Organization of the TC and TE cellular T-DNA regions in *Nicotiana otophora* and functional analysis of three diverged TE-6b genes

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SUMMARY

*Nicotiana otophora* contains *Agrobacterium*-derived T-DNA sequences introduced by horizontal gene transfer (Chen et al., 2014). Sixty-nine contigs were assembled into four different cellular T-DNAs (cT-DNAs) totaling 83 kb. TC and TE result from two successive transformation events, each followed by duplication, yielding two TC and two TE inserts. TC is also found in other *Nicotiana* species, whereas TE is unique to *N. otophora*. Both cT-DNA regions are partially duplicated inverted repeats. Analysis of the cT-DNA divergence patterns allowed reconstruction of the evolution of the TC and TE regions. TC and TE carry 10 intact open reading frames. Three of these are TE-6b genes, derived from a single 6b gene carried by the *Agrobacterium* strain which inserted TE in the *N. otophora* ancestor. 6b genes have so far only been found in *Agrobacterium tumefaciens* or *Agrobacterium vitis* T-DNAs and strongly modify plant growth (Chen and Otten, 2016). The TE-6b genes were expressed in *Nicotiana tabacum* under the constitutive 2×35S promoter. TE-1-6b-R and TE-2-6b led to shorter plants, dark-green leaves, a strong increase in leaf vein development and modified petiole wings. TE-1-6b-L expression led to a similar phenotype, but in addition leaves show outgrowths at the margins, flowers were modified and plants became viviparous, i.e. embryos germinated in the capsules at an early stage of their development. Embryos could be rescued by culture *in vitro*. The TE-6b phenotypes are very different from the earlier described 6b phenotypes and could provide new insight into the mode of action of the 6b genes.

Keywords: *Nicotiana otophora*, cT-DNA, natural transformant, 6b oncogene, plast genes, *Agrobacterium rhizogenes*, vivipary.

INTRODUCTION

Several plant species carry *Agrobacterium* T-DNA sequences (cellular T-DNAs or cT-DNAs) in their genomes and can be considered as ‘natural transformants’. cT-DNAs were most likely introduced by infection with *Agrobacterium rhizogenes* and subsequent regeneration of hairy roots (Matveeva and Lutova, 2014; Chen and Otten, 2017). cT-DNAs have been found in *Nicotiana* (White et al., 1983; Furner et al., 1986; Chen et al., 2014), *Linaria vulgaris* (Matveeva et al., 2012) and *Ipomoea batatas* (Kyndt et al., 2015).

Expression of the *Nicotiana glauca* genes Ngorf13 and NgroIC and of the *Nicotiana tabacum* genes torf13 and trolC in various test plants demonstrated that they have growth-modifying properties similar to their *A. rhizogenes* equivalents (Meyer et al., 1995; Fründt et al., 1998a,b; Aoki and Syono, 1999b,c; Aoki, 2004; Mohajjel-Shoja, 2010; Mohajjel-Shoja et al., 2011). The TB-mas2 gene of *N. tabacum* encodes opine synthesis in tobacco (Chen et al., 2016).

The cT-DNA sequences have been identified and fully assembled using genomic sequence data. Four cT-DNA
inserts (TA, TB, TC and TD) were found in Nicotiana tomentosiformis (Chen et al., 2014; Chen, 2016); these differ from the N. glauca cT-DNA. TA, TB, TC and TD carry partial, inverted repeats. Using repeat divergence, the order of the insertion events could be reconstructed as TC > TB > TD > TA. Members of the section Tomentosae carry different cT-DNA combinations (Chen et al., 2014). From this analysis it was concluded that the N. tomentosiformis ancestors were repeatedly transformed by different A. rhizogenes-like strains. Lack of T-DNA-like sequences in the Nicotiana setchellii transcriptome (Long et al., 2016), and the failure to amplify TC fragments from its genomic DNA (Chen, 2016), indicate that the N. setchellii line split off before the first cT-DNA insertion. The proposed order and distribution of the four cT-DNA inserts is consistent with the phylogeny of section Tomentosae (Knapp et al., 2004).

Here we report on the cT-DNA sequences in Nicotiana otophora Griseb. This species was first described by August Grisebach from a population in Tarija, Cuesta Colorado, South Bolivia (Grisebach, 1879). The name otophora means ‘having auricles’ and refers to the shape of the leaf petioles. Several N. otophora accessions have been collected from Bolivia and Argentina. The majority of the Tomentosae species are day-flowering and pollinated by bees and hummingbirds, but N. otophora is exceptional as it is night-flowering and pollinated by bats and hawkmoths (Nattero et al., 2003). Nectar sugars and amino acids play a role in pollinator choice (Tiedge and Lohaus, 2017). Nicotiana otophora was initially suspected to be the paternal parent of N. tabacum (Goodspeed, 1954), but this idea was later abandoned, favouring instead N. tomentosiformis (Nattero et al., 2003). Nicotiana otophora has been used in tobacco breeding (Reed and Schneider, 1992).

In a preliminary analysis, genomic sequences from N. otophora (Sierro et al., 2014) were searched for cT-DNA sequences. This yielded 25 contigs with TC sequences and 44 contigs with other cT-DNA sequences. The latter were tentatively attributed to a new cT-DNA called TE (Chen et al., 2014). The TC and TE sequences could not be assembled at the time because of unexpected sequence variability, suggesting three or four non-identical copies.

Surprisingly, the TE contigs carried not only A. rhizogenes sequences but also genes typical for Agrobacterium tumefaciens/Agrobacterium vitis: vitopine synthase (vis) and gene 6b. The 6b gene was initially described as an oncogene since it induces tumours on N. glauca (Hooyskaas et al., 1988). Its expression in various plant species causes strong morphological changes (for reviews see Ishibashi et al., 2014; Ito and Machida, 2015). AB-6b from A. vitis strain AB4, and T-6b from A. vitis strain Tm4 have been expressed in tobacco under 2 × 35S promoter control, and induce a specific phenotype, called ‘enation syndrome’ (Helfer et al., 2003; Grémillon et al., 2004). This includes leaf and flower doubling (enations and catacorollas, respectively), ectopic vascular strands, root thickening on succore media and a large number of other abnormalities.

Four basic 6b-induced changes were identified: chlorosis, induction of ectopic vascular strands and leaf primordia, and abnormal cell expansion (Chen and Otten, 2016). Earlier work showed a 6b-induced increase in sucrose uptake and retention, both in leaf discs and root fragments, suggesting that this could be the underlying mechanism for the observed growth changes (Clément et al., 2006, 2007). Other mechanisms have been proposed for 6b gene activity (Wabiko and Minemura, 1986; Gális et al., 2002, 2004; Kitakura et al., 2002, 2008; Kakiuchi et al., 2006; Terakura et al., 2007; Wang et al., 2011; Takahashi et al., 2013; Ishibashi et al., 2014; Ito and Machida, 2015; summarized in Chen, 2016).

The unexpected finding of vis and 6b sequences in N. otophora raised the following questions. Are they connected to the A. rhizogenes-like sequences or do they result from a transformation by A. tumefaciens or A. vitis? Do the cT-DNA sequences of N. otophora contain intact open reading frames? Are the unusual 6b genes intact and, if so, are they biologically active? Preliminary analysis of the contigs showed that TC and TE were duplicated, partial inverted repeats, yielding from one to four copies for different regions. This complex situation resulted in errors in the automatic contig construction and necessitated re-assembly and re-mapping of the original reads. Here we report the maps of the TC and TE inserts, a reconstruction of the evolution of these inserts, and a functional analysis of the N. otophora TE-6b genes.

RESULTS

Properties of N. otophora

We obtained six N. otophora accessions: TW94, TW95, TW96, TW97 (from the US Nicotiana Germplasm Collection), NIC406 (from IPK Gatersleben) and ITB643 (from Imperial Tobacco Bergerac). These accessions show phenotypic differences, particularly in leaf venation (Figure S1a–f in the online Supporting Information; Figure S1g shows N. tabacum cv. Samsun). Accessions with well-developed veins have wrinkled leaves (Figure S1h), petiole wings are large and carry strong veins like the leaf (Figure S1i). These differences were stable and reproducible under our greenhouse conditions. Since N. otophora TW95 was used to obtain genomic and transcriptome sequence data (Sierro et al., 2014), we used this accession for our studies.

Mapping of TC-1 and TC-2

The N. tomentosiformis TC region (TOF-TC) has been described before (Chen et al., 2014). It is a 17-kb imperfect inverted repeat of a DNA fragment which resembles the
left part of the A. rhizogenes A4 TL-DNA (Slightom et al., 1986) with orf2, orf3n, orfB, rolA and rolB sequences. The inverted repeat is flanked by two unique regions: an ocl (octopine synthase-like) gene in the left part of the cT-DNA insert and a gene c-like sequence on the right. TOF-TC carries only one intact open reading frame (ORF). ocl. The ocl and c-like genes are unusual, as they are normally only found in A. tumefaciens or A. vitis.

Nicotiana otophora TW95 carries two TC copies, TC-1 and TC-2 (Figure 1). These differ by 4%, but are inserted in the same plant DNA sequence as TOF-TC and are similarly organized, therefore they are derived from the same insertion event. The N. otophora and N. tomentosiformis TC regions show some interesting differences. TC-1 carries a 3237-nucleotide (nt) plant sequence in the right arm of its inverted repeat, within the orf3n sequence (TC-1 plant sequence; TC-1P, Figure 1a), surrounded by a 16-nt direct repeat (TATCATTCTGCAATCA). TC-1P is 72% identical to a long interspersed nuclear element (LINE-1-like retrotransposon) from Solanum tuberosum (HMO13964.1; Wolters et al., 2010). TC-1P potentially encodes a 782-amino-acid ORF with 76% identity to an RNA-directed DNA polymerase from mobile element Jockey-like from Nicotiana attenuata (XP_019236372.1). TC-1P sequences are also found elsewhere in the N. otophora genome. TC-2 carries a 206-nt plant sequence in the left arm (TC-2P, Figure 1a), surrounded by an imperfect 12-nt direct repeat (TTGTGCAAACTA and TTGTCAAAACTA), 73 nt downstream of the TC-1-orf3n stop codon. This sequence is similar to various Nicotiana short interspersed nuclear element (SINE) sequences (Wenke et al., 2011), and is partially present in TC-1P. TC-2P is 95% identical to SINE TS_N12 (221 nt, HE583509) from N. tabacum and is also found elsewhere in N. otophora. LINES and SINES are reverse transcripts of RNA molecules, integrated in various genomic locations (Wenke et al., 2011). TOF-TC lacks TC-1P and TC-2P. Therefore it is likely that these sequences were inserted into the N. otophora TC regions after the separation of N. otophora and N. tomentosiformis.

The structure of the orf3n–orfB regions of the right and left arms of the TC-1 and TC-2 regions of N. otophora and N. tomentosiformis carry not only TC-1P and TC-2P but also other indels. Together, they are marked ‘a’ to ‘h’ in the sequence alignment shown in Figure S2 and in the schematic in Figure 1b. The distribution of the various indels was used to reconstruct the evolution of this region (Figure 1b). A 35-nt deletion (‘b’) is present in all right arms, and therefore occurred at an early stage after insertion, before TC duplication. A 350-nt deletion in the left arm of TC-1 of both N. otophora and N. tomentosiformis (‘h’) must have occurred prior to the separation of the N. tomentosiformis and N. otophora lines. A 17-nt deletion (‘c’) and the TC-1P insertion (‘a’) in the right arm of TC-1 of N. otophora, but not of N. tomentosiformis, must have occurred after the separation of N. tomentosiformis and N. otophora. Finally, a 9-nt insertion (‘g’) and the TC-2P insertion (‘e’) in the TC-2 left arm, and an 8-nt deletion (‘d’) and 31-nt insertion (‘f’) in the TC-2 right arm are specific for TC-2 and therefore occurred after the TC duplication.

The different TC modifications provide a scenario for the evolution of the TC region (Figure 1b, c). Assembly of the two N. otophora TC regions allowed us to identify their intact ORFs. TC-1 carries two intact ORFs, ocl and orf3n, whereas TC-2 contains none.

Mapping of TE-1 and TE-2

The TE regions were assembled in the same way as were the TC regions. As with TC, two TE copies were found: TE-1 and TE-2 (Figure 2).

The common parts of TE-1 and TE-2 differ by 1.7%, with TE-1 being larger than TE-2. On the left of Figure 2, TE-1 resembles the T1 T-DNA of A. vitis strain S4 (Canaday et al., 1992). However, similarity between TE and S4 T-1 is low, and only the vis part was detected by BLASTN analysis (71% nucleotide identity with region 3122-2084 of M91608.1). The closest TE-6b homolog is T-6b from A. vitis Tm4 (71% nucleotide identity, but only for the central 174–504 part of the 6b gene, on a total of 630 nt). The right end of the TE-1 vis-6b region is part of a partial repeat (R1) with 6b and tryptophan monoxygenase (iaaM) sequences. The left and right arms (R1-L and R1-R) are separated by a unique region with iaaH and agrocinopine synthase (acs) sequences. The organization of R1 is similar to that of the A. tumefaciens and A. vitis T-DNAs of the LB-acs-5-iaaH-iaaM-ipt-6a-6b-ocs/vis-RB type (as in A. tumefaciens A6, or A. vitis Tm4). However, genes 5, ipt and 6a are missing. The right part of TE-1 consists of another inverted repeat (R2) with rolC-orf13-orf13a-orf14-mas2-mas′ sequences on its left and right arms (R2-L and R2-R). R2 is similar in organization and sequence (78% identity) to the right part of the A. rhizogenes strain 8196 T-DNA (Hansen et al., 1991). TE-2 is similar to TE-1, but the right arm of R1-L and the left arm of R2-L are missing. The region between R1-L and R1-R (with iaaH and acs sequences) is inverted with respect to TE-1, and with respect to the normal T-DNA orientation. This is probably due to homologous recombination between the R1 repeats.

A model for the origin and evolution of the TE inserts is shown in Figure 3. The partial inverted repeats were most likely derived from two separate T-DNAs (marked in blue and red): one with acs-iaaH-iaaM-6b-vis and the other with rolB-rolC-orf13-orf13a-orf14-mas2-mas′, linked at their right borders, and (most likely) located on the same plasmid. Alternatively, TE could result from a mixed infection involving two different Agrobacterium strains. Fragments a, b, c and d were probably ligated together before integration to form a composite structure, which was then inserted into the plant genome. Subsequently, this insert

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Figure 1. TC-1 and TC-2.
(a) Maps of TC-1 and TC-2. Horizontal black lines: TC regions. Horizontal orange lines: Agrobacterium rhizogenes A4 TL-DNA (Slightom et al., 1986), with open reading frames (ORFs). Orange blocks: regions of similarity with A4 TL-DNA. Only the intact TC ORFs (TC-orf1 and TC-orf3nt) are shown. R-L and R-R: left and right arms, respectively, of inverted repeats. Green areas: plant DNA. TC-1P and TC-2P: plant insertion elements.

(b) Evolution of the TC orf3nt-orf8 region. This model is derived from indel events (a–h) found in the Nicotiana tomentosiformis TC region (Chen et al., 2014) and in TC-1L, TC-1R, TC-2L and TC-2R from Nicotiana otophora (this work). For sequence alignment see Figure S2. Left and right arms are aligned in the same direction to facilitate comparison. Evolution of regions orf3nt–orf8 is presented from top to bottom. The 350-nucleotide (nt) sequence marked ‘h’ was deleted after the TC duplication event in TC-1 but not in TC-2. Nicotiana tomentosiformis inherited TC-1. TC-1P (a) and TC-2P (e) are plant insertion elements.

(c) Evolution of the TC region. The width of lines indicates zero, one and two copies. After the line leading to Nicotiana setchellii had split off, a single TC region was inserted. This TC region was subsequently duplicated to TC-1 and TC-2. TC-2 was lost in the line leading to Nicotiana tomentosiformis. In Nicotiana tabacum, TC-1 was lost as well.

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was duplicated and both regions underwent further modifications.

Assembly of the two TE maps allowed us to identify eight intact ORFs: TE-1-6b-L, TE-1-6b-R, TE-1-orf14-L, TE-1-orf14-R, TE-2-6b, TE-2-rolC, TE-2-orf13 and TE-2-orf14. All belong to the plast gene family (plast for phenotypic plasticity; Levesque et al., 1988; Helfer et al., 2002). The TE-6B and TE-Orf14 proteins are quite different from their homologs (see below) but TE-Orf13 and TE-RolC are very similar to the *N. glauca* equivalents (Figure S3).

Interestingly, TC carries *orf2-orf3-orf8-rolA-rolB* sequences (as on the left part of the A4 TL-DNA) whereas TE carries *rolC-orf13-orf13a-orf14-mas20-mas10* sequences (as on the right part of the A4 TL-DNA). Thus it seemed possible that both represent two different fragments from the same T-DNA. However, TC and TE show a small 350-nt overlap at the start of the *rolB* gene with only 77% identity. Therefore, TC and TE are derived from two different *A. rhizogenes* strains and were introduced at different times. In total, *N. otophora* contains 83 kb of cT-DNA, distributed over four inserts. No border sequences could be found (see Experimental Procedures). Together, TC-1, TC-2, TE-1 and TE-2 carry 10 intact ORFs.

**Transcription of the TC and TE regions**

The cT-DNA sequences of *N. otophora* were investigated for transcriptional activity (see Experimental Procedures). Transcriptome reads from TW95 leaf and root tissues were mapped on TC-1, TC-2, TE-1 and TE-2 (Figure S4). Overall transcription levels were low, except for TC-1P (13.9 and 6.4 RPKM (reads per kilobase of transcript per million mapped reads) in leaf and root, respectively) and TC-2P (3.4 and 6.8 RPKM in leaf and root, respectively). TC-1 and TC-2 had very low transcription levels (0.01 RPKM for TC-1-ocl in leaf), whereas TE-2 had higher levels than TE-1 (0.41 RPKM for TE-1-6b-L in root, 0.59 and 0.41 RPKM for TE-2-6b in leaf and root respectively, 0.44 RPKM for TE-2-rolC in leaf, 0.22 RPKM for TE-2-orf13 in leaf, and 0.20 and 0.23 RPKM for TE-2-orf14 in leaf and root, respectively).

**Functional analysis of the *N. otophora* 6b genes**

*Nicotiana otophora* TE-1 and TE-2 contain three intact 6b genes, derived from the single 6b gene carried by the original T-DNA (Figure 3). TE-1-6b-L is part of the TE-1 R1-L repeat and TE-2-6b occupies an equivalent position on TE-2. TE-1-6b-R is located on the R1-R part of TE-1; this region is deleted in TE-2. The three predicted 6B protein sequences are very similar to each other, and quite different from the closest homolog, T-6B (CAA39648.1) with an identity value of only 54% (113 out of 208 amino acid residues for TE-1-6B-L). The acidic repeat found in the C-terminal part of various 6B proteins (Helfer et al., 2002) is also present in the TE-6B proteins (Figure S5). However, TE-1-6b-L is shorter by seven E residues. The promoter regions of TE-1-6b-L and TE-2-6b are similar. In contrast, 167 nt upstream from the start codon, the TE-1-6b-R promoter region is linked to the 3' region of the *mas1* gene on the R2-L repeat, and its sequence diverges. The transcription data show very low expression for TE-1-6b-R, and measurable levels for TE-1-6b-L and TE-2-6b, mainly in the leaves (Figure S4).
The three TE-6b genes were placed under the control of the constitutive 2 × 35S promoter (see Experimental Procedures) in order to maximize chances to obtain a phenotype, and tested for biological activity. As several 6b genes induce tumours on *Nicotiana rustica* and *Kalanchoe tubiflora* (Hooykaas et al., 1988; Helfer et al., 2002; Chen and Otten, 2016), we first tested the TE-6b gene constructs on these plants, but no tumours were formed. Subsequently, the TE-6b genes were introduced into *N. tabacum* (see Experimental Procedures).

Figure 3. Model for the evolutionary origin of TE-1 and TE-2.

(a) Octopine T-DNA structure. (b) Hypothetical *Agrobacterium tumefaciens*-like T-DNA (blue) and *Agrobacterium rhizogenes*-like T-DNA (red) of the *Agrobacterium* strain which introduced TE. Most likely the blue and red regions were part of separate T-DNAs from the same Ri plasmid. On the left: an *acs-iaaH-iaaM*-6b-vis region, related to the octopine T-DNA structure *acs-5-iaaH-iaaM-ipt-6a-6b-ocs-vis*, but without genes 5, ipt and 6a. Fragments a and b became part of the TE region. On the right: right part of an *A. rhizogenes*-type T-DNA with genes *rolB-rolC-orf13-orf14-mas2*-mas1 (as in *A. rhizogenes* strain 8196; Hansen et al., 1991). Fragments c and d became part of the TE region. (c) Original TE region obtained by ligation of T-DNA fragments a, b, c and d, shown in (b). It is assumed that originally all open reading frames (ORFs) were intact, except at the junctions between the fragments. (d) Modification of the original TE region by deletions (double-headed arrows), and loss of several ORFs. TE-6b-L, TE-6b-R, TE-orf14-L, TE-orf14-R, TE-rolC and TE-orf13 and TE-orf14-R remained intact. (e) Generation of TE-1 by duplication of the TE region, and loss of TE-1-rolC and TE-1-orf12. (f) Generation of TE-2 by duplication of the TE region, followed by loss of the central part with R1-R and R2-L, and inversion of the region indicated by the magenta arrow.

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severe cases (Figure 5a-f), fan-shaped leaves developed. In some cases, leafy filaments developed on the leaf edge and grew into fan-shaped structures (Figure 5a, b). Leaf margins were broader and irregular and carried numerous large trichomes (Figure 5c, d). Roots grew directly from the leaf base (Figure 5e). Ectopic meristems could be found on the adaxial leaf surface (Figure 5f). Less severely affected, but still highly modified, TE-1-6b-L plants (Figures 6a and S6) developed stems and roots, and although they grew slowly they reached the same height as wild-type tobacco before flowering. Petiole wings formed distinct leaflets (Figure 5b). Veins were strongly developed, up to the leaf edge (Figure 6c-e). Leaf tips of TE-1-6b-L plants were often split (Figure 6f), and leaf edges could be highly irregular (Figure 6g). Leaflets growing from the leaf edges showed white, semiglobular growing points (Figure 6h, i), as on ectopic meristems in dex-T-6b tobacco plants (Chen and Otten, 2016). Ectopic shoot primordia appeared on some leaves (Figure 6j, k). The youngest leaves (Figure S6k) and the base of older leaves (Figure S6c) curled downward at the edges, and bulged between the veins (Figure S6d). Leaf edges on the apical part of the lower leaves were dentate at the apex and folded at the base (Figure S6a, b), and leaflets seemed to grow out from veins growing perpendicular to the leaf edge (Figures 6e-h and S6e-g, i), forming dentate leaves. Dentate leaves are found in numerous plants (such as *Lactuca virosa*; Figure S6h). Young leaves carried numerous large trichomes, giving them a tomentose (woolly) appearance (Figure S6j, k). On some leaves, spikes emerged at junctions of secondary veins (Figure S6i-n).

**RT-qPCR analysis of TE-1-6b-L expression in F1 plants from the single-locus lines S34, S39, S42, S47 and S56** showed that strong phenotypes were correlated with high expression levels (Figure S7a, b). Expression of T-6b leads to sucrose uptake and accumulation (Clément et al., 2006, 2007), causing dramatic root cell expansion in tobacco (Grémillon et al., 2004; Clément et al., 2007; Pasternak et al., 2017). This is considered to be an important characteristic of 6b activity (Chen and Otten, 2016). Homozygous S34 seedlings were therefore grown on M0222 medium with 2% sucrose. S34 seedlings (Figure S7d–f) grew more slowly than the controls (Figure S7c) and had abnormal cotyledons. However, S34 roots were indistinguishable from control roots (Figure S7g–j). Thus, TE-1-6b-L does not induce root expansion on 2% sucrose, unlike T-6b.

**TE-1-6b-L induces vivipary**

Most TE-1-6b-L plants produced flowers with an altered phenotype. Petals were less coloured and curved downwards (Figure 7a, b). Remarkably, most TE-1-6b-L lines were viviparous: their seeds germinated prematurely in the capsules, forming hundreds of embryo-like structures (Figures 7c–h and S8). Capsules from flowers shortly after pollination contained embryo-like structures at the heart stage (Figure S8c, e). These embryos seemed to rupture the seed integuments and grew rapidly in size, forming green tubes. As a consequence, the capsules became larger than normal (Figure 7e). Capsules of a few TE-1-6b-L lines (e.g. S39 and S34) contained both normal seeds and germinating embryos, grouped in patches (Figure S8c, d). At a later stage, capsules burst open and embryos formed long green hypocotyls with cotyledons (Figures S8e and S7k).

**Figure 4. Phenotypes of TE-1-6b-R transformants.**
(a) Wild type tobacco (WT) and seven homozygous single-locus transformants. Lines 5, 14 and 28 are smaller.
(b) RT-qPCR for TE-1-6b-R for the lines shown in (a). Values were calculated relative to the highest value (line 5). The stunted lines 5, 14 and 28 have a high TE-1-6b-R expression level.
(c) Leaf veins in WT tobacco (left) and TE-1-6b-R line 5 (right). The transformed line shows more prominent tertiary and higher-order veins.
(d) Petiole wings from normal tobacco.
(e) Petiole wings from TE-1-6b-R line 5: small leaflets with many trichomes replace the normal petiole wings.
Scale bars: (a) 20 cm; (c) 5 cm; (d), (e) 2 cm.
The embryos eventually turned black and dried out (Figure S8f). Capsules of single-locus TE-1-6b-L lines contained only germinated embryos, showing that premature seed germination is determined by the parental plant. Embryos from viviparous plants looked initially quite similar, but later became heterogeneous in shape (Figure 7h). Physical constraints inside the capsule probably contributed to unequal development. The prematurely germinating structures did not develop into normal plantlets but had a white base and one or two abnormal, dark-green cotyledons. The embryo apex emerged first from the seed coat (Figure S9c, d), contrary to the apex of wild-type seedlings, which emerges last (Figure 7i). No roots were formed (Figures 7j, k and S8–S10). Some embryos formed spherical, stalked structures in the capsules (Figure S9a, b). Heart-shaped early stage embryos could be grown in M0222 medium with 1% sucrose for 3 weeks (Figure S9e, f), and then became necrotic. Stalked structures like those seen in the capsules emerged and grew out into longer structures (Figure S10). Further development (Figure S10h) showed that they were leaf primordia. Thus, TE-1-6b-L embryos initiate shoot apical meristems and leaf primordia, but their development differs considerably from that of normal tobacco embryos. Some shoot meristems formed between the cotyledons (Figure S10a, b), others below the cotyledons on the hypocotyl (Figure S10c, d), or on one of the cotyledons (Figure S10e). Secondary shoot meristems appeared on some embryos (Figure S10b, g). On M0222 medium with 1% sucrose the embryos did not form roots. Instead, a mass of some embryos (Figure S10b, g). On M0222 medium with 1% sucrose for 3 weeks (Figure S9e, f), and then became necrotic. Stalked structures like those seen in the capsules emerged and grew out into longer structures (Figure S10). Further development (Figure S10h) showed that they were leaf primordia. Thus, TE-1-6b-L embryos initiate shoot apical meristems and leaf primordia, but their development differs considerably from that of normal tobacco embryos. Some shoot meristems formed between the cotyledons (Figure S10a, b), others below the cotyledons on the hypocotyl (Figure S10c, d), or on one of the cotyledons (Figure S10e). Secondary shoot meristems appeared on some embryos (Figure S10b, g). On M0222 medium with 1% sucrose the embryos did not form roots. Instead, a mass of cell filaments grew at the base of the embryo (Figure S10a–d), possibly derived from early root hairs (Figure 7i).

**DISCUSSION**

*Nicotiana otophora* contains 83 kb of *Agrobacterium*-derived DNA, consisting of two different cT-DNA types, TC and TE. TC and TE each occur in two copies, due to duplication of the original inserts with their surrounding plant DNA.

TE was inserted after TC, as shown by the divergence of the inverted repeats (1.7% and 6%, respectively). Since the rate of nucleotide divergence in *Nicotiana* is about 6% per million years (Lim *et al.*, 2007), TC and TE are estimated to be 1 and 0.3 million years old, respectively. *Nicotiana tomentosiformis* has only one TC copy which corresponds to TC-1. The loss of a 350-nt T-DNA fragment in TC-1 but not in TC-2, and the inheritance of this TC-1 deletion by *N. tomentosiformis* show that the TC duplication occurred before the separation of the lines leading to *N. otophora* and *N. tomentosiformis*. As the TC-1/TC-2 divergence (4%) is greater than the TE-1/TE-2 divergence (2%), it is likely that TC was duplicated before TE. A better understanding of these duplication events may be obtained by determining the full extent of the duplicated regions, both in *N. otophora* and in other Tomentosae species.

Only two *N. otophora* TC genes are intact: TC-1-ocl and TC-1-orf3n. 35S-A4-orf3n causes growth abnormalities in tobacco (Lemcke and Schmulling, 1998). Thus, TC-1-orf3n should be tested for growth effects and compared with its A4 homolog. *Nicotiana tomentosiformis* TC-ocl is intact, but its overexpression in *N. benthamiana* failed to produce opines (Chen *et al.*, 2014). In *N. tabacum*, TC has been completely deleted (Chen *et al.*, 2014). The highly degenerate nature of the TC region and its absence in *N. tabacum* seem to indicate that most of the initial TC genes lacked a selective advantage. However, they could have played a role at an early stage, favouring regeneration or reproductive isolation, two of the steps postulated for the survival of natural transfectants (Chen and Otten, 2017). TC-1 and TC-2 carry plant transposon-like sequences. TC-1P (3237 nt) is similar to a LINE sequence and is inserted in TC-1-orf3n. TC-2P (206 nt) corresponds to a SINE sequence and is inserted immediately 3' of TC-2-orf3n. The N.

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**Figure 5.** Highly abnormal TE-1-6b-L regenerants.
(a) Fan-shaped leaf-like structure with well-developed vascular system. (b) Detail of the structure in (a); the fan-shaped leaf is connected to another leaf-like structure by a ribbon-like structure (arrow) and appears as an outgrowth from the rim of the older structure. (c) Edge of a normal tobacco leaf. (d) Edge of the TE-1-6b-L leaf shown in (a) and (b). The edge of the TE-1-6b-L leaf is thicker, with many trichomes. (e) Rooting of another abnormal fan-shaped TE-1-6b-L leaf. Inset: roots emerge directly from the leaf. (f) Growth of buds (arrow) on leaf-like structures as in (a) and (d).

Scale bars: (a), (b), (e) 2 cm; (c), (d) 1 cm; (e) inset and (f) 2 mm.
tomentosiformis TC region (similar to TC-1) does not carry such elements, showing that TC-1P was inserted after TC duplication. Plant transposon-like sequences have also been found in a cT-DNA of I. batatas. The IbT-DNA1 region of I. batatas variety ‘Huachano’ carries a 6.8-kb Gypsy 2 type long terminal repeat transposon, but is absent in the variety ‘Xu781’ (Kynadt et al., 2015).

TE-1 and TE-2 harbour eight intact genes with potential morphogenetic activity: three 6b genes, three orf14 genes, one orf13 gene and one rolC gene. All belong to the plast gene family. Expression analysis showed low, but significant, expression for these ORFs, but also for degenerate genes interrupted by stop codons. Transcription of degenerate genes could be due to residual promoter activity.

Among the N. otophora cT-DNA genes, the three 6b genes are especially interesting since they are linked to typical A. rhizogenes T-DNA genes. Agrobacterium plasmids with acs-iaaH-iaaM-6b-vis genes on one T-DNA and rolB-rolC-orf13-orf13a-orf14-mas2-mas† on another T-DNA have not been found so far. However, if such a plasmid survived, its sequences should still be similar to the ancestral plasmid, as indicated by the low level of divergence between the TE repeats. The transfer of the acs-iaaH-iaaM-6b-vis T-DNA from such a plasmid may have induced tumours and the transfer of the rolB-rolC-orf13-orf13a-orf14-mas2-mas† T-DNA may have induced roots. In the case of a combined transfer, one or the other effect could dominate, or intermediate structures could be formed, conferring a hybrid character on such a plasmid. It is therefore impossible to say at this point whether the plasmid carrying these T-DNAs was a Ti or Ri plasmid.

Reconstruction of the evolution of TE shows that the original TE insert contained two identical 6b genes located on the R1 repeat. The insert was then duplicated, yielding four 6b copies, but one of these, TC-2-6b-R, was lost by the deletion of the R1-R/R2-L region (Figure 3). During the evolution of TE the repeats and the 6b copies diverged, both structurally and functionally. Expression in tobacco in the same expression cassette showed that TE-1-6b-L protein has a stronger activity than TE-1-6b-R and TE-2-6b. Site-directed mutagenesis could identify the amino acids responsible for the weak and strong phenotypes.

Unexpectedly, the TE-6b phenotypes were found to differ considerably from the AB-6b, C-6b, AK-6b and T-6b phenotypes. The TE-6b genes did not induce tumours on N. rustica or on K. tubiflora. On media with 2% sucrose, TE-1-6b-L roots grew normally, unlike T-6b roots which strongly expand on such media (Tinland et al., 1989, 1990, 1992; Grémillon et al., 2004). T-6b root expansion is probably due to increased uptake and the accumulation of large amounts of sucrose (Clément et al., 2006, 2007; Pasternak et al., 2017).

In tobacco, TE-6b genes did not induce enations, cataractorllas or ectopic vascular strands. Instead, TE-1-6b-R and TE-2-6b leaves showed strongly developed tertiary and higher-order veins, wrinkling and leaflets growing from petiole wings. Wrinkling is also typical for plants regenerated from hairy roots, although these do not contain 6b.
The dentate leaves of TE-1-6b-L tobacco plants resemble certain mutants of the miR319-jaw-TCP module in Arabidopsis, *Antirrhinum majus* and tomato. Overexpression of *MIR319A* in Arabidopsis (*jaw-D* mutants) or the combined mutation of the five miR319 target genes TCP2, TCP3, TCP4, TCP10 and TCP24, leads to folded and crinkled leaves with outgrowth of the leaf margins. This is due to the abnormal maintenance of the leaf marginal meristem in such mutants (Nath et al., 2003; Palatnik et al., 2003; Alvarez et al., 2016; Bresso et al., 2017). Leaf vasculature is also increased (Alvarez et al., 2016; Bresso et al., 2017) as in the TE-6b plants. Possibly, TE-6b genes affect the miR319/jaw-TCP module; this will require introduction and further study of the TE-6b genes in Arabidopsis. The growth stimulation of higher-order leaf veins in TE-6b plants leads to a dense, regularly branched pattern, up to the leaf rim. This could rigidify the leaf before it is fully expanded, leading to wrinkling and outgrowth of spikes. Most likely, the strongly modified venation patterns redirect sucrose, amino acids and hormones to abnormal sites, affecting source-sink relations. This could explain the slow overall growth, the smaller dark-green leaves of these plants and the large number of glandular trichomes. Vivipary could be due to modifications in the vascular strands of the funiculi, leading to increased transport of nutrients from the placental tissues into the growing embryos. Non-synchronous development of the vascular connections between the placenta and the embryos could lead to patches with normal seeds or germinating embryos within the same capsule. The striking effects of TE-1-6b-L on genes. TE-1-6b-L plants showed similar changes, but in addition formed large amounts of glandular trichomes, leaflets on the leaf edges, leaf spikes, split leaf tips, ectopic shoot primordia and modified cotyledons. Their flowers were modified, and most strikingly they became viviparous. Prematurely germinating embryos formed aberrant plantlets in the seed capsules, a phenomenon which is controlled by the parental plant. TE-1-6b-L plants could become an interesting tool for studying embryo growth. TE-1-6b-L embryos germinate at a very early stage and can be isolated in large numbers from sterilized capsules. This will allow studies on the external factors that influence their growth and development. The stalked spherical structures reported here seem to be an unusual form of leaf primordium. No roots were formed on embryos, even after prolonged growth in vitro. However, when grown from seeds, seedlings with highly abnormal cotyledons formed normal roots. At the base of the embryos, a large spherical mass of colourless filaments appeared: these seem to correspond to root hairs.

Vivipary has been linked to deficiency of abscisic acid (ABA), to ABA insensitivity or to low osmotic potential (Durationini et al., 2008; Wang et al., 2016). It will be worthwhile to investigate these factors in TE-1-6b-L plants.

The induction and development of the apical stem meristems and of the basal filaments, and the formation of veins and dentate leaves are particularly interesting for basic studies on plant morphogenesis. This will necessitate further anatomical and physiological analysis, preferably by using an inducible TE-1-6b-L gene construct.
embryo development may be used to modify seed filling in crop plants.

The complex enation syndrome of T-6b, AB-6b and AKE-6b on the one hand, and the phenotypes of the TE-6b genes described here, are remarkably different. The role of the various amino acid residues in 6B proteins may be explored using hybrid proteins and proteins with mutations in specific residues. This may allow the identification of determinants for the two 6B phenotypes. No fewer than 46 TE-6B amino acid residues (out of 216) are unique compared with the corresponding residues of 18 other 6B sequences. Five residues are fully conserved in the non-TE-6B proteins, but are different in the TE-6B proteins (positions from TE-2-6B, TE-2-6B residues in brackets): Q26 (A), R28 (K), D51 (N), Y54 (C) and I147 (N). These could play a role in generating the observed phenotypic differences. The highly variable TE-2-6B residues in brackets): Q26 (A), R28 (K), D51 (N), Y54 (C) and I147 (N). These could play a role in generating the observed phenotypic differences. The highly variable C-terminal acidic region of 6B proteins (Figure S5) is of special interest. The acidic region of AK-6B is essential for nuclear localization, transactivation and induction of hormone independence in tobacco (Kitakura et al., 2002). The size and composition of this region could have important effects on the morphogenetic activities, which remain to be explored. Amino acid residues found earlier to be essential for T-6B activity (T92, P95, P96, F130 and A132; Helfer et al., 2002) are conserved in the TE-6B proteins.

T-6b plants show enhanced sucrose uptake. If this also occurs in TE-6b plants it is most likely restricted to leaf tissues, since root growth is not modified. A link possibly occurs in TE-6b plants it is most likely restricted to leaf tissues, and the phenotypes of the TE-6b gene. It will also be important to study the cellular localization of the TE-6B genes and their spatio-temporal expression patterns in N. otophora. Expression of the TE-6B genes under their own promoters will provide clues to their respective roles in N. otophora. The expression of at least two TE-6B genes in N. otophora leaves, the strong TE-6B-induced phenotype in leaves of the closely related N. tabacum and the strong veneration of N. otophora leaves all point to a role for these genes in the growth and development of the natural transformant N. otophora.

The other N. otophora plast genes also require functional analysis. The three TE-orf13 genes are especially interesting, since they are different from the usual orf14 genes. The orf14 genes are generally considered to have little or no biological activity (Aoki et al., 1994; Aoki and Syono, 1999a), but their conservation in N. otophora and in other Nicotiana species (Chen et al., 2014) suggests a biological function. The TE-orf13 and TE-roic genes may also have morphogenetic activity, like orf13 and roic from N. glauca and N. tabacum. We have proposed that the highly morphogenic T-DNA plast genes could have led to speciation, an essential step in the establishment of natural transformants (Chen and Otten, 2017). Various unusual N. otophora features, such as flower morphology, nectar production and pollinator choice, and the switch from day flowering to night flowering, could have contributed to speciation. In addition, N. otophora shows prominent tertiary and higher-order leaf veins and leaf wrinkling, similar to TE-6b tobacco plants. It is tempting to speculate that the TC and TE genes could have played a role in the evolutionary origin of these features.

RNA interference or CRISPR-Cas9 studies will provide a better insight in the role of individual N. otophora cT-DNA genes and gene combinations. The combined effect of the TC and TE genes can be studied by their transfer to other plant species or by complete removal from the N. otophora genome.

EXPERIMENTAL PROCEDURES

Mapping of cT-DNA regions and border analysis

For cT-DNA mapping, the N. otophora whole-genome sequence (WGS) AWOL series of contigs (Sierra et al., 2014) was used. Individual contigs were extended by searching the main read collection (SRX3354655) for overlapping sequences. Contigs were further assembled using A. tumefaciens, A. rhizogenes and A. vitis T-DNA regions as models. Mapping of reads on these sequences allowed separation of the different repeats. Subsequently, repetition-specific sequences were identified and used to amplify and sequence specific repeats and transition zones between these repeats and adjacent single-copy regions. Assembled regions were checked by re-mapping the individual reads on the assembled sequences. The TC-1, TC-2, TE-1 and TE-2 sequences are available upon request. A search for T-DNA border sequences was done with the YGRCAAGATATNNNNNKGMTMAWN border consensus (Barrell et al., 2007).

RT-qPCR analysis

The RT-qPCR analysis of leaves of individual plants was done according to Chen et al. (2014). Young leaves (5 cm long) of soil-grown plants were used. For the three TE-6b genes, RT-qPCR forward (agagggcgccgatgtag), and reverse (cctagctcccgatttgtt) primers were used. Expression of the internal standard gene EF-2 was measured with forward (cctgaccagaagcgtggaca) and reverse (ccagatgtagcagccctcaag) primers.

Transcriptional analysis

Seeds of N. otophora (TW95, PI235553) were sterilized with bleach (13% active chlorine) and fuming HCl and sown on rooting medium (Murashige and Skoog medium, Duchefa M0255, https://www.duchefa-biochemie.com/) and cultivated in a phytotronic chamber (Strader, strader.fr) at 24°C in 16-h light/8-h dark for 2 weeks until the plantlets were fully developed. RNA was extracted from roots and leaves using the RNeasy Plant Mini kit from Qiagen, according to the manufacturer’s instructions. RNA
sequencing libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit from Illumina, and sequenced on an Illumina HiSeq2500 in rapid mode. RNA-seq data were mapped to the reference TC-1, TC-2, TE-1 and TE-2 sequences using hisat2 and filtered to keep only perfectly and uniquely mapped unspliced reads. Twenty-eight per cent of the unspliced leaf reads and 33% of the unspliced root reads mapping to the T-DNA were removed when filtering for perfect and unique mapping.

Cloning of TE-6b genes

Cloning of TE-6b genes was carried out in two steps. First, each region carrying a TE-6b gene was amplified separately by PCR using region-specific primers. The three TE-6b ORFs were then amplified with appropriate primers and inserted in the pCK vector carrying a 2×35S promoter and a termination site (Carrington et al., 1991). Constructs were sequenced and TE-6b genes were introduced into the binary vector pBI121.2 (Jefferson et al., 1987). Binary vector derivatives were introduced into LBA4404 (Hoekema et al., 1983) for transformation.

Protein and DNA alignments

DNA sequence alignments for orf3n-orf8 and protein alignments for 6B sequences were done with Multialign (Corpet, 1988). Plastid ORF13 and ORF14 were aligned with MUSCLE (Edgar, 2004).

Inoculation and transformation

Stems of N. rustica, K. tubiflora and Kalanchoe daigremontiana were inoculated as reported (Helfer et al., 1994) for transformation. Samsun nn leaf discs were incubated with the various Agrobacterium strains. Stems of Nicotiana otophora cT-DNAs and TE-6b gene function

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