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Cremart: A new chemical for efficient induction of micronuclei in cells and protoplasts for partial genome transfer

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Summary. Results on efficient induction of micronuclei by Cremart in suspension cells and protoplasts of potato are reported. Cremart is a highly effective phosphoric amide herbicide, which acts on the mitotic spindle, and induces micronuclei through modification of mitosis. After treatment with Cremart, metaphase chromosomes changed directly into micronuclei without centromere division and chromatid separation. When suspension cells were treated with Cremart (3.7 - 15.0 μM) for 48h, and subsequently incubated in a mixture of cell wall-digesting enzymes in the presence of cytochalasin-B and Cremart for 18h, the frequency of micronucleation in the cell/protoplast mixture increased significantly, as compared to that obtained after treatment of suspension cells with Cremart (3.7 - 15.0 μM) for 48 h. Sieving after enzyme incubation resulted in the recovery of protoplasts, showing mass induction of micronuclei. Also synchronized suspension cells of *Nicotiana plumbaginifolia* responded with high frequency of micronucleation after Cremart (3.7 μM) treatment. The application of this procedure for partial genome transfer is discussed.

Abbreviations: APM: Amiprophos-methyl, BAP: 6-Benzyl aminopurine, CB: Cytochalasin-B, MI: Mitotic index, MN: Micronuclei, MS: Murashige and Skoog, NAA: 1-Naphthalene acetic acid

Introduction

The transfer of desirable traits from sexually incompatible wild species or wild relatives to cultivated species is feasible through genetic manipulation at cellular or DNA level (Sybenga 1989). Somatic hybridization is potentially useful for the transfer of desirable traits, especially those controlled by polygenes or uncloned genes from wild species to crop plants, and to generate novel nucleus -cytoplasm combinations, circumventing sexual crossing barriers (reviewed in Gleba and Sytnik 1984; Glimelius 1988; Puite 1992). To achieve partial genome transfer from the donor species, methods for asymmetric somatic hybridization have been developed. Generally, irradiated donor protoplasts have been fused with intact recipient protoplasts. However, this technique has so far not given satisfactory results, because a large segment of the donor genome was still present. Furthermore, several authors reported polyploidization, aneuploidy, poor growth and regeneration, and lack of fertility due to the genetic complexity of the hybrids obtained and the radiation-induced genetic damage (Gleba *et al.* 1988; Sacristan *et al.* 1989; Famelaer *et al.* 1990; Wijbrandi *et al.* 1990; Wolters *et al.* 1991; Derks *et al.* 1992; Puite and Schaart 1993). Alternatively, asymmetric hybrids can be produced through the fusion of microprotoplasts containing one or two chromosomes from the donor with whole protoplasts of the recipient parent. Several micronuclei can be induced per cell by exposure of actively growing cells to spindle toxins, and protoplasts of these cells can be fractionated into microprotoplasts which contain one or a few chromosomes. For successful application of this technique, induction of micronuclei on a mass-scale is essential. Previously, we reported that two microtubule inhibitors APM and oryzalin, belonging to the class of phosphoric amide herbicides, induce micronuclei at high frequencies in plant cells (Ramulu *et al.* 1988, 1991; Verhoeven *et al.* 1990, 1991 a,b). Recently, we found that Cremart (Butamiphos, O-ethyl-O-(3-methyl-6-nitrophenyl) N-*sec*-butylphosphorothioamidate), which also belongs to the class of phosphoric amide herbicides, induces micronuclei at much higher frequencies than APM or oryzalin in potato, enabling the transfer of one or a few chromosomes (partial genome) from potato to *Lycopersicon peruvianum* and the production of highly asymmetric hybrids (Ramulu *et al.* 1994). This article reports data on efficient induction of micronuclei by Cremart in cells and protoplasts of a transformed potato line. Some

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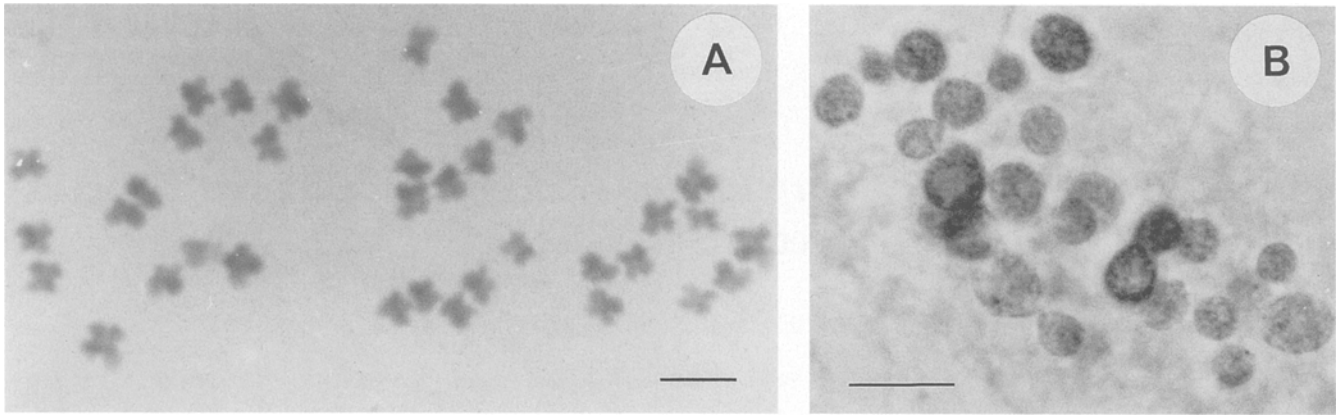

Fig. 1. Micrographs showing metaphase arrest of potato chromosomes after treatment with Cremart (7.5μM) for 4h (A), and a cell with several micronuclei after treatment with Cremart (7.5μM) for 48h (B). Bars = 10μm.

data on enhanced frequency of micronucleation in suspension cells of *Nicotiana plumbaginifolia* treated with Cremart are also reported.

Materials and Methods

Genotype and cell culture: A transformed potato cell line (line 413) was used. It had been derived from the interdihaploid line HH260 (*Solanum tuberosum*, 2n = 2x = 24) after transformation with *Agrobacterium tumefaciens* strain LBA 1060 KG (pRi 1855/pBI 121, binary vector) (De Vries-Uijtewaal *et al.* 1989; Gilissen *et al.* 1991). It carries various genetic markers (kanamycin resistance, ß-glucuronidase activity, opine synthesis, hairy root phenotype and hormone autotrophy), and is a triploid with 2n = 3x = 36. The parent plant from which this cell line derived, was a diploid (2n = 2x = 24). During the establishment of suspension culture, the chromosome number doubled to tetraploid (2n = 4x = 48), and eventually stabilized at triploid level. Cell suspensions were cultured in the dark at 28° C in liquid MS medium (Murashige and Skoog 1962), supplemented with 0.44μM BAP and 2.7μM NAA on a gyratory shaker(120rpm). For sustained cell division activity, subculture was carried out at intervals of 7 days.

Treatments: Cremart was obtained from Sumitomo Chemical Company, Osaka, Japan. A stock solution was prepared at 10 mg.ml^{-1} in water-free dimethylsulfoxide, and actively growing early log-phase suspension cells at one day after subculture in freshly prepared MS-modified medium mentioned above, were treated with Cremart at various concentrations ranging from 1.8-30μM for 6-66h. The control (untreated) cells show mitotic index of about 1% on day 0, 1.4% on day 1 and about 4% (maximum) between days 2 and 3. Both the treated and untreated supension cultures were placed continuously on a gyratory shaker (120rpm) in the dark at 28 °C.

Incubation of cells in the enzyme mixture and chemical treatments: After Cremart treatment for 48h, suspension cells were incubated for 18h in a cell wall-digesting enzyme mixture which consisted of cellulase Onozuka-R10(1.0%), macerozyme Onozuka-R10 (0.2%) (Yakult, Honsha Co. Tokyo, Japan), half-strength V-KM medium (Bokelmann and Roest 1983), with 0.2M glucose and 0.2M mannitol, but no hormones. Before enzyme incubation, the pH was adjusted to 5.6, and osmolality to 500 mOsmol.kg^{-1}. Enzyme incubation was carried out in a 9-cm Petri dish, containing 1.5 ml packed cell volume and 15 ml enzyme solution, on a gyratory shaker (30rpm) at 28°C. Cytochalasin-B (20μM) and Cremart (3.7, 7.5 or 15μM) were added to the enzyme incubation mixture at the time of incubation to prevent the formation of microfilaments and fusion of micronuclei respectively, during the protoplast isolation. After enzyme incubation, the samples were filtered through 297-μm and 88-μm nylon meshes, and repeatedly washed with half-strength V-KM medium (Bokelmann and Roest 1983) with macro-and microelements and 0.24M NaCl (pH 5.6). For comparison suspension cells were treated with the spindle toxin, APM (32μM) for 48h, which induces the maximum percentage of micronuclei in potato line 413, as shown by previous studies (Ramulu *et al.* 1990, 1991) and in *Nicotiana plumbaginifolia* (Verhoeven *et al.* 1990; Ramulu *et al.* 1988, 1993) and subsequently incubated in the enzyme mixture. The procedure of enzyme incubation was exactly the same as described above, except that APM (32μM) was added instead of Cremart to the enzyme mixture, which also contained CB (20μM).

Cytology: To analyse the mitotic index and the frequency of micronucleation, the samples of control and treated suspension cells were fixed in ethanol: acetic acid (3:1 v/v) for 24-48h. The protoplasts isolated after enzyme incubation were washed free from enzymes, fixed by the addition of 30 vol. of 12% (v/v) formaldehyde in 0.2M phosphate buffer (pH 6.0) for 4h, followed by washing and fixation in ethanol: acetic acid (3:1 v/v) for 24h (Ramulu *et al.* 1993). The fixed samples were stained by Feulgen reagent. For slide preparation, a more gentle squashing was used for the protoplasts isolated after enzyme incubation than was used for the suspension cells. The number of cells or protoplasts analysed was 1000 per sample for a given treatment in an experiment. The yield of micronuclei was calculated per total of 1000 cells or protoplasts. Statistical analysis of data from two to three experiments was carried out, using the Student t-test (comparison of two means), or analysis of variance and the Duncan test (comparison of more than two means), and χ^2 tests.

Results

Metaphase arrest and micronucleation after Cremart treatment

The untreated control suspension cells showed normal mitotic divisions with prophase, metaphase, anaphase and telophase. On the other hand, after Cremart treatment metaphase chromosomes lost their regular arrangement on the spindle and were scattered all over the cytoplasm. The chromosomes were arranged in groups of two or more. They were decondensed, developed nuclear membranes around them and then formed micronuclei (Fig. 1). Thus, micronucleation occurred through modification of mitosis, i.e. metaphase chromosomes changed directly into micronuclei without centromere division and chromatid separation. In the control, the mitotic index was 1.4% at the start of the experiment, i.e. at one day after subculture. It increased to 3.1% after 24h and decreased to 2.3% after 60h after the start of the experiment. After treatment with Cremart, the mitotic index gradually increased,

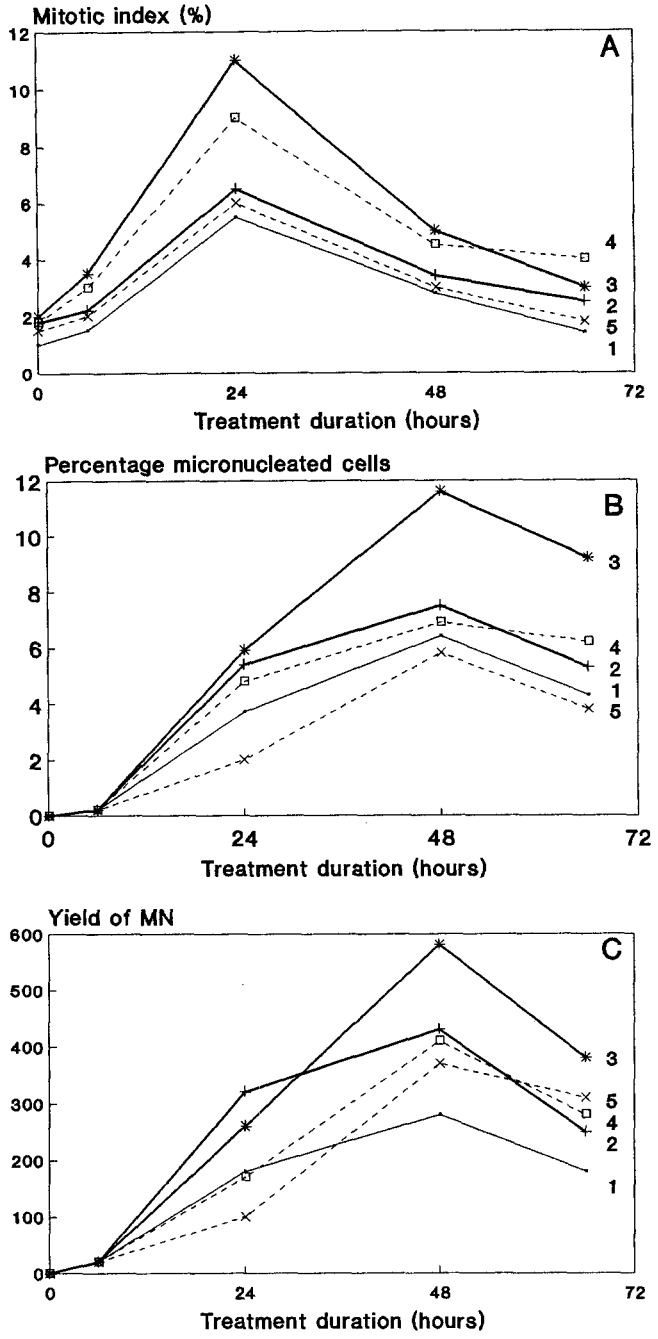

Fig. 2. Mitotic index (MI%)(A), percentage of micronucleated (MN) cells (B) and yield of micronuclei (MN)(C) in suspension cells treated with Cremart for 6-66h at various concentrations. Standard error (SE) (n = 2000-3000 cells analysed per treatment in 2-3 experiments) of the mean ranged from 0.2 to 1.4 for MI or MN cells, and 5-53 for yield of micronuclei. 1: 1.8μM; 2: 3.7μM; 3: 7.5μM; 4: 15μM; 5: 30μM.

showing a maximum at 24h for all the concentrations (Fig. 2A). When the duration of Cremart treatment was increased from 24 to 66h, the mitotic index decreased considerably. The treatment with 7.5μM gave the highest mitotic index (11%), as compared to the other concentrations. Micronuclei were formed from about 6-8h onwards after the

Table 1. Percentage of cells or protoplasts with micronuclei (MN) and yield of micronuclei after treatment with Cremart at 3.7μM and 15μM for 48h, followed by 18h incubation in enzyme solution, containing CB (20μM) and Cremart (3.7μM or 15μM), and sieving. Cells remained on the filters, and protoplasts passed through the filters were analysed (n = 2000 cells or protoplasts per treatment in 2 experiments).

Cremart treatment	*Percentage of cells or protoplasts with MN mean ± SE*	*Yield of MN mean ± SE*
3.7μM		
Cells	10.2 ± 1.3	559 ± 51
Protoplasts	16.1 ± 1.5	1049 ± 64
15μM		
Cells	11.1 ± 0.9	703 ± 49
Protoplasts	16.8 ± 1.7	1178 ± 73

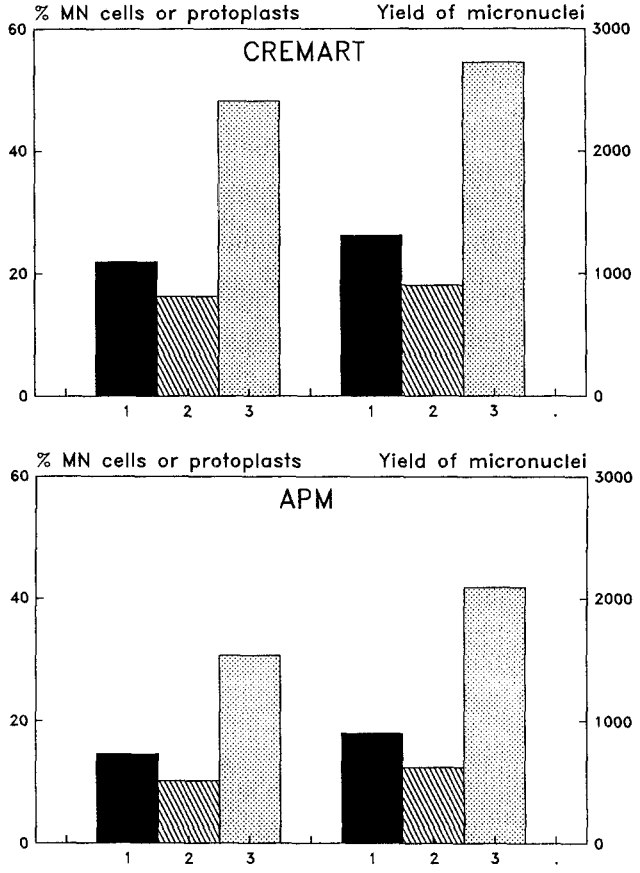

Fig. 3. Percentage of micronucleated (MN) cells or protoplasts, and yield of micronuclei in samples treated with Cremart (7.5μM) or APM (32μM) for 48h, followed by incubation for 18h in enzyme solution containing CB (20μM) and Cremart (7.5μM) or APM (32μM), and sieving through filters. 1: Cell/protoplast mixture before sieving; 2: Cells remaining on the filter after sieving; 3: Suspension of protoplasts passed through the filter after sieving. SE ranged from 2 to 9 for MN cells or protoplasts, and 50-600 for yield of micronuclei (n = 2000 cells or protoplasts analysed per treatment in 2 experiments).

start of the Cremart treatment and the percentage of cells with micronuclei and the yield of micronuclei increased with the treatment duration up to 48h, and afterwards it decreased (Fig. 2B, 2C). The treatment

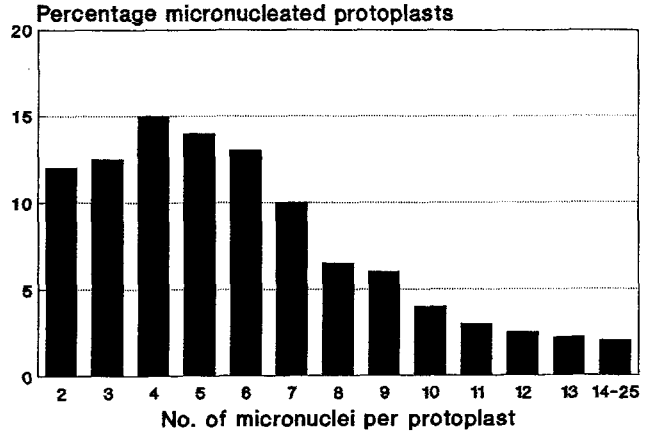

Fig. 4.Frequency distribution of the number of micronuclei per micronucleated protoplast after Cremart treatment (7.5μM, 48h), followed by 18h incubation in enzyme solution containing CB (20μM) and Cremart (7.5μM).

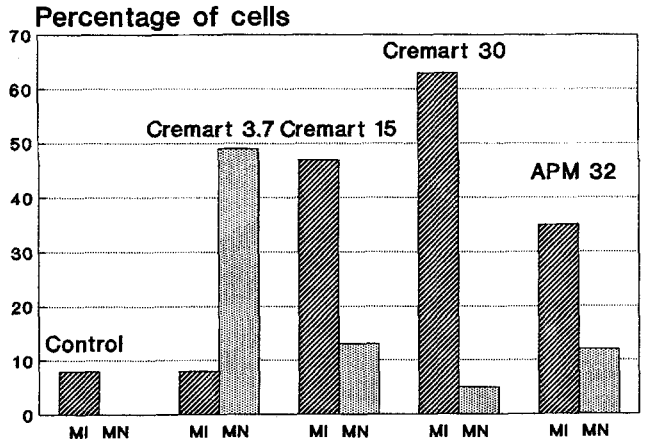

Fig. 5. Mitotic index (MI) and percentage micronucleated (MN) cells after synchronization of suspension cells of *Nicotiana plumbaginifolia* with the DNA synthesis inhibitor hydroxyurea (5mM) for 24h followed by washing and treatment with Cremart (3.7μM, 15μM and 30μM) and APM (32μM) during 24h. Culture conditions are as described in Verhoeven *et al.* 1990.

at 7.5μM gave a significantly ($P<0.05$) higher frequency of micronucleated cells and yield of micronuclei than the other concentrations.

Enzyme and chemical treatments

Figure 3 gives a comparison of data on micronucleation in samples induced by treatment (48h) of suspension cells with Cremart (7.5μM) or APM (32μM), followed by incubation in enzyme solution containing CB and Cremart or APM, and sieving i.e. in cell/protoplast mixture (1), cells remained on the filter (2), and suspension of protoplasts passed through the filter (3).The percentage of micronucleation and yield of micronuclei in protoplasts were significantly ($P < 0.001$) higher than those in cell/protoplast mixture, or in cells remaing on the filter. These data reveal that sieving resulted in the recovery of protoplasts showing mass micronucleation. The treatment with Cremart (7.5μM) gave a higher frequency of micronucleation than that induced by APM (32μM)(Fig.3). The data obtained with other concentrations (3.7 and 15μM) confirm that the frequency of micronucleation was significantly higher in protoplasts than in cells (Table 1). The mitotic index was extremely low (0.1-0.3%) in protoplasts as well as in cells (data not given in Table 1), indicating a rapid change of metaphase chromosomes to micronuclei during the enzyme incubation. Incubation of the untreated control cells in enzyme solution containing CB (20μM) alone, showed no micronucleation in cells or protoplasts. Figure 4 shows the frequency distribution of the number of micronuclei per micronucleated protoplast for Cremart treatment 7.5μM, which gave the highest yield of micronuclei. The number of micronuclei per protoplast varied from 2 to 25, and the percentage of protoplasts showing various numbers of micronuclei ranged from 2 to 15.

Recovery of cell divisions and chromosome doubling after washing

After washing of suspension cells with MS medium (without hormones) at 48h after the initiation of the Cremart treatment (7.5μM), and subcultured for 48h on fresh MS medium supplemented with 0.44μM BAP and 2.7μM NAA, cells recovered showing normal mitotic divisions (prophase, metaphase, anaphase, telophase) and doubling of the chromosome number. The control suspension culture contained 57% triploid (3x=36 chromosomes) and 43% hexaploid (6x=72 chromosomes) cells. The treated cells which were washed and subcultured showed 20% of 3x cells, 60% of 6x cells and 20% of 12x (=144 chromosomes) cells.

Effect of Cremart versus APM on MI and number of micronucleated suspension cells of N.plumbaginifolia

The results of Cremart and APM treatments on synchronized supension cells of *N.plumbaginifolia* are presented in Fig. 5. These results confirm the data obtained in potato line 413, that Cremart at the lowest concentration (3.7μM) is more efficient in the induction of micronuclei than the optimal treatment with APM, and that the percentage increase of MI or MN cells is strongly dependent on the Cremart concentration.

Discussion

The results obtained show that Cremart is highly efficient in inducing micronuclei in cells and protoplasts. To our knowledge, the induction of micronuclei by Cremart has not been previously reported either in plants or animal cell systems. The metaphase chromosomes directly changed into

micronuclei, and this was accompanied by structural and functional changes in chromosomes, resembling the progression of a telophase nucleus in the normal cell cycle. These features were similar to those observed after treatments with APM or oryzalin, but different from that of C-mitosis induced by colchicine which resulted in a low frequency of micronucleation in plant cells (Ramulu *et al.* 1991; Verhoeven *et al.* 1991a,b). When compared to colchicine, APM and oryzalin show a strong binding affinity to plant tubulins (Morejohn and Fosket 1986). The fact that Cremart also belongs to the class of phosphoric amide herbicides and shows the same effect on mitotic process as APM and oryzalin, suggests a similar high binding affinity of Cremart to plant tubulins. After washing and subculture of Cremart-treated cells in a fresh liquid medium, cells showed restoration of spindle function and cell division. As the cells did not undergo centromere division, chromatid separation or anaphase movement in the presence of Cremart, the chromosome number doubled in the next cell division after washing and subculture. It was also evident from previous studies that cells continue proliferation and growth after removal of the spindle toxins, such as APM, oryzalin and colchicine in plant or animal cells (Ramulu *et al.* 1988, 1991; Ege *et al.* 1977).

After incubation of Cremart-treated suspension cells in an enzyme mixture containing CB and Cremart, the percentage of micronucleated protoplasts and the yield of micronuclei greatly increased, whereas the mitotic index drastically decreased. This was due to the fact that micronuclei already formed prior to enzyme incubation were stably maintained without undergoing fusion and restitution. Additionally the chromosomes in the metaphases and possibly in the early prophases from the late dividing cells that have progressed through G2 decondensed forming micronuclei, thus enhancing the percentage of micronucleated protoplasts and the yield of micronuclei. As the cell walls are digested during the enzyme incubation, changes occur in the orientation and organization of microtubules, microfilaments, intermediate filaments and cell polarity (Traas 1990; Simmonds 1991). The presence of Cremart in the enzyme mixture avoids the reformation of microtubules in micronucleated protoplasts, while CB disrupts the microfilaments. The fact that Cremart induces micronuclei at a high frequency also in other species, such as *N. plumbaginifolia* indicates the potential application of Cremart to a broad range of species. Cell synchronization, either induced through treatments with DNA synthesis inhibitors, as in the case of *N.plumbaginifolia*, or spontaneous synchrony occurring, as in the actively growing potato cell line, is highly essential for obtaining mass micronucleation after Cremart treatment. Because of its high efficiency for micronucleation, Cremart is currently used for the production and isolation of sub-diploid microprotoplasts as well as for microprotoplast-protoplast fusions, for which the protocols have been developed (Ramulu *et al.* 1994). The protocol of Cremart treatment described here has enabled partial genome transfer from potato line 413 to *L.peruvianum* through microprotoplast fusion and production of highly asymmetric hybrids containing one or two potato chromosomes and a complete genome of *L.peruvianum* (Ramulu *et al.* 1994).

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