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Genetic diversity among sublines originating from a single somatic hybrid cell of *Duboisia hopwoodii* + *Nicotiana tabacum*

T. Endo [1], T. Komiya [1], M. Mino [1], K. Nakanishi [2], S. Fujita [2] and Y. Yamada [1]

[1] Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan
[2] Department of Pathology, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamikyo-ku, Kyoto 602, Japan

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Summary. The genetic instability of an intertribal hybrid cell line, *Duboisia hopwoodii* + *Nicotiana tabacum*, obtained by mechanical isolation of a single hybrid cell was studied. Ten subclones of calli derived from this hybrid cell line were cultured for 3 years, and their genetic makeup clarified as to nuclear DNA content, chromosome constitution, and peroxidase isozymes. Nuclear DNA content differed in each subclone. In most subclones, mean DNA content was lower than the mean DNA content in the original hybrid cell line determined 1 year after fusion. This decrease in DNA content is partly attributable to the elimination of tobacco chromosomes that occurred in all subclones. The extent to which tobacco chromosomes were eliminated varied among the subclones – evidence that chromosome elimination occurred slowly. Peroxidase isozyme analysis indicated the loss of a tobacco-specific isozyme, thus confirming results obtained by chromosome analysis. Shoots regenerated from two hybrid subclones after 2 years were also heterogeneous in morphology and nuclear DNA content.

Key words: *Duboisia hopwoodii* – *Nicotiana tabacum* – Somatic hybrid – Nuclear DNA content – Genetic instability

Introduction

Parasexual hybridization by protoplast fusion has made it possible to produce hybrid plants between species belonging to different taxonomic tribes of the Solanaceae (Krumbiegel and Schieder 1979, 1981; Gleba et al. 1982, 1983; Skarzhynskaya 1982; Potrykus et al. 1984), the Unbelliferae (Dudits et al. 1979, 1980), and the Cruciferae (Gleba and Hoffmann 1978, 1979, 1980). Somatic hybridization between taxonomically remote species normally produces genetic instability (Gleba and Sytnik 1984).

Hoffmann and Adachi (1981) made a thorough study of genetic diversity among somatic hybrids derived from a single fusion cell of *Arabidopsis thaliana* + *Brassica campestris*. They obtained a large number of regenerants from a hybrid cell line and demonstrated the morphological and chromosomal heterogeneity among those plants. Hybrid plants formed between *Lypopersicon esculentum* and *Solanum rickii* also showed heterogeneity in leaf shape and ploidy, even tough they were offspring of a single fusion product (O'Connell and Hanson 1986). Although the number of examples of intergeneric and interfamilial somatic hybridizations have increased, the mechanisms for these changes in their genetic makeup during subculture are largely unknown.

Recently, we succeeded in culturing protoplasts of *Duboisia hopwoodii* and, subsequently, were able to obtain an intertribal hybrid cell line, HT 1031, between *D. hopwoodii* and *Nicotiana tabacum* by protoplast fusion (Endo et al. 1987). *D. hopwoodii* is one of three *Duboisia* species that are shrubs of the Solanaceae (tribe Salpiglossideae) endemic to Australia (Barnard 1952; Evans 1979). Short *Duboisia* chromosomes are readily distinguishable from the long chromosomes of *Nicotiana*, a phenomenon which facilitates monitoring changes in the genetic constitution of the hybrid cells. About 1 year after fusion, the hybrid cell line contained cells differing in chromosome number. In the present article we describe in detail the genetic instability in this hybrid cell line after 3 years of culture and discuss the mechanisms involved in the genetic instability of somatic hybrids.

Materials and methods

Protoplasts from cell suspension cultures of *Duboisia hopwoodii* F. Muell were fused with mesophyll protoplasts of *Nicotiana*

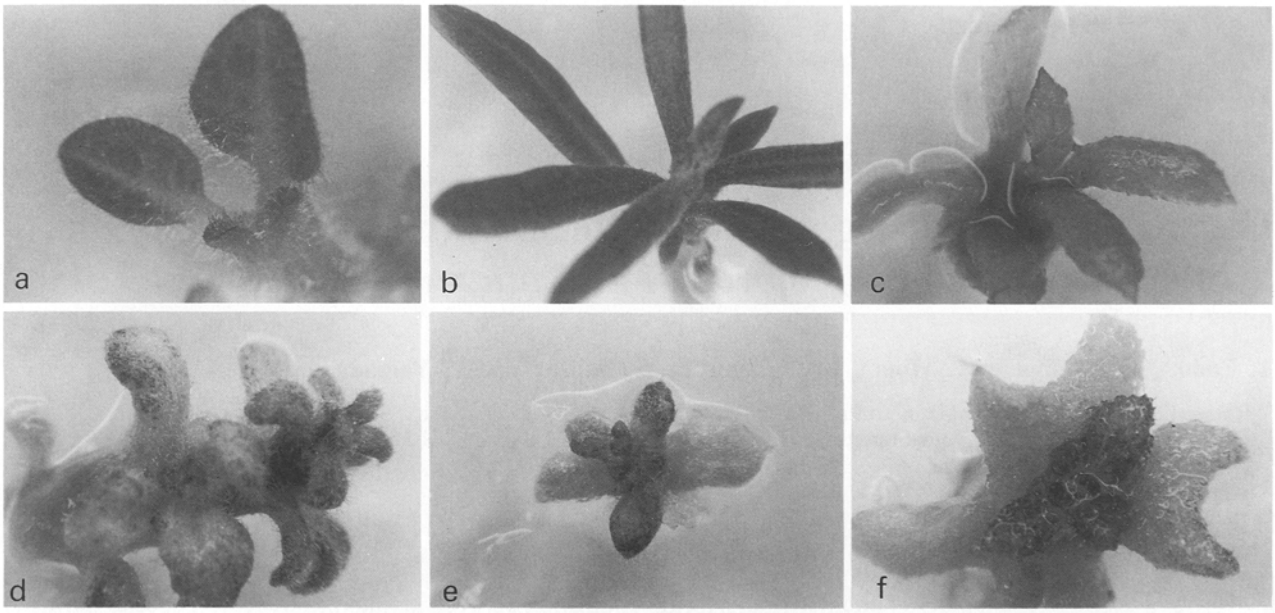

Fig. 1 a–f. Shoot morphologies of *Nicotiana tabacum* **a**, *Duboisia hopwoodii* **b** and their hybrids (**c**, shoot culture 1; **d–f**, shoot culture 2)

tabacum L. cv. 'Samsun' using polyethylene glycol. The hybrid cell line HT 1031 was obtained by mechanical isolation of the fused cells which were then cultured in tobacco nurse cells (Endo et al. 1987). The methods used for the mechanical isolation and nurse culture of individual fusion products have been described previously (Yamada and Morikawa 1985). Approximately 1 year after isolating the hybrid cell, we divided its callus into about 500 small cell aggregates. These have been maintained in light on solidified B5 media (Gamborg et al. 1968) containing 2×10^{-5} *M* 1-naphthaleneacetic acid and 2×10^{-6} *M* 6-benzyladenine. Ten subclones thus obtained showed good growth and were used in the present investigation. For shoot regeneration, calli were placed on B5 media containing 10^{-5} *M* 6-benzyladenine.

Nuclear DNA content was determined by microfluorometry: the fluorescence emitted from the DNA-DAPI complex formed in the nuclei of protoplasts fixed on a slide glass was measured. An enzyme solution containing 4% Cellulase Onozuka RS, 2% Macerozyme R-10 (both from Kinki Yakult, Japan), 4% Diserase (Kyowa Hakko, Japan), 0.1% Pectlyase Y-23 (Seishin Pharmaceutical, Japan), 20 m*M* MES, 5 m*M* $MgCl_2$, and 0.6 *M* sorbitol (pH 5.6) was used to obtain protoplasts from calli. Two percent Cellulase Onozuka RS and 0.5% Macerozyme R-10 was used to isolate mesophyll protoplasts. The isolated protoplasts were washed twice with a solution containing 20 m*M* MES, 5 m*M* $MgCl_2$, and 0.6 *M* sorbitol (pH 5.6), after which 50 µl of the protoplast solution was placed on a slide glass, dehydtrated, and then fixed in a mixture of glacial acetic acid and ethanol (1:3) for 30 min. DNA was stained overnight at 4 °C with a solution containing 50 µg 4', 6-diamino-2-phenylindole dihydrochloride (DAPI), 10 m*M* EDTA-2Na, 10 m*M* 2-mercaptoethylamine hydrochloride and 100 m*M* NaCl in 1,000 ml Tris/HCl buffer (10 m*M*, pH 7.4) (Hamada and Fujita 1983). Fluorescence was monitored and measured under an epifluorescent microfluorometer (Olympus MMSP) equipped with a 365 nm interference excitation filter, a 400 nm dichroic mirror, a 420 nm cut filter, a 450 nm interference band pass filter, an objective lens (100/1.30), and a high pressure mercury lamp (200 W). Nuclear DNA content was expressed relative to that of the tobacco mesophyll cells (2c = 20.0).

For chromosome observation, calli were treated with 0.025% colchicine for 2 h. They were fixed in the acetic acid-ethanol mixture (1:3) for 12 h at 4 °C, then stained with basic fuchsin.

For peroxidase isozyme analysis, a cell-free extract was prepared by first grinding the cells in cold buffer (50 m*M* Tris /HCl, pH 7.4) with a chilled mortar, then centrifuging the slurry (12,000 *g*, 20 min). Isoelectric focusing was carried out for 3 h at a constant voltage of 200 V using gel columns containing 5% polyacrylamide and 2% Ampholine (pH 3.5–10, LKB). Deaminobenzidine tetrahydrochloride was used as the substrate in the staining of peroxidase.

Results

Morphology

Subclones from the hybrid cell line HT 1031 originally showed morphological heterogeneity when first isolated from HT 1031 as small cell aggregated. The color of the calli varied from pale yellow to bright green, and most were friable. Due to cell elongation, some calli had a very hairy appearance that was absent from both parent species; others were compact and hard. A feature common to all subclones was the appearances of necrosis on the surface of the callus 2–3 weeks after subculture. In addition, purple spots frequently appeared on the surface (also seen on *Duboisia* callus).

During longterm maintenance in undifferentiated growth, most subclones became homogeneous in appearance: friable showing a pale green coloration. One subclone (here designated subclone H) accumulated an ex-

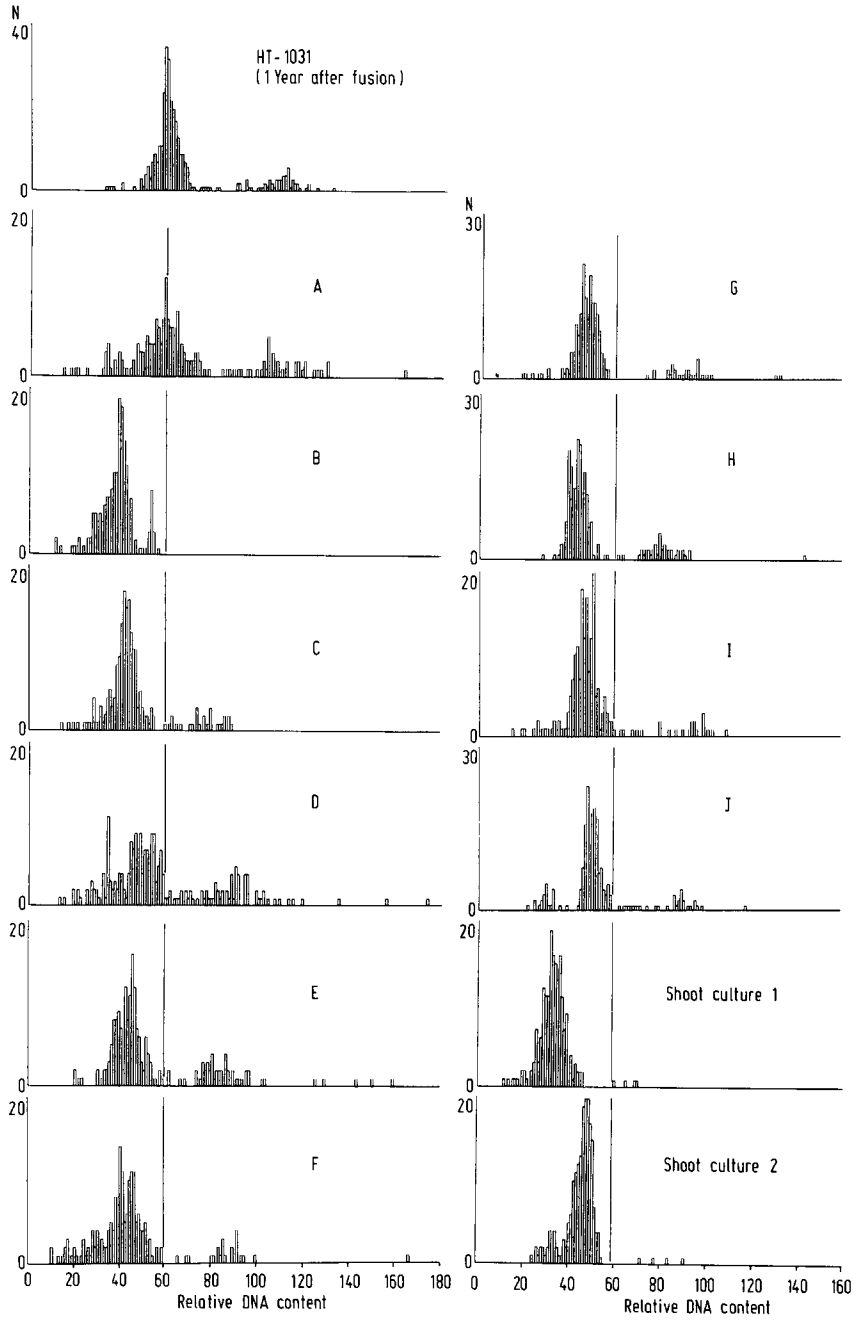

Fig. 2. Distribution of nuclear DNA contents in the HT 1031 line. The DNA content of the original line 1 year after fusion is compared with the DNA contents of subclones *A*–*J* and shoot cultures 3 years after fusion. The *vertical line* indicates the mean value of the main peak of the original HT 1031 (1 year after fusion)

tensive amount of purple pigment, but all other subclones seemed to have lost the ability to produce this pigment. Attempts to induce shoots from the subclones were unsuccessful until 2 years after fusion, when shoots regenerated from two subclones. These shoots, designated shoot culture 1 and 2, were maintained in regeneration media containing 10^{-5} *M* benzyladenine. The morphologies of these shoots are shown in Fig. 1. One had leaves similar to those of *Duboisia* – narrow and pointed (shoot culture 1, Fig. 1c); the other had broad tobacco-like leaves (shoot culture 2, Fig. 1d–f).

When these shoots were treated for 3 days with a high concentration of 3-indolebutyric acid (10^{-4} *M*) and then cultured in hormone-free media, adventitions roots formed at a high frequency. The growth of those plantlets did not, however, go beyond root initiation.

Nuclear DNA content

Nuclear DNA content of *Nicotiana tabacum, Duboisia hopwoodii*, and the hybrid (HT 1031) were investigated approximately 1 year after fusion (Table 1). At that time,

Table 1. Nuclear DNA contents of *Nicotiana tabacum*, *Duboisia hopwoodii* and their hybrid (HT 1031)

	Peak	Mean[a]	SD[a,b]	Frequency in total population (%)	Level of DNA content
N. tabacum					
Mesophyll	1	20.0	2.20	96.0	2c
	2	37.4	2.85	4.0	4c
Callus	1	36.5	4.18	63.1	4c
	2	69.0	4.71	33.0	8c
	3	134.7	20.56	3.9	16c
D. hopwoodii					
Mesophyll	1	7.7	0.64	98.4	2c
	2	14.6	1.94	1.6	4c
Callus	1	15.2	1.63	85.2	4c
	2	29.7	3.11	14.2	8c
	3	60.9	2.18	0.6	16c
HT 1031					
Callus	1	59.3	4.55	76.9	
	2	107.3	8.81	17.0	

[a] Values expressed are relative to the nuclear DNA content of *Nicotiana tabacum* mesophyll cells (2c = 20.0)
[b] SD = standard deviation

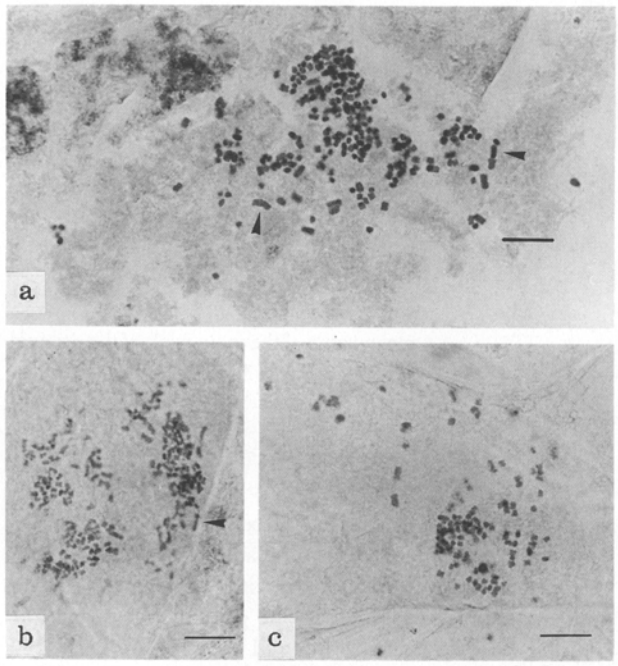

Fig. 3a–c. Mitotic metaphase chromosomes of the hybrid cells *Nicotiana tabacum* + *Duboisia hopwoodii*. *Arrows* indicate a tobacco type chromosome. *Bar*: 10 μm. **a** A cell of subclone B with 150–160 chromosomes; **b** and **c** cells of subclone E, both having 120–130 chromosomes. In plate **b**, some tobacco type chromosomes are present, whereas in plate **c**, no such chromosomes are found

the HT 1031 line was found to be intermediate between the parental species for peroxidase isozyme patterns and species-specific fluorescent compounds (Endo et al. 1987). Calli of *N. tabacum* and *D. hopwoodii* had a 4c nuclear DNA content, an indication that the culture conditions we used favored the doubling of the nuclear DNA content. The mean value of the DNA content for the main peak in the HT 1031 line, which was a fusion product between *N. tabacum* (mesophyll) and *D. hopwoodii* (callus), was much larger than the sum of the 2c value for *N. tabacum* (mesophyll) and the 4c value for *D. hopwoodii* (callus). This hybrid, therefore, was most likely a product of multiple fusion, although such a high DNA content could have been the result of somatic polyploidization after fusion. In addition, multinuclear cells were frequently found in this hybrid.

When the nuclear DNA contents of some subclones derived from the HT 1031 line were investigated approximately 3 years after fusion (Fig. 2), all except subclone A had lower DNA contents than the original HT 1031 line 1 year after fusion. Subclones A and D showed a very broad distribution of their nuclear DNA in comparison to the other subclones. Multinuclear cells were also common in all subclones. A cell containing 22 or 23 nuclei was found in subclone H. Most nuclei in a given multinuclear cell had the same amount of DNA. The two shoot cultures derived from the HT 1031 line contained different amounts of nuclear DNA: shoot culture 1 had much less nuclear DNA than both shoot culture 2 and subclones maintained in an unorganized growth state.

Chromosome constitution

The mitotic metaphase chromosomes of some subclones approximately 3 years after fusion are shown in Fig. 3. The *Nicotiana* type (long) chromosomes had been eliminated in all ten subclones examined, although the observations made approximately 1 year after fusion had indicated no tendency for the specific elimination of chromosomes. No *Nicotiana* type chromosomes were found in subclones C and J. Other lines had fewer than 20 *Nicotiana* type chromosomes per metaphase. Subclones E and H were chimeric in their chromosome constitution: some metaphase plates contained only *Duboisia* type chromosomes, others contained both *Nicotiana* and *Duboisia* type chromosomes. In most of the metaphase plates observed, the chromosome number ranged from 100 to 170.

Peroxidase

Isoelectric focusing of the peroxidase isozymes extracted from the HT 1031-derived subclones 3 years after fusion (Fig. 4) indicated that only subclones E, F, and H showed the *Nicotiana*-specific band. The other lines all showed *Duboisia*-like peroxidase patterns, a reflection of the loss of the *Nicotiana* genes in these hybrid calli.

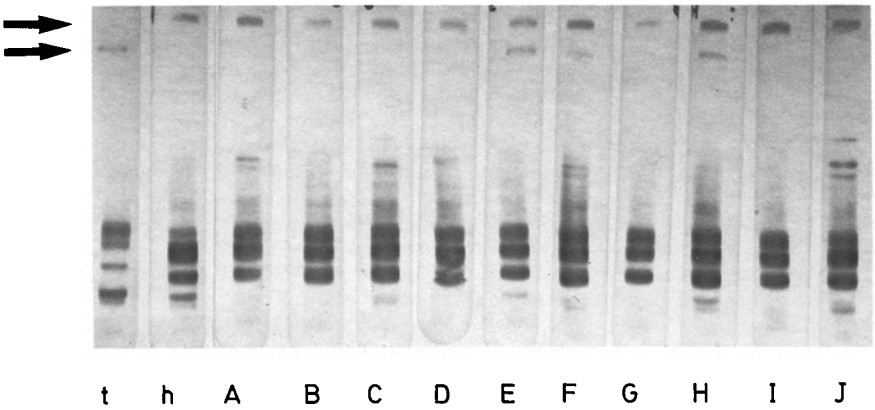

Fig. 4. Peroxidase isozymes from *Duboisia hopwoodii* (*h*), *Nicotiana tabacum* (*t*) and hybrid subclones (*A*–*J*). *Arrows* indicate species-specific bands

Discussion

Ten subclones derived from the intertribal somatic hybrid cell line *Duboisia hopwoodii* + *Nicotiana tabacum* showed considerable genetic instability. Microscopic observation of chromosomes and peroxidase analysis provided evidence that the *Nicotiana* genome had been eliminated. The lower DNA content of these subclones can, therefore, be partly attributed to the absence of the *Nicotiana* genome.

Although elimination of the parental chromosomes is common in intergeneric and interfamilial hybrids, the mechanism for this elimination is not clear. In most cases, species-specific elimination takes place (Kao 1977; Krumbiegel and Schieder 1981; Gleba et al. 1983; Potrykus et al. 1984; Tabaeizadeh et al. 1985). Hoffmann and Adachi (1981), however, showed that both *Arabidopsis*- and *Brassica*-type plants could be obtained from the hybrids of *Arabidopsis* and *Brassica*. This suggests that the elimination of either the *Brassica* or *Arabidopsis* genome occurs in this parental combination. Asymmetric nuclear division seems to be one source of parental genome imbalance in somatic hybrids. Asymmetric distribution of parental chromosomes was evident even at the first division of fused cells of *Nicotiana tabacum* and *Glycine max* (Chien et al. 1982). This phenomenon may produce a genetically heterogeneous cell population and, during subculture, preferential selection of a certain cell type may take place. Another possible explanation for the elimination of a specific genome is preferential chromosome elimination from a homogeneous cell population by an unknown mechanism. In the case of *Duboisia hopwoodii* + *Nicotiana tabacum*, the elimination of the tobacco chromosomes seems to occur slowly. Their elimination was not evident during the early stage of culture and was only manifested 3 years after fusion.

In the HT 1031 hybrid line, attempts to regenerate plants during the early stage of culture were unsuccessful – no regenerants were obtained until 2 years after fusion. This indicates that some genetic incompatibility already present in the early stages of culture prevented the hybrid cells from regenerating plants. After the elimination of the tobacco chromosomes, this incompatibility may diminish or disappear, resulting in shoot regeneration. A similar observation has been reported for *Datura innoxia* + *Atropa belladonna* somatic hybrids (Krumbiegel and Schieder 1981). Genetic instability in interfamilial and intergeneric hybrids often results in hybrid cells incapable of morphogenesis or in the formation of abnormal plants that frequently lack root organogenesis (Dudits et al. 1979; Krumbiegel and Schieder 1979, 1981; Gleba and Hoffmann 1980; Gleba et al. 1982, 1983).

In addition to this imbalance between the parental genomes, the genetic instability common to unorganized cultures of plant cells, which results in the so-called somaclonal variation (Larkin and Scowcroft 1981), must be considered. Somatic hybrids generally are not free fom this type of genetic variation because most fused cells must go through unorganized culture before they regenerate hybrid plants. For example, multinuclear cells common in calli and cell suspension cultures were frequently present in HT 1031 calli.

In conclusion, individual somatic hybrids, even those derived from a single fusion product, should be regarded as unique genetic material which may serve to improve existing plant species and to develop novel plants.

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