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Frequency of Polyploids of *Solanum tuberosum* Dihaploids in 2X × 2X Crosses

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Abstract

When breeding diploid potatoes, tetraploid progeny can result from the union of 2n eggs and 2n pollen in 2x-2x crosses. Thirty-three crosses were made to examine tetraploid progeny frequency in 2x-2x crosses. All crosses were between *S. tuberosum* dihaploids and diploid self-compatible donors, M6 and DRH S6-10-4P17. Using chloroplast counting for ploidy determination, the frequency of tetraploid progeny was as high as 45% in one of the 33 crosses. Based upon single nucleotide polymorphism (SNP) genotyping, the tetraploid progeny were attributed to bilateral sexual polyploidization (BSP), which is caused by the union of 2n egg and 2n pollen. Dihaploids were identified that produce lower frequencies of *2*n eggs. The results of this study suggest that *S. tuberosum* dihaploids with high frequency of 2n eggs should be avoided in 2x - 2x crosses for diploid breeding programs.

Keywords: Potato; Diploid; Tetraploid; 2n eggs; 2n pollen; Bilateral sexual polyploidization.

1. Introduction

Historically, diploid Solanum species have been used to introgress economically important traits into tetraploid cultivated potato *Solanum tuberosum* Grp. Tuberosum L. (*2n = 4x* = 48) [1]. However, self-compatible (SC) diploid germplasm presents opportunities for breeding at the diploid level [2]. Ultimately, the goal for diploid breeding is to develop inbred lines that can be used to generate F1 hybrids as cultivars [2, 3]. Self-compatible *S. chacoense* clone, M6 (previously designated *S. chc* 523-3), is a critical breeding line being used as a source of self-compatibility in diploid breeding programs [2]. Crossing self-compatible donors to dihaploids derived from elite tetraploid varieties and advanced breeding lines can produce breeding lines with self-compatibility and cultivated traits from the dihaploids. However, tetraploid progeny can result from these 2x-2x crosses if both diploid parents produce 2n gametes, enabling bilateral sexual polyploidization [4-10]. In the early stages of our diploid breeding program we observed tetraploid progeny in our 2x-2x crosses between dihaploids crossed to self-compatibility donors. Diploid breeding can be complicated when there is a frequent occurrence of tetraploid progeny in 2x-2x crosses. Therefore, assessing the frequency of tetraploid progeny from 2x parents should be evaluated in a diploid breeding program. The purpose of this study was to assess the frequency of polyploids in 2x-2x crosses and quantify the proportion of polyploid progeny resulting from crossing a set of *S. tuberosum* dihaploids with two diploid SC donors for a diploid potato breeding program. Chloroplast counting and single nucleotide polymorphism (SNP) genotyping has been established as an efficient and reliable method to discriminate between polyploid (triploid and tetraploid) and diploid potatoes [11] and was used to distinguish polyploid and diploid progeny in the 2x-2x crosses. Employing ploidy screening will help choose dihaploids with low frequencies of 2n gametes to use in diploid breeding.

2. Materials and Methods

2.1. Generation of Seed Families by Making 2x by 2x Crosses

A total of 29 dihaploids were derived from *S. tuberosum* cultivars Atlantic, Superior and MSR127-2 (pedigree MSJ167-1 x MSG227-2). For the populations examined in this study, the dihaploids were used as female parents only because of male sterility (Table 1). The male parents were M6, an S_7 inbred line derived by self-pollinating a diploid *S. chacoense* line [2] and DRH S6-10-4P17, an S_6 inbred line derived from a *S. phureja* x *S. tuberosum* hybrid, which also possesses self-compatibility [2]. The male and female parental plants used for 2x-2x crosses were planted in 10 L pots and grown in the greenhouse (20 - 25º C and 16:8 light:dark photoperiod) over a six week period during the winter season beginning December 2016 at Michigan State University, East Lansing, MI. Standard horticultural practices were applied to ensure optimum growth and fertility from the plants. Pollen was collected into 1.5 microfuge tubes and used directly for crossing. One month after the cross was made, the fruits were

harvested, and seeds were extracted and dried. Seeds from each family were germinated in soil. Up to 50 seedlings were grown from each family for ploidy evaluation.

2.2. Leaf Sample Collection and Guard Cell Chloroplast Counting Procedure

Two mature leaves were collected from each seedling one month after emergence for chloroplast counting. Sampled leaves were then placed in a moist Petri dish to prevent wilting. Using a pair of fine tweezers, a strip of epidermis was peeled off the abaxial surface of a leaflet near the midrib and immediately placed on a glass microscope slide. The peeled epidermis strip was then dyed with a drop of 1:1 propidium iodine-potassium iodide (PIPI). The PIPI solution was dissolved as described by Alsahlany, *et al.* [11]. Briefly, 500 mg of propidium iodide was dissolved together with 500 mg of potassium iodide in 50 ml of 70% ethanol. After two minutes, a cover slip was mounted, and the slide was observed under a light microscope (OLYMPUS BX60) at 400x magnification, as described by Alsahlany, *et al.* [11] while adjusting the magnification to the level to obtain a clear view of the guard cell that appears as dark brown or black in color. A picture processing software called SPOT software was used to capture chloroplast images which are transmitted to the computer [12]. Chloroplasts were counted from 20 random guard cells per line to calculate an average, with the exception of the following lines: 19 guard cells from EE905-22, EE920-11, EE923-43, EE926-22, EE937-01, EE939-02, EE941-01; 18 guard cells from EE902-48, EE937-29; and 11 guard cells from EE904-29. A total of 1,546 progeny were sampled following this chloroplast counting procedure [13]. Individuals were tested within each family for an analysis of variance (ANOVA) for chloroplast counts. Chloroplast count means comparisons were conducted with Tukey-Kramer honest significant difference (HSD) and hierarchical clustering of means using the average method to classify ploidy using JMP 15 Pro (SAS Institute, Cary, NC) (data not shown).

2.3. Validating Bilateral Sexual Polyploidization with SNP Genotyping

Seven crosses which exhibited a high level of polyploid progeny (MSEE905, MSEE925, MSEE927, MSEE928, MSEE929, MSEE933 and MSEE939) were chosen for the SNP genotyping to confirm ploidy (Table 1). A subset of 343 progeny from the 33 2x-2x crosses were SNP genotyped. Young leaf tissue was collected for DNA isolation. Additionally, previously collected SNP genotype data from 14 diploid and 14 tetraploid reference lines described by Alsahlany et al., 2019 were used for ploidy comparison. Qiagen DNA extraction kit was used (DNeasy Plant Mini Kit (cat. Nos. 69104 and 69106, QIAGEN kit).

Single nucleotide polymorphism genotyping was conducted using the 22K Infinium potato SNP array [7, 11]. Samples were SNP genotyped using the Illumina GenomeStudio 2.0.4 software (Illumina, San Diego, CA) for three cluster (diploid) and five cluster (tetraploid) marker calling using a custom three cluster calling file and tetraploid custom cluster file based on the PolyGentrain tetraploid calling module of reference samples (unpublished). Hierarchical clustering was performed using diploid genotypes for all samples (compressing tetraploid heterozygosity) using the R package 'ape' version 4.1 [14]. Simplex SNP frequency (AAAB and ABBB) from a given DNA sample can differentiate tetraploid from diploid genotypes as described by Alsahlany, *et al.* [11]. The SNP data can also distinguish triploid progeny from the diploid and tetraploid progeny. Triploid progeny have an exaggerated frequency of no-calls in the sample because the dosage of heterozygous triploids don't cluster with three heterozygous tetraploid clusters or the diploid AB heterozygote cluster. Hierarchical clustering (average method) of simplex frequencies was used to classify ploidy using JMP 15 Pro (SAS Institute, Cary, NC) (data not shown).

3. Results

3.1. Ploidy Determination in 2x-2x Progeny

The chloroplast counts for the 1,546 progeny tested from the 33 families were within these expected ranges for diploid and tetraploid samples (data not shown) based on reports by Ordoñez [13] and Alsahlany, *et al.* [11]. Triploid references were not available for comparison at the time of chloroplast counting. As expected, the majority of the progeny in the 2x-2x crosses were diploid but polyploid progeny were observed at varying levels from 0–45% within each family (Table 1). From the 1,546 progeny examined, 168 were classified as polyploid (Table 1). Approximately 79% of the 24 Atlantic dihaploid families and 71% of the 7 Superior dihaploid families produced polyploid progeny. In two families with dihaploid ATL_M_404 as a female, 67% and 32% of the progeny classified as polyploid (Table 1). There were 15 families that had a polyploid frequency of greater than 10%. Eleven families had a polyploid frequency between 2-10%. MSR127 dihaploid families produced 2% and 9% polyploid progeny (Table 1). Only seven of the 33 families resulted in completely diploid progeny.

Table-1. Ploidy determination of 2x × 2x family based on guard cell chloroplast counts.

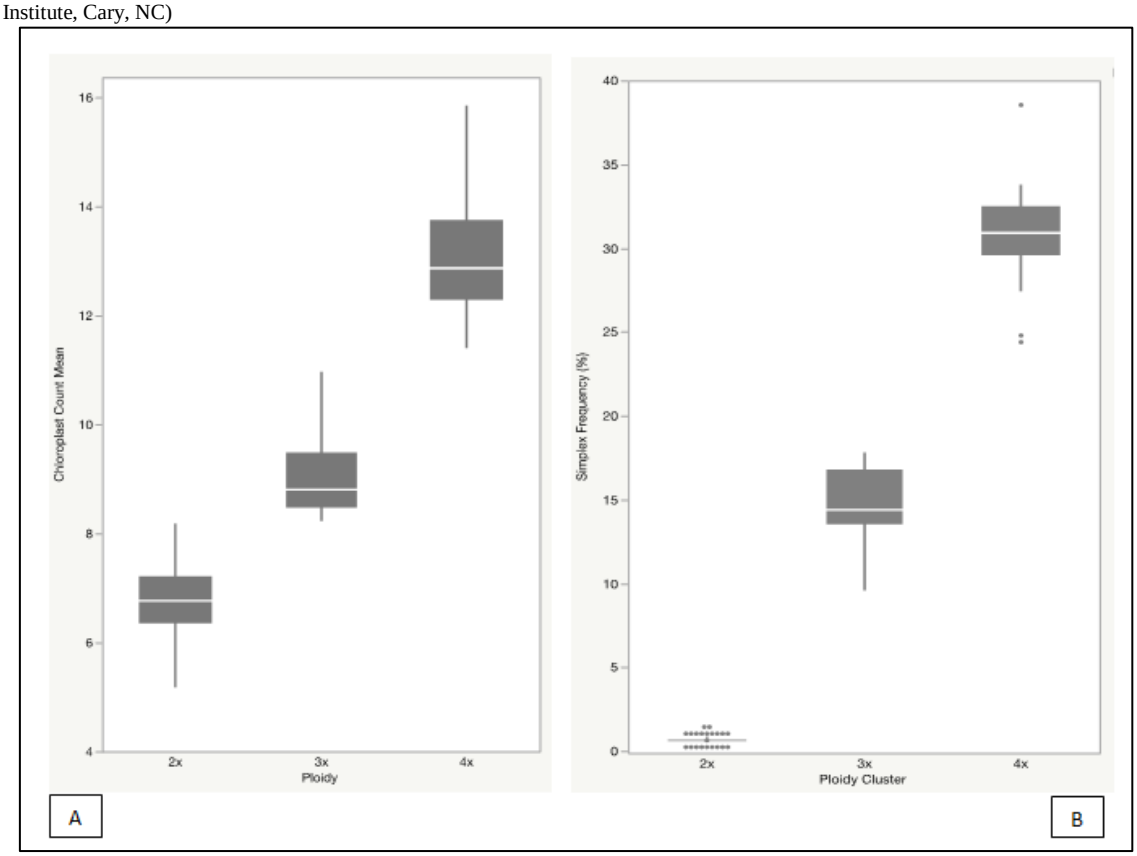
Family	Parent DH	Female	Male	>2x (%)	N
MSEE929	Atlantic	ATL-M-404	M6	40	50
MSEE923	Atlantic	ATL-M-427	DRHS6-10-4P17	32	50
MSEE948	Atlantic	ATL-M-418	M6	31	16
MSEE920	Atlantic	ATL-M-404	DRHS6-10-4P17	24	50
MSEE939	Atlantic	ATL-M-429	M6	22	49
MSEE928	Atlantic	ATL-M-120	M6	20	49
MSEE933	Atlantic	ATL-M-402	M6	16	45
MSEE905	Atlantic	ATL-M-170	M6	14	49

MSEE932	Atlantic	ATL-M-409	M6	12	49
MSEE946	Atlantic	ATL-M-169	M6	10	50
MSEE940	Atlantic	ATL-V-033	M6	10	50
MSEE904	Atlantic	ATL-M-403	M6	8	48
MSEE935	Atlantic	ATL-M-186	M6	8	50
MSEE930	Atlantic	ATL-M-405	M6	6	49
MSEE903	Atlantic	ATL_V_23	DRH S6-10-4P17	6	50
MSEE944	Atlantic	ATL-M-133	M6	5	20
MSEE921	Atlantic	ATL-M-120	DRHS6-10-4P17	5	41
MSEE902	Atlantic	ATL_V_23	M6	4	49
MSEE943	Atlantic	ATL-V-006	M6	2	50
MSEE931	Atlantic	ATL-M-406	M6	0	50
MSEE934	Atlantic	ATL-M-401	M6	0	48
MSEE936	Atlantic	ATL-M-188	M6	0	50
MSEE938	Atlantic	ATL-M-198	M6	0	50
MSEE945	Atlantic	ATL-M-153	M6	0	46
MSEE901	MSR127-2	R127H2	M6	8	50
MSEE900	MSR127-2	R127H1	M6	2	50
MSEE925	Superior	VT-SUP-70	M6	45	38
MSEE927	Superior	VT-SUP-08	M6	18	50
MSEE926	Superior	VT-SUP-19	M6	14	50
MSEE922	Superior	VT-SUP-96	DRHS6-10-4P17	10	50
MSEE942	Superior	VT-SUP-12	M6	2	50
MSEE919	Superior	VT-SUP-96	M6	0	50
MSEE924	Superior	VT-SUP-79	M6	0	50

3.2. SNP Genotyping

A subset of 343 progeny from seven families were SNP genotyped using a five-cluster calling tetraploid model. The simplex marker (AAAB and ABBB) frequency is an indicator of sample ploidy [11, 15]. The 260 diploids had a simplex frequency range of 0.5-1.4% and the 83 polyploid samples simplex frequencies ranged from 9.5-33.8% (Fig. 1B) suggesting that the polyploid progeny are sexually derived. Furthermore, the polyploid progeny formed two clusters. With, the clustering of both the chloroplast counts and the SNP simplex frequencies, we could speculate that triploid progeny may be present, but we did not have any triploid reference samples for comparison. Furthermore, the SNP alleles observed in each cross supported the parental pedigree of each cross.

Figure-1. Comparison of chloroplast count means (A) and SNP genotype simplex frequency (B) and ploidy. Figures generated using JMP 15 Pro (SAS Institute, Cary, NC)



4. Discussion

In this study polyploid occurrence was observed in *S. tuberosum* crosses of female dihaploids and self-compatible males. In this study, the 2x-2x crosses were expected to possess a high number of diploid progeny since it is speculated that 2n pollen is very rare in the self-compatibility donors used [2], but the frequency of polyploid progeny ranged from 0 to 45% in the 33 crosses using 29 different dihaploid females crossed to two different self-compatibility donors (M6 and DRHS6-10-4P17). This occurrence of 4x progeny is attributed to bilateral sexual polyploidization (BSP) where both parents produce 2n gametes thus favoring formation of tetraploid offspring [9, 16, 17]. Alternately, spurious pollen from tetraploids could result in tetraploid progeny. SNP genotyping of 343 progeny (260 diploid and 83 polyploid) from the 2x-2x crosses supports the formation of tetraploid progeny via bilateral sexual polyploidization. The contribution of the male donor traits in the progeny of the crosses also supported the pedigree of the crosses in this study. These results are consistent with reported work from Carputo, *et al.* [6], Peloquin, *et al.* [9], Werner and Peloquin [18] and Younis, *et al.* [19] who have previously observed 2x-2x BSP. Carputo, *et al.* [6], reported that the frequency of 2n gametes varied with diploid Solanum species having 2n pollen frequency from 1.9 - 36.3%, while 2n egg frequency ranges from 4.9 to 22.6%. In this study the polyploid progeny ranged from 0 to 45% indicating that in many of the 2x-2x crosses both parents were producing *2*n gametes. The 4x frequency may reflect *2*n egg frequency in dihaploids and cytological examination of the megaspore would confirm this hypothesis [17, 20].

Recently Graebner, *et al.* [21] observed triploids in 4x-2x crosses despite a triploid block typically leading to a high frequency of tetraploid progeny. Out of the 33 families evaluated, we focused on seven families that had the highest polyploid incidence to compare chloroplast counting to SNP genotyping. SNP genotyping was able to confirm BSP for the detected polyploids in these families and that triploids may exist in these seven families but without reference triploids it is only speculation.

Because of cost and accessibility chloroplast counts can be employed in most cases for ploidy screening in an applied diploid breeding program to ensure the proper ploidy level is maintained. Additionally, early ploidy screening can be used to enhance breeding efficiency in early generation selection stages of breeding programs [11]. For diploid breeding programs, the occurrence of 4x and 3x progeny must be minimized. Hence, breeders need to select parental lines that produce no, or a low frequency, of 2n eggs so that breeding can remain at the diploid level. This study was able to identify dihaploids derived from Atlantic, Superior and MSR127-2 with a zero or low frequency of 2n gamete occurrence that can be useful in diploid breeding programs.

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