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Obtaining citrus hybrids by *in vitro* culture of embryos from mature seeds and early identification of hybrid seedlings by allele-specific PCR

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ABSTRACT

Two trifoliate oranges (*Poncirus trifoliata* var. monstrosa and *P. trifoliata* cv 'Xiaoganzhi') were used separately as pollinators to cross with Shantou-Suanju (*Citrus sunki* Hort. ex. Tan.) for breeding new citrus rootstocks. Embryos from the seeds of mature fruits were rescued and individually cultured *in vitro*. On average, 9.0–10.0 embryos per seed were harvested from the two crosses. The zygotic seedlings were only derived from large- to medium-sized embryos. The small embryos were not able to germinate or develop into seedlings. The numbers of seedlings per seed produced from the two crosses were similar, ranging from 1 to 11 and 1 to 14, at average of 4.17 and 4.90, respectively, with *in vitro* culture in this study. A dominant trifoliate leaf trait from *P. trifoliata* was used as a morphological marker to discriminate hybrid from nucellar seedlings. Of 43 and 14 zygotic seedlings were obtained from 98 and 65 seeds of the two crosses, respectively. In addition to the use of morphological marker, single nucleotide polymorphism based allele specific PCR (AS-PCR) was also successfully employed as a handy tool to confirm and identify zygotic seedlings.

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1. Introduction

Rootstocks play a pivotal role in citrus production. The productivity and fruit quality of scion cultivars are to a large extent affected by the rootstocks. The resistance of scions to many diseases such as *Phytophthora* fungus and tristeza virus is influenced and in some cases even determined by what rootstock is used. The trees' adaptation to different soil conditions and their survival under abiotic stresses are also largely dependent on rootstocks. Thus developing new citrus rootstocks, which was generally neglected in the past, is of great importance to ensure the sustainability of the citrus industry.

Currently, several types of rootstocks are used in citrus production. Trifoliate orange (*Poncirus trifoliata* L. Raf) is one of the most important ones. It has been widely used as a rootstock of citrus for centuries for its possessing many excellent properties such as high tolerance to cold, dwarfing trees and improving productivity and fruit quality. It is also resistant to gummosis disease and tristeza virus. 'Flying Dragon' (*P. trifoliata* var. monstrosa) was reported to be the only true dwarfing rootstock for citrus trees (Cheng and Roose, 1995). The disadvantages of trifoliate orange are sensitive to saline and alkaline soils (Weng, 1990) and not compatible with some scion cultivars such as 'Allen Eureka'. Several hybrids such as 'Carrizo' and 'Troyer' citranges and 'Swingle' citrumelo have been produced from cross of trifoliate orange with other citrus species in order to improve the drawbacks of the rootstock cultivar. Those hybrids generally perform much better than their parents, but still need further improvement on adaptation to saline and calcareous soils (Medina-Urrutia, 2008). Shantou-Suanju (*Citrus sunki* Hort. ex. Tan.) is a traditional citrus rootstock commonly used in Guangdong and other citrus growing regions in south China. This rootstock has a deep root system and is highly tolerant to saline and alkaline soils. Compared to trifoliate orange, trees grafted on Shantou-Suanju are more vigorous and productive, but less tolerant to cold, and also produce slightly poorer quality of fruits. By making crosses between Shantou-Suanju and trifoliate orange, it should be possible to obtain new rootstocks combining the desired traits of both parents.

The production of large numbers of polyembryonic seeds with high germination rate and high percentage of nucellar progenies is one of the essential attributes that a rootstock should possess (Broadbent and Gollnow, 1993). This is because nucellar embryos derived from somatic cells during the early stage of seed development, are genetically identical to their mother plant and can thus ensure the uniformity of the nursery seedlings. Therefore, a citrus rootstock breeding program should usually use at least one polyembryonic parent to ensure the production of polyembryonic hybrids.

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The dilemma is that citrus zygotic embryos are usually weaker than nucellar ones, which have less chance to succeed in the competition for nutrients during their development and germination (Soost and Roose, 1996).

Embryo rescue, a plant tissue culture technique, offers a solution to the above problem. This technique is to culture the weaker embryos *in vitro* before their abortion to increase the survival rate of the embryos. With this method, many triploid progenies were obtained (Jaskani et al., 2005; Song et al., 2005; Viloria et al., 2005). This technique was also successfully used to obtain diploid hybrids from crosses between polyembryonic cultivars (Tan et al., 2007). It was reported that the success of embryo rescue depended to a large extent on the stage of embryo development. Alternatively, Chen and Wang (1990) separated the zygotic embryos from nucellar ones at 50–55 days after pollination and cultured *in vitro* individually, but the success rate was not very high (less than 10%) since most of the embryos at this early stage of development were too delicate to be cultured. In this case, it would be interesting to know how much chance that a zygotic embryo would survive up to fruit maturation and is still able to germinate naturally. If the zygotic embryo develops well in a mature seed but only fails to germinate, it should be possible to rescue it with *in vitro* culture to avoid the failure on germination due to the competition from rival nucellar embryos.

Early identification of hybrid seedlings is important. It allows us to eliminate unwanted nucellar embryo derived plantlets to save land and cost on management since citrus trees are large and with long juvenile period. For this purpose, both morphological and biochemical markers have been developed and used (Agarwal et al., 2008). The dominant nature of the 'trifoliate' leaves of trifoliate orange is a useful morphological marker in the visual identification of hybrids from crosses using trifoliate orange as male parent. Other morphological traits are scarce and not very reliable. Molecular markers are therefore increasingly used because the markers are not influenced by environments. In our previous work, we found that the density of SNPs in ESTs of sweet orange, clementine and ponkan mandarins was averaged approximately at 1 SNP/100 bp (unpublished data). SNP-based allele specific PCR (AS-PCR), also called ARMS (Amplification refractory mutation system) (Newton et al., 1989) or PASA (PCR amplification of specific allele) (Risch and Merikangas, 1996), is a method for rapid genotyping of SNPs and offers the advantages of time- and cost-efficiency, convenience and less false positives.

In the present study, two trifoliate orange cultivars 'Flying Dragon' and 'Xiaoganzhi' were used as paternal parents to cross with Shantou-Suanju. Hybrid seedlings were obtained by separating embryos from the seeds of mature fruits and cultured *in vitro*. Zygotic seedlings with trifoliate leaves were verified using AS-PCR.

2. Materials and methods

2.1. Plant materials

All the plant materials were from the National Citrus Germplasm Repository located at the Citrus Research Institute of Chinese Academy of Agricultural Sciences, Chongqing, China. 'Xiaoganzhi' (*P. trifoliata*) is a landrace of trifoliate orange originated from Hubei, China. It is a moderate-sized rootstock with large size of flower and leaf.

2.2. Pollen collection and hybridization

Pollens were collected from two trifoliate orange cultivars ('Flying Dragon' and 'Xiaoganzhi'), dried with silica gel and kept at 4 °C until use. Crosses were made as follows. Un-opened fully developed flowers of Shantou-Suanju were emasculated and pollinated with dried pollens of trifoliate oranges, then bagged immediately to prevent contaminations from other pollen sources.

2.3. In vitro culture of embryo

Mature fruits from artificially pollinated flowers were harvested in November for collecting seeds. For *in vitro* culture, the seeds were immersed in 1 mol/L sodium hydroxide for 30 min to remove pectin from the seed coat, then washed 3 times with tap water and sterilized in 2% sodium hypochlorite for 15–20 min. The sterilized seeds were washed 3 times with sterilized water and kept at 4 °C for 3–4 days. The embryos (Fig. 1A) from each single seed were cultured on solid MS medium (Sigma) containing 25 g/L sucrose (Fig. 1B) in an order from large to small size. For the control, the seeds were sown in nursery soil in big containers after removal of seed coat. All the cultures were kept at 25 ± 1 °C, 16 h/8 h of photoperiod to facilitate germination. The seedlings with trifoliate leaves were transplanted to the pots in greenhouse at 4–5-leaf stage. One year later, the survived plantlets were transplanted into field.

2.4. Data collection and analysis

The experiments of both *in vitro* culture and the control were carried out in three replications, each with 15–35 seeds. Data were collected 50–60 days after culture and analyzed with ANOVA (Analysis Of Variation) using SAS software at 0.05 of significance. The seedlings with trifoliate leaves were treated as hybrids.

2.5. Isolation of genomic DNA

Genomic DNA was extracted from young leaves of the hybrids, nucellar seedlings and their parents using the *EasyPure*™ Plant Genomic DNA Kit (TransGen. Biotech., Beijing), following the protocol supplied with the kit.

2.6. Primer design and PCR

In our previous work, the genomic full-length of *CHX* (*β-carotene hydroxylase*) gene sequences from more than 50 citrus accessions including trifoliate orange and Shantou-Suanju mandarin were sequenced (unpublished data). A SNP site with an adenosine (A) in trifoliate oranges and a cytosine (C) in Shantou-Suanju mandarin was identified and used as marker to discriminate the hybrids in this study. The reverse primer (AS-R) was 5′-GCCAGAAACATAGTCCAGCAGAAAT-3′ (the underlined indicates the nucleotide complementary to the A-SNP of trifoliate orange). The forward positive control primer (ConF) was 5′-CGTAAACAAACAAAACCCCACCA-3′ and the backward positive control primer (ConR) was 5′-CTATGGCTGGAACTGCGTTGATT-3′ (Fig. 2). All primers were designed using Primer 5 and synthesized by Beijing Genomics of Institute (BGI).

The 10 μL PCR reaction mixture contained 100 ng of genomic DNA, 2 mM dNTPs, 0.25 U HS *Taq* polymerase (TaKaRa), 1× reaction buffer containing Mg^{2+}, 2 μM ConR, 2 μM AS-R and 3.6 μM ConF. The PCR cycles were programmed as follows: 1 cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s, and 1 cycle at 72 °C for 7 min.

3. Results

3.1. In vitro culture and plant regeneration

Two crosses were made using Shantou-Suanju as female parent and two trifoliate orange cultivars 'Flying Dragon' and 'Xiaoganzhi' as male parents. Fruits from these crosses were harvested in