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Progress Report

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Utilization of plant protoplast biotechnologies for the transfer of organelles.

by

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The visit of one of us (E.G.) to the International Potato Center (CIP) in Lima, Peru, resulted in a number of collaborative research plans. During this visit it became clear that organelle transfer can be very helpful to a major breeding effort at CIP, namely, the production of F_1 hybrids of true potato seeds (TPS). Presently such hybrids are produced manually at CIP through tidious emasculations and with pollinator plants which are only partially male-fertile. Consequently it was decided that we (at the Weizmann Institute) should attempt to convert prospective seed-parents into cytoplasmic male steriles (CMS) and also attempt to increase the male-fertility of prospective pollen-parents of certain hybrid combinations. The first phase of such work should be to develop workable methodologies for the regeneration of functional potato plants from protoplasts, in all the breeding-lines involved. The second phase should consist of transferring mitochondria, among the breeding lines, by the donor-recipient protoplast-fusion method. Accordingly we transferred to Israel sterile shoots of the target breeding-lines as well as of the donors of mitochondria.

It should be noted that previous experience indicated that the experimental procedures to regenerate potatoes from protopalsts vary between cultivars. The material obtained from CIP was quite different from standard cultivars. We therefore had to examine a wide range of conditions to attain reproducible results. Thus our research during the past 6 months focussed on isolation, culture and regeneration of the potato lines. The results of this research line are presented as experimental protocols - thus they are phrased in the present tense. In addition we performed donor-recipient protopalst-fusions among the

different potato-lines, as briefly reported below. We started to develop the required methodologies for the analyses of the plastomes (chloroplast genomes) and chondriomes (mitochondrial genomes) of potato species. Such analyses are required to ascertain the organelle compositions of future inter-specific cybrids.

We are pleased to note that the main efforts of the past 6 months resulted in success beyond previous expectations: we found adequate means to regenerate plants from all the breeding-lines.

A. POTATO PROTOPLASTS: ISOLATION, CULTURE AND REGENERATION INTO PLANTS.

Maintenance

The eight potato lines (Table 1), which were obtained from C.I.P. are maintained as shoot-cultures on half strength MS medium (Murashige and Skoog, 1962) containing 1% sucrose and 0.8% agar, at 25°C with 16hr/day illumination (6000 lux). The shoots are harvested for protoplast isolation, 3-4 weeks after transfer to fresh medium.

Protoplast Isolation

After testing many enzyme combinations and other conditions we arrived at the following enzyme digestion mixture which is optimal for all the eight potato lines:

0.1% Driselase, 0.75% Meicelase,

0.025% pectolysase Y-23,

0.1% Cellulase R-10, 0.015% Macerozyme

The enzymes are dissolved in V47 macroelement solution (Binding 1974) containing 0.5M mannitol and 0.5% P.V.P. (M.W. 360000).

Pricked shoots are incubated ($25^{o}c$, dark) in the enzyme solution for about 16hr.

Protoplast Culture

Washed protoplasts are plated in 1.5ml per 5cm dish at about 10^5/ml. protoplasts/ml. During the first 3 days the plates are kept in the dark. Thereafter the plates are transferred gradually to higher light intensities reaching finally 2000 lux. In parallel, from the fourth day and on, the osmoticum of the medium is gradually reduced by the addition of small quantities of half strength MS medium.

All of the following three liquid media were found to stimulate divisions in all the eight potato lines:

1. Medium A. This medium is based on Adams and Townsend (1983) with several modifications (the major ones are replacement of NH_4NO_3 with glutamine plus aspargine and addition of coconut water).

2. Medium TM-2 This is Shahin's medium.(Shahin, 1984)

3. Mixed A + TM-2 medium The two media were mixed at a 1:1 ratio

The hormone composition is identical in all three media: 1 ug/ml NAA, 0.5 ug/ml zeatin and 0.05ug/ml 24D.

The gradual reduction of osmoticum, in all three media, is done by addition of half strength MS medium containing 0.5ug/ml zeatin and 1 ug/ml NAA.

Plant Regeneration.

Three to four weeks after plating about 1-2% of the original protoplasts produced calli of 1-2mm in diameter. At this stage, the calli were transferred onto MS agar medium. (We have tested several different media and found that the hormone combinations used were more critical than media compositions).

Several hormone combinations were found to stimulate plant regeneration (Table 1). Some of the lines responded well to all hormone combinations while others responded only to some combinations. In some of the lines, regeneration took place about 3 weeks after transfer to regeneration medium while with others a second transfer was necessary. In the latter cases, culturing the calli for 3 weeks in low concentration of gibberellic acid (GA_3) followed by increased GA_3,(or GA_{4+7}) concentration for additional 3 weeks improved the regeneration yield considerably.

Cytoplasmic transfer.

Having found appropriate conditions for protoplast culture and regeneration of the eight potato lines we initiated the protoplast-fusion experiments. The rational for these protoplast-fusions were stated above. The fusions are of the 'donor-recipient' type i.e. irradiation of cytoplasmic donor protoplasts (10-12 Krad) prior to fusion. Recipient protoplasts were treated with rhodamine 6G to facilitate mitochondrial transfer by decreasing the mitochondrial content of the recipient protoplasts. (see below).

The following combinations are underway:

Exp.	DONOR	RECIPIENT	GOAL
1.	CIP Y2457	Atzimba	convert to CMS
2.	Serrana	Atzimba	convert to CMS
3.	CIP Y2457	Atlantic	convert to CMS
4.	Desire	LT-1	convert to male fertility

In Exp. 1 the calli started to regenerate into plantlets while the other experiments are at an earlier stage. In the coming months, we expect to transfer several plants from each of the different fusion combinations to the greenhouse and to analyze their plasmones. Concurrently we shall analyze DNA restriction patterns of the plastomes and the chondriomes of the fusion partners as well as of other Solanum species which may serve in future donor-recipient protoplast-fusions.

Table 1. Response of Potato lines to different regeneration conditions

Potato line	First Transfer		Second Transfer	
	Hormone combinations[1]	Regeneration[2]	Hormone combinations[1]	Regeneration[2]
Atzimba (CIP720045)	A, B, C	+++		
DTO-28 (CIP800169)	A, B, C	++		
Serrana	B, C	++		
Atlantic (CIP800827)	A, B, D	++		
LT-1 (CIP377257)	A, B, D	+	H L M N	++
Chata blanca (CIP702514)	F, G		H I	++
Ticahuasi (CIP720019)	A, B, E		H I J	++

1. For hormone combinations see attached appendix
2. Regeneration of about 80% of calli (+++) about 50% of calli (++) and of about calli 1% (+)

Appendix to Table 1. Concentration of hormones are given in mg/liter

Designation of hormone combination	IAA	NAA	Zeatin	Zeatin riboside	GA_3	GA_{4+7}	kinetin	Ca Pantho-tenate	ABA
A		0.01	2	1	0.01				
B	0.2		2		0.01				
C			10		0.1		10		
D		0.1		1	0.01				
E		1	4						
F		0.2	1						
G	0.5		0.5						
H			0.2		0.2				
I			0.01					2	
J			1		0.1				
K			0.2		8			2	
L			0.2		8	0.3		2	
M			0.2			0.3		2	
N			0.2		8			2	0.5

B. RHODAMINE 6G PRETREATMENT: A POSSIBLE WAY TO CONTROL CHONDRIOME COMPOSITIONS OF CYBRIDS.

In our previous report (section III) we suggested a possible way of controlling the chondriome composition of different cybrid plants by rhodamine 6G (R6G) treatment prior to fusion. We proposed that R6G pretreatment of recipient protoplasts should decrease specifically the recipient's mitochondria and therefore increase the proportion of donor mitochondria among cybrid plants and vice versa when the R6G pretreatment is given to donor protoplasts, we expected more retention of the original mitochondria of the recipients in the cybrids.

Detailed mtDNA analysis of cybrid plants, derived from two combinations, in which recipient protoplasts were treated with R6G did not substantiate our supposition.

Fusion combinations:

EXP	DONOR	RECIPIENT
1.	N. rustica rus/rus/rus *	N. tabacum, pig^{-}-35. (R6G) tab/tab pig^{-} tab *
2.	N.tabacum, cms, lin^{R}-17 tab/und Lin^{R}/ und	N.tabacum, pig^{-}-35 (R6G) tab/tab, pig^{-35}/ tab

* For each plant the nuclear, plastome and chondriome composition /.../..../..../ is given from left to right.

In neither of these experiments, was a complete elimination of the (R6G treated) recipient mitochondria observed in the resulting cybrids. In Exp. 1 the mtDNA restriction patterns of cybrid plants (as revealed by Southern-blot hybridizaiton to several mitochondrial probes) were of either N. rustica or N. tabacum type, without any apparent recombiantions. In Exp. 2, most of the cybrid plants exhibited novel restriction patterns. However, it is still possible that a complete elimination of mitochondria could be attained by increased concentration of R6G. Several fusion combinations are underway in which donor protoplasts were treated with increased concentration of R6G. These experiments are still at an early stage.

References

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3. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

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