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Production of a Microspore-derived Callus Population from Sweet Cherry

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Abstract. **Microspore-derived callus cultures were obtained by anther culture of 'Emperor Francis' sweet cherry (*Prunus avium* L.). Branches were removed from the field in January and March and forced in the laboratory. When the microspores reached the uninucleate stage, anthers were placed on modified Quoirin and Lepoivre liquid culture medium containing 4.4 μM BA and 4.5 μM 2,4-D. After ≈60 days, callus that emerged from the anthers was placed on woody plant medium supplemented with 1 μM 2,4-D and 3 μM 2iP and routinely transferred. The resulting 270 callus cultures were screened for two allozymes heterozygous in 'Emperor Francis', *Pgi-2* and 6-*Pgd-1*. Of the 270 callus cultures, 154 expressed only one allele each for *Pgi-2* and *6-Pgd-1*; thus, they were considered microspore-derived. The microspore-derived callus cultures can be used as a linkage mapping population. Chemical names used: 6-benzyladenine (BA); 2,4-dichlorophenoxyacetic acid (2,4-D); *N*6-(2-isopentenyl)-adenine (2iP).**

Plant breeders have traditionally developed improved cultivars by selecting on the basis of phenotype. Now, the use of genetic maps and markers holds promise for increasing the efficiency of the selection-breeding process (Tanksley et al., 1989). DNA polymorphism linkage maps are being constructed for many species, and genes controlling important phenotypic traits are being located on these maps. Such maps allow selection for desirable genes via their linkage to detectable marker(s) (marker-assisted selection). Marker-assisted selection would be especially advantageous for sweet cherry and sour cherry (*Prunus cerasus* L.) breeding. In cherry, seedlings do not flower until they are 3 to 5 years old. Prior knowledge of linkage relationships between marker loci and traits of interest would enable elimination of undesirable genotypes from progeny populations at an early stage and allow more resources (i.e., planting spaces, evaluation time, etc.) to be devoted to promising genotypes.

In gymnosperm tree crops, linkage map development has been accelerated by the use of haploid populations of megagametophytes and molecular markers that are polymerase chain reaction (PCR) amplification products of random DNA segments with single 10 base primers of arbitrary sequences (RAPDs) (Welsh and McClelland, 1990; Williams et al., 1990). In gymnosperms, the haploid megagametophyte is multicellular, and sufficient DNA can be isolated from each megagametophyte for 150 to 250 PCR reactions (Carlson et al., 1991; Tulsieram et al., 1992). In angiosperms, microspore-derived callus cultures, as opposed to individual pollen grains, must be used to provide sufficient DNA so that the haploids can be screened for the entire set of markers.

Anther culture of peach [*P. persica* (L.) Batsch.] (Hammerschlag, 1983; Stiles et al., 1980) and peach and almond (*P. amygdalus* Stokes) (Michellon et al., 1974) resulting in haploid callus has been reported previously. Ploidy of the callus cultures was verified by chromosome counts (Michellon et al., 1974; Stiles et al., 1980) and cytofluorometric methods (Hammerschlag, 1983). Other authors have obtained callus from anther cultures of peach (Ognjanov, 1989; Seirlis et al., 1979; Todorovic et al., 1991), sweet cherry (Jordan, 1974), apricot (*P. armeniaca* L.) (Harn and Kim, 1972), and sour cherry and plum species (*P. salicina* Lindl., *P. cerasifera* Ehrh., *P. domestica* L.) (Seirlis et al., 1979); however, no genetic evidence was presented to verify a microspore origin for the callus cultures.

Prunus avium 'Emperor Francis' was chosen because of its breeding utility and heterozygosity for two allozymes, 6-phosphogluconate dehydrogenase locus 1 (*6-Pgd-1*) and glucose phosphate isomerase locus 2 (*Pgi-2*) (Beaver, 1993; Beaver and Iezzoni, 1993). The genetic origin of individual callus cultures was investigated by isozyme analysis.

Materials and Methods

Plant material. Thirty branches of 'Emperor Francis' sweet cherry, grown at the Michigan State Univ. Clarksville Horticultural Experiment Station, were collected on 13 Jan. and 3 Mar. 1992. The branches were placed in plastic bags and inserted in moist vermiculite and held in a 4C cooler. They were removed from the cooler between 5 Feb. and 21 Apr. The cut ends were placed in distilled water that was changed daily, and the branches were kept at 25C until the microspores reached the uninucleate stage (0 to 3 days of forcing).

To identify microspores in the uninucleate stage, anthers from the flower buds on the forced branches were squashed each day to release the microspores. The microspores were stained with 1% carmine in 45% acetic acid and viewed at ×400 with a light microscope to determine the stage of microsporogenesis. Anthers with uninucleate microspores were cultured immediately or the branches were held at 4C for 1 to 3 days to slow meiotic development until culturing.

Tissue culture. Flower buds from branches with uninucleate microspores were surface-disinfected by immersing in 1) 95% ethanol for 30 sec, 2) then in a mild solution of "Dial" antibacterial soap (Dial Corp., Phoenix) for 30 sec, 3) then in 1.05% NaClO for 20 min, followed by a) three rinses with sterile distilled water, b) 5 min in a 0.14-mM streptomycin sulfate solution containing 200 units/ml of penicillin, c) then three more rinses in sterile distilled water. The two to four flowers within each compound bud were then extracted aseptically. Flowers, which contain 32 anthers each, were placed in 100 × 15-mm petri dishes and macerated with a forceps to release the anthers into 9 ml of modified Quoirin and Lepoivre (1977) liquid culture medium supplemented with (mg·liter^{-1}) 100 myoinositol, 1 thiamine-HCl, 1 nicotinic acid, 1 pyridoxine-HCl, and with 3% sucrose, 4.4 μM BA, and 4.5 μM 2,4-D; pH = 5.0. About 290 anthers were placed in each petri dish. Unwanted flower parts, including the style and petals, were removed from the culture dish.

Petri dishes containing the float-cultured anthers were kept in darkness for 10 days at 23C then moved to a 16-h photoperiod provided by cool-white fluorescent bulbs (45 μmol·m^{-2}·s^{-1}) at 25C. The anthers remained on this medium for 12 to 15 weeks or until they initiated callus. The callus that emerged from the anther lobe was selected under a dissecting microscope at ×15 magnification and placed onto solid modified woody plant medium (WPM) (Lloyd and McCown, 1980) supplemented with 1 μM 2,4-D and 3 μM 2iP, 3% sucrose, 0.8% Sigma Type M agar (Sigma, St. Louis), pH = 5.0 The callus cultures were transferred to fresh medium every 3 to 4 weeks. Once 300 to 400 mg of callus tissue had been generated, a portion of the tissue was removed for isozyme analysis.

Isozyme analysis. Callus sections of 100 to 200 mg were aseptically removed from each anther-derived callus culture and screened for PGI and 6-PGD activity using the procedure of Beaver and Iezzoni (1993). 'Emperor Francis' has two alleles for *Pgi-2* (*Pgi-2*^{82} and *Pgi-2*^{100}) and *6-Pgd-1* (*6-Pgd-1*^{88} and 6-*Pgd-1*^{100}) (Beaver, 1993; Beaver and Iezzoni, 1993). Since PGI and 6-PGD are dimeric enzymes, each heterozygous locus also has a heteromeric band with intermediate mobility. Callus cultures exhibiting only one allele for each isozyme locus were considered to be microspore-derived.

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Results

Of 1255 anthers placed onto WPM, 270 produced sufficient callus (Fig. 1) for isozyme analysis. Of the 270 callus individuals, 108 were discarded because they exhibited both alleles and heteromeric bands for *Pgi-2* and *6-Pgd-1* and were presumed to have arisen from sporophytic tissue. Eight of the 270 calluses were discarded since they exhibited both alleles for *Pgi-2* or *6-Pgd-1* with the absence of the heterodimer. These callus cultures presumably arose from a cluster of two or more microspores with differing allozymes. Of the remaining cultures, 154 exhibited only one allele each for *Pgi-2* and *6-Pgd-1* and were considered microspore-derived (Fig. 2). They represent 12.3% of the total number of anthers plated.

All but one of the microspore-derived callus cultures originated from five branches that were collected in the field on 3 Mar., removed from the cooler on 2 or 7 Apr., and were cultured immediately upon removal from the cooler, or after 1 day of forcing. Only one of the 154 microspore-derived callus cultures resulted from a branch that was collected in the field on 13 Jan., removed from the cooler on 15 Feb., and float-cultured on 20 Feb.

Allelic segregation at *Pgi-2* and *6-Pgd-1* fit the expected 1:1 ratios (Table 1). Joint segregation at both loci fit the expected 1:1:1:1 ratio, indicating that the two allozymes are not linked (Table 2).

Discussion

Helentjaris et al. (1986) demonstrated that a population of 50 fully classified F_2 individuals is sufficient for the construction of a linkage map. Since in the haplotyping procedure each meiotic product is measured separately, our target population size was 100 individuals. We exceeded this target population size by obtaining 154 microspore-derived calluses. Since all but one of the microspore-derived callus cultures resulted from branches collected on 3 Mar. and removed from the cooler on 2 or 7 Apr., timing appears critical to culture success. Thus, further improvement may be obtained by holding the sweet cherry branches in the cooler for a short time, and reducing the amount of time required to reach the uninucleate stage once the branches are removed from the cooler.

If certain chromosome segments are identified that are more prevalent than expected in a microspore-derived callus culture population, then these sections may contain genes that control success in anther culture. Differential transmission of specific chromosome regions associated with the production of microspore-derived haploids has been reported in barley (*Hordeum vulgare* L.) (Thompson et al., 1991; Zivy et al., 1992), rice (*Oryza sativa* L.) (Guiderdoni, 1991), and maize (*Zea mays* L.) (Cowen et al., 1992). In the sweet cherry microspore-derived callus culture population, neither allele at either allozyme locus was preferentially selected. Segregation data for both marker loci, *Pgi-2* and *6-Pgd-1*, fit the expected 1:1 ratio.

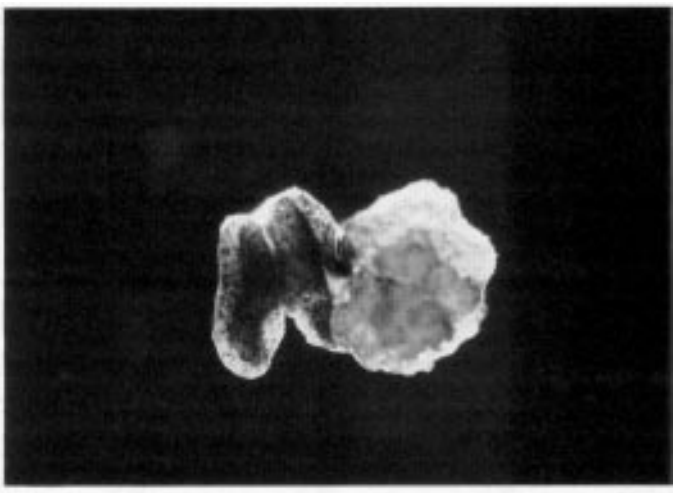

Fig. 1. Callus emerging from 'Emperor Francis' sweet cherry anther lobe.

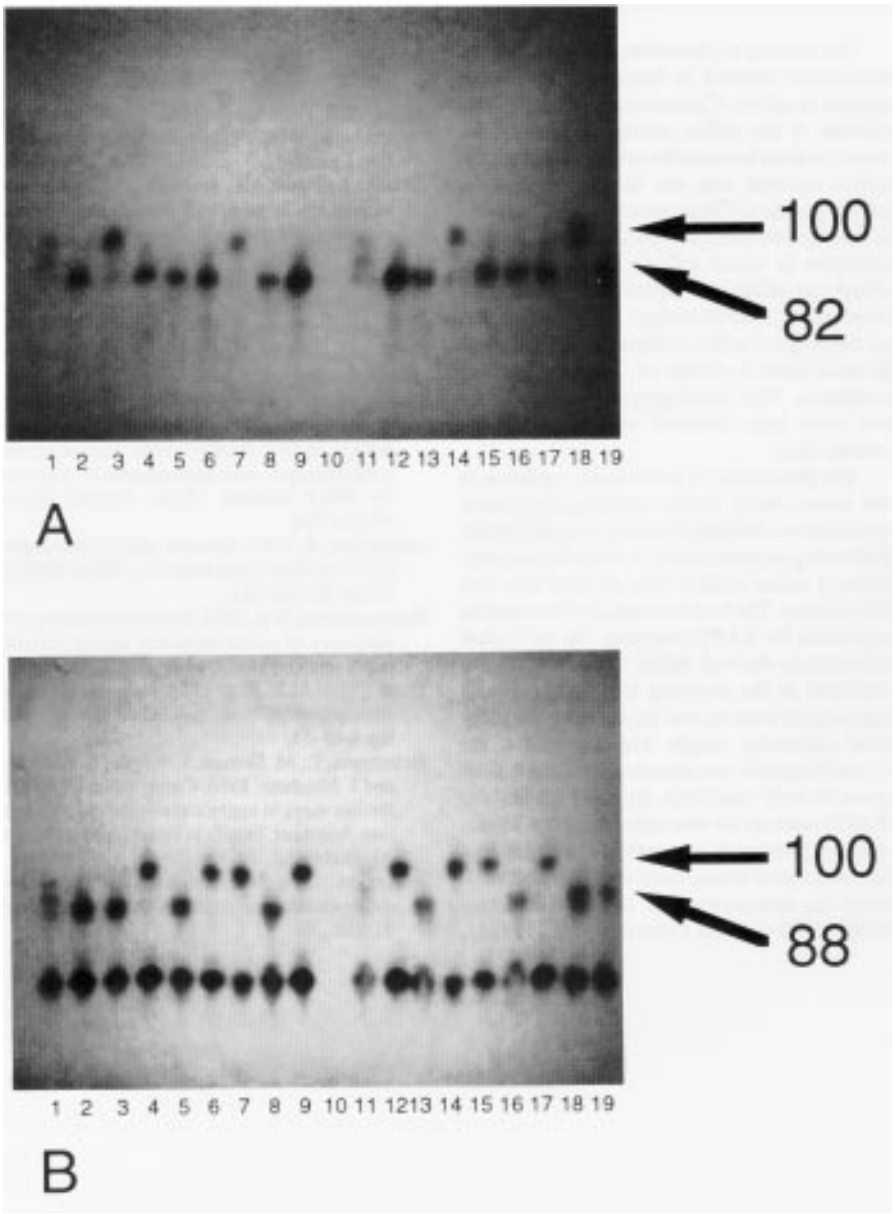

Fig. 2. 'Emperor Francis' sweet cherry callus cultures segregating for alleles at (**A**) *Pgi-2* and (**B**) *6-Pgd-1*. Mobilities are given for bands that represent alleles for (**A**) *Pgi-2*^{100} and *Pgi-2*^{82}, and (**B**) *6-Pgd-1*^{100} and *6-Pgd-1*^{88}. In A and B, lanes 1 and 11 are from sporophytic tissue, while lanes 2–9 and 12–19 are microspore-derived callus cultures, and lane 10 is blank.

Table 1. Segregation of *Pgi-2* and *6-Pgd-1* alleles among microspore-derived callus cultures of 'Emperor Francis' sweet cherry.

	Haploid genotypes			
	Pgi-2		*6-Pgd-1*	
No.	*Pgi-2*^{82}	*Pgi-2*^{100}	*6-Pgd-1*^{88}	*6-Pgd-1*^{100}
Observed	75	79	84	70
Expected (1:1)	77	77	77	77
Total	154		154	
χ^2 (1:1)	0.058		1.097	
P	>0.50		>0.25	

Table 2. Joint segregation of *Pgi-2* and *6-Pgd-1* alleles among microspore-derived callus cultures from 'Emperor Francis' sweet cherry.

	Haploid genotypes (*Pgi-2*, *6-Pgd-1*)			
No.	*Pgi-2*^{100}, *6-Pgd-1*^{100}	*Pgi-2*^{100}, *6-Pgd-1*^{88}	*Pgi-2*^{82}, *6-Pgd-1*^{100}	*Pgi-2*^{82}, *6-Pgd-1*^{88}
Observed	36	43	34	41
Expected (1:1:1:1)	38.5	38.5	38.5	38.5
Total	154			
χ^2 (1:1:1:1)	1.376			
P	>0.50			

Our criteria to determine that a callus was microspore-derived is based on the use of genetic markers. Chromosome counts to determine if the callus cultures were haploid were not done because the mitotic index for the callus cultures was too low to make such counts practical. Chromosome doubling likely occurred; however, polyploidization, which is common in tissue culture, likely does not affect the ability to detect the presence or absence of a RAPD marker. Additionally, 3% of the original callus cultures were presumed to arise from a cluster of two or more microspores. This heterogeneous callus would not have been detected with chromosome counts alone.

The possibility of somaclonal variation in the sweet cherry callus mapping population was addressed during mapping (unpublished). Following isozyme analysis, each microspore-derived callus culture was divided into two subcultures. The two subcultures were used as replicates for RAPD mapping. An individual microspore-derived callus culture was only included in the mapping population if both subcultures were scored the same for the identical molecular weight fragment and if the scored fragment was also amplified from 'Emperor Francis' leaf DNA. By requiring that the RAPD markers for two subcultures be identical, we attempted to eliminate cultures that had undergone somaclonal variation and minimize the misscoring that has been reported with RAPD markers (Weeden et al., 1992).

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