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Evaluation of *In Vitro* Chromosome-doubled Regenerates with Resistance to Potato Tuber Moth [*Phthorimaea operculella* (Zeller)]

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Abstract

In vitro chromosome doubling by the use of the variation induced by zeatin riboside was applied to generate tetraploid individuals to introgress potato tuber moth (PTM) resistance from diploids to tetraploids. This approach is especially important when the expression of desirable traits is strongly influenced by cytoplasmic factors. The levels of PTM resistance and yield performance was evaluated in chromosome-doubled regenerates and their corresponding original diploids. The levels of PTM resistance were maintained in the derivatives. Test of yield performance in two years indicated chromosome-doubled regenerates generally had comparable yield with their diploid original potato clones. These results demonstrated that *in vitro* chromosome doubling could be an effective method to convert valuable 2x potato germplasm into the 4x level without the considerable loss of PTM resistance nor productivity.

1. Introduction

Potato tuber moth [*Phthorimaea operculella* (Zeller), PTM] is one of the most damaging pests of potato in storage and in the field in warmer climates. Tuber infestation by PTM causes dramatic losses, as damaged tubers are attacked by different secondary pests and diseases (Van Rie *et al.* 1994). Resistance to PTM has been reported from host *Solanum* species including *S. sparsipilum* (Raman and Palacios 1988), *S. commersonii*, *S. sucrense*, and *S. tarjense* (Chavez *et al.* 1988). Resistance to PTM in *S. sparsipilum* was reported to be simply inherited and controlled by a few genes (Raman *et al.* 1981, Ortiz *et al.* 1990). However, cytoplasmic factors also influence the expression of the resistance together with nuclear genes (Ortiz *et al.* 1990). In our previous study (Watanabe *et al.* 1998) no resistant progeny was observed among 117 individuals generated from crosses between susceptible 4x female and resistant 2x male parents. This result confirmed the strong cytoplasmic influence in the expression of PTM resistance.

As alternatives to the sexual hybridization to improve populations, the genetic changes generated during callus culture including altered chromosome number, as well as structural changes and/or changes in nucleotide sequences can be applied for potato improvement (Kumar 1994). Somaclonal variation in potato was reviewed by Kumar (1994) with the discussion of the advantages and disadvantages of this technique. Agronomically useful somaclonal variation, such as yield, quality (Matern *et al.* 1978, Thomas *et al.* 1982, Bright *et al.* 1983), tuber shape, maturity date, and morphology of stem, leaf, and flower (Thomas *et al.* 1982, Bright *et al.* 1983) have been reported. Somaclonal variation for resistance to disease includes common scab (*Streptomyces scabies*), potato virus Y, potato leafroll virus, early blight (*Alternaria solani*), potato late blight (*Phytophthora infestans*), Fusarium wilt (*Fusarium oxysporum*) and blackleg (*Erwnia cartivora*) (Matern *et al.* 1978, Thomas *et al.* 1982, Bright *et al.* 1983, Secor and Shepard 1981, Thomson *et al.* 1986, Behnke 1979, Behnke 1980, Shepard 1981, Wenzel and Uhrig 1981, Wenzel 1985).

Quantitative pest resistance traits such on bacterial wilt (*Pseudomonas solanacearum*), root knot nematodes (*Meloidogyne* spp.), and type A grandular trichomes related to insect resistance have been successfully transmitted from diploids to tetraploid lines using first division restitution (FDR) 2*n* pollen (Iwanaga *et al.* 1989, Ortiz *et al.* 1997, Watanabe *et al.* 1992, Watanabe *et al.* 1998). However, the use of FDR 2*n* pollen can not be

applied to transmit PTM resistant from diploids to tetraploids. This is due to the fact that the expression of PTM resistance was strongly affected by cytoplasm (Ortiz *et al.* 1990, Watanabe *et al.* 1998, Harmsen *et al.* 1981), and no major occurrence of 2*n* eggs was observed in the given PTM resistance diploid populations (Chavez *et al.* 1988). Therefore, chromosome doubling may be an alternate way to use diploids as a source of PTM resistance to introgress resistant traits into tetraploid potato clones. Several attempts on *in vitro* chromosome doubling by the use of zeatin roboside or benzilamino purine (BAP) have been made in potato breeding to introduce desirable traits from diploid lines to tetraploid lines (Roest and Bokelmann 1976, 1980, Orrillo and Watanabe 1994).

The objective of this study is to determine whether selected potato clones applied *in vitro* chromosome doubling, derived from PTM resistant diploids, would maintain comparative resistant levels and yield similar to their parental diploids. The applicability of this method as a means to introgress desirable traits from 2x populations to 4x levels is discussed.

2. Materials and Methods

2.1 Plant materials

The genetic background of the diploid genotypes used in this study and their corresponding progenitors are listed in **Table 1** (Orrillo and Watanabe 1994). The level of PTM resistance for each genotype is also stated in the same table.

2.2 Callus induction and plant regeneration

Callus induction was established either from 1) segments of stem internodes 5mm to 10 mm in length, which were cut longitudinally, or 2) leaf segments with middle-rib veins. These segments were placed onto medium containing Murashige Minimal Organic Medium (Gibco Laboratory), 5% (w/v) sucrose, 0.8% (w/v) agar, 8.5 μM zeatin riboside, 11.4 μM indole acetic acid (IAA), and 29.0 μM gibberellic acid at 20 ℃ under 3000 lux light intensity with Daylight Gro-lux lamps for a 16 hour day length. After two weeks, these explants were subcultured in the new medium containing the same composition. This procedure was repeated up to three times.

Generated calli were propagated in the MSA (CIP) medium containing Murashige Minimal Organic Medium and base solution (0.29 μM gibberellic acid, 26.7 μM glycine, 12.2 μM nicotinic acid, 2.4 μM pyridoxine HCl, 1.2 μM thiamin HCl, 2.5 % sucrose, and 0.4 % Phytagel.). For the efficiency of somatic doubling refer to Orrillo and Watanabe (1994). Regenerated plants were then potted and tubers were harvested for the field and storage evaluation.

2.3 Ploidy analysis

The ploidy levels of regenerates were determined by scoring the number of chloroplasts in guard cells of stomata (L1 histogenic layer). The number of chromosomes in L3 histogenic layer in meristematic cells of root tips were counted for the confirmation by the standard acetocarmine squash method (De Main and Fantes 1983). Flow cytometry with FACS Calibur™ (Becton Dickinson) was also employed to infer the occurrence of chimeras. For the procedures for the preparation and measurement refer to Valkonen *et al.* (1994).

Table 1. Genetic background of diploid potato genotypes used for *in vitro* chromosome doubling, and those of their progenitors with respect to species involved [Revised from Orrillo and Watanabe (1994)].

Genotype	Category	pedigree/species involved[1]
84.128.58	hybrid	81M.4.118 × FH.31
CCC1386.26	hybrid	phu intercross
HHI-9	haploid	tbr, adg, chc?
KWPTM-7	hybrid	adg, chc, phu, spl, tbr
KWPTM-18	hybrid	adg, chc, phu, spl, tbr
KWPTM-24	hybrid	adg, chc, phu, spl, tbr
KWPTM-29	hybrid	adg, chc, phu, spl, tbr
M200.32	hybrid	USW2230 × PI473331 (ber)
M200.38	hybrid	USW2230 × PI473331 (ber)
MI49.10	hybrid	adg, chc, phu − stn, spl, tbr

[1] Abbreviations for *Solanum berthaultii* (ber), S. *sparsipilum* (spl), *S. tuberosum* ssp. *tuberosum* (tbr), *S. tuberosum* ssp. *andigena* (adg), *S. chacoense* (chc), *S. phureja* (phu), *S. stenotonum* (stn), respectively.

2.4 Evaluation of PTM resistance on tubers

PTM resistance was evaluated using 5 tubers/replication with 3 replications for each family. Those tubers were evaluated principally using a laboratory test for antibiosis at Lima, Peru, in February, 1994 and a storage testing method for antixenosis at San Ramon, Peru, in May, 1995. Resistance was ranked into five classes and based on the visual score of feeding by insect larvae (Ortiz *et al.* 1990).

2.5 Field trials

Five tubers/replication were used for each regenerate with two replications for the field trial. Regenerates were clonally propagated by nodal cutting and 5 clones for each regenerate were planted in the field, located in Lima, Peru in September 1993 and harvested in December 1993. In 1994, the same procedure was conducted to make regenerates. Tubers were planted in September 1994 and harvested in December 1994.

3. Results and Discussion

3.1 Evaluation PTM resistance levels on tubers in regenerates and their progenitors

Results of antibiosis as well as antixenosis tests to evaluate the levels of PTM resistance on tubers in regenerates and their progenitors are listed in **Table 2**. Principally the regenerates maintained the levels of PTM resistance in their progenitors. Unlike chromosome doubling generated by colchicine treatment with the direct application to apexes, the use of *in vitro* chromosome doubling by agents such as zeatin riboside, benzylamino purine (BAP), did not result in the frequent production of chimera in chromosome-doubled regenerates in potato (Orrillo and Watanabe 1994, Valkonen *et al.* 1994). In our study, no chimera was observed at L1 and L3 levels (**Fig. 1 and Table 2**). *In vitro* chromosome doubling, therefore, has an advantage in obtaining 4x regenerates with desirable traits over colchicine treatment. Moreover, when the expression of desirable traits such as PTM resistance is affected by cytoplasm, this method promises an effective man-

Table 2. The evaluation of resistance to PTM on tubers and mean DNA content (2C value) in leaf nuclei for each progenitor and regenerate

Genotype	Antibiosis test[2,3]	Antixenosis test[2,4]	2C (pg)[5]	Standard deviation
84.128.58	S	S	1.52	0.05
84.128.58CD[1]	S	S	3.16	0.05
CCC1386.26	MR	MR	1.61	0.05
CCC1386.26CD	MR	MR	3.63	0.07
HHI-9	S	S	1.62	0.08
HHI-9CD	S	S	3.28	0.10
KWPTM-7	R	R	1.58	0.07
KWPTM-7CD	R	R	3.18	0.11
KWPTM-18	R	R	1.60	0.06
KWPTM-18CD	R	R	3.26	0.04
KWPTM-24	R	R	1.55	0.06
KWPTM-24CD	R	R	3.40	0.10
KWPTM-29	R	R	1.67	0.05
KWPTM-29CD	R	R	3.38	0.03
M200.32	MR	MR	1.63	0.04
M200.32CD	MR	MR	3.24	0.06
M200.38	MR	MR	1.59	0.05
M200.38CD	MR	MR	3.31	0.07
MI49.10	MR	MR	1.69	0.08
MI49.10CD	MR	MR	3.36	0.08

[1] CD indicates regenerates applied *in vitro* chromosome doubling derived from each progenitor.

[2] Abbreviation for susceptible: S, moderately resistant: MR, resistant: R, respectively. Refer to Chavez *et al.* (1988) and Ortiz *et al.* (1990) on description of the resistance level.

[3] Adverse effect on biology of PTM.

[4] Adverse effect on behavior of pest as non-preference.

[5] 2C values were obtained from around 10,000 nuclei.

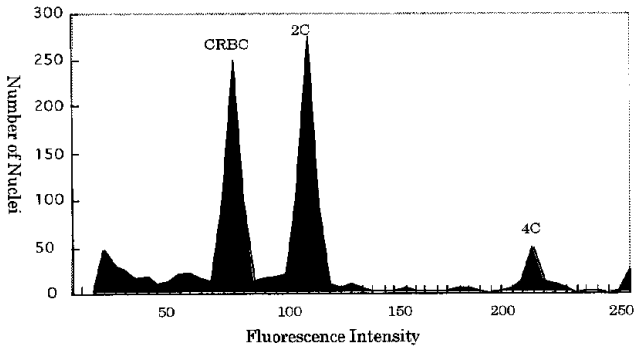

Fig. 1 Histogram of chromosome-doubled KWPTM-7 shows the numbers of propidium iodide (PI)-stained leaf cell nuclei and PI-stained chicken red blood cells (CRBC) as a function of fluorescence intensity resulting from cytometric measurement. The histogram comprises 255 channels which correspond to the liner scale of fluorescence intensity. Signals from nuclei were gated to eliminate most of debris from analysis. The nuclear DNA content was calculated by direct comparison of the modal position of the plant peak to the modal position of the CRBC peak (2.33 pg). The value of 2C peak for chromosome-doubled KWPTM-7 (4x) was 3.18 pg with S. D. 0.11.

ner for introgression of valuable genes from wild or exotic germplasm to the tetraploid potatoes. Cytoplasmic influence on the trasmission of pest resistance was also reported in root knot nematodes (*Meloidogyne* spp.) (Gomez *et al.* 1983). By using resistant genotypes as female parents, the expression of resistance in their progeny could be assisted by the cytoplasm inhereted from female parents.

3.2 Yield performance of regenerates compared with their progenitors

The result of yield performance in the 1993 growing season is summarized in **Table 3**. Since mini-tubers (2-3 cm diameter) from small pots were used, the overall results of the yield trials were lower than normal yield trials with 4x cultivars. Five of six genotypes of resistant regenerates yielded higher than their progenitors on average. Among the regenerates derived from resistant progenitors, those from CCC1386.26 had much higher yield (0.236 kg) than their originals (0.123 kg) on average. In other regenerates, slight increase was observed. Significant increase was observed in regenerates of susceptible 84.128.58. On the other hand, yield in regenerates of resistant KWPTM-24 was significantly lower than its progenitors.

In the 1994 growing season, more variable response to yield was observed than in the 1993 trial (**Table 4**). In general, yield trials conducted in Lima in 1994 besides this study produced decreased crop compared to 1993 (Watanabe *et al.* 1996). This could be due to the difference of the environmental conditions such as water availability and temperature, between the two years. Among regenerates from resistant originals, four genotypes of regenerates obtained higher yield than their originals (HHI-9, KWPTM18, KWPTM-29, and M200.32) on average. In these genotypes, the increase was considerably high for all genotypes. Significant increase was observed in regenerates of susceptible 84.128.58.

Half of genotypes in regenerates derived from resistant or moderately resistant genotypes yielded less than their progenitors in 1994 on average. Among these four genotypes, KWPTM-24 showed lower yield in regenerates than their original, following the same tendency in 1993. However, the degree of decrease in 1994 was much smaller than that in 1993. The rest of genotypes with the yield decrease were from moderately resistant progenitors, CCC 1386.26, M200.38, and MI 49.10. Decrease observed in MI 49.10 was significant at 0.05 level.

Yield performance was tested in two years in six

Table 3. Summary of yield of regenerates derived from PTM resistant 2x, susceptible 2x, and that of their progenitors in 1993.

Genotype	Mean yield of progenitors (kg/plant)	Standard Deviation	Mean yield of regenerates (kg/plant)	Standard Deviation
84.128.58	0.354	0.019	0.563***	0.096
CCC1386.26	0.120	0.022	0.236	0.248
HHI-9	0.100	0.018	0.128	0.073
KWPTM-7	0.302	0.041	0.428	0.129
KWPTM-18	0.282	0.020	0.314	0.109
KWPTM-24	0.466	0.063	0.226***	0.080
KWPTM-29	0.216	0.062	0.262	0.062

*** significant difference between yield of progenitors and that of regenerates derived from them at the 0.001 level.

Table 4. Summary of yield of regenerates derived from PTM resistant 2x, susceptible 2x, and that of their progenitors in 1994.

Genotype	Mean yield of progenitors (kg/plant)	Standard Deviation	Mean yield of regenerates (kg/plant)	Standard Deviation
84.128.58	0.736	0.280	0.746	0.323
CCC1386.26	0.130	0.136	0.071	0.060
HHI-9	0.017	0.019	0.054	0.060
KWPTM-18	0.080	0.080	0.132	0.104
KWPTM-24	0.169	0.111	0.147	0.085
KWPTM-29	0.159	0.086	0.296	0.193
M200.32	0.003	0.009	0.009	0.019
M200.38	0.036	0.062	0.026	0.036
MI49.10	0.384	0.253	0.229*	0.139

* significant difference between yield of progenitors and that of regenerates derived from them at the 0.05 level.

genotypes, 84.128.58, CCC1386.26, HHI-9, KWPTM-18, KWPTM-24, and KWPTM-29. In the rest of genotypes, continuous testing was impossible due to the difficulties of obtaining enough number of tubers. In the comparison between yield performance in 1993 and that in 1994, five genotypes showed the same tendency. Regenerates of resistant genotypes 84.128.58, HHI-9, KWPTM-18 and KWPTM-29 had higher yield than their originals in both years on average. Whereas, KWPTM-24 showed decreased yield in both years. In CCC1386.26, a constant tendency was not observed. Comparison with standard 4x cultivars was not done. Also, *in vitro* nodal cutting was used, so that the comparison with 4x cultivars does not provide an estimate of the performance as 4x. The somatically doubled 4x clones should be regarded as media to transmit the resistance to 4x populations and further consideration as yield progenitors should not be a priority.

Several other investigations were conducted to determine how chromosome doubling affects the yield performance. Rowe (1967) showed decrease in the yield of 31% on average in the 4x derivatives compared with single 2x plants in crosses between 2x *S. phureja* × (di) haploid *S. tuberosum*. On the other hand, De Main found principally no difference in yield (De Main 1984, DE Main 1994) and rate of photosynthesis in leaves of tetraploids (De Main 1984) or tuber characteristics (De Main 1994) compared with their chromosome doubling derivatives. Maris (Maris 1990) reported that vegetatively doubled derivatives generated from crosses between 2x *S. phureja* × (di)haploid *S. tuberosum* generally had significantly taller plants, later maturity, fewer tubers, higher mean tuber weight, more tubers yield and dry matter yield than their counterpart 2x. In these studies, heterozygosity and gene action seems to have a significant effect on yield performance. Our result also indicated that *in vitro* chromosome doubling had no negative effect on yield in selected clones compared with their 2x originals. In addition to the maintained productivity in 4x regenerates, they showed the same levels of PTM resistance with their originals. This study confirmed that *in vitro* chromosome doubling approach is an effective means to introgress desirable traits from 2x to 4x population, especially when the expression of these traits is considerably affected by cytoplasm.

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