# Investigating Evidence for UV-B Induced Mutagenesis in *Dimorphotheca sinuata* Using the *18S* rDNA and *rbc*L Gene Sequences

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**ABSTRACT**

The nuclear *18S* rDNA and chloroplast encoded *rbc*L gene sequences were used to investigate the evidence of residual UV-B induced mutagenesis in *Dimorphotheca sinuata* plants. The probes were generated by the polymerase chain reaction and labelled with a non-radioactive Digoxigenin label and used in Southern hybridization studies. High levels of variability in the *18S* rDNA gene were found, pointing to genome rearrangements and possibly genome instability due to UV-B effects.

***Key words:*** UV-B, DNA damage, recombination, DNA repair, pyrimidine dimers, mutagenesis.

## Introduction

Predicted increases in solar UV-B radiation have served to focus attention on the cytotoxic effects of UV-B on plants[8,29,33,37,38]. This is because DNA is considered a primary absorbing chromophore in plant cells in the UV-B region of the spectrum and DNA is a highly reactive molecule that is prone to damage from a wide range of both physical and chemical agents. Nuclear DNA is present in very low copy number and acts as a template for its own synthesis and because of this, it is a vulnerable target for UV-induced damage[36].

Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone photoproducts make up the majority of UV-induced DNA damage products and both are toxic lesions. CPDs are formed when adjacent pyrimidines become covalently linked by the formation of a four-membered ring upon UV exposure and have a stable confirmation which is resistant to pH and temperature extremes[21,36]. Lesions alter the structure of DNA and consequently interfere with critical aspects of DNA metabolism such as transcription, replication and recombination. Even a single persisting UV-induced lesion can be a potentially lethal event, particularly in haploid tissue such as pollen grains[4-6].

At the molecular level, pyrimidine dimers are known to inhibit the progress of microbial and mammalian DNA polymerases. Since pyrimidine dimers cannot effectively base pair with other nucleotides, they are not directly mutagenic, but instead act as blocks to DNA replication and transcription, as RNA polymerases have been reported to stall at the site of these photoproducts[6]. Unrepaired dimers are lethal to the cells because they deform the DNA helix and interfere with both replication and transcription. Thus a single pyrimidine dimer, if left unrepaired, is sufficient to completely eliminate the expression of a transcriptional unit.

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Although reproductive organs are generally considered to be well protected from UV-B during developmental and maturation phases, long term exposure to high UV-B levels may affect the reproductive structures of plants and cause DNA damage. Mature pollen grains are potentially very susceptible to UV-B induced DNA damage during the short period between anther dehiscence and pollen tube penetration into stigma tissues[14]. It is therefore proposed that radiation damage to DNA might be transmitted via the seeds and pollen[23,24] and result in morphological, physiological and genetic changes in plants[25-27].

UV radiation also affects adjacent thymine bases of DNA, linking them together to form dimers which block restriction endonuclease recognition or cleavage for those enzymes that recognise sites containing adjacent thymines. Since UV-B is known to affect adjacent pyrimidine bases on the DNA by linking them to form dimers, Harlow *et al.*[12] postulated that an assay that targeted pyrimidine dimers would aid in the detection of DNA mutations directly linked to UV-damage. Therefore, genetic analysis of pyrimidine dimer (T=T) formation in *D. sinuata* plants was conducted using the *Dra*I assay method of Harlow *et al.*[41]. The *Dra*I assay is based on Whittaker and Southern's[41] finding that restriction enzyme activity can be inhibited by the presence of DNA damage at the recognition sequence. Since any alteration of the bases within this recognition sequence would be expected to inhibit cleavage, UV irradiation of substrate DNA should destroy potential cleavage sites. Partial DNA digests therefore result when UV-irradiated DNA is digested with enzymes whose recognition sequences contain adjacent thymidines. This assay was developed for comparing induction of UV damage in a *uvh1* (an *Arabidopsis* mutant hypersensitive to UV light and ionising radiation) and wild-type plants[14] and, to determine if DNA was less protected from UV damage in *uvh1* plants than in wild-type plants.

The genetic analysis employed in this study involved investigations of *D. sinuata* chromosomal DNA for evidence of UV-induced mutagenesis. To determine the dimer content of *Dra*I sites or UV-B-induced mutations in specific DNA fragments, suitable hybridisation probes were used, *18S* rDNA and *rbcL* genes in this case. Since the probe sequences do not contain *Dra*I sites, only one band is to be expected following complete digestion of plant DNA with *Dra*I[14]. The appearance of partially digested bands is a function of UV fluence and amount of dimers present in the DNA or UV-B induced mutations at dimer sites. The proportion of damaged molecules thus represents the average frequency of dimer production in one or more of *Dra*I sites that flank the probed region. The advantage of this assay lies in the fact that only specific sites flanking the gene of interest are being investigated unlike, other methods which look at all regions of the genome. Examples of such methods are amplified fragment length polymorphisms (AFLPs), restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA sequences (RAPDs). These methods suffer the main drawback in that they tend to be too general. In this study, plants were grown in the absence of UV-B radiation and the aim of the study was to assay for evidence of UV-B induced mutagenesis.

## Materials and methods

### *Germination of seed material and growth of plants.*

Populations of the desert annual *D. sinuata*, derived from a common seed stock (Generation 0) obtained from populations in the National Botanical Gardens, Kirstenbosch, Cape Town, South Africa, were exposed concurrently over three successive generations to either ambient (representing no stratospheric ozone depletion), or elevated (representing 20% stratospheric ozone depletion) UV-B levels during the complete life cycles [27]. The ambient UV-B group was exposed to UV-B fluences approximating those received daily over the natural growing period of *D. sinuata* at its southerly distribution limit (33°56'S, 18°29'E: Cape Town, South Africa) (a seasonal range of 2.55-8.85 kJ/m$^2$/day), while the enhanced UV-B group was exposed to UV-B fluences simulating those at the northerly distribution limit of this species (26°38'S, 16°18'E: Aus, Namibia) (a seasonal range of 4.70-11.41 kJ/m$^2$/day) (Musil, 1996). The UV treatments lasted over the full species' growth cycle and were given over the natural growing period (mid winter or late spring).

The first two generations were grown in the absence of natural UV-radiation in a polycarbonate-cladded greenhouse (with no transmission below 400 nm) where UV-B radiation at ambient and enhanced levels was supplied exclusively from artificial sources. Peak daily photosynthetic photon flux densities (PFD) in the greenhouse ranged seasonally (spring to midsummer) from 600 to 1800 mmol/m$^2$/s[27]. Lamps above treatment plants were filtered with 0.075 mm thick cellulose acetate film (Coutaulds Chemicals, Derby, UK) with transmission down to 290 nm (which was replaced weekly). For control plants receiving ambient UV-B levels, lamps were filtered with 0.12 mm thick Mylar –D film (DuPont De Nemours, Wilmington, Delaware, USA) with no transmission below 316 nm. This was done in accordance with reports on the importance of UV-A radiation and total photon flux ratios of UV-B:UV-A and UV-B:PFD as mitigating factors in plant responses to UV-B[9,19]. In the third generation

(Generation 3), these population groups were exposed to either ambient or ambient plus elevated levels of UV-B outdoors in an open natural setting, simulating approximate field growth conditions.

Seeds from generation 0 and the third generation were soaked for five minutes in a 5% solution of sodium hypochlorite and rinsed five times in distilled water. The seeds were then placed on five layers of moistened Whatman filter paper on petri dishes and these were sealed with paraffin-wax film to minimise evaporation. Seeds were germinated in the dark for three days before being transferred to potting medium comprising coarse sand, leaf mould and loam (2:1:1, v:v) in 20 cm diameter pots and watered daily thereafter. The standard conditions in the growth room were as follows: temperature = 22°C, relative humidity = 65%, 16 hours light and 8 hours darkness with a light intensity of ± 100 mmol/$m^2$/s. After six weeks, leaf samples were taken for DNA analysis.

*Isolation and quantitation of total plant DNA*

All standard DNA manipulations were performed as described by Sambrook *et al.*[34], with some minor modifications and according to specifications of the manufacturers and suppliers of the DNA modifying enzymes (Roche Diagnostics GmBH, Amersham or Promega). DNA was isolated from the original seed stock (Generation 0) and control (G3A) and test plants (G3H) from Generation 3.

Fresh plant material (100 mg) from *D. sinuata* plants was ground to a fine free flowing powder in a mortar in the presence of liquid nitrogen. The powder was transferred to an eppendorf tube and 750 ml of extraction buffer (100 mM Tris, 50 mM EDTA, 0.5 M NaCl, 10 mM b-mercaptoethanol) and 50 ml of 20% SDS (w/v) were added. This was mixed thoroughly by vortexing. Ten ml of RNAse A (10 mg/ml) was added and the tube was shaken to mix the contents. The tubes were incubated at 65°C in a water bath for 10 minutes. The mixture was then emulsified in an equal volume of phenol/chloroform and centrifuged at 14 000 rpm for 10 minutes. The aqueous phase was then extracted with an equal volume of chloroform and centrifuged for 10 minutes at 14 000 rpm. One-tenth volumes of 3 M sodium acetate, pH 5.2 was added to the aqueous phase which was then precipitated with 2.5 volumes of ice cold ethanol.

The DNA pellet was washed with 70% ethanol and resuspended in TE buffer (pH 8.0). DNA concentrations were determined by measuring the absorbance at 260 nm, or by fractionating DNA aliquots on a gel against known concentrations of l-DNA standards and quantifying densitometrically. Equal amounts (2 mg) of DNA were routinely digested to completion with *Dra*I overnight, separated by electrophoresis and then transferred to positively charged nylon hybridisation membranes (Hybond+) (Roche Diagnostics GmBH, Mannheim, Germany) according to standard procedures[34].

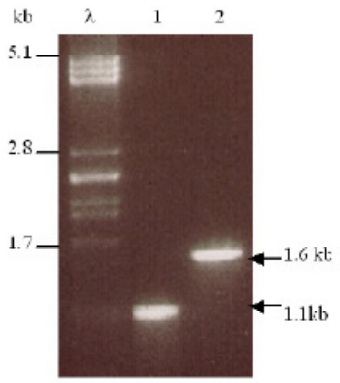


**Fig. 1:** PCR products used as probes in the study. Lane 1 = rbcL, lane 2 = 18S rDNA. The two PCR products were 1.6kb (18S rDNA) and 1.1 kb (rbcL). Lambda DNA digested with PstI (lane) was used as molecular weight standard. The sizes of the different fragments are shown on the right.

*Generation of the 18S rDNA and rbcL probes*

The nuclear-encoded (*18S* rDNA) and chloroplast encoded *rbc*L gene probes were used in the assay. The probes were generated by the polymerase chain reaction (PCR) from seedling genomic DNA of *D. sinuata* (Figure 1). The DNA used for making probes was isolated from plants from Generation 0, the parental seed stock from which all experimental material was derived.

*18S rDNA probe*

Oligonucleotide primers designed to complement an internal fragment of the *18S* rDNA gene sequence from *Arabidopsis thaliana*[39] were used to produce a 1.6-kb fragment of the *18S* rDNA gene. The sequence of the forward primer (primer 1) was 5'- GTG TAA GTA TGA ACG AAT TC–3' and that of the reverse primer (primer 2) was 5' GGAATT CTT CGT TGA AGA CC 3'. The PCR conditions were as follows: 70 ng template DNA; 5 ml of 10 mM primer 1; 5 ml of 10 mM primer 2; 4.0 ml of 5 mM dNTPs; 8 ml of 25 mM $MgCl_2$; 10 ml 10X PCR buffer; 0.5 ml Taq polymerase (5U/ml). Roche

Diagnostics GmBH, Mannheim, Germany, supplied reagents used for PCR. The total reaction volume was made up to 100 ml with distilled water and then overlaid with mineral oil to eliminate evaporation. The PCR cycle profile was as follows: initial denaturation at 94°C for 60 seconds, followed by 30 cycles of denaturation at 94°C for 60 seconds, primer annealing at 50°C for 30 seconds and PCR product extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. PCR products were visualized by running 2 ml of the reaction mixture on a 1% agarose gel.

*rbcL gene probe*

A 1.1-kb fragment of the *D. sinuata rbc*L gene was amplified from genomic DNA by PCR (see Figure 1). Oligonucleotide primers designed to complement an internal fragment of the *rbc*L sequence from the grain amaranth, *Amaranthus hypochondriacus*[17] were used. The sequence of the forward *rbc*L primer (primer 1) was 5'-GAT ATC TTG GCA GCA TTC CG–3' and that for the reverse primer (primer 2) was 5'–TGT CCT AAA GTT CCT CCA CC–3'. The PCR conditions and visualization were as for the *18S* rDNA probe.

*Cloning and sequencing of the 18S rDNA PCR products*

Both PCR products were fractionated in a 1% agarose gel by electrophoresis and the fragment was excised and purified using the Geneclean II® Kit according to the manufacture's protocol (BIO 101 Inc. La Jolla, USA). These were then cloned into the *Eco*RV site of pSK (p-Bluescript) by blunt end ligation and named p18S and prbcL for the *18S* rDNA and *rbc*L fragments, respectively. The authenticity of the cloned *18S* rDNA and *rbc*L gene fragments was determined by end sequencing. DNA sequencing was done by the dideoxy chain-termination method of Sanger *et al.*[35] with a sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). This method is utilised for sequencing clones in M13 based vectors. All reactions were performed according to the manufacturer's instructions and cycle sequenced using the GeneAmp PCR System 9700 amplifier (Perkin Elmer Applied Biosystems). The data was processed by ALFwin version 2.1 software, (Amersham Pharmacia Biotech) and homology searches and sequence analysis was done using the BLAST programme[2].

*Labelling of probes and Southern hybridization*

The probes used for detection were DIG-labelled[13] either by the random-priming method or through PCR incorporation according to the supplier's protocol (Roche Diagnostics GmBH, Mannheim, Germany). All Southern hybridization steps were done according to standard procedures[34]. Hybridizations were done at 42°C overnight in DIG-Easy Hyb solution according to the supplier's protocol (Roche Diagnostics GmBH, Mannheim, Germany). At the end of the hybridisation, the membrane was washed twice, 5 minutes per wash in 2X wash solution (0.3 M NaCl, 30 mM sodium citrate; pH 7.0, containing 0.1% SDS (w/v)) at room temperature. After these low stringency washes, high stringency washes were carried out by washing the membrane twice, 15 minutes per wash in 0.1X wash buffer (15 mM NaCl, 1.5 mM sodium citrate; pH 7.0, containing 0.1% SDS (w/v)) (prewarmed to 65°C) at 65°C.

After the post-hybridisation washes, membranes were treated with blocking reagent for 30 minutes to prevent non-specific attraction of the antibody to the membrane. Membranes were then incubated with a dilution of anti-digoxigenin Fab fragments conjugated to alkaline phosphatase for 30 minutes (Roche Diagnostics GmBH, Mannheim, Germany) followed by two washes in Buffer 1 (100 mM maleic acid, 150 mM NaCl; pH 7.5) + 0.3% Tween® 20 for 15 minutes each at room temperature. The membranes carrying the hybridised probe and bound antibody conjugate were finally equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for two minutes, before being reacted with the chemiluminescent substrate, CSPD and exposed to X-ray film to record the chemiluminescent signal.

## Results

*Generation of the* 18S *rDNA and* rbc*L probes*

The amplified products of the *18S* rDNA and *rbc*L fragments are shown in Figure 1.

*Cloning and sequencing the 18S rDNA and rbcL gene probes*

The *18S* rDNA gene sequence was verified by end-sequencing approximately 400 bp of the 3'-end of the cloned PCR product (plasmid p18S). The *18S* rDNA gene sequence was found to be >95% identical to the reported *A. thaliana 18S* rDNA gene[39]. The *rbc*L fragment (prbcL) was sequenced from both ends and was found to have over 98% identity to a reported sequence of *D. pluvialis*, a close relative at the nucleotide level.

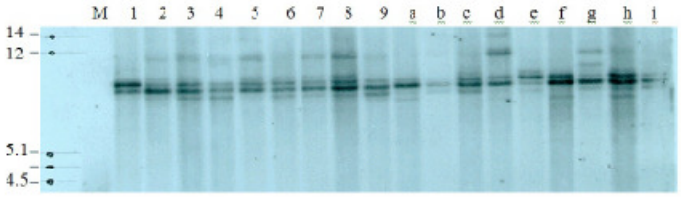


**Fig. 2:** The DraI assay. A representative southern blot showing genomic DNA digested with DraI and probed with a DIG-labelled 18S rDNA probe. Lane M is the molecular weight marker while lanes 1 to 9 represent plants from ambient UV-B (G3A), and lanes a to i are plants from the enhanced UV-B group (G3H). The molecular weight sizes are shown to the left of the figure.


**Fig. 3:** The DraI assay. A representative blot of genomic DNA from Generation 0 digested with DraI and probed with a DIG-labelled rbcL probe.

*The DraI assay*

Gels were blotted overnight and probed with a DIG-labelled *18S* rDNA probe (Figures 2 and 3 respectively). The *18S* rDNA probe hybridised to several high molecular weight bands ranging in size from 7 to 11.5 kb (Figure 2). Hybridizing the same blot with the *rbc*L probe gave a single intense band of approximately 7.51 kb (Figure 3). A total of 101 DNA samples from individual seedlings were analysed. These consisted of 45 samples from the original seed batch, 22 G3A plants (representing three generations of ambient UV-B irradiation) and 34 G3H plants (three generations of enhanced UV-B irradiation. A total of 30 DNA samples isolated from plants grown from the original seed batch (G0 or control plants) and another 30 from the enhanced UV-B group (G3H) were digested with *Dra*I and probed with the *rbc*L gene. Genomic DNA from both enhanced UV-B group and control samples consistently showed a single intense band of approximately 7.5 kb (Figure 3).

**Discussion**

The *Dra*I assay is a technique that is used in assaying genomic DNA for evidence of UV-induced mutations. It was used in this study to analyze variations in the two study populations. The assay is based on the premise that loss of *Dra*I sites would point to evidence for targeted damage by UV. If *Dra*I sites are lost, one would anticipate higher molecular weight bands in plants with an enhanced UV-B exposure history. Both chloroplast (*rbc*L) and nuclear (*18S* rDNA) probes were used. The significance of evaluating a chloroplast as well as a nuclear gene is that mutations in the *rbc*L gene would have been contributed to the seed genetic material through maternal DNA as chloroplasts are maternally inherited[15].

The *18S* rDNA gene is a single copy gene arranged in tandem repeat. For this reason, intense signals were always obtained and the probe routinely produced bands ranging both in size and number. This result, however, is contrary to what was proposed in the work by Harlow *et al.*[12] in which a single band was found when control samples were hybridized with the *18S* rDNA. Harlow *et al.*[12] attributed the appearance of partial bands to UV-B effects. In this study a number of bands were found in the control samples that had no known history of UV-B exposure, indicating the presence of variation in the arrangement of the *18S* rDNA tandem repeats of *D. sinuata*, hence the unsuitability of the *18S* rDNA as a probe in the *Dra*I assay.

One well documented result of UV-B is the appearance of double-strand breaks (DSBs) in DNA [28,18] and pyrimidine dimers. The cyclobutane pyrimidine dimer (CPD) (and possibly DSBs) burden resulting from sublethal doses of UV-B may inhibit plant growth and development by slowing transcription and mitosis through imposing energetic costs associated with DNA repair. These DSBs can be repaired via several repair pathways but the most economical is simple

ligation with another available DNA strand. This pathway is preponderant in higher eukaryotes although it has serious disadvantages since eventually it is accompanied by the loss of genetic material and may even lead to gross chromosomal re-arrangements. The variability observed with the *18S* rDNA probe could have arisen from this. Ries *et al.*[30,31] reported that elevated solar UV-B does increase the frequency of somatic homologous DNA rearrangements in *Arabidopsis* and tobacco plants. Increases in recombination were accompanied by strong induction of photolyase and *Rad*51 gene expression. Both genes are putatively involved in major DNA repair pathways-photo reactivation and recombination[7,40].

Basal variation in Generation 0 samples indicated that there was too much variability stemming from the arrangement of the *18S* rDNA gene for conclusive deductions to be made from the study using this assay. Similar levels of variability were observed when control plants (Generation 3A) were compared with test plants (Generation 3H), suggesting that there is inherent variation in the arrangement of the *18S* rDNA gene and that this was not necessarily a result of stressful growth conditions in the greenhouse. However, the UV-B irradiated samples (G3H), showed slightly greater variability than ambient levels (G3A), even though no bigger bands were evident. This variation could be a result of stress-induced re-arrangement or duplication in the *18S* rDNA gene as opposed to evidence of pyrimidine dimer formation or UV-B induced mutations resulting in loss of *Dra*I sites[22]. Since the maintenance of genetic integrity is essential for cellular survival, it could be that an efficient repair mechanism, such as homologous recombination repair pathways, might be involved in eliminating UV-B induced DNA lesions in the study plants.

Variation in the number of subrepeat elements per repeat unit probably accounts for much of the differences observed when genomic DNA is probed with the *18S* rDNA. The variability in the *18S* rDNA possibly arises from RNA duplications, gene rearrangements and variation in the number of tandem repeats. Only minor variations have been reported in the coding regions of rDNA within a species, though variation is common in the intergenic spacer resulting from a series of repetitive elements in the nontranscribed spacer (NTS) region. Moreover, since rDNA is a repetitive DNA sequence within the genome, individuals may contain different length variants[1,3,32]. The existence of differences between rDNA repeats of a single genotype in pea with respect to the degree of base modification at certain restriction sites and incomplete cleavage of genomic rDNA have been reported resulting mostly from methylation[15]. It would be very interesting to look at the effect of UV-B on DNA methylation as this would also help explain the possibility of sequence variation being the cause of observed differences in this study.

The *rbc*L gene is a single copy chloroplast-encoded gene which, like the *18S* rDNA, gives an intense signal due to the multicopy nature of the chloroplast genome. Since no differences could be detected with this probe, this could be an indication that the actual *rbc*L gene is not mutated or that any original mutations are either lethal or are never seen. Previous studies on biochemical and physiological aspects of *D. sinuata*[27] pointed to a facet of the photosynthetic machinery being possibly affected by UV-B radiation. Therefore, if the *rbc*L gene has not been mutated, perhaps the regulation of genes involved in photosynthesis may have been affected. This could explain the biochemical variations observed in plant assays.

An alternative approach would be to employ the use of a pyrimidine dimer specific endonuclease to detect pyrimidine dimers directly in DNA. However, this can only be used in situations where the DNA is irradiated and then immediately assayed for CPDs as was the case in the study by Harlow *et al,*[12]. The other alternative may be to use the RAPDs to identify polymorphisms in the genome. Cullis *et al.*[11] have studied the occurrence of environmentally induced changes in certain flax varieties and these were found to be accompanied by changes in the genomic DNA. Such techniques look at changes in the whole genome and are not specific. In this instance, we were looking for evidence of residual mutations at dimer sites after the plants had been irradiated over several generations and then grown in a greenhouse in the absence of UV-B radiation.

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## References

1. Agarwal, M.L., J. Aldrich, A. Agarwal and C.A Cullis, 1992. The flax ribosomal RNA-encoding genes are arranged in tandem repeat at a single locus interspersed by "non-rDNA" sequences. Gene, 120: 151-156.

2. Altschul, S.F., T.L. Madden, A.A. Schaffer, Z. Zhang, W. Miller and D.J. Lipman, 1997. PERLINK"http://nar.oupjournals.org/cgi/reprint/25/17/3389.pdf"Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, 25: 3389-3402.
3. Amarger, V. and L. Mercier, 1996. Nuclear ribosomal DNA unit length variation: a putative marker of genetic diversity in jojoba. International Journal of Plant Sciences, 157: 296-302.
4. Britt, A.B., 1995. Repair of DNA damage induced by ultraviolet radiation. Plant Physiology, 108: 891-895.
5. Britt, A.B., 1996. DNA damage and repair in plants. Annual Review of Plant Physiology and Plant Molecular Biology, 47: 75-100.
6. Britt, A.B., 1997. Genetic analysis of DNA repair in plants. In Lumsden P (ed.). *Plants and UV-B: Responses to environmental change*, Cambridge University Press. pp: 77-93.
7. Britt, A.B., 1999. Molecular genetics of DNA repair in plants. Trends in Plant Sciences, 4: 20-24.
8. Caldwell, M.M., 1981. Plant response to solar ultraviolet radiation. Encyclopaedia of Plant Physiology, 12A: 169-197.
9. Caldwell, M.M., S.D. Flint and P.S. Searles, 1994. Spectral balance and UV-B sensitivity of soybean: a field experiment. Plant, Cell and Environment, 17: 267-276.
10. Caldwell, M.M., A.H. Teramura, M. Tevini, J.F. Bornman, L.O. Bjorn and G. Kulandaivelu, 1995. Effects of increased solar ultraviolet radiation on terrestrial plants. Ambio, 24: 166-173.
11. Cullis, C.A., S. Swami and Y. Song, 1999. RAPD polymorphisms detected among the flax genotrophs. Plant Molecular Biology, 41: 795-800.
12. Harlow, G.R., M.E. Jenkins, T.S. Pittalwala and D.W. Mount, 1994. Isolation of *uvh1*, an *Arabidopsis* mutant hypersensitive to ultraviolet light and ionizing radiation. The Plant Cell, 6: 227-235.
13. Holtke, H.J., W. Ankenbauer, K. Muhlegger, R. Rein, G. Sagner, R. Seibel and T. Walter, 1995. The Digoxigenin (DIG) system for non-radioactive labelling and detection of nucleic acids - an overview. Cellular and Molecular Biology, 41: 883-905.
14. Jacksor, J.F., 1987. DNA repair in pollen – A review. Mutation Research, 181: 17-29.
15. Jorgensen, R.A., R.E. Cuellar, W.F. Thompson and T.A. Kavanagh, 1987. Structure and variation in ribosomal RNA genes of pea: characterisation of a cloned rDNA repeat and chromosomal rDNA variants. Plant Molecular Biology, 8: 3-12.
16. McIntosh, L., C. Poulsen and L. Bogorad, 1980. Chloroplast gene sequence of the large subunit of ribulose bisphosphatecarboxylase of maize. Nature 288: 556-560.
17. Michalowski, C.B., H.J. Bohnert, D.F. Klessig and J.O. Berry, 1990. Nucleotide sequence of *rbcL* from *Amaranthus hypochondriachus* chloroplasts. Nucleic Acids Research, 18: 2187.
18. Michel, B., S.D. Ehrlich and M. Uzzest, 1997. DNA double-strand breaks caused by replication arrest. The EMBO Journal, 16: 430-438.
19. Middleton, E.M. and A.H. Teramura, 1993. The role of flavonol glycosides and carotenoids in protecting soybean from UV-B damage. Plant Physiology, 103: 741-752.
20. Midgley, G.F., S.J.E. Wand and C.F. Musil, 1998. Repeated exposure to enhanced UV-B radiation in successive generations increases developmental instability (fluctuating asymmetry) in a desert annual. Plant, Cell and Environment, 21: 437-442.
21. Mitchell, D.L. and R.S. Nairn, 1989. The biology of the (6-4) photoproduct. Photochemistry and Photobiology, 49: 805-819.
22. Mpoloka, S.W., V.A. Abratt, S.G. Mundree, J.A. Thomsom and C.F. Musil, 2007. Potential Effects of Prolonged ultraviolet radiation exposure in plants: chloroplast DNA analysis. American-Eurasian Journal of Agricultural and Environmental Sciences, 2: 437-441.
23. Mulcahy, D.L., 1971. A correlation between gametophytic and sporophytic characteristics in *Zea mays* L. Nature, 171: 1155-1156.
24. Mulcahy, D.L., 1974. Correlation between speed of pollen tube growth and sedling height in *Zea mays* L. Nature, 249: 491493.
25. Musil, C.F. and S.J.E. Wand, 1994. Differential stimulation of an arid-environment winter ephemeral *Dimorphotheca pluvialis* (L.) Moench by ultraviolet-B radiation under nutrient limitation. Plant, Cell and Environment, 17: 245-255.
26. Musil, C.F., 1995. Differential effects of elevated ultraviolet-B radiation on the photochemical and reproductive performances of dicotyledonous and monocotyledonous arid-environment ephemerals. Plant, Cell and Environment, 18: 844-854.
27. Musil, C.F., 1996. Cumulative effect of elevated ultraviolet-B radiation over three generations of the arid environment ephemeral *Dimorphotheca sinuata* DC (Asteraceae). Plant Cell and Environment, 19: 1017-1027.
28. Puchta, H., B. Dujon and B. Hohn, 1995. Two different bu related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proceedings of the National Academy of Sciences, 93: 5055-5060.

29. Quaite, F.E., B.M. Sutherland and J.C. Sutherland, 1992. Action spectrum for DNA damage in alfalfa lowers predicted impact of ozone depletion. Nature, 358: 576-578.
30. Ries, G., W. Heller, H. Puchta, H. Sandermann, H.K. Seidlitz and B. Hohn, 2000a. Elevated UV-B radiation reduces genome stability in plants. Nature, 406: 98-101.
31. Ries, G., G. Buchholz, H. Frohnmeyer and B. Hohn, 2000b. UV-B-mediated induction of homologous recombination in *Arabidopsis* is dependent on photosynthetically active radiation. Proceedings of the National Academy of Sciences, 97: 13425-13429.
32. Rogers, S.O. and A.J. Bendich, 1987. Ribosomal RNA genes in plants:variability in copy number and in the intergenic spacer. Plant Molecular Biology, 9: 509-520.
33. Rozema, J., J. van de Staaij, Bjorn Lo and M.M. Caldwell, 1997. UV-B as an environmental factor in plant life: stress and regulation. Trends in Ecology and Evolution, 12: 22-28.
34. Sambrook, J., E.E. Fritsch and T. Maniatis, 1989. *Molecular cloning: a laboratory manual,* 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Sanger, F., S. Niklen and A.R. Coulson, 1977. Proceedings of the National Academy of Sciences, USA 74: 5463-5467.
36. Taylor, R.M., A.K. Tobin and C.M. Bray, 1997. DNA damage and repair in plants. *Plants and UV-B: Responses to environmental change.* Edited by Lumsden P, Cambridge University Press.
37. Tevini, M., 1993. In *UV-B Radiation and Ozone Depletion: Effects on Humans, Animals and Plants, Microorganisms and Materials.* (ed. Tevini M.) 125-153 (CRC, Press, Boca Raton; Lewis Publishers, USA)
38. Tevini, M. and A.H. Teramura, 1989. UV-B effects on terrestrial plants. Photochemistry and Photobiology, 50: 479-487.
39. Unfried, I., U. Stocker and P. Gruendler, 1989. Nucleotide sequence of the *18S* rRNA gene from *Arabidopsis thaliana* co10. Nucleic Acids Research, 17: 7513.
40. Vispe, S., C. Cazux, C. Lesca and M. Defais, 1998. Over expression of *Rad*51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation. Nucleic acids Research, 26: 2859-2864.
41. Whitaker, P.A. and E.M. Southern, 1986. UV irradiation of DNA: A way of generating partial digests for rapid restriction site mapping. Gene, 41: 129-134.

29. Quaite, F.E., B.M. Sutherland and J.C. Sutherland, 1992. Action spectrum for DNA damage in alfalfa lowers predicted impact of ozone depletion. Nature, 358: 576-578.
30. Ries, G., W. Heller, H. Puchta, H. Sandermann, H.K. Seidlitz and B. Hohn, 2000a. Elevated UV-B radiation reduces genome stability in plants. Nature, 406: 98-101.
31. Ries, G., G. Buchholz, H. Frohnmeyer and B. Hohn, 2000b. UV-B-mediated induction of homologous recombination in *Arabidopsis* is dependent on photosynthetically active radiation. Proceedings of the National Academy of Sciences, 97: 13425-13429.
32. Rogers, S.O. and A.J. Bendich, 1987. Ribosomal RNA genes in plants:variability in copy number and in the intergenic spacer. Plant Molecular Biology, 9: 509-520.
33. Rozema, J., J. van de Staaij, Bjorn Lo and M.M. Caldwell, 1997. UV-B as an environmental factor in plant life: stress and regulation. Trends in Ecology and Evolution, 12: 22-28.
34. Sambrook, J., E.E. Fritsch and T. Maniatis, 1989. *Molecular cloning: a laboratory manual,* 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Sanger, F., S. Niklen and A.R. Coulson, 1977. Proceedings of the National Academy of Sciences, USA 74: 5463-5467.
36. Taylor, R.M., A.K. Tobin and C.M. Bray, 1997. DNA damage and repair in plants. *Plants and UV-B: Responses to environmental change.* Edited by Lumsden P, Cambridge University Press.
37. Tevini, M., 1993. In *UV-B Radiation and Ozone Depletion: Effects on Humans, Animals and Plants, Microorganisms and Materials.* (ed. Tevini M.) 125-153 (CRC, Press, Boca Raton; Lewis Publishers, USA)
38. Tevini, M. and A.H. Teramura, 1989. UV-B effects on terrestrial plants. Photochemistry and Photobiology, 50: 479-487.
39. Unfried, I., U. Stocker and P. Gruendler, 1989. Nucleotide sequence of the *18S* rRNA gene from *Arabidopsis thaliana* co10. Nucleic Acids Research, 17: 7513.
40. Vispe, S., C. Cazux, C. Lesca and M. Defais, 1998. Over expression of *Rad*51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation. Nucleic acids Research, 26: 2859-2864.
41. Whitaker, P.A. and E.M. Southern, 1986. UV irradiation of DNA: A way of generating partial digests for rapid restriction site mapping. Gene, 41: 129-134.