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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 3. F-29
July 1 - Dec 31, 1984

Attachment

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Progress Report

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Grant No. DFE-5542-G-SS-4030-00

UTILIZATION OF PLANT PROTOPLAST BIOTECHNOLOGIES FOR THE TRANSFER OF ORGANELLES (3F-29)

by

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Sciences

Period: July 1 to December 31, 1984.

The following is actually our first semi annual progress report, because the financing of this project was started in July 1985. It should be kept in mind that the main trust of our experimental efforts, such as the 'donor-recipient' protoplast fusions are long-term procedures: fusion-derived plantlets are obtained about three to four months (sometimes even after longer times) after fusion and additional 3 to 4 months are required to attain flowering. If characterization of chloroplast and mitochondrial DNA is required, the final results are obtained after an additional laps of time. Hence, in respect to such experiments we shall only be able to report on the phase which was reached at the time of this writing.

I. Isolation of hetero-fused protoplasts

One of the main problems in the production of somatic-hybrids and cytoplasmic hybrids (cybrids) by protoplast fusion is the selection of the hybrid and cybrid protoplasts from the homo-fused 'parental' protoplasts. There are several approaches to achieve such a selection (e.g. use of nuclear and/or organelle mutants; pre-fusion treatments; X-irradiation). A rather direct approach is to isolate, manually, hetero-fused protoplasts. To achieve this method the hetero-fused protoplasts should be clearly different from either of the parental protoplasts (fusion partners) of the fusion. Although there were a few reports on the isolation of hetero-fusion products by means of fluorescent staining, we were not satisfied with the rate of success of these reported procedures. We therefore had to develop our own procedure.

Actually two questions were asked: (a) can individual protoplasts be cultured up to the regeneration of plants; (b) what are the most appropriate stains which will not only enable clear identification of hetero-fusants but also result in viable protoplasts having the capability to divide and ultimately regenerate plants. In summary, the following results were obtained.

(a) By serial dilutions of Nicotiana mesophyll protoplasts, which were plated in liquid medium containing about 2×10^4 /ml of X-irradiated (i.e. non-dividing feeder-cells) protoplasts we found that even 5 mesophyll protoplasts or less, in a total volume of 3 ml are capable to divide and to regenerate plants. When the feeder cells are from albino plants the identification of green calli resulting from the individual, non-irradiated protoplasts, is rather obvious.

(b) We screened several fluorescence dyes such as flurescein isothiocyanate (FITC) and Rhodamines and dichlorotriazinyl aminofluorescein (DTAF). DTAE was found to be most suitable. We thus developed procedures to stain albino-protoplasts in a way which will enable good staining but will retain the division capability of the stained protoplasts. Figure 1 shows a fusion product between a non-stained N. alala mesophyll protoplast and a protoplast from an albino N. tabacum plant which was stained with DTAF. The red color resulted from the natural fluorescence of the chloroplasts of N. alata while the green-fluorescence is from the DTAF stained N. tabacum protoplast.

The isolation of the hetero-fused protoplast is then performed with a micromanipulator and individual hetero-fusion products are transferred

to petri dishes containing non-dividing feeder protoplasts. The procedure is reproducible and was used routinely.

II. Rhodamine sensitivity

Rhodamine-6G was reported to cause specific repression (or destruction) of mitochondria in cultured mammalian cells. If R6G affects plant protoplasts similarly, R6G treatment of one partner, before fusion, could be instrumental in eliminating the contribution of mitochondria of this fusion partner from the hetero-fusion product, leading to cybrid plants having (mostly or only) the mitochondria of the other (untreated) fusion partner. We thus studied the dose-effect of R6G on various kinds of plant protoplasts and found that mesophyll protoplasts are generally less sensitive to R6G than suspension-culture derived protoplasts. A procedure was thus established by which R6G treatment was performed during the isolation of protoplasts from the leaf-tissue or the suspension culture (i.e. during enzymatic maceration). It should be noted that such treated protoplasts are not capable to divide in culture, but when fused with other protoplasts the hetero-fusants are indeed capable to divide and to regenerate plants.

III. Cybrid plant production : attempts to control organelle composition by pre-fusion treatment of protoplasts.

One of the main crop-improvement applications of protoplast-fusion and cybrid formation is the establishment of plants having the nuclear-

genotype of a given species (or breeding-line) and either or both chloroplasts and mitochondria from another species (or organelle-donor). The detailed rational for such organelle-transfers was presented in our proposal. Transfer of alien mitochondria into a given genotype to produce alloplasmic male sterility and transfer of temperature-tolerant chloroplast into a heat-sensitive cultivar are two examples for the potential use of such transfers.

In our general approach to attempt controlling the transfer of organelles in the donor-recipient protoplast fusion method we quested the efficiency of using specific mutants and pre-fusion treatments, for shifting the organelle-sorting out towards the required organelle composition in the cybrids. The approach will become clearer with the description of the types of fusions and treatments which were performed with Nicotiana as follows.

1. Fusion between N. rustica (rus/rus/rus)* and N. tabacum. - Normal-green N. rustica protoplasts were fused with an albino, plastome-mutated line, of N. tabacum VBW termed (tbc/tbc, pig$^-$1/tbc). The N. rustica protoplasts were X-irradiated before fusion, serving as organelle donors. Two fusions were performed:

(a) rus/rus/rus + tbc /tbc,pig $^-$1/tbc (VBW) without iodoacetate pre-fusion treatment

(b) rus/rus/rus + VBW with iodoacetate treatment.

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*this designation is to abbreviate: N. rustica nuclear genome/ N. rustica plastome/ N. rustica chondriome.

To enable manual isolation of fusion products the recipient protoplasts (VBW) were stained, before fusion, with DTAF. The red-greenish fluorescing hetero-fusion products were then transferred into plates with feeder cells as detailed above. In both cases cybrids were detected by greening of the respective calli (which should contain the green N. rustica chloroplasts but nuclei of VBW, since the VBW plastids are albino and the N. rustica nuclear division was arrested by irradiation). The two fusions differed in respect to the iodoacetate (IA) treatment. If IA causes damage to the recipients'(VBW) organelles it is expected that the mtDNA of the cybrids, resulting form the two fusions will be affected: we should expect more N. rustica mtDNA among (b) cybrids than among (a) cybrids. The number of cybrids obtained was rather small (10 and 14 from (a) and (b) respectively) and they were derived from few calli. The mtDNA analysis is not yet finished but only few of the resulting cybrids had fertile pollen. Chromosome counts showed that most plants were polyploid. although the experiment is not yet concluded - it seems that much greater populations of cybrids (derived from more calli) should be produced in order to evaluate the effect of IA on the recipients mitochondria. We are therefore contemplating to repeat these fusions or testing the effect of IA by another "pair" of fusions.

2. Fusion between N. rustica and a double plastid mutant of N. tabacum. In this fusion we quested the effect of R6G on the organelles. The two fusions were:

(a) rus/rus/rus+ tbc/tbc,str^R-1,pig^-35 / tbc (R6G)

(b) rus/rus/rus (R6G) + tbc/tbc,str^R-1,pig^-35/tbc

The mutant tbc/tbc,str^R-1,pig$^-$35/tbc is a double plastome mutant, produced in our laboratory (Fluhr, Aviv, Galun and Edelman, PNAS, in press). It was derived from a streptomycin-resistant (SR-1) N. tabacum mutant which was mutated again to produce a chlorophyll-deficient (plastome-coded) line which can be reproduced as shoot-cultures in vitro. Staining of the recipient protoplasts, the isolation of the hetero-fusion products and the culture of the fusants was as described for the previously mentioned fusion pair. In this pair either the recipient (in (a)) or the donor (in (b)) protoplasts were treated with R6G before fusion and in both cases the heterofusion products were isolated manually as detailed in I. Fifty and 9 cybrid plants were obtained from (a) and (b) respectively. We hope to regenerate more cybrids, especially from (b). The plants will be analysed in respect to plastome composition (many of the cybrids are variegated and are expected to contain both rus and tbc, str^R1,pig$^-$35 chloroplasts) and mitochondrial (chondriome) composition. This pair and others of it's kind, where R6G is applied to either the donor or the recipient fusion-partner, should furnish an answer to the question whether or not R6G pretreatment specifically reduces the number of functional (i.e. having division capability) chondriomes in the treated protoplasts.

Other fusion experiments in which the donor and/or the recipients were pretreated with R6G and the recipient were treated with IA were performed, but they are in earlier stages : colony formation, regeneration of calli and transfer into the green house.

The above section should be regarded as interim-information, because none of the fusion experiments was yet concluded. We hope to present the results of this direction of our research in the next Progress Report.

III. Regeneration of plants from potato protoplasts

The procedure to regenerate plants from potato cultivars is being developed in the frame of another project in our laboratory (by D. Aviv, J. Gressel and A. Perl). We are utilizing this procedure for the needs of the present project. Most of the work was performed with one potato cultivar Mirka. The procedure is based on published information (by Adams and Townsend, Plant Cell Rep. 2, 165-168, 1983) but was modified to provide better results. The source of the protoplasts are *in vitro* culture shoots. We are therefore preparing such shoot-cultures from additional cultivars as well as from a breeding line (Y245.7) obtained from our collaborators at CIP Lima, Peru. These shoot-cultures will furnish the plant material for future donor-recipient fusion experiments. Additional potato breeding lines, cultivars and wild-relatives of potato will be obtained after the visit of one of us (E.G.) at CIP (see V below).

IV Isolation of plastome mutants from potato cultivars and breeding lines.

As indicated above one of the means to select cybrids having donor organelles is to use recipient protoplsts from albino mutants. Green cybrids resulting from such fusion-combinations are expected to contain only the green chloroplasts of the donor. For this and additional reasons, we initiated a research line to induce plastome mutations in potatoes. The approach is similar to the published procedure (Fluhr, Aviv, Galun and Edelman, PNAS, in press) which was developed for Nicotiana.

We first tested the effect of the mutagen N-nitroso-N-methylarea (NMU) on true seed of potato (PTS) and found that a 3 h treatment with 0 to 75 µg/ml of NMU gave a reasonable dose-response curve. The dose of 25 µg/ml of (freshly prepared) NMU was then used in further experiments.

PTS obtained from CIP, were used for mutagenesis. In a typical experiment 500 seeds were NMU treated and a similar number of seeds were non-treated. The seeds were then disinfected by hyperchloride, washed with sterile water and plated over Nitsch nutrient agar. After four weeks, seedlings with chlorophyll-variegation could be detected in the NMU treated seeds only. Examples of such variegations are shown in Figure 2.

It is thus established that the mutagenic procedure is efficient in potato. Further work in this field will be in three directions: (a) to isolate the seedling sections which will contain only pigment-deffective leaves and then to propagate pure mutant shoots as future source of recipient protoplasts; (b) to test these shoot-lines, whether the mutations are plastome or nuclear coded (this will be relatively simpler

in mutants which are not fully albino and are capable to produce a sexual progeny - otherwise the test will have to be performed by protoplast fusion); (c) to mutagenise those potato lines (which will be obtained from CIP, mainly) which are prospective recipients in future donor-recipient fusion experiments.

V. Collaboration with the International Potato Center (CIP) Lima, Peru

The collaboration with CIP started actually before this project was financed. We initiated a reciprocal correspondence with Dr. Charles Brown, then geneticist at CIP. In this correspondence we discussed which potato plant-material should serve in this project. Consequently we chose the transfer of alien mitochondria into potato breeding lines as one of our objectives. Such a transfer is expected to cause cytoplasmic male sterility (CMS) in the breeding line. The CMS breeding-line would then furnish a future seed-parent in F_1 hybrid potato seed production. The establishment of F_1 hybrid varieties (i.e. PTS F_1) is one of the central aims of potato improvement at CIP because it is intended to supply, in the future, such seeds to the farmers in the tropical highlands, in substitution of potato tubers. Dr. Brown left CIP and Dr. John Dodds a tissue culture specialist, is now our collaborator at CIP. An exchange of letters and telexes is keeping us in contact but it became obvious that a visit at CIP by one of us is essential for the success of the part of our project which will deal with potatoes. The visit, scheduled for early March 1985, will be utilized to get familiar with the main objectives of potato improvement handled at CIP, to reveal genetic traits of economical relevance which are possibly coded by the organelle's genome, to plan future-more efficient-collaboration and to focus on plant material which should serve for both donor of organelles and as recipient in our future protoplast-fusion work.

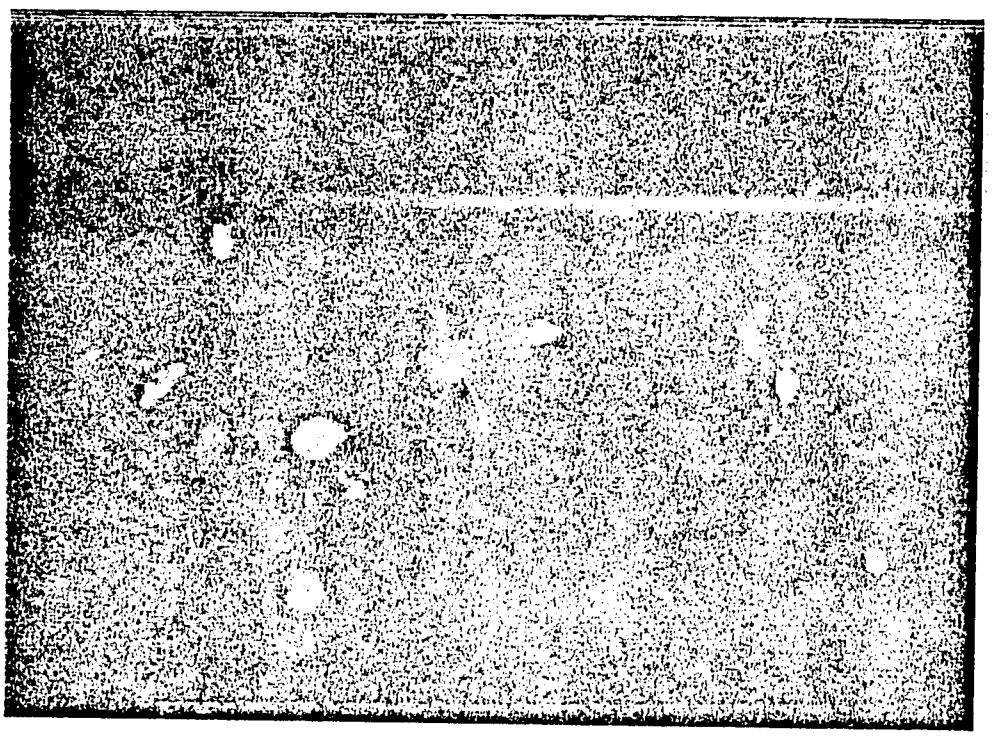

Fig. 1. Fusion between non-stained (and X-irradiated) *N. alata* protoplasts and DTAF stained protoplasts from an albino *N. tabacum* mutant (*tbc*/tbc *str*^{R} 1, *pig*$^{-35}$ /tbc). Fluorescence illumination; note fused protoplast in the center of the photograph - the red is the natural fluorescence of the *N. alata* chloroplasts and the greenish florescence is from the DTAF staining of the *N. tabacum* protoplast.

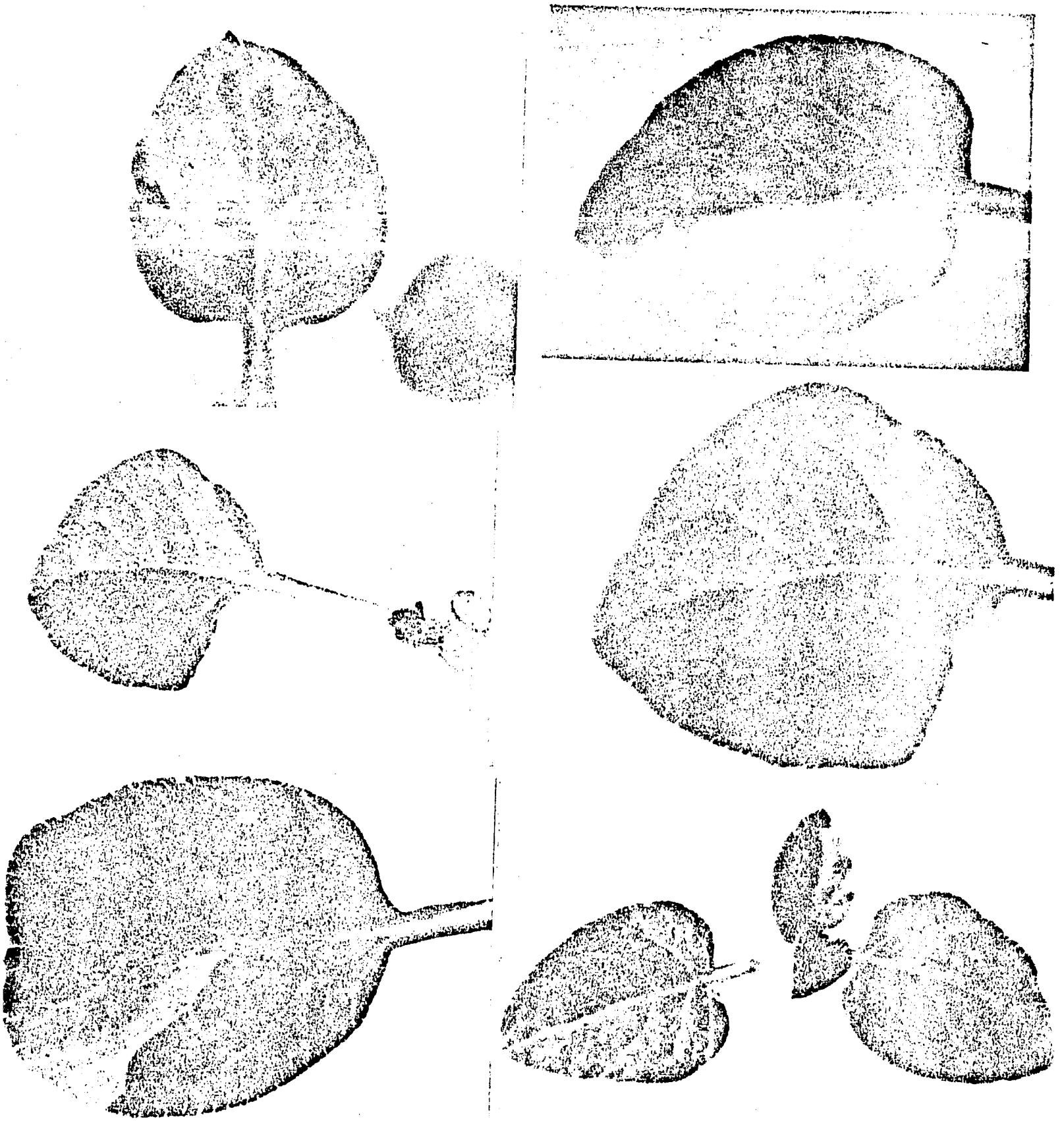

Fig. 2. Examples of variegated leaves from seedlings which developed from NMU treated potato seeds which were plated on Nitsch nutrient medium