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Molecular marker studies in groundnut (*Arachis hypogaea* L.)

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Historical background

Despite being named a 'nut', groundnut or peanut, is actually more a 'pea' than a 'nut' since it is a legume i.e. it bears fruit in the form of pods, though below the ground. Groundnut has acquired prominence because of its economic importance as well as its nutritional value. Today it is grown in all the six continents as an important oil and food crop in approximately 32 million hectares in over 100 countries (lanra.anthro.uga.edu). It is the third major oilseed crop of the world next to soybean and cotton. The botanical name of groundnut, *Arachis hypogaea* L., is derived from two Greek words, *Arachis* (arachos) meaning a weed and *hypogaea* meaning below ground.

Centre of origin

The archaeological records support its cultivation between 300 and 2500 BC in Peruvian desert oases [1, 2]. The first written account of the crop is found with the Spanish entry into Hispanola in the year 1502, where the Arawak cultivated it under the name 'mani'. In Brazil a record exists around the year 1550 indicating it was known there as 'mandubi'. According to Hammons [3] the first probable domestication of groundnut took place in the valleys of Parana and Paraguay river systems in the Grain Chaco area of South America in the area of southern Bolivia and northwest Argentina. Seven centers of diversity (gene centers) have been identified in South America [4]. However, many questions about its history remain unanswered.

Taxonomical background: The present day cultivated groundnut is an allotetraploid (2n = 4x = 40) while most of the wild relatives are diploid (2n = 2x = 20). In addition to the domesticated species, 68 wild species have been described [5] and several additional ones have been collected. The genus *Arachis* has been divided into nine sections. The cultivated species belongs to section 'Arachis' which contains 22 diploid species, two tetraploid species, *A. hypogaea* and *A. monticola* and three aneuploid species (2n = 18). A natural cross between two diploid species coupled with chromosome doubling is believed to have given rise to the cultivated groundnut [6]. Attempts by groundnut breeders to experimentally recreate the interspecific hybrid by using known diploid *Arachis* species as parents have so far been without success. The question of which diploid wild species of *Arachis* have combined to form the cultivated tetraploid *A. hypogaea* has received much attention over the last four decades, both from classical genetics and breeding point of view and from the last decade till today on the basis of molecular studies. Despite much effort on this aspect the answer is still inconclusive. As many as 14 different wild species combinations have been proposed as probable progenitor species using different criteria as shown in Table 1. The probable progenitor species proposed on the basis of molecular data is discussed in the section 'Origin of cultivated species'. Groundnut (*Arachis hypogaea* L.) is a member of the family *Fabaceae* (*Leguminosae*) subfamily *Papillionaceae*, tribe *Aeschynomenae*, subtribe *Stylosantheae* and section *Arachis*.

Habitat: Groundnut is a self pollinating, indeterminate, annual legume. Cross-pollination at low rates of 1 to 6% has been reported [7]. Groundnut is cultivated between latitudes 35° S and 40° N but extending upto 45° N. It is not affected by day length since it is a day neutral plant. Temperatures between 25° and 30° C are optimum for its growth. Although it can be produced with as little as 400 mm of rainfall, the commercial production is done in areas with an average rainfall of 500 to 1000 mm. Rainfall during late stages of development seriously affects those varieties which have little or no dormancy.

Karyotype: Groundnut has 40 small chromosomes, which are mostly metacentric. They range in length between 1.44-4.17 µm. A cytological comparison among all the *A. hypogaea* botanical varieties was able to distinguish all in terms of number of asymmetrical chromosome per cultivar and in the chromosome with a secondary constriction [8]. The genetics and cytogenetics of groundnut have been reviewed earlier [9, 10, 11].

Genome size: The DNA amount in the unreplicated

haploid nuclear genome, the 1C value, for groundnut has been recorded in the range of 5.1 to 5.9 pg. In the diploid species the value ranges from 2.1 to 3.5 pg [12]. A later re-evaluation of genome sizes of *A. hypogaea* and *A. monticola* however, has estimated the amount to be half of the earlier reported value [13]. The 2C DNA amount was found to be 5.914 pg in *A. hypogaea* and 5.979 pg in *A. monticola*. The genome size of groundnut was taken as 3×10^9 bp by Ferguson *et al.* [14].

Origin of cultivated groundnut: Various studies have confirmed genomic differences among the species in the section *Arachis* [5, 15]. Most of the species in this section possess a distinct small pair of chromosome that has been designated as 'chromosome A'. Some other species like *A. batizocoi, A. ipaensis* and *A. williamsii* do not have the A chromosome but instead have a pair of chromosome with secondary constriction and satellite (chromosome B). Only seven wild species (two undescribed) have the B chromosomes. *A. monticola*, a wild tetraploid possesses both the A and B chromosome complement (AABB) just like *A. hypogaea*. Synthetic amphidiploids have been produced by crossing *A. batizocoi* (BB) with various species (AA). But the plants had neither the genome nor the morphology of the cultivated species. However, until 1991 *A. batizocoi* was the most proposed B genome donor species.

When DNA based marker analysis studies were undertaken in *Arachis*, the proposed progenitors changed. RFLP data did not support *A. batizocoi* as one of the progenitor species. Instead *A. ipaensis* was proposed as one of the progenitors with the other being either *A. duranensis* or *A. spegazzinii* [16]. The results also showed that *A. monticola* was virtually identical to *A. hypogaea*. Till today all the molecular data imply that *A. batizocoi* is very distant from *A. hypogaea*. On the basis of DNA studies many possible combinations of diploid species have been proposed. Among the four diploid species compared using RFLP, *A. duranensis* was found to be the closest to *A. hypogaea* [17]. Similar results were obtained by RAPD analysis [18]. Based on RFLP and cytogenetic evidence it was proposed that *A. ipaensis* and *A. duranensis* are the genome donors to the cultivated groundnut and that *A. duranensis* was the female parent [19]. They suggested that the present day cultivated groundnut arose as a result of a hybridization event between the wild species *A. duranensis* (A genome donor) and *A. ipaensis* (B genome donor) followed by chromosome doubling. The latter event could have isolated the tetraploid *Arachis* from its diploid wild relatives. Because of this isolation no further introgression from *Arachis* wild species could occur. Perhaps this has led to the low level of genetic variability observed in the present day groundnut genotypes, even when assessed by various sensitive molecular marker techniques.

RAPD analysis comparing 11 diploid species with the two tetraploid species showed that the three species forming a cluster closest to the tetraploids were *A. chacoense, A. stenosperma* and *A. correntina* [20]. Singh and Smartt [21] revisited the available data and concluded that although *A. duranensis* and *A. ipaensis* seem probable progenitors on the basis of molecular similarities, until an amphidiploid was produced synthetically and was crossed successfully with *A. hypogaea, A. batizocoi* would remain the most probable progenitor species. Comparison of RAPD and ISSR profiles revealed that *A. villosa* and *A. ipaensis* were the most probable genome donors [22]. AFLP analysis also pointed out that *A. duranensis* and *A. ipaensis* were closest to the tetraploid species [23].

Raina and Mukai [24] carried out genomic in situ hybridizations (GISH) to conclude that *A. villosa* and *A. ipaensis* were the progenitors. They also carried out fluorescent *in situ* hybridization (FISH) studies at ribosomal DNA loci using wheat rDNA as a probe [25]. These studies endorsed their earlier GISH results. Jung *et al.* [26] compared the gene sequence for stearoyl-ACP desaturase and oleoyl-PC desaturase among the wild diploid and tetraploid species and found that one of these gene sequences was identical with that from *A. duranensis* and the other was identical to *A. ipaensis* sequence. Hence they suggested that these two species must be the progenitors of tetraploid groundnut. Their comparison also indicated very little homology with *A. monticola* indicating that it is not a direct progenitor of *A. hypogaea*. Bhagwat *et al.* [27] cloned the rDNA repeat from groundnut. Using this as probe, the RFLP analysis among the diploid species revealed that *A. correntina* and *A. cardenasii* were close to *A. hypogaea* but *A. monticola* was distantly related. When a combined phylogenetic tree was generated using 198 RAPD loci, and 33 rDNA RFLP loci, the tetraploid cluster was closest to *A. ipaensis*. The sequence comparison of the two ITS regions along with 5.8s rRNA gene from five closely related diploids with the two tetraploids indicated that *A. ipaensis* sequence is closest to *A. monticola* while *A. hypogaea* was closest to *A. duranensis* [Bhagwat and Krishna, unpublished data]. Probably the two tetraploids share only one of the genome donors.

Burow *et al.* [28] ruled out *A. cardenasii, A. diogoi* and *A. batizocoi* as the probable ancestors of *A. hypogaea* by studying the transmission genetics in a cross involving a synthetic amphidiploid and groundnut. Singh *et al.* [29] compared RFLP at ribosomal loci

among the wild species using wheat and *Vicia faba* rDNA probes and concluded that *A. batizocoi* could not be the progenitor since it lacked the *Hin*dIII site. Seijo *et al.* [30] did physical mapping of 5s and 18s-25s rRNA genes by FISH and concluded that *A. duranensis* and *A. ipaensis* are the most likely progenitors of cultivated groundnut.

Scientists believe that both the diploids and the cultivated species have genetically diverged over time or possibly the 'true progenitor' diploid species is still to be discovered. Given the fact that still new species in the section *Arachis* continue to be identified, it is reasonable to suspect that the true progenitors are yet to be discovered [31]. The main reason behind efforts in identifying the progenitor species in a crop like groundnut is because the cultivated crop lacks the genes for resistance to many diseases and pests that reduce the yield. Many of the wild species screened for these diseases showed resistance [4, 32].

Classical mapping: Genetic information on linked phenotypic characters in groundnut was too meager to construct a linkage map until 1993 when substantial number of polymorphic molecular markers became available in the diploid groundnut species. The first genetic linkage map based on RFLP was constructed using a cross between the two diploid species *A. cardenasii* and *A. stenosperma* [33].

Limitations of classical maps and use of molecular mapping: Basic genetic studies in groundnut, in contrast to other crops, have progressed at a very slow pace. This is at least partially due to the limited acreage devoted to groundnut production, as compared to other major agronomic crops, and the relative importance of the crop as a staple food in less developed countries. Also cytologically it is a difficult crop to work with, due to small chromosome size with few distinctive cytogenetic markers making identification of individual chromosome tedious. Groundnut productivity has many constraints like fungal and viral diseases and a range of pests. A limited source of resistance exists for these traits within the *A. hypogaea* gene pool. Although induced mutation has yielded variability in some of these traits, introgression through wild species has given the limited success achieved so far. Hence, trait utilization with linked molecular markers that can be easily scored at an early stage in the hybrid derivatives is perhaps the key in future for faster progress in groundnut breeding.

Genetic maps: Since the early nineties when molecular marker analysis of the cultivated groundnut began, very low level of polymorphism was detected. Kochert *et al.* [16] observed very low levels of RFLP variability among allotetraploid US cultivars and *A. monticola*. Halward *et al.* [34, 35] expanded the study to unadapted germplasm lines from various South American centers of origin, Africa and China and also analyzed it using three methods RFLP, RAPD and CAPS, but found the same low variability. Lanham et al. [36] identified 49 polymorphic RAPD loci after screening 60 random primers between groundnut cultivar TMV2 and a synthetic amphidiploid $(B \times C)^2$ created from the cross between *A. batizocoi* and *A. chacoense*. He and Prakash [37] used DAF and AFLP techniques to survey the cultivars. They found 43% of AFLP primers and 3% of DAF primers could detect polymorphism. Out of 559 DAF primers 17 detected polymorphism, giving 3.7 polymorphic bands per primer and a total of 63 polymorphic bands. Out of 64 AFLP primer pairs 28 detected polymorphism giving 6.7% of bands polymorphic and a total of 111 AFLP markers. Bhagwat *et al.* [38] analyzed radiation induced direct mutants of cultivar Spanish Improved by RAPD and were able to detect variation among the different plant height mutants and pod size mutants.

Hopkins *et al.* [39] picked up 128 clones hybridizing to microsatellite probes of which 66 had SSRs. From these about 26 primer pairs were synthesized. Five primer pairs produced polymorphic bands on amplification in both cultivated and the wild species. A primer pair amplifying an $(AT)_{18}$ SSR located in the 3' untranslated region of the lectin gene amplified a total of 14 fragments and 10 out of the 20 groundnut accessions could be differentiated. Although only 6 polymorphic SSRs were identified, they could detect more variation in groundnut than any other molecular marker system studied. Subramanian [40] studied RAPD differences among 70 selected genotypes with 48 primers, seven of which detected 27 polymorphic bands out of 408 total bands. Dwivedi *et al.* [41] assessed genetic diversity among 26 accessions using eight random primers and identified five accessions with diverse profiles for mapping and genetic enhancement studies. Bhagwat *et al.* [42] reported a high degree of polymorphism among closely related 14 groundnut genotypes using a single RAPD primer which did not reveal a similar degree of polymorphism in other legumes like mung bean or blackgram. Raina *et al.* [22] carried out RAPD and ISSR analysis among cultivars and found RAPD detected 42.7% while ISSRs detected 54.4% polymorphism among 220 and 124 genetic loci amplified from 13 accessions. Singh *et al.* [29] studied ribosomal DNA repeat unit polymorphism among groundnut accessions and could distinguish the two subspecies using the variable *Bam*HI and *Eco*RV restriction site.

He *et al.* [43] constructed a microsatellite library by an enrichment method. Out of 56 primer pairs designed 19 showed polymorphism. The average number

of alleles per locus was 4.25 and the maximum number of alleles at one locus was 14. GA/CT repeat was the most frequently dispersed microsatellite in groundnut. Moretzohn *et al.* [44] found that of the 67 TTG based microsatellite markers screened only three were polymorphic in cultivated groundnut. In a latest exhaustive study by Ferguson *et al.* [14] about 110 STMS markers have been generated for the cultivated groundnut. This was a result of searching two genomic libraries of 27,548 clones each, one made by *Pst*I digestion and the other by *Sau* 3AI/*Bam* HI. The libraries were screened with two dinucleotide repeats (GT and GA), seven trinucleotide repeats (AAC, ATC, AGT, ATT, CAC, CTT and CTG) and in addition the *Pst*I library was screened with three tetranucleotide repeats (ATCC, GATA and AAAT). The most frequent repeat motif was ATT (29%) followed by GA (28%) which was followed by AT > CTT > GT. About 81% of ATT and 70.8% of GA repeats were polymorphic. They found no consistent relationship between number of repeats and number of alleles, either across all motifs or individually. The widest variation in number of alleles was between 12 and 32 repeats.

In a subsequent paper Ferguson *et al.* [45] used 10 of the STMS markers to analyze the distribution of diversity at molecular level within and among botanical varieties from three continents. The results did not support inclusion of var *peruviana* in the subspecies *fastigiata*. The results also suggested that subspecies *hypogaea* and var *hirsute* should not hold the same subspecies ranking. Using these markers along with the earlier RFLP and RAPD markers construction of a high resolution map in groundnut will soon be possible in a cross involving two diverse tetraploid cultivars.

Molecular marker maps: The first genetic map constructed for groundnut was by Halward *et al.* [33] using a cross between two diploid species *A. stenosperma* and *A. cardenasii*. RFLP markers were used from genomic as well as cDNA libraries of groundnut *A. hypogaea* cv GK7. Partial genomic library was constructed by *Pst*I digestion of genomic DNA and cloning the 1-2 kb fragments. The cDNA libraries were made from shoot and root. The F_2 population (87 individuals) was analyzed at seven restriction sites (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII and *Rsa*I). Out of the 100 genomic and 300 cDNA probes used in the study 15 and 190 respectively, gave polymorphic profile between the parents. Of the 205 probes that showed polymorphism, 132 were analyzed for segregation since the rest 73 revealed complex patterns and hence could not be mapped. Of the 132, 33 loci showed deviation from the expected ratio ($P<0.05$) most having an excess of one or the other parental type, four had excess number of heterozygotes. Of the 117 segregating loci distributed in 11 linkage groups only four were genomic probes. It is interesting to note that the polymorphism revealed by cDNA probes was more than the genomic probes. A map distance of 1400 cM was covered with a 20 cM resolution representing 80% coverage of the groundnut genome.

First generation map: The next map constructed was with one parent being the cultivated groundnut and the other being the diploid species *A. cardenasii* [46]. RFLP and RAPD analysis was carried out to test the marker suitability in studying the introgression of the nematode resistance trait. The introgression lines were derived by colchicine treatment of the triploid F_1 hybrid resulting from the cross. The hexaploids were selfed for five generations when the progeny had 40 chromosomes and morphologically they ranged from almost identical to the tetraploid parent to those similar to the wild species. The introgression lines were examined after nine generation of selfing. This population ($F_{10}BC_9$) was examined with 73 RFLP markers of known map position covering all the linkage groups. The parents were screened with about 270 RAPD primers. About 70 primers that showed *A. cardenasii* specific bands were used to analyse the introgression lines. It was observed that about 360 cM of the diploid genome was introgressed into the tetraploid genome or about 30% of the diploid genome. Three lines did not show any introgression. One line showed the highest introgression, which was 176 cM (16%). A total of 34 cDNA RFLP probes and 45 RAPD primers identified introgressed chromosomal segments in one or more lines. The introgression segments covered 10 out of the 11 linkage groups, smallest of which was a RFLP marker and the largest had 3-4 adjacent markers at a distance of 30-40 cM.

Garcia *et al.* [46] also used a backcross population *A. stenosperma* × (*A. stenosperma* × *A. cardenasii*) and 39 shared RFLPs to place 167 RAPD loci onto the RFLP map. The RAPDs covered a total genetic length of 800 cM and mapped to 11 linkage groups. A reduction in the recombination fraction was observed in the RAPD backcross map as compared to the RFLP map, which was constructed using an F_2 population [47].

Recently the first partial genetic linkage map of the cultivated groundnut was published where both the parents were *A. hypogaea* lines [48]. They screened a total of 308 AFLP primer combinations and identified twenty markers of which 12 mapped to five linkage groups covering a map distance of 139.4 cM.

Second generation maps: The first molecular map representing the entire tetraploid genome of groundnut was constructed by Burow *et al.* [28]. Variability was

introduced from three diploid species into the tetraploid using a synthetic amphidiploid T × AG-6 [*A. batizocoi* × (*A. cardenasii* × *A. diogoi*)]^{4X}. The tetraploid breeding line T × AG-6 was used as a donor parent and *A. hypogaea* cv Florunner as recurrent parent. Seventy eight backcross (BC_1) progenies were used as the mapping population. *Eco*RI and *Hind*III digested genomic DNA of the BC_1 plants were blotted and hybridized to 220 probes. These probes were from root and shoot cDNA libraries of *A. hypogaea* cv GK7 and *Vigna* cDNA clones, together representing 370 RFLP loci. The total map distance covered was 2210 cM which was distributed among 23 linkage groups. These linkage groups composed of nine pairs of homeologous groups, one trio representing a homeologous chromosome pair, one fragment consisting of two markers and one linkage group that was probably an artifact. Given that cultivated groundnut is disomic polyploid (2n = 4x = 40), 20 linkage groups were expected. A total of 917 bands were observed at an average of 4.1 bands per probe. A mean of 1.68 loci per probe were mapped. The total length of the tetraploid map was slightly greater than twice the diploid map. The average spacing of markers was 5.7 cM. With a total of 135 STMS markers now available along with the earlier 352 RFLP and 167 RAPD markers, construction of a high density genetic map in groundnut will soon become a reality.

Gene mapping: Two genes were mapped for resistance to the nematode, *M. arenaria* [49]. They used two F_2 families of 129 and 135 plants each derived from a single F_1 plant from a cross of GA6 which was a nematode resistant introgression line (*A. hypogaea* × *A. cardenasii*, 2n = 40) crossed to susceptible *A. hypogaea* PI 261942. The gene symbol used to describe egg number was *Mae* and for galling was *Mag*. Bulked Segregant Analysis (BSA) was performed on the progeny using 450 RAPD primers. One RAPD marker Z3/265 was found to be linked to both *Mag* and *Mae* at a distance of 10 ± 2.5 cM and 14 ± 2.9 cM respectively. This marker was converted into a Sequence Characterized Amplified Region (SCAR). This marker was mapped to linkage group-1 at 5 cM distance from *Xuga.cr* 239 in the backcross map, in an area where introgression from *A. cardenasii* was previously reported.

Molecular marker assisted introgression: The first practical use of MAS was done by Burow *et al.* [50] who identified three RAPD markers linked to *M. arenaria* resistance in several breeding populations derived from T × AG-7 in the fifth backcross generation. The resistance in each of the population was derived from *A. cardenasii* by a single dominant gene. Choi *et al.* [51] identified three RFLP loci linked to nematode resistance at distance of 4.2, 5.2 and 11.0 cM. Church et al. [52] utilized two RFLP loci linked to a single gene of nematode resistance to select individuals homozygous for resistance in a segregating population. Two of these markers could be used with a high degree of confidence since they were sufficiently close to the trait. Stalker and Mozingo [53] reported association of RAPD markers with a gene conferring resistance to *Cercosporidium arachidicola* sporulation, lesion diameter, defoliation and overall rating. A marker was also associated with resistance to corn rootworm damage. In addition they associated markers with *Cylindrocladium* black rot resistance and sporulation to *C. arachidicola* in a cross between cultivar NC7 and PI 109839, the first report of molecular markers being associated with resistance genes in an *A. hypogaea* × *A. hypogaea* cross. Herselman *et al.* [48] identified AFLP markers linked to aphid resistance. A single recessive gene was mapped on linkage group 1, at a distance of 3.9 cM from a marker originating from the susceptible parent.

Transgenic groundnut: Introduction of single trait by genetic engineering route has been possible in groundnut. Well standardized tissue culture protocols for regeneration are now available [54]. The nucleocapsid protein gene of TSWV has been inserted both by microprojectile bombardment [55, 56] and via *Agrobacterium* mediated route [57]. ICRISAT has developed the first transgenic groundnut, which will undergo field trials for IPCV (Indian Peanut Clump Virus) and another with coat protein gene of Groundnut Rosette Assistor Virus (GRAV) for deployment in Africa.

Future scope of work: So far unambiguous identification of donor parents to the cultivated species has not been possible. More research perhaps with other less studied diploid species could provide an answer. Since more RFLP polymorphism was detected with cDNA probes than genomic probes it might be rewarding to establish a groundnut EST database, identify EST-SSRs and use them as markers. Cultivated groundnut is susceptible to a number of diseases and pests and lacks the resistant sources. It would be worthwhile to study the resistant gene analogues in wild species based on Nucleotide Binding Site domains [58], tag them with markers to aid in marker assisted breeding. Concerted efforts should also be made to establish synteny of groundnut with model legumes like *Lotus* and *Medicago*.

As molecular markers accumulate in groundnut, the resulting high-density linkage map will become a useful tool for breeding programs, as breeders will be able to tag and follow introgression of specific chromosome segments containing desirable genes from either wild species or mutant derivatives into the desired genetic background minimizing the genetic drag. Our understanding of genetic segregation and linkage

Table 1. Donor genomes to cultivated groundnut proposed to date

	Genome A	Genome B	Criterion	Reference
A. hypogaea	*A. cardenasii*	*A. batizocoi*	Phytogeography	[59]
	A. cardenasii	A. batizocoi	Cytogenetics	[60]
A. hypogaea	*A. cardenasii* or *A. duranensis*	A. batizocoi	Arachin Seed storage proteins	[61]
A. hypogaea ssp *fastigiata*	*A. duranensis*	*A. batizocoi*	Cytogenetic	[62]
ssp *hypogaea*	*A. villosa*	*A. batizocoi*	Cytogenetic	[62]
	A. duranensis	*A. cardenasii*	RFLP	[17]
	A. duranensis	?	RAPD	[18]
	A. ipaensis	*A. duranensis*	RFLP + Cytogenetics	[19]
A. hypogaea	*A. duranensis*	*A. batizocoi*	Cytogenetic	[21]
A. hypogaea	*A. ipaensis*	*A. villosa*	GISH	[24]
A. hypogaea	*A. ipaensis*	*A. villosa*	FISH	[25]
A. hypogaea	*A. ipaensis*	*A. villosa*	RAPD+ISSR	[22]
A. hypogaea	*A. duranensis*	*A. ipaensis*	Gene sequence comparison	[26]
A. hypogaea	*A. duranensis*	*A. ipaensis*	FISH of rRNA gene loci	[30]

relationships of useful traits will be greatly enhanced as the map gets saturated, since the major yield controlling traits are quantitative. Incorporating high levels of stress resistance into high yielding cultivars with acceptable market traits will then become a reality.

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