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Research on the application of CRISPR/Cas9 Gene editing technology in traditional Chinese medicine

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Abstract. Traditional Chinese medicine (TCM) is the collective term for the medical practices of all ethnic groups in China (both Han Chinese and ethnic minorities), reflecting the Chinese understanding of life, health, and illness over thousands of years. In addition, traditional herbal medicine has demonstrated its effectiveness in treating complex diseases that have challenged modern Western medicine, such as the use of Artemisia annua extracts to treat malaria, preparations based on traditional Chinese pharmacology to combat large-scale influenza outbreaks, and herbal preparations as an adjunctive therapy to cancer treatment. Currently, the development of traditional Chinese medicine is facing problems such as the loss of prescription knowledge and the degradation of the quality of cultivated species of herbs. This paper uses a literature review to analyze the application of CRISPR/Cas9 gene editing technology in medicinal plants. It also proposes directions that can solve the challenges facing traditional Chinese medicine based on the arrangement of existing literature, and foresees the future application of CRISPR/Cas9 gene editing technology in medicinal plant editing.

Keywords: CRISPR/Cas9, gene function, Traditional Chinese medicine, disease resistance and stress resistance, molecular breeding

1. Introduction

Traditional Chinese medicine (TCM) has shown remarkable potential in addressing some of the challenges of modern medicine, such as cancer and large-scale infectious diseases, as well as its use of natural raw materials that are readily available and relatively low-cost, making it more applicable to the current state of health care in developing country environments. However, traditional Chinese medicine currently faces multiple challenges, the first of which is the dilemma of knowledge transmission and breeding. Influenced by the feudal and paternalistic system, many valuable TCM prescriptions have been lost due to limited inheritance, making modern TCM research largely dependent on medical literature found in archaeological sites. Meanwhile, the surge in market demand for herbal medicines has led to a strain on wild plant resources, making it difficult to satisfy market demand [1]. Even mass production through modernized cultivation methods has triggered problems such as quality degradation and species degradation, and the content of their active ingredients is much lower than that of wild plants. This situation challenges the stability and quality of the efficacy of TCM.

Literature reviews and related studies on the application of CRISPR/Cas-9 technology for cash crops and horticultural crops have entered a stage of systematic development. However, there is rarely relevant literature on research in herbal medicine. Therefore, this paper organizes the literature on CRISPR/Cas-

9 technology and its application in medicinal plants. In addition, while combining the pathological studies of traditional Chinese herbs in fighting cancer and novel coronaviruses, the prospects of traditional Chinese herbs and the potential of CRISPR/Cas-9 technology in improving medicinal plants are discussed, which can provide a reference for future in-depth research.

2. Application of CRISPR/Cas9 gene editing technology in TCM

2.1. CRISPR/Cas9 Gene editing

CRISPR/Cas9 technology originated from a study targeting the IAP gene in Escherichia coli [2]. This study revealed short palindromic repeats spaced regularly in clusters, which were subsequently named CRISPR. Researchers found this particular sequence in other bacteria and archaea and confirmed that Streptococcus thermophilus rapidly resisted phage invasion using the CRISPR/Cas system. Subsequently, the principles of the CRISPR/Cas9 system were systematically elaborated and it was confirmed that Cas9 is a DNA endonuclease guided by two RNAs. The system has great potential for gene function editing.

On this basis, the precise editing of endogenous genomic loci in human and mouse cells were successfully realized by designing two different type II CRISPR/Cas9 systems. And the technology was utilized to complete the protoplast transformation of Arabidopsis thaliana and tobacco of Benjamin [3], as well as the editing and targeted gene mutation of rice genome [4]. As a result, CRISPR/Cas9 technology has been gradually applied to genome editing of plants, animals and microorganisms.

Based on the differences in the sequences of Cas-encoded genes, CRISPR/Cas systems are categorized into types I, II and III. The CRISPR/Cas9 system, which is widely used today, was developed and adapted from the type II system. This system includes Cas9 nucleic acid endonuclease, CRISPR RNA (crRNA), and trans-activated crRNA (tracrRNA). After improvement, Jinek et al. composed crRNA and trans-activated crRNA into single-stranded guide RNA (sgRNA) [5]. This system only needs to design a 20bp target site and synthesize a gRNA expression frame, which is easy to construct and can simultaneously realize multiple sites of a target gene and editing of multiple target genes. The specific principle of implementation is that the CRISPR/Cas9 system generates DSBs and activates the cellular DNA repair mechanism by recognizing the PAM, (the target gene contains a proto-spacer sequence neighboring motif (PAM) consisting of a few bases), and shearing it upstream. DSBs have two repair mechanisms: homologous recombination repair (HDR) and non-homologous end joining (NHEJ). Cells mainly repair DSBs through NHEJ to realize the loss of gene function. In contrast, the presence of homologous sequence templates activates the precise HDR repair pathway, realizing precise gene knock-in, deletion, replacement or mutation. This advanced and efficient gene editing technology is expected to make more breakthroughs in the fields of medicine and botany.

2.2. CRISPR/Cas9 gene editing in plants

In the detailed process of CRISPR/Cas9 gene editing in plants, it is first necessary to obtain the target gene sequence [6]. For species for which whole genome data are available, full-length gene and coding sequences can be retrieved from databases to ensure that exonic sequence regions are identified. Considering the limited availability of complete genome data for many medicinal plants, a stepwise approach can be used to obtain the genome sequences of the target genes. The genome sequence of the target gene is obtained gradually through techniques such as rapid cloning of the cDNA termini and chromosome walking, and the genomes of related plant species are referred to in order to identify exonic regions. sgRNA (single guide RNA) sites were designed for the target genes using online software tools and their off-target risks were simultaneously assessed. Synthetic sgRNA expression vectors and Cas9 genes are constructed, often using methods such as Golden Gate assembly, and cloned into plant expression vectors. At this stage, plant transformation techniques, such as Agrobacterium-mediated transformation, are often used to introduce editing tools into the genes of medicinal plants. Once these steps are completed, mutant plants can be obtained. Genomic DNA is extracted from these mutants for positive identification. The mutation type is further identified by Sanger sequencing or high-throughput sequencing to generate mutant plant lines. This elaborate and systematic process ensures accurate editing of the target gene and generation of the desired plant line [7-8].

2.3. Gene function of medicinal plants

CRISPR/Cas9 technology, an emerging genome editing technology, has not yet established effective vector construction and genetic transformation systems in most medicinal plants. Therefore, researchers usually utilize known reporter genes or other special target genes to verify its effectiveness and feasibility.

2.3.1. Salvia miltiorrhiza (Danshen). Salvia miltiorrhiza (Danshen) is a commonly used Chinese herb enriched with diterpenoids and is a model plant for genetic engineering studies of medicinal plants. The primary bioactive constituents in Danshen include diterpenoids represented by tanshinones and phenolic acids represented by salvianolic acids. Research concerning tanshinones primarily involves the functional identification of cytochrome P450 enzymes in the downstream biosynthetic pathway. Li et al. used CRISPR/Cas9 to precisely knock out the key enzyme gene copalyl diphosphate synthase 1 (CPS1) involved in tanshinone biosynthesis in Danshen. Through Agrobacterium-mediated transformation, they obtained three homozygous mutants and eight chimeric mutants. Experimental results demonstrated that tanshinones were completely absent in homozygous mutants, while other phenolic metabolites were not significantly affected. Salvianolic acid B is a powerful scavenger of free radicals and has strong antioxidant activity. Hu Bei utilized CRISPR/Cas9 gene editing technology to knock out the side-pathway gene SmHPPD involved in salvianolic acid biosynthesis in Danshen hairy root cultures. By reducing the expression of this gene to minimize competing side-pathway synthesis, they promoted the accumulation of salvianolic acid compounds. This experiment validated the influence of the SmHPPD gene on the synthesis of salvianolic acid compounds and increased their content. In addition, Liu [9] found that phytohormone induction (e.g., MeJA) is an effective means to enhance the pharmacodynamic constituents in Salvia miltiorrhiza, including tanshinone and salicylic acid. By analyzing Danshen genomic and transcriptomic data, multiple transcription factors (e.g., bHLH, MYB, WRKY, and AP2/EARF) were revealed to be involved in the pathway of JA signaling, negatively regulating salicylic acid and anthocyanin biosynthesis. Specifically, the SmJAZ9-SmMYB76 complex negatively regulates salvianolic acid biosynthesis. And the SmbHLH60-SmMYC2 complex antagonistically regulated salvianolic acid and anthocyanin biosynthesis, providing new insights into the novel mechanisms of the JA signaling pathway in the regulation of plant metabolism.

2.3.2. CRISPR/Cas9 in medicinal plants. Traditional crossbreeding involves selecting plants with superior genetic phenotypes among various plant varieties and combining favorable genes through crossbreeding to achieve increased crop yields and resistance. However, this approach suffers from long breeding times, complex processes and uncertain results. Exogenous transgenic breeding represents an emerging approach to breeding, but remains controversial due to current regulations and public concerns about its safety. CRISPR/Cas9 gene editing provides the means to precisely insert or knock out target genes at the genomic level, allowing rapid development of desired traits in plants. Also, the technology edits genes in a manner similar to natural variation, which is much more acceptable to the public.

Gene editing techniques in medicinal plants are mainly used to increase the content of bioactive compounds and enhance plant resistance. The DeCsl4 gene encodes a cellulose synthase that is highly expressed in Dendrobium stems. Hu Song et al. obtained a Csl4 mutant strain by constructing a vector to knockdown the DeCsl4 gene using the CRISPR/Cas9 technique, which reduced cellulose synthesis [10]. The lower cellulose content in Dendrobium stems facilitates the extraction of its bioactive components, which further effectively increases the content of active substances in herbal formulations. This study and direction also provide a basis for further research on other medicinal plants.

Enhancing the content of rare bioactive compounds in specific plants is another major concern in the improvement of medicinal plant breeding. Zeng et al. used CRISPR/Cas9 to knockdown the gene for butyramine 6β hydroxylase (H6H) in black currant willow, which is involved in the butyramine transformation pathway [11]. This resulted in a significant increase in butyramine content in H6H-pure mutant plants. However no atropine or butyramine was detected in their extract products.

3. TCM Anti-disease mechanism

3.1. *Anti-cancer mechanism*

Traditional Chinese medicine (TCM) has great potential for adjuvant therapy against cancer and has been successfully applied in clinical adjuvant therapy [12]. As an example of adjuvant therapy for digestive system tumors, Saikosaponin D is the major active compound in the Chinese medicinal herb Chaihu (Radix Bupleuri). It exhibits broad inhibitory effects on tumor cells. Wang et al. demonstrated that Chaihu saponin D could induce autophagic cell death in human hepatocellular carcinoma cells HepG2, SMMC 7721, and MHCC-97H by inhibiting the Akt/mTOR, AMP-activated protein kinase (AMPK)/mTOR, and rapamycin complex 1 (mTORC1) signaling pathways, as well as up-regulating the expression of LC3 and Beclin-1 [13]. In colon cancer SW480 cells, Saikosaponin D induces autophagy to reduce the accumulation of p62 protein and inhibit cell proliferation [14].

Chonglou saponin is primarily extracted from the dried rhizomes and roots of the lily family plant Polygonatum cyrtonema or Disporum sessile. It promotes mitochondrial autophagy and inhibits proliferation in human gastric cancer cells SGC-7901 by upregulating the expression of caspase-3. Additionally, Chonglou saponin can suppress the AMPK/mTOR signaling pathway, inducing autophagic cell death in human colon cancer HCT116 cells [15-16].

Curcumin is a natural phenolic antioxidant. It inhibits autophagic flow by blocking the degradation of autophagosomes in human gastric cancer AGS cells, thereby promoting cell death. Rhodopsin, a derivative of hydroxyanthraquinone, enhances autophagosome formation and ROS expression, upregulates autophagy levels and inhibits proliferation in colon cancer SW480 cells [17]. Picrasidine, derived from the legume Ginseng, induced autophagy in esophageal cancer Ec109 cells by down-regulating the PI3K/Akt/mTOR signaling pathway and by inhibiting cell proliferation [18].

3.2. *COVID-19*

Taking Lianhua Qingwen Granules/Capsules as an example, this traditional Chinese medicine compound formulation is a combination of three classic TCM prescriptions: Mahuang Shigantang, Dayuan Yin, and Yinqiao San, with modifications. It possesses the efficacy of clearing away heat and toxins, as well as dispersing lung heat. In in vitro experiments, researchers have found that Lianhua Qingwen Granules/Capsules exert a broad-spectrum effect against influenza viruses by inhibiting viral replication and regulating immune functions. Simultaneously, they can reduce cytokines associated with cytokine storms in a concentration-dependent manner. In addition, it inhibits COVID-19-induced expression of cytokines and chemokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-X-C chemokine ligand 10 (CXCL10) [19].

Molecular docking simulations suggest that active components in Lianhua Qingwen Granules/Capsules, such as baicalin and glycyrrhizic acid, possess potential ACE2-targeting activity. Compounds like forsythoside, quercetin, and rutin target 3CLpro, which can inhibit the replication of the novel coronavirus. Lianhua Qingwen, when combined with conventional treatment, can shorten the duration of flu-like symptoms, lead to faster improvement in pulmonary imaging results, and enhance the clinical efficacy in COVID-19 patients.

Table 1. Main common components of Lianhua Qingwen Granules/Capsules in the treatment of COVID-19 [19]

Active ingredient	Function
Liquirice	Targeting 3CLpro inhibits viral replication, prevents lung damage, and inhibits inflammation
Quercetin	Inhibits 3CLpro to inhibit new coronavirus replication and regulate immune function
Baicalein	Inhibits pathway-mediated inflammatory responses such as NF-κB
Forsythoside	Affects viral replication and reduces cytokines secreted by cells
Emodin	Inhibits the MAPK pathway, reduces oxidative stress and inflammatory response

4. Discussion

CRISPR/Cas9 technology still faces several limitations in its application to medicinal plants, but it holds immense potential and promising prospects for development. Through precise editing of plant genes, it is expected to address the problems of active ingredient reduction and species degradation at the root, thereby realizing therapeutic efficacy comparable to that of wild Chinese herbs in large-scale cultivation. In the current research, some medicinal plants have already completed the construction of gene editing systems, such as Salvia miltiorrhiza, ginseng, and dihuang. However, most of the bulk medicinal plants have not yet established a complete and efficient gene editing genetic transformation system. Research on gene function of medicinal plants and molecular breeding of medicinal plants is lagging behind, and gene function research is limited to a few model plants. In addition, the application of molecular breeding in medicinal plants is relatively small and the methodology is single.

CRISPR/Cas9 gene editing technology offers new hope for related research, especially in woody plants. Purity screening to verify functional genes is often done in model plants, healing tissues, or prokaryotic cells due to reasons such as long growth years and lack of adult plant phenotypes, which is simplified by obtaining pure mutants in the T0 generation by CRISPR/Cas9. As more medicinal plant genomes are analyzed in the future, CRISPR/Cas9 will be used in a large number of studies on functional genes and metabolic pathways. In addition, CRISPR/Cas multiple gene editing allows for simultaneous editing of multiple target sites in the genome, which greatly shortens the period and difficulty of obtaining higher-order mutants in research. Therefore, the authors believe that one of the most effective ways to address the current problem of declining herbal plant species and low levels of active ingredients is to construct synthetic models for synthesizing active compounds in herbal plants. This would allow targeted gene editing of herbal plants at the time of large-scale cultivation, achieving the same efficacy as wild plants while greatly reducing the number of plants required to produce pharmaceuticals.

Modern molecular biology techniques to study the function of active ingredients in clinically validated herbal preparations are essential for the modernization of the herbal system and its integration into the modern medical system. In the fight against cancer, Chinese medicines can induce tumor cells to undergo autophagy. Some TCM compounds, monomers and their active ingredients can regulate autophagy, thereby inhibiting the proliferation of tumor cells while enhancing sensitivity to radiotherapy, and thus exerting anti-cancer effects. In the treatment of neocoronitis, China has invested in the use of a variety of Chinese medicines and remedies, and has obtained very good results. It has shortened the treatment cycle of patients with neocoronitis pneumonia while reducing the complications of neocoronitis pneumonia, in addition to lowering the mortality rate of neocoronitis-infected patients.

Furthermore, enhancing modern biological explanations of TCM can significantly reduce public skepticism about TCM and promote its global dissemination and development through a scientific approach. The millennia-old prescriptions derived from TCM, which have been continuously improved through clinical practice, offer new hope for solving the challenges of modern medicine, such as cancer and widespread infectious diseases, which are difficult for modern medicine to address.

5. Conclusion

This paper reviews the basic ways in which the application of CRISPR/Cas9 gene editing technology of medicinal plants can solve the problems faced by TCM, and combines the existing successful applications and mechanistic analyses of TCM against cancer and mass infectious diseases to explore the potential of the traditional TCM system in depth.

However, this paper suffers from deficiencies in the breadth and content of the literature, ignoring some of the research in related fields, such as the yeast production of active ingredients of medicinal plants that have been successfully applied, and there are many regrets in the completeness of the content. As the relevant research on medicinal plants mainly focuses on traditional Chinese herbal medicines, there is an extreme lack of relevant English literature, and most of the cited literature is in Chinese, and there are some raw translations and paraphrases of proper nouns in the collation and translation of relevant Chinese literature.

Although the gene editing method of CRISPR/Cas9 is closer to natural mutation and has not been shown to be biologically hazardous in the current study, CRISPR/Cas9 gene editing technology may still be potentially biologically hazardous in the long-term study. Nevertheless, the author still believes that the development of CRISPR/Cas9 technology in medicinal plants has a broad development space and prospect. This technology will realize the sustainable use of medicinal plant resources, combined with the progressive research of traditional Chinese medicine, and ultimately provide a rich material and theoretical basis for human medical treatment and health care.

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