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# A tobacco homologue of the Ri-plasmid *orf13* gene causes cell proliferation in carrot root discs

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**Abstract** The tobacco genome contains genes, called cellular *rol* (*c-rol*) genes, that are very similar in sequence to genes present in the T-DNA of the *Agrobacterium rhizogenes* Ri-plasmid. We have cloned two homologues (*torf13-1* and *torf13-2*) of the Ri-plasmid *orf13* gene from *Nicotiana tabacum* L. cv. Havana 425. The clone *torf13-1* has a 594-bp open reading frame (ORF) which is similar in sequence (77–82% for DNA and 67–77% for the deduced amino acid sequence) to *orf13* genes of the agropine, mikimopine, and mannopine Ri -plasmids and the *N. glauca* homologue *Ngorf13*. Southern analyses showed that there are at least two *torf13* genes derived from the *N. tomentosiformis* ancestor of tobacco, strongly suggesting that *torf13* resulted from an ancient transfer between ancestors of modern *A. rhizogenes* and tobacco. Steady-state expression of *torf13* mRNA is high in sepals, petals, shoot tips and in younger leaves, but considerably lower in stem tissues, lower leaves and roots. Treatment of cultured leaf discs for 5–20 days on medium containing auxin (10.7 μM α-naphthaleneacetic acid) and cytokinin (1.4 μM kinetin) resulted in a marked down-regulation of *torf13* mRNA accumulation. Therefore, *torf13* is transcriptionally active in normal tobacco tissues and the steady-state mRNA level is regulated. Inoculation of carrot-root discs with *A. tumefaciens* strains carrying the mannopine Ri-plasmid *orf13* and *torf13-1* regulated by the strong cauliflower mosaic virus 35S RNA promoter induced the formation of dense green callus on the disc surface. These findings indicate that at least one function of the *orf13* ORF is conserved in the tobacco homologue, and provide direct evidence that a *c-rol* gene can influence cell proliferation.

**Key words** *Agrobacterium rhizogenes* · Horizontal gene transfer · *Nicotiana tabacum* · *Daucus carota* · *rol* genes

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## Introduction

Infection of many dicotyledonous plant species with the soil bacterium *Agrobacterium rhizogenes* results in the neoplastic disease hairy root (reviewed in De Cleene and De Ley 1981; Zupan and Zambryski 1995). Pathogenicity depends upon a large plasmid harbored by the bacteria called the root-inducing (Ri) plasmid. During the infection process, the T-DNA region of the plasmid is transferred from the bacterium to the plant-cell nucleus (Chilton et al. 1982; Sheng and Citovsky 1996). Roots from several plant species, when transformed by an Ri-plasmid, form complete plants when cultured in vitro (Ackermann 1977; Tepfer 1984). These plants exhibit a characteristic "hairy-root syndrome"– wrinkled, leaves, shortened internodes, reduced apical dominance, and small flowers. Both the hairy-root syndrome and the T-DNA are transmitted meiotically through successive seed generations.

Two regions of the Ri plasmid, $T_L$- and $T_R$-DNA, are integrated into the host genome of plants infected with the A4 agropine strain of *A. rhizogenes* (Chilton et al. 1982; White et al. 1982; Willmitzer et al. 1982). Mutational analysis of the Ri plasmid (White et al. 1985) and the introduction of Ri genes alone or in combination into plants (Cardarelli et al. 1987; Offringa et al. 1986; Schmülling et al. 1988; Sinkar et al. 1988; Spanò et al. 1988; van Altvorst et al. 1992) have shown that $T_R$-DNA carries two loci that are functionally and structurally homologous to the *tms1* and *tms2* loci of the Ti-plasmid, which are responsible for the biosynthesis of the auxin indole-3-acetic acid by a metabolic pathway typical of microorganisms (Huffman et al. 1984; White

et al. 1985; Offringa et al. 1986). Genes important for the hairy-root syndrome, *rolA*, *rolB*, *rolC*, *rolD*, *orf13*, and *orf14*, are located in the $T_L$-DNA (White et al. 1985; Spena et al. 1987; Cardarelli et al. 1987; Vilaine et al. 1987; Spanò et al. 1988; Schmülling et al. 1988; Capone et al. 1989). Although the individual genes, when introduced into plants, can give an aberrant phenotype, there is considerable interaction between *rol* genes, and the phenotypes observed depend both on the bacterial strain and the host used (Spena et al. 1987, Capone et al. 1989; Hansen et al. 1991).

Certain plant species contain DNA sequences that are very similar to genes present in the $T_L$-DNA of the Ri-plasmid (White et al. 1982; Spanò et al. 1982; Furner et al. 1986; Aoki et al. 1994; Meyer et al. 1995). It has been proposed that these plant homologues, which we call cellular *rol* genes (*c-rol*), resulted from an ancient, horizontal transfer of genes between plants and an ancestor of *A. rhizogenes* (Furner et al. 1986; Meyer et al. 1995). The finding that *N. glauca* homologues of *rolB*, *rolC orf13*, and *orf14* are expressed in spontaneous teratomas arising in the tumor-prone hybrid *N. glauca* × *N. langsdorffii* (Ichikawa et al. 1990; Aoki et al. 1994) has led to the speculation that these genes might have an oncogenic function (Ichikawa and Syono 1991). There is, however, to date, no direct evidence that the expression of *c-rol* genes can influence the growth or development of plants.

We previously cloned a tobacco homologue of *rolC*, called *trolC*, and showed that this gene exhibits regulated expression in normal tissues (Meyer et al. 1995). Southern blot hybridization established that *trolC* is present in more than one copy and has its origin in the *tomentosiformis* ancestor of tobacco. The present report deals with *orf13*, one of the most highly conserved of the T-DNA genes (Brevet and Tempé 1988). We show by PCR cloning and sequencing that at least two homologues, *torf13-1* and *torf13-2*, are present in the Havana 425 cultivar of tobacco. The *torf13* gene is expressed in normal tissues in a hormonally and developmentally regulated fashion. Inoculation of carrot-root discs with *A. tumefaciens* strains carrying *torf13* or pRi8196 *orf13* genes regulated by the cauliflower mosaic virus (CaMV) 35S RNA promoter results in a marked proliferative response. These findings indicate that at least one function of the *orf13* ORF is conserved in the tobacco homologue, and provide direct evidence that a *c-rol* gene can influence cell proliferation.

## Materials and methods

### Plant materials and tissue culture

Carrots were purchased from Oekologische Produktion, Migros, Basel, Switzerland. *Nicotiana* plants were grown from seed in a greenhouse. Seed of tobacco – the *N. tabacum* L. cultivar Havana 425 – was originally provided by the Agricultural Experiment Station, University of Wisconsin, Madison, Wis.. Seed of *N. tomentosiformis* and *N. sylvestris* was provided by the Institut Experimental du Tabac, Bergerac, France. Tissue-lines were cultured as described (Binns and Meins 1973; Meins et al. 1980). In brief, tissue explants, transferred at 21-day intervals, were cultured in continuous light at 25° C on a modified Linsmaier and Skoog (1965) agar medium containing cytokinin (1.4 μM kinetin) and auxin (10.7 μM α-naphthaleneacetic acid).

### Nucleic acid analysis

Unless indicated otherwise, nucleic acid analyses and molecular cloning were performed using standard methods (Sambrook et al. 1989). Nucleotide and amino acid sequences were analyzed by computer using Genetics Computer Group software (Devereux et al. 1984). The *N. tomentosiformis* genomic DNA used has been described (Sperisen et al. 1991). High-molecular-weight DNA was isolated from leaves by the method of Rogers and Bendich (1988). The probe used for Southern blot hybridizations was a 594-bp fragment containing the *torf13-1* coding region labelled with [α-$^{32}$P]dCTP using a random priming kit (Readyview, Amersham). The final washing conditions for the filters were 2 × 30 min in 0.1 × SSC (1 × SSC is 0.15 NaCl/15 mM sodium citrate) and 1% (w/v) SDS at 67° C. DNA was sequenced by the chain termination method (Sanger et al. 1977) using a Sequenase kit (United States Biochemicals).

Plant RNA was isolated either by a modified Goodall et al. (1990) method (Meyer et al. 1995) or using the Rneasy kit for plants (Qiagen). RNA for RNA blot hybridization was fractionated on formaldehyde-containing agarose gels. Equal amounts of RNA were loaded in each lane and, in the case of total RNA, uniform loading was confirmed by ethidium bromide straining of ribosomal RNAs. Filters were hybridized with antisense RNA probes for *torf13* prepared by transcribing either the fragment W32 (Meyer et al. 1995) cloned in the Bluescript II(SK$^+$)(Stratagene) with T3 RNA polymerase or the complete *torf13-1* coding region in the pCR-Script SK$^+$ (Stratagene) with T7 RNA polymerase. The labelled nucleotide used was [α-$^{32}$P]CTP. The CNT6 probe for a constitutively expressed tobacco mRNA (Memelink et al. 1987) was the 0.8-kb *Eco*RI insert of cDNA CNT6 labelled with a random priming kit (Boehringer). Hybridization was carried out overnight at 55° C in 6 × SSC, 0.1% (w/v) sodium pyrophosphate, 1% (w/v) bovine serum albumin, 150 μg/ml denatured, sonicated salmon sperm DNA, 5 mM EDTA, 50 mM TRIS-HCl pH 7.5, and 50% (v/v) formamide. Blots were finally washed twice for 20 min at 65° C with 0.2 × SSC and 0.5% SDS. Sizes of the hybridizing RNAs were estimated from RNA molecular weight markers (Boehringer).

The *torf13* gene *torf13-1* was cloned by inverse PCR (Triglia et al. 1988). PCR was carried out with genomic DNA prepared from one leaf of a Havana 425 tobacco plant using a Perkin-Elmer Cetus DNA Thermal Cycler and Taq polymerase. The PCR clone W32 (Meyer et al. 1995), which is similar in sequence to the *orf13* ORF of pRiA4b (Slightom et al. 1986), was used as the basis for designing the inner primers. The primers used were:

1. (61) 5′-GGTCCTTGATCCAACGAAGATCAT-3 (38);
2. (414) 5′-TATTTTTTGGCTATCTTCCCAAAT-3′ (498);
3. 5′-CTCTAGTCCACCCTTATTTACTT-3′;
4. (661) 5′-ACCATGAGAATGATGGTGACAC-3′ (683).

The position of sequences in the clone *torf13-1* corresponding to the primers is shown in Fig. 1. The positions of the first and last nucleotides in parenthesis are given relative to the 5′ end of the clone.

Genomic DNA (15 μg) was digested with *Hin*dII, religated and linearized by digestion with *Sca*I, which cuts at a single site in the W32 clone. A fragment was amplified with primers 1 and 2 (directed to regions outside of W32) and then cloned in the TA cloning vector (Invitrogen), resulting in clone pI13. Sequencing of pI13 showed that it contained the two halves of a *torf13* ORF at its 3′- and 5′- ends. The final amplification was made on genomic DNA using primers 3 and 4, corresponding to 3′- and 5′-regions near the *Hin*dII site. The resulting PCR mixture was fractionated

on a 1% (w/v) agarose gel, purified, cloned into the TA vector and sequenced.

### Carrot-root disc inoculation

Carrot-root discs were inoculated with *A. tumefaciens* strains by a modification of the methods of Ryder et al. (1985) and Capone et al. (1989). Carrots were peeled, surface-sterilized, and cut transversely into 5-mm thick discs. The discs were incubated root-apical side up, 6 discs per dish, for several hours in 150-mm diameter × 25 mm deep plastic Petri dishes (Falcon No. 3025) containing 80 ml of 10% (w/v) agar. After the discs had been inoculated with 100 µl of the indicated bacterial suspension on their root-apical (basal) side, the dishes were closed with Parafilm and incubated for 3 days in the dark at 25° C. The discs were then transferred onto fresh medium, incubated for an additional 15 days, and scored for root and callus formation.

The *A. tumefaciens* strains used for inoculation were: LBA4404 without a binary vector (Ooms et al. 1982; Hoekema et al. 1983); CIB200, LBA4404 carrying the binary vector pCIB200 (Neuhaus et al. 1991); R1000 carrying pRiA4b with the complete T-DNA of *A. rhizogenes* strain A4 (White et al. 1985); C58C1 (pGV3850) pGH35SORF13 (Hansen et al. 1993), carrying the *orf13* gene of the mannopine Ri plasmid with a CaMV 35S RNA promoter; and E15 carrying the *rolA*, *rolB*, and *rolC* genes of the agropine strain pRi1855 cloned in the plant vector BIN19 (Capone et al. 1989). Based on partial restriction maps, pRi1855 appears to be identical to pRiA4b (Jouanin 1984). The strain 35S-torf13-1 was obtained by inserting the cauliflower mosaic virus 35S RNA expression cassette containing the entire *torf13-1* ORF into the *Sma*I cloning site of expression vector pCIB200 (Neuhaus et al. 1991).

Cultures (100 ml) of bacteria were grown for 48 h in YEB medium (Leemans et al. 1981) with the appropriate antibiotics. The bacteria were harvested by centrifugation (1400 × *g* for 15 min), washed in 10 ml of water, and resuspended in a final volume of 5 ml of water.

## Results

### The structure of *torf13*

Two tobacco homologues of the Ri plasmid gene *orf13*, designated *torf13-1* and *torf13-2,* were cloned from Havana 425 tobacco genomic DNA. The clone torf13-1 (EMBL database Accession No. AJ007621) was obtained by an inverse-PCR strategy by amplifying undigested genomic DNA using primers corresponding to sequences indicated in Fig. 1. It contained a complete 594-bp ORF, very similar in sequence to that of *orf13* from the $T_L$-DNA of pRiA4b (Slightom et al. 1986), as well as 51-bp of the 5′- and 85 bp of the 3′- untranslated region (Fig. 1). The sequence of this gene was confirmed with a second clone obtained in an independent PCR. The two clones differed at only two positions –15 and 441. A sequence corresponding to primer 3 was present in both the 5′- and the 3′- flanking region of the *torf13-1* ORF. Therefore, the sequence of the *torf13-1* clone shown in Fig. 1 contains only the regions whose sequences are confirmed by the independent clones 2C3 and pI13. The 5′-untranslated region of *torf13-1* showed several features of plant genes (for reviews, see Heidecker and Messing 1986; Katagiri and Chua 1992). Relative to the putative start of translation, there was a TATA-binding motif at position −26, a CAAT-like box at position −43, and a consensus sequence for A/GNNATGG-type eukaryotic translation initiation sites at position −1. As is typical for plant genes, the polypeptide encoded by the ORF started with the dipeptide Met-Ala.

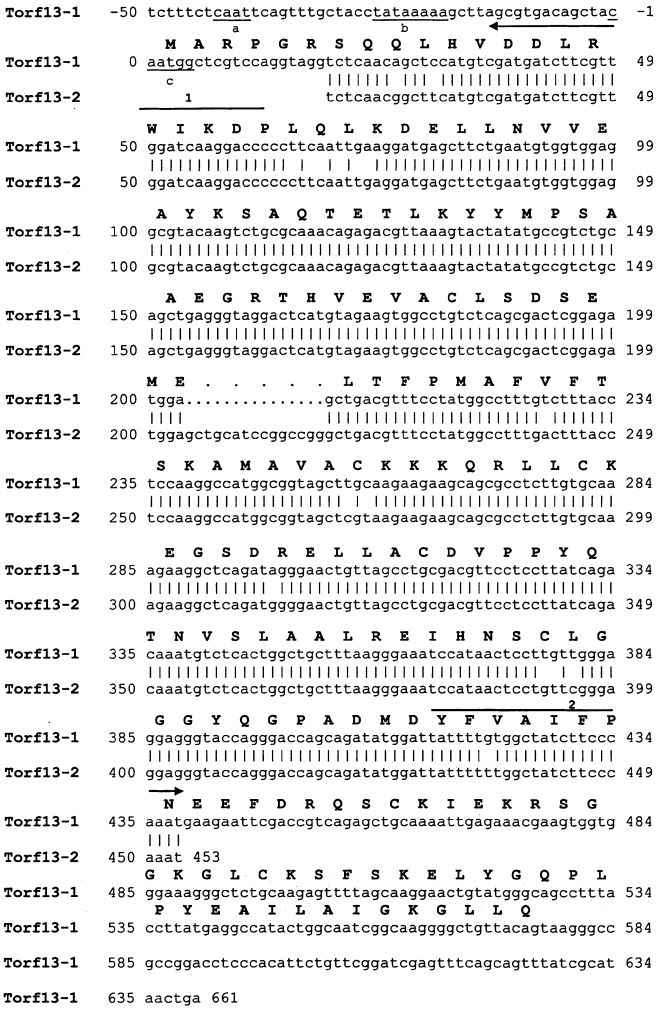


**Fig. 1** Nucleotide and deduced amino acid sequences of tobacco homologues of *orf13*. Torf13-1 is the sequence of a PCR clone containing a complete ORF and 3′- and 5′-untranslated regions. Torf13-2 is the sequence of a partial PCR clone represented by pW32 (Meyer et al. 1995). The clones are numbered from the putative translational start of *torf13-1*. The deduced amino acid sequence of *torf13-1* is shown. There is a CAAT-like box (a) at position −43, a TATA binding motif (b) at position −26, and a consensus sequence for plant translation initiation sites (c) at position −1. The *arrows* indicate the positions of PCR primers 1 and 2

Sequencing of the partial PCR clone pW32 (Meyer et al. 1995), which had been shown to hybridize with a probe for *orf13*, revealed that this clone encompasses about 70% of the ORF, differed in nucleotide sequence from *torf13-1* at 4% of the positions, and contained an insertion of 15 bp at position 254 (Fig. 1). Although the possibility of PCR artifacts cannot be completely ruled out, it is likely that this clone represents a second homologue, which we have designated *torf13-2* (EMBL database Accession No. AJ007622).

The PCR clone 2C3 representing the intergenic region between *trolC* and *torf13* (Meyer et al. 1995) was also sequenced. Examination of the 912-bp sequence (EMBL database Accession No. AJ010794 showed that 117 bp at the 5′ end of 2C3 are identical to the 3′ end of the *trolC* coding region and 102 bp at the 3′ end of 2C3 are identical to the 5′ end of the *torf13* coding region. There was insufficient sequence overlap to determine whether 2C3 represents the *trolC-torf13-1* or the *trolC-trof13-2* intergenic region. The *trolC-torf13* intergenic region was 46% identical to comparable regions of the agropine, mikimopine and mannopine plasmids, and was 53% identical to the *N. glauca NgrolC-Ngorf13* intergenic region.

Figure 2 shows the amino acid sequences deduced from alignments of the sequences of the tobacco *torf13* homologues, the *N. glauca* homologue *Ngorf13*, and *orf13* homologues from agropine, mannopine, and mikimopine Ri-plasmids. The comparison shows that the protein encoded by *torf13-1* is missing a 5-amino acid sequence present in the product of *torf13-2* and in the other homologues. Table 1 shows that the DNA and deduced amino acid sequences of the six homologues are very similar. Sequence identity ranges from 77 to 82% for DNA and 69 to 78% for protein. Based on both the DNA and protein sequences, the *torf13* genes were most closely related to the *orf13* of the mannopine strain Ri plasmid. DNA sequences were more highly conserved in the ORF than in the 5′- and 3′-nontranslated regions, e.g. 43% and 49% identity, respectively, between *torf13-1* and mannopine *orf13*.

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          1                                                  50
Torf13-1  MAR  PGRSQ QLHVDDLRWI KDPLQLKDEL LNVVEAYKSA QTETLKYYMP
Torf13-2  ~~~~~~~~SQ RLHVDDLRWI KDPPSIEDEL LNVVEAYKSA QTETLKYYMP
Ngorf13   MAR.L.GSSP RLHVDNLCWI KEPTRLKAQL MNVVEAYKPA QPETLKYYIP
Agorf13   MARYCSGGSQ RLHVDDFRWI KEPTRLKAQL INVVETYKAA QTETLKYYIS
Miorf13   MARYFGSSSQ RLHVDDFRFI KEPTRLKAQL VNVVETYKAV QTETLKYYIS
Maorf13   MARSY.GSSR RFHIDEFRWI KEPTRLKAQL LNVVEAHKSA QRETLKYYIP
Cons      MAR     S  RLHVDD RWI KEPTRLKAQL  NVVEAYK A QTETLKYYIP

          51                                                 100
Torf13-1  SAAEGRTHVE VACLSDSEME .....LTFPM AFVFTSKAMA VACKKKQRLL
Torf13-2  SAAEGRTHVE VACLSDSEME LHPAGLTFPM AFDFTSKAMA VARKKKQRLL
Ngorf13   SAAEHGTHVE AAYVNDAEME LHPAGLKYPL SFVFTSLAVA KAYKETKHLL
Agorf13   SATERVAHVE AAEVNNAEME LHPAGLKYPL SFVFTSLAVA TACKENKHLL
Miorf13   SATERVAHVE AVHVDNAEMG LHPAGFKYPL SFVFTSLAVA KACKENKHLV
Maorf13   SAAEHWAHAE AAYVNDAEME LHQAGWKYPL SFVFTSLDVA KACKENKHLL
Cons      SAAE   HVE AA V DAEME LH.AGLKYPL SFVFTSLAVA  A KE KHLL

          101                                                150
Torf13-1  CKEGSDRELL ACDVPPYQTN VSLAALREIH NSCLGGGYQG PADMDYFVAI
Torf13-2  CKEGSDGELL ACDVPPYQTN VSLAALREIH NSCSGGGYQG PADMDYFLAI
Ngorf13   CEEHWDGDLL ACVVPPYQTN VSLAALRERH NSFSGGGYQE QTDMDYFVAI
Agorf13   CEEHLEGDLI SCVVPPYQTN VSLAALRELH NSISGGGYQE QADMDYFVAI
Miorf13   CEEHLEGDLI SCVVPPYQTN VSLAALREIH NSVSGGGYLD PTDMDYFVAI
Maorf13   CKEDSDGQLF ACAVPPYQTN VSLAALREIH NSVSGGGEYQE EEDMDYFVAI
Cons      CEE  DG L  AC VPPYQTN VSLAALRE H NS SGGGYQ    DMDYFVAI

          151                                                200
Torf13-1  FPNEEFDRQS CKIEKRSGGK GLCKSFSKEL YGQPLPYEAI LAIGKGLLQ*
Torf13-2  FPN~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Ngorf13   LPDEKFGYQS LEIETRS.RK GLCKIYSREL .GGPLAYDAI LAIGKVLLE*
Agorf13   IPNDNFDYQS CEIDTRSCGK GLCKIYSREL GGQPLAYDAI LAIGKVLLLE*
Miorf13   LPDENFDYQS CEIVTKNGGE GLCKIYSRQL DG.PLAYEAI LAIGKVLLE*
Maorf13   FPNENFNYES CEIVTRSGGK GICKIYSREL .GEPLAYDAI LAIGKVLLE*
Cons       PNE F YQS CEI T S GK GLCKI SREL  G PLAY AI LAIGKVLL.
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**Fig. 2** Comparison of the deduced amino acid sequences of *orf13* homologues of tobacco (*torf13-1* and *torf13-2*), *N. glauca* (*Ngorf13*; Aoki et al. 1994); the agropine Ri plasmid (Agorf13; Slightom et al. 1986); the mikimopine Ri plasmid (Miorf13; Kiyokawa et al. 1994); and the mannopine Ri plasmid (Maorf13; Hansen et al. 1991). Deletions in the sequences aligned using the BESTFIT algorithm are indicated by *dots*. *Dashes* indicate that no sequence information is available. The criterion for the consensus sequence (Cons) is that identical amino acids be present in at least four of the sequences compared. Positions at which all the amino acids are identical are shown in *bold*

## Southern hybridization analysis

We verified that *torf13* is of tobacco origin by Southern hybridization. To facilitate comparison with earlier *trolC* results (Meyer et al. 1995), the same membranes were stripped and rehybridized with the probe for *torf13-1*. Strong hybridization signals were obtained with restriction fragments of Havana 425 tobacco DNA, but not with maize DNA used as a negative control (Fig. 3A). In agreement with the sequences of *torf13-1* and *torf13-2*, tobacco DNA digested with *Hin*dIII, *Sac*I, and *Bam*HI, which do not cut within these clones, each gave single bands; whereas, *Eco*RI, which cuts at one site in *torf13-1*, gave two hybridizing bands. Two bands were also obtained with *Xho*I, which does not cut in the sequenced regions. These results indicate that the Havana 425 tobacco genome contains at least two *torf13* genes. This conclusion is consistent with the finding that *trof13-1* and *trof13-2* differ substantially in sequence, and with earlier studies of the copy number of the *c-rol* gene region in tobacco (Meyer 1995; Meyer et al. 1995).

Tobacco is an amphidiploid species derived from ancestors that were most closely related to the present-day species *N. sylvestris* and *N. tomentosiformis* (see review by Gerstel 1976). To investigate the origins of *torf13*, genomic DNA of Havana 425 tobacco, *N. sylvestris* and *N. tomentosiformis* was digested with *Hin*dIII and *Xho*I and hybridized with a probe for *torf13-1*. The identity of the DNA was verified by using a membrane that had been hybridized with a probe for the tobacco chitinase genes *CHN48*, which gives diagnostic restriction fragments for the three *Nicotiana* species (Meyer et al. 1995). The same patterns of hybridizing bands were found for Havana 425 and *N. tomentosiformis* DNA; but, no signal was detected with *N. sylvestris* DNA (Fig. 3B). This strongly suggests that the *torf13* sequences present in tobacco are of *N. tomentosiformis* origin.

## Regulation of *torf13* expression

We examined the pattern of *torf13* expression in different tissues of Havana 425 tobacco plants by RNA blot hybridization using probes for the *torf13* ORE. As a control for loading, blots were also rehybridized with a probe for *CNT6*, a gene which shows constitutive expression in tobacco tissues (Memelink et al. 1987). Strong hybridization signals for *torf13* transcripts were obtained with RNA prepared from the shoot tip, an upper leaf, and a middle leaf of a mature plant (Fig. 4). Far weaker signals were obtained with the stem,

**Table 1** Sequence comparisons of the coding regions of Ri-plasmid and plant *orf13* genes

| | Positions identical/similar (%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Tobacco | | *N. glauca* | *A. rhizogenes Ri plasmids* | | |
| | Torf13-1 | Torf13-2 | Ngorf13[b] | Agropine[c] | Mikimopine[d] | Mannopine[e] |
| Torf13-1 | 100 | 96 (94) | 82 (72) | 79 (73) | 77 (68) | 82 (77) |
| Torf13-2 | | 100 | 81 (72) | 80 (72) | 77 (67) | 81 (72) |
| Ngorf13 | | | 100 | 89 (88) | 88 (85) | 87 (89) |
| Agropine | | | | 100 | 93 (89) | 87 (87) |
| Mikimopine | | | | | 100 | 87 (84) |
| Mannopine | | | | | | 100 |

[a] Nucleotide sequences aligned using the BESTFIT algorithm with default settings were analyzed for nucleotide sequence identity and deduced amino acid sequence similarity. Values in *parentheses* are for amino acid sequences. Values for Torf13-2 are for the sequenced region of the incomplete ORF (see Fig. 1)

[b] Aoki et al. (1994)
[c] Slightom et al. (1986)
[d] Kiyokawa et al. (1994)
[e] Hansen et al. (1991)

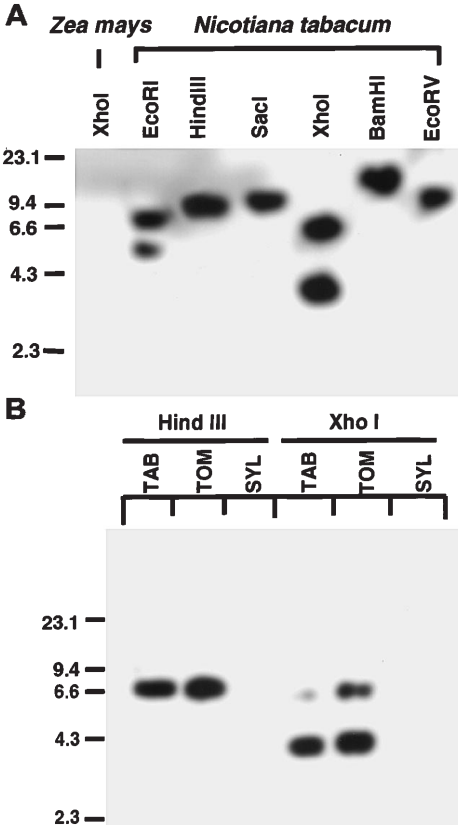


**Fig. 3 A, B** Southern analysis of *Zea mays* and Havana 425 tobacco (TAB), *N. tomentosiformis* (TOM), and *N. sylvestris* (SYL) DNA, digested with the restriction enzymes indicated. Membranes were hybridized with a *torf13-1* coding region probe. To permit a direct comparison of results, the same membranes probed for *trolC* (Meyer et al. 1995) were used. Size standards (in kb) are shown on the left. **A** Each lane was loaded with 30 μg of DNA. **B** The TOM and SYL lanes were loaded with half the amount of DNA in the TAB lane (20 μg)

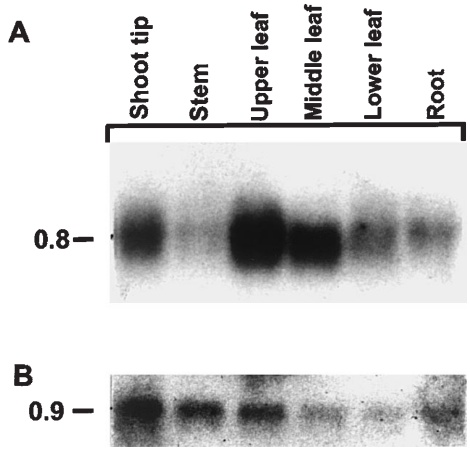


**Fig. 4 A, B** RNA blot analysis of total RNA prepared from organs of a mature, 75 cm tall Havana 425 tobacco plant with 14 expanded leaves. The sources of the tissues was: shoot tip, the upper-most region of the stem with the apical meristem leaves < 5 mm in length; upper leaf, leaves 12–14 counting from the bottom of the plant; stem, the internode between leaves 12 and 13; middle leaf, leaf 7; lower leaf, leaf 3; and roots. **A** Filter hybridized with a *torf13-2* (W32) RNA probe. **B** Filter rehybridized with a probe for the constitutively expressed *CNT6* gene. Equal amounts of RNA (11 μg) were loaded in each lane. The scale shows the approximate sizes of hybridizing transcripts in kb

lower-leaf, and root samples. The signals corresponded to a 800-nucleotide transcript, which is some 200 nucleotides longer than the *torf13-1* coding region. *torf13* also showed tissue-specific regulation in different flower parts. Strong signals were obtained with total RNA prepared from petals and sepals; no signals were detected with total RNA prepared from stigmas, ovaries, or stamens (Fig. 5). These results show that *torf-13* is transcriptionally active in normal tobacco tissues and

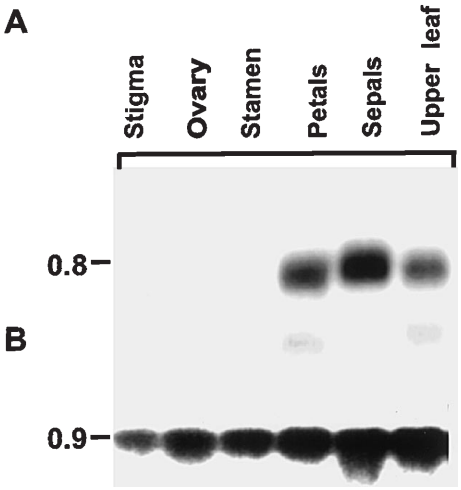


**Fig. 5A, B** RNA-blot of total RNA prepared from floral organs of a flowering Havana 425 tobacco plant. The source of the upper-leaf tissues was leaves 14–17 counting from the bottom of the plant. **A** Filter hybridized with a *torf13-1* RNA probe. **B** Filter rehybridized with a probe for the constitutively expressed *CNT6* gene. Equal amounts of total RNA (11 μg) were loaded in each lane. The scale shows the approximate sizes of hybridizing transcripts in kb

that the steady-state mRNA level is developmentally regulated.

To detect effects of auxin and cytokinin on *torf13* mRNA accumulation, discs excised from a middle leaf of Havana 425 tobacco plants were incubated for up to 20 days on media containing different combinations of NAA and kinetin. Under these conditions, only leaf discs cultured on medium containing both hormones grew appreciably. As judged from the strength of the hybridization signal, the *torf13* mRNA content of leaf discs cultured on hormone-free medium or auxin-containing medium declined over a period of 8 days and was below the limit of detection by day 13 (Fig. 6). The decline in *torf13* mRNA content was appreciably slower in leaf discs incubated on cytokinin-containing medium. Finally, leaf discs cultured on medium containing both auxin and cytokinin showed a marked decrease in *torf13* mRNA: only a weak signal was detected on day 5 and no signals were detected for later time points.

## Inoculation of carrot-root discs with *A. tumefaciens* carrying *orf13* and *torf13*

The important question that arises is whether or not *orf13* and *torf13* have similar biological effects when expressed in plant cells. To address this question, we used a carrot-root disc system to assess root and callus formation in response to combinations of *rol* genes and *orf13*. Carrot-root discs were inoculated with *A. tumefaciens* strains carrying 35S·orf13 from pRi8196 (pGH35SORF13) (Hansen et al. 1991) and *torf13-1* in the CaMV 35S RNA expression vector PCIB200 (Neuhaus et al. 1991). Because co-inoculation experiments had shown that the agropine pRi1855 *orf13* in combination with *rolA*, *rolB*, and *rolC* gave a weak rooting response (Capone et al. 1989), we also compared the effect of 35S·torf13-1 and 35S·orf13 alone and in combination with pRi1855 *rolA·rolB·rolC* (E15) (Cardarelli et al. 1987). Strain R1000 carrying the *A. rhizogenes* pRiA4b (White et al. 1985) and strain CIB200 carrying the empty expression vector pCIB200 were used as controls.

The results obtained for 2–3 independent experiments are summarized in Table 2. Although root discs inoculated with the empty vector pCIB200 occasionally formed a few roots or a small amount of white, friable callus, most of the discs, 87%, showed no discernible response (Fig. 7A). In contrast, more than 80% of the discs inoculated with pRiA4b formed numerous roots and dense, green-white callus (Fig. 7B). Inoculation with the *rolA*·rolB·rolC plasmid gave very weak root and callus responses comparable to those obtained with the empty vector. Co-inoculation of this plasmid with either 35S·orf13 or 35S·torf13-1 gave roughly the same, weak rooting response obtained with *rolA*·rolB·rolC alone.

The striking feature of the root discs inoculated with either 35S·orf13 or 35S·torf13-1 was the marked callus response: more than 84% of the discs formed a ring of dense, dark-green callus starting in the region of the vascular cambium and eventually covering the entire surface of the discs (Fig. 7C, D). The important point is that both the *orf13* and *torf13-1* strains elicited this response and that the incidence of callus formation did not differ significantly (T-test of means, $P = 0.45$).

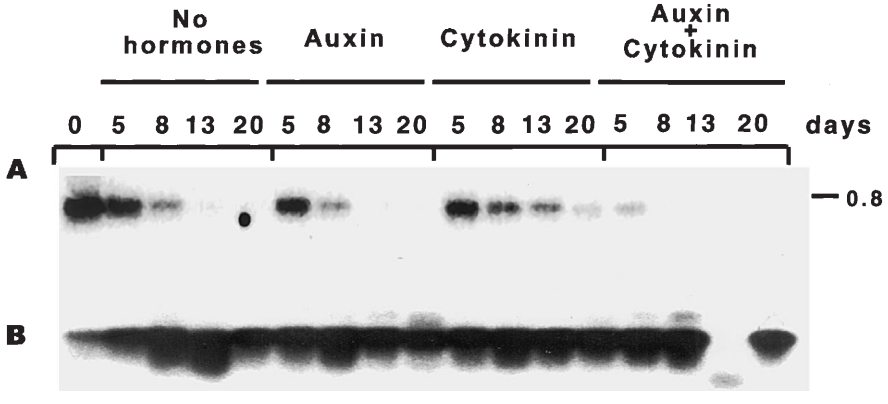


**Fig. 6 A, B** RNA-blot hybridization of RNA from leaf discs of Havana 425 tobacco cultured on medium containing various combinations of auxin and cytokinin. **A** Filter hybridized with a *torf13-1* coding region probe. Equal amounts (11 μg) of total RNA were loaded in each lane. The size of the hybridizing transcripts (in kb) is indicated. **B** Filter rehybridized with a probe for ribosomal RNA. Note that the RNA sample for leaf discs incubated for 13 days on auxin + cytokinin medium was lost

**Table 2** Response of carrot-root discs inoculated with *A. tumefaciens* strains carrying *torf13-1* and *orf13*

| Treatment[b] | Average number of discs scored per experiment | Percentage of discs scored[a] | | |
|---|---|---|---|---|
| | | No response | Callus | Roots |
| CIB200 | 22 | 87 ± 6.1[c] | 8.8 ± 4.0 | 4.0 ± 4.2 |
| pRiA4b | 21 | 13 ± 0.6 | 84 ± 2.5 | 82 ± 1.6 |
| rolA·rolB·rolC | 30 | 91 ± 4.0 | 7.8 ± 4.4 | 4.5 ± 1.2 |
| rolA·rolB·rolC + 35S·orf13 | 32 | 11 ± 1.0 | 84 ± 1.1 | 5.2 ± 1.0 |
| rolA·rolB·rolC + 35S·torf13–1 | 32 | 12 ± 1.2 | 85 ± 2.8 | 2.0 ± 1.0 |
| 35S·orf13 | 30 | 13 ± 0.0 | 85 ± 1.7 | 1.7 ± 1.7 |
| 35S·torf13-1 | 31 | 14 ± 4.4 | 84 ± 5.9 | 4.9 ± 1.8 |

[a] Carrot-discs were inoculated with *A. tumefaciens* strains CIB200, carrying the empty expression vector pCIB200 used with the *orf13* and *torf13–1* constructs (Neuhaus et al. 1991); R1000, carrying pRiA4b (White et al. 1985); 35S·orf13, carrying the pRi8196 *orf13* with the CaMV 35S RNA promoters in pBI121 (Hansen et al. 1993); 35S·torf13–1, carrying *torf13–1* with CaMV 35S RNA expression signals in pCIB200; and, rolA·rolB·rolC, carrying the pRi1855 *rolA*, *rolB*, and *rolC* genes in the vector Bin 19 (Capone et al. 1989). Co-inoculation with two strains is indicated by +

[b] Carrot discs scored 18 days after inoculation for formation of dense white or green callus and the formation of at least one root on each disc.

[c] Mean values ± SEM for 2–3 independent experiments

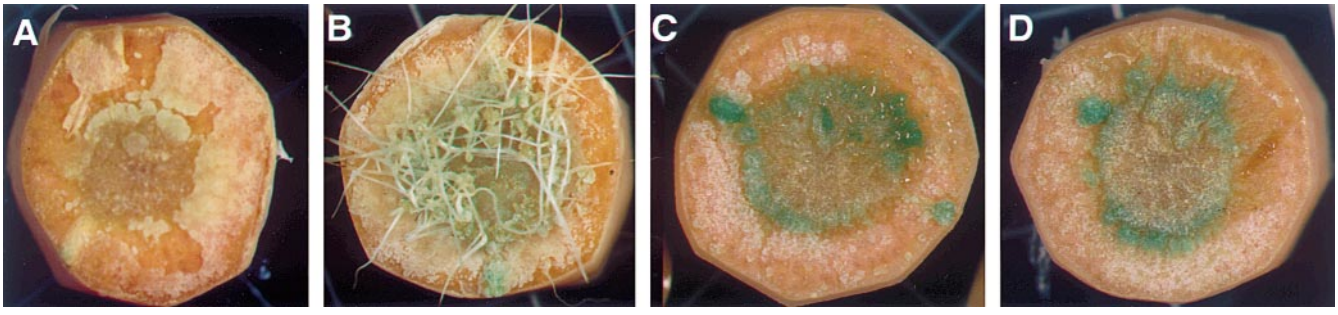


**Fig. 7A–D** Representative carrot-root discs inoculated with *A. tumefaciens* strains 35S·orf13 and 35S·torf13-1. **A** Inoculation with the empty-vector strain CIB200. **B** Inoculation with strain R1000. **C** Inoculation with strain 35S·torf13-1. **D** Inoculation with strain 35S·orf13. Discs were photographed 21 days after inoculation. Magnification: 2×

Co-inoculation with *rolA*·rolB·rolC did not significantly affect the incidence of the callus response (T-test of means, $P = 0.60$ and $P = 0.10$, respectively), but it did increase the amount of callus formed (data not shown). Taken together, these results indicate that the Ri-plasmid *orf13* gene and the tobacco *torf13-1* gene regulated by CaMV 35S expression signals are equally effective in inducing the formation of callus.

## Discussion

Several *c-rol* genes in *N. glauca* and tobacco have now been cloned and shown to be expressed in a regulated fashion (Ichikawa et al. 1990; Aoki et al. 1994; Meyer et al. 1995; Meyer 1995; for review, see Fründt et al. 1997). Nevertheless, direct evidence that they affect growth and development is still lacking. Our most important finding was that inoculation of carrot-root discs with the *orf13* from a mannopine strain or *torf13-1*, regulated by CaMV 35S RNA expression signals, induces a proliferative callus response – and the incidence of this response was comparable for the two genes. These findings indicate that at least one function of the *orf13* ORF is conserved in the tobacco homologue and provide direct evidence that a *c-rol* gene can influence cell proliferation.

Little is known about the function and regulation of *orf13*. Xanthi tobacco transformed with the pRi8196 *orf13* regulated by the CaMV 35S RNA promoter develops abnormally (Hansen et al. 1993). These transformants have dark-green, abnormally shaped leaves, shortened internodes, reduced apical dominance, reduced growth, and abnormal flowers. Similar phenotypes have been described for auxin-resistant tobacco (Wilson et al. 1990) and for *ipt* transformants of tobacco producing high levels of cytokinins (Medford et al. 1989, Hewelt et al. 1994). Of particular interest is the observation that the abnormal phenotype can be transmitted by grafting from *35S*·orf13 transformants to normal tobacco, suggesting that *orf13* might specify production of a diffusable, hormone-like factor (Hansen et al. 1993). Exogenous auxin is required for the formation of roots on carrot-root discs inoculated with bacteria carrying pRi1855 *rolB*, or the combination of *rolA, rolB*, and *rolC* (Capone et al. 1989). In contrast, co-inoculation of these strains with strains carrying *orf13*, *orf14*, and intervening T-DNA sequences results in a robust, cell-autonomous, rooting response without added auxin. At present it is not clear whether the loss of auxin re-

quirement reported for the co-cultivation experiments is due to *orf13*, *orf14*, a combination of both genes, or other elements in the T-DNA sequences used.

Our results bear on the possible functions of *c-rol* genes in neoplastic growth. Sexual hybrids between *N. glauca* and *N. langsdorffii* (GGLL) form teratomatous, shooty tumors in response to wounding and spontaneously at the onset of flowering (reviewed in Ichikawa and Syono 1991; Smith 1988; Bayer 1983). *NgrolB*, *NgrolC*, *Ngorf13*, and *Ngorf14* are expressed in the tumor-prone GGLL hybrid but expression has not been detected in the *N. glauca* parent, which is not tumor prone (Ichikawa et al. 1990; Aoki et al. 1994; Nagata et al. 1995, 1996). Findings of this type led Ichikawa and Syono (1991) to propose that tumor-specific *c-rol* gene expression is important for tumorigenesis. Our finding that *torf13-1* expression can promote cell proliferation is consistent with this hypothesis. However, in the case of tobacco, the *rolC* (Meyer et al. 1995) and *orf13* homologues are transcribed in organs of mature, normal plants. Thus, *c-rol* gene expression is not an exclusive property of tumorous or tumor-prone cells. Sense and antisense transformation with cloned *c-rol* genes might provide a fruitful approach for investigating the possible oncogenic effects of these genes.

RNA blot hybridization showed that *torf13* expression is regulated. Steady-state mRNA levels are high in sepals, petals, shoot tips and in younger leaves, but considerably lower in stem tissues, lower leaves and roots. In addition, expression of *torf13* mRNA was strongly down-regulated in leaf discs treated with combinations of auxin and cytokinin. Transcript accumulation was measured over a period of days, under conditions in which it had been demonstrated that hormones affect *c-rol* gene expression (Meyer etal. 1995). At present we cannot distinguish between specific effects on *trof13* expression and more indirect effects resulting from the growth-promoting action of the hormones. It is intriguing that the plant defense-related class I chitinases and $\beta$-1,3-glucanases show a similar pattern of down-regulation in response to auxin and cytokinin (for review, see Meins et al. 1992).

Although *torf13* is expressed, tobacco plants show neither the hairy-root phenotype or the extreme developmental abnormalities characteristic of *35S*·orf13 transformants (Hansen et al. 1993). One plausible explanation is that *torf13*, *orf13*, and the chimeric *35S*·orf13 constructs show different levels and patterns of expression in tobacco. Thus, as judged from reporter gene experiments, activity of the pRi8196 *orf13* promoter is considerably higher in roots than in the stem of Xanthi tobacco plants (Hansen et al. 1997). Promoter activity in the stem showed an increased gradient in vascular tissues from the base to the top of the plant. Wound-induced activity of the promoter was also stimulated in root explants by treatment with auxin.

Sequences homologous to *rol* genes have been found by Southern analysis in petunia, carrot, and in six out of 17 *Nicotiana* species surveyed (White et al. 1982, Spanó et al. 1982, Furner et al. 1986). The region of homology in the *N. glauca* genome extends for about 11 kb and corresponds to the core region of the Ri-plasmid $T_L$-DNA, with homologous genes present in the same arrangement and including a partial inverted repeat of that region (Furner et al. 1986). The present study, considered together with our earlier results (Meyer et al. 1995), suggests a plausible model for the arrangement of the *c-rol* region in the tobacco genome. Cloning showed that the tobacco genome contains intergenic regions homologous to *rolB-rolC* and *rolC-orf13*. Sequencing of the intragenic region *trolC-torf13* present in clone 2C3 established that *torf13* is located 3′ of *trolC*. *Bam*HI does not cut in *trolC*, *torf13,* or the *trolC-torf13-1* intergenic region. Digestion of tobacco DNA with this enzyme gave a single ≈16-kb fragment which hybridized with probes for *trolC* and *torf13*. Thus, all pairs of *trolC* and *torf13* genes from the two gene families are closely linked in a ≈16-kb region. *Sac*I, which cuts at a single site in *trolC*, and *Hind*III, which cuts at a single site in the *trolC-torf13-1* intergenic region, each gave a hybridizing fragment with the *torf13* probe. This suggests that the tobacco homologues are not present as an inverted repeat as reported for the *N. glauca* homologues (Furner et al. 1986).

The arrangement and high sequence conservation of *rol* homologues in different Ri plasmids, in *N. glauca*, and in tobacco are consistent with the hypothesis that horizontal transfer of genes occurred from an ancestral *A. rhizogenes* to a plant during the history of the genus *Nicotiana* (for review, see Fründt et al. 1998). The plasmid progenitors of *c-rol* genes are not known and evolutionary relationships in the genus *Nicotiana* are still uncertain (Goodspeed 1954). Thus, it is difficult to establish the evolutionary relationship of *c-rol* sequences present in *N. glauca* and tobacco. The sexual hybridization of *tomentosiformis* and *sylvestris* progenitors to form tobacco is thought to have occurred in South America less than 6 Myr years ago and probably in pre-Columbian times (Gerstel 1976; Okamuro and Goldberg 1985). Our Southern hybridizations with a *torf13* ORF probe, and earlier studies with probes for *trolC* and the *trolB-trolC* intergenic region (Meyer et al. 1995), strongly suggest that tobacco *c-rol* DNA is of *tomentosiformis* origin and that the *Hind*III and *Xho*I cleavage sites were conserved in tobacco. Therefore, *c-rol* genes present in tobacco are likely to have resulted from a transformation event which occurred before the emergence of the modern tobacco. It has been proposed that the multiple copies of the *c-rol* region in tobacco and *N. glauca* resulted from an ancient Ri-plasmid mediated gene transfer (Fründt et al. 1998). If this is the case, then the small (4%) divergence in sequence between *torf13-1* and *torf13-2* suggests that the transfer of *torf13* to the *tomentosiformis* ancestral line might have been a relatively recent event.

In conclusion, we have shown that the tobacco genome contains the complete ORF corresponding to a *orf13* homologue with eukaryotic expression signals,

that this gene is expressed in a developmentally regulated fashion, and that expression of this gene has at least some biological effects similar to those of its Ri-plasmid homologue. This provides strong evidence that a bacterial gene introduced into plants during evolution is still functional.

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