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RESEARCH ARTICLE

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A poplar chimera with stable differentiation regulation

Abstract *Populus shenzhou*, a chimera, is divided into three types: A, B, and C. Among them, Type B is the chimera of Type A and Type C. Type B of *P. shenzhou*, as a progenitive material was cut and differentiated stably into Type A, Type B, and Type C, at an average differentiation rate of approximately 79.6%, 18.3%, and 2.2%, respectively. The studies on SSR identification of *P. shenzhou* showed that all tissues, including the bud, leafstalk, leaf nervation, phloem, and xylem, were a chimera. There were two kinds of color roots of the three types. The rufous roots were identified as Type A tissue and the yellow roots as Type C tissue by the SSR molecular markers.

Keywords chimera, *P. shenzhou*, differentiation regulation, molecular identification

1 Introduction

Populus tomentosa is one of the important forestation tree species in North China. Since it's not easy for the Chinese white poplar and other species and varieties in the section of *P. tomentosa* to take root by hardwood cutting, their current reproduction mainly relies on grafting, seedling-burying, softwood cutting, and other methods with a long breeding cycle, a low survival rate of seedlings, and a high nursery cost, which limit its rapid development. In the past, the breeding works mainly targeted fast-growing, improvement of wood properties, and increase of resistance, but seldom targeted the rooting ability. Thus, field hardwood cutting breeding for new varieties of *P. tomentosa* on a large-scale (Su et al., 2004) has not yet been conducted. Therefore, the selection of new variant species with simple propagation methods at a high survival rate by root cutting is of great significance to enrich germplasm resources, speed up propagation, reduce nursery costs, and finally improve the quality of seedlings in widespread production and application.

As an access to new varieties of a simple and effective method, chimera has been widely used in fruit breeding but seldom in the breeding of timber species. *P. tomentosa* chimera is a variant species which was first found in Ningjin County, Hebei Province, China (Song et al., 1998, 2001). It is afforested as a dominant variety in that area after being selected and cultivated. Moreover, it has formed a fine species chimera called *Populus shenzhou* with regulated differentiation.

This chimera has been widely used in Hengshui Area of Hebei Province and in the meanwhile, it has already been adopted as a tree variety by the Hebei Province Tree Variety Examining Committee. In our investigation, based on the biological characteristics and SSR identifications, the differentiation regulation of *P. shenzhou* was studied in order to provide a theoretical basis for exploring chimeric mechanisms and to promote the application in the production of *P. shenzhou.*

2 Materials and methods

2.1 Plant materials

The test materials were one-year-old seedlings and adult trees of *P. shenzhou* (Type A, B, and C). *Populus daquansis* was used as rootstocks. Also, a variety was found using whip budding, which was identified as a chimera of *P. tomentosa* and *P. daquansis*, and used as a native variety in production and application. This species of *P. shenzhou* has attracted much attention of the local government and relevant experts. Through screening, purification, and analyses of the growth characteristics, biological characteristics, and differentiation mechanisms, a new fine chimera species was gained with stable differentiation regulation, easy asexual reproduction, and rapid growth.

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2.2 Research methods

2.2.1 Morphologic traits

Conventional indicators of morphological traits of the adult *P. shenzhou* including floss, arris and color of the bough, size and color of the bud, shape and floss of leaf, shape of leaf base, lenticel and leafstalk, and so on, were investigated according to Zhang et al. (1999).

2.2.2 Differentiation regulation of *P. shenzhou*

In the hardwood cutting breeding field of *P. shenzhou* Type B in Datun Village, Shenxian County, Hebei Province, 10 lines were selected randomly, and 100 individual trees were investigated in each line to analyze the separation of *P. shenzhou* Type B. According to the different morphologic characteristics of Type A, B, and C, the total number and the proportion of Type A, B and, C were recorded in order to find their differentiation regulation.

2.2.3 Molecular identification

One-year-old seedlings of the three types (Type A, B, and C) were analyzed by SSR molecular markers. Total genomic DNA from tissues (leaf, bud, phloem, xylem, and root) was extracted following the protocol described by Doyle and Doyle (1987) and Pan et al. (2004). The selected primer PN I297289 (5′-ACACGACCAGCAG-CAGTA-3′, 5′-TCCGATGATGACCCTTTA-3′) rooting in the microsatellite sequence of *Populus nigra* L. in the GenBank was designed by the software of Primer Premier 5.0. PCR amplifications were performed in a 20-μL volume containing 30 ng of genomic DNA, 10 mmol·L^{-1} Tris-HCl (pH 8.3), 0.25 mmol·L^{-1} of each dNTP, 1.5 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} KCl, 0.5 μmol·L^{-1} of each of forward and reverse primers, and 1 U of *Taq* polymerase (TaKaRa). Amplification conditions on a Biometa T1 thermocycler included an initial denaturation step at 94°C for 5 min followed by 30 cycles of 94°C for 50 s, 44°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 7 min (Liang et al., 2005). Amplified products (4 μL each) were electrophoresed on 5% nondenaturing polyacrylamide gel with 1×TBE. Electrophoresis was performed for about 1 h at 40 mA. A 100 bp DNA ladder 3kb marker was used as a size standard. After electrophoresis, the gels were stained with silver according to the Silver Sequence protocol described by Rajora et al. (2000) with some modifications.

3 Results

3.1 Differentiation types and morphological characteristics of *P. shenzhou*

Three types of of *P. shenzhou* (Type A, B, and C) had distinct different morphological characteristics (Fig. 1).

Type A, similar to *P. tomentosa* is a male clone, whose trunk is straight, smooth, nonedge, bark is dark-green, taupe lenticel is diamond-shaped, and has a scattered distribution. The angle between the trunk and branches was 40°–50°. The branches including the sprout branches

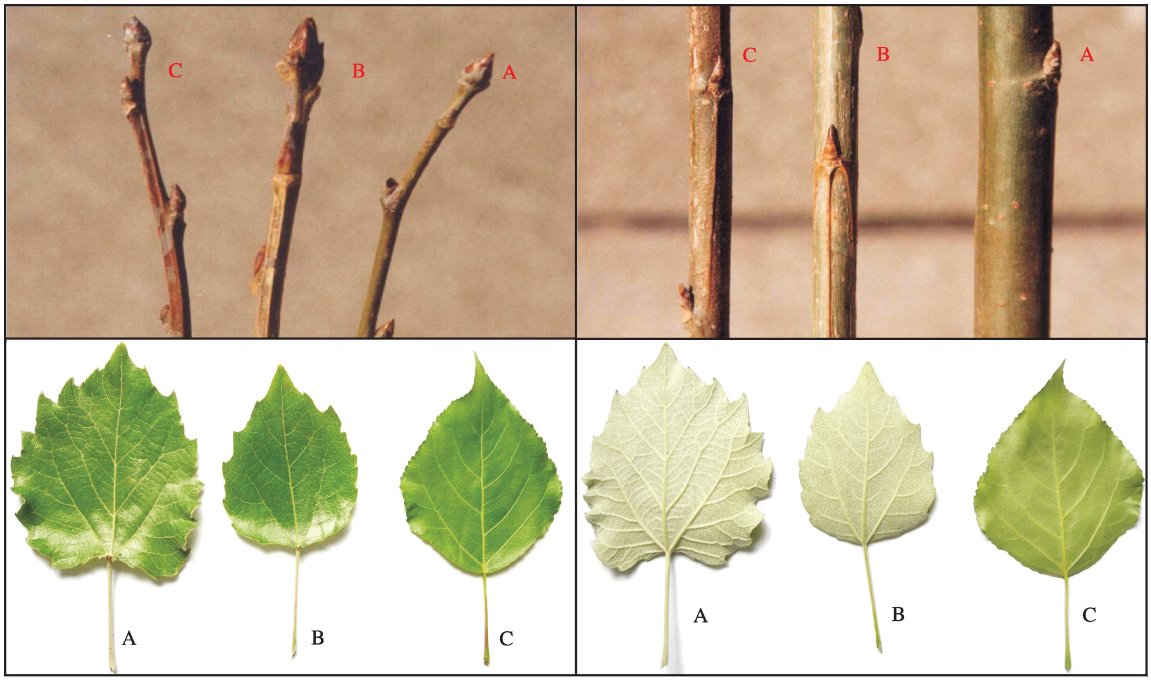

Fig. 1 Morphology comparison of tissues and organs in three types of *P. shenzhou*

stretched sideway with short villi at the beginning and then the villi fell off gradually. The leaves of the tree were in triangle or oval shape with white villi. The male inflorescence is of catkin with an average length of 10–13 cm. Type A is considered derived from the *P. tomentosa*.

Type B is also a male clone. Its bark is celadon and the lenticel is diamond-shaped or in circular and dust color, with most of lenticels distributing into pieces. The long branches and the sprout branches are in celadon, without obvious edges, but with short villi at the beginning and then the villi fell off gradually. The twigs stretched sideway and the lateral buds were full. Type B of *P. shenzhou*, as a progenitive material for the chimera, was cut and differentiated into Type A, B, and C.

For Type C, its bark is hoar with obvious arris. The branches also stretched sideway, but had no villi. The shape of leaves is diamond-shaped, with no villi on both sides. The leave stalk is red with no villi, either. It has been observed that Type C is developed from the upper terminal callus of Type B. As one-year-old seedlings had lots of side-branches, they are no use in production. Type C is the hybrid clone of Black poplar and Cathay poplar, and no longer differentiates after hardwood cutting, which is considered as another chimera of *P. daquansis*.

3.2 The differentiation regulation of Type B

3.2.1 Cuttage investigation

Type A, whose hardwood cutting propagation is difficult and the formative clones remains stable with no new differentiation, can be used as a choice production of clones in application. Type B of *P. shenzhou*, as a progenitive material, was cut and differentiated into Type A (79.60%), Type B (18.30%), and Type C (2.20%), and the proportion was stable (Table 1). Because of its stable differentiation, Type B can not only provide Type A seedlings continually, but also ensure the characteristic of easy rooting, which is called "reproductive system" in production.

From Table 1, we can see that Type B hardwood cuttings can develop into $18.30\% \pm 2.36\%$ of Type B plants, $79.60\% \pm 2.32\%$ Type A and $2.20\% \pm 1.75\%$ Type C, respectively at a steady differentiation regulation rate. Type A and B developed from Type B's lateral bud, and Type C was from the top of the callus (Fig. 2).

3.2.2 Rooting investigation of *P. shenzhou*

When investigating the roots of the three types developed from Type B, we only found rufous and yellow roots. Both of them could be found in lenticels of the cuttings, while only the yellow roots could be found in callus.

We also carried out the root-burying experiment for the three types, suggesting that all of the rufous roots could develop into Type A and the yellow roots could develop into Type C. The results indicated that the genetic composition of rufous was similar to that of Type A of *P. shenzhou*, while the genetic composition of yellow roots was similar to that of Type C of *P. daquansis*.

Through the above test analysis, the differential regulation can be drawn in Fig. 3.

3.3 Molecular identification of *P. shenzhou*

We did SSR identification on the leaf, bud, phloem, xylem, and root of the three types of *P. shenzhou*. By comparison of different tissues and organs, we found the chimera. Through DNA comparison of different tissues and organs of Type B plants, the chimeric site was preliminarily located; and DNA comparison of different organizations of Type A and Type C plants revealed the purity of plant.

Figure 4 shows that the DNA electrophoresis bands of *P. shenzhou* Type A and C were the same as compared with *P. shenzhou*, and the mixed DNA electrophoresis bands of *P. shenzhou* Type A and Type C were the same as Type B.

Table 1 Differentiation rate of *P. shenzhou* Type B by cottage

plot No.	surveyed trees	Type A rate/%	Type B rate/%	Type C rate/%
1	100	84	14	2
2	100	78	20	2
3	100	80	17	3
4	100	76	19	6
5	100	82	17	1
6	100	80	19	1
7	100	77	23	0
8	100	80	18	2
9	100	79	17	4
10	100	80	19	1
average	100	79.60±2.32	18.30±2.36	2.20±1.75
coefficient of variability/%	—	2.91	12.89	79.60

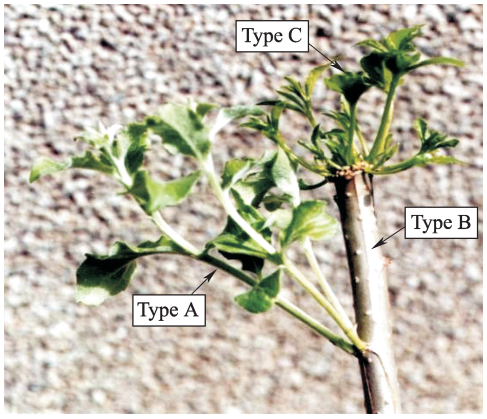

Fig. 2 The differentiation of *P. shenzhou* Type B

Therefore, we can conclude that the buds of *P. shenzhou* Type A and Type C were pure, and that of Type B was in the chimeric state.

Figure 5 indicates that the leaves of Type A and Type C were pure, the leafstalk and main nervation were in a chimeric state, while the DNA of Type B's leaves with no main nervation was the same as that of Type A, which was accorded with the leaf characteristics of Type A and Type B.

Figure 6 shows that the xylem and phloem electrophoresises of DNA bands of *P. shenzhou* Type A and Type C were the same as compared with *P. shenzhou*, and the mixed electrophoresis of DNA bands of *P. shenzhou* Type A and Type C was the same as that of Type B, therefore we can make a conclusion that the xylem and phloem of Type A and Type C are pure and the xylem and phloem of Type B are in a chimeric state.

Figure 7 reveals that the shoot tip of the lateral branches of Type B, whose DNA electrophoresis bands were similar to that of *P. shenzhou* the A-C mixed trees, was also in a chimeric state.

By the SSR identification on the two types of root, we got the results showed in Fig. 8. The electrophoresis bands for the rufous roots were the same as compared with that of Type A, while the electrophoresis bands of yellow roots were similar to that of Type C, indicating that the rufous root of *P. shenzhou* has the same genetic composition as that of Type A, and belongs to the pure organization of Type A, and the yellow root, sharing the same genetic composition with Type C and belonging to the pure organization of Type C. Therefore, the roots of *P. shenzhou* of the three types belong to the mosaic organization comprising the pure roots of Type A and Type C.

4 Discussion

Many reports on the application of chimera in the production of fruit trees have been published (Hu et al., 1996; Zhou et al., 1999; Li et al., 2004). There are mainly two ways for chimera to form.

One is through sprout mutation that in some meristematic cells, gene mutations and chromosome variations in structure and quantity may and at last develop the chimera (Hartmann and Keister, 1983). The meristem of the plant growth point has a dermatogen cell layer, a periblem cell layer, and a plerome cell layer. The cells of each layer arrange regularly, divide towards different directions, and then differentiate into special tissues. Sprout mutation usually happen in some individual cells of one layer, followed by the enlargement of the mutation part, finally formed chimera tissues (Simon, 2001). Chimeras generally can divide into a periclinal chimera, a sectorial chimare and a mericlinal chimera (Li and He, 2005). The sooner growing point appears when the meristem mutates, the larger the part of the variation when it grows, and the more frequently it will become a periclinal chimera. The later the variation time appears, the narrower variation of site the sectorial chimera will have.

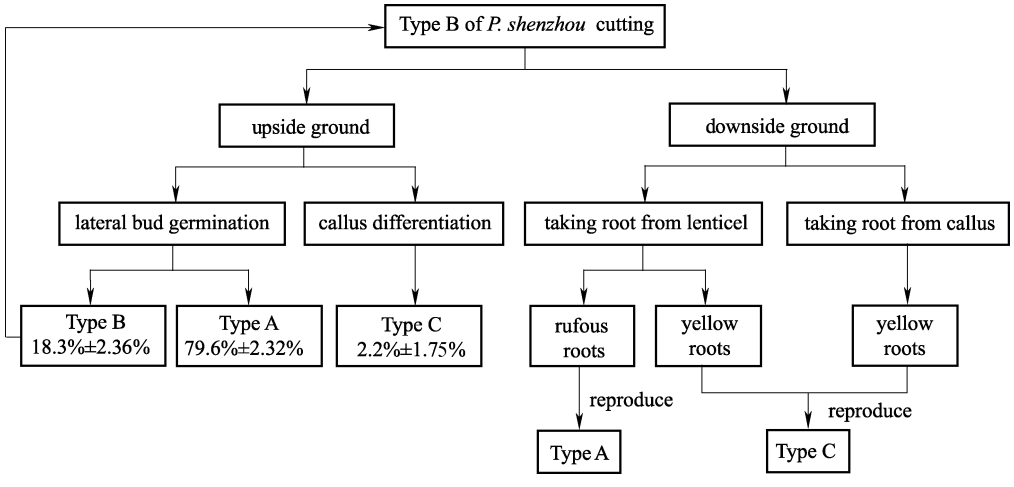

Fig. 3 The differentiated types and proportion of *P. shenzhou* Type B after cutting

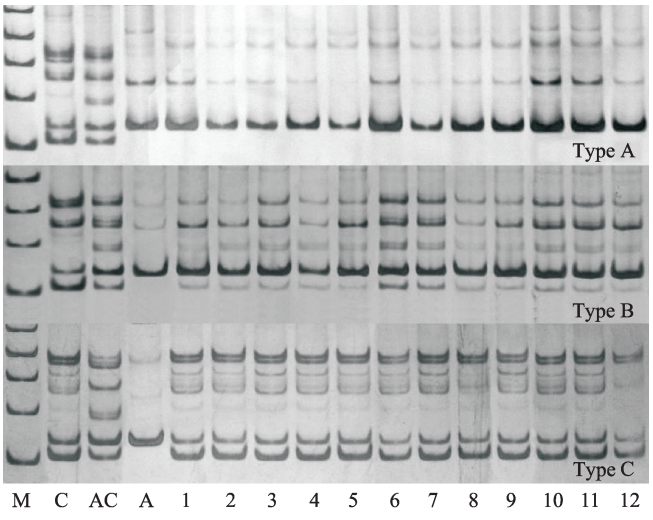

Fig. 4 The SSR results of *P. shenzhou*'s buds
Note: M, C, AC and A represent 100 bp DNA ladder 3 kb Marker, DNA from Type C of *P. shenzhou*, DNA from Types A and C of *P. shenzhou*, and DNA from Type A of *P. shenzhou*. 1–12 represent DNA from the buds of *P. shenzhou*.

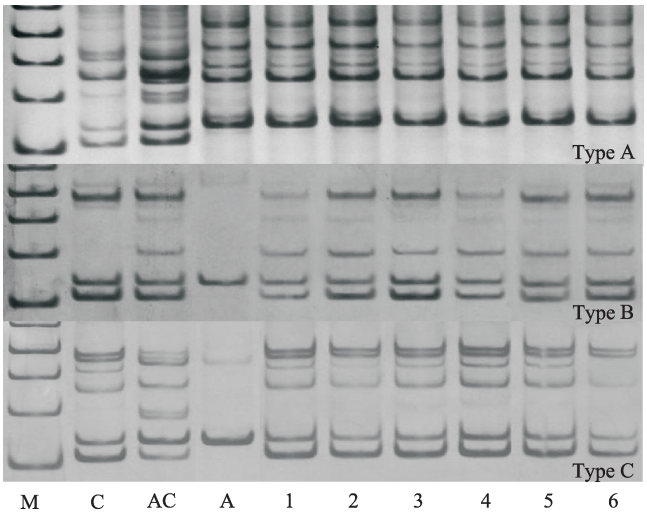

Fig. 6 The SSR results of *P. shenzhou*'s phloem and xylem
Note: M, C, AC and A represent 100 bp DNA ladder 3 kb Marker, DNA from Type C of *P. shenzhou*, DNA from Types A and C of *P. shenzhou*, DNA from Type A of *P. shenzhou*. 1–3 are DNA from the phloem of *P. shenzhou*; 4–6 are DNA from the xylem of *P. shenzhou*.

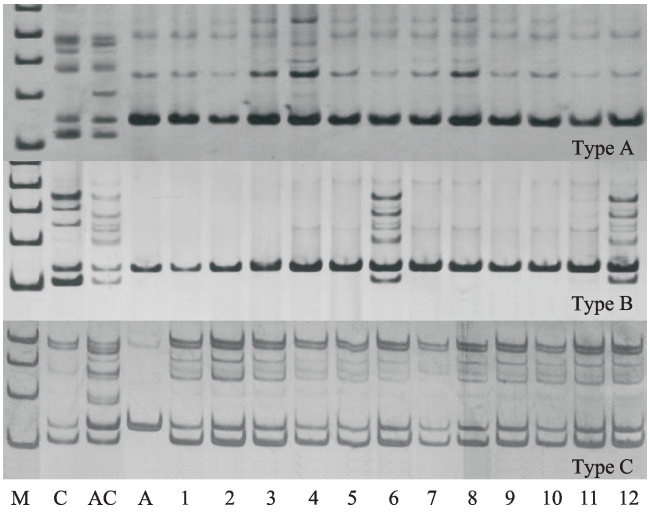

Fig. 5 The SSR results of *P. shenzhou*'s leaves
Note: M, C, AC and A represent 100 bp DNA ladder 3 kb Marker, DNA from Type C of *P. shenzhou*, DNA from Types A and C of *P. shenzhou*, DNA from Type A of *P. shenzhou*. 1–12 represent DNA from the leaves of *P. shenzhou* in Types A and C. In Type B, 1–5 and 7–11 represent DNA from leaves with no chief vein. 6 is DNA from the leafstalk and 12 is DNA from the chief vein of leaf.

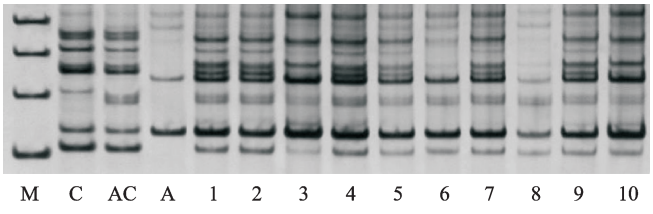

Fig. 7 The SSR results of Type B's cane top on lateral branch of *P. shenzhou*
Note: M, C, AC and A represent 100 bp DNA ladder 3 kb Marker, DNA from Type C of *P. shenzhou*, DNA from Types A and C of *P. shenzhou*, DNA from Type A of *P. shenzhou*. 1–10 are DNA from the cane top on lateral branch of Type B.

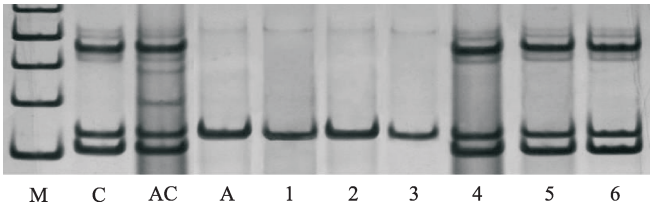

Fig. 8 The SSR results of *P. shenzhou*'s roots
Note: M, C, AC and A represent 100 bp DNA ladder 3 kb Marker, DNA from Type C of *P. shenzhou*, DNA from Types A and C of *P. shenzhou* and DNA from Type A of *P. shenzhou*, respectively. 1–6 represent DNA from rufous roots of Type A, DNA from rufous roots of Type B, DNA from rufous roots of Type C, DNA from yellow roots of Type A, DNA from yellow roots of Type B and DNA from yellow roots of Type C.

The other is through grafting; two different kinds of plants for grafting can produce grafted hybrids at interfaces in the cell fusion or chimera (Yang and Lu, 1995; Luo et al., 1996; Liu, 1999). Darwin, Michurin, and other famous scientists had proved the existence of the grafted hybrids. Pandey (1985) proved the authenticity of the grafted hybrids in theory based on mass data. Ohta (1991) and Yagishita et al. (1990) in their respective grafted hybrid tests got the grafted hybrids of pepper, eggplant, and other vegetables. Meng and Lu (1989) achieved the

new potato-mung varieties by grafting mung to potato, Chen et al. (2006) obtained the interspecific chimeras between tuber mustard and red cabbage by an in vitro graft-culture method. Graft chimeras can be obtained from many trees, mainly in most citrus, such as Kobayashi Citrus, Kinkoji Citrus, Casella chimera, Citrus medica, and so on (Lu and Lin, 1995).

It is believed that the formation mechanism of the graft chimera is when grafting, the calli of rootstock and scion mix together at the interface and then develop into adventitious buds with two different genetic compositions of the rootstock and scion. Therefore, the cells will develop into the graft chimera with expressed characteristics of both parents. These graft chimeras possess not only the expression of two components of the grafted characters, but also the intermediate traits, even with new characters such as "trifoliate orange-poon kan" a kind of citrus graft chimera, which has unifoliate, bifoliolate, and trifoliolate leaves in some branches. However, bifoliolate leaves cannot exist in the rootstock and scion (Liu, 1999). Liu (1999) successfully realized the tube grafting of hawthorn and apple, induced the plantlet from the callus, and provided a new way of artificial induction of grafting chimera using in vitro culture techniques, including aseptic seedling obtaining, tube grafting, callus multiplication culture, and plant regeneration from the callus. However, the chimera previously reported produced a stable material, through clones or sexual reproduction to maintain the genetic stability of chimera, avoiding chimera reseparation. In addition, there have been fewer reports about chimera of timber species.

P. shenzhou is a new chimera of two poplar species, different from previous reports, which had a stable differentiation regulation and could be divided into Type A, B, and C. Type B of *P. shenzhou* as a progenitive material was cut and differentiated into Type A, Type B, and Type C at a steady differentiation regulation rate of about 80%, 20%, and 2% respectively. Type B, for its stable differentiation, could not only provide Type A seedlings continually, but also guarantee the characteristic of easy rooting, and therefore it is called "reproductive system" in production. From the mentioned above, we conclude that Type B should belong to cell or tissue chimera, without cell fusion and generic material transfer, and therefore, could differentiate stably. The discovery of the poplar chimera provided the new types and examples for the study of chimera. However, the true mechanism of differentiation needs further studies.

The studies on SSR identification of *P. shenzhou* showed that Type B had bicomponents from Type A and Type C, belonging to chimeric tissues. Through observation, the lateral branches of Type B also had the characteristics itself. Consequently, Type B as the progenitive material, would be differentiated into Type A, B, and C, while the lateral buds of the one-year-old seedlings was still chimeristic with no differentiation. What's more, the lateral buds of the one-year-old seedlings with no shoot bud sprouted into Type A seedlings. Through analysis, we suggest that it depends on the shoot buds whether the lateral buds sprout into Type A or Type B. If in existence, they will form into Type B, if not, they will form into Type A of *P. shenzhou*. Therefore, we guess that the shoot buds and the lateral buds are closely related and the shoot buds control the lateral buds. Furthermore, we could speculate that there is a sharp close incretion transportation between the shoots and lateral buds, and the poplar is one of the plants with relatively topmost advantages. When the shoot buds exist, they first germinate, then produce a quantity of auxin and transport it into the lateral buds to restrain and slow down the growth of the lateral buds. Hence, the incretion material is produced when the shoot buds sprout, which would differently keep the growth and develop of cells with different types and could balance the growth of the cells and retain the chimeristic since. When lacking the shoot buds, the growth of Type A would be promoted or the growth of Type C would be restrained till the gradual disappearance of the cells, and thereafter the lateral buds may form into Type A. The above is only the phenomenon presumed, the true causation needs further studies. The studies on the separation mechanism of the poplar chimera are only staying in infer without enough experiments and documents to confirm.

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