Supplementary material

Molecular analysis of the T-DNA insertion mutants identified putative regulatory elements in the \textit{AtTERT} gene
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\textbf{RT-PCR analysis of T-DNA borders in mutant lines F\textunderscore385 and F\textunderscore493}

The \textit{AtTERT}-specific primers (1F, 2F, 5Rev) and primers recommended for genotyping of FLAG lines (INRA-LB4, INRA-Tag3) were used (Table S1, Fig. S1B,C) to exclude/confirm transcription driven by the 35S promoter from T-DNA. In addition, we also used primer barFw as the second internal control in RT-PCR and genotyping PCR reactions. Genotyping of F\textunderscore385 showed the presence of two inverted T-DNA copies embedded by left border (LB) sequences; the line F\textunderscore493 possesses one T-DNA insertion (Fig. S1B,C). The RT-PCR analysis clearly showed that the transcription upstream and downstream of T-DNA insertions (compare to Fig. 6, 7) is not caused by a leaking terminator 3' g7 (Fig. S1B,C).

\textbf{Root elongation analysis}

Seeds were surface sterilized and placed on 0.8 % agar plates with ½ MS supplemented with 0.5 g.l\textsuperscript{-1} MES, 0.1 g.l\textsuperscript{-1} myoinositol and 10 g.l\textsuperscript{-1} sucrose. After 3 days at 4°C, the plates were placed vertically and plants were grown under conditions of 16 h light, 21°C and 8 h dark, 19°C, illumination 100 \textmu mol.m\textsuperscript{-2}.s\textsuperscript{-1}. Since the third day of plant growth, the main root length was determined every 24 h for 10 days and evaluated as cumulative root length for every day of growth. Plants grown on the same plate were compared. The following T-DNA accessions were used: S\textunderscore048, S\textunderscore126 (upstream lines); S\textunderscore050, S\textunderscore284 (gene lines) and S\textunderscore1287 (control line, T-DNA insertion in putative 3'UTR of \textit{AtPPR} gene, Figures 1C, S1). The root phenotype of homozygous mutant plants was compared to segregated wild type
plants (wt) of the same plant line and generation. G3 progeny of three separate plants from each accession were analyzed, one line being analyzed in two technical replicates, adding up totally to 4 independent experimental replicates. All data were analyzed statistically using the two-tailed unpaired Student’s t-test with $\sigma_A \neq \sigma_B$.

The results did not show any apparent phenotype difference in the root growth between the wild type and mutant plants. Fig. S2 shows a representative example of the analysis for the two gene lines S_050 and S_284. Although some differences in the final root length in the 13-day plants were statistically significant, this difference lacked any trend and failed to be confirmed in the remaining experimental replicates (Fig. S3).

In addition, no apparent phenotype was observed when we compared the meristem length and the root cap morphology of homozygous mutant plants (S_126, S_048, S_050, F_385, F_493) to segregated wild type plants (not shown).

**Analysis of TRAP products from T-DNA insertion lines using CAMV substrate primer and modifications of the TRAP assay**

In telomerase-negative extracts from gene lines, longer bands as a result of the TRAP analysis using the CAMV and TELPR primer sets were observed (Fig. 3). TRAP products from this assay were cloned into pCRRIITOPO vector (Invitrogen) and sequenced. The sequences were aligned manually (Fig. S4) and used as queries in a BLAST search of Genbank databases. The BLAST search identified the EST sequence EG442858 with similarity to cloned TRAP products that started in the CAMV primer region. TRAP products contained degenerated telomere sequence motifs, were asymmetric in nucleotide distribution (Fig. S4), and probably contained amplified unknown RNA sequence (see below).

In addition to standard TRAP assays with CAMV and TELPR primers, a series of control reactions was performed: (i) testing of RNase sensitivity - protein extracts were treated by RNase A at 37°C for 10 min before the extension step (Fig. S5A); (ii) testing of
Taq polymerase - hot-start Taq Polymerase (AB Gene) was used in the PCR step instead of DyNAzyme II (Finnzymes) (Fig. S5B); (iii) testing of primer specificity - the substrate primer CAMV was replaced by CAMVGG with reduced homology with the EST sequence (Table S1) or by TS21 (Fig. S5C). The analysis showed (i) the synthesis of long TRAP products was sensitive to RNAse digestion (Fig. S5A) suggesting their RNA origin; (ii) the products were created in the PCR step and their appearance was dependent on the Taq polymerase used (Fig. S5B). The products disappeared when the substrate primer CAMV was replaced by TS21 (Fig. S5C). These observations are interesting because the RNA template after heat-treatment was stable enough to serve as template for DyNAzyme II. Thus, control experiments showed that the TRAP products originated from RNA; these products apparently do not represent common artifacts generated during TRAP assays via primer catenation, and they occur when the combination of substrate primer CAMV and DyNAzyme II polymerase is used (Figures S4, S5).

*Analysis of telomerase activity and AtTERT expression in the individual S_041/3 mutant lines*

Telomerase activity was not detected in seedlings and buds of all gene lines except for seedlings of the G4 and G5 generations of the individual mutant line S_041/3 (Fig. S6B). Detailed analysis showed that the protein extracts from buds and seedlings collected from individual plants were negative in the TRAP assay, but extracts from pooled seedlings of later generations displayed mild telomerase activity (Fig. S6). As we carefully genotyped each plant used for analysis, we found occasionally a heterozygous plant in later generations (not shown) which may have caused the weak telomerase activity detected in extracts prepared from pooled seedlings. Nevertheless, the occasional presence of a heterozygous individual in pooled seedlings cannot be a cause of different AtTERT transcription patterns in the S_041/3 individual mutant line in comparison to other gene lines including the “sister” mutant line.
S_041/2 (Table 1). In S_041/3, $AtTERT$ transcription is slightly but reproducibly increased in regions upstream of the T-DNA insertion and comparable to Columbia wild type in the exon 12 located downstream of the insertion (Table S2). Genotyping of line S_041 revealed the presence of two copies of T-DNA in head-to-head orientation. However, sequencing of the T-DNA borders revealed no difference between “sister” lines S_041/2 and S_041/3. Analysis of more S_041 individual mutant lines is necessary to discriminate whether S_041/3 represents an artifact or if this $AtTERT$ transcription pattern is typical for plants bearing a T-DNA insertion in this region, pointing out the possibility of the presence of another regulatory element similar to that in the S_048 line.
| PRIMER | REACTION | 5′→3′ sequence |
| --- | --- | --- |
| CAMV | TRAP | CGTTTCAAAGAATGGATT |
| TS21 | TRAP | GACAATCCGTGAGCAGAGTT |
| CAMVGG | TRAP | ATTCGTCTTCAAAGCAGTGGAGG |
| TELPR | TRAP | CGGATTTCCAACCTAAACCCTAAACCCTAAACCCTAAACC |
| 1F | AtTERT exon 1 expression, genotyping FLAG_385G01, FLAG_493F06 | TCTCTGTGACCACCAAGATGTTGGAGAG |
| 1R | AtTERT exon 1 expression | CCATCGACTGCCAGGAAGATCTAATAG |
| 10F2 | AtTERT exon 10 expression | GACACAAAAGGTAGTGATCATCAATAAATCTCAGTAAAC |
| 12F | AtTERT exon 12 expression | GCAGCTATCTCGGAATATTGATAT |
| 12R | AtTERT exon 12 expression, genotyping SAIL_284_B07, SALK_050921 | ACCTAAGTTTCGATGATAATTGATAGTCAATGAGTCAGGTT |
| 2F | AtTERT exon 2-5 expression | CATGGTTTATCTCTTCAACGAAACATC |
| 4F | AtTERT exon 4-5 expression | CTTTTTCGGTTGCTGCTGAC |
| 5R | AtTERT exon 2-5, 4-5 expression | CTTTTATGGGGAATCTCATTAGTAC |
| 5-6F | AtTERT alternative splicing | GTTCATCTGATITTTGTAAAGCAGGCTAAAC |
| 7-8R | AtTERT alternative splicing | AGACTTGAGCAATGAGTCATCAATGAGTCAGGTT |
| 6iF | AtTERT alternative splicing | TCAATCTTCTTACTTCGGAATTTGAG |
| 6iR | AtTERT alternative splicing | TGATATCATAAAGGAGATCCATTCACAATGCAGAAG |
| ubqFw | ubiquitin expression | ACGGAAAGAGAGCATTAC |
| ubqRev | ubiquitin expression | ACAAGATGAAGGGTGGAC |
| PPR_3kbFw | genotyping SAIL_1287_C04 | GCCAAGGGGATTGATAATAAGCACGT |
| PPR_3kbRev | genotyping SAIL_1287_C04 | GCCAAGGGGATTGATAATAAGCACGT |
| RRM_Fw | genotyping SALK_110053 | GGCTCAAGCTTCTTGCTGCTGGA |
| RRM_Rev | genotyping SALK_110053 | CAAAAGAGAAGGTAGTGAAGTCGAGCAA |
| PPR_2kbFw | genotyping SAIL_575_F07, expression of At5g16860 | TGGAAAAGCAACCCCATACCAGAC |
| PPR_2kbRev | genotyping SAIL_575_F07, expression of At5g16860 | TGGAAAAGCAACCCCATACCAGAC |
| PPR | genotyping FLAG_490/492, SALK_048471 | ACTTGGACTGATGATAGTGGATACGT |
| promFw | genotyping SALK_126201 | CTGCGTTAATCAAAACACTTTACGT |
| promRev | genotyping SALK_126201, SALK_048471, FLAG_490/492 | TGATATCATAAAGGAGATCCATTCACAATGCAGAAG |
| 10exFw | genotyping SALK_041265 | AAGCCTACTCAGGATATAGTGGATACGT |
| 10exRev | genotyping SALK_041265 | GGGGACAGATTTACATCCTCGTTATGAGTGGGTT |
| 11exFw | genotyping SAIL_284_B07, SALK_050921 | CATCCTACTTATGAGTGGATACGTAATCCTCACCTACGATCAGT |
| 4iRev | genotyping FLAG_385G01, FLAG_493F06 | AAACACAAAGAGAAGCTCGGATACAAGT |
| 10exFw | genotyping SALK_041265 | GGGGACAGATTTACATCCTCGTTATGAGTGGGTT |
| 10exRev | genotyping SALK_041265 | GGGGACAGATTTACATCCTCGTTATGAGTGGGTT |
| 11exFw | genotyping SAIL_284_B07, SALK_050921 | CATCCTACTTATGAGTGGATACGTAATCCTCACCTACGATCAGT |
| Primer   | Function                        | Sequence                           |
|----------|---------------------------------|------------------------------------|
| Tinz     | genotyping SALK lines           | CAACACTCAACCCTATCTCGGG             |
| SAIL_LB3 | genotyping SAIL lines           | TAGCATCTGAATTTCATAACCAATCTCGATACAC |
| INRA_LB4 | genotyping FLAG lines           | CGTGTGCCAGGTGCCACCGGAATAGT         |
| INRA_Tag3| genotyping FLAG lines           | CTGATACCGACGTTGCCCGCATAA          |
| barFw    | genotyping FLAG lines, T-DNA expression | CATCGAGACAGCAGGTCAACTTC         |

Table S1: Sequences of primers used in analysis of telomerase activity (TRAP), genotyping of mutant lines, and analysis of *AtTERT* transcription.
| T-DNA insertion line | tissue/generation | exon 1      | exon 10     | exon 12     |
|---------------------|-------------------|-------------|-------------|-------------|
| SALK_041265 / 3     | SG2               | 3.39±1.22   | 4.21±0.83   | N.A.        |
|                     | SG3               | 3.52±0.25   | 2.8±0.25    | 1.05±0.10   |
|                     | SG4               | 3.06±0.13   | 2.75±0.33   | 1.06±0.17   |
|                     | BG4               | 4.22±0.83   | 3.87±1.05   | N.A.        |
|                     | LG4               | N.D.        | N.A.        | N.A.        |

Table S2: *AtTERT* transcription in seedlings (S), buds (B) and matured leaves (L) in the S_041/3 mutant line. Transcription levels are expressed relative to that in the respective wild type tissue. Data were taken from analyses of at least two biological replicates. N.A., not analyzed; N.D., not detected (expression below the detection limit).
Fig. S1. Description of the T-DNA lines. (A) Positions and orientation of T-DNA insertions along the genes At5g16840 (AtRRM), At5g16850 (AtTERT) and At5g16860 (AtPPR). Data were taken from the T-DNA Express database established by the Salk Institute Genomic Analysis Laboratory [http://signal.salk.edu/cgi-bin/tdnaexpres]. Most of the T-DNA insertion sites were identified using the left border primers (arrows, except FLAG_490D05). The AtTERT and AtPPR genes share 5'UTR/promoter region; the AtTERT and AtRRM genes share the 3'UTR region. (B) Map of the pGKB5 T-DNA according [http://dbsgap.versailles.inra.fr/portail/] (on top) and arrangement of T-DNA insertions in the FLAG lines identified by genotyping with the AtTERT specific primers (1F, 4iRev, PPR, promRev) and T-DNA specific primers (LB4, Tag3), shown in italics. (C) Detailed analysis of T-DNA borders of the lines F_385 and F_493 by genotyping PCR using genomic DNA template (gDNA) and by RT-PCR (cDNA) with the T-DNA and the AtTERT specific primers clearly shows the overexpression upstream and downstream of T-DNA insertions is not caused by leaking 3’g7 terminator.
**Fig. S2.** Root growth analysis in S_050/4 (segregated wild type) and S_050/13 (-/-, gene line).  

A: Example of the analyzed plate on the 13th day of the growth.  

B: Analysis of the root length during 13 days of the plants cultivations. Fourth independent plates of S_050/4 segregated wild type (black circles) and S_050/13 individual gene line (grey squares) were analyzed. Seeds collected from 3 plants (plant 1 – plates 1a and 1b; plant 2 - plate 2; plant 3 - plate 3) were subjected to the analysis. Error bars represent SD.
Fig. S3. Root length of 13-day old plants of mutant lines. Length of roots of two upstream (S_048, S_126) and two gene mutant lines (S_050, S_284; grey columns) and respective segregated wild types (black columns) were compared. 10 – 16 representatives of each individual mutant line and segregated wild type were analyzed. Mutant line with the T-DNA insertion out of the AtTERT gene (S_1287, Fig. 1C) was used as a control. Error bars represent SD. Asterisks represent statistical significance in two-tailed unpaired Student’s t-test with $\sigma_A \neq \sigma_B$: * - $p < 0.05$, ** - $p < 0.001$. 
Fig. S4. Sequence alignment of cloned products from the TRAP assay in telomerase negative gene lines. Independently of their origin (the clones were named according the T-DNA line number) they show T/G-rich sequence with degenerated telomeric motifs and similarity to the EST sequence EG442858. Sequence of the CAMV substrate primer (note the 11-base homology with EST sequence) and of the TELPR reverse primer are highlighted; regions containing at least one plant telomeric motif TTTAGGG are shown in green; depicted C bases (pink circles) reflect asymmetric base content in the cloned TRAP products. The clones 041-7-T7 and 284-22-T7 are partial sequences.
Fig. S5. RNA nature of TRAP products obtained using the CAMV substrate primer in telomerase negative gene lines (homozygous for T-DNA insertions).

(A) TRAP assay using non-treated (- lines) and RNaseA treated (+ lines) protein extracts. Presence of both telomerase-specific bands in the upstream lines and non-specific high molecular weight products in the gene lines was dependent at the RNaseA treatment; TSR8,
control template from the TRAPEze® XL Telomerase Detection Kit (Millipore).

(B) Analysis of the telomerase activity using hot start Taq polymerase (ABgene, left panel) and DyNAzyme II polymerase (Finnzymes, right panel) using the CAMVGG substrate primer with lower level of homology with the EST sequence (Table S1). High molecular weight product disappeared when a hot start Taq polymerase was used for amplification of extension products. Col, positive control (protein extract from Columbia seedlings); NC, negative control (no protein extract in the TRAP reaction).

(C) Telomerase activity assay of telomerase-positive tissues (seedlings, buds) collected from T-DNA insertion lines. Telomerase activity was determined in the upstream lines (top panels) and the gene lines (bottom panels) according to the TRAP protocol using the TS21 and TelPr primer set (Table S1). Regular ladder of bands in upstream lines evidenced telomerase activity comparable to that in seedlings of respective wild types (Col, WS4). No telomerase activity was detected in the gene lines (data of the 041/3 individual mutant line see Fig. S6). G2, G3, and G4, mark the second, the third and the fourth generation, respectively; NC, negative control.
**Fig. S6.** *In vitro* telomerase activity assay in buds (top panels) and seedlings (bottom panels) collected from the individual mutant line S_041/3.

(A) Telomerase activity was analyzed in pooled buds of three generations of the S_041/3 plants (on left) and from individuals in the fourth generation (G4). (B) Seedlings pooled from ten plants (on left) and pooled seedlings originated from individual plants (on middle and right panel) were examined up to the fifth generation (G5). The numbers above lines identify individual samples; NC, negative control.