Biological Activity of the *Agrobacterium rhizogenes*–Derived *trnC* Gene of *Nicotiana tabacum* and Its Functional Relation to Other *plast* Genes

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Agrobacterium rhizogenes induces hairy roots through the activity of three essential T-DNA genes, *rolA*, *rolB*, and *rolC*, whereas the *orf13* gene acts as an accessory root-inducing gene. *rolB*, *rolC*, and *orf13* belong to the highly diverged *plast* gene family with remotely related representatives in the endomycorrhizal basidiomycete *Laccaria bicolor*. *Nicotiana glauca* and *N. tabacum* contain *A. rhizogenes*–derived T-DNAs with active *plast* genes. Here, we report on the properties of a *rolC* homolog in *N. tabacum*, *trnC*. Dexamethasone-inducible *trnC* and *A4-rolC* genes from *A. rhizogenes* A4 induce comparable, strong growth effects affecting all parts of the plants. Several have not been described earlier and were found to be very similar to the effects of the distantly related *plast* gene *6b*. They include leaf chlorosis and starch accumulation, enations, increase of sucrose-dependent leaf disk expansion, growth of isolated roots on low-sucrose media, and stimulation of sucrose uptake by small root fragments. Collectively, our findings indicate that enhancement of sucrose uptake plays an important role in generating the complex *6b* and *rolC* phenotypes and might be an ancestral property of the *plast* genes.

Agrobacterium rhizogenes is well-known for its capacity to induce hairy roots on a large number of plant species. Different strains harbor root-inducing plasmids (pRi) with different T-DNA structures, but all have a conserved central portion carrying the root locus (*rol*) genes *rolA*, *rolB*, and *rolC*. These three genes are both necessary and sufficient for hairy-root induction, although pRi8196 does not require *rolA* (Hansen et al. 1991). *orf13* and *orf14* enhance *rolA*-, *rolB*-, and *rolC*-induced root growth and are considered to be accessory (Aoki and Syono 1999b; Capone et al. 1989; Costantino et al. 1994; Meyer et al. 2000; Spena et al. 1987). *rolA*, *rolB*, *rolC*, and *orf13* modify growth when expressed individually, whereas *orf14* does not (Lemcke and Schmittling 1998).

Among the *A. rhizogenes* T-DNA genes, *rolB*, *rolC*, *orf13*, *orf14*, and the 5' part of *orf8* belong to a family of highly diverged genes without detectable DNA similarity but with sta-

tistically significant similarity at the protein level. They have been called *plast* genes (for phenotypic plasticity) (Levesque et al. 1988); *plast*-like genes also occur on the T-DNAs of *A. tumefaciens* and *A. vitis* (*b*, *c'*, *d*, *e*, *3'*, *5*, *6a*, *6b*, *7*, *lso*, and the 5' part of *iaaM*; Helfer et al. 2002), but until now, none were reported outside a T-DNA context. The mechanism of action of *plast* genes remains controversial, especially since the earlier proposed glucosidase activities of *rolB* and *rolC* have been questioned (Faiss et al. 1996; Meyer et al. 2000; Nilsson and Olsson 1997; Nilsson et al. 1993).

T-DNAs (called cellular [c]T-DNAs) also occur in *Nicotiana glauca* and *N. tabacum* and result from ancient transformation events (Aoki 2004; Furner et al. 1986; Intrieri and Buiatti 2001; Meyer et al. 1995; White et al. 1983). The *N. glauca* cT-DNA (Aoki 2004; Aoki et al. 1994; Furner et al. 1986) has been fully sequenced and consists of two inverted, incomplete T-DNA fragments of a pRi1724-like cT-DNA (Fig. 1). *NgroIC* (Aoki and Syono 1999a; Nagata et al. 1995) and *Ngorf13* (Aoki and Syono 1999c) are transcribed and have morphogenetic activity, whereas *NgroIB* is mutated. In the case of *N. tabacum*, a *trnC* (Meyer et al. 1995), a complete and a partial *trf13* (Fründt et al. 1998) and a *trf14* (Intrieri and Buiatti 2001) sequence have been reported. Mikimopine synthase (*mis*)-like sequences were identified by Southern analysis (Suzuki et al. 2002). The *trf13* gene of cultivar Havana 425 induces callus on carrot disks (Fründt et al. 1998), but the activity of *trnC* has not been tested. Here, we show that *N. tabacum* cv. Samsun nn has a mutated *trf13* gene and contains only one intact *rol* gene, *trnC*. Its growth-modifying properties are very similar to those of the *A. rhizogenes* A4-*rolC* gene and partially similar to those of the distantly related *6b plast* gene, suggesting conservation of an ancestral function.

RESULTS

N. tabacum cT-DNA contains the three essential *rol* genes.

Since the publication of the tobacco *trnC*, *trf13*, and *trf14* sequences, a large number of tobacco expressed sequence tags (EST) and genomic survey sequences (GSS) have been reported. Among these, we found three *trf8* and two *trf13* fragments (Table 1). The new tobacco sequences and the *N. glauca* cT-DNA were used to design primers allowing polymerase chain reaction (PCR) amplification of two cT-DNA fragments from *N. tabacum* cv. Samsun nn. The first fragment carries *trf8*, *trnC*, *trf13*, and *trf14*, the second *trf14* and *trf13*

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(Fig. 1; accession numbers FN667969 and FN667970, respectively). The two sequences align with the *A. rhizogenes* TL-DNA of pRi1724, but no fragments could be amplified between *torf13* and *torf14*. The *torf8-torf13* region is most closely related to sequences from A4 and *N. glauca* (70 and 80% homology, respectively), whereas *torf14-tmis* is 96% homologous to the *N. glauca* sequence up to the right border, but homology does not extend beyond, confirming that *torf14-tmis* is not inserted in the same region as *Ngorf14-Ngmis* in *N. glauca*, as earlier deduced from Southern analysis (Suzuki et al. 2002). Contrary to *N. glauca*, which lacks *rolA*, *N. tabacum* contains all three essential *rol* genes (*rolA*, *rolB*, and *rolC*). However, *rolA* has an internal stop codon at position 112 (normal open reading frame [ORF] = 270 nucleotides [nt]), *rolB* carries a 40-nt direct repeat (TCAACGCTT-(T/A)-CACCCAAGAAATCTCACTCGAGCATTGAGC) at position 24 with one mismatch, thereby causing an early frameshift (normal ORF size = 783 nt), and only *rolC* is intact. Interestingly, cv. Samsun nn *torf13* (*S-torf13*) differs from cv. Havana 425 *torf13-1* (*H-torf13-1*) (AJ007621). *H-torf13-1* is intact, but *S-torf13* is truncated beyond nt 400, as shown by PCR experiments with *H-torf13-1* primers downstream from position 400 (not shown). The *torf13* genes of *N. tabacum* cv. Wisconsin 38 and *N. tomentosiformis* are intact, whereas those of *N. tabacum* cv. Basma Drama 2, Samsun, and Xanthi are truncated (results not shown). *torf8* has a stop codon at position 1,873 (normal ORF size = 2,286 nt). *torf14* is intact, and *tmis* has a stop codon at position 199 (normal ORF size = 987 nt). Thus, *rolC* is the only potentially growth-modifying cT-DNA gene in cv. Samsun nn. This gene is expressed as shown by reverse transcription quantitative (RT-q)PCR analysis. In medium-sized leaves of *N. tabacum* Samsun nn plants (height 20 cm), the *rolC/EF2*

expression ratio (tested with appropriate primers as discussed below) is 1.0×10^{-4} (standard deviation = 0.3×10^{-4}).

Inducible expression of *dex-rolC-HAHIS* and *dex-A4-rolC-HAHIS* in tobacco.

Earlier studies showed that *rolC* is transcribed (Meyer et al. 1995), but its biological activity has not been investigated. The *A. rhizogenes* A4-*rolC* gene causes dwarfing in tobacco and pale-green lanceolated leaves (Schmülling et al. 1988). Therefore, we used A4-*rolC* as a standard to test *rolC*. In order to detect the proteins, we added an HAHIS tag (Thomas et al. 2006) at the 3' end of both genes. Experiments in our laboratory with other inducible HAHIS constructs showed that this construct does not modify tobacco growth, even at high expression levels, as detected with the anti-HAHIS antibody (results not shown). We therefore felt confident that this tag would not disturb plant growth. The tagged constructs were placed under inducible promoter control using the pTA7002 vector (Aoyama and Chua 1997), as earlier done for the *T-6b plast* gene (Grémillon et al. 2004). The pTA7002 vector allows controlled gene induction by addition of dexamethasone (dex) to the medium or by spraying plants with a dex solution. *N. tabacum* plants carrying a dex-GFP gene in the same pTA7002 vector (Shen 2001) and induced to high GFP levels did not show any particular growth effects under the conditions used in this work (results not shown). Dex-inducible *dex-rolC-HAHIS* and *dex-A4-rolC-HAHIS* genes were introduced in *N. tabacum* cv. Samsun nn (discussed below). Several independent transgenic lines showed high levels of tRoC-HAHIS or A4-RoC-HAHIS protein 24 h after leaf infiltration with dex. Five homozygous single-locus F2 lines (dex-*rolC*-HAHIS-7, dex-*rolC*-HAHIS-13, and dex-A4-*rolC*-HAHIS-6, dex-A4-*rolC*-HAHIS-7, and dex-A4-*rolC*-HAHIS-10) with high expression levels (Fig. 2A) were chosen for further studies. When seedlings of these lines were grown on inducing medium with 3 μ M dex, they showed a comparable growth reduction, reproducing the earlier reported *rolC* dwarfing phenotype (Fig. 2B). The dex-*rolC*-HAHIS-7 and dex-A4-*rolC*-HAHIS-7 lines were selected for further detailed studies. The phenotypic range for *rolC* and A4-*rolC* was determined with different inducer concentrations. Seedlings were grown on nylon filters on vertical plates containing media with dex concentrations ranging from 0 to 3 μ M. Results are shown in Figure 3. tRoC-HAHIS and A4-RoC-HAHIS proteins reached maximal levels at 0.3 μ M dex (Fig. 3A). At more than 0.03 μ M dex, cotyledons and leaves were smaller and pale green (Fig. 3B). Root hairs of dex-*rolC*-HAHIS-7 and dex-A4-*rolC*-HAHIS-7 seed-

Fig. 1. T-DNA and cellular (c)T-DNA maps. pRi1724: TL-DNA of *Agrobacterium rhizogenes* 1724 (NC_002575.1). *N. glauca*: cT-DNA of *Nicotiana glauca* (Suzuki et al. 2002). *N. tabacum*: cT-DNA fragments of *N. tabacum* (this work). Arrows: open reading frames. 10, 11 and 12: *rolA*, *rolB*, and *rolC*. mis: mikimopine synthase gene. Thick horizontal lines: earlier published sequences from *N. tabacum*.

Table 1. Overview of *Nicotiana tabacum* cellular (c)T-DNA sequences

cT-DNA region	Tobacco cultivar	Accession no.	Reference	DNA type
torf8	Petit Havana	EH664027	Millar et al., ^a unpublished	c-DNA
torf8	Hicks Broadleaf	FH900730	Opperman et al., ^b unpublished	Genomic DNA
torf8	Hicks Broadleaf	FH901074	Opperman et al., unpublished	Genomic DNA
torf8	Samsun nn	FN667969	This paper	Polymerase chain reaction (PCR)
rolA	Samsun nn	FN667969	This paper	PCR
rolB	Petit Havana	EH664294	Millar et al., unpublished	c-DNA
rolB	Hicks Broadleaf	FH925392	Opperman et al., unpublished	Genomic DNA
rolB	Samsun nn	FN667969	This paper	PCR
rolC	Havana 425	X91881	Meyer et al. 1995	PCR
rolC	Samsun nn	FN667969	This paper	PCR
rolC-torf13	Havana 425	AJ010794, AJ007621, AJ007622	Fründt et al. 1998	PCR
torf13	Not specified	AF281242	Intrieri and Buiatti 2001	PCR
torf13	Samsun nn	FN667969	This paper	PCR
torf14	Not specified	AF281246	Intrieri and Buiatti 2001	PCR
torf14	Samsun nn	FN667970	This paper	PCR
tmis	Samsun nn	FN667970	This paper	PCR

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lings became shorter at 0.01 μM dex and disappeared at 0.3 μM (Fig. 3C), and root growth slowed down (Fig. 3D) from 0.1 μM dex onwards. At later stages, cotyledons and first true leaves became chlorotic, thick, and epinastic. However, subsequent leaves and lateral roots of dex-troIC-HAHIS-7 seedlings normalized contrary to those of dex-A4-rolC-HAHIS-7 seedlings (Fig. 3E and F, also discussed below).

In order to compare *rolC* and *A4-rolC* effects on plants growing under greenhouse conditions, dex-troIC-HAHIS-7, and dex-A4-rolC-HAHIS-7 seeds were germinated in soil and were sprayed with 3 μM dex at the two-cotyledon stage. Dex-sprayed *rolC* and *A4-rolC* plants showed considerable growth reduction compared with wild-type plants (Fig. 4A). At later stages, side shoots replaced the main shoots, which appeared to be blocked. Surprisingly, several plants showed enations along the central leaf veins (Fig. 4B). Enations are a rare type of plant growth modification found in some viral diseases and in tobacco plants that express 2x35S-AB-6b (Helfer et al. 2003) or dex-T-6b (Grémillon et al. 2004) constructs derived from two *6b* genes from the *A. vitis* nopaline strain AB4 and the *A. vitis* octopine and cucumopine strain Tm4, respectively. The induction of enations reveals an unsuspected, highly specific

Fig. 2. Initial characterization of transformed homozygous lines. **A**, Expression of transgene-encoded proteins 24 h after leaf infiltration with 10 mM MgSO_4 (–) or with 3 μM dexamethasone (dex) in 10 mM MgSO_4 (+). Each sample contains the same total amount of protein. **B**, Phenotypes of induced seedlings 8 days after transfer to medium with 3 μM dex. Growth of induced *rolC* and *A4-rolC* lines has slowed down. WT = rC6, rC7, rC10, trC7 and trC13; wild-type = dex-A4-rolC-HAHIS-6, dex-A4-rolC-HAHIS-7, dex-A4-rolC-HAHIS-10, dex-troIC-HAHIS-7, and dex-troIC-HAHIS-13, respectively.

relation between *rolC* and *6b* *plast* genes. When dex-troIC-HAHIS-7 and dex-A4-rolC-HAHIS-7 plants at a height of 20 cm were sprayed with 3 μM dex, narrow leaves with irregular chlorosis patterns developed (Fig. 4C), demonstrating that *rolC* and *A4-rolC* not only interfere with early development but also induce changes in older plants. Chlorosis could be

Fig. 3. Control of dex-A4-rolC-HAHIS and dex-troIC-HAHIS expression by different dex (dexamethasone) concentrations and associated phenotypes. Dex-A4-rolC-HAHIS-7 and dex-troIC-HAHIS-7 seedlings were induced on media with 0, 0.01, 0.03, 0.1, 0.3, 1, and 3 μM dex (lanes 1 to 7, respectively). **A**, Western analysis of A4-rolC-HAHIS and trolC-HAHIS proteins with an antihemagglutinin antibody, 24 h after induction. Top: A4-rolC-HAHIS and trolC-HAHIS proteins. Bottom: loading control. A4-rolC and trolC proteins are detectable from 0.1 μM dex onwards. **B**, Seedlings 8 days after induction. Shoots are chlorotic and smaller from 0.1 μM dex onwards. Bar = 2 cm. **C**, Roots 8 days after induction. Root hairs are reduced from 0.03 μM dex onwards. Bar = 0.25 cm. **D**, Root growth (mm/day) of seedlings decreases with increasing dex concentration. Squares = dex-troIC-HAHIS-7, triangles = dex-A4-rolC-HAHIS-7. Values are means $\pm$ standard deviation. For each timepoint, 24 seedlings were measured. Experiments were repeated three times. Experimental errors were less than 3%. **E**, Wild type, dex-A4-rolC-HAHIS-7, and dex-troIC-HAHIS-7 seedlings 18 days after induction. Dex-A4-rolC-HAHIS-7 seedlings remain highly abnormal and yellow but dex-troIC-HAHIS-7 seedlings normalize, i.e., new leaves are normal and dark green and new lateral roots have normal root hairs. Bar = 1 cm. **F**, Wild type, dex-A4-rolC-HAHIS-7, and dex-troIC-HAHIS-7 seedlings 28 days after induction. Dex-A4-rolC-HAHIS-7 seedlings continue to generate abnormal leaves, whereas dex-troIC-HAHIS-7 seedlings produce normal leaves. Bar = 1 cm.

Fig. 4. *trcC* and *A4-rolC* modify growth of tobacco plants in soil. Wild type (WT), dexamethasone (dex)-*trcC*-HAHIS-7 (trC), and dex-*A4-rolC*-HAHIS-7 (rC) plants were sprayed with 3 μ M dex solution. **A**, Seedlings after induction at the 2-cotyledon stage on day 0, 8 and 20. Wild-type plants develop normally, dex-*A4-rolC*-HAHIS-7 and dex-*trcC*-HAHIS-7 plants show reduced growth. Bar = 3 cm. **B**, Enations (black arrows) on dex-*trcC*-HAHIS-7 (trC, on the left) and dex-*A4-rolC*-HAHIS-7 (rC, on the right) plants 40 days after induction at the two-cotyledon stage. no, abax = normal leaf, abaxial side. en, abax = enation, abaxial side. Enations are abnormal, extra leaf blades that are partially fused at their basal side to a normal leaf along the central leaf veins and are oriented in a mirror-wise fashion. Red arrows point to locations on the midveins where normal leaves and enations separate. **C**, Leaves of plants induced at an intermediate, nonflowering stage, 7 days after induction. Dex-*trcC*-HAHIS-7 (trC) and dex-*A4-rolC*-HAHIS-7 (rC) leaves show irregular chlorosis patterns and are reduced in width compared with a wild-type leaf (WT). Bar = 2 cm. **D**, Iodine staining revealing starch (black areas) in dex-*trcC*-HAHIS-7 (trC) and dex-*A4-rolC*-HAHIS-7 (rC) leaves, 7 days after local induction with a dex-containing lanolin sphere placed at the center. Upper two disks were treated with lanolin spheres without dex; lower two disks were treated with lanolin spheres with 10 μ M dex. Starch accumulates within a small zone around the inducing lanolin sphere. Bar = 1 cm; the small black dot shows size of the lanolin sphere. **E**, Starch accumulation in a dex-sprayed dex-*A4-rolC*-HAHIS-7 leaf. Left: Irregular chlorosis as in C. Right: Same area cleared with hot 70% ethanol and stained with iodine for starch; starch appears black. Chlorotic and starch-containing areas coincide.

induced locally by placing small lanolin spheres containing 10 μ M dex on well developed leaves; the induced areas not only became chlorotic but also accumulated high levels of starch, as revealed by iodine staining (Fig. 4D), showing that *trcC* and *A4-rolC* can act directly on mature leaf tissues. The chlorotic areas of uniformly sprayed leaves also correspond to high starch zones (Fig. 4E). Starch accumulation has also been reported for *6b* (Clément et al. 2006; Helfer et al. 2003) and for the *rolB*-like 5' part of *orf8* (Umber et al. 2002). The local induction of starch accumulation by lanolin spheres might suggest that *trcC* and *A4-rolC* directly affect leaf-specific processes (like photosynthesis). The root changes observed in seedlings could then be an indirect result of these leaf changes. Alternatively, *trcC* and *A4-rolC* act on processes that are found in both leaves and roots, which would indicate a more general cellular target. As a next step, we therefore tested the effects of *trcC* and *A4-rolC* on isolated shoots and roots by using leaf disks and isolated roots.

trcC and *A4-rolC* induce changes in leaf disks.

Induction of dex-*trcC*-HAHIS and dex-*A4-rolC*-HAHIS seedlings strongly reduces shoot and root growth. When roots were removed before induction, isolated induced shoots still slowed down their growth and became chlorotic, confirming that the action of *trcC* and *A4-rolC* on leaves is direct and does not require previous modification of roots. Local starch accumulation in leaves is most likely due to sucrose accumulation, which was analyzed using a sucrose-dependent leaf-disk expansion assay. Tobacco leaf disks from immature leaves floated on a simple medium (Keller and Van Volkenburgh 1997) will expand to a limited extent on medium without sucrose. When 3% sucrose is added, expansion increases by about 60%. The expansion of dex-*trcC*-HAHIS-7 and dex-*A4-rolC*-HAHIS-7 leaf disks in 3% sucrose was further increased upon induction with dex, whereas no increase occurred when the inducing medium contained no sucrose (Fig. 5). It can therefore be hypothesized that *A4-rolC* and *trcC* stimulate the uptake of sucrose from the medium, which then leads to increased

Fig. 5. Increase of leaf disk expansion by *trcC* and *A4-rolC* and dependence on sucrose. Disks of wild-type, dexamethasone (dex)-*A4-rolC*-HAHIS-7 and dex-*trcC*-HAHIS-7 lines were placed on liquid medium, and expansion after 5 days was expressed as the ratio of fresh weight at day 5 over the fresh weight at day 0, indicated as relative fresh weight. White bars = no induction, black bars = induction by 3 μ M dex. Induced dex-*A4-rolC*-HAHIS and dex-*trcC*-HAHIS disks show increased expansion but only on sucrose-containing media. Values are means $\pm$ standard deviation. For each medium, 12 disks were measured per line. Experiments were repeated twice. Experimental errors were less than 1%.

expansion. Since *T-6b* also stimulates tobacco-leaf disk expansion in the presence of sucrose (Clément et al. 2006), this further confirms the functional relation between *rolC* and *6b* (discussed above).

trcC and *A4-rolC* induce changes in isolated roots.

Isolated tobacco roots do not grow on medium with 1% sucrose. However, on the same medium, isolated dex-induced *trcC* and *A4-rolC* roots continued to grow, although they lost their root hairs, as in the case of intact seedlings. Sensitivity to sucrose was tested by placing dex-*trcC*-HAHIS-7 and dex-*A4-rolC*-HAHIS-7 roots on media with different sucrose concentrations (from 0 to 2%) with or without 3 μ M dex. Growth did not occur on medium without sucrose but was already visible at 0.1%; both lines showed similar sensitivity (Fig. 6A). Interestingly, dex-*trcC*-HAHIS-7 roots grew, initially, as fast as dex-*A4-rolC*-HAHIS-7 roots but ceased growth at day 5, whereas dex-*A4-rolC*-HAHIS-7 roots continued their growth for more than 10 days, an example (3 μ M dex and 1% sucrose) is shown in Figure 6B. Growth of isolated roots on low sucrose has also been demonstrated for dex-T-6b roots, and absorption of radioactively marked sucrose by these roots increased significantly upon induction (Clément et al. 2007). Sucrose uptake by dex-*trcC*-HAHIS-7 and dex-*A4-rolC*-HAHIS-7 root

Fig. 6. Influence of *rolC* and *trcC* on root growth and sucrose uptake. **A**, Growth of dexamethasone (dex)-*A4-rolC*-HAHIS-7 (squares) and dex-*trcC*-HAHIS-7 roots (triangles) grown on different sucrose concentrations, shown in mm/day over a 5-day period. Continuous lines = noninducing medium, broken lines = inducing medium. **B**, Length increase (in mm) of dex-*A4-rolC*-HAHIS-7 (squares) and dex-*trcC*-HAHIS-7 roots (triangles) on medium with 1% sucrose and 3 μ M dex, during a 10-day induction period. Dex-*A4-rolC*-HAHIS-7 roots show regular growth; growth in dex-*trcC*-HAHIS-7 roots slows down and stops at day 5. For **A** and **B**, values are means $\pm$ standard deviation. For each condition, 10 roots were measured per line. Experiments were repeated three times. Experimental errors were less than 3%. **C**, Sucrose uptake by subapical 3-mm-long root fragments of wild-type (WT), dex-T-6b (T-6b), dex-*A4-rolC*-HAHIS-7 (rC), and dex-*trcC*-HAHIS-7 (trC) lines, without induction (white bars) or after induction with 3 μ M dex (black bars). Dex-T-6b, dex-*A4-rolC*-HAHIS-7, and dex-*trcC*-HAHIS-7 lines show a clear increase in sucrose uptake, while the WT control does not. Data are expressed as percentage of sucrose taken up after 24 h. Values are means $\pm$ standard error. Root fragments were pooled in groups of five; ten groups were used per line. Experiments were repeated two times. Experimental errors were less than 10%.

fragments was measured in the same way, using wild-type tobacco and the dex-T-6b line D6Nt17 (Clément et al. 2007) as negative and positive controls, respectively. Sucrose uptake of wild-type tobacco-root fragments was not modified by dex treatment, whereas root fragments of induced dex-T-6b, dex-*trcC*-HAHIS-7, and dex-*A4-rolC*-HAHIS-7 seedlings showed a clear increase in sucrose uptake (Fig. 6C).

Expression of *dex-trcC*-HAHIS is affected by silencing.

In the course of our induction studies, some *dex-trcC*-HAHIS-induced growth changes rapidly reversed to normal in spite of the continued presence of inducer; shoots and lateral roots normalized (Fig. 3E and F), whereas isolated roots stopped their growth on 1% sucrose (Fig. 6B). Dex-*A4-rolC*-HAHIS changes, on the contrary, appeared stable. This difference in phenotype stability might be due to differences in tRoC and A4-RoC protein stability. Protein levels were compared for different induction times. Whereas A4-RoC-HAHIS protein levels remained stable for 2 weeks, tRoC-HAHIS protein showed a decrease starting after day 1, resulting in very low levels after 2 weeks (Fig. 7A). Transgene transcripts measured by RT-qPCR were found to decrease in dex-*trcC*-HAHIS-7 and dex-*A4-rolC*-HAHIS-7 seedlings, but more rapidly and to lower levels in dex-*trcC*-HAHIS-7 (Fig. 7B). Dex-*trcC*-HAHIS-7 plants showed increasing amounts of a 21-nt siRNA, demonstrating post-transcriptional gene silencing; dex-*A4-rolC*-HAHIS-7 plants did not (Fig. 7C).

Expression of *trcC* and *A4-rolC* in *Arabidopsis thaliana*.

Our results show that *dex-trcC*-HAHIS expression is rapidly silenced in tobacco. Expression of exogenous *trcC* and *A4-rolC* genes in tobacco might therefore lead to silencing of the endogenous tobacco *trcC* gene and make it difficult to distinguish between the effects of exogenous and endogenous *rolC* genes. In order to confirm *trcC* activity in a plant species lacking *rolC*-like genes, we transformed *Arabidopsis thaliana*

Fig. 7. Progressive silencing of dexamethasone (dex)-*A4-rolC*-HAHIS-7 and dex-*trcC*-HAHIS-7 after induction. Seedlings were placed on medium with 3 μ M dex and were analyzed 0, 1, 3, 5, 7, and 14 days later. **A**, Western analysis. Top, A4-RoC-HAHIS and tRoC-HAHIS proteins. Bottom, loading control. Whereas A4-RoC-HAHIS amounts remain stable, tRoC-HAHIS levels rapidly decline. **B**, Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The same samples shown in **A** were analyzed for mRNA levels by RT-qPCR. Units = relative expression levels $\times$ 0.0001 (calculated with respect to values at day 0, set at 1). Dex-*A4-rolC*-HAHIS mRNA levels decline slowly, whereas dex-*trcC*-HAHIS mRNA levels decline more rapidly. **C**, siRNA analysis. Top, dex-*A4-rolC*-HAHIS samples show no signal, whereas dex-*trcC*-HAHIS samples show increasing quantities of a 21-nucleotide siRNA (arrow). Bottom, RNA loading control obtained by hybridizing the filter to a U6 RNA probe (Gy et al. 2007).

ecotype Col-0 with *2x35S-trolC*, *2x35S-A4-rolC*, *2x35S-trolC-HAHIS*, *2x35S-A4-rolC-HAHIS*, and the pBI121.2 vector control. Plants of the T3 generation expressing *trolC* or *A4-rolC* (with or without HAHIS tag) showed similar phenotypes. Root growth was strongly reduced (Fig. 8A), flowering occurred early, both in vitro and in vivo (Fig. 8B and C, respectively), and plants were slightly less green than control plants. Thus, *trolC* and *A4-rolC* are biologically active in a plant species without an endogenous *rolC* gene.

Plast-like proteins in another eukaryote, *Laccaria bicolor*.

So far, *plast* genes have only been reported in *Agrobacterium* species and a few *Nicotiana* species. In the latter group, they are clearly derived from *Agrobacterium* species by horizontal transfer. The plast proteins similarly constitute an isolated group of unknown origin (Studholme et al. 2005). However, a recent databank search identified five *Laccaria bicolor* S238N-H82 proteins with unknown function as possible plast-like proteins: EDR08145.1 (National Center for Biotechnology Information number XP_001881215.1, 491 amino acids [aa], EDR04342.1 (XP_001884861.1, 451 aa), EDR04443.1 (XP_001884962.1, 273 aa), EDR04444.1 (XP_001884963.1, 284 aa), and EDR04445.1 (XP_001884964.1, 409 aa) (Martin et al. 2008). Four of the five proteins (XP_001884861.1, XP_001884962.1, XP_001884963.1, and XP_001884964.1) are coded by genes clustered within a 33-kb region. The common region of the five *Laccaria* proteins is about 250 amino acids in size, corresponding to the average size of the *Agrobacterium* plast proteins (Fig. 9A), and similarity values between the common regions range from 56 to 84%. XP_001884962.1 aligns with almost all plast proteins above statistically significant levels (not shown), whereas the four others only show significant homology to plast proteins C', D, and 5. Similarity values between plast and *Laccaria* proteins are in the range of inter-plast similarity values (about 20%). An alignment of XP_001884962.1 and the *Agrobacterium* plast protein with the highest similarity score (protein 5) is shown in Figure 9B.

Fig. 8. Expression of *A4-rolC* and *trolC* in *Arabidopsis thaliana*. Tagged and nontagged versions of *A4-rolC* and *trolC* under 2x35S promoter control and the empty pBI121.2 vector construct were used to transform *Arabidopsis thaliana* ecotype Col-0. T3 plants are shown. **A**, At 10 days after germination in vitro. Both the tagged and nontagged *A4-rolC* and *trolC* versions cause strong root growth inhibition, pBI121.2 vector plants are normal. **B**, At 21 days after germination in vitro. Early flowering of plants with tagged and nontagged versions of *A4-rolC* and *trolC*. **C**, At 32 days after germination in soil. Early flowering of plants with tagged and nontagged versions of *A4-rolC* and *trolC*. Bars in A and B = 1 cm, in C = 2 cm.

DISCUSSION

The *Nicotiana* cT-DNAs constitute the only known examples of naturally occurring DNA transfer in plants. Among the many unsolved questions regarding this phenomenon are those concerning the relation between the *N. tabacum* and *N. glauca* cT-DNAs, the present-day role of the cT-DNA genes, and the survival of the initial regenerants. The relation between the *Nicotiana tabacum* cT-DNAs and those of *N. glauca* are more complex than suspected. Two nonadjacent sequences occur in *N. tabacum*. The first carries *torf8* to *torf13* (with possibly an extension to the left) and is moderately related (70 to 80% homology) to the corresponding sequences from *A. rhizogenes* A4 and *N. glauca*. The second one carries *torf14* and *tmis* and extends up to the right border; this sequence is practically identical (96% homology) to the *N. glauca* *Ngorf14-Ngmis* region but is inserted in a different region. It should be noted that the evolution of *Nicotiana* species is extremely complex because of the occurrence of interspecific hybrids. Trees for different *Nicotiana* genes do not always coincide, so that no consensus has yet emerged for trees that reflect the evolution of the entire species (Kelly et al. 2010). Therefore, the tree we present in Figure 10A only reflects the evolution of the cT-DNA regions in the different species and does not pretend to represent species evolution.

To account for the present data, we propose the following evolutionary scenario (Fig. 10A). First, the two arms of the inverted repeat of the cT-DNA of *N. glauca* are clearly derived from a single transformation event (Fig. 10A, arrow 1, the inverted repeat structure is indicated by a) and, therefore, had initially the same sequence. The 5% divergence between the two arms (Furner et al. 1986) is due to divergent evolution between the two arms. The *torf14-tmis* sequence of *N. tabacum* shows 4% divergence with respect to the two *Ngorf14-Ngmis* sequences but is located in another site. The easiest assumption is that the *torf14-tmis* fragment (Fig. 10A, fragment b) de-

Fig. 9. Plast-like proteins in *Laccaria bicolor*. **A**, Schematic alignment of five *Laccaria bicolor* plast-like proteins with the *Agrobacterium* plast protein AAD30487 (gene 5 protein from *A. tumefaciens* C58). Double-headed arrow indicates the common part of plast proteins, about 250 amino acids. **B**, Alignment of protein XP_001884962.1 from *Laccaria bicolor* (top line) with gene 5 protein (bottom line). Bold indicates residues conserved in the *Agrobacterium* plast family.

rives from the same transformation event and T-DNA as fragment a but was inserted elsewhere. Later, fragment a and b separated and are now found in two different *Nicotiana* species. Secondly, since *trdB-torf13* (Fig. 10A, fragment c) and *NgroB-Ngorf13* differ by about 20%, we propose that *trdB-torf13* (fragment c) originates from another T-DNA than *NgroB-Ngorf13* and, therefore, from another transformation event (Fig. 10A, arrow 2). The order of entry (1 before 2 or 2 before 1) is unknown, and both fragments might have been combined by crosses rather than by a secondary transformation. Thirdly, the *trf8-torf13* fragment underwent a modification at the *trf13* end (Fig. 10A, c to c', modification indicated by arrow 3). The tobacco cultivars with a truncated *trf13* gene belong

Fig. 10. Evolutionary models. **A**, Origin of *Nicotiana* cellular (c)T-DNAs. TOM = *N. tomentosiformis*, TAB-Sa = *N. tabacum* cv. Samsun, TAB-Ha = *N. tabacum* cv. Havana, GL = *N. glauca*, SYL = *N. sylvestris*. Circles indicate present-day species, squares ancient species. Arrow 1, Transformation by a 1,724-like mikimopine strain, insertion of an inverted repeat inherited by GL (fragment a) and an *orf14-mis* fragment inherited by TOM and TAB (fragment b). *N. tabacum* is a hybrid of *N. tomentosiformis* and *N. sylvestris*. Arrow 2, Insertion of a second cT-DNA with *orf8-orf13* genes by an unidentified *A. rhizogenes* strain and inherited by TOM and TAB (fragment c). Arrow 3, Partial deletion of *trf13* in one group of TAB cultivars, inherited by TAB-Sa and related tobaccos (c to c'). **B**, Origin of *plast* genes. Arrow 1, Hypothetical transfer of *plast* genes from a fungus to *Agrobacterium* spp. by horizontal gene transfer. Subsequent divergence of *plast* genes between *Agrobacterium* spp. and fungi and within *Agrobacterium* spp. reduced DNA homology to undetectable levels and left only weak protein similarity. Arrows 2 and 3, Recent horizontal transfers of *Agrobacterium plast* genes to *Nicotiana* spp., DNA and protein are still significantly homologous. Thick lines indicate species with *plast* or *plast*-like genes.

to the oriental tobacco group. It would be interesting to test the influence of the wild-type *trf13* gene from Havana 425 on cv. Samsun nn and other oriental cultivars, as this may provide some ideas on the consequences of the loss of *trf13*. We believe our evolutionary model can provide a framework for further studies, especially with regard to cT-DNAs from other *Nicotiana* species.

N. tabacum carries the three essential hairy-root genes *rolA*, *rolB*, and *rolC*, but *trdB* and *trf13* are mutated. *trf13* has strong morphogenetic activity, very similar to that of *A4-rolC* from *A. rhizogenes*. Functional comparison was facilitated by the use of dex-inducible constructs, avoiding the accumulation of growth abnormalities, as in the case of constitutive genes.

In dex-*trf13*-HAHIS but not in dex-*A4-rolC*-HAHIS plants, post-transcriptional gene silencing led to a rapid and strong decrease in transgene mRNA and protein levels, with concomitant reversion to normal phenotypes. The strong *trf13*-HAHIS silencing merits further investigation, as it might also affect endogenous *trf13* expression. At the protein level, tRfC-HAHIS might compete with putative tRfC partners for binding or other types of interaction, thereby producing dominant negative effects. In addition, it is possible that the various abnormalities induced by strong dex-*trf13*-HAHIS expression produce indirect changes in expression of the endogenous *trf13* gene. These possibilities remain to be studied. However, the data from *Arabidopsis* show that the *trf13*-HAHIS gene construct does not need to interact with an endogenous *trf13* gene to produce a strong biological effect.

In this work, we have used several new *rolC* assays, monitoring growth changes after induction of seedlings, intact plants, leaf disks, or isolated roots. These assays have revealed hitherto unsuspected functional similarities between *rolC* and *6b*. In spite of the fact that 6B and RolC proteins show only 23% similarity, *AB-6b*, *T-6b*, *trf13*, and *rolC* all induce enations. Enations are secondary leaf blades arising in a mirror-wise fashion along the veins of the abaxial leaf side. Since these growth modifications are very unusual, we believe this provides a strong indication that *rolC* and *6b* impact similar pathways. *rolC* and *6b* also induce leaf chlorosis and starch accumulation. They stimulate leaf-disk expansion on sucrose-containing media, reduce root-hair development and root growth, and stimulate sucrose uptake by root fragments. In view of these results, we propose that the various *rolC* and *6b* effects are caused by enhanced sucrose absorption and retention. RolC is cytosolic (Estruch et al. 1991; Oono et al. 1991) and, therefore, probably not a transporter itself. The regulation of *rolC* transcription by sucrose and increased hexose levels in *rolC* transgenic potato has earlier led to the suggestion that *rolC* plays a role in sucrose metabolism, transport, or both (Nilsson and Olsson 1997). Our studies provide strong experimental support for this idea and indicate a role in sucrose uptake. In natural *A. rhizogenes* infections, sucrose accumulation may stimulate initiation and outgrowth of hairy roots. Since the tobacco *trf13* gene is expressed (Meyer et al. 1995) and functionally active in tobacco (this study), it could play a significant role in tobacco growth and physiology.

In spite of their similarities, *6b* and *rolC* plants also show characteristic differences. *6b* plants have dramatically expanded roots and show ectopic divisions in the root stele, around leaf veins, and on seedling stems (Grémillon et al. 2004; Helfer et al. 2003); contrary to this, *trf13* or *A4-rolC* plants do not. Instead, *trf13* and *A4-rolC* leaves are pale green and narrow, show wrinkled edges and irregular chlorosis, and are epinastic in seedlings. Possibly, these differences are due to differences in plast protein mobility. The *6b* phenotype is graft-transmissible (Helfer et al. 2003) and the 6B protein moves in leaves (Grémillon et al. 2004), whereas *rolC* effects are cell autonomous (Fladung

and Ahuja 1997; Fladung et al. 2004; Gidoni et al. 2001; Spena et al. 1989). 6B has also been shown to interact with the NtSIP1 protein (Kitakura et al. 2002) and to enter the nucleus (in which it acts as a histone H3 chaperone protein; Terakura et al. 2007). In view of the partial functional similarities between the RolC/tRolC and 6B proteins, it will be interesting to test the interaction of RolC/tRolC with NtSIP1 and H3 and their possible localization in the nucleus. Whether other *plast* genes share activities with *rolC* and *6b* remains to be tested. Interestingly, the *rolB*-like 5' part of *orf8* expressed under 2x35S promoter control causes dwarfing, irregular leaf chlorosis, and starch accumulation in tobacco with a strong reduction of sucrose export (Otten and Helfer 2001; Umber et al. 2002).

Our studies confirm that *rolC* accelerates flowering as noted in tobacco and in other plant species (Casanova et al. 2005; Faiss et al. 1996). Possibly, this *rolC* property led to rapid speciation of the initial *Nicotiana* transformants.

The occurrence of *plast*-like genes in the ectomycorrhizal basidiomycete *Laccaria bicolor* is of considerable interest. The close association of *Agrobacterium* spp. and *Laccaria* spp. with plant roots and their common interest in plant-derived metabolites (López et al. 2008) might have favored horizontal DNA transfer from the fungus to the bacterium (Fig. 10B, arrow 1a). A long period of divergence then generated the many different *Agrobacterium plast* genes. Although a transfer from the bacterium to the fungus cannot be excluded, the lack of other T-DNA genes, like opine synthesis genes (often found close to the right T-DNA borders and, therefore, likely to be cotransferred) and hormone synthesis genes in the two chromosomal *Laccaria* regions that contain *plast*-like genes, makes this less likely. More recently, at least two transfers occurred from *Agrobacterium* spp. to *Nicotiana* spp. (Fig. 10B, arrow 2 and 3). The distribution of *plast*-like genes in other fungi, their role in *Laccaria* spp. and their effects on plants should constitute topics for further studies.

MATERIALS AND METHODS

A4-*rolC* and *tolC* constructs.

For constitutive expression, A4-*rolC* and *tolC* coding regions were cloned in a 2x35S promoter cassette by PCR and were subcloned into the pBI121.2 binary vector (Jefferson et al. 1987) as described for other constructs (Otten and Helfer 2001). Dex-inducible constructs were prepared as for *dex-T-6b* (Grémillon et al. 2004), using the pTA7002 binary vector (Aoyama and Chua 1997). At the locations indicated, a HAHis tag from pNTL2104 (Thomas et al. 2006) was added at the 3' end, to permit protein detection using antihemagglutinin antibodies. All constructs were checked by sequencing before subcloning. Binary vectors with constructs were introduced in the disarmed *Agrobacterium* helper strain LBA4404 (Hoekema et al. 1983).

Transformation and regeneration.

Tobacco transformation and regeneration was as described (Otten and Helfer 2001). *Arabidopsis thaliana* ecotype Colombia (Col-0) plants were transformed by the floral dip method (Clough and Bent 1998).

Induction by dex.

Plants were induced with dex in different ways. Mature leaves were infiltrated with a dex solution in 10 mM MgSO₄, using a 1-ml syringe without a needle. Seedlings were germinated on MS255 (Duchefa, Haarlem, The Netherlands) agar plates with 1% sucrose and, 10 days later, were placed on nylon filters on vertical plates with the same medium and, 3 days after that, were transferred to inducing vertical plates. Leaf disks

were induced by floating them upside down on a solution of 10 mM KCl, 0.5 mM HEPES buffer, pH 6.0, with varying concentrations of sucrose and were weighed at 1-day intervals (Clément et al. 2006; Keller and Van Volkenburgh 1997). Intact small or medium-sized plants in soil were sprayed on 2 consecutive days with 3 µM dex and 0.05% Tween-20 in water. Leaves of different sizes were locally induced by placing lanolin spheres (1 mm in diameter) containing 10 µM dex on the upper leaf surface. One week later, disks were removed and stained for starch with iodine.

Sucrose absorption.

Sucrose absorption was tested with 3-mm-long root fragments situated originally at 9 to 12 mm from the root apex. These fragments were obtained from intact seedlings grown for 3 days on inducing medium on vertical plates. Five fragments were floated on a 15-µl drop of MS255 medium with 1 mM ¹⁴C-sucrose in microtiter plates (Clément et al. 2007). Radioactivity in the medium was measured at the start of the experiment and after 24 h; all experiments were carried out in triplicate.

Arabidopsis thaliana seed germination.

Arabidopsis thaliana seeds (generation T3) were germinated on MS255 medium in the presence of 50 mg of kanamycin per liter and 1% sucrose for 8 days and were then transferred to soil or to nylon filters on vertical agar plates containing the same medium.

RNA extraction and siRNA gel blotting.

RNA was extracted from leaves of tobacco seedlings using Tri-Reagent (Sigma, St. Louis). Leaves of 15 plants (two leaves per plant) were ground in liquid nitrogen. The resulting powder was homogenized in 1 ml of Trizol and 200 µl of chloroform was added. The upper phase was precipitated with 1 volume of isopropanol. After washing with 70% ethanol (1 ml), RNAs were taken up in RNase-free H₂O. RNA gel-blot analysis of low-molecular-weight RNA was conducted on 25 µg of total RNA as described (Akbergenov et al. 2006). The A4-*rolC* coding sequence and the 183 to 395 fragment of the *tolC* coding sequence were used as probes. Blots were stripped and were rehybridized with a probe complementary to U6 RNA as a loading control (Gy et al. 2007).

RNA extraction and RT-quantitative PCR.

RNA was extracted as described above. After being treated with RNase-free DNase kit (Qiagen, Hilden, Germany), cDNA was synthesized with 5 µg of total RNA, using the Superscript III reverse transcription kit (Invitrogen, Carlsbad, CA, U.S.A.). Real-time qPCR reactions (10 µl) were performed in 384-well optical plates on a BioRad i-cycler apparatus using PCR master 2x mix (Roche Molecular Systems, Branchburg, NJ, U.S.A.) containing 480 SYBER Green I fluorescein reporter with gene-specific primers. Quantification was performed in triplicate. *Nicotiana tabacum* translation elongation factor gene (*EF2*; GenBank accession number AJ299248.1) was used as an internal standard for equalization of RNA levels. Quantifications were then normalized to noninduced tobacco plantlets. Experiments were repeated three times in a biologically independent manner.

RT-qPCR primers.

Primers used for RT-qPCR are as follows: *EF2*-Forward, 5'-ctgaaccagaagcgtggaca-3'; *EF2*-Reverse, 5'-ccagatgtacgagccctc aag-3'; *tolC*-Forward, 5'-ctgaaccagaagcgtggaca-3'; *tolC*-Reverse, 5'-ccctgacattcagctttatgcac-3'; *rolC*-Forward, 5'-gggcagt cgacgtagagg-3'; *rolC*-Reverse, 5'-cgatggatattgacgaagaagg-3'.

Sequencing primers.

The sequencing primers for the *torf8-torf13* fragment were: D1958, 5'-gaatggcagcagggcaaccattattt-3' (from EH664027); D1686, 5'-tgcagagattatcggtcga-3' (primer 10 in Meyer and associates [1995]); D1734, same as D1686 but reverse complement; D1884, 5'-cccgaacaggagttatgga-3' (from AF281242.1). The sequencing primers for the *torf14-tmis* fragment were: D1917, 5'-tgagcatgccgtcttgac-3' (from AB071334.1); D1865, 5'-cccttttcgttaataagatggacctt-3' (from AB071334.1).

General PCR conditions were: 5 min at 94°C, and then, 30 cycles at 94°C (20 s), 60°C (20 s), and 72°C (30 s), followed by 10 min at 72°C. Conditions were adapted with respect to primer sequence and expected fragment length.

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