Embryo Rescue and Genetic Transformation

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INTRODUCTION

A part of the plant breeding program at the USDA's Agricultural Research Service in Fresno is to develop early-ripening fresh market stone fruit and seedless grapes for the table and raisin industry. The California stone fruit industry needs earlier ripening cultivars which have fruit with good size, color, and eating qualities (i.e., high sugar and firm texture). For California's table and raisin grape industry early-, mid- and late-ripening seedless cultivars are needed with good fruit characteristics. Using conventional breeding the presence of immature embryos in early-ripening stone fruit and seedless grapes only allows their use as male parents. Therefore, in stone fruit, mid-season selections that develop mature seeds are used as female parents. For grapes, seeded females are used and only about 15% of the hybrid offspring are seedless. These small hybrid populations of early-ripening stone fruit and seedless grapes make the development of new cultivars slow and inefficient.

The immature embryos found in early-ripening stone fruit are due to the flesh ripening before the embryo has had sufficient time to mature within the seed. During the ripening process embryo abortion will occur. In stenospermy seedless grapes pollination and fertilization occurs but embryo and seed development becomes arrested during the early stages of development. The use of embryo rescue would allow the maturation of immature embryos found in early-ripening stone fruit and seedless grapes. This would permit the use of selections from these crops as female parents. When both parents are early-ripening and/or seedless a greater number of the hybrid population will possess these desired characteristics. This increased efficiency in a breeding program would allow the rapid development of early-ripening stone fruit and seedless grapes.

In addition, grape embryo rescue has opened another strategy of genetic improvement via genetic transformation of somatic embryos derived from zygotic embryos. If these somatic embryos develop into stable transgenic plants they could be used as parents to transmit inserted beneficial genes by traditional or nontraditional breeding methods. This approach will allow a breeder to enhance germplasm with beneficial genes previously not present in that germplasm.

EMBRYO RESCUE PROTOCOLS

Stone Fruit. The stone fruit embryo rescue program at Fresno was started in 1975. Over the years it has developed into a two-part procedure. The first part involves in-ovulo embryo rescue of our earliest-ripening selections. These ovules contain embryos ranging in length from 0.5 - 3.0 mm. Fruit is harvested before the ripening process begins and surface sterilized with 70% ethyl alcohol for 1 min then 5 min with 10% bleach. Ovules are removed aseptically and cultured in vitro on liquid Stewart and Hsu medium (Stewart, 1979) plus 6.0% sucrose from 2 to 4 weeks at 27C in the dark. During this time embryos will enlarge to lengths of 5.0 to 10.0 mm. To further develop these embryos they are excised from their ovules and placed into test

tubes containing Woody Plant Medium (WPM) (Llyod and McCown, 1981) plus 3% sucrose. These embryos are transferred to a dark cold room for 60 days of chilling at 1C. The second part of embryo rescue consists of culturing embryos that are 5.0 mm in length or larger directly from immature seeds of female parent selections. Fruit is opened and the seed is removed and placed into Petri dishes. These are then taken to an aseptic environment where each seed is surface sterilized by dipping into 95% ethyl alcohol and flamed using an alcohol lamp. Once the flame is out the seed coat is slit open and the embryo is removed and placed into a test tube. Embryos that are 10.0 mm or less in length are cultured in test tubes containing WPM with 3.0% sucrose. Embryo lengths larger than 10.0 mm are cultured on Smith, Bailey, and Hough medium (Smith et al., 1969) plus 2.0% sucrose. Upon culture these embryos are placed in a dark cold room and chilled for 60 days at 1C. On the completion of chilling embryos are germinated at 20C under fluorescent cool-white lights with a 12-h photoperiod. When shoots are the length of the cotyledons the tubes are transferred to a temperature setting of 24-25C with the same lighting regime. Once shoot lengths are from 50 to 60 mm, plants are transplanted to soil.

Using the above embryo rescue protocols the USDA has released five early-ripening stone fruit cultivars (Table 1). Depending on the early-ripening parentage used, fruit ripening dates have been advanced 10 to 30 days (Table 2).

Table 1. Cultivars developed by the USDA's peach (*Prunus persica*) embryo rescue program.

Cultivar	Fruit type	Year released
Goldcrest	Peach	1983
Mayfire	Nectarine	1983
Spring Gem	Peach (freestone)	1995
Spring Baby	Peach	1996
Crimson Baby	Nectarine	1996

Grape. The USDA's Fresno laboratory reported on the first plants produced from a stenospermic seedless grape (Emershad and Ramming, 1982) via in-ovulo embryo rescue. This pioneering research has evolved into a protocol which is used exclusively for the breeding of table and raisin grapes at our facility. Fruit is harvested 6 weeks after cross pollinations have occurred. The berries are surface sterilized with 70% ethyl alcohol for 1 min then 5 min with 50% bleach followed by three rinses with sterile water. Seed traces are removed aseptically and cultured onto Emershad/Ramming medium (Emershad and Ramming, 1994) with 6.0% sucrose for two months at 25C under fluorescent cool-white lights with a 12-h. photoperiod. After 2 months of culture, embryos are excised aseptically from seed traces and placed into test tubes containing WPM plus 1.5% sucrose plus 1.0 μM BAP to germinate and grow into plants. Test tubes are placed inside a chamber set at 26C under fluorescent cool white lights with a 12 h photoperiod. Once 4-5 true leaves have formed, plants are transplanted to soil.

Table 2. Fruit ripening dates of parents and the earliest-ripening peach selections from their progeny

Parentage	Ripening dates: Parents	Ripening dates: Earliest-ripening selection
Maybelle × OP	5/19/87	4/29/93
Junegold × Maycrest	6/4/87 × 5/18/87	4/28/92
P34-147 × P83-48	5/28/95 × 5.26.95	4/29/92
P57-56 × P107-78	6/1/87 × 5/28/83	5/9/94
P46-74 × P30-129	5/24/96 × 5/8/96	5/2/96
P45-142 × P45-141	5/15/96 × 5/3/96	5/6/96

Table 3. Summary of embryo production from 336 seedless × seedless crosses from 1989 to 1992.

No. female genotypes	108
No. *male genotypes*	128
No. ovules cultured	112,903
No. zygotic embryos found	21,567
No. zygotic embryos forming somatic embryos	2589
% zygotic embryos forming somatic embryos	12
% female parents forming somatic embryos	89

GRAPE GENETIC TRANSFORMATION

Occasionally when a seed trace is opened the zygotic embryo will have proliferated somatic embryos (Emershad and Ramming, 1984) (Table 3). Similar observations of somatic embryogenesis from zygotic embryos have been reported by others (Gray, 1992; Stamp and Meredith, 1988). Somatic embryogenesis from zygotic embryos found in cultured stenospermic grape seed have been shown to proliferate from epidermal cells of larger embryos (Margosan et al., 1994). The successful transformation of somatic embryos of walnut (McGranahan et al., 1988) and soybean (Finer and McMullen, 1991) by *Agrobacterium* and particle bombardment, respectively, suggest that these transformation/regeneration systems may also be feasible in transforming these epidermal cells. Transformed cells could then develop into embryos and eventually stable transgenic plants. A highly proliferative somatic embryogenic and plant development system was developed from our zygotic embryos (Emershad and Ramming, 1994). Using this system in cooperation with another USDA research facility we were successful in the transformation of somatic embryos and their regeneration into transgenic grape plants (Scorza et al., 1995). From the success of this research another collaborative project produced transgenic *Vitis vinifera* 'Sultana' (syn. 'Thompson Seedless') grape plants (Scorza et al., 1996). The source of somatic embryos used in this research were derived from in-vitro-grown leaves. The genes transferred include a viral coat protein gene and a lytic peptide gene. Briefly the steps followed by the researchers were: 1) microprojectile bombardment wounding of somatic embryos with gold particles using the Biolistic

PDS-1000/He device (Bio-Rad laboratories); 2) cocultivate wounded somatic embryos with *A. tumefaciens* / plasmid for 15 to 20 min (wounding allows for easier entry of *Agrobacterium* into cells); 3) rinse; 4) transfer of somatic embryos to cocultivation medium (proliferation medium containing 100 µM acetosyringone) for 2 days; 5) wash and transfer to proliferation medium for two passages (3 weeks each); and 6) transfer embryos for selection on proliferation medium containing kanamycin.

SUMMARY

In the development of improved selections and cultivars for early-ripening stone fruit and seedless grapes embryo rescue has been a successful tool. Genetic transformation is a relatively new technology, but rapid advances are being made in this field. In either case plant breeders are having more and more new breeding tools made available for crop improvement.

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