COMPARISON OF HOMOEOLOGOUS RECOMBINATION FREQUENCY BETWEEN MITOTIC AND MEIOTIC POLYPLOIDIZATION IN BC_1 PROGENY OF INTERSPECIFIC LILY HYBRIDS

Ki-Byung Lim, M.S. Ramanna and J.M. van Tuyl
Plant Research International, Business Unit Genetics and Breeding,
P.O. Box 16
6700 AA, Wageningen
The Netherlands
k.b.lim@plant.wag-ur.nl

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Abstract

Wide interspecific hybrids of lily generally show F_1-sterility. For overcoming this crossing barrier mitotic polyploidization (artificial chromosome doubling) or meiotic polyploidization through unreduced gametes can be applied. For introgression of the desirable characters from a donor into the recipient, homoeologous recombination is essential. 2x-gametes and 2n-gametes were compared with respect to the frequency of homoeologous recombination between parental chromosomes, in the F_1-hybrids as well as in BC_1 progenies.
GISH (genomic *in situ* hybridization) revealed that the **LR** and **LA** hybrids (2n=2x=24) composted of each genome set of parental chromosomes. These two types of F_1-hybrids (**LR** and **LA**) were used as a male parent to *L. longiflorum* and Asiatic hybrids. Since the **LR**-hybrid was absolute sterile, the chromosome number of this hybrid was doubled artificially, which recovered its fertility. GISH results revealed that the BC_1 individuals (2n=3x=36), **LLR** and **ALA**, differed on homoeologous recombination frequency. Recombinant chromosomes were not be demonstrated in the mitotic chromosome complements of the **LLR**-hybrids. This lack of exchange between homoeologous chromosomes caused by complete preferential pairing of the **L**-**L** and **R**-**R** chromosomes during the meiosis of F_1-hybrid (**LLRR**). On the contrary, **ALA**-hybrids derived from functioning of 2n-gamete of **LA** hybrids showed a range of homoeologous recombinations between **L** and **A** chromosomes. Homoeologous recombinations were obtained by homoeologous pairing between **L** and **A** chromosomes during meiosis. As a conclusion, 2n-gametes producing F_1 interspecific hybrids have a high potential to introgress certain characteristics selectively.

1. Introduction

The genus *Lilium* has been classified into seven sections with over 80 species (Comber, 1949). Among them *L. longiflorum* belonging to section Leucolirion, Asiatics belonging to section Sinomartagon, and Orientals belonging to section Archelirion, are the most important species or sections for lily breeding. All three sections have several distinctive characters such as flower color and size, plant height, disease resistance (Lim, 2000). Due to such desirable traits belong to the different sections, interspecific hybridization has been carried out as breeding tool to introduce species-specific traits into other species or cultivars. Wide interspecific F_1-hybrids in general show sterility both male and female due to chromosome disturbances during meiosis. Mitotic chromosome doubling by treatment with colchicine and oryzalin is one way to overcome this problem (Van Tuyl *et al*., 1992). In this case, full chromosome sets are duplicated without any chromosome. Therefore, homologous chromosomes can pair and disjoin to the both poles as normal meiotic division. Unreduced gametes as an alternative can be employed to overcome F_1 sterility of wide interspecific lily hybrid (Lim, 2000). For the successful introgression of distantly related chromosome segments into cultivar or other genotypes, the use of unreduced

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gametes of interspecific allodiploid is preferred (Lim, 2000). Unreduced gametes are rarely produced during micro- and megasporogenesis (Ramanna, 1979). In Monocotyledonae, few genera such as Bromeliaceae, Gramineae, Musaceae and Orchidaceae are known to produce 2n-gametes (Ramanna, 1983; Veilleux, 1985). However, to retain maximum heterozygosity of parental chromosomes into the subsequent generation the mitotic polyploidization is preferred (Veilleux, 1985; Lim *et al*., 2000). In this case, whole chromosome(s) can be transmitted rather than chromosome segment(s) (Lim, 2000).

2n-gametes can be used for relatively efficient breeding schemes, which consist of elite diploid germplasm to tetraploids cultivar through unilateral (4x × 2x) and bilateral (2x × 2x) crosses in potato (Mendiburu *et al*., 1974; Peloquin, 1982). However, the use of 2n-gametes for the efficient introgression of useful genes in lily breeding program is not set up yet. We only found several 2n-gametes producing plants from interspecific hybridization between *L. longiflorum* and Asiatic hybrid (Lim, 2000).

Homoeologous recombination represents one of the most reliable ways of achieving stable gene introgression (Koebner and Shephaerd, 1986; Islam and Shepherd, 1992). It delivers desirable genes within a segment of DNA whose physical environment, in terms of chromatin structure and configuration, matches that of the recipient homoeologous genome to a high extent. The resulting homoeologous chromosomes usually have a hom(oe)ologous pairing partner at the next meiosis and, therefore, can be stable incorporated into gametes.

GISH provides a new tool for effective parental genome analysis in both sexual and somatic hybrids. This technique also allows detecting translocations involving chromosomes from different genomes (Kenton *et al*., 1993). Therefore, the extent of introgression in BC_1 progenies between different species can be analyzed by GISH analysis.

In this study, BC_1 progenies derived from 2n-gametes of interspecific diploid (meiotic polyploidization) and 2x-gametes of amphidiploid plant (mitotic polyploidization) were compared to analyze homoeologous recombination frequency by genomic *in situ* hybridization.

2. Material and methods

2.1. Plant materials

Two different pollen, 2n-gametes from interspecific **LA**-hybrids and 2x-gametes from artificially chromosome-doubled amphidiploid **LLRR**, were used for the subsequent progenies (Fig 1). The detailed information on the materials is described earlier (Lim, 2000). Since F_1-hybrids (*L. longiflorum* 'Gelria' × *L. rubellum*) showed absolute sterility, mitotic polyploidization was performed by *in vitro* treatment of the bulb scales in 0.003 % oryzalin (3,5-dinitro-N^4, N^4-dipropylsulfanilamide) solution for 3 hours. The selected amphidiploid (**LLRR**) plants resulting from artificial chromosome doubling were checked for pollen viability by lactophenol-fuchsin staining and pollen germination test. The **LLRR**-hybrids were used as male parent in backcrossing with both *L. longiflorum* and Oriental hybrids (2n=2x=24). Over a hundred BC_1 plants (**LLR**) were obtained and nine of them were used for GISH analysis (Table 1).

Three diploid (2n=2x=24) 2n-gametes producing interspecific hybrids of *L. longiflorum* (**L**) 'Gelria' × Asiatic hybrid (**A**) 'Whilito' as well as their backcross progenies were used for GISH analysis. The hybrids were produced through integrated pollination and embryo rescue methods (Van Tuyl *et al*., 1991). **LA**-hybrids (F_1) were known to produce viable 2n-pollen ranging about 15 %. In order to produce backcross progenies, four different Asiatic hybrids (all diploid) were used as female parents and back crossed to the three above mentioned **LA**-hybrids. Among four female parents, three were cultivars ('Montreux', 'Puccini' and 'Meribel') and one genotype was a breeding parent ('78251'). Plants were grown in a greenhouse at 20 25 °C during the day and 14 – 18 °C during the night.

2.2. Chromosome preparation

In the early morning roots were collected in the saturated α-Bromonaphtalene solution,

allowed overnight at 4 °C, fixed in ethanol – acetic acid solution (3:1) for at least 2 hours. Root tips were treated with 10mM citric acid buffer containing 0.3 % pectolytic enzyme mixture for about 1 hour at 37 °C, washed with mQ water for twice and squashed with fine needles after dropping 60 % acetic acid solution. The slide preparations were quickly frozen by dipping in liquid nitrogen and cover slips were removed by razor blade from the slides. The slides were then dehydrated in absolute ethanol for a few minutes, dried and stored at –20 °C until use.

2.3. DNA isolation and probe preparation

The methods for the isolation of genomic DNA, sonication, nick translation for labeling of probe DNA were the same as described by Lim *et al*. (2000).

2.4. *In situ* hybridization

In situ hybridization protocols were carried out according to Lim (2000). Hybridization mixture consisted of 2x SSC, 50 % formamide, 10% (W/V) sodium dextran sulfate, 0.25 % SDS, 3.0 ng/µl *L. longiflorum* 'Snow Queen' as a probe DNA, 50-60 ng/µl herring sperm DNA as block DNA. Hybridization mixture was heated at 70 °C for 10 min and placed on ice for about 10 min and 40 ul hybridization mixture to each slide was applied, denatured at 80 °C for 10 min, and incubated overnight at 37 °C. Digoxigenin labeled probe DNA was detected with FITC-antidigoxigenin detection system (Boeringer Mannheim, Germany). All slides were counterstained with DAPI (4,6-diaminido-2-phenylindole) and/or PI (propidium iodide). Photographs were taken with ASA400 color negative film.

3. Results

3.1. Morphological observation

All F_1 interspecific hybrids showed intermediate phenotypic characters between the parents. However, the BC_1 progenies derived from 2n-gametes showed morphological segregation with respect to their parental characteristics. All **LLR**-hybrids (BC_1) showed intermediate in their phenotypic characters such as plant height, leaf shape and flower color (Lim *et al*., 2000). The physiological characters such as flowering time and disease resistance were similar among progenies.

3.2. Meiosis and pollen fertility of the F_1-hybrids

Three **LA**-hybrids produced about 5 - 30 % of viable 2n-gametes (Fig. 2c) while **LR**-hybrid was absolute sterile. For further crossing chromosome number of **LR**-hybrid has been doubled by treatment of oryzalin as mitotic polyploidization method and the **LLRR**-hybrid produced about 30 % fertile 2x-gametes. **LA**-hybrids have been known to produce FDR or IMR 2n-gametes (Lim, 2000). Therefore, pollen from **LA**-hybrids was used for crossing for subsequent generations.

The meiotic chromosome behavior of hybrids, **LA**- and **LR**-hybrid (Fig. 2a), demonstrated normal division until metaphase I. From anaphase I stage, however, chromosomes of **LA**-hybrid divided equationally as in anaphase II (speudohomoeotypic division; Gustafsson, 1935) (Fig. 2b). At the final stage of meiosis, about 30 % of cells were 2n-gametes and 12 % was germinated, respectively.

Meiotic chromosome disjoining of **LR**-hybrid at anaphase I was chaotic and showed disturbed division. At the end of meiosis, tetrads were formed with unbalanced chromosome amount and all of pollen was sterile. However, the meiosis of **LLRR**-hybrid showed perfect pairing between homologous chromosomes as bivalent and regular disjoining at anaphase I. At the end of meiosis, tetrad were formed with 2x-gametes.

3.3. GISH analysis of backcross progenies

2n-gametes producing interspecific hybrids has been successfully crossed with Asiatic hybrids for back cross program, however only few seedlings were available via embryo rescue method which carried out just before embryo is aborted.

LLRR (amphidiploid) has been satisfactory recovered for the pollen fertility by artificial chromosome doubling and lead to embryo formation. Over 100 plants from *L. longiflorum* 'Gelria' as female parent crossed with **LLRR** (amphidiploid) as male parent has been successfully produced by embryo culture.

The somatic chromosome complement of **LLR** (BC_1, allotriploid, 2n=2x=36) plants consisted of 12 chromosomes from *L. rubellum* and 24 chromosomes from *L. longiflorum* (Fig. 2e). No recombination on **LLR**, allotiploid BC_1 progenies, has been shown. The meiotic chromosome behavior of BC_1 plants (**LLR**) showed preferential pairing as bivalents between **L-L** and univalents (**R**) (Fig. 2f).

The BC_1 progeny (**ALA**) from **LA**-hybrids revealed homoeologous recombination between **L-A** chromosomes whereas the progeny (**LLR**) from amphidiploid (**LLRR**) showed no recombination between **L-R** chromosomes (Fig 2d, e). These progenies from amphidiploid showed intermediate phenotypic characters between parents like F_1.

LA-hybrid and amphidiploid (**LLRR**) has been crossed to Oriental hybrids to generate allotriploids (**OLA** and **OLR**) (Fig. 2g, Table 1). There was no homoeologous recombination detected by GISH. Because of few plants were available to analyze on these crossing combinations, the frequency of homoeologous recombination was not clearly determined.

4. Discussion

Interspecific hybridization has been employed on many crops to bring useful traits from wild species into the cultivar assortment. Once interspecific hybrids were made by embryo rescue methods, there is a problem with sterility of interspecific hybrid. Mitotic polyploidization has long been employed to overcome F_1-sterility of wide interspeicfic hybrids. However, this method has one great disadvantage in which hardly any homoeologous recombination takes place between two genome chromosomes. This fact revealed more clearly by Lim *et al*. (2000), that the main differences of mitotic and meiotic polyploidization methods were demonstrated in relation to introgression of alien chromosome segments from wild species into cultivars. Mitotic polyploidization method is still widely used for breeding purpose. As has seen in this result mitotic polyploidization hinders the homoeologous chromosome pairing and recombinations (Table 1). As expected, after somatic chromosome doubling, the amphidiploid (**LLRR**) F_1-hybrid showed only bivalent formation between **L-L** and **R-R** homologous. Because of their duplicated homologous chromosome, pairing condition is predominant for preferential (homologous) chromosome pairing than homoeologous pairing. However, in case of **LA**-hybrid the forced pairing is obligated between two different genome chromosomes. This type of forced pairing was also seen in **LR**-hybrid, which was the situation before chromosome doubling. Although a high degree of genomic differentiation was found, the **LR**-hybrid formed 3.2_{II} on average with a maximum of five bivalents (Fig. 2a).

One great disadvantage of the somatic chromosome doubling is the occurrence of homologous pairing in the amphidiploid, which reduces the prospects for intergenomic recombination dramatically. This explains why recombinant chromosomes are absent in all the BC_1 plants analyzed. Furthermore, the BC_1 plants (**LLR**) showed perfect preferential pairing which resulted to 12_{II} (**L-L**) + 12_I (**R**) (Fig. 2f) which lead to aneuploidy gametes which is transmitted into next generation (BC_2). All BC_2 progenies indeed showed aneuploid in their somatic chromosome number (Lim *et al.*, 2000).

In our results of **LA**-hybrid, five out of nine BC_1 progenies have shown homoeologous recombinations with different level of crossover from 3 to 5 breakpoints. These homoeologous recombinant segments could be transmitted into next generation by forming fertile 2n-gametes through abnormal meiotic division processes such as FDR and IMR. Successful use of FDR gametes for transfer of a specific trait like resistance gene to the root-knot nematodes of 2x germplasm into 4x level was performed in potatoes (Iwanaga *et al*., 1989). Among BC_2

population, we could see a range of genetic variation such as color of leaf and bulb in a young seedling stage.

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Table 1. A comparison of the extent of homoeologous recombination estimated in the BC_1 progenies derived from meiotic and mitotic polyploidization of **LA** and **LR** F_1-hybrids.

Crossing types Type of crosses [z]	2n-gametes		2x-gametes	
	AA × LA	**O × LA**	**LL × LLRR**	**OO × LLRR**
Genome constitution (BC_1)	**A LA**	**O LA**	**L LR**	**O LR**
No. of progenies analyzed	8	1	9	2
No. of progenies with homoeologous recombinations	5	0	0	0
% of progenies with recombinant chromosomes	55.5		0.0	

[z] **AA**=diploid *L.* Asiatic hybrid, **LA**=2n-gamete producing **LA**-hybrid, **OO**=*L.* Oriental hybrid, **LL**=*L. longiflorum* hybrid, and **LLRR**=tetraploid **LR**-hybrid from mitotic chromosome doubling.

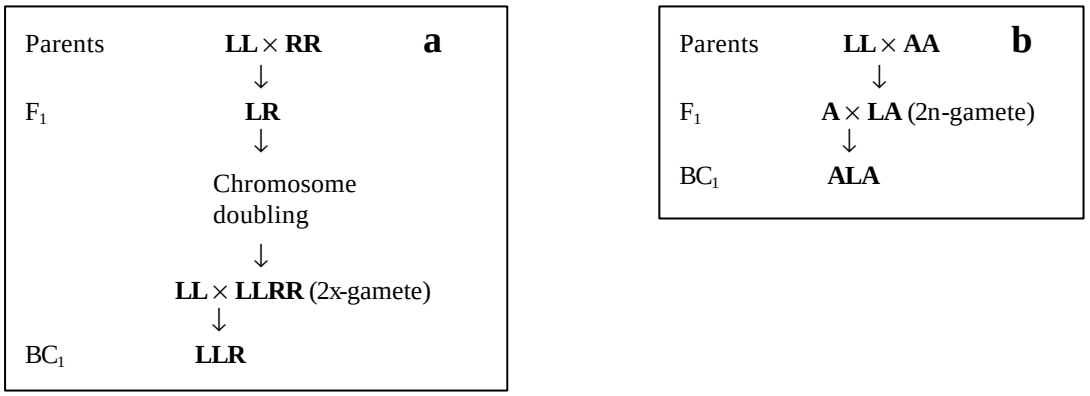
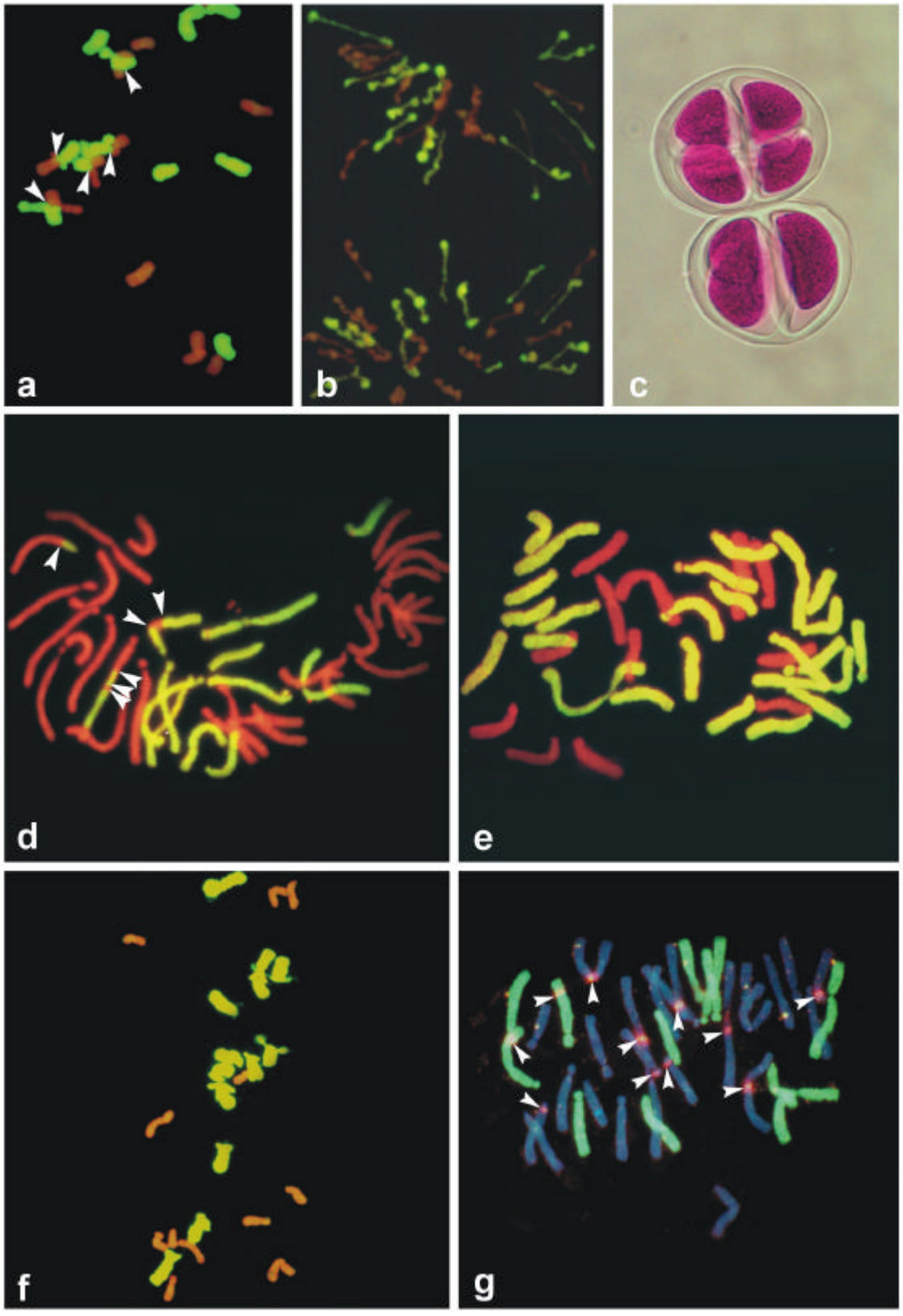


Figure. 1. Diagram of parentage of two different ways of polyploidization. **a**: Parentage of mitotic polyploidization. **b**: Parentage of meiotic polyploidization.

Figure 2. Meiotic stages and mitotic chromosome complements in the F_1-hybrids of **LR**- and **LA**-hybrids as well as the backcross progenies **ALA**, **LLR** and **OLR**. (**a**) Chromosome differentiation due to GISH in metaphase I of **LR**-hybrid, showing 5 bivalents (arrowheads) and 14 univalents. (**b**) A modified anaphase I stage showing equational division in the pollen mother cells of **LA**-hybrid. (**c**) A spore tetrad and dyad in **LA**-hybrid. (**d**) Somatic chromosomes of **ALA**-hybrid (BC_1) plant showing 24 **A** (red fluorescence) and 12 **L** (yellow fluorescence) chromosomes. Note the presence of four homoeologous recombinant segments (arrowheads). (**e**) Triploid chromosome constitution of BC_1, **LLR**-plant, 24 **L** chromosomes (yellow fluorescence) and 12 **R** chromosomes (red fluorescence). Note the total absence of recombinant segment in this plant. (**f**) Meiotic metaphase I stage in **LLR**-hybrid showing 12 bivalents of **L**-**L** (yellow fluorescence) and 12 univalents of **R** (red fluorescence). Absence of trivalent formation in such plants confirms that there is no homoeologous recombination. (**g**) Mitotic chromosome complement of **OLR**-hybrid with 24 chromosomes of **O** and **R** genomes (blue fluorescence) and 12 chromosomes of **L** genome (yellow green fluorescence). Because the genomic DNA of **R** genome was used as a probe, the closely related **O** and **R** genomes (both of the section Archelirion) in the trispecific hybrid was not differentiated due to GISH. Note the absence homoeologous recombination between **L** and **R** chromosomes. Red signals (arrowheads) represent 45S rDNA position on the *L. longiflorum* chromosome and *L*. Oriental including *L. rubellum* chromosome.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