

Genetic Characterization of *Quercus suber* L. 1. Preliminary Detection of Histone Promoter Variabilities

David Brás*, Filomena Nóbrega, Rui Vidal***, Raúl Sardinha**** e Raúl Bruno de Sousa*******

* Estagiário

Instituto PIAGET-ISEIT/Almada. Av. João Paulo II, Lote 544-2º, 1900-726 LISBOA

** Investigador Auxiliar

Departamento de Ecofisiologia e Melhoramento Florestal. Estação Florestal Nacional,
Rua do Borja nº 2, 1399-051 LISBOA

*** Professor Associado

Departamento de Engenharia Genética. Faculdade de Farmácia da Universidade de
Lisboa, Av. das Forças Armadas, 1649-019 LISBOA

**** Professor Catedrático

Centro Internacional de Epistemologia e Reflexão Transdisciplinar. Instituto Piaget.
Av. João Paulo II, Lote 544 - 2º, 1900-726 LISBOA

***** Professor Catedrático

Instituto Superior de Agronomia da Universidade Técnica de Lisboa. Tapada da
Ajuda, 1349-018 LISBOA

Abstract. The genetic variability of *Quercus suber* L. has been verified and examined by several authors, mainly through the evaluation of the restriction fragment length polymorphisms (RFLPs), isoenzymatic polymorphisms, suberin and waxes compositions, and, also, suberin's triglyceride contents in order its potential use in the improvement of the cork quality could be established. In this context, total DNA extracts obtained from two Portuguese cork oaks have been amplified by the polymerase chain reaction (PCR), using a specific, well-conserved molecular marker that occurs in the proximal promoter region of the plant histone H3 genes (type I element, CCACGTCACCGATCCGCG). A comparison of the electrophoretic profiles of the PCR products has allowed the detection of genetic variabilities between the studied trees whose potential implications may be associated with the cork quality. These results, that are here reported for the first time as far as known, although preliminary suggest that, at least, the molecular marker above mentioned may be used for screening, and characterization of the *Quercus suber* L. genetic variabilities. This is the objective of several studies that are actually in course by this team.

Key words: cork oak; molecular marker; PCR; cork quality

Sumário. A variabilidade genética de *Quercus suber* L. tem sido detectada e estudada por diversos autores, nomeadamente pela avaliação do polimorfismo das dimensões dos fragmentos de restrição (RFLP), do polimorfismo isoenzimático e, ainda, da composição da suberina e de ceras, tendo em vista a sua possível utilização no melhoramento da qualidade da

Corresponding Author E-mail: FilomenaNobrega@mail.telepac.pt

cortiça. Neste contexto, recorrendo-se a um marcador molecular específico, muito conservado, contido nas regiões promotoras dos genes codificantes das histonas H3 de plantas (elemento do tipo I, CCACGTCACCGATCCGCG), extractos totais de DNAs obtidos a partir de 2 sobreiros Portugueses foram submetidos à reacção em cadeia pela polimerase de DNA (PCR). Os perfis electroforéticos dos produtos gerados permitiram a detecção de variabilidades genéticas entre as árvores estudadas cujas potenciais implicações na qualidade da cortiça são discutidas. Estes resultados, que tanto quanto se conhece se publicam pela 1ª vez neste artigo, embora preliminares sugerem que aquele marcador genético pode ser, pelo menos, utilizado no rastreio e caracterização das variabilidades genéticas de *Quercus suber* L. o que constitui, a par com a procura do significado funcional, o objectivo dos estudos actualmente em curso por esta equipa.

Palavras-chave: sobreiro; marcador molecular; PCR; qualidade da cortiça

Résumé. Au Portugal, la variabilité génétique de *Quercus suber* L. a été détectée et étudiée par plusieurs auteurs, notamment à travers l'évaluation de polymorphismes des dimensions des fragments de restriction (RFLP), de polymorphismes isoenzymatiques, des compositions de subérine et de cires, et également des quantités de triglicérides de la subérine en ayant pour but son utilisation dans l'amélioration de la qualité du liège. Dans ce contexte, et en ayant recours à un marqueur moléculaire spécifique, très conservé, qui existe dans les régions promotrices des gènes codificateurs des histones H3 de plantes (élément du type I, CCACGTCACCGATCCGCG), des extraits totaux d'ADN obtenus à partir de deux chênes-lièges ont été soumis à la réaction en chaîne par la polymérase d'ADN (PCR). Les profils électrophorétiques des produits engendrés ont permis la détection de variabilité génétique parmi les arbres étudiés, dont les implications potentielles sur la qualité du liège sont discutées. Ces résultats, qui, nous semble-t-il, sont publiés pour la première fois dans cet article, bien que préliminaires, suggèrent que ce marqueur génétique peut être utilisé du moins dans la recherche et la caractérisation des variabilités génétiques de *Quercus suber* L., ce qui constitue, parallèlement à la recherche de la signification fonctionnelle, l'objectif des études actuellement menées par cette équipe.

Mots clés: chênes-lièges; marqueur moléculaire; PCR; qualité du liège

Introduction

Nowadays, it is well known that within the eukaryotic nucleus, including those from higher plants, the chromosomal DNA is wrapped around an octamer of two copies each of the four core histones H2A, H2B, H3, and H4 to form nucleosomes, the fundamental structural subunit of the chromatin. One copy of a fifth histone, known as the linker histone, H1 or H5, binds to this assembly of approximately equal masses of DNA and histones. Chromatin not only ensures the compaction necessary for DNA packaging inside the cell nucleus, but also provides the framework for gene-regulated expression,

owing to its structural flexibility at both higher order structure and nucleosome levels (COOPER, 1997). DNA sequencing analyses of about 40 plant histone genes have demonstrated their extreme conservations in plant and animal kingdoms (CHABOUTE, 1993) although phylogenetic studies of histone H3 protein sequences have recently indicated the independent origin of the replacement histone H3 genes in animals and in plants (WATERBORG and ROBERTSON, 1996).

In this context, since the promoter regions of the plant histone genes have been intensively examined, it was demonstrated that they harbour one or more types of highly conserved, specific

sequences (motifs) which may be used as molecular markers (BRIGNON and CHAUBET, 1993). One of them, the type I element (CCACGTCANCGATCCGCG) composed of 2 independent *cis*-acting sequences of the Hex motif (ACGTCA-box) and the reverse-oriented Oct motif (GATCCGCG-box), is one well-conserved regulatory element found in proximal promoter region of wheat histone H3 genes (TERADA *et al.*, 1995, MINAMI, MESHI and IWABUCHI, 2000).

This means that any point mutation or other type of alteration on the sequence of the type I element may be reflected in the first instance on the genetic expression of those histone genes, and then probably of other genes. Consequently, to further characterize the genetic variability of *Quercus suber* L. that has been detected through the determination of RFLP¹ profiles (ALMEIDA *et al.*, 1998) that, in turn, may be related with variabilities that have been also found with several isoenzymatic polymorphisms (NÓBREGA, 1997, ELENA-ROSELLO, SANTAMARIA and CARDIEL, 1998), suberin (BENTO *et al.*, 1998) and waxes compositions (CONDE *et al.*, 1998), and suberin's triglyceride contents (NÓBREGA *et al.*, 2000), the type I element was used as a molecular marker in the PCR carried out on total DNAs extracted from two Portuguese cork oaks in order to detect any possibility of the correlation between the PCR profiles and the qualitative characteristics of the cork tissue. The results² that have been obtained, for the first time as far as it is known, are here reported and briefly discussed.

Materials and methods

In May 2000, young leaves (15 days

old) were collected from 2 cork oaks (B7-5 and B7-7) from Ermidas - Sado (Herdade Monte Fava), immediately transported to the laboratory, and frozen at -70°C. Their total DNA extracts were prepared by the CTAB method as described by Doyle & Doyle (1987), and aliquots of them were maintained at -20°C until use.

PCR amplifications were done by using the QIAGEN Taq PCR Master Mix Kit under instructions specified by the supplier, the type I element CCACGTC-ACCGATCCGCG (from MWG-Biotec AG, Germain) as primer, and the Hybaid Thermal Cycler Express according to the following reaction conditions: an initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 3 min, with a final extension at 72°C for 10 min. For each reaction (total volume of 50 µL), 25 µL of Taq PCR Master Mix (containing 2.5 units of Taq DNA polymerase, 200 µM each dNTP, and 1.5 mM magnesium chloride dissolved in 1x concentrated QIAGEN PCR buffer), 3.5 µL of 50 mM magnesium chloride solution, 3 µL of primer solution (containing 0.4 µM primer dissolved in bi-sterilized Milli Q water), 17.5 µL of bi-sterilized Milli Q water, and 1 µL of DNA solution (corresponding to 500 ng DNA or less amount) were mixed in that order, and immediately submitted to PCR amplifications.

PCR products were simultaneously resolved with the 123 bp DNA Ladder (from Life Technologies, USA) by electrophoresis on 1.2% (w/v) agarose gels. These were prepared, and run at room temperature according to the standard methods (SAMBROOK, FRITSCH and MANIATIS, 1989) until the tracking dye (bromophenol blue) has neared the

bottom of each gel since it migrates just ahead of the 123 bp DNA Ladder. After electrophoresis, the gels were stained with ethidium bromide, and photographed, using 667 Polaroid films, according to those standard methods. The molecular dimensions of the PCR products were deduced by extrapolation of their electrophoretic mobilities on a calibration (linear regression) curve defined for each gel. For this, the electrophoretic mobilities of the DNA Ladder bands were plotted against the decimal logarithmic values of their correspondent molecular dimensions, using a suitable computer program (Cricket Graph III, Version 1.01).

Results

In 3 independent experiments, reproducible results have been found where, at least, about twelve PCR products were obtained from both B7-5 and B7-7 DNAs amplified by PCR, after being simultaneously fractionated by AGE as shown in Figure 1. However, a careful visual inspection of the photographic registers of the gels have revealed two types of the PCR products according to the relative intensities of their ethidium bromide-fluorescences: one concerning the major products (that is, with the higher fluorescence), and another being the minor ones (with the lower fluorescence).

The molecular sizes of the PCR products with electrophoretic mobilities between those of the 492 bp and 1,695 bp DNA Ladder bands were deduced after the linear regression calibration curve for each gel has been established. The results are shown in Table 1 with the correspondent relative values of the ethidium bromide-fluorescence intensities.

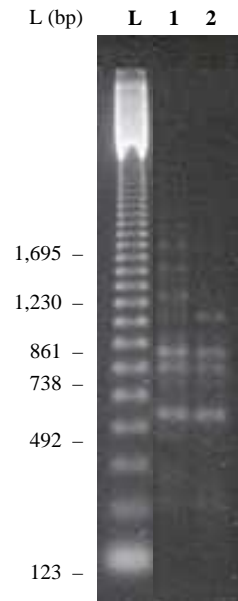


Figure 1 - PCR products from B7-5 DNA (1) and B7-7 DNA (2) simultaneously fractionated with 123 bp DNA Ladder (L) by 1.2% AGE in TBE (0.5x 90 mM Tris.Borate, 2 mM EDTA, pH 8,3) at 2.8 V/cm at room temperature

From these results, the following four main observations could be made:

i) more than one PCR product was found on both amplified DNAs, totalizing about 4-5 major bands on AGE gels (Figure 1) with molecular sizes between 550 and 1,279 bp. Since only one primer was used instead of two as usual, this means that on both DNAs the target sequence occurs twice repeated, at least;

ii) the molecular size of the PCR products found in one profile was exactly the same in the other one. This means that both amplified DNAs apparently contain the same genetic complexity based on the primer sequence. Otherwise, one or more PCR products will be present in one profile, and absent in the other. This also reflects the high specificity of the primer;

Table 1 - Molecular sizes, and relative fluorescence intensities of the PCR products

PCR bands (Figure 1)								
Relative intensity on each profile ¹		Molecular size (bp)						
		deduced ²	differential between the major bands					
B7-5	B7-7		1,259	1,156	1,021	811	740	550
.....1x	1,559						
.....2x	1,379						
=====	1,279	—	123	135			
=====	1,156			258			
.....	=====	1,021						
.....	895						
=====2x	=====	811				—	71	190
=====	=====	740						261
.....	644						
=====	=====	550						

1. full lines and broken lines correspond to the major and the minor PCR products, respectively; the numbers represent the relative proportional increases in the ethidium bromide-fluorescence intensities between PCR products of the same molecular size as deduced by visual inspection of both PCR profiles;

2. the calibration curve of the gel shown in Figure 1 was as follows: $\log \text{bp} = 3.6867 - 0.1633 \cdot \text{cm}$ ($r^2 = 0.99992$).

iii) the most abundant major PCR products were 550, 740, and 811 bp long, this being common to both profiles. However, the amount of the major 811 bp PCR product obtained from B7-5 DNA was approximatively twice higher than the corresponding product amplified from B7-7 DNA;

iv) from the remaining major PCR products, only one containing 1,021 bp was found on amplified B7-7 DNA while two with 1,156 and 1,279 bp were detected on amplified B7-5 DNA. This observation, once conjugated with the previous one about the 811 bp PCR product, represents at least two types of genetic variabilities, one of them concerning the most abundant PCR

products, and the other the less abundant ones. In any case, these results suggest that the primer has allowed not only the amplification of the promoter regions of the histone H3 genes, but also other regions for which it seems to be also somewhat specific.

Discussion

The type I element (CCACGTCANC-GATCCGCG), consisting of 2 independent *cis*-acting elements of the hexamer CCA-CGTCA (Hex motif, also named ACGTCA-box) and the octamer GATCCGCG (reverse-oriented Oct motif, also called GATCCGCG-box), is one well-conserved regulatory element found in proximal

promoter region of the plant histone H3 genes, being necessary and sufficient to confer the S phase-specific transcription of those genes (NAKAYAMA *et al.*, 1992; MIKAMI, SAKAMOTO and IWABUCHI, 1994; TERADA *et al.*, 1995; TAOKA *et al.*, 1998; MINAMI, MESHU and IWABUCHI, 2000). In plants, multiple copies of the histone genes have been detected which, being organized into multigenic families, are highly dispersed in their genomes (CHABOUTE *et al.*, 1993). However, the core histone genes occur in a dozen of clusters of H2A-H2B and H3-H4 pairs, in which each gene pair shows outwardly divergent transcription from one short (lower than 300 bp) intercistronic region, these intercistronic regions containing typically conserved promoter elements (FABRY, 1995). Consequently, it seems that the data here reported agree with these observations since: *i*) the total number of the observed PCR bands was about twelve (Figure 1), and *ii*) a mean value of 260 bp was deduced for the difference between the highest and the lowest molecular size in each one of both sub-groups of the major bands (Table 1).

In the wheat histone H3 gene promoter, which has its upstream sequence at to -1,717 bp position (relative to the histone H3 gene initiation site as +1), the distal region from -909 to -1,711 bp (corresponding to a differential size of 802 bp) contains positive *cis*-acting element(s) while in the proximal region (up to -185 bp) occur the conserved hexamer and octamer motifs (TERADA *et al.*, 1993). Also, it has been noted that all the consensus motifs identified in plant histone gene promoters have been detected within 200 bp upstream from the typical TATA box of the H3 promoter, and in this region two or three copies of the type I element were found from

which two of them occur at -87 and -191 positions (corresponding to a differential size position of 104 bp) in tobacco cells, the more distal one having a degenerated octamer instead of the highly conserved Oct-motif (REICHHELD, GIGOT and CHAUBET-GIGOT, 1998). On the other hand, truncated derivatives or variants of histone H3 gene promoter have been detected, for example, in maize (ATANASSOVA *et al.*, 1998), alfalfa (ROBERTSON, KAPROS and WATERBORG, 1997), soybean, barley, wheat (KANAZIN, BLAKE and SHOEMAKER, 1996), and *Arabidopsis thaliana* (CHAUBET, CLEMENT and GIGOT, 1992). However, a comparison of the nucleotide sequences of 23 genes and 14 cDNAs from 8 plant different species have shown the extreme conservation of histones H3 in plants (CHABOUTE *et al.*, 1993). This means that the type I element could occur either degenerated or truncated at different positions, apparently with no serious effect on the plant histone H3 codifying sequences.

In the present work, the most abundant major PCR product was 811 bp long, that is, with a molecular size very closed to that one described for the differential size (802 bp) of the distal region of the wheat histone H3 gene promoter above mentioned. Also, this 811 bp PCR product differed from the next (with 740 bp) only by 71 bp, and from a third one (with 550 bp) by 261 bp (Table 1). According to these observations, it is most probable that both PCR products with 811 and 740 bp as well as the smaller one (with 550 bp) have resulted from each *Quercus suber* promoter region of the histone H3 genes. In any case, the most relevant result concerns to the relative amounts of the 811 bp PCR product that was higher

(about twice) on the B7-5 profile than on the B7-7 one. This indicates a particular (quantitative) type of genetic variability between the two studied cork tree DNAs, one of them being more rich on the PCR target sequence than the other.

Further genetic variabilities between those DNAs were also shown by the subgroup of the less abundant major PCR products (Table 1) since the 1,021 bp PCR product was only present on the B7-7 DNA while both 1,156 and 1,279 products were only contained on the B7-5 DNA. As the molecular size of the 1,021 bp PCR product was the same of that one reported for the truncated derivative form of the maize histone H3C4 gene promoter that is 1,023 bp long (ATANASSOVA *et al.*, 1998), these results demonstrate another type (qualitative) of genetic variabilities between the two studied cork tree DNAs whose biological consequences are unknown at moment.

Conclusions

In this work, genetic variabilities between two *Quercus suber* DNAs have been detected by PCR by using an unique primer, the type I element (CCACGTCACCGATCCGCG) that is one well-conserved regulatory element found in proximal promoter region of plant histone H3 genes. From about twelve PCR products that have been observed after their resolutions by AGE, one most abundant major product containing 811 bp, and three less abundant ones containing 1,021, 1,156 and 1,279 bp have allowed a clear differentiation between the two examined *Quercus suber* DNAs, designated by B7-5, and B7-7. As histones not only ensure the compaction necessary for DNA packaging inside the cell nucleus, but also provide the framework for gene-

regulated expressions, these genetic variabilities may play a crucial role in the S phase-specific regulation of one or more gene expressions, some of which could be associated later on with the cork quality.

As a consequence, the validation of the PCR methodology here described and a better understanding of these results are the immediate objectives of the works actually in course.

References

- ALMEIDA, M.H., *et al.*, 1998. Estudo da variabilidade genética do sobreiro em Portugal. In *Cork Oak and Cork* (European Conference on Cork Oak and Cork, 5-7 May 1997, Lisbon, Portugal), ed. CEF - Centro de Estudos Florestais, pp. 237-247.
- ATANASSOVA, R., *et al.*, 1998. Analysis of the promoter region of a maize (*Zea mays* L.) H3 histone gene in transgenic *Arabidopsis thaliana*. *Plant Mol. Biol.* **37**(2) : 275-285.
- BENTO, M.F., *et al.*, 1998. Variability of suberin in cork from *Quercus suber* L. In *Cork Oak and Cork* (European Conference on Cork Oak and Cork, 5-7 May 1997, Lisbon, Portugal), ed. CEF - Centro de Estudos Florestais, pp. 378-386.
- BRIGNON, P., CHAUBET, N., 1993. Constitutive and cell-division-inducible protein-DNA interactions in two maize histone gene promoters. *Plant* **4** (3): 445-457.
- CHAUBET, N., CLEMENT, B., GIGOT, C., 1992. Genes encoding a histone H3.3-like variant in *Arabidopsis* contain intervening sequences. *J. Mol. Biol.* **225**(2) : 569-574.
- CHABOUTE, M.E., 1993. Histones and histone genes in higher plants: structure and genomic organization. *Biochimie* **75**(7) : 523-531.
- CONDE, E., *et al.*, 1998. Estudo da variabilidade genética do sobreiro em Portugal. In *Cork Oak and Cork* (European Conference on Cork Oak and Cork, 5-7 May 1997, Lisbon, Portugal), ed. CEF - Centro de Estudos Florestais, pp. 413-418.

- COOPER, G.M., 1997. *The Cell. A Molecular Approach*. ASM Press. 673 pp.
- DOYLE, J.J., DOYLE, J.L., 1987. Isolation of plant DNA from fresh tissue. *Focus*, **12**: 13-15.
- ELENA-ROSELLO, J.A., SANTAMARIA, I.G., CARDIEL, P.J.C., 1998. Variabilidad genética (Isoenzimática) en poblaciones mixtas de *Quercus suber* L. y *Q. rotundifolia* Lam.: evidencia de introgresión. In *Cork Oak and Cork* (European Conference on Cork Oak and Cork, 5-7 May 1997, Lisbon, Portugal), ed. CEF - Centro de Estudos Florestais, pp. 229-236.
- FABRY, S., 1995. The organization structure and regulatory elements of *Chlamydomonas* histone genes reveal features linking plant and animal genes. *Curr. Genet.* **28**(4) : 333-345.
- KANAZIN, V., BLAKE, T., SHOEMAKER, R.C., 1996. Organization of the histone H3 genes in soybean, barley and wheat. *Mol. Gen. Genet.* **250**(2) : 137-147.
- MIKAMI, K., SAKAMOTO, A., IWABUCHI, M., 1994. The HBP-1 family of wheat basic/leucine zipper proteins interacts with overlapping cis-acting hexamer motifs of plant histone genes. *J. Biol. Chem.* **269** (13): 9974-9985.
- MINAMI, M., MESHI, T., IWABUCHI, M., 2000. S phase-specific DNA-binding proteins interacting with the Hex and Oct motifs in type I element of the wheat histone H3 promoter. *Gene* **241**(2) : 333-339.
- NAKAYAMA, T., et al., 1992. Highly conserved hexamer, octamer and nonamer motifs are positive cis-regulatory elements of the wheat histone H3 gene. *FEBS Lett.* **300**(2): 167-170.
- NÓBREGA, F., 1997. O Polimorfismo Isoenzimático na Caracterização do Sobreiro. *Silva Lusitana* **5**(1) : 29-58.
- NÓBREGA, F., et al., 2000. Estudos moleculares no sobreiro, *Quercus suber* L.. Detecção de proteínas associadas com a suberina. *Silva Lusitana* **8**(2) : 135-148.
- REICHHELD, J.P., GIGOT, C., CHAUBET-GIGOT, N., 1998. Multilevel regulation of histone gene expression during the cell cycle in tobacco cells. *Nucl. Acids Res.* **26** (13): 3255-3262.
- ROBERTSON, A.J., KAPROS, T., WATERBORG, J.H., 1997. A cell cycle-regulated histone H3 gene of alfalfa with an atypical promoter structure. *DNA Seq.* **7** (3-4): 209-216.
- SAMBROOK, J., FRITSCH, E. F., MANIATIS, T., 1989. *Molecular Cloning. A Laboratory Manual*. 2nd Edition. Cold Spring Harbor Laboratory Press
- TAOKA, K., et al., 1998. The modular structure and function of the wheat H1 promoter with S phase-specific activity. *Plant Cell Physiol.* **39** (3): 294-306.
- TERADA, R., et al., 1993. A wheat histone H3 promoter confers cell division-dependent and independent expression of the gus A gene in transgenic rice plants. *Plant J.* **3** (2): 241-252.
- TERADA, R., et al., 1995. A type I element composed of the hexamer (ACGTCA) and octamer (CGCGGATC) motifs plays a role(s) in meristematic expression of a wheat histone H3 gene in transgenic rice plants *Plant Mol. Biol.* **27**(1) : 17-26.
- WATERBORG, J.H., ROBERTSON, A.J., 1996. Common features of analogous replacement histone H3 genes in animals and plants. *J. Mol. Evol.* **43** (3): 194-206.

Submetido para publicação em Setembro de 2001

Aceite para publicação em Outubro de 2001

¹ The abbreviations used are: AGE, agarose gel electrophoresis; bp, base pair; CTAB, N-cetyl-N,N,N-trimethylammonium bromide; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

² This work was supported by Project PIDDAC 210

