Journal of Tropical Forest Science 12(1):149–163 (2000)

MOLECULAR STABILITY ASSESSMENTS OF TREES REGENERATED FROM CRYOPRESERVED MAHOGANY (*SWIETENIA MACROPHYLLA*) SEED GERMPLASM USING NON-RADIOACTIVE TECHNIQUES TO EXAMINE THE CHROMATIN STRUCTURE AND DNA METHYLATION STATUS OF THE RIBOSOMAL RNA GENES

K. Harding,

Crop Genetics, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, United Kingdom

M. Marzalina, B. Krishnapillay, N. A. Nashatul Zaimah,

Forest Research Institute Malaysia, Kepong, 52109 Kuala Lumpur, Malaysia

M. N. Normah

Department of Botany, Faculty of Life Sciences, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor Darul Ehsan, Malaysia

&

E. E. Benson

Plant Conservation Biotechnology Group, Division of Molecular and Life Sciences, School of Science and Engineering, University of Abertay Dundee, Bell Street, Dundee DD1 1HG, Scotland, United Kingdom

Received June 1998

HARDING, K., MARZALINA, M., KRISHNAPILLAY, B., NASHATUL ZAIMAH, N. A., NORMAH, M. N. & BENSON, E. E. 2000. Molecular stability assessments of trees regenerated from cryopreserved mahogany (*Swietenia macrophylla*) seed germplasm using non-radioactive techniques to examine the chromatin structure and DNA methylation status of the ribosomal RNA genes. The cryopreservation technique employing desiccation-slow cooling procedure was applied for the *in vitro* conservation of mahogany (*Swietenia macrophylla*). An examination of DNA from trees regenerating from cryopreserved embryos showed identical Restriction Fragment Length Polymorphisms (RFLPs) after Hpa II/Msp I digestion in genomic DNA sequences and Hpa II ribosomal RNA gene (rDNA) sequences as compared to the DNA of non-cryopreserved mahogany trees. DNA-DNA hybridisation analysis

showed that the CCGG target site in rDNA was preferentially methylated (CmCGG) in trees regenerated from cryopreserved germplasm. An examination of the DNA methylation status between the mother tree and the trees regenerated from cryopreservation showed little evidence to support a correlation between methylation status and changes in chromatin structure. There were some differences in chromatin structure throughout the nuclear genome and within the rDNA sequence between the mother tree and the trees regenerated from cryopreserved embryos. While RFLP profiles signify genetic stability in mahogany trees derived from cryopreserved seed germplasm, the chromatin and DNA methylation changes may have significance for patterns of gene expression.

Key words: Molecular stability - cryopreservation - *Swietenia macrophylla* - chromatin - DNA methylation - ribosomal RNA genes - conservation

HARDING, K., MARZALINA, M., KRISHNAPILLAY, B., NASHATUL ZAIMAH, N. A., NORMAH, M. N. & BENSON, E. E. 2000. Penilaian kestabilan molekul bagi pokok- pokok yang dipulihkan daripada germplasma biji benih mahogany (*Switienia macrophylla*) yang dikrioawet menggunakan teknik tak radioaktif untuk memeriksa struktur kromatin dan status pemetilan DNA bagi gen RNA ribosom. Teknik krioawetan yang menggunakan prosedur pendinginan pengeringan perlahan digunakan untuk perlindungan *in vitro* pokok mahogany (*Switienia macrophylla*). Pemeriksaan DNA ke atas pokok-pokok yang dipulihkan daripada embrio yang dikrioawet menunjukkan Polimorfisme Panjang Serpihan Terbatas (RFLPs) yang seiras selepas pencernaan Hpa II/Msp 1 dalam turutan DNA genomik dan turutan Hpa II ribosom gen RNA (rDNA) berbanding dengan DNA bagi pokok mahogany yang tak dikrioawet. Analisis penghibridan DNA-DNA menunjukkan bahawa tapak sasaran CCGG dalam rDNA adalah bermetil utama (CmCGG) di dalam pokok yang dipulihkan daripada germplasma krioawetan. Satu ujian status pemetilan DNA antara pokok induk dan pokok yang dipulihkan daripada krioawetan menunjukkan sedikit bukti untuk menyokong korelasi antara status pemetilan dan perubahan dalam struktur kromatin. Terdapat beberapa perbezaan dalam struktur kromatin di seluruh genom nuklear dan dalam turutan rDNA antara pokok induk dan pokok-pokok yang dipulihkan daripada embrio yang dikrioawet. Manakala profil RFLP menunjukkan kestabilan gen dalam pokok mahogany yang diperoleh daripada germplasma biji benih yang dikrioawet, perubahan kromatin dan perubahan pemetilan DNA mungkin bererti bagi pola ekspresi gen.

Introduction

Swietenia macrophylla King (large-leaf mahogany) is indigenous to tropical America and has a natural distribution within the central Americas, south Florida, Peru, Bolivia and Brazil (Soerianegara & Lemmens 1992). It is cultivated in Southeast Asia, including Malaysia, and is considered to produce one of the world's finest timbers. Mahogany is one of the most important tropical timber trees, and as such, attention must be given to the management of its genetic resources. Its introduction into Malaysia came from an unknown seed source planted in 1903 within the Bukit Bruang Forest Reserve in Malacca (Appanah & Weinland 1993). Since 1928, local seeds from this source have been used to establish plantations throughout Malaysia, including those trees at the Forest Research Institute of Malaysia (FRIM). Mayhew and Newton (1998) provide an excellent description of this species. *Swietenia macrophylla* is monoecious with unisexual flowers, which

are pollinated by insects, bees and moths being the main pollen vectors, although on occasion, some individual trees have been reported to be dioecious (Soerianegara & Lemmens 1992). The flowers are branched inflorescences including both male and female flowers; studies suggest it is an obligate outbreeder and self-compatible under experimental conditions (Mayhew & Newton 1998). After approximately 15 years, trees are capable of reproductive development and set fruit once a year. The seeds are winged and are wind dispersed. According to Soerianegara and Lemmens (1992), short- and medium-term seed storage is possible, for two months to one year respectively, if the seeds are stored hydrated, under refrigeration (2–5 °C and 45% RH). Mahogany seeds have been categorised as semi-recalcitrant by Marzalina (1995) and are, therefore, difficult to conserve by traditional seed banking methods.

Cryopreservation offers an alternative option for the conservation of mahogany seed germplasm and this process has been extensively explored at FRIM. Marzalina *et al.* (1994) initially monitored the ultrastructural changes which can occur in mahogany embryos exposed to cryoprotective desiccation treatments (to a moisture content of about 7–10% on a fresh weight basis) followed by exposure to cryogenic conditions. Thus, embryos exposed to liquid nitrogen were highly vacuolated and had cytoplasm closely appressed to the cell wall, indicating that the cells had undergone cryo-protective desiccation thereby reducing ice formation. Marzalina *et al.* (1994) demonstrated that the embryos were capable of surviving the cryogenic process and electron microscopy profiles of the post-thaw recovery period indicated new cell division and repair of the endoplasmic reticulm.

To date, FRIM seed technologists have applied a range of different cryogenic methods to mahogany embryos extracted from seed germplasm. Normah and Marzalina (1996) report a survival rate of 63% for whole, cryopreserved mahogany seeds exposed to cryoprotective desiccation treatments (to a moisture content of 5–6%). The same survival rate was also observed for cryopreserved embryonic axes of mahogany which were exposed to cryoprotective encapsulation and desiccation treatments (to a moisture content of 5–8%) (Marzalina 1995, Normah & Marzalina 1996). Embryos excised from mahogany seeds are thus able to withstand embryo rescue, cryoprotection and exposure to liquid nitrogen and all these storage manipulations may be performed aseptically under *in vitro* conditions.

One important aspect of *in vitro* cryo-conservation concerns the assessment of genetic stability in plants recovered from *in vitro* cryogenic storage. There are several approaches to study the genetic stability of plants recovered from *in vitro* conservation (Harding 1996); some molecular biological techniques are helpful (Powell *et al.* 1997) but rarely produce useful information regarding the function of DNA sequences. The ribosomal RNA genes have been used to examine genetic changes regenerated from cryopreserved seed germplasm (Harding 1991) and DNA methylation changes in tissue cultures (Harding *et al.* 1996). The rDNA sequences contain coding regions within the transcriptional (repeat) unit comprising the 25S, 5.8S and 18S genes and are organised as a tandem array located in the (NOR) nucleous organiser region (Wanzenbock *et al.*1997). This region is associated with DNA methylation changes and has a role in the ordering

of nucleosome structures in chromatin (Lopez-Leon *et al.* 1995, Bestor 1998). These RNA genes are transcribed within the nucleus, after the splicing process; transcripts are transported to the cytoplasm and aggregated with ribosomal proteins to complete the assembly of a functional ribosome (Liang & Fournier, 1997). The impairment of rDNA expression reduces the pool of available RNA for the maintenance of cytoplasmic ribosomes and the transcription of essential mRNAs for desirable traits. This is likely to have consequences for protein synthesis, cellular growth and plant development (Lambe *et al.* 1997).

In this paper, the objective was to evaluate rDNA stability in mahogany trees regenerated from cryopreserved germplasm for changes in base sequence, DNA methylation and chromatin structure. This combined genetic analysis may be especially important for the assessment of tree genetic resources which have been conserved using *in vitro* techniques. Tropical tree species have complex developmental patterns and it is appropriate to assess those aspects of genetic stability which may influence gene expression.

Materials and methods

Preparation of embryos for cryopreservation

Fresh mahogany seeds were cleaned and washed three times in 70% alcohol. The testas were removed and the embryos dissected aseptically by cutting to a size of 2 × 2 mm at the point of cotelydon attachment. The embryos were placed in a sterile laminar flow to desiccate for an optimal period of 6 h at which point the moisture content was 4.59 ± 0.25%. The embryos were placed in cryovials (1.8 ml NUNC) which were then tied to cryocanes and cryopreserved by a slow cooling method using a Cryomed Programmable Freezing Chamber (model 1010, New Baltimore, Michigan, USA). The cooling steps were set at 0 °C (initial temperature), -1 °C min^{-1} (rate of cooling), -35 °C (final temperature) and a 35 min hold period. Following the hold period, the cryocanes were directly placed into a liquid nitrogen dewar. After 24 h of storage in the liquid phase (-196 °C), the cryovials were removed and thawed slowly in a laminar flow bench. Embryos were aseptically placed onto Murashige and Skoog basal medium containing 0.5 mg l^{-1} 6-benzyl aminopurine (BAP). Using this method, 87% survival rates were achieved (Marzalina 1995). The embryos germinated and established roots and shoots after only one week. Developed plantlets were removed from *in vitro* culture and placed into polyethylene bags containing soil for two months. During this period the plantlets were maintained under high humidity (using clear plastic bags over the polyethylene bags). The environmental temperature was increased slowly (hardening process) from 25 to 30 °C by transferring the seedlings from the laboratory to the nursery. The seedlings were placed under shade and later transferred to open nursery conditions, where they were maintained using standard practices and given fertiliser. At six months old, ten plants were selected for transfer to the

arboretum for standard field maintenance and further monitoring of tree growth. Due to animal predation, only two trees survived after the 2.5 years of post-conservation maintenance; these were used for genetic analysis. At the time of analysis, the mother tree was 45 years old.

Preparation of leaf material

Prior to DNA extraction, 5–10 g of leaves selected from the mother tree and the cryopreserved regenerants were washed in tap water, distilled water, 20 mM potassium chloride and finally in 5 mM ethylene diamine tetraacetic acid (EDTA).

Preparation of chromatin and DNAse I assay

Healthy expanded leaves were cleaned as described above and 5 g were homogenised in a Waring blender in 50 to 100 ml of pre-chilled 4 °C buffer. The homogenate was filtered through several layers of myracloth (Sigma) and the filtrate centrifuged (Sorvall RC5 with SLA 1500 rotor) at 1500 × g to pellet the nuclei. This crude nuclear pellet was suspended in 3 ml nuclease buffer as described elsewhere (Paul & Ferl 1988).

Nuclei were dispensed in 500 µl aliquots. A DNase I (bovine, 2000 units.mg Sigma) dilution series was prepared (0, 10, 20, 40, 80 and 200 µg ml^{-1}) where 10 µl of each dilution was added to each nuclei preparation. These were incubated at 37 °C for 1 min then at 30 °C for 9 min. Nuclei were pelleted after 10 sec at 10 000 × g (Microfuge) then suspended in 300 µl of cold (4°C) extraction buffer with 5 µl of 20 mg ml^{-1} proteinase K and 50 µl of 10% sodium dodecyl sulfate (Paul & Ferl 1988). Incubation was performed at 65 °C for 45 to 60 min. Following this, 100 µl of 5 M potassium acetate was added and the mixture was held on ice for 30 min. The mixture was then centrifuged for 10 min at 10 000 × g and the supernatant transferred to Eppendorf tubes in which they were stored at -20 °C for 1h following the addition of 600 µl isopropanol. The DNA was centrifuged for 10 min at 10 000 × g; the supernatant removed and the pellet suspended in 25 to 50 µl of TE (10 mM Tris.HCl; 1mM EDTA pH 8.0) buffer. These samples were sequentially loaded onto an agarose 1.2% gel with 10 µl of bromophenol blue marker dye. Gel electrophoresis and Southern blotting were done as described below.

Preparation of nuclear DNA

DNA was extracted from isolated nuclei as described (Paul & Ferl 1988). DNA pellets were resuspended in 25 to 50 µl of TE buffer prior to restriction enzyme digestion. DNA preparations were treated with 1 µl RNase A (1 mg ml^{-1}) for 30 min at 37 °C.

Restriction enzyme digestion, gel electrophoresis and Southern blotting

Digestion of DNA was performed with 10 units of each restriction enzyme overnight at conditions described for the enzyme in the appropriate buffer (BRL) or at 37 °C in potassium glutamate buffer (Harding *et al.* 1996). Digested DNA (5 μg/track) was fractionated in a 1.2% agarose gel, the gel was depurinated, denatured, neutralised and Southern blotted onto neutral nylon membrane filters (Tropilon, Tropix) as described by Harding and Benson (1995).

Hybridisation conditions and plasmid biotin labelling procedure

These techniques were performed as described by Harding and Benson (1995). Briefly, membranes were incubated for 4 h in pre-hybridisation solution. Plasmid pTa 71 containing the ribosomal genes derived from wheat (Gerlach & Bedbrook 1979) was biotin labelled and heat denatured. Hybridisation was performed for 16–20 h at 65 °C under the same conditions as pre-hybridisation, after hybridisation filters were washed, once in the fresh pre-hybridisation solution, then in 4 × SSC, 0.1% SDS for 5 min at 65 °C and 2 × SSC at 20 °C for 2 min.

Detection of chemiluminescent DNA sequences

Chemiluminescence was performed with hybridised membranes washed in blocking buffer for 30 min (Harding 1992). Avidin-alkaline phosphatase conjugate (1mg, Sigma) was diluted (1:15 000) in conjugate buffer and the membrane incubated for 30 min. Approximately 20 ml of diluted avidin conjugate was used for 10 cm^2 of membrane. The membrane was rinsed in washing solution, then washed in assay buffer prior to incubation with the dioxetane substrate AMPPD for 5 min. Chemiluminescent detection was performed in cassettes containing X-ray film (Fuji HR-G) without screens at room temperature.

Assay for methylated DNA sequences

The methylation status of DNA sequences can be examined by comparing the activities of an isoschizomer pair of restriction enzymes (Harding *et al.* 1996).

Results

DNAse I assay

Figure 1 shows an ethidium bromide stained gel containing nuclei derived from the mahogany mother tree and two mahogany plants (trees) derived from cryopreserved germplasm incubated with increasing amounts of DNase I. The same fresh weight of mahogany leaves was used in all nuclei preparations taken

from the mother tree and the two trees recovered from cryopreservation. Identical solutions and DNA extraction procedures were applied to the nuclei preparation, therefore the equal DNA volumes loaded onto the gels reflects the physiological status of the leaf material, the conditions of the nuclei during nuclease assay and the efficiency of DNA extraction within each experimental sample. Nuclei derived from young leaf material of cryopreserved plant 1 were completely degraded by 200 μg ml^{-1} DNase I, in comparison to the older leaf material which was degraded with 40–80 μg ml^{-1} DNase I. Unlike plant 1, cryopreserved plant 2 showed young leaf DNA material degraded after a treatment of 20 μg ml^{-1} DNase I, while nuclei derived from the older leaves were readily degraded in the absence of nuclease. The regular repeating pattern of DNA fluorescence (older leaves cryo plant 2) reflects the nucleosome ordering pattern and the distance between these structures. The upper DNA fluorescence is approximately 1.6 kb and the next lower DNA fluorescence is 1.4 kb, giving a characteristic DNA-chromatin strand interval of 200 bp. The mature mahogany tree of some 45 years shows nuclei digested after 80–200 μg ml^{-1} DNase I, similar to plant 1 recovered from cryopreservation. DNase I profiles of the mother tree also show a nucleosomal ordering pattern in nuclease digested chromatin. These results show a high molecular 'residual' fragment, at 21.2 kb resistant to nuclease digestion at higher concentration (200 μg ml^{-1}) for the mother tree and cryopreserved samples.

Analysis of ribosomal RNA genes

Figure 2 represents a luminograph directly taken from a Southern blot of the gel shown in Figure 1 after hybridisation with an rDNA probe (size markers were removed from Figure 2 but distances correspond to those in Figure 1). The luminograph shows rDNA profiles of nuclei derived from the mother tree and the two trees regenerated from cryopreserved germplasm. A comparison of relative differences in nuclease-digestion profiles between these experimental leaf samples shows the residual high molecular weight rDNA complex at the top of the gel, this corresponds to the same high molecular weight (approximately 21.2 kb) complex in Figure 1. This nuclease-digestion 'relic' represents an rDNA fraction within the NOR region resistant to digestion by DNase I. Nuclease-sensitive domains within the rDNA gene array in young and old leaves of cryopreserved plant 1 were completely digested at relatively high concentrations of DNase I (80–200 μg ml^{-1}). A similar degradation pattern of rDNA genes can be seen in the nuclease-digestion cascade between the young leaves of cryo-preserved plant 1 and the mother tree. Nuclei derived from both young and old leaf material of cryopreserved plant 2 were more sensitive to nuclease treatment compared to cryopreserved plant 1, in particular the nuclease-sensitive rDNA domains in older material were degraded in the absence of DNase I, possibly reflecting intrinsic differences in physiological status of these trees.

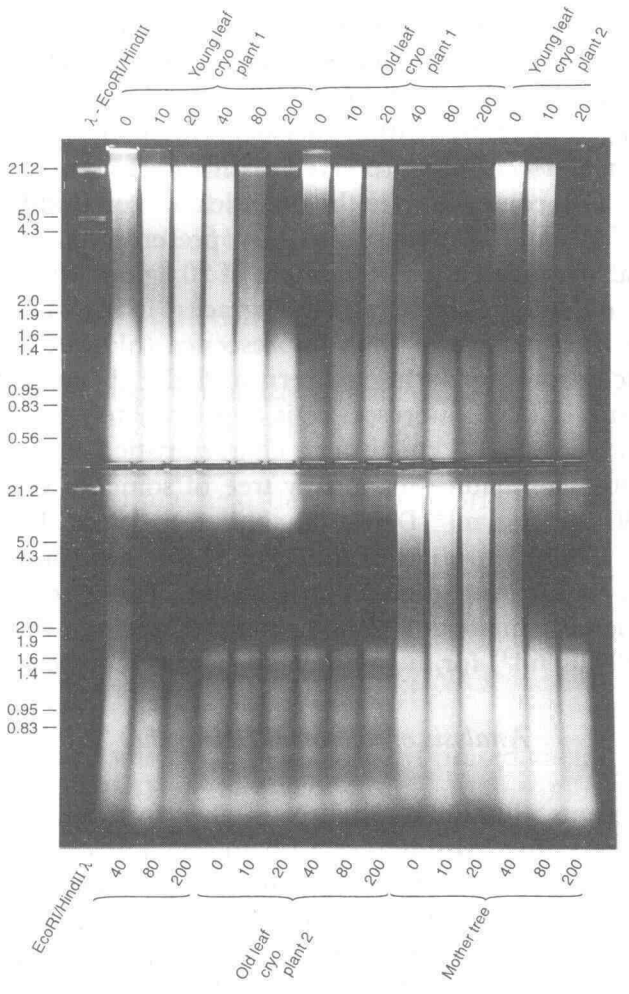

Figure 1. An ethidium bromide stained gel containing nuclei derived from mahogany mother tree and two plants derived from cryopreservation after sequentially digested with increasing amounts of DNase I (0, 10, 20, 40, 80 and 200 μg ml^{-1}). The molecular weight marker is represented by an lambda Eco RI/Hind III digestion

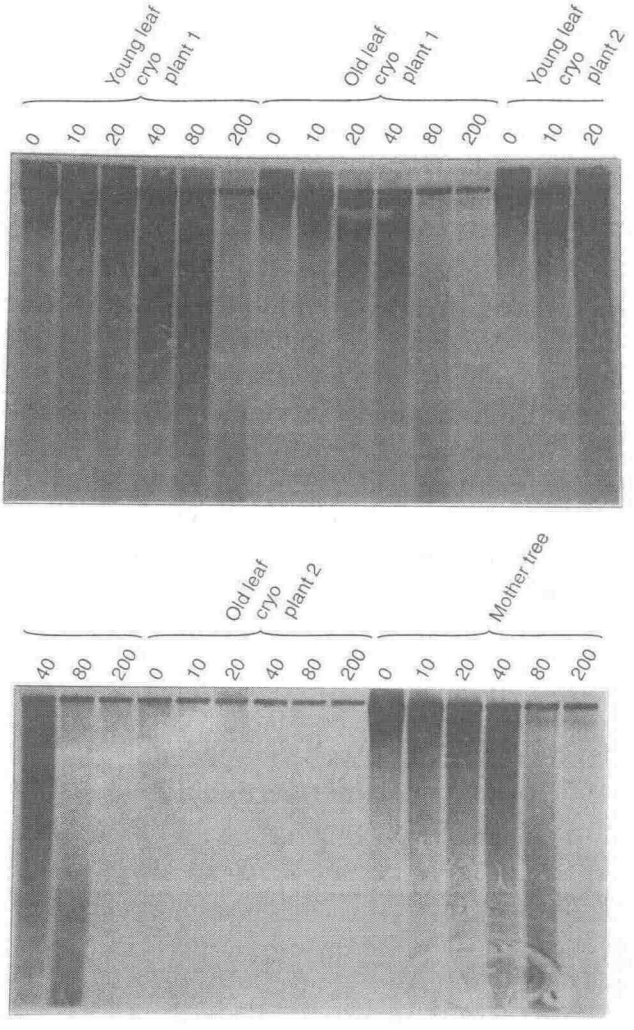

Figure 2. Luminograph of rDNA profiles of nuclei derived from mahogany mother tree and two plants derived from cryopreservation after sequentially digested with increasing amounts of DNase I (0, 10, 20, 40, 80 and 200 µg ml^{-1}) following Southern blotting and hybridised to the rDNA probe pTa 71.

Analysis of methylated genomic DNA sequences

Figure 3 shows an ethidium bromide stained gel containing DNA derived from the mahogany mother tree and the two mahogany trees derived from cryopreservation after digestion with Eco RV, Eco RV/Hpa II and Eco RV/Msp I restriction enzymes. The lambda marker (λ - EL, left side of gel) is a Hind III partial

digest of lambda DNA, end-labelled with biotin, as a positive chemiluminescence control; the size fragments include (from top to bottom): 23.1, 9.4, 6.5, 4.3, 2.0 and 1.0 kb. The right side of the gel contains a Eco RI/Hind III digest of lambda DNA with the following size markers: 21.2, 5.1/5.0, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 0.95, 0.83 and 0.56 kb. The pTa 71- Eco RI digest produces fragments 10 and 3.6 kb as a positive rDNA hybridisation control, and the biotin end-labelled p35S - neo EL is a positive chemiluminescence control containing the size fragments: 2.5, 1.3, 1.1, 1.0, 0.4 and 0.2 kb. The methylation sensitive Hpa II, and insensitive Msp I recognise the same target sequence CCGG; however, Hpa II activity is inhibited by methylation of the internal cytosine (CmCGG), whereas Msp I activity recognises and cleaves this methylated site but it is inhibited by methylation of the external cytosine in the same target sequence (mCCGG or CmCGG). Eco RV is not a methylation sensitive enzyme, it recognises sites within the rDNA repeating unit to produce two main rDNA fragments necessary to assess the internal target sequence CCGG for cytosine methylation in rDNA genes (see below). This assay is described elsewhere (Harding *et al.* 1996). An examination of the Eco RV, the double restriction enzyme Eco RV/Hpa II and Msp I digestion profiles revealed an identical genomic fragment pattern. There were no obvious differences between these samples. This observation indicates a similar pattern in the methylation of the cytosine bases within the target sites of these restriction enzymes for genomic sequences.

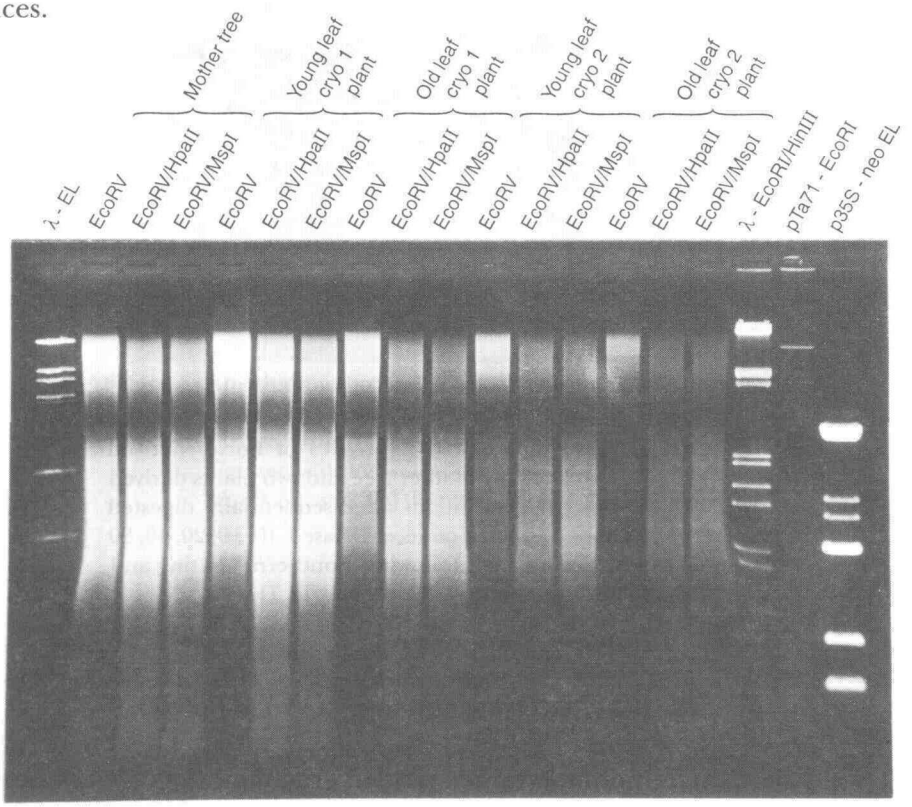

Figure 3. An ethidium bromide stained gel containing nuclei derived from mahogany mother tree and two plants derived from cryopreservation after digestion with Eco RV; Eco RV/Hpa II and Eco RV/Msp I. The molecular weight marker is represented by an lambda Eco RI/Hind III digestion.

Analysis of methylated ribosomal RNA genes

Figure 4 is a luminograph showing rDNA profiles of the mahogany mother tree and two trees derived from cryopreservation after digestion with Eco RV, Eco RV/ Hpa II and Eco RV/MsP I and hybridisation to pTa 71. As this luminograph is directly derived from a Southern blot of the gel shown in Figure 3, after hybridisation to an rDNA probe, the molecular weight markers and positive hybridisation and chemiluminescence controls are described in the text above. A comparison of the Eco RV digestions in all samples show identical rDNA profiles, the two rDNA fragments (8.0 and 2.5 kb) represent the repeating unit approximately 10.5 kb. The Eco RV/Hpa II and Eco RV/ Msp I were identical in the mother tree indicating methylation within the rDNA sequences. Eco RV/Hpa II digestion profiles of the young and old leaf material derived from cryopreserved plants 1 and 2 were identical and the rDNA fragments match those of the mother tree. However, Eco RV/MspI digestion profiles of the young and old leaf material derived from cryopreserved plants 1 and 2 were identical but differed from that of the mother tree. This comparison indicates distinct differences in patterns for methylation, CmCGG for cryopreserved plants 1 and 2 and mCmCGG for the mother tree. The fainter lower molecular weight fragments are less visible in cryopreserved plant 2, this was probably due to poor DNA transfer during Southern blotting and inefficient hybridisation to the rDNA probe. Nonetheless, a comparison of the Eco RV/Hpa II with the Eco RV/Msp I digestion profile of the young and old leaf material derived from cryopreserved plants 1 and 2 show complete digestion with Msp I suggesting preferentially methylation (CmCGG) of these rDNA sequences.

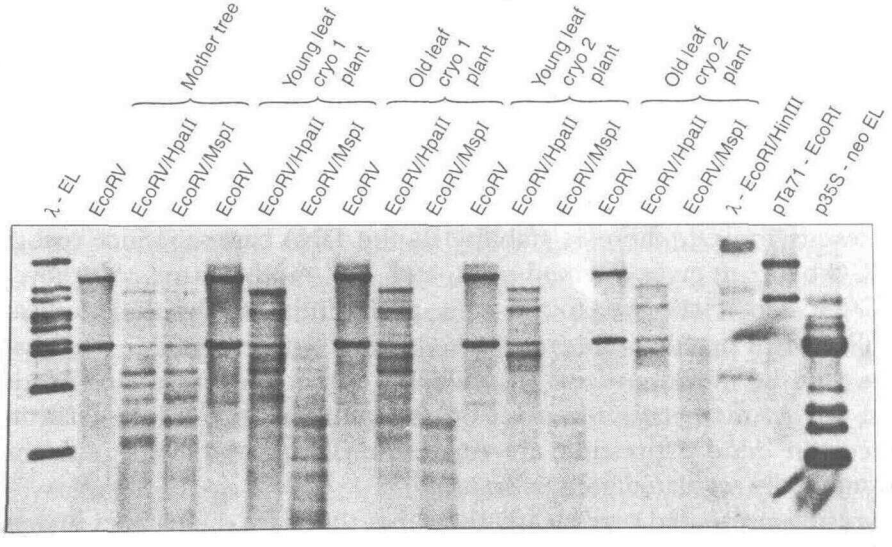

Figure 4. Luminograph showing rDNA profiles of mahogany mother tree and two plants derived from cryopreservation after digestion with Eco RV, Eco RV/Hpa II and Eco RV/Msp I and hybridisation to pTa 71. The molecular weight marker is represented by the biotin-end labelled lambda partial Hind III digestion.

Discussion

Cryopreservation involves tissue culture, pre-growth, cryoprotection, freezing, thawing, recovery (re-growth) and plant regeneration . The success of a cryogenic storage method is usually judged by the survival of the plant tissue and its ability to regenerate into morphologically normal and complete plants. However, as a consequence of the tissue culture-regeneration system, surviving plants may be subject to the effects of somaclonal variation (Scowcroft 1984, Scowcroft *et al.* 1984, Scowcroft 1985). Care must be taken to ensure that cryopreserved germplasm is regenerated using *in vitro* methods which do not predispose the material to genetic instability. In addition, the time taken to regenerate plants and the quality of germplasm recovered after cryopreservation are likely to be important features for the exploitation of germplasm recovered from cryogenic storage (Harding & Benson 1994).

Several molecular biological studies have been applied to detect genetic stability after cryopreservation by randomly amplified polymorphic DNA (RAPD-DNA) fingerprinting in the endangered shrub species, *Grevillea scapigera* (Touchell & Dixon 1996). RFLP analysis of 161 regrown plants representing old potato varieties from cryopreservation did not show abnormal banding patterns (Schafer *et al.* 1996). This offers considerable confidence to support the use of cryogenic storage methods for the conservation of plant genetic resources. Stability of the ribosomal RNA genes (rDNA) in potato plants after cryopreservation (Harding 1991) has been noted; however, variable rDNA hybridisation signals were also detected in some potato plants recovered from cryopreservation (Harding 1997) and are likely to be attributed to DNA methylation (Harding 1994).

The object of this study was to examine potential RFLP changes and possible alterations in chromatin structure and DNA methylation status of the ribosomal RNA genes in mahogany trees derived from cryopreservation. Following the use of the methylation assay successfully applied elsewhere (Harding *et al.* 1996), a comparison of the restriction enzyme (Hpa II/Msp I) digestion products (Figure 3) showed identical genomic fragments in DNA samples of the mother tree and cryopreserved trees. The rDNA fragments (Figure 4) showed identical (Hpa II) profiles for both cryopreserved trees and the mother mahogany tree. These results indicate there is stability in the DNA base sequence recognition site CCGG between cryopreserved trees and the mother tree. Moreover, the rDNA CCGG target site was found to be preferential methylated, indicated by the EcoRV/MspI profile in trees recovered from cryopreservation. This evidence suggests that the overall process (tissue culture to cryopreservation) produces a residual DNA methylation imprint on the genome; in the long-term its consequences on gene expression are unknown. This may have significance for developmentally regulated genetic traits.

The results presented here clearly show that there are differences in sensitivity to nuclease in chromatin between the mother tree and the trees recovered from cryopreservation (Figures 1 and 2). The DNase I activity will preferentially digest exposed linking DNA strands between histone proteins but also, at high

concentrations, it is active against the DNA strands surrounding these proteins (van Blokland *et al.* 1997). The higher the degree of resistance to DNAse I, the tighter the binding of the DNA strands to histone proteins within chromatin indicating organisational differences in higher order structures. The differences in nucleosomal patterns may well be reflective of changes at the chromosomal level which may alter the temporal sequence for active gene expression. The age of leaves (young or old) show differences in nucleosomal patterns. The ageing process appears to increase sensitivity to DNase I digestion to produce a regular pattern of relatively low molecular weight DNA fragments (2000–200 bp), as shown in plant 2 compared with plant 1 (Figure 1). This regular ordering is seen in the older mother tree. There are differences in chromatin structure between the young and old leaf samples. These differences in chromatin structure are due to ageing resulting from normal growth, development and senescence and not cryopreservation *per se*. This evidence indicates the importance of correct sampling for this type of analysis. The ageing process has been correlated with increasing levels of DNA methylation (Harding *et al.* 1996, Lambe *et al.* 1997, van Blokland *et al.* 1997). While this may be true for the 45-y-old mother tree (having a mCmCGG imprint), the trees recovered from cryopreservation revealed little evidence to support a correlation between DNA methylation and changes in chromatin structure. This study expands the use of molecular tools and apart from marking the 'bands on a gel' to confirm stability, it focuses on the application of techniques to examine those functional attributes essential for plant growth and development (Lambe *et al.* 1997). For example, plant characters, vigour, yield, maturity and height, are key features in agronomic performance required in plant breeding programmes, these features may well be influenced by factors affecting gene expression and chromatin structures. These investigations are especially important for tree species which have long life cycles.

The DNA profiles after Hpa II/Msp I digestion in old and young leaves of plants after cryopreservation show similar digestion products suggesting DNA methylation does not play a direct role in the ageing phenomenon (Figures 3 and 4). These observations do not preclude increases in DNA methylation of specific regions of the genome essential for the expression of transcriptional factors. The chromosomal regions of transcriptional inactivity have been associated with increases in DNA methylation (Finnegan *et al.* 1993, Bestor 1998). The imprinting of additional DNA methylation patterns with the normal methylation imprint on the genetic code of species may have long-term implications for gene expression if it is passed via seed or vegetative structures to the next generation of individuals (Harding 1994, Harding 1996, Harding *et al.* 1996). In the case of outbreeding trees, theoretically the F1 seedlings may well exhibit a new range of phenotypes according to the sequence and pattern of gene expression.

The research findings here show the stability and utility of ribosomal RNA gene sequences in trees regenerated from cryopreserved mahogany germplasm. Also, this work demonstrates the use of rDNA probes as effective molecular tools to examine trees regenerated from cryopreservation. Stability of these rDNA sequences supports the use of this cryopreservation method to conserve mahogany germ-

plasm. Moreover, rDNA markers can detect differential DNA methylation profiles and changes in chromatin structure. Future studies requiring the application of cryogenic methods to conserve tree species should consider these aspects of long-term, post-storage molecular stability. This is important as the application of *in vitro* cryo-conservation technologies is the most appropriate means of conserving mahogany seed germplasm which is recalcitrant to standard procedures in seed genebanks.

Acknowledgements

This work was supported by a British Council Academic CICHE Link between the Forest Research Institute Malaysia, the Universiti Kebangsaan Malaysia, the University of Abertay Dundee and the Scottish Crop Research Institute. The authors are very grateful to the British Council Office, Kuala Lumpur, for all their support.

References

APPANAH, S. & WEINLAND, G. 1993. In : *Planting Quality Timber Trees in Peninsular Malaysia - A Review.* Malayan Forest Records No. 38. ISBN 983-9592-17-3. Forest Research Institute Malaysia, Kepong, 52109, Kuala Lumpur, Malaysia.

BESTOR, T. H. 1998. Methylation meets acetylation. *Nature* 393:311–312.

FINNEGAN, E. J., BRETTELL, R. I. S. & DENNIS, E. S. 1993. The role of DNA methylation in the regulation of plant gene expression. Pp. 218–261 in Jost, J. P. & Saluz, H. P. (Eds.) *DNA Methylation:Molecular Biology and Biological Significance.* Birkhauser Verlag, Basel.

GERLACH, W. L. & BEDBROOK, J. R. 1979. Cloning and characterisation of ribosomal RNA genes from wheat and barley. *Nucleic Acids Research* 7(7):1869–1885.

HARDING, K. 1991. Molecular stability of the ribosomal RNA genes in *Solanum tuberosum* plants recovered from slow growth and cryopreservation. *Euphytica* 55:141–146.

HARDING, K. 1992. Detection of ribosomal RNA genes by chemiluminescence in *Solanum tuberosum* L.: a rapid and non-radioactive procedure for the characterisation of potato germplasm. *Potato Research* 35:199–204.

HARDING, K. 1994. The methylation status of DNA derived from potato plants recovered from slow growth. *Plant Cell, Tissue Organ Culture* 37:31–38.

HARDING, K. 1996. Approaches to assess the genetic stability of plants recovered from *in vitro* culture. Pp. 137–170 in Normah, M. N., Narimah, M. K. & Clyde, M. M. (Eds.) *Proceedings of the International Workshop on In Vitro Conservation of Plant Genetic Resources.* Plant Biotechnology Laboratory, Faculty of Life Sciences, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

HARDING, K. 1997. Stability of the ribosomal RNA genes in *Solanum tuberosum* L. plants recovered from cryopreservation. *Cryo-Letters* 18:217– 230.

HARDING, K. & BENSON, E. E. 1994. A study of growth, flowering, and tuberisation in plants derived from cryopreserved potato shoot-tips: implications for *in vitro* germplasm collections. *Cryo-Letters* 15:59–66.

HARDING, K. & Benson, E. E. 1995. Biochemical and molecular methods for assessing damage, recovery and stability in cryopreserved plant germplasm. Pp. 103–151 in Grout, B.W.W. (Ed.) *Genetic Preservation of Plant Cells In Vitro.* Springer-Verlag, Heidelberg.

HARDING, K., BENSON, E. E. & ROUBELAKIS-ANGELAKIS, K.A. 1996. Changes in genomic DNA and rDNA associated with the tissue culture of *Vitis vinifera*: a possible molecular basis for recalcitrance. *Vitis* 35(2):79–85.

LAMBE, P., MUTAMBEL, H. S. N., FOUCHE, J-G., DELTOUR, R., FOIDART, J-M. & GASPAR, T. 1997. DNA methylation as a key process in regulation of organogenic totipotency and plant neoplastic progression. *In Vitro Cellular & Developmental Biology* 33(3):155–162.

LIANG, W-Q. & FOURNIER, M. J. 1997. Synthesis of functional eukaryotic ribosomal RNAs in trans: development of a novel *in vivo* rDNA system for dissection ribosome biogenesis. *Proceeding National Academy of Science USA* 94:2864–2868.

LOPEZ-LEON, M. D., CABRERO, J. & CAMACHO, J. P. M. 1995. Changes in DNA methylation during development in the B chromosome NOR of the grasshopper *Eyprepocnemis plorans. Heredity* 74:296–302.

MAYHEW, J. E. & NEWTON, A. C. 1998. *The Silviculture of Mahogany.* ISBN 0-85199-307-9. CABI Publishing, CAB International, New York, USA.

MARZALINA, M. 1995. Penyimpanan Biji Benih Mahogani (*Swietenia macrophylla* King). Ph.D thesis, Universiti Kebangsaan, Malaysia.

MARZALINA, M., NORMAH, M. N., KRISHNAPILLAY, B., HO, O. K. & JUSOH, A. 1994. Ultrastructural changes in cryopreserved embryos of *Swietenia macrophylla.* In *Proceedings of the 4th Scientific Conference of the Electron Microscopy Society of Malaysia.* Malaysia.

NORMAH, M. N. & MARZALINA, M. 1996. Achievements and prospects of *in vitro* plant conservation for tree germplasm. Pp. 253–261 in Normah, M. N., Narimah, M. K. & Clyde, M. M. (Eds.) *Proceedings of the International Workshop on In Vitro Conservation of Plant Genetic Resources.* Plant Biotechnology Laboratory, Faculty of Life Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

PAUL, A. & FERL, R. J. 1988. Assays for studying chromatin structure. *Plant Molecular Biology Manual* B2:1–11.

POWELL, W., MORGANTE, M., ANDRE, C., HANAFEY, M., VOGEL, J., TINGEY, S. & RAFALSKI, A. 1997. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding* 2:225– 238.

VAN BLOKLAND, R., TEN LOHUIS, M. & MEYER, P. 1997. Condensation of chromatin in transcriptional regions of an inactivated plant transgene:evidence for an active role of transcription in gene silencing. *Molecular and General Genetics* 257:1–13.

SCHAFER, A., MULLER, E. & MIX-WAGNER, G. 1996. Cryopreservation: an alternative for the long-term storage of old potato varieties. *Potato Research* 39:507–513.

SCOWCROFT, W. R. 1984. *Genetic Variability in Tissue Culture: Impact on Germplasm Conservation and Utilisation.* Report (AGPG:IBPGR/84/152). International Board for Plant Genetic Resources, Rome.

SCOWCROFT, W. R. 1985. Somaclonal variation: the myth of clonal uniformity. Pp. 217–243 in Hohn, B. & Dennis, E. S. (Eds.) *Genetic Flux in Plants.* Springer-Verlag, Wien, New York.

SCOWCROFT, W. R., RYAN, S. A., BRETTEL, R. I. S. & LARKIN, P. J. 1984. Somaclonal variation: a 'new' genetic resource. Pp. 258–267 in Holden, J. H. W. & Williams, J. T. (Eds.) *Crop Genetic Resources: Conservation and Evaluation.* George Allen and Unwin Ltd., London.

SOERIANEGARA, I. & LEMMENS, R. H. M. J. 1992. *Prosea 5.* Pudoc Scientific Publishers, Wageningen, Holland.

TOUCHELL, D. H. & DIXON, K. W. 1996. Cryopreservation for the conservation of Australian endangered plants. Pp. 169–180 in Normah, M. N., Narimah, M. K. & Clyde, M. M. (Eds.) *Proceedings of the International Workshop on In Vitro Conservation of Plant Genetic Resources.* Plant Biotechnology Laboratory, Faculty of Life Sciences, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

WANZENBOCK, E-M., SCHOFER, C., SCHWEIZER, D. & BACHMAIR, A. 1997. Ribosomal transcription units integrated via T-DNA transformation associate with the nucleolus and do not require upstream repeat sequences for activity in *Arabidopsis thaliana. Plant Journal* 11(5):1007–1016.