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A Dwarf Mutant of *Arabidopsis* Generated by T-DNA Insertion Mutagenesis

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Most plant genes that control complex traits of tissues, organs, and whole plants are uncharacterized. Plant height, structure of reproductive organs, seed development and germination, for example, are traits of great agronomic importance. However, in the absence of knowledge of the gene products, current molecular approaches to isolate these important genes are limited. Infection of germinating seeds of *Arabidopsis thaliana* with *Agrobacterium* results in transformed lines in which the integrated T-DNA from *Agrobacterium* and its associated kanamycin-resistance trait cosegregate with stable, phenotypic alterations. A survey of 136 transformed lines produced plants segregating in a manner consistent with Mendelian predictions for phenotypes altered in height, flower structure, trichomes, gametogenesis, embryogenesis, and seedling development. This report is the characterization of a dwarf mutant in which the phenotype is inherited as a single recessive nuclear mutation that cosegregates with both the kanamycin-resistance trait and the T-DNA insert.

TRANSPOSABLE ELEMENTS HAVE been successfully used to tag genes in a limited number of eukaryotic organisms. Transposons simultaneously mutagenize and tag genes when insertion of the transposable element into a DNA region modifies expression of the gene. A number of genes have been identified and isolated by this technique in *Caenorhabditis elegans* (*1*), *Drosophila* (*2*), *Antirrhinum* (*3*), *Zea mays* (*4*), and mouse (*5*). However, because most transposons in plants occur in multiple copies with low transposition frequencies, detection of the insert responsible for the altered phenotype is difficult. Also, relatively few plant species contain endogenous transposons. Recently, the potential of using a transposable element from maize to tag genetic loci in transgenic plants was demonstrated (*6*).

An alternative method of tagging genes in plants is the use of the T-DNA of *Agrobacterium tumefaciens* as the insertional mutagen. A large number of plant species can be transformed with *Agrobacterium*, which integrates a portion of its DNA (T-DNA) into the plant genome (*7*). However, insertional mutagenesis via *Agrobacterium*-mediated transformation is inefficient in most higher plant species because of the abundance of noncoding DNA, which decreases the probability of insertion into a functional gene, and also because of the space required to grow hundreds of thousands of transformants and the plethora of variations introduced by the tissue culture process—that is, somaclonal variation (*8*). The use of *Arabidopsis thaliana* as an experimental organism to develop an *Agrobacterium*-mediated insertional mutagenesis system has several advantages. *Arabidopsis* has a haploid genome size of 70,000 kb consisting of only 10% to 14% highly repetitive DNA (*9*) and is sufficiently small that thousands of individually transformed plants can be grown separately to maturity in growth chambers or in a small greenhouse in a short time (4 to 6 weeks). However, somaclonal variation is a major obstacle in regenerating *Arabidopsis* (*10*), making it difficult to separate the effect of the T-DNA insert from the effect of the tissue culture process, even under the best transformation and regeneration conditions (*11*).

We previously reported a non–tissue culture method for transformation, which involves infecting germinating seeds of *Arabidopsis* with *Agrobacterium* and results in transformants that do not exhibit somaclonal variation (*12*). An initial population of more than 1000 transformants containing an average of 1.4 functional inserts was generated by the successful application of this seed transformation technique in three separate infection experiments (*12, 13*). Of the 156 families scored for resistance to kanamycin, 20 lines contained inserts that failed to segregate in a Mendelian fashion on selective medium, produced families with no kanamycin-resistant (Kan^R) progeny, or were lost (*12, 14*). The remaining 136 lines segregated for at least 190 independent functional inserts when scored on kanamycin. Screening of the 136 transformed lines in growth chamber conditions (*15*) produced 36 lines that segregated in a Mendelian fashion for a variety of altered phenotypes. These phenotypes include a dwarf (Fig. 1A) and other height variants, floral structure variants (agamous, Fig. 1B), stems lacking trichomes (glabrous, Fig. 1C), and embryo lethals (Fig. 1D). Genetic and molecular analyses of the dwarf mutant described in this report are consistent with the hypothesis that the T-DNA was inserted into a gene that in the homozygous state confers dwarfism to the plant.

The dwarf, in almost all characteristics, is a miniature of the wild-type plant, 7 to 8 cm in height compared to 30 to 40 cm for the wild type. The hypocotyls of dwarf seedlings are reduced in length, and the dwarf cotyledons have a square shape in comparison to the round, wild-type cotyledons. The leaves are short, dark green, and have an epinastic growth habit (Fig. 1A). The number of rosette leaves (six to eight; refer to cover) is similar to that of the wild type. Wild-type plants have three to five primary inflorescences when grown separately in 3-inch pots. Although dwarf plants initially have only one inflorescence, additional inflorescences arise as the plants age, so that by the time the plants senesce there may be more than 40 inflorescences arising from the base of the plant (*14*). This phenotype indicates a reduction in apical dominance. Seed set is reduced, owing in part to a failure of the stamens to elongate, thus decreasing the amount of pollen shed on the stigma. Seeds are reduced in size, more concave than those of the wild type, and have decreased viability with increasing age (*14*). Wild-type plants complete senescence in 8 to 9 weeks under the described growth conditions, whereas aging of dwarf plants is delayed 1 month.

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Physiological tests demonstrate that 2,4-dichlorophenoxyacetic acid, indoleacetic acid, or gibberellic acid do not correct the phenotype when given in the medium or sprayed on the foliage.

Genetic analysis of progeny from selfed and backcrossed plants demonstrate that the T-DNA is tightly linked to the *dwf* mutation (Tables 1 and 2) (*16*). More than 300 seeds from the single segregating Dwf family (T3-31) were plated on nonselective medium. Five days after germination, the seedlings were scored for wild-type (tall) or Dwf stature. A deficiency of Dwf seedlings was observed from the expected 3:1 ratio of tall to Dwf plants predicted for a single recessive nuclear mutation (237 tall, 57 Dwf). Subsequent testing of aging seeds from Dwf plants showed that reduced germination accounts for this difference (*14*). All further genetic crosses (T4 through T6 families) were done with fresh seeds to avoid this complication. To test for the segregation of the Kan^R marker in this family, tall and Dwf seedlings were transferred separately to medium containing kanamycin. The ratio of Kan^R to Kan^S (kanamycin-sensitive) tall plants was 2:1 (58 Kan^R:31 Kan^S), whereas the Dwf seedlings were all Kan^R. The T4 families resulting from selfing Kan^R tall T3 plants (heterozygotes) segregated 1 Kan^R Dwf:2 Kan^R tall:1 Kan^S tall (Table 1). When Dwf plants were selfed they produced homogeneous families of Dwf plants (Table 1). Kan^R tall plants were selfed for two additional generations and the families that resulted (T5 and T6) continued to segregate 1:2:1 [4850 progeny scored (*14*)].

Dwf plants were reciprocally crossed to wild-type plants (Table 2). The F_1 families were all Kan^R and wild type for height, showing that the *dwf*::K^RTi mutation (*16*) is recessive to *DWF*, and the Kan^R marker is dominant to the null allele (Table 2). The 1:2:1 segregation of F_2 families is consistent with the hypothesis that an insert containing a dominant marker has created a recessive mutation (Table 2). A recombination event between these two loci (generating a Kan^S Dwf) has never been observed. The cumulative data from both the backcrosses and selfs (6708 progeny) indicate that the two loci (Dwf and Kan^R) are tightly linked.

Table 1. Segregation of T4 families from selfed T3 Kan^R plants (*20*).

T_4	Segregation of T4 families				Mendelian ratio tested
	Kan^R Dwf	Kan^R tall	Kan^S tall	Kan^S Dwf	
		From Kan^R tall T3 plants			
T4-31-1*	41	81	34	0	1:2:1
T4-31-2	54	104	52	0	1:2:1
T4-31-3	62	142	54	0	1:2:1
T4-31-4	50	125	50	0	1:2:1
T4-31-5	46	131	52	0	1:2:1
		From Kan^R Dwf T3 plants			
T4-31d-1	47	0	0	0	1:0
T4-31d-2	50	0	0	0	1:0
T4-31d-3	41	0	0	0	1:0
T4-31d-4	85	0	0	0	1:0
T4-31d-5	49	0	0	0	1:0

*Chi-square tests against hypotheses were not significant.

Table 2. Segregation of the Kan^R and Dwf traits in reciprocal crosses to wild-type (wt) plants (*20*).

Cross		Family	Phenotype				Mendelian ratio tested
Female	Male		Kan^R Dwf	Kan^R tall	Kan^S tall	Kan^S Dwf	
				F1			
wt	Dwf		0	64	0	0	0:1:0*
Dwf	wt		0	137	0	0	0:1:0
				F2			
		1	38	78	36	0	1:2:1
		2	17	45	14	0	1:2:1
		3	16	33	16	0	1:2:1
		4	23	62	32	0	1:2:1
		5	24	50	30	0	1:2:1
		6	12	27	16	0	1:2:1
		7	20	69	32	0	1:2:1
		8	21	46	28	0	1:2:1
		Total	171	410	204	0	1:2:1

*Chi-square tests against hypotheses were not significant.

DNA hybridization analysis of Dwf plants indicates that the insert is a concatamer of four to six T-DNAs in direct and inverted repeats, typical of transformants made by C58 derivatives of *A. tumefaciens* (*12, 17*). Evidence for direct repeats comes from probing Hind III–cut genomic DNA from Dwf plants with DNA homologous to each of the borders of the T-DNA. The left and right border probes hybridize to a common enzyme fragment of 5.8-kb, a length appropriate for a left-right border junction (Fig. 2). In addition, the 6.1-kb fragment (LB,*) and 5.1-kb fragment (RB,*) are of the appropriate sizes to be inverted repeats of the left and right borders, respectively. The 2.7-kb fragment hybridizing to the RB probe is the T-DNA right border–plant junction fragment. The 6.7-kb fragment hybridizing to DNA homologous to the LB may represent the other T-DNA plant junction. The 2.9-kb fragment (RB) may be a rearranged piece of DNA as is the 2.8-kb fragment that hybridizes to pBR sequences (Dwarf, pBR lane). We have observed similar rearrangements in slightly more than 50% of the transformants (*18*). Two internal fragments of 7.5- and 4.5-kb (Dwf-pBR lane) hybridize to DNA sequences homologous to pBR, as expected. The 7.5-kb fragment hybridizing to the RB probe results from the homology of the 3′ end of the nopaline synthase (*nos*) gene in the probe with the *nos* 3′ end of the *npt* II gene in the 7.5-kb Hind III fragment.

To test for silent inserts or rearrangements of the insert, we isolated DNA from single dwarf plants of a T4 family segregating for the dwarf phenotype, restricted it with Hind III and probed it with DNA homologous to the right border. Seventeen single T4 Dwf plants have hybridization patterns identical to those of the original T3 Dwf::K^RTi isolates (*14*). We examined two additional generations of plants from the dwarf family (T5 and T6), both Kan^R tall heterozygotes and dwarf homozygotes, and the insertional pattern remained unchanged (*14*).

From the relatively small pool of transformants examined, we identified 36 lines from T3 families segregating in a Mendelian fashion for obvious morphological or physiological alterations. We showed that one altered phenotype (Dwf) cosegregates with a T-DNA insert. Other mutations examined (glabrous, agamous, and those affected in gametogenesis) also cosegregate with the Kan^R marker (*18*). If all 36 mutants identified in the population examined (136 T3 families) are insertional mutants, we will have been able to identify an altered phenotype induced by 19% of the inserts (36/190).

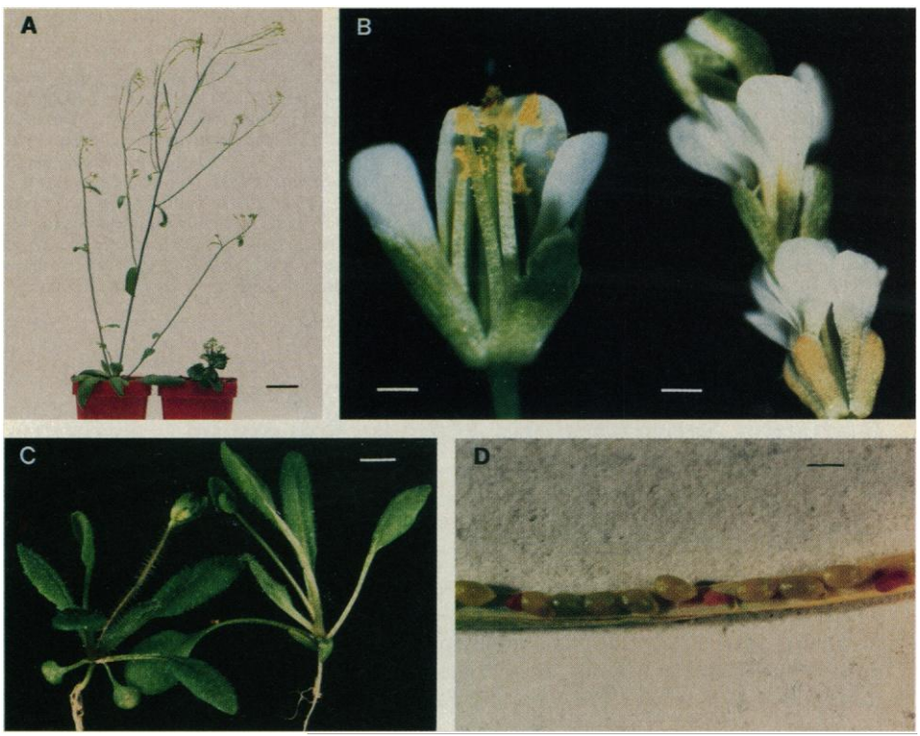

Fig. 1. Mutants generated by T-DNA insertional mutagenesis. (**A**) Four-week-old Dwf plant (bar, 1 cm). (**B**) Wild-type flowers (left bar, 0.3 mm) and agamous mutant (right bar, 0.5 mm). (**C**) Two-week-old wild-type (left) and glabrous plants (bar, 0.5 cm). (**D**) Siliques segregating for embryo lethality (bar, 0.8 mm).

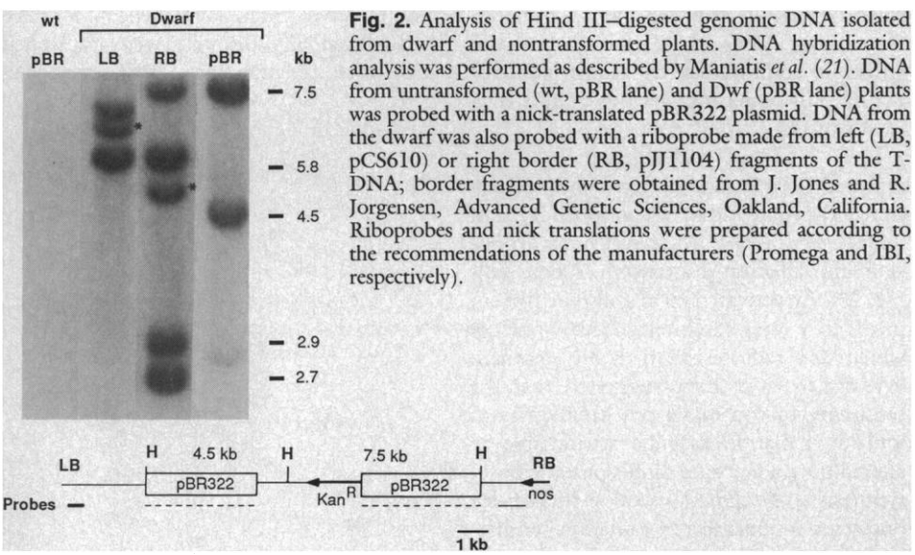

Fig. 2. Analysis of Hind III–digested genomic DNA isolated from dwarf and nontransformed plants. DNA hybridization analysis was performed as described by Maniatis *et al.* (*21*). DNA from untransformed (wt, pBR lane) and Dwf (pBR lane) plants was probed with a nick-translated pBR322 plasmid. DNA from the dwarf was also probed with a riboprobe made from left (LB, pCS610) or right border (RB, pJJ1104) fragments of the T-DNA; border fragments were obtained from J. Jones and R. Jorgensen, Advanced Genetic Sciences, Oakland, California. Riboprobes and nick translations were prepared according to the recommendations of the manufacturers (Promega and IBI, respectively).

These results demonstrate the utility of using the seed transformation protocol for generating insertional mutants. However, the isolation and characterization of specific mutants of interest requires a much larger population of transformants. Under the growth conditions used to generate the T2 progeny, we typically harvest 250,000 T2 seeds from 1000 T1 plants. From this population of T2 seeds we can expect to generate 50 Kan^R transformed lines (*14, 19*). To generate a population large enough (>75,000 unique transformants) to have a 95% probability of having an insert every 2 kb (assuming random insertion) would require 1.5 million T1 seeds to be treated. Given the small size and fecundity of *Arabidopsis* this number can be realized in a relatively short time.

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13. Transformants were made by treating germinating seeds of *Arabidopsis thaliana*, L. Heynh. (ecotype Wassilewskija) with a nononcogenic strain of *Agrobacterium tumefaciens*. The *Agrobacterium* strain used (C58C1rif) contained the 3850:1003 Ti plasmid with the chimeric NPTII gene (conferring Kan^R) (J. Velten and J. Schell, *Nucleic Acids Res.* **13**, 6981 (1985). The introduced Kan^R is a dominant selectable marker. Progeny (T2) resulting from infected seeds (T1) were plated on Kan medium (*15*). Kan^R plants (T2) were transferred to growth chamber conditions and grown to maturity. Selfed progeny (T3) from individual T2 plants were designated T3-1, -2, -31, . . . , according to the order in which they were collected.
14. K. A. Feldmann, unpublished data.
15. To screen for mutant phenotypes, we planted about 40 seeds from T3 families segregating for Kan^R on Metro-Mix (E. C. Geiger, Harleysville, PA) presoaked with Hoagland's solution (*12*) and transferred them to 22° to 25°C, with 16 hours of fluorescent and incandescent light (8000 lux). The plants were subirrigated with water or Hoagland's solution as necessary. The plants were scored for segregating phenotypes shortly after germination, at

the rosette stage, shortly after bolting, during flowering, and again at senescence.

16. We use the designation Dwf or Dwf::K^RTi for the dwarf phenotype, *dwf*::K^RTi for the disrupted allele, and *DWF* for the wild-type allele. We have not crossed the Dwf mutant described in this report to previously mapped dwarf mutants.
17. D. G. Jones, E. D. Gilbert, K. L. Grady, R. A. Jorgensen, *Mol. Gen. Genet.* **207**, 478 (1987); R. A. Jorgensen, C. Snyder, J. D. G. Jones, *ibid.*, p. 471.
18. M. D. Marks and K. A. Feldmann, unpublished data.
19. The transformation of genetically effective cells in the T1 plants occurs in such a way that only one or the other of the meiotic products is transformed. We have never observed siblings of any of the mutant phenotypes.
20. To assay for Kan^R or Kan^S, we surface-sterilized seeds in pure Clorox (5.25% wt/vol sodium hypochlorite) for 8 min and rinsed three times with sterile water. After seeds were dry, they were sprinkled on agar solidified (0.8%) medium containing M&S salts and vitamins, 1% sucrose (*12*), and kanamycin-sulfate at 50 mg/liter (Sigma). Kanamycin was eliminated from the medium for nonselective conditions. The plates containing the seeds were transferred to the dark at 4°C for 48 hours. The plates were transferred to growth chamber conditions and observed after 10 to 12 days. (A) Tall and Dwf seedlings were identified on nonselective medium and transferred to kanamycin-containing medium. (B) Individual Kan^R T3 seedlings (tall and Dwf) were identified and grown to maturity, and the selfed progeny, T4, were observed after growth on kanamycin.
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Effect of Antisense c-*raf*-1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma

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Antisense RNA–mediated inhibition of gene expression was used to investigate the biological function of the c-*raf*-1 gene in a radiation-resistant human squamous carcinoma cell line, SQ-20B. S1 nuclease protection assays revealed that transfection of full-length *raf* complementary DNA in the antisense orientation (AS) leads to a specific reduction (greater than tenfold) of steady-state levels of the endogenous c-*raf*-1 sense (S) transcript in SQ-20B cells. In nude mice, the malignant potential of SQ-20B cells transfected with *raf* (S) was significantly increased relative to that of SQ-20B cells transfected with *raf* (AS). SQ-20B cells containing transfected *raf* (S) maintained a radiation-resistant phenotype as compared to those cells harboring the AS version, which appeared to have enhanced radiation sensitivity. These data indicate that the reduced expression of endogenous c-*raf*-1 is sufficient to modulate the tumorigenicity and the radiation-resistant phenotype of SQ-20B cells, thus implicating c-*raf*-1 in a pathway important to the genesis of this type of cancer.

CELLULAR RADIATION SENSITIVITY is a complex function of genetic and environmental factors, not fully understood at the molecular level (*1, 2*). Only recently have applications of genetic engineering permitted probing of genomic events associated with radiation sensitivity or resistance (*3*). In the treatment of cancer, the sensitivity or resistance of tumor cells to ionizing radiation has substantial clinical consequences (*4, 5*). Using DNA-mediated gene transfer, we have observed the association of the *raf* oncogene in three radioresistant cancers of the head and neck (*6, 7*). Furthermore, Chang *et al.* have associated the expression of *raf* and *myc* oncogenes with radioresistant fibroblasts from a patient with the Li-Fraumeni syndrome (*8*). Others have reported correlations between *myc* expression and a more radioresistant phenotype of lung carcinomas (*9, 10*), as well as *ras* expression and radiation resistance in rodent cells (*11, 12*). An activated *raf* may also be present in oat cell lung carcinomas (*13*), some of which are radioresistant. More recently, Wasserman *et al.* have suggested that the sensitivity of squamous cell carcinomas to ionizing radiation may be attributable to altered poly(adenosine diphosphate–ribose) synthesis (*14*). Unequivocal evidence for oncogene modulation of radiation sensitivity has not been reported.

It has been recently discovered that a specific messenger RNA can be functionally inactivated by hybridization to complementary RNA or antisense RNA (*15, 16*). A number of investigators have now used antisense RNA to study the functional regulation of gene expression including cellular oncogenes. We have investigated the consequences of introduction of sense or antisense human c-*raf*-1 cDNA sequences on two major biological parameters in radiation-resistant human laryngeal squamous carcinoma cells (SQ-20B): tumor growth and radiation survival.

We have used a 3.05-kb Bam HI fragment of human c-*raf*-1 cDNA (*17*) that was isolated from an Okayama-Berg human fibroblast cDNA library (*18*) to construct various recombinant DNA molecules in the eukaryotic expression vector pADMLP. This vector contains an origin of replication and transcriptional initiation sequences of the adenovirus 2 major late promoter. Three different types of vector-insert plasmid DNA constructs were generated: (i) 3.05-kb Bam HI cDNA fragment in the "sense" orientation, downstream from the ad promoter, namely RAF (S); (ii) 3.05-kb Bam HI cDNA fragment in the reversed orientation, RAF (AS); and, (iii) 1.3-kb Bam HI–Bgl II cDNA fragment in the antisense orientation representing *raf* 3′ terminal sequences, 3′ RAF (AS). From SQ-20B, cells were transfected with these recombinant DNA molecules

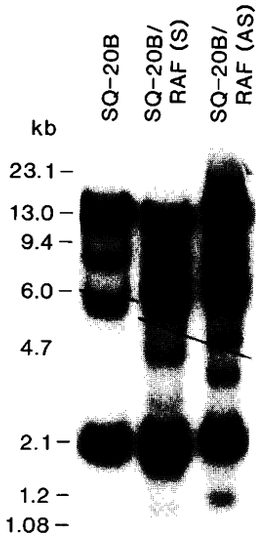

Fig. 1. Southern blot hybridization analysis of DNA isolated from various cell types. DNA samples (10 μg per lane) were digested to completion with Bgl II and hybridized to a ^{32}P-labeled 1.09-kb Ava II fragment of c-*raf*-1 cDNA (*6*). DNA isolation, restriction endonuclease digestion, 0.8% agarose gel electrophoresis, Southern blotting onto nitrocellulose, and ^{32}P-labeling of probes were all performed as described (*27*). Hybridization conditions and wash conditions have also been previously described (*6*).

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