SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
TELOMERES
DNA METHYLATION
PCR-BASED TECHNIQUES

Received
2 December 2013

Accepted
16 June 2014

Published
2 July 2014

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Determination of *Arabidopsis thaliana* telomere length by PCR

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In humans, telomere length studies have acquired great relevance because the length of telomeres has been related to natural processes like disease, aging and cancer. However, very little is known about the influence of telomere length on the biology of wild type plants. The length of plant telomeres has been usually studied by Terminal Restriction Fragment (TRF) analyses. This technique requires high amounts of tissue, including multiple cell types, which might be the reason why very little is known about the influence of telomere length on plant natural processes. In contrast, many of the human telomere length studies have focused on homogenous cell populations. Most of these studies have been performed by PCR, using telomeric degenerated primers, which allow the determination of telomere length from small amounts of human cells. Here, we have adapted the human PCR procedure to analyze the length of *Arabidopsis thaliana* telomeres. This PCR approach will facilitate the analysis of telomere length from low amounts of tissue. We have used it to determine that CG and non CG DNA methylation positively regulates Arabidopsis telomere length.

Telomeres usually contain short G/C-rich tandem telomeric repeats that can be also found at interstitial chromosomal loci[1–11]. The length of telomeres is regulated, which is essential to guaranty genomic stability[1–3]. This has been clearly established in studies where mutants lacking of telomerase, the enzyme that replicates telomeres, were analyzed[12–14].

In humans, telomere length studies have acquired great relevance because the length of telomeres has been related to diseases, aging and cancer[15]. Human telomere length measurements are essentially achieved by three different methods[16]: 1) Analysis of Terminal Restriction Fragments (TRF). 2) Analysis of Fluorescence *in situ* Hybridization (FISH). 3) PCR analysis (STELA and telomeric PCR).

TRF analysis allows the study of the average telomere length of all the telomeres in a cell population. It is the oldest method used to measure telomere length and is based on the digestion of genomic DNA with frequently cutting restriction enzymes, the resolution of the digested DNA in agarose gels and the further hybridization with a telomeric probe. This approach allows the determination of telomere length using appropriate markers. Since telomeres are essentially composed of perfect telomeric repeats they are not cut by the restriction enzymes. In contrast, Interstitial Telomeric Sequences (ITSs) are usually degenerated and cut with the restriction enzymes. Therefore, they do not generally interfere with the determination of telomere length[17].

FISH is based on the labeling of telomeres with fluorescent probes and is generally performed in two different ways: Q-FISH that allows length determinations of all the telomeres independently in a single cell and F-FISH, which allows the determination of the average telomere length, also in a single cell.

STELA PCR studies involve the use of primers containing perfect telomeric repeats and subtelomeric primers. This technique allows the analysis of the average telomere length of specific telomeres, as far as their subtelomeric regions contain unique sequences. It usually involves hybridization with subtelomeric probes.

Telomeric PCR analyses are based on the use of human telomeric degenerated primers that amplify telomeres more efficiently than ITSs. This method involves the amplification of single copy sequences as DNA quantity reference. It was initially described by Richard Cawthon[18] and further refined by the same author following a Monochrome Multiplex Quantitative PCR approach (MMQPCR)[19]. MMQPCR allows the detection of telomeres and of a single copy DNA sequence in the same reaction mixture using SYBR Green.

Arabidopsis mutants lacking of telomerase acquire a complex pleiotropic phenotype, including genome instability, and become sterile after several self-pollination cycles[14]. However, the influence of telomere length on the biology of wild type plants remains largely unknown although a correlation between telomere length and embryo viability has been described in rye[20,21]. Two methods have been mainly used to study telomere length in

plants: TRF analysis and PETRA. PETRA is quite similar to STELA and allows the study of a single telomere length, as far as its sub-telomeric region contains unique sequences[22].

The human telomeric degenerated primers cannot amplify the plant type telomeric sequences (TTTAGGG/CCCTAAA) because plants, in general, contain one T/A more than humans in their telomeric repeats. Here, we have designed two telomeric degenerated primers that amplify Arabidopsis telomeres more efficiently than ITSs. Using these primers and primers that amplify a single copy DNA sequence, we have performed MMQPCR reactions to measure the length of telomeres. In addition, we have used the MMQPCR technique to investigate the influence of DNA methylation on the homeostasis of telomere length in Arabidopsis.

Eukaryotic DNA methylation is mainly found in cytosines and plays essential roles in cell biology. In plants, cytosine methylation can be found in three different contexts: CG, CHG and CHH, where H can be A, G or T. The methylation of Arabidopsis cytosines involves a complex interplay of different DNA methyltransferases and of additional proteins[23]. MET1 is the main DNA methyltransferase in Arabidopsis and collaborates with DDM1 to establish CG methylation. MET1 and DDM1 also influence non CG methylation, which is achieved by other DNA methyltransferases including CMT3 (CHG methylation) and DRM1 or DRM2 (CHH and also CHG methylation). We have analyzed the length of telomeres in wild type Arabidopsis plants, in single mutants with impaired MET1 or DDM1 functions and also in a triple mutant affected in DRM1, DRM2 and CMT3 activities. All these methylation mutants have shorter telomeres than the wild type strain. Hence, both, CG and non CG methylation, positively regulates the homeostasis of telomere length in Arabidopsis.

Results

Design of primers to amplify Arabidopsis telomeres. Primers containing perfect telomeric repeats organized in tandem cannot be used to amplify telomeres by PCR. They would amplify themselves at earlier PCR cycles than they would amplify telomeres from genomic DNA. Therefore, the analysis of telomeres by PCR requires the use of degenerated telomeric primers. We have designed two primers, TelA and TelB, to amplify Arabidopsis telomeres following the principles previously described by Cawthon[18] but considering the sequence of the plant type telomeric repeats and trying to minimize ITSs amplification (Fig. 1). These primers contain several degenerated copies of Arabidopsis telomeric repeats in their 3′ end and several non-telomeric nucleotides in their 5′ end. Both primers establish many hydrogen bonds with Arabidopsis telomeres except in their 5′ end. In addition, once they anneal with Arabidopsis telomeres, they can prime DNA synthesis because their last 3′ nucleotide is complementary to telomeres. Although these primers can anneal with themselves and form primer dimers, they establish a number of hydrogen bonds much lower than they do with telomeres and, in addition, they should not prime DNA synthesis because their 3′ ends are not complementary. Therefore, these primer dimers are not expected to amplify themselves. Primers TelA and TelB were designed with more than 4 degenerated telomeric repeats because we have previously found that the sequence $(CCCTAAA)_4$ is fundamentally present at Arabidopsis telomeres (in 98% of the cases), whereas it is very infrequent at ITSs (only in 2% of the cases)[24]. Therefore, primers TelA and TelB should amplify telomeres more efficiently than perfect ITSs. However, they are also expected to amplify imperfect ITSs with certain level of efficiency. Therefore, the capability of primers TelA and TelB to amplify telomeres more efficiently than ITSs should be empirically tested (see below).

Visualization of the telomeric amplification products generated by primers TelA and TelB. Before performing the MMQPCR reactions for telomere length measurements, we wanted to visualize the amplification products generated by primers TelA and TelB. We performed standard PCR reactions using Arabidopsis wild type bulk genomic DNA samples (isolated from multiple plants). As a control, we also performed PCR reactions using samples without DNA. Both kinds of samples were subjected to different number of consecutive PCR cycles and the reaction products were resolved by agarose gel electrophoresis and stained with ethidium bromide (Fig. 2). When DNA was present in the PCR reactions, primers TelA and TelB generated a smear starting with a band of low molecular weight. The size of this band match the sum of the length corresponding to the two telomeric primers, as previously reported for humans[18]. In addition, the intensity of the band and the smear increased exponentially with the number of PCR cycles

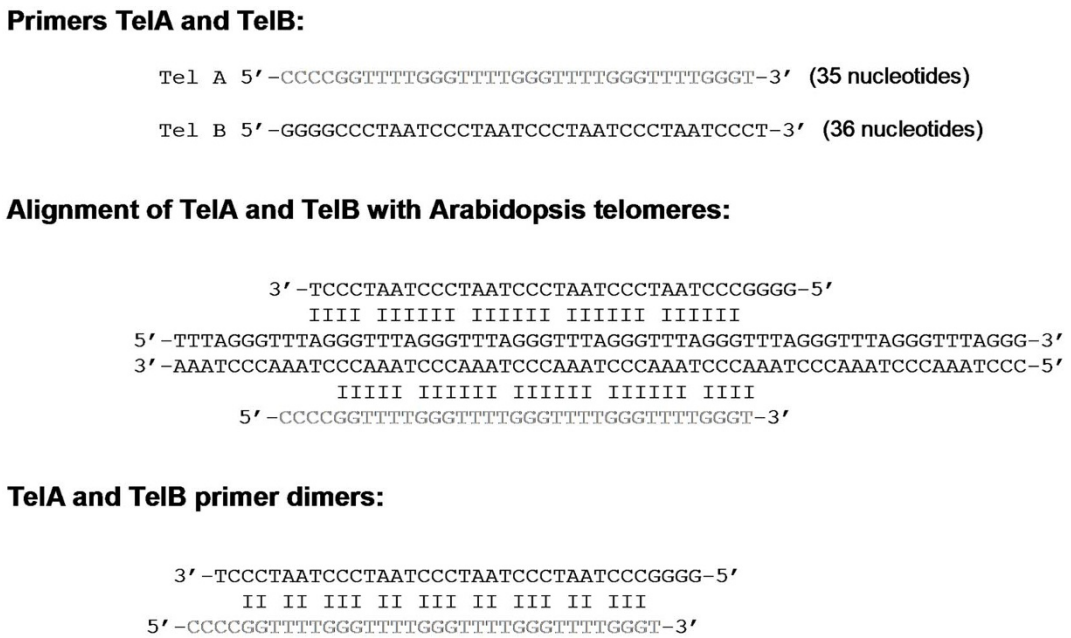

Figure 1 | Primers designed to amplify Arabidopsis telomeres. The sequences of primers TelA and TelB are shown as well as their alignment with telomeres and between themselves.

revealing that they were telomeric amplification products. In contrast, primers TelA and TelB did not generate products of amplification in the absence of DNA indicating that they did not amplify themselves.

MMQPCR analysis of telomeric sequences. We followed the method previously described by Cawthon to perform the MMQPCR experiments[19]. This method allows detecting telomeres and a single copy DNA sequence simultaneously in the same PCR reaction using SYBR Green. Since telomeric sequences are very abundant, they are amplified at earlier cycles than the single copy sequence in the MMQPCR reactions. In turn, the telomeric amplification products dissociate at lower temperatures than the single copy product. Single copy products are very stable because the primers used to amplify them in the MMQPCR reactions contain GC-rich sequences at their 5′ ends[19]. Therefore, the MMQPCR procedure allows detecting telomeres and a single copy sequence at different temperatures. Based on the dissociation curves obtained after amplifying Arabidopsis wild type bulk genomic DNA with TelA and TelB or with primers designed to amplify a unique sequence from the *CYP5* gene, we selected 74°C to determine the Ct (threshold cycle) for the telomeric sequences and 85°C for *CYP5* (Supplementary Fig. 1).

We performed monoplex amplification reactions of Arabidopsis wild type bulk genomic DNA with the telomeric and with the *CYP5* primers. In addition, we also performed multiplex amplification reactions (MMQPCR) with both primer pairs. The resulting amplification curves are shown in Fig. 3. At 74°C, the *CYP5* amplification signal was still at baseline when the Ct values for the telomeric amplification products were recorded. In turn, the telomeric products were dissociated at 85°C when the Ct values for the *CYP5* sequence were obtained.

Arabidopsis telomeres are detected with TelA and TelB by MMQPCR. To determine whether telomeres were amplified more efficiently than ITSs by the MMQPCR procedure, we first analyzed Arabidopsis wild type bulk genomic DNA samples digested with Bal 31. Bal 31 is an exonuclease that digests telomeres before digesting significant amounts of internal DNA sequences[4]. After performing a time course digestion of bulk genomic DNA with Bal 31 and analyzing the resulting DNA samples by MMQPCR, we determined the relative ratios of telomeric versus *CYP5* amplification products (T/S) following the standard curve method (Supplementary Fig. 2). We found that the T/S ratio decreased as telomeres were digested by Bal 31, which could be verified by TRF analysis (Fig. 4). Hence, the MMQPCR procedure amplifies wild type Arabidopsis telomeres more efficiently than ITSs. However, we cannot rule out that the MMQPCR procedure amplifies ITSs more efficiently than extremely short telomeres.

To further analyze whether the length of Arabidopsis telomeres can be measured by MMQPCR, we decided to study two Arabidopsis mutants altered in proteins that control telomere length. These proteins are KU70, which participates in the double strand DNA repair response, and DDM1, a chromatin remodeling protein involved in the establishment of DNA methylation[25–32]. The Columbia ecotype of *Arabidopsis thaliana* (Col-0) has been found to have an average telomere length of about 3.75 Kbp (between 2 and 5 Kbp) as estimated by TRF analyses[4,25]. We have previously published that a *ku70* mutant isolated from Col-0 (hereinafter reported as *ku70*) has an average TRF telomere length of about 12 Kbp[10], which is consistent with data formerly reported[25–27]. Here, we have found that the TRF telomere length of a mutant altered in DDM1 (hereinafter reported as *ddm1*) is about 0.6 times lower than the length of its wild type Col-0 parental strain (Supplementary Fig. 3). This result is in agreement

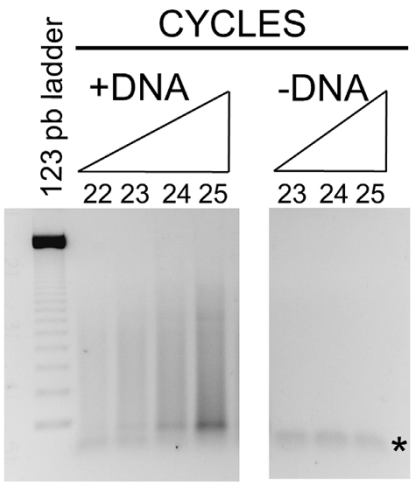

Figure 2 | Visualization of telomeric amplification products. PCR reactions were performed using primers TelA and TelB under standard conditions as previously reported[34]. Arabidopsis wild type bulk genomic DNA samples and control samples without DNA were subjected to different number of consecutive PCR cycles. The final concentration of DNA in the PCR reactions was 2 pg/μL. The reaction products were resolved on a 1.5% agarose gel and stained with ethidium bromide. The left panel displays a 123 bp ladder and the amplification products of PCR reactions that included DNA. The right panel displays the amplification products of PCR reactions that did not include DNA. The number of PCR cycles performed is indicated above the panels and the asterisk marks putative primer dimers.

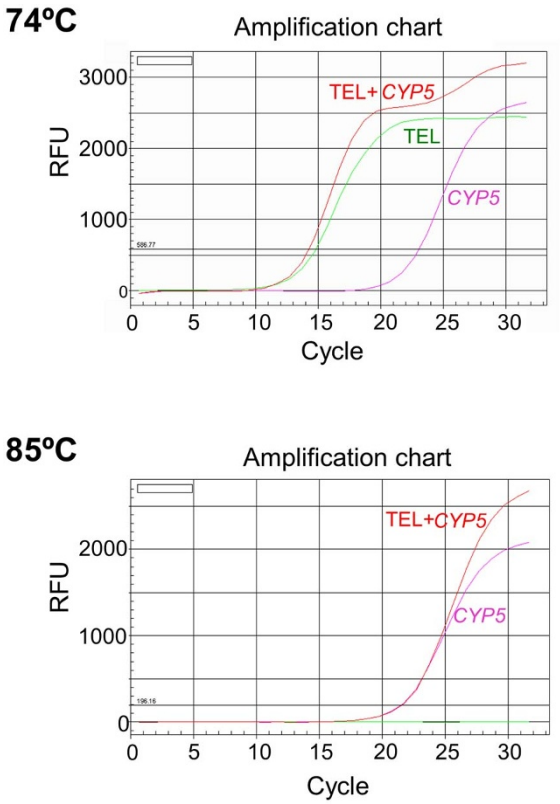

Figure 3 | MMQPCR amplification of Arabidopsis telomeric sequences and a single copy sequence from the *CYP5* gene. Monoplex amplifications of telomeric sequences (green) and of *CYP5* (pink) as well as multiplex amplification of both (red) were performed using Arabidopsis bulk genomic DNA as template. The amplification curves obtained at 74°C and at 85°C are shown. This figure has been performed as previously reported for humans[19].

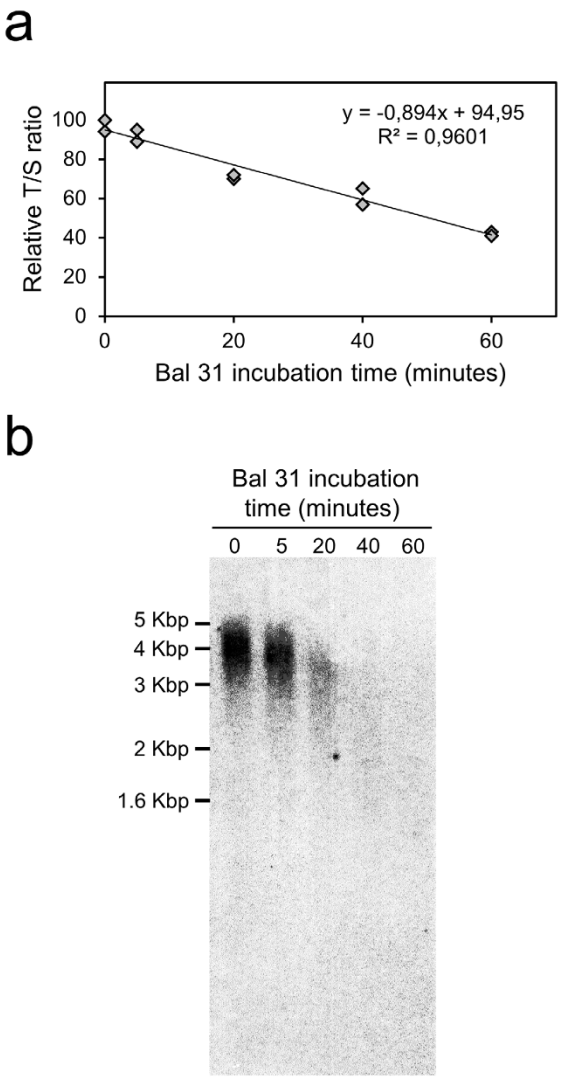

Figure 4 | MMQPCR and TRF analyses of genomic DNA samples digested with Bal 31. (a) Evolution of the relative T/S ratio during a Bal 31 time course digestion. Arabidopsis wild type bulk genomic DNA samples were digested with Bal 31 during different periods of time and analyzed by MMQPCR. The ratios of telomeric versus *CYP5* amplification products (T/S) were determined following the standard curve method. Two different MMQPCR reactions, rendering two different T/S ratios, were performed for each incubation time with Bal 31. A graphic representation of relative T/S ratio versus Bal 31 incubation time is shown together with a regression line. Relative T/S ratios represent the percentage of T/S ratio remaining after each Bal 31 incubation time. (b) TRF analyses of the Bal 31 digested DNA samples. The same samples used to perform the MMQPCR amplifications were analyzed by Southern blot hybridization with a telomeric probe after digestion with the restriction enzyme Tru9I. The migration distances of molecular size markers are indicated in the left. The contrast of the image has been adjusted to better detect the limits of the hybridization signals corresponding to 40 and 60 minutes as they are very faint due to their high level of spreading. Therefore, the intensities of the hybridization signals do not reflect DNA amounts. Note that the lower limit of the hybridization signal at 40 minutes is around 1.6 Kbp whereas the lower limit at 60 minutes is ill-defined.

with recently published data showing that a different mutant lacking of DDM1 also has short telomeres[28].

Since we had estimated by TRF the telomere lengths of the *ku70* and *ddm1* mutants, we used these strains to study whether primers TelA and TelB can be used to measure telomere length by MMQPCR.

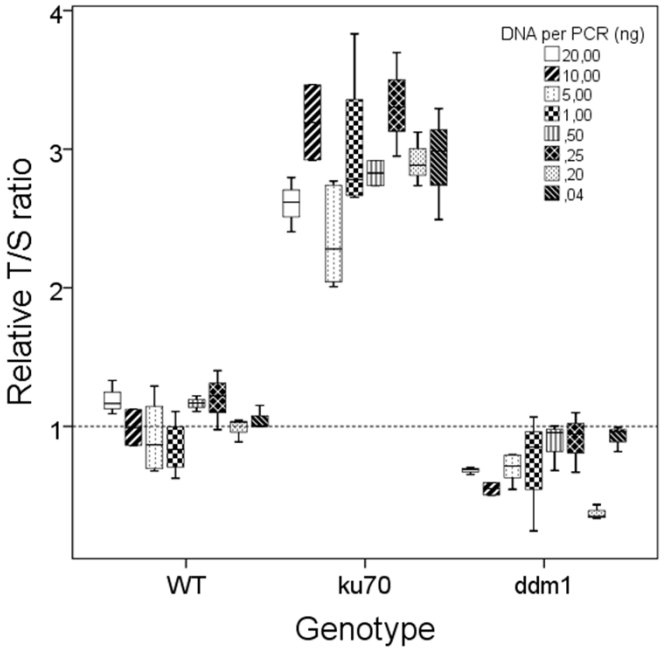

Figure 5 | Relative T/S ratios determined for the Arabidopsis wild type strain and for its *ku70* and *ddm1* mutant derivatives. A box plot representation is shown. For each strain, MMQPCR reactions were performed using eight different amounts of bulk genomic DNA per PCR reaction, ranging from 20 ng to 0.04 ng. Six different technical repetitions were performed for each DNA amount. Therefore, 48 different T/S ratios were determined for every strain (corresponding to 8 DNA amounts and 6 technical repetitions). All the T/S ratios were normalized by dividing them between by the average T/S value obtained for all the wild type DNA amounts and technical repetitions. The resulting T/S values were used to perform the box plot representation. For statistical analyses, all the T/S values corresponding to each strain were pooled together. Then, the significance of differences between the wild type strain and each of the mutants were determined using the Student's two-tailed t-test ($p < 0.001$ for both comparisons).

We performed MMQPCR analyses using bulk genomic DNA isolated from the wild type and from the mutant strains and determined relative ratios of telomeric versus *CYP5* amplification products (T/S) following the standard curve method. Different amounts of DNA per PCR reaction, ranging from 20 ng to 40 pg, were used to perform these analyses (Fig. 5, Supplementary Fig. 4). The T/S ratios determined for each strain using different amounts of DNA were, in general, quite consistent, being 1, 2.8 and 0.7 the average T/S ratios estimated for the wild type, *ku70* and *ddm1*, respectively (pvalues $<$ 0.001). These results are in close agreement with our TRF telomere length determinations, which were 1, 3.2 (12/3.75) and 0.6 for the wild type, *ku70* and *ddm1*, respectively. Therefore, primers TelA and TelB can be used to measure telomere length in *Arabidopsis thaliana* using low amounts of DNA. The relative T/S ratios determined by MMQPCR represent relative telomere length measurements.

To estimate the correlation existing between the relative telomere length measurements determined by TRF and by MMQPCR, we analyzed several Arabidopsis wild type and *ddm1* individual plants following both procedures (Fig. 6a). The coefficient of determination estimated after comparing both kinds of measurements was 0.87 (Fig. 6b). This result further supports the validity of primers TelA and TelB to measure the average telomere length of a population of cells by MMQPCR. However, unlike the TRF technique, the MMQPCR procedure does not allow determining the telomere size distribution within the cell population, thus limiting the identification of critically short telomeres. Nevertheless, short telomeres can be better assessed by Q-FISH in individual cells.

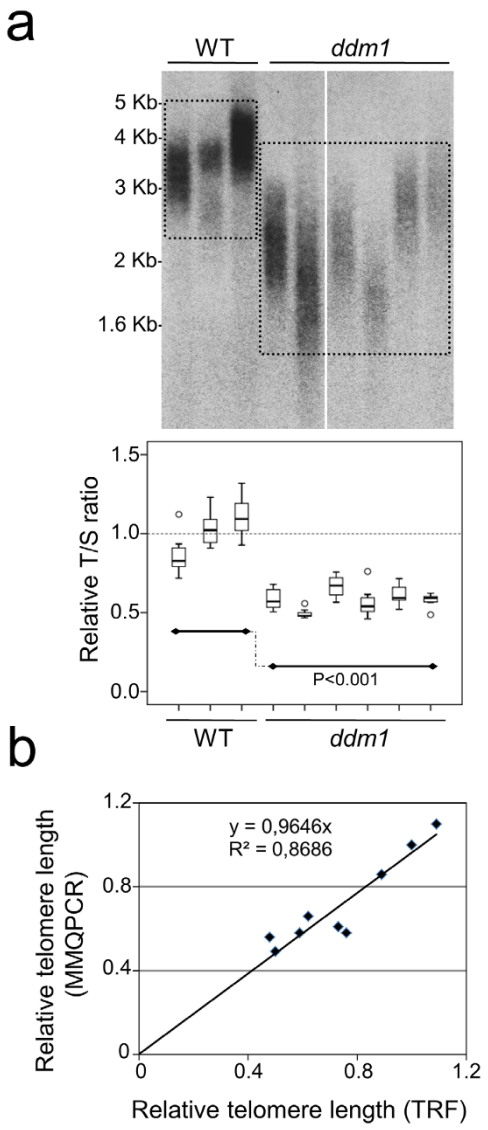

Figure 6 | Individual *ddm1* plants have shorter telomeres than individual wild type plants. (a) TRF and MMQPCR analyses of relative telomere length in Arabidopsis wild type and *ddm1* individual plants. The upper panel shows the Southern blot of genomic DNA samples digested with the restriction enzyme Tru9I and hybridized with a telomeric probe. The migration distances of molecular size markers are indicated in the left. The lower panel shows the box plot representation of relative T/S ratios corresponding to the same individuals analyzed in the upper panel and in the same order. MMQPCR reactions were performed using about 2 ng of DNA per PCR reaction. Eight different technical repetitions were performed for each plant, rendering eight different T/S ratios. All the T/S ratios were normalized by dividing them between the average T/S value obtained for all the wild type plants. The resulting T/S values were used to perform the box plot representation. The statistical significances of differences between any of the wild type plants and any of the mutant plants were determined using the Student's two-tailed t-test ($p < 0.001$ for all the comparisons). (b) Correlation of relative telomere length values estimated by TRF and by MMQPCR. The values represented correspond to the same individuals analyzed in (a).

DNA methylation regulates telomere length in Arabidopsis. Since the DDM1 protein is involved in the establishment of DNA methylation, we decided to determine the length of telomeres in different DNA methyltransferase mutants by MMQPCR. The same group that has recently reported the presence of short telomeres in plants lacking of DDM1 has also described that a *met1-3* mutant has shorter telomeres than the wild type strain[28]. Even more, they have demonstrated that the wild type progeny of a *met1-3* heterozygous mutant can have short telomeres. We have essentially found the same results by analyzing the telomere length of a homozygous *met1-7* mutant and of wild type plants segregated from a heterozygous *met1-7* plant (Fig. 7a). Hence, our results corroborate the influence of the CG methyltransferase MET1 on telomere length homeostasis.

To gain insight into the relationship existing between DNA methylation and telomere length, we analyzed the length of telomeres in a mutant that was simultaneously altered in three non CG DNA methyltransferases: DRM1, DRM2 and CMT3. We found that individual *drm1-2, drm2-2 and cmt3-11* mutant plants have also shorter telomeres than wild type plants (Fig. 7b). Therefore, different types of DNA methyltransferases are required to maintain the homeostasis of telomere length in Arabidopsis.

Discussion

Analysis of plant telomere length by PCR. We have designed two telomeric degenerated primers that amplify Arabidopsis telomeres by MMQPCR. These primers allow the analysis of telomere length using low amounts of DNA, which could be useful to study small organs with uniform cell types. Taking into account that the Arabidopsis diploid genome contains about 2.5×10^8 bp, and that the average mass of a bp is 660 Da, we reach to the conclusion that the telomere length of approximately 100 cells can be determined by MMQPCR when 40 pg of genomic DNA are amplified. Therefore, the telomere length of homogeneous cell populations isolated by laser microdissection could be estimated. We believe that the MMQPCR procedure could help to understand the biological relevance of telomere length in wild type Arabidopsis plants.

The length of other plants telomeres could also be analyzed by PCR using primers TelA and TelB or similar to them. The design of these primers will require the previous analysis of the ITSs present in the plants as the nature and amount of ITSs condition the number of degenerated telomeric repeats that the telomeric primers require at their 3′ end. In addition, primers that amplify a single copy sequence from the genome of the plants of interest should also be designed. Finally, the technique should be validated and the MMQPCR results should be statistically tested.

Very little is known about the influence of telomere length on natural plant processes, although a correlation between the length of rye embryo telomeres and seed viability has been established[20,21]. The MMQPCR procedure could help to ascertain whether the length of telomeres influences crop and trees traits through the proliferative capability of their stem cells.

DNA methylation, heterochromatin and telomere length in Arabidopsis. We have previously reported that whereas Arabidopsis telomeres exhibit euchromatic features, subtelomeric regions and ITSs, as an average, are heterochromatic[24,33,34]. With regard to DNA methylation, we have reported that the methylation levels of Arabidopsis telomeres are lower than those found at ITSs[34]. However, other authors have proposed that Arabidopsis telomeres exhibit heterochromatic features and have levels of DNA methylation similar to ITSs[28,35]. These discrepancies should arise from the different technologies that we have used to analyze telomeric chromatin structure. We have discussed these technologies elsewhere[34,36–38]. Currently, we believe that DNA methylation is essentially absent from Arabidopsis telomeres (our unpublished results).

As mentioned above, subtelomeric regions in Arabidopsis are heterochromatic. These regions have levels of DNA methylation similar to those found in the heterochromatic *Ta3* retrotransposon[34]. We have previously reported that the *ddm1* mutant analyzed here has lower levels of subtelomeric CG methylation than the wild type strain[34]. Therefore, DDM1 is required for the methylation of subtelomeric regions. In addition, we have shown here that *ddm1* and also

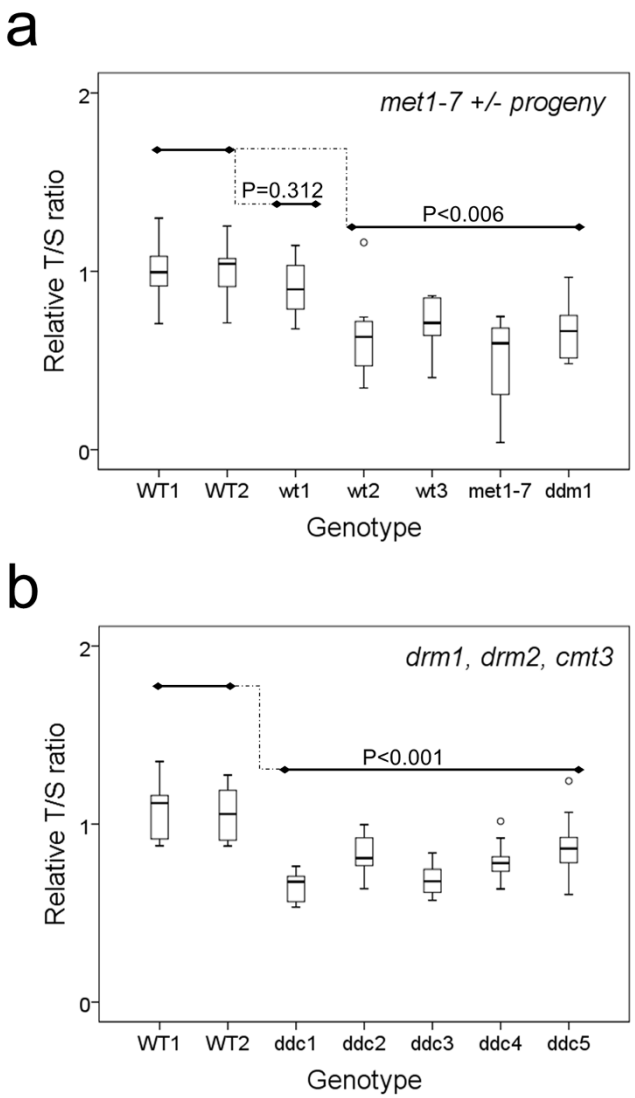

Figure 7 | DNA methylation mutants have shorter telomeres than the wild type strain. (a) Box plot representation of relative T/S ratios determined for wild type plants (WT1 and WT2) and for a *ddm1* mutant (ddm1) using bulk genomic DNA. The T/S ratios of individual plants segregated from a heterozygous *met1-7* mutant are also shown. The progeny of the *met1-7* heterozygous includes three wild type plants (wt1, wt2 and wt3) and one homozygous mutant plant (met1-7). MMQPCR reactions were performed using about 2 ng of DNA per reaction. At least five different technical repetitions were performed for each bulk or individual plant DNA sample. All the T/S ratios were normalized by dividing them between the average T/S value obtained for the WT1 and WT2 DNA samples. The resulting T/S values were used to perform the box plot representation. Statistical significances of differences between the T/S values obtained with the DNA samples of WT1 or WT2 and the other DNA samples were determined using the Student's two-tailed t-test. The highest p values obtained for each couple of comparisons (with WT1 or WT2) are indicated in the panel. (b) Box plot representation of relative T/S ratios determined for wild type plants (WT1 and WT2) using bulk genomic DNA and for individual *drm1-2, drm2-2* and *cmt3-11* triple mutant plants (ddc1 to ddc5). MMQPCR and statistics analyses were performed as indicated in (a).

a *met1-7* mutant, both have shorter telomeres than the wild type strain. These results are in agreement with recently reported data[28].

Since DDM1 and MET1 drive CG methylation in heterochromatin and also in euchromatic genes, it is difficult to ascribe the telomere length phenotypes of the *ddm1* and *met1* mutants to a specific chromatin status. To gain insight into this issue we analyzed the telomere length of a triple mutant altered in non CG methylation, which is almost exclusively found in heterochromatin[23]. Since we found that this triple mutant (*drm1-2, drm2-2 and cmt3-11*) has shorter telomeres than the wild type strain, we concluded that heterochromatic DNA methylation positively regulates telomere length in Arabidopsis.

Different Arabidopsis genome-wide DNA methylation studies have been previously reported (see for example references 39–42). More recently, 86 Arabidopsis silencing mutants have been analyzed by bisulfite genomic sequencing[43]. The genome-wide methylation status of all these mutants can be accessed at http://genomes.mcdb.ucla.edu/AthBSseq. We have used this Web resource to visualize the levels of subtelomeric DNA methylation in mutants related to the study presented here (*met1-3*, the wild type progeny of a *met1-3* heterozygous mutant and the triple *drm1-2*, *drm2-2* and *cmt3-11* mutant). These mutants have lower levels of subtelomeric DNA methylation than the wild type strain and exhibit DNA methylation patterns that are consistent with those observed at genome wide level (see for example the left subtelomeric regions of chromosomes I, III and V)[43]. Therefore, *met1-3*, the wild type progeny of a *met1-3* heterozygous mutant, *ddm1* and the triple *drm1-2*, *drm2-2* and *cmt3-11* mutant all have altered subtelomeric DNA methylation and short telomeres. Since we have not found heterochromatic features at Arabidopsis telomeres[24,33,34], we believe that heterochromatic DNA methylation at subtelomeric regions might regulate the homeostasis of telomere length in Arabidopsis. Alternatively, heterochromatin might regulate proteins that control telomere length homeostasis in Arabidopsis. However, no evidence of such a control has been reported to date.

Methods

Strains, culture conditions and DNA isolation. Wild type *Arabidopsis thaliana* Columbia ecotype (Col-0) and mutant derivatives were studied. The *ku70* (line SALK_123114) and the *ddm1* mutants have been previously used to analyze telomere length and subtelomeric chromatin structure, repectively[10,34]. The *ddm1* mutant was originally provided by Dr. Ingo Hofmann and contains a translational stop signal at position 2301. *met1-7* heterozygous seeds were obtained from the Nottingham Arabidopsis Stock Center (NASC code N16384) and segregated to obtain wild type and homozygous mutant siblings that were verified by PCR. The triple *drm1-2, drm2-2 and cmt3-11* mutant[40] was obtained from NASC (code N16384). Plants were grown on soil at 22°C and with a 70% of humidity during three weeks under long day conditions (16 hours with light).

Genomic DNA was purified from rosette leaves following a protocol based on the use of CTAB[10,44]. DNA samples isolated from multiple plants are referred as bulk genomic DNA in the text. They were isolated from at least ten plants.

TRF analyses. Southern blot analyses were performed using DNA isolated as previously indicated. DNA samples were digested with the restriction enzyme Tru9I, resolved on agarose gels, transferred to HybondTM-XL membranes (GE Healthcare) and hybridized with a telomeric probe, as previously reported[10]. Mean telomere lengths were measured as previously described[17].

Bal 31 time course digestion. Samples containing 100 ng of Arabidopsis wild type bulk genomic DNA were digested with 1 Unit of nuclease Bal 31 from New England BioLabs (catalogue #M0213S) at 30°C, in the presence of 1× Bal 31 reaction buffer and in a final volume of 20 µL during different periods of time. The reactions were stopped by adding 0.8 µL of EGTA 0.5 M pH 8.0 and incubating at 65°C during 5 minutes. Then, 280 µL of water were added to all the samples, which were phenol/chloroform extracted, precipitated with ethanol and resuspended in 30 µL of water. 2.5 µL were used for each MMQPCR reaction and 8 µL were used for TRF analyses.

MMQPCR amplification. The MMQPCR procedure was essentially performed as previously described[19]. Primers TelA and TelB were used to amplify telomeric sequences. In turn, a single copy sequence from the *CYP5* gene was amplified using previously reported primers[34] fused to the GC-rich stretches designed to stabilize human single copy sequences in MMQPCR experiments[19]. The sequences of these primers are

5′-CGGCGGCGGGCGGCGCGGGCTGGGCGGGGAGAAGACACAAATG-GTTCGC-3′ and 5′-GCCCGGCCCGCCGCGCCCGTCCCGCCGTCCATTCCTT-GCACCACTTTC-3′. Each MMQPCR reaction contained 0.75 µL H_2O; 2.5 µL 10× SYBR Green I (Life technologies); 2.5 µL ImmoBuffer 10×; 1.25 µL $MgCl_2$ 50 mM; 2 µL dNTPs mix 2.5 mM; 4 × 1.5 µL each primer 10 mM (TelA, TelB and the pair of primers used to amplify *CYP5*); 0.125 µL Immolase™ DNA polymerase (Bioline) and 10 µL of the DNA. 20 ng of DNA per PCR were used unless otherwise stated. Samples

were run at the Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. The PCR conditions were as follow: Stage 1) 15 minutes at 95°C; Stage 2) Two cycles of 15 seconds at 94°C and 15 seconds at 49°C; Stage 3) 32 cycles of 15 seconds at 94°C, 10 seconds at 62°C, 15 seconds at 74°C with signal acquisition, 10 seconds at 84°C and 15 seconds at 85°C with signal acquisition; Stage 4) 1 minute at 72°C.

The 74°C reads provided the Ct values for the amplification of telomeric sequences, while the 85°C reads provided the Ct values for the amplification of *CYP5*. The results were analyzed using the Bio-Rad iQ5 2.1 Standard Edition Optical System Software and the ratios of telomeric versus *CYP5* amplification products (T/S ratios) were determined using standard curves (Supplementary Fig. 2). Relative T/S ratios were calculated as indicated in the legend of the figures and used for graphic representations. Statistical significances of differences were calculated using the Student's two-tailed t-test.

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Acknowledgments

We thank Ingo Hoffman for his kind gift of *ddm1* seeds and the Pellegrini and Jacobsen labs for making available bisulfite genomic sequencing data at http://genomes.mcdb.ucla.edu/AthBSseq. We also acknowledge support from the Spanish Ministry of Economy and Competitiveness (Grant BIO2011-24794).

Author contributions

M.A.V. designed experiments and M.I.V. and M.A.V. performed experiments, accomplished analyses and wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Vaquero-Sedas, M.I. & Vega-Palas, M.A. Determination of *Arabidopsis thaliana* telomere length by PCR. *Sci. Rep.* **4**, 5540; DOI:10.1038/srep05540 (2014).

SCIENTIFIC REPORTS

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Corrigendum: Determination of *Arabidopsis thaliana* telomere length by PCR

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Scientific Reports **4**:5540; doi: 10.1038/srep05540; published online 02 July 2014; updated 22 November 2016

This Article contains a typographical error in the Methods section under the subheading 'MMQPCR amplification', where

"4 × 1.5 μL each primer 10 mM (TelA, TelB and the pair of primers used to amplify *CYP5*)"

should read:

"4 × 1.5 μL each primer 10 μM (TelA, TelB and the pair of primers used to amplify *CYP5*)".