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A MICROSPORE-DERIVED LINKAGE MAPPING POPULATION

IN SWEET CHERRY (*Prunus avium* L.)

presented by

Christopher Michael Long

has been accepted towards fulfillment of the requirements for

Master of Science degree in Horticulture
Plant Breeding & Genetics

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A MICROSPORE-DERIVED LINKAGE MAPPING POPULATION IN SWEET CHERRY (*Prunus avium* L.)

By

Christopher Michael Long

A THESIS

Submitted to
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in partial fulfillment of the requirements
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Plant Breeding and Genetics

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ABSTRACT

A MICROSPORE-DERIVED LINKAGE MAPPING POPULATION IN SWEET CHERRY (*Prunus avium* L.)

By

Christopher Michael Long

A linkage mapping population was developed for sweet cherry (*Prunus avium* L.), using microspore-derived callus cultures. The sweet cherry cultivar 'Emperor Francis' was used, since it is heterozygous at the glucose phosphate isomerase locus 2 (*Pgi* -2) and the 6-phosphogluconate dehydrogenase locus 1 (6-*Pgd* -1). From the anthers cultured, 154 calli were produced which exhibited one or the other allele for *Pgi* -2 and 6-*Pgd* -1. The alleles at each locus segregated 1:1 and fit a X^2 value $P > 0.05$. Based on this allozyme information, the 154 calli are thought to be microspore-derived and useful for the development of a genetic linkage map.

Four RAPD markers which were previously found to map to the same location were tested in an additional 82 haploid calli. Segregation for each marker fit a 1:2 ratio (presence : absence) X^2 value $P > 0.05$. The map order was found to be AL-03_{760}, AI-03_{1300}, B-18_{1300}, E-20_{1400} at 0, 1.5, 1.3 cM map units between each, respectively.

For since the creation of the world
God's invisible qualities - His eternal power
and divine nature - have been
clearly seen, being understood from what
has been made, so that men are without excuse.
ROMANS 1:20

My hope is that God is manifested through this work. That people can recognize His great power, and see His hand at work in the world today. God has created all things for His glory, so He can be seen in all the earth.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Amy Iezzoni for her help and support during my Masters' work. I admire her abilities as a scientist and appreciate her for being an understanding friend. Thanks goes to Dr. Sink, and Dr. Whallon for serving on my committee. To my mom and dad: simply I say I love you both! Thank you so much. To Kathy, my wife to be: Thank you for your Christ centered love. To my friends: I appreciate each of you and I want to thank you for your friendship.

Thank you Lord for all that you have taught me about your creation. Truly it is You who has prepared me for this earthly life.

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CHAPTER 1. PRODUCTION OF A MICROSPORE-DERIVED CALLUS POPULATION OF SWEET CHERRY

INTRODUCTION

Plant breeders have traditionally developed improved cultivars by selecting on the basis of phenotype. Recently, the use of genetic maps and markers holds promise for increasing the efficiency of the breeding process (Tanksley et al., 1989). Molecular linkage maps are being constructed and genes controlling important phenotypic traits are also 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 (*Prunus avium* L.) and sour cherry (*P. cerasus* L.) breeding. Cherry seedlings mature in three to five years. Prior knowledge of linkage relationships between marker loci and traits of interest would enable selection of some desirable genotypes from progeny populations at an early stage and allow more resources (i.e., planting spaces, evaluation time, etc.) to be devoted to these promising individuals.

To date, 26 allozyme loci and one morphological trait have been mapped in sweet cherry (Tobutt and Nicoll, 1992; K. Tobutt, pers. comm.). Molecular map construction in sour cherry has not been reported. Linkage map development in sour cherry is complicated due to polyploidy. The presumed progenitor species of the tetraploid sour cherry (2n=4x=32) are the diploid sweet cherry (2n=2x=16), and the tetraploid ground cherry (*P. fruticosa* Pall., 2n=4x=32) (Olden and Nybom, 1968). Sour cherry was derived from the fusion of an unreduced gamete of sweet cherry (1n=2x=16) and a reduced gamete of ground cherry (1n=2x=16). Because of the difficulties of developing a linkage map of an allopolyploid, the objective was to develop a linkage map in sweet cherry. This would be essentially equivalent to mapping one genome in sour cherry. The approach was to develop a microspore-derived callus population for linkage mapping in sweet cherry because a molecular linkage mapping population for sweet cherry was not available. Additionally, the correlation of horticultural traits with the molecular markers, which is not possible without regenerating plants from the callus cultures, was not an objective. Instead, the goal is to eventually use the markers mapped in sweet cherry as possible single gene markers (Wu et al., 1992) to develop a linkage map in the tetraploid sour cherry.

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

LITERATURE REVIEW

The production of haploid or doubled-haploid plants is a valuable means for generating homozygous lines. In some crop species, inbred lines are most easily obtained by self-pollination. However, with highly heterozygous crop species that have long generation times, such as apple or cherry, doubled haploids from anther culture may be a more efficient avenue to reach homozygosity for a particular trait or traits of interest.

Guha and Maheshwari (1964) were the first to regenerate haploid plants from anthers of jimson weed, *Datura* . Bourgin and Nitsch (1967), working with tobacco, were the first to regenerate haploids from a cultivated species. Further studies indicated that the success of anther culture depends upon the stage of microsporogenesis. Anther culture is most successful when the microspores contained within the anthers are between the late uninucleate and early binucleate stages of microspore development (Harn and Kim, 1972). At these developmental stages it is thought that the microspores have the ability to differentiate into callus as opposed to continuing on the developmental pathway to becoming mature pollen (Stiles et al., 1980). At these points of microsporogenesis, investigators have been able to alter the developmental direction of the microspores to differentiate into embryos or, most often, into non-morphogenic callus.

Anther culture has been reported in eight species of *Prunus* : almond, *P. amygdalus* Stokes (Michellon et al., 1974); apricot, *P. armeniaca* (Harn and Kim, 1972); sweet cherry, *P. avium* L. (Jordan, 1974); sour cherry, *P. cerasus.* (Seirlis et al., 1979); plum, *P. cerasifera* , *P. domestica* , *P. salicina* (Seirlis et al., 1979); and peach, *P. persica* L. (Hammerschlag, 1983; Michellon et al., 1974; Ognjanov, 1989; Seirlis et al., 1979; Stiles et al., 1980, Todorovic et al., 1991). A variety of different basal salt mediums were used in these studies. The media varied in a number of characteristics, but all yielded calli. The hormone combinations used were found to be of importance. Common auxin-cytokinin combinations used in conjunction with these basal salt mediums are: 1) 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP); 2) 2,4-D, 6-furfurylaminopurine (Kinetin); 3) naphthaleneacetic acid (NAA), Kinetin; 4) NAA, 6-benzyladenine (BA); 5) indole-3-acetic acid (IAA), 6-[4-Hydroxy-3-methylbut-2-enylamino] purine (Zeatin); 6) NAA, Zeatin; 7) 2,4-D, Zeatin; 8) 2,4-D, BA; 9) 2,4-D, N6- (2-isopentenyl) -adenine (2iP).

Ognjanov (1989) and Todorovic et al. (1991) cultured anthers in 24 h. darkness. Hammerschlag (1983) used a 14 day period of darkness following culturing as an acclimation period before placing anther cultures in the light. Neither of these methods were consistent practices among all the authors, but all reported successful results.

Anther culture is a means by which haploid individuals can be developed, but the ploidy level or homozygous nature of this tissue must be established using cytology, isozyme or molecular marker assisted analysis. If embryos can be generated directly from microspores in the anther culture system, it is reported that little chromosomal aberration occurs, but when anthers, pollen grains or microspores yield callus, which in turn is induced to produce plantlets, a much greater chance of chromosomal change will occur (Singh, 1993). Ploidy of some callus cultures was verified by chromosome counts (Michellon et al., 1974; Stiles et al., 1980) and cytofluorometric methods (Hammerschlag, 1983). Callus that appears to grow at an abnormally slow rate in culture is thought to be haploid (i.e. a lower mitotic index) (Harn and Kim, 1972). Other authors reported obtaining haploid callus, however, but published no genetic evidence to verify the microspore origin of the callus cultures.

Each gametophyte is genetically unique and, therefore, may respond to the environment differently than other gametes. Mature gametes, pollen grains, compete on the stigma surface to fertilize the ovary (Mulcahy, 1979). In a culture system there exists a variety of selection pressures that are not present in the normal sexual reproduction cycle. Media composition, temperature and humidity of the culture environment, length of time on a particular medium, stage of microspore development before culturing, and light intensity are a few of the selection pressures that are applied to the microspores within the anthers. There are a number of chemical and biochemical selection pressures in *in vitro* culture systems that at present are not understood. A concern that is not addressed in the *Prunus* anther culture literature, but is reported in other plant species, is the possibility of segregation distortion in anther and microspore culture systems which may result in skewed distributions.

It was reported by DeBuyser et al. (1985) that the culture system increased the development of abnormal gametes. This would cause the development of a microspore-derived population with a broader array of diversity, but would not be representative of a natural population. DeBuyser et al. (1989) were able to show that plants derived from anther culture of wheat (*Triticum aestivum* L.) represented the same biased sample of the male gamete population as that observed in a pollination event using pollen from a monosomic individual. Thus, gametic transmission in anther culture of monosomic and disomic plants is not significantly different from that in a disomic by monosomic cross. This would suggest that a 2n genetically random individual could be obtained by doubling a haploid gametic product, in contrast to making a disomic by monosomic cross and selecting for the 2n progeny.

To investigate the presence of selection pressure one can test for segregation distortions (Zivy, 1992). This can be done by evaluating isozyme, restriction fragment length polymorphisms (RFLP), and/or random amplified polymorphic DNA (RAPD) segregation data. For a gene that has two alleles we would expect to see a 1:1 segregation pattern. Guiderdoni (1991) investigated segregation of heterozygous isozyme markers in microspore-derived callus lines of rice (*Oryza sativa* L.) to test for segregational skewing. The results of this study revealed that segregation distortion for allozymes, in the anther culture-derived plants, demonstrate the existence of *in vitro* gametic selection. An additional point of interest that is raised from this study is at what developmental stage of microsporogenesis does gametophytic selection actually occur. From the results presented, the later in microsporogenesis differentiation occurs, the greater likelihood there would be of segregation distortion. This is also correlated with organogenesis in that the earlier in microsporogenesis the gametes are cultured, the greater chance they will have to produce organs such as roots and shoots.

Gametophytic selection has also been reported in barley (*Hordeum vulgare* L.) microspore culture. The goal of Thompson et al. (1991) was to regenerate a random array of gametes that could be chemically doubled to produce double haploid lines. These lines then could be used as parents in hybrid crosses. The segregation of alleles at specific loci was used to test for the random assortment of genetic information in microspore-derived lines of four spring barley crosses and their parents (Thompson et al., 1991). Thompson et al. (1991) used biochemical, molecular and morphological markers located on five of seven chromosome pairs to monitor segregation of alleles in the microspore-derived lines. Six of ten markers screened fit expected Mendelian ratios. The remaining four markers exhibited skewed segregation in the microspore-derived population. These four markers are present in a parent that is responsive to anther culture. This suggests that the selection of a particular genotype was induced by the *in vitro* culture system. Finally, this author and others suggested that there are some varieties or cultivars of plants that simply respond better in the culture environment based on recombination of favorable alleles.

Zivy et al. (1992) also studied the occurrence of selection pressure during the process of double haploid production in barley by scoring segregation distortions among protein markers separated by two-dimensional electrophoresis in a population of 62 doubled haploid lines derived from a single F_1. The analysis of this study revealed there was some segregation distortion on two chromosomes. The author raised a point as to the difficulty in showing a relationship between preferential selection of one chromosome and the physiological action that is actually being selected (i.e. double haploidization, embryo formation).

Cowen et al. (1992) were able to identify chromosomal regions that condition high anther culture response in maize using RFLP analysis.

MATERIALS AND METHODS

Plant material. Thirty branches of the sweet cherry cultivar 'Emperor Francis', grown in an orchard plot at the Michigan State University Clarksville Horticultural Experiment Station, Clarksville, Michigan were collected on two dates, 13 January and 3 March, 1992. The branches were placed in plastic bags with moist Vermiculite and then held at 4 °C for approximately one month. The branches were removed from the cooler between 5 February and 21 April and kept at 25 °C 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 400X with a light microscope to determine the stage of microsporogenesis. Branches having anthers with uninucleate microspores were cultured immediately or the branches were held at 4 °C for 1 to 3 days to slow meiotic development until the anthers were cultured.

Tissue culture. Flower buds from those branches exhibiting uninucleate microspores were surface sterilized by immersing for 30 sec. in 95% ethanol, then in a mild solution of "Dial" anti-bacterial soap for 30 sec., then in 1.05% Na hypochlorite, for 20 min., followed by three rinses with sterile distilled water, and 5 min. in a 0.14 mM streptomycin sulfate solution containing 200 units/ml of penicillin for 5 min., followed by three rinses in sterile distilled water. The two to four flowers within each bud were then extracted aseptically. Flowers, which contained 32 anthers each, were placed in 100 x 15 mm Petri dishes and macerated to release the anthers into 9 ml of modified Quoirin and Lepoivre (1977) liquid culture medium supplemented with 100 mg/l myo-inositol, 1 mg/l thiamine-HCl, 1 mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 3% sucrose, 4.5 μM 2, 4-D and 4.4 μM BA, pH = 5.0. Approximately 288 anthers were plated in each 100 x 15 mm Petri dish containing 9 ml of Quoirin and Lepoivre medium. Undesirable flower parts, including the style and petals, were removed from the culture dish.

Petri dishes containing the float cultured anthers were kept in the dark for 10 days at 23 °C, then moved to a 16 h photoperiod provided by cool white fluorescent bulbs (45 μmol $m^{-2}sec^{-1}$ irradiance) at 25 °C. The anthers remained in this medium for 7 to 9 weeks or until they began to produce calli at which time the callus was observed under a dissection microscope at 15X magnification. The calli that burst from the anther lobes were selected and placed onto modified Woody Plant Medium (WPM; Lloyd and McCown, 1981) 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 three to four 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 pieces of 100 to 200 mg were aseptically removed from each anther-derived callus culture and screened for glucose phosphate isomerase (PGI) and 6-phosphogluconate dehydrogenase (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 two alleles for 6-*Pgd* -1 (6-*Pgd* -1^{88} and 6-*Pgd* -1^{100}) (Beaver, 1993). Since PGI and 6-PGD are dimeric enzymes, each heterozygous locus also has a heteromeric band with intermediate mobility. Those callus cultures exhibiting only one band for each allozyme were considered to be microspore-derived.

Chromosome counts. Five of the 154 calli, which were determined to have one allele each for *Pgi* -2 and 6-*Pgd* -1, were used for chromosome counts. The callus was evaluated approximately 13 months after the initial culture date. For each individual screened, approximately 15 mg. of white, friable, well-growing callus was removed aseptically from culture and placed into a french square bottle containing 20 ml. of Farmer's solution (3 parts absolute ethanol to 1 part glacial acetic acid) for 24 h. The Farmer's solution was drained off and replaced with 70% ethanol. The callus was then stored at 4 °C until it was ready to be examined. Prior to viewing, the callus was placed in 1N HCl to soften the tissue in order to aid in slide preparation. Callus remained in the 1N HCl for 45 min. or longer depending on its response to squashing. Slight warming of the slide over a small flame also aided in this process. Once the tissue softened, the HCl was removed and the callus tissue was rinsed with distilled H_2O to rehydrate the cells.

A very small 2 x 2 mm piece of callus was placed onto a precleaned, glass microscope slide. One to two drops of 1% carmine in 45% acetic acid was added. A needle was used to macerate the tissue and a cover slip was placed over the tissue. Heat and pressure were used to obtain a single cell layer on the glass slide. The slides were viewed at 400X - 1000X magnification on a light microscope. Chromosome counts were performed on 1 - 3 cells during anaphase of mitosis at 1000X magnification for each of the 5 callus individuals.

RESULTS

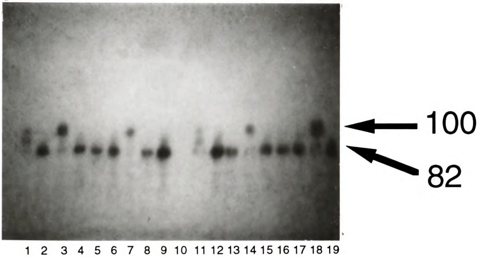
Of 1255 anthers placed onto WPM, 270 produced sufficient callus for isozyme analysis (Figure 1). Of the 270 calli, 108 individuals were discarded because they presumably arose from sporophytic tissue by exhibiting both allelic and heteromeric bands for *Pgi* -2 and 6-*Pgd* -1. Eight of the 270 callus individuals were discarded because they exhibited both alleles for *Pgi* -2 and/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 band for *Pgi* -2 and 6-*Pgd* -1 and were, therefore, considered to be microspore-derived (Figure 2). These 154 calli represent 12.3% of the total number of anthers plated.

Of the 154 individual calli, chromosome counts were done on only 5 calli. Chromosome number for each callus line ranged from 12 to 31 (n=8 was expected). Additionally, chromosome number between cells within a callus line varied.

All but one of the microspore-derived callus cultures were from five branches that were collected in the field on March 3, removed from the cooler on April 2 or 7, and were cultured immediately upon removal from the cooler, or after one day of forcing. Only one of the 154 microspore-derived callus cultures resulted from a branch collected in the field on 13 January, removed

Figure 1. Callus emerging from 'Emperor Francis' anther lobe.

Figure 2. 'Emperor Francis' 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^{82} and *Pgi* -2^{100}, and (B) 6-*Pgd* -1^{88} and 6-*Pgd* -1^{100}. In both 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.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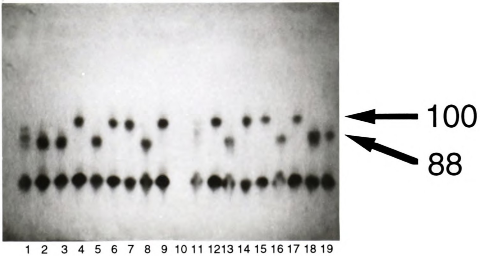
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B

from the cooler on 15 February, and float cultured on 20 February (Table A1).

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

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

	Haploid Genotypes			
	Pgi -2		6-*Pgd* -1	
	Pgi -2^{82}	*Pgi* -2^{100}	6-*Pgd* -1^{88}	6-*Pgd* -1^{100}
No. observed	75	79	84	70
No. expected (1:1)	77	77	77	77
Total	154		154	
X^2 (1:1)	0.058		1.097	
P	> 0.5		> 0.2	

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

	Haploid Genotypes (*Pgi* -2, 6-*Pgd* -1)			
	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}
No. observed	36	43	34	41
No. expected (1:1:1:1)	38.5	38.5	38.5	38.5
Total	154			
X^2 (1:1:1:1)	1.376			
P	> 0.5			

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, the target population size was 100 individuals. This target population size was exceeded by obtaining 154 microspore-derived callus cultures. Since all but one of the microspore-derived callus cultures resulted from branches collected on March 3 and removed from the cooler on April 2 and 7, timing appears to be a critical factor. These findings suggest that anther culture could be maximized by holding the branches in the cooler for a short time, thus, reducing the amount of time required to reach the uninucleate stage once the branches were removed from the cooler.

If certain chromosome arms are identified that are more prevalent than expected in a microspore-derived callus culture population, it suggests that these arms 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 (Thompson et al.,1991; Zivy et al.,1992), rice (Guiderdoni, 1991), and maize (Cowen et al., 1992). In the sweet cherry microspore-derived callus culture population, neither allele at either locus was preferentially selected. Segregation data for both marker loci, *Pgi* -2 and 6-*Pgd*

-1, fit the expected 1:1 ratio. To fully evaluate a genome for segregation distortion caused by the culture system, molecular markers need to be located on each chromosome and at a level of saturation that would reduce the chance of missing a region of a chromosome that exhibits genetic variation.

The criteria used to determine if a callus was microspore-derived was based on the use of genetic markers. Chromosome counts to determine if the callus cultures were haploid were performed on 5 of 154 putative haploid callus cultures. These 5 callus lines varied in chromosome number from 12 to 31 (n=8 was expected). No consistency in chromosome number was found within or between calli. The mitotic index for the callus cultures was low and made chromosome counts problematic. It is likely that chromosome doubling and loss of duplicated chromosomes occurred. The auxin 2,4-D is known to be a chemical mutagen and will induce chromosomal aberrations in tissue grown for an extended period of time on medium containing this compound. Polyploidization, which is common in tissue cultures, should not affect the ability to detect the presence or absence of a RAPD marker since RAPDs are dominant markers. Additionally, 3% of the original callus cultures were presumed to arise from a cluster of 2 or more microspores. This heterogeneous callus would not have been detected with chromosome counts alone.

The possibility of somaclonal variation in a tissue culture mapping population was addressed during the mapping process. 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 sub-cultures were scored identical for a RAPD marker and if the scored band amplified 'Emperor Francis' leaf DNA. By requiring that the RAPD markers for two subcultures be identical, we eliminated those cultures that had undergone somaclonal variation due to culture and minimized "misscoring" (Weeden et al., 1992). The presence of somaclonal variation was only detected 2 - 5 times in the 82 individuals screened. This result, however, is more likely due to incorrect loading order on the gel.

CHAPTER 2. RAPD ANALYSIS OF A LINKAGE GROUP IN SWEET CHERRY

INTRODUCTION

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 ten 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 - 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 so the haploids can be screened for the entire set of markers.

The haplotyping procedure using RAPD markers has the following advantages. The genotype of each meiotic product is analyzed directly, thus a family structure (i.e. a segregating seedling population) is not required. Because the tissue to be screened would be microspore-derived, heterozygotes, which cannot be scored in a majority of the random primer amplifications (Williams et al., 1990), would not occur, and the efficiency of the linkage analysis would be increased. A genetic linkage map has been generated using RAPD and isozyme analysis for sweet cherry (Stockinger et al., in prep.).

The objective of this work was to analyze a linkage group that is present within linkage group fifteen of the sweet cherry genetic linkage map. This linkage group consists of 11 genetic markers of which one is an isozyme marker and the other ten are PCR amplification products. Of these 11, 5 are completely linked, 3 markers in one group are designated (A) and 2 markers in another are designated (B). Group (A) is closely linked to one additional marker where only one recombination separates it from linkage group (A). Linkage between marker AI-03_{1300} and linkage group (A), AL-03_{760}, B-18_{1300}, E-20_{1400} was studied. The objective was to screen 82 additional microspore-derived individuals in an attempt to examine the linkage between AI-03_{1300} and linkage group (A), as well as, the linkages within group (A), so more accurate recombination distances could be established between these markers.

LITERATURE REVIEW

Random amplified polymorphic DNA (RAPD) markers (Williams et al., 1990) are polymerase chain reaction (PCR) products derived by using 10 base pair DNA primers of arbitrary sequences. Polymorphisms, if detected among amplification products, are useful as genetic markers, and can be visualized on an ethidium bromide-stained agarose gel. From such results, it is unlikely that each amplification product is from perfect pairing between the primer and the native DNA template. But, if the amplified DNA segments are reproducible and exhibit simple segregation patterns they are useful in map construction. Thus, RAPD markers can be used as a means to determine the segregation of genetic sequences in a plant population.

Williams et al. (1990) discovered that amplification polymorphisms can provide DNA markers in genomic regions which are not accessible to random fragment length polymorphisms (RFLP) analysis due to the presence of repetitive DNA sequences. They also suggested that it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies) with a dominant RAPD marker. Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, rarely are detected. Most RAPD markers are generally dominant.

Welsh et al. (1990) reported that PCR requires less than 1/100 the amount of genomic DNA per lane compared to that needed to prepare a Southern blot. Thus, many more PCR reactions may be run from a given DNA extraction adding to the efficiency of PCR analysis.

Information obtained from one RAPD marker is very low. Markers need to be utilized that amplify regions of DNA randomly throughout the entire genome. This is important in showing linkages between markers, as well as, for investigation of preferential selection of markers due to selection pressures (i.e. culture systems, growing medium). RAPDs exhibit a higher degree of polymorphism then do RFLPs; therefore, they appear to be more useful to plant breeding programs (Weeden et al., 1992).

RAPD markers used for linkage mapping must first be tested for monogenic inheritance. Weeden et al. (1992) analyzed pea and apple populations for RAPD marker segregation and found that nearly every RAPD scored reflected true genetic variation that could be placed onto the respective linkage maps. Weeden et al. (1992) suggested that lower annealing temperatures reduce the incidence of scoring errors. Thus, the bands obtained are highly amplified and appear more pronounced when stained in the agarose gel making them easier to score. A change in the ramp times of the reaction might shorten the reaction time, but decreasing the rate of each cycle can completely change the RAPD phenotype of an individual (Weeden et al., 1992). It is also reported that the majority of errors incurred in screening a plant population with RAPD markers occurs not in the reactions themselves, but in the scoring of amplification products that are alluded in the gel. Weeden et al. (1992) concluded that, with a good DNA extraction protocol, reasonable care of amplification and electrophoresis conditions, and a conservative scoring of the observed segregation patterns, the intrinsic error rate in scoring segregating RAPDs can be kept below 4%. It is suggested that each sample to be screened should be duplicated and screened and only those fragments amplified in both samples be considered in the summary analysis. Finally, when using RAPD markers it is believed that scoring errors lead to increased map distances between adjacent markers, as well as, possibly causing changes in marker map order. Using duplicated individuals when screening a population reduces or eliminates these types of errors.

In tree crops, marker assisted selection methods are particularly useful. Due to long generation times, large progeny populations are rarely maintained and homozygous tree populations are not available. The major problem in tree breeding is time. Methods to improve the efficiency of early selection in trees would be of considerable value (Grattapaglia et al., 1992).

Linkage maps are not available in many tree species. Linkage analysis in tree crops using isozyme markers are limited by the number of polymorphic allozyme loci. An advantage of the RAPD technique with conifers is the use of haploid DNA, which is available in the megagmetophyte of conifer seeds (Tulsieram et al., 1992). Tulsieram et al. also stated that RAPD marker screening should be equally applicable in those Angiosperms for which microspore-derived cell culture, dihaploid plants or recombinant inbred lines are available. The fact that such a small amount of template DNA is

required to perform the RAPD analysis helps to make the screening of haploid cell cultures possible.

MATERIALS AND METHODS

Plant material. Of 154 'Emperor Francis', microspore-derived callus individuals described previously, 82 were used in this experiment. The production of this microspore-derived callus was previously explained (Chapter 1).

DNA isolation. Total genomic DNA was isolated from 'Emperor Francis' leaves and the callus lines by using a modification of the Murray and Thompson (1980) extraction protocol. Approximately 0.1 - 0.3 g of fresh leaf or white, friable callus tissue was placed into microfuge tubes and placed in a -80 °C freezer for 1 h then lyophilized for 24 h. The tissue was then used or stored at -20 °C until needed. The tissue was ground to a fine powder, in a microfuge tube, in the absence of liquid nitrogen or buffer, using a 1 ml plastic pipette tip with a flame-sealed end. After 30 sec. of vigorous grinding, 400 µl of extraction buffer was added. The extraction buffer was composed of; 0.2 M Tris pH = 8.5, 5 mM EDTA, 0.7 M NaCl, 2.5% CTAB, 125 mM Sorbitol and 2% 2-mercaptoethanol. Once the tissue was completely dispersed in the buffer, the samples were incubated at 60 °C for 20 - 30 min., after which, 400 µl of chloroform/isoamyl alcohol (24:1) was added. The samples were then gently inverted until complete mixing was achieved. After the samples were centrifuged at 18,000 x g. for 10 min., the aqueous phase was removed to a fresh tube. In addition, 1 ml of 5% CTAB was added, and the samples were inverted several times, to completely dissolve the pellet, then centrifuged at 2000 x g. for 5 min. The supernatant was discarded and the precipitate was resuspended in 400 µl of TNE (10 mM Tris pH = 8.0, 0.7 M NaCl, 0.5 mM EDTA). Once the pellet was resuspended, 920 µl of 100% ethanol was added. The samples were inverted several times and then centrifuged at 18,000 x g. for 2 min. The DNA pellet was washed twice with 1 ml of 70% ethanol, dried and resuspended overnight in 600 µl of TE (10 mM Tris pH = 8.0, 0.5 mM EDTA). The DNAs were kept at 4 °C until used. By comparing sample DNA concentrations to known quantity of Lambda DNA, the sample DNA concentration was approximated to be 10 ng/µl (Stockinger et al., in prep.).

PCR protocol. All PCR reactions were carried out in 25 µl reaction volumes using 25 ng of template DNA, 16.5 ng decanucleotide primer DNA (Operon Technologies, Alameda, CA), 10 mM Tris pH = 8.5 , 50 mM KCl, 0.2 mM of dNTP, 3 mM MgCl, 0.13 units Taq DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) overlaid with two drops of mineral oil. A 7 min. denaturation at 94 °C was followed by 47 cycles using the conditions established by Williams et al. (1990): 94 °C for 1 min., 36 °C for 1 min., and 72 °C for 2 min. A 5 min. incubation at 72 °C followed the final cycle. Amplifications were performed on a Perkin Elmer Cetus (Emeryville, CA) DNA Thermal Cycler model 480. Amplified products were loaded onto 1.2% agarose gels, in 2 mm wells, and electrophoresed in TEA buffer (40 mM Tris-acetate pH = 8.0, 1 mM EDTA) for 450 to 500 Volt hours. The gels were stained for 30 min. in 40 µg/400 ml EtBr stain.

RESULTS

Five bands were scored presence : absence for the 82 microspore-derived callus population. These bands were: a 900 base pair (bp) fragment amplified by primer AI-03, (AI-03_{900}); a 1300 bp. fragment amplified by AI-03, (AI-03_{1300}); a 760 bp. fragment amplified by primer AL-03, (AL-03_{760}); a 1300 bp. fragment amplified by primer B-18, (B-18_{1300}); a 1400 bp. fragment amplified by primer E-20, (E-20_{1400}) (Figure 3). Each of the segregating markers was tested for independent segregation using X^2 analysis to a 1:1 and a 1:2 ratio (Table 3). All markers did not fit the 1:1 ratio $P > 0.05$, but did fit the 1:2 ratio, $P > 0.2$. The data for each marker exhibit an excess of the absence class.

Joint segregation comparisons were made between a marker and all additional markers (Tables B1 - B14). Each comparison was tested for fit to a 1:1:1:1 ratio. All comparisons failed to fit this ratio $P < 0.001$, suggesting linkage among all markers. Six of the ten comparisons exhibited obvious parental and recombinant classes: AI-03_{1300}, AL-03_{760}; AI-03_{1300}, B-18_{1300}; AI-03_{1300}, E-20_{1400}; AL-03_{760}, B-18_{1300}; AL-03_{760}, E-20_{1400}; B-18_{1300}, E-20_{1400}. The presence of parental and recombinant classes indicated linkage between these pairs of markers. These loci are in the coupling phase (i.e., ++ or --).

Figure 3. A segregating population of 7 haploid callus individuals (numbered 1-7) scored with primer E-20 (Lanes 5 - 18). (A, B) are replicates of the same individual. Lane 1 and 19 are Lambda Hind III molecular weight marker. Lane 2, H_2O used as template DNA. Lane 3, 'Emperor Francis' leaf DNA with no primer. Lane 4, 'Emperor Francis' leaf DNA as template. The arrow shown indicates the polymorphic band scored (E-20_{1400}).

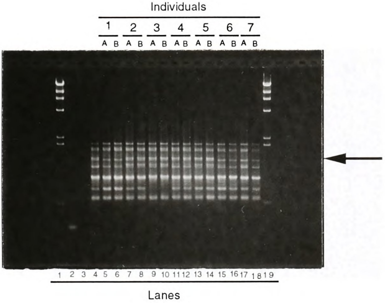
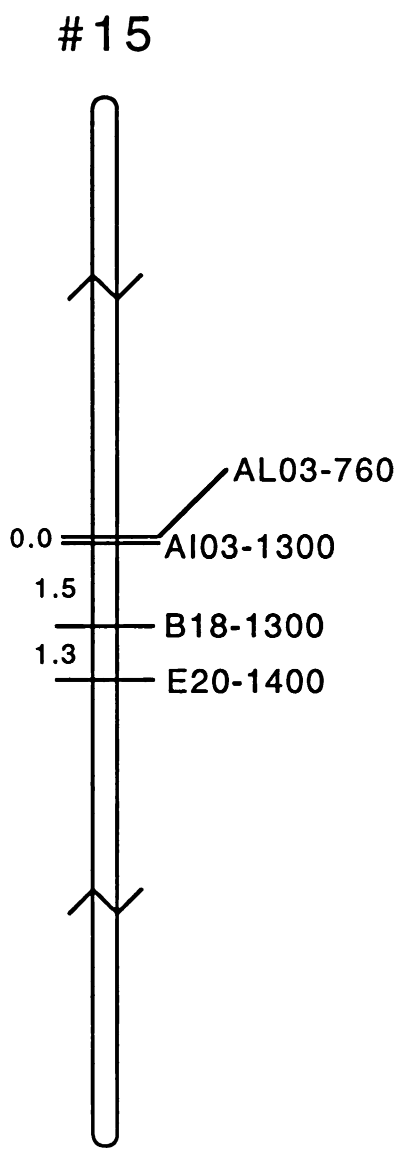

Individuals
1 2 3 4 5 6 7
A B A B A B A B A B A B A B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Lanes

Table 3. Segregation of $Al\text{-}03_{900}$, $Al\text{-}03_{1300}$, $AL\text{-}03_{760}$, $B\text{-}18_{1300}$, $E\text{-}20_{1400}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes									
	$Al\text{-}03_{900}$		$Al\text{-}03_{1300}$		$AL\text{-}03_{760}$		$B\text{-}18_{1300}$		$E\text{-}20_{1400}$	
	+	-	+	-	+	-	+	-	+	-
No. observed	22	58	22	48	15	38	28	53	27	51
Total	80		70		53		81		78	
No. expected (1:1)	40	40	35	35	26.5	26.5	40.5	40.5	39	39
X^2 (1:1)	15.3		8.93		9.13		7.11		6.78	
P	< 0.001		> 0.001		> 0.001		> 0.001		> 0.001	
No. expected (1:2)	26.4	52.8	23.3	46.7	17.7	35.3	27.0	54.0	26.0	52.0
X^2 (1:2)	0.994		0.042		0.397		0.014		0.015	
P	> 0.2		> 0.5		> 0.5		> 0.9		> 0.9	

Parental and recombinant classes were not distinguishable for the four remaining pairs involving Al-03_{900}: Al-03_{1300}, Al-03_{900}; Al-03_{900}, AL-03_{760}; Al-03_{900}, B-18_{1300}; Al-03_{900}, E-20_{1400}. Therefore, these pairs of markers were tested against a 1:2:2:4 ratio that accounted for skewed parental and recombinant outcomes. These four pairs fit the 1:2:2:4 ratio $P > 0.5$ proving that Al-03_{900} is not linked to the other 4 markers. Recombination distances for the six pairs of linked markers, are presented in Figure 4.

Figure 4. RAPD linkage group #15 of sweet cherry cultivar 'Emperor Francis'.



DISCUSSION

Segregation for the 5 marker loci fit a 1:2 ratio presence : absence demonstrating the presence of segregational skewing. This skewing could be the result of preferential selection in the anther culture environment for the absence of these marker loci or the result of genes linked to these marker loci. The absence class for all markers occurs more frequently as the presence class, suggesting that the absence alleles are linked to a gene or genes that express survivability in culture or the ability to produce haploid callus. These results support the findings of Thompson et al. (1991) who demonstrated the presence of gametophytic selection in barley anther culture.

These findings are in contrast to the results presented earlier in this thesis in regard to the independent segregation of alleles of both *Pgi* -2 and 6-*Pgd* -1. To accurately evaluate any population for preferential selection due to culture environment, a higher level of marker saturation would be needed.

Joint segregation analyses for 5 markers were tested for fit to a 1:1:1:1 ratio. Six of the 10 comparisons exhibited obvious parental and recombinant classes which is indicative of linkage between these pairs of markers. The map order was found to be AL-03_{760}, Al-03_{1300}, B-18_{1300}, E-20_{1400} with 0, 1.5, 1.3 cM map units between each marker, respectively.

Four of the ten comparisons did not exhibit obvious parental and recombinant classes and were tested against a 1:2:2:4 skewed, joint segregation ratio. These comparisons, all including marker AI-03_{900}, were accepted $P > 0.5$ for fit to a 1:2:2:4 ratio. Also, marker AI-03_{900} was not found to be closely linked to the above markers. This new linkage group order fits closely that reported by Stockinger et al. (in prep.). Only one recombinant had previously been identified between marker loci AI-03_{1300} and each of the following: AL-03_{760}, B-18_{1300}, E-20_{1400}. This suggests markers AL-03_{760}, B-18_{1300}, and E-20_{1400} could map to the same region of a chromosome. However, no recombination was found between marker loci AI-03_{1300} and AL-03_{760} in the additional 82 individuals screened. Recombination was found between AI-03_{1300} and both B-18_{1300} and E-20_{1400}. Recombination events have occurred between AL-03_{760}, B-18_{1300}, and E-20_{1400}. This allowed us to calculate map distances between these marker loci.

APPENDICES

APPENDIX A

Table A1. Haploid callus origin as related to culture history of 'Emperor Francis' anthers.

Branch Number	Date Collected	Out of Cooler	Back in Cooler	Date Plated	Plate Number	# of flower buds/plate	Date in Light	# of haploid
3	1/13/92	2/15/92	2/18/92	2/20/92	45	9	3/2/92	1
23	3/3/92	4/2/92	none	4/3/92	383	10	4/13/92	1
23	3/3/92	4/2/92	none	4/3/92	384	9	4/13/92	1
23	3/3/92	4/2/92	none	4/3/92	387	9	4/13/92	2
24	3/3/92	4/2/92	none	4/3/92	388	8	4/13/92	4
24	3/3/92	4/2/92	none	4/3/92	389	7	4/13/92	16
24	3/3/92	4/2/92	none	4/3/92	391	6	4/13/92	13
24	3/3/92	4/2/92	none	4/3/92	392	10	4/13/92	9
25	3/3/92	4/2/92	4/3/92	4/6/92	395	9	4/16/92	1
26	3/3/92	4/2/92	4/3/92	4/6/92	412	9	4/16/92	3
26	3/3/92	4/2/92	4/3/92	4/6/92	419	9	4/16/92	2
26	3/3/92	4/2/92	4/3/92	4/6/92	420	9	4/16/92	20
26	3/3/92	4/2/92	4/3/92	4/6/92	421	11	4/16/92	8
26	3/3/92	4/2/92	4/3/92	4/6/92	422	9	4/16/92	1
28	3/3/92	4/7/92	4/8/92	4/10/92	429	9	4/20/92	15
28	3/3/92	4/7/92	4/8/92	4/8/92	430	9	4/20/92	4
28	3/3/92	4/7/92	4/8/92	4/8/92	431	9	4/20/92	13
28	3/3/92	4/7/92	4/8/92	4/8/92	432	9	4/20/92	12
28	3/3/92	4/7/92	4/8/92	4/8/92	433	9	4/20/92	5
28	3/3/92	4/7/92	4/8/92	4/8/92	434	9	4/20/92	11
28	3/3/92	4/7/92	4/8/92	4/8/92	438	9	4/20/92	10
28	3/3/92	4/7/92	4/8/92	4/8/92	439	9	4/20/92	2
								Total = 154

APPENDIX B

Tables showing X^2 analyses of joint segregation of five RAPD markers.

Table B1. Joint segregation of $Al\text{-}03_{900}$ and $AL\text{-}03_{760}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($Al\text{-}03_{900}$, $AL\text{-}03_{760}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	4	12	11	26
No. expected (1:1:1:1)	13.5	13.5	13.5	13.5
Total	53			
X^2 (1:1:1:1)	18.89			
P	< 0.001			

Table B2. Joint segregation of $AI\text{-}03_{900}$ and $B\text{-}18_{1300}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($AI\text{-}03_{900}$, $B\text{-}18_{1300}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	8	14	20	36
No. expected (1:1:1:1)	19.5	19.5	19.5	19.5
Total	78			
X^2 (1:1:1:1)	22.30			
P	< 0.001			

Table B3. Joint segregation of $AI\text{-}03_{900}$ and $E\text{-}20_{1400}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($AI\text{-}03_{900}$, $E\text{-}20_{1400}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	8	14	19	34
No. expected (1:1:1:1)	18.75	18.75	18.75	18.75
Total		75		
X^2 (1:1:1:1)		19.76		
P		< 0.001		

Table B4. Joint segregation of $Al\text{-}03_{1300}$ and $AL\text{-}03_{760}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($Al\text{-}03_{1300}$, $AL\text{-}03_{760}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	11	0	0	32
No. expected (1:1:1:1)	10.5	10.5	10.5	10.5
Total		43		
X^2 (1:1:1:1)		44.05		
P		< 0.001		

Table B5. Joint segregation of $AI\text{-}03_{1300}$ and $B\text{-}18_{1300}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($AI\text{-}03_{1300}$, $B\text{-}18_{1300}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	22	0	1	47
No. expected (1:1:1:1)	17.5	17.5	17.5	17.5
Total		70		
X^2 (1:1:1:1)		66.45		
P		< 0.001		

Table B6. Joint segregation of $AI\text{-}03_{1300}$ and $E\text{-}20_{1400}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($AI\text{-}03_{1300}$, $E\text{-}20_{1400}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	21	0	2	45
No. expected (1:1:1:1)	17	17	17	17
Total	68			
X^2 (1:1:1:1)	60.3			
P	< 0.001			

Table B7. Joint segregation of $AI\text{-}03_{1300}$ and $AI\text{-}03_{900}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes	($AI\text{-}03_{1300}$, $AI\text{-}03_{900}$)		
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	6	16	13	33
No. expected (1:1:1:1)	17	17	17	17
Total		68		
X^2 (1:1:1:1)		23.18		
P		< 0.001		

Table B8. Joint segregation of $AL\text{-}03_{760}$ and $B\text{-}18_{1300}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($AL\text{-}03_{760}$, $B\text{-}18_{1300}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	15	0	1	36
No. expected (1:1:1:1)	13	13	13	13
Total	52			
X^2 (1:1:1:1)	52.08			
P	< 0.001			

Table B9. Joint segregation of $AL\text{-}03_{760}$ and $E\text{-}20_{1400}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes	($AL\text{-}03_{760}$, $E\text{-}20_{1400}$)		
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	14	0	2	35
No. expected (1:1:1:1)	12.75	12.75	12.75	12.75
Total		51		
X^2 (1:1:1:1)		48.01		
P		< 0.001		

Table B10. Joint segregation of B-18_{1300} and E-20_{1400} alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes (B-18_{1300}, E-20_{1400})			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	16	0	1	51
No. expected (1:1:1:1)	17	17	17	17
Total		68		
X^2 (1:1:1:1)		83.12		
P		< 0.001		

Table B11. Skewed joint segregation of $AI\text{-}03_{1300}$ and $AI\text{-}03_{900}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes	($AI\text{-}03_{1300}$, $AI\text{-}03_{900}$)		
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	6	16	13	33
No. expected (1:2:2:4)	7.55	15.1	15.1	30.2
Total		68		
X^2 (1:2:2:4)		0.923		
P		> 0.5		

Table B12. Skewed joint segregation of $Al\text{-}03_{900}$ and $AL\text{-}03_{760}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($Al\text{-}03_{900}$, $AL\text{-}03_{760}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	4	12	11	26
No. expected (1:2:2:4)	5.89	11.78	11.78	23.56
Total		53		
X^2 (1:2:2:4)		0.915		
P		> 0.5		

Table B13. Skewed joint segregation of AI-03 $_{900}$ and B-18 $_{1300}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes (AI-03 $_{900}$, B-18 $_{1300}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	8	14	20	36
No. expected (1:2:2:4)	8.67	17.33	17.33	34.67
Total		78		
X^2 (1:2:2:4)		1.15		
P		> 0.5		

Table B14. Skewed joint segregation of $AI\text{-}03_{900}$ and $E\text{-}20_{1400}$ alleles, presence (+) absence (-), among the 82 calli mapping population.

	Haploid Genotypes ($AI\text{-}03_{900}$, $E\text{-}20_{1400}$)			
	(+ , +)	(+ , -)	(- , +)	(- , -)
No. observed	8	14	19	34
No. expected (1:2:2:4)	8.33	16.67	16.67	33.33
Total	75			
X^2 (1:2:2:4)	0.782			
P	> 0.5			

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LITERATURE CITED

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