REVIEW

The Application of Biotechnology in Medicinal Plants Breeding Research in China*

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ABSTRACT Breeding is not only an important area of medicinal plants research but also the foundation for the superior varieties acquirement of medicinal plants. The rise of modern biotechnology provides good opportunities and new means for medicinal plants breeding research in China. Biotechnology shows its technical advantages and new development prospects in breeding of new medicinal plants varieties with high and stable yield, good quality, as well as stress-resistance. In this paper, we describe recent advances, problems, and development prospects about the application of modern biotechnology in medicinal plants breeding research in China.

KEYWORDS biotechnology, medicinal plants, breeding, application, China

In recent years, with the trend of return to nature and the improvement of people's living standards, the demand for natural herbal has surged.[1] However, the wild resources of medicinal plants are limited. Owing to years of excessive and unplanned excavation, not only were the resources destructed but also the growth environment was affected. Wild resources of medicinal plants are facing shortage crisis.[2] Besides, the cultivated varieties of medicinal plants have been degrading in the process of long-term cultivation which resulted in reducing the yield and quality of medicinal material.[3,4] It is necessary to carry out the research on the sustainable use of medicinal plants resources in order to protect the wild resources, and enrich germplasm resources. The sustainable use of medicinal plant resources is not only the need of the biological diversity conservation and the ecological balance maintenance, but also plays an important role for sustainable development of traditional Chinese medicine industry in China.[5]

Research on the breeding of superior varieties is essential to medicinal plants resources sustainable use. The rise of modern biotechnology provides good opportunities and new means for medicinal plants breeding research in China.[6] The rise of modern biotechnology was in the 1970s in China, which was rapidly developed in the last twenty years and has been widely used in agriculture,[7,8] food industry,[9,10] environmental protection[11,12] and so on. In the field of plant breeding, biotechnology was mainly applied to the crops in early period.[13-15] With the rapid development of molecular biology, bioengineering and other disciplines, and the matureness of plant cell engineering, genetic engineering and other technologies, biotechnology has been penetrated into various fields of medicinal plants research, and also promotes breeding research progress of medicinal plants in China.[16]

The modern biotechnology has broad application prospects for medicinal plant breeding research. In this paper, we describe the application of modern biotechnology from the advances of medicinal plants breeding research in China.

Application of Tissue Culture in Medicinal Plants Breeding

Since regenerate plant from single, isolated cell was obtained in 1965, tissue culture technology has been widely used in plant breeding.[17]

The Superior Varieties Asexual Rapid Propagation

Rapid propagation of medicinal plants by tissue

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culture can shorten the time to cultivate new varieties, and improve breeding efficiency. Propagation technology had been successfully applied in many medicinal plants. Kong, et al[18] achieved embryo germination, stem growth, bud rapid proliferation, and root germination via explants such as seeds, stem segments with axillary bud of *Ligustrun lucidum* Ait by tissue culture. Taking shoot tip as explant, Tao, et al[19] successfully implemented the rapid propagation of *Atractylodes macrocephala* Koidz. Gao, et al[20] also established a rapid propagation system of *Scutellaria baicalensis* Georgi with tender shoot tips, stem segments of test-tube plantlets from seed.

Germplasm Conservation

Germplasm resources are genetic material foundation for cultivating superior varieties. Currently, the majority of medicinal plants such as *Dioscorea batata*,[21] *Fritillaria cirrhosa*,[22] and *Dendrobium officinale*[23] are subcultured at room temperature through tissue culture. However, the subculture cycle is short at room temperature, which easily leads to contamination of materials. It is advantageous to extend preservation time, improve preservation effect by appropriately increasing the osmotic pressure of medium, supplementing with growth inhibitor, lowering culture temperature, under the condition of tissue culture. Through adjusting the contents of sucrose, mannitol, and supplementing with growth regulators, when *in vitro* buds of *Dioscorea zingiberensis* were inoculated onto Murashige and Skoog media, in an illuminated chamber at 15 ℃ for six months, the survival rate was over 60% with a good growth recovery and genetic stability.[24] The results of this study can be used for medium-term germplasm preservation *in vitro* of *D. zingiberensis*. In addition, the best survival rate of shoot tips with cryopreservation of *Rhodiola sachalinensis* was close to 100%, and the regenerated plants grew normally.[25]

Removal of Plant Viruses

Virus disease is an important factor which affects the yield and quality of medicinal plants. Significant reduction of yields caused by viral diseases, has become a major obstacle to the production of medicinal plants. Apical meristem culture usually has good effects of virus removal.[26,27] Virus-free plantlets can be obtained by plant apical meristem culture, and then through micropropagation, a lot of good seedlings with virus-free can be gotten for production application. For example, the excellent seedlings of *Rehmannia glutinosa* with virus-free could be obtained by apical meristem culture.[28] Furthermore, through research on virus removal technology of *Lilium brownii*, a good virus removal effect has been achieved.[29]

Protoplast Culture in Medicinal Plants Breeding

Protoplast, which was bare cell surrounded by wall membrane after removing cell wall, was obtained by mechanical preparation in early years.[30] However, only a small number of protoplasts could be obtained with species restrictions. The British scientist fullfilled large scale preparation of protoplasts for the first time by enzyme in 1960.[31] Protoplasts with totipotency intake foreign genetic material, organelle and other vectors easily. Bare protoplasts fusion does not only result in somatic cell hybrids but also creates a new way for plant breeding. Currently, a lot of researches on protoplast preparation, culture and fusion have been done.[32] In order to establish regenerate system of *Nervilia fordii*, Zhang, et al[33] researched on preparation and purification of protoplast from *N. fordii*, taking fresh leaves as materials; and a large number of complete and clear protoplasts with few cell debris were obtained. Xing, et al[34] carried out the study on protoplast electrofusion conditions of *Aloe vera* by electrical field stimulation which provided a basis to study somatic hybridization. In course of study on protoplast isolation, purification and electric fusion of *Reynoutria japonica*, Zhou, et al[35] achieved the protoplast survival rate of 75%, and the fusion rate of 20.2%. Liang, et al[36] obtained fusion recombinants of *Poria cocos* by protoplast fusion technology.

Haploidy Breeding

Haploid Breeding of Medicinal Plants

Haploid only containing one set of parental chromosome group is almost infertile. The importance of haploid in breeding is that the homozygous diploid with genetic stability can be gotten by doubling chromosome. A lot of researches on haploid breeding of medicinal plants were carried out in China.[37-39] The anthers or pollens of medicinal plants such as *Platycodon grandiflorum*,[40] *Liriope spicata*,[41] *Echinacea purpurea*,[42] *Lycium barbarum*,[6] and so on were successively cultured, and the regenerated plants were obtained.

Polyploid Breeding of Medicinal Plants

Polyploid plant with cell containing three or more sets of chromosomes, can be obtained with colchicine treating callus, embryoids or cluster buds. The polyploid plants often show giant characteristics of roots, stems, leaves, flowers and fruits, and high contents of effective compounds compared with diploids, which is desired goal of medicinal plants breeding. Thus, polyploid breeding of medicinal plants has a high application value and production potential.[43]

The polyploid breeding has been widely used as one of the important medicinal plants breeding methods. Since the 1980s, over 30 new varieties of polyploid medicinal plants such as *Angelica sinensis*, *A. dahurica*, *Saposhnikovia divaricata*, *Atractylodes macrocephala*, and so on have been bred in China.[44] The polyploid breeding research has been frequently reported in recent years. When colchicine with appropriate concentration was added to the medium, the chromosome of immature seeds of *Salvia miltiorrhiza* Bunge *f. alba* was doubled in the process of germination.[45] Then, large quantities of autotetraploid could be obtained by tissue culture. The polyploid of *Isatis indigotica* has higher resistance to insect, cold, drought, disease than diploid which was found in experiment.[46] When the stem, seed, radicel of *Saussurea involucrata* were induced with colchicine and 2% dimethyl sulfoxide, the regenerated plants with cell rate of doubled chromosome over 90% were obtained.[47] After measuring pyrethrin contents of 16 autotetraploid lines of *Pyrethrum cinerariifolium*, Zhang, et al[48] found that the total pyrethrin content in dried flowers of seven tetraploid lines reached 1.473%, and that of diploid lines was only 1.126%.

Compared with diploid, triploid plants exhibit characteristics with big fruit, seedless or less seed, which is very useful to some medicinal plants. Triploid breeding has been successfully applied in crop production of watermelon, banana, grape, and so on.[49] However, the triploid breeding of medicinal plants started fairly late. Early triploid breeding mainly depended on reciprocal crosses of diploid and tetraploid. Currently, many scholars try to get triploid medicinal plants by endosperm culture, and have made some progress.[50-52] In order to obtain triploid of *Lycium barbarum*, Mi, et al[53] took the endosperm of five genotypes as induction materials, and the callus was successfully induced with the highest induction rate of endosperm callus 80.0%. Peng, et al[54] selected young fruits of loquat with artificial pollination for 5 to 8 weeks as materials to culture endosperm of cell formation period. The highest induction rate of callus reached 33.3% when 30 pieces of endosperm were inoculated on media. The highest sprouts differentiation rate reached 18.04% when 10 pieces of callus were inoculated on media. When *in vitro* endosperm of *Akebia trifoliata* was cultured, the callus induction and proliferation were achieved, but the differentiations of the buds, stems, roots were not occurred.[55]

In general, endosperm culture technology has not been mature. In order to promote the success rate of endosperm culture *in vitro*, improvement of endosperm culture methods, rational use of plant hormones, and determination of growing period of cultured material are needed.

Radiation Mutagenesis Biotechnology in Medicinal Plants Breeding

Radiation breeding technique has rapidly developed since Muller found that the X-ray could greatly increase the mutation rate of *Drosophila melanogaster* in 1927.[56] More and more radiation means such as X-ray, β-ray, γ-ray, neutron, proton, and other source of radiation were introduced to the field of medicinal plants breeding. However, γ-ray was the major mutagenic factor which was widely used in radiation breeding of medicinal plants. It was reported that callus structure was changed, the color became shallow after ^{60}Co-γ ray irradiation for callus of *Ephedra intermdia*, cell proliferation was significantly increased, and split exuberant period was advanced under the condition of suspension culture.[57] The mutagenesis provided possibility to increase synthesis of active ingredient in cell. The cell lines of high yield were obtained by irradiating callus of *Panax quinquefolium* with ^{60}Co-γ ray.[58] Furthermore, Fang, et al[59] obtained 16 variant lines of *Mentha haplocaylx* by chemical mutagenesis and ^{60}Co-γ ray irradiation under the condition of tissue culture. The yields of 8 lines, the total volatile oil contents of 10 lines, and the menthol contents of 12 lines were higher than those of the control lines.

Ion beam biotechnology has opened up a new field for study on medicinal plants breeding since the biological effects of ion beams were found in the mid-1980s.[60] Chinese scholars launched breeding research on *Ginkgo biloba*,[61] *Ephedra Sinica*,[62]

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and *Glycyrrhiza uralensis*,(63) which showed good application prospects of ion beam biotechnology. In addition, control gene with good traits can be cloned, and transformed into other materials with different genetic backgrounds to create new superior varieties by ion beam-mediated technique combining with gene cloning technique. By ion beam mediated superdistant hybridization, the hybridization fusion of whole genome DNA from Ginkgo and watermelon had been achieved, and ginkgolide was detected in the offspring of watermelon.(64) Ion beam-mediated hybridization could be achieved because of the role of modification and processing of injected ions to DNA molecules in cells which provides a recombination opportunity for receptor genomic DNA and donor genomic DNA.

That tissue culture combined with radiation mutagenesis has great potential for the expansion of genetic variation, improvement of breeding efficiency, and acceleration of breeding process. Thus, the breeding with radiation mutagenesis biotechnology has become an important area of breeding research.

Genetic Engineering in Medicinal Plants Breeding

After the advent of the first case of transgenic plants in 1983, plant genetic engineering was rapidly developed around the world.(65) Genetic engineering is playing an increasingly important role in medicinal plants breeding in China.

Disease-Resistant Genetic Engineering

Traditional breeding for disease-resistance has defects of long cycle, heavy workload, and genetic instability. With the rapid development of plant genetic engineering technology, and constant identification and cloning of disease-resistance genes,(66,67) it is possible to enhance resistance to diseases by transforming exogenous resistance genes into the target plant.

In medicinal plants breeding research, the important role of the disease resistant genetic engineering is increasingly evident. The disease-resistant genetic engineering research on citrus tristeza virus,(68-70) barley yellow dwarf virus,(71) and *Rhizoctonia solani* from *Atractylodes macrocephala*,(72) was conducted, respectively. There were reports that gastrodia antifungal protein-1 (GAFP-1) had been isolated,(73) and cDNA of GAFP-1 also been cloned.(74) Because GAFP-1 significantly inhibits plant pathogenic fungi such as soft rot bacteria of pear, *Rhizoctonia solani*, and *Botrytis cinerea*, related results of researches were expected to be applied to against fungal diseases genetic engineering of medicinal plants.

Anti-insect Genetic Engineering

The rapid development of plant genetic engineering has brought revolutionary change for pest control. Research progress of medicinal plants genetic engineering relating to insecticidal protein, and insect hormone had been made.(75,76) It was reported that Luo, et al(77) used *Agrobacterium*-mediated transformation system to transform exogenous snowdrop lectin enzyme gene, and obtained transgenic *Lycium barbarum* with aphid resistance. When exogenous *Pinellia ternata* agglutinin (PTA) gene was transformed into tetraploid genome of *Isatis indigotica*, the new lines of transgenic *I. indigotica* with pest resistance could be obtained by using Agrobacterium-mediated transformation system.(78,79) In addition, the lines of *I. indigotica* with anti-*Plutella xylostella* could be obtained when Bacillus thuringiensis crystal protein gene Cry IA (c) and cow pea trypsin inhibitor gene Cp TI were transformed into tetraploid genome.(80) The study indicated that co-transformation of Cry IA (c) and CpTI genes is an effective strategy to enhance the resistance of tetraploid of *I. indigotica* to moths.

Herbicide-resistant Genetic Engineering

Herbicides have been widely used in agricultural production,(81,82) and also have brought some harm to crop growth. It is meaningful to carry out the research on herbicide-resistant gene engineering.

Herbicide-resistant genetic engineering research began in the 1980s in China, which was often reported on crops.(83-85) However, only a few cases of the research on herbicide-resistant transgenic medicinal plants have been reported to date. In order to improve the resistance of tetraploid of *Isatis indigotica* to herbicide, Chinese scholars used *Agrobacterium tumefaciens* strain EHA 105 as engineering bacterium to transfer the plant binary expression vector pCAMBIA 3300 carrying bar gene, and obtained the transgenic tetraploid with herbicide-resistance.(86)

Stress-Resistant Genetic Engineering

The abiotic stress inhibits plant growth, accelerates plant senescence, causes cell death, and reduces yield of plant. The significance of stress resistant genetic engineering lies in improving plant varieties, and enhancing the adaptability of plant. For medicinal plants breeding, stress resistant genetic engineering not only can enhance stress-resistance of varieties, but also may bring changes of the specific chemical compound.

The stress-resistant genetic engineering in plant, which was later developed, belongs to the resistance genetic engineering. Currently, stress resistant genetic engineering research mainly focuses on some economic crops such as rice,(87) tomato,(88) soybean,(89) and so on. For medicinal plants breeding research, stress-resistant genetic engineering involves only some medicinal and edible plants, such as barley,(90) kiwi,(91) and so on. Taking immature embryo and embryogenic callus of maize as transformation materials, Yang, et al(92) obtained transgenic plants of maize with drought resistance through cloning the dehydration-responsive transcription factor CBF4 gene from *Arabidopsis thaliana*, and constructing the expression vector pBAC146.

Stress-resistance in plants is usually not determined by a single gene, but controlled by multiple genes.(93) A series of genes are transferred by genetic engineering, which can comprehensively improve stress resistance of plants, and has broad application prospect.(94)

DNA Molecular Markers Technology in Medicinal Plants Breeding

DNA molecular markers technology has been rapidly developed since Bostein took the differences of DNA sequences among individual organisms as markers for construction of genetic linkage map in 1980.(95) Currently, DNA molecular marking technology has been widely used for medicinal plants breeding research in China.

Plant-based Sources Identification

Clear plant-based source is premise of medicinal plants breeding. Some useful explorations for plant-based sources identification of closely related species with similar shape by DNA molecular marking technology were made. Zheng, et al(96) effectively distinguished *Polygonum multiflorum* from its adulterants *P. ciliinerve*, *Pteroxygonum giraldii*, and *Cynanchum auriculatum* by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis. After analyzing the differences of chloroplast psbA-trnH region sequences of *Artemisia annua*, *A. capillaris*, and *A. carvifolia*, Liu, et al(97) found that valid distinction can be implemented relying on their unique loci, although the psbA-trnH regions among the three species were highly similar. In the study of DNA barcode identification of medicinal plants, Zhu, et al(98) found that ITS2 could be used to accurately identify medicinal plants of the genus *Paris*.

Study on Genuineness

Authentic medicinal herbs are comprehensive standards for evaluation of medicinal quality which was summarized in long-term clinical practice. The local specialization genotype from long-term evolution of species and ecological adaptation process is internal factor of authentic medicinal herbs.

The early authentic medicinal herbs research focused on the chemistry and pharmacology. The literature which elaborated authentic medicinal herbs from the molecular level of DNA was limited. In order to explore the phylogeography and molecular mechanisms of authentic medicinal herbs formation of *Morinda officinalis*, Ding, et al(99) detected the sequence of rDNA ITS gene of *M. officinalis* from different origins by PCR sequencing technique, and found a correlation between the phylogenetic relationship among groups and geographic distribution. Lai, et al(100) analyzed six different populations of *Paeonia lactiflora* by random amplified polymorphic DNA (RAPD) analysis, and found a wealth of genetic diversity among different producing areas. Cao, et al(101) took advantage of RAPD molecular markers to analyze 48 samples of *Fructus Evodiae* collected from five different producing areas, and probed into the genetic background related to genuineness.

Genetic variation in authentic medicinal herbs can be revealed by using molecular genetic markers, and authentic medicinal herbs formation can be elaborated from the molecular level of DNA, which will provide beneficial ideas for authentic medicinal herbs breeding, cultivation, and utilization.

Genetic Diversity and Germplasm Resource

Biodiversity protection has become an international hot issue, of which the core is protection of genetic diversity. Genetic diversity research has

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become an important foundation for medicinal plants breeding which is closely related to the germplasm resource classification and resource innovation. Screening the appropriate random primers and using RAPD analysis, Zhou, et al[102] analyzed the genetic diversity of *Gymostemma Pentaphyllum* and constructed DNA fingerprints. Amplifying the valuable loci by using RAPD technique, Mo, et al[103] analyzed the genetic diversity and differentiation degree of five populations of *Lonicera confusa*, and thought that geographic isolation strongly influenced genetic differentiation among populations. Wu, et al[104] carried out the research on genetic diversity of *Sinopodophyllum hexandrum* in Yunnan of China using direct amplification of length polymorphism (DALP) analysis, and found significant genetic differentiation and blocked gene flow among populations, which was closely related with the breeding system and population habitat. The study provided a basis to protect and exploit the resource of *S. hexandrum* based on molecular genetic theory.

Evolution of Species and Genetic Relationship

By the molecular marking technique of RAPD, RFLP, amplified fragment length polymorphism (AFLP), and gene sequencing technique, the differences of genetic background, species evolution and genetic relationship of organism can be effectively clarified from DNA level. The DNA polymorphisms of six species, of the genus *Polygonatum* were detected by RAPD molecular markers, which revealed that the classified features of genus were obvious while the classification within the genus was crossed.[105] Seven species of medicinal plants from *Asteraceae* were analyzed by RAPD molecular markers, and phylogenetic tree was constructed with the data of the amplified DNA fragments by unweighted pair group method with arithmetic mean clustering method. The clustering results showed that the phylogenetic tree was consistent with the traditional classification system among the genuses, of the same family.[106] The chloroplast DNA trnK gene of five species, of the genus *Atractylodes* were sequenced, and the evolutionary relationship of five species was established, which revealed that the trnK of the genus *Atractylodes* was conservative.[107]

Molecular Genetic Linkage Map

Molecular genetic linkage map is important for gene localization, gene cloning, and the research on structure and function of the genome, which is closely related to genetic breeding and genetic engineering research. Thence, the construction of molecular genetic linkage map is a key step of the genome research, and also becomes focus of medicinal plants breeding research.

The use of genetic linkage map can guide the breeding to accelerate the breeding process. The genetic maps of some medicinal plants such as *Silene vulgaris*,[108] *Cynara cardunculus*,[109] *Trifolium repens*,[110] and so on has been constructed abroad. The molecular techniques were applied later in China, only a handful of genetic maps of the medicinal plants were constructed.[111] In the future, the genetic map construction of some common, and important medicinal plants such as *Panax ginseng*, *P. notoginseng*, *Gastrodia elata*, *Salvia miltiorrhiza*, *Glycyrrhiza uralensis*, should be priority, which will lay a good foundation for the superior varieties breeding of medicinal plants (Table 1).

Table 1. List of Biotechnologies in Medicinal Plants Breeding Research

No.	Name of the technology	Reference
1	Tissue culture	(17–29)
2	Protoplast culture	(30–36)
3	Ploidy breeding	(37–55)
4	Radiation mutagenesis	(56–64)
5	Genetic engineering	(65–94)
6	DNA molecular markers	(95–111)

Conclusions and Future Perspectives

Medicinal plants breeding had been using conventional breeding technique of crops for a long time resulting in its slow development in China.[112] In recent years, with the rapid development of modern science and technology, biotechnology has been gradually penetrated into various fields of medicinal plant resource research. Rapid progress has been made in medicinal plants breeding research, and superior varieties are constantly springing up with high yield and quality, and good genetic stability.[113-115]

Modern biotechnology shows broad application prospects in medicinal plants breeding but still faces some challenges. From the view of current study status, tissue culture and polyploid breeding technology are relatively mature, and have been used in the breeding of medicinal plants more.[116,117] However, large-scale production has not been yet achieved on account of unstable genetic traits and low seedling survival rate. The protoplast fusion,(118) genetic engineering,(119) molecular markers technology,(120) and so on, need continual improvement.

Therefore, for medicinal plant breeding research in future, modern biotechnology will still need to be combined with conventional breeding technique so as to promote the modernization process of development and utilization of medicinal plants resources.

Conflict of Interest

The authors have no personal or financial conflicts of interest associated with this work.

Authors' Contributions

All the authors made contributions to this paper. All authors read and approved the final manuscript.

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