Genetics and Molecular Research

In vitro embryo rescue culture of F1 progenies from crosses between different ploidy grapes

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Genet. Mol. Res. 14 (4): 18616-18622 (2015)
Received August 19, 2015
Accepted October 14, 2015
Published December 28, 2015
DOI http://dx.doi.org/10.4238/2015.December.28.10

ABSTRACT. Crossing different ploidy grapes is an effective way to obtain new seedless cultivars. Although embryo rescue has been extensively applied in breeding seedless and triploid grapes, only a few improved cultivars have been developed. Based on preliminary studies, we set five crosses between tetraploid and diploid grape varieties to obtain new hybrid triploid germplasms. Additionally, we compared two different methods of performing *in vitro* embryo rescue and sowing in the development of hybrid triploid grape plants. The results showed that the germination rate of hybrid seeds was much lower (0-22.8%) than that of the self-pollinated seeds (50.9-61.2%) obtained though the same method of *in vitro* culture. Meanwhile, the seed germination rates of all crosses obtain through *in vitro* culture (0-61.2%) were higher than those obtained through sowing (0-42.0%). Identification of ploidy level confirmed that three lines obtained from the crosses of 'Ruby Seedless (2x) x Black Olympia (4x)' and 'Big black (2x) x Kyoho (4x)' were triploid, and one line from the cross of 'Big black (2x) x Kyoho (4x)' was haploid, and the others were diploid, tetraploid, or aneuploidy plants.

Moreover, 4 haploid and 42 triploid surviving grape seedlings were planted in a vineyard after propagation. Therefore, an efficient system of breeding triploid seedless grapes using embryo rescue was established and 42 hybrid triploid germplasms were obtained for use in future studies.

Key words: Grape; New germplasms; Embryo rescue; Triploid; Seedless

INTRODUCTION

Grape (*Vitis vinifera* L.) is one of the most important fruit crops in the world and intense breeding efforts are underway to develop new seedless cultivars (Li et al., 2014). Triploid grapes are typically bred by hybridizing tetraploid and diploid grapes, and seedlessness results from their unbalanced chromosome sets. However, traditional breeding of triploid grapes is difficult due to the low success rate of crossing tetraploid and diploid grapes. Seeds tend to abort due to endosperm degeneration during early embryogenesis. Thus, embryo rescue is required for survival of subsequent generations (Lulsdorf et al., 2014). In addition, *in vitro* embryo rescue in grape breeding projects assures breeding efficiency by reducing the time taken to develop seedless grape cultivars by 6-8 years (Singh et al., 2011). Emershad and Ramming (1982) first reported that stenospermic grapes could generate plants via ovule culture, and, Yamashita et al. (1993) was the first to generate triploid hybrid plants by embryo rescue. Since then, the embryo rescue technique has been widely applied to embryo germination of seedless grape cultivars in different ploidy hybridization programs (Yamashita et al., 1998; Bessho et al., 2000; Bharathy et al., 2005; Tang et al., 2009; Sun et al., 2011, Ji et al., 2013a).

Embryo rescue of hybridized progeny occurs in three phases: further embryo development in the ovule, embryo germination, and plant development. Many factors influence in ovulo embryo rescue during this procedure, including genotype, age of the ovule upon removal, and the nature of the culture medium (Tian et al., 2008; Ji et al., 2013b). Therefore, although embryo rescue has been applied extensively to breeding seedless grapes and triploid grapes, only a few improved cultivars have been developed.

Over the past 10 years, our group has been committed to breeding new seedless cultivars by means of *in vitro* embryo rescue. On the basis of preliminary studies, we carried out this project with the aim of: 1) comparing two different ways of *in vitro* embryo rescue and sowing in triploid grape plant development; 2) obtaining new hybrid triploid germplasms.

MATERIAL AND METHODS

Hybridization

All grape plants were grown in a vineyard at a germplasm nursery of Shanxi Agricultural University and Pomology Instltute, Shanxi Academy of Agrlcultual Sciences, located in Taigu County, Shanxi province of P.R. China (112°32-58'E, 37°23'-42'N). Plants aged 8-10 years old were used and planted with a spacing of 1.5 m x 2.5 m. Emasculation was conducted 3 days before anthesis, followed by washing and bagging of the inflorescence. When the stigma had secreted mucus, artificial pollination was conducted using a mass of cotton with pollen that had been collected earlier from the male parents and stored at 4°C, followed by immediate bagging and marking of the inflorescence. The genotypes used in this study are listed in Table 1.

Table 1. Cross combinations.

Crosses	Female characteristics	Male characteristics
Ruby Seedless x Black Olympia	*V.vinifera*; stenospermic; diploid	*V. vinifera* x *V. labrusca* hybrid; seeded; tetraploid
Flame Seedless x Hutai NO.8	*V.vinifera*; stenospermic; diploid	*V. vinifera* x *V. labrusca* hybrid; seeded; tetraploid
Big black x Kyoho	*V. vinifera*; stenospermic; diploid	*V. vinifera* x *V. labrusca* hybrid; seeded; tetraploid
Centennial Seedless x Blush Seedless	*V. vinifera*; stenospermic; tetraploid	*V.vinifera*; stenospermic; diploid
Wuhe Cuibao × Jingyou	*V.vinifera*; hybrid by Guibao x Wuhe Baijixin; stenospermic; diploid	*V. vinifera* x *V. labrusca* hybrid; seeded; tetraploid

Embryo rescue and plant development

Immature fruits were collected at different times after pollination and surface-sterilized with 70% ethanol for 30 s, followed by 0.1% $HgCl_2$ for 6 min and three washes in sterilized water for one minute each time. Ovules were excised and cultured in Erlenmeyer flasks containing embryo formation medium (MM4 + 500 mg/L mashed banana), with each flask containing 10-15 ovules (Figure 1a, 1b). After 14-weeks *in vitro* culture (Figure 1c, 1d), the germinated embryos were counted, and then transferred onto rooting media (MS + 0.1 mg/L IBA + 0.4 mg/L 6-BA) for plant regeneration (Figure 1e). The pH of the medium was adjusted to 6.0 before autoclaving. All cultures were grown at 25°C under a 16-h photoperiod with a light intensity of 40 μE·s/m provided by a cool white fluorescent light. Sub-culture was performed every 4 weeks. After 4-weeks of culture, the well-rooted clones were recorded and transplanted into pots containing a mixture of perlite-peat-soil (3:1:1 v/v). These pots were covered with plastic cups to maintain humidity (Figure 1f), and the cups were then uncovered gradually to enable plant hardening (Figure 1g). The hardened plants were moved to a greenhouse under natural daylight for acclimatization. The surviving progenies were established in soil (Figure 1h).

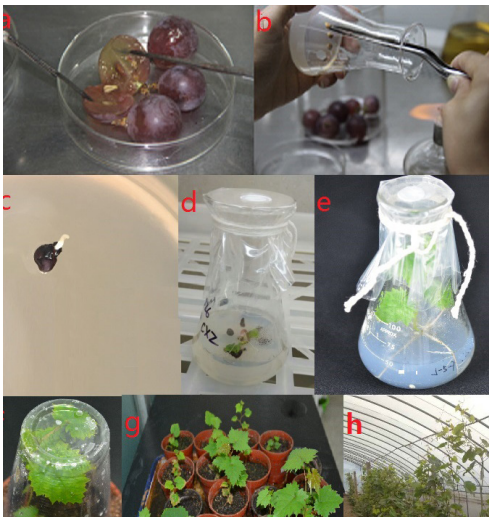

Figure 1. *In vitro* culture of hybrid seeds from crosses of diploid and tetraploid seeds. a) Ovules were excised under sterile conditions; b) ovules were cultured on embryo formation medium; c) seed germination; d) formation of two true leaf; e) single embryo rescue seedlings were transferred to fresh rooting medium; f) a single plant was transplanted into pots containing matrix and covered with plastic cups; g) the cups were uncovered for plant hardening; h) the surviving progenies were transferred to soil.

Stratification and sowing from crosses of diploid and tetraploid

Grape berries were collected during the late-maturing period (2013/09/23-26). The seeds were removed from berries, washed with running water, packed in nylon mesh bags, and hung to dry in a laboratory of Shanxi Agricultural University (Figure 2a). Next, seed stratification was carried out in the grape germplasm resources garden of Shanxi Agricultural University on 2013/12/10 (Figure 2b and c). Until 2014/03/28, seeds were unearthed and the blighted grains were discarded. The filled seeds were counted, transferred to dishes containing three layers of wet filter paper (Figure 2d), and covered by wet gauze to provide moisture (Figure 2e). The accelerated germination disposals were carried out at 25°C. The seeds were cleaned once every other day until 30% exposed white coloring. Next, seeds were sown in a plug with a wet nutrition matrix, and placed in a greenhouse under natural daylight to allow acclimatization in the grape germplasm resources garden of Shanxi Agricultural University (Figure 2f). Each seed was sown into one grid of a plug. Irrigation was performed once every three days. On 2014/05/20, the germinated hybrid seedlings with 4-5 leaves were moved to soil.

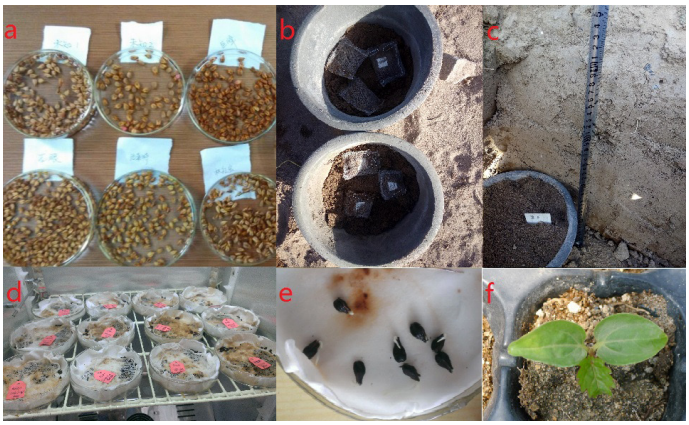

Figure 2. Hybrid seed germination and survival of crosses between diploid and tetraploid seeds. (**a**) All hybrid seeds were marked after cleaning; (**b-c**) seed stratification; (**d-e**) accelerated germination of seeds; (**f**) hybrid seed germination and seedlings formation.

Identification of F1 posterity hybrid ploidy level

We identified the chromosome ploidy level of F1 posterity hybrids, which were obtained from crosses between diploid and tetraploid grape varieties set in this experiment. The identification was carried out with a ploidy analysis instrument (Partec, Germany).

Data analysis

A completely randomized design was used in all experiments, with three replicates per treatment. Rates of embryo formation, embryo germination, and plant development were analyzed by *t*-test. When significant differences at the 5% level were found, they were compared using Duncan's new multiple range test. Analysis of variance with fixed main effects was carried out using SAS software (Cary, NC, USA).

RESULTS

Comparison of hybrid seed germination rate between the different cultivation method of *in vitro* and sowing

The rate of hybrid seed germination ('Big black x Kyoho', 14.1%) was much lower than self-pollinated seed germination ('Kyoho natural pollination', 61.2%), although the same method of *in vitro* culture was used. Furthermore, the seed germination rates of all crosses cultured under *in vitro* conditions were higher than those cultured under sowing conditions. In two-out-of-five crosses (viz., 'Centennial Seedless x Blush Seedless', 'Flame Seedless x Hutai NO.8'), no seedlings survived under either *in vitro* culture or sowing conditions (Table 2).

Table 2 Comparison of hybrid seed germination rates between sowing and *in vitro* culture from crosses of diploid and tetraploid seeds.

Crosses	No. of seeds *in vitro* cultured	No. of seeds sown	Seed germination rate (%)	
			in vitro culture	sown seeds
Kyoho (4x) open pollination	100	100	61.2	42.0
Hutai NO.8 (4x) open pollination	100	100	50.9	36.6
Centennial Seedless (4x) X Blush Seedless (2x)	522	513	/	/
Ruby Seedless (2x) × Black Olympia (4x)	233	275	22.8	2.8
Wuhe Cuibao (2x) × Jingyou (4x)	298	262	10.1	/
Flame Seedless (2x) × Hutai NO.8 (4x)	279	241	/	/
Big black (2x) × Kyoho (4x)	341	302	14.1	1.3

[1]Rate of germinated seeds = No. of germinated seeds/No. of total seeds 100X%.

Identification of ploidy level in grape hybrids plants

Identification of ploidy levels showed that three lines from the crosses of 'Ruby Seedless x Black Olympia' and 'Big black x Kyoho' were triploid, and one line from the cross of 'Big black x Kyoho' was haploid (Figure 3). The other lines were identified as diploid, tetraploid, or aneuploidy plants. Moreover, 4 haploid and 42 triploid surviving grape seedlings were planted in a vineyard after propagation (Table 3).

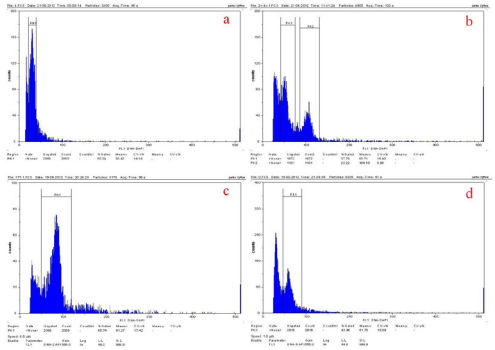

Figure 3. Flow cytometry histograms of hybrid seedlings from crosses between diploid and tetraploid grape cultivars. (**a**) Haploid; (**b**) diploid and tetraploid; (**c**) triploid and (**d**) aneuploidy plants. Relative DNA content and ploidy levels were calculated from the ratio of sample peaks to that of the internal standard, "Xinyu" (a known diploid, *2X* peak = 53.71). Mode of diploid (**a**) x peak = 30.42; (**b**) *2X* peak = 53.71, *4X* peak = 104.51; (**c**) *3X* peak = 81.27; (**d**) *nx* peak = 61.75.

Table 3. Chromosome ploidy detection of grape hybrid seedlings.

Crosses	No. of plants for detection	No. of haploid	No. of triploid	No. of surviving grape seedlings planted in vineyard after propagation
Ruby Seedless x Black Olympia	68	/	2	37
Wuhe Cuibao x Jingyou	22	/	/	/
Flame Seedless x Hutai NO.8	19	/	/	/
Big black x Kyoho	33	1	1	9
∑	142	1	3	46

DISCUSSION

Consumer demand for seedless grapes has increased in the global market year-by-year. To date, embryo rescue has been utilized in grape breeding for more than three decades (Bessho et al., 2000; Zhao et al., 2004; Guo et al., 2011; Li et al., 2014). Thus, our group has strived to cultivate excellent seedless grape varieties by embryo rescue for more than ten years (Tian et al., 2008; Tang 2010; Ji et al., 2013a, 2013b; Li et al., 2014). Due to the advantages of seedlessness, large grains, and good resistance, the creation of new triploid grape germplasm is a target of our research (Ji et al., 2013a). In this study, we set five crosses with diploid and tetraploid lines and obtained 9 haploid and 37 triploid surviving hybrid seedlings (Table 3). We found that the germination rate of hybrids (0-22.8%) was much lower than that of self-pollinated seeds (50.9-61.2%), although the same method of *in vitro* culture was used (Table 2). This finding is consistent with Johnston's hypothesis of endosperm balance number, which states that the endosperm will develop healthily into a seed when the gamete ratio of the parents is 2:1, otherwise disordered chromosome pairing will occur, and the endosperm will cease to develop, leading to abortion (Johnston et al., 1980). Therefore, it was necessary to obtain new triploid germplasm by means of the *in vitro* embryo rescue technique to overcome this mating obstacle in crosses between diploid and tetraploid grape (Xu et al., 2005; Guo et al., 2011). The results of our study also showed that the seed germination rates obtained through *in vitro* culture were higher than those obtained through sowing (Table 2). This might be caused by the additional nutritional supplements derived from media, which complement the endosperm as the sole nutrition source for hybrid seed embryo development. In addition, it is easier to harvest hybrid progeny when using diploids as female parents (Guo et al., 2011). Sun et al. (2011) also assumed that the ovule fertility of tetraploids was lower than that of diploids. Our earlier study indicated that the 4X x 2X crosses had lost their germinability and no seedlings were obtained. Therefore, the diploids were used as female parents in our experiment. Furthermore, developmental studies still have to be done in aneuploidy breeding (Park et al. 1999, 2002; Druart, 2006; Tucker et al., 2010; Reisch et al., 2012). In this study, we obtained 46 surviving grape seedlings that were planted in a vineyard after propagation. These can be used as parent materials in an aneuploidy breeding plan, which is the focus of our future research. In fact, breeding triploid seedless grapes by embryo rescue is a long-term task, and this study is only a preliminary report. The results obtained clearly indicate the feasibility and perspective of our protocol.

Conflicts of interest

The authors declare they have no conflict of interest.

ACKNOWLEDGMENTS

Research supported by National Natural Science Foundation of China (Grant #31401834); State Key Laboratory of Crop Stress Biology in Arid Areas research fund (Grant #CSBAA2015002); Program for the Top Young Innovative Talents of Shanxi Agricultural University (Grant #TYIT201401). The useful suggestions and comments from the anonymous reviewers are acknowledged here.

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