图 8　薄壳山核桃 35S::*CiGA2ox1*::GFP 再生植株表型特分析

Fig. 8　Analysis of phenotypic characteristics of *Carya illinoinensis* 35S::*CiGA2ox1*::GFP regenerated plants

果表明，薄壳山核桃35S::*CiGA2ox1*::GFP再生植株与野生型植株相比，再生植株高度较矮，节间长度和叶片长度更短。

提取野生型及薄壳山核桃35S::*CiGA2ox1*::GFP再生植株的RNA进行*CiGA2ox1*基因相对表达量检测，实时荧光定量PCR结果显示，薄壳山核桃35S::*CiGA2ox1*::GFP再生株系*CiGA2ox1*基因相对表达量均显著高于野生型植株。其中，35S::*CiGA2ox1*::

GFP-1株系的相对表达量为野生型的12倍，35S::*CiGA2ox1*::GFP-2株系相对表达量为野生型的12.36倍，35S::*CiGA2ox1*::GFP-3株系的相对表达量为野生型的13.67倍，差异均极显著（图9）。

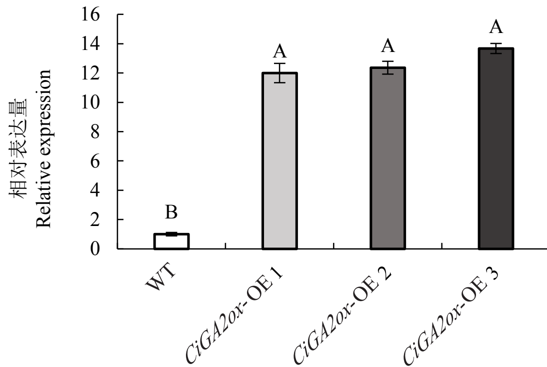


图9　薄壳山核桃35S::*CiGA2ox1*::GFP再生植株的相对表达量分析

Fig. 9　Relative expression analysis of regenerated plants of *Carya illinoinensis* 35S::*CiGA2ox1*::GFP

2.5.2　薄壳山核桃*CiGA2ox*基因过表达株系叶绿素含量的测定　进一步研究发现，薄壳山核桃35S::*CiGA2ox1*::GFP转基因再生植株叶色变深。选取3个阳性再生株系进行叶绿素含量测定（图10）。结果显示，35S::*CiGA2ox1*::GFP-1的叶绿素a、叶绿素b以及总叶绿素含量分别是野生型植株的1.40、1.30和1.40倍，35S::*CiGA2ox1*::GFP-2的叶绿素a、叶绿素b以及总叶绿素含量分别是野生型植株的1.51、1.50和1.52倍，35S::*CiGA2ox1*::GFP-3的叶绿素a、叶绿素b以及总叶绿素含量分别是野生型植株的1.56、1.62和1.67倍。以上指标均显著提高。由此可以发现，过表达*GA2ox1*基因使得薄壳山核桃组培

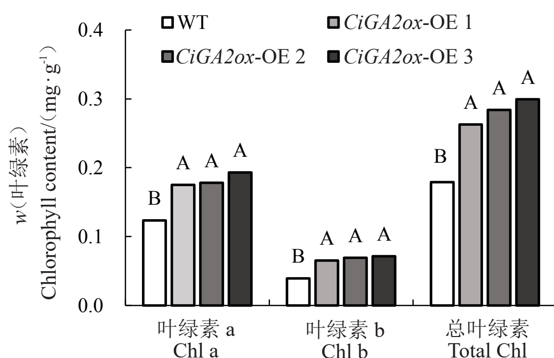


图10　薄壳山核桃35S::*CiGA2ox1*::GFP再生植株的叶绿素含量分析

Fig. 10　Analysis of chlorophyll contents of regenerated plants in *Carya illinoinensis* 35S::*CiGA2ox1*::GFP

苗呈现更绿的叶片是因为更多叶绿素的累积。

3　讨　论

GA生物合成代谢途径在高等植物的整个生命周期中有着极为重要的作用。GA的代谢和分解过程中涉及多种酶。这些酶通过影响生物合成与降解间的平衡，在特定发育阶段维持生物活性水平，保证植物生长发育过程的正常进行。其中，GA2ox蛋白是一种与GA失活相关编码氧化酶，由多个基因编码[54]。通过过表达*GA2ox*基因可以降低内源GA含量，使植株矮化，延长种子的休眠时间，还可以改变植物体内叶绿素的含量[42, 55]。本研究中克隆得到薄壳山核桃*GA2ox*基因，经分析发现薄壳山核桃*CiGA2ox1*基因全长1053 bp，编码350个氨基酸。与核桃*JrGA2ox*基因亲缘关系最近，同源率达到96.69%。亚细胞定位结果显示，*CiGA2ox1*基因编码的蛋白主要位于细胞核及细胞膜中行使功能，这同核桃*JrGA2ox1*基因编码的蛋白亚细胞定位相似[48]。这表明*CiGA2ox1*基因编码的蛋白可能对细胞质膜具有催化功能[54]。

半矮化和矮化树木可以高密度种植以便管理。在许多树种中，树木矮化是通过广泛使用矮化砧木实现的。砧木诱导矮化的机制已经得到了广泛的研究，但仍知之甚少[56-57]。研究表明，赤霉素代谢通路在诱导矮化砧木中起着重要作用[58]。在香波罗（*Artocarpus odoratissimus*）砧木上生长的面包果树（*A. altilis*）植株矮小，在嫁接24个月后，总植株高度减少约60%，节间长度减少约80%。这表明嫁接在香波罗砧木上面包果的矮化表型可能与内源GA含量降低有关[30]。在GA代谢中，*GA2ox*基因可以催化活性GA并将其转化为非活性产物。*GA2ox*基因在矮化中的功能已经在一些物种中得到证实[27, 59-60]。Liu等[61]报道了在柑橘中*GA2ox1*的表达与植物的生长呈负相关。在转基因烟草中过表达*DkGA2ox1*基因会导致矮秆表型和其他矮秆性状[58]。此外，*GA2ox*基因转录水平升高，矮化李子杂交种的节间缩短，茎伸长减少，当用作砧木时，降低了接穗中生物活性GAs的水平[60]。笔者在本研究中通过农杆菌介导转化法获得了薄壳山核桃转基因株系，植株高度约为野生型的0.64倍。本研究结果显示，*CiGA2ox1*基因过表达株系引起植株的半矮化、节间缩短和叶片长宽比降低，这与前人研究结果相符，表

明薄壳山核桃*CiGA2ox1*基因对植株高度具有负调节作用。

研究发现，叶绿素的降解与内源GA含量有关[62]。以往的研究表明，内源激素含量与光合效率之间存在着一定的关系，施用烯效唑(uniconazole，UCZ)抑制了GA，而较低的GA含量提高了少根紫萍的ABA和玉米素核苷(trans-Zeatin-riboside，ZR)含量，从而提高了少根紫萍的叶绿素含量和净光合速率[63]。NAP转录因子通过与DELLA蛋白互作，参与GA介导的叶绿素降解[64]。在拟南芥和水稻中过表达*GA2ox*基因会导致活性GAs水平降低，植株表型呈矮化和小的深绿色叶片[42, 65-67]。Yan等[68]发现在拟南芥中过度表达*BngGA2ox6*基因会导致GA缺乏症状，同时增强总叶绿素的积累。同时，水稻半矮杆*GA2ox*基因突变体叶片呈深绿色，而叶片的总叶绿素含量增加，光合作用增强[69]。本试验中，与野生型相比，*CiGA2ox1*基因过表达再生株系的总叶绿素含量极显著升高，这与以上研究结果相符。这表明GA是调节叶绿素生物合成的重要信号，通过过表达GA2-氧化酶可以降低内源GA含量，进而增加植物体内叶绿素的含量[70]。

薄壳山核桃作为营养价值较高的经济作物，其种质资源的优化、生长发育的调控及品种的选育等相关的研究备受关注。由于嫁接技术的发展，薄壳山核桃半矮化/矮化栽培成为生产发展的趋势。半矮化/矮化栽培具有管理方便、适应性强等优点，为薄壳山核桃生产发展提供了更好的条件和优势。半矮化/矮化砧木种类稀少且自根苗繁殖困难，严重制约了矮化密植果树生产的进展。随着分子生物学研究发展，相关研究将越来越多转向半矮化砧木调控机制方面。植物激素对于果树的生长与发育存在着非常重要的影响。因此，通过分析探究GA对果树半矮化的调控作用，进一步提高果树产量、品质，增强其抗逆性，可更好实现半矮化砧木的选育及应用。

4 结 论

薄壳山核桃*CiGA2ox1*基因与核桃*JrGA2ox1*基因亲缘关系最近，*CiGA2ox1*基因编码的蛋白主要定位于细胞核及细胞膜中。通过遗传转化及功能验证初步表明，薄壳山核桃再生植株中*CiGA2ox1*基因相对表达量升高，可以导致薄壳山核桃植株矮化，叶绿素含量升高。为进一步开发新的半矮化薄壳山核桃突变体提供有价值的参考数据。

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