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What is base excision repair good for?: knockout mutants for FPG and OGG glycosylase genes in *Arabidopsis*

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Strains of *Arabidopsis thaliana* that lack a DNA glycosylase to recognize and remove 7,8-dihydro-8-oxoguanine from their DNA are expected to be compromised in their ability to deal with this highly mutagenic base, which is formed in the presence of reactive oxygen species (ROS). We have identified two strains, one containing a *Ds* insertion in an exon of the gene that codes for oxoguanine glycosylase and one containing a T-DNA insertion in the gene that codes for formamidopyrimidine glycosylase (both EC 3.2.2.23), and have crossed them to produce the double mutant. The homozygous mutant strains showed no phenotypic difference from the wild type in growth, development or reproductive potential under either normal conditions or conditions known to induce the formation of ROS. The lack of phenotype may be ascribed to the redundant nature of the base excision repair pathway in *Arabidopsis*. Longer multigenerational studies may be needed to determine the quantitative selective advantage of individual DNA glycosylase genes.

Introduction

There are several environmental stresses that cause an increase in the concentration of reactive oxygen species (ROS) in plant cells. These include growth under high-salt conditions, drought or drought followed by watering, toxic heavy metals, chilling (Burdon et al. 1996) and the combination of high photosynthetic light and cold (Michaeli et al. 2001). In plant cells, as in animals and fungi, sources of ROS include mitochondrial electron transport, peroxidases and oxidases (Mahalingam and Fedoroff 2003). In addition, plants have photosynthetic systems that produce superoxide and singlet oxygen, particularly when carbon fixation rates are limited by temperature or CO_2 supply.

The generation of ROS can have genetic consequences. For instance, singlet oxygen reacts with guanine to produce 7,8-dihydro-8-oxoguanine (8-oxoguanine), which is strongly mutagenic because it pairs with dATP as well as dCTP during DNA synthesis (Shibutani et al. 1991). Thus the removal of 8-oxoguanine from DNA is important for genetic integrity.

The removal of 8-oxoguanine and certain other modified purines from DNA involves the base excision repair pathway (Wilson et al. 2003). The initial excision of 8-oxoguanine from deoxyribose is accomplished by formamidopyrimidine-DNA glycosylase (EC 3.2.2.23, FPG) in bacteria and oxoguanine glycosylase (EC 3.2.2.23, OGG) in animals and fungi. Plants, uniquely, have genes for both FPG and OGG (Ohtsubo et al. 1998, Dany and Tissier 2001, Garcia-Ortiz et al. 2001, Murphy and Gao 2001). The reason why plants have retained these apparently redundant enzymes is not known. The two enzymes may have evolved different substrate specificities, or they may be localized in different compartments of cells, as are the nuclear and mitochondrial forms of OGG in human cells (Nishioka et al. 1999). It is possible that the photosynthetic system creates a uniquely mutagenic environment that requires the presence and activity of both enzymes.

As an initial step to determine the importance of the glycosylases in limiting ROS-generated mutagenesis in

Abbreviations – Fapy-A, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; FPG, formamidopyrimidine-DNA glycosylase; OGG, oxoguanine glycosylase; 8-oxo-G, 8-oxo-7,8-dihydroguanine; PCR, polymerase chain reaction; ROS, reactive oxygen species.

plants, a *Ds*-transposon-induced knockout mutant for OGG and a T-DNA-induced knockout mutant of FPG were identified in *Arabidopsis thaliana*. These strains were crossed and homozygous double mutants from the F2 generation were identified. Here, the characterization of the phenotypes of these mutants is reported under normal and stressful conditions.

Materials and methods

Isolation of the mutants

A strain of *Arabidopsis thaliana* (var. Landsberg erecta) containing a *Ds* element insert in the *Ogg* gene was identified using BLASTN from the database of the collection of transgenics produced at the Institute of Molecular Agrobiology, Singapore, as described by Parinov et al. (1999) (Fig. 1A). Seeds of the identified strain (SGT 5453) were obtained from Professor V. Sundaresan. Seedlings were selected by growth on kanamycin, survivors were propagated and, from seeds of the resulting plants, homozygous mutants and wild types, as well as heterozygotes, were identified by polymerase chain reaction (PCR) (Fig. 1B). The position of the insert was confirmed by determining the sequence of the PCR fragment produced with O14 and Ds32a primers (Figs. 1A and B) and an analogous fragment at the other end of the insert produced with primers O1 and Ds52a (not shown). Reverse transcriptase-PCR applied to RNA isolated from strain Ogg2 demonstrated that the insert resulted in a lack of detectable wild-type *Ogg* mRNA (Fig. 1C).

Seeds of *Arabidopsis thaliana* (var. Columbia-0) containing a T-DNA insert of the *AtMMH* (*Fpg*) gene (Salk_076932) were obtained from the Salk Institute Genomic Analysis Laboratory's *Arabidopsis* sequence indexed collection (Alonso et al. 2003) through the Arabidopsis Biological Resource Center. The left border of the T-DNA was in the first intron of the gene (462 bases from the initiating ATG codon), determined by the sequence of the LBb1 and Ftor PCR fragment; the position of the right border could not be confirmed because the portion of the T-DNA complementary to known primers was missing (Fig. 1A). From seeds of the self-crossed plants, homozygous mutants and wild types were identified by PCR (Fig. 1B) using primers F1, LBb1 and F5. Reverse transcriptase-PCR applied to RNA isolated from strains Salk4 and Salk5 demonstrated that the insert resulted in a lack of detectable wild-type *AtMMH* mRNA (Fig. 1C).

Reciprocal crosses were made between homozygous mutants Ogg2 and Salk4 and, from the F2 generation, homozygous double mutants and wild types were selected by PCR. Three double mutants (No. 17, A4β, C9β) and three wild types (B1β, D5a, D5β) have been propagated, as of this writing, to the F5 generation.

Growth conditions

For the measurement of root growth (Table 2, see later), seeds were sown on medium containing half-strength

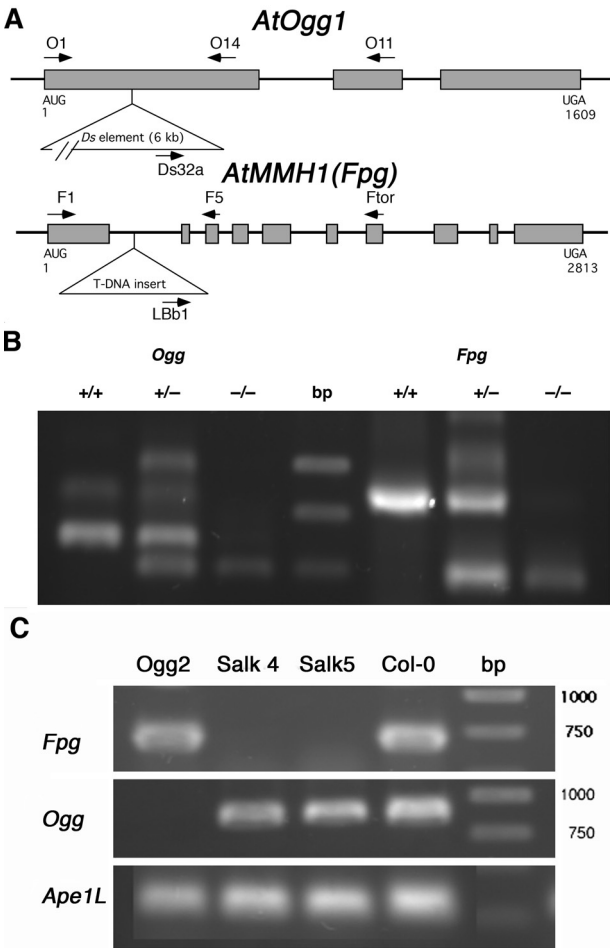

Fig. 1. (A) Location of the *Ds* insert in the *Arabidopsis thaliana Ogg* gene and T-DNA insert in the *Fpg* gene. The forward and reverse primers used to confirm the presence and location of the insertions are shown. (B) Polymerase chain reaction (PCR) fragments of segregants from an original heterozygote, using as primers O1, Ds32a and O14 (left three lanes) and F1, LBb1 and Ftor (right three lanes). (C) Reverse transcriptase-polymerase chain reaction (RT-PCR) of RNA extracted from various strains. The Ogg2 (*Ogg*–/–) strain showed no band with primers O1 and O11. The Salk4 and Salk5 (*Fpg*–/–) strains showed no band with primers F1 and Ftor. All preparations showed a band using primers for an abasic endonuclease, *Ape1L* (another potential enzyme in the base excision repair pathway), indicating that the RNA in the preparations was intact.

Murashige–Skoog salts (Gibco-Invitrogen, Carlsbad, CA) and solidified with 0.6% Phytagel (Sigma, St. Louis, MO) in a square Petri dish, and incubated at room temperature (*c.* 23°C) under continuous cool-white fluorescent light with the dish placed on edge. At day 3, the dish was turned 90°, and treatments were begun (irradiation with UV-A or a spray with 5 m*M* H_2O_2 solution); after one more day (day 4), the dish was returned to its original position and incubated for an additional day (day 5). The dishes were then photographed, and the roots were measured (Fig. 2). Gamma-irradiated seeds were grown under the same conditions (but the dishes were not turned).

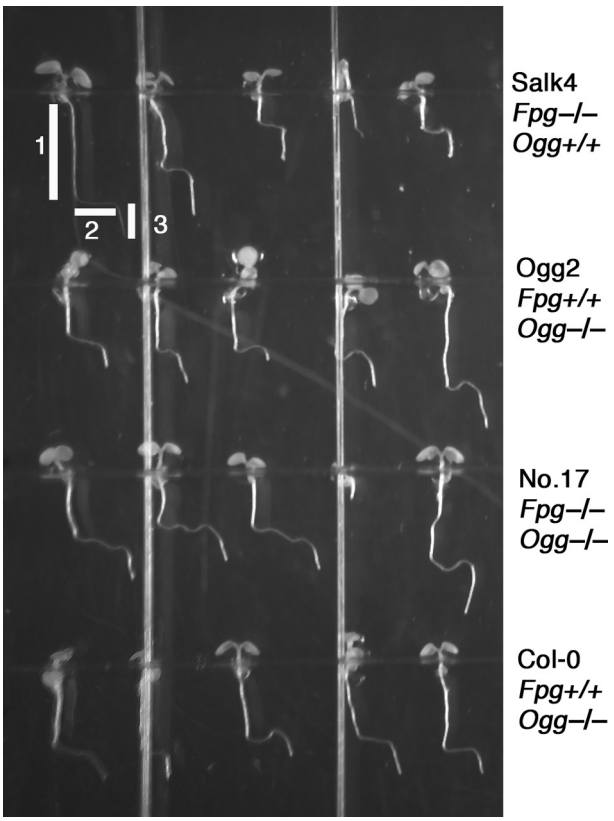

Fig. 2. Examples of root growth assay for seedlings subjected to UV-A irradiation stress. Seeds were germinated and grown on vertical plates containing Phytagel plus half-strength Murashige–Skoog salts at room temperature under constant white fluorescent light. After 3 days, the plates were turned 90° and exposed to UV-A light. One day later, the UV-A was removed and the plates were returned to their original position for one additional day. Measurements were made by eye using a millimetre scale. The bars at the top left seedling show representative measurements.

For standard propagation, seeds were sown in soil (Premier Promix BX, Premier Horticulture, Oceanside, CA), chilled for 2 days and then placed at 20°C under continuous cool-white fluorescent light. For measurement of seed set (Table 4, see later), siliques were randomly chosen from treated plants that had been transplanted to soil and grown as above.

Irradiations

UV-A radiation was obtained from a BPESL15T/BLB fluorescent lamp (Feit Electric, Pica Rivera, CA) placed 12 cm from the polystyrene dish containing the seedlings. This lamp produced one major peak from *c*. 345–405 nm, centred at 370 nm, superimposed on the Hg peaks at 365, 405 and 415 nm. A spectroradiometer (Ocean Optics, Dunedin, FL model, USB2000) detected no radiation below 345 nm. The fluence rate penetrating the lid of the Petri dish, measured with a radiometer (model UXB, UVP, Inc., San Gabriel, CA) ranged from 350 to 450 $\mu W\ cm^{-2}$, depending on the orientation of the lamp.

Gamma radiation at 759 $R\ min^{-1}$ was provided by a ^{137}Cs source (Institute for Toxicology and Environmental Health, UC Davis, CA). Seeds were imbibed with water for 24 h before irradiation and planted immediately after irradiation.

Results

UV radiation, particularly UV-A (320–400 nm), has been shown to induce oxygen-dependent damage to DNA and G to T transversions in bacteria (Palmer et al. 1997). Gamma radiation, amongst its multiple effects, forms 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-A) and 8-oxo-7,8-dihydroguanine (8-oxo-G) (Douki et al. 1997, Sutherland et al. 2000). These were chosen, together with the direct application of H_2O_2, as oxidative stresses. For preliminary experiments, chilling and intense visible light applied to plants in the cold were also used.

Preliminary experiments with Ogg2 (*Fpg+/+*,*Ogg–/–*) and Salk4 (*Fpg–/–*,*Ogg+/+*) failed to show any differences from the wild type with regard to viability, fertility or tolerance to stress. Because FPG from bacteria and OGG from mammalian cells have similar enzyme activities, the lack of phenotype of single homozygous knockout mutants could reflect functional redundancy of the genes and proteins in *Arabidopsis*. This hypothesis was tested on the double knockout mutant formed by crossing Ogg2 and Salk4.

An analysis of the progeny of the F2 generation provided the first indication that double mutants showed no lack of viability under normal growth conditions. Seventy-five F2 plants were tested for *Ogg* and *Fpg* genotypes by PCR. The distribution of the nine possible genotypes did not differ significantly from the expected distribution (Table 1).

A sensitive test for the response of DNA function to genotoxic stress was performed by measuring the growth of roots (Fig. 2). The small size and lack of pigmentation of *Arabidopsis* roots allow UV-A radiation to impinge directly on the meristems. However, the irradiation treatment did not inhibit root growth significantly: a *t*-test comparing the mean growth of control and UV-A-irradiated roots through day 4 (a period of constant

Table 1. Analysis of F2 progeny of Ogg2 × Salk4. NS, not significant.

Genotype (*Fpg*,*Ogg*)	Observed	Expected
+/+,+/+	4	4.69
+/–,+/+	8	9.38
–/–,+/+	7	4.69
+/+,+/–	11	9.38
+/–,+/–	21	18.75
–/–,+/–	9	9.38
+/+,–/–	4	4.69
+/–,–/–	6	9.38
–/–,–/–	5	4.69
Totals	75	75
Chi-squared	3.3	NS

irradiation), using data for all strains combined (Table 2), failed to show a difference ($P > 0.5$). In addition, there was no significant difference between mutants and wild types in the growth of roots under UV-A radiation (Table 2).

The same experimental system was used to test the effect of a spray of H_2O_2 solution. The spray, applied at the beginning of day 4, significantly inhibited the growth of roots ($P < 0.01$). There was a significant difference in the responses of the different strains to 5 m*M* H_2O_2 (Table 2), but inspection of the data indicated that the differences did not support a clear hypothesis that the glycosylase genes were responsible. The wild-type and the double-mutant strains tolerated the treatment better than either single mutant; 10 m*M* H_2O_2 completely inhibited root growth in all strains (data not shown).

A second series of experiments tested the effect of gamma radiation on seedling growth. Seeds imbibed for 1 day were exposed to radiation from a ^{137}Cs source and planted on Phytagel containing half-strength Murashige–Skoog salts. As measured by the growth of roots after 6 days, seedlings irradiated with 5 or 15 kR showed no significant inhibition compared with control seedlings (data not shown); however, seedlings irradiated with 50 kR (480 Gy) showed only 23% of the root elongation of untreated seedlings (Table 3). Although there were minor differences between strains, both untreated ($P < 0.05$) and irradiated ($P < 0.01$), there was no systematic effect attributable to *Fpg* or *Ogg* genotype. One double-mutant line was more sensitive, but two others were less sensitive, than the wild-type strains.

It has been known for 50 years that the cells of gamma-irradiated seedlings can expand, producing 'gamma plantlets', but if cell division in the shoot meristem is inhibited, no true leaves will be formed. True leaves formed more slowly in 50 kR-irradiated seedlings than in controls (except in Salk4, Table 4), but again there was no systematic effect related to *Fpg* or *Ogg*. When surviving plants were transplanted to soil and grown until they produced siliques, there was no systematic effect related to *Fpg* or *Ogg* on the number of seeds per silique (Table 4).

Discussion

8-Oxoguanine is one of the most common environmentally induced DNA alterations (Grollman and Moriya 1993), and its ability to base pair with either C or A makes it very mutagenic (Thomas et al. 1996).

In *Escherichia coli* and other bacteria, the system that deals with guanine oxidation involves proteins coded by *MutM* (FPG), *MutY* and *MutT* (Michaels and Miller 1992). The three enzymes coded by these genes have distinct roles. The *MutT* enzyme hydrolyses the nucleotide triphosphate of 8-oxoguanine. The *MutY* glycosylase removes A when it is paired with 8-oxoguanine. FPG removes 8-oxoguanine from DNA when it is paired with C, although not when it is paired with A. In Archaea, the function of FPG is handled by another, non-homologous enzyme, OGG. The presence of FPG in bacteria and OGG in Archaea demonstrates that the need for such repair enzymes arose early, after the divergence of the two prokaryotic groups, but before the evolution of eukaryotes. OGG is the enzyme found in fungi and mammals, reflecting the evolution of eukaryotic nuclei from

Table 2. Effects of stress on root growth. Seeds were planted on 0.6% Phytagel containing half-strength Murashige–Skoog salts, and the plates were placed on edge. At the end of the third day, the seeds had germinated and roots were growing downwards along the surface of the agar; the plates were turned 90° and were exposed to a stress or left untreated. Plates treated with UV-A were irradiated continuously for 1 day; plates treated with H_2O_2 were sprayed twice to full coverage with 5 m*M* solution. At the end of the fourth day, the plates were turned back to their original position. Plates were photographed at the end of the fifth day. The 'Day 3' (pre-stress) measurement is the distance from the root–stem junction to the first bend; the 'Day 4' (stress) measurement is the distance from the first to the second bend; the 'Day 5' (post-stress) measurement is the distance from the second bend to the root tip (see Fig. 2 for an example). All values are in millimetres (means ± SE). The last line gives the results of an analysis of variance (ANOVA) test for difference between the strains. NS, not significant.

Strain	n	Root length (mm)		
		Day 3	Day 4	Day 5
Control: no treatment				
Salk4 (*Fpg–/–,Ogg+/+*)	20	2.1 ± 0.2	2.1 ± 0.2	1.4 ± 0.3
Ogg2 (*Fpg+/+,Ogg–/–*)	20	2.3 ± 0.1	2.0 ± 0.2	1.2 ± 0.3
No. 17 (*Fpg–/–,Ogg–/–*)	18	2.5 ± 0.2	2.2 ± 0.4	1.2 ± 0.4
Col-0 (*Fpg+/+,Ogg+/+*)	20	2.6 ± 0.2	2.2 ± 0.2	1.6 ± 0.3
ANOVA		$P < 0.05$	NS	NS
5 m*M* H_2O_2				
Salk4 (*Fpg–/–,Ogg+/+*)	29	2.4 ± 0.1	1.6 ± 0.2	0.6 ± 0.2
Ogg2 (*Fpg+/+,Ogg–/–*)	27	2.7 ± 0.2	0.6 ± 0.1	1.0 ± 0.2
No. 17 (*Fpg–/–,Ogg–/–*)	28	3.6 ± 0.3	1.2 ± 0.2	1.7 ± 0.3
Col-0 (*Fpg+/+,Ogg+/+*)	28	3.9 ± 0.2	1.9 ± 0.2	2.3 ± 0.3
ANOVA		$P < 0.01$	$P < 0.01$	$P < 0.01$
UV-A				
Salk4 (*Fpg–/–,Ogg+/+*)	56	3.1 ± 0.2	1.8 ± 0.1	1.5 ± 0.1
Ogg2 (*Fpg+/+,Ogg–/–*)	30	4.6 ± 0.2	2.0 ± 0.2	1.7 ± 0.1
No. 17 (*Fpg–/–,Ogg–/–*)	56	3.1 ± 0.2	2.2 ± 0.1	1.8 ± 0.2
Col-0 (*Fpg+/+,Ogg+/+*)	58	3.9 ± 0.2	1.6 ± 0.1	1.4 ± 0.1
Ler (*Fpg+/+,Ogg+/+*)	30	2.9 ± 0.1	1.7 ± 0.1	1.0 ± 0.1
ANOVA		$P < 0.01$	NS	NS

Table 3. Effect of gamma radiation on root growth. Seeds were imbibed overnight, and then one set was irradiated with 50 kR (480 Gy). Thirty-three irradiated and control seeds were then planted on the surface of 0.6% Phytagel containing half-strength Murashige–Skoog salts, and the plates were placed on edge. Roots were measured on day 6; data show the mean ± SE (n = number germinated). The last line gives the results of an analysis of variance (ANOVA) test for difference between the strains.

Strain	Root length (mm)		Irradiated/control ratio
	Irradiated	Control	
Salk4 (*Fpg–/–,Ogg+/+*)	1.6 ± 0.1 (33)	5.3 ± 0.7 (30)	0.30
Ogg2 (*Fpg+/+,Ogg–/–*)	1.3 ± 0.1 (25)	5.2 ± 0.6 (31)	0.25
No. 17 (*Fpg–/–,Ogg–/–*)	1.6 ± 0.1 (31)	6.7 ± 0.8 (33)	0.24
A4β (*Fpg–/–,Ogg–/–*)	1.2 ± 0.2 (22)	7.4 ± 0.9 (27)	0.16
C9β (*Fpg–/–,Ogg–/–*)	2.1 ± 0.1 (27)	8.6 ± 0.6 (24)	0.24
B1β (*Fpg+/+,Ogg+/+*)	1.4 ± 0.1 (23)	6.9 ± 0.7 (26)	0.20
D5a (*Fpg+/+,Ogg+/+*)	1.4 ± 0.1 (30)	6.5 ± 0.6 (30)	0.22
ANOVA	$P < 0.01$	$P < 0.05$	

Archaea. Plants have genes for both OGG and FPG. *Arabidopsis* has a single nuclear gene for each enzyme (Ohtsubo et al. 1998, Dany and Tissier 2001).

The data described here demonstrate that *Arabidopsis* plants lacking the *Ogg* and *MutM* homologue genes do not show a clear phenotype that distinguishes them from the wild type, even under conditions that produce oxidative stress. Although surprising, this finding is not unprecedented. Mice engineered to lack the *Ogg* gene also lacked any indication of pathology, although they accumulated 8-oxoguanine in their nuclear genomes and showed a slightly elevated mutation rate in an indicator transgene in liver cells (Klungland et al. 1999). There are other cases in which the deletion of mouse repair genes – presumably maintained in the wild-type genome by positive selection – produced no phenotype (Friedberg and Meira 2003).

Table 4. Effect of gamma radiation on plant development. The seedlings described in Table 3 were inspected for the presence of true leaves: the second and third columns give the percentage of plants with true leaves on day 19 (n = 22–34). Surviving irradiated plants were transplanted to soil and grown under standard growth chamber conditions. The number of seeds in 10 randomly chosen siliques was counted: data in the fourth column give the mean ± SE.

Strain	% of plants with true leaves		Seeds/ silique
	Irradiated	Control	Irradiated
Salk4 (*Fpg–/–,Ogg+/+*)	76	83	19 ± 2.6
Ogg2 (*Fpg+/+,Ogg–/–*)	38	78	34 ± 4.2
No. 17 (*Fpg–/–,Ogg–/–*)	52	62	17 ± 4.0
A4β (*Fpg–/–,Ogg–/–*)	71	81	35 ± 6.6
C9β (*Fpg–/–,Ogg–/–*)	50	77	15 ± 2.1
B1β (*Fpg+/+,Ogg+/+*)	55	100	28 ± 4.2
D5a (*Fpg+/+,Ogg+/+*)	55	87	32 ± 4.1
D5β (*Fpg+/+,Ogg+/+*)	17	58	18 ± 2.0

One explanation for the lack of phenotype in the *Arabidopsis* double mutants could be that the conditions imposed did not produce 8-oxoguanine in DNA. UV-A produces G to T transversions in *E. coli* (Palmer et al. 1997) through the production of 8-oxoguanine, and the fluences used in the present study were 2.5 times those applied by Palmer et al. (1997). Landry et al. (1995) demonstrated that the presence of phenolics, especially hydroxycinnamates, reduced the susceptibility of *Arabidopsis* to UV-B radiation. However, hydroxycinnamates do not absorb UV-A effectively. Even if UV-A was attenuated in plant cells, this explanation does not apply to H_2O_2 and gamma radiation. As little as 100 μM H_2O_2 produced high levels of 8-oxoguanine, Fapy-G and Fapy-A in human respiratory epithelial cells (Spencer et al. 1996). Avidin binds to oxidized purines in the nuclei of mammalian cells treated with UV-A, H_2O_2 and gamma radiation (Struthers et al. 1998, Chen et al. 2001). We attempted to use the assay of Chen et al. (2001) to confirm the formation of oxidized purines in the nuclei of *Arabidopsis* cells, but high backgrounds obscured the signal.

It is possible that, in *Arabidopsis,* other enzymes besides OGG and FPG might be able to initiate repair of the damage normally handled by these two glycosylases. The case of *Ogg–/–* mice (Klungland et al. 1999) provides one example; in cell lines derived from *Ogg–/–* mice, FPG-sensitive sites (presumably including 8-oxoguanines) were removed, albeit slowly. The *Arabidopsis* genome contains 16 homologues to DNA glycosylase genes, one or more of which may have activity towards 8-oxoguanine (Arabidopsis Genome Initiative 2000).

Finally, it is possible that base substitutions, even if unrepaired, need to accumulate over several generations before deleterious phenotypes appear. In this case, it is likely that longer term, multigeneration tests comparing mutation rates in *Ogg–/–*, *MutM–/–* and wild types will be needed to identify the significance of these repair genes.

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