Cultivation, Development and Utilization of Bupleurum Based on Ribosomal Its Sequence

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Abstract: The protoplasts of Bupleurum angustifolia were fused with grape protoplasts after being irradiated with 240 μ W/ cm2 ultraviolet rays for 0 ,1 ,2 and 3min. The phenotypes, isozymes, chromosomes and 5S rDNA spacer sequences of 10 single cell clones regenerated by fusion were analyzed. The results showed that 10 different IT S sequences were detected in the germplasm resources of Bupleurum chinense DC., which ranged in size from 625 BP to 626 BP, with 99.18% sequence identity, 99.04% similarity with the ITS sequence of Bupleurum chinense DC., and the genetic distance was 0.036. All these indicated that the genetic differences of ITS sequences between artificially cultivated and wild Bupleurum chinense DC. were small and the genetic relationship was close.

1. Introduction

Bupleurum is a herbaceous plant of Umbelliferae. Other names are Bupleurum chinense, Bupleurum chinense, Bupleurum chinense, Bupleurum chinense, Bupleurum chinense, Shancai, etc., which are commonly used Chinese herbal medicines. In traditional Chinese medicine, dried roots are used as medicine. Mainly produced in Northeast China, North China, East China, Inner Mongolia, Henan, Shaanxi and other places. Planting Bupleurum is one of the ways to adjust agricultural industrial structure and increase farmers' income. In recent years, injection, tablet, granule and other dosage forms have been widely developed in Bupleurum chinense [1]. With the increasing consumption of Bupleurum chinense, its price has been rising. At present, it has been reported that different species of Bupleurum have been identified by lipase isozyme bands, RAPD methods and ITS sequence analysis [2-3]. For Bupleurum, its wild resources are decreasing day by day, and breeding of cultivated varieties is also urgent. Therefore, screening breeding materials of Bupleurum and early selection and identification of its hybrid offspring by means of molecular markers can reduce the blindness of breeding and accelerate the breeding process.

2. Efficient Cultivation Techniques of Bupleurum Chinense Dc

2.1 Land Selection and Preparation

Because Bupleurum has the characteristics of cold resistance and drought tolerance, but is afraid of flooding, it is more suitable to choose sandy land with deep soil layer, fertile soil, loose soil and favorable irrigation and drainage. Before sowing, apply plantar fertilizer. Generally, apply organic fertilizer 2500~3000kg per mu, turn over 25 ~ 30 cm deep, level and rake fine, pick up stones or weeds, and plot or plot in autumn for sowing. The border is 1.2m wide, 1.5m high and 10 ~ 20m long. It is better to have a width of 60cm.

2.2 Seed Selection and Treatment

Seeds from robust plants should be selected, and seeds with full grain surface, perfect development and no diseases and insect pests should be selected because of the short life span of Bupleurum chinense seeds, which are generally not used every other year. Bupleurum chinense is usually propagated by seeds, directly broadcast or transplanted by seedlings. The germination rate

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of seeds is about 60%, the temperature is about 15 ~ 20℃, the humidity is about 60%, and the seedlings can emerge 7 ~ 10 days after sowing. The seeds of 2-year-old robust plants without diseases and insect pests are harvested from September to October every year. Immediately laminate the seeds at low temperature, lay one layer of spun yarn on the seeds in a wooden box, lay the spun yarn on the top layer, keep the water content at 65%, store the seeds and wet sand in the environment of 0 ~ 5℃, and the embryo will mature after 5 months, and the germination rate can reach 70%, otherwise the germination rate will be less than 50%.

2.3 Sowing and Seedling Raising

Sowing in late April can be done by broadcasting or drilling. Before sowing, the seedling bed was watered with water, and the seeds after stratification were soaked in warm water at 35℃ for 12 hours. After being taken out and dried in the sun, they were sown and covered with fine soil for 1 ~ 2 cm. According to the row spacing of 23-27 cm, open a shallow ditch with a depth of 1-1.5 cm, mix the seeds with fire ash, spread them evenly into the ditch, cover the soil for about 1-3m, and water them after a little suppression. Pay attention to keep the soil moist after sowing to facilitate the emergence of seedlings. Mulching with plastic film can be sown from late February to mid-March, and it is better to cultivate with high ridge, and then mulching with plastic film immediately after sowing. When the seedlings are just unearthed, pay attention to breaking the membrane to prevent the seedlings from being burnt. It is also possible to set up a small arch shed to make the seedlings grow in the small arch shed for a period of time, and then remove the film after the external temperature is stable, which is more conducive to the growth of Bupleurum chinense seedlings.

2.4 Field Management

After emergence or transplantation, Bupleurum chinense should loosen soil frequently to keep soil permeability. Weeding is mainly to control weeds competing with seedlings for water and nutrients. Weeding should be done at least three times in nursery or field to keep the field clean and free of weeds. When the seedlings are 1-2 inches long, the thinning should be carried out in combination with weeding, and the too dense, thin and weak seedlings should be pulled out, and the seedlings should be fixed according to 1-2 inches of plant spacing and 5-6 inches of row spacing. 20kg of calcium superphosphate and 10-15kg of ammonium sulfate should be applied in the stage of division of evil, and the application should be carried out by trenching between rows, and then the soil should be covered tightly; Bupleurum chinense stems are weak and easy to fall down in case of wind and rain, so root ridging can be carried out in combination with intertillage weeding; When the plant height is 40 cm, it should be topped, and the redundant clustered basal buds should be removed continuously, so as to control the growth of stems, promote the rapid growth of roots and improve the yield and quality. In the first year, only basal leaves grow in Bupleurum chinense DC., which are arranged on both sides. In the second year, it begins to take out moss and blossom. If planting continues to be expanded, attention should be paid to reserving seeds. If no seeds are left, moss should be picked in time, nutrients should be concentrated, and roots should be caught for growth.

2.5 Limping Pest Control

Common diseases include rust and spot blight. Rust pathogen is a basidiomycete of fungi, which damages stems and leaves, and forms rust yellow summer spore piles on the back and base of leaves. After breaking, yellow powder flies in the wind. The injured part caused perforation, and the stems and leaves withered early, which mostly occurred in flowering period (June). Aphids usually occur in the seedling stage of Bupleurum chinense and the rejuvenation period of old plants in early spring, which are harmful to the leaves. They can be prevented by spraying 2.50% deltamethrin emulsifiable concentrate with a concentration of 2500-3000 times, which should be sprayed once

every 5-7 days and continuously for 2-3 times; Spraying 25% triadimefon with 1000 times solution at the early stage of rust. Root rot is easy to occur when it is hot and rainy, and the drainage is poor. It is necessary to pay attention to ditching and drainage, and to rotate crops in rotation, preferably with gramineous crops.

2.6 Harvesting and Processing

From September to October of the second year after sowing, the Bupleurum chinense is harvested when the plant withers, after the roots are dug out, the stems and leaves are removed, the soil is shaken and dried in the sun. The products are thick, long, neat, hard, not easy to break, and have no residual stems and fibrous roots. In some places, stems and leaves are used as medicine, which can be cut in the autumn of the sowing year and harvested in the second year and dried in the sun. Processing in time after harvest, do not pile up for too long, shake off the soil and dry in the shade in time. When 70% to 80% of the harvested Hu's roots are dry, cut off the stubble, remove the fibrous roots, straighten the roots, and bundle them into small bundles to dry.

3. Materials and Methods

3.1 Material

In this study, 14 artificially cultivated Bupleurum chinense and 1 wild Bupleurum chinense (No.215) germplasm resources were selected. Seeds of artificially cultivated Bupleurum chinense were collected from planting areas in autumn of 2019.

3.2 Hybrid Identification

Compare the shape and color of the fused regenerated callus and young leaves with their parents. (ⅱ) Karyotype analysis. The fused and control callus and young leaves were treated at 4℃ for 16h, fixed with a fixed solution of methanol: glacial acetic acid = 3: 1, and sliced by flame drying with wall-removed and low permeability [4].

3.3 Cloning and Sequencing of Its Fragments

The purified PCR product was connected with PMD-18T Vector, then transformed into competent cells of Escherichia coli JM109, screened with plates containing X-Gal, IPTG and Amp LB, cultured with white colonies, and extracted plasmids for PCR detection [5]. After successfully cloning, the strain samples were sent to Biotechnology Co., Ltd., and sequencing was performed on ABI 3700 self-operated sequencer. Forward and reverse sequencing was performed, and BioEdit software was used to splice the complete sequence.

4. Results and Analysis

The sequencing results of 33 strains of Bupleurum chinense from 15 samples were compared, and it was found that the ITS sequence consistency of 33 strains of Bupleurum chinense was 99.18%. The size of ITS sequences ranged from 625 to 626bp, among which 215-3 and 215-5 were 625bp, and other plants had ITS sequences of 626 BP. There are 12 SNP sites in ITS sequences of 34 strains of Bupleurum chinense from 15 germplasm resources, including 49,246 and 578bp C-A variation, 216,492,579 and 592bp G-A variation, 9,145,490 and 575bp C-T variation and 215-3, respectively The regenerated calli of clones 6,7,11 and 18 produced by fusion newly differentiated plantlets with complete roots and leaves, and grew rapidly. In addition, some early differentiated young leaves grew roots to form complete plantlets. Grape protoplasts as control could only divide into small cell clusters, while Bupleurum chinense protoplast regenerated calli did not differentiate.

MEGA software was used to analyze the K2P genetic distance between two Bupleurum samples

and ITS sequences with high homology (over 90%). The results are shown in Table 1. The smallest distance from sample 1 is Bupleurum chinense, the second is Bupleurum chinense, the distance is 0.063 and 0.036 respectively, the third is Bupleurum chinense, and the farthest distance is Bupleurum chinense.

Table 1 Genetic Distance Of K2p between Species and within Species of Bupleurum Chinense Its Sequence

Type	Genetic distance						
	1	2	3	4	5	6	7
2	0.025						
3	0.047	0.050					
4	0.056	0.063	0.064				
5	0.052	0.054	0.063	0.057			
6	0.050	0.051	0.061	0.052	0.057		
7	0.048	0.053	0.055	0.063	0.053	0.037	
8	0.046	0.052	0.053	0.055	0.054	0.036	0.021

Sequence data sorted by Clustal W have a total of 1604 feature sites. in order to make the length of different sequence data basically consistent, 52 bases at the 5' end of the sequence and 928 base sites at the 3' end of the sequence were excised, and there are still 724 feature sites. At the same time, 104 loci in fuzzy Alignment sequence region were excluded for systematic analysis, and finally the sequence matrix used for systematic analysis was 47 classification units and 620 characteristic loci. From the genetic point of view, the genetic relationship between Bupleurum chinense and Bupleurum chinense is also very close. Chen fanling et al. [6] analyzed the karyotypes of 6 Bupleurum plants, which showed that the genetic relationship between Bupleurum chinense and Bupleurum chinense was close, with the chromosome number of 2n=12, and the karyotype approximate coefficient between Bupleurum chinense and Bupleurum chinense reached 0.992, and the karyotype evolution distance was the smallest (0.008), indicating that the genetic relationship between Bupleurum chinense and Bupleurum chinense was very close, which was the same as the experimental results.

Due to the wide distribution of Bupleurum species and the high similarity of phenotypic characteristics, there are many suspicious groups and the unclear classification among their subordinate species, which makes it difficult to identify and utilize Bupleurum resources [7]. According to its related species Pseudostellaria heterophylla, the length and GC content of ITS1, 5. 8S and ITS2 in three regions were determined, in which ITS1 fragments were all 243 bp and GC content was 54.42% ~ 54.61%. 5. 8S fragments are all 156 bp, and GC content is 54.36% ~ 54.47%. ITS2 fragment is 226~227 bp, and GC content is 55.03% ~ 56.37% (Table 2). According to the available data, it is still uncertain whether the strains PHZAU2, PHZAU3 and No.4 Bailing from China can truly represent Pleurotus nebrodensis. In addition, whether the name of PHZAU20 is correct or not and the relationship among P. eryngii, P. eryngii var. ferulae and P. nebrodensis need to be further discussed by obtaining reliable strains.

Table 2 Size and Gc Content of Each Fragment in 10 Its Sequences

Serial number	ITS1/bp (GC content/%)	5. 8S/bp (GC content/%)	ITS2/bp (GC content/%)
Sequence 1	241(54.61)	155(54.36)	225(56.37)
Sequence 2	241(54.02)	155(53.06)	225(54.17)
Sequence 3	241(54.03)	155(54.66)	225(54.16)
Sequence 4	241(54.02)	155(54.36)	225(56.37)
Sequence 5	241(55.11)	155(54.36)	225(53.74)
Sequence 6	241(53.62)	155(54.12)	225(56.37)
Sequence 7	241(53.03)	155(54.66)	225(56.37)
Sequence 8	241(54.42)	155(54.36)	225(55.03)
Sequence 9	241(54.09)	155(54.47)	225(55.03)

Although ITS sequence can be used to analyze the differences between different populations within the genus and within the species, ITS sequence length of Bupleurum chinense is only 625 ~

626 BP, which can not fully reflect the genetic differences among these germplasm resources, and it is difficult to explain the differences in total flavonoids content among these germplasm resources by using the differences of its sequences. For the real reasons, more detailed molecular biology research should be carried out.

5. Discussion

Identification methods of Chinese medicinal materials, such as primitive identification, character identification, microscopic identification, physical and chemical identification, chromatography identification, etc., have played an important role in the research of identification and quality evaluation of Chinese medicinal materials. However, with the rapid development of modern molecular biology technology, a variety of DNA molecular identification technologies of Chinese medicinal materials have emerged. On the one hand, the area suitable for growing with Bupleurum chinense is small, and the range of artificial planting is mainly concentrated in the areas from Tongxin Yuwang and Pengyang in Ningxia to Longxi in Gansu Province. On the other hand, Bupleurum chinense was introduced into different areas during artificial planting, which made the genetic relationship of germplasm resources unclear, resulting in the phenomenon of germplasm confusion. Even lower than the phenomenon of the receptor side [8]. This experiment proves once again that reducing the imbalance of genetic material between parents by chromosome reduction in distant hybridization is one of the prerequisites for regeneration of plants from hybrid callus [9]. Hybrid plants with phenotype completely like grapes were obtained from clones 6 and 18. Because grapes can be propagated by cutting, these hybrid plants can be used to improve the breeding of grape germplasm after growing up.

Because its region has more base information and is conservative in length, it is particularly suitable for phylogeny and taxonomic study at genus and group level. In particular, it can provide the basis for molecular identification of traditional Chinese medicine. The similarity between its sequence 1 and its sequence of Bupleurum chinense was 99.03%, and the genetic distance was 0.003. Cluster analysis showed that the sample containing its sequence 1 was Bupleurum chinense; Due to the deterioration of the growth environment and excessive mining, the germplasm resources of wild silver Bupleurum are in danger. However, there are great differences between the artificial planting environment and the wild environment, so the yield and quality of the cultivated species are obviously different from those of the wild species. Therefore, it can be considered that the effect of UV on chromosome damage and instability is gradually revealed during the growth and development of the fusion. At the same time, the growth and development characteristics of hybrids also changed, and plants with phenotype exactly like receptor appeared.

6. Application of Bupleurum Chinense

The “dry root” of medicinal parts of Bupleurum chinense was used in all editions of Pharmacopoeia, so the aerial parts were not fully utilized. According to the research, the stems and leaves of several kinds of Bupleurum chinense, such as Bupleurum chinense, Allium tuberosum, Bupleurum chinense, Luxi Bupleurum chinense and Bupleurum chinense with stem and stalk, contain basically the same saponins. There are 120 species of Bupleurum plants in the world and 40 species in China. There is great potential for the development of traditional Chinese medicine. At present, there are compound Bupleurum tablets, compound Bupleurum injection, Chaihuang tablets, Xiaoyao pills, Chaitong tablets and so on.

7. Conclusions

With the rapid development of molecular biology, people will have a more extensive and in-depth study on ITS sequence and its value in plant molecular systematics. Except for the unknown growth years of wild Bupleurum chinense, the artificially cultivated Bupleurum chinense grew in the same habitat for two years, which basically eliminated the influence of environment on its

content. Therefore, it can be considered that the difference of total flavonoids content among Bupleurum chinense germplasm resources from different sources is caused by genetic differences. The base of young leaves of partially asymmetric hybrid began to take root, and the callus of four asymmetric hybrid clones newly differentiated into small plants with complete roots and leaves, and the growth speed was obviously faster than that of young leaves at the initial stage of culture. At this time, the chromosome number of somatic hybrid decreased obviously and distributed between 18 and 28.

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