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Parasexual Interspecific Plant Hybridization

(*Nicotiana*/leaf mesophyll/plant tissue culture/genetics/selective media)

PETER S. CARLSON, HAROLD H. SMITH, AND ROSEMARIE D. DEARING

Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

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ABSTRACT **Interspecific plant hybrids have been produced by parasexual procedures. Protoplasts of *Nicotiana glauca* and *N. langsdorffii* were isolated, fused, and induced to regenerate into plants. The somatic hybrids were recovered from a mixed population of parental and fused protoplasts by a selective screening method that relies on differential growth of the hybrid on defined culture media. The biochemical and morphological characteristics of the somatically produced hybrid were identical to those of the sexually produced amphiploid.**

Recent advances in plant cell culture have demonstrated that protoplasts isolated from leaf mesophyll cells can be induced to regenerate into entire plants (1, 2), and that they can be stimulated to fuse by defined experimental manipulations (3). A combination of these two techniques should permit the fusion of protoplasts isolated from two different species and the regeneration of a somatically produced hybrid plant without having to involve a normal sexual cycle. This paper reports the successful parasexual production of a hybrid between two different species of *Nicotiana*, *N. glauca* Grah. and *N. langsdorffii* Weinm. The amphiploid hybrid between these two species has been produced by sexual means, and the characteristics of the hybrid plant have been thoroughly studied (4–7). Known biological differences between the hybrid and its parental species have been used in a selective screen to recover preferentially regenerated fused hybrid protoplasts from a mixed population of protoplasts. We have also used the distincitive characteristics of the hybrid tissue to verify that parasexual hybridization was achieved.

MATERIALS AND METHODS

The species used were *Nicotiana glauca* ($2n = 24$), *N. langsdorffii* ($2n = 18$), and the amphiploid ($2n = 42$) of the tumorous hybrid of these species.

Protoplasts were isolated from leaf mesophyll cells by stripping the lower epidermis from sterilized, young, expanding leaves. Stripped leaf pieces were placed in an enzyme solution consisting of 4% cellulase (Onozuka SS, All Japan Biochemicals Co. Ltd.), 0.4% macreozyme (All Japan Biochemicals Co. Ltd.), and 0.6 M of sucrose at pH 5.7. Flasks containing the leaf pieces in the enzyme solution were evacuated briefly, then returned to standard atmospheric pressure to facilitate penetration of the enzyme solution into the intercellular spaces. These flasks were incubated for 4–6 hr at 37°, after which the protoplasts were harvested by low-speed centrifugation ($<100 \times g$).

Experimental conditions and regeneration medium used for protoplast culture were exactly those described by Nagata and Takebe (2). Protoplast density was always greater than 5×10^3 protoplasts per ml. In the Nagata and Takebe medium, proroplasts of *N. glauca* and *N. langsdorffii* will regenerate a cell wall and occasionally go through one division cycle. Protoplasts of these two species were never observed to regenerate into a callus. Protoplasts of the amphiploid hybrid react similarly; however, about 0.01% of the protoplasts will continue to divide and give rise to a callus mass of cells. The different growth characteristics of protoplasts from the two parental species and from the amphiploid hybrid on Nagata and Takebe medium constitutes a selection method with which to recover preferentially hybrid individuals from a mixed population of protoplasts. Since protoplasts from both parental species are unable to regenerate on the Nagata and Takebe medium, the only protoplasts capable of forming viable colonies will be those with a hybrid genetic constitution.

Mixed populations of protoplasts of *N. glauca* and *N. langsdorffii* in an approximate 1:1 ratio were stimulated to fuse by their suspension in 0.25 M of $NaNO_3$ for 30 min, and then pelleted by low-speed centrifugation. This pellet was then resuspended in the regeneration medium and plated in petri dishes. After the fusion procedure, the population consisted of protoplasts of both parental types and fused clumps of protoplasts involving various numbers of cells. About 25% of the protoplasts were involved in a fusion event. On the regeneration medium, only the cells containing the genetic information of both parental species were able to regenerate into calli. Regenerated calli were removed from the regeneration medium and placed on the medium of Linsmaier and Skoog (8), which was solidified with agar and contained no hormones. This constitutes a further selective step, since tissue from neither parental species is able to grow on a medium without added hormones, while the amphiploid hybrid grows vigorously without exogenous hormones present (6).

Recovered calli formed rudimentary shoots and leaves in culture, but failed to form roots. In order to obtain further differentiation of presumed hybrid tissue, the regenerated shoots were grafted onto the freshly cut stem surface of young plants of *N. glauca*. The grafts were wrapped with parafilm and were kept under high moisture conditions in the mist propagation section of a greenhouse until the graft had taken and a few leaves had developed.

The chromosomes were prepared from young leaves by the method of Burns (9). Electrophoresis and staining for peroxidase isozymes was as described by Smith *et al.* (10, 11).

RESULTS

More than 10^7 protoplasts of *N. glauca* and 10^7 protoplasts of *N. langsdorffii* were taken through the fusion procedure and plated on a regeneration medium that permits the growth of only cells containing the genetic information of both parental species. 33 Regenerated calli were recovered after 6 weeks, and placed on a medium containing no added hor-

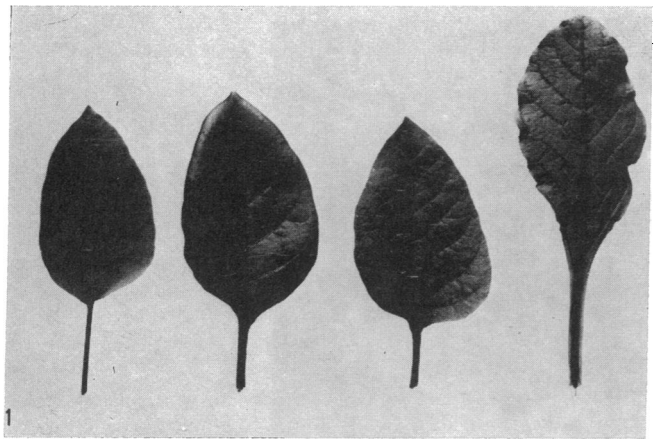


FIG. 1. Leaves typical of (*left* to *right*): *N. glauca*, amphiploid *N. glauca* × *N. langsdorffii*, somatic hybrid *N. glauca–N. langsdorffii*, and *N. langsdorffii*.

mones. All 33 isolates grew vigorously with no exogenous hormone source. This observation provided circumstantial evidence that the recovered calli had a hybrid genetic composition. Several of the recovered calli that were presumed parasexual hybrids were chosen for further tests to confirm their hybrid genetic composition.

The following analysis was completed with three isolates. Photographs and figures are presented for only one, although the described characteristics were common to all three.

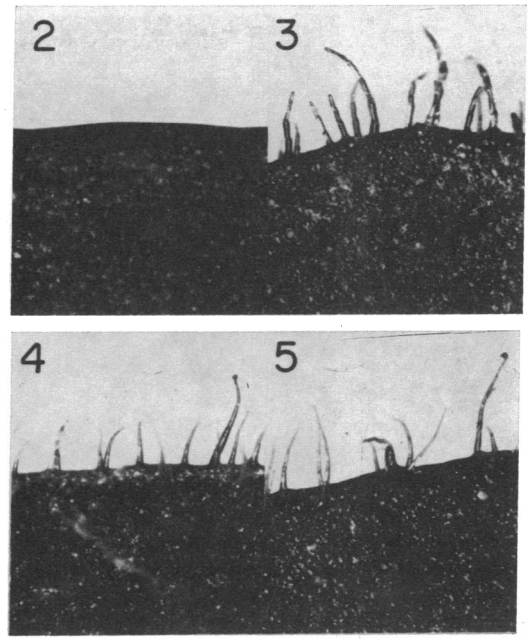
Morphology of the Tissue in Culture. The characteristic tissue morphology and growth requirements of the somatically produced hybrid are identical to those of the sexually produced hybrid. Tissues from both sources grow vigorously in culture in the absence of exogenous hormones. On an agar medium containing no hormones, both tissues form a semifriable callus. In a liquid medium containing no hormones, both tissues will regenerate shoots and leaves. Tissue from either parental species is not capable of growth and differentiation in medium lacking added hormones.

Morphology of the Leaf. The morphology of leaves regenerated on the somatically produced hybrid is identical to that of the sexually produced hybrid, and distinct from either parental type (Fig. 1). The leaves of *N. langsdorffii* are sessile, those of *N. glauca* are petiolate, and the hybrid has a leaf of intermediate morphology (12).

Trichome Characteristics. The leaves of *N. langsdorffii* are densely covered with trichomes, while leaves of *N. glauca* are glabrous without trichomes (12). On leaves of both the somatically and sexually produced hybrid trichomes are present, but in a much lower density (Figs. 2–5).

Tumor Formation. The somatically produced hybrid spontaneously forms tumorous outgrowths on the stem (Fig. 6). Spontaneous tumor formation is a genetically determined trait that is characteristic of the F_1 hybrid and amphiploid, but is not found in either parent species, and is not transmitted across a graft union (7).

Chromosome Numbers. A somatic chromosome number of 42 (Figs. 7 and 8) was determined for the somatically produced hybrid. This is a summation of the diploid somatic numbers of



FIGS. 2–5. Glabrous leaf surface of *N. glauca* (2), dense trichomes on *N. langsdorffii* leaf (3), intermediate trichrome condition on leaves of amphiploid (4), and somatic hybrid (5).

the parental species (24 + 18), and is distinct from a whole ploidy change in either parental type. The sexually produced amphiploid has been shown to contain a chromosome number of 42 (12). Although the somatically produced hybrids all

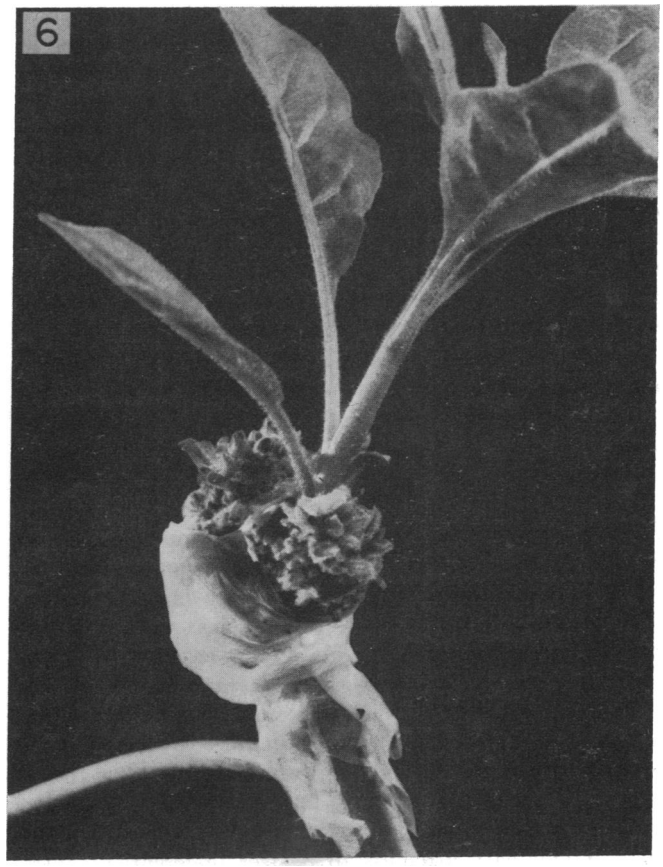
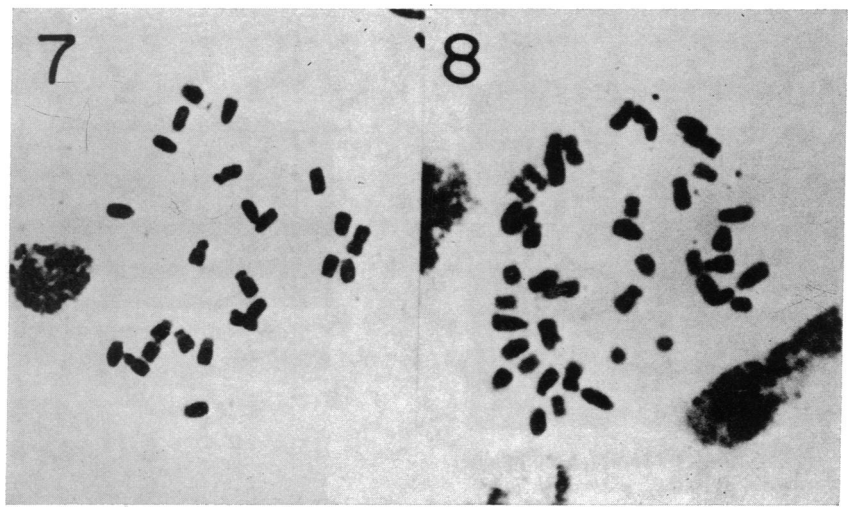


FIG. 6. Tumor formation on scion of somatic hybrid, *N. glauca–N. lagsdorffii*, grafted onto stock of *N. glauca*.



FIGS. 7–8. Metaphase chromosomes in cell of young leaf of *N. glauca* $2n = 24$ (Fig. 7), and of somatic hybrid (Fig. 8) with additional 18 *N. langsdorffii* chromosomes, giving a total of 42 chromosomes.

demonstrated a chromosome number of 42, deviations from simple addition of the parental chromosome numbers might be expected to occur due to the complexity of the fusion event and divisions after fusion.

Peroxidase Isozymes. The leaf peroxidase isozymes in the somatically produced hybrid are identical to those of the amphiploid. The isozyme bands of the hybrid are a summation of those found in the parental species.

The characteristics of the somatic hybrid are not due to a chimerial association of cells. Single cells derived from calli of the somatically produced hybrid were regenerated into calli, and their characteristics were analyzed. In every case, the regenerated callus displayed characteristics of hybrid tissue, and was distinct from either parantal type. Hence, the characteristics of the somatically produced hybrid are not due to a chimerial association of cells of the parental species. All cells of the somatically produced hybrid contained only one nucleus. The possibility that the somatically produced hybrid is due to contamination by sexually produced amphiploid cells is ruled out by the experimental procedure used.

Summation of this evidence leaves no doubt that the calli and plants recovered from fused cells are of a hybrid genetic constitution corresponding to the sexually produced amphiploid.

DISCUSSION

Each of the individual steps in the procedure of protoplast isolation, fusion, and regeneration has, as noted above, already been performed. The successful recovery and analysis of a parasexually produced hybrid, as reported here, has depended primarily on the availability of a selective technique to permit preferential recovery of fused hybrid cells, and recognition of known distinctive characteristics of the hybrid amphiploid. Further attempts to produce a somatic interspecific hybrid and hybrids between more distantly related species in our laboratory have been hampered by a lack of familiarity with the kind of characteristics the tissue will display. Preliminary attempts to recover preferentially intra- and interspecific and intergeneric hybrids with the available auxotrophic mutants of *N. tabacum* (13) have been inconclusive. Many of the auxotrophic protoplasts will grow at a reduced rate in minimal medium when mixed with other auxotrophic mutants or with wild-type protoplasts of other species, presumably due to the effect of crossfeeding between the cell types. We are investigating one further method for preferentially recovering parasexually produced hybrids. Protoplasts containing potentially complementing recessive nuclear albino mutations from different species are isolated and fused. Only calli that have regenerated from fused cells should appear as green colonies on the petri plate.

In general, the potential offered by somatic hybridization may be expected to exceed the limitations imposed by sexual processes, and extend the possibilities of combining widely divergent genotypes of plants.

Note added in proof

The somatic hybird has produced flowers and fertile seed capsules that are identical with the *N. glauca* × *N. langsdorffii* amphiploid.

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