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The Use of Amino Acid Analog Resistance and Plant Regeneration Ability to Select Somatic Hybrids Between *Nicotiana tabacum* and *N. glutinosa*

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Summary. Protoplasts isolated from a 5-methyltryptophan resistant nonregenerable *Nicotiana tabacum* (L.) Xanthi suspension culture were fused via the dextran method (Kameya et al. 1981) with protoplasts isolated from *N. glutinosa* L. leaf mesophyll cells. Prospective somatic hybrids were selected by their ability to produce chlorophyll and to grow on medium containing 5-methyltryptophan and eventually to regenerate into complete plants. Twenty-eight plants regenerated from selected colonies were classified into three groups: (1) Four plants had intermediate morphology, were sterile, had hybrid isozyme patterns for four different enzymes, and contained fraction 1 protein large subunit patterns indicative of a *N. glutinosa* chloroplast origin. (2) One plant had *N. tabacum* morphology, was fertile, had two hybrid isozyme patterns, and contained fraction 1 protein large subunits indicative of the *N. tabacum* chloroplast type. Although this hybrid contained both parental small subunit patterns of fraction 1 protein, two-dimensional gel electrophoresis showed the *N. glutinosa*-type small subunit to consist of several molecular weights suggestive of errors in the processing steps. Progeny of the Type 2 hybrid (selfed) contained no *N. glutinosa* characteristics. (3) Twenty-three plants were *N. glutinosa* escapes. Chromosome numbers ranged from 60 (some chromosome loss) in two hybrids to 34–35 (extensive chromosome loss) in three hybrids.

These results suggest that amino acid analog resistance and regeneration capability can be utilized as complementing markers for selecting and identifying somatic hybrids after protoplast fusion. Mesophyll protoplasts from *N. glutinosa* can also be regenerated into morphologically normal fertile plants.

Introduction

In recent years there have been many reports of somatic hybrid formation via fusion of plant protoplasts (Thomas et al. 1979) using many different methods to select the hybrids (Widholm 1982). Recently, we published a report in which each of the parental lines had a biochemical marker which allowed easy identification of fusion hybrids when plated on a suitable medium (Kameya et al. 1981). Specifically, amino acid analog-resistance has been shown to be useful as biochemical markers (White and Vasil 1979; Harms et al. 1981, 1982; Kameya et al. 1981). In this report we extend this selection system to two *Nicotiana* species, *N. tabacum* and *N. glutinosa* and describe two types of somatic hybrids which were produced as a result of protoplast fusion.

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Abbreviations. 5MT, DL-5-methyltryptophan; GOT, glutamate oxaloacetate transaminase; LAP, leucine aminopeptidase; PER, peroxidase; RuBPCase, ribulose bisphosphate carboxylase; PVP, polyvinylpyrolidone; IEF, isoelectric focusing; PVPP, polyvinylpolypyrolidone

Materials and Methods

Protoplast Isolation, Fusion, Selection and Culture. Protoplasts were prepared from 5MT-resistant *N. tabacum* L. (var. Xanthi) suspension cultures (TX3; Widholm 1972) and *N. glutinosa* greenhouse grown leaf mesophyll as described previously for carrot (Kameya et al. 1981; Kameya 1982). The TX3 suspension cultures were 5MT-resistant and would not regenerate plants or turn green (Fig. 1). Cultures derived from *N. glutinosa* leaf mesophyll protoplasts were 5MT-sensitive, remained green and would regenerate plants. Approximately 5×10^4 protoplasts were isolated from each parent and were fused as described previously (Kameya et al. 1981). After fusion the protoplasts were incubated for 10 days in nonselective medium (MS medium [Murashige and Skoog 1962] with 5% glucose) and then diluted with an equal volume of fresh medium containing 5MT for a final concentration of 229 μM. After 51 days any colonies that were green and grew well on this medium were regenerated as described below (Fig. 1).

Culture Initiation and Plant Regeneration. N. glutinosa and somatic hybrid cultures were obtained by placing leaf pieces on MS agar medium (with 0.4 $mg \cdot l^{-1}$ 2,4-dichlorophenoxyacetic acid) after surface sterilization and transferring the resulting callus to the same liquid MS medium. Plant regeneration was induced by transferring callus or suspension cultured cells onto MS agar medium containing 1 $mg \cdot l^{-1}$ IAA and 0.64 $mg \cdot l^{-1}$ kinetin in the light, and the resultant shoots were then transferred to MS medium containing no hormones to induce root formation.

Chromosome Counting. Ten ml aliquots of two day old suspension cultures growing on a 4-day subculturing regime were placed in 10 cm plastic petri dishes to which 5 mg

colchicine was added. After shaking on a gyratory shaker at 40 rpm for 2 h the cells were harvested on Miracloth and washed continuously for 5 min with water. The cells were scraped into a centrifuge tube filled with 5 N HCl, incubated for 15 min, spun for 5 min at 200 × *g*, rinsed and spun again. The cells were stained with 5% modified carbol fuchsin, squashed and the chromosomes observed (Kao 1975).

Electrophoresis and Enzyme Analysis. Isozyme analysis was performed as described previously (Kameya et al. 1981). The cell cultures were in constant logarithmic growth via a 4 day-subculturing regimen. Isozymes were examined on day 4. The enzyme staining methods are as follows: GOT (Brewbaker et al. 1968 as modified by Wetter 1977), LAP (Brewbaker et al. 1968), PER (Brewbaker et al. 1968). RuBPCase was isolated by grinding leaf tissue using a mortar and pestle in 50 mM TRIS, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 3.33 ml concentrated HCl, 2% PVPP and 5 mM DTT (pH 7.5 at room temperature) in a ratio of 2 g to 4 ml grinding medium. After centrifugation, 1 ml of supernatant was layered atop a 16 ml 10 to 30% linear sucrose gradient dissolved in the grinding buffer minus the PVPP. The gradients were centrifuged for 24–26 h at 27,000 rpm using a Sorvall AH627 rotor and then fractionated while monitoring protein at 260 nm. The RuBPCase peak was collected and subjected to isoelectric focusing without prior S-carboxymethylation according to the method of O'Farrell (1975). The gels were removed and stained for 1 h in a 0.2% bromophenol blue solution (30:3:27 ethanol:acetate: H_2O) and then destained in a 6:1:13 ethanol:acetate:H_2O solution until clearing progressed and then destained further with 10% acetate. The gels were then stained for 2 h in a 0.25 $g \cdot l^{-1}$ Coomassie Blue R-250 solution (25:10:13 2-propanol:acetate:H_2O) and destained in 10% acetate. The gels were scanned at 600 nm by a Beckman 34 spectrophotometer equipped with a scanning attachment. The second dimension of 2-D gels were run according to Chua (1980) but stained according to O'Farrell (1975). Anthranilate synthase from suspension cultures was assayed as described previously (Widholm 1973, as modified in Kameya et al. 1981).

Results

Using the selection scheme in Fig. 1, 14 green colonies were recovered along with about 1500 white colonies from 10^5 protoplasts. The latter were most likely TX3 colonies which were able to grow on 5MT medium, but which would not turn green. Eleven of the 14 green colonies were transferred to regeneration medium and 28 plants formed which could be potted in soil. These 28 plants could be classified into three types:

Type 1: Four plants had intermediate leaf morphology, flower color and size (Fig. 2), inflorescence arrangement (not shown) and were self-sterile. These plants were designated T3g 1C, 1K, 1U, and 1V. After flowering, the plants quickly senesced and died in a fashion similar to that observed in sexual hybrids between *N. glutinosa* (♀) and *N. tabacum* (♂). Three of these plants were from one selected colony and the other from a different colony.

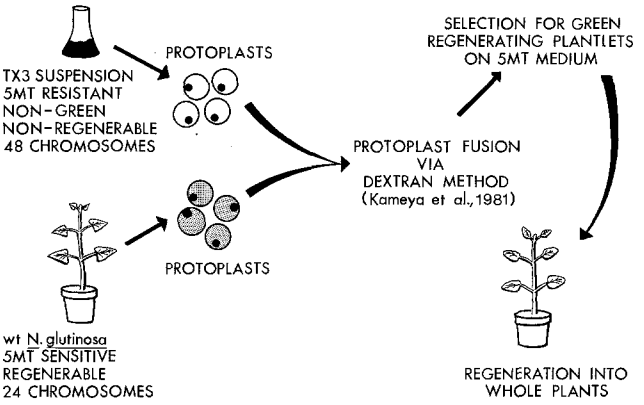

Fig. 1. Selection scheme for T3g hybrids. Protoplasts were isolated and fused by the method described in Kameya et al. 1981

Type 2: One plant had *N. tabacum* morphology, was fertile and also senesced soon after flowering and setting seed. This plant was designated T3g 1CC.

Type 3: 23 plants had *N. glutinosa* morphology, were fertile and did not senesce drastically after flowering.

Type 1 Plants

Type 1 plants were rescued by initiating callus cultures from leaves prior to flowering. The plants regenerated from these calli appeared to be identical to those from which the calli had been obtained. Suspension cultures derived from the calli were used for isozyme analysis and chromosome counts; leaf tissue from the regenerated plants was used as the RuBPCase source.

The GOT isozyme pattern from the four *Type 1* lines showed not only the three activity bands expected for a hybrid, if additive, but also an extra band positioned between the slow bands of the parents (Fig. 3). This would suggest in vivo mixing of subunits to form heterodimers. The intermediate band is not seen when the two parental extracts are mixed and run on polyacrylamide gels (Fig. 3). Intermediate hybrid GOT isozymes have been described previously in somatic fusion products (Evans et al. 1980; Douglas et al. 1981).

PER isozyme patterns also provided evidence of hybridity for the 4 *Type 1* lines (Fig. 4). The TX3 extract showed no anodal bands but a fast and an intermediate cathodal band. *N. glutinosa* exhibited one slow and one fast anodal band and one fast cathodal band different from the fast TX3 cathodal band. All four *Type 1* hybrids had both *N. glutinosa* anodal bands and either two or three cathodal bands (Fig. 4). LAP isozyme analysis also showed the four *Type 1* lines to be hybrids (data not shown).

RuBPCase patterns on IEF gels showed that the four *Type 1* hybrids had *N. glutinosa* chloroplasts since only *N. glutinosa* large subunits were seen (Fig. 5). The small subunit bands of both parents were observed in the hybrids but in reduced amounts compared to the parents. Total small subunit peak area is equivalent, however, to either parent's total small subunit peak area.

Two of the *Type 1* hybrids had chromosome numbers of near 60 while the other two had 30 and 34, respectively (Table 1). The TX3 parent had 48 chromosomes while the *N. glutinosa* parent plant had 24. However, in culture *N. glutinosa* was observed to have only 20 chromosomes.

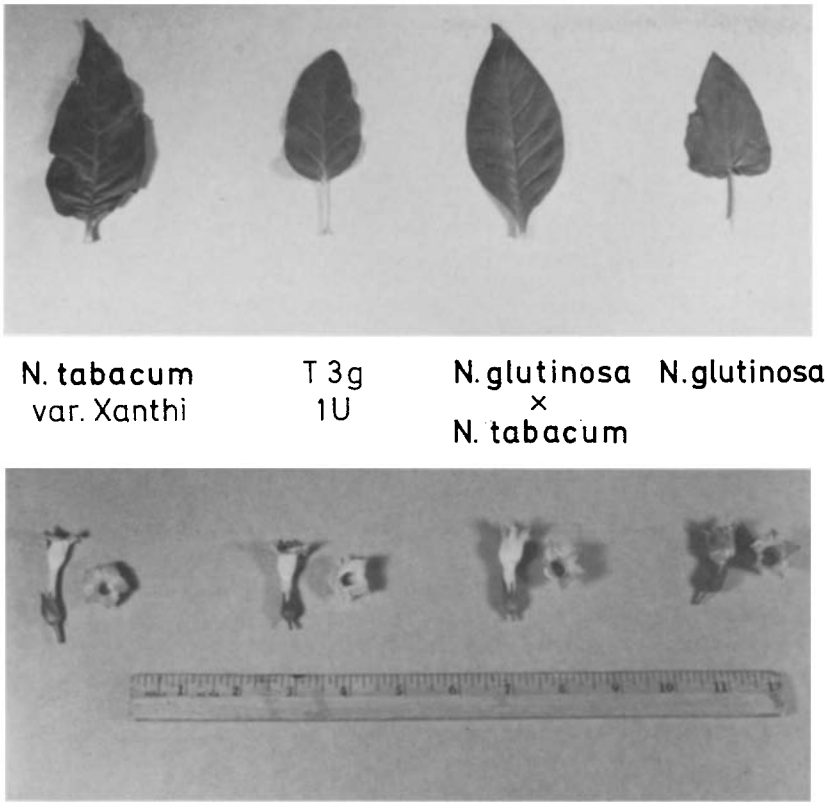

Fig. 2. Plant part morphology of T3g hybrids, parent lines and sexual hybrid; Top Leaves. Bottom Flowers

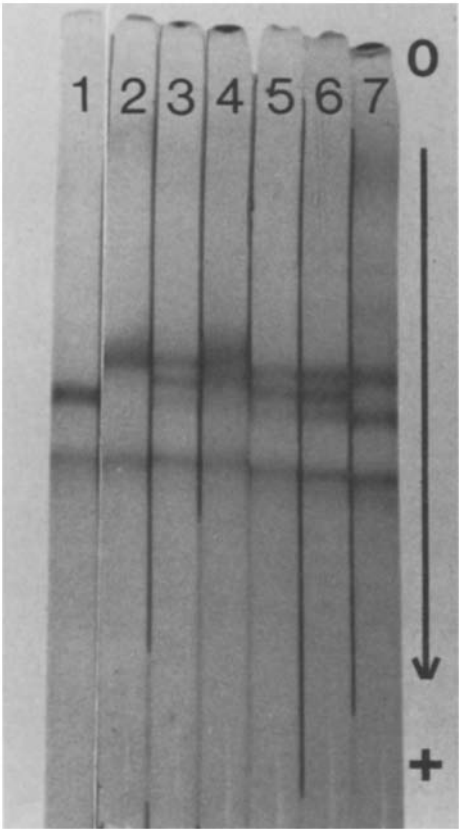

Fig. 3. GOT isozyme patterns from extracts of suspension cultures of T3g hybrids and parental lines. *1 N. glutinosa*, *2* TX3, *3* T3g1C, *4* T3g1K, *5* T3g1U, *6* T3g1V, *7* Mixture of *N. glutinosa* and TX3. Gels were 7.5% polyacrylamide with a 5% polyacrylamide stacking gel

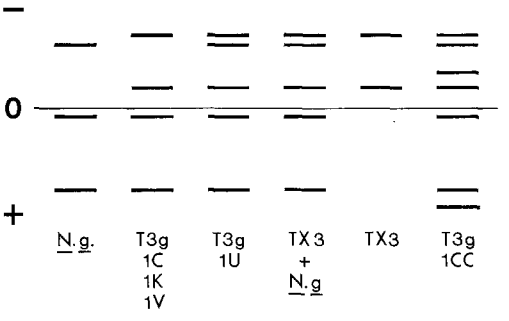

Fig. 4. PER isozyme patterns from extracts of suspension cultures of T3g hybrids and parental lines following starch gel electrophoresis

Thus, the additive chromosome number for the parents would be 68 to 72. The sexual hybrid (*N. glutinosa* × *N. tabacum*) had 36 chromosomes as a plant and 35 in culture (Table 1).

Type 2 Hybrid

The *Type 2* hybrid (T3g 1CC) in contrast to the *Type 1* hybrids, showed GOT, and LAP patterns identical to TX3 (data not shown). PER isozymes analysis gave a hybrid pattern plus two new bands (Fig. 4). The RuBPCase large subunit bands were identical to those of *N. tabacum* (TX3) as shown in Fig. 5. The small subunit bands of *N. tabacum* were also present but in the region where *N. glutinosa* small subunit band should have been there was only one broad band. Assuming this is *N. glutinosa* small subunit protein, total small subunit peak area is roughly 70% of the parental total small subunit peak area. Although our RuBPCase protein preparations are not pure, no contamination has ever been seen in this area of a *N. tabacum* IEF gel.

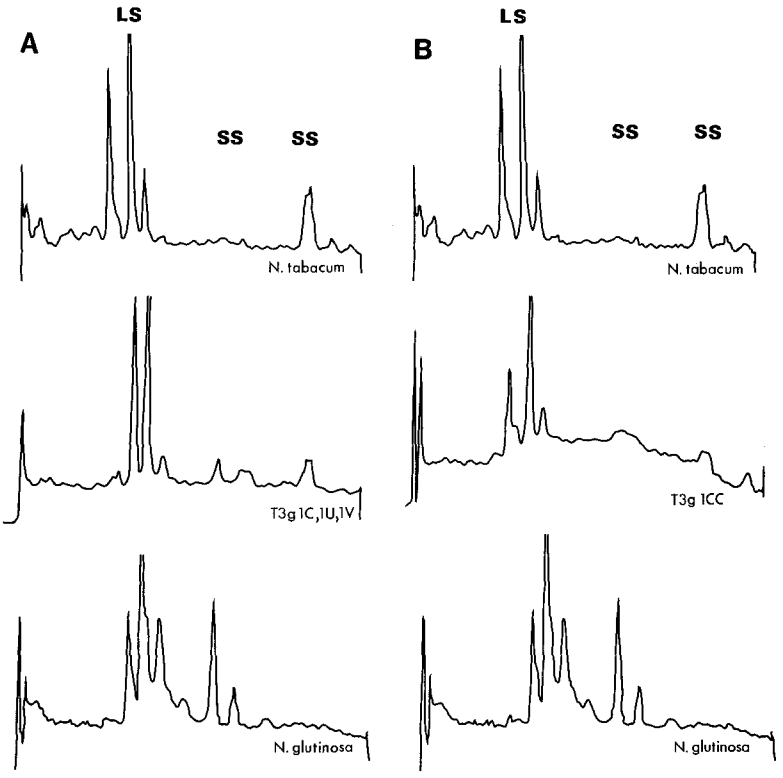

Fig. 5 A, B. Spectrophotometric scans of RuBPCase on IEF gels.
A Comparison of parental lines with T3g1U (representative of *Type 1* hybrids).
B Comparison of parental lines with T3g1CC (Type 2). RuBPCase small subunits (SS) and large subunits (LS) are as indicated

Table 1. Chromosome numbers in suspension cultured cells

Cell line	Number of counts	$\bar{X}$	s.d.
TX3	25	47.45	7.87
N. glutinosa	25	20.36	1.85
T3g 1C	25	59.70	1.48
T3g 1K (Type 1)	25	34.10	1.30
T3g 1U	18	30.33	3.68
T3g 1V	25	60.50	1.89
T3g 1CC (Type 2)	12	34.17	2.82
*N. glutinosa*1*N. tabacum*	25	34.85	1.62

s.d. = standard deviation

To further analyze the presumptive *N. glutinosa* small subunit band, two dimensional gel electrophoresis was performed using a linear polyacrylamide gradient (7.5 to 15%) containing SDS for the second dimension (Fig. 6). This resolved the band in question into several spots of various molecular weights including three which lie close to or at 12–13 kilodaltons, the correct small subunit molecular weight.

Seeds collected from this fertile *Type 2* hybrid plant were germinated and the RuBPCase of 43 progeny plants was examined by IEF gels. All showed large and small subunits of only *N. tabacum* with normal quantities of the latter (data not shown). A random sampling of eight of these plants showed PER to also be of *N. tabacum* type.

The chromosome number of this hybrid in culture was 34, similar to two of the four *Type 1* hybrids.

Type 3 Plants

The 24 remaining plants showed GOT and LAP isozymes patterns, as well as plant morphology, to be identical to those of *N. glutinosa*. These plants were concluded to be *N. glutinosa* escapes and were not examined further. The reason for the occurrence of the *N. glutinosa* escapes was a higher tolerance to 229 μM 5MT than was originally believed.

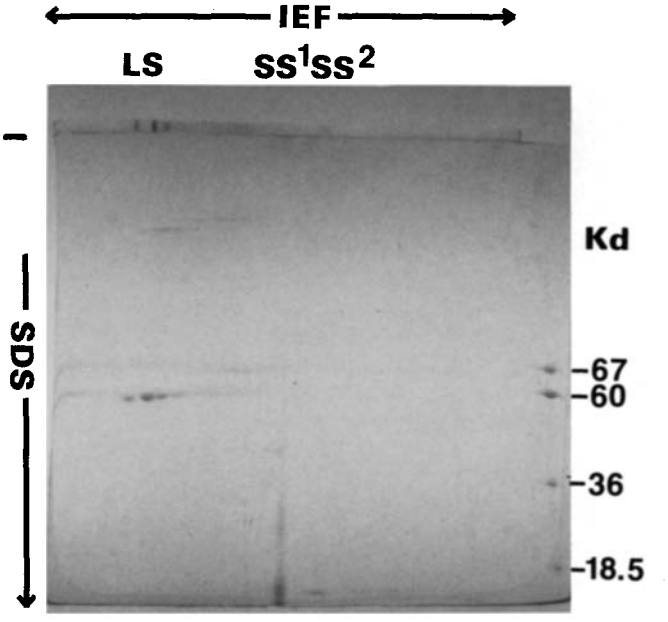

Fig. 6. Two-dimensional electrophoresis of Type 2 hybrid RuBPCase. The first dimension was a standard IEF tube gel shown stained at the top of the figure. A second unstained IEF gel was placed atop a SDS slab gel containing a 7.5 to 15% linear polyacrylamide gradient and a 5% polyacrylamide stacking gel (Chua 1980) and was run at 20 mA per gel for approximately 9 h. Molecular weight marker proteins are shown at the extreme right. LS = *N. tabacum* large subunits, SS^1 = *N. glutinosa* small subunits, SS^2 = *N. tabacum* small subunits

Table 2. Anthranilate synthase activity versus tryptophan concentration. TX1 is the 5MT-sensitive *N. tabacum* line from which TX3 was isolated

(μM) Trypto-phan	TX1	TX3	*Nico-tiana gluti-nosa*	T3g 1C	T3g 1K	T3g 1U	T3g 1V	T3g 1CC
0	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
1	87	87	96	83	77	70	82	79
6	40	83	67	54	35	11	0	33
10	0	72	47	11	0	0		0
60		57	0	0				
100		57						
1000		47						

5MT-Resistance in Hybrids

All five hybrids (four *Type 1* and one *Type 2*) lost resistance to 5MT when regenerated to plants as determined by sensitivity of anthranilate synthase activity to tryptophan. Extracts from both the plants and cultures derived from the hybrid plants showed sensitivity to tryptophan that appeared similar in magnitude to wild type *N. tabacum* (TX1, Table 2), i.e. more sensitive than either TX3 or *N. glutinosa*.

Discussion

Clearly, the *Type 1* plants are somatic hybrids as judged by plant morphology, isozyme patterns and infertility; and a senescence pattern similar to sexual hybrids. The RuBPCase patterns of these plants shows that the chloroplasts originated from the *N. glutinosa* parent in all four cases. There was variability among even the *Type 1* cell lines as shown by PER isozymes (Fig. 4). Other isozymes also showed variability (band intensity, etc.) between the lines, e.g. malate dehydrogenase, superoxide dismutase and esterase (data not shown), but these were not useful in discerning hybridity due to the similarity of the parental patterns. A few enzymes such as endopeptidase had activity patterns that were identical among both parents and hybrids (data not shown). It is obvious that many enzymes need to be examined when analyzing putative somatic hybrids.

Several features in the *Type 1* somatic hybrids are of interest. Two of the four hybrid lines had 60 chromosomes which is less than the additive number of chromosomes of the parents (72). Such changes are not unexpected since chromosome instability has been observed in fusion hybrids (Douglas et al. 1981; Wetter 1977). Two of the hybrids, however, had 30 and 34 chromosomes, respectively (Table 1). Obviously, extreme chromosome loss occurred but it should be noted that these numbers are just below that found in the sexual hybrid (*N. glutinosa* × *N. tabacum*, 2n = 36) and if standard deviations are taken into account the differences are not statistically significant (Table 1). Perhaps the chromosome number of 36 is a stable plateau for this particular cross during chromosome elimination.

The single *Type 2* hybrid (T3g 1CC) is predominantly *N. tabacum* in character with respect to flower color and morphology, fertility and most isozymes. PER isozymes, RuBPCase small subunits of both parents and its rapid senescence after setting seed showed that it is probably a partial nuclear hybrid. Its chromosome number of 34 is similar to those of two of the *Type 1* hybrids. More importantly, the progeny resulting from self pollination are free of any easily detected *N. glutinosa* characteristic. This observation may have important consequences in the future usage of somatic hybridization for crop improvement. Specifically, if the chromosomes of one parent are lost during sexual reproduction another one or more selection steps may be required to maintain the desired hybrid characteristic. It is of interest that the only fertile somatic hybrid is one in which the chloroplast origin complements the dominant nuclear characteristics, i.e. both *N. tabacum* in origin. Thus, we have apparently managed to obtain *N. tabacum* plants from the otherwise non-regenerable TX3 line via protoplast fusion. Use of regeneration as a dominant trait in protoplast fusion has been reported previously (Kameya et al. 1981). In another report concerning the somatic hybridization of *N. glutinosa* with *N. tabacum* (containing *N. debneyi* cytoplasm), no hybrids were found which contained *N. tabacum* (actually *N. debneyi*)-type RuBPCase large subunits (Uchimiya 1982; Uchimiya et al. 1982).

The heterogenous *N. glutinosa*-like RuBPCase small subunit in the type 2 hybrid raises a question as to the ability of multisubunit enzymes to complement in somatic hybrids. It would appear that there are abnormalities created when *N. glutinosa* RuBPCase small subunit protein is made in cells which apparently contain only *N. tabacum* chloroplast genomes (Fig. 6). The reverse situation (*N. tabacum* small subunit with *N. glutinosa* large subunit) does not show similar protein abnormalities (Fig. 5). Thus, certain nucleus-cytoplasm combinations may result in abnormal proteins, the Type 2 RuBPCase being one easily identified example.

Neither the T3g 1CC plant nor cultures started from this plant had the 5MT-resistance trait of T3X. Moreover, anthranilate synthase from all five hybrids was more sensitive to feedback inhibition by tryptophan than either parent and closely resembled wild-type *N. tabacum*. This loss of resistance could be due to several possibilities, including chromosome loss or an epigenetic basis for the resistance. This loss is in contrast to what was observed when a different 5MT-resistant cell line was regenerated into plants and then cultures re-initiated from the plants (Widholm 1980). In this case the altered anthranilate synthase was not expressed in the plant but was again found in the re-initiated cultures. Despite the loss of 5MT-resistance in the regenerated hybrids, 5MT-resistance was useful as a selection criterion in the newly fused protoplasts.

We conclude that amino acid analog resistance and plant regeneration capability are effective selection tools in protoplast fusion studies. The fusion products obtained exhibited variability in terms of isozymes and the type of parental chloroplast retained. Despite the occurrence of many *N. glutinosa* escapes, the selection scheme utilized here resulted in a relatively efficient selection of hybrid plants thereby avoiding time- and space-consuming screening procedures. Regeneration of fertile plants from *N. glutinosa* mesophyll protoplasts was also demonstrated for the first time.

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