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ORIGINAL PAPER

Age-associated alterations in the somatic mutation level in *Arabidopsis thaliana*

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Abstract Age-related accumulation of somatic mutations has been implicated as an important mechanism contributing to ageing and various diseases in animals. However, it is unclear whether the somatic mutation frequency changes as plants age and to what extent somatic mutagenesis contributes to plant ageing or senescence. The contribution of somatic mutagenesis to plant ageing and senescence was not investigated. We used the species *Arabidopsis thaliana* to study whether an increasing chronological age of an annual plant species influences the total amount and molecular spectrum of somatic DNA mutations. The number of small-scale somatic mutations was studied in nine randomly chosen *A. thaliana* control DNA regions, including those in the nuclear genome (*Actin2* coding region, *Actin2* 3′-UTR region, *CMT3* methyltrasferase gene, *tRNAPro,* and *ITS1-5.8rRNA-ITS2*), mitochondrial DNA (cytochrome oxidase C, a gene encoding an unnamed protein, and a noncoding mitochondrial DNA region), and chloroplast DNA (*rbcL* gene). We found that the frequency of single nucleotide substitutions, insertions, and deletions increased considerably during the *A. thaliana* growth and development from the seeds to the 12-week-old plants. The vast majority of nucleotide substitutions (~86 %) were transitions with A:T→G:C transitions being the most frequent type of substitution. The data indicate that at least some plant DNA regions accumulate point mutations during plant aging.

Keywords Plant ageing · Somatic mutations · Nucleotide substitutions · Insertions · Deletions · *Arabidopsis thaliana*

Introduction

The molecular mechanisms of ageing are actively being investigated in animals. Genomic instability, including DNA damage and mutation accumulation, is considered to be a major stochastic mechanism of animal ageing (Vijg 2004; Lombard et al. 2005). According to the somatic mutation theories, the first of which was proposed by Failla and Szilard (Failla 1958; Szilard 1959), the age-related accumulation of somatic mutations randomly inactivates genes important for the functioning of the somatic cells in a multicellular organism, which results in age-related degeneration and the eventual death of the organism. Somatic mutations are known to be caused by environmental factors such as exposure to ultraviolet radiation, oxygen-free radicals or to certain chemicals as well as by some endogenous factors that include mutagenic defects occurring during mitosis (Smith 1992).

For animals, the somatic mutation theory of ageing, supported by experimental evidence from multiple sources, states that the levels of somatic mutations in the animal mitochondrial and nuclear genomes increase with age (Akiyama et al. 1995; Jones et al. 1995; Tucker et al. 1999; Dolle et al. 2000; Vijg 2004; Rotskaya et al. 2010; Kennedy et al. 2012). For plants, there is a lack of experimental data on ageing and senescence mechanisms, and a lack of clarity still exists

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concerning the theoretical interpretation of the phenomena. That ageing leading to eventual death is considered to be thermodynamically unavoidable for all plants, whereas plant senescence has evolved as a developmental strategy, enabling the individual plant to control its own viability and integrity over the course its life cycle (Thomas 2013). Plant ageing and senescence differ from that in animals in some fundamental ways, and it has even been suggested that plants do not age in the strict gerontological sense (Thomas 2002; Munne-Bosch 2008).

The current theories of plant ageing are based on the accumulation of damage in plant tissues with age, plant growth, and size limitations or on a limited number of plant cell divisions as a consequence of telomere shortening (Thomas 2013; Brutovská et al. 2013). Oxidative damage to biomolecules caused by reactive oxygen species and DNA damage due to various causes are the two main types of damage considered in these theories. It has been proposed that unrepaired DNA damage and DNA polymerase errors may accumulate in plant cells and lead to an increased somatic mutation rate and alterations in transcription (Brutovská et al. 2013). The theory of somatic mutations appears especially appropriate for plant meristems, which undergo many divisions during the life of a plant, thus increasing the possibility of somatic mutations (Brutovská et al. 2013). Only a limited number of attempts have been made to analyze whether somatic mutation frequencies vary during the plant growth and ageing. A knowledge of the somatic mutation rate and spectrum is essential for unraveling the mechanisms of ageing and understanding the underlying evolutionary processes. Investigating somatic mutation levels during plant ageing might provide information that could be used for the efficient introduction of somatic mutations to a given gene, at a given time, and in a specific cell type, which would facilitate the study of plant gene functions and the generation of plants with desirable properties.

Using synthetic microsatellites introduced into *Arabidopsis thaliana* plants and histochemical staining, Golubov et al. (2010) found that microsatellite instability increased dramatically with the age of *A. thaliana* plants. The authors found that deletions, insertions, and nucleotide substitutions were gradually accumulating in the microsatellite repeats during the development of *A. thaliana* from 3-day-old seedlings to 22-day-old plants. Caetano-anollés (1999) measured the mutation rates of bermudagrass using a DNA amplification strategy that generates arbitrary signatures from amplification profiles during vegetative growth and across the population annually. The annual mutation rate across the population was 3.7–3.8 $\times$ 10^{-7} changes per nucleotide per year, which was similar to those found in *Drosophila* and mammals (reviewed in Drake et al. 1998; Kondrashov 1998). However, they were one or two orders of magnitude higher than the reported mutation rate for a bermudagrass generation, assuming that a bermudagrass spring constitutes a generation of growth (Caetano-anollés 1999). The age-related somatic mutation rates in *Arabidopsis* reported by Golubov et al. (2010) were markedly higher than the reported transgenerational mutation rates for an *Arabidopsis* or bermudagrass generation (Caetano-anollés 1999; Ossowski et al. 2010). Using DNA sequencing, Pla et al. (2000) demonstrated that there was a large accumulation of point mutations in the DNA and RNA of a heat shock protein in *Quercus suber* cork, a senescent plant tissue, in comparison to that in the normally growing young tissue, with the A:T→T:A transversion being the most frequently observed mutation (33.3 %). The data suggest that genetic ingegrity is not maintained in senescent plant tissue under oxidative stress. Performing a microsatellite analysis, Ally et al. (2010) found that somatic mutations considerably reduced relative male fertility in clonal aspen populations, resulting in an 8 % reduction of viable pollen grains. The authors suggest that even long-lived clonal plants are vulnerable to senescence. It has also been shown that single nucleotide substitutions accumulate in several endogenous genes and transgenes during the long-term cultivation of plant cell cultures (Noro et al. 2007; Kiselev et al. 2009, 2011, 2013). To our knowledge, there is no other experimental data where somatic mutagenesis has been investigated in relation to ageing in plants or plant cell cultures. Thus, there is currently scarce experimental data on the somatic mutation rate and spectrum occurring during the plant life span.

The purpose of this study was to elucidate whether somatic mutation level changes at nine randomly chosen coding and noncoding regions of nuclear, mitochondrial, and chloroplast DNA at different stages of growth and development during the *A. thaliana* life cycle. We analyzed the frequency of small-scale mutations (single nucleotide substitutions, deletions, and insertions) per 1,000 bp of the *Actin2* coding and 3′-untranslated (UTR) regions, and the *CMT* methyltrasferase*, tRNAPro,* and *ITS1–5.8rRNA–ITS2* regions (nuclear genome); cytochrome *c* oxidase, an unnamed protein, and a noncoding mitochondrial DNA region, and the ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) chloroplast gene. These sequences were obtained for DNA extracted from seeds and 1-, 4-, 8-, and 12-week-old *A. thaliana* plants. We found that both the frequency of single nucleotide substitutions and the frequency of insertions/deletions (indels) gradually increased in the nine DNA control regions during the development of *A. thaliana* from seed to 12-week-old plant.

Materials and methods

Plant material and growth conditions

Plants (*Arabidopsis thaliana* ecotype Columbia L., stored by our lab) were grown in pots filled with commercially available

rich soil in a controlled environmental chamber at +22 °C (KS-200 SPU, Smolensk, Russia) kept on a 16/8 h day/night cycle at a light intensity of ~70 μmol m^{-2} s^{-1}. To compare the frequency and types of somatic mutations at different stages of *A. thaliana* life cycle, three *A. thaliana* plants were collected every 1, 4, 8, and 12 weeks after seed sowing. We also collected 100 seeds of *A. thaliana* from the same parent plant to analyze the level of mutations present in the used seed stock.

DNA extraction and PCR analysis

For DNA purifications, we collected the above-ground vegetative tissues of *A. thaliana* 1 week post-sowing (seedlings with two cotyledons), 4 weeks post-sowing (full rosette with emerging flower shoots), 8 weeks post-sowing (full rosette with developed flower shoots and developing siliques), and 12 weeks post-sowing (seed maturation and plant senescence). We also purified DNA from 100 seeds of *A. thaliana* to estimate the frequency of mutations in the *A. thaliana* seed stock used in this study and compare it with the rate of somatic mutation in the *A. thaliana* plants which were germinated from the seeds and collected at further stages of *Arabidopsis* life cycle. The 100 seeds and the seeds used for germination were obtained from the same plant of *A. thaliana*.

The total DNA was extracted as described (Echt et al. 1992) with some modifications. Dried tissues of each plant were ground in a mortar for DNA isolation and thoroughly mixed. 25 mg of the obtained powder for the 4-, 8-, and 12-week-old plants and a whole seedling for the 1-week-old *A. thaliana* were mixed with 800 μl of the homogenization buffer containing 0.2 % mercaptoethanol, 100 mM Tris (pH 7.5–8.0), 0.7 M NaCl, 40 mM EDTA (pH 7.5–8.0), and 1 % cetyltrimethylammonium bromide (CTAB). The mixture was incubated at +60 °C for 1 h under stirring. Then, the samples were mixed with 300 μl of chlorophorm and centrifuged at 13–14,000 rpm for 5 min. 400 μl of the aqueous phase were precipitated with 2.5 V 96 % ethanol (at −20 °C for 20 min) and pelleted by spinning in a microcentrifuge at 13–14,000 rpm for 5 min. The DNA samples were dried at +37 °C and dissolved in 100–300 μl of distilled water.

PCR was used to amplify partial sequences of the nuclear *Actin2* gene (*Actin-C3* and *Actin-U1*); nuclear chromomethylase (*CMT*) methyltransferase gene sequence; nuclear *tRNAPro*; the internal transcribed spacer sequence *ITS1* and *ITS2* of ribosomal DNA with 5.8S rRNA sequence between them (region *ITS*); an unnamed mitochondrial protein (*Mitoh-2*); mitochondrial cytochrome *c* oxidase (*Mitoh-3*); a noncoding mitochondrial DNA region (*Mitoh-4*); and a chloroplast gene encoding ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*). The primer sequences are shown in the Table S1. The primers *Actin-U1* F and R were designed based on the *Actin2* gene of *A. thaliana* and were used for amplification of a 401-bp product of the *Actin2* gene (31 bp of the 3′ coding region and 370 bp of the 3′-UTR), annealing temperature (Ta) 53 °C, elongation time 23 s. The primers *Actin-C3* F and R were designed based on the *Actin2* gene of *A. thaliana* and were used for amplification of a 442-bp product of the *Actin2* gene (86 bp of the intron region and 356 bp of the exon region), Ta 51 °C, elongation time 30 s. The primers *CMT* F and R were designed based on the *A. thaliana CMT3* methyltransferase coding sequence and were used for amplification of a 317-bp product from the *CMT* gene, Ta 52 °C, elongation time 35 s. The primers *tRNA* F and R were designed based on the nuclear *tRNAPro* sequence and were used for amplification of a 576-bp product from the nuclear DNA encoding for *tRNAPro*, Ta 54 °C, elongation time 33 s. The primers *ITS* F and R were designed based on the *A. thaliana ITS1-5.8S rRNA-ITS2* sequence and were used for amplification of a 728-bp product from the ribosomal DNA region, Ta 53 °C, elongation time 42 s. The primers *Mitoh-2* F and R were designed based on the *A. thaliana* mitochondrial DNA encoding for an unnamed protein orf153a and were used for amplification of a 460-bp fragment of the gene, Ta 53 °C, elongation time 30 s. The primers *Mitoh-3* F and R were designed based on the *A. thaliana* mitochondrial cytochrome *c* oxidase coding sequence and were used for amplification of a 471-bp product from the gene of cytochrome *c* oxidase subunit 3, Ta 53 °C, elongation time 35 s. The primers *Mitoh-4* F and R were designed based on a noncoding mitochondrial DNA region of *A. thaliana* and were used for amplification of a 414-bp product from the noncoding sequence, Ta 53 °C, elongation time 30 s. The primers *rbcL* F and R were designed based on the *A. thaliana rbcL* large subunit nucleotide sequence and were used for amplification of a 599-bp product from the *rbcL* gene, Ta 52 °C, elongation time 35 s. The amplification reactions were performed as described (Kiselev et al. 2013). For the PCR reactions, we used a mix (1:6) of Pfu and Taq polymerases (Silex M, Russia). Previously, we demonstrated that this blend of polymerases had an error rate of 0.08–0.09 nt per 1,000 nt (Kiselev et al. 2009).

Cloning and DNA sequencing

The PCR products were isolated from agarose gels using a Cleanup Mini Kit (Eurogene, Moscow, Russia) and subcloned as described (Kiselev et al. 2012). The clones were amplified and sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following the manuphacturer's protocol and recommendations as described (Kiselev et al. 2013). The Basic Local Alignment Search Tool (BLAST) program was used for

sequence analysis. The multiple sequence alignments (e.g., Fig. S1) were performed using the ClustalX program (Altschul et al. 1990). To test whether the detected nucleotide variations are sequencing artifacts or not, we also sequenced the antiparallel strands, and the results repeated.

Screening of the *Actin-U1, Actin-C3*, *CMT*, *tRNA*, *ITS*, *Mitoh-2*, *Mitoh*-3, *Mitoh-4*, and *rbcL* clones

24 clones of the *Actin-U1, Actin-C3*, *CMT*, *tRNA*, *ITS*, *Mitoh-2*, *Mitoh*-3, *Mitoh-4,* and *rbcL* DNA regions were sequenced from the DNA of seeds and 1 to 12-week-old plants of *A. thaliana* using the M13 forward and reverse sequencing primers. The 24 clones for each DNA region were obtained from three biological replicates (8 clones per each plant). Two biological replicates were presented by whole individual plants for each time point, and the third biological replicate was presented by the leaves of the same plant for each time point. For the individual plants, we purified DNA from 25 mg of dried and mixed tissues (all types of tissues were mixed) or from one whole seedling for the 1-week time point. For the third biological replicate, we used one cotyledon (1 week) or leaves (4, 8, and 12 weeks). The mutation frequencies for the same plant used as the third biological replicate were approximately equal in comparison with that obtained for the individual plants. Therefore, we combined the data on the third plant with the data on the two other biological replicates. If a sequenced PCR product differed by one or two nucleotides from the previously described candidates for the *Actin-U1, Actin-C3*, *CMT*, *tRNA*, *ITS*, *Mitoh-2*, *Mitoh*-3, *Mitoh-4,* and *rbcL* DNA regions, we considered it as a novel nucleotide substitution. The nucleotide variations described as nucleotide substitutions or deletions/insertions in the sequenced gene parts were usually a single change per analyzed clone, or a maximum of two or three, which were not repeated in several independent clone collections. Since the polymerase (a mix 1:6 of Pfu and Taq polymerases) error rate is much lower (Kiselev et al. 2009) than the frequency of nucleotide substitutions and deletions/insertions detected in the present work, we did not distinguish possible polymerase errors from the detected somatic mutations.

The total level of small-scale mutations (single nucleotide substitutions, insertions and deletions) per 1,000 nt was determined using the following formula:

$$\frac{(Ns + Ni + Nd) \times 1{,}000}{(L - P) \times Nc}$$

Ns is the total number of nucleotide substitutions in all clones obtained from a certain time point; Ni is the total number of insertions in all clones obtained from a certain time point; Nd is the total number of deletions in all clones obtained from a certain time point; L is the length of the analyzed gene fragment; P is the length of the primers (in nucleotides) used for amplification; and Nc is the total number of clones for the sequenced target gene (Kiselev et al. 2009, 2011, 2013).

The frequency of nucleotide substitutions per 1,000 nt was determined using the following formula:

$$\frac{(Ns \times 1{,}000)}{(L - P) \times Nc}$$

The frequency of insertion/deletion per 1,000 nt was determined using the following formula:

$$\frac{(Ni + Nd) \times 1{,}000}{(L - P) \times Nc}$$

Besides the somatic mutations, the obtained mutation rate in our experiments included the errors of the used Pfu/Taq polymerase blend and the differences in the DNA sequences of the used plants as biological replicates. Although it is not possible to divide the true somatic mutation frequencies from these additional sequence variations, we can provide the information about the error rate of the used polymerase blend (0.08–0.09 nt per 1,000 nt; Kiselev et al. 2009) and about the existing differences in the DNA regions of the used seed stock and the plants of *A. thaliana*. For this purpose, we also included the seeds of *Arabidopsis* in our analysis.

Statistical analysis

The statistical analysis was carried out using the Statistica 10.0 program. The data are presented as mean ± standard error of the mean (SE) and were tested for statistical significance by paired Student's *t* test. $p < 0.05$ was selected as the point of minimal statistical significance in all analyses.

Results and discussion

Age-dependent sequence variation and the frequency of somatic mutations in *A. thaliana*

To estimate the rate at which somatic mutations are accumulated during the *Arabidopsis* life cycle, we analyzed the somatic mutation frequencies and mutation types at nine DNA regions in *A. thaliana* plant tissues of different ages, collected 1, 4, 8, and 12 weeks after seed sowing, and compared them with the mutation frequencies in the *A. thaliana* seeds used. For that comparison, we collected 100 *A. thaliana* seeds from one plant to estimate the level of mutations in the seed stock used to germinate the *Arabidopsis* plants in this study.

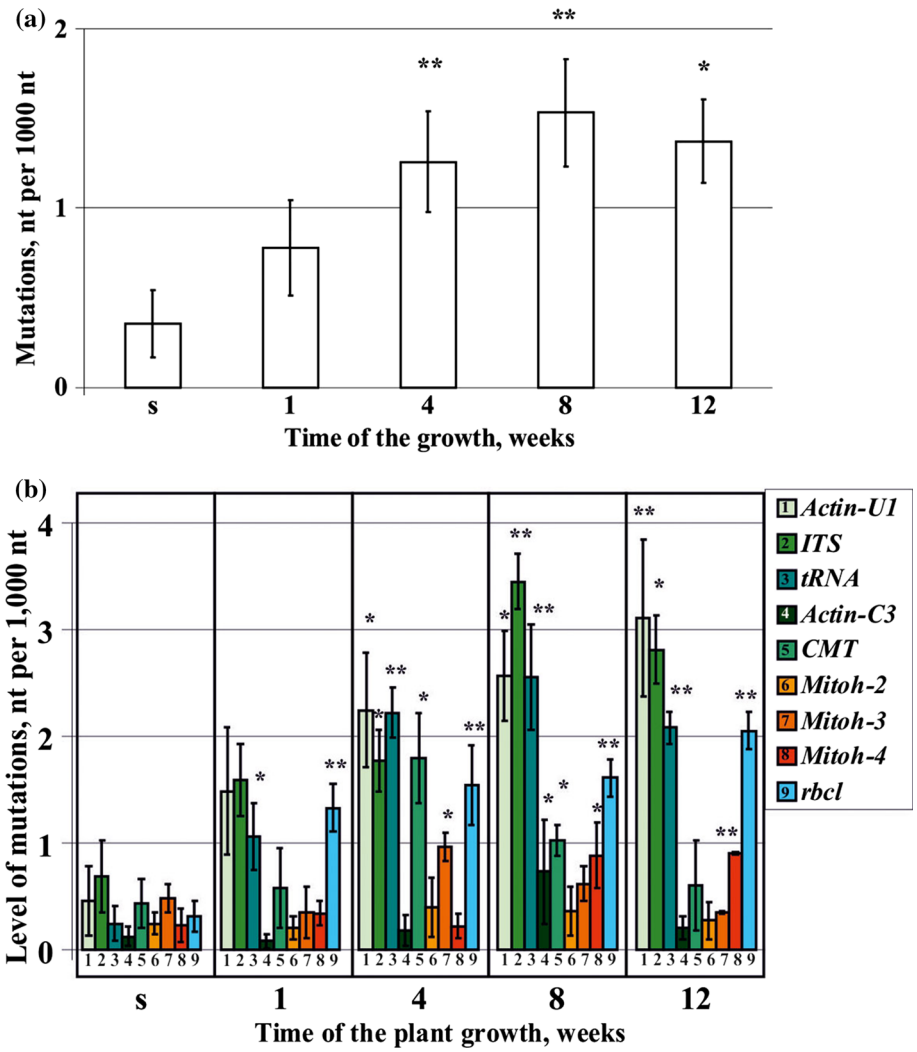

Fig. 1 The frequency of small-scale somatic mutations (nucleotide substitutions, deletions, and insertions) in the partial *Actin-U1, Actin-C3*, *rbcL*, *ITS*, *tRNA*, *CMT*, *Mitoh-2*, *Mitoh-3*, *Mitoh-4*, and *rbcL* DNA regions of *A. thaliana*. The data are presented as the average level of mutations (**a**) and the level of mutations for each analyzed DNA region (**b**). Data are presented as mean ± SE for the seeds (s) or plants collected 1, 4, 8, and 12 weeks after seed sowing. $*p < 0.05$, $**p < 0.01$ versus values of mutation frequency in the DNA of 100 seeds of *A. thaliana* (s)

To compare the amount of sequence variation during the growth and ageing of the *A. thaliana* plants, we sequenced nine randomly chosen DNA regions from the nuclear, mitochondrial, and chloroplast genomes: a portion of the *Actin2* 3′-UTR (*Actin-U1*), a portion of the *Actin2* 3′coding region (*Actin-C3*), the *CMT3* methyltransferase coding region (*CMT*), the *ITS1–5.8 rRNA–ITS2* itergenic region (*ITS*), the *tRNAPro* coding DNA (*tRNA*), an unnamed protein (*Mitoh-2*), cytochrome *c* oxidase (*Mitoh-3*), a noncoding mitochondrial DNA region (*Mitoh-4*), and the ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL)* gene. The gene regions and intergenic sequences are found in the nuclear (*Actin-C3*, *Actin-U1*, *CMT*, *tRNA, ITS*), mitochondrial (*Mitoh-2*, *Mitoh-3*, *Mitoh-4*), and chloroplast (*rbcL*) genomes of *A. thaliana* (Table S1). We detected single nucleotide substitutions and small insertions and deletions (indels) in the DNA regions analyzed. These could not be attributed to mistakes in the DNA sequencing (Fig. S2), because we also sequenced the antiparallel strands to test whether these variations were sequencing artifacts, and the results repeated.

We analyzed the frequency of the small-scale mutations (single nucleotide substitutions, deletions, and insertions) per 1,000 nucleotides (nt) of the DNA sequences obtained from the cloned *A. thaliana* DNA regions (Figs. 1, 2). The analysis revealed that the somatic mutation frequency gradually increased with age from 0.8 mutations per 1,000 nt in the 1-week-old seedlings to 1.4 mutations per 1,000 nt in the 12-week-old plants (Fig. 1a, b). The level of sequence variation present in the 1 to 12-week-old plants was higher than that observed in the seed stock used (Fig. 1a). Notably, we found no indels in the DNA purified from the seeds, and the level of the nucleotide substitutions was 0.35 ± 0.18 nt per 1,000 nt analyzed (Figs. 1, 2). We attribute this mutation frequency in the seeds to differences existing between the analyzed DNA sequences of the individual seeds and to errors from the polymerase blend used.

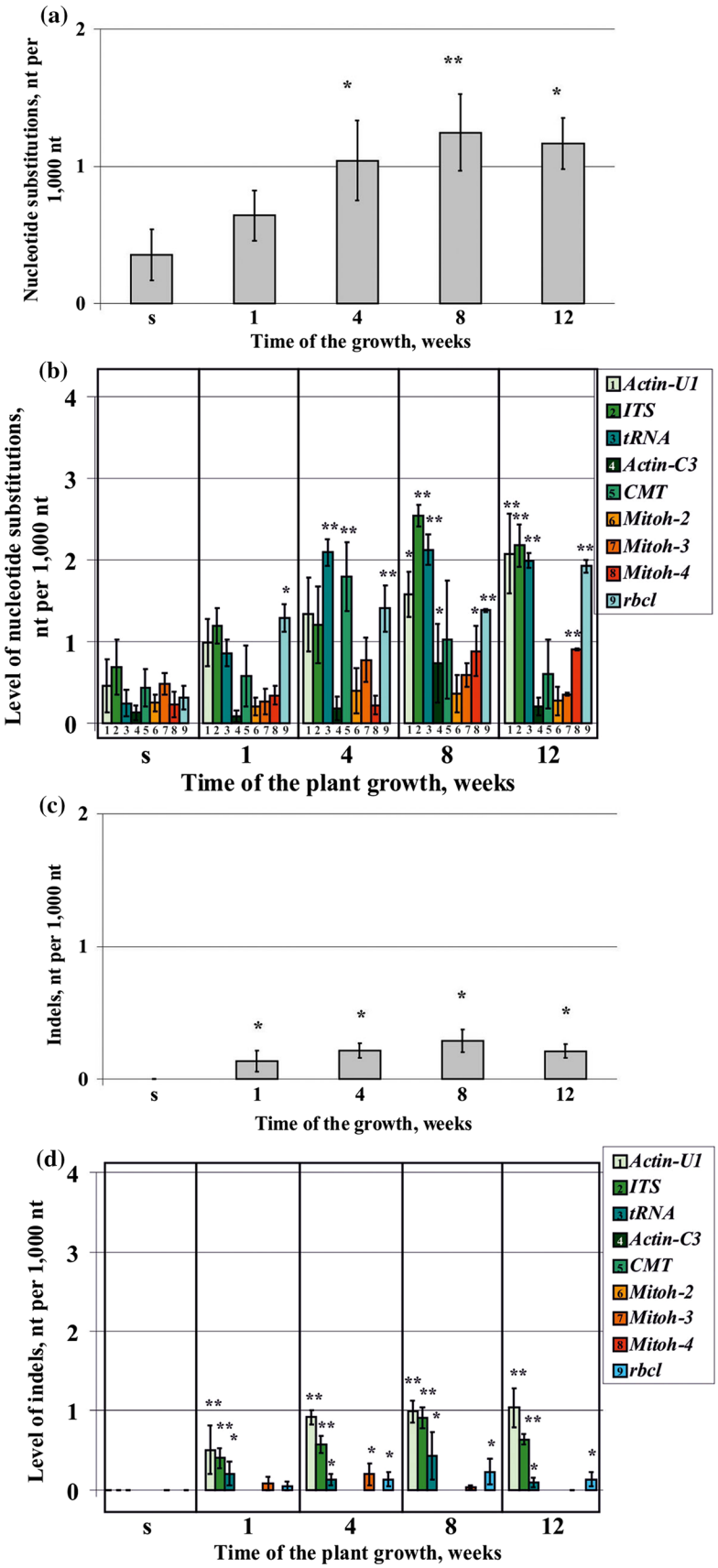

Fig. 2 The frequency of nucleotide substitutions (**a**, **b**) or indels (**c**, **d**) in the *Actin-U1*, *Actin-C3*, *rbcL*, *ITS*, *tRNA*, *CMT*, *Mitoh-2*, *Mitoh-3*, *Mitoh-4*, and *rbcL* DNA regions of *A. thaliana*. The data are presented as average level of mutations (**a**, **c**) and the level of mutations for each analyzed DNA region (**b**, **d**). Data are as mean ± SE for the seeds (s) or plants collected 1, 4, 8, and 12 weeks after seed sowing. $*p < 0.05$, $**p < 0.01$ versus values of mutation frequency in the DNA of 100 seeds of *A. thaliana* (s)

It should be noted that the obtained mutation frequencies obtained for the *Arabidopsis* plants were artificially increased due to Taq/Pfu polymerase blend errors (0.08–0.09 nt per 1,000 nt; Kiselev et al. 2009) in combination with the differences in the plant DNA sequences of the biological replicates. Thus, a part of the mutation rate is attributable to these variations. However, since the somatic mutation rates detected are considerably higher than the level of the Taq/Pfu polymerase error rate and the detected sequence variation in the seed stock, it can be concluded that the majority of the observed mutation frequencies should be attributed to true somatic mutations accumulating in the *Arabidopsis* plants over time.

Both the frequency of single nucleotide substitutions (Fig. 2a, b) and the frequency of indels (Fig. 2c, d) markedly increased during the growth and development of *A. thaliana* from seeds to 12-week-old plants. The frequency of single nucleotide substitutions in the 12-week-old plants was threefold higher than that in the seeds used of *A. thaliana* and twofold higher than that in the 1-week-old seedlings (Fig. 2a), and the frequency of indels was 1.5–2 times higher in the 8- and 12-week-old plants than that in the 1-week-old seedlings (Fig. 2c). We did not detect any indels in the DNA purified from the *A. thaliana* seeds (Fig. 2c). Most of the detected indels were found in the noncoding *Actin-U1* and *ITS* DNA regions (Figs. 2d, S3). The highest mutation rates found were for the noncoding *ITS* and *Actin-U1* regions, while the lowest were for the *Actin-C3* and mitochondrial *Mitoh-2* and *Mitoh-3* coding regions (Fig. 1b).

This study shows that the number of somatic mutations, including single nucleotide substitutions and small deletions and insertions, gradually increased throughout the growth and ageing of *A. thaliana* in six of the DNA regions analyzed (*Actin-U1, CMT, ITS, tRNAPro, Mitoh-4,* and *rbcL*), while it increased only at one stage of development in the other three DNA regions (*Actin-C3, Mitoh-2*, and *Mitoh-3*). The number of somatic mutations in the 1- to 12-week-old *A. thaliana* plants was considerably higher than that observed in the seed stock used (Figs. 1, 2). We believe this gradual increase in the mutation frequencies is an age-related accumulation of somatic mutations in the DNA regions analyzed, because it could be attributed neither to amplification errors nor to the sequence variation between the plants used as biological replicates; the substitutions and indels could also not be attributed to errors in the DNA sequencing (Fig. S2). The data also suggest that the somatic mutations accumulate at a higher rate in the noncoding DNA than in the protein-coding DNA regions during the plant life span. The accumulation of somatic mutations in the noncoding DNA could have considerable consequences for cellular function, since various noncoding DNA regions are known to effect the regulation of gene activity, chromatin organization, recombination, or DNA replication.

We noted that the mutation level in the 12-week-old plants was slightly lower than that in the 8-week-old ones (Figs. 1, 2), possibly due to the presence of seeds in the dried plant tissue prepared for the DNA purification. However, it is important to note that the differences in the mutation frequencies of the 8- and 12-week-old plants were not statistically significant (Fig. 1a, b).

For animals, it is well established that the somatic mutation levels in both the coding and noncoding regions of the nuclear and mitochondrial genomes gradually increase with ageing (Vijg 2000; Kennedy et al. 2012; Vijg and Suh 2013; Moskalev et al. 2013). To our knowledge, there has been only one investigation of the somatic mutation rate during the plant life cycle. Golubov et al. (2010) showed that older *Arabidopsis* plants have a higher level of microsatellite instability relative to that in younger plants. Deletions, insertions, and nucleotide substitutions have been actively accumulating in *Arabidopsis* microsatellites over time. To obtain the mutation rates, Golubov et al. (2010) compared the number of events per single cell genome and found that the rate increased 64.2-fold, from 1.77×10^{-7} to 1.14×10^{-5} in 3- and 22-day-old *Arabidopsis* plants, respectively. To our knowledge, our study is the first report employing DNA sequencing to investigate whether somatic mutations accumulate in plant DNA regions other than microsatellites.

Types of somatic mutations

The vast majority of nucleotide substitutions were transitions (~86 %), with A→G and T→C transitions being the most frequent (Table 1). In addition, we detected a number of transversions in most DNA regions analyzed (Table 1). The rate of transversions was much higher in the *ITS* intergenic region in comparison to those in the other DNA sequences analyzed, with A→C transversions being the most frequent nucleotide substitution type (~23 %) in the *ITS* DNA. We also noted that approximately 50 % of the nucleotide substitutions in the protein coding DNA regions (*Actin-C3*, *CMT*, *Mitoh-2*, *Mitoh-3*, and *rbcL*,) were silent mutations, not altering the amino acid sequence of the protein products.

The distribution of the detected single nucleotide substitutions appeared random (e.g., Fig. S1). In contrast, the indels detected were not randomly distributed in the DNA regions analyzed (Fig. S3). For example, the insertions and deletions were found in the poly-A region in the *Actin-U1* region (Fig. S3), while the deletions were found in the poly-T regions in the *tRNA* and *Mitoh-3* sequences. For the *ITS* region, the insertions detected were in the 492–503-bp region, which was reach in C. At the same time, there were

Table 1 Percent ratio and number (in parentheses) of single nucleotide substitutions in the *Actin-U1*, *Actin-C3*, *CMT*, *tRNA*, *ITS*, *Mitoh-2*, *Mitoh-3*, *Mitoh-4*, and *rbcL* sequences of *A. thaliana*

Type of substitution	Substitution	Total	*Actin-U1*	*Actin-C3*	*CMT*	*tRNA*	*ITS*	*Mitoh-2*	*Mitoh-3*	*Mitoh-4*	*rbcL*
Transitions	A→G	30.7 (226)	35.9 (22)	36.7 (8)	14.3 (4)	40.1 (56)	20.4 (58)	41.6 (8)	22.2 (4)	22.6 (6)	42.5 (60)
	G→A	7.8 (74)	2.2 (1)	8.4 (2)	15.5 (5)	5.9 (8)	14.8 (45)	12.5 (3)	5.5 (2)	0 (0)	5.7 (8)
	T→C	37.5 (204)	47.3 (38)	36.7 (8)	38.1 (10)	27.6 (40)	11.8 (34)	37.5 (7)	66.7 (16)	45.2 (13)	26.6 (38)
	C→T	10.1 (92)	4.2 (2)	8.4 (2)	15.5 (5)	12.9 (18)	12.6 (40)	8.4 (2)	0 (0)	15.5 (3)	13.8 (20)
Transversions	A→T	2.6 (24)	0 (0)	0 (0)	0 (0)	0 (0)	5.4 (14)	0 (0)	5.5 (2)	8.4 (2)	4.2 (6)
	A→C	4.7 (70)	0 (0)	0 (0)	16.7 (6)	2.4 (4)	23.1 (64)	0 (0)	0 (0)	0 (0)	0 (0)
	T→A	2.5 (26)	10.5 (6)	0 (0)	0 (0)	5.3 (8)	2.1 (6)	0 (0)	0 (0)	0 (0)	4.4 (6)
	T→G	2.6 (14)	0 (0)	10 (3)	0 (0)	1.8 (2)	3.2 (7)	0 (0)	0 (0)	8.4 (2)	0 (0)
	G→T	0.7 (12)	0 (0)	0 (0)	0 (0)	1.2 (2)	2.1 (6)	0 (0)	0 (0)	0 (0)	2.7 (4)
	G→C	0.3 (6)	0 (0)	0 (0)	0 (0)	1.2 (2)	1.7 (4)	0 (0)	0 (0)	0 (0)	0 (0)
	C→A	0.2 (4)	0 (0)	0 (0)	0 (0)	0 (0)	1.7 (4)	0 (0)	0 (0)	0 (0)	0 (0)
	C→G	0.3 (5)	0 (0)	0 (0)	0 (0)	1.8 (3)	1.1 (2)	0 (0)	0 (0)	0 (0)	0 (0)

single indels that were not repeatedly detected, e.g., for the *rbcL* region. Fig. S3 presents all of the indels detected in the *A. thaliana* DNA sequences analyzed. The *Actin-U1* region contained both deletions and insertions, while the *ITS*, *tRNA*, and *Mitoh-3* regions contained either insertions or deletions.

Ossowski et al. (2010) investigated the rate and molecular spectrum of spontaneous mutations in the complete nuclear genomes of five *A. thaliana* individuals derived by 30 generations of single-seed descent from the reference strain. The authors reported a spontaneous mutation rate of 7×10^{-9} base substitutions per site per generation, and the majority of nucleotide substitutions were G:C→A:T transitions. The authors proposed that the deamination of methylated cytosines and ultraviolet light-induced mutagenesis resulted in the spontaneous germ-line mutagenesis. In our analysis, we found that most of the detected single nucleotide substitutions that accumulated during *A. thaliana* growth and aging were A:T→G:C transitions, which suggests that the transitions did not result from an ultraviolet light-induced mutagenesis. The data correlate with observations that most of the single nucleotide substitutions that accumulated during the long-term cultivation of *Panax ginseng* and *Oryza sativa* callus cultures were also A:T→G:C transitions (Noro et al. 2007; Kiselev et al. 2009, 2011, 2013). However, in contrast to the somatic mutagenesis observed for the *A. thaliana* plants in this study, no insertions or deletions were detected in the endogenous gene sequences analyzed for *P. ginseng* in cell cultures that had been propagated for more than 20 years (Kiselev et al. 2009, 2011, 2013).

It is known that the DNA of plants and other eukaryotes can unexpectedly mutate due to spontaneous DNA polymerase errors, recombination errors, DNA repair errors, a lack of DNA protective proteins, or oxidative metabolism (Smith 1992). An efficient system of DNA repair has evolved in plants to maintain mutation levels at an optimal level to allow them to avoid deleterious effects on fitness in diverse environments (Britt 1996; Tuteja et al. 2001). However, even the most precise genome maintenance system can undergo occasional failure and result in a variety of mutations. In addition, plants are sessile organisms and cannot avoid the influence of unfavorable environments, and various DNA mutations are well-known to be induced at a marked rate by naturally occurring environmental mutagens such as UV light, ionizing radiation, and oxygen-free radicals as well as by various anthropogenic factors (Smith 1992; Müller et al. 2000; Lombard et al. 2005; Tuteja et al. 2001).

In summary, we found that somatic mutations accumulated during the *Arabidopsis* life-cycle in the DNA regions analyzed. The data support the hypothesis that somatic mutations can accumulate during plant growth and ageing and contribute to age-related deterioration of the plant and its eventual death. Plants are known to form their reproductive structures and gametes from somatic meristems late in development (Buss 1983), and the somatic mutations and other genomic changes that occur in those somatic cells can therefore be passed on to subsequent plant generations. This can potentially influence a plant species' ecological and evolutionary fitness. Further studies are needed to show how the age-related accumulation of somatic mutations in plant tissues influences its vegetative and reproductive traits.

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