New disease-resistant, seedless grapes are developed using embryo rescue and molecular markers

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Abstract

Improving the operation of embryo rescue technology is a highly efficient way to breed new high-quality cultivars of seedless, disease-resistant grapes when using a stenospermocarpic *Vitis vinifera* L. (female parents) hybrid with Chinese wild *Vitis* (male parents). In this study, sampling time had a significant effect on embryo recovery. Four crosses were investigated to improve the embryo rescue efficiency by determining the best sampling time, which was found to be 40, 50, 55, and 57 days after pollination (DAP) for ‘Flame seedless’ × ‘Ruby seedless’, ‘Kunxiang seedless’ × ‘Flame seedless’, ‘Ruby seedless’ self-pollination and ‘Ruby seedless’ × ‘Flame seedless’, respectively. The highest percentage embryo germination ranged from 60.83 to 89.10% for four cross-combinations when the embryo was germinated on woody plant medium (WPM), with 1.0-μM thidiazuron (TDZ, a potent cytokinin). For 11 cross-combinations, the embryo recovery rate varied from 4.8 to 29.6% and the plant development rate varied from 17.1 to 78.9%. ‘Ruby seedless’ was the best female parent followed by ‘Flame seedless’. The Grape Seedless gene Probe 1 (GSLP1) and SCF27 molecular markers were used to assist the identification of the seedless traits of the 388 progeny. A total of 309 plantlets were amplified, with the specific bands at 569-bp and 2k-bp being preliminarily considered as seedless. The S382-615 marker was used to identify the downy mildew resistance of the ‘Ruby seedless’ × ‘Beichun’ cross. Two F1 progeny were amplified, with the specific band at 615-bp being identified as downy mildew resistance.

Keywords Grapevine · Seedlessness · Disease resistant · Embryo rescue · Molecular marker-assisted selection

Abbreviations

IAA Indole-3-acetic acid
6-BA 6-Benzyladenine

MS Murashige and Skoog (1962) medium
WPM Woody plant medium
AC Activated charcoal
TDZ Thidiazuron
PGR Plant growth regulator
DAP Days after pollination
SCAR Sequence characterized amplified region
RAPD Random amplified polymorphic DNA

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Introduction

The European grapevine, *Vitis vinifera* L., is grown extensively around the world. The seedless grape is preferred for consumption as table grapes (fresh) and raisins (dried). There are two types of seedless grapes, parthenocarpic and stenospermocarpic (Stout 1936). In the stenospermocarpic genotype, the embryo abort after fertilization during development, and they are commonly used as parents when breeding seedless grapes (Ledbetter and Ramming 1989). Parthenocarpic grapes are small berries that develop without

fertilization. The *V. vinifera* grapes have a very high value and versatility and the vine has the ability to thrive in a wide range of climates. However, *V. vinifera* grapes are particularly susceptible to fungal disease and insect pests, especially those prevalent in cool, moist, and climates (Alleweldt and Possingham 1988).

China is one of the main centers of origin for *Vitis* species. Its genotypes also offer many useful sources of disease resistance. However, most of the Chinese species, while offering high resistance to stress from disease, chilling, moisture, and other environmental extremes, offer rather poor fruit quality compared with *V. vinifera* (He et al. 1991). In a previous study, cultivars of *Vitis amurensis* as one of the Chinese wild grape germplasms were evaluated to determine their resistance to disease and cold (Wang et al. 1998; Wan et al. 2008). ‘Beichun’ is derived from ‘Muscat Hamburg’ (*V. vinifera*) × ‘Heilongjiang’ (*V. amurensis*) and exhibits a significantly higher level of resistance to downy mildew and anthracnose than its wild parent (Li et al. 1983; Liu and Li 2004). ‘Shuangyou’ is also a cultivar of *V. amurensis* and is resistant to downy mildew disease (Yu et al. 2012). Previous studies have shown that the resistance of these wild germplasms can be controlled by the genetics of the progeny, with some exhibiting both seedlessness and also significantly improved disease resistance (Tian and Wang 2008). Breeding of seedless and disease-resistant new grape cultivars with large berries is one of the important goals in grape growing (Akkurt et al. 2019). To improve the quality and disease resistance of new cultivars, we bred new cultivars using the wild species ‘Shuangyou’ and ‘Beichun’ as male parent hybrids with various *V. vinifera* seedless cultivars as female parents to increase the number of progenies (Ji and Wang 2013; Li et al. 2014).

The in vitro embryo rescue technique from stenospermocarpic grape has been reported to improve the efficiency of breeding seedless grapes compared with the traditional hybrid technology, and the proportion of seedless progeny obtained from seedless × seedless crosses by embryo rescue is 85% (Cain and Emershad 1983; Agüero et al. 1995). Hence, embryo rescue is now widely used for hybridizing seedless grapes (Bouquet et al. 1989; Emershad and Ramming 1994; Bouquet and Danglot 1996; Ramming et al. 2000; Notsuka et al. 2001; Tian et al. 2008; Tang et al. 2009; Ji et al. 2013; Li et al. 2015, 2018).

It has been shown that the success of embryo recovery depends on the genotype of the parents (Tian and Wang 2008), sampling time (Gray et al. 1990), and composition of the culture medium (Cain and Emershad 1983; Spiegel-Roy et al. 1985; Bouquet et al. 1989; Emershad et al. 1989). The female and male parents affect both the capacity for embryo formation and the germination of the rescued embryo (Spiegel-Roy et al. 1985; Li et al. 2015, 2018; Liu et al. 2015). The seed trace of different stenospermocarpic

grapes can be classified into three types: small, medium, and large. The larger the seed trace, the easier it is to rescue before embryo abortion (Pommer et al. 1995).

Careful timing of sampling has a significant effect on the efficiency of embryo rescue (Li et al. 2014). During the early stages of development, the embryo is not well developed and in vitro culture embryo rescue is difficult, while in the later stages, embryo rescue can fail before the embryos abort (Singh and Brar 1992). A suitable embryo rescue time is 40–60 days after blooming, before the embryo abortion (Cain and Emershad 1983; Spiegel-Roy et al. 1985; Emershad et al. 1989; Gray et al. 1990).

The medium composition is a critical factor in embryo rescue, and supplementation with appropriate additions of hormones can improve the efficiency (Tian et al. 2008; Li et al. 2014). The percentage embryo germination is influenced by exogenous plant regulators, such as auxin and cytokinins, which are incorporated in WPM as a basal medium (Emershad and Ramming 1994; Liu et al. 2003).

A model to explain the genetic mechanism of seedlessness has been proposed in which three independent and complementary recessive genes are regulated by the dominant locus seed development inhibitor (*SDI*) on linkage group (LG) 18 (Bouquet and Danglot 1996; Lahogue et al. 1998). Two simple sequence repeat (SSR) markers, VMC7f2 and p3_VvAGL11, are closely located near the *SDI* locus (Pellerone et al. 2001; Mejía et al. 2011). The two markers are in the promoter region of the *AGMOUS-like 11* gene of the *V. vinifera* L. (*VviAGL11*), but there is a region (~430 bp) between them (Mejía et al. 2011). A high linkage disequilibrium of VMC7f2 and p3_VvAGL11 has been shown (Li et al. 2018). The *SDI* locus makes them major candidate markers for the assisted selection of seedlessness and shows a low number of false positives (Bergamini et al. 2013; Benicci et al. 2019). The *VviAGL11* gene belongs to the D-lineage of MADS-box genes, which control the ovule identity, and is the major functional candidate gene for seedless grape morphogenesis (Costantini et al. 2008; Ocarez and Mejía 2016). Further research has indicated *VviAGL11* transcript accumulation in the dual endotesta layer of the seeds in seeded grapes, which is a necessary step to determine the final seed size and lignification, but the expression is decreased in seed coats, inducing abnormal seed development in ‘Sultanina’ (Malabarba et al. 2017, 2018). Molecular analyses of seedlessness have revealed a single-nucleotide variation, causing the arginine-197-to-leucine substitution in the *VviAGL11* gene of stenospermocarpic grapes (Royo et al. 2018).

There are two sequence characterized amplified region (SCAR) markers SCC8 (Lahogue et al. 1998) and SCF27 (Mejía and Hinrichsen 2003), that are important in the development of seedlessness. The SCC8 marker was successfully used for the segregation of seeded × seedless hybrid

genotypes (Akkurt et al. 2019), and the expected distribution ratio could be obtained from different crosses (Fatahi et al. 2004). Mejía and Hinrichsen (2003) found that only 42% of the F1 hybrids of a seedless ('Ruby seedless') × seedless ('Sultanina') cross amplified a band using SCC8, but the SCF27 which they developed was strongly associated (81%) with the seedlessness trait in the F1 hybrids.

Marker efficiency depends on the genetic background when screening the seedless trait. When the lignified seed is absent in seedless grapes, the p3_VvAGL11 marker is able to correctly determine seedlessness in about 85% of the progeny (Conner et al. 2018). When comparing the markers VvIn16, VMC7f2, p3_VvAGL11, and SCF27 to test seedlessness selected germplasms under different genetic backgrounds, the results showed that use of the VMC7f2 and p3_VvAGL11 markers for a validation assay provided the most accurate allelic variability, and when the frequencies were analyzed by the best two markers, the false positives and negatives were different (Bennici et al. 2019). It is also necessary for each parental combination to be evaluated by seedlessness-related markers (Bennici et al. 2019).

The Grape Seedless gene Probe 1 (GSLP1) with an 18-bp sequence can amplify a 569-bp specific band of seedless grapes (Wang and Lamikanra 2002), which has been widely used to analyze the seedless trait (Ji and Wang 2013). It can amplify the specific band in progeny derived from 'Sultanina', but does not present the band in seeded (Li et al. 2015, 2018).

In our laboratory, grape germplasms with disease resistance have been screened by molecular markers in Chinese wild species (Wang et al. 2010). During this screening process, a series of molecular markers related to disease resistance have been developed. Random amplified polymorphic DNA (RAPD) markers, such as OPWO2-1756, have been developed to analyze grapes for powdery mildew resistance (Zhang et al. 2008a). Three RAPD markers, S416-1224, S294-369, and S382-615, have been developed for downy mildew resistance (Zhang et al. 2008b) and OPS03-1354 has been developed for anthracnose resistance (Zhang et al. 2010b). A total of 127 hybrid progeny were found from six crosses tested by the markers GSLP1, OPS03-1354, and S416-1224, and 27 hybrids with a seedless trait, 28 hybrids with anthracnose resistance, and 19 hybrids with downy mildew resistance were identified (Zhang et al. 2010a).

In this study, we selected 'Beichun' and 'Shuangyou' as male parents and a number of good-quality stenospemic table grapes were selected as female parents, with the aim of generating number of new, high-quality, disease-resistant, seedless grape genotypes. This study also confirmed that embryo rescue combined with molecular markers and a specific culture medium containing thidiazuron (TDZ, a potent synthetic cytokinin) can be an efficient route for breeding new grape cultivars.

Materials and methods

Plant materials

Two cultivars, 'Beichun' and 'Shuangyou', of the Chinese wild species *V. vinifera* L. × *V. amurensis* Rupr., respectively, have high levels of disease resistance. These were grown at Northwest A&F University, Yangling, Shaanxi Province, China. In 2016, we used them as male parents in a cross with seven *V. vinifera* female parents. The cultivars were 'Flame seedless', 'Ruby seedless', 'Kunxiang seedless', 'Huozhouhongyu', 'Crimson seedless', 'Centennial seedless', and 'Zixiang seedless'. All were grown in a vineyard at the Xinjiang Development and Research Center for Grapes and Melons, Shanshan County, Xinjiang Uygur, Autonomous Region of China. These vines were over 10 years old, planted at with a spacing of 1.0 × 4.0 m and their canopies trellised (Tang et al. 2009). For the detailed characteristics of these vines, see Table 1.

Emasculation and hybridization

Emasculation was conducted 3 days before anthesis, with each inflorescence immediately enclosed in a paper bag (Fig. 1b, d). Pollen was collected as the flowers started to open (Fig. 1a). Hand pollination conducted with cotton wool, using pollen collected from the male parents (Tian et al. 2008) (Fig. 1c). Pollination was conducted between 8 and 11 o'clock. Self-pollination of the 'Ruby seedless' cultivar was achieved simply by enclosing the inflorescence in a paper bag before anthesis.

Sampling time

Embryo rescue timing was investigated as the number of days after pollination (DAP) (Li et al. 2014). In 2016, immature berries of 'Ruby seedless' self-pollination and 'Ruby seedless' × 'Flame seedless' were harvested at 52, 53, 54, 55, 56, 57, and 58 DAP. The immature berries of 'Flame seedless' × 'Ruby seedless' were harvested at 37, 38, 39, 40, 41, 42, and 43 DAP. The immature berries of 'Kunxiang seedless' × 'Flame seedless' were harvested at 47, 48, 49, 50, 51, 52, and 53 DAP. In each case, 300 healthy immature berries were collected, from which the ovules were excised for culture. The ovules were inoculated on modified medium 3 (MM3), supplemented with 6% (w/v) sucrose, 0.05% (w/v) complex protein hydrolysate, 0.01% inositol, 0.3% activated charcoal (AC), and 0.7% agar.

Ovule culture and embryo development

Immature berries were surface sterilized with 75% (v/v) alcohol for 30 s and 3% (w/v) NaClO for 15 min, and then

Table 1 The characteristics of the male and female parents

Genotype	Species	Characteristics ^a
‘Shuangyou’	<i>V. amurensis</i> Rupr.	Seeded; very resistant to anthracnose (<i>Sphaceloma ampelinum</i> de Bary.), resistant to downy mildew [<i>Plasmopara viticola</i> (Berk. Et Curtis) Berl. Et de Toni] and cold
‘Beichun’	<i>V. vinifera</i> L. × <i>V. amurensis</i>	Seeded; very resistant to downy mildew [<i>Plasmopara viticola</i> (Berk. Et Curtis) Berl. Et de Toni], anthracnose (<i>Sphaceloma ampelinum</i> de Bary.), ripe rot (<i>Gloeosporium fructigenum</i> Berk.) and white rot [<i>Coniothyrium diplodiella</i> (Speg.) Sacc.]
‘Flame seedless’	<i>V. vinifera</i> L.	Stenospermic, large and branched conical, red, early maturing
‘Ruby seedless’	<i>V. vinifera</i> L.	Stenospermic, large and branched conical, red, good quality; late-maturing
‘Kunxiang seedless’	<i>V. vinifera</i> L.	Stenospermic, golden yellow, aroma of Muscat; mid-maturing
‘Huozhouhongyu’	<i>V. vinifera</i> L.	Stenospermic; early maturing
‘Crimson seedless’	<i>V. vinifera</i> L.	Stenospermic; large and branched conical, fruit elliptic, late-maturing
‘Zixiang seedless’	<i>V. vinifera</i> L.	Stenospermic; late-maturing
‘Centennial seedless’	<i>V. vinifera</i> L.	Stenospermic; mid-early maturing, large and branched conical, green

^aRefer to Tian et al. (2008) and Li et al. (2014, 2018)

Fig. 1 Hybrid progeny from *Vitis vinifera* grapes × wild Chinese *vitis* species in the field. **a** The inflorescence of Shuangyou; **b** emasculated inflorescence; **c** hand pollination; **d** bagging the inflorescence

washed three times with sterile water. After excision, the ovules were inoculated on to the MM3, supplemented with MM3, 6% (w/v) sucrose, 0.05% (w/v) complex protein hydrolysate, 0.01% (w/v) inositol, 0.3% (w/v) AC, and 0.7% (w/v) agar, and pH adjusted with NaOH to 5.8–6.0. Each culture flask contained 15 ovules (Fig. 2a). After 9 weeks of culturing, the ovules were examined under a stereomicroscope model (Fig. 2b). The naked embryos were then transferred to the plant development medium (i.e., WPM), which contained 2% (w/v) sucrose, 1 μM 6-BA, 7% (w/v) agar, and 2% (w/v) AC (Fig. 2c, d). The plantlets were allowed to develop for 1–2 months before being transferred to an optimal media, 1/2 MS, containing: IAA (0.3 mg/L), and grown in Erlenmeyer flasks (100 mL). Regenerated plantlets were transplanted to pots (120 × 100 mm) containing a synthetic soil mixture (perlite:peat:soil = 3:1:1, v/v/v) and grown under greenhouse conditions (Fig. 2e) (Ji and Wang

2013). Initially, plants were covered with a plastic cup, but this was partially opened after 2 weeks to reduce the humidity (Fig. 2f). After 42 days of hardening, the plants were cultivated in the vineyard (Fig. 2g).

Plant growth regulator effects on embryo germination

After the ovules of ‘Flame seedless’ × ‘Shuangyou’, ‘Flame seedless’ × ‘Ruby seedless’, ‘Kunxiang seedless’ × ‘Flame seedless’, and ‘Ruby seedless’ × ‘Beichun’ had been cultured on the MM3 for 8 weeks, the developed embryos were peeled from the ovules under a stereomicroscope. Embryos were selected as a cohort, each consisting of 21–88 embryos, with three replicates. The embryos were randomly cultured on WPM containing: 0.15 (w/v) AC, 2% (w/v) sucrose, and 0.7% (w/v) agar

Fig. 2 Embryo rescue protocol for hybrid progeny. **a** Ovules inoculated onto MM3 medium; **b–d** embryo inoculated onto germination medium; **e–f** plant in a pot covered with a plastic cup in the greenhouse; **g** surviving plants growing in soil

at pH 5.8, and supplemented with a PGR. The control medium was WPM with no additional PGR. Medium 1 was WPM with 5.7 μM IAA, 4.4 μM 6-BA, and 1.4 μM GA₃ (Li et al. 2014); medium 2 was WPM with 1.0 μM 6-BA (Tian et al. 2008); and medium 3 was WPM with 1.0 μM TDZ. Embryo germination numbers and abnormal plantlet development numbers were recorded after 6 weeks of culturing.

Marker-assisted selection

Samples of DNA were isolated from the fresh leaves of all 11 parents used for hybridization and from all progeny. A modified cetyltrimethylammonium bromide (CTAB) method was employed and the DNA was stored at -80°C (Wang and Lamikanra 2002; Li et al. 2015). Eight primers were designed, with sequences and references summarized in Table 2. All PCR amplifications were conducted with 20 μL reaction volumes containing 100 ng DNA, 0.2 μM of each primer (10 μM), 7 μL of 2 $\times$ Taq PCR Master Mix, and

Table 2 The eight primers: designations, sequences, and references

Gene name	Forward primer sequence 5'–3'	Reverse primer sequence 5'–3'	T _m (°C)	Size of amplified product (bp)	References
SCC8	GGTGTCAAGTTGGAAGATGG	TATGCCAAAAACATCCCC	60	1018	Lahogue et al. (1998)
SCF27	CAGGTGGGAGTAGTGGAATG	CAGGTGGGAGTAAGATTTGT	62	2000	Mejía and Hinrichsen (2003)
P3_VvAGL11	CTCCCTTTCCTCTCCCTCT	AAACGCGTATCCCAATGAG	Touch down	198/188	Bergamini et al. (2013)
VMC7F2	AAGAAAGTTTGCAGTTTATGGTG	AAGATGACAATAGCGAGAGAA	61	198	Adam-Blondon et al. (2001)
GSLP1	CCAGTTCGCCCCGTAAATG		32	569	Wang and Lamikanra (2002)
ScORA7-760	GAAACGGGTGTGAGGCAAGGTGG	GGCCATTAG GAAATCAACATTAC	56	760	Akkurt et al. (2007)
OPB 15 ₁₂₇₄	CGGCAGACCTTACTGAAAGGATGAGT	CAACCACCATCCAATGATGGCGGGCTTC	37	1274	Kim et al. (2008)
S382-615	TGGGCGTCAA		36	615	Zhang et al. (2008a, b)

10 μL ddH₂O. Then, PCR products were separated on 1% agarose and the results were photographed.

Statistical analysis

All experiments were repeated three times. The data were subjected to an analysis of variance (ANOVA) using a randomized complete block design and the differences between the means were compared by a Duncan's test ($P \leq 0.05$) using SPSS software, version 19.0.

Results

Effects of sampling time on embryo rescue efficiency

The optimal sampling time differed significantly among the crosses (Fig. 3). The highest embryo recovery rate (35.33%) and plant development rate (71.18%) for 'Ruby seedless' $\times$ 'Flame seedless' were on DAP 53 (Fig. 3b). For 'Ruby seedless', the highest self-pollination plant development rate (73.73%) was on DAP 55. When 'Ruby seedless' was the female parent, DAP 55 was the best sampling time for embryo rescue. Embryo rescue was limited by immaturity before this time, and was then followed by natural abortion. The morphology of larger seed traces ('Ruby

Fig. 3 Effect of different sampling times on embryo recovery rate in different crosses. **a(1)**, the ovules on cultures of 'Flame seedless' $\times$ 'Ruby seedless' from DAP 37 to 43; **a(2)**, the ovules on cultures of 'Ruby seedless' $\times$ 'Flame seedless' from DAP 52 to 58. The red arrow indicates the aborted ovule. **b(1)–(4)**, the embryo recovery rate of four crosses; **b(5)–(8)**, the embryo germination rate of four

crosses; **b(9)–(12)**, the plant development rate of four crosses. Values are mean $\pm$ SE of three replicates. Means with the same letter within a column are not significantly different (Duncan's multiple range test, $P \leq 0.05$). Embryo recovery rate (%) = the number of embryos developed/the number of ovules cultured $\times$ 100%

seedless' × 'Flame seedless') did not change during the culture stage (Fig. 3a(2)). For 'Kunxiang seedless' × 'Flame seedless', the highest plant development rate (59.72%) was on DAP 50. The highest plant development rate (43.92%) of 'Flame seedless' × 'Ruby seedless' was on DAP 39 (Fig. 3b). The ovules of 'Flame seedless' × 'Ruby seedless' were cultured from DAP 37 to DAP 43. After DAP 42, the ovules turned brown and the seed coat was wrinkled (Fig. 3a(1)).

Effect of PGRs on embryo germination rate

The PGRs in the WPM medium had significant effects on embryo germination for all four crosses, i.e., 'Flame seedless' × 'Ruby seedless', 'Flame seedless' × 'Shuangyou', 'Kunxiang seedless' × 'Flame seedless', and 'Ruby seedless' × 'Beichun' (Table 3). The most suitable medium was the WPM with 1.0 µM TDZ, which had a significant effect on embryo germination in the four cross-combinations, with the percentage embryo germination ranging from 60.83% to 89.10%. The WPM medium with 5.7 µM IAA, 4.4 µM 6-BA, and 1.4 µM GA₃ generated a callus (Fig. 4). The embryo of 'Centennial seedless' × 'Shuangyou' differentiated into a callus without growing a root (data not shown), and the formation of the callus was also influenced by genotype. The lowest percentage embryo germination of 'Flame seedless' × 'Shuangyou' was 20.51% when cultured on the medium 1 (Fig. 4). Clearly, the WPM medium did not need many PGRs, and just adding cytokinins (e.g., 1.0 µM TDZ) could promote embryo germination.

Effect of cross- and self-pollination on embryo rescue efficiency

There were 11 different cross-combinations used to determine the embryo rescue efficiency with different female parents (Table 4). When seedless cultivars were used as the female parents, the percentage embryo recovery ranged from 4.8 to 29.6% and the plant development rate ranged from 17.1 to 78.9%. The 'Ruby seedless' self-pollination had the highest plant development rate, but not the highest embryo recovery rate. In the two reciprocal crosses, the embryo recovery rate and the plant development rate of 'Ruby seedless' × 'Flame seedless' were higher than 'Flame seedless' × 'Ruby seedless'. When the male parent 'Shuangyou' was crossed with female parents of 'Flame seedless', 'Centennial seedless', and 'Ruby seedless', the highest rates of embryo recovery and plant development were observed in 'Ruby seedless' × 'Shuangyou' (22.9% and 61.4%, respectively), followed by 'Centennial seedless' (13.6% and 17.1%, respectively) and 'Flame seedless' (9.0% and 27.9%, respectively). When the male parent 'Beichun' was crossed with 'Huozhouhongyu', 'Crimson seedless', 'Zixiang seedless', and 'Ruby seedless', the highest rates of embryo recovery and plant development were in 'Ruby seedless' × 'Beichun' (29.6% and 49.2%, respectively), followed by 'Crimson seedless' (7.3% and 30.4%, respectively), 'Huozhouhongyu' (12.6% and 19.4%, respectively), and 'Zixiang seedless' (4.8% and 28.3%, respectively). 'Ruby seedless' was the most suitable as the female parent.

Table 3 The effect of different plant growth regulators on embryo germination

Cross-combinations	The medium type	The number of development embryo	The number of germinated embryos	The rate of germinated embryos (%)
'Flame seedless' × 'Ruby seedless'	Control	21	4.33 ± 0.33 ^{bc}	42.12 ± 4.08 ^{bc}
	1	28	3.33 ± 0.33 ^c	33.23 ± 1.84 ^c
	2	31	5.00 ± 0.58 ^b	48.18 ± 4.30 ^b
	3	24	6.33 ± 0.33 ^a	61.21 ± 1.21 ^a
'Flame seedless' × 'Shuangyou'	Control	33	5.33 ± 0.33 ^b	48.48 ± 3.03 ^b
	1	39	2.67 ± 0.33 ^c	20.51 ± 2.57 ^c
	2	34	6.00 ± 0.58 ^b	52.78 ± 3.82 ^{ab}
	3	46	9.33 ± 0.33 ^a	60.83 ± 0.83 ^a
'Kunxiang seedless' × 'Flame seedless'	Control	31	7.00 ± 0.58 ^b	67.57 ± 3.87 ^b
	1	34	7.67 ± 0.33 ^b	67.68 ± 2.67 ^b
	2	31	7.00 ± 0.58 ^b	67.58 ± 3.87 ^b
	3	19	9.33 ± 0.33 ^a	85.05 ± 2.51 ^a
'Ruby seedless' × 'Beichun'	Control	27	7.33 ± 0.33 ^c	70.91 ± 0.91 ^b
	1	88	14.33 ± 0.67 ^a	53.71 ± 1.03 ^c
	2	65	11.00 ± 0.58 ^b	49.21 ± 0.79 ^c
	3	37	11.00 ± 0.36 ^b	89.10 ± 2.89 ^a

Values are mean ± SE of three replicates. Mean values with the same letter within a column are not significantly different (Duncan's multiple range test, $P \leq 0.05$). The rate of germinated embryos (%) = the number of germinated embryos/the number of development embryo × 100

Fig. 4 The embryos germinated from four different cross-combinations in different media

Table 4 Effect of genotype on embryo recovery and plant development in vitro

Cross (genotype)	No. of ovules cultured	No. of embryos excised	Embryo recovery rate (%)	No. of plants developed	Plant development rate (%)
'Flame seedless' × 'Shuangyou'	1359	40.7 ± 1.5 ^{cd}	9.0 ± 0.5 ^g	11.3 ± 0.9 ^f	27.9 ± 1.9 ^d
'Flame seedless' × 'Ruby seedless'	1534	55.0 ± 1.7 ^c	10.8 ± 0.1 ^f	19.7 ± 2.0 ^e	35.6 ± 2.6 ^d
'Ruby seedless' × 'Shuangyou'	2091	159.7 ± 1.3 ^b	22.9 ± 0.1 ^b	98 ± 2.9 ^b	61.4 ± 1.4 ^b
'Ruby seedless' selfing	736	49.3 ± 2.1 ^{cd}	20.1 ± 0.25 ^c	39.0 ± 3.0 ^d	78.9 ± 3.3 ^a
'Ruby seedless' × 'Flame seedless'	3620	291.7 ± 25.7 ^a	24.2 ± 0.1 ^b	179.7 ± 7.3 ^a	62.1 ± 3.0 ^b
'Kunxiang seedless' × 'Flame seedless'	742	28.7 ± 0.4 ^{de}	11.6 ± 0.1 ^{ef}	9.7 ± 0.3 ^{fg}	33.7 ± 0.8 ^d
'Huozhouhongyu' × 'Beichun'	284	12.0 ± 1.1 ^f	12.6 ± 1.0 ^{de}	2.3 ± 0.3 ^g	19.4 ± 1.4 ^e
'Crimson seedless' × 'Beichun'	313	7.7 ± 0.3 ^f	7.3 ± 0.2 ^h	2.3 ± 0.3 ^g	30.4 ± 3.7 ^d
'Zixiang seedless' × 'Beichun'	293	4.7 ± 0.3 ^f	4.8 ± 0.3 ⁱ	1.3 ± 0.4 ^g	28.3 ± 6.0 ^d
'Ruby seedless' × 'Beichun'	521	154.3 ± 0.6 ^b	29.6 ± 0.1 ^a	76.0 ± 0.6 ^c	49.2 ± 0.2 ^c
'Centennial seedless' × 'Shuangyou'	214	29.0 ± 1.8 ^{de}	13.6 ± 1.1 ^d	5.0 ± 0.6 ^{fg}	17.1 ± 0.9 ^e

Values are mean ± SE of three replicates. Mean values with the same letter within a column are not significantly different (Duncan's multiple range test, $P \leq 0.05$). Embryo recovery rate (%) = the number of embryos developed/the number of ovules cultured × 100. Plant development rate (%) = the number of plantlets recovered/the number of ovules cultured × 100

Marker-assisted selection of seedless hybrid progeny following embryo rescue

To test the seedlessness, each parental combination had to be evaluated by the related markers (Bennici et al. 2019). Previously, we analyzed the two markers SCF27 and GSLP1 that were linked to the seedlessness of the seven seedless parents ('Zixiang', 'Centennial seedless', 'Crimson seedless', 'Flame seedless', 'Kunxiang', 'Ruby seedless', and 'Huozhouhongyu') and the two seeded parents ('Beichun' and 'Shuangyou') (Supplementary Figs. S1 and S2). The results showed that in 'Flame seedless', the GSLP1 marker amplified the specific band of 569-bp that is linked to the seedless gene. The 750-bp and 1300-bp bands were amplified in 'Shuangyou' and were linked to the seeded gene. Hence, the GSLP1 marker was considered suitable for identifying the seedless descendants of 'Flame seedless' × 'Shuangyou' in this study. Among the 23 descendants of the 'Flame seedless' × 'Shuangyou' cross-combination, there were 17 individuals with a 569-bp band; the seedlessness ratio was 73.91% (Supplementary Fig. S3). The molecular marker SCF27 was present in the 2k-bp specified band in 'Ruby seedless' and 'Kunxiang seedless', but was absent in 'Flame seedless' (Supplementary Fig. S2). Among the descendants of 'Kunxiang seedless' × 'Flame seedless' and 'Ruby seedless' × 'Flame seedless', there were 17 and 339 hybrids with a 2k-bp specified band, and the seedlessness ratio was 62.96% and 71.09%, respectively (Supplementary Figs. S4 and S5). These descendants with specific bands were identified as seedless.

We also investigated the S382-615 molecular marker of disease resistance. The 615-bp specific band was found in the male parent of 'Beichun' (Supplementary Fig. S6), and two F1 progeny in the cross of 'Ruby seedless' × 'Beichun' also presented the 615-bp specific band. We propose that the two cultivars may possess resistance to link to downy mildew (Supplementary Fig. S7).

Discussion

In recent years, the development of a seedless grape with disease resistance by embryo rescue has been a target for the table grape breeding industry (Tian and Wang 2008; Li et al. 2014; Akkurt et al. 2019). The genotype of male and female parents has a significant effect on embryo rescue (Cain and Emershad 1983; Spiegel-Roy et al. 1985; Burger and Gousard 1996; Ramming et al. 2000; Valdez 2005; Tian and Wang 2008; Li et al. 2014, 2015, 2018). Female parents with larger seed traces tend to be more easily rescued, and therefore, tend to produce larger numbers of ovules with viable embryos (Pommer et al. 1995). The female parent of 'Ruby seedless' is late ripening and has large seed traces,

and the embryo and endosperm abort slowly and are relatively easy to rescue. Ji et al. (2013) reported that 'Ruby seedless' × 'Black Olympia' had a higher plant development rate than 11 other cross-combinations. We obtained the same result, with the highest plant development rate observed for female parents of 'Ruby seedless'. Further evidence was provided by seeds exhibiting poor germination efficiency in early ripening grapes due to nutritional and physiological deficiencies (Li et al. 2015). While 'Flame seedless' is early ripening and has a medium seed trace, the rates of embryo recovery were lower than in late-ripening cultivars. 'Crimson seedless' has small seed traces and is associated with 'Flame seedless' and it also offers a low embryo rescue efficiency.

In seedless grape breeding, reciprocal crossing is a useful way to increase the rate of embryo rescue (Park et al. 1999). Among the cultivars tested here, the highest plant development rate was observed for the 'Ruby seedless' self-crossing, but the embryo recovery rate was not the highest. There were three factors affecting the rate of cultured embryos: the medium, interactions between the genotype and medium, and culture period and medium (Cain and Emershad 1983; Gray et al. 1990; Gribaudo et al. 1993). Although the embryo recovery was not high, self-pollination was beneficial for the development of the embryo following normal germination.

The optimal sampling time is suggested to be during the fruit development stage, 7–14 days prior to version (Amaral et al. 2001; Hiramatsu et al. 2003). A previous study suggested that the suitable embryo rescue time was 40–60 days after blooming, before the embryo abortion (Gray et al. 1990). Other studies have suggested that the optimum time for berry sampling for in vitro embryo rescue is 50 ± 10 days after flowering (Notsuka et al. 2001). When the sampling time is too early, it will result in poor embryo development and also poor seed germination (Sharma and Kaur 1996). Li et al. (2014) found that the optimal time was dependent on the number of days between pollination and the maximum percentage embryo formation. We were able to narrow down the optimal time for the early ripening 'Flame seedless' to about 39–40 DAP, for the mid-ripening 'Kunxiang seedless' to about 48–50 DAP and for the late-ripening 'Ruby seedless' to about 55–58 DAP. Our results also showed that small seed traces shrank and became brown more quickly, such as in 'Flame seedless', where most ovules became brown at 40 DAP. This result was reported by Liu et al. (2003) to occur in cultures at 70 days after flowering.

It has recently been shown that auxin, gibberellins (GAs), and cytokinin play an important role in regulating fruit development (Kumar et al. 2014). The seeds are stores of auxin, GAs, and cytokinins, which stimulate the growth of surrounding tissues and determine the fruit size (Crane 1964; Ozga and Reinecke 2003). Other evidence has

suggested that there is a correlation between high cytokinin levels in developing seeds and cell division activity in the surrounding tissue (Abdel-Rahman 1977). It is important for seed/embryo germination to break the embryo dormancy, which can be achieved by exogenous cytokinins, such as benzyladenine (BA) (Gray et al. 1990; Notsuka et al. 2001). Embryo germination is a key factor limiting the efficiency of in ovulo embryo culture, which is used in breeding seedless grapes (Liu et al. 2003). The WPM, which contained exogenous cytokinins and auxins, could influence the percentage embryo germination (Emershad and Ramming 1994; Liu et al. 2003). It has been reported that the 5.7 μM IAA, 1.4 μM GA₃, and 4.4 μM ABA with WPM produce the highest percentage embryo germination (Li et al. 2014). The WPM medium with 1.0 μM 6-BA has also been shown to produce a high percentage embryo germination (Tian et al. 2008; Li et al. 2015). Comparing the two embryo germination mediums, our results showed that by adding just 1.0 μM TDZ to WPM, rapid germination could be stimulated and the number of abnormal plantlets was reduced. It has been reported that high concentrations of TDZ can be used for mature and immature seed regeneration; however, unlike other active compounds, low concentrations of TDZ can stimulate shoot develop (Huetteman and Preece 1993). Hence, low concentrations of TDZ will induce embryo germination and generate high levels of activity.

The DNA probe GSLP1 was derived from ‘Sultanina’. The GSLP1 probe has been used to test seedlessness in seven seedless $\times$ seeded crosses, and 17 lines that could amplify the 569-bp band were considered seedless (Ji and Wang 2013). Li et al. (2015) used three markers, SCC8, SCF27, and GSLP1, to analyze the seedlessness of 311 progeny, with the results showing that only GSLP1 could be used. Li et al. (2018) identified 14 individuals from 259 progeny of 10 cross-combinations using the GSLP1, and the specific band at 569-bp was only present in seedless grapes. Our results indicated that in ‘Flame seedless’, the GSLP1 marker was only present in the 569-bp band and was not present in ‘Shuangyou’ seeded grape. The SCAR marker SCF27 is linked to *sd1⁺*, which identifies seedlessness. It was used in the evaluation of 127 of the progeny of a ‘Ruby seedless’ $\times$ ‘Sultanina’ cross, and a 3:1 seedless/seeded ratio was obtained (Mejía and Hinrichsen 2003). The SCF27 marker was later used to test 47 of 57 progeny with seedlessness and with a specific band at 2k-bp in three different seedless $\times$ seedless crosses. The seeded $\times$ seedless cross was analyzed and fitted the expected ratio of 1:3 (seedless/seeded) (Akkurt et al. 2012; Korpás et al. 2015). We also used the SCF27 marker to identify the seedless individuals from two crosses, ‘Kunxiang seedless’ $\times$ ‘Flame seedless’, and ‘Ruby seedless’ $\times$ ‘Flame seedless’, and the frequency of seedless/seeded coincided

with the expected ratio of 3:1 (data not shown). Therefore, GSLP1 and SCF27 are suitable markers for the selection of traits in grape breeding. In future research, the seedless trait will be tested to ascertain whether these grapes are entirely seedless.

A number of molecular markers have been developed for the selection of disease resistance. The SCAR markers, ScORA7-760 and ScORN3, are used to test resistance to powdery mildew (Akkurt et al. 2007). The SCAR marker OPB15₁₂₇₄ has been linked to anthracnose resistance in grapes (Kim et al. 2008). The S416-1224 and S382-615 markers have been found to be tested suitable for marker-assisted selection for downy mildew resistance (Zhang et al. 2008b), while OPS03-1354 can select for anthracnose resistance (Zhang et al. 2010b). However, in our results, these markers could not amplify specific bands in parents and progeny, and they were not considered suitable (data not shown). It was proposed that every marker has a certain limitation and could only be applied in some specific circumstances. In a preliminary laboratory study, we developed many markers for disease resistance, which were reported to assist in selection in the F1 hybrids derived from *V. vinifera* $\times$ ‘Beichun’ and *V. vinifera* $\times$ ‘Shuangyou’ (Guo et al. 2005). In the present study, the same marker S382-615 was also suitable for the detection of two hybrids with specific bands from the ‘Ruby seedless’ $\times$ ‘Beichun’ cross. However, it takes a long time to breed new grape cultivars for disease resistance and our work is only a preliminary study. There are some disease-resistant progeny that we have not detected. In future, more work will be undertaken to evaluate the characteristics of disease resistance in the field.

Conclusion

Embryo rescue is a quick and efficient way to improve the breeding of new grape germplasm. This study investigated some of the factors affecting the success of embryo rescue. New hybrid progeny with seedless and disease-resistant characteristics were identified using molecular markers, and further studies will polymerize the molecular markers, with field identification for the selection of seedlessness and disease-resistance traits. Meanwhile, it is important to develop new molecular markers for the early selection of offspring with seedlessness and disease-resistance traits.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

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