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CRISPR/Cas9技术在药用植物中的应用与展望

朱瑞艳[1,2], 许莹[2], 时敏[2*], 开国银[2*]

1. 沈阳农业大学园艺学院, 沈阳 110866
2. 浙江中医药大学药学院, 杭州 310053
* 联系人, E-mail: kaiguoyin@163.com; min_shi@zcmu.edu.cn

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摘要 药用植物是最古老的药物形式之一, 次生代谢物的含量是判定药用植物品质的重要指标, 但由于仅在特定组织中积累且含量较低, 其应用受到限制. 成簇的规律间隔的短回文重复序列及其相关蛋白(clusters of regularly spaced short palindromic repeats/CRISPR-associated proteins, CRISPR/Cas)基因编辑技术可以对目标基因实现定点替换、插入和缺失, 从而影响基因的表达和功能. 目前, CRISPR/Cas9技术已成为药用植物中探索基因功能和解析代谢途径的重要手段, 在提高次生代谢物产量和改良药用植物品质方面具有至关重要的作用. 而相对于农作物和园艺植物等, CRISPR/Cas9在药用植物中的应用仍然面临编辑效率低和应用范围小等问题. 本文综述了CRISPR/Cas9技术在次生代谢物合成途径及转录调控方面的应用, 结合CRISPR/Cas9面临的问题, 对技术的发展方向进行了展望, 以为其在药用植物领域的推广使用提供参考.

关键词 药用植物, 次生代谢产物, CRISPR/Cas9, 生物合成, 转录调控

基因编辑技术利用位点特异性核酸酶对基因组中某一特定DNA片段进行剪切后产生双链断裂(double-strandbreak, DSB), 然后利用生物体自身的修复系统实现特定DNA片段的删除、插入、替换及定点突变, 从而实现靶基因的序列改变, 进而影响基因的表达和功能[1]. 成簇的规律间隔的短回文重复序列系统(clusted regularly interspaced short palindromic repeats, CRISPR)/CRISPR相关蛋白(CRISPR-associated proteins, Cas)自问世以来就成为了生物科学领域最重要的基因编辑工具之一. 与前两代基因编辑技术相比, CRISPR/Cas系统利用RNA-DNA识别原理, 构建较容易、效率高且细胞毒性低[2].

依据Cas编码基因序列的差异将CRISPR/Cas系统分为Ⅰ、Ⅱ和Ⅲ型. Ⅰ和Ⅲ型通常形成多亚基蛋白-CRISPR RNA复合物, 而Ⅱ型仅由一个单一的多域蛋白行使功能. Cas9蛋白是一种核心单酶, 目前研究最为广泛的CRISPR/Cas9系统就是在Ⅱ型基础上发展而来[3,4]. CRISPR/Cas9系统工作原理: 反式激活crRNA (*trans*-activating crRNA, tracrRNA)和crRNA(CRISPR RNA)被人为整合成一个RNA转录出来, 通过碱基互补配对作用组成单链向导RNA(single-guide RNA, sgRNA), 与Cas9结合发挥作用. sgRNA扫描识别靶基因前间隔序列邻近基序(protospacer adjacent motif, PAM)序列, 指导Cas9蛋白在PAM序列上游第3~4个碱基处进行剪切. Cas9具有两个核酸酶结构域: HNH结构域负责切割靶序列, RuvC结构域负责靶序列互补链的切割(图1)[5,6]. DNA双链断裂, 激活同源重组修复(homology-directed repair, HDR)和非同源末端连接(nonhomologous end-joining, NHEJ)两种自身损伤修复机制[7]. HDR修复允许在外源同源DNA模板存在的情

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况下插入或者替换特定的核苷酸序列，可以实现靶基因的精准编辑，但是HDR修复因为供体DNA模板的递送性较差，其发生概率较低. NHEJ是指将断裂的两个DNA直接连接起来，可能造成核苷酸插入或缺失，碱基错配率较高. 细胞自身修复后，造成靶基因处碱基的替换、插入或缺失，影响基因的正常转录与翻译，进而破坏靶基因功能(图1).

2013年，CRISPR/Cas9技术首次被应用于烟草(*Nicotiana tabacum*)和拟南芥(*Arabidopsis thaliana*)中，自此以后为在不同物种中进行不依赖标记基因和抗生素选择的高精度基因组工程提供了基础[8]. Zeng等人[9]利用CRISPR同时编辑水稻(*Oryza sativa*)穗长相关基因*OsPIN5b*、谷粒大小相关基因*GS3*和耐寒性相关基因*OsMYB30*，获得了2个*ospin5b*/*gs3*/*osmyb30*三重突变体. 与野生型相比，2个突变体的T_2代表现出更高的产量和耐寒性[9]. 利用CRISPR/Cas9技术破坏生长素响应因子*SlARF4*的功能后，番茄(*Solanum lycopersicum*)的生长及气孔功能均改变，对盐分和渗透胁迫的耐受性也提高，证实了*SlARF4*参与番茄对盐和渗透胁迫的耐性[10]. 通过CRISPR/Cas9损坏木薯(*Manihot esculenta* Crantz)

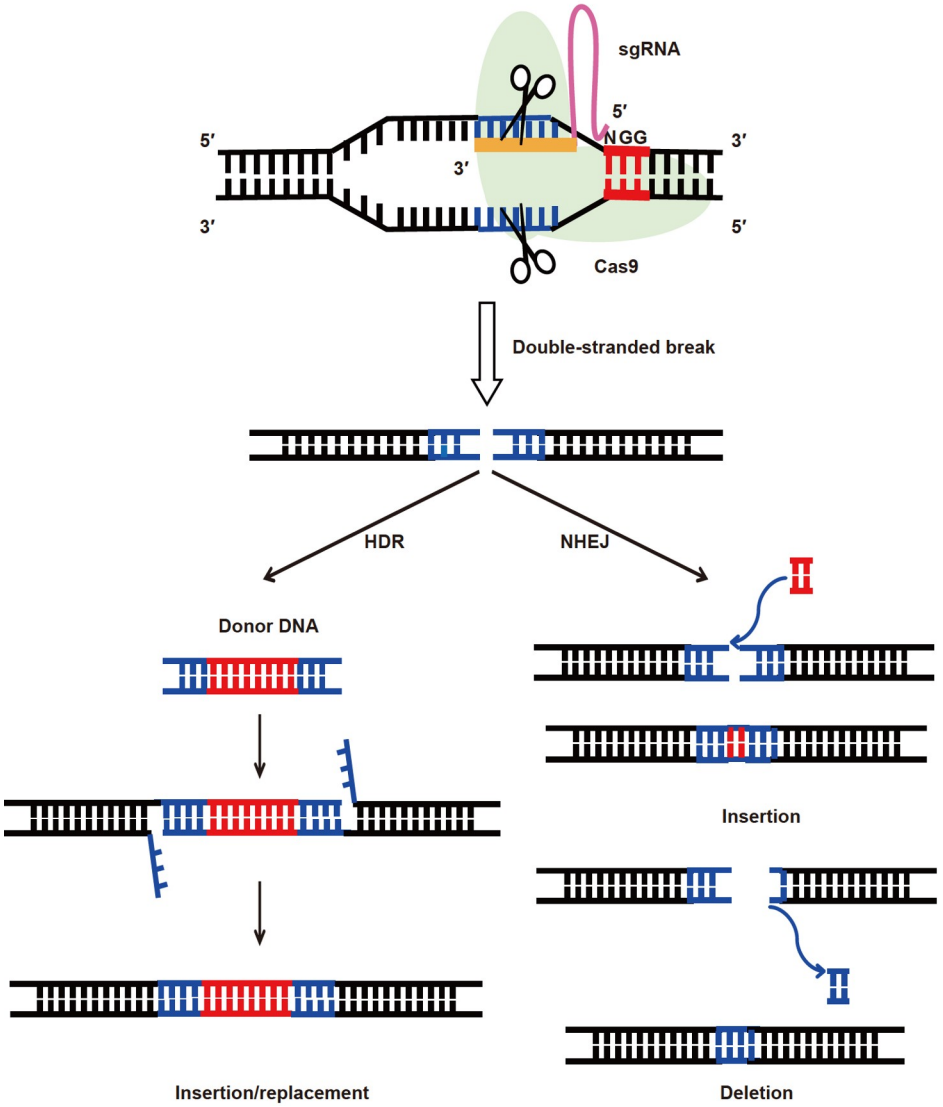

图 1 (网络版彩色)CRISPR/Cas9系统工作原理. 绿色结构代表Cas9蛋白, 紫色代表sgRNA, 橙色代表sgRNA上的识别序列, 红色代表PAM序列. HDR, 同源重组修复; NHEJ, 非同源末端连接

Figure 1 (Color online) Working mechanism of CRISPR/Cas9 system. The green structure represents the Cas9 protein, purple represents the sgRNA, orange represents the recognition sequence on the sgRNA, and red represents the PAM sequence. HDR, homology-directed repair; NHEJ, nonhomologous end-joining

中*MeSWEET10a*基因启动子中的EBE结合元件, 突变品系获得抗病菌的能力, 且具有和野生型一样的正常形态和产量, 这为选育抗病菌木薯品种提供了研究基础[11]. 此外, 越来越多的文献总结了CRISPR技术在粮食、园艺作物、水果作物和油料作物等领域的应用进展, 并对其后续的研究方向进行了展望[12~16].

药用植物中的次生代谢物质在临床上具有预防和治疗疾病的功效, 其含量高低是评判药用植物品质的指标之一. 从药用植物中提取得到的次生代谢物展现出抗癌、抗病毒、抗疟疾、抗炎症等活性, 临床应用广泛[17]. 由于过度采挖, 野生植物资源逐渐枯竭. 随着农业和医药学的发展, 药用植物逐渐成为栽培植物, 但其在生产上仍存在着许多问题, 如产量和品质的不稳定性、连作障碍、抗逆性差等. CRISPR/Cas9技术已经在植物的产量提升、抗生物和非生物胁迫方面等方面取得了一定的进展. CRISPR/Cas9结合合成生物学在生产药用植物次生代谢物方面展示了巨大的潜力. 本文主要介绍了CRISPR/Cas9基因编辑技术在药用植物次生代谢物生产中的应用情况, 并对其在药用植物领域的应用前景进行展望, 为推动该研究领域的前进提供参考.

1 CRISPR/Cas9技术在药用植物次生代谢物生产中的应用

次生代谢产物在调节植物的生长发育和胁迫响应等方面发挥重要作用[18]. 药用植物中的次生代谢产物按其生物合成途径, 可分为萜类、生物碱类和苯丙烷类等类型(图2)[19]. 代谢途径中的合成酶通过氧化还原、羟基化、甲基化、糖基化等作用将初生代谢底物转化为有药用价值的次生代谢产物. CRISPR/Cas9是一种简单、高效和具有靶向功能的基因编辑技术, 已经成为探究未知功能的合成酶及转录因子的主要手段, 在解析代谢途径和提高代谢产物的含量方面得到广泛应用. 本部分主要介绍了CRISPR/Cas9技术在萜类、生物碱类和苯丙烷类次生代谢物生产中的应用(图3, 表1[20~45]).

1.1 CRISPR/Cas9技术在萜类物质生产中的应用

萜类化合物是自然界最丰富的天然产物之一, 已有超过40000种萜类化合物被报道, 且具有重要药用价值[46~49]. 萜类化合物的合成主要分为3个阶段. 第一阶段: 2-C-甲基-D-赤藓醇-4-磷酸(2-C-methyl-D-erythritol-4-phosphate, MEP)途径起始于3-磷酸甘油醛(glyceraldehyde-3-phosphate, G3P)和丙酮酸(pyruvate), 在1-脱氧-D-木酮糖-5-磷酸合成酶(1-deoxy-D-xylulose-5-phosphate synthase, DXS)、1-脱氧-D-木酮糖5-磷酸还原异构酶(1-deoxy-D-xylulose-5-phosphate reductoisomerase, DXR)等酶的连续催化下生成异戊烯焦磷酸(isopentenyl pyrophosphate, IPP)/二甲基烯丙基焦磷酸(dimethylallyl pyrophosphate, DMAPP)[50,51]; 甲羟戊酸(mevalonate, MVA)途径起始于乙酰辅酶A(acetyl CoA), 在乙酰辅酶A合酶(acetyl CoA synthase, AACT)、3-羟基-3-甲基戊二酸单CoA合酶(3-hydroxy-3-methylglutaryl CoA synthase, HMGS)等酶的催化作用生成IPP/DMAPP. 异戊烯基焦磷酸异构酶(isopentenyl pyrophosphate isomerase, IDI)可以催化IPP和DMAPP的转换[52]. 第二阶段: 1分子DMAPP与不同分子的IPP在牻牛儿基二磷酸合成酶(geranyl diphosphate synthetase, GPS)、法尼基二磷酸合酶(farnesyl diphosphate synthase, FPS)和牻牛儿基牻牛儿基二磷酸合酶(geranylgeranyl diphosphate synthase, GGPPS)的催化下分别形成单萜前体牻牛儿基二磷酸(geranyl pyrophosphate, GPP)、倍半萜/三萜的前体物质法尼基焦磷酸(farnesyl pyrophosphate, FPP)和二萜/四萜的前体物质牻牛儿基牻牛儿基二磷酸(geranylgeranyl diphosphate, GGPP)[53~55]. 第三阶段: 不同的萜类合酶(terpene synthase, TPS)能催化GPP、FPP和GGPP形成特异性的萜类化合物[56~62]. 但TPS作用下形成的代谢物大多具有挥发性, 需细胞P450氧化酶、甲基转移酶、糖基转移酶、脱氨酶和酰基转移酶等酶的修饰形成最终产物[63~67]. 随着组学技术的发展, 越来越多参与调控萜类代谢物合成的转录因子也被挖掘出来[68,69].

大多数萜类化合物虽具有重要的药理活性, 但由于天然资源短缺且在植物体内的合成具有组织特异性, 使其含量极低, 限制了使用. 例如, 青蒿素是典型的倍半萜类化合物之一. 中国药学家屠呦呦从黄花蒿(*Artemisia annua*)中提取出了青蒿素并开创疟疾治疗的新方法, 也因此获得了2015年诺贝尔生理学或医学奖. 至今为止, 青蒿素类药物仍是治疗疟疾的首选药物. 但由于黄花蒿中青蒿素主要在腺毛积累, 含量极低, 如何结合分子生物学手段提高其含量是目前的研究热点. 丹参酮是来源于模式药用植物丹参(*Salvia miltiorrhiza*)中的脂溶性二萜类化合物, 具备预防和治疗心脑血管疾病的功效. 以丹参为主要成分的复方丹参滴丸是美国

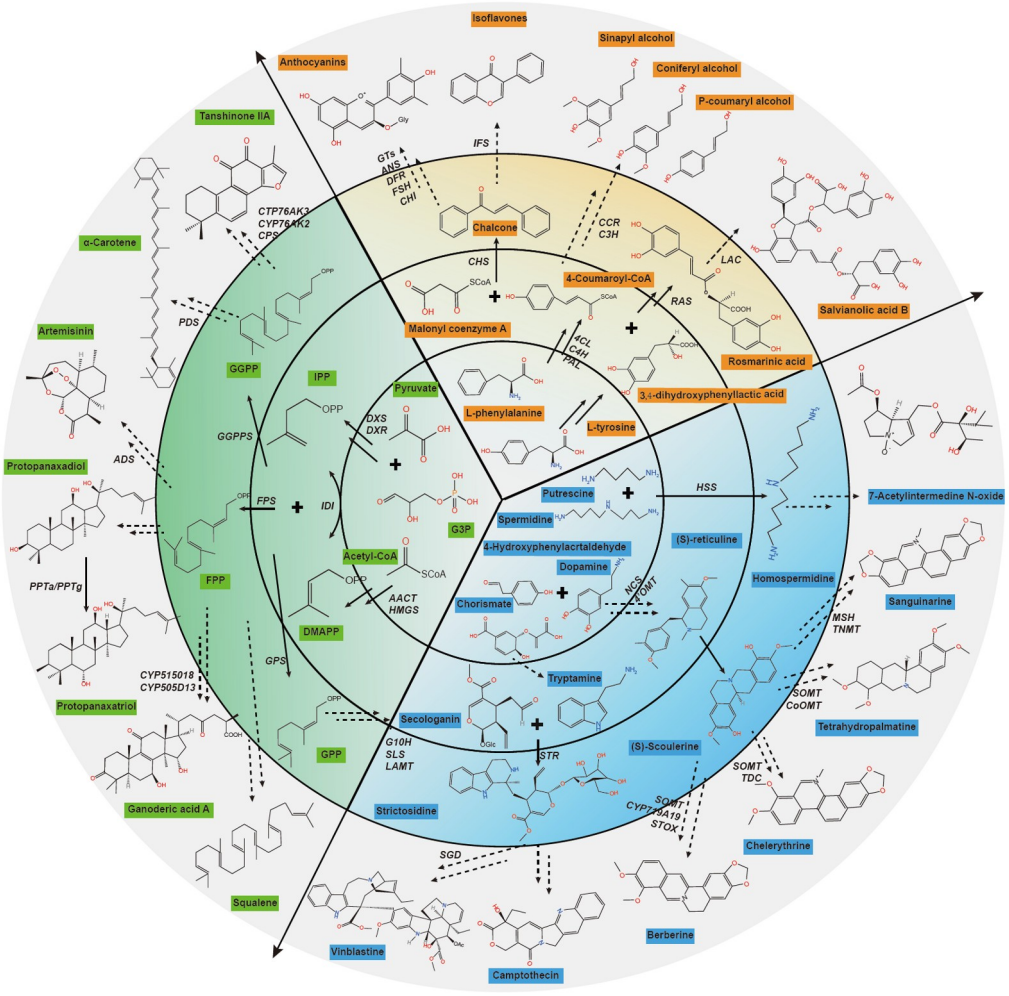

图 2 (网络版彩色)萜类、生物碱类、苯丙烷类生物合成途径. 萜类生物合成途径分为3个阶段. 第一阶段: 以G3P和丙酮酸或以乙酰辅酶A为底物生成IPP或DMAPP; 第二阶段: 以IPP和DMAPP为底物生成前体物质GPP、FPP和GGPP; 第三阶段: GPP、FPP和GGPP在TPS和修饰酶作用下生成特异性萜类化合物. 参与萜类合成途径的酶包括: DXS、DXR、AACT、HMGS、IDI、GPS、FPS、GGPPS、ADS、CPS、CYP76AK2、CYP76AK3、PDS、PPTa/g、cyp5150l8、cyp505d13. 生物碱类以氨基酸为合成前体. 4-羟基苯乙醛和多巴胺合成苄基异喹啉类生物碱的前体物质(*S*)-金黄紫堇碱. 分支酸经过吲哚途径合成色胺, IPP/DMAPP经过环烯醚萜途径转化为开环马钱子苷. 色胺和开环马钱子苷合成单萜吲哚类等生物碱的共同前体异胡豆苷. 参与生物碱合成途径的酶包括: NCS、TNMT、MSH、SOMT、TDC、CYP719A19、STOX、CoOMT、STR、SGD、4′OMT、G10H、SLS、LAMT、HSS. 苯丙烷合成途径起始于苯丙氨酸. 苯丙氨酸被催化为4-香豆酰辅酶A, 后者与丙二酰辅酶A作用形成黄酮类物质, 与3,4-二羟基苯乳酸作用形成酚酸类物质. 参与苯丙烷合成途径的酶包括: PAL、C4H、4CL、CHS、IFS、CHI、F3H、DFR、ANS、GTs、C3H、CCR、RAS、LACs; 黄色色块代表苯丙烷类; 蓝色色块代表生物碱类; 绿色色块代表萜类; 实线代表已知途径; 虚线代表未知途径; 两个实线/虚线代表多步反应

Figure 2 (Color online) Biosynthetic pathways of terpenoids, alkaloids and phenylpropane. The pathway of terpenoid biosynthesis can be divided into three stages. The first stage: IPP or DMAPP is produced by G3P and pyruvate or acetyl-CoA as substrate; the second stage, IPP and DMAPP were used as substrates to generate terpenes precursor GPP, FPP and GGPP; the third stage: GPP, FPP and GGPP generate specific terpenoids under the action of TPS and modifying enzymes. Enzymes involved in the terpenoid synthesis pathway include: DXS, DXR, AACT, HMGS, IDI, GPS, FPS, GGPPS, ADS, CPS, CYP76AK2, CYP76AK3, PDS, PPTa/g, cyp5150l8, and cyp505d13. Alkaloids use amino acids as their precursors. 4-ydroxyphenylacetaldehyde and dopamine are converted to (*S*)-scoulerine, which is the precursor of benzyl isoquinoline alkaloids; tryptamine is synthesized from branched acid through indole pathway, and IPP/DMAPP is converted into secologanin through iridoid pathway. Tryptamine and secologanin are converted to strictosidine, which is a common precursor of monoterpene indole alkaloids. Enzymes involved in the alkaloid synthesis pathway include: NCS, TNMT, MSH, SOMT, TDC, CYP719A19, STOX, CoOMT, STR, SGD, 4′OMT, G10H, SLS, LAMT, and HSS. The phenylpropane synthesis pathway begins with phenylalanine. Phenylalanine is catalyzed to 4-coumaryl CoA, which reacts with malonyl-CoA to form flavonoids, and with 3,4-dihydroxyphenyllactic acid to form phenolic acids. Enzymes involved in the phenylpropane synthesis pathway include: PAL, C4H, 4CL, CHS, IFS, CHI, F3H, DFR, ANS, GTs, C3H, CCR, RAS, and LACs; the yellow color blocks represents phenylpropane; the blue blocks represent alkaloids; the green blocks represents terpenes; solid lines represent known pathways; dotted lines represent unknown pathways; the two solid/dashed lines represent a multistep reactions

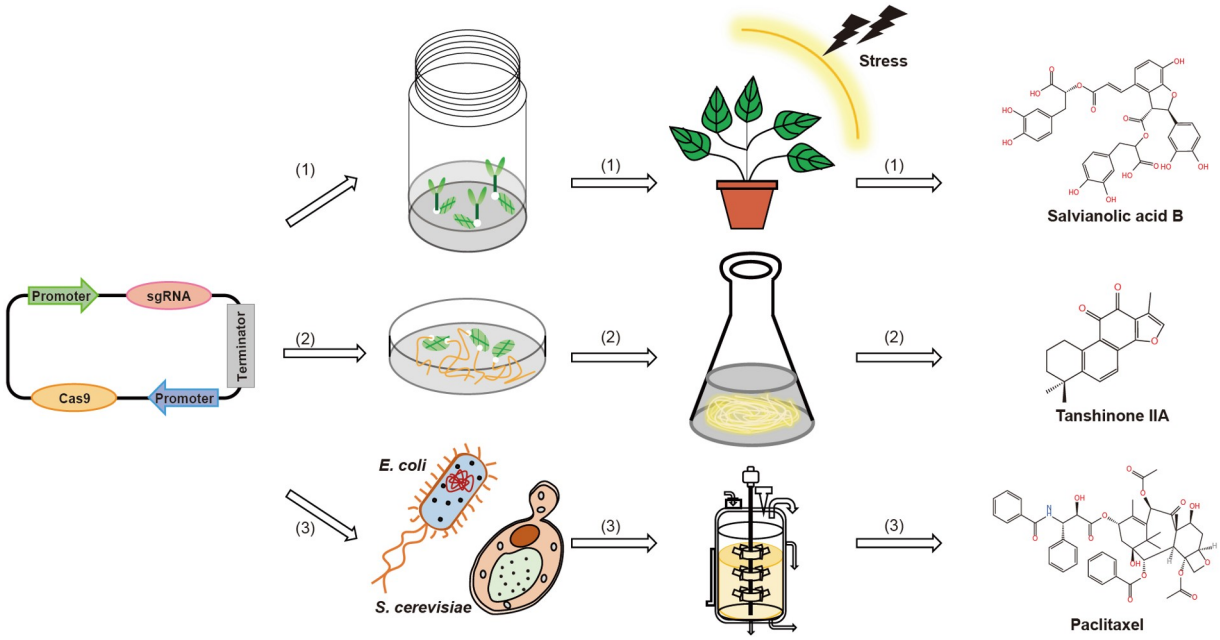

图 3 (网络版彩色)CRISPR/Cas9系统编辑药用植物基因获得目标产物. (1)基因编辑载体的构建; 利用农杆菌GV3101侵染植物外植体, 获得转基因植株材料; 根据目的基因的不同, 经过编辑后的植株获得更强的抗胁迫能力, 同时能够高效生产某些次生代谢产物如丹酚酸B等. (2) 利用农杆菌C58C1侵染植物外植体, 通过植物细胞全能性获得转基因毛状根材料. 摇瓶培养编辑成功的毛状根株系, 可获得目标产物如二萜类丹参酮IIA等. (3) 将携带sgRNA和Cas9的载体直接转化大肠杆菌和酿酒酵母等底盘细胞, 经过摇瓶发酵得到得到高产量的目标产物如紫杉醇等

Figure 3 (Color online) Application of CRISPR/Cas9 system editing genes in medicinal plant for targeted bioactive compounds. (1) Construction of gene editing vector; *Agrobacterium* GV3101 was used to infect plant explants to obtain transgenic plant materials. According to the different target genes, the edited plants were more resistant to stress and could efficiently produce some secondary metabolites such as salvianolic acid B. (2) *Agrobacterium* C58C1 was used to infect plant explants, and transgenic hairy root materials were obtained through plant cell totipotency. Target products, such as diterpenoid tanshinone IIA, can be obtained by shaking the edited hairy root lines. (3) The vectors carrying sgRNA and Cas9 were directly transformed into chassis cells, such as *E. coli* and *S. cerevisiae*, and high-yield target products such as paclitaxel were obtained through shaker fermentation

食品和药物监督管理局(US Food and Drug Administration, FDA)批准的第一个复方中药, 丹参酮因为仅在丹参根部的周皮中产生使其应用受限. 人参(*Panax ginseng*)、三七(*Panax notoginseng*)和灵芝(*Ganoderma lucidum*)均是具有保健功能的名贵中药材, 人参更是被誉为“百草之王”, 三者的主要成分均为三萜皂苷类. 随着野生资源的枯竭, 采用分子生物学技术提高这些次生代谢物含量成为主要手段之一.

目前, CRISPR/Cas9技术在次生代谢物生产上多用于增加次生代谢物含量. 枯草芽孢杆菌(*Bacillus subtilis*)因能够生成前体物质IPP/DMAPP, 所以被用来作为底盘细胞生产萜类物质. 紫穗槐二烯合酶(amorpha diene synthase, ADS)能够催化IPP/DMAPP形成倍半萜/三萜的前体FPP. Song等人[70]利用CRISPR/Cas9技术将带有绿色荧光蛋白(green fluorescent protein, GFP)融合*ADS*(共2.5 kb)整合到枯草芽孢杆菌的基因组中, 20℃下培养24 h得到较高含量的紫穗槐二烯. 紫穗槐二烯是倍半萜青蒿素的合成前体. CRISPR/Cas9技术的基因插入能力为实现青蒿素的高产提供了新思路.

此外, CRISPR/Cas9技术在次生代谢物生产上也用于验证潜在基因或转录因子功能, 以此为实现次生代谢物的高产提供更多的靶标. 丹参酮是药用植物丹参中特有的一类二萜化合物. 在丹参中, 柯巴基焦磷酸合酶(copalyl diphosphate synthase, SmCPS1)将代谢流引向二萜丹参酮生物合成, 细胞色素P450(cytochromes P450, SmCYP76AH1、SmCYP76AH3、SmCYP76AK1等)对后续产物进行修饰, 得到终产物丹参酮[63~65]. Li等人[20]在*SmCPS1*的第1、4、11位外显子各设计了一个靶点, 银染实验和DNA测序结果显示前两个靶标均脱靶, 第三个靶标得到11.5%的纯合突变率和30.8%的杂合突变率. 分析结果表明, 纯合及杂合突变体相较于野生型, 丹参酮的含量降低. 敲除*SmCYP76AK2*株系表现出碱基替换、插入和缺失, 且丹参酮含量降低[21]. 敲除*SmCYP76AK3*获得14个阳性的突变株系中所产丹参酮

表 1　CRISPR/Cas9技术在药用植物中的应用[a)]

Table 1　Applications of CRISPR/Cas9 technology in medicinal plants

基因名称	物种	转化体系	Cas9/sgRNA启动子	第几外显子	编辑类型	次生代谢物	编辑效果	参考文献
SmCPS1	丹参	毛状根	2×35S/AtU6	1、4、11	插入/缺失	丹参酮(tanshinones)	下降	[20]
SmCYP76AK2	丹参	植株	–	–	插入/缺失/替换	丹参酮	下降	[21]
SmCYP76AK3	丹参	植株	–	–	缺失	丹参酮	下降	[21]
SmbZIP1	丹参	毛状根	35S/AtU6	1	缺失/替换	丹酚酸(salvianolic acids) 丹参酮	下降 上升	[22]
SmWRKY34	丹参	毛状根	35S/AtU6	1	缺失/替换	丹参酮	上升	[23]
SmbZIP3	丹参	毛状根	35S/AtU6	1	缺失	丹参酮	下降	[23]
SmJAZ9	丹参	毛状根	AtUBQ/AtU6	1	插入/缺失	丹参酮	上升	[24]
SmMYB76	丹参	毛状根	AtUBQ/AtU6	1	插入/缺失	丹参酮	下降	[24]
SmbHLH60	丹参	毛状根	AtUBQ/AtU6	1	插入/缺失	丹参酮	上升	[25]
PPTa/PPTg	人参	毛状根	35S/AtU6	1	插入/缺失	原人参二醇(protopanaxadiol)	下降	[26]
PnMYB4	三七	愈伤	–	–	–	三七皂苷(notoginsenoside)	上升	[27]
Dzfps	盾叶薯蓣	植株	35S/OsU3	1	缺失	角鲨烯(squalene)	下降	[28]
CsPDS	大麻	植株	–	6	缺失	类胡萝卜素(carotene)	白化	[29]
RcPDS1	天目地黄	植株	2×35S/AtU6	4	插入/缺失/替换	类胡萝卜素	白化	[30]
4'OMT2	罂粟	植株	35S/AtU6	–	插入/缺失	苄基异喹啉类生物碱(benzylisoquinoline alkaloids)	下降	[31]
OpG10H	短小蛇根草	毛状根	35S/AtU6	1	插入/缺失	喜树碱(camptothecin)	下降	[32]
OpSLS	短小蛇根草	毛状根	35S/AtU6	1	插入/缺失	喜树碱	下降	[32]
OpLAMT1	短小蛇根草	毛状根	35S/AtU6	1	插入/缺失	喜树碱	下降	[33]
OpNAC1	短小蛇根草	毛状根	35S/AtU6	1	插入/缺失	喜树碱	上升	[33]
OpWRKY6	短小蛇根草	毛状根	35S/AtU6	1	缺失	喜树碱	上升	[34]
HSS	聚合草	植株	AtU6	3、7、8	插入/缺失	吡咯里西啶类生物碱(pyrrolizidine alkaloids)	下降	[35]
AbH6H	颠茄	植株	35S/AtU6	2	插入/缺失	莨菪碱(hyoscyamine)	上升	[36]
NtERF189	烟草	植株	–	–	插入/缺失	尼古丁(nicotine)	下降	[37]
NtERF199	烟草	植株	–	–	插入/缺失	尼古丁	下降	[37]
IFS1	红车轴草	植株	–	–	缺失	异黄酮(isoflavones)	下降	[38]
NtMYB4a	烟草	植株	35S	1、3	插入/缺失/替换	花青素(anthocyanins)	下降	[39]
SnAN2	龙葵	植株	–	–	插入/缺失	花青素	下降	[40]
RcMYB3	洋地黄	植株	–	–	–	花青素	下降	[41]
C3H、*C4H*、*4CL*、*CCR*、*IRX*	铁皮石斛	植株	35S/OsU3	–	插入/缺失/替换	木质素(lignin)	下降	[42]
SmRAS	丹参	毛状根	35S/AtU6、OsU3	–	–	丹酚酸	下降	[43]
SmLAC	丹参	毛状根	AtUBQ/AtU6	–	插入/缺失	木质素 丹酚酸	下降	[44]
SmHPPD	丹参	毛状根	–	–	缺失/替换	丹酚酸	上升	[45]

含量与对照相比显著下降[21]. 研究发现, 植物激素脱落酸(abscisic acid, ABA)促进丹参中丹参酮的积累. *SmbZIP1*是从丹参ABA差异转录组中筛到的转录因子, 可能参与调控丹参酮的合成. 构建敲除载体遗传转化丹参, 由35S驱动Cas9, AtU6驱动sgRNA, 敲除成功的概率为12%, 表现出碱基缺失和替换两种突变类型[22]. 敲除毛状根中丹参酮含量上升, 证实*SmbZIP1*负调控丹参酮的功能. Shi等人[23]从丹参ABA差异转录组中筛选到两个可能参与调控丹参酮合成的转录因子: *SmWRKY34*和*SmbZIP3*. 构建敲除株系, *SmWRKY34*株

系表现出替换和缺失两种突变类型，丹参酮比对照增加3倍，*SmbZIP3*株系在靶标序列发现碱基缺失，丹参酮和丹酚酸含量均下降. 进一步研究表明，*SmWRKY34*可以通过结合*SmbZIP3*启动子中的W-box，抑制其转录活性，影响丹参活性成分含量. 以上结论证实SmWRKY34-SmbZIP3模块介导丹酚酸和丹参酮生物合成. JAZ蛋白已被报道负调控茉莉酸介导的丹参酮合成[24]. Liu等人[24]构建*SmJAZ9*的敲除毛状根株系，其中位于第一外显子的sgRNA由AtU6驱动，而Cas9由AtUBQ驱动. 阳性敲除株系表现出丹参酮含量上升. 筛选获得SmJAZ9的互作蛋白SmMYB76. *SmMYB76*的敲除株系中丹参酮含量上升，而*SmJAZ9*促进*SmMYB76*转录水平的表达. 以上结果证实，SmJAZ9-SmMYB76模块负调控丹参酮的合成. 借助CRISPR/Cas9技术，*bHLH60*等转录因子也被证明调控丹参酮生物合成[25].

三萜是传统中药材人参中的主要活性成分，存在两种形式: 原人参二醇(protopanaxadiol, PPD)型和原人参三醇(protopanaxatiol, PPT)型皂苷. 在人参中, *PPTa*及其同源序列*PPTg*均能编码PPT合酶, 后者催化PPD转化为PPT. 位于第一外显子中的sgRNA1和sgRNA4是*PPTa*和*PPTg*共有的靶标. CRISPR/Cas9编辑后获得了3个纯合突变株系和2个杂合株系[26]. 这些株系表现出插入和缺失两种突变类型, 较野生型相比PPT型皂苷成分减少、PPD型皂苷成分增加, 而总皂苷成分没有明显变化[26].

三七皂苷类成分是三七的主要药效成分，具有活血、降血压等药理活性. 研究表明, 三七主根中三七皂苷的含量显著高于支根. 对三七的主根及支根进行转录组测序及分析, *PnMYB4*被预测可能参与调控三七皂苷的合成. 利用CRISPR/Cas9系统对三七*PnMYB4*进行精准编辑，突变株系有2~3个碱基替换，敲除株系中三七皂苷含量上升，表明*PnMYB4*负调控三七皂苷的生物合成[27].

灵芝酸是一种来源于灵芝的三萜物质. 由于缺乏对灵芝中RNA聚合酶Ⅲ启动子的了解，很大程度限制了CRISPR/Cas9技术的应用[71]. 2020年, Wang等人比较预测得到的5个内源性的启动子(pU6-0、pU6-1、pU6-2、pU6-3、pU6-4), 结果显示pU6-3启动子具有更高的编辑效率. 为了实现gRNA精确的转录终止，Wang等人[72]在gRNA的3′末端引入肝炎病毒(hepatitis delta virus, HDV)核酶，成功构建了pU6-3-gRNA-HDV系统. 将该系统应用于2个CYP基因，*cyp5150l8*(参与灵芝酸的生物合成)和*cyp505d13*(参与角鲨烯型三萜的生物合成)，碱基的缺失和替换使基因突变，造成灵芝酸含量下降.

*Dzfps*基因编码的合成酶催化盾叶薯蓣(*Dioscorea zingiberensis*)中三萜前体FPP形成[28]. Feng等人[28]在*Dzfps*的第一外显子设计靶标，由水稻的OsU3驱动表达. 60%株系的靶标序列表现出碱基缺失，且角鲨烯含量明显减少. 此外, Zhou等人[73]以酵母为底盘细胞，利用CRISPR/Cas9技术验证了雷公藤(*Tripterygium wilfordii*)*TwSEs1-4*基因(角鲨烯环氧化酶)催化角鲨烯形成环氧角鲨烯的能力.

类胡萝卜素是一类四萜化合物，具有抗氧化、抗癌、延缓衰老等功效. 八氢番茄红素脱氢酶(phytoene desaturase, PDS)是类胡萝卜素生物合成途径中的限速酶. 因敲除植物体内的*PDS*基因会引起白化现象, 所以其常被用来作为检测基因编辑体系成功与否的标记[74~76]. Zhang等人[29]在大麻(*Cannabis sativa*)中设计了6个靶向*CsPDS*的sgRNA. 位于第6外显子中的sgRNA获得了83个嵌合体和4个纯合体，这些突变株系表现出6种基因缺失类型，均呈现出白化现象. 经过两轮筛选后，仅有5个株系表现出T-DNA插入，表明一些突变株系并未实现真正的基因编辑. 左鑫等人[30]将CRISPR/Cas9技术成功应用于天目地黄(*Rehmannia chingii*), 其sgRNA由拟南芥的AtU6启动子驱动, Cas9由35S驱动. 天目地黄*RcPDS*第四外显子上靶标的成功敲除获得了完全/嵌合白化株系，其中以碱基缺失形式的编辑类型最多.

1.2 CRISPR/Cas9技术在生物碱类物质生产中的应用

生物碱存在于约20%的植物中, 包括异喹啉类生物碱、吲哚类生物碱、吡啶类生物碱、莨菪烷类生物碱和有机胺类生物碱，具有广泛的药理活性[77~82]. 例如, 秋水仙碱可抑制痛风，麻黄碱可用于治疗习惯性支气管哮喘和预防哮喘发作[83,84]. 生物碱的生物合成一般都以氨基酸作为初始前体，在一系列酶的催化作用下生成不同产物. 两种酪氨酸衍生物4-羟基苯乙醛(4-hydroxyphenylacrtaldehyde)和多巴胺(dopamine)在去甲乌药碱合成酶(norcoclaurine synthase, NCS)、4′-甲基转移酶(4′-methyltransferase, 4′OMT)等酶的连续催化下生成中间体(*S*)-金黄紫堇碱[85~87]. (*S*)-金黄紫堇碱在四氢化原小檗碱-*cis*-N-甲基转移酶(tetrahydroprotoberberine-

cis-*N*-methyltransferase, TNMT)、P450依赖型*N*-甲基刺罂粟碱14-羟化酶(P450-dependent *N*-methylpapaverine 14-hydroxylase, MSH)等酶催化下形成血根碱(sanguinarine); 在(*S*)-金黄紫堇碱9-*O*-甲基转移酶((*S*)-corydaline 9-*O*-methyltransferase, SOMT)、(*S*)-四氢小檗碱合酶((*S*)-tetrahydroberberine synthase, TDC)催化下形成白屈菜红碱(chelerythrine)[88,89]; 在SOMT的作用下得到四氢非洲防己碱, 后者在细胞色素P450(cytochromes P450, CYP719A19)和四氢原小檗碱氧化酶((*S*)-tetrahydroprotoberberine oxidase, STOX)催化下生成小檗碱(berberine)[90]. CoOMT催化四氢非洲防己碱转化为延胡索乙素(tetrahydropalmatine). 异胡豆苷(strictosidine)是单萜吲哚类生物碱的关键中间体, 来源于环烯醚萜合成途径和吲哚合成途径[91]. 吲哚合成途径以分支酸为前体, 最终转化为色胺. 环烯醚萜途径以IPP/DMAPP为前体, 在香叶醇-10-羟化酶(geraniol 10-hydroxylase, G10H)、开环马钱子苷合成酶(secologanin synthase, SLS)、马钱子酸甲基转移酶(loganic acid methyltransferase, LAMT)等酶的催化下形成开环马钱子苷[92]. 开环马钱子苷和色胺在异胡豆苷合酶(strictosidine synthase, STR)的催化下形成异胡豆苷[93]. 异胡豆苷在异胡豆苷-β-D型葡萄糖苷酶(strictosidine β-D-glucosidase, SGD)等酶作用下形成长春碱, 而经过氧化、还原、糖基化等反应可得到喜树碱[94,95]. 高珂等人[96]综述了AP2/ERF、WRKY、bHLH、bZIP、ZCT、WD40类转录因子调控生物碱类次生代谢物的研究进展.

罂粟(*Papaver somniferum*)富含多种可应用于生物医药领域的苄基异喹啉类生物碱(benzylisoquinoline alkaloids, BIAs), 例如用于止咳的可待因、抗癌的诺斯卡平、抗痉挛的罂粟碱、镇痛的吗啡等[31]. 这些生物碱的生物合成都可追溯到中间产物(*S*)-牛心果碱(*S*-reticuline), 由4′OMT催化去甲乌药碱形成. Alagoz等人[31]构建靶向4′*OMT2*序列的敲除载体, 得到序列突变范围为1~4 bp插入/缺失的阳性株系. 这些株系中不仅BIAs成分(吗啡、可待因、诺斯卡平、*S*-牛心果碱、蒂巴因、劳丹素、罂粟碱)显著减少, 而且还有新型的未鉴定生物碱成分.

喜树(*Camptotheca acuminata*)与短小蛇根草(*Ophiorrhiza pumila*)均可产生单萜吲哚类生物碱喜树碱. Shi等人[32]在2个喜树碱合成酶基因*OpG10H*和*OpSLS*的第一外显子中设计了sgRNA, 由AtU6驱动. 敲除成功的转基因毛状根株系中CPT含量明显减少. Hao等人[33]报道了短小蛇根草的高质量基因组, 结合转录组分析, 鉴定了能将马钱子酸转化为马钱子素的O-甲基转移酶 OpLAMT1. 获取了*OpLAMT1*基因的敲除毛状根株系, 含量检测结果表明, OpLAMT1参与了马钱子素和喜树碱的生物合成. 此外, 在*OpNAC1*的靶标序列中产生碱基缺失和插入的株系, 喜树碱含量上升, 与过表达株系中喜树碱含量下降趋势一致, 表明*OpNAC1*负调控喜树碱生物合成的功能[33]. Wang等人[34]从短小蛇根草的基因组中筛选到46个WRKY家族成员, 通过喜树碱生物合成途径基因与*OpWRKY*基因的相关性分析, *OpWRKY6*被推测可能参与生物碱生物合成调控. 与对照相比, 3个碱基缺失的敲除株系(1个纯合、2个杂合)中喜树碱含量和产量显著增加, 表明*OpWRKY6*负调控喜树碱的生物合成[34].

吡咯里西啶类生物碱(pyrrolizidine alkaloids, PAs)是存在于部分中草药中的一类毒性成分. 因其毒性的存在, 很多含有此类成分的中草药都被限制内用, 例如紫草(*Lithospermum erythrorhizon*)、聚合草(*Symphytum officinale*)等. 同亚精胺合酶(homospermidine synthase, *HSS*)负责PAs生物合成的第一步催化, Zakaria等人[35]在聚合草*hss*基因的第3、7和8外显子中设计了sgRNA, sgRNA由拟南芥的AtU6驱动. 敲低株系的靶标序列产生插入和缺失2种突变. 只编辑2个等位基因中的一个同亚精胺合酶和PAs含量显著降低, 而2个等位基因均被编辑的株系中检测不到生物碱成分. 补充同亚精胺到*hss*敲除株系中, 再一次检测到PAs, 证明CRISPR/Cas9方法在验证基因功能及分子育种中的巨大潜力[35].

莨菪碱及其衍生物山莨菪碱和东莨菪碱均属于莨菪烷类生物碱, 三者虽然结构相似, 但却可用于不同疾病的治疗[97]. 颠茄(*Atropa belladonna*)是3种莨菪烷类生物碱成分的主要来源, 但由于含量较低且三者结构相似, 想从颠茄中分离莨菪烷类成分难度大且成本高. 莨菪碱-6β-羟化酶(hyoscyamine 6β-hydroxylase, H6H)是一种双功能加氧酶, 既能将莨菪碱转化为山莨菪碱, 也能环氧化山莨菪碱形成东莨菪碱. Zeng等人[36]利用CRISPR/Cas9技术敲除*AbH6H*第二外显子的靶标序列, 纯合突变株系中莨菪碱含量升高, 未产生山莨菪碱和东莨菪碱. 为实现颠茄中莨菪碱成分的分离提供了参考方法. Hasebe等人[98]利用基因敲除的方法验证了颠茄中*Abpyks*催化莨菪烷骨架构建的重要功能.

来源于烟草叶片的烟碱属于吡啶类生物碱, 一对

响应茉莉酸的AP2/ERF家族转录因子*NtERF189*和*NtERF199*被鉴定可能参与尼古丁的生物合成[37]. 为证明二者的功能, Hayashi等人[37]在2个ERF基因相同区域设计3个靶标序列, 引入用于表达多重sgRNA的载体. 通过农杆菌介导的转化, 获得了长度不等的碱基插入和缺失的株系. 与对照相比, 突变体叶片的生物碱含量下降到2%~4%, 根部的生物碱含量下降到13%~27%.

1.3 CRISPR/Cas9技术在苯丙烷类物质生产中的应用

苯丙烷类代谢物包括酚酸类、黄酮类和香豆素及其衍生物等, 具备抗氧化、抗炎等活性[99,100]. 柚皮苷、槲皮素均具有抗动脉粥样硬化作用[101,102]. 香豆素及其衍生物具有抗病毒、抗凝血等功能[103]. 苯丙烷代谢途径始于苯丙氨酸(phenylalanine), 在苯丙氨酸解氨酶(phenylalnine ammonialyase, PAL)、肉桂酸-4-羟化酶(cinnamic acid 4-hydroxylase, C4H)、4-香豆酸辅酶A连接酶(4-coumaric acid coA ligase, 4CL)的连续催化下生成苯丙烷途径的共用中间体——4-香豆酰辅酶A(4-coumaroyl-CoA)[104~106]. 4-香豆酰辅酶A(4-coumaroyl-CoA)与丙二酰辅酶A(malonyl coenzyme A)在查尔酮合酶(chalcone synthase, CHS)、异黄酮合成酶(isoflavone synthase, IFS)、查尔酮异构酶(chalcone isomerase, CHI)、黄烷酮3-羟化酶(flavanone 3-hydroxylase, F3H)、二氢黄酮醇-4-还原酶(dihydroflavonol-4-reductase, DFR)、花青素合成酶(anthocyanidin synthase, ANS)、糖基转移酶(glycosyltransferase, GTs)等酶作用下生成异黄酮、黄烷酮、二氢黄酮醇、花青素等黄酮类物质[107~111]. 以4-香豆酰辅酶A为前体, 在肉桂酰辅酶A还原酶(cinnamyl CoA reductase, CCR)等酶的催化下, 4-香豆酰辅酶A转化为H型木质素的单体. 4-香豆酰辅酶A在羟基肉桂酰-CoA 莽草酸/奎宁酸羟基肉桂酰转移酶(hydroxycinnamyl CoA shikimic acid/quinic acid hydroxycinnamyl transferase, HCT)的引导下进入S型和L型木质素的合成途径. 在对香豆酸-3-羟化酶(P-coumarate 3-hydroxylase, C3H)、肉桂酰辅酶A还原酶(cinnamoyl-CoA reductase, CCR)等酶的催化作用下得到S型木质素和G型木质素[106,112]. 研究表明, AP2/ERF、WRKY、bHLH、bZIP、MYB和NAC等转录因子家族介导药用植物活性成分合成调控[113,114].

已有研究表明, 豆科植物的根瘤固氮现象与其产生的黄酮类物质有关. 红车轴草(*Trifolium pratense*)根、茎、花中富含异黄酮类物质, 且具有根瘤固氮现象, 但很少研究证明二者之间的关系. 借助CRISPR/Cas9技术, Dinkins等人[38]将IFS的功能破坏, 结果显示突变植株中异黄酮类物质含量显著减少, 但并未影响根部结节的产生.

花青素是广泛存在于植物中的一种黄酮类物质, *NtMYB4a*调控烟草中的花青素的积累[39]. 蒋悦等人[39]分别在*NtMYB4a*第1和第3外显子设计靶标Y1和B1, Cas9蛋白由35S启动子驱动, 构建CRISPR/Cas9系统的双靶标敲除载体. 利用农杆菌介导进行转化, 获得29株阳性植株(阳性率24.37%). 其中15株在靶点和非靶点处突变不一致, 呈现4种突变类型. 突变株系相较于野生型株高下降、花色变浅、花青素含量减少和花青素合成相关酶基因*DFR*、*ANS*、*4CL*、*C4H*、*PAL*表达量下调[39]. 龙葵(*Solanum nigrum*)黑色果实中富含花青素, 具有作为食物使用的潜力. Heo等人[40]对龙葵的黑色果实进行转录组测序, 筛选到显著富集的R2R3-MYB转录因子*SnAN2*. *SnAN2*可能是参与龙葵果实中花青素合成的潜在因子. 在*SnAN2*的5′区域设计4个sgRNA, 敲除株系因不能产生花青素, 果实呈现黄色和绿色. 地黄(*Rehmannia glutinosa*)是亚洲最受欢迎的草药之一. 为揭示3种地黄物种中花青素组成和含量差异的原因, Zuo等人[41]对其进行转录组测序后发现, *RcMYB*3可能通过激活花青素合成酶(anthocyanidin synthase, ANS)基因表达来调控花青素合成的靶基因. 利用CRISPR/Cas9技术敲除*RcMYB*3基因, 编辑成功的株系中花冠裂片减少、花青素含量降低, 以上结果证明了*RcMYB*3正调控花青素合成的功能.

Kui等人[42]在药用植物铁皮石斛(*Dendrobium officinale*)中构建并优化了CRISPR/Cas9技术体系, 以35S驱动Cas9蛋白的表达, 水稻OsU3驱动sgRNA的表达. 利用该系统编辑了木质素生物合成途径5个关键酶(C3H、C4H、4CL、CCR、IRX)基因序列, 阳性株系有插入、缺失和替换3种突变类型, 编辑成功的概率在10%~100%之间, 展现出分子遗传学研究中的应用潜力[42].

丹酚酸B(salvianolic acid B, SalB)是药用植物丹参的主要次生代谢物之一, 属于酚酸类物质. 迷迭香酸(rosmarinic acid, RA)是丹酚酸B的合成前体, 其在植物体内的合成途径已被解析, 但RA如何转化为SalB还尚不清楚. Zhou等人[43]在丹参中筛选到可能参与RA生物合成的候选迷迭香酸合酶(rosmarinic acid synthase,

RAS)，利用35S启动Cas9蛋白的表达，2个启动子AtU6、OsU3驱动sgRNA的表达. 突变体中RA和SalB含量显著下降，而RA合成前体3,4-二羟基苯乳酸的含量增加. 漆酶家族成员被推测可能参与RA到SalB的转化[115]. Zhou等人[44]在29个丹参漆酶基因(*SmLAC*)的保守结构域中设计两个敲除靶标，由AtU6启动子驱动表达. 突变株系表现出生长发育受阻、毛状根木质部细胞变大和变松. 另外, SalB含量及木质素含量在突变株系中均显著下降. 上述研究结果证明了*SmLAC*影响丹参丹酚酸和木质素合成的功能. 丹参4-羟基苯丙酮酸双氧化酶(4-hydroxyphenylpyruvate dioxidase, HPPD)与SalB合成途径中的4-羟基苯丙酮酸还原酶(4-hydroxyphenylpyruvate reductase, HPPR)竞争底物. Hu[45]根据HPPD序列设计了6个靶标(H1、H2、H3、H4、H5、H6)，成功获得H2、H3、H4的编辑毛状根. 检测了*HPPD*的7个编辑株系中RA和SalB的含量，编辑毛状根中两种成分的含量均增加. 可见，利用CRISPR/Cas9基因编辑技术敲除旁支代谢途径基因，可以有效增加活性成分的含量.

CRISPR/Cas9作为应用最广泛的基因编辑技术之一, 已经成功应用于多种药用植物. 操作简单、效率高和周期短等优点使CRISPR/Cas9技术成为解析未知基因功能和代谢途径的有力工具. 药用植物基因组学和分子生物学技术的迅速发展为CRISPR/Cas9基因编辑提供了越来越多的靶基因和技术支持. 未来，CRISPR/Cas9技术结合高质量的基因组学将助力更多功能基因的挖掘，而在合成生物学、遗传改良和种质创新等方向也期待得到更多的应用与完善.

2 总结与展望

2.1 CRISPR/Cas9技术应用于研究基因功能

CRISPR/Cas9通过靶向特定基因, 实现基因的定点敲除、敲入和突变，从而揭示了基因在生长、发育、代谢和抗逆等生物学过程中的作用. 近年来, 多种药用植物高质量基因组已被发布，越来越多的潜在功能基因可以作为CRISPR/Cas9系统的靶基因. 但许多药用植物的基因组杂合度非常高，很难进行深入的基因组分析及功能基因的挖掘. 此外, 由于药用植物具有复杂的代谢调控网络，很多合成酶基因具有底物杂泛性和功能冗余, 因此在验证基因功能时, 需敲除某家族的多个基因来检验效果, 这使得CRISPR/Cas9系统在药用植物中的应用依旧具有挑战性.

2.2 CRISPR/Cas9技术应用于次生代谢物的微生物生产

虽然已经利用CRISPR/Cas9技术在微生物系统实现了多种药用植物次生代谢物的工程化生产，但Cas9蛋白对于某些微生物如蓝细菌却具有毒性，而Cas12蛋白相较于Cas9蛋白毒性较低，具体的机理尚不可知[116~119]. 因此，可开发更多适合次生代谢物生产的微生物细胞及找到适配的CRISPR/Cas系统来高效生产高价值的次生代谢物.

2.3 CRISPR/Cas9技术应用于药用植物品种选育

药用植物在生长过程中会面临多种环境胁迫，且会在适应环境的过程中具备相应能力, 例如, 生长在喀斯特的地枫皮(*Illicium difengpi*), 对于干旱/极端干旱的耐性较强, 是研究植物适应极端干旱的代表性材料[120]. 生长在青藏高原低温地区的马蔺(*Iris lactealacteal pall*)、问荆(*Equisetum arvense*)等，具备极强的抗低温适应能力[121]，利用CRISPR/Cas9技术挖掘药用植物关键抗逆基因，可为改善中药材品质和加速药用植物的驯化提供重要助力.

2.4 CRISPR/Cas9技术编辑效率的提高及工具的递送

目前, CRISPR/Cas9技术仍旧具有编辑效率低和工具的递送较困难等问题. PAM序列的依赖性是CRISPR/Cas9编辑效率较低的原因之一. 不同来源的CRISPR/Cas9系统识别不同的PAM序列，可一定程度缓解PAM序列的依赖性，因此可尝试在药用植物中使用不同物种来源的PAM序列[122,123]. 在编辑药用植物基因时，常通过基因枪法和农杆菌介导将CRISPR/Cas元件递送到细胞中, 而这两种方法都需要组织培养的程序, 严重制约了CRISPR/Cas系统在药用植物中的应用. 因此需要开发不通过组织培养即将CRISPR/Cas9递送至靶细胞的方法, 实现更高效率的基因编辑.

综上, CRISPR/Cas9是一项简便、高效的基因编辑技术，已成功在多种药用植物中应用. CRISPR/Cas9及其衍生技术在其他作物中的应用，也为药用植物建立完善的基因编辑体系提供了宝贵的经验. 因此，CRISPR/Cas9是助力药用植物次生代谢物生产、品种改良和种质创新等的重要力量.

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Summary for “CRISPR/Cas9技术在药用植物中的应用与展望”

Application and prospect of CRISPR/Cas9 technology in medicinal plants

Ruiyan Zhu[1,2], Ying Xu[2], Min Shi[2*] & Guoyin Kai[2*]

[1] *College of Horticulture, Shenyang Agricultural University, Shenyang 110866, China*
[2] *College of Pharmacy, Zhejiang Chinese Medicine University, Hangzhou 310053, China*
* Corresponding authors, E-mail: kaiguoyin@163.com; min_shi@zcmu.edu.cn

China is one of the countries with the richest resources of medicinal plants, and the discovery, use and cultivation of medicinal plants have a long history. Medicinal plants are one of the oldest forms of medicine, and since ancient times, people have used medicinal plants as a source of food, healing and industry. Secondary metabolites are a class of non-essential small-molecule organic compounds produced by secondary metabolism for the normal functioning of cell life activities or plant growth and development, and their production and distribution are usually species, organ, tissue, and growth and development period specific. The content of secondary metabolites is an important index to determine the quality of medicinal plants, and secondary metabolites from medicinal plants have become potential clues to explore new drugs to improve human mortality and morbidity. Due to the long growth cycle of medicinal plants and their local nature, the accumulation of secondary metabolites is spatiotemporally specific, which makes the content of secondary metabolites in medicinal plants low, thus limiting their application in medicine. Classical molecular biology techniques such as overexpression and gene silencing have been successfully applied to regulate plant secondary metabolism pathways to enhance the production of valuable secondary metabolites. So far, protein-based editing tools, namely zinc finger nucleases (ZFNs) and transcription activator-like endonuclease enzymes (TALENs) have been promoted for transcriptional genome manipulation. Cluster of regularly spaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas) is the third generation gene editing tool after the ZFNs and TALENs, and is a cheap, simple and efficient gene editing technique. This technology mainly uses sequence-specific nucleases to identify and cut the target sites on the genome, causing DNA double strand breaks (DSBs), and further induces two repair mechanisms, Nonhomologous end-joining (NHEJ) and homology-directed Repair (HDR), to repair the broken DNA double strand, achieving site-specific substitutions, insertions and deletions, thus affecting gene expression and function. It is widely used in metabolic engineering, synthetic biology and medical research. At present, CRISPR/Cas9 technology has become an important tool for exploring gene function and analyzing metabolic pathways in medicinal plants, and plays a crucial role in improving the production of secondary metabolites and improving the quality of medicinal plants. Compared with crops and horticultural plants, the application of CRISPR/Cas9 in medicinal plants still faces problems such as low editing efficiency and small application range. This study briefly describes the principle of CRISPR/Cas9 technology, focuses on the application of CRISPR/Cas9 technology in the three main secondary metabolites: terpenoids, alkaloids and phenylpropanoids, and enumerates the studies on CRISPR/Cas9 technology for exploring gene function and analyzing metabolic pathways in medicinal plants. This paper summarizes the application of CRISPR/Cas9 technology in the analysis of secondary metabolic pathways and secondary metabolite production, and points out that the application of CRISPR/Cas9 technology in medicinal plants faces problems such as wide application, PAM dependence, delivery efficiency and off-target effect. Finally, the research directions of CRISPR/Cas9 technology for secondary metabolite production, variety improvement and germplasm innovation of medicinal plants were prospected. This review is expected to provide references for the widespread application of CRISPR/Cas9 technology in the field of medicinal plants.

medicinal plants, secondary metabolites, CRISPR/Cas9, biosynthesis, transcriptional regulation

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