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Summary

Procedures are now available for the exploitation of protoplast technology for the genetic manipulation of lettuce and for studies of lettuce/virus interactions. These techniques have made it possible to overcome interspecific barriers by protoplast fusion. Following the procedures we developed, we were able to obtain hybrids between *Lactuca sativa* and two wild *Lactuca* species *(L. tatarica* and *L. perennis*). Moreover, protoplast technology can overcome some of the limitations in the application of mutation techniques in crops. In this paper, we demonstrated the feasibility and the power of our *in vitro* techniques, in combination with induced mutations, for creating new variability in lettuce. Furthermore, we adapted protocols that can provide the opportunity to start studies on mechanisms associated with the different LMV-resistance genes at the cellular level. It is presently possible to obtain protoplasts from different *Lactuca* species containing different resistance genes. A protocol for specific staining by immunofluorescence of infected lettuce protoplasts is described and preliminary electroporation experiments in some *Lactuca* protoplasts were performed.

Lactuca sativa, wild *Lactuca* spp., protoplast isolation and fusion, LMV-resistance genes, immunofluorescence

Introduction

The fruits of protoplast technology can be very helpful for breeding programs. These technology may offer plant breeders the opportunity to extend the accessible genetic variation and to accumulate more efficiently resistance genes in one genotype in relation to their mechanism of action.

Although a large genetic variation might exist in germplasm, the desired character is not always available for a breeding program; the interesting genes might only be available in incompatible species. Protoplast fusion has made it possible to overcome sexual compatibility barriers and then to transfer traits between distant species (20).

When existing germplasm fails to provide the desired trait, it is necessary to resort to other sources of variation. Techniques to induce mutations provide tools to increase variability of crops (13). There are some important limitations in the applications of these techniques that can be greatly facilitated by the development of protoplast techniques. Among the limitations, the most important is the unrealistically large but necessary size of the population to be mutated. The size of this population is calculated on the basis of an expected frequency of mutation for a desired characteristic and according

to the size of the crop genome. Protoplast techniques allow growth of millions of cells in Petri-dishes. This potentially leads to the production of millions of plants. Moreover, if an *in vitro* selection system for the desired character is available, the mutated population can be rapidly screened in a small volume.

Another question for the breeder is to decide the best strategy for use of the different disease resistance genes available against one pathogen. There are many types of virus resistance mechanisms in plants and a better knowledge of those could help to build durable resistance. The ability to isolate plant protoplasts and infect them with virus has been fundamental in the knowledge of plant-virus interactions. This technique was particularly used in studies of virus replication and in studies of the mode of action of resistance genes (3, 14, 15, 17, 18, 21).

Protoplast culture and regeneration has been achieved for several crops since many years. But use of protoplast techniques for interspecific crosses and mutagenesis requires efficient and reproducible control of the various steps from protoplast isolation to regenerated plants. This level of efficiency has been effective in lettuce only after modifications of the standard procedure (6) initially developed for various *Nicotiana* spp.

Disease resistance is one of the most important goals in lettuce breeding. Useful genes have been identified in wild *Lactuca* species. But only few species, including *L. serriola*, *L. saligna* and *L. virosa*, are sexually compatible with lettuce (8,10, 19, 22). In order to extend the available gene pool by protoplast fusion, a *Lactuca* universal hybridizer was produced (7) and used for hybridization with *L. tatarica* (12).

Furthermore, the ability of our protoplast technique to be used in mutation strategy in order to create new variability was tested for UV-induced mutations. In this preliminary experiment, the selected characteristic was resistance to chlorsulfuron exclusively because it is easy to screen *in vitro* and it constitutes a good model. The technique must be applied in the future for agronomicaly useful traits which are not hazardous for environment as herbicide resistance.

Moreover, in preliminary experiments, protocols for evaluation of virus spread in infected plants were adapted to *Lactuca*. Two complementary approaches were developed: 1) Observation, from virus-inoculated leaves, of the number of contaminated protoplasts at various times after infection, and 2) Observation of the virus replication after *in vitro* infection of protoplasts. These techniques are now available for fundamental studies of the interaction between LMV and different resistance genes in lettuce. The percentage of infected cells in different leaves of the plant and the multiplication of the virus in *in vitro* inoculated protoplats can provide more information about the nature of resistance observed at the whole plant level, e.g. whether resistance is the consequence of the inhibition of viral movement or the inhibition of viral replication (17).

Increasing of available germplasm in lettuce by protoplast fusion

To facilitate selection of somatic hybrids, a universal hybridizer, carrying both a dominant marker, kanamycin resistance (Kan^R), and a recessive one, albinos (a), was developed (7). Protoplasts were prepared from *in vitro* grown leaflets, one to two months

after sowing on B medium (5) with 100mg/l kanamycin. For good preparation of protoplasts from albino lettuce, we adapted, for lettuce seedlings, the medium reported earlier for isolating protoplasts from albino tobacco (2): medium B complemented with 0.01 mg/l NAA, 0.01 mg/l BA, 100 mg/l adenosine and 7% sucrose (7). Hybrid plants between this universal hybridizer and two wild *Lactuca* species *(L. tatarica* and *L. perennis*) were regenerated. Modification of environmental conditions for the cultivation of these plants (25 °C, 16 h per day) allowed growth and bolting of some of the somatic hybrids in a growth chamber.

A fusion with protoplasts from one plant of *L. perennis* produced 51 regenerated plants derived from 13 different fusions (cluster of buds). Blue flowers were observed on only 8 plants regenerated from 6 different fusions. A few pollinations by *L. sativa* were attempted but were not successful.

After several generations of sterile plants derived from hybrids between *L. sativa* and *L. tatarica* (Table 1), few fertile plants were obtained. These data show an increase in the percentage of plants with flowering and in the sexual compatibility with *L. sativa* of these plants after BC_2 generation (11, 42 and 107 harvested seeds for 100 pollinated capitula on BC_2, BC_3 and BC_4 plants, respectively). The fertility of these plants is very sensitive to environmental conditions. Indeed, no plant, including 91 BC_2 plants and 22 BC_3 plants, was fertile for two years, and then in the next year some were fertile. This apparition of fertility was unexpected, specially with this very good production of F_2 seeds (between 3 to 28 g of seeds per plant). The restoration of fertility was complete with a normal seed production in crosses with several lettuce cultivars used as female as well as male parents.

Table 1. Progeny of somatic hybrids between *L. sativa* and *L. tatarica* (PIVT1163)

Generation of interspecific plants	F_1	BC_1	BC_2	BC_3	BC_4
No. of studied plants	47	33	102	73	80
No. of flowering plants					
Sterile plants	15	21	56	66	67
Fertile plants	0	0	1	4	11
Pollination of sterile plants					
No. of pollinated capitula	439	1301	2036	3498	3339
No. of harvested BC seeds	80	138	221	1484	3560

These results suggested the possibility of transfer of a character from PIVT1163 to fertile *L. sativa*-like plants. But the likelihood of production of interesting plants for a desired character in the present material is very low because all plants of the BC population were derived from one unique BC_1 plant. A study of recombination between the chromosomes of both species would be very useful to evaluate if it will be easy to transfer a resistance gene from the wild species without undesirable characteristics. In different BC plants, segregation of different traits was observed and then the transfer of resistance gene can be expected. The F_2 populations have to be tested for resistance to *Bremia lactucae*.

Increasing of available germplasm in lettuce by protoplast mutagenesis

In a preliminary experiment, 2 x 10^6 lettuce protoplasts were exposed to ultraviolet irradiation according to Bourgin (4). After a screen on liquid medium with 200 nM chlorsulfuron, four green buds were regenerated on a selective medium with 50 nM chlorsulfuron. Eleven M_0 plants derived from these four mutagenesis events were grown in insect-proof greenhouses. The majority were weak and sterile or with very low fertility; only 3 M_1 seeds were harvested. After cross-pollination of M_0 plants with lettuce cultivars, few F_1 seeds were harvested on 15 M_0 plants derived from three different mutated buds. A test for resistance to chlorsulfuron of F_1 or F_2 derived seedlings from these hybrids showed a good efficiency of the screening. A Mendelian inheritance of resistance was observed in the analysed F_3 progenies derived from one mutant with some resistant (e.g. 25 resistant / 0 susceptible seedlings) and some segregating (e.g. 20 resistant / 7 susceptible) progenies.

These data suggested that the efficiency of our protoplast technique was enough to create, via *in vitro* mutation, new variability for desired character if an *in vitro* screening is available for this trait.

Development of protoplast techniques for plant-pathogen interaction studies

Plant material and isolation of protoplasts

We have developed a procedure for isolation of leaf protoplasts of lettuce. As for lettuce protoplast fusion, the donor plants were cultivated in a growth chamber under conditions that avoid water stress and large environmental fluctuations (75 % humidity, 18/ 21 °C night/day temperatures, 8 h per day, low light intensity of 75 $mEm^{-2}s^{-1}$). Protoplasts were prepared from young but fully developed leaves. Leaves were gently chopped and then incubated for 14 to 20 hours at 25 °C in a maceration medium (final concentration 0.02% Macerozyme R10, 0.1% Onozuka R10, 0.5 % Driselase) as described by Chupeau et al. (6).

This procedure was effective for a collection of different cultivars, susceptible (butterhead: Trocadéro or Girelle; iceberg: Salinas or Vanguard) or resistant to LMV (butterhead: Mantilia; iceberg: Salinas88 or Vanguard75, Malika) and for two *L. virosa* accessions. The most difficult genotype is *L. virosa* (yield of 2 x 10^5 protoplasts per 0.5 g of leaf tissue). Moreover, we were able to obtain protoplasts (isolated from inoculated leaves, 3 to 15 days after inoculation, and from upper leaves) from infected susceptible genotypes. These protoplasts were used as positive controls in immunofluorescence staining studies.

Immunofluorescence staining of infected protoplasts

After mechanical inoculation of plants according to a previous work (11), LMV infection of protoplasts was revealed by fluorescence antibody staining using anti-LMV polyclonal rabbit anti-serum and sheep anti-rabbit IgG (H+L) FITC conjugated antibody (ICN Biomedicals, Inc.).

Two techniques were compared, a procedure used for Solanaceae protoplast studies (1, 16) and another protocol. In the Solanaceae procedure, the different incubations with reagents were conduced in liquid state using phosphate-buffered saline (PBS)

solution; in the other protocol, incubation with antibodies was conduced after fixation of protoplasts on microscope slides. The second procedure was the best; it will be described in some details.

A drop of the protoplast suspension was added to teflon-coated microscope slides (HTC super cured green slides, 10 wells, 7 mm, Polylabo) and allowed to air dry. The protoplasts were fixed by dipping the slide into 95% ethanol for 15 min at room temperature and washed overnight in PBS containing 1% BSA. One drop of a polyclonal antibody for LMV was added, and the protoplasts were incubated for 2 h at room temperature in a moist chamber. To remove the excess antibody, the protoplasts were first washed in PBS and secondly in PBS containing 1% BSA in a bath for at least 30 min on a slow moving orbital agitator. One drop of fluorescein isothiocyanate-conjugated antibody was added, and the protoplasts were incubated in a moist chamber for an additional 2 h at room temperature. To remove the excess antibody, the protoplasts were washed in PBS for at least 30 min at room temperature on an orbital agitator. The protoplasts were mounted in one drop of glycerol/PBS (1/1). Specific immunofluorescence was detected by microscopy under UV light.

In preliminary experiments, by this technique applied on inoculated susceptible genotypes, fluorescence was observed in nearly 100% of protoplasts from systemic infected leaves and about 10 to 50% of protoplasts from mechanically inoculated leaves (the percentage depended on the sample and the time after inoculation). In contrast, there was no coloration in protoplasts from uninoculated plants.

In vitro inoculation of protoplasts

Systems are now been developed to infect protoplasts from two susceptible genotypes, butterhead lettuce cv. Trocadero and *L. virosa* accession PIVT280. The procedure described by Chupeau et al. (6) was efficient for electroporation of cv. Trocadero protoplasts with chimeric DNA containing a GUS-reporter gene. On the contrary, the result of this procedure of electroporation was very poor for *L. virosa* protoplasts.

In order to improve the electroporation efficiency for the *L. virosa* genotype, different voltage conditions are beeing tested with chimeric GUS-reporter gene, before infection of these protoplasts with viral RNA or infectious clones of LMV cDNA.

Conclusions

The development of protoplast techniques for several genotypes containing different LMV resistance genes (*Mo3*, *mo1*^{1} and *mo1*^{2}) (9) will provide the opportunity to study resistance mechanisms at the cellular level. The protoplast techniques should complete the knowledge of these genes obtained by DAS-ELISA studies of virus multiplication in whole plants (9, 11). Indeed these protoplast studies should give more information concerning the first steps of virus infection (multiplication in inoculated cells, migration from cell to cell) in plants with immunity gene (*Mo3*) and in plant with tolerance genes (*mo1*^{1} and *mo1*^{2}).

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