FEBS Letters 472 (2000) 14–16 FEBS 23560

Large accumulation of mRNA and DNA point modifications in a plant senescent tissue

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Received 26 January 2000

Edited by Takashi Gojobori

Abstract Although nucleic acids are the paradigm of genetic information conservation, they are inherently unstable molecules that suffer intrinsic and environmental damage. Oxidative stress has been related to senescence and aging and, recently, it has been shown that mutations accumulate at high frequency in mitochondrial DNA with age. We investigated RNA and DNA modifications in cork, a senescent plant tissue under high endogenous oxidative stress conditions. When compared to normally growing young tissue, cork revealed an unexpected high frequency of point modifications in both cDNA (Pn = 1/1784) and nuclear DNA (Pn = 1/1520). Cork should be viewed as a mosaic of genetically heterogeneous cells. This has biological implications: it supports somatic mutation models for aging and challenges 'single cDNA clone' as descriptor for the molecular genetics of senescent tissues.

Key words: mRNA damage; DNA damage; Point mutation; Senescence; Oxidative stress; Heat-shock protein

1. Introduction

The stratified outermost covering tissues, such as the keratinized skin epidermis in animals or the suberized cork tissue in plants, are the most directly exposed to the external oxidative agents (mainly UV radiation). Besides, cork is subjected to high endogenous oxidative stress conditions due to its metabolic activity. In these tissues, cells undergo terminal differentiation and die. It is known that oxidative stress increases DNA mutation rates in bacteria [1]. Also, transcriptional mutations occur at high frequency in specific post-mitotic neuron subpopulations with high metabolic activity and the number of neurons showing mutations increases with age [2]. Free radicals and other chemicals are capable to modify ribonucleotides (rNTPs), deoxyribonucleotides (dNTPs), single-stranded RNA (ssRNA) and double-stranded DNA (dsDNA). Oxidized rNTPs and dNTPs are readily incorporated in vitro into RNA and DNA [3]; from DNA templates containing uracil and other altered bases, mutant mRNA transcripts are made with high efficiency resulting in production of mutant proteins [4]. Thus, in cells under high oxidative stress conditions and/or with nucleic acids repair-defective mechanisms, nucleic acid modifications may accumulate and lead to an alteration of proteins and, ultimately, to cell death [5].

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We investigated the frequency of cDNA modification in cork (phellem) compared to a normally growing young tissue (root tip) using cork-oak (*Quercus suber*) as a model system. For this purpose, we analyzed a population of *Qs_hsp17* mRNA sequences (reverse transcription PCR products form position 32–401, AC AJ000691) in cork and in root tip tissue [6]. Cork (phellem) is an external layer of protective tissue, consisting of several layers of cells that deposit large amounts of suberin and undergo programmed cell death. Due to phenoxy radicals generated during suberin synthesis [7,8], cork cells are subjected to high endogenous oxidative stress. *Qs_hsp17* codes a cytoplasmic class I small heat-shock protein (HSP) which is constitutively over-expressed in cork and other cells with endogenous oxidative stress and in apical meristems; and it can be induced in other tissues by different stresses including oxidative [9]. HSPs are an important subset of chaperones, proteins that bind to nascent or unfolded polypeptides and ensure correct folding or transport [10]. Recently, a new role has been proposed for HSPs: to stabilize mutant proteins to keep them working properly allowing the organisms to have a reservoir of mutations that might be revealed when HSPs are out of commission [11].

2. Materials and methods

2.1. Plant material

A sheet of pure cork (phellem) tissue was peeled off from inner bark and woody tissues (cork) from an actively growing shoot of a selected cork-oak tree (*Q. suber*), during June. Root tip tissue was obtained from the primary root of a single acorn from the same selected cork tree by germination in water-soaked peat. The seedling was allowed to grow for 1 month. The standard growth conditions were 22°C at 70% humidity in a light/dark cycle of 16/8 h. Then, a segment of 1.5 cm up from the root cap junction was sectioned for analysis. For details in apical organization of the primary root, see [6].

2.2. mRNA and DNA extraction

RNA was extracted from cork cells as [12]. Poly(A)$^+$ RNA was purified with polyA Tract system 1000 (Promega) according to the instructions of the manufacturer. DNA was extracted with CTAB mini-DNA extraction for PCR [13] with some modifications: 2% PVP was added to CTAB buffer and DNA was further purified through Qiagen PCR purification kit (Qiagen) according to the instructions of the manufacturer.

2.3. Qs_hsp17 cDNAs and genomic DNAs cloning and sequencing

Poly(A)$^+$ RNA was reverse-transcribed with oligo d(T) (15 min at 42°C, 3 min at 95°C and 5 min at 5°C). *Qs_hsp17* cDNAs and genomic DNAs were amplified with oligonucleotide primers HS3 (5′-CTCTGAATTCGCAACGTGTTCGACCCTTTC-3′) and HS4 (5′-CTCTGAATTCGTGAGCACTCCATTCTCCAT-3′) containing *Eco*RI restriction sites (underlined). This yields a fragment of 390 bp. PCR amplifications were performed in 100 μl reaction volumes in the presence of the appropiate Tris-based buffer, using 1 unit

PII: S0014-5793(00)01424-1

of High Fidelity Pfu DNA polymerase (Stratagene), 150 μM of each dNTP and 23 pmol of each primer. PCR mixtures were denatured at 94°C for 1 min and 30 cycles were performed using the following conditions: 1 min at 94°C, 2 min at 55°C, 1 min at 72°C (in the last cycle 7 min at 72°C). The amplified fragments were digested with *Eco*RI and ligated into λgt11 arms. The ligation reactions were packaged and the λ phage used to infect *Escherichia coli* 1090r− host strain. Aliquots of infected cells were plated on LB plates with top agar containing X-gal (0.3 mg/ml). Plates were individually collected in 300 μl SM medium and 20 μl chloroform and 2–4 μl of lysate was directly subjected to PCR amplification. Sequencing was performed using the ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit. The sequencing device was Applied Biosystems ABI PRISM 310. Regions corresponding to PCR oligonucleotides were excluded from the analysis.

3. Results and discussion

Sequence comparison of 59 independent clones of cork *Qs_hsp17* cDNAs obtained from a single shoot showed a distribution containing 51 (86.5%) identical sequences (master sequence) and eight (13.5%) mutant sequences differing from each other and from the master sequence in 1–2 single-base substitutions distributed at random or in a single-base deletion (G^{256}). The number of polymorphic sites divided by the number of nucleotides sequenced (Pn) gave rise to a value of 1/1784 (Table 1). Mutated sequences showed at least four of the six possible base substitution mutations (A:T to T:A transversion was the most frequently observed mutation (33.3%); C:G to A:T transversion and T:A to C:G and C:G to T:A transitions accounted for 22.2% each). Diversity of the mutant sequences ranged from silent mutations (30%) to in-frame stop codons (10%) and included both conservative (20%) and non-conservative (40%) amino acid substitutions. All the above values are close to the polymorphism degree and the mutant frequency found in quick evolving RNA viral quasispecies [14–16]. In contrast to cork data, all of the sequences of the 48 independent clones of *Qs_hsp17* cDNAs from a single root tip were identical to the master sequence (Pn lower than 1/12 250). This excludes PCR and cloning artefacts as potential source for the observed cDNA mutations. Pn values from cork and root tissue are statistically different ($\chi^2 = 6.87$, df = 1, $P = 0.009$).

Point mutations in cDNA clones might be due to nucleotide misincorporation opposite to modified DNA bases. Since RNA polymerase cannot bypass certain DNA modifications [4], mutations at the RNA level might be underestimated. If eventually truncated mRNAs were generated, they could not be detected by our experimental approach.

Because mutations in cDNA may either arise at mRNA or at DNA levels, we cloned and sequenced PCR-amplified cork genomic *Qs_hsp17* (Table 1). When the sequences of 66 independent clones were compared, 54 sequences (81.8%) were identical (master sequence) and 12 (18.2%) showed a spectrum of point mutants. Point mutations gave rise to a Pn of 1/1520. These genomic DNA mutations were also distributed at random and showed at least three of the six possible base substitutions (T:A to C:G (54.5%) and C:G to T:A (27.3%) transitions and A:T to T:A (18.2%) transversion). Mutant sequences included silent mutations (41.7%), conservative (8.3%) and non-conservative (33%) amino acid substitutions and in-frame stop codons (16.7%). Variability values were not significantly different from those of mRNA in cork tissue ($\chi^2 = 0.14$, df = 1, $P = 0.708$).

Several types of mutation we found in cork *Qs_hsp17* cDNA and genomic DNA have been associated to oxidative damage. Thus, T:A to A:T transversion is the base substitution mutation most frequently caused by chemical oxidants [17] and it is also the predominant mutation in bacteria subjected to oxidative stress [1]. The oxidation of GTP to 8-oxo-GTP may result in C:G to A:T transversion [3]. Free rNTPs, dNTPs and ssRNA are more susceptible to oxidative damage than dsDNA [18]. Enzymes that specifically degrade oxidized nucleotides in the cellular pool or in nucleic acids have been characterized in bacteria [3] and several eukaryote species [19]. These enzymes belong to the cell machinery of DNA replication and transcription fidelity systems and are highly redundant in the genome of the radioresistant bacterium *Deinococcus radiodurans* R1 [20]. In addition, the C:G to T:A transition is the mutation most frequently induced in p53 DNA by sunlight in epidermal actinic keratoses [21]. On the other hand, in Alzheimer disease, dinucleotide deletions were found within (in β amyloid precursor protein mRNA) or adjacent to (in ubiquitin-B mRNA) GAGAG motifs. These deletions were apparently promoted by enhanced transcription and by high cellular metabolic activity [22]. Note that the only single-base deletion that we observed in cork *Qs_hsp17* cDNA occurs in the same motif: 5′-GAGAGgag-3′. This supports the idea that transcript mutation may be a widely occurring phenomenon in transcripts containing a susceptible motif such as GAGAG [23].

Our data provide evidence for high rates of mutation in a complex plant genome. Cork *Qs_hsp17* cDNA shows, as a minimum, a 7-fold higher frequency of mutation compared to normally growing young tissue. From the higher relative proportion of variant sequences in the cDNA fragment sequenced (about two thirds of the coding region), we estimate that about one third of *Qs_hsp17* mRNAs in cork might contain aberrant messages. This finding shows that in a plant senescent tissue under oxidative stress, the genetic integrity is not maintained.

Specific modifications in somatic DNA and mRNA have

Table 1
Sequence complexity of *Qs_hsp17* cDNA and genomic DNA in cork and in root tip tissue in cork-oak (*Q. suber*)

Tissue	Nucleic acid	No. of clones sequenced	No. of different nucleic acid sequences	Point mutations	No. of different amino acid sequences	No. of nucleotides sequenced	Pn (maximum)
Cork	RNA	59	8 (13.5%)	10[a]	6 (10.2%)	17 842	1/1 784
Root tip	RNA	48	0	0	0	12 252	< 1/12 252[c]
Cork	DNA	66	12 (18.2%)	12[b]	6 (9.1%)	18 237	1/1 520

A or G at positions 111 and 195 represent allelic segregation and were excluded for the analysis. Accession numbers for mutated sequences: AJ272617–AJ272639. Pn: number of polymorphic sites/number of nucleotides sequenced.
[a]Nine base pair substitutions (five transitions and four transversions) and one single-base deletion.
[b]Twelve base pair substitutions (nine transitions and two transversions).
[c]Only a maximum value can be calculated.

been associated to aging [24,25] and to pathological processes such as Alzheimer disease [23,26], Down syndrome [23,26] and xeroderma pigmentosum disease [27]. Mutation in somatic cells is not subjected to Darwinian laws and does not provide survival advantage for the individual, although putative adaptive mutations in bacteria subjected to stress have been speculated ([28] but see [29]). The essential difficulty to propose a positive role for somatic non-specific mutations arises from the extremely low probability of creating a new molecular function by pure chance. At high mutation rates, a generalized disruption of gene function may be the case. If damage was extensive to mechanisms critical for cell survival, such as the control region for mitochondrial DNA replication [24] or chaperone synthesis [11], dramatic changes may occur leading to a concatenated catastrophe. However, in tissues at the boundary of the external environment, such as cork or the keratinized skin epidermis, a cell death strategy mediated by gene hypermutation could help to preserve the body structures and result in a selective advantage.

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