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Somatic hybridization between potato and *Nicotiana plumbaginifolia*

2. Karyotypic modification and segregation of genetic markers in hybrid suspension cultures and sublines

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Summary. Several hybrid callus lines were produced through somatic hybridization between the diploid transformed *Solanum tuberosum* plant clone 413 (2n = 2x = 24) and a diploid wild-type plant clone of *Nicotiana plumbaginifolia* (2n = 2x = 20). The hybrid callus lines with subdiploid numbers of potato chromosomes were studied for karyotypic evolution as well as for segregation of the transformation marker characters (i.e. hormone autotrophy, opine synthesis, kanamycin resistance and β-glucuronidase activity). Initially, these hybrids (cultured in kanamycin-containing medium) expressed all of the transformation characters. Six callus lines were selected for the establishment of cell suspension cultures; two of these were also used to initiate sublines, one from single cells of a suspension culture, and the other from callus-derived protoplasts. The cell suspension cultures and the sublines were cultured in kanamycin-free medium. After prolonged culture, karyotypic analysis of the various cell suspension lines revealed independent evolution of both parental genomes. Out of the six suspension lines, four showed a considerably reduced number of potato chromosomes as compared to the original hybrid callus lines, whereas the karyotypes of the individual sublines generally reflected the karyotypic diversity of the original cultures. The fate of the marker characters in various suspension cultures and sublines revealed independent segregation of the markers of TL-DNA (hormone autotrophy) and vector T-DNA (kanamycin resistance and β-glucuronidase activity). Loss of the TR-DNA marker (opine synthesis) was observed only in combination with the simultaneous loss of the TL-DNA marker and the vector T-DNA markers. The results on segregation patterns of marker characters are discussed in the light of specific chromosome loss in the hybrid lines and gene linkage relationships.

Key words: *Nicotiana plumbaginifolia* – *Solanum tuberosum* – Hybrid cell suspension cultures – Hybrid callus sublines – Karyotypic modifications – Segregation of transformation marker characters

Introduction

Cell cultures generally show reduced genetic stability, and an alteration in the physiological conditions of the cultures can further increase this instability (Negrutiu et al. 1989; Pijnacker and Sree Ramulu 1990). In hybrid cell cultures, genetic instability can result in chromosome elimination and the concomitant loss of marker characters. This phenomenon has been exploited extensively to study gene linkage relationships, e.g. to map the human genome (Kao 1983).

In the present study attention is focussed on the modification in karyotypic composition in various hybrid callus lines resulting from altered culture conditions. The lines were produced through fusions between leaf protoplasts of diploid transformed *Solanum tuberosum* clone 413 (De Vries-Uijtewaal et al. 1989) and a diploid wild-type clone of *Nicotiana plumbaginifolia* (Gilissen et al. 1992). The altered culture conditions included the establishment of suspension cultures from hybrid callus lines and the initiation of individual sublines from isolated single hybrid suspension cells or from hybrid callus protoplasts. In all cases, kanamycin was omitted from the culture medium to enable karyotypic evolution independent of selection pressure.

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In addition, the fate of the transformation marker characters was analysed in the hybrids after prolonged culture (under altered conditions) to elucidate the linkage relationships in the transformed potato parent between the TL- and TR-DNA (carrying the characters of hormone autotrophy and opine synthesis, respectively) and vector T-DNA (with the genes coding for kanamycin resistance and β-glucuronidase activity).

Materials and methods

Plant materials

Plants of the diploid transformed clone 413 of *Solanum tuberosum* ($2n \times 2x = 24$) carrying various genetic markers (hormone autotrophy, opine synthesis, kanamycin resistance and β-glucuronidase (GUS) activity) (De Vries-Uijtewaal et al. 1989; Gilissen et al. 1991) and a diploid wild-type clone of *Nicotiana plumbaginifolia* ($2n = 2x = 20$) were used as parents in somatic hybridization experiments. The culture of parental plants, isolation of leaf protoplasts, electrofusion and hybrid selection have been described previously (Gilissen et al. 1992).

Cell suspension cultures

Cell suspension cultures were established from the calli of six hybrid lines (H1, H6, H24, H25, H57 and H60). For details on the hybrid lines, see Gilissen et al. (1992). At 3 weeks after subculturing, approximately 5 cm^3 of actively growing 3-week-old callus was pressed through a 10-ml syringe minus needle (Terumo Corp., Japan) into 45 ml kanamycin-free MS medium (Murashige and Skoog 1962) containing 5 mg/l NAA and 0.1 mg/l BAP in a 250-ml Erlenmeyer flask. The flasks were placed on a gyratory shaker (120 rpm, amplitude 3 cm) at 28 °C in darkness, The suspension cultures were subcultured weekly.

Sublines

Sublines were produced from hybrids H1 and H60. In the case of H1, the cell suspension culture was sieved through nylon gauze (mesh diameter 80 μm) to isolate free cells and small aggregates. After sieving, the cells were collected by gentle centrifugation (400 rpm in a swing-out table centrifuge) and then diluted to a concentration of 10^5 units (i.e. single cells plus small aggregates) in MS medium containing 5 mg/l NAA and 0.1 mg/l BAP. This "single cell" suspension was cultured in 1.5-ml aliquots in 5-cm Petri dishes at 24 °C and under continuous light (1 klm/m^2, TL type F48T12). At 7 and 14 days of culture, fresh medium was added in equal amounts to the Petri dishes. After 4 weeks, single calli were isolated and subcultured monthly as individual sublines in 9-cm Petri dishes containing MS medium with 5 mg/l NAA and 0.1 mg/l BAP and solidified with 0.8% (w/v) agar (Oxoid). Culture took place under controlled conditions of 24 °C and 16 h/day light (1 klm/m^2, TL type FTD58W33).

In the case of H60, sublines were established from protoplasts isolated from callus and incubated overnight in ½ V-KM medium without hormones (Bokelmann and Roest 1983) containing 1% (w/v) Cellulase R10 (Onozuka) and 0.2% (w/v) Macerozyme R10 (Yakult), in 9-cm Petri dishes placed on a gyratory shaker (30 rpm, amplitude 2.5 cm). After successive washings in 0.4 *M* mannitol, and purification on a 0.4 *M* sucrose layer by gentle centrifugation (400 rpm in a swing-out table centrifuge), the protoplasts were collected, diluted to a concentration of 10^5 per millilitre and cultured to individual callus sublines, a described for the "single cell" suspension of H1.

Karyotypic analysis

Karyotypic composition (i.e. the numbers of *S. tuberosum* and *N. plumbaginifolia* chromosomes) of each hybrid cell suspension and subline was determined in a number (a maximum of 25) of well-spread Feulgen-stained metaphase cells (Sree Ramulu et al. 1985) at 3 days after subculture. At the time of karyotypic analysis, the ages of the suspension cultures were 18 months for H1 and H6 and 12 months for H24, H25, H57 and H60, and the ages of the sublines of H1 and H60 were 15 and 12 months, respectively. For details on the karyotypic composition of the original hybrid calli at the time of initiation of the suspension cultures and sublines, reference is made to Gilissen et al. (1992).

Hormone autotrophy, opine synthesis, kanamycin resistance and GUS-activity

Hormone autotrophy and kanamycin resistance of the cell suspensions were measured by direct determination of the increase over time (1 week) of the packed cell volume during two or three successive subcultures in the Erlenmeyer flasks (Gilissen et al. 1983) on MS medium without hormones, or on MS medium with hormones (5 mg/l NAA and 0.1 mg/l BAP) supplemented with 100 mg/l kanamycin. For the callus of the original hybrids and individual sublines, hormone autotrophy was determined by omitting hormones from the solid MS medium, and kanamycin resistance was determined by adding kanamycin (100 mg/l) to the medium during two successive culture periods. The increase in callus diameters during culture on selective media was compared to the increase observed on control media (control 1: MS medium with hormones; control 2: MS medium without hormones but with 100 mg/l kanamycin). The presence of agropine and mannopine was determined by paper electrophoresis and silver staining according to Petit et al. (1983). GUS-activity was assayed by fluorimetry using 4-methyl umbelliferyl glucuronide (MUG) (Jefferson et al. 1986, 1987) and expressed as n*M* methyl-umbelliferone produced per milligram protein per minute (n*M* MU/mg protein/min). The protein concentration of the cell extracts was determined by the method of Bradford (1976) with a reagent supplied by Bio-Rad Laboratories. Expression of the marker characters in the original hybrid callus lines was assayed at various times during cultures and at the time of initiation of the suspension cultures and sublines.

Flow cytometric determination of nuclear DNA content

The nuclear DNA contents of hybrid suspension cells were determined by flow cytometric measurements of DAPI-stained, isolated interphase nuclei (Sree Ramulu and Dijkhuis 1986). Expression of the nuclear DNA content in C-values is according to Bennett and Smith (1976). During flow cytometric measurements, freshly isolated nuclei from leaves of *N. plumbaginifolia* were used as an internal standard. The coefficient of variation (CV value), indicating the degree of statistical and spontaneous variation, was determined by the formula (Super 1979):

$$CV = \frac{\text{width of the histogram peak at half height}}{\text{position of the histogram peak on X axis times } 2.355} \times 100\%$$

Results

Karyotypic analysis

Cell suspension cultures of hybrid lines. Karyotypic analysis of six hybrid suspension cultures were carried out at 12 months (H24, H25, H57, H60) or 18 months (H1, H6)

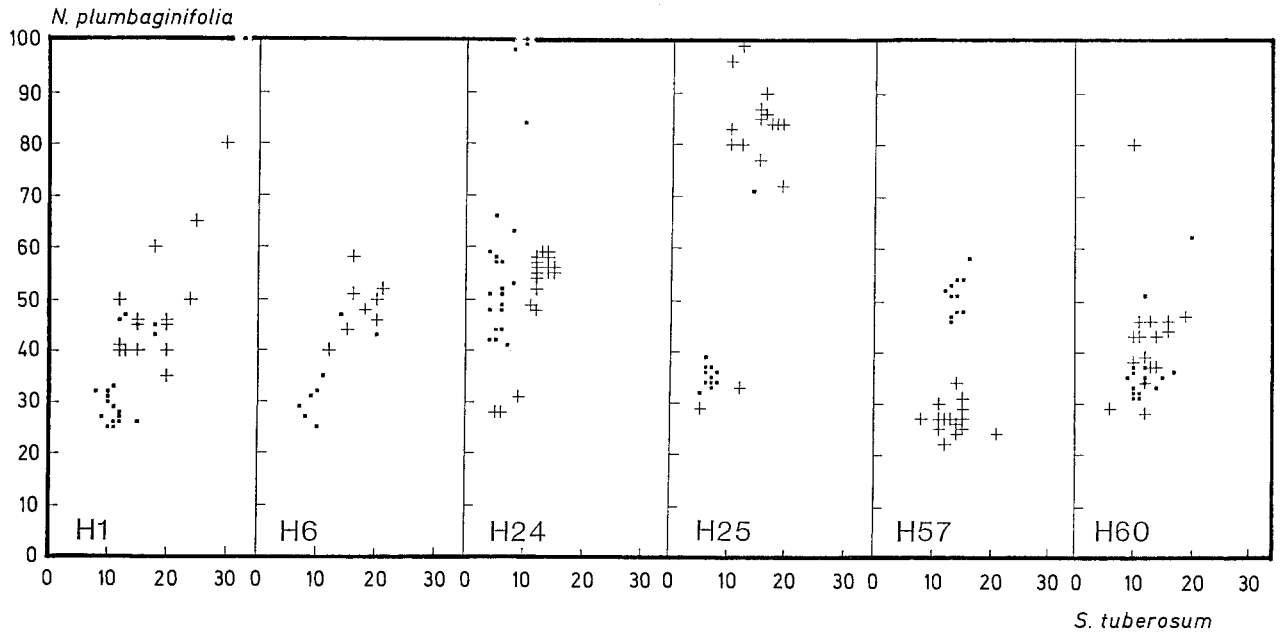

Fig. 1. Karyotypic composition (numbers of potato and *N. plumbaginifolia* chromosomes) of six hybrid suspension cultures and their original hybrid callus lines obtained after somatic hybridization of the diploid, transformed *S. tuberosum* clone 413 and a diploid wild-type clone of *N. plumbaginifolia*. The *X axis* gives the numbers of potato chromosomes, and the *Y axis* gives the numbers of *N. plumbaginifolia* chromosomes. ■ Karyotypic composition of an individual cell from the suspension culture, + karyotypic composition of an individual cell from the original callus at the time of initiation of the suspension culture

after their establishment from the callus cultures. Figure 1 shows that the karyotypic composition of the cell suspension of each hybrid was different from that of the original callus cultures. The type of difference appeared to be specific to each hybrid. Cell populations of H1, H6 and H25 predominantly contained about half the number of potato and *N. plumbaginifolia* chromosomes as the original hybrid callus lines. In H25, minor population of 2C cells was already present in the original culture (Fig. 1). In H24, the cell suspension had a very low number of potato chromosomes, but a similar number of *N. plumbaginifolia* chromosomes as the original line. In H57, the suspension cells showed a doubling of *N. plumbaginifolia* chromosomes, whereas the number of potato chromosomes remained the same. On the other hand, in H60 there was only a slight reduction in the number of *N. plumbaginifolia* chromosomes and no change in the number of potato chromosomes. All hybrid suspension cultures (except H57) contained polyploid (4C, >4C) cells in addition to the main population of 2C cells (Fig. 1). The coefficients of variation (CV) from flow cytometric analysis of the nuclear DNA content of the suspension cells of H1, H25, H57 and H60 were equal to that of the control (i.e. the CV of the DNA contents of leaf cells of diploid *N. plumbaginifolia*), but in H6 and H24 the CV were 2 times higher than the CV of the control (Table 1). This indicates that the latter two hybrid suspension cultures were chimeric (i.e. contained different numbers of chromosomes in the cells),

Table 1. Relative nuclear DNA contents and their coefficient of variation (CV) in cell suspension cultures of various somatic hybrids between *S. tuberosum* and *N. plumbaginifolia* at 12 months (H24, H25, H57, H60) or 18 months (H1, H6) after establishment from hybrid callus lines

Materials	Relative DNA content (in % of control *N. plumbaginifolia*)	CV
Controls:		
N. plumbaginifolia	100	8.8
S. tuberosum	34.8	7.2
Hybrids:		
H25	115	8.2
H1	122	8.5
H6	126	17.5
H57	156	8.1
H60	161	8.2
H24	193	14.7

whereas the former suspensions had approximately the same numbers of chromosomes in each cell.

Sublines from isolated cells of a hybrid (H1) suspension culture. Twelve callus lines were initiated from the culture of isolated single cells of the suspension culture of hybrid H1. Karyotypic analysis, carried out at 15 months after initiation, revealed that the mean number of potato chro-

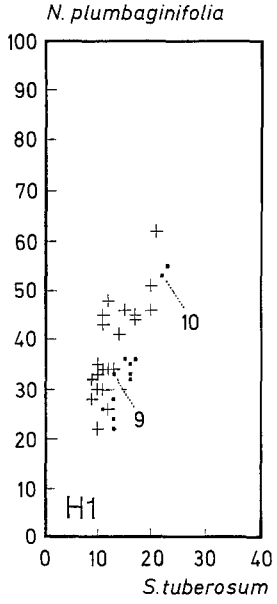

Fig. 2. Karyotypic composition of sublines of hybrid callus line H1 derived from isolated single suspension cells. Karyotypic analysis was carried out at 15 months after subline initiation. Mean variation in the parental chromosome numbers within the individual sublines of the hybrid was 3 for potato and 4 for *N. plumbaginifolia*. The *numbers* in the figure (9, 10) refer to the hybrid subline numbers which showed a loss of expression of marker characters. ■ Mean karyotypic composition of a subline; a maximum of 25 metaphase cells was analysed in each subline, + karyotypic composition of an individual cell from the original suspension culture of H1 at the time of subline initiation

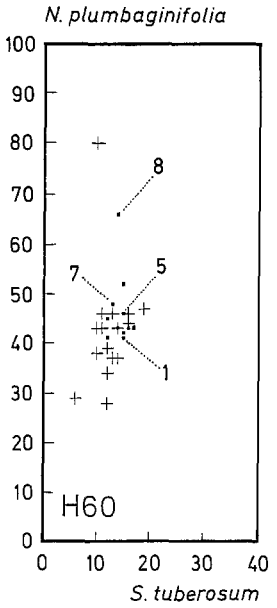

Fig. 3. Karyotypic composition of sublines of hybrid line H60 derived from callus protoplasts. Karyotypic analysis was carried out at 12 months after subline initiation. Mean variation in the parental chromosome numbers within the individual sublines of the hybrid was 3 for potato and 4 for *N. plumbaginifolia*. The *numbers* in the figure (1, 5, 7, 8) refer to the hybrid subline numbers which showed a loss of expression of marker characters. ■ Mean karyotypic composition of a subline; a maximum of 25 metaphase cells was analysed in each subline, + karyotypic composition of an individual cell from the original suspension culture of H60 at the time of subline initiation

mosomes was slightly increased in most sublines as compared to that of the original suspension culture (individual suspension cells analysed at the time of single cell isolation) (Fig. 2). Two sublines showed relatively higher numbers of chromosomes (polyploidization) from both parents. The deviation from the mean number of chromosomes in the sublines ranged from one to three for potato, and from two to four for *N. plumbaginifolia*.

Sublines from protoplasts of a hybrid (H60) callus line. The isolation and culture of protoplasts from hybrid callus line H60 resulted in 12 sublines. At 1 year after initiation of the sublines, the mean karyotypic composition (i.e. the mean numbers of potato and *N. plumbaginifolia* chromosomes) of 10 sublines was approximately similar to that of the original callus of H60 (individual callus cells analysed at the time of protoplast isolation). One subline showed an increased number of *N. plumbaginifolia* chromosomes (Fig. 3). The deviation from the mean number of chromosomes in the sublines was similar to those found in the sublines of H1.

Fate of genetic marker characters

The fate of the genetic markers introduced into the hybrids via transformed potato parent clone 413 was analysed in 24 hybrid callus cultures, including those used for the establishment of the cell suspension cultures and the sublines at various periods during culture. Hormone autotrophy, opine synthesis, kanamycin resistance and GUS activity remained expressed throughout the culture period in all the hybrids, even in those that showed the elimination of a few or many potato chromosomes (i.e. category I and III hybrids, see Gilissen et al. 1992).

The expression of all marker characters was observed in each original hybrid culture at the time of initiation of the hybrid suspension cultures and sublines. However, after prolonged culture (i.e. 12 months for H24, H25, H57, H60 and 18 months for H1 and H6, in kanamycin-free medium), four of the six hybrid suspension cultures showed the absence of expression of one or more transformation marker characters, i.e. hormone autotrophy in H24, H25 and H60, opine synthesis in H24 and kanamycin resistance and GUS activity in H6 and H24 (Table 2).

Ten out of the 12 sublines obtained from isolated suspension cells of the hybrid H1 were tested for expression of the transformation marker characters after 15 months of culture. Two sublines, H1-9 and H1-10 (Fig. 2), had lost hormone autotrophy (Table 2).

Table 2. Expression of transformation marker characters in suspension cultures and sublines of various somatic hybrids between *S. tuberosum* and *N. plumbaginifolia* after prolonged culture (18 months for H1 and H6; 15 months for the sublines of H1; 12 months for the other suspension cultures and sublines of H60) in kanamycin-free MS medium

Suspension cultures and sublines	Marker characters			
	Hormone autotrophy	Opine synthesis	Kanamycin resistance	GUS activity
Suspension cultures:				
H6	+	+	−	−
H24	−	−	−	−
H25	−	+	+	+
H60	−	+	+	+
H1, H57	+	+	+	+
Sublines from isolated hybrid (H1) suspension cells:				
H1-9	−	+	+	+[a]
H1-10	−	+	+	+
Other 8 sublines	+	+	+	+
Sublines from isolated hybrid (H60) protoplasts:				
H60-1	+	+	−	−
H60-5	−	−	−	−
H60-7	−	+	+	+
H60-8	−	+	+	−
Other 8 sublines	+	+	+	+

+: present, −: absent
[a] GUS-activity was very low but still detectable

From the 12 sublines of hybrid H60, the sublines H60-5, H60-7 and H60-8, derived from protoplasts of hybrid H60 callus, showed the absence of hormone autotrophy after 1 year of culture. Subline H60-5 had also lost the character of opine synthesis. The absence of kanamycin resistance and GUS activity was observed in sublines H60-1 and H60-5 (Table 2).

GUS activity in the parental potato plant and hybrid lines ranged between 3.0 and 10.0 n*M* MU/mg protein per minute. In three hybrid sublines (H1-9, H60-2, H60-12) strongly reduced GUS activities were observed (i.e. 0.13, 0.29 and 0.09 n*M* MU/mg protein per minute, respectively), whereas kanamycin resistance remained unchanged.

Discussion

The results show that the modes of altered karyotypic composition were different among the six suspension cultures obtained from various hybrids between potato and *N. plumbaginifolia* after prolonged culture in the absence of kanamycin. Some suspension cultures (H1, H6, H25) showed approximately half the numbers of both potato and *N. plumbaginifolia* chromosomes as the original callus lines, suggesting the occurrence, during prolonged suspension culture, of a strong preferential selection for cells with reduced biparental chromosome numbers; these reduced numbers were probably already present at a low frequency in the original hybrid callus culture (e.g. in H25; see Fig. 1). Each of the other three hybrid suspension cultures showed different karyotypic modifications: the number of chromosomes of one parent (i.e. *N. plumbaginifolia* in H24, and *S. tuberosum* in H57 and H60) remained unchanged, whereas the chromosome numbers of the other parent were altered. These results clearly indicate that changes in the growth conditions of the hybrids, i.e. shortening of the cell-cycle time due to the transition from callus to suspension culture, greatly influenced the evolution of the hybrid karyotype. Strikingly, the parental genomes in the various hybrid suspension cultures developed independently of each other, thus showing the maintenance of the parental genome identity within the hybrid nucleus (see also Gilissen et al. 1992).

The sublines derived from isolated suspension cells and protoplasts of hybrids H1 and H60 showed a different karyotypic composition, which reflected to a high degree the karyotypic diversity (chimerism) already present in the original callus cultures of the hybrids. Two years after culture initiation, Endo et al. (1988) also observed a high degree of genetic diversity (i.e. various levels of reduction in DNA contents) among sublines derived from a hybrid callus line between *Duboisia hopwoodii* (+) *Nicotiana tabacum*. However, these authors did not correlate this diversity to karyotypic variation between the individual cells of the original callus line, instead they ascribed the phenomenon to a gradual elimination of *N. tabacum* chromosomes.

With regard to the transformation marker characters, the absence of expression of one or more markers was observed in various hybrid suspension cultures, whereas the original hybrid callus cultures maintained all of the markers. These results strongly suggest that the absence of marker characters in the hybrid suspension cultures is due to the elimination of specific potato chromosomes (e.g. in H24), but not to silenced gene expression. However, in various suspension cultures and sublines (e.g. in H60, H1-10, H60-5) the elimination of potato chromosomes could not be demonstrated unambiguously to be the cause of marker loss because of the occurrence of variations in chromosome numbers or polyploidization (H1-10). In this regard, chromosome identification using Giemsa C-banding (Pijnacker and Ferwerda 1984; Mouras et al. 1986) and chromosome-specific probes (Wijbrandi et al. 1990) will be useful in establishing a relationship between marker loss and elimination of specific chromosomes.

In three sublines (H1-9, H60-2, H60-12) GUS activity was considerably reduced, but still detectable, whereas kanamycin resistance in these sublines remained unchanged. The independent loss or reduction of GUS activity was also observed to occur at high frequencies in transformed root clones from diploid and tetraploid potato genotypes (Gilissen et al. 1991; Ottaviani and Hänisch ten Cate 1991), and this can be attributed to altered expression of the CAMV35S promoter of the GUS gene.

The patterns of the loss of marker characters in the hybrid suspension cultures and sublines indicate that the TL-DNA marker (hormone autotrophy) segregates independently from the T-DNA markers of the vector (kanamycin resistance and GUS activity). Since the loss of the TR-DNA marker (opine synthesis) was only found to occur in combination with the simultaneous loss of the TL-DNA markers and vector markers, the TR-DNA is suggested to be linked to the TL-DNA, and also to the vector T-DNA, but on a different chromosome. Therefore, these results are complementary to those of Visser et al. (1989), who from the inheritance pattern of individual transformation marker characters in progeny from crosses with untransformed plants suggested that in diploid potato the insertions of both Ri-T-DNAs and vector T-DNA occurs only at the same genetic locus.

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