Origin and Fates of TERT Gene Copies in Polyploid Plants

Petr Fajkus 1, Vratislav Peška 1, Jiří Fajkus 1,2,3,* and Eva Sýkorová 1,*

1 Institute of Biophysics of the Czech Academy of Sciences, Královopolská 135, CZ-61265 Brno, Czech Republic; fajkuspe@ibp.cz (P.F.); vpeska@ibp.cz (V.P.)
2 Laboratory of Functional Genomics and Proteomics, NCBR, Faculty of Science, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic
3 Mendel Centre for Plant Genomics and Proteomics, CEITEC, Masaryk University, Kamenice 5, CZ-62500 Brno, Czech Republic
* Correspondence: fajkus@ibp.cz (J.F.); evin@ibp.cz (E.S.); Tel.: +420-541-517-183 (J.F.); +420-541-517-199 (E.S.)

Abstract: The gene coding for the telomerase reverse transcriptase (TERT) is essential for the maintenance of telomeres. Previously we described the presence of three TERT paralogs in the allotetraploid plant Nicotiana tabacum, while a single TERT copy was identified in the paleopolyploid model plant Arabidopsis thaliana. Here we examine the presence, origin and functional status of TERT variants in allotetraploid Nicotiana species of diverse evolutionary ages and their parental genome donors, as well as in other diploid and polyploid plant species. A combination of experimental and in silico bottom-up analyses of TERT gene copies in Nicotiana polyploids revealed various patterns of retention or loss of parental TERT variants and divergence in their functions. RT-qPCR results confirmed the expression of all the identified TERT variants. In representative plant and green algal genomes, our synteny analyses show that their TERT genes were located in a conserved locus that became advantageous after the divergence of eudicots, and the gene was later translocated in several plant groups. In various diploid and polyploid species, translocation of TERT became fixed in target loci that show ancient synapomorphy.

Keywords: polyploidy; Nicotiana; telomerase; gene evolution; synteny

1. Introduction

Flowering plants (angiosperms) are important for the existence of many terrestrial organisms, including humans, and a long history of plant breeding has taught us that polyploidization can be advantageous in terms of quantitative traits of crops. Gains and losses of paralogs, their neofunctionalization and sub-functionalization, have all been associated with the generation of duplicate gene copies, e.g., by whole-genome duplications (WGDs) and further rounds of genome duplication/reduction, resulting in genetic diversity upon which the fittest combinations thrived in a competitive environment [1–4]. An ancient WGD has been reconstructed at the base of seed plants, another at the base of angiosperms [5–7] and numerous additional, subsequent WGD events were associated with the divergence of many angiosperm lineages [3]. Polyploidy is usually associated with many genetic and epigenetic changes, including chromosomal rearrangements, expansions of transposable elements and changes in gene expression [8,9]. At the gene level, polyploids can tolerate the presence of paralogs or eliminate a copy of the spare gene. Thus, evolutionary forces result in an equilibrium defined by gene dosage [10]. Studies of model plants have mostly focused on genes important for crop production; however, genes that are critical for genome stability are extremely important for understanding repeated polyploidization events during natural selection, and these remain underexplored.

Telomerase reverse transcriptase (TERT) is involved in the maintenance of telomeres, nucleoprotein structures that are essential for genome stability [11–13]. Telomer-
ase adds telomere repeats to the ends of eukaryotic chromosomes, thereby elongating telomeres and compensating for their shortening due to incomplete end-replication. When telomerase is not active, telomeres become shortened, and their function in the protection of chromosomes is disrupted. The extreme evolutionary success of telomerase-based mechanisms of telomere maintenance is illustrated by current findings in plants (reviewed in [14]). Even among apparent exceptions in telomere sequences, in plant genera *Allium* (Asparagales) and *Cestrum* (Solanales) [15–18], recent research has revealed that novel, unusual telomere DNA sequences are synthesized by telomerase [16,18,19] and not by alternative mechanisms as had been suggested previously (reviewed in [20]). Moreover, we recently demonstrated that changes in the template region of the telomerase RNA subunit directed the observed evolutionary transitions in telomere DNA sequences [14,21,22]. In contrast to the RNA subunit, the protein subunit TERT is evolutionary well conserved and possesses a central reverse transcriptase domain essential for its catalytic function [23,24]. Plant TERTs are structurally similar to human, ciliate or yeast TERTs with a telomerase-specific T motif [25–29]. The gene encoding TERT is usually expressed at low mRNA levels even in telomerase-positive tissues and is maintained as a single copy gene in most eukaryotic genomes. However, the natural allotetraploid *Nicotiana tabacum* possesses three sequence variants of the TERT gene [30]. Various allopolyploidization events among closely and distantly related diploid parental species (Figure 1) in *Nicotiana* make the genus an ideal experimental model system to study the long-term evolution of TERT following natural gene duplication. The increasing number of publicly available assembled plant genomes enables the exploration of TERT genomic loci, gene copy numbers and gene synteny in diverse plant species for comparisons with the data from *Nicotiana* polyploids and the diploid species most closely related to their progenitors (hereafter called progenitor diploids). The *Nicotiana* genus [31–35] comprises relatively young polyploids (i) *N. tabacum* (section Nicotianae), *N. rustica* (sect. Rusticae), *N. arentsii* (sect. Undulatae) that formed approx. 0.4–0.6 million years ago, (ii) *N. clevelandii* and *N. quadrivalvis* (ca. 1.5 million years ago, sect. Polydicliae), (iii) four species from the 4–5 million years old section Repandae (*N. nudicaulis*, *N. repanda*, *N. nesophila* and *N. stocktonii*), and (iv) ~35 species including the model *N. benthamiana* from the oldest section Suaveolentes formed about 6 million years ago [31]. Among these species, members of sections Suaveolentes and Repandae are of interest because, with *N. tabacum*, they share an ancient genome donor, *N. sylvestris*, and these speciation events happened at different times. In *N. tabacum*, two TERT variants originated from the maternal *N. sylvestris* genome (TERT_Cs, TERT_D) and one from the *N. tomentosiformis* paternal genome (TERT_Ct). Variants TERT_Cs and TERT_Ct code for a full-length functional protein, while the TERT_D variant is truncated and contains several indels resulting in premature stop codons, suggesting that it is a pseudogene [30]. All three variants are nevertheless transcribed and show distinct, tissue-dependent levels of mRNA transcripts, indicating a sub-functionalization of TERT variants [30,36].
Figure 1. Overview of experimental results and illustration of phylogenetic relationships of Nicotiana species used in this study. Phylogeny and the proposed origin of polyploids were adapted from [31,32]. An uncertain parental genome donor for section Suaveolentes is indicated by a question mark. Summary of experimental and in silico results (squares, analyses of genomic DNA; circles, expression of TERT variants investigated by RT–qPCR) is shown in boxes of Nicotiana sections, the origin of TERT variant in polyploids is depicted by color of respective parental diploids, and variants that were not identified are depicted with open squares. Nicotiana accessions used in the experimental analyses are listed in Table S1, genomic assemblies and genomic/transcriptomic SRA data used for in silico analyses are listed in Material and Methods. For the purposes of this paper, we refer to a TERT copy that does not code for a catalytically active protein as a putative pseudogene (dashed symbols) in contrast to a functional TERT gene copy (open symbols), n.a. not analyzed.

Based on previously described TERT variants in N. tabacum, we explored the fate of TERT paralogs in other Nicotiana polyploids to determine whether both parental TERT genes are conserved in allotetraploid genomes, whether they are transcribed, present in syntenic, collinear arrays with their progenitor diploids, and whether any relationship exists between telomere lengths in polyploids and their progenitor diploids. Of particular interest in this study was to clarify the origin of the presumed pseudogene variant TERT_D in N. sylvestris, a diploid genome donor of N. tabacum, as well as of even older species from sections Repandae and Suaveolentes. In addition, we investigated in silico whether diploid and polyploid plants outside of the family Solanaceae sustained TERT paralogs/pseudogenes in their genomes, and we explored syntenic relationships of genes adjacent to TERT to interpret the evolutionary success of TERT copies after translocation.

2. Results
2.1. Number of TERT Variants in Nicotiana Polyploids as a Case Study

At the beginning of this project, there was limited genomic sequence data available for the majority of Nicotiana allopolyploids and their parents. To characterize experimentally the number, identity and origin of TERT copies in genomes of polyploid Nicotiana species and representatives of their diploid progenitors, we employed several primer combinations derived from conserved TERT regions of the evolutionarily distant relatives N. sylvestris and N. tomentosiformis (Figure 1), designed originally for amplification of N. tabacum TERT variants [30,36]. These PCR primers (Figure 2A, Table S2) amplify TERT regions nonspecifically, i.e., all variants are produced in a single PCR. Sequencing of PCR products then identifies single nucleotide polymorphisms (SNPs) and/or indels evidencing the presence of multiple TERT variants. Primer positions were with respect to Nicotiana TERT gene structure with 13 exons (Figure 2A), which differed
Figure 2. Experimental identification of TERT variants and analysis of gene expression in Nicotiana polyploids. (A) Conserved te-
lomerase specific motifs (T2, NLS, CP, QFP, T) and reverse transcriptase motifs (1, 2, A–E) are highlighted in protein and mRNA of Nicotiana TERT (modified from [30]). Positions of primers used for screening experiments (blue arrows) and TERT-variant-specific primers (black arrows) are indicated at corresponding TERT mRNA regions (primers are listed in Table S2). The triangle within exon 9 shows the position of a 102 nt long deletion that was identified in N. repanda, N. nesophila and N. stocktonii and represents a specific TERT-variant of N. obtusifolia origin. (B, C) Validation of primer specificity for TERT variants in N. repanda (B) and N. nudicaulis (C). PCR products amplified with primers 9exF1 and 9exR1 show two bands corresponding to TERT variants that differ by a 102 bp long deletion. Specific amplification of TERT_O and TERT_Cs variants was demonstrated using the 9exF1 primer in combination with variant-specific reverse primers 9exR_Nrepa/O and 9exR_Nrepa/S, respectively. (C) For validation of qPCR primers and to distinguish three TERT variants in N. nudicaulis, the PCR products amplified with indicated qPCR primer combinations were digested with Msel. A specific cut of the TERT_O variant that possesses the restriction site for Msel within the amplified region confirmed the specificity of amplified TERT-variants. (D) Relative mRNA levels of specific TERT variants were determined by RT–qPCR in N. nudicaulis, N. repanda and N. rustica. Relative mRNA abundance of particular parental TERT variants (in %) was calculated by the delta Ct method [37]. Ct values were normalized using the reaction efficiency calculated from a standard curve analysis (Table S3).

from the prevalent 12-exon structure of plant TERTs [23]. As expected, a successful amplification was achieved mostly using primers derived from the more conserved sequences at the 3’ end of TERT genes (Table S3). As the first screening experiment, we applied this approach to six diploid Nicotiana species investigated as representatives of parental genome donors, including N. sylvestris as a control, and to nine polyploid Nicotiana species (Figure 1). Among parental diploids, we detected one TERT variant in N. alata, N. attenuata, N. undulata, N. wigandaoides, N. paniculata and N. obtusifolia (Supplementary A1), and two TERT variants (TERT_C and TERT_D) in N. sylvestris [30]. In the case of N. attenuata and N. obtusifolia, species representing parents of polyploid sections Polycdioleae and Repandae, we further confirmed our results by in silico analysis using genome assemblies (GenBank accessions: GCA_001879085.1 and GCA_002018475.1, respectively). To complete the set of representative parental species, we assembled available transcriptomic SRA data of N. noctiflora (GenBank accession: SRR2106514) and identified one TERT variant. In conclusion, our results show the presence of more than one TERT variant in diploid N. sylvestris [30], an exception among parental species of Nicotiana polyploids.

The same experimental approach applied to representative Nicotiana polyploids detected variant-specific SNPs and/or indels, demonstrating the presence of two TERT variants in 5 of 9 polyploid species investigated (N. arensii, N. rustica, N. repanda, N. nesophila, N. stocktonii) and three variants were identified in N. nudicaulis (summarized in Figure 1, Table 1, see below for details). While PCR products obtained from N. clevelandii, N. quadrivalvis and N. benthamiana genomic DNA revealed the presence of a single copy of the TERT gene, our search for TERT variants in raw transcriptomic data from N. clevelandii showed the occurrence of two gene variants. To avoid possible errors in comparison of experimental and in silico data that could be caused, e.g., by possible incorrect mapping of TERT reads to the raw genome/transcriptome data, assembly version or allele sequence, we analyzed in detail individual SNPs in sequences from each polyploid species and its progenitor diploids (see Supplemental Text S1, Figure S1, Table S4). Results deduced from sequence similarity (in %, Table 1) and individual SNPs (Table S4) were in agreement in all cases analyzed.
Table 1. Origin of telomerase reverse transcriptase (TERT) variants in polyploid Nicotiana species determined by sequence similarity with representative progenitor diploids.

| Allopolyploids | GeneBank Accessions | Sequence Similarity [%] | Analyzed Region |
|----------------|---------------------|-------------------------|----------------|
|                | Maternal Parent     | Paternal Parent         |                |
| SUAVEOLENTES   | **N. alata**        | **N. noctiflora** 2      |                |
|                | N. benthamiana      | 96.3                    | 97.5           |
|                | 27g01116.1          | n.a.                    | 97.4           |
|                |                     |                         | exon 4 to 5    |
| REPANDAE       | **N. syl. C var.**  | **N. syl. D var.**      |                |
|                | N. repanda          | 97.9                    | 96.4           |
|                | MG242402 1          | 91.6                    | 97.0           |
|                |                     |                         | exon 9         |
|                | N. stocktonii       | 98.6                    | 97.6           |
|                | MG242408 1          | 93.1                    | 97.0           |
|                |                     |                         | exon 9         |
|                | N. nesophila        | 95.2                    | 97.0           |
|                | MG242406 1          | 91.6                    | 97.0           |
|                |                     |                         | exon 9         |
|                | N. nudicaulis       | 98.6                    | 94.4           |
|                | MG545647 1          | 94.3                    | 94.4           |
|                |                     |                         | exon 10 to 12  |
| POLYDICLIAE    | **N. obtusifolia**  | **N. attenuata**        |                |
|                | N. clevelandii      | 94.3                    | 99.3           |
|                | var1 2              | 97.3                    | 98.9           |
|                | var2 2              | 99.2                    | 97.3           |
|                |                     |                         | exon 9         |
|                | N. quadrivalvis     | 94.9                    | 98.6           |
|                | MG242423 1          |                         | exon 4 to 5    |
| ARENTSII       | **N. undulata**     | **N. wigandiodes**      |                |
|                | N. arenstii         | 99.5                    | 98.4           |
|                | MG242419 1          | 98.8                    | 99.8           |
|                |                     |                         | exon 9         |
|                | **N. paniculata**   | **N. undulata**         |                |
|                | N. rustica          | 100.0                   | 98.2           |
|                | MG242413 1          | 98.2                    | 99.8           |
|                |                     |                         | exon 9         |

1 all sequences cloned in this work are in Supplementary A1, including corresponding sequences cloned from progenitor diploids; 2 regions mapped to raw RNAseq data or extracted from genome assembly (Supplementary A1).

2.2. Origin of TERT Genes in Polyploids with the Ancestral N. Sylvestris Donor Genome

An N. sylvestris progenitor is considered to be a progenitor diploid of the allopolyploid sections Suaveolentes, Repandae and Nicotianae (Figure 1). The evolutionary history of TERT associated with allopolyploidy is inferred for each of these sections.

2.2.1. Suaveolentes

In the section Suaveolentes, we used the model plant N. benthamiana as a representative species of the section, and N. alata and N. noctiflora as recent relatives of the putative maternal lineage originating from sections Alatae or Noctiflorae, respectively (Figure 1, [31,32,38]). We detected a single copy of the N. benthamiana TERT experimentally, and this result was confirmed in silico (Table 2) using (i) an N. benthamiana genome assembly based on deep sequencing (N. benthamiana Genome v1.0.1) and (ii) analysis of raw genomic NGS reads [39] by BLAST followed by read-mapping back to the query. Comparison of corresponding regions of N. benthamiana TERT and representative parental TERT sequences (Table 1, Table S4) revealed that the N. benthamiana TERT sequence (accession number NbS00010427g0116.1) was more similar to N. sylvestris TERT_C variant than to the TERT sequence cloned from N. alata (GenBank accession MG242421) or deduced from N. noctiflora SRA data (Supplementary A1). Thus, we conclude an N. sylvestris origin of N. benthamiana TERT and a loss of the second parental TERT copy during the evolution of N. benthamiana.
Table 2. Number of TERT gene copies in *Nicotiana* species determined in silico.

| Species/Genome Dataset Accession | Total No. of TERT Reads | Expected Genome Coverage (Depth) | No. of Detected TERT Variants | Read Counts Corresponding to Known TERT Variants | Ratio of TERT Variants in Genome |
|-----------------------------------|-------------------------|---------------------------------|-------------------------------|---------------------------------|----------------------------------|
| *N. tabacum* SRX338107            | 1259                    | 35×                             | 3                             | NtTERT-Cs 425 NtTERT-D 424 NtTERT-Ct 410 | 1:1:1                             |
| *N. sylvestris* ERX248848         | 644                     | 26×                             | 2                             | NsTERT-C 332 NsTERT-D 312          | 1:1                               |
| *N. tomentosiformis* ERX248865    | 203                     | 15×                             | 1                             | NtomTERT 203                        | -                                |
| *N. benthamiana* (raw data from [39]) | 286                     | 20×                             | 1                             | NbenTERT 286                        | -                                |

2.2.2. Repandae

In contrast to the more ancient polyploids from the section Suaveolentes that originated from a single polyploidization event, followed by a reduction in genome size and chromosome number (2n ranges from 30 to 48), all other *Nicotiana* allopolyploids are 2n = 4x = 48, representing a doubling of the diploid chromosomal number for the genus (2n = 2x = 24) [31,32,35]. The monophyletic section Repandae originated from diploid progenitors closely related to *N. sylvestris* (the maternal genome donor) and *N. obtusifolia* (the paternal genome donor), and it comprises four species—*N. nudicaulis*, sister to and distinct (both morphologically and genetically) from the remaining three species—*N. repanda*, *N. nesophila* and *N. stocktonii* [32,33]. We captured two TERT sequences in cloned PCR products from *N. repanda*, *N. nesophila* and *N. stocktonii* and three TERT sequences in *N. nudicaulis* (Table 1, Supplementary A1, Figure 3). Using TERT variant-specific PCR primers for amplification of respective TERT variants and/or variant-specific restriction enzyme digestion of PCR products (Figure 2A–C), we confirmed the occurrence of all TERT variants identified in Repandae species, including the TERT_D variant detected in *N. nudicaulis* that could be assumed to be of *N. sylvestris* origin (Table 1). Moreover, using qPCR with *N. nudicaulis* genomic DNA as a template and specific primers (Supplemental Text S1, Table S3), we determined that the TERT_Cs, TERT_D and TERT_O variants occurred in the ratio 1:1:1. Interestingly, SNPs in the *N. obtusifolia*-like TERT sequence (TERT_O) differed between *N. nudicaulis* and three other species. The TERT_O variants from *N. repanda*, *N. nesophila* and *N. stocktonii* shared a striking 102 nt-long in-frame deletion within exon 9 (Figure 2A,B) that shortens the protein linker sequence between reverse transcriptase motifs 2 and A (details in Figure 3A and Supplementary A2). The TERT_O variants in these species also share a 4 nt long deletion within exon 9, resulting in a premature stop codon in proximity to motif A. In addition, we detected a stop codon within exon 11 of the TERT_O variant from *N. repanda* (MG242415, Supplementary A1), caused by a nucleotide transition from G to A. The TERT_O sequence from *N. nudicaulis* (MG242410) showed a nucleotide transition from G to A that would change the essential residue Asp (D) to Asn (N) within the motif C (details in Figure 3B and Supplementary A3) and the presence of several indels in exons 10, 11 and 12, resulting in out-of-frame mutations. These results suggest that currently existing TERT gene variants that originated from an ancient *N. obtusifolia* parent cannot produce a catalytically active TERT protein and could represent a pseudogene in all Repandae species.
Figure 3. Comparison of TERT variants. (A) Alignment of representative TERT variants from Nicotiana polyploids identified here, and previously in *N. tabacum* [30], clearly illustrates the occurrence of conserved structural motifs, the telomerase specific motif T and reverse transcriptase motifs (magenta arrows), and variant-specific sequence characteristics (boxed). Polyploid variants are marked according to the parental origin of specific variants (Cs for *N. sylvestris*, O for *N.
obtusifolia, A for N. attenuata, W for N. wigandiodes, U for N. undulata, P for N. paniculata parent). Notably, N. repanda, N. nesophila and N. stocktonii (all Repandae) possess the TERT_O variant of N. obtusifolia with an in-frame mutation caused by a 102-nt-long deletion that occurs in the protein linker region that is suggested as important for telomerase catalytic function in humans. However, another 4-nt-long deletion within exon 9 results in an out-of-frame mutation in the same variant (stop codon is highlighted, translation of possible truncated variant is shown). (B) Comparison of three TERT variants from N. tabacum and N. nudicaulis shows the occurrence of TERT_Cs and TERT_D variants of N. sylvestris origin in both polyploids and mutations in the N. nudicaulis TERT_O variant. The TERT_O variant shows an amino acid transition D > N within motif C (two of three Asp residues essential for telomerase activity are depicted by triangles in motif C, and the remaining Asp residue is marked in motif A above) and a 4-nt-long deletion in the protein linker region proximal to motif D resulting in a stop codon. Structurally important regions from exon 9 (A) and exons 10, 11 and 12 (B) of TERT variants from polyploids and their progenitor diploids are shown, including nucleotide and protein consensus sequences (numbering of nucleotide sequence on top, full alignments are in Supplementary A2 and A3).

2.2.3. Nicotianae

The section Nicotianae is represented by N. tabacum, which originated from the most recent polyploidization event [31,40]. Three TERT variants (TERT_Cs, TERT_D of N. sylvestris origin and TERT_Ct of N. tomentosiformis origin) were characterized in detail experimentally [30,36]. To get a better insight into the origin and evolutionary fate of the TERT_D variant that is transcriptionally active and developmentally regulated in N. sylvestris and N. tabacum [36] but cannot produce a functional protein, we analyzed the N. sylvestris genome assembly (TW136) in silico. BLAST search identified three contigs (Figure 4). Two of them comprised sequences matching the previously identified (i) TERT_C variant (NW_009540950) and (ii) TERT_D variant (NW_009367114). A comparison of (i) and (ii) TERT variants revealed a TERT_D similarity with TERT_Cs, starting from the repetitive sequence region within intron 7 and ending within exon 12. (iii) In addition, we found a 92 nt region homologous to the 3’ end of exon 12 and also a putative 3’UTR region of TERT within contig NW_009526057 that apparently represents a part of the TERT sequence (Figure 4, TERT_12exD) that is missing in the TERT_D variant within the contig (ii). A BLAST search for the TERT_12D sequence in N. tabacum cv. TN90 genome assembly identified an unplaced genomic scaffold NW_015807891 with a sequence similar to TERT_12exD, thus indicating that N. tabacum retained all TERT-like sequences of N. sylvestris origin. To verify the number of TERT-like copies in silico, we analyzed raw data from SRA archives of N. tabacum, N. tomentosiformis and N. sylvestris genome sequencing projects. Quantification of a number of mapped SRA reads corresponding to respective TERT variants revealed equal occurrences of TERT_Cs:TERT_Ct:TERT_D in N. tabacum, and TERT_C:TERT_D variants in N. sylvestris, and a single TERT gene copy in the N. tomentosiformis (Table 2). In addition, we analyzed experimentally five available N. sylvestris accessions for the presence of TERT_C and TERT_D paralogs because previous genome sequencing [41] reported differences among accessions, including a burst of sequence amplification and/or homogenization. Using qPCR, we demonstrated the same relative abundance of TERT_C and TERT_D in all N. sylvestris accessions investigated, including the reference genome accession TW136 (Table 3, Supplemental Text S1).
Figure 4. Arrangement of TERT-like sequences identified in the N. sylvestris genome assembly. Ancient TERT gene duplication is documented in contigs identified by BLAST search. The TERT_C variant represents a functional TERT copy. Two more contigs contained TERT-like sequences (TERT_D, TERT_12exD) that probably originated from TERT duplication and translocation because the TERT_12exD sequence stands for the 92 nt long region homologous to the end of exon 12 that is missing in the TERT_D variant. Mutual comparison of three TERT contigs in GEvo revealed the presence of the delta subunit of mitochondrial ATP synthase (MtATP0) that is often associated with TERT. Moreover, two repetitive sequences and retrotransposon Ogre-SD1_I that may be responsible for the split of TERT_D and TERT_12D sequences were identified. Sequence similarities (in %) are illustrated by a color scale relative to the reference sequence (Ref., 100%), as indicated.

Table 3. Number of TERT copies in N. sylvestris accessions determined by qPCR.

| N. sylvestris Accession | NsTERT_C | NsTERT_C 1/2x (Control) | NsTERT_D | ΔCt (C-C1/2x) | ΔCt (C-D) | C:D Ratio |
|-------------------------|----------|-------------------------|----------|---------------|----------|-----------|
| A04750326               | 16.41 (±0.036) | 16.91 (±0.085) | 16.41 (±0.065) | −0.5 | 0.00 | 1:1 |
| 934750005               | 16.65 (±0.052) | 17.12 (±0.043) | 16.59 (±0.049) | −0.47 | 0.06 | 1:1 |
| ITB626                  | 17.65 (±0.067) | 18.12 (±0.051) | 17.66 (±0.035) | −0.47 | −0.01 | 1:1 |
| TW136                   | 17.25 (±0.02) | 17.68 (±0.015) | 17.13 (±0.043) | −0.43 | 0.12 | 1:1 |
| Ducrettet 101-268       | 17.31 (±0.023) | 17.95 (±0.063) | 17.26 (±0.08) | −0.64 | 0.05 | 1:1 |

2.3. Origin of the TERT Gene in Polyploid Sections Polydicliae, Rusticae and Undulatae

2.3.1. Polydicliae

Two allopolyploid species, N. quadrivalvis and N. clevelandii, probably originated in polyploidization events that involved the same diploid parents [34,38,42]. The ancestor of N. obtusifolia functioned as a maternal genome donor and a progenitor of current N. attenuata as a paternal genome donor. We investigated the presence of TERT variants with different primer combinations covering exon 4 to exon 5, exon 9 and exon 10 regions (Tables S2 and S3). Experimentally, we detected a single TERT copy in both N. quadrivalvis and N. clevelandii, covering the genomic region from exon 4 to exon 5 that showed similarity to TERT from the N. attenuata lineage. Other primer combinations failed in N. clevelandii. However, during this study, transcriptomic SRA data from N. clevelandii became available, and a search for TERT variants uncovered the occurrence of two TERT variants that originated from both parental lineages (Table 1, deduced sequences used for analysis are in Supplementary A1).
2.3.2. Undulatae and Rusticae

Two independent and relatively recent polyploidization events gave rise to *N. arentsii* (Undulatae) and *N. rustica* (Rusticae) (Figure 1). *N. arentsii* is an intrasectional polyploid that arose from related diploid parents closely related to *N. undulata* and *N. wigandoides* (all belonging to section Undulatae) [31,40]. Experimentally, we confirmed two distinct TERT variants in *N. arentsii* by sequencing of cloned PCR products and assigned them to their parental origins (Table 1, Supplementary A1).

*N. rustica* was formed from diploid species closely related to *N. paniculata* (maternal genome donor) and *N. undulata* (paternal genome donor, [40]). Experimentally, we detected both parental TERT copies (*TERT_P* and *TERT_U*, respectively) in the *N. rustica* genome (Table 1, Supplementary A1).

2.4. Expression of TERT Variants in Nicotiana Polyploids

We further focused on the question of whether multiple variants of the TERT gene were transcribed (results summarized in Figure 1), especially in Repandae, where the *N. obtusifolia*-like TERT variant in all four polyploid species contains premature stop codons. We designed specific qPCR primers (Tables S2 and S3) spanning the 4-nt-long deletion to distinguish between parental TERT variants in *N. repanda*. Surprisingly, RT–qPCR analysis (Figure 2D) revealed that both variants, *N. sylvestris*-like (*TERT_Cs*) and *N. obtusifolia*-like (*TERT_O*), were transcribed at a comparable level in *N. repanda* seedlings. Using TERT-variant-specific primers in *N. nudicaulis*, RT–qPCR analysis revealed a higher transcript level of *TERT_O* in comparison with the *TERT_Cs* variant, while *TERT_D* transcripts were detectable but heavily under-represented. In *N. rustica* (Rusticae) seedlings, our RT–qPCR analyses showed comparable transcript levels of both TERT variants, *TERT_P* of maternal origin and *TERT_U* of paternal origin.

2.5. In Silico Analysis of N. Sylvestris Genome Assembly Illustrates a Possible Evolutionary Scenario and the Origin of Subsequent Multiple TERT Loci

We detected both *N. sylvestris*-like TERT_Cs and TERT_D variants in *N. tabacum* [30] and *N. nudicaulis* experimentally (Table 1 and Figure 2C,D), but not in *N. benthamiana*. Looking at the time scale of polyploidization events (Figure 1) that gave rise to these species, this result suggests the occurrence of a TERT_D variant in ancient *N. sylvestris*, the progenitor of the section Repandae. The question is why the putative pseudogene variant is maintained in the current genome of *N. sylvestris*, as demonstrated in five accessions by qPCR (Table 3) and in its ancient polyploid offsprings.

A comparison of three TERT-containing contigs from *N. sylvestris* (Figure 4) using the GEvo tool (https://genomevolution.org/seq/GEvo.pl) showed that the Ogre-SD1_I sequence was associated with both TERT_D and TERT_12exD sequences. RepeatMasker [43] classifies this highly repetitive sequence as an LTR/Gypsy retrotransposon. The position of Ogre-SD1_I suggests that TERT_12exD and TERT_D originated from the same ancient TERT locus. We presume that either retrotransposition- or transposon-facilitated ectopic recombination shattered the TERT_12exD and TERT_D sequences and transferred TERT_D to a completely new genomic locus. Another possibility is that the change within the ancestral TERT locus was mediated by a 1.3 kb long repeated sequence that is interspersed all over the *N. sylvestris* genome, including all three TERT contigs (Figure 4), and possibly serving as a hot spot for recombination [44]. Crucially, it is difficult to distinguish which of the copies, TERT_C or TERT_D, was derived from an ancestral copy because all three contigs are relatively short, and the *N. sylvestris* genome assembly is not complete. Moreover, TERT_12exD and TERT_C contigs show regions of sequence similarity downstream of the TERTs, corresponding to the gene for mitochondrial ATP synthase subunit delta (*MtATPO*) (Figure 4). A copy of *MtATPO* within the TERT_C contig is annotated as a pseudogene, while the TERT_12exD contig contains two copies of the *MtATPO* gene, one representing a putative functional copy while the other (inverted)
copy is a pseudogene. MtATPO is usually a single-copy gene, and it is often associated with TERT within land plants, including N. tomentosiformis (contig NW008896550.1, compare in Figure 5). Thus, the presence of a functional MtATPO gene copy may be the reason the genomic loci containing the TERT_12exD variant were retained (see Supplemental Text S1).

Figure 5. Graphical overview of TERT loci synteny across selected Angiosperms. Simplified presentation of TERT synteny is based on syntelogs visualized in GEvo among indicated plant TERT loci identified directly by SynFind in CoGe or by BLAST in NCBI genome databases. Dominant eudicot-like synteny (green) and three other types of shared gene synteny among close relatives, as indicated (yellow, cyan, purple), were identified across tested species. Source data...
used for GEvo analysis are listed in Table S5. SynFind parameters—algorithm:last; gene window size:30; minimum number of genes:5.

2.6. Genomic TERT Loci Analysis Defines Ancestral Synteny within Flowering Plants

The turbulent history of TERT copies within *Nicotiana* polyploid genomes directed our attention to the question of the genomic arrangement of the TERT locus. TERT-containing contigs in *N. sylvestris* are short and/or gene-free (Figure 4), so there are no checkpoints for comparison of microsynteny, and only short TERT-containing contigs could be analyzed in *N. tomentosiformis* (Figure 5), the progenitor diploid of *N. tabacum*. Thus, we focused on a comprehensive analysis of the gene order (syntelogs) of TERT neighboring genes across representative plant genomes (Table S5). Syntelog is a special case of gene homology where sets of genes are derived from the same ancestral genomic region. This homology may arise from speciation events or through whole or partial genome duplication events. Initially, we asked whether there was any syntelog of the genes neighboring TERT within the Solanaceae species. Then we examined species from plant clades closely or distantly related to the Solanaceae family. Using SynFind [45] and the *Solanum penellii* genome assembly as a reference, we identified syntelogs among Solanaceae species tested, and these were subsequently visualized by GEvo. TERT microsynteny similar to *S. penellii* was found in 18 of 49 genomes analyzed, including ancient polyploids and evolutionarily distantly angiosperms (Figure 5). In addition, many TERT loci showed co-linearity. These were representatives of large taxonomic groups (Asterids and Fabids) and species representing basal clades—*Vitis vinifera* (Vitales, basal for rosids), and *Nelumbo nucifera* (Proteales, basal eudicots), but not *Amborella* (early diverging angiosperm lineage) or green algae, suggesting that the detected TERT microsynteny may have originated in eudicots subsequent to the divergence of the *Amborella* lineage. Interestingly, 16 of 18 genes neighboring TERT in *S. penellii* were almost co-linear within the *Vitis* TERT locus, and 14 of 18 genes were shared in the *Nelumbo* TERT locus. Thus, with the exception of the carbohydrate esterase gene that is exclusive to the TERT loci in Solanaceae, these genes occurred within the TERT locus in species grouped by ancestral eudicot-like microsynteny (green, Figure 5). This microsynteny of the TERT locus was secondarily lost in several plant lineages that show the TERT gene translocated into completely different genomic regions. We reciprocally compared these novel TERT genomic regions to determine whether any other TERT syntelogs were evolutionarily conserved within Angiosperms. We identified TERT microsynteny among closely related species from Brassicales and Malpighiales within eudicots (Figure 5). With the exception of two species from Poales, no other conserved TERT syntelogs were detected among monocots or within other particular eudicot species/clades. Thus, novel genomic TERT loci show microsynteny restricted to closely related species.

We then asked how conserved was the ancestral eudicot locus that accommodated the TERT gene with respect to its occurrence in current genomes. To answer this question, we searched for the presence of genomic loci involving only syntenic genes neighboring TERT, i.e., with eudicot-like microsynteny (green, Figure 6A). We identified such original loci (without a TERT gene) in eudicots (except *A. thaliana*) and *Amborella*, but not in monocots or representatives of other basal clades (*Physcomitrella*, *Selaginella*, green algae). Moreover, several genomes showed the occurrence of more loci with eudicot-like microsynteny, either complete (UD, Figure 6A) or comprising downstream neighboring gene pairs (D, Figure 6A).

A reciprocal search was then carried out using syntenic genes neighboring TERT in novel genomic loci that accommodated TERT in current genomes (termed here as, e.g., *Arabidopsis*-like, *Populus*-like, *Oryza*-like synteny, Figure 6B). This identified these loci in eudicots, but not in monocots and basal clades (*Physcomitrella*, green algae). For example, the syntelog derived from the TERT locus of *Citrus sinensis* (*Citrus*-like) occurred in *Amborella*, *Nelumbo*, *Vitis*, *Eucalyptus* and *Fragaria* genomes. Within the *Citrus sinensis* genome, loci with the eudicot-like (represented by *Vitis*-like query) and *Arabidopsis*-like
synteny were present. Interestingly, *Theobroma cacao* (Malvales) that harbors the species-specific TERT locus contained loci with eudicot-like, *Ricinus*-like, *Eucalyptus*-like and *Arabidopsis*-like synteny, but loci with *Theobroma*-like synteny did not occur in the other genomes investigated.

**Figure 6.** Occurrence of TERT syntelogs in Angiosperms. (A) Detailed analysis of the eudicot-like type of synteny (represented by *Vitis* syntelog as a query) in indicated genomes shows the presence of syntenic regions with/without the TERT gene in the majority of investigated eudicots and *Amborella*. The number of syntelogs and synteny categories are shown for each species. (B) The occurrence of conserved syntenic regions corresponding to the species-specific TERT query was investigated in representative genomes. Co-occurrence of syntelogs in more species suggests an ancient origin of target loci that accommodated TERT in current species. Analyses were carried out using CoGe, GEvo and SynFind. SynFind parameters—algorithm: last; Gene window size: 30; minimum number of genes: 5.
2.7. The Occurrence of TERT Homologs in Model Species Illustrates a Possible Origin of TERT Variants

The same questions about genomic arrangement of the TERT locus and number of gene copies were asked about polyploid model species Glycine max, Gossypium hirsutum, Brassica napus, Camelina sativa, Mimulus luteus and Actinidia chinensis (Figure 6, Supplemental Text S1, Table S5, summarized in Figure 7 and Figure S2).

Actinidia (x = 29, Ericales) is a paleotetraploid derived from an ancestor with x = 14 [46,47] that resembles the ancient N. benthamiana speciation, with changes in chromosome number in the section Suaveolentes. One TERT locus and three additional loci with eudicot-like synteny were present in the A. chinensis genome (Figure 6A). Glycine max (Fabales) is a paleopolyploid with a highly duplicated genome, where nearly 75% of the genes are present in multiple copies [48]. The TERT gene is no exception, and we identified two loci on chromosomes 15 and 8 (LOC100790649 and LOC100776816, respectively) that contain functional copies of TERT. The TERT locus on chromosome 15 shares a full eudicot-like synteny, while the arrangement of the TERT locus on chromosome 8 was similar to the eudicot-like synteny only in the region upstream of syntenic TERT (S and Su, respectively, Figure 6A). Additionally, two regions with similarities to eudicot-like synteny, but without a copy of the TERT gene, were located on chromosomes 12 and 13 (UD, Figure 6A). The occurrence of eudicot-like synteny in Mimulus luteus (Lamiales) resembles Glycine max with two TERT loci (S and Sd) and two additional loci without a TERT gene (UD and D, Figure 6A). The number of TERT gene copies in Brassicaceae reflects ploidy level with 2 and 3 TERT genes in tetraploid Brassica napus and hexaploid Camelina sativa, respectively (Table S5). However, one of the TERT copies in C. sativa is a putative pseudogene. The tetraploid genome of Gossypium hirsutum that originated ca. 1–2 Myr ago [2,49] contains two TERT genes and four additional loci without a TERT gene that share eudicot-like microsynteny (Figure 6A).

Intriguingly, during silico identification of TERT loci, we noticed that the analyzed diploid species, Populus trichocarpa, Mimulus guttatus, Amborella trichopoda, and Vigna radiata, contained more than one TERT-like sequence, which could illustrate maintenance of TERT variants (details in Supplemental Text S1, Table S5, Figure S3). In Amborella trichopoda (Figure S3A), the search for TERT revealed the full-length TERT gene (LOC18433477) and a truncated TERT-like sequence (LOC18443854) covering 208 amino acids from the N-terminal part of the TERT protein. However, the origin of the truncated TERT-like sequence is unclear (see Supplemental Text S1). In P. trichocarpa, M. guttatus and V. radiata, the additional TERT-like copy could be classified as a pseudogene or may function as an ncRNA (Figure S3B–D).

3. Discussion

To test experimentally and in silico TERT gene balance following ancient polyploidization events, we identified and characterized TERT copies in genomes of polyploid Nicotiana species and representatives of their diploid progenitors. We also investigated the expression of TERT variants identified in the polyploids using RT–qPCR. We found that the N. sylvestris progenitor was a very successful parent of sections Suaveolentes, Repandae and Nicotianae because the TERT_Cs variant of N. sylvestris origin was identified in all polyploid genomes investigated (Figure 1), and high levels of its transcripts were detected. Moreover, an additive occurrence of TERT copies observed in N. tabacum and N. nudicaulis suggests that gene/genome duplication resulting in the formation of TERT_C and TERT_D variants in N. sylvestris had occurred at least before the formation of the section Repandae. The TERT_D transcripts were detectable but heavily under-represented in N. nudicaulis (Figure 2D), similar to TERT_D expression in N. sylvestris and N. tabacum [30,36]. In contrast to the success of the N. sylvestris progenitor, the TERT_O variant of N. obtusifolia origin was pseudogenized in all four polyploid species from Repandae. A 102-nt-long in-frame deletion within exon 9 would shorten the linker
region between motif 2 and motif A, including protein motif GSSVF that is well-conserved in plant TERTs. This region, termed as motif 3 in human TERT, was found to be crucial for telomerase catalytic functions [50]; however, its absence is not the only problem in TERT_O variants. Various indels found across TERT_O variants from Repandae would result in out-of-frame mutations, and interestingly, a nucleotide transition found within motif C of N. nudicaulis TERT_O would disrupt one of three Asp residues that are essential for the catalytic function of any telomerase [28,29]. However, mRNA levels of the TERT_O variant revealed expression comparable to the TERT Cs variant in N. nudicaulis and N. repanda (Figure 2D). Comparable transcript levels of parental TERT variants coding for the functional TERT protein were detected in the relatively young polyploid, N. rustica (TERT_P and TERT_U, Figure 2D), and similarly in N. tabacum [36].

Our experimental analyses were accompanied by an in silico approach to answering the question on the origin and fate of the TERT_D variant in the N. sylvestris genome and, for a wider perspective, in other polyploid plant genomes. Our experimentally estimated ratio 1:1 of TERT_C and TERT_D gene copies in five N. sylvestris accessions was confirmed by in silico analysis of raw data from the N. sylvestris genome sequencing project (Table 2). Moreover, we identified a part of the TERT_D variant sequence (TERT_12D) associated with high-copy repetitive sequences, and the MtATPO gene, within a novel genomic locus in the N. sylvestris genome (Figure 4). An unplaced genomic scaffold arranged similarly to the TERT_12D locus was identified in N. tabacum, suggesting that an ancestral split of the TERT_D copy had occurred at least before the formation of N. tabacum. There is no information about a species-specific WGD event or an additional genome donor in N. sylvestris, but the increase in transposable elements and repeats was reported [41]. Moreover, activation of transposable elements was observed as a stress response to genome instability that may have been caused by a polyploidization event or environmental stress [41,51]. We presume, therefore, that the ancestral TERT_D locus (including TERT_12exD and MtATPO) originated as a result of gene/segment duplication of the TERT_C (plus MtATPO) locus or vice versa (Supplemental Text S1, Figure S2). Both loci were pseudogenized—the TERT_C locus within the MtATPO region and the TERT_D locus within the TERT region—and later, the TERT_D locus was split and translocated by Ogre/SD1-I. Currently, the mutual positioning of TERT_C, TERT_D and TERT_12exD within the genome of N. sylvestris is not known; however, similar scenarios could have resulted in pseudogenization and/or neofunctionalization of an additional TERT gene copy that we found in diploid species Populus trichocarpa, Vigna radiata and Mimulus guttatus. These TERT-like sequences may illustrate possible scenarios leading to the formation of TERT pseudogene variants in Nicotiana and the progression of TERT gene elimination after gene/genome duplication: (i) A large-scale segment/genome duplication event had created an additional TERT locus, presumably encoding a TERT pseudogene on chromosome 1 in Populus, (ii) two TERT copies placed on the same scaffold in Mimulus. (iii) A completely different arrangement comprising an additional TERT variant of Vigna radiata that is formed by two adjacent inverted copies of exon 9 of TERT, and this TERT-like sequence was annotated as ncRNA (summarized in Figure S2). Multiple TERT copies were present in some, but not all polyploid species investigated, and toleration of more TERT copies after young polyploidization events is obvious (Figure 7).
Figure 7. Origin of syntenic TERT loci in angiosperms. An ancestral locus with eudicot-like synteny that is present in the *Amborella* genome adopted the TERT gene in basal eudicots (*Nelumbo*). This ancestral locus with eudicot-like synteny occurs within the genomes of eudicots with the exception of the model plant *Arabidopsis thaliana* (for simplicity, diagrams on right panels show occurrence and arrangement of loci with eudicot-type synteny and with a specific synteny if present). The TERT gene was translocated several times into novel loci with a conserved synteny observed in current species (termed here as *Citrus*-like, *Populus*-like, *Eucalyptus*-like, *Ricinus*-like, *Arabidopsis*-like syntelogs) that had already occurred in ancestors (nodes depicting synapomorphic relationships of specific synteny-types and thus the putative origin of ancestral syntenic loci are shown above respective phylogeny nodes). As an exception, Malpighiales (*Theobroma, Gossypium*) show the TERT gene translocated into novel species-specific loci. These genomes nevertheless still contain the ancient loci with conserved synteny (details in Figure 6). TERT is mostly maintained as a single copy gene, but polyploid species can tolerate more copies (*M. luteus, G. max, G. hirsutum* are shown as representatives). Copies of genomic loci with the original synteny remain present after TERT gene elimination, e.g., in *Actinidia chinensis*, where it is difficult to distinguish which of the ohnologous loci (ohnologs = paralogs derived by WGD) have lost their TERT gene copy (see Supplemental Text S1, Figure S2). Phylogeny was adapted from APG IV [52], WGDs were mapped according to [7] in eudicots, and according to [53] in *Actinidia*.

Regarding the origin and evolution of the TERT loci in eudicots, comparison of eudicot phylogeny relationships [52] with the occurrence of syntenic loci that adopted the TERT gene demonstrated ancient synapomorphies, i.e., loci preserved in current ge-
nomes are assumed to have been present in their most recent common ancestor (nodes are depicted in Figure 7). The eudicot-like synteny locus emerged in early eudicots (Amborella) and adopted the TERT gene later in the ancestral parent of Nelumbo. The original Amborella TERT locus was probably fragmented. Another translocation of the TERT gene into novel loci grouped in all investigated malvids and Malphigiales (in fabids), and further translocations to other loci, took place later on. Interestingly, in several cases, we detected a translocation into loci that had already existed in ancestral genomes for a long time, e.g., the locus with the Citrus-like synteny originated in early eudicots, as assumed from the locus synapomorphy. The first TERT translocation from a locus with eudicot-type synteny was not caused by locus fragmentation because these loci occur in current eudicots (Figure 6B), the only exception being A. thaliana. Moreover, destabilization of the TERT position within the eudicot-like synteny locus was probably not caused by gene rearrangement because the predicted TERT gene structure with 10 exons is specific for Populus and does not occur in other Malphigiales, and TERTs with 13 exons were found in Solanaceae [30] that share eudicot-like synteny. Thus, it could be speculated that the successful TERT translocation event was more likely into target loci that show ancient synapomorphy (Figure 7). The only exceptions from this observed pattern are the Theobroma and Gossypium loci that were not syntenic to other genomes. This could indicate that these species-specific translocations are relatively recent.

In conclusion, our results show that natural Nicotiana polyploids tolerate more TERT copies and, similarly to other polyploid genomes investigated, retention of various copies is obvious in species formed by young polyploidization events. A comparison of TERT locus arrangement in current genomes suggests that the TERT gene was placed in a conserved locus that became advantageous following the emergence of basal eudicots (Figure 7). The gene was relocated later in several plant groups where only a narrow syntenic relationship restricted to closely related species could be found. Various evolutionary scenarios took place in ancestral genomes with multiple TERT copies resulting in elimination, pseudogenization and/or fragmentation, and neofunctionalization of novel TERT copies that could also illustrate the origin and fate of N. sylvestris and polyploid Nicotiana TERT variants (Figure S2).

4. Materials and Methods

4.1. Isolation of Plant Material, Genomic DNA and RNA

Nicotiana species and their accessions are listed in Table S1. N. sylvestris (accessions 934750005, TW136), N. obtusifolia, N. nesophila and N. stocktonii were kind gifts from Prof. Marie-Angèle Grandbastien (INRA, France). N. sylvestris (accession A04750326), N. re-panda, N. nudicaulis, N. paniculata, N. undulata, N. rustica, N. clevelandii, N. attenuata and N. alata were gifts from Prof. Andrew Leitch (Queen Mary University London, UK). N. sylvestris (accessions 626, Ducretet 101–268), N. quadrivalvis, N. wigandoides and N. arenstii were purchased from Imperial Tobacco Bergerac (France). Plants were grown in growth chambers under conditions of 16 h light, 22 °C and 8 h dark, 19 °C, illumination 150 μmol.m⁻².s⁻¹. DNA for qPCR experiments was isolated from plant leaves, according to Dellaporta et al. [54]. Total RNA was isolated from seedlings or young leaves using NucleoSpin® RNA kit (Macherey-Nagel, Dueren, Germany). RNA was purified by DNase I treatment (Turbo DNA-free, Life Technologies), and its integrity was checked by electrophoresis on a 1% (w/v) agarose gel. RNA concentration was measured using a spectrophotometer (Nanodrop).

4.2. PCR Amplification of TERT Sequence Variants

For detection of variant TERT sequences in a single PCR reaction (25 μl), we used 200 ng of genomic DNA from various Nicotiana species as a template, KAPA Taq DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA) and primer combinations listed in Tables S2 and S3. Thermocycling conditions for PCR reactions were as follows: 1 min at
95 °C, 35 cycles of 15 sec at 95 °C, 15 sec at 56 °C, 2 min at 72 °C, final extension 7 min at 72 °C. PCR products were checked on a 1% (w/v) agarose gel. For sequencing (Macrogen Europe B.V., Amsterdam, Netherlands), we purified PCR products using a QIuquick PCR purification kit (Qiagen, Valencia, CA, USA). In the case of N. repanda, N. nesophila and N. stocktonii, PCR products were purified from an agarose gel using a QIuquick gel purification kit). Alternatively, PCR products were cloned into the pCRITOPo vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced to analyze individual TERT variants. Representative sequences were submitted to GenBank (all accessions are specified in Table S3 and Supplementary A1). All primers are listed in Table S2.

4.3. Quantitative PCR and RT–qPCR

The number of TERT copies in genomes of N. sylvestris accessions was investigated using qPCR and TERT variant-specific primers under qPCR conditions described in [36].

For RT–qPCR, we prepared cDNA from 2 µg of total RNA according to the manufacturer’s protocol using M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA) and random nonamers (Sigma, Saint Louis, MO, USA). Quantification of the relative transcription levels of the TERT paralogs in 10 day-old seedlings of N. rustica, N. repanda and N. nudicaulis was performed in three technical replicates using TERT variant-specific primers and KAPA SYBR FAST qPCR master mix (Kapa Biosystems) in a Rotorgene 6000 cycler (Qiagen, Valencia, CA, USA). For comparative quantification of paralogous TERT transcripts in N. repanda and N. rustica, we optimized qPCR conditions to reach the same efficiency for TERT variant-specific reactions (Table S3). Optimized qPCR conditions were as follows: 5 min at 95 °C, 40 cycles of 5 sec at 95 °C, 20 sec at 62 °C, 15 sec at 72 °C, final extension 3 min at 72 °C. RT–qPCR for three TERT variants in N. nudicaulis was performed using specific primer combinations and EliZyme Green MIX AddROX (Elisabeth Pharmacon, Brno, Czech Republic) in a Rotorgene 6000 cycler under the following conditions: 3 min at 95 °C, 45 cycles of 5 sec at 95 °C, 20 sec at 61 °C, 15 sec at 72 °C, final extension 3 min at 72 °C. The proportion of TERT transcripts in allopolyploids were calculated by the delta Ct method [37] and normalized according to PCR efficiency determined from calibration curves.

4.4. In Silico Identification of TERT Variants in Nicotiana Species

TERT gene sequences were constructed in silico using genome assembly data from N. attenuata and N. obtusifolia (accessions: GCA_001879085.1 and GCA_002018475.1, respectively) and transcriptomic SRA data from N. noctiflora (SRR2106514) and N. clevelandii (SRX3866257).

For identification of TERT variants in N. benthamiana, we carried out a BLAST (BLASTn) search in genome assembly (Genome ID: 20448, [39] using full-length CDS of N. sylvestris TERT (LOC104217220) as a query. Subsequently, we analyzed raw whole-genome NGS data (kindly provided by Prof. Aureliano Bombarely and Prof. Gregory Martin, leaders of the BTI Nicotiana benthamiana genome Project): two PE libraries comprising 4 files, 2 × 100 nt; 500 bp insert size; 16 Gb compressed file size/each corresponding to ≥ 30x genome coverage. We built a nucleotide BLAST database on Meta-Centrum using BLAST+ command-line applications. TERT-like reads were identified by BLAST and mapped back to the query using Geneious software (Biomatters Ltd., Auckland, New Zealand). As a proof of concept, this approach was tested on publicly available genomic SRA data from N. tabacum, N. tomentosiformis and N. sylvestris (SRX338107, ERX248865 and ERX248848, respectively). Identification of similarities among TERT contigs from N. sylvestris and repetitive elements was performed using GEvo with/without masking of non-CDS regions and validated manually. To determine the parental origin of TERT variants in polyploids, cloned fragments and/or corresponding sequences reconstructed from genomic/transcriptomic databases were aligned with
TERT sequences from their progenitor diploids. Alignments were generated, and pairwise % identities (shown in Table 1) were calculated using Geneious software.

4.5. Analysis of Gene Synteny of the TERT Locus within Angiosperms

We selected 49 representative species (Table S5) across the Angiosperm phylogeny and basal clades with deeply sequenced and well-annotated genomes from CoGe [55] or publicly available at GeneBank. TERT-containing genomic regions were identified using FeatView (in CoGe genomes) or BLAST (www.ncbi.nlm.nih.gov) for subsequent syntenic analysis. The syntenic analysis of the TERT loci (Figure 5) was performed in two steps. First, we used the SynFind tool [45] as a screening approach for the existence of syntenic relationships between the tested TERT genomic region from one species against genomes (CoGe) of other Angiosperms. SynFind identified syntenic regions using a TERT locus query from one genome against any set of genomes. Then TERT genomic regions from species that shared gene synteny were grouped and analyzed using the GEvo tool in CoGe [55]. GEvo served for comparison of multiple large genomic regions, identification and visualization of local BLAST hits. The GEvo setup for analysis of synteny, i.e., sequence masking, was: non-CDS—used for reference sequence; algorithm: BLASTz; word size: 8; gap start penalty: 400; score threshold: 3000; minimum HSP length: 40. Information necessary for SynFind and GEvo analyses including genome accessions and TERT loci) are listed in Table S5. Identification of species-specific loci containing TERT neighboring genes, but not the TERT gene in other species, was performed using SynFind (setup: comparison algorithm—last; gene window size—30; minimum number of genes—5). We used representative TERT loci for each synteny type (Figure 5, Supplemental Text S1, Table S5) as a query against the genomes tested. SynFind outputs were further analyzed in GEvo for visualization and manual syntelog classification.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/22/4/1783/s1.

Author Contributions: P.F. and E.S. designed the study. P.F. performed all experimental work, E.S. was involved in primer design and synteny interpretation. Data assemblies and in silico analyses were done by P.F. and V.P. P.F., and J.F. drafted the primary version of the manuscript, E.S. compiled data and wrote the final manuscript. All authors contributed to and approved the final manuscript for publication. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Czech Science Foundation (18–07027S) and by the project SYMBIT CZ.02.1.01/0.0/0.0/15_003/0000477 financed by the ERDF.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequences were submitted to Genbank (NCBI, www.ncbi.nlm.nih.gov) under accession numbers MG242402-MG242425 and MG545647.

Acknowledgments: We would like to thank Miloslava Fojtová (Institute of Biophysics, Czech Republic) for helpful comments on qPCR evaluation, Marie-Angèle Grandbastien (INRA, France) for help with Nicotiana material and fruitful discussion, and Andrew Leitch (QMUL, London, UK) for kind guidance in Nicotiana taxonomy and comments on the manuscript draft.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

BLASTn  Basic local alignment search tool, nucleotide to nucleotide
LTR      Long terminal repeat
MtATPO   Mitochondrial ATP synthase subunit delta
NGS      Next-generation sequencing
qPCR     Quantitative PCR
SNP      Single nucleotide polymorphism
SRA  Short read archive  
TERT  Telomerase reverse transcriptase  
WGD  Whole-genome duplication  

References
1.  Ohno, S. Evolution by Gene Duplication; Springer: New York, NY, USA, 1970.
2.  Soltis, D.E.; Visger, C.J.; Marchant, D.B.; Soltis, P.S. Polyploidy: Pitfalls and paths to a paradigm. Am. J. Bot. 2016, 103, 1146–1166, doi:10.3732/ajb.1500501.
3.  Lohaus, R.; Van de Peer, Y. Of dups and dinos: Evolution at the K/Pg boundary. Curr. Opin. Plant Biol. 2016, 30, 62–69, doi:10.1016/j.pbi.2016.01.006.
4.  Barker, M.S.; Husband, B.C.; Pires, J.C. Spreading Wings and flying high: The evolutionary importance of polyploidy after a century of study. Am. J. Bot. 2016, 103, 1139–1145, doi:10.3732/ajb.1600272.
5.  Jiao, Y.; Wickett, N.J.; Ayyampalayam, S.; Chandler, A.S.; Landherr, L.; Ralph, P.E.; Tomsho, L.P.; Hu, Y.; Liang, H.; Soltis, P.S.; et al. Ancestral polyploidy in seed plants and angiosperms. Nature 2011, 473, 97–100, doi:10.1038/nature09916.
6.  Jiao, Y.; Leebens-Mack, J.; Ayyampalayam, S.; Bowers, J.E.; McKain, M.R.; McNeal, J.; Rolf, M.; Ruzicka, D.R.; Wafula, E.; Wickett, N.J.; et al. A genome triplication associated with early diversification of the core eudicots. Genome Biol. 2012, 13, R3, doi:10.1186/gb-2012-13-1-r3.
7.  Murat, F.; Armero, A.; Pont, C.; Klopp, C.; Salse, J. Reconstructing the genome of the most recent common ancestor of flowering plants. Nat. Genet. 2017, 49, 490–496, doi:10.1038/ng.3813.
8.  Flagel, L.; Udall, J.; Nettleton, D.; Wendel, J. Duplicate gene expression in allopolyploid Gossypium reveals two temporally distinct phases of expression evolution. BMC Biol. 2008, 6, 16, doi:10.1186/1741-7007-6-16.
9.  Parisod, C.; Mhiiri, C.; Lim, K.Y.; Clarkson, J.J.; Chase, M.W.; Leitch, A.R.; Grandbastien, M.A. Differential dynamics of transposable elements during long-term diploidization of Nicotiana section Repandae (Solanaceae) allopolyploid genomes. PLoS ONE 2012, 7, e50352, doi:10.1371/journal.pone.0050352.
10.  Birchler, J.A.; Veitia, R.A. The gene balance hypothesis: From classical genetics to modern genomics. Plant Cell 2007, 19, 395–402, doi:10.1105/tpc.106.049338.
11.  McClintock, B. The fusion of broken chromosome ends of sister half-chromatids following chromatid breakage at meiotic anaphases. Mo. Agric. Exp. Stn. Res. Bull. 1938, 290, 1–48.
12.  Blackburn, E.H.; Gall, J.G. Tandemly Repeated Sequence at Termini of Extrachromosomal Ribosomal-Rna Genes in Tetrahyemen. J. Mol. Biol. 1978, 120, 33–53.
13.  Greider, C.W.; Blackburn, E.H. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1983, 45, 403–413.
14.  Peska, V.; Garcia, S. Origin, Diversity, and Evolution of Telomere Sequences in Plants. Front. Plant Sci. 2020, 11, 117, doi:10.3389/fpls.2020.00117.
15.  Sykorova, E.; Lim, K.Y.; Kunicka, Z.; Chase, M.W.; Bennett, M.D.; Fajkus, J.; Leitch, A.R. Telomere variability in the monocotyledonous plant order Asparagales. Proc. R. Soc. Lond. B Biol. Sci. 2003, 270, 1893–1904, doi:10.1098/rspb.2003.2446.
16.  Fajkus, P.; Peska, V.; Sitova, Z.; Fulneckova, J.; Dvorackova, M.; Gogela, R.; Sykorova, E.; Hapala, J.; Fajkus, J. Allium telomeres unmasked: The unusual telomeric sequence (CTCCGTATAGGG)n is synthesized by telomerase. Plant J. 2016, 85, 337–347, doi:10.1111/tpj.13115.
17.  Sykorova, E.; Lim, K.Y.; Chase, M.W.; Knapp, S.; Leitch, I.J.; Leitch, A.R.; Fajkus, J. The absence of Arabidopsis-type telomeres in Cestrum and closely related genera Vestia and Sessea (Solanaceae): First evidence from eudicots. Plant J. 2003, 34, 283–291.
18.  Peska, V.; Fajkus, P.; Fojtova, M.; Dvorackova, M.; Hapala, J.; Dvoraec, V.; Polanska, P.; Leitch, A.R.; Sykorova, E.; Fajkus, J. Characterisation of an unusual telomere motif (TTTTTATAGGG)n in the plant Cestrum elegans (Solanaceae), a species with a large genome. Plant J. 2015, 82, 644–654, doi:10.1111/tpj.12839.
19.  Peska, V.; Sitova, Z.; Fajkus, P.; Fajkus, J. BAL3I-NGS approach for identification of telomeres de novo in large genomes. Methods 2017, 114, 16–27, doi:10.1016/j.ymeth.2016.08.017.
20.  Fajkus, J.; Sykorova, E.; Leitch, A.R. Telomeres in evolution and evolution of telomeres. Chromosome Res. 2005, 13, 469–479, doi:10.1007/s10577-005-0997-2.
21.  Fajkus, P.; Peska, V.; Zavodnik, M.; Fojtova, M.; Fulneckova, J.; Dobias, S.; Kilar, A.; Dvorackova, M.; Zachova, D.; Nescasova, I.; et al. Telomererase RNAs in land plants. Nucleic Acids Res. 2019, 47, 9842–9856, doi:10.1093/nar/gkz695.
22.  Peska, V.; Matl, M.; Mandakova, T.; Vitales, D.; Fajkus, P.; Fajkus, J.; Garcia, S. Human-like telomeres in Zostera marina reveal a mode of transition from the plant to the human telomeric sequences. J. Exp. Bot. 2020, 71, 5786–5793, doi:10.1093/jxb/eraa293.
23.  Sykorova, E.; Fajkus, J. Structure-Function relationships in telomerase genes. Biol. Cell 2009, 101, 375–392, doi:10.1042/BC20080205.
24.  Belfort, M.; Curcio, M.J.; Luo, N.F. Telomerase and retrotransposons: Reverse transcriptases that shaped genomes. Proc. Natl. Acad. Sci. USA 2011, 108, 20304–20310, doi:10.1073/pnas.1100269109.
25.  Fitzgerald, M.S.; Riha, K.; Gao, F.; Ren, S.; McKnight, T.D.; Shippen, D.E. Disruption of the telomerase catalytic subunit gene from Arabidopsis inactivates telomerase and leads to a slow loss of telomeric DNA. Proc. Natl. Acad. Sci. USA 1999, 96, 14813–14818.
26. Oguchi, K.; Liu, H.; Tamura, K.; Takahashi, H. Molecular cloning and characterization of AtTERT, a telomerase reverse transcriptase homolog in Arabidopsis thaliana. FEBS Lett. 1999, 457, 465–469.

27. Harrington, L.; Zhou, W.; McPhail, T.; Oulton, R.; Yeung, D.S.; Mar, V.; Bass, M.B.; Robinson, M.O. Human telomerase contains evolutionarily conserved catalytic and structural subunits. Genes Dev. 1997, 11, 3109–3115, doi:10.1101/gad.11.23.3109.

28. Lingner, J.; Hughes, T.R.; Shchepchenko, A.; Mann, M.; Lundblad, V.; Cech, T.R. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 1997, 276, 561–567, doi:10.1126/science.276.5312.561.

29. Nakamura, T.M.; Morin, G.B.; Chapman, K.B.; Weinrich, S.L.; Andrews, W.H.; Lingner, J.; Harley, C.B.; Cech, T.R. Telomerase catalytic subunit homologs from fission yeast and human. Science 1997, 277, 955–959.

30. Sykorova, E.; Fulneckova, J.; Mokros, P.; Fajkus, J.; Fojtova, M.; Pesa, V. Three TERT genes in Nicotiana tabacum. Chromosome Res. 2012, 20, 381–394, doi:10.1007/s10577-012-9282-3.

31. Clarkson, J.J.; Dodsworth, S.; Chase, M.W. Time-Calibrated phylogenetic trees establish a lag between polyploidisation and diversification in Nicotiana (Solanaceae). Plant Syst. Evol. 2017, 303, 1001–1012, doi:10.1007/s00606-017-1416-9.

32. Leitch, I.J.; Hanson, L.; Lim, K.Y.; Kovarik, A.; Chase, M.W.; Clarkson, J.J.; Leitch, A.R. The ups and downs of genome size evolution in polyploid species of Nicotiana (Solanaceae). Ann. Bot. 2008, 101, 805–814, doi:10.1093/aob/mcm326.

33. Clarkson, J.J.; Leitch, I.J.; Kovarik, A.; Chase, M.W.; Knapp, S.; Leitch, A.R. Long-Term genome diploidization in allopolyploid Nicotiana sectio Repandae (Solanaceae). New Phytol. 2005, 168, 241–252, doi:10.1111/j.1469-8137.2005.01480.x.

34. Knapp, S.; Lughadha, E.N.; Paton, A. Taxonomic inflation, species concepts and global species lists. Trends Ecol. Evol. 2005, 20, 7–8, doi:10.1016/j.tree.2004.11.001.

35. Kelly, L.J.; Leitch, I.J.; Clarkson, A.R.; Knapp, S.; Chase, M.W. Reconstructing the complex evolutionary origin of wild allopolyploid tobaccos (Nicotiana section suaveolentes). Evolution 2013, 67, 80–94, doi:10.1111/j.1558-5646.2012.01748.x.

36. Jureckova, J.F.; Sykorova, E.; Hafidh, S.; Honys, D.; Fajkus, J.; Fojtova, M. Tissue-Specific expression of telomerase reverse transcriptase gene variants in Nicotiana tabacum. PLoS ONE 2017, 245, 549–561, doi:10.1007/s00425-016-2624-1.

37. Pfaffl, M.W. Quantification strategies in real-time PCR. In A-Z of Quantitative PCR; Bustin, S.A., Ed.; International University Line: La Jolla, CA, USA, 2004; pp. 87–112.

38. Clarkson, J.J.; Knapp, S.; Garcia, V.F.; Olmstead, R.G.; Leitch, A.R.; Chase, M.W. Phylogenetic relationships in Nicotiana (Solanaceae) inferred from multiple plastid DNA regions. Mol. Phylogenet. Evol. 2004, 33, 75–90, doi:10.1016/j.ympev.2004.05.002.

39. Bomarely, A.; Rosli, H.G.; Vrebakov, J.; Moffett, P.; Mueller, L.A.; Martin, G.B. A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. Mol. Plant Microbe Interact. 2012, 25, 1523–1530, doi:10.1094/MPMI-06-12-0148-TA.

40. Lim, K.Y.; Matyasek, R.; Kovarik, A.; Leitch, A.R. Genome evolution in allotetraploid Nicotiana. Biol. J. Linn. Soc. 2004, 82, 599–606.

41. Renny-Byfield, S.; Chester, M.; Kovarik, A.; Le Comber, S.C.; Grandbastien, M.A.; Delogier, M.; Nichols, R.A.; Macas, J.; Novak, P.; Chase, M.W.; et al. Next generation sequencing reveals genome downsizing in allotetraploid Nicotiana tabacum, predominantly through the elimination of paternally derived repetitive DNAs. Mol. Biol. Evol. 2011, 28, 2843–2854, doi:10.1093/molbev/msr112.

42. Chase, M.W.; Knapp, S.; Cox, A.V.; Clarkson, J.J.; Butsko, Y.; Joseph, J.; Savolainen, V.; Parokony, A.S. Molecular systematics, GISH and the origin of hybrid taxa in Nicotiana (Solanaceae). Ann. Bot. 2003, 92, 107–127, doi:10.1093/aob/mcg087.

43. Kohany, O.; Gentles, A.J.; Hankus, L.; Jurka, J. Annotation, submission and screening of repetitive elements in Repbase: RepbaseSubmitter and Censor. BMC Bioinform. 2006, 7, 474, doi:10.1186/1471-2105-7-474.

44. Gordenin, D.A.; Lobachev, K.S.; Degtjareva, N.P.; Malkova, A.L.; Perkins, E.; Resnick, M.A. Inverted DNA repeats: A source of eukaryotic genomic instability. Mol. Cell. Biol. 1993, 13, 5315–5322.

45. Tang, H.; Bomhoff, M.D.; Briones, E.; Zhang, L.; Schnable, J.C.; Lyons, E. SynFind: Compiling Syntenic Regions across Any Set of Genomes on Demand. Genome Biol. Evol. 2017, 5, 3286–3298, doi:10.1093/gbe/evv219.

46. He, Z.C.; Li, J.Q.; Cai, Q.; Wang, Q. The cytology of Actinidia, Saurauia and Clematoclethra (Actinidiaceae). Bot. J. Linn. Soc. 2005, 147, 369–374, doi:10.1111/j.1095-8339.2005.00372.x.

47. Shi, T.; Huang, H.W.; Barker, M.S. Ancient genome duplications during the evolution of kiwifruit (Actinidia) and related Ericales. Ann. Bot. 2010, 106, 497–504, doi:10.1093/aob/mcq129.

48. Schmutz, J.; Cannon, S.B.; Schlueter, J.; Ma, J.; Mitros, T.; Nelson, W.; Hyten, D.L.; Song, Q.; Thelen, J.J.; Cheng, J.; et al. Genome sequence of the paleoepipolyploid soybean. Nature 2010, 463, 178–183, doi:10.1038/nature08670.

49. Zhang, T.; Hu, Y.; Jiang, W.; Fang, L.; Guan, X.; Chen, J.; Zhang, J.; Saski, C.A.; Scheffler, B.E.; Stelly, D.M.; et al. Sequencing of allotetraploid cotton (Gossypium hirsutum L. acc. TM-1) provides a resource for fiber improvement. Nat. Biotechnol. 2015, 33, 531–537, doi:10.1038/nbt.3207.

50. Xie, M.; Podlevsky, J.D.; Qb, X.; Bley, C.J.; Chen, J.J. A novel motif in telomerase reverse transcriptase regulates telomere repeat addition rate and processivity. Nucleic Acids Res. 2010, 38, 1982–1996, doi:10.1093/nar/gkp1198.

51. Madlung, A.; Tyagi, A.P.; Watson, B.; Jiang, H.; Kagochi, T.; Doerge, R.W.; Martienssen, R.; Comai, L. Genomic changes in synthetic Arabidopsis polyploids. Plant J. 2005, 41, 221–230, doi:10.1111/j.1365-313X.2004.02297.x.

52. Byng, J.W.; Chase, M.W.; Christenhusz, M.J.M.; Fay, M.F.; Judd, W.S.; Mabberley, D.J.; Sennikov, A.N.; Soltis, D.E.; Soltis, P.S.; Stevens, P.F.; et al. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Bot. J. Linn. Soc. 2016, 181, 1–20, doi:10.1111/bij.12385.
53. Huang, S.; Ding, J.; Deng, D.; Tang, W.; Sun, H.; Liu, D.; Zhang, L.; Niu, X.; Zhang, X.; Meng, M.; et al. Draft genome of the kiwifruit Actinidia chinensis. Nat. Commun. 2013, 4, 2640, doi:10.1038/ncomms3640.

54. Dellaporta, S.L.; Wood, J.; Hicks, J.B. A plant DNA minipreparation: Version II. Plant Mol. Biol. Report. 1983, 1, 19–21.

55. Fajkus, J.; Dvorackova, M.; Sykorova, E. Analysis of telomeres and telomerase. Methods Mol. Biol. 2008, 463, 267–296, doi:10.1007/978-1-59745-406-3_17.